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Title: Quantification of myosin heavy chain isoform mRNA transcripts in the supraspinatus muscle of vertical clinger primates

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Running title: Supraspinatus in vertical clingers

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ABSTRACT

Vertical clinging is a specialized form of locomotion characteristic of the primate family Callitrichidae. Vertical clinging requires these pronograde primates to maintain a vertical posture, so the protraction of their forelimbs must resist gravity. Since pronograde primates usually move as horizontal quadrupeds, we hypothesized that the supraspinatus muscle of vertical clingers would present specific characteristics related to the functional requirements imposed on the shoulder area by vertical clinging. To test this hypothesis, we quantified by real time quantitative polymerase chain reaction the mRNA transcripts of myosin heavy chain isoforms in the supraspinatus muscle of fifteen species of pronograde primates, including vertical clingers. Our results indicate that the supraspinatus of vertical clingers has a specific pattern of expression of the myosin heavy chain isoforms, with a low expression of the transcripts of the slow MHC-I isoform and a high expression of the transcripts of the fast MHC-II isoforms. We conclude that these differences can be related to the particular functional characteristics of the shoulder in vertical clingers, but also to other anatomical adaptations of these primates, such as their small body size.

Keywords: supraspinatus, myosin heavy chain, vertical clinging
INTRODUCTION

Vertical clinging [Napier and Walker 1967] refers to a specialized form of locomotion and posture that is characteristic of the primate family Callitrichidae [Gebo 2014]. The Callitrichidae comprises the smallest of the New World primates, including marmosets (genus Callimico, Cebuella and Callithrix) and tamarins (genus Leontopithecus and Saguinus) [Rowe 1996]. The callitrichids are pronograde primates whose main locomotor modes are quadrupedal walking/running and leaping [Fleagle 1999]. They are also able to cling to thick tree trunks when collecting the insects, gums and other tree secretions that constitute a fundamental part of their diet [Garber 1992; Schmitt 2003; Gebo 2014]. The anatomic adaptations that enable this particular form of locomotion include the development of claw-like nails on their fingers and toes, except the hallux, which allow them to cling to large vertical supports [Garber 1992; Porter 2004].

Vertical clinging is used to a greater or lesser extent by different species of callitrichids. For example, the smaller callitrichids, such as Cebuella pygmaea, use vertical clinging approximately 65.5-89.6% of the time [Youlatos 1999; Jackson 2011]. Other callitrichids, such as Callimico goeldii, also use vertical clinging as much as 45% of the time, mostly on thinner vertical supports while gathering insects [Porter 2004]. In contrast, callitrichids of the genus Saguinus use vertical clinging only 23% (Saguinus fuscicollis) or 3% (Saguinus labiatus) of the time [Porter 2004].

The vertical clinging locomotion of the callitrichids is interesting from both an anatomic and a functional point of view, since it requires a vertical posture in primates with a clear pronograde anatomy. The anatomical differences between pronograde and
orthograde primates [Gebo 2014] affect the muscles of the shoulder, especially the supraspinatus. The supraspinatus muscle arises from the supraspinatus fossa of the scapula and inserts in the greater tubercle of the humerus, passing between the acromion and the glenohumeral joint. Together with the subscapularis, infraspinatus and teres minor muscles, it forms the rotator cuff, which is the main stabilizing structure of the glenohumeral joint. In pronogrades (Figure 1), the supraspinatus is an antigravitational muscle that prevents the collapse of the glenohumeral joint in retraction [Larson and Stern 1989; Larson and Stern 1992; Anapol and Gray 2003; Preuschoft et al. 2010]. In the orthogrades (superfamily Hominoidea), the supraspinatus helps the deltoid during the elevation of the forelimb in the scapular plane [Inman et al. 1944; Tuttle and Basmajian 1978; Basmajian and de Luca 1985; Larson and Stern 1986; Alpert et al. 2000].

The functional characteristics of the supraspinatus, such as speed of contraction, power, and resistance to fatigue, are also reflected in the pattern of expression of myosin heavy chain (MHC) isoforms [Bottinelli and Reggiani 2000]. The skeletal muscles of adult mammals express three types of MHC isoforms in varying proportions: the slow MHC-I, the fast MHC-IIa, and the fastest MHC-IIx. The muscles of small mammals also express a fourth isoform, MHC-IIb [Baldwin and Haddad 2001; Toniolo et al. 2005; Kohn et al. 2007; Resnicow et al. 2010]. The muscle fibers that express the MHC-IIx isoform at higher levels are faster and more powerful but less resistant to fatigue than those that express the MHC-IIa isoform at higher levels, while those that express the MHC-I isoform at high levels are less fast and powerful but more resistant to fatigue [Bottinelli et al. 1999; Pette and Staron 2000]. In general, postural muscles primarily express the slow MHC-I isoform and, at lower levels, the MHC-IIa isoform,
but they do not express the fastest MHC-IIx isoform, while fast and powerful muscles express both fast isoforms – MHC-IIa and MHC-IIx [Baldwin and Haddad 2001]. Thus, the supraspinatus of pronograde primates does not express the fastest MHC-IIx isoform but has a high expression of the slow MHC-I isoform (about 50%), while the supraspinatus of orthograde primates expresses more than 60% of the two fast MHC-II isoforms, MHC-IIa and MHC-IIx [Potau et al. 2011]. These proportions of slow and fast MHC isoforms mirror the postural function of the supraspinatus in the pronogrades and its elevator function in the orthogrades.

Vertical clingers have specific anatomic adaptations that could affect the function of the supraspinatus as well as the expression of the MHC isoforms. For example, their small body size could well influence the expression of MHC isoforms, since studies in several species of animals have found that heavier weight is associated with higher expression of the slow MHC-I isoform and a lower expression of the fast MHC-II isoforms [Pette and Staron 2000]. In addition, the vertical posture required in vertical clinging means that the protraction of the glenohumeral joint must be carried out against gravity more frequently than in quadrupedal walking. Electromyographic studies in vervets (Cercopithecus aethiops) have found that, as occurs in orthograde primates, the supraspinatus muscle protracts the forelimb against gravity during voluntary overhead reaching and the swing phase of climbing, while it does not participate in the protraction of the forelimb during quadrupedal walking [Larson and Stern 1989]. We can therefore speculate that the expression patterns of MHC isoforms in vertical clingers are closer to those of arboreal than of terrestrial primates.

In order to increase our understanding of the role of the supraspinatus in vertical clingers, we have quantified in different primate species the mRNA transcripts of MHC
isoforms in the supraspinatus by real time quantitative polymerase chain reaction (RT-qPCR). We then compared our findings in vertical clingers with those in other pronograde primates with different types of locomotion (arboreal quadrupeds and semiterrestrial quadrupeds). We envisioned three possibilities: 1) that the supraspinatus of vertical clingers would be similar to that of pronograde primates, based on the pronograde body pattern of vertical clingers; 2) that it would be similar to that of orthograde primates, based on the vertical posture and the greater protraction of the forelimb against gravity; or 3) that it would be unique, adapted to the specific physical characteristics of vertical clingers, including their small body size.

METHODS

Supraspinatus muscle samples

We included six adult vertical clingers in the study (Table 1), all of which came from Spanish zoological parks and had died from causes unrelated to the present study. The cadavers were transferred to the Anatomy Museum of the University of Valladolid (Valladolid, Spain) and frozen without fixation until dissection. The six vertical clingers studied were from members of the family Callitrichidae: one female Cebuella pygmaea; one female Callithrix geoffroyi; one female Callithrix jacchus; one female Saguinus imperator; one female Saguinus oedipus; and one male Leontopithecus chrysomelas.

The same investigator (JMP) systematically dissected the rotator cuff of each primate. He weighed the supraspinatus and obtained samples of 2 mm³ with a scalpel of its central region with respect to the length, width and thickness of the muscle (Figure 2). These samples were then frozen in saline solution for 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 µl 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 25°C, 48 min 30 ºC and 5 min 95 ºC.

Gene expression and quantification by RT-qPCR

Applied Biosystems supplied primers and probes. We labeled primers at the 5’ end with the reporter dye molecule FAM. We analyzed MYH-I (Hs00165276_m1), MYH-IIa (Hs00430042_m1), MYH-IIx (Hs00428600_m1) and MYH-IIb (Hs00757977_m1) genes. 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 °C, 15s 95 °C) and 1 min 60 °C. 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−ΔΔCt. In order to avoid any possible effects of post-mortem mRNA degradation, we normalized the mRNA values for each of the MHC isoforms using the endogenous gene 18S.

Finally, we calculated the percentage of expression of the transcripts of each MHC isoform relative to the total expression of the transcripts of all MHC isoforms (%MHC-I, %MHC-IIa, %MHC-IIx, and %MHC-IIb) and compared the expression of the transcripts of the three fast MHC-II isoforms taken together (%MHC-II) to the expression of the transcripts of the slow MHC-I isoform (%MHC-I).

Comparison with other primate groups

We compared the findings of the present study in vertical clingers with those of two other primate groups: eight arboreal quadrupeds and seven semiterrestrial quadrupeds [Schmitt 2010] (Table 1). For this purpose, we used the results previously published by our team [Potau et al. 2011] and we added new results obtained from one male *Macaca fascicularis* (crab-eating macaque), one female *Miopithecus talapoin* (Angolan talapoin), one female *Saimiri sciureus* (common squirrel monkey) and one male *Cercocebus atys lunulatus* (white-collared mangabey). The first three of these new four specimens came from the Anatomy Museum of the University of Valladolid and the last came from the Barcelona Zoological Park (Barcelona, Spain).

Statistical analyses

We used the non-parametric Kruskall-Wallis one-way ANOVA test to compare the median values among the three primate groups and the Median Test within the
Kruskall-Wallis ANOVA for the pair-wise post-hoc comparisons; adjusted p-values were automatically provided by SPSS within the Kruskall-Wallis test. In addition, we analyzed the relation between the expression of the transcripts of the MHC isoforms and the species mean value of the log-transformed weight of the supraspinatus muscle (log-SUP) with the Spearman correlation coefficient test. We also derived a phylogenetic independent contrast [Garland et al. 1992] of supraspinatus weight versus the percentage of expression of the MCH-I isoform by species. The molecular phylogeny of the species considered was derived from the 10kTrees website [Arnold et al. 2010]. Rank-transformed phylogenetic contrasts were obtained and the relationship between the expression of the MHC isoforms and supraspinatus weight were computed again. We used SPSS 22 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 %MHC-I was 33.4% ± 7.2 in vertical clingers, compared to 40.7% ± 10.1 in arboreal quadrupeds and 49.5% ± 3.0 in semiterrestrial quadrupeds (Table 2). Overall comparisons showed significant differences among groups (K=10.475, P=0.005, N=21) for %MHC-I. However, pair-wise significant differences were only found between vertical clingers and semiterrestrial quadrupeds (χ²=9.551, P=0.002, adjusted P=0.006). In contrast, the differences observed in the expression of the mRNA
transcripts of the MHC-I isoform between arboreal quadrupeds and vertical clingers \( (\chi^2=1.167, \ P=0.280, \ \text{adjusted}-P=0.840) \) and between arboreal quadrupeds and semiterrestrial quadrupeds were not significant \( (\chi^2=3.233, \ P=0.072, \ \text{adjusted}-P=0.216) \).

All specimens expressed transcripts of the MHC-IIa isoform but none of them expressed transcripts of the MHC-IIx or MHC-IIb isoforms (Table 1). The mean %MHC-II was 66.6% ± 7.2 for the vertical clingers, 59.3% ± 10.1 for the arboreal quadrupeds, and 50.5% ± 3.0 for the semiterrestrial quadrupeds (Table 2). Overall comparisons showed significant differences among groups \( (K=10.475, \ P=0.005, \ N=21) \) for %MHC-IIa, but pair-wise significant differences were only found between vertical clingers and semiterrestrial quadrupeds \( (\chi^2=13.000, \ P=0.000, \ \text{adjusted} \ P=0.001) \) for %MHC-IIa. The differences observed in the expression of the mRNA transcripts of the MHC-IIa isoform between arboreal quadrupeds and vertical clingers \( (\chi^2=1.167, \ P=0.280, \ \text{adjusted} \ P=0.840) \) and between arboreal quadrupeds and semiterrestrial quadrupeds were non-significant \( (\chi^2=1.727, \ P=0.189, \ \text{adjusted} \ P=0.566) \).

Twenty of the 21 primates in the study had available data on the supraspinatus weight (SUP) (Table 1). For *Saimiri sciureus*, this information was not available. For these 20 primates, included in 14 different species, we observed a significant positive correlation between %MHC-I and log-SUP (Spearman correlation coefficient test: \( \rho=0.632, \ P=0.015, \ N=14 \)) (Figure 3). Molecular data on 10kTrees for the 14 species considered was used to derive the phylogenetic independent contrasts analysis. In this case, correlation between the two variables was not significant \( (\rho=0.456, \ P=0.117, \ N=14) \).
DISCUSSION

We found no significant differences in the expression pattern of the MHC-I and the MHC-II isoform transcripts between vertical clingers and arboreal quadrupeds, but in the former the percentage of expression of the MHC-I isoform transcripts was lower and the percentage of expression of the MHC-II isoforms transcripts was higher than in arboreal quadrupeds. On the other hand, the pattern of expression of the MHC-I and MHC-II isoforms transcripts in semiterrestrial quadrupeds was different from that of both arboreal quadrupeds and vertical clingers (Table 2), although these differences were only significant in comparison with vertical clingers. In vertical clingers, the %MHC-I mean was a low 33.4% while the %MHC-II mean – MHC-IIa – was a high 66.6%. These results contrast with those in arboreal quadrupeds (40.7% MHC-I and 59.3% MHC-II) and in semiterrestrial quadrupeds (49.5% MHC-I and 50.5% MHC-II), but are more similar to those in orthograde primates (30-40% MHC-I and 64.6% MHC-II) [Potau et al. 2011]. There is, however, an important difference in the MHC-II transcripts expression between vertical clingers and orthograde primates, since vertical clingers do not express the MHC-IIx transcripts. The low percentage of expression of the MHC-I transcripts and the high percentage of expression of the MHC-IIa transcripts in vertical clingers could be related to their posture, which necessitates a more frequent protraction of the forelimb against gravity than other pronograde primates. On the other hand, the high expression of the MHC-I transcripts in semiterrestrial quadrupeds confirms the important postural and anti-gravity function of the supraspinatus in this form of locomotion [Larson and Stern 1989; Larson and Stern 1992]. In comparison with the semiterrestrial quadrupeds, the arboreal quadrupeds had a lower %MHC-I and a higher %MHC-II (Table 2). These differences between arboreal and semiterrestrial
quadrupeds, though not statistically significant, may be due to the different functional demands of the supraspinatus in quadrupedal terrestrial and in arboreal climbing locomotion, as different electromyographic studies have shown [Larson and Stern 1989; Larson and Stern 1992]. These studies indicate that the supraspinatus is inactive during the swing phase of quadrupedal walking but is active during the swing phase of climbing.

One limitation of our study is that we were only able to analyze samples from the central region of the supraspinatus although the distribution of muscle fibers is generally heterogeneous in different regions of the same muscle. Schmidt and Schilling (2007) reported a heterogeneous distribution of type I fibers in the supraspinatus of Saguinus oedipus and Saimiri sciureus, with a larger proportion of type I fibers in the distal region of the muscle and near the scapular spine. However, in a previous study by our group using RT-qPCR, we found no significant differences in the expression patterns of the MHC isoforms in the different regions of the supraspinatus muscle in Macaca fascicularis or Gorilla gorilla [Potau et al. 2011].

The initial positive correlation between the weight of the supraspinatus and the %MHC-I (ρ=0.632, P=0.015) (Figure 3) suggests that the low %MHC-I observed in vertical clingers may also be related to its smaller body size. This is in line with previous findings showing an association between higher body weight and higher expression of the slow isoform and lower expression of the fast isoforms [Pette and Staron 2000]. Nevertheless, when correcting for phylogenetic non-independence of data points the association becomes not significant. This could be either due to the reduced sample size available or to the fact that the myosin expression-supraspinatus weight relationship has a strong phylogenetic signal. Although it is true that the smaller
supraspinatus muscles had the lowest expression of MHC-I (Figure 3), there was a great deal of dispersion among the species studied and not all the “small” muscles showed low expression levels of MHC-I transcripts, and some outliers for the weight might be distorting the distribution (which was corrected by the rank-transformation of data). Since we did not know the total body weight of our specimens, we used the weight of the supraspinatus muscle as an indication of body weight. Our results should therefore be interpreted with caution since muscle weight depends on other anatomical and functional factors, although it is generally related to body weight. In addition, the fact that our specimens were all from zoos limits the generalization of our results, since patterns of locomotion and behavior of captive primates may well differ from those of primates living in the wild.

In summary, the supraspinatus of vertical clingers shares characteristics with that of other pronograde primates using different forms of locomotion, such as the lack of expression of the MHC-IIx transcripts. At the same time, other characteristics of the supraspinatus of vertical clingers differ from that of other pronogrades, although its pattern of expression does not present significant differences with respect to that observed in arboreal quadruped primates. For example, the supraspinatus of vertical clingers expressed the transcripts of the slow MHC-I isoform at low percentages and the transcripts of the fast MHC-II isoform at high percentages. These differences can be related to the vertical posture of vertical clingers in general and to a smaller body size.

**CONFLICT OF INTEREST**

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

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TABLE LEGENDS

Table 1. Means of the supraspinatus weight and of the mRNA transcripts expression of MHC isoforms in the supraspinatus muscle (SUP=supraspinatus weight in grams; VC=vertical clinger; AQ=arboreal quadruped; STQ=semiterrestrial quadruped).

* Potau et al., 2011
NA = not available

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<th>SPECIES</th>
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Table 2. Means, standard deviations (SD) and statistical significance (pair-wise post-hoc adjusted non-parametric P-value) of the expression of the MHC isoforms mRNA transcripts in the supraspinatus muscle of the three locomotor groups analyzed (VC=vertical clinger; AQ=arboreal quadruped; STQ=semiterrestrial quadruped).

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</table>
FIGURE LEGENDS

Figure 1. Patterns of locomotion of (a) pronograde and (b) orthograde primates, highlighting the location of the supraspinatus muscle (*).
Figure 2. Dissection of the muscles of the rotator cuff in a vertical clinger primate (*Callithrix jacchus*). a) Ventral view (1=subscapularis; 2=teres major; 3=latissimus dorsi; 4=biceps brachii; 5=triceps brachii); b) Dorsal view (1=supraspinatus; 2=infraspinatus; 3=teres minor; 4=teres major; 5=latissimus dorsi; 6=triceps brachii (caput longum); 7=triceps brachii (caput laterale); 8=brachialis; 9=biceps brachii; 10=humerus). * Location where muscle samples were obtained.
Figure 3. Regression plot between the log-transformed supraspinatus weight (log-SUP) and the percentage of expression of the MHC-I isoform mRNA transcripts (%MHC-I) in 14 primate species.