Phyllomedusa 8(1):51-58, **2009**

ISSN 1519-1397

Anuran artifacts of preservation: 27 years later

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Abstract

Anuran artifacts of preservation: 27 years later. Measurements made on preserved anuran specimens are often used in studies of systematics, ecology and evolution. Here, we examine the effect of preservation on one of the most common measurement of frogs, snout-urostyle length (SUL). Preservation had significant effects on the SUL of 13 of the 14 species of North American frogs included in this study, with all species decreasing in SUL by 0.31-5.62%. Smaller frog species did not shrink proportionally more or less than larger species. Absolute shrinkage was correlated with SUL and was greater in larger species. Within species, percent shrinkage was not significantly correlated with SUL in 10 species, but significantly greater for larger individuals in 3 species, and decreased with size in 1 species. Absolute shrinkage was statistically greater for larger individuals in 4 species. Our results agree with studies of morphological permutations in fish which show that most preservation-related changes take place within the first few months after initial preservation. We suggest that the potential consequences of using preserved specimens in research must be considered and that future studies continue to examine preservation effects, not only on frogs, but on all preserved specimens used in scientific investigations.

Keywords: Anura, morphology, museum collections, snout-urostyle length, shrinkage.

Received 9 December 2008.
Accepted 25 May 2009.
Distributed July 2009.

Resumo

Artefatos de preservação em anuros: 27 anos depois. Medidas tomadas em espécimes preservados são frequentemente usadas em estudos de sistemática, ecologia e evolução. Examinamos aqui o efeito da preservação sobre uma das medidas mais comumente feitas em anuros, o comprimento rostro-clocal (CRC). A preservação teve efeitos significativos sobre o CRC de 13 das 14 espécies de anuros norte-americanos incluídas no estudo, com uma diminuição de 0,31 a 5,62% em todas as espécies. As espécies de menor porte não encolheram proporcionalmente mais nem menos que as espécies maiores. A redução absoluta de tamanho mostrou-se correlacionada com o CRC, tendo sido maior em espécies de maior porte. Dentro de cada espécie, a porcentagem de redução de tamanho não se mostrou significativamente correlacionada com o CRC em 10 espécies, mas foi significativamente maior para os indivíduos de maior porte em três espécies e diminuiu com o tamanho em uma espécie. A redução absoluta de tamanho foi estatisticamente maior para os indivíduos maiores em quatro espécies. Nossos resultados concordam com os dados obtidos em estudos de permutações morfológicas em peixes, que mostraram que a maioria das modificações relacionadas à preservação ocorre durante os primeiros meses após a preservação. Sugerimos que as consequências potenciais do uso de espécimes preservados na pesquisa devem ser consideradas, e que estudos futuros continuem a examinar os efeitos da preservação, não apenas em anuros, mas em todos os espécimes preservados utilizados em investigações científicas.

Palavras-chave: Anura, morfologia, coleções taxonômicas, comprimento rostro-clocal, redução de tamanho.

Introduction

Approximately 27 years ago, Julian Lee (1982) published the first-ever article detailing changes in linear measurements and allometries associated with preservation of an anuran. Since then, there have been no subsequent studies of frog changes in preservation (Hayek *et al.* 2001). Perhaps more problematic is that researchers have continued the long history of assuming that character transformations between live and preserved specimens are negligible or that they are commensurate across individuals of a species (e.g., Emerson 1978, Withers and Hillman 2001).

Such ongoing assumptions emerge from a practical reality. Preserved specimens are important tools for scientific studies of evolution, ecology and systematics, and museum wet collections house several million anuran specimens worldwide (HerpNET 2007). Although these specimens have been collected and preserved using a variety of techniques over the years, many collectors have moved towards more uniform methods of preservation over the last few decades, and today most anurans are fixed in formalin and then transferred to alcohol for storage (Simmons 2002, National Park Service 2006).

While measurements of preserved specimens continue to be applied to live animals, little is known about the effects of preservation on morphology of frogs. Obviously, biomass of preserved frogs can easily misrepresent live biomass, but how much do linear measurements such as snout-urostyle length change during preservation? Lee's (1982) seminal study of a single species, *Rhinella marina* (*Bufo marinus*), revealed that all 14 linear measurements changed after preservation; some increased while others decreased.

Preservation artifacts are better known in fishes, although relatively few species have been studied, mostly those of commercial value (e.g., Burgner 1962, Parker 1963, Stobo 1972, Engel 1974, Yeh and Hodson 1975, Billy 1982, Leslie and Moore 1986, Jennings 1991, Sagnes 1997, Fey 1999). The types of osmotic influences and autolysis that change lengths in fishes in preservation may produce similar

effects in frogs, given that lengths of both taxa encompass skin and cartilage over an internal skeleton. Changes in fish length with preservation are not uniform and tend to be species-specific and vary among size classes and the preservative used (Billy 1982, Jennings 1991, Fey 1999). The general trend is a decrease in length with most of the shrinkage occurring within the first 40 days after preservation (Jennings 1991, Sagnes 1997).

Here, we evaluate one of the most widely measured characters in anurans, snout-urostyle length (SUL), before and after preservation, for 14 species of frogs to test for interspecific differences in response to preservation. Whereas Lee (1982) measured numerous characters on one species, we have measured one character on numerous species. We address four questions: (1) Does SUL change with preservation? (2) How is change in SUL related to body size within and among species? (3) Is the change proportionally different in large or small individuals and species? (4) Do specimens continue to change over time in preservative?

Materials and Methods

We examined and re-measured SUL of frog species that had been stored at the Louisiana State University Museum of Natural History (LSUMZ). Initially the live frogs had been anesthetized with chloretone and snout-urostyle length (SUL1) was measured to the nearest 0.1 mm using dial calipers. Then, the frogs were fixed using 10% formalin and subsequently transferred to either 70% ethanol or 55% isopropanol for long term storage. The preserved frogs were measured a second time (SUL2) between August and October, 2006. This represented at least one month and not more than 5 years after the initial measurement (SUL1). Then, SUL3 was measured in November 2007 (13 months after SUL2) for 3 species (*Incilius nebulifer*, *Hyla cinerea* and *Acris gryllus*) to monitor secondary storage changes, aside from initial preservation.

Initial SUL1 was measured by JB, whereas SUL2 and SUL3 were measured by JLD. In order to monitor potential researcher bias in measurements, both JB and JLD were asked to measure the same set of 10 frogs for 3 species of different sizes at SUL2—*Acris gryllus*, *Hyla cinerea* and *Incilius nebulifer*.

Because the time between SUL1 and SUL2 was not uniform, we tested for a correlation between the absolute value of change in preservation (SUL2-SUL1) and time in preservation in order to determine whether time since initial preservation had an effect on overall SUL change per individual frog. We then used paired t-tests to test for differences between pairs of measurements of SUL1, SUL2 and SUL3 and between researchers for SUL2. Pearson's correlation coefficient was used to investigate changes in SUL as a function of frog size among species and within species. We tested to see if the percent change or the absolute change in size was correlated with frog size. Clearly, a significant percent correlation might imply an absolute correlation as well, albeit curvilinear. Likewise, a linear absolute change in size should generally preclude a significant percent change. It is statistically possible that correlations of both absolute and percent shrinkage might be statistically significant when larger individuals shrink more than smaller ones, but not proportionally more. Also possible is neither an absolute size change nor a percent size change with frog size. Unless otherwise indicated, all statistical tests were performed using SAS 9.1 (Cary, NC).

Results

For the three species measured by both researchers, there was a statistically nonsignificant difference in measurement bias. The mean percent difference in measured lengths between the researchers (JLD-JB) was -0.68% for *Incilius nebulifer*, -0.46% for *Hyla cinerea* and -1.09% for *Acris crepitans* (Table 1). These differences were not significant in any

Table 1 - Size range of frogs measured and the difference in measurements between researchers (JLD-JB).

Family	Species	N	SUL range (mm)	Difference range (mm)	Mean difference (SD)	*P*-value	Mean % difference
Bufonidae	*Incilius nebulifer*	10	33.7 – 70.3	1.9 – -2.2	-0.41 (1.33)	0.356	-0.68
Hylidae	*Hyla cinerea*	10	24.4 – 55.0	0.9 – -0.9	-0.18 (0.46)	0.246	-0.46
Hylidae	*Acris gryllus*	10	15.6 – 26.5	1.0 – -0.3	-0.20 (0.39)	0.138	-1.09

of the three (Table 1, $P = 0.36$, 0.25, and 0.14, respectively), nor were they significant taken together ($P > 0.10$ Fisher's Combined Probability Test, Sokal and Rohlf, 1969). Despite no statistical significance, the differences represent a mean difference in measurements of 0.74%, which may be biologically important (Hayek and Heyer 2005).

Tests for differences due to preservation (SUL2-SUL1) in each species resulted in negative differences (i.e., shrinkage) for all 14 species (Table 2). The mean shrinkage was statistically significant in 13 of the 14 species measured and nearly significant in the 14th species, *Hyla femoralis* ($P = 0.08$), whose sample size was small with only 8 individuals (Table 2). Over the 14 species, the magnitude of shrinkage in SUL varied from 1.05% to 6.36% (Table 2). Shrinkage was independent of time in preservation across all individuals (Pearson's R = 0.08, $P = 0.14$).

Across species, there was no indication that smaller species shrank proportionately more or less than larger species (Pearson's R = 0.0002, $P = 0.96$; Figure 1A). Alternatively, absolute shrinkage did increase in larger anuran species, with a small coefficient of determination (Pearson's R = 0.28, $P = 0.05$; Figure 1B). Sample size was not associated with absolute shrinkage (Pearson's R = 0.02, $P = 0.64$) nor with percent shrinkage (Pearson's R = 0.06, $P = 0.38$).

Within species, results were somewhat mixed, but generally showed no consistent evidence for size-related shrinkage (Table 3). Nine species showed neither a significant correlation between absolute shrinkage and SUL nor between percent shrinkage and SUL. Three species showed both significantly more absolute shrinkage and more percent shrinkage with greater size, respectively: *Acris crepitans* (Pearson's R = 0.17 for absolute shrinkage and Pearson's R = 0.14 for percent shrinkage),

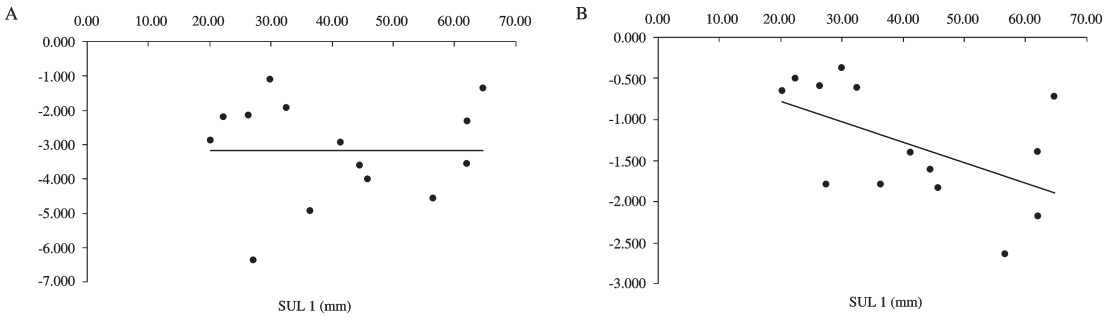

Figure 1 - The relationship between mean SUL of 14 species before preservation and (A) the mean percent of shrinkage and (B) the mean absolute degree of shrinkage after preservation.

Table 2 - Ranges and means of SUL1 (before preservation) and the change in SUL from the first measurement to the second measurement (SUL2-SUL1).

Family	Species	N	SUL range (mm)	Mean SUL (mm)	Difference range (mm)	Mean difference (mm)	*P*-value	Mean % difference
Bufonidae	*Incilius nebulifer*	26	36.0 - 79.9	64.73	3.9 – -3.7	-0.71	0.046	-1.32
Ranidae	*Lithobates sphenocephala*	15	34.6 - 82.6	62.09	1.0 – -5.5	-2.17	0.002	-3.55
Bufonidae	*Anaxyrus fowleri*	12	43.4 - 73.8	61.99	2.4 – -3.7	-1.38	0.010	-2.29
Scaphiopodidae	*Scaphiopus holbrookii*	6	46.6 - 63.6	56.57	-1.3 – -5.2	-2.63	0.012	-4.56
Ranidae	*Lithobates clamitans*	56	23.6 - 82.1	45.71	2.5 – -7.3	-1.83	0.001	-3.99
Hylidae	*Hyla cinerea*	23	25.0 - 56.3	44.45	0.3 – -4.4	-1.60	0.001	-3.58
Hylidae	*Hyla chrysoscelis*	27	19.6 - 52.5	41.39	3.0 – -5.5	-1.38	0.001	-2.96
Hylidae	*Hyla avivoca*	13	25.2 - 44.3	36.31	0.9 – -4.6	-1.78	0.001	-4.89
Hylidae	*Hyla femoralis*	8	28.6 - 37.0	32.46	0.5 – -2.1	-0.60	0.080	-1.90
Hylidae	*Hyla squirella*	35	22.0 - 35.1	29.83	2.1 – -2.0	-0.37	0.044	-1.05
Microhylidae	*Gastrophryne carolinensis*	17	24.2 - 32.3	27.35	0.4 – -4.1	-1.79	0.001	-6.36
Hylidae	*Pseudacris crucifer*	14	13.4 - 33.1	26.36	1.1 – -2.5	-0.57	0.046	-2.14
Hylidae	*Acris gryllus*	24	16.5 - 27.5	22.33	0.8 – -1.5	-0.50	0.001	-2.19
Hylidae	*Acris crepitans*	52	11.1 - 25.6	20.28	2.5 – -2.5	-0.65	0.001	-2.85

Gastrophryne carolinensis (Pearson's R = 0.56, Pearson's R = 0.45), and *Hyla chrysoscelis* (Pearson's R = 0.21, Pearson's R = 0.26). One species, *Lithobates clamitans*, showed significantly more absolute shrinkage with size (Pearson's R = 0.16), but not more percent shrinkage with size. Another species, *Incilius nebulifer*, showed significantly less percent shrinkage with size (Pearson's R =0.21), but no difference in absolute shrinkage with size.

Changes over the additional 13 months of storage, measured as differences between SUL2 and SUL3, were greatly reduced relative to the original shrinkage in preservative. For the three species measured, there was no significant change in *Acris gryllus*, a significant 0.30% increase in *Incilius nebulifer* and a significant 0.45% decrease in *Hyla cinerea* (Table 4).

Discussion

Because the original live SUL1 measurements were performed by JB, and the recent SUL2 measurements by JLD, we tested for differences in inter-observer mensuration. These differences were not statistically significant although perhaps larger sample sizes would have proven them to be. However, our goal here was to determine the potential magnitude of researcher bias in order to compare it to the measured shrinkage, not to prove that research bias can exist (Hayek *et al.* 2001). Mean shrinkage from the original SUL1 to SUL2 in the three co-measured species could be adjusted accordingly for each of those species as follows: *Incilius nebulif*er (1.32% - 0.68% = 0.64% shrinkage), *Hyla cinerea* (3.58% - 0.46% = 3.12% shrinkage), and *Acris gryllus* (2.19% - 1.09% = 1.10% shrinkage). For the remaining species, we do not have co-measurements from both observers, but we suggest that the percent shrinkage values in Table 2 can be reduced by the mean inter-observer bias of 0.74%. Doing so would adjust the range of percent shrinkage values to 0.31% - 5.62% across the 14 species and adjust the mean percent shrinkage to 2.38%.

Table 3 - Pearson's Correlation Coefficient for the relationship between percent change and SUL1 and between absolute change and SUL1 within species. * = $P<0.05$.

		Correlation coefficient	
Family	**Species**	**Percent change**	**Absolute change**
Bufonidae	*Anaxyrus fowleri*	0.16	-0.08
Bufonidae	*Incilius nebulifer*	0.46*	0.23
Hylidae	*Acris crepitans*	-0.37*	-0.41*
Hylidae	*Acris gryllus*	-0.23	-0.37
Hylidae	*Hyla avivoca*	-0.01	-0.07
Hylidae	*Hyla chrysoscelis*	-0.50*	-0.46*
Hylidae	*Hyla cinerea*	-0.05	-0.20
Hylidae	*Hyla femoralis*	0.31	0.26
Hylidae	*Hyla squirella*	-0.28	-0.29
Hylidae	*Pseudacris crucifer*	-0.04	-0.25
Microhylidae	*Gastrophryne carolinensis*	-0.67*	-0.75*
Ranidae	*Lithobates clamitans*	-0.01	-0.41*
Ranidae	*Lithobates sphenocephala*	0.08	-0.27
Scaphiopodidae	*Scaphiopus holbrookii*	-0.43	-0.53

One other study of inter-observer measurements on a set of 88 individuals of *Vanzolinius discodactylus*, showed a statistically significant difference of 1.4% between two observers for SUL (Hayek *et al.* 2001). That study found significant differences in 13 of 14 characters studied, although the variable measured most consistently and with the greatest precision was SUL (Hayek *et al.* 2001). Here, we have achieved less inter-observer variability because we, in fact, attempted to standardize our SUL measurements by having the observers converse and compare preliminary measurements. In short, our goal was to minimize inter-observer differences, not monitor independent measurements as was the case in Hayek *et al.* (2001). It should be no surprise then that our inter-observer difference (0.74%) is about half that (1.4%) found by Hayek *et al.* (2001).

Across the 14 species studied here adjusted shrinkage averaged 2.38% of SUL, and ranged from 0.31% to 5.62%, a range which is quite comparable to the range of shrinkage in fishes

Table 4 - SUL differences between two measurements taken after preservation (SUL3-SUL2).

Family	**Species**	**N**	**Difference range (mm)**	**Mean difference (mm)**	***P*-value**	**Mean % difference**
Bufonidae	*Incilius nebulifer*	26	1.10 – -0.50	0.223	0.008	0.30
Hylidae	*Hyla cinerea*	23	0.05 – -0.60	-0.196	0.001	-0.45
Hylidae	*Acris gryllus*	24	0.40 – -0.40	-0.042	0.304	-0.22

of 1% to 6.8% (Lee 1982). In contrast, Lee's measurements on *Rhinella marina* showed an increase in SUL with preservation, not a decrease (+1.9% in males and +1.2% in females). Of his 14 characters, Lee (1982) reported 6 increases and 8 decreases in preservation. Given our records for 14 frog species and numerous reports for fishes, the increase in length in preservation of *Rhinella marina* appears to be unusual among both frogs and fishes, and as such it may be particular to that species, as *Rhinella marina* is among the largest and "hardiest" of anurans. However, it should be no surprise to find species-specific differences in frogs at least as great as those in fishes, given the magnitude of morphological variation among species in both taxa. For example, our greatest degree of adjusted shrinkage, 5.62%, occurred in *Gastrophryne carolinensis*, which coincidentally was the only species where size explained about half the shrinkage; clearly, this species is more susceptible to shrinkage than the others we studied, as it shrank more and showed more size-related shrinkage. Further investigations examining species-specific rates of change in preservation are necessary to prevent potential biases in conclusions drawn from studies involving museum specimens.

For our 14 species, variation in shrinkage was not correlated with species size, sample size, time in preservation, nor was it associated with family or genus. Within species there was some tendency for shrinkage to increase with frog size, but the majority, 9 of 14, showed no evidence for shrinkage, absolute or percent, as a function of frog size (Table 2). This result could reflect no change or changes too small to be detected with our sample sizes. Interestingly, 8 of these 9 species exhibited the lowest sample sizes in the study, suggesting that the lack of significance here may be related to the number of frogs measured. These results contradict the general trend in fishes where shrinkage is proportionately greater in smaller fish (Burgner 1962, Stobo 1972, Yeh and Hodson 1975, Billy 1982). Consequently, the only working hypothesis for the degree of shrinkage associated with preservation in anurans is that there are species-specific differences.

This hypothesis offers both positive and negative factors for anuran biologists. On the negative side, we are unable to simply assume no changes occur in SUL with preservation, nor can we assume some constant absolute or proportional shrinkage across all species. On the positive side, we can establish useful guidelines for SUL measurements on preserved specimens:

(1) SUL measurements on preserved specimens are likely to be different from SUL measurements on live individuals, on the order of 0-6%;

(2) SUL measurements on preserved specimens can be adjusted by a species-specific proportional factor that can be determined by measuring SUL on live, anaesthetized individuals, preserving them through standard protocol, and re-measuring SUL after a moderate period of time, about 2-3 months, based on data here and from Lee (1982);

(3) As the proportional factor is likely to be in the range of 0-6%, applications using preserved SUL can either accept such error without adjustment, or provide proportional adjustment as needed. For example, in systematic studies, where frog measurements are used to distinguish cryptic species, this error may not be tolerable. However, in ecological studies, such as estimation of anuran community biomass (Deichmann *et al.* 2008), a 6% error in SUL measurements could translate into approximately a 15% error in mass estimates for most species, or 15 grams out of 100. Whether or not this difference in SUL measurements is acceptable will depend on the ultimate goals of the study.

These guidelines are obviously tentative as we have measured preservation effects on only 14 of the globe's 5000 species. Still, expanding the universe of monitored preservation effects from one (Lee 1982) to 14 merits a modicum of tentative generalization.

Acknowledgements

We thank the Louisiana State Museum of Natural Science and C. Austin for access to the collection. All frogs were collected by J. Boundy during work hours for the Louisiana Department of Wildlife and Fisheries for the purpose of developing SUL/mass curves, to obtain tissue samples for DNA sequencing, and to provide locality vouchers. This work was supported in part by funding from the U.S. National Science Foundation (DEB: 0639114) and a grant from the Conservation, Food and Health Foundation.

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