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The effect of preservation on lizard morphometrics – an experimental study

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Abstract. The millions of conserved biological specimens that are stored upon the shelves of Natural History Museums across the world constitute a capital of biological information that is becoming increasingly accessible to students of various disciplines. Most students have taken measures of body size and shape of preserved museum specimens to test various elements of ecological and evolutionary theory. One possible hazard of using morphological measurements of museum specimens is that fixation and preservation may deform bodies or body parts, but most researchers implicitely assume that the magnitude of conservation-induced distortions are insufficient to jeopardize their analyses. However, no study to our knowledge has clearly quantified those possible distortions. In this study, we have measured 17 morphological variables on a set of 65 green iguanas (*Iguana iguana*), starting shortly after their death and then repeatedly over a two month period, a period during which they were fixated and preserved. Our aims were (1) to quantify and compare the deformation in different morphometrics frequently used in evolutionary studies; (2) to determine the amount of temporal variation that can be attributed to reader variability; and (3) to build conversion equations that should improve the reliability of morphological comparisons of life and conserved specimens. Conserved lizards revealed major reduction in snout vent length and body weight. Changes in other measured traits are more subtle, but persistent. These facts disturb analyses when using relative measurements, especially when comparing (often small) intraspecific differences or even morphological differences within populations in a temporal frame. We urge caution in using museum specimens as direct proxies for living organisms in ecological and taxonomic studies.

Keywords: alcohol collections, artifacts of preservation, deformation, *Iguana iguana*, spirit specimens

Introduction

The millions of preserved biological specimens that are stored upon the shelves of Natural History Museums across the world constitute a capital of biological information that is becoming increasingly accessible to students of various disciplines. For instance, the HerpNET network currently connects 49 institutions and provides available data for over 5.5 million of herpetological specimens. Museum specimens have traditionally played a major role in the fields of taxonomy and phylogeny, but more recently have also proved valuable in areas such as phylogeography (e.g. Godoy et al., 2004), epidemiology (e.g. Persing et al., 1990; Garrett, 1994), toxicology (e.g. Hayes et al., 2002), agricultural sciences (e.g. Davies, Villablanca and Roderick, 1999), conservation biology (e.g. Roy et al., 1994; Parmesan, 1996) and evolutionary morphology (e.g. Irschick et al., 1997; Losos and De Quieroz, 1997).

Students in the latter discipline have taken measurements of body size and shape of conserved museum specimens to test various elements of ecological and evolutionary theory, such as character displacement (e.g. Yom-tov, 1991), ecological radiation (e.g. Irschick et al., 1997), sexual selection (e.g. Gienger and Beck, 2007), Bergmann's rule (e.g. Ochocinska and Taylor, 2003), Allen's rule (e.g. Laiolo and Rolando, 2001) and Cope's rule (e.g. Moen, 2006). Conserved specimens are used when live animals are difficult to obtain, or when data is required from large numbers of species or populations, e.g. for comparative analyses. As collections grow older and begin to span significant periods of time, they also offer the fascinating opportunity to observe temporal changes in mean body size and shape, thus allowing direct tests of morphological character plasticity

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and evolution (e.g. Pergams and Ashley, 1999; Yom-tov, 2003; Millien et al., 2006).

One possible hazard of using morphological measurements of museum specimens is that fixation and preservation may deform bodies or body parts. This artificial distortion may confound analyses of changes in body size or shape, especially if specimens are being contrasted to live individuals, or when museum specimens that have been conserved differently, or for different periods of time are being compared with each other. Preservation effects on size and shape have been described for adult crabs (Rufino, Abello and Yule, 2004), fish (e.g. Shetter, 1936; Parker, 1963; Stobo, 1972; Shields and Carlson, 1996; Sagnes, 1997), frogs (Lee, 1982) and snakes (Klauber, 1943). Information for lizards is scant. Several authors have used ad-hoc solutions to overcome the potential dangers of preservation artefacts (e.g. Lazell, 1972; Irschick et al., 1997; Losos et al., 1997). Others have explicitly (e.g. Irschick et al., 1996) or tacitly (e.g. Zani, 2000; Gienger and Beck, 2007) assumed that the effects of conservation are sufficiently small with respect to the factors under study. However, no study has investigated in detail how and when lizard body size measurements change during fixation and preservation. This is surprising, since lizards enjoy a reputation as prime model organisms in ecological morphology (Garland and Losos, 1994) and evolutionary biology (Vitt and Pianka, 1994; Fox, McCoy and Baird, 2003; Reilly, McBrayer and Miles, 2007).

In this study, we have measured 17 morphological variables on a set of 65 green iguanas (*Iguana iguana*), starting shortly after their death and then repeatedly over a period of two months, a period during which they were fixated and preserved. Our aims were (1) to quantify and compare the deformation in different morphometrics frequently used in evolutionary studies; (2) to determine the amount of temporal variation that can be attributed to reader variability; and (3) to build conversion equations that should improve the reliability of morphological comparisons of live and preserved specimens.

Materials and methods

Study animals and measurements

We obtained 93 young specimens of green iguanas (*Iguana iguana*) that had died through oxygen deficiency during transportation to a local commercial pet trader. From these 93 specimens we took 65 specimens with similar fresh post mortem status. No animals were killed for the purpose of this study. All lizards were measured and fixated within one day following their death. Snout-vent length (SVL) varied between 67.59 and 108.7 mm.

One of us (BV) measured the SVL and 15 external morphological variables (table 1) of each individual lizard to the nearest 0.01 mm using electronic callipers (CD-20PP, Mitutuyo Corporation, Japan). Body mass was determined to the nearest 0.05 g with an electronic balance (Kern fkb), after drying the specimen with cloth paper. We took measurements prior to fixation (day 0), shortly after fixation (day 3) and subsequently on days 5, 10, 15, 20, 30, 40, 50, 60 and 68. Specimens were fixated and preserved in the laboratory with an ambient temperature of 20°C.

Fixation and preservation

In most professional collections, specimens are first fixed to arrest the physical and chemical changes that would occur upon death of a tissue (Humason, 1981). Subsequently, specimens are submerged in a preserving liquid that protects them against decay or deterioration and conserves as much as possible its 'natural' appearance (Stoddard, 1989).

Table 1. Repeatability of measurement of the linear morphometric variables considered. Each characteristic was measured five times on 20 individuals.

Trait	Repeatability (%)
SVL	86.0
Tail length	97.0
Tail base width	78.0
Head length	98.0
Head width	95.0
Head heigth	92.4
Lower jaw length	97.9
External quadratum	98.5
External coronoid	96.1
Upper fore limb length	62.6
Lower fore limb length	61.6
Manus length	60.3
Length third digit manus	67.3
Upper hind limb length	77.7
Lower hind limb length	58.1
Pes length	75.7
Length fourth toe pes	85.6

The chemicals and solutions used for fixation and preservation vary among museums (e.g. Etheridge, 1958; Sturgess and Nicola, 1975; Gotte and Reynolds, 1997). After having surveyed the literature on specimen handling procedures, we opted to fixate our specimens in buffered 10% formalin (i.e. an aqueous formaldehyde solution) for 48 hours, which is probably the most commonly used method for fixation of reptiles. The buffering is necessary because acid solutions will decalcify the skeleton, while basic solutions will cause excessive clearing of the specimens. We used a sodium phosphate monobasic/sodium phosphate dibasic (anhydrous) buffer to maintain the 10% formalin at a pH of 7.0 (Etheridge, 1958; Quay, 1974). We immersed the specimens in recipients containing at least a 1 : 3 specimen-to-formalin ratio by volume because there is evidence that with higher ratios, the formalin will turn acid faster (Gotte and Reynolds, 1997). After fixation, we preserved the animals in 70% ethanol. We checked the alcohol percentage on each measurement day and added 100% ethanol to restore it to 70% if necessary.

Statistical analyses

We estimated the repeatability of our measurements by measuring each of the morphological variables five times on a subset of 20 lizards. The measurements took place on the same day, and we randomised the order in which the individuals were processed. We calculated repeatability as the between-individual variation divided by the total variation (the sum of between and within-individual variation, Lessells and Boag, 1987).

The effect of fixation on the morphometric variables (changes between day 0 and day 3) was assessed using repeated measures analysis of paired-t-tests. We carried out analyses both for absolute and relative measurements (trait divided by SVL of each separate measurement day). The consequences of preservation (changes between day 3 and day 60) was analysed in two different ways. Firstly, we used repeated measures analysis of variance to compare average SVL and body weight at each measurement day to the measurements made at day 60. Second, we fitted a modified Janoscheck growth model (Janoscheck, 1957; Sager, 1978) through the measurements of SVL and body weight of each individual lizard, in order to ascertain if the specimens become fixed over a certain period (i.e. becoming in osmotic balance with the surrounding preservative). The equation of this model is

$$W = A - S * \exp(-k * t^{p}),$$

where W is the corresponding measurement at time t (in days). In order to correct for initial SVL and body weight, we divided the raw measurements by the equivalent measures at day 3. In this model, the parameter A reflects the asymptotic degree of change, S indicates the amount of change, k is a form parameter, and p determines the shape of the exponential change. Between-individual variation in the parameters A and S were added as random effects to the model. This enables us to evaluate to what extent the changes in SVL and weight varies amongst individuals, after correction for individual size. Finally, we analysed the changes in morphological variables from day 0 until day 60, in order to obtain information regarding the possible deformation during the total process of conservation (sum of fixation and preservation) and provide conversion equations that should improve the reliability of morphological comparisons of life and preserved specimens. All statistics were performed using SAS (version 9.1).

Results

Measurement error varied significantly among the variables considered (table 1). Repeatability was high for measures of SVL, tail length and head dimensions, but somewhat lower for the different elements of the limbs. However, the error due to within-reader variation was sufficiently small enough to enable assessment of the effects of fixation and preservation.

The parameters of the modified Janoschek model were estimated as; for SVL (proportional decrease of SVL, propSVL, as dependent variable): $A = 3.19 \pm 0.143$, $S = 3.104$, $k = 0.0854 \pm 0.0336$ and $P = 1.055 \pm 0.139$ and for weight (proportional decrease of weight, propWeight, as dependent variable): $A = 13.397 \pm 0.522$, $S = 7.137 \pm 0.481$, $k = 0.000448 \pm 0.00049$ and $P = 3.2018 \pm 0.4519$. Application of modified Janoschek models resulted in average estimated asymptotic reductions of 3.2% in SVL and of 13.4% in body weight. Surprisingly, for both variables, between-individual variation contributed significantly to the total variation explained (both $P < 0.001$, fig. 1). The degree to which individual specimens shrunk over the experimental period varied considerably (SVL: 1 to 7%, body weight: 4-20%). As the models were run on size-corrected SVL and weight, these inter-individual differences in shrinkage cannot be explained by initial differences in size. Also, individual proportional changes in SVL and body weight were not correlated ($r = 0.01$; $P = 0.91$). We conclude that these spirit specimens become fixed after spending several weeks in preservative.

Fixation caused an average shrinkage of 0.7%, and the subsequent preservation in alcohol caused a further 3% reduction in SVL (ab-

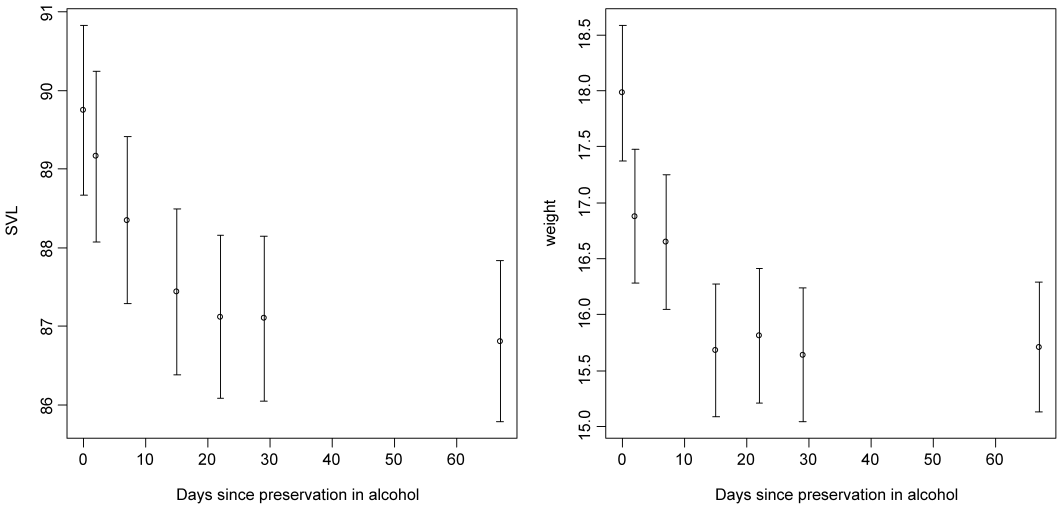

Figure 1. Mean SVL (mm) and body weight (g) of the 65 specimens of *Iguana iguana* used in the tests. Error bars indicate standard errors.

Table 2. Effect of fixation and preservation on the **absolute** morphometric variables considered. The t- and p-values indicate the significance of differences in absolute measures between day 0 and day 3 (fixation) and between day 3 and day 60 (preservation). Significant p-values are in bold. The average difference is expressed in percentage of the initial value (on day 0 and day 3, respectively).

Trait	Fixation			Preservation		
	t_{64}	p	% change	t_{64}	p	% change
SVL	7.93	<**0.0001**	−0.70	10.23	<**0.0001**	−3.19
Body weight	−3.28	<**0.05**	+1.68	10.65	<**0.0001**	−13.39
Tail length	−3.46	<**0.05**	+0.73	0.02	0.9820	−0.0092
Tail base width	2.35	<**0.05**	−1.29	8.45	<**0.0001**	−9.01
Head length	−0.29	0.774	+0.033	−1.63	0.1073	+0.271
Head width	−3.24	<**0.05**	+0.428	1.66	0.1026	−0.00519
Head heigth	−3.84	<**0.05**	+0.697	2.94	<**0.05**	−1.0037
Lower jaw length	−0.45	0.485	+0.058	−3.32	<**0.05**	+1.268
External quadratum	−0.58	0.5607	+0.067	−6.62	<**0.0001**	+1.472
External coronoid	−0.87	0.3857	+0.195	−1.83	0.0722	+1.160
Upper fore limb length	−5.22	<**0.0001**	+2.55	2.87	<**0.05**	−1.855
Lower fore limb length	−3.99	<**0.05**	+2.25	4.97	<**0.0001**	−3.823
Manus length	−1.03	0.305	+0.542	3.88	0.1080	−4.255
Length third digit manus	−0.84	0.406	+0.836	2.16	<**0.05**	−2.446
Upper hind limb length	−1.86	0.0669	+1.112	3.98	<**0.05**	−2.836
Lower hind limb length	−2.03	<**0.05**	+0.953	6.67	<**0.0001**	−3.3712
Pes length	−0.01	0.994	+0.0069	3.17	<**0.05**	−3.330
Length fourth toe pes	−1.25	0.2176	+0.449	2.58	<**0.05**	−1.6292

solute measurements: table 2, relative measurements: table 3). Body weight increased with fixation (1.7%), but then decreased by 7% during preservation. With the exception of tail base, all other body parts tended to grow larger as a result of fixation, although the effect was statistically significant for tail length, head height, upper and lower arm length and upper leg length only (table 2). In contrast, preservation made most body parts shrink. Only head length and the jaw length measures increased over the 57 days preservation period.

Pair-wise comparisons indicate that the decrease in body weight was non-linear and

Table 3. Effect of fixation and preservation on **relative** (to SVL) morphometric measures. The t- and p-values indicate the significance of differences in relative measures between day 0 and day 3 (fixation) and between day 3 and day 60 (preservation). Significant p-values are in bold. The average difference is expressed in percentage of the initial value (on day 0 and day 3, respectively).

Trait	Fixation			Preservation		
	t_{64}	p	% change	t_{64}	p	% change
Body weight	−4.54	<**0.0001**	+2.384	18.38	<**0.0001**	−9.822
Tail length	−6.44	<**0.0001**	+1.43	−9.46	<**0.0001**	+3.376
Tail base width	1.07	0.2906	−0.564	5.24	<**0.0001**	−6.092
Head length	−4.99	<**0.0001**	+0.73	−15.86	<**0.0001**	+3.63
Head width	−6.93	<**0.0001**	+1.132	−8.41	<**0.0001**	+2.797
Head heigth	−7.43	<**0.0001**	+1.41	−6.40	<**0.0001**	+2.473
Lower jaw length	−5.33	<**0.0001**	+0.038	−10.33	<**0.0001**	+3.308
External quadratum	−4.98	<**0.0001**	+0.782	−18.63	<**0.0001**	+4.915
External coronoid	−3.75	<**0.05**	+0.965	−7.03	<**0.0001**	+4.461
Upper fore limb length	−6.49	<**0.0001**	+3.314	−2.19	<**0.05**	+1.604
Lower fore limb length	−5.18	<**0.0001**	+3.055	0.71	0.4797	−0.593
Manus length	−2.49	**0.0153**	+1.323	0.83	0.4093	−0.9053
Length third digit manus	−1.53	0.1307	+1.629	−0.52	0.606	+0.675
Upper hind limb length	−2.99	<**0.05**	+1.774	−0.75	0.4540	0.536
Lower hind limb length	−3.53	<**0.05**	1.663	0.32	0.7473	−0.158
Pes length	−0.89	0.3781	−2.86	0.02	0.985	−0.0693
Length fourth toe pes	−3.03	<**0.05**	1.135	−2.74	<**0.05**	+1.8566

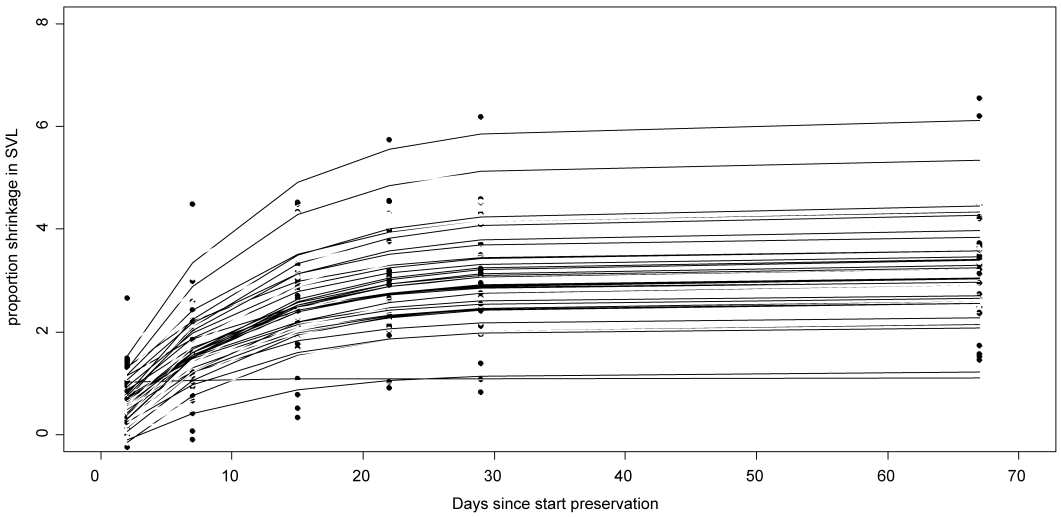

Figure 2. Observed (dots) and expected (lines) proportional reduction in SVL due to preservation as estimated by the modified Janoschek model. Different individuals are indicated by different colours.

reached an asymptote after 15 days (fig. 2). The reduction in SVL was also greatest in the first two weeks, but remained statistically significant until day 30. Because of the large variation between individuals we analysed the effects of SVL (SVL $*$ TOTday $-$ effect) on the magnitude of distortions. However, except for body weight ($t_{64} = 2.02$; $P = 0.0481$), none of the traits were SVL-dependent (all $P > 0.05$)

Comparisons between day 0 (fresh) and day 60 (conserved) allows us to recalculate fresh body size and shape dimensions, at least on population level (for absolute measurements and relative measurements, table 4). The con-

Table 4. Effect of conservation on absolute and relative (to SVL) morphometric measures. The t- and p-values indicate the significance of differences in relative measures between day 0 and day 60. Significant p-values are in bold. The average difference is expressed in percentage of the initial value.

Trait	Absolute change of conservation			Relative change of conservation		
	t_{64}	p	% change	t_{64}	p	% change
SVL	25.57	<**0.0001**	−3.71	/	/	/
Body weight	27.64	<**0.0001**	−13.39	39.01	<**0.0001**	−7.629
Tail length	−2.18	<**0.05**	+0.725	−12.12	<**0.0001**	+4.851
Tail base width	8.12	<**0.0001**	−10.19	4.95	<**0.0001**	−6.631
Head length	−1.77	0.0807	+0.305	−18.81	<**0.0001**	+4.429
Head width	0.27	0.7883	−0.093	−10.99	<**0.0001**	+3.982
Head heigth	0.92	0.3625	−0.313	−10.11	<**0.0001**	+3.845
Lower jaw length	1.23	0.2845	−0.278	−15.86	<**0.0001**	+4.187
External quadratum	−6.67	<**0.0001**	+1.54	−20.55	<**0.0001**	+5.735
External coronoid	−2.16	<**0.05**	+1.36	−8.33	<**0.0001**	+5.476
Upper fore limb length	−0.91	0.3653	+0.652	−6.63	<**0.0001**	+4.922
Lower fore limb length	1.98	0.0515	−1.657	−2.91	<**0.05**	+2.468
Manus length	3.31	<**0.05**	−3.735	0.14	0.7129	+0.406
Length third digit manus	2.05	<**0.05**	−1.631	−2.81	<**0.05**	+2.289
Upper hind limb length	2.09	<**0.05**	−1.756	−2.69	<**0.05**	+2.302
Lower hind limb length	4.55	<**0.0001**	−2.449	−2.65	<**0.05**	+1.487
Pes length	2.68	<**0.05**	−3.323	−0.65	0.5151	+0.811
Length fourth toe pes	1.64	0.1065	−1.187	−4.01	<**0.05**	+2.999

version equations that should improve the reliability of morphological comparisons of life and conserved specimens on population level for SVL: [SVL(museum specimens)+SVL(museum specimens) × 0.037] = SVL(fresh).

Discussion

Repeatability was shown to be small in (relatively) large traits such as SVL, tail and fourth toe pes length as was the case with 'bony' trais such as those of head dimensions (table 1). Consequently, the more rigid and larger morphological variables are more useful than others in the study of intraspecific divergence and (adaptive) differentiation. This investigation clearly showed effects of preservation on most morphological variables. The most pronounced differences were observed in the snout vent length (SVL), on the other hand, measurements of the head had firstly the smallest measurement error and secondly were not affected by preservation. But, most studies use relative head dimensions and because SVL is heavily influenced by preservative, head dimensions must be corrected with the suggested factor when comparing fresh and preserved material. The only morphological variables that did not change during the entire period (conservation) were pes and manus. It should be noted that both traits were not very repeatable. It should also be noted that not every preservative will have the same effects (Shields and Carlson, 1996). In this study, we used the most common fixative and preservative. Some collections are stored in pure formaldehyde, while others use additives in their preservatives. In the natural history museum of Vienna (Austria) reptiles are preserved in 70% ethanol, denaturated with the addition of 2% methylalcohol (pers. comm. Dr. Franz Tiedemann). As a fixing agent, formalin has been judged as the best solution to use (Sturgess and Nicola, 1975). Because specimens become rigid in fixative sometimes bent or twisted specimens, this can have had effects on the exacteness of measurement; caused by the replacement of water in tissues by preservative (Sturgess and Nicola, 1975). Ethanol is known as a chemical that penetrates tissues and clearly dehydrates (Sturgess and Nicola, 1975). There can also be raised some temporal aspects

of the used procedures of fixation and preservation. Most specimens in museums worldwide are collected during divergent times and we did not test for possible differences due to long term preservation. However, we found that specimens shrink asymptotically, getting probably in osmotic equilibrium with the surrounding preservative. This suggest that specimens become fixed over a period of time after a while. In general, few studies looked at the possible temporal aspects, Guilette et al. (1988) showed that the majority of weight change in reptilian eggs occured within two weeks of preservation. Shields and Carlson (1996) reported differences of 1.9% for lengths and 6.4% for weights due to preservation in alcohol. Parker (1963) reported a gain in weight between 10-20% in formalin for *Oncorhynchus nerka*, also Yeh and Hodson (1975) and the study of Stobo (1972) found a significant increase in weight due to preservation in 10% formalin. Others reported weight losses in fish during preservation (Clutter and Whitesel, 1956; Parker, 1963 for *Oncorhynchus gorbuscha*; Billy, 1982). Reports of preservation also vary for length: Burgner (1962) and Parker (1963) found a shrinkage of live lengths, but Billy (1982) found no shrinkage. These relatively high rates can be due to the different water content between the tissues of reptilians and fishes.

Alcohol is known to dehydratate tissues (i.e. Glenn and Mathias, 1987), consequently, our findings on weight loss in samples stored in alcohol are comparable to other studies where the majority of shrinkage occurred very soon after placement in the preservative (e.g. Glenn and Mathias, 1987; Shields and Carlson, 1996).

Billy (1982) recorded that the weight of formalin-preserved fish were reduced considerably within days of being transferred to alcohol. Shields and Carlson (1996) noted that weight loss apperared to stabilize after 16 days, which may indicate long-term stability with preservation in alcohol. The choice of preservative should be dependent on the objective of the study, for example in osteological research, alcohol is necessary because it does not decalcify bone (Sturgess and Nicola, 1975).

Finally, we have some remarks about the utility of museum specimens. Collections in natural history museums are the work of a variety of collectors and collection dates. Most of them were preserved in isopropyl alcohol at the time of examination, but without specific testing we cannot be certain if they were originally fixed in formaldehyde, generally no treatment documentation exists. Specimens available at different locations may be of an inappropriate age, sex, location. Many anatomical and histological characteristics i.e. the reproductive tract is known to decompose faster than many other internal systems (Beford, 1975; personal communication Dr. Wolfgang Böhme). In general, the delay between death and fixation may be the most frequent cause of deteriorated tissues and fixation was often poor in many older specimens (e.g. Berger, 1955; Burton, 1978). Currently, virtually all specimens are fixed in formalin (Quay, 1974), but many eralier specimens were fixed in other solutions (Williams and Hawks, 1987). This is because formalin, which can be regarded as the standard museum fixative (Quay, 1974; Fink et al., 1978) has some advantages over other fixatives such as Bouin's solution, Gilson's solution, accidified formalin, acetic acid-formalin-alcohol, or glutaraldehyde.

We suggest that the conversion equations we produced can be safely used for data collected from alcohol-preserved specimens of this species. However, when comparisons between inter and intra annual weight evolutions are made, they may be overshadowed, because of the large differences within populations between seasons (Vervust, unpublished). Thus, small (intraspecific) differences can be unvisible because specimens of different localities are sampled during different seasons. Also morphology-performance relationships can vary considerably between seasons (e.g. Irschick et al., 2006; unpublished data). These equations can be study-species specific, because of the large differences in water content (which can be

removed from tissues) between desert and tropical adapted species.

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