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Fixation and preservation contribute to distortion in vertebrate museum specimens: a 10-year study with the lizard *Anolis sagrei*

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Preservation of museum specimens depends on chemical fixation and preservation, processes that might distort the original material. Relatively few studies have examined the effects of preservation in potentially susceptible soft-bodied taxa, such as herpetofauna, and those that have rarely extend over more than a few months. We collected six common morphological measurements from the same set of radiographed specimens of the Neotropical lizard *Anolis sagrei* over nearly 10 years to investigate whether morphometric changes result from fixation in formalin and/or subsequent long-term preservation in ethanol. Snout–vent length declined by 3.5% on average over 10 years, starting almost immediately with fixation and continuing to decline during fluid preservation, eventually levelling off at 40 weeks and beyond. The mostly ossified component of snout–vent length, spine length, declined by 2% on average, but the decline did not begin until fluid preservation commenced and continued throughout the duration of the study. Other characters showed significant decline over the course of the study. Our findings suggest caution when combining fresh and preserved specimens or specimens of different preservation ages, because a decline in snout–vent length but not in other allometrically proportional characters will introduce error when correcting characters for body size in preserved animals.

ADDITIONAL KEYWORDS: body size – ethanol – formalin – morphometrics – reptile – squamates.

INTRODUCTION

Natural history collections aggregate and showcase the great diversity of form found in nature, providing a crucial foundation for our understanding of the organization, function, anatomy and distribution of organisms (Winker *et al.*, 1991; Shaffer *et al.*, 1998; Rocha *et al.*, 2014). They give contemporary researchers the unique ability to peer into the past and to undertake investigations that the original collectors could not have anticipated. Specimens amassed during the past few centuries serve a remarkable variety of fields, including taxonomy (Cooper, 1996; Amato, 1999), conservation (Shaffer, 1998; Cameron, 2011), epidemiology (Ávila-Arcos *et al.*, 2012; Hewson, 2014), population genetics and genomics (Roy, 1994; Bouzat *et al.*, 1998; Rosenbaum *et al.*, 2000; Miller *et al.*, 2003; Card *et al.*, 2021) and palaeontology (Höss, 1996; Hofreiter, 2001). Collections provide access to species, populations and lineages that are now extinct or difficult to study and offer the opportunity to reveal undescribed diversity among the troves of yet-unexamined specimens (Bebber, 2010; Kemp, 2015). As such, collections consisting of preserved specimens are a vital resource for scientific inquiry.

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Preservation ensures that the specimens housed in these collections continue to remain useful through time and involves a variety of methods, materials and techniques depending on the organism and the era when the collection was made. As soft-bodied animals, reptiles are especially susceptible to tissue decay; thus, a usual first step in their preservation, at least since the early to mid-20th century, is fixation in a formalin solution, which preserves physical tone and prevents cellular degradation (Pisani, 1973; Simmons, 2015). Specimens can then be transferred to long-term storage in > 70% ethanol (EtOH).

Although chemical treatments are vital to maintaining the structural integrity of a specimen, they might result in morphological distortions. Formaldehyde-based fixation solutions, such as 3.7% formalin (= '10%' formalin, because formalin is usually sold at 37% concentration), might either hydrate or shrink specimens, potentially causing expansion or contraction of specific morphological characters, whereas subsequent preservation in > 70% ethanol causes dehydration and might lead to shrinkage (Simmons, 2014). Such distortions, or 'preservation effects', might subsequently affect the findings drawn from the specimens (Hedrick *et al.*, 2018).

Preservations effects, especially changes in size and shape, are known potentially to be confounding in studies of morphology (Cato *et al.*, 2001) and vary in severity across different taxa (Simmons, 2014). For example, wing aspect ratios derived from live and preserved bats were found to differ, with implications for flight style predictions (Bininda-Emonds, 1994), and salamanders have been found to vary in mass and head shape depending on the preservation method used (Pierson *et al.*, 2020). Several studies document morphological distortions in fish (e.g. Fowler & Smith, 1983; Fox, 1996; Paradis *et al.*, 2007; Martinez *et al.*, 2013). Only a handful of studies on herpetofauna have been carried out, focusing primarily on preserved frogs and snakes examined over relatively short time periods (Klauber, 1943; Lee, 1982; Scott & Aquino-Shuster, 1989; Reed, 2001; Deichmann *et al.*, 2009; Shu *et al.*, 2017), although a study on salamanders spanned 18 months (Pierson *et al.*, 2020). Remarkably, only one published study has measured preservation effects explicitly in any lizard (*Iguana iguana*; Vervust *et al.*, 2009), albeit over the course of only 2 months. Given that many herpetological specimens have been in collections for decades or even centuries, no study has yet examined longer-term possibilities for preservation effects.

Here, we assess the effects of fixation and nearly 10 years of preservation on the lizard *Anolis sagrei* Duméril & Bibron (1837), one of the best-studied species from a genus of ~400. As the subjects of intensive study during the past several decades, *Anolis* lizards have emerged as a model system for work integrating morphology with ecology, evolution, behaviour, physiology and many other lines of inquiry (Losos, 2009). Much of this work has relied on museum specimens (e.g. Mahler *et al.*, 2010; Kolbe *et al.*, 2011; Sanger *et al.*, 2011; Revell *et al.*, 2015; Reynolds *et al.*, 2020; Yuan, 2021). Importantly, preservation effects in anoles have been explored only briefly (Lazell, 1972; Irschick *et al.*, 1997; Losos & de Queiroz, 1997); hence, we lack a focused and long-term evaluation of the impact of preservation on morphometric analyses in this important group.

We made repeated measurements of a set of *A. sagrei* specimens over the course of a decade (2000–2010) to assess whether key morphological characters differed between the time when the specimens were freshly euthanized, immediately after they had been formalin fixed and after subsequent preservation in ethanol for increasing lengths of time. Specifically, we asked which characters were altered, at what times during preservation they were most affected and how severely they were distorted. Using rulers and radiographs taken from a series of time points during our study, we were able to measure and characterize distortions of important quantitative character traits that are used most frequently by *Anolis* researchers. By characterizing the practical implications of fixation and preservation for museum specimens, we hope to expand the utility of museum resources to scientific study.

MATERIAL AND METHODS

Specimen fixation and preservation

We gathered live adult female *Anolis sagrei* (N = 52; Fig. 1) in the year 2000 after completion of an unrelated experiment, then euthanized them following standard protocols established by the American Society of Ichthyologists and Herpetologists Guidelines for Live Amphibians and Reptiles in Field and Laboratory Research. Euthanasia was required by our permits and project design at the end of the previous experiment because the species is non-native in Florida, where the specimens had been collected. We fixed specimens in a 3.7% aqueous neutral-buffered formalin solution (one part 37% formalin to nine parts water) for 7 days, then rinsed them in water and transferred each individual to long-term preservation via immersion in 70% ethanol.

Measurement procedures

Immediately after euthanasia (and before fixation), we obtained radiographs of the specimens using a Faxitron

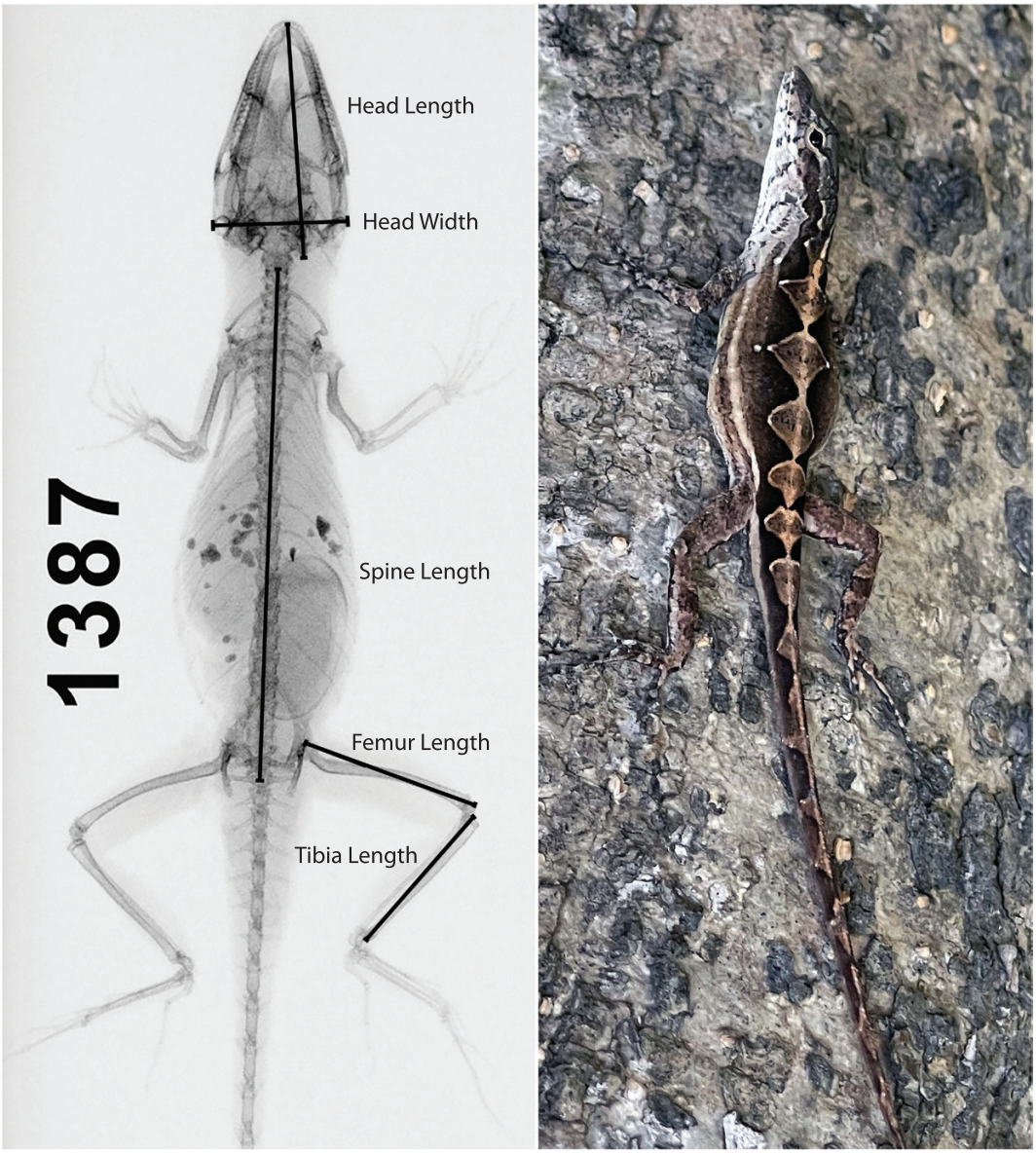

Figure 1. Left, X-ray image of a female *Anolis sagrei* showing landmarks for head length, head width, spine length, femur length and tibia length. Right, adult female *A. sagrei* from New Providence Island, Bahamas. Photograph by R. Graham Reynolds.

43805N radiography system at Washington University in St. Louis or a Thermo Kevex cabinet X-ray system (model PX510-16W) at the Museum of Comparative Zoology, Harvard University, with settings of 30 µA and 30 kV and a metal object for scale on the detector plate. We then re-radiographed specimens at 1 week (after formalin fixation) and subsequently at 16, 40, 232 and 464 weeks of ethanol preservation. The hindlimbs of each individual were taped to the detector plate before each radiography session. We converted radiographs to digital images using Varian Image Viewing and Acquisition software (v.2.0; Varian Paxscan Medical Systems). Before capturing each set of radiographs, one researcher (J.B.L.) measured snout–vent length (SVL; edge of upper jaw to the centre of the cloacal slit) on each individual using a hand-held ruler to avoid inter-observer bias (e.g. Roitberg *et al.*, 2011); after all specimens had been measured to the nearest 0.5 mm for the first time, they were measured a second time. Specimens for which the measurements were not identical were remeasured multiple times until a consistent result was obtained.

After all radiographic images were collected at the end of the study period (465 weeks after euthanasia), we then measured five additional linear morphological

characters of interest from the specimen radiographs (Fig. 1): spine length (SL; centre of first cervical vertebra to centre of last lumbar vertebra), head length (HL; centre of the quadrate bone 'U-curve' to the distal tip of the premaxilla), head width (HW; centre of the quadrate 'U-curve' on the left to that on right side), and femur length (FL) and tibia length (TL; maximum distance from proximal to distal ends of bones). The same researcher (E.A.L.) measured each character three times on each image using the program ImageJ (Abràmoff *et al.*, 2004), calibrating linear measurements with the metal scale included in each radiograph (Fig. 1). No images were measured until all radiographs from all time points had been collected; the order of images was randomized during measurement sessions, and the measurer was blinded with respect to the dates of the images. We took each of the three repeated measurements after the full batch of radiographs were measured once; hence, any given measurements on an individual lizard were taken several weeks apart by the same researcher. We remeasured a character if the maximum and minimum measurements among the three sessions differed by 1 mm or more. We took measurements on both sides (left and right of the sagittal plane) of the animal for head length, femur length and tibia length, then averaged the two sides. If the body was later found to be twisted on an image, such that exact measurements could not be made, we excluded character data for that given time point. For this reason, some characters at some time points included fewer than $N = 51$. We had to exclude head length data for ten specimens from the 232-week time point because some of the radiographs did not capture the skull up to the tip of the snout, a procedural error that was detected at a later date.

Statistical analysis

Data quality

We conducted all analyses in R v.4.1.2 (R Core Team, 2021) and examined character measurement distributions using Shapiro–Wilk tests for normality. To verify that measurements made on radiographs could be used reliably in further analyses, we investigated measurement repeatability using the *icc()* function in the R package psy (Falissard, 2012) by calculating the widely used intraclass correlation coefficient (ICC; Wolak *et al.*, 2012). We analysed each of the six time-point sets for each character for repeatability separately to obviate the effects of non-independence. We then calculated the average of the three replicate measurements for all characters with acceptable repeatability (> 95%) for use in subsequent analyses.

Changes incurred during fixation

Preservation effects can be characterized as either fixation effects (the impact of formalin treatment on the specimens) or fluid preservation effects (the impact of immersion in an ethanol preservative) or both. Initially, to determine whether the characters examined changed during fixation, we conducted Student's paired *t*-tests (controlling for multiple measurements of the same animal) comparing each set of $\log_{10}$-transformed measurements taken immediately post-mortem with those taken after 1 week of fixation in 3.7% formalin.

Overall preservation effects

After 1 week of formalin fixation, we rinsed and transferred specimens to 70% ethanol for long-term fluid preservation and obtained measurements at the four subsequent time points (16, 40, 232 and 464 weeks in ethanol). We assessed proportional trends in morphological distortion by calculating the percentage deviation of each character from freshly euthanized specimens at each of the five time points (1, 16, 40, 232 and 464 weeks) and plotting the results with a quadratic linear model.

To determine whether characters of individual lizards changed at different rates, we compared two linear mixed models that differed in terms of slope delimitation per individual (i.e. constrained vs. random slopes). Both models represented the relationship between the logarithm of trait value and time point for the different morphological characters, while accounting for non-independence of measurements taken from each specimen. We used a likelihood ratio test to determine which model was a more appropriate fit for our data. We constructed linear mixed models using the lme4 package in R (Bates *et al.*, 2015).

To test for differences in the overall means of each trait, we used a repeated measures linear mixed effect model controlling for non-independence across sampling sessions, because we could not assume sphericity for our dataset given some missing data and the different time intervals. We used the *lmer()* and *anova()* functions from the packages lme4 (Bates *et al.*, 2015) and lmerTest (Kuznetsova *et al.*, 2017), respectively, with $\log_{10}$-transformation on characters that deviated from a normal distribution as assessed using the function *Shapiro.test()*.

Character shifts through time

Given that our sample size was large enough (Kline, 2004), we used the *cohens_d* function in the rstatix package (Kassambara, 2021) in R to compute Cohen's *d* (and its 95% confidence intervals, using 100 bootstrap replicates), a measure of the effect size of change in

each character between the first time point (fresh) and each subsequent time point. We also calculated overall effect sizes for changes in characters from freshly euthanized specimens to the end of the study 465 weeks later.

Relative change in shape through time

Many studies examine size-corrected morphological variables using methods that relate the focal variable to one or more other variables. As such, size-corrected variables could be influenced by preservation changes in any of the variables used in the size-correction procedure. To examine how size-corrected variables changed through time in our dataset, we calculated residuals from $\log_{10}$-transformed measurements regressed against SVL. We did this for specimens measured directly after euthanasia (fresh), using the SVL at that time, and at the end of the study (465 weeks later), using the SVL measured at that time. We compared these sets of residuals for each of the five characters using Student's paired *t*-tests.

Many studies of morphology do not commonly use individual linear measurement data, as we have done here. Instead, multivariate analyses, such as principal components (PC) analysis (PCA), are performed on the residuals of $\log_{10}$-transformed data regressed on SVL, to account for differences associated with allometry (e.g. Irschick *et al.*, 1997; Beuttell & Losos, 1999; Macrini *et al.*, 2003; Pinto *et al.*, 2008). To investigate whether overall specimen shape changes during fixation and preservation, we analysed our dataset in a multivariate PCA framework. In this case, we treated the starting and ending time points as 'population samples', ignoring, for the moment, the correlated nature (non-independence) of the datasets, to ask simply whether these 'populations' changed through time. Our null hypothesis was that overall shape, as defined by orthogonalized axes from PCA, at time point 0 (immediately post-mortem) does not differ from the final time point 465 weeks later. We conducted the PC analyses with the residuals obtained from $\log_{10}$-transformed measurements regressed against SVL for both time points as above (i.e. measurements from time point 0 are regressed against SVL measurements from time point 0). We then conducted a MANOVA on combined PC axes and ANOVAs on the individual PC axes comparing time point 0 with the final time point.

RESULTS

Repeatability analyses

All morphological characters we examined showed very high repeatability across three independent measurement sessions of the same set of radiographic images (mean ICC = 0.988, median ICC = 0.989, standard deviation ICC = 0.006; Supporting Information, Table S1). Measurement variance was small and was, in all cases, dwarfed by subject variance (Supporting Information, Table S1). One sampled lizard was found to have measurements that were significant outliers, and this lizard was excluded, giving a total of 51 specimens used in the analyses.

Changes incurred during fixation

Overall, we found a significant reduction in SVL among the specimens (mean = –1.457%; $t = 9.732$, $P < 0.0001$) after 1 week of formalin fixation, although ten of the 51 specimens (19.6%) did not exhibit a decline in SVL during fixation (Supporting Information, Fig. S1). Tibia length appeared to shrink during fixation in some specimens and increased in others. Student's paired *t*-test suggested that, on average, specimens increased by 0.87% ($t = –2.196$, $P = 0.033$) in TL during fixation; other characters did not exhibit a significant change during the fixation period (Table 1; Fig. 1).

Overall preservation effects

We examined among-specimen changes in linear morphometric characters across the 464 weeks of fluid (ethanol) preservation using linear mixed models. By comparing two models (constrained vs. random slopes), both accounting for non-independence of measurements within each individual specimen but varying with respect to constraints on random slopes, we selected the model constraining all specimens to the same slope per character (likelihood ratio test, $P > 0.05$). On the basis of this outcome, we concluded that there is no significant variation among specimens with respect to rate of change over time.

Proportional changes in all six characters are apparent from Figures 2 and 3 (and Table S2), and our repeated measures linear mixed effect models

Table 1. Student's paired *t*-tests for fresh vs. fixed (1 week in 3.7% formalin) specimens reveal a significant decline in $\log_{10}$-transformed snout–vent length and tibia length

Character	*t*	d.f.	*P*-value	%Δ
Snout–vent length	9.59	50	**< 0.001**	–1.457
Spine length	0.41	50	0.655	–0.07
log head width	–1.37	49	0.178	+0.31
Head length	1.30	50	0.201	–0.63
Femur length	1.54	50	0.131	–0.54
Tibia length	–2.20	50	**0.033**	+0.87

Characters were $\log_{10}$-transformed if they deviated from a normal distribution. Significant values at $\alpha = 0.05$ are in bold. The percentage change (%Δ) is shown for the difference in means between fresh and fixed states.

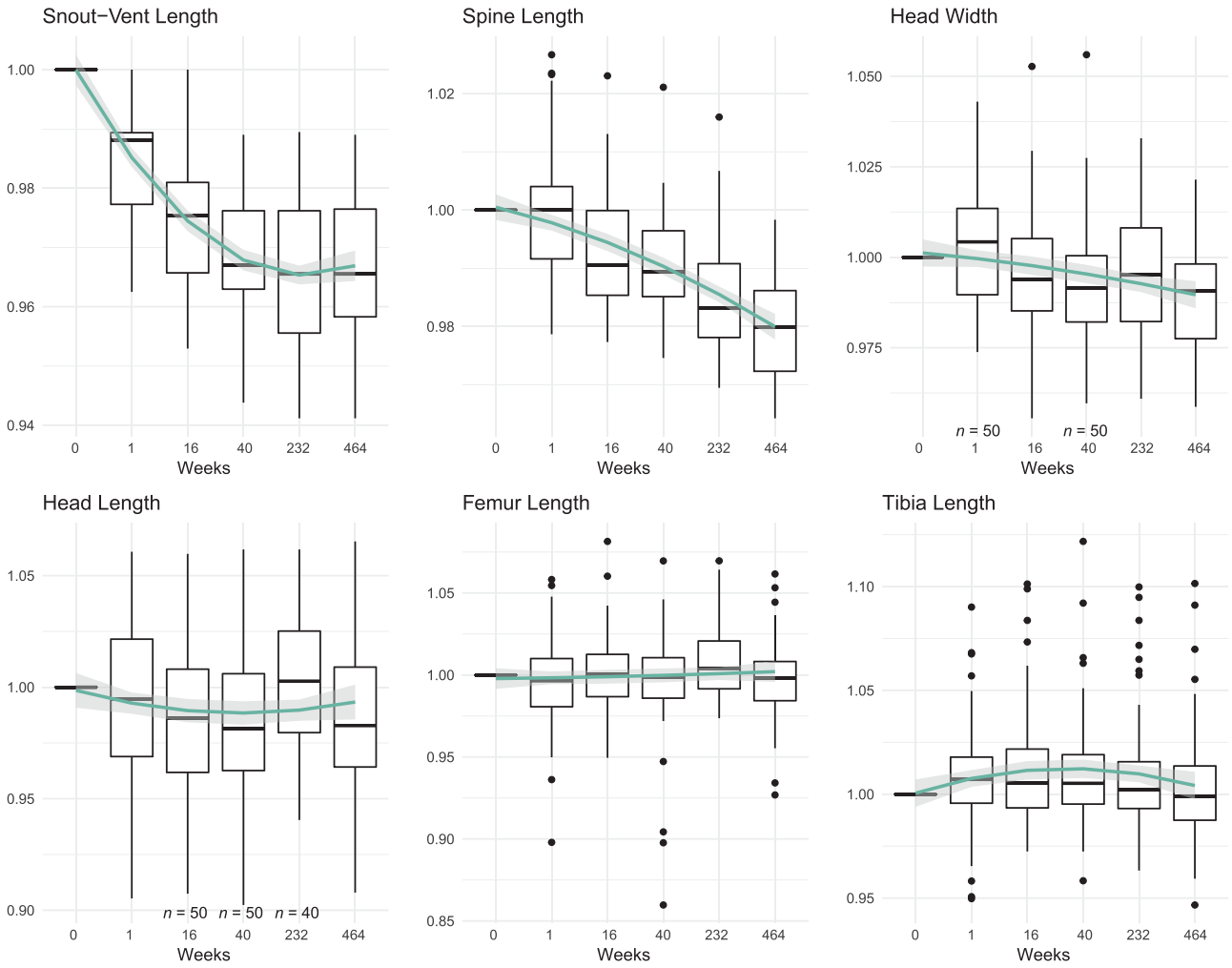

Figure 2. Boxplots of proportional changes in specimens between the start of the study and each subsequent time point. Quadratic linear models plus 95% confidence intervals (grey shading) are shown. Note that the *y*-axes are on different scales, and that weeks on the x axis represents the number of weeks in ethanol.

revealed that each character changed significantly over the 465-week study (Tables 2 and 3), with every character except tibia length decreasing in mean size.

Character size changes through time

To examine effect sizes, we used Cohen's *d* (Supporting Information, Table S3) and found the largest overall effect sizes for SVL (Cohen's $d = -2.759$) and SL (Cohen's $d = -2.378$, $P < 0.0001$), both of which reflected shrinking from when the specimens were fresh. Fixation had the largest effect on SVL (Cohen's $d = -1.317$; *t*-test $P < 0.001$). For SL, preservation effects were not detected during fixation (*t*-test $t = -0.018$; $P = 0.69$) but appeared to manifest at week 16 of fluid preservation (Cohen's $d = -0.683$) and continued throughout the study (week 465; Cohen's $d = -1.297$). We found effects for other characters (Supporting Information, Table S3), but these were harder to interpret given that effects were observed for alternating expansion and contraction of characters during the course of the study (Fig. 3; see Discussion).

Relative change in shape through time

Decline of specimen SVL significantly altered residuals calculated from characters regressed against SVL from the start to the end of the study. Every size-corrected character except for spine length showed a significant difference between the start of the study (fresh specimens) and the end of the study 465 weeks later (Table 4), demonstrating that significant error can be introduced by using shrunken SVL to correct for characters that themselves did not shrink (or did not shrink to a similar extent). The exception was spine length; we did not observe a correction effect owing

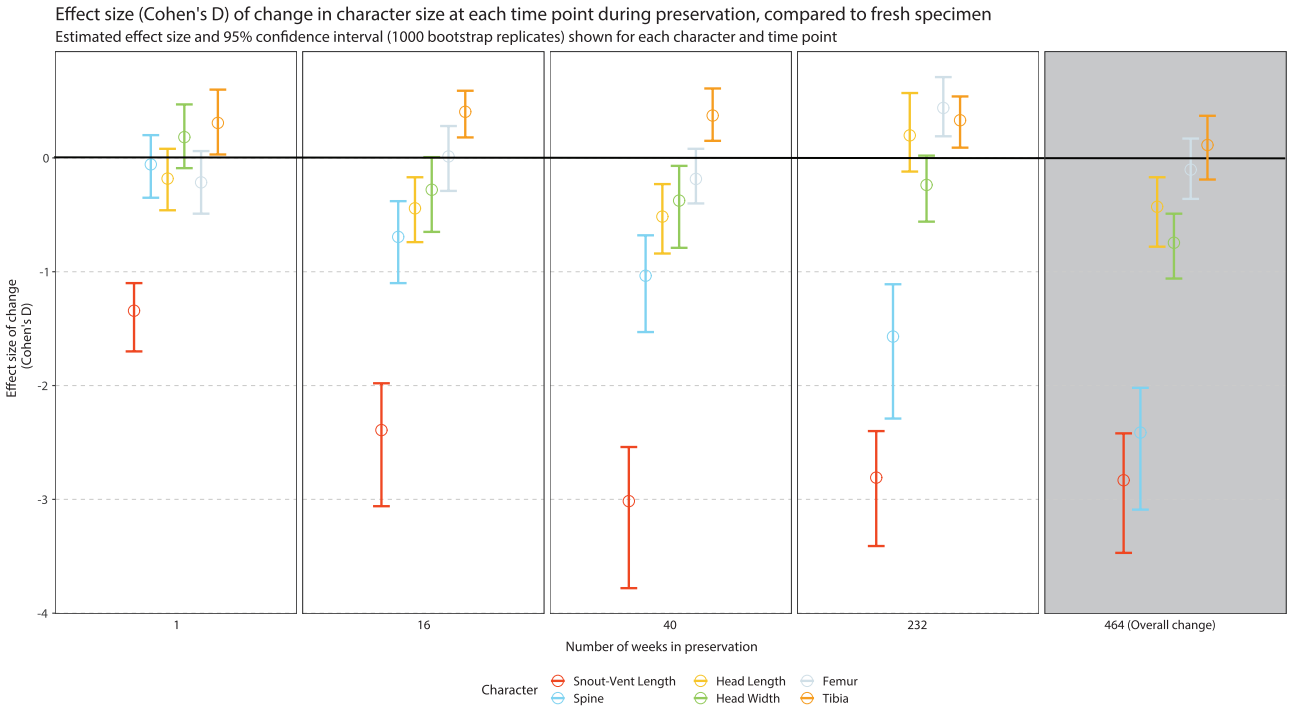

Figure 3. Effect sizes (mean Cohen's *d* and 95% confidence intervals) of the differences between time point 0 (fresh) and each subsequent time point, with values closer to zero (horizontal continuous line) indicating a smaller change between time points. The overall change between the first and last time points are to the right (shaded grey box). Note that intervals between weeks on the *x*-axis are not shown to scale, and that the last week is after 464 weeks in ethanol.

Table 2. Repeated measures linear mixed effects models for all time points and all characters in the study

Character	*F*	d.f.	*P*-value
log snout–vent length	224.43	5	**< 0.001**
log spine length	78.86	5	**< 0.001**
log head width	10.19	5	**< 0.001**
Head length	11.04	5	**< 0.001**
Femur length	4.05	5	**0.002**
log tibia length	3.70	5	**0.003**

Characters were $\log_{10}$-transformed if they deviated from a normal distribution. Significant values at $\alpha = 0.05$ are in bold.

to the fact that spine length also appeared to shrink almost in proportion to SVL (and was tightly correlated with SVL because it is the ossified component of this latter measurement).

We examined whether overall shape, defined by a multivariate analysis of the residuals from regressions against SVL of our five linear measurements, was significantly altered by preservation using a PCA. Using MANOVA to test for changes in shape among all PC axes, we found a significant difference between fresh specimens and specimens at the end of the study 465 weeks later (d.f. = 1; Wilks' $\lambda = 0.64$; $P < 0.0001$). Using ANOVA, we found a significant change in shape over the course of the study for PC axis 1 (72.8% of the variance; d.f. = 100; $F = 29.35$; $P < 0.0001$) but not for PC axis 2 (12.2% of the variance; d.f. = 100; $F = 0.565$; $P = 0.454$) or PC axis 3 (7.9% of the variance; d.f. = 100; $F = 1.38$; $P = 0.243$).

DISCUSSION

Studies that incorporate data from specimens of varying states or ages of preservation often assume that the effects of fixation and preservation are negligible or consistent across specimens, and thus do not account for these effects. Our results show that this is a flawed supposition. Overall, body size (often inferred using SVL as a proxy) can shrink significantly, while other characters do not (Fig. 2; Table 2). Given that many studies of character evolution rely on size correction using body size, we show that this will introduce significant error into a dataset using preserved specimens of differing ages (Tables 2 and 3) or preservation methods (Tables 1 and 2). Our study adds evidence that preservation effects should be acknowledged and addressed directly when using preserved soft-bodied specimens of any age.

Table 3. Sequential comparisons of linear mixed effect models for consecutive time points

Week	d.f.	SVL	SL	HW	HL	FL	TL
1	250	−11.28 (**< 0.01**)	−0.63 (0.53)	1.38 (0.17)	−1.73 (0.09)	−1.48 (0.14)	2.54 (**0.01**)
16	250	−20.46 (**< 0.01**)	−5.58 (**< 0.01**)	−2.04 (**0.04**)	−4.01 (**< 0.01**)	0.07 (0.94)	3.35 (**< 0.01**)
40	250	−24.95 (**< 0.01**)	−7.41 (**< 0.01**)	−3.17 (**< 0.01**)	−4.57 (**< 0.01**)	−1.65 (0.10)	3.21 (**< 0.01**)
232	250	−26.47 (**< 0.01**)	−11.70 (**< 0.01**)	−1.88 (0.62)	2.28 (**0.02**)	2.26 (**0.02**)	3.08 (**< 0.01**)
465	250	−26.12 (**< 0.01**)	−16.13 (**< 0.01**)	−5.09 (**< 0.01**)	−3.61 (**< 0.01**)	−0.64 (0.52)	1.11 (0.27)

Each week shows the test statistic comparing that week and the one previous. Week 1 is a comparison of week 0 with week 1. Test statistics (t) are reported, with the P-value in parentheses. Significant values at $\alpha = 0.05$ are in bold.
Abbreviations: FL, femur length; HL, head length; HW, head width; SL, spine length; SVL, snout-vent length; TL, tibia length.

Table 4. Student's paired t-tests for fresh vs. end-of-study residuals from a regression of each character against snout-vent length measured at that time point

Character	t	d.f.	P-value
Spine length	7.51	50	**< 0.001**
Head width	8.26	50	**< 0.001**
log Head length	6.11	50	**< 0.001**
Femur length	9.13	50	**< 0.001**
Tibia length	11.67	50	**< 0.001**

All characters appear to have shifted at a population level, but this is largely a consequence of the change in snout-vent length distorting the residual calculations. Significant values at $\alpha = 0.05$ are in bold.

By repeatedly measuring *A. sagrei* specimens before fixation, after fixation and through nearly a decade of preservation, we found that although the specimens themselves responded in a similar manner to fixation and fluid preservation, consistent preservation effects occurred. Specifically, one week of fixation in formalin led to significant expansion in tibia length and decline in SVL (Table 1). The latter result was contrary to what we anticipated, because formalin might be expected to hydrate and thus potentially elongate soft tissues (Simmons, 2014). However, studies examining preservation methods with fish found comparable reductions in body length within the first few days in formalin solution, suggesting that significant hydration might take place more gradually at the whole-body scale or that hydration is expressed more in terms of weight gain than in terms of body elongation (Glenn & Mathias, 1987; DiStefano *et al.*, 1994). Conversely, some traits, such as rodent volar pads, do not shrink during fixation and preservation (Kingston, 2018). It is unclear why we observed an elongation of tibia length, although this was negated after immersion in ethanol (Fig. 2).

The first 16 weeks in ethanol preservation led to significant declines in SVL (−1.17%), spine length (−0.62%), head length and tibia length (Table 4; Supporting Information, Table S2). These proportions are cumulative, such that by the end of week 16 of ethanol immersion (week 17 of the study), SVL had already shrunk by 2.63% and SL by 0.69% on average, in comparison to measurements taken before fixation and preservation. Shrinkage in ethanol but not in formalin is expected for SL, given that there is relatively less soft tissue that might be subject to shrinking in this character than in SVL (Hedrick *et al.*, 2018; Leonard *et al.*, 2022). As preservation continued, size declines persisted such that by the end of the study (465 weeks after euthanasia), SVL had declined by 3.35% and SL by 2.0% on average (Table 4; Fig. 2; Supporting Information, Table S2). A recent study of fish specimens found that fish also continue to shrink in ethanol over a short period of 8 weeks (Sotola *et al.*, 2019), a finding both corroborated and expanded upon by our present study. In our study, SVL and spine length (characters that include a substantial cartilaginous component and are not independent of each other) both appeared to shrink during fixation and preservation, emphasizing the importance for such long-term scrutiny across multiple characters. This is a particularly troubling revelation if researchers are expecting to detect changes in body size in a population through time. If specimens are collected at a time in the past, then collected fresh in the present, a researcher might erroneously conclude that body size has increased in the population through time, although the population had a consistent distribution of SVL through time.

Our analysis of size-corrected residuals provided some strong evidence for the introduction of bias in studies using these types of data from preserved specimens. Given that SVL shrank significantly while other characters did not, we found that when the other characters were size-corrected by regression against SVL measured at either the start or the end of the study, they differed significantly, purely because of the decrease in SVL during preservation (Table 4). Furthermore, we showed that at the end of study, animals were significantly different in overall shape in a multivariate PCA when compared with the fresh specimens (d.f. = 1; Wilks' $\lambda = 0.64$; $P < 0.0001$), although they were the same animals and should be

identical if there had been no impact of preservation on character size. Even though this shape analysis consisted of repeated measures, the two 'populations' of 'fresh' vs. 'preserved for 465 weeks' appeared significantly different in a PCA, a result driven largely by PC1 (d.f. = 100; $F = 29.46$; $P < 0.0001$). Distortions of SVL owing to preservation effects are thus especially problematic and can influence the way in which species or populations are characterized, be it in terms of sexual dimorphism, ecological specialization, functional morphology or broader diversification patterns (e.g. Losos & Miles, 1994; Butler *et al.*, 2007; Thomas *et al.*, 2009). The implication is that the size correction of characters by body size is fraught when SVL has shrunk but other characters have not. This is a likely source of bias that has gone largely unappreciated in the literature.

We have no mechanistic explanation for the fluctuations in head shape or long bone lengths that we observed during the study, and we do not believe that these fluctuations represent real changes in size. Given that we exercised such great caution in reducing measurement bias and error, we do not believe that error during measurement contributed to the observations. Instead, the fluctuations we observed are most probably attributable either to temporary compression of individuals in the jar (individuals would have been reshuffled in the jar after each measurement session and would therefore not be expected to retain any compression effects) or to error introduced when positioning and/or radiographing specimens. This is an important finding, because it suggests that even when researchers are doing their best to maintain consistency in radiographing and measuring museum specimens, there is a high likelihood that errors will come into the dataset. Given that the same animals (in some cases) show fluctuations up or down in head measurements and long bone lengths, we conclude that this is attributable to one of these types of error. Many studies of vertebrate evolution rely on absolute precision in measurement of relevant traits, with only a millimetre or less sometimes providing the evidence for significant directional evolution in quantitative traits (e.g. Boag & Grant, 1981). If we suspect that error is more prevalent than we tend to believe, as our findings suggest, we must exercise even more caution when obtaining quantitative trait measurements, particularly on preserved specimens. Thus, we recommend building in error-mitigation strategies, such as repeatedly measuring images (as we did here), repeatedly imaging animals to account for the effects of repositioning, or developing tools to increase the consistency with which animals are prepared and imaged. We also urge caution when drawing inferences on the basis of small differences between specimens or experimental groups when preserved specimens are involved.

Although our study shows the presence of preservation effects, our data are limited to females of a single species of lizard and to slightly less than a decade of storage. Given the taxon-specific nature of these effects (as has been found in anurans and fishes; Deichmann *et al.*, 2009), we acknowledge that fixation and preservation will not influence other species in exactly the same manner; however, it is likely that similar effects might occur in other species of anoles and even in other lizard genera. In addition, all ethanol-preserved collections are subject to differential evaporation of alcohol and water from storage containers, which could lead to fluctuations in preservative concentration (Simmons, 2014) unless containers are sealed properly (J. Rosado, pers. comm.). We therefore recognize the limitations of our findings but recommend using them as cautionary guide when preparing, preserving, imaging, measuring and interpreting findings drawn from valuable museum specimens.

Conclusion

We identify some major issues and sources of error when using preserved specimens in biological quantitative trait research, but we also offer some hope for combating this error.

First, we found that body size (measured as the length of the animal; SVL, in our case) shrinks both during fixation in formalin and during subsequent immersion in ethanol, eventually becoming asymptotic at a shrunken state (3.35% reduction) after 40 weeks in ethanol. A subset of this measurement, spine length, does not shrink during fixation but does start to decline during ethanol immersion and continues to shrink all the way to 464 weeks. We show that common methods to analyse quantitative traits using size correction will produce significantly biased results when SVL has shrunk but other characters have not.

Second, we find that despite our best efforts, we detect noise in the measurements of other characters at various times. This suggests that errors are introduced in studies using these types of specimens when obtaining radiographs, even when all other sources of measurement error are reduced as much as possible. Jar effects could be the cause of this, whereby individuals become distorted in the jar and upon being reshuffled lose the distortion. When precision is very important for detecting minute differences in quantitative traits that are thought to be attributable to processes such as selection, researchers must be more forthcoming about the reality of errors introduced by these sources.

Happily, by examining a well-controlled 10-year preservation process, we can offer some insight into how to correct against bias. For example, researchers

ought to acknowledge their own limitations in preventing all error from being introduced into these types of datasets. We might also suggest that high-quality morphological data should be obtained before fixation and preservation of museum specimens. Furthermore, we suggest that, for *Anolis* lizards, an SVL correction might be applied to correct for shrunken SVL in older museum specimens when using SVL as a body size proxy to correct other traits that do not shrink, but we encourage other researchers to examine the magnitude of the change in size among *Anolis* species. We do not wish to discourage use of preserved specimens, but instead acknowledge and account for the existence of error, making this vastly important museum resource more accessible to sound scientific study.

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DATA AVAILABILITY

All data and R code are available on DRYAD (https://doi.org/10.5061/dryad.sxksn035 (Maayan *et al.*, 2022)).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Proportional changes in snout–vent lengths of individual lizards at each time point during fixation and preservation relative to when the specimen was fresh.

Table S1. Repeatability parameters calculated for three repeated measurements taken from digitized radiographs of *Anolis sagrei*. Denotation of (R) or (L) indicates measurement on the left or right side of the sagittal plane for bilateral traits.

Table S2. Mean proportional change (as a percentage) in character values from the fresh specimens to each subsequent time point. Refer to Figure 2.

Table S3. Effect sizes (and their 95% confidence intervals) of the differences between character measurements of fresh specimens and cumulative change in size through subsequent time points (in weeks post-mortem). The overall change is between the first and last time point, corresponding to weeks 0 and 465, respectively. Week 1 encompasses fixation effects and weeks 16–464 encompass ethanol preservation. Effect sizes are Cohen's *d* for paired samples, with values closer to zero indicating a smaller change, and negative values indicating that the change was negative ('shrinking'). The anomalous change in head length is likely to be attributable to reduced sample size (40 instead of 51) at the penultimate time point (232 weeks).