



Sexual dimorphism in the limb muscles of the dark-spotted frog, *Pelophylax nigromaculata*

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It has been shown in many anurans that males with larger body size have a mating advantage. However, robust forelimb muscles rather than large body size per se might increase the likelihood of mating. To this end, we investigated sexual dimorphism of nine forelimb muscles and nine hindlimb muscles of *Pelophylax nigromaculata*. We found that three forelimb muscles involved in axillary amplexus were heavier in males compared to females, whereas five forelimb muscles not involved in axillary amplexus showed no sexual differences in muscle mass. The observed muscle dimorphism was allometric, and could be the result of sexual selection. The analysis on hindlimb muscle mass revealed a largely female-biased sexual dimorphism, likely related to females being heavier. There was a positive correlation between forelimb and hindlimb muscle mass and SVL for both sexes. There was no significant sexual difference in the contents of water in musculature except for the extensor carpi radialis.

Key words: muscles, *Pelophylax nigromaculata*, sex selection, sexual dimorphism

INTRODUCTION

Sexual dimorphism in size and morphology is widespread in the animal kingdom, and has attracted considerable attention from evolutionary biologists (Darwin, 1874; Ralls, 1976; Hedrick & Temeles, 1989; Andersson, 1994; Abouheif & Fairbairn, 1997; Bonnet et al., 1998; Liao & Chen, 2012; Liao & Lu, 2012; Liao, 2013). Three major hypotheses have been proposed to explain the evolution of dimorphism in secondary sexual characters: mechanisms of sexual selection, intersexual food competition and reproductive role division (Hedrick & Temeles, 1989; Shine, 1988; Andersson, 1994). In amphibians, females attain larger body size than males in 61% of urodeles and 90% of anuran species (Shine, 1979; Monnet & Cherry, 2002). In addition to body size, sexual dimorphism has been described with regard to vocal sacs and nuptial pads (Duellman & Trueb, 1986; Glaw & Vences, 2006), structures used as weapons for male-male combat (Katsikaros & Shine, 1997; Tsuji & Matsui, 2002), colour and pattern (Buchanan, 1994), as well as some muscles with specific functions (Sassoon et al., 1987).

The amplexus is an important stage of the reproductive process in many frogs and toads (Clark & Peters, 2006). Clasping usually lasts for hours, days or even weeks until spawning occurs (Duellman, 1992; Peters & Aulner,

2000). The ability to perform amplexus suggests the presence of specialisations in the male's forelimbs. Some forelimb muscles involved in amplexus have been found to be larger, heavier and less fatigable in males than in females (Oka et al., 1984; Yekta & Blackburn, 1992; Peters & Aulner, 2000; Clark & Peters, 2006; Navas & James, 2007; Liao et al., 2012b; Mi, 2012). Larger and less fatigable forelimb muscles in males are normally assumed to produce and maintain a relatively larger force, which is advantageous to clasp the females or repel other rivals (Peters & Aulner, 2000). It is thought that the amplexus had resulted in the evolution of sexual dimorphism in forelimb muscles (Oka et al., 1984; Clark & Peters, 2006). However, not all muscles involved in clasping action are dimorphic in mass between the sexes, and there are further interspecific differences in muscle dimorphism (Oka et al., 1984; Yekta & Blackburn, 1992; Mi, 2012). Mi (2012) found that some antagonistic muscles required for axillary amplexus are also sexually dimorphic, with muscle mass of males exceeding that of females.

The hindlimbs of frogs and toads are mainly responsible for hopping, kicking and swimming (Peters et al., 1996). So far, little attention has been paid to sexual dimorphism of hindlimb muscles (Lee & Corrales, 2002; Liao et al., 2012a; Mi, 2013). Lee and Corrales (2002, in *Bufo marinus*) and Mi (2013, in *Bufo gargarizans*) found certain hindlimb muscles of males being heavier than

those of females, whereas Liao et al. (2012a, with *Bufo andrewsi*) discovered that the homologous muscles of females were significantly larger than that of males.

The dark spotted frog, *Pelophylax nigromaculata*, is a widespread species in temperate East Asia but has been classified as Near Threatened according to the IUCN Red List (Zhao & Adler, 1993; Fei, 1999). It inhabits river pools, channels, lakes, ponds, swamps, ditches and paddy fields (Zhao & Adler, 1993; Zhao, 1999). *Pelophylax nigromaculata* is regarded as an explosive breeder, with a breeding season lasting from late April to mid-May (Khonsue et al., 2001; Liao et al., 2010). Males use their forelimbs to clasp females by means of axillary amplexus. Dark spotted frogs were chosen due to previous work on the location, morphology and function of limbs muscles (Zhou, 1956). The purpose of the study was to describe sexual differences in forelimb and hindlimb muscle mass, and explore the evolution of sexual dimorphism in mass of limbs muscles in *P. nigromaculata*.

MATERIALS AND METHODS

A total of 84 individuals (45 males and 39 females) of *P. nigromaculata* were collected in a suburb of Nanchong (30°50'N, 106°07'E, 338 m a.s.l.), northeastern Sichuan, China, in late April 2011 during the breeding season. Before dissecting these frogs, the snout-vent length (SVL) was measured with a digital vernier caliper to the nearest 0.1 mm. Nine forelimb muscles and nine hindlimb muscles on the left and right side of each individual were investigated (forelimb muscles: deltoideus, flexor carpi radialis, flexor carpi ulnaris, abductor indicis longus, triceps brachii, extensor carpi radialis, extensor carpi ulnaris, extensor digitorum communis, flexor digitorum communis; hindlimb muscles: sartorius, triceps femoris, rectus internus major, semimembranosus, adductor magnus, gastrocnemius, peroneus, tibialis anterior, extensor cruris). Because the dry/wet weight ratio of muscles represents the proportion of water in muscles, both wet weight and dry weight of each muscle were weighed. Firstly, we dissected and collected all selected muscles discreetly, then rolled them on a blotting paper to get rid of excess liquid and weighed to the nearest 0.1 mg using an electronic balance (wet weight). Secondly, we put these muscles into a thermostat drier (60°C) for

24 hours before re-weighing them (dry weight).

The data were tested for normal distribution by the Kolmogorov-Smirnov procedure. Snout-vent length (SVL) between sexes were examined using *t*-tests. Differences of muscle mass between the sexes were analysed by general linear models (GLMs) with muscles mass as dependent variables, sexes as a fixed factor, SVL as a covariate. We regressed the muscle mass on SVL for each muscle and total forelimb or hindlimb muscles, and compared regression coefficients between males and females with regression analysis for homogeneity. The experiment-wise error rate was set as 0.05, and data were given as means±SD.

RESULTS

We measured all muscles of 45 males (SVL ranging from 54.9 to 80.6 mm) and 39 females (SVL ranging from 56.1 to 88.9 mm). Average body size of females (69.8±8.8 mm) was larger than that of males (66.9±6.3 mm), but the difference did not reach significance ($t=1.71$, $p=0.09$).

After controlling for the influence of body size, three of nine forelimb muscles (flexor carpi radialis, flexor carpi ulnaris and abductor indicis longus) had a mean weight which was significantly larger ($p<0.01$) in males than in females (Table 1). For all forelimb muscles, wet and dry weight was correlated with SVL for both sexes ($p<0.001$, Table 3). Slopes were homogeneous ($p>0.05$) for all comparison except for the dry and wet mass of flexor carpi radialis and abductor indicis longus ($p<0.001$), as well as the dry mass of extensor digitorum communis ($p<0.01$, Table 3). Linear regression of the dry weight of nine forelimb muscles on SVL was highly significant for both sexes ($p<0.001$), and the slopes were not homogenous ($p<0.01$, Table 3); the same was the case for the wet weight of the nine forelimb muscles (Fig. 1, males: $r^2=0.858$, $F=259.05$, $p<0.001$; females: $r^2=0.836$, $F=189.26$, $p<0.001$). The dry/wet muscle weight ratio did not differ between the sexes except for the extensor carpi radialis (Table 1). The total dry/total wet weight ratio of the nine muscles highly correlated with SVL in both sexes (Fig. 3, males: $r^2=0.506$, $F=44.01$, $p<0.001$; females: $r^2=0.164$, $F=7.28$, $p=0.01$), and the slopes were not homogenous ($p=0.01$).

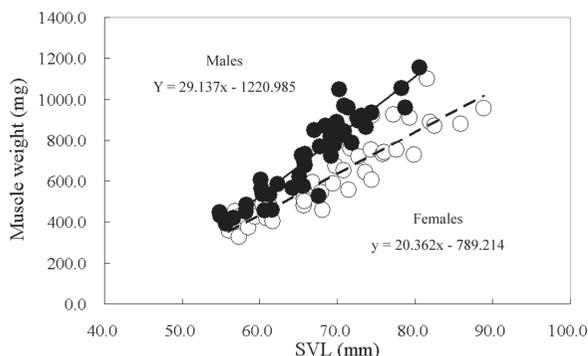


Fig. 1. Linear regression of total wet weight of forelimb muscles on SVL for males (solid circles, solid line) and females (open circles, broken line) of *Pelophylax nigromaculata*.

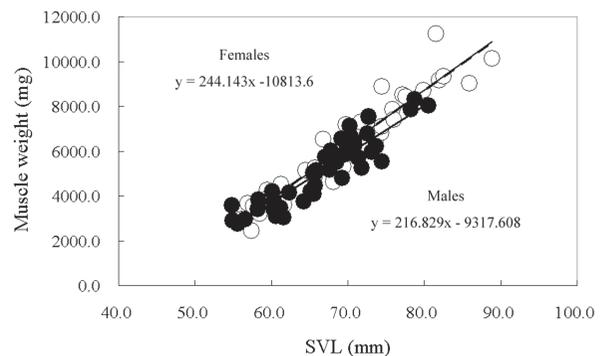


Fig. 2. Linear regression of total wet weight of hindlimb muscles on SVL for males (solid circles, solid line) and females (open circles, broken line) of *Pelophylax nigromaculata*.

Table 1. Difference in mass of forelimb muscles between sexes in *Pelophylax nigromaculata*.

Muscles	Dry weight (mg)			Wet weight (mg)			100 × ratio (dry/wet weight)		
	Males (n=45)	Females (n=39)	ANOVA F p	Males (n=45)	Females (n=39)	ANOVA F p	Males	Females	p
Deltoides	34.8±8.8	38.0±13.0	0.13 0.72	165.6±44.7	178.0±65.5	1.03 0.31	21.17±1.48	21.59±1.97	0.10
Triceps brachii	44.3±12.2	46.5±13.8	2.75 0.10	200.7±57.4	214.5±68.9	1.28 0.26	22.21±1.61	21.90±1.67	0.65
Extensor carpi radialis	8.6±2.3	8.8±3.0	2.15 0.15	28.2±6.9	32.2±10.7	1.14 0.29	30.61±4.41	27.38±3.70	0.001
Flexor carpi radialis	30.8±12.1	6.1±1.6	240.19 <0.001	122.3±55.9	23.0±6.2	184.52 <0.001	26.18±3.59	26.70±3.63	0.24
Extensor carpi ulnaris	8.1±2.2	9.4±3.1	1.88 0.17	32.8±9.6	39.5±14.1	3.30 0.07	24.99±3.10	24.08±2.50	0.28
Flexor carpi ulnaris	5.5±1.7	4.9±1.3	11.29 0.001	23.0±7.1	22.0±6.8	6.60 0.01	24.11±4.32	23.07±4.21	0.40
Extensor digitorum communis	9.5±2.3	9.9±4.1	0.34 0.56	34.1±8.9	37.0±14.5	0.00 0.99	28.36±3.71	26.85±3.62	0.13
Flexor digitorum communis	16.6±3.8	18.9±5.8	1.66 0.20	63.1±18.1	71.5±23.3	0.43 0.52	27.01±5.13	26.89±4.11	0.43
Abductor indicis longus	14.6±4.4	3.9±1.3	371.95 <0.001	58.7±19.1	14.8±4.0	320.50 <0.001	25.27±3.77	26.84±6.68	0.28
Total	172.7±41.2	146.3±41.7	111.00 <0.001	728.7±199.2	632.5±196.2	77.47 <0.001	23.98±1.37	23.34±1.40	0.19

Table 2. Difference in mass of hindlimb muscles between sexes in *Pelophylax nigromaculata*.

Muscles	Dry weight (mg)			Wet weight (mg)			100 × ratio (dry/wet weight)		
	Males (n=45)	Females (n=39)	ANOVA F p	Males (n=45)	Females (n=39)	ANOVA F p	Males	Females	p
Sartorius	31.1±8.4	34.2±11.1	0.05 0.83	137.5±40.5	149.7±52.8	0.91 0.34	22.87±1.99	23.14±1.88	0.15
Triceps femoris	378.3±102.3	449.2±152.4	6.14 0.02	1656.3±477.6	1994.1±712.7	6.65 0.01	22.98±1.02	22.72±1.29	0.70
Rectus internus major	153.3±43.0	178.8±63.2	2.11 0.15	640.1±201.7	754.5±287.1	1.69 0.20	24.24±1.37	24.02±1.55	0.79
Semimembranosus	172.5±52.0	205.9±70.1	5.17 0.03	740.5±236.2	897.8±333.4	5.31 0.02	23.46±1.28	23.23±1.33	0.97
Adductor magnus	147.4±38.4	174.7±65.3	2.48 0.12	623.1±168.1	771.2±306.8	5.18 0.03	23.76±1.26	22.97±1.99	0.09
Gastrocnemius	221.9±65.3	262.7±96.4	3.12 0.08	932.4±294.1	1127.2±428.5	4.70 0.03	24.04±2.04	23.51±1.46	0.36
Peroneus	71.4±18.2	82.3±29.4	1.29 0.26	300.3±86.0	355.1±137.2	2.30 0.13	24.05±1.68	23.52±1.79	0.53
Tibialis anterior	20.9±5.1	22.4±7.3	0.58 0.45	73.4±20.8	83.8±29.5	0.53 0.47	29.13±3.73	27.21±3.44	0.07
Extensor cruris	23.7±5.0	25.6±7.2	0.002 0.96	87.5±23.4	99.1±37.4	0.20 0.65	27.65±3.23	26.94±4.37	0.84
Total	1220.5±325.1	1435.7±488.8	4.59 0.04	5191.0±1494.2	6232.5±2262.3	6.15 0.02	23.69±1.11	23.27±1.19	0.39

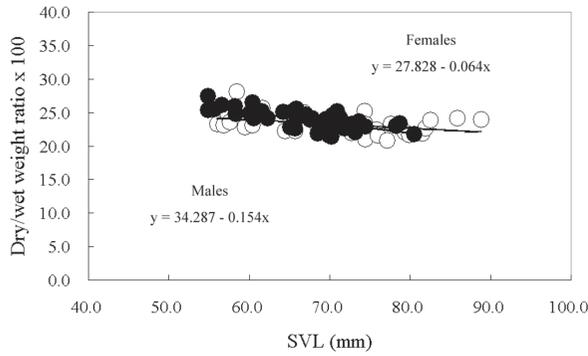


Fig. 3. Linear regression of total dry/total wet weight ratio of forelimb muscles on SVL for males (solid circles, solid line) and females (open circles, broken line) of *Pelophylax nigromaculata*.

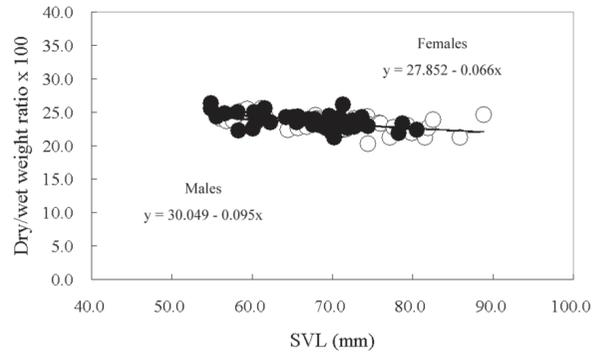


Fig. 4. Linear regression of total dry/total wet weight ratio of hindlimb muscles on SVL for males (solid circles, solid line) and females (open circles, broken line) of *Pelophylax nigromaculata*.

Of the nine hindlimb muscles, the triceps femoris and semimembranosus significantly differed between the sexes in wet and dry weight ($p < 0.05$). The adductor magnus and gastrocnemius were only significantly differed in their dry weight ($p < 0.05$), with muscle mass in females being heavier than in males independent of SVL (Table 2). When the nine hindlimb muscles were considered together, females had overall significantly higher muscle mass than males ($p < 0.05$, Table 2). No significant intersexual difference was found in other hindlimb muscles in dry mass and/or wet mass (Table 2). The mass of all hindlimb muscles was correlated with SVL in both sexes ($p < 0.001$); the slopes were homogenous ($p > 0.05$) except for wet mass of the adductor magnus ($p = 0.04$) and dry mass of peroneus ($p = 0.04$, Table 4). Total dry and wet mass of the nine hindlimb muscles also regressed significantly on SVL within each sex (Table 4, Fig. 2, for total wet weight: males, $r^2 = 0.844$, $F = 232.77$, $p < 0.001$; females, $r^2 = 0.905$, $F = 351.56$, $p < 0.001$), at homogenous slopes (dry: $p = 0.143$, wet: $p = 0.167$). The dry/wet weight ratio did not significantly differ between

sexes (Table 2). The total dry/total wet weight ratio was correlated with SVL for males and females (Fig. 4, males: $r^2 = 0.294$, $F = 17.87$, $p < 0.001$; females: $r^2 = 0.236$, $F = 11.42$, $p = 0.002$), and the slopes were homogenous ($p = 0.329$).

DISCUSSION

Previous studies had found that female *P. nigromaculata* are larger than males (Khonsue et al., 2001; Liao et al., 2010; Mao et al., 2012), in agreement with the present study. Sexual size dimorphism is widespread among anurans (Shine, 1979; Liao et al., 2010; Chen et al., 2011; Liao, 2011; Liao et al., 2011; Liu et al., 2012; Lou et al., 2012; Li et al., 2013), and linked to intersexual differences in growth rate, time of sexual maturation, age structure and mortality schedules (Halliday & Tejedo, 1995; Lee, 2001; Mao et al., 2012; Liao et al., 2013a, b). With respect to *P. nigromaculata*, females usually reach sexual maturity later than males, and adult males are younger than females (Khonsue et al., 2001; Liao et al., 2010; Mao et al., 2012). Individuals will devote most energy to

Table 3. Linear regression analysing of forelimb muscle dry mass (mg) on SVL (mm) and slopes comparison between the sexes of *Pelophylax nigromaculata*. $p < 0.001$ for all linear regressions.

Muscle	Males (n=45)				Females (n=39)			slope homogeneity	
	Equation	r^2	F	Equation	r^2	F	t	P	
Deltoides	Y=1.181x-44.213	0.730	116.511	Y=1.274x-50.959	0.743	107.161	0.543	0.59	
Triceps brachii	Y=1.696x-69.144	0.776	148.784	Y=1.400x-51.275	0.805	152.628	-1.641	0.11	
Extensor carpi radialis	Y=0.249x-8.098	0.478	39.407	Y=0.258x-9.227	0.588	52.865	0.149	0.88	
Flexor carpi radialis	Y=1.448x-66.053	0.572	57.524	Y=0.114x-1.896	0.420	26.796	-7.468	<0.001	
Extensor carpi ulnaris	Y=0.206x-5.663	0.359	24.125	Y=0.269x-9.419	0.593	53.914	1.131	0.26	
Flexor carpi ulnaris	Y=0.149x-4.527	0.301	18.479	Y=0.120x-3.452	0.633	63.952	-0.801	0.43	
Extensor digitorum communis	Y=0.112x-2.065	0.095	4.524	Y=0.320x-12.464	0.484	34.719	2.690	0.01	
Flexor digitorum communis	Y=0.334x-5.794	0.304	18.784	Y=0.539x-18.114	0.645	67.365	1.932	0.06	
Abductor indicis longus	Y=0.568x-23.460	0.666	85.626	Y=0.095x-2.670	0.411	25.790	-7.823	<0.001	
Total	Y=5.943x-224.886	0.834	216.291	Y=4.379x-159.476	0.856	220.150	-3.140	<0.01	

Table 4. Linear regression analysing of hindlimb muscle dry mass (mg) on SVL (mm) and slopes comparison between the sexes of *Pelophylax nigromaculata*. $p < 0.001$ for all linear regressions.

Muscle	males (n=45)			females (n=39)			Slope homogeneity	
	Equation	r^2	F	Equation	r^2	F	t	p
Sartorius	Y=1.136x-44.901	0.728	115.159	Y=1.160x-46.783	0.842	197.589	0.183	0.86
Triceps femoris	Y=14.783x-610.898	0.837	221.312	Y=16.423x-697.503	0.902	339.475	1.212	0.23
Rectus internus major	Y=6.060x-252.260	0.798	170.033	Y=6.734x-291.383	0.883	280.241	1.085	0.28
Semimembranosus	Y=7.263x-313.502	0.781	153.690	Y=7.593x-324.305	0.912	382.525	0.475	0.64
Adductor magnus	Y=4.402x-147.110	0.526	47.667	Y=6.159x-255.250	0.692	83.107	1.838	0.07
Gastrocnemius	Y=9.208x-394.226	0.798	170.141	Y=10.381x-462.063	0.901	338.138	1.293	0.20
Peroneus	Y=2.552x-99.395	0.787	159.218	Y=3.145x-137.306	0.890	299.364	2.149	0.04
Tibialis anterior	Y=0.656x-22.969	0.653	80.777	Y=0.710x-27.187	0.743	107.042	0.526	0.60
Extensor cruris	Y=0.631x-18.509	0.636	75.231	Y=0.680x-21.914	0.701	86.777	0.469	0.64
Total	Y=46.692x-1903.771	0.827	204.988	Y=52.984x-2263.692	0.913	388.402	1.480	0.14

reproduction rather than growth after reaching sexual maturity, resulting in decreased growth (Hemelaar, 1988). Earlier time of maturation and younger ages of male *P. nigromaculata* are the likely underlying reasons for their smaller body size. Individuals from our study population reach maturity at the age of 2–3 years (unpublished data). The lack of a statistically significant size dimorphism may be due to the lack of old individuals in our sample.

Comparisons between sexes based on dry muscle mass were largely in line with those based on wet muscle mass. The three dimorphic muscles all participate in the action of clasping females during amplexus (the flexor carpi radialis and flexor carpi ulnaris are flexing the wrist, and the abductor indicis longus is abducting the second digit). Moreover, the five muscles not involved in amplexus (triceps brachii, extensor carpi radialis, extensor carpi ulnaris, extensor digitorum communis and flexor digitorum communis being antagonistic muscles of axillary amplexus) were non-dimorphic. Since robust forelimb muscles are important for males to grasp females firmly and to resist take-over attempts from competitors, and because there is a positive correlation between robust forelimbs and reproductive success of males (Wells, 1977; Howard, 1984; Lamb, 1984; Howard & Kluge, 1985), it was suggested that the sexual dimorphism of forelimb muscles could be the results of sexual selection and male-male competition (Lee, 2001; Navas & James 2007; Liao et al., 2012b; Mi, 2012). Our findings largely confirm this evidence, although we failed to find intersexual differences in the deltoideus, which adducts upper limbs (Mi, 2012). This may imply that the deltoideus of *P. nigromaculata* does not play an important role for the axillary amplexus.

For the nine forelimb muscles, wet or dry mass highly correlated with SVL in both sexes. The slopes were not homogenous between sexes for two of the three dimorphic muscles (exception: flexor carpi ulnaris), with

the regression coefficients of males exceeding that of females. The slopes were however homogenous for the six non-dimorphic muscles. This finding may indicate that certain muscles grow in an allometric way (faster in males than females), perhaps in line with increased androgens and their receptors in male muscles.

Lee & Corrales (2002, in *B. marinus*) and Mi (2013, in *B. gargarizans*) found that males surpassed females in hindlimb muscle mass, and that hindlimb muscle mass was related to male reproductive success. In *P. nigromaculata*, however, female muscle mass was overall higher than that of males. Although in some hindlimb muscles, no statistically significant intersexual differences were found, despite a mean which was larger in females than males, similar to the case of *B. andrewsi* (Liao et al., 2012a). This suggests that male-male competition and maintenance of amplexus in *P. nigromaculata* might not rely on the hindlimb muscle mass of males (Liao et al., 2012a). As for females, robust hindlimbs may be related to a heavier body.

The dry/wet weight ratio, a measurement of the proportion of water in muscles (Slack et al., 1973; Lipar & Ketterson, 2000), is responsible for the intracellular and intercellular differences in ionic concentrations (Hamon et al., 1976). The dry/wet weight ratio was indifferent across sexes except for the extensor carpi radialis. Our results suggest that there is no difference in ionic concentration between males and females, also implying that either dry or wet mass can be used to study sexual dimorphism in muscle size. The proportionally higher water content of muscles by larger individuals might be linked to higher levels of metabolism in bigger individuals.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for critical comments on the earlier draft of this manuscript. We appreciate the improvements in English language style

made by Dr. Robert Jehle. Financial support was provided by the follow-up grants program for the outstanding young foregoer of academic and technology of Sichuan Province (2011JQ0056), the key laboratory program of Sichuan Province Education Department (10ZX007) and the Innovative Team Foundation of China West Normal University. We declare that all animals used in the study were treated humanely and ethically following all applicable institutional Animal Care guidelines in China.

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Accepted: 22 January 2014