

## Pushing the limit: masticatory stress and adaptive plasticity in mammalian craniomandibular joints

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### Summary

Excessive, repetitive and altered loading have been implicated in the initiation of a series of soft- and hard-tissue responses or 'functional adaptations' of masticatory and locomotor elements. Such adaptive plasticity in tissue types appears designed to maintain a sufficient safety factor, and thus the integrity of given element or system, for a predominant loading environment(s). Employing a mammalian species for which considerable *in vivo* data on masticatory behaviors are available, genetically similar domestic white rabbits were raised on diets of different mechanical properties so as to develop an experimental model of joint function in a normal range of physiological loads. These integrative experiments are used to unravel the dynamic inter-relationships among mechanical loading, tissue adaptive plasticity, norms of reaction and performance in two cranial joint systems: the mandibular symphysis and temporomandibular joint (TMJ).

Here, we argue that a critical component of current and future research on adaptive plasticity in the skull, and especially cranial joints, should employ a multifaceted characterization of a functional system, one that incorporates data on myriad tissues so as to evaluate the role of altered load *versus* differential tissue response on the anatomical, cellular and molecular processes that

contribute to the strength of such composite structures. Our study also suggests that the short-term duration of earlier analyses of cranial joint tissues may offer a limited notion of the complex process of developmental plasticity, especially as it relates to the effects of long-term variation in mechanical loads, when a joint is increasingly characterized by adaptive and degradative changes in tissue structure and composition. Indeed, it is likely that a component of the adaptive increases in rabbit TMJ and symphyseal proportions and biomineralization represent a compensatory mechanism to cartilage degradation that serves to maintain the overall functional integrity of each joint system. Therefore, while variation in cranial joint anatomy and performance among sister taxa is, in part, an epiphenomenon of interspecific differences in diet-induced masticatory stresses characterizing the individual ontogenies of the members of a species, this behavioral signal may be increasingly mitigated in over-loaded and perhaps older organisms by the interplay between adaptive and degradative tissue responses.

Key words: temporomandibular joint (TMJ), symphysis, mechanical properties, MicroCT, microanatomy, rabbit, masticatory stress/load, adaptive plasticity, functional adaptation, degradation.

### Introduction

Of late, adaptive plasticity has attracted considerable attention in myriad fields of biology (Gotthard and Nylin, 1995; Agrawal, 2001; Holden and Vogel, 2002; West-Eberhard, 2003). Adaptive plasticity refers to the ability of an organism to respond ontogenetically to an altered environmental condition(s) (Gotthard and Nylin, 1995). Thus, it is intimately related to the concept of functional adaptation, which typically refers to the dynamic coordinated series of cellular, tissue and biochemical processes of modeling and remodeling that occur to maintain a sufficient safety factor of a given element or

system to routine peak stresses (Lanyon and Rubin, 1985; Biewener, 1993; Bouvier and Hylander, 1996a; Bouvier and Hylander, 1996b; Vinyard and Ravosa, 1998; Hamrick, 1999). In the case of cortical bone, the link between altered loading patterns and functional adaptation is reasonably well documented for vertebrate limb elements and the mammalian mandibular corpus (Bouvier and Hylander, 1981; Lanyon and Rubin, 1985; Biewener et al., 1986; Biewener and Bertram, 1993). Studies regarding the ontogeny of locomotor performance have been equally fundamental for identifying behavioral, anatomical and physiological adaptations and

constraints specific to particular ages (Carrier, 1996). A common goal of these and other investigations is to analyze, under naturalistic conditions, the range of behaviors an organism employs with a given morphology as well as the role of adaptive plasticity in fine-tuning the fit between form and behavior during an organism's lifespan (Grant and Grant, 1989; Losos, 1990). In doing so, such analyses of the biological role of a feature or system directly address one or more facets of the important inter-relationships among behavior, morphology, performance, fitness and evolution, information critical for understanding ontogenetic and interspecific variation in character-state transformations (Bock and von Walhert, 1965; Losos, 1990; Wainwright and Riley, 1994; Lauder, 1995).

For those interested in the evolution of craniomandibular variation, an understanding of the short- and long-term effects of dynamic alterations in masticatory stress is critically important for interpreting the behavioral and/or ecological correlates of fossil form, functional adaptation in routinely loaded systems/elements, as well as the onset and progression of joint disease and dysfunction. Most information on plasticity in the mammalian masticatory complex is derived from alert organisms subjected to variation in jaw-loading patterns *via* the postnatal manipulation of dietary properties (e.g. Bouvier and Hylander, 1981; Bouvier and Hylander, 1982; Bouvier and Hylander, 1984; Bouvier and Hylander, 1996a; Bouvier and Hylander, 1996b). This methodology has proven beneficial because masticatory stresses due to jaw-adductor, bite and reaction forces are elevated during the processing of relatively tough and/or resistant foods (Herring and Scapino, 1973; Thexton et al., 1980; Weijs et al., 1987; Weijs et al., 1989; Gans et al., 1990; Dessem and Druzinsky, 1992; Hylander et al., 1992; Hylander et al., 1998; Hylander et al., 2000; Hylander et al., 2005; Ravosa et al., 2000), and mammals with such diets typically possess relatively larger mandibular dimensions (Freeman, 1979; Freeman, 1981; Freeman, 1988; Hylander, 1979b; Bouvier, 1986; Daegling, 1989; Daegling, 1992; Ravosa, 1991a; Ravosa, 1991b; Ravosa, 1996; Biknevičius and Ruff, 1992; Ravosa and Hylander, 1994; Spencer, 1995; Biknevičius and Van Valkenburgh, 1996; Hogue, 2004; Ravosa and Hogue, 2004).

Indeed, early research on masticatory plasticity observed that growing monkeys raised on an over-use diet of hard/resistant items exhibit greater cortical bone remodeling as well as greater mandibular depth and cortical bone thickness (Bouvier and Hylander, 1981). Compared to the temporomandibular joint (TMJ) of under-use/soft-diet macaques, over-use/hard-diet macaques of the same age also develop a higher density of connective tissue and subchondral bone as well as thicker condylar articular cartilage (Bouvier and Hylander, 1982). Similar patterns characterize condylar and craniofacial dimensions as well as articular cartilage thickness in rats and rabbits raised postnatally on different diets (Beecher and Corruccini, 1981; Bouvier and Hylander, 1984; Kiliardis et al., 1985; Bouvier, 1987; Bouvier, 1988; Bouvier and Zimny, 1987; Block et al., 1988). Increased alkaline phosphatase activity associated with biomineralization of TMJ condylar

tissues, and changes in osteoclastic and osteoblastic activity also have been noted (Bouvier, 1988; Kim et al., 2003). In addition, altering TMJ force application by varying masticatory loading regime, tooth extraction, unilateral bite raise or corticotomy has been shown to result in gene expression changes and elevated glycosaminoglycan (GAG) content in condylar cartilage (Copray et al., 1985; Carvalho et al., 1995; Holmvall et al., 1995; Pirttiniemi et al., 1996; Mao et al., 1998; Agarwal et al., 2001; Huang et al., 2002; Huang et al., 2003). Lastly, changes in expression of type I and type II collagen vary in response to joint loads, further supporting the hypothesis that mechanotransduction signals changes in gene expression that alter tissue proliferation, composition and function as a response to induced degeneration of the cartilage matrix (Mizoguchi et al., 1996; Pirttiniemi et al., 1996; Grodzinsky et al., 2000; Honda et al., 2000; Lee et al., 2000; Huang et al., 2003; Kim et al., 2003; Wong and Carter, 2003).

The use of naturalistic experimental approaches ensures that potential tissue, cellular and biochemical responses do not result from aberrant behaviors and/or surgical artifacts, thus facilitating the identification of a range of physiological responses or norms of reaction of joint components to variation in masticatory loads. However, despite the fact that an understanding of the performance and integrity of the mandibular symphysis and TMJ hinges on the ability of individual tissues of such composite structures to adapt to applied stresses, no comprehensive comparative data exist regarding the dynamic cascade of anatomical, biochemical and biomechanical responses of bone and cartilage tissues of these cranial joints *vis-à-vis* long-term alteration of masticatory loads. Moreover, due to the presence of considerable variation in loading conditions across various experimental studies to date as well as a dearth of evidence regarding adaptive plasticity in cranial arthroses such as the mammalian mandibular symphysis, it has been difficult to compare norms of reactions for different masticatory elements or systems.

In this regard, symphyseal and TMJ tissues from diet-modified rabbits were analyzed for changes in (i) joint proportions and cortical bone thickness associated with the ability to counter increased joint stresses; (ii) biomineralization *via* microcomputed tomography (microCT) of articular, subarticular and cortical bone linked to the compressive strength of bone; and (iii) histology and immunohistochemistry of articular cartilage extracellular matrix (ECM) composition related to a primary role of joint cartilage in resisting compressive loads. The underlying hypothesis is that dynamic alterations in masticatory stresses during chewing and biting will induce postweaning variation in gross proportions, bony and connective tissue anatomy, tissue properties and biochemistry, a series of changes which serve to maintain the strength and integrity of the mammalian symphysis and TMJ. In particular, rabbits subjected to elevated masticatory loads are predicted to develop: (i) relatively larger symphyses, condyles, corpora and jaw-adductor muscles; (ii) greater symphyseal cortical bone thickness; (iii) elevated bone-density levels along the

symphysis and TMJ condyle; and (iv) increased type II collagen and proteoglycan expression in the symphyseal fibrocartilage (FC) pad and TMJ articular cartilage. These analyses address a related goal, which is to uniquely conduct a long-term study of adaptive plasticity and norms of reaction in comparable tissue types from two cranial joints in the same model organism subjected to the same loading conditions.

Evidence on anatomical, structural and biochemical patterns of variation are used to address several additional outstanding issues regarding masticatory function in mammals: (i) the absence of data on adaptive plasticity for cranial arthroses (symphysis), joints with highly disparate functional and structural constraints as compared to synovial joints (TMJs) and syndesmoses (sutures); (ii) the correlational nature of *in vivo* and morphological support for models of symphyseal fusion, and a related claim that symphyseal strength is unrelated to variation in fusion; and (iii) the preponderance of experimental information on symphyseal fusion for members of only a single mammalian order (Primates). Therefore, in identifying dynamic determinants of joint growth, form and function in a model organism, this experimental research develops an integrative, ontogenetic framework for investigating important inter-relationships among mechanobiology, adaptive plasticity and performance in the mammalian skull and masticatory system.

## Materials and methods

### *Experimental model*

To evaluate plasticity of masticatory elements *vis-à-vis* altered loading levels, 20 New Zealand white rabbits (*Oryctolagus cuniculus* L.) were obtained as weanlings (4 weeks old) from an approved commercial source and housed in the AALAC-accredited Center for Comparative Medicine (Northwestern University, IL, USA) for 15 weeks until attaining subadult status at 19 weeks old (Sorensen et al., 1968; Yardin, 1974). To control for variation in genetics and thus ensure the response to loading modification occurred postnatally, only siblings were used. Two dietary cohorts of ten rabbits each were established to induce postweaning variation in jaw-adductor activity and masticatory loads. Weaning was chosen as the starting point for dietary manipulation because plasticity may decrease with age (Bouvier, 1988) and because we sought to minimize the confounding influence of postweaning diets other than those utilized herein. Weanlings were fed *ad lib* either a 'soft' diet of ground pellets to model under-use (U) of the chewing complex or a 'tough/hard' diet of Harlan TekLad (Madison, WI, USA) rabbit pellets supplemented daily with two 2-cm hay blocks to model over-use (O). The inclusion of pellets in the diet of all weanling rabbits ensured adequate nutrition for normal growth. In this regard, behavioral analyses and observations indicate that U-diet rabbits did not exhibit failure to thrive nor did they develop incisor malocclusions; 90% of the U-diet sample is within the skull-length range for ten O-diet rabbits. Procedures for dietary alteration, animal monitoring and euthanasia under heavy

sedation were conducted in accordance with an ACUC-approved protocol for M.J.R.

A major benefit of domestic white rabbits is that considerable *in vivo* data exist regarding jaw-adductor muscle activity, jaw-kinematic and jaw-loading patterns, masticatory function during ontogeny, and the link between masticatory behaviors and diet (Weijs et al., 1987; Weijs et al., 1989; Langenbach et al., 1991; Langenbach et al., 1992; Langenbach et al., 2001; Langenbach and van Eijden, 2001). Similar to a variety of mammals, rabbit jaw-adductor activity patterns vary with dietary properties, such that harder, more resistant foods (pellets) and more non-brittle, tough foods with higher elastic moduli (hay) require absolutely larger jaw-adductor forces during biting and chewing (Weijs et al., 1989; Hylander et al., 1992; Hylander et al., 2000; Hylander et al., 2005). In rabbits and other mammals, this results in elevated peak strains along the mandible and higher TMJ reaction forces (Weijs and de Jongh, 1977; Hylander, 1979a; Hylander, 1979b; Hylander, 1979c; Hylander, 1992; Hylander et al., 1998; Ravosa et al., 2000). Like marsupials, rodents, carnivorans, artiodactyls and primates, rabbits exhibit postnatal variation in the size and conformation of the articular surface and connective tissues of the symphysis, beginning as an amphiarthrosis (unfused) in neonates and developing into a synarthrosis (partially fused) by adulthood (Trevisan and Scapino, 1976a; Trevisan and Scapino, 1976b; Beecher, 1977; Beecher, 1979; Hirschfeld et al., 1977; Scapino, 1981; Weijs and Dantuma, 1981; Ravosa and Simons, 1994; Ravosa, 1996; Ravosa, 1999; Hogue and Ravosa, 2001; Hogue, 2004).

### *Material properties of experimental foods*

Using a portable food tester (Darvell et al., 1996; Lucas et al., 2001), the material properties of pellets and hay were assessed (Table 1) and were routinely monitored to ensure consistency (Wainright 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. While the properties of crushed pellets differ little from intact pellets, the latter entail greater repetitive loading due to a longer processing time. Thus, the sequence from crushed pellets to whole pellets (only) to pellets with hay tracks diets with longer preparation time and progressively greater elastic moduli, hardness and toughness (well known to result in increasingly elevated masticatory stresses – see above). As the between-cohort comparisons largely accentuate the duration of oral processing (i.e. crushed pellets exhibit similar properties to whole pellets), U-diet rabbits are posited to more closely resemble normal/non-pathological loading conditions. Indeed, unlike the anatomy of O-diet rabbits, masticatory joints of U-diet rabbits are similar to those for a limited number of 6-month old adult rabbits raised on a 'normal/control' diet of intact pellets (M.J.R., unpublished observation).

Table 1. Material properties of rabbit experimental diets measured with portable food tester

Food items	<i>N</i>	Young's modulus <i>E</i> (MPa)	Toughness <i>R</i> (J m <sup>-2</sup> )	Hardness <i>H</i> (MPa)
Pellets	10	29.2 (17.0–41.0)	–	11.8 (6.3–19.9)
Wet hay	15	277.8 (124.9–451.0)	1759.2 (643.6–3251.9)	–
Dry hay	15	3335.6 (1476.8–6711.4)	2759.8 (434.0–6625.5)	–

Values are means (range).

#### Morphometry of masticatory elements

Following euthanasia, rabbit skulls were detached at the vertebral column and jaw-adductor muscles exposed and carefully dissected from their attachments. Left and right mandibles were detached from the skull and fixed in 10% buffered formalin. All specimens were weighed (to 0.01 g), with digital calipers used to obtain mandible length/breadth, symphysis length/width, corpus height/width, condyle width/length and masseter mass (Ravosa, 1991b; Nicholson et al., 2006). Such morphometric data also were used to control for size-related variation in the skull and masticatory apparatus in comparisons of loading cohorts (Bouvier and Hylander, 1981; Bouvier and Hylander, 1982; Bouvier and Hylander, 1984). Subsequently, symphyseal and TMJ samples were employed in microcomputed tomography (microCT) analyses of biomineralization and cortical bone thickness, followed by histology and immunohistochemistry.

#### MicroCT analysis of skeletal biomineralization

Intra- and between-group variation in joint structure was assessed *via* microCT (Wong et al., 1995; Nuzzo et al., 2002; Patel et al., 2003; Stock et al., 2003; Morenko et al., 2004; Nicholson et al., 2006; Ravosa et al., 2007a; Ravosa et al., 2007b). Using a Scanco Medical MicroCT 40 (PA, USA), the microfocuss X-ray tube was operated at 70 kV and 57  $\mu$ A, and the beam passed through a 0.13 mm thick beryllium window on the X-ray tube and through a 0.50 mm thick aluminum filter before encountering a sample. With this cone beam system, data from fixed specimens were collected with the longest integration time (0.30 s per view) and the highest sensitivity mode (1000 projections over 180°, 2048 samples per projection). Reconstruction was with 8  $\mu$ m voxels (volume elements). The linear attenuation coefficient ( $\mu$ ) was measured in reconstructed slices parallel to the coronal plane: five equidistant sites per symphysis (labial, anterior, middle, posterior, lingual) and three equidistant sites per TMJ (anterior, middle, posterior). For each joint site, 40 contiguous slices covering 0.31 mm were imaged, with one such slice chosen to represent a given site. At each symphyseal site,  $\mu$  was sampled at a total of nine locations: five equidistant points along the articular surface and four equidistant points along the external cortical bone (Fig. 1A). At each condylar site,  $\mu$  was sampled a total of 15 locations: five equidistant points along the articular surface, four equidistant subchondral points and three equidistant points per side along cortical bone of the condylar neck (Fig. 1B). Values of linear attenuation were pooled for each specimen and used to characterize between-group

variation in biomineralization or local tissue mineral density along the symphysis and TMJ (Fig. 1) (Nicholson et al., 2006; Ravosa et al., 2007a; Ravosa et al., 2007b). For symphyseal coronal sections, linear data on joint height and width as well as articular surface thickness and three measures of cortical bone thickness were collected in each slice.

In order to compare measured values of  $\mu$  for the rabbit symphysis and condyle with values expected for bone, one first

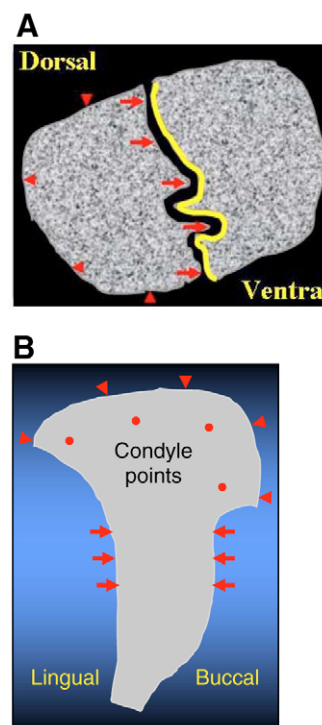


Fig. 1. MicroCT analysis of symphyseal (A) and TMJ (B) structure in rabbits. (A) Tracing of a coronal section of the middle joint site. In each of five coronal sections, biomineralization levels were evaluated with computer assisted image analysis at five equidistant points along the articular surface (arrows) and four equidistant points along the lateral, superior and inferior cortical bone regions (arrowheads). Symphysis height and width also were quantified (not shown). (B) Tracing of the coronal section of the middle condylar site. In each of three coronal sections, bone-density levels were evaluated with computer-assisted image analysis at five equidistant points along the articular surface (arrowheads), four equidistant subchondral bone locations below the condylar articular cartilage (circles) and three equidistant points along each side of the condylar neck below the articular surface (arrows).

has to consider the characteristics of the X-rays incident on the sample. Any X-ray tube produces a spectrum of X-rays modified by any filters or windows between the X-ray source and the sample. This quantity is generally not well known for a given tube, and one should note that each wavelength is absorbed differently by a sample. In practice, it is generally adequate to determine an effective X-ray energy for the tube operated at a specific voltage and base comparisons on tabulated values of the attenuation coefficients at this energy (Stock et al., 2003). A sample of aluminum of known composition (and roughly the same linear attenuation coefficients,  $2.92 < \mu < 2.96 \text{ cm}^{-1}$ , as the TMJ,  $2.6 < \mu < 3.3 \text{ cm}^{-1}$ , at 70 kV) was used to determine the effective energy. Using the NIST tabulation of mass attenuation coefficients (Hubbell and Selzer, 2001), the effective energy for Northwestern University's Scanco MicroCT 40 operated at 70 kV is about 30 keV.

#### *Histology and immunohistochemistry of cartilage composition*

Histological and immunohistochemical analysis of symphyseal and TMJ tissues followed standard procedures (Scapino, 1981; Trevisan and Scapino, 1976a; Trevisan and Scapino, 1976b; Beecher, 1977; Beecher, 1979; Hirschfeld et al., 1977; Bouvier and Hylander, 1982; Bouvier and Hylander, 1984; Bouvier, 1987; Bouvier, 1988; Kiernan, 1999; Huang et al., 2002; Kim et al., 2003; Ravosa and Hogue, 2004). Joints were fixed in 10% neutral buffered formalin. Once analyzed *via* microCT, a specimen was decalcified *via* formic acid and sodium citrate. The oxalate test was used to verify the endpoint of decalcification. Subsequently, a joint was dehydrated in a series of increasingly concentrated ethanol baths, washed in xylene, and then embedded in paraffin. Special care was exercised to maintain symphyseal and TMJ, and ultimately section, orientation parallel to the surface of the paraffin block. At five equidistant sites per symphysis (labial, anterior, middle, posterior, lingual) and at three equidistant sites per TMJ (anterior, middle, posterior), 4–6  $\mu\text{m}$  sections were obtained with a Reichert–Jung autocut microtome in the coronal plane, i.e. orthogonal to craniomandibular long axis. Once floated on a water bath, collected on a coated slide, dried and finally deparaffinized, each section then was stained by one of several methods.

The cationic dye Safranin O was used to evaluate relative GAG content in the symphyseal fibrocartilaginous pad and TMJ articular/hyaline cartilage (Kiernan, 1999; Huang et al., 2002). Primary antibodies directed at variation in cartilage type II collagen were employed to assess collagen and proteoglycan relative expression pattern (i.e. change in staining localization) as a function of masticatory loads (Type II Collagen Staining Kit; Chondrex Inc., Redmond, WA, USA). Lastly, tunel-staining was employed to track variation in DNA fragmentation and chondrocyte apoptosis in response to joint loading (Apoptosis Detection Kit; Chemicon Inc., Temecula, CA, USA). Although not presented here, H&E was utilized to distinguish the FC pad and ligaments of the symphysis as well as the articular cartilage layers of the TMJ. Definitions of

progressively deeper zones of TMJ articular cartilage are as follows: articular, filamentous network of elongate cells densely packed and tangentially arranged (high  $\text{H}_2\text{O}$ , low proteoglycan, collagen rich); proliferative, ovoid or circular cells random in distribution (proteoglycan/protein production area); chondroblastic, large cell bundles arranged in columns (tidemark separates this from subjacent layer); hypertrophic chondrocyte/calcified, cells heavily encrusted in apatitic salts (Mankin et al., 1971; Newton and Nunamaker, 1985; Ostergaard et al., 1999). To facilitate a characterization of the integrated suite of dynamic adaptive and degradative responses of skeletal and connective tissues to altered mechanical loads, similar sample sections and locations were used for microCT, histology and immunohistochemistry.

#### *Statistical analysis and predictions*

The first step in the analysis of the linear data on symphyseal and TMJ proportions from morphometry and microCT was to adjust for variation in masticatory or body/skull size between loading cohorts. This occurred by calculating the ratio of a given linear dimension, or cube root of a volumetric measure, *versus* jaw length (Bouvier and Hylander, 1981; Bouvier and Hylander, 1982; Bouvier and Hylander, 1984; Bouvier, 1986; Ravosa and Hylander, 1994; Ravosa and Hogue, 2004). To facilitate the comparison of specific masticatory parameters and to characterize the magnitude of difference between dietary cohorts, all between-group differences in metric and microCT data were tested *via* non-parametric ANOVA (Mann–Whitney *U*-test,  $P < 0.05$ ); in the case of metric data, this consisted of analyses of size-adjusted masticatory proportions (means, s.d.). To provide a confirmatory, multivariate characterization of differences in bone-density levels between loading cohorts, discriminant function analysis was employed. This procedure was used to evaluate if, based on a series of biomineralization parameters, a given joint was correctly identified as belonging to its dietary cohort, thus offering a quantitative measure of overall morphological distinctness and adaptive plasticity between loading groups (Nicholson et al., 2006; Ravosa et al., 2007a).

## **Results**

### *Morphometry*

After 15 weeks of dietary manipulation, ANOVAs indicate that size-adjusted measures of the corpus, condyle, symphysis and masseter muscle are significantly larger in 10 O-diet *versus* 10 U-diet (19-week old) domestic white rabbits (Table 2). Thus, the TMJ and corpus findings correspond to earlier studies, whereas the symphyseal data provide the first such evidence regarding plasticity of joint proportions in mammals.

### *MicroCT*

The influence of routine joint over-use and under-use on symphyseal and TMJ biomineralization, and on internal symphyseal proportions, was evaluated *via* microCT. MicroCT analyses of the articular surface, subarticular bone and cortical

Table 2. Comparison of size-adjusted measures of load-resisting and force-generating masticatory elements between rabbit dietary cohorts

Variable	O-diet	U-diet	% Difference
Condyle			
AP length (mm)	0.178±0.016	0.150±0.014	18.7*
ML width (mm)	0.074±0.010	0.069±0.005	7.3*
Corpus			
Height (mm)	0.242±0.023	0.237±0.020	2.1
Width (mm)	0.096±0.009	0.090±0.008	6.7*
Symphysis			
Length (mm)	0.396±0.034	0.351±0.046	12.8*
Width (mm)	0.148±0.016	0.129±0.014	14.7*
Articular breadth (mm)	0.173±0.027	0.140±0.031	23.6*
Superior cortical depth (mm)	0.176±0.045	0.134±0.042	31.3*
Lateral cortical depth (mm)	0.089±0.009	0.058±0.008	53.4**
Masseter mass (wet; g)	0.135±0.016	0.112±0.023	20.5*

Values are means ± s.d. for each variable, indicated by loading cohort;  $N=10$  for each diet.

O-diet rabbits develop relatively larger bony proportions and jaw adductor muscles as well as thicker cortical bone along the symphyseal articular and external surfaces. Asterisks indicate significant differences, \* $P<0.05$ , \*\* $P<0.01$ ; Mann-Whitney  $U$ -test.

bone along the symphysis and TMJ condyle indicate that significant variation develops in joint density and anatomy between O-diet and U-diet rabbits, with the former group exhibiting significantly higher levels of biomineralization (Table 3, Table 4). Using linear attenuation coefficients ( $\mu$ ) for 9 symphyseal and 15 TMJ sites, discriminant function analysis was performed for each joint to summarize patterns of variation in bone-density levels between U-diet and O-diet rabbits. Much as expected based on the univariate ANOVAs, these multivariate analyses of biomineralization for each joint correctly classified all members of each dietary group (as such, these redundant results are not presented). ANOVAs also indicate the presence of significantly thicker cortical bone along the symphyseal outer and articular surfaces in O-diet rabbits (Fig. 2; Table 2). These

Table 3. Comparison of symphyseal biomineralization levels ( $\mu$ ) between rabbit dietary cohorts

Variable	O-diet	U-diet	% Difference
Symphysis			
Top	2.243±0.219	1.822±0.160	23.1**
Upper	2.005±0.103	1.610±0.118	24.5**
Middle	2.055±0.201	1.623±0.151	26.6**
Lower	1.910±0.064	1.613±0.076	18.4**
Bottom	1.904±0.156	1.673±0.168	13.8**
Corpus			
Inferior	2.163±0.208	1.762±0.152	22.8**
Inf./lat.	2.655±0.164	2.262±0.211	17.4**
Lateral	2.607±0.135	2.437±0.137	7.0*
Superior	2.611±0.228	2.281±0.220	14.5**

Values are means ± s.d. for each variable, indicated by loading cohort;  $N=7$  for each diet. O-diet rabbits develop elevated bone-density levels along the symphyseal articular and external cortical bone surfaces. Asterisks indicate significant differences, \* $P<0.05$ , \*\* $P<0.01$ ; Mann-Whitney  $U$ -test.

findings underscore the significant influence of dietary material properties on adaptive plasticity in masticatory proportions, tissue structure and bone mineral density.

#### Histology and immunohistochemistry

Sulfated GAGs are expressed in tissues regularly exposed to loads, and rat TMJ chondrocytes have been shown to increase

Table 4. Comparison of TMJ biomineralization levels ( $\mu$ ) between rabbit dietary cohorts

Variable	O-diet	U-diet	% Increase
Outer			
1	1.490±0.196	1.482±0.076	1.0
2	1.561±0.241	1.181±0.184	32.2**
3	1.485±0.231	1.314±0.165	13.0*
4	1.554±0.239	1.243±0.116	25.0*
5	1.618±0.175	1.425±0.168	13.3*
Inner			
1	2.187±0.231	1.946±0.185	12.4*
2	2.180±0.196	1.787±0.209	22.0**
3	2.102±0.155	1.776±0.159	18.4**
4	2.111±0.140	1.953±0.263	8.1*
Neck			
1	1.995±0.225	1.710±0.186	16.7*
2	2.009±0.164	1.746±0.168	15.1*
3	1.971±0.057	1.626±0.132	21.2**
4	2.051±0.198	1.807±0.079	13.5*
5	1.990±0.182	1.815±0.147	9.6*
6	2.008±0.178	1.648±0.173	21.8**

Values are means ± s.d. for each variable, indicated by loading cohort;  $N=8$  for each diet.

O-diet rabbits develop elevated bone-density levels along the articular surface, subchondral region and external cortical surface of the condylar neck. Asterisks indicate significant differences, \* $P<0.05$ , \*\* $P<0.01$ ; Mann-Whitney  $U$ -test.

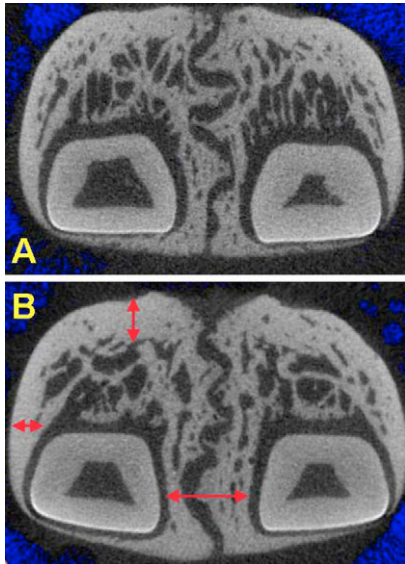


Fig. 2. Symphyseal cortical bone thickness. Coronal sections of 'middle' joint sites from U-diet (A) and O-diet (B) subadult rabbits obtained *via* microCT. Comparisons of three size-adjusted measures of internal joint proportions indicate that O-diet rabbits develop significantly thicker cortical bone along the superior, lateral and articular surfaces of the symphysis (red lines with double arrowheads) (see Table 2).

GAG synthesis in response to mechanical force (Coprav et al., 1985; Carvalho et al., 1995). Strong Safranin O staining is indicative of keratan sulfate-containing proteoglycans and chondroitin sulfate, which in turn increases the viscoelastic ability of cartilage for resisting compressive stresses. Type II collagen has a distinct fibrillar organization and associates strongly with water and proteoglycans, important for tissues subjected to compression, tension and shear, such as the symphyseal FC pad and TMJ articular cartilage (Mizoguchi et al., 1996; Pirttiniemi et al., 1996; Benjamin and Ralphs, 1998; Tanaka et al., 2000).

Histological analyses of U-diet and O-diet subadults indicate more intense Safranin O staining in the symphyseal FC pad (compare 'A' vs 'B' in Fig. 3) and TMJ condylar articular cartilage of the U-diet rabbit (compare 'A' vs 'B' in Fig. 4). Lower proteoglycan content throughout the FC pad and in the lower two layers of the condylar cartilage of O-diet rabbits mirrors findings for the articular surface of mammal limb elements, where age-related onset of cartilage degradation is linked to decreases in proteoglycan content (Mankin et al., 1971; Newton and Nunamaker, 1985; Haskin et al., 1995; Ostergaard et al., 1999). Due to the elevated viscoelasticity of proteoglycan-rich tissues in joints subjected to cumulatively low postnatal stresses (*i.e.* U-diet), analyses suggest that articular cartilage and fibrocartilage of such organisms are able to resist greater compressive stresses than that of repetitively over-loaded cranial joints. As proteoglycan content is most pronounced in the two innermost layers of TMJ articular

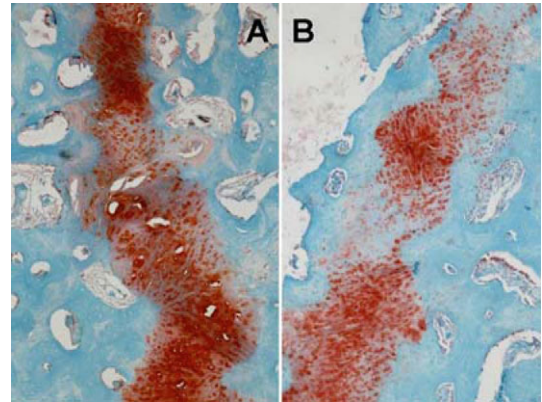


Fig. 3. Symphyseal proteoglycan content. Coronal sections (6  $\mu\text{m}$ ) of 'middle' joint sites from U-diet (A) and O-diet (B) subadults stained with Safranin O to identify GAG content in the FC pad. In the high-power views of the ventral joint with the FC pad, darker staining in A vs B indicates lower proteoglycan content and thus decreased FC pad viscoelasticity in O-diet rabbits. In B note also the corresponding development of bony rugosities ('blue' bone, nearly completely traversing the 'red' FC pad).

cartilage (chondroblastic and hypertrophic/calcified chondrocyte), this suggests it is critical to account for regional variation in this and other ECM components in evaluating the biomechanical significance of cartilage properties and proportions.

Immunohistochemical data for U-diet *versus* O-diet subadult rabbits demonstrate a more widespread distribution of type II collagen in the symphyseal FC pad and TMJ condylar articular cartilage of U-diet rabbits (Fig. 5, Fig. 6). Expression of collagen II has been noted in the ECM of mature chondrocytes and inner cartilage layers such as the hypertrophic and chondroblastic zones of the TMJ. Type II collagen has a distinct fibrillar organization and associates more strongly with proteoglycans, and both ECM components are important in tissues subjected to compressive loads during biting and chewing. These comparisons suggest that, much as the case for the well-documented TMJ, symphyseal adaptive plasticity is characterized by similar patterns of postweaning variation in type II collagen *and* proteoglycan content (Figs 2, 3).

In the FC pad and TMJ articular cartilage, tunnel staining indicates a greater number of apoptotic chondrocytes in O-diet *versus* U-diet rabbits (Figs 7, 8). In fact, as symphyseal fibrocartilage is characterized by fewer chondrocytes than hyaline cartilage of the TMJ, it is exceedingly difficult to identify apoptotic cells in the U-diet symphysis. This pattern in both joints suggests that routine overloading induces accelerated cell death and increased cartilage degradation. In TMJ articular cartilage, O-diet rabbits appear to develop more hypertrophic chondrocytes (Fig. 8). In the growth plate of a joint, apoptosis is a normal terminal event for hypertrophic chondrocytes, and such cells express angiogenic factors initiating vascular invasion, erosion of mineralized cartilage and bone formation (Gerber et al., 1999). Thus, increased

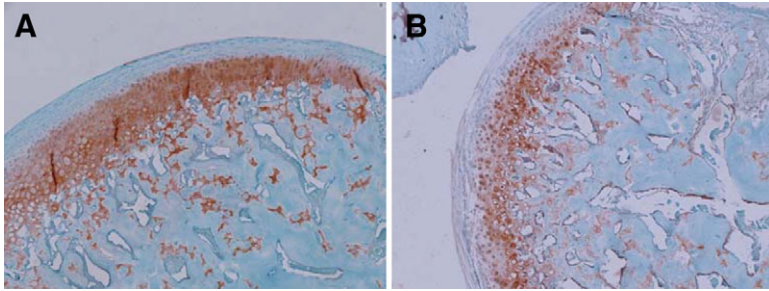


Fig. 4. TMJ proteoglycan content. Coronal sections (6  $\mu\text{m}$ ) of middle joint sites from U-diet (A) and O-diet (B) 4-month old subadults were stained with Safranin O to identify GAG content in the articular cartilage. In low-power views of the articular surface and underlying subchondral bone, more intense staining in A vs B indicates lower proteoglycan content and thus diminished articular cartilage viscoelasticity in O-diet rabbits.

numbers of apoptotic hypertrophic chondrocytes appear related to advance of the subchondral mineralizing front.

### Discussion

#### *Adaptive plasticity and degradation in masticatory tissues*

*In vivo* and comparative analyses indicate that the postnatal development of masticatory elements and tissues is influenced by variation in jaw-loading patterns, with weaning being a particularly important life-history stage (Ravosa and Simons, 1994; Ravosa, 1996; Ravosa, 1999; Ravosa and Hogue, 2004). Once weaned, mammals ingest 'adult' food items (e.g. Watts, 1985; Tarnaud, 2004) and develop 'adult' jaw-adductor activity patterns (Herring and Wineski, 1986; Weijs et al., 1987; Herring et al., 1991; Iinuma et al., 1991; Langenbach et al., 1991; Langenbach et al., 1992; Langenbach et al., 2001; Westneat and Hall, 1992; Huang et al., 1994), with associated skeletal and soft-tissue responses to 'adult' jaw-loading regimes (Ravosa, 1991a; Ravosa, 1992; Ravosa, 1996; Ravosa, 1999; Cole, 1992; Biknevičius and Leigh, 1997; Vinyard and Ravosa, 1998; Taylor et al., 2006).

Early experimental studies of postweaning plasticity in the mammalian masticatory apparatus often focused on the

mandibular corpus and TMJ articular cartilage (cf. Bouvier and Hylander, 1981; Bouvier and Hylander, 1982; Bouvier and Hylander, 1984). More recent work provides considerable support for the hypothesis that cartilage of the mandibular condyle and TMJ articular disc is affected by local biomechanical effects. Indeed, chondrocytes are highly sensitive to 3-D microenvironment and exhibit changes in differentiation status in response to environmental cues (Lemare et al., 1988; Goldring, 2004a; Goldring, 2004b), with expression of cartilage ECM elements likely reflecting regional variation due to differential loading patterns in distinct joint regions (Bayliss et al., 1983; Nakano and Scott, 1989; Mow et al., 1990; Hamrick, 1999; Tanaka et al., 2000). In this regard, it is interesting that collagen- and proteoglycan-degrading proteinases have been reported in TMJ tissues and synovial fluids (Kiyoshima et al., 1993; Kiyoshima et al., 1994; Marchetti et al., 1999; Puzas et al., 2001; Srinivas et al., 2001).

A general conclusion is that growth responses of the mandibular condyle following alteration of local biomechanical conditions (both increased and decreased loads) can lead to hyperplastic or hypoplastic changes in TMJ cartilage and bone (Bouvier and Hylander, 1984; Nicholson et al., 2006). Based largely on short experimental periods in growing mammals (<2 months), these studies support the hypothesis that altered, excessive and/or repetitive forces induce secondary osteonal remodeling of mandibular cortical bone and chondroblastic activity of articular cartilage, a suite of physiological responses or functional adaptations that maintain a sufficient safety factor for the tissues of a cranial element or joint complex to routine peak masticatory loads (cf. Lanyon and Rubin, 1985; Biewener, 1993; Bouvier and Hylander, 1996a; Bouvier and Hylander, 1996b; Vinyard and Ravosa, 1998; Hamrick, 1999; Ravosa et al., 2000). These investigations also suggest that a minimum loading level and frequency is required for the growth and maintenance of normal adult skull form and function (Beecher et al., 1983; Bouvier and Hylander, 1984). Interestingly, the magnitude of such responses appears to be age-dependent and may be underlain by genetic and epigenetic factors that vary systemically and interspecifically (Bouvier, 1988; Bouvier and Hylander, 1996a; Bouvier and Hylander, 1996b).

Employing an animal model for which considerable *in vivo* data on feeding behavior are available, we performed a series of integrative experiments to probe the longer-term dynamic

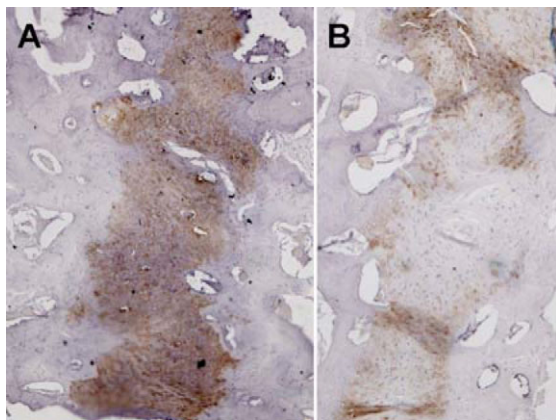


Fig. 5. Symphyseal type II collagen. Coronal sections (6  $\mu\text{m}$ ) of the 'middle' joint site in 4-month old U-diet (A) and O-diet (B) subadults stained with a primary antibody directed against type II collagen. In the high-power views of the ventral joint, darker staining in the FC pad of A vs B demonstrates less type II collagen and thus lower viscoelasticity in O-diet rabbits.



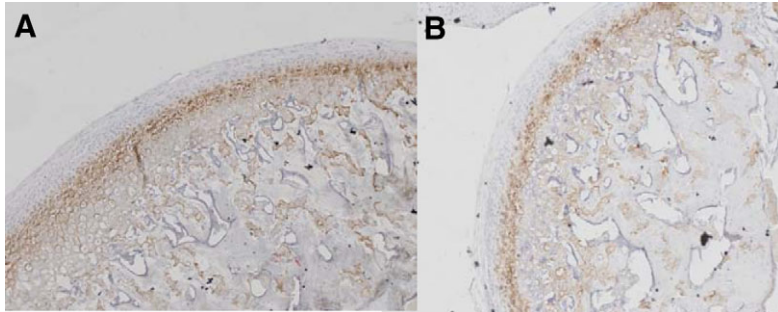


Fig. 6. TMJ type II collagen. Coronal sections ( $6\ \mu\text{m}$ ) of middle sites in 4-month old U-diet (A) and O-diet (B) subadults were stained with a primary antibody directed against type II collagen. In the low-power views of the articular cartilage and subchondral bone, more intense staining of TMJ articular cartilage in A vs B indicates less type II collagen and thus diminished viscoelasticity in O-diet rabbits.

links among mechanical loading, tissue adaptive plasticity, norms of reaction and performance in two mammalian masticatory joint systems. The mandibular symphysis and TMJ are highly specialized joints capable of both rotational and translational movements, and thus encounter multidirectional compressive, shear and tensile forces during biting and chewing (Rigler and Mlinsek, 1968; Beecher, 1977; Beecher, 1979; Hylander, 1979a; Hylander, 1979b; Hylander, 1979c; Hylander, 1992; Scapino, 1981; Ravosa and Hogue, 2004). In addition to cortical and trabecular bone, TMJs and symphyses are composed of cartilage, ligaments and dense fibrous tissue containing proteoglycans and collagens (Figs 2–8). As the symphyseal FC pad and TMJ articular cartilage are anchored into subarticular bone, their stress distributions are constrained respectively by movements between dentaries (symphysis) or between the mandibular condyle and temporal bone (TMJ).

Analyses of rabbits represent the first case where plasticity is assessed at two different joints in the same model organism. In this experimental model, symphyses and TMJs of overloaded joints develop larger joint proportions and higher bone-density levels, coupled with lower proteoglycan content, lower type II collagen and greater chondrocyte apoptosis.

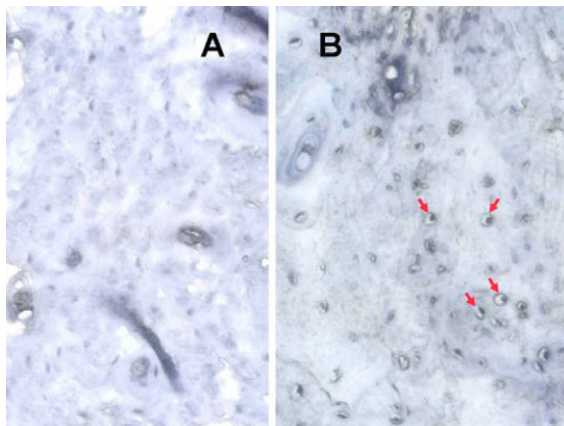


Fig. 7. Symphyseal apoptosis. Coronal sections ( $6\ \mu\text{m}$ ) of 'middle' joint sites from U-diet (A) and O-diet (B) subadults were stained to identify fragmented DNA of apoptotic chondrocytes in the FC pad. In the high-power views of the ventral joint with the FC pad, O-diet rabbits exhibit numerous apoptotic chondrocytes vs U-diet rabbits (red arrows).

Interestingly, although symphyseal fibrocartilage is more acellular, it exhibits responses similar to that for hyaline cartilage of the TMJ articular surface. However, while the gross anatomical and bone biomineralization data are much as predicted, findings for the ECM composition of joint cartilage seemingly contradict shorter-term experimental studies cited above. In light of this earlier work, it is reasonable to interpret the rabbit cartilage patterns as the result of degradative changes due to long-term joint over-loading. Thus, we do not and cannot refute the fact that cartilage exhibits a compensatory adaptive response to joint over-loading. Rather, the duration of dietary manipulation in our study greatly exceeds that of previous investigations and it is well known that cartilage exhibits accelerated degradation in response to elevated and/or repetitive loading (Guerne et al., 1994; Guerne et al., 1995; Bae et al., 1998). Such changes in cartilage composition reflect the early onset and progression of degenerative effects that compromise the structural integrity of a joint (Mankin et al., 1971; Newton and Nunamaker, 1985; Haskin et al., 1995; Kamelchuk and Major, 1995; Ishibashi et al., 1996; Ostergaard et al., 1999; Fujimura et al., 2005). This interpretation is consistent with patterns of change noted for rabbit TMJ

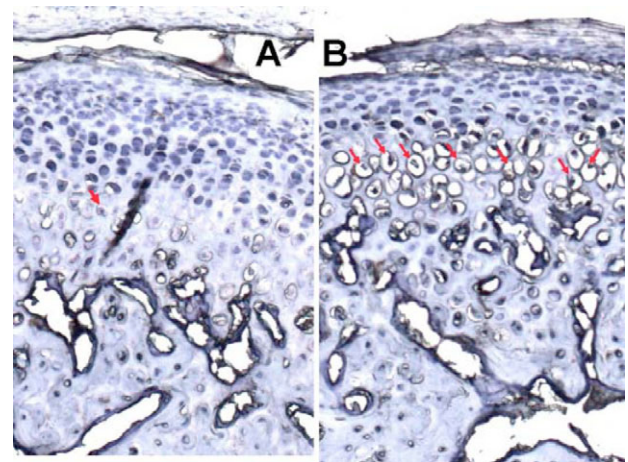


Fig. 8. TMJ apoptosis. Coronal sections ( $6\ \mu\text{m}$ ) of middle joint sites from U-diet (A) and O-diet (B) 4-month old subadults were stained to identify cellular apoptosis in articular cartilage. In high-power views of the articular surface and subchondral bone, O-diet rabbits exhibit elevated chondrocyte apoptosis vs U-diet rabbits (red arrows).

connective tissues. In fact, it is likely that a component of the adaptive changes in mammalian joint proportions and biomineralization represents a compensatory mechanism to cartilage degradation that maintains the overall functional integrity of such composite tissue systems.

In the case of the rabbit symphysis, the development of bony rugosities, larger joint surfaces due to thicker cortical bone and greater bone density, all represent adaptive responses to joint over-loading (Tables 2, 3; Figs 2, 3, 5). However, repetitive joint over-loading results in the FC pad eventually becoming less viscoelastic, which diminishes its ability to resist compressive stresses. As joint ossification clearly does not compromise symphyseal function as it would with the TMJ, the disparate long-term responses of symphyseal soft *versus* hard tissues may explain a common (but poorly understood) intraspecific trend of older mammals developing increased fusion (cf. Beecher, 1977; Beecher, 1979; Scapino, 1981; Ravosa and Simons, 1994; Ravosa, 1996; Ravosa, 1999; Hogue and Ravosa, 2001). Thus, age-related changes in fusion, especially in old adults, may represent a compensatory osteogenic response to load-induced degradation of the FC pad and perhaps other connective tissues.

The rabbit findings are similar to recent analyses of myostatin-deficient mice documenting greater differentiation of symphyseal parameters in response to elevated physiological loads (Ravosa et al., 2007a), which is suggestive of greater tissue plasticity or norms of reaction for this joint *versus* elsewhere in the masticatory system (Tables 2–4). It is thus interesting that the symphysis experiences relatively higher bone-strain levels during biting and chewing, and is characterized by strong positive allometry of joint proportions (Hylander, 1979a; Hylander, 1979b; Ravosa, 1991a; Ravosa, 1991b; Ravosa, 1992; Ravosa, 1996; Ravosa and Hylander, 1994; Hylander et al., 1998; Vinyard and Ravosa, 1998; Hogue and Ravosa, 2001; Hogue, 2004; Ravosa and Hogue, 2004; Ravosa et al., 2000), two additional factors that likely contribute to the potential for increased symphyseal plasticity.

As alluded to above, our research uniquely suggests that the short-term duration of earlier analyses of cranial joint tissues may offer a limited notion of the complex process of developmental plasticity, especially as it relates to the effects of long-term alterations in mechanical loads, when a joint is increasingly characterized by adaptive and degradative changes in tissue structure, composition and function. Perhaps not surprisingly, we also sound a cautionary note that the assessment of masticatory plasticity based solely on external joint proportions can under-represent the amount of change in individual tissues. For instance, the magnitude of the plasticity response differs between loading cohorts according to the level of analysis, e.g. external joint proportions vary less between dietary groups (Table 2) than in comparisons of skeletal biomineralization (Tables 3, 4).

#### *Adaptive plasticity and symphyseal function*

Though it is well known that *in vivo* information is best for detailing how an animal functions during normal behaviors

such as biting and chewing (Bock and von Walhert, 1965; Hylander, 1979a; Hylander, 1979b; Wake, 1992; Wainright and Reilly, 1994; Lauder, 1995), there is perhaps one shortcoming of the evidence for symphyseal fusion based on studies of craniomandibular bone strain and jaw-adductor muscle activity. Apart from sound theoretical arguments, the best *in vivo* support for a functional link between symphyseal stress and symphyseal fusion is essentially correlational, in linking character-state variation to the way an adult organism loads, or is posited to load, a masticatory structure (Ravosa and Hogue, 2004). While this does not invalidate or diminish the unique and important role of *in vivo* data for testing hypotheses regarding the biological role and performance of cranial elements, it does imply that when evaluating masticatory function during growth or across a clade, presently one must assume that variation in symphyseal fusion corresponds to specific differences in jaw-loading and jaw-adductor muscle patterns. Indeed, this gap in our knowledge has abetted arguments that variation in symphyseal fusion is unrelated to variation in symphyseal loading levels during mastication, with an unfused joint being sufficiently strong to routinely counter significant stresses (Dessem, 1989; Lieberman and Crompton, 2000). It follows from such an interpretation that the tissues of an unfused symphysis would be unresponsive to postnatal variation in long-term, repetitive loads.

This controversy exists because an integrative biomechanical, cellular and biochemical analysis of adaptive plasticity heretofore had been applied only to cranial synovial joints (TMJ) (Bouvier and Hylander, 1982; Bouvier and Hylander, 1984; Huang et al., 2002; Huang et al., 2003) and syndesmoses (sutures) (Byron et al., 2004). To this end, data on tissue plasticity for a cranial arthrosis (rabbit symphysis) offer a novel perspective on the dynamic inter-relationships among symphyseal fusion, joint performance and feeding behaviors. In support of prior research (Hylander, 1979a; Hylander, 1979b; Hylander et al., 1998; Hylander et al., 2000; Hylander et al., 2005; Ravosa and Hylander, 1994), our analyses where both rabbit cohorts used their incisors similarly, but differed largely in diet-related forces experienced during postcanine chewing and biting, highlights the significant role of stresses during mastication on postnatal and phylogenetic variation in symphyseal anatomy across diverse mammal clades (e.g. Tables 2, 3). Moreover, evidence regarding functional adaptation in symphyseal proportions, morphology and bony properties supports the hypothesis that dynamic alterations in masticatory loads positively influence developmental variation in symphyseal joint strength, integrity and performance. As argued elsewhere, these findings are inconsistent with alternative claims that fusion occurs to stiffen, rather than strengthen, the symphyseal joint during mastication (Hogue and Ravosa, 2001; Ravosa and Hogue, 2004).

#### *Conclusion*

By selecting similar section/site samples for morphometric, microCT, immunohistochemical and histological comparisons,

our experimental research facilitated a characterization of the coordinated series of dynamic functional adaptations as well as the onset of degradative responses of cranial joint tissues *vis-à-vis* altered masticatory stresses. Results suggest that evolutionary variation in symphysis and TMJ morphology, and thus by inference joint performance, among sister taxa is in part an epiphenomenon of interspecific differences in (diet-induced) jaw-loading patterns characterizing the individual ontogenies of the members of a species (Vinyard and Ravosa, 1998; Ravosa and Hogue, 2004). However, this interspecific behavioral signal may be increasingly mitigated among aging adults by the (potentially species-specific) interplay between adaptive and degradative tissue responses. Therefore, current and future research on adaptive plasticity in the skull, and especially joints, should employ a multifaceted characterization of a given functional network, one that incorporates data on myriad tissues so as to evaluate the role of altered loading *versus* differential tissue response on functional adaptation of such composite structures. As tissue degradation is the failure of the adaptive process to adequately respond to altered and/or excessive loading conditions, this integrative perspective is also fundamental for unraveling the etiology of joint disease.

#### List of symbols and abbreviations

TMJ	temporomandibular joint
FC pad	fibrocartilage pad
ECM	extracellular matrix
GAG	glycosaminoglycan
U-diet	under-use diet
O-diet	over-use diet
microCT	microcomputed tomography
$\mu$	linear attenuation coefficient
$E$	elastic or Young's modulus (MPa)
$R$	toughness ( $\text{J m}^{-2}$ )
$H$	hardness (MPa)

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