Of Mice, Monkeys, and Men: Physiological and Morphological Evidence for Evolutionary Divergence of Function in Mimetic Musculature

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ABSTRACT

Facial expression is a universal means of visual communication in humans and many other primates. Humans have the most complex facial display repertoire among primates; however, gross morphological studies have not found greater complexity in human mimetic musculature. This study examines the microanatomical aspects of mimetic musculature to test the hypotheses related to human mimetic musculature physiology, function, and evolutionary morphology. Samples from the orbicularis oris muscle (OOM) and the zygomaticus major (ZM) muscle in laboratory mice ($N = 3$), rhesus macaques ($N = 3$), and humans ($N = 3$) were collected. Fiber type proportions (slow-twitch and fast-twitch), fiber cross-sectional area, diameter, and length were calculated, and means were statistically compared among groups. Results showed that macaques had the greatest percentage of fast fibers in both muscles (followed by humans) and that humans had the greatest percentage of slow fibers in both muscles. Macaques and humans typically did not differ from one another in morphometrics except for fiber length where humans had longer fibers. Although sample sizes are low, results from this study may indicate that the rhesus macaque OOM and ZM muscle are specialized primarily to assist with maintenance of the rigid dominance hierarchy via rapid facial displays of submission and aggression, whereas human musculature may have evolved not only under pressure to work in facial expressions but also in development of speech. Anat Rec, 00:000–000, 2014.

Key words: facial muscle; orbicularis oris; zygomaticus major; speech; fiber type

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INTRODUCTION

The human face has long been regarded as unique relative to the faces of all other mammals, even among other members of the order Primates (Darwin, 1872; Huber, 1931; Schmidt and Cohn, 2001; Burrows, 2008; Burrows and Cohn, in press). Within humans, face recognition is associated with an assortment of cognitive and neural specializations, suggesting that it has played an important role in our evolution (van Hooff, 1962, 1972; Parr and de Waal, 1999; Parr et al., 2000; Sherwood et al., 2005; Taubert, 2010; Dobson and Sherwood, 2011a,b; Parr, 2011). The human face is the primary mechanism of our social interactions and person identification (Ekman, 1973; Ekman and Keltner, 1997; Schmidt and Cohn, 2001; Burrows and Cohn, in press) and is itself a visible signal of social intentions, motivations, and kin/individual identity (Schmidt and Cohn, 2001; Burrows, 2008). Clearly, the structural integrity of the human face is a hallmark of our species, and we depend on it for normal social interactions and a normal human lifestyle.

Facial expressions and movements are controlled by facial expression (mimetic) musculature. Mimetic muscles are branchiomeric in nature, being derived from the second (or hyoid) branchial arch, and are, as such, innervated by the seventh (facial) cranial nerve (Young, 1957; Sperber, 2001). This musculature is both morphologically and physiologically unique when compared with the striated musculature of the limbs and trunk (Standring, 2008). Unlike other striated muscles that typically attach to bony landmarks, mimetic musculature attaches into the dermis of the face, neck, and external ears. When the musculature contracts, it deforms the facial mask, producing actions associated with facial expression of emotion or intent as well as functions related to nutrient intake and vocalizations/speech. Human mimetic musculature is dominated by Type II (fast-twitch) fibers to a much higher percentage than the fast/slow distribution found in limb and trunk musculature (Schwarting et al., 1982; Stål, 1987, 1990; Happak et al., 1988; Freilinger et al., 1990; Cheng et al., 2007). This unique physiological attribute may assist in the production of spontaneous facial expressions comprising quick muscle contractions that last only a few seconds (Ekman and Friesen, 1982; Schmidt et al., 2003).

Numerous recent studies have shown that the gross anatomical aspects of human facial expression musculature, including number of muscles and attachments, are not so derived when compared with other primates (Burrows and Smith, 2003; Burrows et al., 2006, 2009, 2011; Burrows, 2008; Diogo and Wood, 2012). Yet, humans clearly have a uniquely wide range of graded, salient facial expressions and have a greater expertise at facial processing than any other primate investigated to date (van Hooff, 1962; Dobson, 2009; Parr et al., 2010; Parr, 2011; Waller et al., 2012; Caeiro et al., 2013). At the microanatomical level, previous comparative and functional studies of the orbicularis oris muscle (OOM) in humans, chimpanzees, and gibbons showed that the arrangement of muscle fibers and muscle fiber morphometrics are reflective of the evolutionary divergence of lip function among these taxa (Rogers et al., 2009; Burrows et al., 2011). Similarly, Burrows and Smith (2003) demonstrated that muscles of the external ear in galagos are microanatomically arranged more tightly with less connective tissue than musculature associated with other regions of the face. Galagos are noted in part for their discrete and complex external ear movements (e.g., Charles-Dominique, 1977). Unmistakably, aspects of mimetic musculature including muscle fiber type, fiber cross-sectional area, diameter, and length could assist our efforts at conceptualizing the functional and evolutionary differences between human facial expression musculature and that of other primates.

The contractile properties of any skeletal muscle depend largely on the proportion of slow-twitch to fast-twitch fibers. Generally, slow-twitch (Type I) fibers are slow to generate a contraction but have a high level of endurance and fatigue slowly. Fast-twitch (Type II) fibers quickly generate a contraction but have a low level of endurance and resist fatigue poorly. The ability of a muscle to do work is reflected in part by the cross-sectional area, fiber diameter, and length of the muscle fibers (Bodine et al., 1982; Gans, 1982; Lieber and Fridén, 2000; Eng et al., 2008). The length of a muscle fiber can be increased by adding sarcomeres so that a longer fiber can contract at a higher velocity (Lieber, 2002; Lieber and Fridén, 2000). Therefore, fiber length can inform our understanding of the potential speed at which a muscle can contract. Muscles that undergo repeated increased use can experience hypertrophy, and this can be accomplished by increasing the size and amount of contractile proteins within the myofibrils. This increases muscle fiber diameter and cross-sectional area, which can themselves inform our understanding of the potential contraction force that a muscle can produce (Lieber and Fridén, 2000; Paul and Rosenthal, 2002).

The relationship between muscle function and fiber type distribution is well known in mammals, and most of our understanding of this relationship comes from studies of limb and paravertebral musculature. Mammals that move quickly and engage in terrestrial quadrupedal postures tend to have limb and spinal musculature dominated by Type II (fast-twitch) fibers, whereas mammals that move slowly and engage in suspensory/bridging behaviors tend to have musculature dominated by Type I (slow-twitch) fibers (Schilling, 2005; Schmidt and Schilling, 2007; Kohn et al., 2011; Myatt et al., 2011; Curry et al., 2012). These sorts of relationships are unknown in mimetic musculature; however, a similar insight would be helpful in understanding how human facial expression muscles vary with respect to those of other primates and other mammals, the evolution of human facial expression and speech/language, and would even be useful in optimizing animal models of human disease, disorders, and associated surgical interventions such as Parkinson's disease, autism, and face transplants.

Animal models for most of these disorders and interventions use murids (rats and mice) or rhesus macaques. Previous studies have documented relatively great complexity in rhesus macaque facial displays and the corresponding mimetic musculature but not in rats/mice. It is largely unknown whether murids, or even rhesus macaques, represent adequate models of the complex function of human mimetic musculature and facial displays. By examining the aspects of mimetic musculature physiology and morphometrics at the microanatomical level, this study aims to address this question.

Hypotheses

This study examines the relationship between fiber type characteristics and mimetic musculature function using a murid (the laboratory mouse, *Mus musculus*), an Old World monkey (the rhesus macaque, *Macaca mulatta*), and humans. The mouse and macaque are species often used as experimental models of human diseases, disorders, and in surgical interventions. They also represent broadly separated phylogenetic positions relative to one another and to humans, and they occupy varied social and ecologic niches relative to humans. Mice (Class Rodentia) are nocturnal, small-bodied (about 20 g), and can live in large social groups; however, they have not been documented to use facial expressions as a means of social communication. Instead, they rely heavily on olfactory communication and "face touch," tactile sensory exploration via the vibrissae (e.g., Vander Wall et al., 2003; Wilson and Reeder, 2005; Merritt, 2010). Rhesus macaques are phylogenetically much closer to humans, are diurnal, medium-sized (about 7,700 g), live in complex, large groups, and, like humans, rely heavily on facial expression as a means of social communication but not, reportedly, to the same extent as in humans (Thierry, 1990, 2000; Fooden, 2000; Parr et al., 2010).

To better understand the physiological and morphological specializations at the microanatomical level associated with human mimetic musculature function, we test the following hypotheses related to mimetic muscle physiology.

Hypothesis 1: Fiber type proportions. Because of the frequent use of facial expressions of emotion in humans and to a lesser extent in rhesus macaques (Parr et al., 2010), we hypothesize that humans will have the significantly ($P < 0.05$) greatest proportion of fast-twitch fibers followed by rhesus macaques (human > macaque > mouse). Because facial expressions occur rapidly and last only a few seconds (Ekman and Friesen, 1982; Schmidt et al., 2003; Parr et al., 2010), we further hypothesize that there will be no significant difference ($P > 0.05$) among the groups in percentage of slow-twitch muscle fibers among groups.

Hypothesis 2: Fiber morphometrics. Because of the frequent use of facial expressions of emotion in humans and to a lesser extent in rhesus macaques (Parr et al., 2010), we hypothesize that humans will have significantly greater cross-sectional area, diameter, and length of fast-twitch fibers than macaques and macaques will have greater values than mice (human > macaque > mouse). Furthermore, we hypothesize that there will be no significant difference in cross-sectional area, diameter, and length of slow-twitch fibers (human = macaque = mouse). If mimetic muscle morphometrics are not adaptive but are instead influenced primarily by body size differences, then we hypothesize that there will be significant differences in morphometrics of slow-twitch fibers in all cases with humans > macaques > mice.

MATERIALS AND METHODS

Specimens and Muscle Samples

To test the hypotheses, two mimetic muscles were sampled. The OOM (upper fibers) and the zygomaticus major (ZM) muscle were selected because of their function in facial expressions documented for the rhesus macaque and humans and the ability to isolate them for sampling in all three study taxa. These muscles are both involved in generating the human smile and in supporting the structural integrity of the lips, typical targets of biomedical research that include appearance and function of the face. Sections from the upper lip and the ZM muscle with overlying skin and dermis were sampled from humans ($N = 3$), rhesus macaques ($N = 3$), and mice ($N = 3$). All human specimens were gathered from cadavers at the Duquesne University and the Slippery Rock University Gross Anatomy Laboratories and had been fixed by the Human Gifts Registry programs using a variety of formaldehyde-based methods. Only cadavers that had teeth intact were chosen because of the possibility of muscle atrophy of the OOM in individuals with missing teeth. Rhesus macaque specimens were obtained from the Yerkes National Primate Research Center after the animals died from natural causes. Wild-type mouse cadavers were obtained from the School of Pharmacy at Duquesne University. These animals were euthanized as part of a different study that was not expected to contribute confounding factors to the current study. All macaque and mouse specimens were fixed with 10% buffered formalin. No specimen in this study had been previously frozen or fixed in alcohols.

The OOM samples were derived from the right side of the upper lip immediately inferior/rostral to the nostril (Fig. 1). This location represents an area where no other musculature is blending into the upper fibers of the OOM, and thus, we were able to obtain samples that only contained the OOM (see Rogers et al., 2009). The ZM muscle and overlying tissue were sampled at a point mid-way along the muscle (Fig. 1). This again represented an area that contained only ZM fibers.

For all specimens, serial ethanols were used to dehydrate the formalin-fixed muscles that were then clarified with xylene and infiltrated with paraffin wax. Tissue samples were embedded in paraffin for sectioning on a traditional microtome in both sagittal and transverse orientations. Serial sections were cut from each sample at 6–10 μm, and every fifth section was mounted on Superfrost Plus slides (Fisher Scientific) with 150–300 sections generated from each muscle in each specimen. Using this methodology generated sections that were representative of the entire muscle, not just one end of the sectioning block. Because of the varying length of time each sample had been exposed to formalin, there was concern that excessive hardening of tissue could cause some difficulty in achieving representative cross sections of muscle fibers. To mitigate possible artifact from the lengthy fixation in formalin that some of the muscle samples experienced, many sections from various depths were analyzed for each muscle sample. Additionally, such differing fixation times would make consistent identification of fiber subtypes unreliable in these samples.

Immunohistochemistry

Immunohistochemistry was used to differentiate slow-twitch fibers (Type I) and fast-twitch fibers (Type II). Mouse monoclonal antibodies, raised against human or rabbit muscle myosin, were used as primary antibodies to slow myosin (ab11083, Clone NOQ7.5.4D; Abcam) and

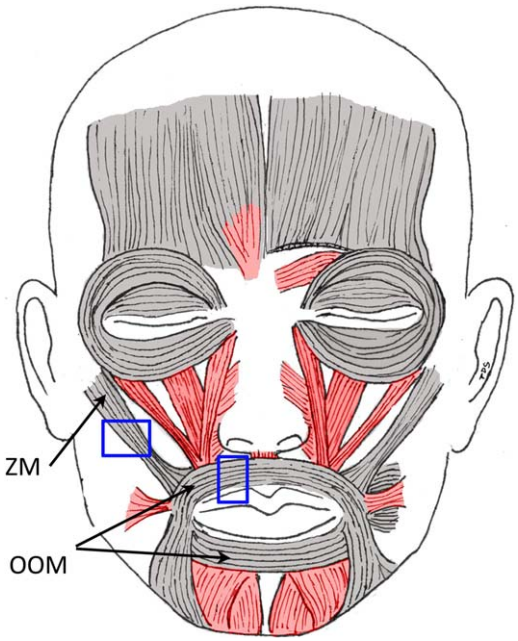

Fig. 1. Original line drawing of human facial expression musculature showing the two sample sites for the zygomaticus major (ZM) muscle and the orbicularis oris (OOM) muscle. The areas captured by the blue rectangles indicate the position of sampling. Although this diagram shows a human only, it is representative of the relative locations of sampling for the rhesus macaque and mouse as well. Note that these areas of sampling exclude other facial expression muscles from the sample.

fast myosin (ab7784, Clone MY-32; Abcam). A random selection of 3–5 slides per individual containing three to four muscle sections per slide were chosen for immunohistochemistry using each primary antibody. This yielded 27–60 sections for each muscle in each of the study groups.

To prepare tissues for immunohistochemistry, sections were immersed in xylene to remove the paraffin, and then sections were rehydrated to distilled water using graded alcohols. Sections were then subjected to enzymatic retrieval with 0.5% trypsin in water for 15 min at 37°C for slow myosin or to an overnight epitope retrieval with Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) at 60°C for fast myosin staining. Endogenous peroxidase activity was blocked by 0.9% hydrogen peroxide in methanol for 20 min at room temperature. Sections were then pretreated with 5% normal goat serum in phosphate-buffered saline (PBS) for 20 min at room temperature. The primary antibodies to slow myosin (1:2,000) and fast myosin (1:1,500) were diluted in 5% normal goat serum and were incubated on sections overnight at 4°C in a humidified chamber. After three washes with PBS, biotinylated goat anti-mouse antibody diluted 1:200 in 5% normal goat serum was applied for 60 min at room temperature. Sections were again washed three times with PBS and then were incubated with Vectastain ABC reagent (Vector Laboratories) for 30 min at room temperature. Finally, sections were exposed to 3,3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) for 2 min. The reaction was stopped with water, and the sections were dehydrated in graded ethanol washes, cleared in xylenes, and mounted with permount (Fisher Scientific).

For the mouse muscle sample sections, the same antibody-specific enzymatic retrieval (slow myosin) and epitope retrieval (fast myosin) methods were used. Once the antigen retrieval steps were accomplished, sections were washed in tap water, and then endogenous peroxidase was blocked via incubation with 3% hydrogen peroxide in water for 5 min at room temperature. After two washes in PBS, sections were incubated with MOM mouse IgG blocking reagent (Vector Laboratories) overnight at 4°C in a humidified chamber. The MOM mouse IgG blocking reagent was replaced after 12 h for another 30-min incubation at room temperature. Mouse muscle tissue sections were then washed twice with PBS and then incubated at room temperature for 5 min with MOM diluent working solution (Vector Laboratories) and with the primary antibody (slow myosin 1:1,000, fast myosin 1:750) diluted in MOM diluent working solution. Sections were then washed twice with PBS and incubated with MOM biotinylated anti-mouse IgG (Vector Laboratories) for 10 min. This incubation was followed with another two PBS washes and then with a 30-min room temperature incubation with Vectastain ABC reagent (Vector Laboratories). Finally, like the human and macaque sections, mouse muscle sections were exposed to DAB for 2 min. The reaction was again stopped with water, and the sections were dehydrated in progressive ethanol washes, cleared in xylenes, and mounted with permount.

Determination of Fiber Type Proportions and Fiber Measurements

The proportions and size of the fiber types were determined by selecting 3–10 sections stained for identification of each fiber type for each individual for both the OOM and ZM muscle. Photographs of the entire cross section of each muscle sample were taken at magnifications ranging from 40× to 200× using a digital camera attached to an Olympus BH-2 light microscope. When needed, multiple photos were taken to later create an image composite in Adobe Photoshop of the entire muscle sample section. Measurements of fiber cross-sectional areas, diameters, lengths, and the ratio of slow/fast muscle fibers in each composite were taken using Image J (NIH). For each composite image created and used for determining fiber type proportions, a quadrant was drawn on the image and a random 10% of the reactive fibers in that image were measured for fiber cross-sectional area, diameter, and length. Fiber length was measured only using fibers that were longitudinally oriented. Fiber cross-sectional area was measured to the nearest 0.01 μm^2. Maximum fiber diameter and length were measured to the nearest 0.01 μm.

Because ratios are not usually normally distributed, each ratio was transformed using the arcsine transformation (Sokal and Rohlf, 1995). Once the arcsine transformation was used, a Kolmogorov-Smirnov test for normalcy (SPSS v. 20) revealed that the transformed data were indeed normally distributed. The mean-transformed values of fast and slow fiber types for the OOM and ZM muscle were then statistically compared among the three groups using one-way ANOVAs in SPSS (v. 20). Where significant group-wide mean

TABLE 1. Percentage of fiber types, cross-sectional area (CSA), fiber diameter, and fiber lengths among groups

	Orbicularis oris muscle			Zygomaticus major muscle		
	Mouse	Macaque	Human	Mouse	Macaque	Human
Type I Percentage	**16.5**%† (0.03)	**10.7**% (0.23)	**20.4**%† (0.03)	**11.2**%† (0.02)	**4.7**% (0.01)	**14.6**%† (0.2)
Type II Percentage	**57.8**% (0.09)	**88.5**%† (0.04)	**90.9**%† (0.02)	**58.7**%† (0.05)	**80.5**% (0.03)	**59.8**%† (0.08)
Type I CSA ($\mu m^2 \times 10^2$)	0.064† (0.002)	**0.060**† (0.006)	0.259 (0.008)	**0.118** (0.007)	0.361† (0.052)	0.331† (0.021)
Type II CSA ($\mu m^2 \times 10^2$)	0.066 (0.002)	**0.116** (0.005)	0.277 (0.013)	**0.016** (0.005)	0.549 (0.020)	0.396 (0.015)
Type I Fiber diameter ($\mu m^2 \times 10^2$)	**3.00** (0.08)	**2.72** (0.07)	**4.48** (0.08)	**4.71**† (0.13)	5.56†‡ (0.47)	**5.85**‡ (0.17)
Type II Fiber diameter ($\mu m^2 \times 10^2$)	**3.34**† (0.05)	**3.46**† (0.04)	**4.24** (0.08)	**5.91**† (0.09)	6.01† (0.08)	**8.93** (0.20)
Type I Fiber length ($\mu m^2 \times 10^2$)	**18.59** (1.41)	41.62 (2.88)	51.93 (4.73)	17.04† (1.57)	19.94† (4.14)	**102.63** (11.44)
Type II Fiber length ($\mu m^2 \times 10^2$)	**27.39** (1.91)	38.76 (1.98)	58.72 (3.12)	19.31† (1.71)	27.87† (2.38)	**174.16** (16.4)

Note: Values in parentheses represent the standard error of the mean. Means with the same superscript symbol next to them in each row do not differ from one another at the $P < 0.05$ level of significance. Within each variable category between means for Type I and Type II fibers, pair means in boldface indicate that the differences between those mean values are statistically different at the $P < 0.05$ level of significance. Mean raw percentages of slow- and fast-twitch fibers are shown; however, statistical analyses were performed using arcsine-transformed measurements.

differences existed, a least squares difference *post hoc* test was used. In addition, mean-transformed values of fast percentage *versus* slow percentage were compared within each taxon using Student's independent *t* tests.

Data for cross-sectional area, fiber diameter, and length of fast and slow fiber types for the OOM and ZM muscle were subjected to a Kolmogorov-Smirnov test for normalcy, which indicated that the data were normally distributed. As these data were normally distributed, they were statistically compared among the three groups using a one-way ANOVA because the data were normally distributed. Where significant group-wide mean differences existed, a least squares difference *post hoc* test was used. In addition, mean fiber diameter and length were compared within each taxon using a Student's independent *t* test. In this study, all mean differences were considered to be statistically significant if $P < 0.05$.

RESULTS

Fiber Type Proportions and Distributions

All muscle sections in the three groups contained both slow-twitch (Type I) and fast-twitch (Type II) fibers. However, the proportions and distribution of the fiber types varied greatly both among the groups and between the muscles (Table 1 and Figs. 2 and 3). There were no distribution biases in either slow- or fast-reactive fibers in any of the groups for the OOM and the ZM muscle except for the human OOM. Figure 4 shows sections through the upper lip of one of the human cadavers. Slow-reactive fibers in the human OOM tended to be more superficially located, much closer to the skin, whereas fast-reactive fibers tended to be distributed more evenly throughout the sections.

The arcsine-transformed mean percentage of slow-reactive fibers in the OOM was significantly ($P < 0.05$) highest in humans and mice and lowest in macaques (humans = mice > macaques), whereas the mean arcsine-transformed percentage of fast-reactive fibers was significantly highest in humans and macaques (humans = macaques > mice; Table 1 and Figs. 2 and 5). Note that raw mean percentages are shown in Table 1; however, statistical testing was done on the arcsine-transformed data. As shown in Figure 2, both the mouse and rhesus macaque tend to show poor reactivity to the slow antibodies in the OOM, whereas humans reacted more strongly. Reactivity to the fast antibodies was especially strong in the macaques followed by humans.

The arcsine-transformed mean percentage of slow-reactive fibers in the ZM muscle was significantly highest in humans and mice (humans = mice > macaques), and the mean arcsine-transformed percentage of fast-reactive fibers was significantly highest in macaques (macaques > humans = mice; Table 1 and Figs. 3 and 5). As shown in Figure 3, all three study groups reacted weakly to the slow antibodies; however, humans reacted to the slow antibodies, the strongest among the groups.

Within groups, all had a significantly higher mean percentage of fast-reactive fibers in both the OOM and the ZM muscle relative to slow-reactive fibers (Table 1).

Fiber Morphometrics

There were significant differences ($P < 0.05$) in slow-reactive OOM mean fiber cross-sectional area (humans > mice = macaques) and fast-reactive OOM mean cross-sectional area (human > macaque > mouse) as shown in Table 1. Significant differences in mean fiber diameters for slow-reactive OOM (human > mouse > macaque) and fast-reactive OOM (human > macaque = mouse) were found (human > mouse > macaque; Table 1). Significant differences were also found in the mean fiber lengths of slow- and fast-reactive OOM (human > macaque > mouse; Table 1).

Slow-reactive ZM muscle mean cross-sectional area was greatest in humans and macaques

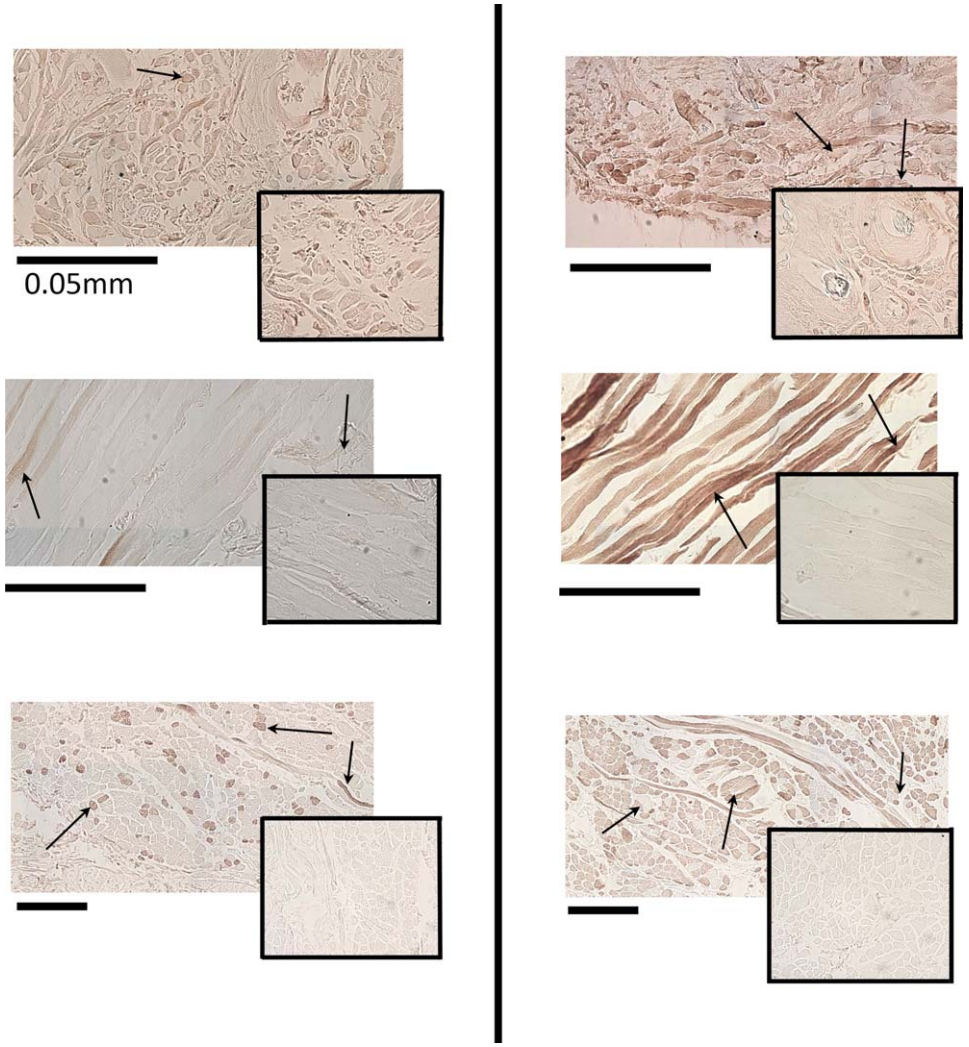

Fig. 2. Microanatomical images of slow (left) and fast (right) myosin ATPase reactivity in the orbicularis oris muscle. Top row: mouse; middle row: rhesus macaque; bottom row: human. Black arrows show examples of reactive fibers, and blue arrows show examples of non-reactive fibers. Small inset boxes are control images. All scale bars represent a length of 50microns. Note in the macaque that slow myosin ATPase signal is above background but weak when compared with other specimens.

(human = macaque > mouse); however, macaques had the greatest mean cross-sectional area in the fast-reactive fibers (macaque > human > mouse). Slow-reactive ZM muscle mean fiber diameter was greatest in both humans and macaques (human = macaque > mouse), and humans had a significantly greater mean fiber diameter in the fast-reactive fibers (human-> mouse = macaque; Table 1). Slow- and fast-reactive ZM muscle mean fiber lengths were greatest in the human with no differences between macaque and mouse (human > macaque = mouse; Table 1).

Within groups, all three had significantly greater mean fast-reactive fiber diameter in the OOM relative to slow-reactive fiber diameter. In the ZM muscle, only humans and mice showed significantly greater mean fast-reactive fiber diameter relative to mean slow-reactive fiber diameter (Table 1). Mean differences in fiber length within groups was found only in the mouse for the OOM with fast-reactive fibers being longer than slow- reactive fibers. For the ZM muscle, only humans had a significantly longer mean fast-reactive fiber length with no differences between mean fiber lengths within any other group (Table 1).

One of the defining anatomical characteristics of facial expression musculature in all mammals is their attachments (at least partially) into one another as shown in Figure 1 (Young, 1957; Burrows, 2008). Because of the partial attachments of the ZM muscle and OOM into one another and into other facial expression muscles, it is not reasonable or reliable to attempt to isolate these muscles for muscle belly mass measurements or other direct measures. Although such muscle properties are typically desirable, the very nature of facial expression musculature does not lend itself to these sorts of data. Thus, fiber morphometrics are not scaled here using whole-muscle measurements.

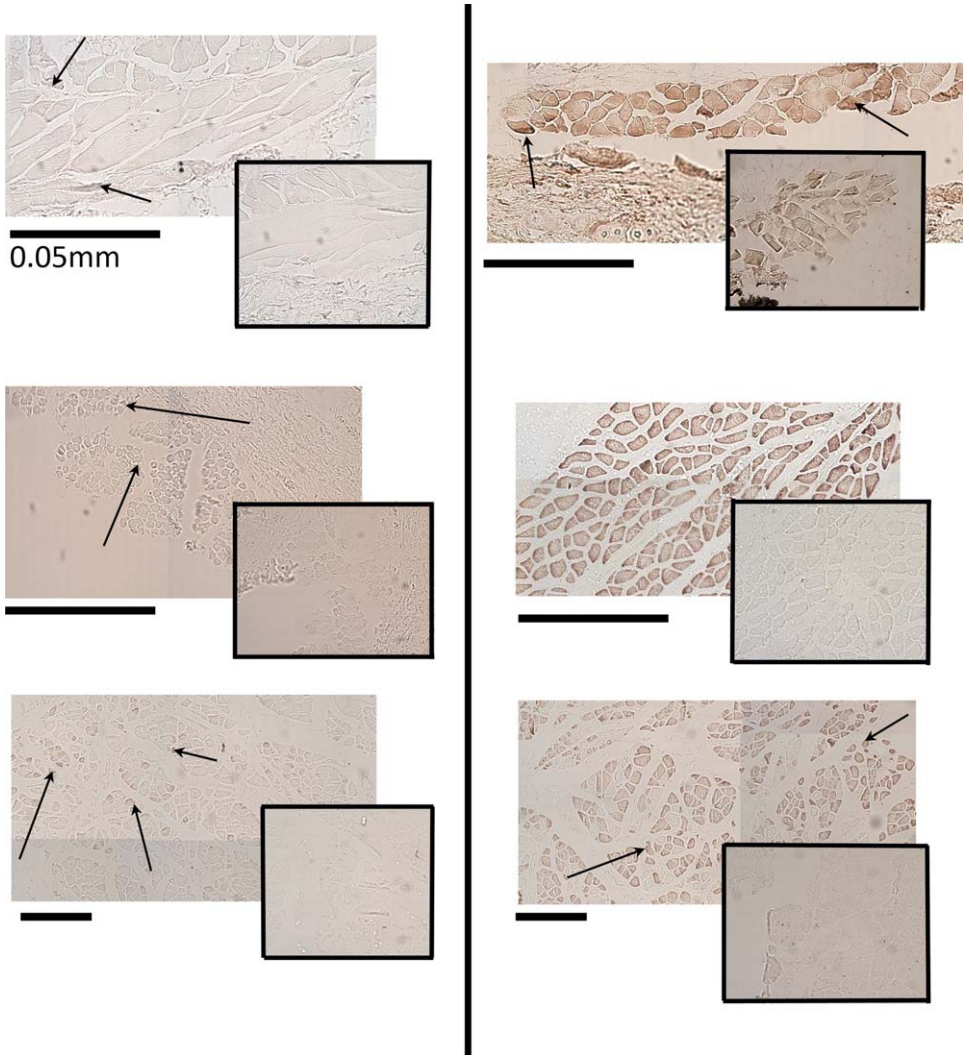

Fig. 3. Microanatomical images of slow (left) and fast (right) myosin ATPase reactivity in the zygomaticus major muscle. Top row: mouse; middle row: rhesus macaque; bottom row: human. Black arrows show examples of reactive fibers, and blue arrows show examples of nonreactive fibers. Small inset boxes are control images. All scale bars represent 50 microns. Note in the mouse and the macaque that slow myosin ATPase signals are above background but weak when compared with other specimens and the stronger reactivity of human fibers relative to mouse and macaque. In addition, note the absence of nonreactive fibers in the macaque to fast antibodies.

On the basis of the previous studies using limbs (e.g., Eng et al., 2008), we chose body size as an exploratory scaler in the current study. Among the three study groups, body mass differs dramatically. The average body size for the mouse (*Mus musculus*) is about 20 g (Wilson and Reeder, 2005; Merritt, 2010); the average body size for the rhesus macaque was 7,700 g (Fooden, 2000); and the average body size for the human sample was 60,000 g (Walpole et al., 2012). Alexander and Ker (1990) found that skeletal muscle fiber lengths do not scale with body size, but it is possible that cross-sectional area and diameter may scale with body size. To see if these metrics scaled with body size in the current study, we also scaled each measurement by average body size as reported in the literature. Evaluation of direct body masses from our specimens was not possible because of the nature of each sample, and therefore, the above-cited averages taken from the literature were

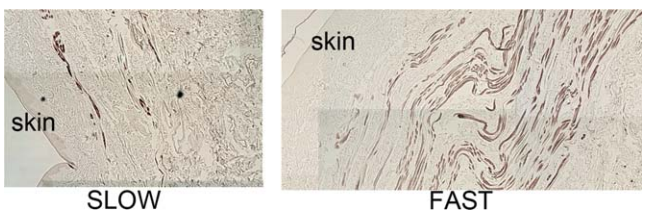

Fig. 4. Microanatomical images of slow (left) and fast (right) myosin ATPase reactivity in the human orbicularis oris muscle showing the skewed distribution of slow-reactive fibers. On the left, the slow-reactive fibers are clearly skewed toward the superficial aspect of the section. On the right, the fast-reactive fibers are much more evenly distributed throughout the thickness of the section. “Skin” indicates the superficial direction of the sections near the dermis and epidermis of the lip.

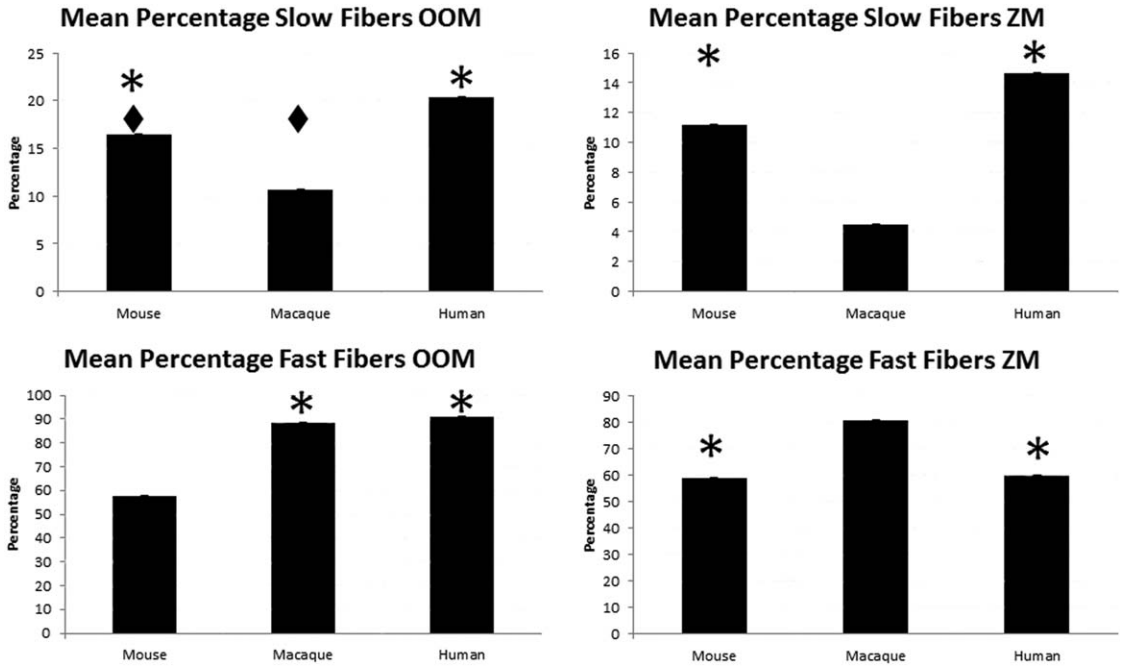

Fig. 5. Proportions of fiber types from the orbicularis oris muscle (OOM) and zygomaticus major (ZM) muscle among the three groups. Columns that share the same. Symbol indicate that the mean percentages were not significantly different ($P > 0.05$). The percentage of slow fibers in the OOM was greatest in both mice and humans, whereas the percentage of fast fibers was greatest in macaques and humans. The percentage of slow fibers in the ZM was also greatest in both mice and humans, whereas the percentage of fast fibers was greatest in the macaque. Bars indicate standard error of the mean. Note: Statistical comparisons among groups were executed on the arcsine-transformed percentages, but these figures show mean percentages from the raw data.

used. Data from these scaled measurements were subjected to a Kolmogorov-Smirnov test for normality using SPSS (v. 20). All data were normally distributed except for Type II fiber diameter in the ZM muscle. This variable was statistically compared among groups using a Kruskal-Wallis Test, whereas all other data were compared among groups using a one-way ANOVA. For all statistical analyses in this study, mean differences were considered to be statistically significant if $P < 0.05$. Because fiber morphometrics among the three groups were relatively similar, scaling by body size always resulted in the mouse group having the significantly greatest measurements with the macaque and human groups never having significant mean differences. Clearly, scaling fiber measurements is inappropriate and reveals minimal useful information.

DISCUSSION

This study tested the hypotheses related to functional aspects of mimetic musculature physiology and fiber morphometrics in humans, rhesus macaques, and mice, groups that occupy varying phylogenetic positions and use differing ecological and social behaviors. The results of these tests reflected both functional and phylogenetic influences and provided evidence of the adaptive nature of mimetic muscles.

Hypothesis 1: Fiber Type Proportions

We hypothesized that humans would have the greatest percentage of fast-twitch fibers in both muscles followed by rhesus macaques. In this study, humans and macaques instead shared the highest percentage of fast-twitch fibers in the OOM, but, surprisingly, it was macaques that had the significantly highest percentage of fast-twitch fibers in the ZM muscle. Rhesus macaques use stereotyped, rapid facial displays in their social interactions with one another in the context of a rigid, complex dominance hierarchy; a highly "despotic" species of macaque (Thierry, 1990, 2000; Parr et al., 2010). The high proportion of fast-twitch fibers in the macaque relative to the human may be reflective of these facial displays. Although humans universally use facial expressions in social communication, there is no rigid social hierarchy seen in rhesus macaques with stereotyped facial displays (Ekman, 1973; Schmidt and Cohn, 2001; Burrows, 2008). Mimetic muscles in humans and rhesus macaques contract quickly in spontaneous facial displays, and the results of the current study demonstrate that mimetic musculature fiber type proportions are consistent with the use of such facial displays in both species.

Unexpectedly, humans had the significantly highest proportion of slow-twitch fibers in both muscles (along with mice in the ZM muscle). Given the intensive use of facial expression in humans, this result was surprising but may be related to the development of human speech. Sanders et al. (in press) compared fiber proportions in tongue musculature of humans and rhesus macaques and found that humans had a far greater percentage of slow-twitch fibers than macaques, relating this to the development of relatively slow tongue movements used in human speech.

All three study groups had a higher percentage of fast-twitch fibers relative to slow-twitch fibers in agreement with our hypothesis. This bias, at least in humans, is itself in agreement with the previous studies of mimetic musculature (Schwarting et al., 1982; Stål, 1987, 1990; Happak et al., 1988; Freilinger et al., 1990; Cheng et al., 2007). The results from the current study support phylogenetic conservation of this bias.

Hypothesis 2: Fiber Morphometrics

Means from fiber cross-sectional area, diameter, and length among groups revealed specific significant differences. When scaled means were statistically tested among groups using body mass as a scaler, the mouse was significantly larger in all cases. This is no surprise given the very small body size of the mouse. Thus, scaling by body mass is an inappropriate methodology here. An examination of results shown in Table 1 make it clear that in many cases, the means for mouse and macaque were statistically the same (e.g., fiber diameter in the OOM fast-twitch fibers) or means for the macaque and human were statistically the same (e.g., cross-sectional area in the ZM muscle slow-twitch fibers). Thus, it appears that scaling by body mass is inappropriate for these fiber dimensions, in at least partial agreement with the results from Alexander and Ker (1990) on fiber length.

The results were mixed in both cross-sectional area and fiber diameter. In the OOM, humans had the greatest cross-sectional area and diameter for both fast- and slow-twitch fibers, whereas macaques and mice did not differ from one another. In the ZM muscle for both fast- and slow-twitch fibers, humans and macaques typically shared the greatest area and diameter, whereas mice always had the lowest means. These results support our hypothesis that humans would have the greatest area and diameter in fast-twitch fibers; however, these results do not support our hypothesis that there would be no differences in the slow-twitch fibers.

Fiber cross-sectional area and diameter give a cautious morphological indicator of the contractile ability of a given muscle fiber (Gans, 1982; Otten, 1988; Lieber, 2002; van Wassenbergh et al., 2007). Humans and macaques typically had the greatest fast and slow fiber cross-sectional areas and diameters. It is unlikely that this is simply due to body size differences because the mouse and macaque displayed no significant differences from one another in several variables. In some cases (such as slow-twitch fiber diameter in the OOM), mean values in the mouse exceeded those of the macaque. The greater cross-sectional areas and diameters in humans and macaques may again be reflective of the frequent use of facial expressions. Although all groups had a greater fast-twitch fiber diameter than slow-twitch diameter (except for the rhesus macaque ZM muscle), differences within groups for cross-sectional area were rare.

We further hypothesized that humans would have longer fast-twitch muscle fibers than macaques and mice; however, this hypothesis is partially confirmed. The results of this study show that humans have the longest fast-twitch fibers followed by macaques for both the OOM and the ZM muscle (human > macaque > mouse in OOM; human > macaque = mouse in ZM muscle). In slow-twitch fibers, humans had the longest fibers; however, the mouse and macaque did not differ from one another in the ZM muscle. Within groups, only the mouse OOM and the human ZM muscle had a significant difference in fiber lengths between slow-twitch and fast-twitch fibers, with the fast fibers being significantly longer than the slow fibers.

Fiber length is connected to muscle contraction velocity such that longer fibers may indicate a greater potential contraction velocity (Gans, 1982; Lieber, 2002). The greater length of fast fibers in humans may be related to both the quick contractions used in spontaneous facial expressions and in speech. Only in the OOM did the macaque have a longer fast fiber than mice, and this is surprising considering that macaques make intensive use of both the OOM and ZM muscle in their facial display repertoire (Parr et al., 2010).

Overall, macaques presented with the highest proportion of fast-twitch fibers, whereas humans had the highest percentage of slow-twitch fibers. Humans and macaques shared characteristics of fast-twitch fibers that may demonstrate adaptations for quick contractions with the potential for generating greater forces than mice. Although they also share some slow-twitch fiber characteristics (such as greater cross-sectional area and diameter in the ZM muscle than mice), they separate on other slow-twitch characters (humans have a greater cross-sectional area and fiber diameter in the OOM). Although the great proportion of slow-twitch fibers in the human sample may reflect adaptations for human speech, the greater cross-sectional area and diameter in the human slow-twitch OOM are unexpected. Greater cross-sectional fiber area and diameter may point to the potential for increased muscle force in the OOM; however, the movements of the upper lip in human speech do not involve a high contractile force from the OOM. Numerous studies have shown that only a small fraction of the available force in the OOM is generated during speech (Rastatter and DeJarnette, 1984; Barlow and Muller, 1991; Hinton and Arokiasamy, 1997; Regalo et al., 2005).

Fiber Morphometrics and Body Size

The results of this study may be at least partially influenced by the vast differences in body size; however, if this was the case, then we should have seen significant differences in slow-twitch fiber morphometrics throughout with human > macaque > mouse. Instead, the results were quite mixed in the slow-twitch fiber morphometrics with the human and mouse sample sometimes being the same (such as slow-twitch cross-sectional area for the ZM muscle) and the macaque and mouse sample sometimes being the same (such as slow-twitch fiber length for the ZM muscle). Although body size may in fact partially influence these morphometrics, there are clear indications that fiber cross-sectional area, diameter, and length are at least partially adaptive in nature.

Adaptive Characteristics of Mimetic Musculature

The results of this study point to some similarities between humans and macaques in mimetic musculature physiology and morphometrics; however, in general,

there is support for an evolutionary divergence of mimetic muscle function. Macaques had a higher percentage of fast-twitch fibers and a far lower percentage of slow-twitch fibers than humans but smaller cross-sectional area, fiber diameter, and length in some cases. The mosaic nature of these results may be reflective not only of the differential use of facial displays between these species but also of the muscular requirements of human speech. Both the ZM muscle and the OOM move the upper lip as part of the supralaryngeal portion of the vocal tract to modify/articulate speech sounds and as an aid in the visual perception of human speech (McGurk and MacDonald, 1976; Titze, 1994; Lieberman, 2007; Raphael et al., 2007). These speech movements are typically quick but may not rise to the level of speed used in rhesus macaque facial expressions where the rigid, despotic dominance hierarchy requires fast facial displays in social interactions (Parr et al., 2010). In addition, a recent study comparing fiber types in tongue musculature of humans and rhesus macaques found that humans had a far greater percentage of slow-twitch fibers than macaques, relating this to the development of tongue movements in human speech (Sanders et al., in press). Lastly, the fiber type distribution in the human OOM was skewed with slow-twitch fibers tending to be located superficially. Previous studies have found differences in fiber type distribution in limb and paravertebral musculature that may reflect timing of muscle fiber recruitment in muscle contraction (Eng et al., 2008; Schilling, 2011; Hazimihalis et al., 2013). A recent study on the human tongue found similar locational biases of muscle fiber distributions, relating these to the specializations of the human tongue for speech (Sanders et al., 2013). The results on the distribution pattern of the human OOM may also be related to its unique use in speech.

Lastly, the mouse results did not follow any clear hypotheses. Fiber type proportions grouped them either with humans or standing alone, fiber cross-sectional areas and diameters mostly grouped them with macaques, and fiber lengths grouped them with macaques or standing alone. These results may reflect their generally low use of facial expression and the use of the mystacial pad in tactile face touch. The mystacial pad of vibrissae in rodents is controlled by both intrinsic musculature and extrinsic musculature, which is composed partly of the mimetic musculature surrounding the lips (Dörfl, 1982; Muchlinski et al., 2013). The mosaic nature of the current results on mice may be reflective of the specialized use of the OOM and ZM muscle in this group and they may not be the best animal biomedical model for the human face in terms of function.

This study clearly had a number of limitations. Because mimetic musculature does not lend itself to direct belly lengths or mass measurements, we could not explore the potential of scaling raw measurements by these variables. Although it does not seem that mimetic muscle fibers vary by body size, morphometric results in the current study should be taken with great caution. Future studies would be needed to confirm our results. Sample size may also be an issue in this study. A large sample of sections was generated here but from a low number of individuals. Ideally, a greater number of individuals could be used in future studies as well as expanding the number of muscles sampled that might include facial regions other than the upper lip. Lastly, exploring the MHC isoforms of fast-twitch fibers may be of interest in future studies to further delineate potential velocity of muscle contractions across both a phylogenetic and functional range of species.

CONCLUSIONS

Previous studies have noted overall similarity in the gross morphology of mimetic musculature among many species of primates, including humans despite the higher complexity of human facial displays and facial processing. This study finds evidence that some of this increased complexity in humans may be derived from the microanatomical characters of mimetic musculature. Based on the fiber type percentages, fiber diameter, and fiber length of the OOM and ZM muscle, humans seem to have a greater potential to generate fast muscle contractions with greater force than mice, but do not differ greatly when compared with the rhesus macaque. This may make the macaque a desirable animal model in biomedical research that involves the face but leaves room for future investigations into the peripheral characteristics of the facial nerve and the neuromuscular junction.

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