



Testing the Role of Environmental Harshness and Sexual Selection in Limb Muscle Mass in Anurans

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OPEN ACCESS

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Specialty section:

This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 20 February 2022

Accepted: 10 May 2022

Published: 26 May 2022

Citation:

Yang YJ, Jiang Y, Mi ZP and
Liao WB (2022) Testing the Role
of Environmental Harshness
and Sexual Selection in Limb Muscle
Mass in Anurans.
Front. Ecol. Evol. 10:879885.
doi: 10.3389/fevo.2022.879885

Sexual dimorphism is regarded as the consequence of differential responses by males and females to selection pressures. Limb muscle plays a very important role during amplexus, which is likely to be under both natural and sexual selection in anurans. Here, we studied the effects of natural and sexual selection on limb muscle mass in males and females across 64 species of anurans. The results showed that there were non-significant differences in relative limb muscle mass between the sexes among species, exhibiting no sexual dimorphism in limb muscle. Absolute and relative limb muscle mass positively displayed correlations with snout-vent length (SVL) for both sexes. However, neither male-biased operational sex ratio (OSR) nor environmental harshness [e.g., coefficient of variation (CV) in temperature and CV in rainfall] can explain relative limb muscle mass (e.g., forelimb, hindlimb, and total limb muscle) within each sex. The findings suggest that environmental harshness and sexual selection cannot play important roles in promoting variations in limb muscle among anuran species.

Keywords: anurans, limb muscles, operational sex ratio, selection pressure, sexual dimorphism

INTRODUCTION

Sexual dimorphism is widespread in nature, and it results from differential responses by males and females to selection pressures (Darwin, 1871; Andersson, 1994; Blanckenhorn, 2005). Most studies have shown that sexual dimorphism evolution arises as a consequence of sexual selection and natural selection (Shine, 1989; Andersson, 1994; Liao et al., 2013a,b; Olarte et al., 2020). On the one hand, sexual selection promotes evolution of the morphology and behavior that enhance opportunities of male mating success (Andersson, 1994; Liao and Lu, 2011). On the other hand, natural selection favors sex-specific adaptations through reducing competition between the sexes for habitats or preys (Shine, 1989; Liao and Chen, 2012; Liao, 2013; Clifton et al., 2020). Under the two selections, females and males diverge in a variety of morphological traits (e.g., body size, head dimensions, body shape, and hind muscles mass) over evolutionary time (Andersson, 1994; Lee and Corrales, 2002; Liao et al., 2015; Zhang et al., 2020; Munoz-munoz et al., 2021).

In anurans, males and females often differ in limb muscle mass as well as body size (Lee and Corrales, 2002; Liao et al., 2012a,b). Limb muscles are important organs associated with amplexus during the male-male competition (Lee, 2001; Liao et al., 2012a,b; Mi, 2012). Stronger forelimb muscle in males can be assumed to produce a relatively larger force where males have an advantageous to clasping the female or repel other rivals

(Peters and Aulner, 2000; Liao and Lu, 2011; Mi, 2012; Gastón and Vaira, 2020) while stronger hindlimb muscle are responded to prevent being replaced by another male through kicking other rivals (Lee and Corrales, 2002; Mi, 2013). As a result, a conspicuous sexual dimorphism in fore- and hind-limb muscle mass is expected in frogs where males have much stronger limb muscle than females (Lee, 2001; Liao et al., 2012a; Mi, 2013). However, some studies also reveal that females have stronger hindlimb muscle than males (Liao et al., 2012b; Zhang et al., 2020) while a previous study found a non-significant differences in hindlimb musculomass (Zhang et al., 2013).

Environmental pressures (i.e., resource availability, competition, or predator risk) are considered as a main evolutionary force in shaping the differentiation of organs size, such as brain size, livers, testes mass, digestive tract length and muscles mass between the sexes and among populations (Lüpold et al., 2011; Jiang et al., 2015; Mai et al., 2017, 2019; Zhong et al., 2017; Tang et al., 2018; Zhao et al., 2019; Obregón et al., 2021; Sowersby et al., 2021; Zamora-Camacho, 2021). For instance, variations in food resources availability among different habitats affect energy intake and energy allocation, and thereby leading to differences in digestive tract length, testes mass and limb muscle mass between the sexes among populations (Jin et al., 2016; Yang et al., 2017; Adams et al., 2020; Cai et al., 2020). Meanwhile, because the intensity of sexual selection is positively associated with male-biased operational sex ratio (OSR, calculated as the number of males to the number of fertilizable females in a breeding aggregation at a given time; Emlen and Oring, 1977) among different environmental conditions, the stronger male-male competition is predicted to be related to select for larger limb muscle mass in a toad (Yang et al., 2017). Hence, both natural and sexual selection can promote variations in limb muscle mass in males and females, thus possibly predicting sexual dimorphism in limb muscle in anurans (Yekta and Blackburn, 1992).

In the present study, we investigate the effects of natural and sexual selection on sexual dimorphism in limb muscle mass across 64 species of anurans. We first investigate whether sexual dimorphism in limb muscle mass occurs in anurans. We then investigate whether the limb muscle mass is correlated with body size within each sex. Finally, we investigate whether male-biased OSR and environmental harshness (e.g., variations in mean annual temperature and rainfall) affect the variations in limb muscle mass for both sexes among species.

MATERIALS AND METHODS

Data Collection

We collected a total of 64 anuran species during the breeding seasons between 2010 and 2020 in China. We sampled each species at a single site using a sampling-lined method. All individuals were captured within each sampling line at night using a 12-V flashlight. We confirmed their sexes through secondary sexual traits (e.g., nuptial pads in males and eggs in females). Sample size per species ranged from 1 to 6 individuals

within each sex (**Supplementary Table 1**). Body size (snout-vent length: SVL) of all individuals was measured to the nearest 0.01 mm with a caliper. All frogs were stored in 4% neutral buffered formalin for dissections. After 2 months, we extracted muscles involving in the clasping of the female by the male during the axillary amplexus (Lee, 2001) and preventing being replaced by another male through kicking other rivals (Lee and Corrales, 2002), including four forelimb muscles (pectoralis series: pectoralis epicoracoides, pectoralis sternalis, pectoralis abdominalis; deltoideus triangularis, triceps branchii, and flexor carpi radialis, Liao et al., 2012a; Diogo and Molnar, 2014) and four hindlimb muscles (sartorius, plantaris longus, triceps femoris, and gastrocnemius, Liao et al., 2012b; Diogo and Ziermann, 2014) from all individuals (**Supplementary Table 2**). We then dried these muscles using a thermostat drier of 60°C for 48 h before quantifying their dry weight to the nearest 0.1 mg using an electronic balance (Liao et al., 2012a,b). We calculated relative limb muscle mass as a model: $RLM = \log_{10}(\text{limb muscle mass})/\log_{10}(\text{SVL})$ (Liao et al., 2012a).

We calculated the species-specific male-biased OSR [expressed as the proportion of males among all adult individuals (Liao et al., 2018)] for every sampling line as the number of males divided by the number of females for three nights using marked recapture methods (Mai et al., 2020). Environmental harshness (e.g., variations in average annual temperature and rainfall) has been hypothesized to affect energy intake and energy allocation, and thus resulting in difference in limb muscle mass in anurans (Yang et al., 2017). Hence, we used coefficient of variation ($CV = SD/\text{mean}$) of temperature and rainfall to measure environmental harshness (Van Woerden et al., 2012; Luo et al., 2017). We calculated CV of temperature and rainfall based on average temperature and rainfall collected at each site in everyday from Chinese Meteorological Stations¹ between 2013 and 2017 (**Supplementary Table 1**).

Phylogeny Reconstruction

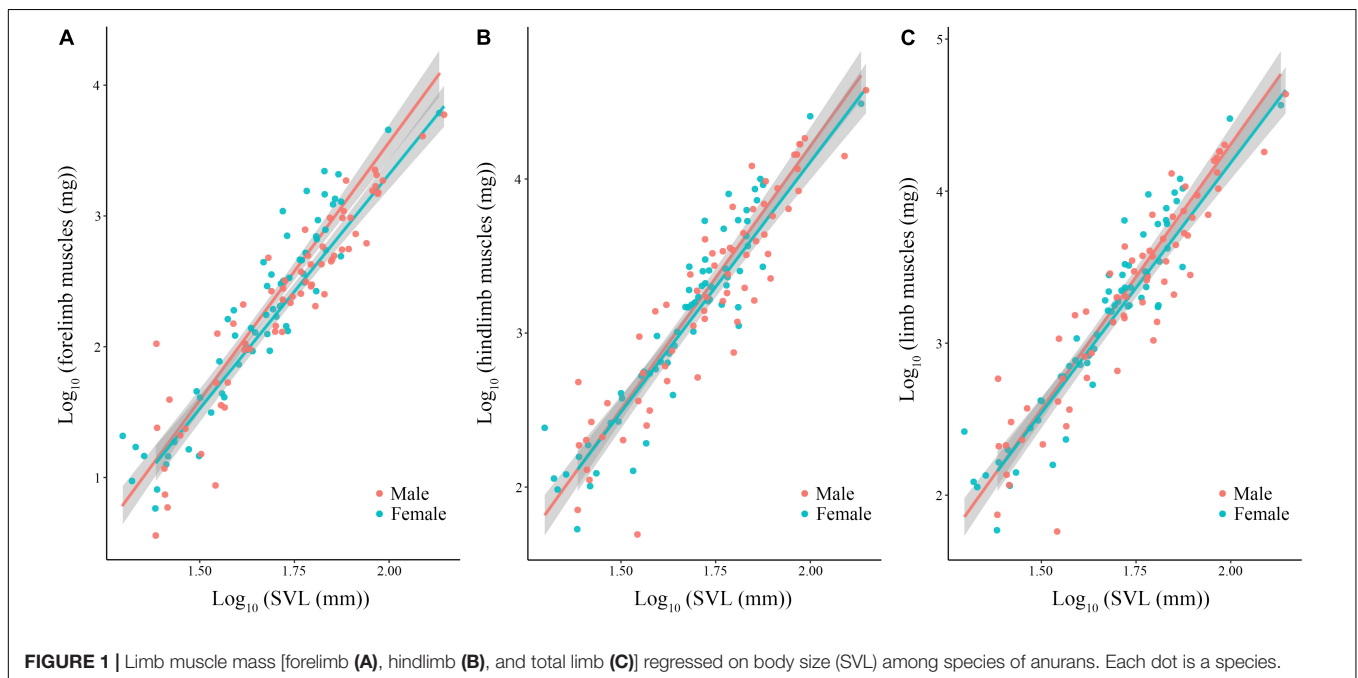
To reconstruct the phylogeny for 64 anuran species, we used the sequences of six mitochondrial ribosome genes (CYTB, 12S, 16S, COI, ND2, and ND4) and three nuclear genes (RAG1, TYR, and RHOD) from GenBank [for accession numbers and sequence coverage (see **Supplementary Table 3**)]. All sequences for each taxon were obtained from the same specimen. For each locus, we aligned the sequences using the multi-sequence alignment (MUSCLE) in MEGA v.10.2.2, and saved the aligned sequences in .fas format (Tamura et al., 2021). We then used the modelTest() function in the *phangorn* package (Schliep, 2011) in the R 4.1.0 software (R Development Core Team, 2021) based on the corrected Akaike Information Criterion (AICc) to determine the best nucleotide substitution model for each gene. The best nucleotide substitution model was GTR + Γ + I for all genes except RHOD, for which HKY + Γ + I had stronger support, and GTR + Γ + I was thus used as the best substitution model for all genes.

Using BEAUTi and BEAST v.1.8.3 (Drummond et al., 2012; also see details in Jiang et al., 2022), we constructed the phylogeny

¹<http://www.lishi.tianqi.com>

TABLE 1 | Relationship between limb muscle mass and body size (SVL) in both males and females among species of anurans.

Dependent variable	Predictor	Sex	λ	$B \pm SE$	t	R^2	P
Forelimb	SVL	Male	0.584 ^{1.000, <0.001}	3.738 \pm 0.204	18.343	0.844	<0.001
		Female	0.000 ^{1.000, <0.001}	3.585 \pm 0.172	20.854	0.875	<0.001
Hindlimb	SVL	Male	0.792 ^{1.000, <0.001}	3.399 \pm 0.172	19.815	0.864	<0.001
		Female	0.482 ^{1.000, <0.001}	3.155 \pm 0.192	16.390	0.813	<0.001
Total limb	SVL	Male	0.839 ^{1.000, <0.001}	3.434 \pm 0.163	21.005	0.877	<0.001
		Female	0.341 ^{1.000, <0.001}	3.205 \pm 0.181	17.674	0.834	<0.001



with unlinked substitution models, a relaxed uncorrelated log-normal clock, a Yule speciation process, and the best-supported nucleotide substitution models. We omitted time calibration due to a lack of fossil dates. We ran the Markov Chain Monte Carlo (MCMC) simulation for 100 million generations where every 10,000th tree was sampled using the BEAST implementation in the CIPRES Science Gateway.² For all tree statistics, the effective sample size values exceeding 200 showed satisfying convergence of the Bayesian chain and adequate model mixing in the program Tracer v.1.6.0 (Rambaut and Drummond, 2014; Chen et al., 2021a,b). We used TreeAnnotator v.1.8.3 (Drummond et al., 2012) to generate maximum clade credibility trees with mean node heights and a 10% burn-in for all species (Supplementary Figure 1).

Statistical Analysis

All analyses were conducted using phylogeny-controlled general least squares (PGLS) models in R statistical version 4.1.0 (R Development Core Team, 2021). In each model, we used the maximum-likelihood estimate of phylogenetic dependence (Pagel's λ). The scale of λ -values ranges from 0 (i.e.,

phylogenetic independence) to 1 (i.e., complete phylogenetic non-independence) (Freckleton et al., 2002). First, we applied PGLS models to analyze relationships between limb muscle mass and SVL within each sex using 64 species. Then we ran phylogenetic paired t -test to test differences in mean mass of limb muscle between males and females when controlling for effects of body size of the respective species. Finally, we ran multi-predictor models to test the associations between relative limb muscle mass and either male-biased OSR or environmental harshness controlling for SVL effect. All numeric variables were \log_{10} -transformed before analyze to make parameter estimates comparable, and model assumptions were also met.

RESULTS

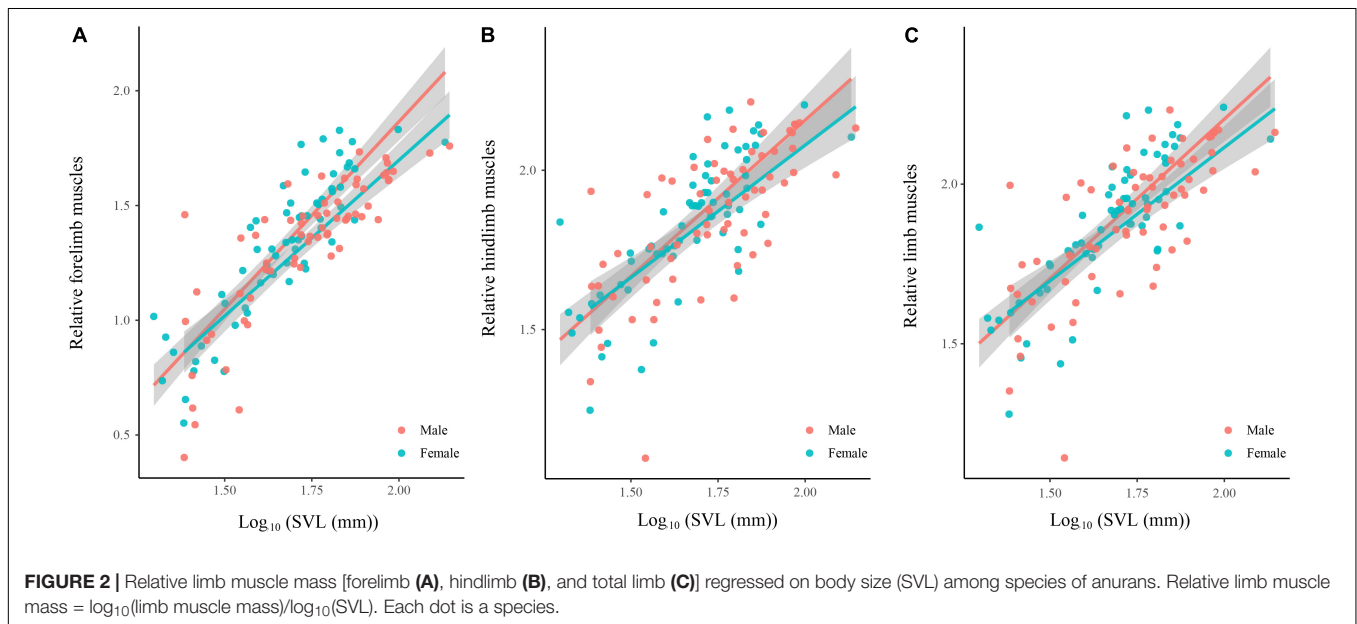
Body size of females was significantly larger than males (phylogenetic paired t -test: $t_{61} = -3.450$, $P = 0.001$; phylogenetic scaling parameter, $\lambda = 0.074$). Total limb mass and forelimb muscle mass of females was significantly heavier than those of males (total limb: $t_{61} = 2.022$, $P = 0.048$, $\lambda = 0.064$; forelimb: $t_{61} = 2.357$, $P = 0.022$, $\lambda < 0.001$). Hindlimb muscle mass of females tended to be larger than those of males ($t_{61} = 1.978$,

²<http://www.phylo.org>

TABLE 2 | Relationship between relative limb muscle mass and body size (SVL) in males and females among species of anurans.

Dependent variable	Predictor	Sex	λ	$B \pm SE$	t	R^2	P
Relative forelimb	SVL	Male	0.681 ^{1.000, <0.001}	1.440 \pm 0.128	11.223	0.670	<0.001
		Female	0.000 ^{1.000, <0.001}	1.358 \pm 0.114	11.880	0.695	<0.001
Relative hindlimb	SVL	Male	0.873 ^{1.000, <0.001}	0.952 \pm 0.104	9.181	0.576	<0.001
		Female	0.728 ^{1.000, <0.001}	0.753 \pm 0.120	6.255	0.387	<0.001
Relative total limb	SVL	Male	0.905 ^{1.000, <0.001}	0.941 \pm 0.099	9.544	0.595	<0.001
		Female	0.346 ^{1.000, <0.001}	0.775 \pm 0.117	6.942	0.437	<0.001

Relative limb muscle mass = $\log_{10}(\text{limb muscle mass})/\log_{10}(\text{body size})$.



$P = 0.052$, $\lambda = 0.071$). Forelimb, hindlimb and total limb muscle mass significantly regressed on SVL within each sex (Table 1 and Figure 1). When the influence of SVL was controlled, relative limb muscle mass did not differ between the sexes (total limb: $t_{61} = -1.030$, $P = 0.307$; forelimb: $t_{61} = -0.503$, $P = 0.617$; hindlimb: $t_{61} = -1.257$, $P = 0.214$). However, relative forelimb, hindlimb and total limb muscle mass increased with SVL for both sexes (Table 2 and Figure 2).

Phylogeny-controlled general least squares models revealed that relative forelimb, hindlimb and total limb muscle mass were not positively correlated with male-biased OSR in males and females, respectively, when controlling for the effects of SVL and phylogeny (Table 3). Meanwhile, relative limb muscle mass (e.g., forelimb, hindlimb, and total limb) cannot be explained by environmental harshness (e.g., CV in temperature and rainfall) for both sexes when the effects of SVL and phylogeny were controlled (Table 3).

DISCUSSION

Our results demonstrate that absolute and relative forelimb, hindlimb and total limb muscle mass increase with increasing SVL within each sex among 64 species of anurans. There is

a lack of sexual dimorphism in relative limb muscle mass although females have larger SVL than males. Moreover, neither male-biased OSR nor environmental harshness can explain variations in relative forelimb, hindlimb and total limb muscle mass for both sexes.

Consistent with the previous studies on limb muscle-SVL relationship in anurans (Lee, 2001; Lee and Corrales, 2002; Liao et al., 2012a,b; Mi, 2012, 2013; Zhang et al., 2013; Mao et al., 2014; Petrović et al., 2017), we found that absolute and relative mass of forelimb, hindlimb and total limb muscle was positively correlated with SVL for both sexes when considering the effects of SVL and phylogeny. This pattern suggested that large-bodied species had heavier limb muscle than small-bodied species because larger-bodied species need more heavier limb muscle to sustain heavier bodies.

For anurans, adult females significantly exceeded adult males in body size in most species (Shine, 1979; Monnet and Cherry, 2002). In this study, we found that females were larger body sizes than males in 53 species, exhibiting a female-biased sexual size dimorphism, which can be result of fecundity selection on female body size (Liao et al., 2013b, 2015). Previous studies have shown that sexual dimorphism in limb muscle is probably correlated with their use by the males during amplexus and male-male competition because limb muscle involved in amplexus in

larger individuals can produce greater forces (Lee, 2001; Clark and Peters, 2006; Mi, 2012, 2013; Zamora-Camacho, 2018). Our exploratory study revealed that relative limb muscles mass did not differ between males and females, when removing SVL effect. This finding is different from previous studies that sexual dimorphism in limb muscle mass in frogs may be attributable to the importance which those limb muscle help males to act on clasping females and develop to resist attempted take-overs by competing males (Lee, 2001; Liao et al., 2012a; Mi, 2012). Furthermore, a lacking sexual dimorphism in limb muscle mass across 64 species may be attributed to the fact that the main weakness in this study is that all these species are considered equally in their mating system and type of amplexus. Indeed, sexual selection favors more robust forearms in a scramble-type mating system and other characteristics are more important

in inter and intersexual selection in leks or territorial systems (Andersson, 1994). Hence, it is possible that we cannot detect the importance of the muscle mass of the forearms in those species where it does matter at the time of acquiring a mate. Meanwhile, these species displaying the type of amplexus also affect variations in limb muscle mass because the robust forearms are related to axillary amplexus where the male surrounds the female and holds her firmly (Lee, 2001). In this study, the only forearm muscle used was the flexor carpi radialis. However, other muscles, especially from the forearm, have already been associated to the clasping behavior of males by Gaupp (1896), with clear differences in flexor carpi radialis, abductor indicis longus, and extensor carpi radialis caput superius between sexes for *Pelophylax cf. esculenta*. Oka et al. (1984) found that sexual differences in forearm muscles (e.g., flexor antibrachii medialis caput superius, abductor

TABLE 3 | Effects of male-biased operational sex ratio (OSR) and environmental harshness [coefficient of variation (CV) in precipitation and coefficient of variation in temperature] on limb muscle mass among species of anurans.

Dependent variable	Sex	Predictor	λ	B ± SE	t	R ²	P
Forelimb	Male	OSR	0.563 ^{1.000, <0.001}	0.075 ± 0.116	0.644	0.006	0.522
		SVL		3.742 ± 0.204	18.332	0.846	<0.001
		CV in precipitation	0.583 ^{1.000, <0.001}	0.002 ± 0.172	0.010	<0.001	0.992
		SVL		3.738 ± 0.205	18.196	0.844	<0.001
		CV in temperature	0.541 ^{1.000, <0.001}	0.055 ± 0.067	0.823	0.011	0.414
		SVL		3.753 ± 0.203	18.500	0.849	<0.001
	Female	OSR	0.000 ^{1.000, <0.001}	0.035 ± 0.148	0.236	0.001	0.814
		SVL		3.567 ± 0.191	18.714	0.852	<0.001
		CV in precipitation	0.000 ^{1.000, <0.001}	-0.174 ± 0.184	-0.944	0.014	0.349
		SVL		3.570 ± 0.173	20.655	0.875	<0.001
		CV in temperature	0.000 ^{1.000, <0.001}	0.034 ± 0.077	0.441	0.003	0.661
		SVL		3.579 ± 0.174	20.604	0.874	<0.001
Hindlimb	Male	OSR	0.822 ^{1.000, <0.001}	0.123 ± 0.087	1.401	0.031	0.166
		SVL		3.404 ± 0.171	19.882	0.866	<0.001
		CV in precipitation	0.805 ^{1.000, <0.001}	0.181 ± 0.135	1.337	0.028	0.186
		SVL		3.396 ± 0.171	19.870	0.866	<0.001
		CV in temperature	0.814 ^{1.000, <0.001}	-0.060 ± 0.047	-1.263	0.026	0.211
		SVL		3.389 ± 0.172	19.702	0.864	<0.001
	Female	OSR	0.491 ^{1.000, <0.001}	0.022 ± 0.136	0.164	0.0004	0.871
		SVL		3.140 ± 0.214	14.706	0.780	<0.001
		CV in precipitation	0.477 ^{1.000, <0.001}	-0.014 ± 0.181	-0.077	<0.001	0.939
		SVL		3.155 ± 0.194	16.270	0.813	<0.001
		CV in temperature	0.578 ^{1.000, <0.001}	-0.078 ± 0.069	-1.126	0.020	0.265
		SVL		3.139 ± 0.195	16.062	0.809	<0.001
Limb	Male	OSR	0.856 ^{1.000, <0.001}	0.108 ± 0.082	1.320	0.028	0.192
		SVL		3.433 ± 0.163	21.060	0.879	<0.001
		CV in precipitation	0.847 ^{1.000, <0.001}	0.149 ± 0.127	1.170	0.022	0.247
		SVL		3.429 ± 0.163	20.993	0.878	<0.001
		CV in temperature	0.842 ^{1.000, <0.001}	-0.038 ± 0.044	-0.864	0.012	0.391
		SVL		3.421 ± 0.165	20.774	0.876	<0.001
	Female	OSR	0.346 ^{1.000, <0.001}	0.012 ± 0.133	0.089	0.0001	0.93
		SVL		3.198 ± 0.200	15.951	0.807	<0.001
		CV in precipitation	0.325 ^{1.000, <0.001}	-0.041 ± 0.175	-0.232	0.001	0.817
		SVL		3.206 ± 0.182	17.591	0.835	<0.001
		CV in temperature	0.431 ^{1.000, <0.001}	-0.069 ± 0.069	-0.998	0.016	0.322
		SVL		3.195 ± 0.184	17.326	0.831	<0.001

indicialis longus, extensor carpi radialis caput superius, and flexor antibrachii lateralis superficialis caput superius) that might also participate in the amplexus in *Bufo japonicus*. Therefore, forearm muscles in more frogs species should also be tested for sexual dimorphism in similar future studies.

Robust limb muscle benefit males to grasp females firmly and resist take-over attempts from competitors during the male-male competition process, thus promoting male mating success in anurans (Wells, 1977; Howard and Kluge, 1985). Male-biased OSR is positively related to the intensity of male-male competition (Wells, 2007). Consequently, relative limb muscle mass is predicted to be positively correlated with male-biased OSR in frogs and toads. Inconsistent with the prediction that the relative mass of limb muscle should increase with increasing male-biased OSR among different environments (Yang et al., 2017), we found male-biased OSR variation did not display a correlation with relative mass of limb muscle in males among species, suggesting that stronger male-male competition cannot promote relative larger limb muscle mass to avoid competitors from replacing.

Environmental harshness such as CV in temperature and rainfall affects directly active seasons length and food availability in anurans (Wells, 2007; Mai et al., 2019). As a result, species living longer active season and accessing more food resources can grow stronger limb muscle than species living shorter active season and accessing fewer food resources in frogs (Yang et al., 2017). In this study, we found that the mass of forelimb muscle, hindlimb muscle and total combinedlimb muscle was not affected by CV in temperature and rainfall, suggesting that when available food resources is reduced in increased environmental harshness, the rate of survival success mainly depends on decreased active season and increased hibernation to decline energetic reserves of limb muscle in frogs (our unpublished data).

In conclusion, we illustrate the positive association between absolute and relative limb muscle mass and SVL across anuran species. Anuran limb muscle mass does not differ between males and females, thus displaying a lack of sexual dimorphism in limb muscles. Also, variations in limb muscle mass cannot be shaped by neither male-biased OSR nor CV in temperature and rainfall, suggesting that environmental harshness and sexual selection cannot shape variations in limb muscle mass in anurans.

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DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The Animal Ethics Committee at China West Normal University approved the sacrifice of animals.

AUTHOR CONTRIBUTIONS

YJY, YJ, and WBL conceived, wrote, and edited the manuscript. WBL and ZPM secured funding. All authors contributed to editing the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Sciences Foundation of China (32170429 and 31970393), the Key Project of Science and Technology of Sichuan Province (22NSFSC2743). The reported experiments comply with the current laws of China concerning animal experimentation, and permit to collect amphibians from the Ethical Committee for Animal Experiments in China Council on Animal Care (CCAC) was in hand at the time of sampling.

ACKNOWLEDGMENTS

We thank Chunlan Mai, Wei Liu, Shengnan Chen, and Chuan Chen to help collecting samplings in fieldwork.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.879885/full#supplementary-material>

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