Using “Mighty Mouse” to understand masticatory plasticity: myostatin-deficient mice and musculoskeletal function

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Synopsis Knockout mice lacking myostatin (Mstn), a negative regulator of the growth of skeletal muscle, develop significant increases in the relative mass of masticatory muscles as well as the ability to generate higher maximal muscle forces. Wild-type and Mstn-deficient mice were compared to investigate the postnatal influence of elevated masticatory loads due to increased jaw-adductor and bite forces on the biomineralization of mandibular articular and cortical bone, the internal structure of the jaw joints, and the composition of temporomandibular joint (TMJ) articular cartilage. To provide an interspecific perspective on the long-term responses of mammalian jaw joints to altered loading conditions, the findings on mice were compared to similar data for growing rabbits subjected to long-term dietary manipulation. Statistically significant differences in joint proportions and bone mineral density between normal and Mstn-deficient mice, which are similar to those observed between rabbit loading cohorts, underscore the need for a comprehensive analysis of masticatory tissue plasticity vis-à-vis altered mechanical loads, one in which variation in external and internal structure are considered. Differences in the expression of proteoglycans and type-II collagen in TMJ articular cartilage between the mouse and rabbit comparisons suggest that the duration and magnitude of the loading stimulus will significantly affect patterns of adaptive and degradative responses. These data on mammals subjected to long-term loading conditions offer novel insights regarding variation in ontogeny, life history, and the ecomorphology of the feeding apparatus.

Introduction

For the greater part of the past century, the mouse has figured heavily as a model organism in studies of an evolutionary and translational nature. Its small size, short gestation time, the ease with which it can be housed, and the genetic proximity to humans, are just some of the myriad benefits of employing mouse models in experimental research. Given the recent sequencing of the mouse genome, studies of this organism will increase exponentially (Waterston et al. 2002). Here, our use of a mouse model relates to long-standing questions regarding the evolutionary morphology of the mammalian skull and masticatory apparatus. In an attempt to expand the implications of laboratory-based studies of plasticity for understanding evolution in the wild, we compare and contrast data on the responses of mammalian jaw joints to long-term, naturalistic variation in masticatory stresses as well as evaluate the relevance of a unique mouse model of masticatory over-use [myostatin (Mstn)-deficient mice] versus more traditional models of masticatory plasticity (e.g., diet-modified rabbits). For those interested in craniodentomandibular evolution, an understanding of the short-term and long-term influence of altered masticatory stresses on tissues of the mandibular symphysis and temporomandibular joint (TMJ) is critical for interpreting the behavioral and/or ecological correlates of variation in the morphology of extant and extinct taxa as well as for understanding the biomechanics and performance of routinely loaded systems and elements. Prior to presenting the two experimental models, we briefly discuss the concept of adaptive plasticity and recount the role of masticatory stress as a determinant of phenotypic variation in mammals.
Plasticity and masticatory stress

Adaptive plasticity refers to the ability of an organism to respond during its ontogeny to altered environmental conditions (Gotthard and Nylin 1995; Agrawal 2001; Holden and Vogel 2002; West-Eberhard 2003). It is thus intimately related to the concept of functional adaptation, which is the dynamic, coordinated series of cellular, tissue and biochemical processes of modeling and remodeling that maintain a sufficient safety factor of a given element or system to routine stresses (Bouvier and Hylander 1981, 1996; Lanyon and Rubin 1985; Biewener et al. 1986; Biewener 1993; Biewener and Bertram 1993; Vinyard and Ravosa 1998; Hamrick 1999; Ravosa et al. 2000). A common goal of these laboratory and other field-based investigations is to analyze, under naturalistic conditions, the range of behaviors an organism employs with a given morphology, as well as the role of plasticity in fine-tuning the fit between form and behavior during an organism’s life history (Grant and Grant 1989; Losos 1990; Carrier 1996). Such analyses of the biological role of a feature or complex address one or more facets of the important links among behavior, morphology, growth, performance, fitness and evolution, information critical for understanding ontogenetic, and interspecific patterns of phenotypic variation (Bock and von Walhert 1965; Wake 1982; Losos 1990; Wainwright and Riley 1994; Lauder 1995).

Most evidence regarding plasticity of soft and hard tissues in the mammalian feeding apparatus has resulted from analyses of alert animals subjected to dynamic variation in jaw-loading patterns via the postweaning manipulation of dietary properties (Bouvier and Hylander 1981, 1982, 1984). This methodology is intuitively appealing because masticatory stresses due to jaw-adductor, bite, and reaction forces are elevated during the processing of relatively tough and/or hard foods (Herring and Scapino 1973; Luschei and Goodwin 1974; Thexton et al. 1980; Wejs et al. 1987, 1989; Hylander et al. 1992; Ravosa et al. 2000). Correspondingly, mammals with such diets typically exhibit relatively larger musculoskeletal dimensions (Freeman 1979, 1981, 1988; Bouvier 1986; Daegling 1989, 1992; Ravosa 1991a, 1991b; Ravosa and Hylander 1994; Spencer 1995; Biknevicius and Van Valkenburgh 1996; Ravosa and Hogue 2004; Taylor et al. 2006; Menegaz et al. 2008). Such a naturalistic approach also ensures that potential tissue, cellular, and biochemical responses do not result from aberrant behaviors and/or surgical artifacts, which in turn facilitates the identification of a range of physiological responses or reaction norms to altered masticatory loads.

Growth responses of the mandible in macaques following alteration of local biomechanical conditions to elevated loads can lead to increases in cortical bone remodeling, greater mandibular depth and cortical bone thickness, a higher density of TMJ connective tissue and subchondral bone, and thicker TMJ articular cartilage (Bouvier and Hylander 1981, 1982). Similar patterns characterize craniomandibular proportions and thickness of the TMJ articular cartilage in diet-manipulated rats as well as thickness of the TMJ articular disc in over-use rabbits (Beecher and Corruccini 1981; Bouvier and Hylander 1984; Bouvier 1987, 1988; Bouvier and Zimny 1987; Yamada and Kimmel 1991).

As chondrocytes are sensitive to 3-D microenvironments and exhibit changes in differentiation status in response to regional cues (Goldring 2004), expression of extracellular elements in cartilage likely exhibits local variation reflecting differential loading patterns in distinct joint regions (Bayliss et al. 1983; Nakano and Scott 1989; Mow et al. 1990; Tanaka et al. 2000). Altering TMJ force application has been shown to result in changes in gene expression and in elevated content of proteoglycans or glycosaminoglycans (GAGs) in TMJ articular cartilage (Copray et al. 1989; Carvalho et al. 1995; Holmval et al. 1995; Pirttiniemi et al. 1996; Mao et al. 1998; Huang et al. 2002, 2003). Increased alkaline phosphatase activity associated with biomineralization of condylar cartilage and changes in osteoclastic and osteoblastic activity also have been noted (Bouvier 1988; Kim et al. 2003). Changes in expression of type-I and type-II collagen vary in response to joint loads, which further supports the hypothesis that mechanotransduction may signal changes in gene expression that alter the composition and function of tissues 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; Huang et al. 2003; Wong and Carter 2003).

One can conclude that bony and cartilaginous components of the mammalian feeding apparatus respond developmentally to dynamic changes in masticatory loads with altered proliferation and changes in gene expression (Kim et al., 2003). Based largely on short experimental periods in growing mammals of less than 2 months, these studies support the hypothesis that altered, excessive and/or repetitive forces induce secondary osteonal remodeling of cortical bone and chondroblastic activity of articular cartilage, a suite of physiological responses or functional adaptations that maintain a sufficient safety factor for a cranial element or joint system to routine masticatory loads (Bouvier and Hylander 1981, 1996; Lanyon and Rubin 1985;
Biewener 1993; Vinyard and Ravosa 1998; Ravosa et al. 2000). This work also suggests that a minimum loading level and frequency is necessary for the growth and maintenance of normal adult cranial form and function (Beecher et al. 1983; Bouvier and Hylander 1984). Interestingly, the magnitude of such responses appears to be age-dependent and perhaps is underlain by genetic and epigenetic factors that vary systemically and interspecifically (Bouvier 1988; Bouvier and Hylander 1996; Ravosa et al. 2008; see also Meyer 1987 on fish). Although a biological and theoretical understanding of the performance and integrity of the TMJ and symphysis rests on the ability of individual tissues of such composite organs to adapt to applied stresses, little comparative evidence exists regarding the dynamic cascade of anatomical, biochemical, and biomechanical responses to altered masticatory loads of the bone and cartilage in mammalian jaw joints. Indeed, as tissue degradation is the failure of the adaptive process to adequately respond to altered and/or excessive loading conditions, an integrative perspective is important for relating data on jaw joint plasticity in the laboratory to understanding phenotypic variation in natural settings.

To address this problem, the TMJ and symphysis of mice and rabbits were analyzed for: (1) changes in joint proportions, (2) biomineralization of articular, subarticular and cortical bone, and thickness of cortical bone via microcomputed tomography (microCT), and (3) histology and immunohistochemistry of articular cartilage extracellular matrix (ECM) composition in the TMJ. Organisms subjected to elevated masticatory loads are predicted to develop: relatively larger musculoskeletal elements; greater thickness of symphyseal cortical bone; elevated bone–density levels along the TMJ and symphysis; and increased type-II collagen and proteoglycan expression of TMJ articular cartilage (similar data on ECM cartilage composition of the rabbit symphysis are presented elsewhere—Ravosa et al. 2007b). The benefit of collecting similar data for mice and rabbits is that one can compare and contrast norms of reaction between species so as to characterize patterns of variation in long-term dynamic determinants of joint formation as well as compare tissue responses between traditional and knock-out models of musculoskeletal plasticity. In doing so, we aim to develop a hierarchical framework for comparative analyses of the important links among plasticity, mechanobiology, and performance which, in turn, can be used to investigate the development and evolution of the mammalian skull and masticatory system.

Materials and methods
Experimental models

Our laboratory has employed two animal models to evaluate plasticity of jaw joints vis-a-vis long-term variation in masticatory loads. One sample consisted of Mstn-deficient and wild-type domestic mice (Mus musculus) (Nicholson et al. 2006; Ravosa et al. 2007a). To provide a comparative perspective on jaw joint responses in a knockout (KO) mouse model, a second sample consisted of New Zealand domestic white rabbits (Oryctolagus cuniculus) subjected to variation in dietary material properties (Ravosa et al. 2007b). The loading cohorts did not otherwise differ behaviorally, such that it is unlikely paramasticatory uses of the feeding apparatus affected variation between groups. To control for variation in genetics and thus ensure the response to loading modification was established postnatally, only siblings were chosen.

The mouse sample consisted of genetically similar, domestic Mstn-deficient (Mstn ÷/−) and control (Mstn +/+ ) mice (for additional details regarding this mouse model—McPherron et al. 1997; Ji et al. 1998; McPherron and Lee 2002). In accord with an ACUC-approved protocol, 23 male mice were bred by MWH and kept in the AALAC-accredited MCG Laboratory Animal Facility for 6 months until attaining adulthood. From a skeletal perspective, these mice exhibit peak bone mass and mechanical properties (Ferguson et al. 2003). Mstn-deficient mice (n = 12) bred on the CD-1 background were used to model masticatory over-loading relative to controls (n = 11) (Nicholson et al. 2006; Ravosa et al. 2007a). To control for the effects of dietary properties on masticatory plasticity, mice in both loading cohorts were fed Harlan TekLad rodent chow ad libitum. Mstn is a negative regulator of the growth of skeletal muscle, with KO mice developing masseter and temporalis muscles over 50% larger in mass due to larger cross-sections of the muscle fibers and increased numbers of muscle cells (McPherron et al. 1997; Byron et al. 2004). This elevated muscle mass and physiological cross-section increases contractile forces, greater maximal bite forces (when jaw adductors are stimulated to tetanus) and attachment size of the jaw muscles compared to similar-sized normal mice (Byron et al. 2004, 2006; Nicholson et al. 2006). Though there are no data regarding bite forces during routine biting and chewing, it is assumed that variation in the form and composition of the jaw joint between mouse loading cohorts results from differences in jaw-adductor muscle forces. Of course, altered loads in KO mice may not be due to increased force production by the muscles per se, but rather to...
changes in muscle configuration. While this alternative explanation requires further study, it would have to characterize all skeletal muscles given the similarity of findings on elevated bone mineral density of postcranial (Hamrick 2003; Hamrick et al. 2003) and cranial elements in KO mice (below). Lastly, while it is possible that Mstn deficiency directly affects skeletal development, higher bone density in such organisms appears due solely to increased relative muscle forces as the Mstn receptor, the type-IIIB activin receptor, is not expressed at significant levels in skeletal tissues (Shuto et al. 1997).

The comparative sample consisted of 20 rabbits obtained as weanlings (4-weeks-old) and housed in the AALAC-accredited NU Center for Comparative Medicine for 15 weeks until attaining subadult status at 19 weeks of age. In accord with an ACUC-approved protocol, two dietary cohorts of 10 rabbits each were established by MJR to induce postweaning variation in jaw-adductor forces and masticatory loads (Ravosa et al. 2007b). Weaning was chosen as the starting point for dietary manipulation because plasticity decreases with age (Bouvier 1988) and because we sought to minimize the confounding influence of postweaning diets other than those used in our protocol. Weanlings were fed ad libitum either a “soft” diet of ground pellets to model under-use (U) of the chewing complex or a “tough/hard” diet of Harlan TekLad rabbit pellets supplemented daily with two 2 cm hay blocks to model over-use (O). Resistant food (pellets) and tougher foods with higher elastic moduli (hay) require absolutely larger jaw-adductor forces and increased preparation time during biting and chewing, and both factors affect jaw-loading patterns and ultimately joint form. Behavioral analyses indicate U-diet rabbits did not show failure to thrive nor did they develop incisor malocclusions (Ravosa et al. 2007b).

Morphometry of masticatory elements

After euthanasia, mouse and rabbit skulls were detached at the vertebral column and the 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) and measured with digital calipers (rabbits) or an optical scope (mice) to obtain dimensions of the mandible (length/breadth), symphysis (length/width), corpus (height/width), and condyle (width/length) (Nicholson et al. 2006; Ravosa et al. 2007b). Metric data were used to control for size-related variation in the skull and masticatory apparatus in comparisons between loading cohorts (Bouvier and Hylander 1981, 1982, 1984). TMJ samples then were used for microCT analyses of bone mineral density followed by histology and immunohistochemistry of cartilage ECM composition. Symphyses were also examined via microCT, however the absence of intact symphseal joints in mice precluded micro-anatomical analyses and comparisons of cartilage composition.

MicroCT analysis of skeletal biomineralization

MicroCT was performed on fixed jaw joints to assess variation in the density or biomineralization of the articular surface, subarticular bone and cortical bone as a function of masticatory stress (Nicholson et al. 2006; Ravosa et al. 2007a, 2007b, 2008). Using a Scanco Medical MicroCT 40 (PA, USA), the micro-focus X-ray tube was operated at 70 kV and 57 μA with an effective energy of 30 keV, 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/view) and the highest sensitivity mode (1000 projections over 180°, 2048 samples per projection). Reconstruction was with 8 μm voxels (volume elements). The linear attenuation coefficient (μ) was measured in reconstructed slices parallel to the coronal plane: five equidistant sites per rabbit symphysis (labial, anterior, middle, posterior, lingual—Fig. 1), two equidistant sites per mouse symphysis (anterior, posterior), and three equidistant sites per rabbit and mouse TMJ (anterior, middle, posterior—Fig. 2). At each joint site, 40 contiguous slices covering 0.31 mm were imaged, with one slice chosen to represent a given site. Values of “μ” were pooled for each specimen and used to characterize between-group variation in bone mineral density along the TMJ and symphysis (Nicholson et al. 2006; Ravosa et al. 2007a, 2007b). For symphseal sections, height and width of the joint as well as thickness of the articular surface and cortical bone were collected. In mouse TMJ sections, articular cartilage thickness was obtained. To facilitate comparisons of the evidence for mice and rabbits, microCT data are summarized across joint sites.

Histology and immunohistochemistry of cartilage composition

Histology and immunohistochemistry of TMJ con-dyalar cartilage followed standard methods (Ravosa et al. 2007b). Once analyzed via microCT, each fixed
joint was decalcified, dehydrated, washed and then embedded in paraffin. At three equidistant sites per TMJ (anterior, middle, posterior), 4–6 μm sections were obtained with a Reichert–Jung autocut microtome in the coronal plane, i.e., orthogonal to the jaw’s long axis. As sulfated GAGs are expressed in chondrocytes regularly exposed to loads, the cationic dye safranin O was used to assess relative GAG content in TMJ cartilage (Copray et al. 1985; Carvalho et al. 1995; Huang et al. 2002). Strong safranin-O staining is indicative of proteoglycans containing chondroitin sulfate and keratan sulfate, which in turn increases the viscoelasticity of cartilage vis-à-vis compressive stresses. Type-II collagen has a distinct fibrillar organization and associates strongly with proteoglycans and water, important for tissues subjected to compression, tension and shear, such as the TMJ articular cartilage (Mizoguchi et al. 1996; Pirttiniemi et al. 1996; Tanaka et al. 2000). Thus, primary antibodies directed at variation in cartilage type-II collagen were used to evaluate the relative expression pattern of collagen and proteoglycan (i.e., change in staining localization) as a function of masticatory load.

**Statistical analysis and predictions**

Based on earlier work, it was expected that subtle differences among treatment cohorts would bias the results toward supporting the predictions (due spuriously to variation in skull size between loading cohorts—Bouvier and Hylander 1984). To ensure the primary signal is one regarding masticatory plasticity, the first step in the morphometric analysis was to calculate the ratio of a given dimension versus mandibular length (Bouvier and Hylander 1981, 1982, 1984; Bouvier 1986; Ravosa and Hylander 1994; Ravosa and Hogue 2004). The comparison of masticatory parameters between loading groups was investigated using non-parametric ANOVA (Mann–Whitney U-test, *P*<0.05) (Nicholson et al. 2006; Ravosa et al. 2007a, 2007b, 2008).

**Results**

**Proportions of masticatory elements**

In mice, ANOVAs indicate that the majority of size-adjusted measures of the TMJ condyle, corpus, symphysis, and jaw adductors are significantly larger in 12 *Mstn*-deficient versus 11 normal mice (Table 1) (Nicholson et al. 2006). ANOVAs also indicate the presence of significantly thicker cortical bone along the symphyseal articular surface in *Mstn*-deficient mice, whereas the thickness of the superior cortical bone is greater, but not significantly so, in this loading cohort. In rabbits, ANOVAs indicate that all but one size-adjusted measure of the TMJ condyle, corpus, symphysis, and jaw adductors is significantly larger in 10 O-diet versus 10 U-diet rabbits (Table 1) (Ravosa et al. 2007b). ANOVAs further highlight the presence of significantly thicker cortical bone along the external and articular surfaces of the symphysis in O-diet rabbits. These findings largely support predictions that jaw joints routinely
subjected to elevated loads and/or greater cyclical loading will develop relatively larger proportions.

**Bone mineral density and internal anatomy of the TMJ and symphysis**

Using linear attenuation coefficients summed over the slices for both joints, ANOVAs demonstrate significant differences between mouse loading cohorts in two TMJ regions—articular surface and condylar neck cortical bone—and both regions of the symphysis—articular surface and external cortical bone (Table 2). Thus, except in the TMJ subchondral region, Mstn-deficient mice exhibit overall greater levels of joint biomimelarization than do wild-type mice (Nicholson et al. 2006; Ravosa et al. 2007a). Interestingly, microCT analyses highlight the degradation of TMJ subchondral bone among KO mice and corresponding increases in articular cartilage height (Fig. 3).

Comparisons of dietary loading cohorts in rabbits indicate similar patterns of variation in tissue mineral density of the articular surface, subarticular bone, and cortical bone along the symphysis and TMJ condyle, with O-diet rabbits exhibiting significantly greater levels of biomimelarization than U-diet rabbits (Table 2) (Ravosa et al. 2007b). These findings underscore a significant effect of dietary properties on adaptive plasticity in masticatory proportions, tissue structure, and levels of bone mineral density.

**ECM composition of TMJ articular cartilage**

Histological analyses of 6-month-old adult KO (Mstn −/−) and CD-1 control (Mstn +/+ ) mice indicate more intense safranin-O staining in the condylar articular cartilage of KO mice (compare “A” with “B” in upper panel pair of Fig. 4). Higher proteoglycan content in the TMJ cartilage of KO mice indicates the presence of adaptive changes in the ECM unlike articular cartilage of older mammalian joints, which experience a lower and more restricted distribution of proteoglycans (Mankin et al. 1971; Newton and Nunamaker 1985; Haskin et al. 1995; Ostergaard et al. 1999; Ravosa et al. 2008).

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**Table 1** Comparison of size-adjusted measures of load-resisting and force-generating structures between loading cohorts

<table>
<thead>
<tr>
<th>Mouse variables</th>
<th>Mstn −/− Mean (SD)</th>
<th>Mstn +/+ Mean (SD)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMJ condyle ML length</td>
<td>0.067 (0.006)</td>
<td>0.061 (0.005)</td>
<td>9.8*</td>
</tr>
<tr>
<td>TMJ condyle AP width</td>
<td>0.134 (0.015)</td>
<td>0.136 (0.012)</td>
<td>−1.5</td>
</tr>
<tr>
<td>Corpus height</td>
<td>0.301 (0.026)</td>
<td>0.292 (0.013)</td>
<td>3.1</td>
</tr>
<tr>
<td>Corpus width</td>
<td>0.122 (0.006)</td>
<td>0.116 (0.008)</td>
<td>5.2*</td>
</tr>
<tr>
<td>Symphysis length</td>
<td>0.339 (0.051)</td>
<td>0.330 (0.048)</td>
<td>2.8</td>
</tr>
<tr>
<td>Symphysis width</td>
<td>0.239 (0.025)</td>
<td>0.232 (0.026)</td>
<td>3.0</td>
</tr>
<tr>
<td>Symphysis articular breadth</td>
<td>0.152 (0.040)</td>
<td>0.125 (0.031)</td>
<td>21.6*</td>
</tr>
<tr>
<td>Symphysis superior cortical width</td>
<td>0.160 (0.086)</td>
<td>0.140 (0.039)</td>
<td>14.3</td>
</tr>
<tr>
<td>Temporalis insertion</td>
<td>0.324 (0.006)</td>
<td>0.296 (0.013)</td>
<td>9.5*</td>
</tr>
<tr>
<td>Masseter insertion</td>
<td>0.327 (0.024)</td>
<td>0.290 (0.022)</td>
<td>12.8*</td>
</tr>
<tr>
<td>Masseter muscle mass</td>
<td>0.016 (0.002)</td>
<td>0.009 (0.001)</td>
<td>77.8*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rabbit variables</th>
<th>O-diet Mean (SD)</th>
<th>U-diet Mean (SD)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMJ condyle AP length</td>
<td>0.178 (0.016)</td>
<td>0.150 (0.014)</td>
<td>18.7*</td>
</tr>
<tr>
<td>TMJ condyle ML length</td>
<td>0.074 (0.010)</td>
<td>0.069 (0.005)</td>
<td>7.3*</td>
</tr>
<tr>
<td>Corpus height</td>
<td>0.242 (0.023)</td>
<td>0.237 (0.020)</td>
<td>2.1</td>
</tr>
<tr>
<td>Corpus width</td>
<td>0.096 (0.009)</td>
<td>0.090 (0.008)</td>
<td>6.7*</td>
</tr>
<tr>
<td>Symphysis length</td>
<td>0.396 (0.034)</td>
<td>0.351 (0.046)</td>
<td>12.8*</td>
</tr>
<tr>
<td>Symphysis width</td>
<td>0.148 (0.016)</td>
<td>0.129 (0.014)</td>
<td>14.7*</td>
</tr>
<tr>
<td>Symphysis articular breadth</td>
<td>0.173 (0.027)</td>
<td>0.140 (0.031)</td>
<td>23.6*</td>
</tr>
<tr>
<td>Symphysis superior cortical width</td>
<td>0.176 (0.045)</td>
<td>0.134 (0.042)</td>
<td>31.3*</td>
</tr>
<tr>
<td>Symphysis lateral cortical width</td>
<td>0.089 (0.009)</td>
<td>0.058 (0.008)</td>
<td>53.4**</td>
</tr>
<tr>
<td>Masseter muscle mass</td>
<td>0.135 (0.016)</td>
<td>0.112 (0.023)</td>
<td>20.5*</td>
</tr>
</tbody>
</table>

In most comparisons, Mstn-deficient mice and rabbits on an O-diet develop relatively larger musculoskeletal proportions and thicker cortical bone along the articular and external surfaces of the symphysis (Mann–Whitney U-test, *P* < 0.01, ′′′*P* < 0.05) (Nicholson et al. 2006; Ravosa et al. 2007b).
Immunohistochemical data for KO versus control young adults indicate a more widespread distribution of type-II collagen in TMJ articular cartilage of KO mice (compare “C” with “D” in lower panel pair of Fig. 4). Note also that proteoglycan content and expression of type-II collagen are most pronounced in the two innermost layers of articular cartilage, which are the chondroblastic and hypertrophic/calcified chondrocyte zones. Due to the elevated viscoelasticity of tissues rich in proteoglycans and type-II collagen subjected to moderately higher masticatory stresses (i.e., KO mice), our analyses suggest that the TMJ articular cartilage of young adults initially develops increases in the ability to resist greater compressive stresses than that of normally loaded adult joints.

Histology of subadult rabbits on U-diets and O-diets indicate more intense safranin-O staining in the condylar articular cartilage (compare “A” with “B” in upper panel pair of Fig. 5) of rabbits on a U-diet (Ravosa et al. 2007b). Lower proteoglycan content in the TMJ condylar cartilage’s lower two layers of rabbits on O-diets mirrors findings for the articular surface of mammalian limb elements, which experience age-related decreases in proteoglycan content. Immunohistochemistry of subadults on U-diets versus O-diets indicate a more widespread distribution of type-II collagen in the condylar articular cartilage of rabbits on U-diets (compare “C” with “D” in lower panel pair of Fig. 5). Due to the greater viscoelasticity of joint tissues rich in proteoglycans and type-II collagen subjected to cumulatively low postnatal stresses (i.e., U-diet), our analyses suggest that the TMJ articular cartilage of such organisms is able to resist greater compressive stresses than that of repetitively over-loaded adult joints. Moreover, as proteoglycan content and type-II collagen is pronounced in the two innermost layers of TMJ articular cartilage—chondroblastic and hypertrophic/calcified chondrocyte—this suggests it is critical to account for regional variation and covariation in this and other ECM components in evaluating the biomechanical significance of variation in the properties, proportions, and plasticity of cartilage (Ravosa et al. 2007b).

Table 2  Comparison of linear attenuation coefficients of the jaw joints between loading cohorts

<table>
<thead>
<tr>
<th>Mouse variables</th>
<th>Mstn −/− Mean (SD)</th>
<th>Mstn +/+ Mean (SD)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMJ surface</td>
<td>2.929 (0.179)</td>
<td>2.806 (0.144)</td>
<td>4.4**</td>
</tr>
<tr>
<td>TMJ subchondral</td>
<td>3.259 (0.160)</td>
<td>3.289 (0.162)</td>
<td>≈0.0</td>
</tr>
<tr>
<td>TMJ neck</td>
<td>3.256 (0.161)</td>
<td>3.113 (0.139)</td>
<td>4.6***</td>
</tr>
<tr>
<td>Symphysis surface</td>
<td>3.264 (0.178)</td>
<td>3.183 (0.141)</td>
<td>2.6*</td>
</tr>
<tr>
<td>Symphysis cortex</td>
<td>3.652 (0.324)</td>
<td>3.452 (0.330)</td>
<td>5.8*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rabbit variables</th>
<th>O-diet Mean (SD)</th>
<th>U-diet Mean (SD)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMJ surface</td>
<td>1.542 (0.147)</td>
<td>1.329 (0.067)</td>
<td>16.0**</td>
</tr>
<tr>
<td>TMJ subchondral</td>
<td>2.162 (0.096)</td>
<td>1.865 (0.095)</td>
<td>15.9**</td>
</tr>
<tr>
<td>TMJ neck</td>
<td>2.004 (0.127)</td>
<td>1.725 (0.075)</td>
<td>16.2**</td>
</tr>
<tr>
<td>Symphysis surface</td>
<td>2.024 (0.081)</td>
<td>1.668 (0.090)</td>
<td>21.3**</td>
</tr>
<tr>
<td>Symphysis cortex</td>
<td>2.624 (0.148)</td>
<td>2.326 (0.136)</td>
<td>12.8**</td>
</tr>
</tbody>
</table>

In all but one case, Mstn-deficient mice and rabbits on an O-diet exhibit significantly higher levels of bone mineral density (Mann–Whitney U-test, **P < 0.001, *P < 0.01, *P < 0.05) (Nicholson et al. 2006; Ravosa et al. 2007a, 2007b).
Discussion

Jaw joints of the mammalian masticatory apparatus are highly specialized, being capable of both rotational and translational movements and thus encounter multidirectional compressive, shear, and tensile forces during biting and chewing (Hylander 1992; Ravosa and Hogue 2004). In addition to articular, subarticular, cortical, and trabecular bone, TMJs and symphyses are comprised of cartilage, ligaments, and dense fibrous tissue containing collagens and proteoglycans. As articular cartilage in both jaw joints is anchored in subarticular bone, the distribution of stresses is constrained, respectively, by movements between dentaries (symphysis) or between the mandible and temporal bone (TMJ). Employing animal models for which in vivo data on feeding behavior are already available (Wejjs and de Jongh 1977; Wejjs et al. 1987, 1989; Langenbach et al. 1991, 1992, 2001; Kobayashi et al. 2002), we performed a series of integrative experiments to probe the longer-term, more naturalistic dynamic hierarchical relations among mechanical loading, adaptive plasticity, norms of reaction, and performance in mammalian jaw joints (Nicholson et al. 2006; Ravosa et al. 2007a, 2007b).

Fig. 4 Mouse TMJ articular cartilage composition. Coronal sections of middle joint sites for KO (A, C) and control (B, D) adults stained with: safranin O to identify GAG content (upper panel pair) and a primary antibody directed against type-II collagen (lower panel pair). In views of the TMJ articular surface and subchondral bone, more intense staining in “A, C” versus “B, D” indicates higher proteoglycan content, more type-II collagen and thus greater viscoelasticity of articular cartilage in KO mice.
Revisiting our analyses of a suite of similar craniomandibular parameters in rabbits and mice represents the first case in which long-term adaptive plasticity in experimental organisms can be more readily compared between species. In the symphysis subjected to higher and/or more frequent loads, mice and rabbits develop relatively larger joint dimensions and elevated bone mineral density. In the over-loaded TMJ, mice and rabbits develop larger joint proportions and higher levels of biomineralization. However, these species differ in terms of the response of TMJ articular cartilage to long-term over-loading. Expression of proteoglycan and type-II collagen is increased in Mstn-deficient versus control mice, whereas over-use diet rabbits develop lower levels of proteoglycan and type-II collagen. Thus, the mouse comparisons indicate that TMJ articular cartilage exhibits a stereotypical adaptive response of elevated viscoelasticity that counters joint over-loading, much as is the case for proportions and levels of biomineralization in the TMJ of both taxa (and similar to short-term loading modification—see above). One way to reconcile the seemingly disparate interspecific differences regarding the effects of increased loading on TMJ articular cartilage is via recourse to the plasticity data on joint proportions and biomineralization. In contrast to the results for mice, the magnitude of the difference between rabbit loading cohorts is nearly always greater, particularly with regard to bone mineral density. This suggests that, while Mstn-deficient mice likely generate larger jaw-adductor, bite, and reaction forces than do

Fig. 5 Rabbit TMJ articular cartilage composition. Coronal sections of middle joint sites from U-diet (A, C) and O-diet (B, D) subadults stained with: safranin O to identify GAG content (upper panel pair) and a primary antibody directed against type-II collagen (lower panel pair). In views of the articular surface and underlying subchondral bone, more intense staining in “A, C” versus “B, D” indicates lower proteoglycan content, less type-II collagen and thus diminished viscoelasticity of articular cartilage in rabbits on the O-diet (Ravosa et al. 2007b).
normal mice during biting and chewing (despite being raised on the same diet), dietary manipulation in rabbits apparently drives a larger wedge between cohorts in terms of the magnitude and/or frequency of joint loads, with corresponding increases in reaction norms of rabbit bony tissues. Thus, much as observed in shorter-term studies of soft-tissue responses to altered loads, we interpret changes in the ECM composition of articular cartilage in the mice as a compensatory adaptive response to moderate over-loading of the TMJ. In contrast, cartilage patterns in rabbits are interpreted to be the result of degradative changes due to long-term significant joint over-loading (Ravosa et al. 2007b). As emphasized above, the duration of loading manipulation in our projects typically exceeded that of earlier investigations and it is well known that cartilage exhibits accelerated degradation in response to significant elevated and/or repetitive loading (Guerne et al. 1995; Bae et al. 1998). It is likely that such changes in rabbit TMJ cartilage composition reflect the early onset and progression of degenerative diseases such as osteoarthritis (OA) that compromise the structural integrity of synovial joints (Mankin et al. 1971; Newton and Nunamaker 1985; Haskin et al. 1995; Ostergaard et al. 1999; Fujimura et al. 2005).

As joints are organs comprised of soft and hard tissues that respond individually and interactively, arguably a component of the adaptive changes in rabbit TMJ proportions, and biomineralization represents a compensatory response to cartilage degradation that maintains the overall functional integrity of such composite joint systems. It thus remains to be determined how much of the bony reaction norm between dietary loading cohorts is a direct adaptive response to joint over-loading versus an adaptive response to decreases in cartilage viscoelasticity that is only indirectly related to the external loading stimulus. In the mouse model of joint over-loading, it is similarly possible that a smaller component of the adaptive response of mouse TMJ articular cartilage, both the altered ECM composition and especially the increased thickness of cartilage along the buccal articular surface, is associated with less subchondral bone along the buccal aspect of the TMJ. Additional ontogenetic data would be necessary to ascertain if the postnatal onset of joint over-loading in the Mstn-deficient mice (versus that for rabbits) might differentially inhibit subchondral bone development/biomineralization or if we are sampling an early stage of OA where the subchondral bone is experiencing degenerative resorption (Radin et al. 1984; Farkas et al. 1987).

As alluded to above, our study uniquely suggests that the shorter-term duration of prior analyses of joint tissues may offer a limited notion of the complex process of developmental plasticity, especially as it relates to the influence of long-term alterations in masticatory loads, when a cranial joint is increasingly characterized by adaptive and degradative changes in tissue structure, composition, and function. Perhaps not surprisingly, this indicates that assessments of masticatory plasticity based solely on external joint proportions under-represent the amount of change in individual joint tissues. For instance, the magnitude of the plasticity response differs between loading cohorts according to the level of analysis, with external joint proportions varying less between loading cohorts than in comparisons of skeletal biomineralization or internal proportions.

The findings regarding joint plasticity allow us to speculate regarding the broader implications for several realms of organismal biology: ecomorphology of the mammalian feeding apparatus, relative growth and variation in life history, limits of paleobiological inference, and quantitative genetics. By focusing on long-term responses of jaw joints which more closely model conditions in a natural setting, our studies contribute to an understanding of the ecology and evolution of vertebrate feeding by importantly documenting likely morphological correlates of intraspecific variation in dietary proclivities, data relatively rare for mammals (Wainwright and Reilly 1994). If an organism develops relatively larger masticatory structures due to diet-induced plasticity, and there is no variation in the duration of ontogeny between sister taxa with different diets, coefficients of relative growth for such elements will be higher in organisms that focus on tougher and/or harder diets (Bernays 1986). This suggests that organisms specializing on harder/tougher forage will develop morphological configurations that allow them to exploit a broader range of foods than do similar-aged sister taxa focusing on softer diets (Robinson and Wilson 1998; Wright 2004). From a life-history and ecomorphological standpoint, an accelerated growth strategy would be advantageous during seasonal fluctuations and/or skews in the distribution of the material properties of available food items. This would be particularly important for increasing the fitness of juveniles, a growth stage often subject to higher mortality rates (Stearns and Koella 1986; Ravosa et al. 1993). As the mammalian mandibular symphysis experiences relatively higher levels of bone strain during biting and chewing, and is often
characterized by strong positive allometry of joint proportions (Hylander 1985; Ravosa 1991a, 1991b; Ravosa and Hylander 1994; Vinyard and Ravosa 1998; Ravosa et al. 2000; Ravosa and Hogue 2004), this joint has the potential for significant plasticity relative to other masticatory elements. In this regard, our investigation contributes to a larger body of research on the inter-relations among ecomorphology, growth, fitness, and feeding performance in mammals, insects, fish, and birds (Bernays 1986; Meyer 1987; Grant and Grant 1989; Wainwright and Reilly 1994; Robinson and Wilson 1998; Miller and German 1999; Wright 2004).

As noted previously, the novel, long-term modification of loading parameters employed herein is a strategy that more closely approximates naturalistic conditions. Therefore, our project offers the requisite experimental data argued by Lauder (1995) to be fundamental for the use of anatomical structure to infer behavior and performance in the fossil record and in the field. In particular, as reaction norms vary according to the level of analysis in the mammalian jaw joint, characterization of masticatory plasticity based solely on external dimensions of a joint is not proportional to the amount of change in specific tissues. For instance, gross dimensions differ less between loading cohorts than do comparisons of levels of bone density or internal measurements, while soft tissues experience degradative responses (that cannot be examined in fossils). Thus, one cannot model jaw joints in living or extinct taxa as optimally designed based solely on external morphology. Neontological and paleobiological inferences regarding feeding behavior will be further confounded by the annual frequency of resource use, specifically the relative amount of tough versus hard food items. For example, taxa that exploit tough/hard objects on a more seasonal basis are less likely to experience degradative responses of certain joint tissues, with other components largely experiencing adaptive responses to the external loads. Conversely, more prolonged annual exploitation of tough/hard foods will result in responses whereby the magnitude of both the adaptive and degradative responses of joint tissues will be elevated, which in turn will differentially impact patterns of variation in masticatory form.

Data on dietary plasticity are likewise important for analyses of the quantitative genetics of evolution. As plasticity can affect phenotypic covariance structure, this can alter the way in which hereditary variation and selection interact to produce evolutionary change (Gupta and Lewontin 1982; Cheverud et al. 1983; Stearns 1989). In considering the role of masticatory plasticity and variation in mammalian skull form, since only lower facial structures are differentially affected by masticatory stresses (Ravosa et al. 2000, 2007b; Menegaz et al. 2008), this suggests that patterns of phenotypic covariation will differ between lower and upper facial skulls for sister taxa that differ greatly in dietary properties or in loading levels/loading frequency. Of course, cranial plasticity also will vary as a function of the degree of positive scaling of growth trajectories (e.g., symphysis—Vinyard and Ravosa 1998; circumorbit—Ravosa 1991a) as well as diet-induced (Bernays 1986; Meyer 1987) and/or nutritional (Miller and German 1999) influences on patterns of relative growth.

While an increasingly detailed understanding of the mouse genome emboldens us to further utilize mouse models for addressing important questions in evolutionary and translational research (Waterston et al. 2002), one also should be cognizant of the pitfalls of phenotypic inference based on the potentially simplistic developmental effects of knock-in or knock-out models (Smith and Schneider 1998). Aside from the detailed evidence regarding the specific effect of the Mstn KO on only myotome derivatives (McPherron et al. 1997; Ji et al. 1998), our comparative study indicates similar responses of the bony tissues of jaw joints in both mice and rabbits vis-à-vis altered loading conditions. In considering patterns of variation in biomineralization of the jaw joints in both species, it is interesting that the TMJ surface exhibits the lowest levels of tissue mineral density whereas the highest levels are observed along cortical bone of the symphysis.

**Summary and conclusions**

Adaptive plasticity has attracted considerable attention from diverse fields of biology (Gotthard and Nylin 1995; Agrawal 2001; Holden and Vogel 2002; West-Eberhard 2003). Here, the collection of similar data on the plasticity of the symphys and TMJ in mice and rabbits facilitates important comparisons of plasticity in joint tissues subjected to long-term alterations in loading. To date, the lack of hierarchical perspective regarding joints as composite structures that vary postnatally has been equally problematic for assessing ontogenetic patterns of adaptive and degradative variation in joint tissues. Arguably, this issue has plagued organismal and translational research on tissue interactions in skeletal joints. By choosing similar joint locations for microCT and microanatomy, our experimental analyses facilitated a characterization of the integrated suite of dynamic responses (both adaptive
and degradative) of skeletal and connective tissues to altered loads. Viewed from an evolutionary perspective, this study suggests that variation in the morphology and performance of the symphysis and TMJ among sister taxa is, in part, an epiphenomenon of interspecific differences in 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 certain individuals by the potentially species-specific interaction between adaptive and degradative responses of joint tissues (Ravosa et al. 2007b, 2008). Thus, a consideration of plasticity in the skull, and especially joints, should employ a multifaceted characterization of the functional network, one that incorporates data on tissue components so as to evaluate the role of altered load versus the differential responses of tissues on functional adaptation of such composite structures. Indeed, plasticity is likely to be a function of the properties of food items as well as the annual duration and/or frequency of the loading stimulus. Considered more broadly, our data on long-term responses of mammalian jaw joints have potential implications for understanding myriad aspects of the biology and evolution of organisms in natural settings. To the extent possible, this integrative perspective should guide our use of animal models, whether one employs genetically modified organisms or more traditional experimental protocols.

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