Title: Expression of MyHC isoforms mRNA transcripts in different regions of the masseter and medial pterygoid muscles in chimpanzees

Authors: Neus Ciurana[a*], Rosa Artells[a], Carmen Muñoz[a], Júlia Arias-Martorell[a,e], Gaëlle Bello-Hellegouarch[a,b], Alejandro Pérez-Pérez[c], Juan Francisco Pastor[d], Josep Maria Potau[a]

Affiliations: [a] Unit of Human Anatomy and Embryology, University of Barcelona, C/Casanova 143, 08036 Barcelona, Spain; [b] Department of Biology, FFCLRP, University of São Paulo, Avenida Bandeirantes, 3900, Bairro Monte Alegre, Ribeirão Preto, São Paulo, Brazil; [c] Department of Evolutionary Biology, Ecology and Environmental Sciences, Section of Zoology and Biological Anthropology, University of Barcelona, Av/Diagonal 643, 08028 Barcelona, Spain; [d] Department of Anatomy and Radiology, University of Valladolid, C/Ramón y Cajal 7, 47005, Valladolid, Spain. [e] Animal Postcranial Evolution (APE) Lab, Skeletal Biology Research Centre, School of Anthropology and Conservation, University of Kent, Canterbury UK, CT2 7NR.

***Corresponding author:** Neus Ciurana MD
Unit of Human Anatomy and Embryology
University of Barcelona
C/Casanova 143
08036 Barcelona
Spain
Email: nc_confidential@hotmail.com
Tel: +34 934021906
Fax: +34 934035263

Running title: Masticatory muscles of chimpanzees

ABSTRACT

Objective: The aim of this study is to examine the expression pattern of the different myosin heavy chain (MyHC) isoforms in the masseter and medial pterygoid muscles by real time quantitative polymerase chain reaction (RT-qPCR) to obtain information at molecular level which can be related to the functional characteristics of these two muscles.

Design: The masseter, deep and superficial portion, and medial pterygoid muscles of five adult Pan troglodytes were dissected in order to obtain samples of the anterior and posterior regions of each portion of the masseter and of the medial pterygoid. The expression of MyHC isoforms mRNA transcripts was analysed by RT-qPCR.

Results: No significant differences in expression of MyHC isoforms between the masseter and the medial pterygoid were found. In contrast, when comparing the superficial and the deep portion of the masseter, we found that the MyHC-IIM isoform was expressed at a significantly higher level in the superficial portion.

Conclusions: The superficial portion of the masseter and the medial pterygoid muscle have the same expression pattern regarding the different MyHC isoforms. On the other hand, the deep portion of the masseter, which is activated mainly during lateral and repositioning movements of the mandible, has a lower MyHC-IIM isoform expression than the superficial portion. Our findings provide new data on functional aspects of the masseter and medial pterygoid that can complement results obtained by other techniques.

Keywords: masseter, medial pterygoid, myosin heavy chain, RT-qPCR

INTRODUCTION

The masseter and medial pterygoid, together with the temporalis and the lateral pterygoid, are the masticatory muscles that move the jaw during chewing and biting (Taylor & Vinyard, 2013). The masseter has a superficial and a deep portion extending from the zygomatic arch to the angle and lateral side of the ramus of the mandible (Williams & Warwick, 1980). The two portions are easy to separate in chimpanzees due to the presence of a dense fascia between them (Aiello & Dean, 1990; Diogo et al., 2013; Swindler & Wood, 1982). The medial pterygoid arises from the pterygoid fossa and the lateral pterygoid plate and inserts in the medial surface of the ramus and angle of the mandible (Williams & Warwick, 1980). Although both the masseter and the medial pterygoid act on the two halves of the ramus of the mandible, several electromyographic studies in humans and other primates have identified slight functional differences between the two muscles, as well as between the two portions of the masseter, during the chewing cycle (Basmajian & de Luca, 1985; Blanksma, van Eijden, van Ruijven, & Weijs, 1997; Guzmán-Venegas, Biotti Picand, & de la Rosa, 2015; Hylander, Johnson, & Crompton, 1987; Hylander, Ravosa, Ross, Wall, & Johnson, 2000; Schindler, Rues, Türp, & Lenz, 2006; Vinyard, Wall, Williams, & Hylander, 2008; Wall, Vinyard, Johnson, Williams, & Hylander, 2006; Williams et al., 2011).

In addition to electromyographic studies, the analysis of the expression of myosin heavy chain (MyHC) isoforms can provide excellent insight into the functional characteristics of muscles (Bottinelli & Regianni, 2000). MyHC-I, MyHC-IIa and MyHC-IIx isoforms are expressed in the skeletal muscles of all mammals, while a fourth isoform, MyHC-IIb, is expressed only in small mammals (Baldwin & Haddad, 2001). The MyHC-I isoform is expressed at higher levels in tonic and postural muscles, which have a slow contractile speed and high resistance to fatigue. The MyHC-IIa and MyHC-IIx isoforms are expressed at higher levels in phasic muscles, which have great force and

a fast contractile speed but lower resistance to fatigue. The MyHC-IIx isoform is associated with muscles of greater force, faster speed, and lower resistance than the MyHC-IIa isoform (Bottinelli, Pellegrino, Canepari, Rossi, & Reggiani, 1999; Pette & Staron, 2000). Besides these three MyHC isoforms, masticatory muscles express the MyHC-IIM isoform (Rowlerson, Mascarello, Veggetti, & Carpene, 1983; Stedman et al., 2004), which is associated with a moderate contractile speed (similar to that of MyHC-IIa) and greater force (Hoh, 2002; Toniolo et al., 2008).

Several methods can be used to analyze the expression patterns of MyHC isoforms. For example, ATPase staining can quantify the percentage of muscular fibers expressing each of the MyHC isoforms, while immunohistochemistry (IHC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be used to quantify the protein expression of MyHC isoforms. A third method, real-time quantitative polymerase chain reaction (RT-qPCR), which provides mRNA transcripts of MyHC isoforms, has several advantages over ATPase staining, IHC and SDS-PAGE. For example, RT-qPCR can be used with very small samples of muscles obtained from cryopreserved cadavers, since any potential post-mortem degradation of the mRNA can be accounted for by normalizing the values of each MyHC isoform with those of an endogenous gene (Bahar et al., 2007). Moreover, RT-qPCR can precisely quantify the percentages of expression of each individual MyHC isoform mRNA regardless of the presence of hybrid muscle fibers that generally express more than one type of MyHC isoform (Korfage, Koolstra, Langenbach, & van Eijden, 2005; Pette & Staron, 2000). Finally, RT-qPCR is highly reliable, since the transcriptional control of the expression of MyHC isoforms provides a good correlation between the mRNA and protein expression patterns (Cox & Buckingham, 1992; Eizema et al., 2005; Hemmings et al., 2009; Men,

Deng, Tao, Qi, & Xu, 2016; Short et al., 2005; Wright, Haddad, Qin, & Baldwin, 1997; Zurmanova & Soukup, 2013).

Few electromyographic or molecular studies have examined the masseter muscle in Pan troglodytes, a hominid that is phylogenetically closely related to Homo sapiens. In fact, to the best of our knowledge, only one study (Rowlerson et al., 1983) has analyzed MyHC isoforms in the masseter of Pan troglodytes. In this study, ATPase staining identified 31-57% of type I fibers, which express only the MyHC-I isoform, and 43-69% of type IIM fibers, which express only the MyHC-IIM isoform. However, this study did not analyze the superficial and deep portions of the masseter separately, nor did it examine the medial pterygoid. Moreover, the authors did not report information on hybrid fibers that can express different types of MyHC isoforms. In other species of primates, however, electromyographic studies have identified functional differences between the superficial and deep portions of the masseter (Basmajian & de Luca, 1985; Blanksma et al., 1997; Guzmán-Venegas et al., 2015; Hylander et al., 2000; Vinyard et al., 2008) and between the anterior and posterior regions of the two portions in humans (Basmajian & de Luca, 1985; Blanksma et al., 1997; Guzmán-Venegas et al., 2015). In contrast, no functional differences were detected between the masseter and the medial pterygoid muscles – either in humans (Basmajian & de Luca, 1985; Schindler et al., 2006) or in non-hominid primates (Hylander et al., 1987; Wall et al., 2006; Williams et al., 2011).

In order to shed further light on this issue, we have quantified the percentages of mRNA transcripts of the MyHC isoforms MyHC-I, MyHC-IIa, MyHC-IIx and MyHC-IIM by RT-qPCR in the anterior and posterior regions of the superficial and deep portions of the masseter and in the medial pterygoid in five adult chimpanzees (Pan troglodytes). The main objective of our study was to find differences in the expression patterns of the MyHC isoforms that could be related to functional differences identified in humans and

other primate species by electromyography between the superficial and deep portions of the masseter and between the anterior and posterior regions of each of the masseter portions.

MATERIALS & METHODS

Muscle samples

We dissected the right masseter and medial pterygoid muscles of five adult chimpanzees (Pan troglodytes) obtained from the Anatomy Museum of the University of Valladolid, Spain. There were two males and three females, all of which had died from causes unrelated to the present study at different Spanish zoos. All bodies had been cryopreserved without chemical fixation within 24-48 hours after death.

The same investigator dissected all samples. The adipose and conjunctive tissue were removed to identify and isolate the superficial and deep portions of the masseter and the medial pterygoid (Fig. 1) and the muscles were weighed on a precision scale. One sample of 0.5 cm^3 was obtained of the anterior and posterior regions of each portion of the masseter and of the medial pterygoid and cryopreserved in physiological saline solution for later molecular analysis.

RNA isolation and cDNA synthesis

We extracted the RNA from the muscle samples using the commercial RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. We used a NanoDrop 1000 Spectrophotometer to determine the concentration, purity and amount of RNA and electrophoresis on 1% agarose gel to assess the integrity and quality of RNA.

We used TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA) to synthesize cDNA. We performed reverse transcription using 330 ng of total

RNA in 10 µl of RT Buffer, 22 ml of 25 mM magnesium chloride, 20 µl dNTPs, 5 µl Random Hexamers, 2 µl RNAse Inhibitor, 2.5 µl MultiScribe Reverse Transcription and RNA sample plus RNAse-free water, for a final volume of 100 µl, in the following thermal cycler conditions: 10 min 25ºC, 48 min 30 ºC and 5 min 95 ºC.

Gene expression and quantification by RT-qPCR

Applied Biosystems supplied primers and probes. Primers are labeled at the 5' end with the reporter dye molecule FAM. MYH-I (Hs00165276_m1), MYH-IIa (Hs00430042_m1), MYH-IIx (Hs00428600_m1) and MYH-IIM (Hs01385213_m1) genes were analyzed. We used 18s gene probe labeled at the 5' end with the reporter dye molecule FAM (Hs99999901_s1) as housekeeping gene.

We performed RT-qPCR in a total volume of 20 µl in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the following master mix conditions: 10 µl of the TaqMan Universal PCR Master Mix, 1 µl of the primers and probes, 2 µl of the cDNA and 7 µl of the RNAse-free water. We ran all samples for each gene in duplicate using this thermal cycler conditions: two min 50 ºC, 40x (10 min 95 ºC, 15 s 95 ºC) and 1 min 60 ºC. We used genomic DNA as negative control in each run. We captured fluorescent emission data and quantified mRNA concentrations by using the critical threshold value and $2^{-\Delta\Delta Ct}$. In order to avoid any possible effects of post-mortem mRNA degradation, the mRNA values for each of the MyHC isoforms were normalized using the endogenous gene 18S, which is preserved intact for up to eight days after death in skeletal muscle (Bahar et al., 2007).

Finally, we calculated the percentage of expression of each MyHC isoform mRNA transcript relative to the total expression of all MyHC isoforms (%MyHC-I, %MyHC-IIa, %MyHC-IIx and %MyHC-IIM).

Statistical analyses

We used the non-parametric Mann-Whitney test to compare the expression patterns of the MyHC isoforms mRNA in the muscles and regions analyzed. We used PASW Statistics 18 for all statistical analyses and set statistical significance at $P<0.05$.

Ethical note

The research complied with protocols approved by the Institutional Animal Care and Use Committee of the University of Barcelona and adhered to the legal requirements of Spain.

RESULTS

The mean mass of the masseter was 2.6 times greater than that of the medial pterygoid (62 vs 24 g) (Table 1). The mean mass of the superficial portion was 2.8 times greater than that of the deep portion (45.5 vs 16.5 g). The superficial portion accounted for 73.4% and the deep portion for 26.6% of the total mass of the masseter. The superficial portion accounted for 52.9%, the deep portion for 19.2%, and the medial pterygoid for 27.9% of the total mass of the muscles analyzed.

The mRNA expression pattern of the MyHC isoforms was the same in the superficial and deep portions of the masseter and in the medial pterygoid: MyHC-IIM>MyHC-IIa>MyHC-I>MyHC-IIx (Table 1). There were no significant differences in the percentages of expression of any of the four MyHC isoforms either between the anterior and posterior regions of the portions of the masseter or between the portions of the masseter and the medial pterygoid (Table 1). When we compared the mRNA expression of the MyHC isoforms in the superficial and the deep portions of the masseter, we found no differences in the expression of MyHC-I, MyHC-IIa or MyHC-IIx, but the

192 expression of MyHC-IIM was significantly greater in the superficial than in the deep
193 portion (40.2% vs 34.2%; p=0.032).

194 **DISCUSSION**

195 In the present study, we have found that the two portions of the masseter and the
196 medial pterygoid of Pan troglodytes have a MyHC mRNA expression pattern
197 characteristic of phasic muscles (Table 1), with less than 50% of MyHC-I and a
198 significant expression level of MyHC-IIx (Bottinelli et al., 1999; Pette & Staron, 2000).
199 This pattern is to be expected in muscles that act directly on the mandibular ramus to
200 elevate the mandible for fast and powerful chewing (Basmajian & de Luca, 1985;
201 Schindler et al., 2006). The great force of these muscles in chimpanzees is reflected in
202 their high %MyHC-IIM, associated with a great contractile force (Hoh, 2002; Rowlerson
203 et al., 1983; Toniolo et al., 2008), in the superficial portion of the masseter (40.2%), in
204 the deep portion of the masseter (34.2%) and in the medial pterygoid (37.7%). This
205 molecular pattern showing the powerful force of the two muscles corresponds with their
206 structural pattern: both are multipennate muscles with large physiological cross-sectional
207 areas (Horton et al., 2001; van Eijden, Korfage, & Brugman, 1997), which is
208 characteristic of muscles with great contractile force.

209 Intriguingly, we have observed few inter- or intra-muscular differences in the
210 percentages of expression of the MyHC isoforms in our chimpanzee specimens. In
211 contrast, studies of the masseter and medial pterygoid muscles in humans, using
212 histochemistry (HC) or IHC (Eriksson & Thornell, 1983; Korfage, Brugman & van
213 Eijden, 2000; Korfage & van Eijden, 2000; Monemi, Eriksson, Eriksson, & Thornell,
214 1998; Österlund, Thornell, & Eriksson, 2011; Österlund Lindström, Thornell, & Eriksson,
215 2012; Thornell, Bitlleter, Eriksson, & Ringqvist, 1984) identified a more heterogeneous

inter- and intra-muscular pattern in the distribution of types of muscle fibers and in the percentages of expression of the MyHC isoforms. This difference may be due to several factors. Hybrid muscle fibers, which can account for more than 40% of muscle fibers in the masseter and medial pterygoid muscles in humans (Korfage et al., 2000; Korfage & van Eijden, 2000; Österlund et al., 2012), express more than one MyHC isoform, which can affect the determination and quantification of types of muscle fibers when using HC or IHC. In contrast, RT-qPCR directly quantifies the mRNA transcripts of the MyHC isoforms regardless of the type of fiber in which they are expressed, thus providing a more reliable indication of the total percentages of expression of each of the isoforms.

The mRNA expression pattern of the MyHC isoforms was similar in the masseter and the medial pterygoid in chimpanzees (Table 1), which indicates that both muscles cooperate and counteract each other in jaw activities. Electromyographic studies have also found that the two muscles have a similar form of action during the elevation of the mandible, both in humans (Basmajian & de Luca, 1985; Schindler et al., 2006) and in other primates, such as Macaca fascicularis and Papio anubis (Hylander et al., 1987; Wall et al., 2006; Williams et al., 2011). Electromyographic studies in humans and other primates (Basmajian & de Luca, 1985; Blanksma et al., 1997; Hylander et al., 2000; Vinyard et al., 2008) have also reported functional differences between the superficial and the deep portion of the masseter. The superficial portion acts primarily to elevate the mandible, while the deep portion exerts a relatively larger component of lateral force to reposition the temporomandibular joint. These functional differences between the two portions of the masseter are also reflected in the expression of MyHC isoforms mRNA transcripts in chimpanzees (Table 1). The superficial portion expresses a higher percentage of the most powerful isoform (MyHC-IIM), while the deep portion expresses a slightly higher percentage of the less powerful isoforms (MyHC-IIa and MyHC-I)

241 (Table 1). However, the only difference reaching statistical significance is that of MyHC-
242 IIM (P=0.032). These findings are in line with those of electomyographic studies in other
243 species of primates, which have found that the superficial portion exerts a greater
244 contractile force than the deep portion (Hylander et al., 2000; Vinyard et al., 2008). The
245 greater contractile force of the superficial portion in chimpanzees may also be related to
246 its relatively larger mass. In our samples, the superficial portion represented 73.4% of the
247 total mass of the masseter, while the deep portion accounted for only 26.6%. These
248 findings are in line with those of Taylor and Vinyard (2013), who found that the
249 superficial portion constituted 82.8% and the deep portion 17.2% of the total mass of the
250 masseter. Nevertheless, functional differences between the anterior and posterior regions
251 of the masseter observed in electromyographic studies in humans (Blanksma et al., 1997;
252 Guzmán-Venegas et al., 2015) have not been borne out by our findings on the percentage
253 of expression of MyHC isoforms mRNA transcripts in chimpanzees (Table 1).

254 The percentages of mRNA expression of MyHC isoforms observed in our study
255 of Pan troglodytes are similar to – but slightly lower than – the percentages of types of
256 muscle fibers identified by ATPase staining (43-69% of type IIM vs 31-57% of type I
257 fibers) (Rowlerson et al., 1983). These differences may be due to the fact that the ATPase
258 staining study did not take into account the MyHC-IIa and MyHC-IIx isoforms.

259 Our findings can also have implications for the fields of anthropology and human
260 evolution, since Pan troglodytes is one of the animal species most closely related
261 phylogenetically to Homo sapiens. Several studies have analyzed the expression of the
262 MyHC isoforms in human masseter and medial pterygoid muscles, using ATPase staining
263 and IHC (Eriksson & Thornell, 1983; Korfage et al., 2000; Korfage & van Eijden, 2000;
264 Monemi et al., 1998; Österlund et al., 2011; Österlund et al., 2012; Sciote, Rowlerson,
265 Hopper, & Hunt, 1994; Serratrice, Pellissier, Vignon, & Baret, 1976; Shaughnessy,

Fields, & Westbury, 1989; Stal, Eriksson, Schiaffino, Butler-Browne, & Thornell, 1994; Thornell et al., 1984). Korfage et al. (2000) found no differences between the two muscles but significant differences between the two portions of the masseter muscle, where the superficial portion had a higher expression of MyHC-IIx, while the deep portion had a higher expression of MyHC-I and MyHC-IIa. Along these same lines, we also found differences between the two portions of the masseter muscle; however, these differences were in the expression of MyHC-IIM, which is not expressed in the masticatory muscles of humans (Korfage et al., 2000; Korfage & van Eijden, 2000; Sciote et al., 1994). This absence of MyHC-IIM has been related to the appearance of a mutation 2.4 million years ago, which could have led to the decrease in the size of type II fibers in human masticatory muscles. This decrease, in turn, could explain one of the defining characteristics of Homo sapiens (Aiello & Dean, 1990) – the size reduction of the masticatory muscles that has occurred over the course of human evolution since the time of Homo habilis (Stedman et al., 2004).

In summary, we found no differences in the expression of the MyHC isoforms mRNA transcripts between the two portions of the masseter and the medial pterygoid in Pan troglodytes, indicating that the two muscles work together to elevate the mandible as has been seen in electromyography. Nevertheless, we did find slight differences between the superficial and deep portions of the masseter, perhaps related to the different functions of the portions reported in electromyographic studies in humans and other primate species (Basmajian & de Luca, 1985; Blanksma et al., 1997; Guzmán-Venegas et al., 2015). These differences may well be related to functional differences between the two portions, where the superficial portion primarily exerts a great elevating force on the mandible while the deep portion uses a greater lateral force to reposition the temporomandibular joint.

Conclusions

In conclusion, our findings by RT-qPCR provide novel information on functional aspects of the masseter and medial pterygoid of chimpanzees that can complement results obtained with ATPase staining, IHC or electromyography. These findings will provide useful stepping-stones for further studies both in primatology and in physical anthropology.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE LEGEND

Fig. 1 Dissection of the superficial portion of masseter **(a)**, deep portion of masseter **(b)** and the medial pterygoid muscle **(c)** in Pan troglodytes. * Locations where muscle samples were obtained.

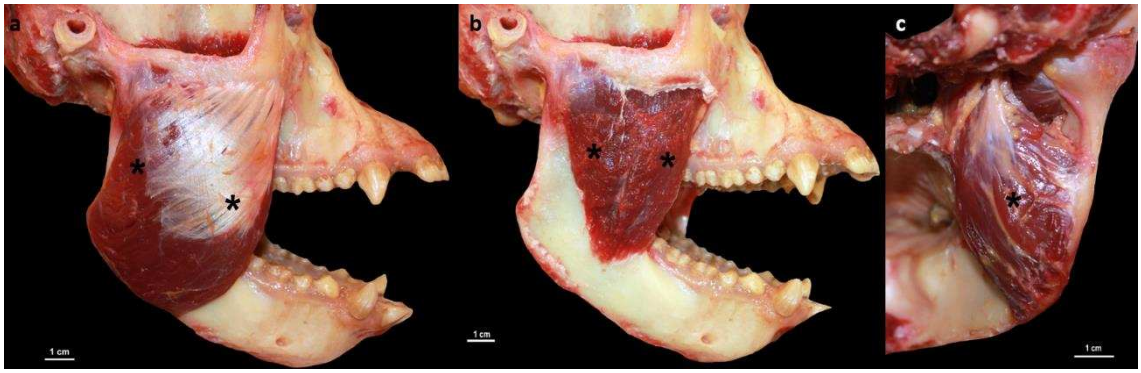

TABLE LEGEND

Table 1. Mean and standard deviations of weight (in grams) and percentages of expression of MyHC isoforms in the masseter and medial pterygoid muscles of five Pan troglodytes.

Table 1.

Muscle	Weight mean (SD)	%MyHC-I mean (SD)	%MyHC-IIa mean (SD)	%MyHC-IIx mean (SD)	%MyHC-IIM mean (SD)
Masseter	62 (27.2)	21.5 (4.9)	28.6 (2.2)	12.8 (5.1)	37.2 (3.7)
Superficial portion	45.5 (19.9)	20.3 (5.1)	26.7 (2.5)	12.8 (6.5)	40.2 (5.1)
Anterior region		20.3 (5.6)	27.4 (2.0)	13.1 (6.7)	39.3 (6.0)
Posterior region		20.3 (4.6)	26.0 (2.9)	12.6 (6.7)	41.1 (4.9)
Deep portion	16.5 (7.5)	22.7 (5.1)	30.4 (2.2)	12.7 (4.0)	34.2 (2.7)
Anterior region		22.6 (5.5)	30.9 (2.3)	12.7 (5.8)	33.8 (1.6)
Posterior region		22.8 (4.8)	30.0 (3.5)	12.6 (4.7)	34.7 (3.9)
Medial pterygoid	24 (8.6)	22.2 (3.0)	28.3 (1.4)	11.8 (7.4)	37.7 (8.1)