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Effects of long-term preservation on amphibian body conditions: Implications for historical morphological research

Guocheng Shu [1], **Yuzhou Gong** [1], **Feng Xie** [1], **Nicholas C. Wu** [2], **Cheng Li** Corresp. [1]

[1] Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan, China

[2] School of Biological Sciences, University of Queensland, Brisbane, Queensland, Australia

Corresponding Author: Cheng Li
Email address: licheng@cib.ac.cn

Measurements of historical specimens are widely applied in studies of taxonomy, systematics, and ecology, but biologists often assume that the effects of preservative chemicals on the morphology of amphibian specimens do not affect their analyses. We compared the body length and body mass of 14 live and preserved (up to 10 years) amphibian species and found that the body length and body mass of preserved specimens significantly decreased by 7.1% and 26.7%, respectively, compared to those measurements of their live counterparts. Additionally, there was greater body length (3.6%) and body mass (6.6%) shrinkage in the order Urodela than in the order Anura, but there were no significant differences in body length and body mass shrinkage between males and females. Furthermore, preservation apparently distorted the magnitude of the intersexual and interspecific differences in body length observed in the fresh specimens. When species were compared, we found that the shrinkage was proportionately greater in longer species, while the body mass of heavier individuals shrank proportionately less than that of lighter individuals. Due to the effects of preservation on amphibian morphology, we propose parsimonious conversion equations to back-calculate the original body length and body mass of study animals for researchers working with historical data because morphological data from preserved specimens may lead to incorrect biological interpretations. Therefore, researchers must correct for errors due to preservation effects that may lead to the misinterpretation of results.

Effects of long-term preservation on amphibian body conditions: Implications for historical morphological research

Guocheng Shu[1,2], Yuzhou Gong[1,2], Feng Xie[1], Nicholas C. Wu[3] and Cheng Li[1]

1. Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan, China

2. University of Chinese Academy of Sciences, Beijing, China

3. School of Biological Sciences, University of Queensland, Brisbane, Queensland, Australia

Corresponding author:

Cheng Li[1]

E-mail address: licheng@cib.ac.cn

Abstract

Measurements of historical specimens are widely applied in studies of taxonomy, systematics, and ecology, but biologists often assume that the effects of preservative chemicals on the morphology of amphibian specimens do not affect their analyses. We compared the body length and body mass of 14 live and preserved (up to 10 years) amphibian species and found that the body length and body mass of preserved specimens significantly decreased by 7.1% and 26.7%, respectively, compared to those measurements of their live counterparts. Additionally, there was greater body length (3.6%) and body mass (6.6%) shrinkage in the order Urodela than in the order Anura, but there were no significant differences in body length and body mass shrinkage between males and females. Furthermore, preservation apparently distorted the magnitude of the intersexual and interspecific differences in body length observed in the fresh specimens. When

species were compared, we found that the shrinkage was proportionately greater in longer species, while the body mass of heavier individuals shrank proportionately less than that of lighter individuals. Due to the effects of preservation on amphibian morphology, we propose parsimonious conversion equations to back-calculate the original body length and body mass of study animals for researchers working with historical data because morphological data from preserved specimens may lead to incorrect biological interpretations. Therefore, researchers must correct for errors due to preservation effects that may lead to the misinterpretation of results.

Introduction

Common preservative chemicals, such as formalin and ethanol, are widely used in museum collections, especially for amphibian and reptile specimens (Simmons, 2002). However, due to the health risks to researchers from formalin (NRC, 1995) and the DNA degradation in formalin-fixed tissues (Wirgin et al., 1997), ethanol is more suitable for preserving amphibian specimens.

As biodiversity is rapidly declining around the world, museum specimens play an increasingly important role in biological research related to taxonomy (Arratia & Quezada-Romegialli, 2017), systematics (Huang et al., 2016), phylogeography (Godoy et al., 2004; Jaffe, Campbell-Staton & Losos, 2016), conservation biology (Forster, Hirst & Atkinson, 2012), evolution (Losos & De Quieroz, 1997) and ecology (Ochocinska & Taylor, 2003; Irschick et al., 2006; Moen, 2006). Measurements of preserved specimens are often compared to those of live amphibians, especially when comparing historical records and evolutionary changes in morphology (Vervust, Van Dongen & Van Damme, 2009), but this method should be used with caution, as preservation may alter the overall appearance of animal specimens (Stuart, 1995). For example, the Australian

green tree frog (*Litoria caerulea*) was originally described as the blue frog (*Rana caerulea*) because the preservation procedure removed the yellow pigments from the skin leaving only the blue and green pigments behind; the Latin name for blue is *caerulea* (Walls, 1995). Additionally, the body shape (length and mass) of preserved specimens may also change in over time (Fey & Hare, 2005; Melo et al., 2010). Thus, analyzing the morphological data collected directly from historical specimens may generate misleading results.

Although a few researchers have examined the effects of preservation techniques on animal specimens, most herpetologists have not considered this problem. Scott and Aquino-Shuster (1989) reported that frozen *Rana pipiens* specimens transferred to 40% isopropanol were softer with a duller overall appearance than non-frozen specimens, and they found that the snout-vent length (SVL) shrank 0-10% after one year, although the sample size was low (n = 7). Lee (1982) showed significant changes in 14 morphometric characters of *Rhinella marina* after six months of preservation in 70% ethanol; six morphological characters (e.g., SVL) increased, while the other eight decreased (e.g., axilla-groin length (AGL)). Surprisingly, repeated measurements after additional eight months showed that the changes in the 14 morphological traits (e.g., SVL) reversed compared to the first six months. Lee (1982) also found that preservation reduced the magnitude of the intersexual differences in fresh specimens and generated a new "sexual dimorphism". In addition, not all characters were measurable with equal precision, and there was a highly significant correlation between precision and the inter-individual variation in characters. Another important confounding factor is inter-observer effects; Hayek, Heyer and Gascon (2001) examined researcher measurement error in frog morphometry in terms of both inter-observer effects on single measurements and intra-observer effects on repeated measurements of 14

characters of *Vanzolinius discodactylus* specimens. Based on statistical modeling, they argued that inter- and intra-observer differences in measurements may lead to different biological interpretations of results, and they also suggested that biologists should separately analyze data by sex and select the most appropriate statistical model for each data set. Meanwhile, large morphological characters (e.g., SVL or total length (TL)) have a lower intra-observer coefficient of variation and a greater precision than small characters (Yezerinac, Lougheed & Handford, 1992).

To date, most preservation studies have focused on one species with many morphological characteristics, except Deichmann, Boundy and Williamson (2009) who reported changes in the SVL of 14 anuran species in response to preservation. Their results revealed that 13 of these 14 species were significantly affected by the preservative with the SVL of all species decreasing by 0.31-5.62%, and across species, there was no evidence that smaller species shrank proportionately more or less than larger species. The authors also argued that most preservation-related changes occurred in the first several months after initial preservation, but they did not report on the long-term effects of preservation (> 5 years).

All of the above researchers only examined the effects of preservation on the morphometric characters of anurans, excluding the order Urodela, which is an important amphibian taxon. Additionally, they examined the effects of short-term preservation on specimen morphology and neglected long-term effects, which are especially important in historical studies because many specimens are preserved for longer than has been previously reported. In this context, the objectives of this study were 1) to estimate the effects of long-term (10 years) ethanol preservation on amphibian (anurans and urodeles) body conditions, 2) to determine how

differences in the change in body conditions are grouped (interorder, intersexual and interspecific), and 3) to provide conversion equations to correct for the body length and mass of preserved specimens and allow for a more accurate estimation of the body conditions of historical specimens.

Materials and Methods

Ethics Statement

The conducted research is in compliance with laws and ethical standards of the country. All animal procedures were approved by the Animal Care and Use Committee of the Chengdu Institute of Biology, Chinese Academy of Sciences (CIB2006062003). All field work with animals was conducted according to relevant national and international guide-lines. Chengdu Institute of Biology issued permit number CIB#2006-18 for field work.

Sample Disposal

A total of 253 specimens representing 14 amphibian species, 7 families, and 2 orders (Table 1) were collected in northwestern Sichuan in 2006 and stored at the Herpetological Museum of the Chengdu Institute of Biology, the Chinese Academy of Sciences (CAS), where they were verified by amphibian experts. Sexual dimorphism was evident in a variety of the morphological traits of the frogs, such as body size, shape, and coloration; thus, male specimens were distinguished from female specimens according to their secondary sexual traits, including keratinized nuptial pads on the fingers, keratinized spines on the fingers and breast, cloacal dimorphism, a vocal sac, and the gonads.

For consistency and to reduce measurement errors due to the position of the limbs and the position of the specimens fixed during preservation, we measured body mass (g) and SVL (mm) for anurans and TL for urodeles. Hereafter, both SVL and TL are termed "body length". The live body length (L_l) and live mass (M_l) of the specimens was measured during their initial capture while they were anesthetized. Body length was measured to the nearest 0.01 mm with dial calipers, and each individual was weighed using an electronic scale with a precision of 0.1 g. All study animals were euthanized via immersion in chloretone and water in compliance with the standards of the Animal Care and Use Committee of the Chengdu Institute of Biology, CAS.

All euthanized individuals were transferred to the Herpetological Museum, and the standard museum procedures were as follows: specimens were fixed in 10% formalin for 24 hours, placed in standing water for an additional 24 hours and subsequently transferred to 70% ethanol for long-term storage (Heyer et al., 1994; Simmons, 2002). In 2015, the same preserved specimens were measured again, including their preserved body length (L_p) and preserved mass (M_p), using the same dial calipers and scale. To determine the occurrence of inter-observer bias, two recorders measured two amphibian species independently, *Pseudorana weiningensis* (n = 8) and *Batrachuperus pinchonii* (n = 20), and the results were compared.

Statistical Analysis

We examined the body length and body mass of 14 amphibian species and compared the changes between their live and preserved states. All data sets were tested for normality prior to other analyses, and a paired sampled *t-test* was applied to test for differences between L_l and L_p, M_l and M_p, and inter-observer variations in the measurements. Wilcoxon signed-rank tests were

used to statistically analyze non-normal matched-pair data, and the analysis of covariance was preformed to compare interspecific differences in response to preservation. Levene's test was conducted to test the homogeneity of variances, and Tamhane's T2 were used for multiple comparisons in fresh specimens and preserved specimens respectively to ensure whether the interspecific differences and intersexual differences were changed during the preservation. An independent sample *t-test* was carried out to analyze changes in body length and body mass between different orders and sexes, and linear correlation analysis was performed to test for relationships between the changes in body length or mass and the live length or mass of the amphibians. Finally, conversion equations were constructed to correct the body length and body mass measurements of preserved amphibian specimens through multiple-linear regression analysis, and the best models were determined by backward stepwise regression. A *t-test* was used to ascertain significant differences between the linear coefficient (y-intercept) and zero and the angular coefficient (slope) and one. All statistical tests were performed using SPSS (version 20.0) (SPSS IBM, 2011), and differences were considered significant when $p < 0.05$. All of the results in the text are presented as the mean ± SD (standard deviation), except in the error bar charts, which show the mean ± SE (standard error).

Results

Reductions in Body Length and Mass

There were no significant differences in both the body length and mass of the two species (*Pseudorana weiningensis*: $p = 0.314$, 0.077; *Batrachuperus pinchonii*: $p = 0.351$, 0.351;

combined: p = 0.314, 0.351) measured by each recorder (Table S1); thus, there is no inter-observer bias in our measurements. Overall, the Wilcoxon signed-rank test revealed highly significant differences in body length (t = 18.538, $p < 0.001$) and body mass (t = 19.436, $p < 0.001$) between the live and preserved specimens, where the body length and body mass of the preserved specimens significantly decreased relative to those of the fresh specimens. The changes in body length and mass also exhibited highly significant variations between species (body length: absolute change, $F_{13,239} = 58.250$, $p < 0.001$; percent change, $F_{13,239} = 11.163$, $p < 0.001$; body mass: absolute change, $F_{13,239} = 12.379$, $p < 0.001$; percent change, $F_{13,239} = 29.655$, $p < 0.001$). Furthermore, the results showed that the mean body length or mass of each sampled species were significantly (at least $p < 0.05$) different between pre-preservation and post-preservation (Tables S2). The body lengths of five species significantly ($p < 0.05$) decreased during preservation; those of four species were markedly significantly ($p < 0.01$) reduced; and those of five species were highly significantly ($p < 0.001$) reduced. The body mass results were similar (Table S3). The mean reductions in body length and body mass varied between species by 7.07 ± 3.45% and 26.68 ± 9.31%, respectively. Both the body length and mass of *Odorrana margaretae* shrank the least with length shrinking by 3.70 ± 3.81% and mass by 16.57 ± 9.26%, and the body length of *Batrachuperus pinchonii* shrank the most by 9.68 ± 2.71%. Surprisingly, *Nanorana pleskei* shrank by 48.43% on average, roughly half its entire body mass (Figure 1).

Shrinkage Variation by Order and Sex

The reduction in body length and body mass varied with order; the effects of ethanol preservation on body length (including percent change and absolute change) and body mass

169 (percent change) were significantly greater in Urodela than in Anura. However, the absolute
170 change in body mass (ACM) was greater in Anura than in Urodela (Table 2). Although
171 preservation resulted in different changes in body condition between orders, it did not alter the
172 magnitude of the differences.

173 The effects of preservation on body length did not differ significantly between sexes, but the
174 percent change in body mass (PCM) after preservation was greater in males (27.80 ± 9.74%) than
175 females (23.18 ± 9.29%; Table 2). Although the absolute change in length (ACL), percent change
176 in length (PCL) and ACM did not differ significantly between males and females, the average
177 female body length became highly significantly larger (p = 0.009 vs. p = 0.017) than that of
178 males in preserved specimens. Thus, preservation apparently increased the magnitude of the
179 intersexual difference in body length detected in the fresh specimens (Table 2).

180 Distortion of Interspecific Differences with Long-term Preservation

181 We conducted paired comparisons of the body characteristics of each species when fresh and
182 after preservation; in total, there were 182 pairs (91 species counterparts, and each counterpart
183 had 2 indicators, i.e., length and mass). Multiple comparisons indicated that long-term
184 preservation significantly distorted the interspecific differences in 14 of the 182 species pairs, and
185 seven preserved pairs exhibited greater interspecific differences than detected in their live
186 counterparts. However, the seven pairs presented interspecific differences of lower magnitude
187 than in the live specimens (Table S4). Furthermore, when comparing body length, the significant
188 differences in 7 pairs disappeared after preservation; a marginally significant difference (p <
189 0.05) in one pair increased to a markedly significant difference (p < 0.01); and differences in

three pairs increased from being markedly significant to highly significant ($p < 0.001$). In body mass, by contrast, a significant difference occurred in one pair after preservation; the difference in one pair disappeared; and the difference increased to marked significance from marginal significance in one pair and to high significance from marked significance in another. The magnitudes of these changes in interspecific differences are shown in Table S4.

Correlation Analysis and Correction Equations

Because of the great reduction in body length, we determined whether L_p, ACL, and PCL were correlated with L_l (Table 3) in all individuals, and the relationships between M_l and M_p and between shrinkage (ACM and PCM) and M_l were also determined. The results indicated that there was a strong correlation between L_l and L_p ($R = 0.995$, $p = 0.000$) and between L_l and ACL ($R = 0.500$, $p = 0.000$) in anuran species as well as between M_l and M_p ($R = 0.993$, $p = 0.000$) and between ACM and M_l ($R = 0.843$, $p = 0.000$); similar correlations were also observed in urodele species. However, PCL was not associated with L_l in both anuran and urodele species. The results showed that PCM was negatively significantly correlated with M_l ($R = -0.481$, $p = 0.000$) in anuran species, but there was no correlation between PCM and M_l in urodeles. In other words, longer individuals showed a greater reduction (absolute change) in body length than shorter individuals, and the body mass of heavier individuals was reduced more (absolute change) than that of lighter individuals. However, in anuran species, the body mass of heavier individuals shrank (percent change) proportionately less than that of lighter individuals, but the PCM was not correlated with PCL in urodele species ($R = 0.136$, $p = 0.258$). Thus, the shrinkages in body length and body mass were not parallel.

Based on the results of the correlation analysis, relative conserved correction equations were

212 constructed to estimate the live length and mass of amphibians from preserved specimens via
213 multiple-linear regression analysis (Figure 2). The most optimal models are presented in table 4.
214 Although the linear association was strong in three of the models (R2 > 0.97), we recommend
215 equation (1) and equation (3) to predict the live length or live mass from ethanol-preserved
216 amphibian specimens because we cannot reject the hypothesis that the angular coefficient (slope)
217 in equation (2) is equal to one by a t-test (t = 1.597, p = 0.115), which may be due to the sample
218 size.

219 Discussion

220 Long-term preservation caused pronounced reductions in body measurements in both anuran
221 and urodele specimens; for all 14 species studied, there were significant differences in body
222 length and mass between live specimens and preserved specimens. Interestingly, the sample sizes
223 of the species in which there were no highly significant pre-preservation and post-preservation
224 differences, such as *R. dugritei, P. nigromaculatus, F. multistriata* and *H. gongshanensis*, were
225 the smallest in the study, which suggested that the number of animals sampled needs to be taken
226 into account, as observed by Deichmann, Boundy and Williamson (2009). The differences in
227 shrinkage between species ranged from 3.70% to 9.68% in body length and 16.57% to 48.43% in
228 body mass, which were comparable to those of other studies. For example, Lee (1982) found that
229 the SVL of *Rhinella marina* increased by 1.68% after 14 months of preservation in 70% ethanol,
230 and Deichmann, Boundy and Williamson (2009) reported that preservation in 70% ethanol
231 significantly influenced the SVL of 13 of 14 species that had been preserved for 5 years or less,
232 with 0.31-5.62% SVL shrinkage. However, the findings of Deichmann, Boundy and Williamson

(2009) were not consistent with our results; our long-term study showed greater shrinkage in body length and body mass, which suggests that the duration of preservation can drastically influence body characteristics and lead to variations in shrinkage. The specimens in our study exhibited greater changes in body length and mass over longer preservation periods, so uninformed researchers focusing on taxonomy, phylogeny, ecology and evolutionary morphology could potentially get misleading results if they use direct measurements of preserved specimens to infer the characteristics of their live counterparts.

Preservation impacted interspecific differences, which varied with preservation time, as well as differences related to order and sex. Because ethanol penetrates and dehydrates tissues (Sturgess & Nicola, 1975), the shrinkage of specimens mainly resulted from the loss of water during the process of preservation. Male amphibians shrank more in both body length and mass compared to females. Typically, female amphibians are larger than males, although *Scutiger mammatus* and *Batrachuperus pinchonii* were exceptions in this study (Fairbairn, Blanckenhorn & Székely, 2007; Fei, Ye & Jiang, 2012). Therefore, body size influences the rate of ethanol dehydration due to the scaling exponent of the surface area to volume ratio (Klein et al., 2016), where smaller animals have a disproportionally larger surface area to volume ratio (exponent 0.68). Urodeles and anurans also differ in body shape, and urodeles have a larger surface area to body volume ratio due to their tails. Thus, the differences in shrinkage in this study can be explained by differences in body shape between sexes or orders.

Among the 14 species studied, more aquatic species (urodeles) generally showed greater reductions in body length and mass than semiaquatic species (anurans). Arboreal species had the highest resistance to evaporative water loss, aquatic species tended to have little or no resistance,

and terrestrial species tended to have resistance between those of arboreal and aquatic frogs. (Young et al., 2005), which may explain the differences in the rate of shrinkage in this study. *Batrachuperus pinchonii* (an urodele species) live in the moist habitats, while all of the anurans sampled in this study belonged to the semi-aquatic group, whose habitats are relatively drier (IUCN, 2016). So, the rate of shrinkage may also relate to habitat selection by the two orders. Schmid (1965) reported that nine amphibian species exhibited marked variation in their habitat preferences in terms of the availability of water. Thus, dehydration due to the process of ethanol preservation might increase cutaneous water diffusion in terrestrial species at a greater rate than aquatic species and result in different degrees of shrinkage.

Various skin characteristics can also change the rate of water loss, including skin texture, thickness, and the presence of cutaneous glands (Toledo & Jared, 1993; Torri et al., 2014), and Schmid and Barden (1965) found an inverse correlation between the permeability to water and the lipid content of skin. The lipid content of the skin might influence the rate of water loss during long-term preservation, resulting in interspecific differences in shrinkage. The SVL of green iguanas (*Iguana iguana*) preserved in 70% ethanol shrank between 1-7% over a two-month period (Vervust, Van Dongen & Van Damme, 2009), and Reed (2001) similarly reported that the SVL of snakes (41 species) decreased by 6-7% in 70% ethanol (16 years, 4 years and < 1 year), while there was little reduction in mass (ranged from 0.772 to 1.267 grams). This suggests that although the longest preservation time studied by Reed (2001) was longer than ours, using the same type and concentration of preservatives, the maximum SVL shrinkage in snakes was less than in amphibians. Thus, skin structure plays an important role in the rate of water loss during long-term preservation because snakes have a thicker epidermis than amphibians with numerous

277 differentiated layers above the *stratum germinativum* (Vitt & Caldwell, 2013). Lee (1982)
278 speculated that intersexual differences in shrinkage might also be related to the reproductive
279 condition of females, which can carry a rich complement of eggs, but this remains to be tested.
280 The variations in body shrinkage observed in this study could potentially be explained by
281 complex interactions among the factors mentioned above. (Arratia & Quezada-Romegialli 2017)
282 Based on this research, we conclude that long-term storage greatly deforms the body
283 characteristics of amphibians, which will confound the results of research if historical specimens
284 are directly measured. For example, we found that preservation apparently changed the
285 magnitude of the intersexual and interspecific differences in body condition detected in fresh
286 specimens, which was consistent with the results of Lee (1982), and correlation analysis indicated
287 that L_p and ACL were significantly correlated with L_l. Overall, the reduction in body length or
288 mass increased with specimen size or mass; the body length or mass of longer or larger preserved
289 individuals will decrease at a greater rate. However, the percent body length shrinkage values
290 were not associated with the live length in both Anura and Urodela, which implies that
291 differences among and within species may be obliterated during long-term preservation, or
292 preservation might exaggerate similarities or differences between large and small specimens
293 (Lee, 1982; Hayek, Heyer & Gascon, 2001). Consequently, preserved specimens are unlikely to
294 accurately reflect the morphologies of live specimens, which may lead to different biological
295 interpretations and result in false conclusions.
296 In summary, long-term preservation significantly influences the body characteristics of
297 amphibian specimens and can distort intersexual, interspecific and inter-individual differences.
298 Therefore, researchers need to consider the influence of preservation on morphology when

299 interpreting the results of ecological and taxonomic studies involving historical specimens,
300 especially if the sampled specimens are being compared with fresh individuals. Fortunately, the
301 length and mass of living individuals can be predicted from preserved specimens using
302 conversion equations, as has been done with medusae (Thibault-Botha & Bowen, 2004), lizards
303 (Vervust, Van Dongen & Van Damme, 2009), insects (Lee, Kodama & Horiguchi, 2012), and
304 especially fish (Shields & Carlson, 1996; Porter, Brown & Bailey, 2001; Fey & Hare, 2005;
305 Thorstad et al., 2007; Melo et al., 2010). Therefore, we suggest parsimonious conversion
306 equations to estimate the fresh length and mass from amphibian specimens preserved in 70%
307 ethanol over the long term to improve the reliability of morphological data from historical
308 specimens. Museums are valuable resources for old and rare specimens; thus, obtaining accurate
309 measurements from preserved specimens will allow for accurate interpretations of biological
310 change over the short or long terms.

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427 **Table 1. Information on the samples used in this study.**

Family	Species	N	Location
Ranidae	*Amolops loloensis*	26	Zhaojue etc, Sichuan
	Pelophylax nigromaculatus	5	Miyi etc, Sichuan
	Pseudorana weiningensis	8	Zhaojue, Sichuan
	Odorrana margaretae	9	Dujiangyan etc, Sichuan
Dicroglossidae	*Nanorana pleskei*	10	Jiulong, Sichuan
	Fejervarya multistriata	5	Xichang, Sichuan
Megophryidae	*Scutiger glandulatus*	15	Kangding, Sichaun
	Scutiger mammatus	28	Jiulong etc, Sichuan
	Oreolalax pingii	14	Zhaojue, Sichuan
	Megophrys shapingensis	18	Zhaojue etc, Sichuan
Hylidae	*Hyla gongshanensis*	6	Xichang etc, Sichuan
Bufonidae	*Bufo gargarizans*	33	Xichang etc, Sichuan
Rhacophoridae	*Rhacophorus dugritei*	5	Mianning, Sichuan
Hynobiidae	*Batrachuperus pinchonii*	71	Mianning, Sichuan
7	14	253	

Table 2. Differences in measurements by order (Anura and Urodela) and sex (male and female).

Object	Interorder comparison			Intersexual comparison		
	Anura (N = 182)	Urodela (N = 71)	*t*	Male (N = 102)	Female (N = 83)	*t*
ACL(mm)	3.53 ± 2.18	13.92 ± 4.38	-19.075***	6.43 ± 5.73	6.97 ± 5.59	0.644
PCL (%)	6.06 ± 3.18	9.68 ± 2.71	-8.468***	7.05 ± 2.87	6.54 ± 3.48	-1.077
ACM (g)	5.35 ± 4.54	3.77 ± 1.67	4.039***	5.70 ± 4.05	6.33 ± 4.25	1.020
PCM (%)	24.82 ± 10.11	31.47 ± 4.02	-7.448***	27.80 ± 9.74	23.18 ± 9.29	-3.279**
L_l	59.70 ± 20.43	144.61 ± 26.80	-27.098***	81.97 ± 47.38	97.38 ± 37.54	-2.410*
L_p	56.17 ± 19.44	130.69 ± 24.66	-25.329***	75.54 ± 42.09	90.41 ± 33.09	-2.625**
M_l	25.42 ± 21.41	12.02 ± 5.33	7.842***	23.25 ± 17.12	31.78 ± 21.98	-2.896**
M_p	20.07 ± 17.75	8.25 ± 3.78	8.501***	17.54 ± 13.51	25.45 ± 19.16	-3.174**

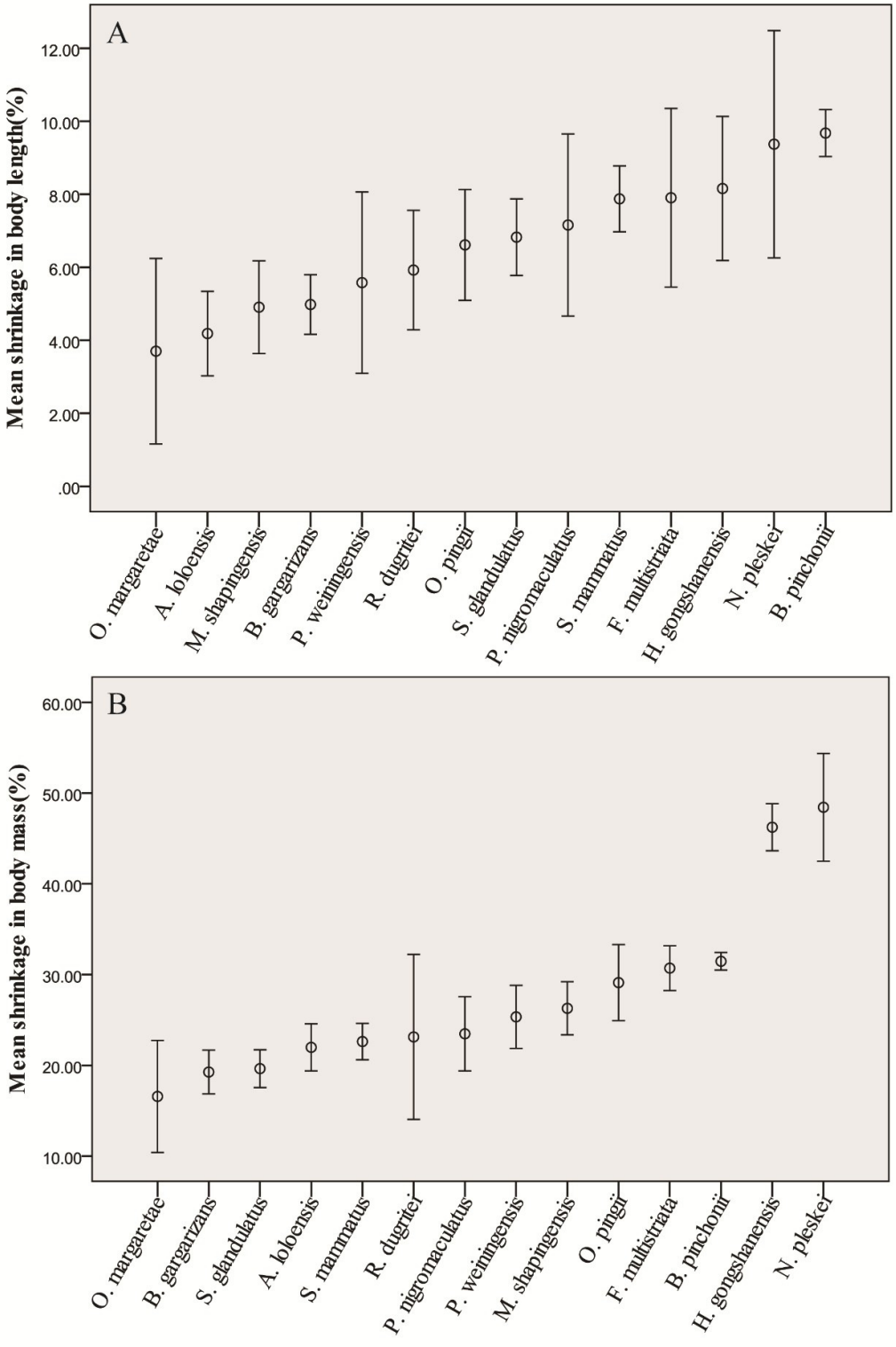
*** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$. ACL -absolute change in length, PCL -percent change in length, ACM-absolute change in mass, PCM-percent change in mass, L_l-live body length, L_p-preserved body length, M_l-live body mass, M_p-preserved body mass.

433 **Table 3. Correlation coefficient for the relationships between L_p and L_l; shrinkage (ACL**
434 **and PCL) and L_l; M_p and M_l; shrinkage (ACM and PCM) and M_l; ACL and ACM; PCL**
435 **and PCM.**

Trait	Anura		Urodela	
	Correlation Coefficient	*P*-value	Correlation Coefficient	*P*-value
L_p and L_l	0.995	0.000	0.989	0.000
ACL and L_l	0.500	0.000	0.947	0.000
PCL and L_l	-0.129	0.084	-0.069	0.565
M_p and M_l	0.993	0.000	0.990	0.000
ACM and M_l	0.843	0.000	0.947	0.000
PCM and M_l	-0.481	0.000	-0.069	0.565
ACL and ACM	0.545	0.000	0.533	0.000
PCL and PCM	0.299	0.000	0.136	0.258

436 **Table 4. Correction equations to estimate the fresh body length and body mass for**
437 **amphibian specimens.**

Taxa	Regression equation	N	R^2	*t-test* slope = 1	*P*-value	*t-test* y-intercept = 0	*P*-value	No.
Anura	$L_l=1.046L_p+0.934$	182	0.991	2.070	0.000	137.754	0.040	(1)
	$M_l=1.195M_p+1.438$	182	0.981	4.340	0.000	96.506	0.000	
Urodela	$L_l=1.075L_p+4.122$	71	0.978	1.579	0.115	55.385	0.000	(2)
	$M_l=1.394M_p+0.518$	71	0.980	2.379	0.020	58.033	0.000	
Two Orders	$L_l=1.116L_p–2.519$	253	0.995	-5.936	0.000	227.892	0.000	(3)
	$M_l=1.191M_p+1.702$	253	0.982	7.240	0.000	117.573	0.000	



438 **Figure 1. The meanshrinkage in (A) body length (%), and (B) body mass (g) across 14**
439 **amphibian species. Error bars indicate standard errors (SE).**

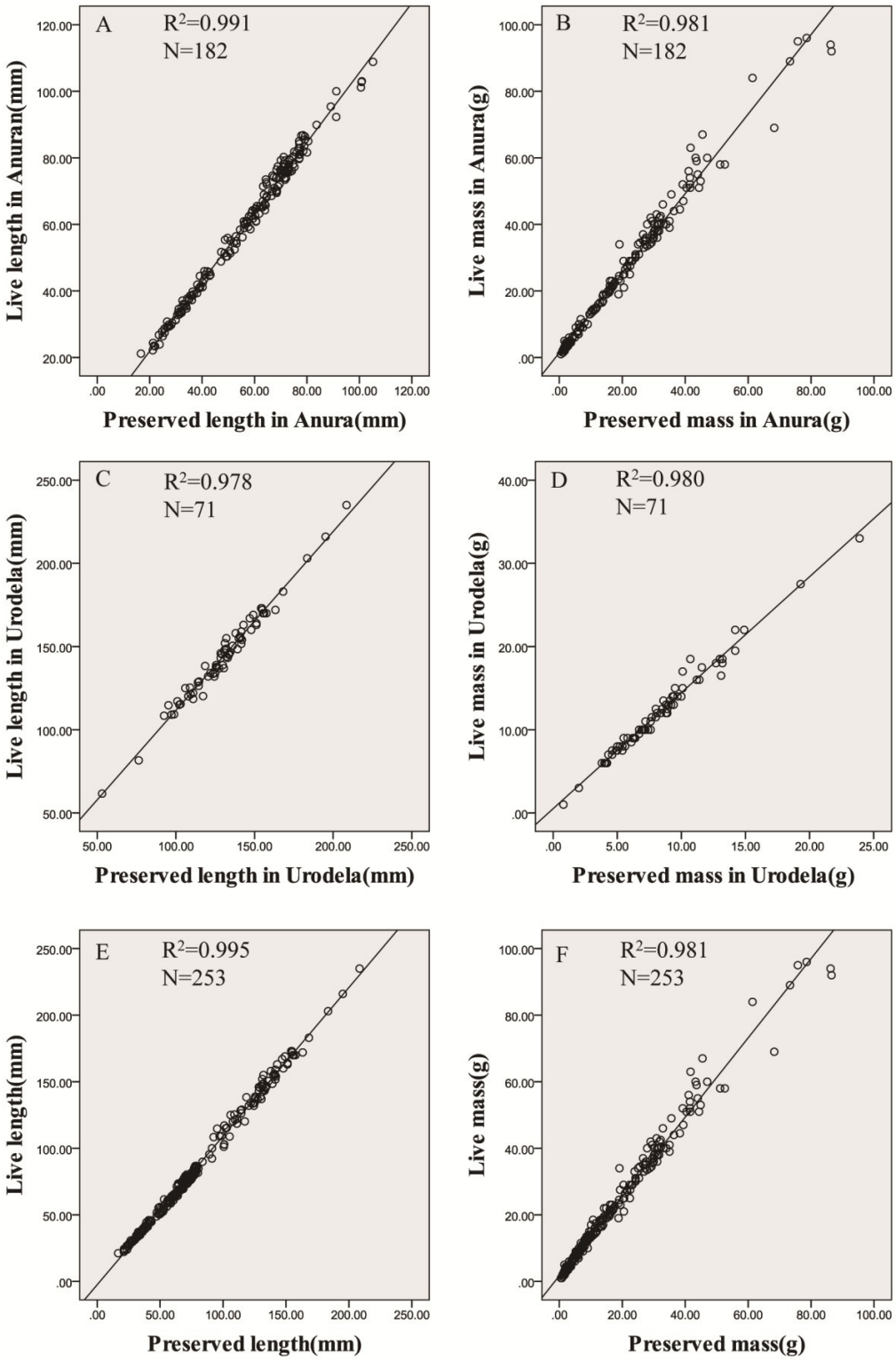


Figure 2. Relationships between live length/mass and preserved length/mass in all 14 amphibian species recorded for all 14 species recorded. A (length) and B (mass) for anuran species, C (length) and D (mass) for urodele species, and E (length) and F (mass).