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The masticatory system under varying functional load. Part 1: structural adaptation of rabbit jaw muscles to reduced masticatory load

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SUMMARY Skeletal muscle fibres can change their myosin heavy-chain (MyHC) isoform and cross-sectional area, which determine their contraction velocity and maximum force generation, respectively, to adapt to varying functional loads. In general, reduced muscle activity induces transition towards faster fibres and a decrease in fibre cross-sectional area.

In order to investigate the effect of a reduction in masticatory load on three functionally different jaw muscles, the MyHC composition and the corresponding cross-sectional area of fibres were determined in the superficial masseter, superficial temporalis, and digastric muscles of male juvenile New Zealand White rabbits that had been raised on a soft diet ($n = 8$) from 8 to 20 weeks of age and in those of normal diet controls ($n = 8$). Differences between groups were tested for statistical significance using a Mann–Whitney rank sum test.

The proportion and cross-sectional area of fibres co-expressing MyHC-I and MyHC-cardiac alpha were significantly smaller in the masseter muscles of the animals that had been fed soft food than in those of the controls. In contrast, the proportions and cross-sectional areas of the various fibre types in the temporalis and digastric muscles did not differ significantly between the groups.

The results suggest that reducing the masticatory load during development affects the contraction velocity and maximum force generation of the jaw-closing muscles that are primarily responsible for force generation during chewing. These muscles adapt structurally to the reduced functional load with changes in the MyHC composition and cross-sectional area mainly within their slow fibre compartment.

Introduction

Skeletal muscles contain a mixture of fibres with different contractile properties, such as contraction velocity and maximum force generation. Muscle fibres have been classified on the basis of the myosin heavy-chain (MyHC) isoforms they contain (Schiaffino and Reggiani, 1994). The MyHC isoform composition of muscle fibres determines their unloaded shortening velocity (Larsson and Moss, 1993), which increases in the sequence of fibres expressing MyHC-I, MyHC-cardiac alpha, MyHC-IIA, MyHC-IIX, and MyHC-IIB (Sciote and Kentish, 1996). The maximum force a muscle fibre can generate is proportional to its cross-sectional area. This area increases with the amount of resistance that is experienced during contraction (McCall *et al.*, 1996).

The dynamic nature of muscle fibres enables them to adapt to altered functional requirements by changing their MyHC isoform and cross-sectional area (Pette, 2002). Although these changes may occur under the influence of various factors and conditions (Grünheid *et al.*, 2009), muscular activity plays an essential role in modulating the phenotypic properties of muscle fibres (Roy *et al.*, 1991). Increased muscular activity elicits transition towards slower more fatigue-resistant fibre types and enlargement of fibre cross-sectional area (Jarvis *et al.*, 1996), whereas reduced muscular activity induces transition towards faster more fatigable fibre types and a decrease in fibre cross-sectional area (Grossman *et al.*, 1998).

The activity of jaw muscles can be altered experimentally by changing the consistency of the available food. A continuous intake of a soft diet, which requires less masticatory effort, has been shown to reduce the functional capacity of jaw muscles (Kiliaridis and Shyu, 1988; Liu *et al.*, 1998). Although this experimental approach has been widely used, information on the associated changes in MyHC composition and fibre cross-sectional area is scarce and, at least in part, appears contradictory. For instance, in studies on rats, which had been raised on diets of different hardness, Kiliaridis *et al.* (1988) found a significant influence of dietary consistency on the MyHC composition

and cross-sectional area of the fibres in the masseter but not on those in the digastric muscle, while Sfondrini *et al.* (1996) did not find any changes in the phenotypic properties of the fibres in the masseter but in those of the temporalis and digastric muscles after feeding the animals a diet of reduced consistency. Similar investigations on other species, such as the mouse (Maeda *et al.*, 1987) and the rabbit (Negoro *et al.*, 2001; Langenbach *et al.*, 2003; Kitagawa *et al.*, 2004), were limited to the masseter muscle.

The purpose of the present study was to investigate the effect of a reduction in the dietary consistency on MyHC composition and fibre cross-sectional area of the rabbit masseter, temporalis, and digastric muscles. As these jaw muscles are functionally distinct, it was hypothesized that they would be affected differently by a change in masticatory functional load.

Materials and methods

The experiment had been approved by the local Animal Ethics Committee and was performed in accordance with the animal care and welfare guidelines of the National Institute of Health.

Animals

Sixteen male juvenile New Zealand White rabbits (*Oryctolagus cuniculus*; Harlan, Horst, The Netherlands) were used. When obtained, they were 6 weeks old and weighed 1402 ± 189 g. The animals were housed individually in metal cages with perforated plastic floors, kept in a climate controlled room (22.0 ± 0.9°C) with a 12 hour light–dark cycle and fed a commercially manufactured pelleted diet (Arie Blok, Woerden, the Netherlands) and water *ad libitum*. All animals were kept under identical conditions before they were randomly divided into two equal-sized groups at the age of 8 weeks.

Experimental design

The experimental group was fed a diet of soft pellets requiring significantly reduced peak loadings (10 N/cm^2), and thus the level of jaw muscle contractions, to break the pellet in comparison with the standard pellets (120 N/cm^2) fed to the control group. The pellets did not differ in size or nutritional value. No environmental enrichment was provided in order to prevent the animals from gnawing. Body weight and physical condition were checked weekly to monitor the growth and health of the animals. At 20 weeks of age, the animals were sedated with 0.6 ml/kg body weight of a 1:3 mixture of xylazine (Sedazine; AST Farma, Oudewater, The Netherlands) and ketamine (Ketamine; Alfasan, Woerden, The Netherlands) and killed by an intravenous overdose of sodium pentobarbital (Euthesate; Ceva Sante Animale, Naaldwijk, The Netherlands).

Analysis of muscle fibres

The fibres of the superficial masseter, superficial temporalis, and digastric muscles were analysed according to the procedure described in detail elsewhere (Korfage *et al.*, 2006a). The muscles were cut from their attachment sites with the jaws of the animals closed to ensure that the muscles were not stretched. All muscles were obtained within 8 hours *post mortem*, rapidly frozen in liquid nitrogen-cooled isopentane, and stored at −80°C for further processing as below.

Serial sections (10 μm) were cut perpendicular to the main fibre direction of the muscles in a cryomicrotome (CM 1850; Leica Microsystems, Nussloch, Germany), mounted on glass slides coated with 3-aminopropyltriethoxysilane, and fixed overnight in a mixture of methanol, acetone, acetic acid, and water (35:35:5:25) at −20°C. The sections were incubated with five monoclonal antibodies raised against different MyHC isoforms: 219-1D1 detected MyHC-I, 249-5A4 detected MyHC-cardiac alpha, 333-7H1 detected MyHC-IIA, 332-3D4 detected MyHC-IIA and MyHC-IIX, and 340-3B5 detected MyHC-IIA, MyHC-IIX, and MyHC-IIB. The specificity of these antibodies has been confirmed previously (Sant'ana Pereira *et al.*, 1995). The binding of the antibodies was visualized using the indirect unconjugated immunoperoxidase technique with nickel-diaminobenzidine as a substrate (Hancock, 1986).

The MyHC composition of muscle fibres was determined on five consecutive sections of each muscle per animal. The sections were evaluated at ×100 magnification using a light microscope equipped with a digital camera (Orthoplan; Leitz, Wetzlar, Germany). Depending on the cross-sectional area of the muscle, either two (temporalis and digastric) or six (masseter) areas of each section were evaluated. All fibres that could clearly be identified in each of the five sections were classified according to their reaction to the various antibodies (Figure 1). A total of 19 139 fibres were analysed.

On the basis of their MyHC composition, and thus contraction velocity, the fibres were divided into three groups: fibres containing MyHC-I, MyHC-cardiac alpha, or a combination of these MyHCs were considered slow fibres (Galler *et al.*, 2002); fibres containing MyHC-IIA, MyHC-IIX, MyHC-IIB, or a combination of these MyHCs were considered fast fibres; and fibres containing a combination of the MyHC types belonging to slow and fast groups were considered intermediate fibres (Pette and Staron, 2001).

The cross-sectional areas of the muscle fibres, which had been classified on the basis of the MyHCs they expressed, were quantified using a custom-made computer program that converted the number of pixels into square micrometres (Korfage *et al.*, 2006a).

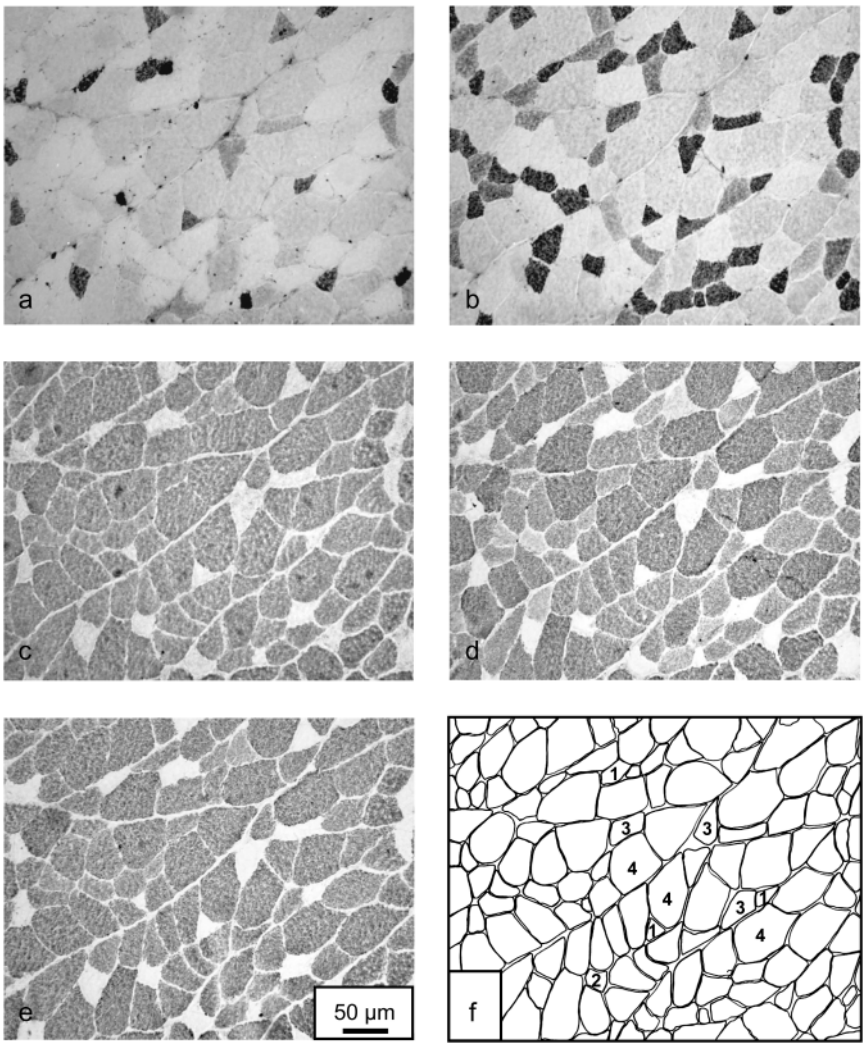

Figure 1 Example of an area of the superficial masseter muscle incubated with antibodies against (a) myosin heavy chain (MyHC)-I, (b) MyHC-cardiac alpha, (c) MyHC-IIA, (d) MyHC-IIA and MyHC-IIX, and (e) MyHC-IIA, MyHC-IIX, and MyHC-IIB isoforms. The drawing (f) shows some of the fibre types: (1) MyHC type I + cardiac alpha, (2) MyHC type cardiac alpha, (3) MyHC type cardiac alpha + IIA, and (4) MyHC type IIA.

Statistical analysis

Mean values and standard deviations (SDs) of fibre-type proportions and fibre cross-sectional areas of the masseter, temporalis, and digastric muscles were calculated for each group of animals. Differences between the experimental and control group were tested for statistical significance, for each muscle separately, using a Mann–Whitney rank sum test after the data had been tested for normality (Kolmogorov–Smirnov test). Statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., Point Richmond, California, USA) with *P*-values of less than 0.05 considered statistically significant.

Results

All animals grew continuously throughout the experimental period. Their body weight increased from 1930 ± 269 g at 8 weeks of age, when the change in dietary consistency was introduced, to 3589 ± 375 g at 20 weeks of age in the experimental group and from 1901 ± 167 g at 8 weeks of age to 3529 ± 183 g at 20 weeks of age in the control group. The body weight of the animals did not differ significantly between the groups at any time. The animals in the experimental group did not show any change in their masticatory pattern in response to the reduced food hardness.

Mean values and SDs of the proportions and corresponding cross-sectional areas of the various fibre types in the masseter, temporalis, and digastric muscles under conditions of varying functional load are shown in Tables 1 and 2. No fibres expressing MyHC-IIB were detected in any of the muscles studied.

Statistical testing revealed significant differences in the population of slow fibres between the groups: the fibres

Table 1 Mean and standard deviations of fibre-type proportions in rabbit masseter, temporalis, and digastric muscles under conditions of varying functional load.

	Fibre-type proportion (%)					
	Masseter		Temporalis		Digastric	
	Experimental	Control	Experimental	Control	Experimental	Control
Slow fibres						
I[a]	0 ± 0	0.03 ± 0.09	0.04 ± 0.10	0.07 ± 0.13	5.76 ± 3.57	7.68 ± 4.80
α[a]	9.70 ± 8.38	7.81 ± 5.61	0.43 ± 0.74	0.69 ± 1.26	0.08 ± 0.15	0.14 ± 0.20
I + α[a]	10.29 ± 4.72*	17.58 ± 5.48*	3.56 ± 3.19	5.75 ± 6.60	24.19 ± 8.67	19.09 ± 6.08
Intermediate fibres						
I + α + IIA[a]	0.94 ± 1.00	0.50 ± 0.79	1.64 ± 1.35	2.35 ± 3.22	1.68 ± 1.13	2.06 ± 1.25
I + α + IIX[a]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.99 ± 1.91	0.71 ± 0.62
I + IIA[a]	0.92 ± 1.65	0 ± 0	1.15 ± 2.99	0.67 ± 1.17	0.17 ± 0.38	0.21 ± 0.18
I + IIX[a]	0 ± 0	0 ± 0	0.03 ± 0.08	0 ± 0	0 ± 0	0 ± 0
α + IIA[a]	17.81 ± 8.42	14.08 ± 4.63	1.78 ± 1.37	2.23 ± 1.81	1.32 ± 1.42	1.28 ± 0.83
α + IIX[a]	0.04 ± 0.12	0.27 ± 0.52	0 ± 0	0.05 ± 0.15	0 ± 0	0 ± 0
Fast fibres						
IIA[a]	59.70 ± 9.05	57.28 ± 9.94	71.05 ± 15.61	72.18 ± 19.60	65.72 ± 8.45	67.47 ± 9.56
IIX[a]	0.60 ± 1.21	2.45 ± 3.13	20.32 ± 15.03	16.01 ± 14.57	0.09 ± 0.25	1.36 ± 3.70

[a]Myosin heavy-chain isoform. α, cardiac alpha.
*Statistically significant difference between groups, Mann–Whitney rank sum test $P < 0.05$.

Table 2 Mean and standard deviations of cross-sectional area of the various fibre types in rabbit masseter, temporalis, and digastric muscles under conditions of varying functional load.

	Fibre cross-sectional area (μm^2)					
	Masseter		Temporalis		Digastric	
	Experimental	Control	Experimental	Control	Experimental	Control
Slow fibres						
I[a]		231 ± 0	110 ± 0	221 ± 0	1142 ± 210	1104 ± 404
α[a]	1759 ± 263	1624 ± 566	363 ± 114	438 ± 139	348 ± 13	500 ± 387
I + α[a]	1085 ± 90*	1258 ± 289*	439 ± 268	566 ± 264	1158 ± 231	1111 ± 377
Intermediate fibres						
I + α + IIA[a]	1856 ± 1166	1614 ± 623	765 ± 773	977 ± 665	1125 ± 285	1184 ± 399
I + α + IIX[a]					1169 ± 135	920 ± 382
I + IIA[a]	3677 ± 3246		312 ± 280	723 ± 204	635 ± 120	923 ± 535
I + IIX[a]			488 ± 0			
α + IIA[a]	1896 ± 485	2019 ± 588	410 ± 228	592 ± 325	1537 ± 365	1220 ± 364
α + IIX[a]	1976 ± 0	2342 ± 265		5287 ± 0		
Fast fibres						
IIA[a]	4748 ± 615	4775 ± 1255	1531 ± 692	1853 ± 621	2762 ± 516	2474 ± 813
IIX[a]	6116 ± 4669	6749 ± 809	3141 ± 1120	3637 ± 1218	3976 ± 0	2026 ± 6

[a]Myosin heavy-chain isoform. α, cardiac alpha.
*Statistically significant difference between groups, Mann–Whitney rank sum test $P < 0.05$.

co-expressing MyHC-I and MyHC-cardiac alpha accounted for a smaller proportion in the masseter muscles of the experimental animals than in those of the controls. These fibres also had smaller cross-sectional areas in the experimental group than in the control group. The proportions and cross-sectional areas of the various fibre types in the temporalis and digastric muscles did not differ significantly between experimental and control animals. The relatively large SDs indicate substantial interindividual variation in fibre-type composition and fibre cross-sectional area in both groups.

Discussion

The results suggest that the reduction in dietary consistency, as performed in the present study, induced changes in the phenotypic properties of fibres in the rabbit masseter muscle. The muscle adapted structurally to the reduced functional load by decreasing the proportion and cross-sectional area of fibres in the slow fibre compartment.

Animals are used within science as models for the study of biological responses. In the present investigation, rabbits were used as, similar to humans, their jaw muscles contain fibres expressing MyHC-cardiac alpha and there is heterogeneous expression of myosin isoforms in some individual fibres (Bredman *et al.*, 1991). The immunohistochemical determination of their MyHC composition allowed accurate characterization of the muscle fibres (Sant'ana Pereira *et al.*, 1995) and, for the first time, the assessment of changes in fibres expressing the cardiac alpha MyHC isoform. However, it should be noted that, with the antibody panel used in the present study, it was not possible to detect hybrid fibres that co-express MyHC-IIA and MyHC-IIX. These fibres might have been misclassified as type IIA fibres.

The proportions and cross-sectional areas of the various fibre types in the masseter, temporalis, and digastric muscles, as determined in the present study, are in accordance with those reported in previous investigations of developing rabbit jaw muscles (van Wessel *et al.*, 2005; Korfage *et al.*, 2006a,b, 2009). Similar to previous investigations, the present study did not detect any fibres expressing MyHC-IIB, which are rarely found in rabbit jaw muscles (Bredman *et al.*, 1992). Also the degree of interindividual variation in fibre-type composition and fibre cross-sectional area was similar to that reported earlier (Korfage *et al.*, 2006a,b, 2009). A number of factors contribute to the large variability in fibre-type composition that are typically found among individuals (Korfage *et al.*, 2005). As in the present study the animals were matched for age and gender in order to exclude influences of ageing and sexual dimorphism (English *et al.*, 1999), the interindividual variation was most likely the result of other factors influencing the phenotypic properties of jaw muscle fibres, such as individually different masticatory patterns (Kemsley *et al.*, 2003) or levels of testosterone (Reader *et al.*, 2001).

The reduction in dietary consistency induced changes in the MyHC composition and fibre cross-sectional area of the masseter muscle, while those of the temporalis and digastric muscles remained unchanged. This disparity is most likely based on the different function of these muscles during mastication. The masseter muscle elevates the mandible and generates occlusal force during the power stroke (Widmer *et al.*, 2003). The superficial temporalis and digastric muscles stabilize and open the jaw, respectively (Weijs *et al.*, 1989). During chewing, it requires less muscle force to break soft food than to break hard food but the force necessary to stabilize or open the jaw is, in all likelihood, independent from the hardness of the ingested food. It appears that the reduction in dietary consistency altered only the functional loading of the muscles that generate the force necessary to crush the pellets. Therefore, only the masseter muscle adjusted its fibre-type composition and fibre cross-sectional area, while the temporalis and digastric muscles were unaffected by the change in dietary consistency.

Of the various fibre types in the masseter muscle, reduced masticatory function induced significant changes only in the slow fibre compartment, most likely as a consequence of the frequency of their recruitment during chewing. Following the so-called 'size principle' (Henneman, 1981), the motor units of jaw muscles are recruited in a strict hierarchical order (Scutter and Türker, 1998). Small motor units with predominantly slow fibres are recruited at lower force threshold levels than the larger ones with faster fibres. Mastication of pellets requires comparatively low forces. It has been previously shown by means of electromyography that the majority of jaw muscle contractions during chewing in rabbits generate only 20 per cent of the maximum force (Langenbach *et al.*, 2004). In order to generate this force level, predominantly small motor units are recruited as their fibres are optimally suited for sustained contractions requiring relatively low force (Maxwell *et al.*, 1980). It can be assumed that especially fibres of these motor units change their MyHC composition and cross-sectional area as an adaptive response to reduced masticatory load. Larger motor units with faster fibres, which are only used during larger efforts for brief periods of time, are not recruited during chewing and consequently not affected by an alteration in food hardness.

The present findings are, on the whole, in agreement with those of other studies that investigated the effect of reduced masticatory function on the phenotypic properties of rabbit jaw muscles. For instance, the masseter muscles of rabbits fed a liquefied diet after puberty adapted to the reduced dietary consistency by decreasing the cross-sectional area of their slow fibres (Langenbach *et al.*, 2003). When the animals were fed a powdered diet soon after weaning, the masseter muscles also responded with a decrease in the percentage of type I fibres and a concomitant increase in type IIA fibres (Negoro *et al.*, 2001; Kitagawa *et al.*, 2004), which indicates a shift in the fibre-type composition towards a higher proportion of fast fibres. The particular adaptive response thus seems to vary with the exact nature of the stimulus and the age at which it is introduced.

The effect of reduced masticatory functional load on the temporalis and digastric muscles has hitherto not been investigated in rabbits. Studies on ferrets (He *et al.*, 2004) and rats (Kiliaridis *et al.*, 1988) found, in accord with the present investigation, no significant differences in the fibre-type composition of the temporalis and digastric muscles

after the normal diet had been changed to a liquefied one. In contrast, when rats were fed by gastric gavage (Sfondrini *et al.*, 1996), the population of IIB fibres in both the temporalis and the digastric muscles increased while the population of IIA and IIX fibres decreased. It seems that the complete elimination of masticatory movements led to severe disuse atrophy of all jaw muscles. This hypothesis is supported by the finding of a lower weight of the muscles affected when compared with those of the control group (Sfondrini *et al.*, 1996). The severity of jaw muscle atrophy induced by a liquefied diet is much lower as shown, for instance, in a mouse model (Urushiyama *et al.*, 2004).

The different experimental designs, species, and ages of the animals used in the various studies complicate a direct comparison of their results. The relative sizes, functions, and orientation of action lines of jaw muscles vary greatly among species (Herring, 2007) and with them their phenotypic properties. For instance, the muscles of large animals generally contain a higher proportion of fibres expressing MyHC-I than those of small animals (Pellegrino *et al.*, 2003). Furthermore, the ability of skeletal muscle to adapt to inactivity attenuates with age (Husom *et al.*, 2005). Nevertheless, when taking the findings of the various studies together, it appears that a reduction in masticatory function, induced by reducing the dietary consistency, leads to selective disuse atrophy of the muscles that are less recruited as a consequence of the lower force level necessary to crush the food. The less recruited fibres decrease their cross-sectional area and start to express faster MyHC isoforms, resulting in a decrease in the proportion of slow fibres in the muscle.

Changes in the phenotypic fibre properties, similar to those observed in the masseter muscle in the present study, are typically found during inactivation of skeletal muscles. For instance, hindlimb suspension, a common experimental model for chronically reduced neuromuscular activity, induces atrophic changes in the rat soleus muscle, which responds to the inactivity with a decrease in the proportion of type I fibres and a concomitant increase in IIA fibres (Oishi *et al.*, 1998). Spinal cord isolation, which leads to complete neuromuscular inactivity of the hindlimb muscles, decreases the cross-sectional area of both type I and type II fibres in addition to the reduction in the proportion of slow fibres (Grossman *et al.*, 1998).

Considering the findings of the present and other studies, it is reasonable to assume that atrophic changes in the jaw muscles, such as decreases in the proportion and cross-sectional area of slow fibres, might also occur in humans under conditions during which muscular activity is chronically reduced, such as sustained intermaxillary fixation (Ingervall *et al.*, 1979), chronic muscle pain (Svensson *et al.*, 2004), or complete tooth loss (Raustia *et al.*, 1996).

Conclusions

The results of the present study suggest that long-term reduction in masticatory functional load contributes to selective disuse resulting in structural adaptation of the masseter muscle, reflected in decreases in the proportion, and the cross-sectional area of its slow fibres.

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Reference

The full list of references is available in Part 2 (doi:10.1093/ ejo/cjq084).