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Preservation-Induced Morphological Change in Salamanders and Failed DNA Extraction from a Decades-Old Museum Specimen: Implications for *Plethodon ainsworthi*

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Abstract.—**Natural history collections are important data repositories, but different chemical treatments of specimens can influence morphological measurements and DNA extraction, complicating taxonomic and conservation decisions dependent upon these data. One such example is the Bay Springs Salamander (*Plethodon ainsworthi*), the only United States amphibian categorized as Extinct by the International Union for Conservation of Nature (IUCN). Recent research has proposed that *P. ainsworthi* is an invalid taxon, arguing that the 55-yr-old type specimens' morphological distinctiveness from syntopic *Plethodon mississippi* is a preservation artifact. To address this controversy, we tested for morphological changes across five experimental treatments in proxy *Plethodon shermani* specimens and we re-examined the datasets used to support the invalidity of *P. ainsworthi*. We also tested recently developed DNA extraction techniques on the putatively formalin-fixed *P. ainsworthi* holotype. We used Bayesian models to demonstrate that preservation method can differentially bias morphological measurements, with most methods causing lower estimates of mass and modestly higher estimates of snout–vent length : head width ratio. These results are broadly consistent with previous studies of other vertebrates, but inconsistent with the hypothesis that *P. ainsworthi* type specimens are actually poorly preserved *P. mississippi*. Attempts to extract DNA from the *P. ainsworthi* holotype unfortunately proved unsuccessful, preventing conclusive resolution of its status and emphasizing the limitations of promising new methods. Nonetheless, we tentatively recommend continued recognition of *P. ainsworthi* as a valid but possibly extinct taxon. More generally, we invite all authors who study preserved specimens to recognize and report how certain chemical treatments might impact their results.**

Natural history collections have significant and long-lasting social, economic, and scientific value. Benefits derived from vertebrate specimens, in particular, have been extensively documented (Shaffer et al., 1998; Suarez and Tsutsui, 2004; Winker, 2004; Holmes et al., 2016; Schmitt et al., 2018). However, the scientific utility of museum specimens often depends upon the choice of preservation method, which is typically based on tradition rather than scientific experimentation (Simmons, 1991, 2015). For fluid-preserved vertebrates such as herpetofauna, the standard protocol for fixation and preservation is the initial treatment of specimens with neutral-buffered 3.7% formalin (often referred to as "10% formalin," but actually a 1 : 10 volume dilution of "full strength" 37% formalin) followed by sequential water baths and permanent storage in ethyl alcohol (EtOH) or isopropyl alcohol (Simmons, 2015). Prior to the standardization of this protocol, numerous historical specimens were treated differently with physical consequences that are not well understood. A survey of 229 museum professionals identified the "impact of preparation methodologies on physical properties of specimens" as a high research priority (Cato et al., 2001).

The reliability of specimen measurements remains vital to taxonomists and morphologists who study fluid-preserved herpetofauna (e.g., Kaliontzopoulou, 2011; Kubicki, 2016; Watters et al., 2016), yet it is commonly assumed that characters in preserved specimens reflect those of living organisms (Deichmann et al., 2009) and that distortion related to preservation treatment is negligible (Lee, 1982; Vervust et al., 2009). Available research actually shows regular, and occasionally dramatic, posttreatment shrinkage in fluid-preserved vertebrates, with decreases in various linear measurements usually ranging from 3–10% in fishes (e.g., Cunningham et al., 2000; Paradis et al., 2007; Melo et al., 2010), anurans (Lee, 1982; Deichmann et al., 2009; Shu et al., 2017), and squamates (Klauber, 1943; Reed, 2001; Vervust et al., 2009). The effect of chemical treatments on morphological measurements in fluid-preserved salamanders remains unexamined, and it is unclear how well the results from previous vertebrate studies might translate.

In addition to morphology, different preservation treatments can also dramatically affect DNA quality. Because formalin cross-links DNA, formalin-fixed specimens have traditionally proved unusable for DNA extraction, amplification, and sequencing. However, recent novel techniques for extracting and sequencing DNA from formalin-fixed and/or decades-old specimens (Hykin et al., 2015; Ruane and Austin, 2017; McGuire et al., 2018) show great promise for overcoming these technical challenges. Such advances have already resolved otherwise intractable taxonomic problems for enigmatic species known only from limited historical material (Ruane and Austin, 2017; McGuire et al., 2018).

A prime example of a taxonomic puzzle dependent upon the interpretation of museum specimens is the Bay Springs Salamander (*Plethodon ainsworthi*). This species is known only from two poorly preserved specimens collected in Jasper County, Mississippi in 1964 and rediscovered decades later in a museum collection (Lazell, 1998). Targeted surveys at and near the type locality have yielded no detections of *P. ainsworthi* (Lazell, 1998, 2005; Folt et al., 2013; Himes and Beckett, 2013), and the International Union for Conservation of Nature (IUCN)

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Red List of Threatened Species categorizes it as the only extinct United States amphibian (Hammerson, 2004). Compared to the widespread and syntopic congener *Plethodon mississippi*, the *P. ainsworthi* type specimens have shorter limbs and are more attenuate, having greater snout–vent length (SVL) : head width (HW) ratios (Lazell, 1998). Recent authors have nonetheless interpreted these poorly preserved types as actually representing distorted specimens of *P. mississippi*, suggesting that *P. ainsworthi* is invalid (Himes and Beckett, 2013; Hammerson, 2015). Thus, *P. ainsworthi* is a prime candidate for applied experimental investigation, either directly or by proxy, of the implications of preservation-induced morphological change and the efficacy of new genomic tools for obtaining DNA from historical material.

Here, we present the first test of the effect of fixation and preservation method upon morphological measurements in salamanders, and we also test promising recent protocols for extracting DNA from old and/or formalin-fixed specimens. Our first objective was to compare morphological change across *Plethodon shermani* specimens in five different treatments: 1) fixation and long-term storage in 37% neutral-buffered formalin, 2) fixation and long-term storage in 3.7% neutral-buffered formalin, 3) fixation in 3.7% neutral-buffered formalin and long-term storage in 70% ethyl alcohol (EtOH), 4) fixation in 3.7% neutral-buffered formalin followed by short-term storage in 50% EtOH and long-term storage in 70% EtOH, and 5) fixation and long-term storage in 70% EtOH. These five treatments span a range of techniques used or recommended by historical or contemporary herpetologists (Simmons, 2002, 2015). For each treatment, we analyzed changes in mass, SVL, and HW in the salamander specimens, using repeated measurements over 18 mo, to determine the timing of any observed changes. Our second objective was to test new techniques for extracting and sequencing DNA from long-preserved and/or formalin-fixed specimens using tissue from the *P. ainsworthi* holotype. Our final objective was to re-examine the datasets used by Himes and Beckett (2013) to question the validity of *P. ainsworthi*. We remark upon the relevance of our results for those who work with fluid-preserved museum specimens and discuss their implications for the contested taxonomic status of *P. ainsworthi*.

Materials and Methods

Collection, Preservation, and Measurement.—We collected juvenile and adult salamanders ($n = 32$) from the southern Appalachian Mountains at the Coweeta Hydrologic Laboratory in Macon County, North Carolina, USA (35.04°N, 83.45°W; datum WGS 84) in June 2014. This site supports a complex, elevation-linked hybrid swarm between *P. shermani* and *Plethodon teyahalee* (Hairston, 1992). Because these salamanders are closely related and are similar in body proportion (SVL : HW ratio = 4.9–7.2) to *P. mississippi* and *P. ainsworthi*, we consider them valid proxies for studying morphological changes in the latter two species. We retained all specimens from an unrelated ecological experiment with euthanasia as the predetermined endpoint. Within 72 h of collection, we euthanized the salamanders by immersion in an aqueous solution of tricaine methanesulfonate. We measured wet mass, snout to posterior angle of vent (SVL), and head width at its widest point (HW) of all specimens immediately post euthanasia and we randomly assigned them to one of five fixation/preservation treatments (described previously) while ensuring qualitatively similar distributions of initial SVL across treatments. We re-measured all specimens after 6, 23, 38, 66, 153, and 556 days. After the 6-day interval, we removed specimens in treatments 3 and 4 from the 3.7% buffered formalin, rinsed them thoroughly in water, and transferred them to their respective EtOH solutions. After the 23-day interval, we transferred specimens in treatment 4 from 50% to 70% EtOH. We measured wet mass using a Sargent Welch WLS 2648 scale (VWR International, Radnor, PA, USA) and we measured SVL and HW using pocket digital calipers (Fowler High Precision, Newton, MA, USA). To test for observer measurement bias, two authors (TJK and NLC) independently measured SVL and HW for every specimen at each time interval. We quantified inter-observer reliability of each measurement by estimating the correlation between the two datasets in R v3.5.1 (R Core Team, 2018). All specimens remain stored in their respective final preservation fluids and are deposited in the Georgia Museum of Natural History (GMNH 52020–52051).

Statistical Analyses.—For each measurement period, we calculated mean SVL and HW of each individual salamander from the paired measurements, and we calculated proportional change between the first and last measurements for use in all models. We used Bayesian models in Stan (Carpenter et al., 2017) as implemented in the package rStan v2.18.2 (Stan Development Team, 2018) in R v3.5.1 (R Core Team, 2018) to estimate parameters describing our data. We designed these models to reflect the underlying mechanism being studied, in which loss of specimen mass (presumably because of dehydration but also potentially because of decalcification and/or additional factors) drives changes in HW and SVL. We estimated five parameters ($\alpha_{1–5}$) for the effects of the five fixation/preservation treatments, and these parameters were shared across models of proportional change in mass, SVL, and HW. We also estimated three parameters (β_{mass}, $\beta_{SVL,}$ β_{HW}) for the effects of initial SVL on each of our response variables, and in the latter two models we included a final parameter (ρ_{SVL}, ρ_{HW}) serving to scale α parameters. We ran these models for 1,000 iterations and evaluated convergence using R-hat. We used parameter estimates for proportional changes in SVL (i.e., $\alpha_{1–5} \times \rho_{SVL}$) and HW (i.e., $\alpha_{1–5} \times \rho_{HW}$) to estimate proportional change in the SVL : HW ratio. We compared means of posterior distributions and estimated statistical significance with overlap in 95% and 80% credible intervals around mean parameter estimates and calculated values. To visualize the timing of changes, we plotted proportional change in mass over time. Our morphological measurements, R scripts, and Stan file are all available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.63xsj3tzd.

DNA Extraction and PCR.—We obtained an approximately 50-mg sample from the tail of the *P. ainsworthi* holotype (MCZ:Herp:A-125869) stored in 70% ethanol. We used sterile razor blades to segment the tissue for the two extraction methods (~1/8 for Qiagen; ~7/8 for phenol:chloroform:isoamyl alcohol [PCI]) and allowed ethanol to evaporate before proceeding following Hykin et al. (2015) and Ruane and Austin (2017). We attempted two different extraction methods and included a negative control (i.e., digest buffer only) in each.

For the first method, we placed the tissue in a digest buffer (180 µL Qiagen DNeasy ATL buffer with 20 µL ProK). We allowed the tissue to digest at 56°C for 48 h and we extracted it following the Qiagen DNeasy Blood and Tissue Kit protocol with elution in 100 µL AE buffer. For the second method, we used an alkali digest and PCI extraction similar to Hykin et al. (2015), with a few alterations. We conducted the alkali digestion with a heat block set to 100°C, conducted centrifugation steps at

TABLE 1. Parameter estimates and 95% and 80% credible intervals for Bayesian models of Δmass, ΔSVL, and ΔHW for *P. shermani* specimens.

Parameter	Parameter estimates (95% credible intervals; 80% credible intervals)
α_1	−0.30 (−0.40 to −0.20; −0.37 to −0.24)
α_2	−0.01 (−0.11–0.09; −0.08–0.05)
α_3	−0.18 (−0.28 to −0.08; −0.24 to −0.12)
α_4	−0.25 (−0.36 to −0.16; −0.33 to −0.19)
α_5	−0.43 (−0.54 to −0.33; −0.50 to −0.37)
β_{mass}	0.0007 (−0.0009–0.0026; −0.0003–0.0020)
β_{SVL}	0.0003 (−0.0001–0.0009; 0.0000–0.0007)
β_{HW}	0.0003 (−0.0008–0.0016; −0.0004–0.0011)
ρ_{SVL}	0.14 (0.05–0.24; 0.08–0.20)
ρ_{HW}	0.30 (0.09–0.49; 0.17–0.43)

approximately 13,700 × *g*, and conducted DNA precipitation with 2X volume ice-cold 95% EtOH (instead of isopropanol) followed immediately by a 20-min hold in a −20°C freezer before proceeding to the EtOH wash. We concluded with a 5-min centrifugation in a SpeedVac to evaporate remaining EtOH and resuspended DNA in 30 µL H_2O. We quantified all DNA extracts with a Qubit fluorometer. After quantifying the two PCI DNA extracts, we combined them and removed three quarters of the volume (i.e., 42 µL) for cleanup with SpeedBeads at a 2.5 : 1 SpeedBead : DNA volume ratio and resuspended in 50 µL H_2O (see Glenn et al., 2019a for preparation methods). We further evaluated the second extraction on an Agilent 2100 BioAnalyzer.

We conducted PCRs with two different primer sets: forward (5′-AAAAAAGTCAGGTCAAGG-3′) and reverse (5′-GGTGACGGGCGGTGTGTG-3′) primers previously designed to amplify small mtDNA amplicons for environmental DNA metabarcoding of plethodontid salamanders (Glenn et al., 2019b) and forward (5′-TGACAAAANCTNGCCCC-3′) and reverse (5′-AAAGTGTTTGAGTTGCATTCA-3′) primers previously designed for phylogenetic studies of *Plethodon* (Weisrock et al., 2005; Kozak et al., 2006). We conducted all PCRs with KAPA HiFi reagents at one-half reaction size using the following thermocycler conditions: 98°C for 2 min, then 20 cycles of 95°C for 20 sec, 60–50°C touchdown for 30 sec (i.e., decrease 0.5°C each cycle), 72°C for 1 min; then 20 cycles of 95°C for 20 sec, 55°C for 30 sec, and 72°C for 1 min; then 72°C for 5 min.

Additionally, we made Illumina genomic libraries with a KAPA Hyper Prep Kit (KR0961 v1.14). We used a SpeedVac to concentrate DNA extracts to 25 µL. We used this full volume in a one-half reaction size library preparation following the manufacturer's protocol with minor changes. After ligation, we used a 2.72 : 1 SpeedBead : DNA volume ratio and reconstituted in 100 µL H_2O. We then used a SpeedVac to concentrate this product to 20 µL. We then conducted a PCR using iTru5 and iTru7 primers (Glenn et al., 2019a) with the following thermocycler conditions: 98°C for 2 min; then 18 cycles of 98°C for 20 sec, 60°C for 30 sec, 72°C for 1 min; then 72°C for 5 min. We examined all PCR products on a 1.5% agarose gel.

RESULTS

Morphological Measurements.—We calculated a correlation between paired measurements of 0.997 for SVL and 0.975 for HW, suggesting high inter-observer measurement reliability and validating our decision to use average values for all subsequent analyses.

We estimated negative values for all five α parameters, with only α_2 having a 95% or 80% credible interval overlapping with zero (Table 1). These results indicated significant mass loss from all treatments except for the 3.7% formalin treatment. The most negative estimates were for α_1 and α_5, which were the 37% formalin and 70% EtOH treatments, respectively. We estimated positive values for all three β parameters, but 95% credible intervals all overlapped with zero. This suggested greater proportional mass loss in smaller salamanders, although this effect was neither large nor significant. Finally, we estimated values of 0.14 for ρ_{SVL} and 0.30 for ρ_{HW}. This suggested a greater effect of mass loss on changes in HW than in SVL, although credible intervals for these parameter estimates overlapped each other. Thus, calculated estimates of proportional change in SVL : HW ratio were positive for all treatments; although 95% credible intervals overlapped with zero for all treatments, 80% credible intervals only overlapped with zero for the 3.7% formalin treatment (Fig. 1). All changes driven by mass loss appeared to happen quickly, with mass restabilizing within 4 wk, and remaining consistent thereafter (Fig. 2).

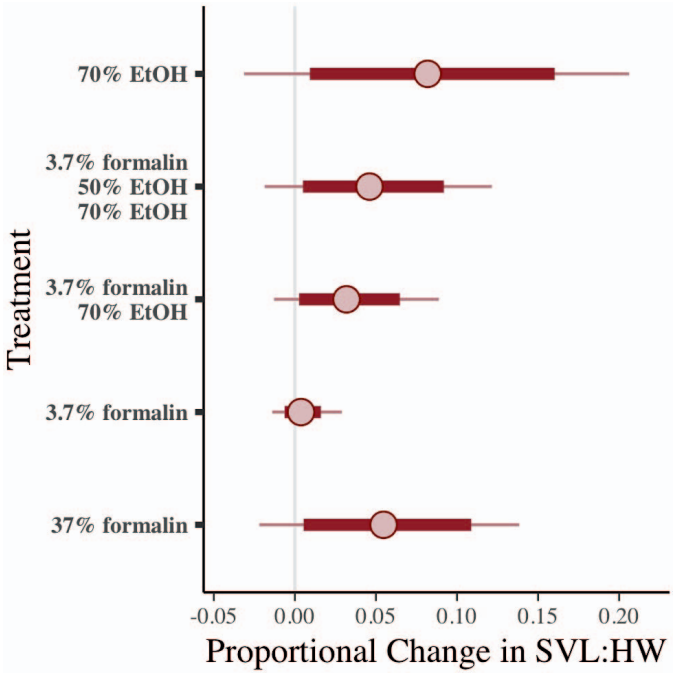

FIG. 1. Proportional change in SVL : HW ratios of *P. shermani* specimens for five chemical treatments. Thick bars represent 80% credible intervals and thinner lines represent 95% credible intervals. Estimates are derived from posterior distributions of parameter estimates of proportional changes in SVL and HW from a Bayesian model.

DNA Extraction and PCR.—We measured the Qiagen DNA extract at 0.14 ng/µL, the first PCI DNA extract at 1.74 ng/µL, and the second PCI DNA extract at 0.44 ng/µL. Negative control extractions were too low to quantify. On the agarose gel, none of the PCR products produced a visible band at the expected size, and neither of the genomic library products produced a smear at the expected size. The BioAnalyzer results revealed an average fragment size of 1 base pair, suggesting low-quality and unusable DNA.

DISCUSSION

Our results generally corroborate previous studies of fixation- and preservation-induced changes in morphological characters of fluid-preserved vertebrate specimens. Save for the 3.7%

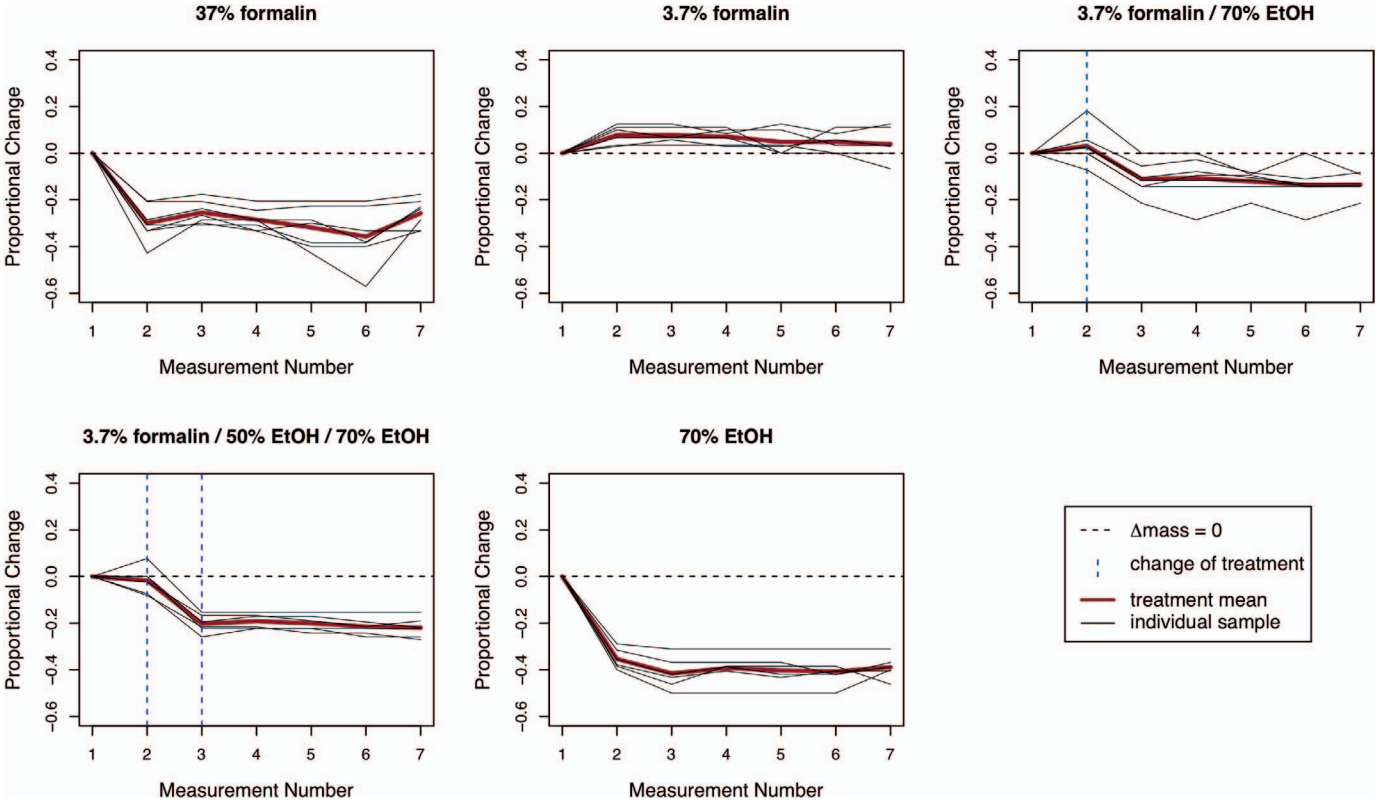

FIG. 2. Change in mass of *P. shermani* specimens across multiple measurement intervals for five chemical treatments. Thin, solid lines represent individual specimens and thick, solid lines represent treatment means. Horizontal, dashed lines represent no proportional change. Vertical, dashed lines indicate the measurements after which treatments were changed (e.g., change from 3.7% formalin to 70% EtOH). Tick marks on the x-axis do not represent equal time intervals; see the text for more information.

formalin treatment, all experimental groups showed an average proportional mass loss of 0.1–0.4 (i.e., 10–40%; Fig. 2) and an increase in SVL : HW ratio of 0.03–0.07 (Fig. 1). The magnitude of these changes appeared to vary between treatments, but we lacked sufficient power (with only 5–7 specimens per treatment group) to resolve this statistically. Consistent with prior work on fish (Cunningham et al., 2000; Melo et al., 2010), specimen mass restabilized after the second (23-day) measurement interval and showed no sign of long-term change out to 18 mo, suggesting that our study duration did not truncate relevant results. Interestingly, our experiment also suggests that transferring formalin-fixed specimens into progressively more concentrated EtOH solutions may do little to reduce morphological impact, contrary to recent recommendations (Simmons, 2002, 2015).

Although other factors may have played a role, our observed morphological changes are likely driven by water loss because the most hypertonic solutions (70% EtOH and 37% formalin) resulted in the greatest proportional changes. Consistent with this explanation, we observed a smaller proportional change in a bony morphological character (SVL) than in a character containing more soft tissue (HW), as indicated by the increased SVL : HW ratios following most treatments (Fig. 1). The treatment with the smallest proportional change in SVL : HW ratio—and the only treatment with an 80% credible interval overlapping with zero—was the 3.7% formalin treatment, our least-hypertonic solution. The mass measurements in this treatment group also displayed the smallest proportional change over time (Fig. 2).

These results underscore the need to account for potential biases when comparing morphology between amphibian specimens fixed and preserved with different methods (Lee, 1982; Deichmann et al., 2009; Shu et al., 2017). We invite researchers to always report the exact method of fixation and preservation applied to vertebrate specimens and to apply appropriate correction factors to morphological measurements if taken after chemical treatment. Guidelines for documenting the exact methods of fixation and preservation have been proposed and widely adopted among natural history museums (e.g., Garrett et al., 1989). However, the utility of these records depends upon accurate reporting by scientists collecting and depositing specimens, and the responsibility for appropriately interpreting morphological comparisons again rests with the scientists using the data. One possible method for detecting otherwise unrecognized biases is to standardize how fixation and preservation methods are reported in peer-reviewed scientific journals.

Turning to the specific case of *P. ainsworthi*, these considerations prove especially relevant. The *P. ainsworthi* type specimens were presumably stored long-term in "strong formalin" (Lazell, 1998; Himes and Beckett, 2013). Paradoxically, strong formalin was identified as most likely being 3.7% formalin, and the type specimens were actually rediscovered in 70% EtOH (Lazell, pers. comm.), so the exact preservation history of these specimens is uncertain. Himes and Beckett (2013) speculated that this strong formalin exposure may have caused distortions to the specimens including "limb shrinkage" and "the distinctively elongated body." One possible mechanism for these proposed changes is water loss, but our data do not support this hypothesis. Our least-hypertonic treatment (3.7% formalin) actually caused the least mass loss and smallest

change in SVL : HW ratio, followed by our 3.7% formalin/70% EtOH treatment, although credible intervals for these parameter estimates overlapped in our models (Figs. 1, 2). An alternative mechanism for possible distortion of the *P. ainsworthi* type specimens was voiced by Lazell (1998), who indicated that their bones were "completely decalcified by long submersion in strong formalin." In particular, unbuffered formaldehyde readily oxidizes in solution to produce formic acid (Fox et al., 1985, Stoddart, 1989). This acid is potentially damaging to preserved tissue, especially bone (Quay, 1974, Simmons, 1995), although to our knowledge only Taylor (1977) has quantified these effects. We used only neutral-buffered formalin in our experiment, which limits its relevance to the decalcification hypothesis. Nonetheless, despite our 37% formalin treatment having nearly comparable mass loss to a treatment with twice the solute concentration (70% EtOH) (Fig. 2), suggesting that decalcification may have occurred, neither our 37% nor 3.7% formalin treatments caused disproportionate shifts in the SVL : HW ratio relative to other treatments (Fig. 1). Lacking direct knowledge of the true chemical treatment of the *P. ainsworthi* type specimens, and absent strong corroboration of either proposed mechanism for how fixation/preservation could produce their diagnostic high SVL : HW ratios and small limbs, the claim that strong formalin caused the distortion of the type specimens is speculative.

Lazell (1998) described the *P. ainsworthi* type specimens as having SVL : HW ratios of 7.9 and 8.4 and reported that an unspecified number of measured *P. mississippi* never surpassed an SVL : HW ratio of 7.2. Himes and Beckett (2013) subsequently examined the damaged *P. ainsworthi* holotype and confirmed its morphological distinctiveness, but the paratype had been virtually destroyed (Lazell, 1998, 2013) and was unmeasurable. Himes and Beckett (2013) also provided measurements of SVL : HW ratios in *P. mississippi* from three sources: 1) their own collections at and near the *P. ainsworthi* type locality, 2) historical museum collections, and 3) literature records from Carr (1996). Himes and Beckett (2013) personally measured 81 adult *P. mississippi* specimens, reporting SVL : HW ratios in excess of 7.2 for nine specimens including a ratio of 8.2 in one "poorly preserved" specimen, plus an average SVL : HW ratio of 7.7 from an additional 24 *P. mississippi* specimens measured by Carr (1996). They used these data to argue that the *P. ainsworthi* type specimens are simply *P. mississippi* distorted by poor preservation, and they concluded that *P. ainsworthi* is likely an invalid taxon.

Our experimental results, coupled with a re-examination of the data presented in Himes and Beckett (2013), suggest that their conclusion was premature. Himes and Beckett (2013; pers. comm.) likely did not fix the specimens they collected in formalin but instead preserved them directly in 70% EtOH. Our experiment indicates that this treatment likely biased their measurements toward higher SVL : HW ratios, with values inflated by roughly 10% (Fig. 1). Still, 0/24 of these specimens had SVL : HW ratios within the range of the *P. ainsworthi* type specimens. The fixation and preservation methods for the 57 additional museum specimens they measured were unreported. Still, just one specimen (and thus only 1/87 total specimens directly measured by Himes and Beckett [2013]) had an SVL : HW ratio within the range of *P. ainsworthi*. Notably, this one specimen lacked the diagnostic short limbs of *P. ainsworthi* and was "contorted" and in "very poor condition" (Himes and Beckett 2013), potentially preventing accurate measurement.

Moreover, the data from Carr (1996) summarized in Himes and Beckett (2013) may have been inappropriate for this purpose. Carr (1996) used specimens that were fixed in 3.7% formalin and stored long-term in 70% EtOH, but he measured HW as "the width of the animal's head at the eyes" (Carr, pers. comm.). This definition may explain the large SVL : HW ratios calculated from these data, and reported in Himes and Beckett (2013), because it is necessarily smaller than or equal to the more common definition of HW (i.e., maximum width of the head) that we used in this study. To show the relevance of this discrepancy, we used ImageJ v1.51 (Schneider et al., 2012) to measure these two characters on the *P. ainsworthi* holotype using the photograph (and scale) provided in Lazell (1998). We found that the width of the head at the eyes was 4.77 mm while the maximum width of the head was 5.87 mm (a difference of >20%). Even smaller differences could still explain the unusually high mean SVL : HW ratio of 7.7 in *P. mississippi* that Himes and Beckett (2013) calculated from these data. However, because the HW measurement protocol used by both Himes and Beckett (2013) and Lazell (1998) was unspecified, this is uncertain. Furthermore, Himes and Beckett (2013) used the mean SVL and mean HW published in Carr (1996) and reported the ratio of these two means, but for all other datasets they calculated the SVL : HW ratio independently for each salamander and reported the mean of these ratios. These two summary statistics (the mean of ratios and ratio of means) are not interchangeable (Stinnett and Paltiel, 1997). For example, the mean of the ratios of our initial SVL and HW measurements is 6.29, but the ratio of means is 6.34. While the exact magnitude of the bias these two factors introduced into SVL : HW ratios calculated from Carr (1996) and reported in Himes and Beckett (2013) is unknown, they provide clear justification for skepticism.

We failed to extract usable DNA from the *P. ainsworthi* holotype, but neither extraction protocol that we mimicked has been universally successful in past applications, with failure rates ranging from 43% (Ruane and Austin, 2017) to 50% (Hykin et al., 2015). Furthermore, results from Ruane and Austin (2017) and McGuire et al. (2018) suggest that 100+ yr-old specimens fixed and preserved solely in EtOH may be even more challenging for DNA extraction than are formalin-fixed specimens. Given the uncertain fixation/preservation history of the *P. ainsworthi* holotype and the uncertain cause of extraction failures in previous work, we refrain from further speculation on the cause of our failure here. We nonetheless encourage future extraction attempts, particularly those that apply new experimental protocols, include the paratype specimen, or involve different tissue types (e.g., liver or other internal organs). Our results underscore the importance of dedicated tissue collections in modern museums, yet also emphasize the need for continued experimental testing of novel extraction protocols—and perhaps more measured enthusiasm for the potential of these new methods.

In summary, we disagree that Himes and Beckett (2013) provided "compelling evidence that *P. ainsworthi* is not a valid taxon." A stronger argument against its validity is the failure to relocate *P. ainsworthi* despite repeated surveys that detected all sympatric plethodontid salamander species (Lazell, 2005; Folt et al., 2013), especially in the absence of a plausible mechanism for the extirpation of only this species from the type locality. Barring the discovery of new *P. ainsworthi* material, conclusive resolution of its taxonomic status will necessitate acquiring evidence from the type specimens. Due to the condition of these

specimens, additional gross morphological data may be unavailable or unreliable, but new, noninvasive methods for gathering fine-scale morphological data (e.g., microCT; Broeckhoven and du Plessis, 2018) provide a potential alternative. However, a micro CT scan of the *P. ainsworthi* holotype (MCZ:Herp:A-125869) appears to corroborate the assessment by Lazell (1998) that nearly all bones are decalcified, thus greatly reducing the potential utility of these data. Finally, while our attempts to extract usable DNA from the holotype proved unsuccessful, it is possible that future advances in these extraction and sequencing methods could still salvage genetic material from the type specimens.

At this time, we recommend that *P. ainsworthi* be tentatively recognized as a valid, possibly extinct taxon. If *P. ainsworthi* is valid and extant, it clearly warrants great conservation attention, and a poorly supported dismissal of its validity could have negative conservation consequences. We thus echo Folt et al. (2013) in encouraging additional efforts to locate *P. ainsworthi* in previously unsurveyed, intact habitat at or near the type locality, particularly at night during warm, wet conditions. Ultimately, our review of this particular case study exemplifies the sometimes-overlooked importance of the effect of chemical treatments on fluid-preserved specimens and how those effects can influence real-world problems of broad import. We hope that this contribution will improve the herpetological community's awareness of these issues and potential solutions.

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