Title: Expression of myosin heavy chain isoforms mRNA transcripts in the temporalis muscle of common chimpanzees (*Pan troglodytes*)

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ABSTRACT

Purpose: The common chimpanzee (*Pan troglodytes*) is the primate that is phylogenetically most closely related to humans (*Homo sapiens*). In order to shed light on the anatomy and function of the temporalis muscle in the chimpanzee, we have analyzed the expression patterns of the mRNA transcripts of the myosin heavy chain (MyHC) isoforms in different parts of the muscle.

Basic procedures: We dissected the superficial, deep and sphenomandibularis portions of the temporalis muscle in five adult *Pan troglodytes* and quantified by real-time quantitative polymerase chain reaction the expression of the mRNA transcripts of the MyHC isoforms in each portion.

Main findings: We observed significant differences in the patterns of expression of the mRNA transcripts of the MyHC-IIM isoform between the sphenomandibularis portion and the anterior superficial temporalis (33.6% vs 47.0%; P=0.032) and between the sphenomandibularis portion and the anterior deep temporalis (33.6% vs 43.0; P=0.016). We also observed non-significant differences between the patterns of expression in the anterior and posterior superficial temporalis.

Principal conclusions: The differential expression patterns of the mRNA transcripts of the MyHC isoforms in the temporalis muscle in *Pan troglodytes* may be related to the functional differences that have been observed in electromyographic studies in other species of primates. Our findings can be applicable to the fields of comparative anatomy, evolutionary anatomy, and anthropology.

Keywords: temporalis muscle, *Pan troglodytes*, myosin heavy chain, RT-qPCR, sphenomandibularis

1. INTRODUCTION

The temporalis, masseter, and medial pterygoid muscles are the jaw-closing muscles that produce the occlusion of the mandible and other movements associated with chewing and biting (Taylor and Vinyard, 2013). The temporalis arises from the side of the skull and inserts onto the coronoid process of the mandible and the anterior edge of the mandibular ramus (Aiello and Dean, 1990). In the common chimpanzee (*Pan troglodytes*), the temporalis comprises a superficial and a deep portion that can be easily separated (Aiello and Dean, 1990; Oxnard and Franklin, 2008). In contrast, in humans (*Homo sapiens*), the temporalis has the equivalent of the deep temporalis in *Pan troglodytes*, while the superficial temporalis is either completely lacking or only vestigially present (Oxnard and Franklin, 2008; Lee et al., 2012). This loss of the superficial temporalis in humans is a result of the general reduction of the masticatory apparatus that occurred in the evolution of the genus *Homo* (Aiello and Dean, 1990), which may in turn be due to a relatively soft diet (Oxnard and Franklin, 2008).

Some authors make a distinction between the sphenomandibularis and the temporalis muscles in humans and consider the sphenomandibularis to be a separate muscle arising from the maxillary surface of the sphenoid bone and inserting on the temporal crest of the mandible (Dunn et al., 1996). Others, however, consider the sphenomandibularis to be a part of the temporalis, without its own specific vascularization and innervation (Türp et al., 1997; Shimokawa et al., 1998; Schön-Ybarra and Bauer, 2001; Geers et al., 2005; Sedlmayr et al., 2009). In non-human primates, such as the *Macaca mulatta*, the sphenomandibularis has been described as part of the deep temporalis (Skinner and Aziz, 2003).

Electromyographic studies of the temporalis in *Homo sapiens* and in the non-human primates *Macaca fuscata* and *Papio anubis* have found that the temporalis acts in the occlusal phase of the masticatory cycle, with different fibers acting at different points of the cycle (Blanksma et al., 1997; Hylander et al., 2005; Wall et al., 2008). The contraction of the anterior fibers, which run vertically, results in elevation of the mandible, closing the mouth. The anterior fibers reach their peak of maximum activity during the power stroke (Blanksma et al., 1997; Hylander et al., 2005; Vinyard et al., 2008; Vinyard and Taylor, 2010). The posterior fibers run horizontally and their contraction retracts the mandible, pulling the jaw posteriorly. The posterior fibers act during a later phase of the power stroke in which the mandible moves laterally to reposition the molar teeth (Ahlgren et al., 1985; Blanksma et al., 1997; Hylander et al., 2005; Wall et al., 2008; Williams et al., 2011). The consistency of the food being chewed also influences which muscle fibers are called into play (Grünheid et al., 2009). In *Macaca fuscata*, *Papio anubis*, and *Homo sapiens*, more muscle fibers in the anterior than in the posterior temporalis are used when chewing tough food (Blanksma et al., 1997; Ross and Hylander, 2000; Hylander et al., 2005; Vinyard et al., 2008). In *Papio anubis*, the increase in electromyographic activity in the anterior region of the temporalis occurred primarily in the superficial temporalis, which has a greater capacity to produce force (Wall et al., 2008). However, when chewing relatively softer food, there was greater electromyographic activity in the posterior temporalis in *Macaca fuscata* and *Papio anubis* (Hylander et al., 2005; Vinyard et al., 2008). In electromyographic studies in *Homo sapiens*, the sphenomandibularis portion of the deep temporalis worked together with the lateral pterygoid muscle to produce lateral movements of the mandible and also helped maintain the mandible in a stable position (Wood, 1986; Fuentes et al., 2012).

The functional characteristics of the temporalis can also be analyzed in terms of the expression of the myosin heavy chain (MyHC) isoforms (Bottinelli and Reggiani, 2000). Different types of muscle fibers have different contractile properties (force, contraction velocity, and resistance to fatigue) that are related to the expression patterns of MyHC isoforms (Pette and Staron, 2000; Bottinelli et al., 1996; Harridge et al., 1996). The main MyHC isoforms expressed in the skeletal muscles of mammals are MyHC-I, MyHC-IIA, and MyHC-IIX (Sciote and Morris, 2000). The MyHC-I isoform is mainly expressed in slow-twitch oxidative fibers and is the predominant isoform in slow-twitch (type I) muscles (Schiaffino and Reggiani, 2011), which are characterized by low force production and high resistance to fatigue. The MyHC-IIA and MyHC-IIX isoforms are primarily expressed in fast-twitch oxidative glycolytic fibers and fast-twitch glycolytic fibers, respectively, and are the predominant isoforms in fast-twitch (type II) muscles (Schiaffino and Reggiani, 2011). Type IIX fibers are characterized by very high force production and low resistance to fatigue, while type IIA fibers fall in between the type I and type IIX fibers. In addition to the MyHC-I, MyHC-IIA, and MyHC-IIX isoforms, the masticatory muscles of non-human primates also express the MyHC-IIM isoform in type IIM muscle fibers (Rowlerson et al., 1983), which have a moderate contraction velocity, similar to that of the type IIA fibers, and a high force production, greater than that of the type IIX fibers (Hoh, 2002; Toniolo et al., 2008).

The composition of muscle fibers and the expression patterns of the MyHC isoforms in the temporalis muscle of non-human primates have been studied by histochemistry in *Macaca mulatta* (Maxwell et al., 1979; Miller and Farias, 1988) and *Cebus apella* (Andreo et al., 2002), by immunohistochemistry in *Macaca irus* (Rowlerson et al., 1983), and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in *Papio anubis* (Wall et al., 2013). Using ATPase

staining, Miller and Farias (1988) found that the temporalis muscle of *Macaca mulatta* had 47.9-58.5% of type IIX fibers, 27.3-42.6% of type IIA fibers, and 6.6-21.3% of type I fibers. When they compared the anterior and posterior superficial temporalis, they found no significant differences in the percentage of type IIA and IIX fibers, while the percentage of type I fibers was greater in the anterior than in the posterior superficial temporalis (13.1% vs 6.6%). They also found that the posterior superficial temporalis had a lower proportion of type I and IIA fibers (6.6% and 34.9%, respectively) than the posterior deep temporalis (9.5% and 42.6%, respectively). Maxwell et al. (1979) also used ATPase staining to study the temporalis muscle in *Macaca mulatta* and found a higher proportion of type I fibers in the anterior than in the posterior superficial temporalis (49.95% vs 20.65%). In contrast, Andreo et al. (2002), using ATPase staining to study the *Cebus apella*, found no significant differences between the anterior and posterior superficial temporalis. Importantly, none of these studies examined type IIM fibers, which are the most frequent type in the temporalis of non-human primates (Rowlerson et al., 1983). Using ATPase staining together with indirect immunoperoxidase staining, Rowlerson et al. (1983) found that the central temporalis in *Macaca irus* had 86% of type IIM fibers and 14% of type I fibers. A study using SDS-PAGE to analyze the temporalis in *Papio anubis* found 87-88.7% expression of the MyHC-IIM isoform in the anterior and 85.2-90.8% expression in the posterior superficial temporalis, while the MyHC-I isoform was the only other isoform expressed (Wall et al., 2013). In the deep temporalis, the MyHC-IIM isoform was expressed at 60.7% in males and 14.4% in females, while the MyHC-IIA isoform was expressed at less than 5% and the MyHC-I isoform at 38.1% in males and 83.3% in females (Wall et al., 2013).

To the best of our knowledge, no electromyographic or molecular studies have examined the temporalis muscle in *Pan troglodytes*, which together with *Pan paniscus*, is the primate that is phylogenetically most closely related to *Homo sapiens*. In order to shed further light on the anatomy and function of the temporalis muscle in the common chimpanzee, we have dissected the temporalis muscles of five adult chimpanzees and quantified the mRNA transcripts of the MyHC-I, MyHC-IIA, MyHC-IIX, and MyHC-IIM isoforms by real time quantitative polymerase chain reaction (RT-qPCR) in the anterior and posterior portions of the superficial and deep temporalis as well as in the sphenomandibularis portion of the temporalis. Our primary objective was to identify differences in the expression patterns of the MyHC isoforms among the different parts of the temporalis muscle, especially in the sphenomandibularis portion, which could be associated with the functional differences identified by electromyographic studies in humans and other primates.

2. MATERIAL AND METHODS

2.1 Muscle samples

A total of five adult *Pan troglodytes* were included in the study – two males and three females. All five chimpanzees came from Spanish zoos and had died from causes unrelated to the present study. All bodies had been cryopreserved without chemical fixation within 24-48 hours after death. We dissected the right temporalis muscles of four of the chimpanzees and the left temporalis muscle of one female chimpanzee (specimen 03), since the right temporalis of this female had been damaged in a prior necroscopy. All dissections were performed by the same investigator at the Anatomy Museum of the University of Valladolid (Valladolid, Spain) (Table 1).

The investigator removed the adipose and conjunctive tissue to identify and isolate the superficial, deep, and sphenomandibularis portions of the temporalis muscle (Figures 1 and 2). He then weighed each of these portions on a precision scale, with the exception of specimen 03, in which it was not possible to weigh the different portions. In all five specimens, the investigator also obtained 0.5-cm^3 samples of the anterior and posterior regions of the superficial and deep portions of the temporalis and of the sphenomandibularis portion, which were cryopreserved in physiological saline solution for later molecular analysis at the Unit of Human Anatomy and Embryology of the University of Barcelona (Barcelona, Spain).

2.2 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.

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.

2.3 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 human ACTB (Beta Actin) gene probe labeled at the 5' end with the reporter dye molecule FAM (Hs99999903_m1) as endogenous control.

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 human ACTB.

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

2.4 Statistical analyses

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

3. RESULTS

In the four specimens in which it was possible to weigh the different portions of the temporalis, the average total weight of the temporalis was 120.5±45.9 grams. On

average, the superficial temporalis accounted for 33.1±9.2% of the total weight, the deep temporalis 57.0±6.1%, and the sphenomandibularis 9.9±3.5%. In all four specimens, the sphenomandibularis portion was clearly separate from the deep temporalis, arising from the temporal surface of the greater wing of the sphenoid bone and inserting in the temporal crest of the internal surface of the coronoid process of the mandible (Figures 2 and 3).

The expression of the mRNA transcripts of the MyHC isoforms followed the pattern MyHC-IIM > MyHC-IIA > MyHC-I > MyHC-IIX in all the portions of the temporalis except the posterior superficial temporalis, where the pattern was slightly different, with a higher percentage of the MyHC-I isoform and a lower percentage of the MyHC-IIA isoform: MyHC-IIM > MyHC-I > MyHC-IIA > MyHC-IIX. Although the expression of the MyHC isoforms in the sphenomandibularis followed the general pattern, the actual percentage of expression of the MyHC-IIM isoform was lower than in other portions of the temporalis. This difference was significant when comparing the sphenomandibularis with the anterior superficial temporalis (33.6±3.2% vs 47.0±10.4%; P=0.032) and with the anterior deep temporalis (33.6±3.2% vs 43.0±4.9%; P=0.016). In addition, the percentage of expression of the MyHC-IIA isoform in the sphenomandibularis was higher than in other portions of the temporalis and showed a trend towards significance when comparing the sphenomandibularis with the anterior superficial temporalis (31.5±8.0% vs 22.5±5.6%; P=0.056) and with the posterior superficial temporalis (31.5±8.0% vs 18.6±7.5%; P=0.056). Although there were no significant differences between the anterior and posterior superficial temporalis in the expression pattern of the mRNA transcripts of the MyHC isoforms, interestingly, the percentage of expression of the MyHC-IIM isoform was higher in the anterior than in the posterior superficial temporalis (47.0% vs 40.5%), while the percentage of

241 expression of the MyHC-I isoform was lower in the anterior than in the posterior
242 superficial temporalis (19.9% vs 26.9%) (Tables 2 and 3).

243 **4. DISCUSSION**

244 The average weight of the temporalis muscle in our *Pan troglodytes* specimens
245 (120.5 grams) is similar to that reported by other authors (111 grams) (Taylor and
246 Vinyard, 2013). Interestingly, the deep temporalis accounted for 57.0% of the total
247 weight, while the superficial temporalis accounted for only 33.1%. In *Homo sapiens*,
248 the temporalis has only the equivalent of the deep temporalis in *Pan troglodytes* without
249 the superficial portion (Oxnard and Franklin, 2008; Lee et al., 2012), and it is much
250 smaller than in *Pan troglodytes*, with an average weight of only 34.79 grams (Taylor
251 and Vinyard, 2013). This reduced size is the result of a generalized process of reduction
252 of the masticatory apparatus during the evolution of the genus *Homo* (Aiello and Dean,
253 1990). Stedman et al. (2004) attribute this reduction in size to a frameshifting mutation
254 in the MYH16 gene that inactivated the gene, causing it to lose its ability to encode the
255 MyHC-IIM protein isoform. The loss of this protein is associated with a marked
256 reduction in the size of type II fibers and in the overall size of the masticatory muscles
257 (Stedman et al., 2004).

258 We observed a clear predominance of the mRNA transcripts of the MyHC-II
259 isoforms (>70%) in the temporalis muscle of our *Pan troglodytes* specimens. This
260 expression pattern is to be expected in a muscle that produces the force necessary for
261 the chimpanzee to chew food. The muscular force needed for the dietary habits and life
262 style of *Pan troglodytes* is reflected in the high percentage of expression of the MyHC-
263 IIM isoform, especially in the anterior superficial temporalis (47.0%). The MyHC-IIM
264 isoform has been shown to have a high ATPase activity, which is related to the capacity

of type IIM muscle fibers to produce a contractile force greater than that produced by type IIX fibers (Hoh, 2002; Toniolo et al., 2008).

The functional differences between the anterior and posterior temporalis observed in electromyographic studies of humans and other primates (Blanksma et al., 1997; Ross and Hylander, 2000; Hylander et al., 2005; Vinyard et al., 2008; Wall et al., 2008) are reflected in the mRNA transcripts of the MyHC isoforms in our *Pan troglodytes* specimens. Although the differences were not significant, the MyHC-IIM isoform was expressed at a higher percentage in the anterior than in the posterior superficial temporalis (47.0% vs. 40.5%), while the MyHC-I isoform was expressed at a higher percentage in the posterior than in the anterior superficial temporalis (26.9% vs. 19.9%). While the MyHC-IIM isoform is related to greater muscle force, the MyHC-I isoform is related to a lesser degree of muscle force, a slower contraction velocity and a greater resistance to fatigue. These characteristics are in line with the function of the posterior superficial temporalis in recentering the mandible that has been observed in electromyographic studies (Hylander et al., 2005). The functional differences observed in electromyographic studies may also be related to structural differences between the anterior and posterior temporalis identified in humans. The anterior temporalis has a greater physiological cross sectional area than the posterior temporalis, which is related to its greater capacity of force production (Van Eijden et al., 1997). Interestingly, there were no differences between the anterior and posterior deep temporalis in the expression patterns of the MyHC isoforms.

The sphenomandibularis is considered by some authors to be an independent muscle in humans (Dunn et al., 1996), while others consider it to be the deep bundle of the temporalis (Türp et al., 1997; Shimokawa et al., 1998; Schön-Ybarra and Bauer, 2001; Geers et al., 2005; Sedlmayr et al., 2009). In our five chimpanzees, we were able

to identify a clearly differentiated sphenomandibularis separate from the deep temporalis. However, the innervation of the sphenomandibularis in our specimens was not separate from the anterior temporalis; rather, it was innervated by a branch of the anterior deep temporal nerve (Figure 4). The mRNA transcripts of the MyHC-IIM isoform was significantly lower in the sphenomandibularis than in the anterior superficial temporalis (33.6% vs. 47.0%, P=0.032) and in the anterior deep temporalis (33.6% vs. 43.0%, P=0.016). This expression pattern suggests that the sphenomandibularis has less capacity of force production, which may be related to its role in the lateral movement and stabilization of the mandible observed in electromyographic studies in *Homo sapiens* (Wood, 1986; Fuentes et al., 2012).

Our findings on the mRNA transcripts of the MyHC isoforms in *Pan troglodytes* are markedly different from those of other investigators analyzing the percentages of different types of muscle fibers by histochemistry and immunohistochemistry in *Macaca mulatta* (Maxwell et al., 1979; Miller and Farias, 1988), *Macaca irus* (Rowlerson et al., 1983), *Cebus apella* (Andreo et al., 2002), and *Papio anubis* (Wall et al., 2013). These differences may be due to the presence of hybrid fibers expressing more than one MyHC isoform, which can hinder the determination and quantification of each type of fiber (Pette and Staron, 2000; Korfage and Van Eijden, 2003). Moreover, the studies using ATPase staining did not identify or quantify type IIM fibers (Maxwell et al., 1979; Miller and Farias, 1988; Andreo et al., 2002), while in the study by Wall et al. (2013) using SDS-PAGE, the MyHC-IIX isoform was not detected. The MyHC-IIX isoform had the lowest percentage of expression in our specimens, which may have limited the capacity of SDS-PAGE to detect it.

In summary, when comparing the sphenomandibularis with the anterior superficial and deep temporalis, we found significant differences in the mRNA

transcripts of the MyHC-IIM isoform, which may be related to the specific functional role of the sphenomandibularis observed in electromyographic studies of other species of primates. Moreover, we identified differences in the pattern of the mRNA transcripts of the MyHC isoforms between the anterior and posterior superficial temporalis that may also be related to the functional differences observed in electromyographic studies of other species of primates. The fact that the differences we observed are not statistically significant may be due to the small number of specimens included in our study, which in turn is the result of the difficulty inherent in obtaining cadavers of chimpanzees with undamaged temporalis muscles. Further studies with larger samples and other analytical techniques are warranted to determine if the functional differences between the anterior and posterior superficial temporalis are due to molecular or structural factors or both.

5. CONCLUSIONS

The use of RT-qPCR to quantify the mRNA transcripts of the MyHC isoforms has enabled us to identify differences between the portions of the temporalis muscle in *Pan troglodytes* that may be related to functional differences observed in other species of primates in electromyographic studies. These findings provide further knowledge of the anatomical and functional characteristics of this muscle in the primate that is phylogenetically most closely related to *Homo sapiens*. Our results can thus be applicable to the fields of comparative anatomy, evolutionary anatomy, and anthropology.

ACKNOWLEDGMENTS

We thank Renee Grupp for assistance in drafting the manuscript. We would also like to thank the two anonymous reviewers for their helpful comments and suggestions, which have greatly improved the manuscript.

FUNDING

This study was supported by the Ministerio de Economía y Competitividad of Spain (projects CGL2014-52611-C2-1-P to APP and CGL2014-52611-C2-2-P to JMP) and by the European Union (FEDER). The funding source had no involvement in the conduct of the research or preparation of the article.

ETHICAL NOTE

The research complied with protocols approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and adhered to the legal requirements of Spain.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Dissection of the superficial (a) and deep (b) temporalis muscle in a male *Pan troglodytes*.

Figure 2. Dissection of the sphenomandibularis portion of the temporalis in a female *Pan troglodytes*.

Figure 3. Detailed image of the origin and insertion point of the sphenomandibularis. The origin is in the temporal surface of the greater wing of the sphenoid bone (a) and the insertion is in the temporal crest of the mandible (b).

Figure 4. Dissection of a female *Pan troglodytes* showing the innervation of the sphenomandibularis by a branch of the anterior deep temporal nerve. (a) sphenomandibularis; (b) medial pterygoid muscle; (*) anterior deep temporal nerve.

TABLE LEGENDS

Table 1. Characteristics and sources of specimens included in the study.

Table 2. Means and standard deviations (SD) of the expression of the MyHC isoforms mRNA transcripts in the different portions of the temporalis muscle of *Pan troglodytes* (AST=anterior superficial temporalis; PST=posterior superficial temporalis; ADT=anterior deep temporalis; PDT=posterior deep temporalis; SMP=sphenomandibularis portion).

Table 3. Statistical significance (P) of the expression of the MyHC isoforms mRNA transcripts in the different portions of the temporalis muscle of *Pan troglodytes* (AST=anterior superficial temporalis; PST=posterior superficial temporalis; ADT=anterior deep temporalis; PDT=posterior deep temporalis; SMP=sphenomandibularis portion). * Statistical significance at $P<0.05$