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

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24 **Running title:** Masticatory muscles of chimpanzees

25 **ABSTRACT**

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

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

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

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

45

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

47 **INTRODUCTION**

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

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

73 a fast contractile speed but lower resistance to fatigue. The MyHC-IIx isoform is  
74 associated with muscles of greater force, faster speed, and lower resistance than the  
75 MyHC-IIa isoform (Bottinelli, Pellegrino, Canepari, Rossi, & Reggiani, 1999; Pette &  
76 Staron, 2000). Besides these three MyHC isoforms, masticatory muscles express the  
77 MyHC-IIIM isoform (Rowlerson, Mascarello, Veggetti, & Carpena, 1983; Stedman et al.,  
78 2004), which is associated with a moderate contractile speed (similar to that of MyHC-  
79 IIa) and greater force (Hoh, 2002; Toniolo et al., 2008).

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

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

99         Few electromyographic or molecular studies have examined the masseter muscle  
100 in *Pan troglodytes*, a hominid that is phylogenetically closely related to *Homo sapiens*.  
101 In fact, to the best of our knowledge, only one study (Rowlerson et al., 1983) has analyzed  
102 MyHC isoforms in the masseter of *Pan troglodytes*. In this study, ATPase staining  
103 identified 31-57% of type I fibers, which express only the MyHC-I isoform, and 43-69%  
104 of type IIM fibers, which express only the MyHC-IIM isoform. However, this study did  
105 not analyze the superficial and deep portions of the masseter separately, nor did it examine  
106 the medial pterygoid. Moreover, the authors did not report information on hybrid fibers  
107 that can express different types of MyHC isoforms. In other species of primates, however,  
108 electromyographic studies have identified functional differences between the superficial  
109 and deep portions of the masseter (Basmajian & de Luca, 1985; Blanksma et al., 1997;  
110 Guzmán-Venegas et al., 2015; Hylander et al., 2000; Vinyard et al., 2008) and between  
111 the anterior and posterior regions of the two portions in humans (Basmajian & de Luca,  
112 1985; Blanksma et al., 1997; Guzmán-Venegas et al., 2015). In contrast, no functional  
113 differences were detected between the masseter and the medial pterygoid muscles – either  
114 in humans (Basmajian & de Luca, 1985; Schindler et al., 2006) or in non-hominid  
115 primates (Hylander et al., 1987; Wall et al., 2006; Williams et al., 2011).

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

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

## 125 **MATERIALS & METHODS**

### 126 **Muscle samples**

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

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

### 138 **RNA isolation and cDNA synthesis**

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

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

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

### 149 **Gene expression and quantification by RT-qPCR**

150 Applied Biosystems supplied primers and probes. Primers are labeled at the 5' end  
151 with the reporter dye molecule FAM. MYH-I (Hs00165276\_m1), MYH-IIa  
152 (Hs00430042\_m1), MYH-IIx (Hs00428600\_m1) and MYH-IIM (Hs01385213\_m1)  
153 genes were analyzed. We used 18s gene probe labeled at the 5' end with the reporter dye  
154 molecule FAM (Hs99999901\_s1) as housekeeping gene.

155 We performed RT-qPCR in a total volume of 20 µl in the ABI Prism 7700  
156 Sequence Detection System (Applied Biosystems) using the following master mix  
157 conditions: 10 µl of the TaqMan Universal PCR Master Mix, 1 µl of the primers and  
158 probes, 2 µl of the cDNA and 7 µl of the RNase-free water. We ran all samples for each  
159 gene in duplicate using this thermal cycler conditions: two min 50 °C, 40x (10 min 95 °C,  
160 15 s 95 °C) and 1 min 60 °C. We used genomic DNA as negative control in each run. We  
161 captured fluorescent emission data and quantified mRNA concentrations by using the  
162 critical threshold value and  $2^{-\Delta\Delta C_t}$ . In order to avoid any possible effects of post-mortem  
163 mRNA degradation, the mRNA values for each of the MyHC isoforms were normalized  
164 using the endogenous gene 18S, which is preserved intact for up to eight days after death  
165 in skeletal muscle (Bahar et al., 2007).

166 Finally, we calculated the percentage of expression of each MyHC isoform mRNA  
167 transcript relative to the total expression of all MyHC isoforms (%MyHC-I, %MyHC-IIa,  
168 %MyHC-IIx and %MyHC-IIM).



169 **Statistical analyses**

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

173 **Ethical note**

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

177 **RESULTS**

178 The mean mass of the masseter was 2.6 times greater than that of the medial  
179 pterygoid (62 vs 24 g) (Table 1). The mean mass of the superficial portion was 2.8 times  
180 greater than that of the deep portion (45.5 vs 16.5 g). The superficial portion accounted  
181 for 73.4% and the deep portion for 26.6% of the total mass of the masseter. The superficial  
182 portion accounted for 52.9%, the deep portion for 19.2%, and the medial pterygoid for  
183 27.9% of the total mass of the muscles analyzed.

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

192 expression of MyHC-IIIM 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-IIIM, associated with a great contractile force (Hoh, 2002; Rowleson  
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, Bittleter, Eriksson, & Ringqvist, 1984) identified a more heterogeneous

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

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

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

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

291 **Conclusions**

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

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308 **CONFLICT OF INTEREST**

309 The authors declare that they have no conflict of interest.

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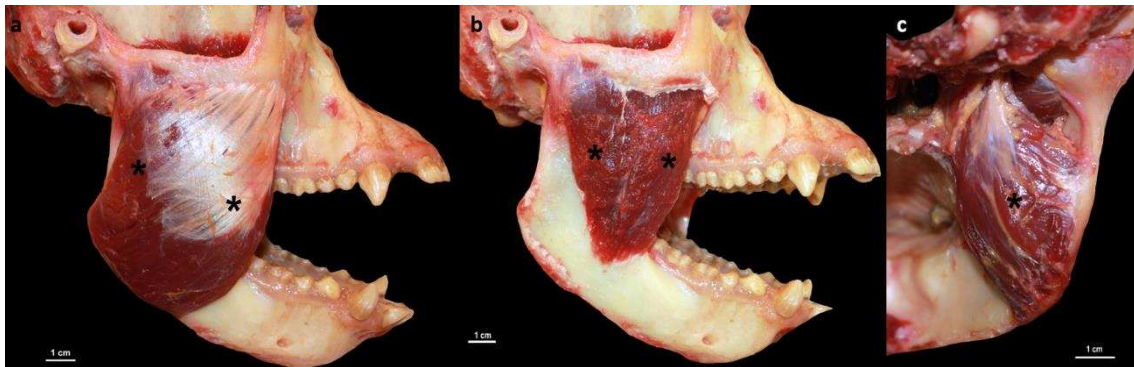
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440 **FIGURE LEGEND**

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

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456 **TABLE LEGEND**

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

460

461 **Table 1.**

<b>Muscle</b>	<b>Weight mean (SD)</b>	<b>%MyHC-I mean (SD)</b>	<b>%MyHC-IIa mean (SD)</b>	<b>%MyHC-IIx mean (SD)</b>	<b>%MyHC-IIIM mean (SD)</b>
<b>Masseter</b>	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)
<b>Medial pterygoid</b>	24 (8.6)	22.2 (3.0)	28.3 (1.4)	11.8 (7.4)	37.7 (8.1)

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