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Research Article

Fresh *vs.* Preserved Specimens: Length-Weight Relationships of Fishes from the Western Amazon (Napo Basin, Ecuador)

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Length-weight relationship (LWR) studies are important for fish taxonomical analysis, ecological assessments, management, and conservation practices. Although LWR studies can use measurements of either fresh or preserved specimens, few studies have directly compared these methods. This study analyzed the effect of preservation on LWR of ten small-sized freshwater fish species from the Curaray River basin (Napo Basin), eastern Ecuador. 255 wild specimens were measured, weighed, fixed in formalin, and finally preserved in 70% ethanol. 287 specimens from the same species batch were measured again after preservation. We estimated LWR curves with linear regressions and compared slopes and intercepts between fresh and preserved specimens. The preservation process altered weight significantly for all ten species and altered both weight and length for two species. The magnitude of change varied according to the morphology and maximum size of each species. Smaller individuals exhibited greater proportional weight losses. LWR estimates for eight of the species are new to science. These results present new evidence of the preservation effects in LWR studies as well as a compilation of the varying results reported in the literature. We conclude that measurements on fresh specimens should be encouraged for LWR studies. However, recognizing that this is not always feasible, studies using preserved specimens should consider the effects of preservation on body length and weight.

1. Introduction

Studies analyzing the variation of fish shape, size, and weight are often performed with preserved specimens in museum collections [1]. Fixation methods include freezing [2, 3], smoking [4], Davidson's B, Borealene [5], and isopropyl alcohol; however, the most common and accepted form to preserve fish specimens is with fixation in 10% formalin (HCOH) and then transfer to 70% ethanol (EtOH) for long-term museum storage [5, 6]. Despite HCOH and EtOH fixation being one of the best methods for maintaining the physical properties of tissues, these methods may alter the morphology of fish specimens [4, 6, 7]. For some species, preservation affects fish length, weight, and body form [1, 8]. Nevertheless, results vary throughout the literature. Fish weight can increase [9, 10] or decrease [8, 11] after preservation. Similarly, length can increase [5, 9] or decrease [4, 8, 11]. Still other studies did not find a significant difference in either length or weight after preservation [12, 13].

This variability of results has been attributed to several factors that influence the shape and size of preserved specimens (Table S1). Factors include the species being

analyzed, preservation technique [9] or capture method, and amount of handling [14, 15]. The size of specimens has been suggested to have a strong relationship with the amount of weight loss, with smaller individuals presenting greater losses [3, 11, 16, 17]. Brinkley [12] suggested that body type (compressiform vs. fusiform) may affect the shrinkage rate on adult stream fishes. Studies have also tested the influence of concentration or type of the fixative solution (HCOH and EtOH) and the effect of using freshwater *vs.* marine water as the HCOH solvent [11, 16, 18, 19].

LWR employs various methodologies that use fresh and/or preserved specimens (Table S1). However, LWR studies give little or no attention to the percentage of bias (from both under- and overestimation) that could influence analyses [7]. Beamish [13] reported no significant differences when comparing the slopes and intercepts from length-weight regressions of fresh and preserved fish of the Thailand freshwater catfish *Glyptothorax major*. However, the study found significant differences in the slopes but not the intercepts for Cypriniformes *Homalopteroides smithi* and *Schistura kohchangensis*; all were fixed in 10% HCOH followed by preservation in 70% EtOH. Similarly, Ogle [20] found that weights predicted from LWR from thawed specimens of Eurasian ruffe (*Gymnocephalus cernua*) had five times the weight measurement error than those estimated from fresh specimens LWRs. Correction factors are often suggested to correct or adjust the observed weight of an individual based on the length measurement. These are helpful to account for the variation in measurements that arise from factors such as sex, age, or preservation methods, generally used in well-documented species (e.g., [21]). Nonetheless, correction factors should be applied in specific contexts, closely following the methodology that provided the correction, and they can still create biased approximations [9, 16].

Due to the considerable variability of the results reported in the literature, plus the tendency of smaller fish species to present a greater magnitude of change, we considered important to analyze data from small fish species that are underrepresented in the literature. In this study, our objectives were as follows: (1) assess potential disparities between LWR slopes and intercepts in fresh and preserved specimens of ten small fish species in the Ecuadorian Amazon, (2) provide LWR values for eight previously unreported species, and (3) provide a literature review table on the effects of fixation and preservation methods (Table S1). This information contributes to improve the accuracy, consistency, and reliability of the data used for LWR studies.

2. Materials and Methods

2.1. Sampling. Fishes were sampled at 28 sites along an altitude gradient of 192 to 737 m.a.s.l in the Curaray River basin, eastern Ecuador. Sampling locations included pristine to slightly disturbed areas of the Curaray, Nushiño, and Villano rivers (Table S2). Ten fish species distributed across eight families and six orders (Table S3, Figure 1) were collected through September to November (dry season) of 2018. Fishes were collected in lagoons, rivers, and forest streams with electrofishing, beach seines, and hand nets. Collecting permits were issued by the Ministry of Environment of Ecuador (Ministerio del Ambiente of Ecuador -MAE, 019-2018-IC-FAU-DNB/MAE).

Standard length (SL) was measured in the field from fresh specimens. For Gymnotiformes, SL, was considered the length from snout to the last radius of the caudal fin. Fresh specimens were weighed in a pocket balance (Kern CM 60-2N, ±0.01 g) after softly removing the surface-excess water with a paper towel. Later, fishes were euthanized by overdose with clove oil and subsequently fixed for 48 h in 10% diluted formalin (HCOH) with freshwater and then preserved in 70% ethanol (EtOH) [7]. Due to in-field sampling limitations, fish specimens were not individually tagged, thus we were not able to identify each individual and match them to their fresh length and weight. Thus, individuals from the same lots were measured and weighed again after four months at the Laboratory of Aquatic Ecology, Universidad San Francisco de Quito. To avoid bias, all specimens were handled and measured by the same researchers during sampling and at the laboratory.

Species were identified using original descriptions and taxonomic keys for species in the region [22, 23] and published fish lists of Ecuador [24–26].

2.2. Data Analysis. Number of individuals per species varied from 10 to 58 for each state (fresh or preserved). All LWR analyses were based on Ogle's [27] "Weight-Length Relationship" chapter and were run in R Studio (R core team, 2018). The FSA [27], car [28], magrittr [29], and dplyr [30] packages were used for analyses.

LWR curves were fitted from $\log_{10}$-transformed variables, for each species per treatment, using the lm() function in *R*, based on the equation $W = (b * SL) + a + ei$ [27]. W indicates weight, *b* is the slope, SL is the standard length, *a* is the intercept, and *ei* is the standard error. An ANCOVA was performed for each species and treatment to confirm that *a* and *b* parameters significantly explain the majority of the variability of the response variable (weight). Parameters *a* and *b*, 95% confidence limits, coefficients of determination (R^2), and residual standard errors (S_{res}) were estimated from the linear regressions of the log-transformed W and SL. We assessed the assumptions of linear regressions (linearity, normality, and constant variance) by analyzing residual plots and residual histograms (outliers were removed only for *Astroblepus* cf. *pholeter*), as per the FSA package [27]. Thirteen species were initially considered, but only ten species which met these assumptions were analyzed further.

For statistical comparison of LWR parameters (slope-a and intercept-b's differences) among treatment groups (fresh or preserved), a dummy variable regression was fitted to allow us to represent multiple groups in a simple regression. The detailed explanation can be found in Ogle [27]. Assumption checking followed the same process described above. Comparison of *a* and *b* between treatments, per species, and determination of the significance of the interaction variable was tested with an ANCOVA using linear models [27]. ANCOVA results were interpreted based on *b*:

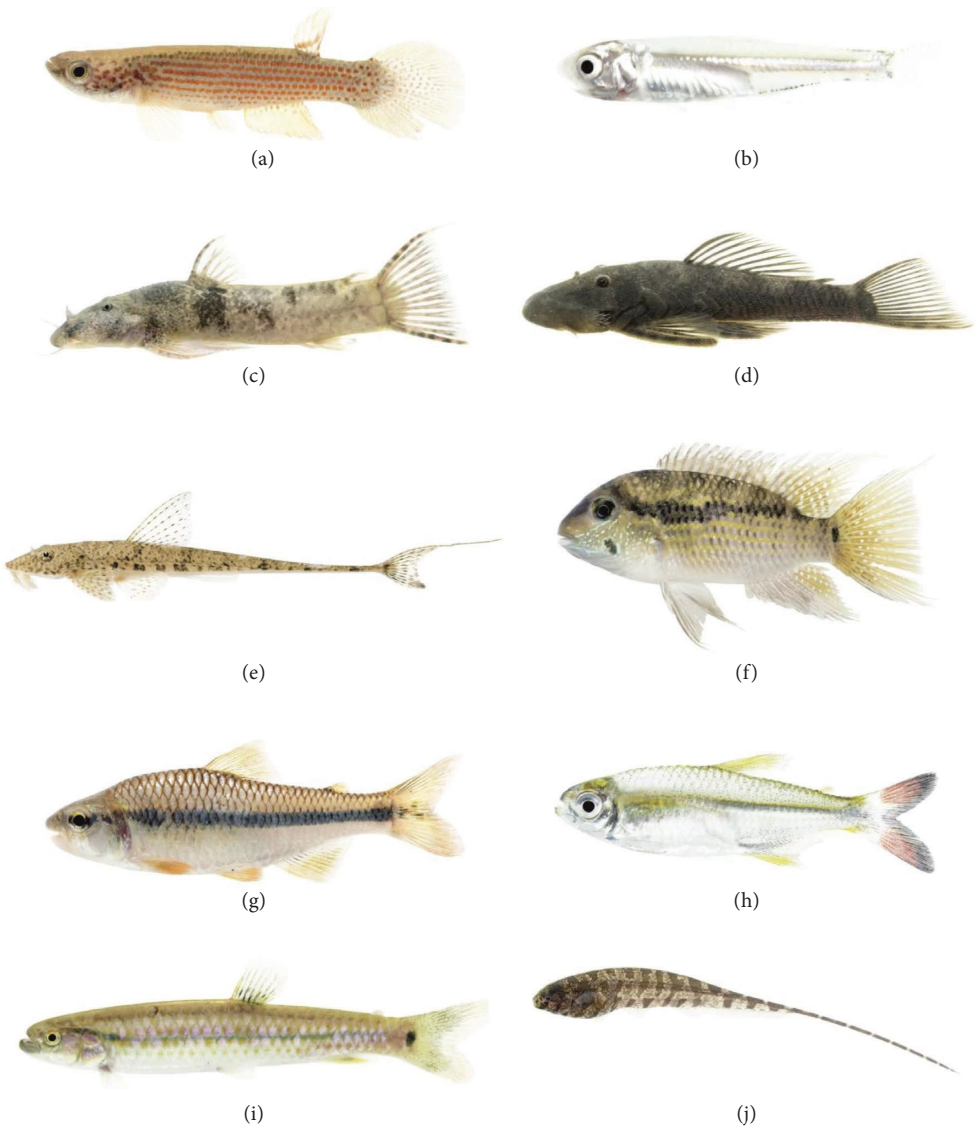

Figure 1: Analyzed species from the Curaray River: (a) *Anablepsoides limoncochae* (mean SL = 2.59 cm), (b) *Anchoviella jamesi* (mean SL = 2.57 cm), (c) *Astroblepus* cf. *pholeter* (mean SL = 4.97 cm), (d) *Chaetostoma microps* (mean SL = 4.85 cm), (e) *Limatulichthys griseus* (mean SL = 9.56 cm), (f) *Bujurquina moriorum* (mean SL = 6.51 cm), (g) *Creagrutus amoenus* (mean SL = 5.39 cm), (h) *Moenkhausia intermedia* (mean SL = 3.87 cm), (i) *Lebiasina elongate* (mean SL = 7.31 cm), and (j) *Hypopygus lepturus* (mean SL = 8.33 cm).

a interaction (Table 1) as per Ogle [27]. Parameter estimates and confidence intervals were calculated using the previously described packages.

Regressions of fresh vs. preserved specimens were plotted for each species to visualize the direction of variation within treatments. To analyze variation of predicted weight at various sizes, we also estimated predicted weights at the 5th, 25th, 50th, 75th, and 95th percentiles of SL with the function *lwCompPreds* from the FSA package [31].

We chose not to report correction factors in this study because they often only apply in a study-specific context that closely follows a specific methodology, often producing unreliable results [9, 16], although we consider correction factors to be valuable when used within consistent methodologies and species with well-reported LWR curves.

3. Results

LWRs are reported for ten small Amazonian fish species [32]. LWRs' estimators calculated from the linear regressions are reported for fresh (Table 2) and preserved (Table 1) specimens. LWR estimators are already available for five species (*Anchoviella jamesi, Limatulichthys griseus, Hypopygus lepturus, Creagrutus amoenus*, *and Moenkhausia intermedia*) [33–35]. For the first three, *Anchoviella jamesi, Hypopygus lepturus,* and *Limatulichthys griseus*, this information was extrapolated from survey studies, which Fishbase's life history tools automatically calculate using Bayesian inferences from length-weight data for the genus and family [36]. Real estimations for eight of the analyzed species are new to science. For all species, ANCOVA indicated that *a* and *b* parameters significantly explain most of

Table 1: Sample size (N), slopes (b), intercepts (a), 95% confidence limits, coefficients of determination (R^2), and residual standard error (S_{res}) for regressions of the log-transformed weight and standard length on preserved specimens.

Species	N	Length range (cm)	Weight range (g)	b (95% CL range)	a (95% CL range)	R^2	S_{res}	F			p		
								b	a	b:a	b	a	b:a
A. limoncochae	13	1.6–3.9	0.05–0.68	2.92 (2.25, 3.60)	−1.74 (−2.02, −1.45)	0.88	0.12	*1.51*	*710.30*	*0.38*	0.23	**0**	0.55
A. jamesi	30	2.2–2.7	0.13–0.26	2.85 (2.24, 3.47)	−1.87 (−2.11, −1.63)	0.75	0.04	*149.62*	*146.95*	*1.69*	**0**	**0**	0.20
A. cf. *pholeter*	45	2.0–6.5	0.15–7.58	3.20 (3.10, 3.30)	−1.78 (−1.83, −1.72)	0.99	0.05	*1.60*	*1021.70*	*6.19*	0.21	**0**	**0.02**
C. microps	52	1.9–7.5	0.17–11.60	2.95 (2.89, 3.01)	−1.51 (−1.54, −1.47)	0.99	0.03	*73.52*	*11423.42*	*13.55*	**0**	**0**	**0**
L. griseus	22	3.8–13.9	0.20–15.20	3.23 (3.02, 3.43)	−2.61 (−2.81, −2.41)	0.98	0.07	*27.54*	*3719.37*	*3.23*	**0**	**0**	0.78
B. moriorum	28	1.5–10.2	0.11–40.54	3.05 (2.86, 3.24)	−1.51 (−1.63, −1.39)	0.98	0.12	*10.75*	*4260.23*	*0.18*	**0**	**0**	0.67
C. amoenus	31	2.1–8.4	0.17–13.72	3.06 (2.98, 3.15)	−1.70 (−1.75, −1.64)	0.99	0.05	*7.01*	*8639.31*	*0.21*	**0.01**	**0**	0.65
M. intermedia	25	2.5–4.3	0.32–1.37	2.77 (2.65, 2.88)	−1.62 (−1.68, −1.56)	0.99	0.02	*12.21*	*412.37*	*0.45*	**0**	**0**	0.51
L. elongata	19	2.1–12	0.24–24.32	2.67 (2.47, 2.88)	−1.55 (−1.72, −1.38)	0.98	0.08	*0.06*	*2111.39*	*0.07*	0.81	**0**	0.80
H. lepturus	22	6.2–10	0.38–0.90	2.71 (2.24, 3.18)	−2.14 (−2.46, −1.81)	0.87	0.05	*153.18*	*299.29*	*2.32*	**0**	**0**	0.14

In italics are F and p values from ANCOVA comparison of fresh and preserved specimens. Bold values are statistically significant.

Table 2: Sample size (N), slopes (b), intercepts (a), 95% confidence limits and coefficients of determination (R^2), and residual standard error (S_{res}) for regressions of the log-transformed weight and standard length on fresh specimens.

Species	N	Length range (cm)	Weight range (g)	b (95% CL range)	a (95% CL range)	R^2	S_{res}
A. limoncochae	16	1.6–3.6	0.07–0.75	3.25 (2.91, 3.59)	−1.87 (−2.01, −1.73)	0.97	0.06
A. jamesi	10	2.2–2.7	0.13–0.26	3.56 (2.44, 4.68)	−1.98 (−2.44, −1.52)	0.86	0.04
A. cf. pholeter	23	2.1–7.2	0.21–7.20	2.67 (2.04, 3.29)	−1.47 (−1.90, −1.04)	0.79	0.19
C. microps	58	2.7–7.2	0.76–11.59	2.74 (2.65, 2.84)	−1.31 (−1.37, −1.24)	0.98	0.04
L. griseus	33	3.8–14.9	0.25–19.32	3.04 (2.92, 3.15)	−2.34 (−2.46, −2.23)	0.99	0.05
B. moriorum	40	1.7–10.2	0.20–41.30	3.01 (2.92, 3.09)	−1.41 (−1.48, −1.34)	0.99	0.06
C. amoenus	24	2.7–7	0.48–8.58	3.02 (2.88, 3.16)	−1.64 (−1.74, −1.54)	0.99	0.03
M. intermedia	13	2.6–5.1	0.40–2.80	2.59 (1.88, 3.29)	−1.45(−1.86, −1.04)	0.84	0.09
L. elongata	22	2.4–12.7	0.24–28.80	2.71 (2.55, 2.86)	−1.58 (−1.71, −1.45)	0.99	0.07
H. lepturus	16	6.7–10.2	0.44–1.16	2.28 (1.90, 2.65)	−2.22 (−2.57, −1.88)	0.92	0.04

the variability in W. Regressions for seven species, per treatment (fresh vs. preserved) were significant ($P < 0.05$), with high coefficients of determination ($R^2 \geq 0.80$) (Table 2) signifying that the predictor variable ($\log_{10}$L) effectively explains the predicted value of the response variable ($\log_{10}$W). Coefficient of determination (R^2) values were inferior to 0.80 for *Astroblepus* cf. *pholeter* (fresh $R^2 \geq 0.79$) and *Anchoviella jamesi* (preserved $R^2 \geq 0.75$). A similar case was observed with the gymnotiformes *Hypopygus lepturus*, where coefficient determination (state preserved $R^2 \geq 0.77$) and slopes (state preserved $b = 1.90$, state fresh $b = 2.28$) had inferior values than other species and for what is expected for LWR assessments [36, 37]. This could be explained by inaccurate measurements of length to anal fin due to loss of small fin segments in preserved specimens. To verify this, we weighted and remeasured the same preserved individuals excluding the caudal portion (length to last radius of the caudal fin). These new results fell within expected ranges ($R^2 \geq 0.87$; $b = 2.71$).

The slopes of seven species ranged from 2.71 to 3.23, all within the expected range for LWR assessments [36, 37]. All species met normality, linearity, and constant variance assumptions.

Significant differences in b:a indicate that groups have different slopes, a indicates whether groups have the same intercept, and b indicates whether groups have significant shared slopes. Our results show that most species did not have significantly different slopes (Table 1), except for *Astroblepus* cf. *pholeter* and *Chaetostoma microps*. For the latter species, length changed after preservation (Table 1, Figure 2). Critically, all species had a significantly different intercept after preservation. Because the slopes were the same between the groups, this shows that difference in the intercepts is a measure of the difference in the mean $\log_{10}$(W) at all values of $\log_{10}$(SL) [27]. Therefore, the preservation process, for most species, altered specimens by reducing weight but not the length (Figure 2). For *A.* cf. *pholeter* and *C. microps*, the preservation process decreased both length and weight.

Although we found significant differences on both parameters a and b between treatments for *C. microps* (mean SL = 4.85 cm), predicted weights at different percentiles of SL show that for sizes above 5.6 cm, the effect of preservation on weight is reduced (Figure 3(b)). The same pattern was also evident in *A. jamesi* (Figure 3(a)), where due to its small size (mean SL = 2.57 cm), the difference in preserved weight was lower at all lengths (Figure 3(b)). In contrast, the weight of *Lebiasina elongata* was almost unaffected at all lengths (Figure 3(d)) probably because it is one of the largest species in our study (mean SL = 7.31 cm). Interestingly, for *A.* cf. *pholeter* (mean SL = 4.97 cm), preserved individuals exhibited reduced weight in specimens below 2.8 cm but increased weight for individuals above 4.4 cm of length.

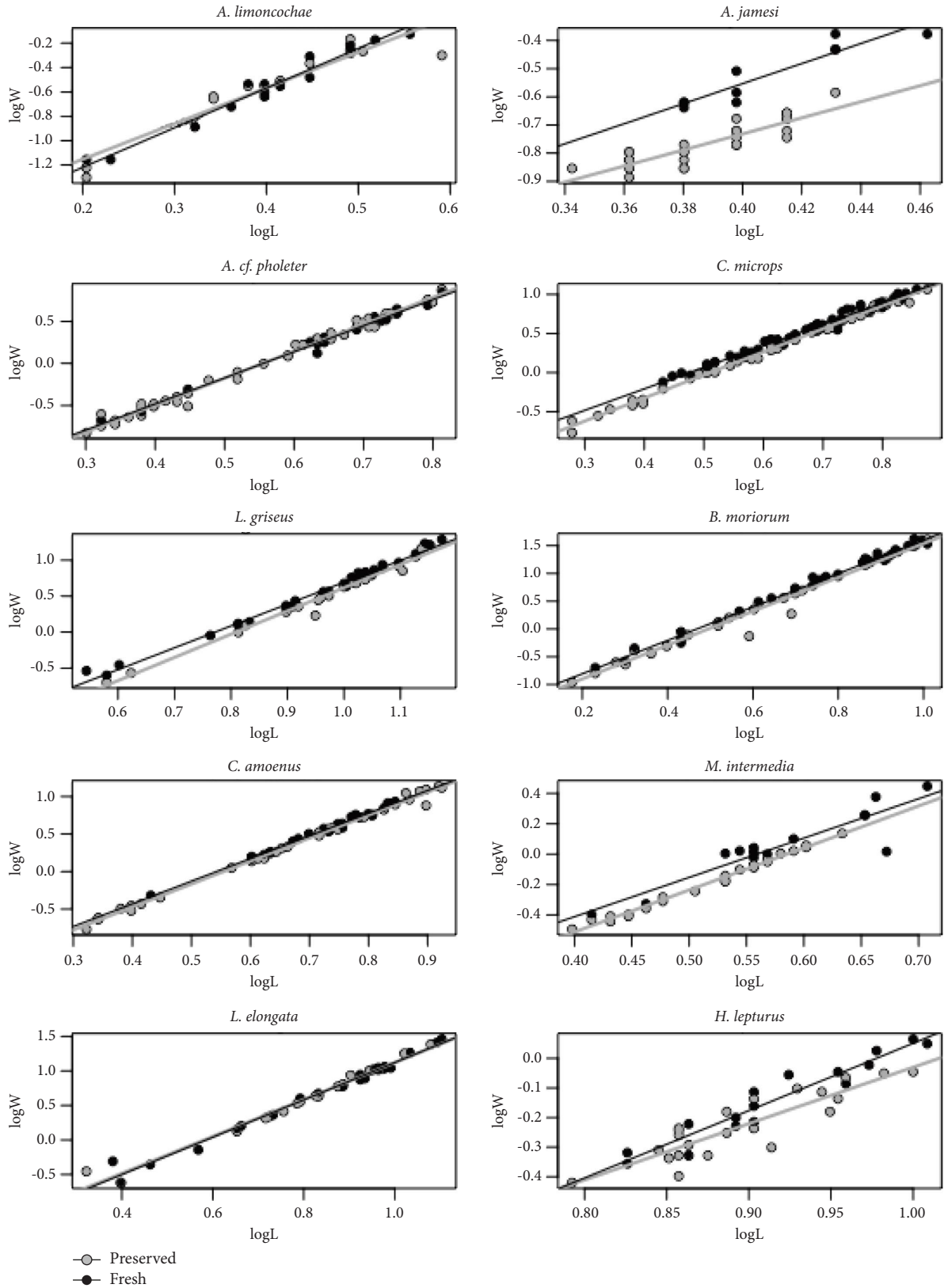

Figure 2: Linear regressions between fresh and preserved states for ten species from the Curaray River basin.

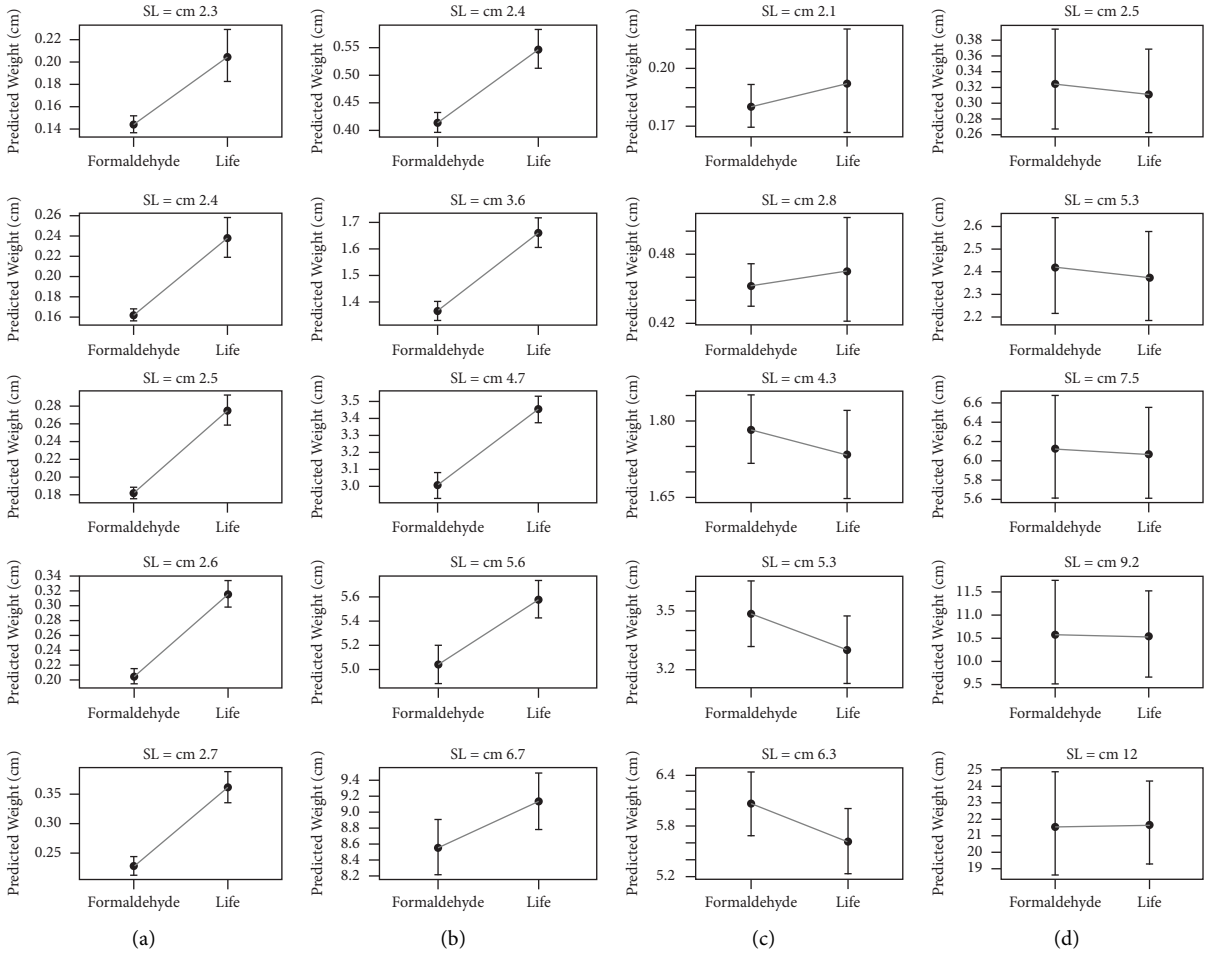

Figure 3: Predicted weights, with 95% confidence intervals, at the 5th, 25th, 50th, 75th, and 95th percentiles of standard lengths (SL) for (a) *Anchoviella jamesi*, (b) *Chaetostoma microps*, (c) *Astroblepus* cf. *pholeter*, and (d) *Lebiasina elongate*.

4. Discussion

4.1. Variable Preservation Effects across Species. Our wide range of results shows that the preservation process is remarkably variable both between and within species.

A low coefficient of determination in LWR regressions for four species could be explained by different factors. For *A. jamesi*, disparate length measures did not allow an adequate distribution of data. In addition, weight values after preservation could have been altered by loss of scales in individuals, which could provide an additional factor of variation independent of the change related to the preservation effect, thus resulting in a minor R^2 value. For *A.* cf. *pholeter*, the lower values of R^2 in fresh individuals could be related to a lower number of individuals of medium sizes, which was corrected when performing measurements on preserved individuals. For *Hypopygus lepturus*, low R^2 values increased when remeasured excluding the caudal portion, suggesting that low slope and R^2 values could have been produced by damage to the caudal portion. Lower or higher *b* values could also indicate allometric growth, which is also seen in gymnotiformes. For *Moenkhausia intermedia*, low R^2 values were corrected by increasing the number of measured specimens. In general, for all species, increasing the number of analyzed individuals could increase the accuracy of the estimators.

Two species, *Chaetostoma microps and Astroblepus cf. pholeter,* presented changes in both weight and length. The reduction in the weight of *Chaetostoma*, an armored fish covered with dermal plates with a high concentration of calcium phosphate, could be related to the oxidation caused by the HCOH. This process produces formic acid, which decreases the degree of calcium in bones, or dermal plates in the case of Loricariidae, and eventually turns the specimens into soft tissues [38]. In addition, size reduction could occur in the collagen-enriched fleshy mouth, present in both *Chaetostoma* and *Astroblepus* [39, 40]. Field observations have shown that this structure is more prominent in live specimens and is reduced in fixed specimens. Our results are in agreement with studies that found body weight loss and length shrinkage, when using similar preservation techniques, in small- and medium-size marine pearlside, glacier lantern fish, and Brazilian silverside [11, 41]. Parker [16] also reported a decrease of 4% in size after 40 days of fixation in 3.8% HCOH but an increase of 5–11% from initial weight in salmonid specimens.

Our results also indicated that magnitude of effect of preservation is strongly dependent on fish size, with smaller individuals/species exhibiting greater weight losses than larger ones, as demonstrated for *C. microps, A. jamesi*, and *A. cf. pholeter*. This pattern is concordant with the literature [3, 11, 16, 42, 43]. Increase in weight after the preservation process has also been previously reported [9, 44].

4.2. Preservation Effects in Small Freshwater Fishes. Previously, studies analyzing body shape variation due to preservation techniques have examined mainly medium-size marine or freshwater species from North America, Europe, Asia, Australia, and Africa (Table S1). Our study presents new evidence on the effects of fixation and preservation in weight and length variation on small fish species of the Amazon basin which are underrepresented on the topic-related available literature. Studies using morphometric approaches involving LWRs should be cautious when using preserved specimens. In species where fixation-based changes occur, these are usually not enough to produce complications in taxonomical differentiation of sister species [1]. However, studies have demonstrated that variation in body shape attributable to preservation could be higher than variation attributable to biological factors such as sex or individual provenance [45]. Thus, ecological studies should avoid using data that use measurements on both fresh and preserved specimens at once as differences attributed by ecological factors could be masked by the effects of preservation.

A recent review showed that body mass is affected after fixation and preservation, and length measurements appear to be slightly negatively affected [18]. Because of the large number of inaccuracies in body mass measurements from preserved specimens, estimates of mass and condition factors based on preserved specimens of small fish species should be discouraged.

Overall, we recommend that measurements are to be performed on fresh specimens using a standardized methodology and that further studies analyzing the effect of preservation methods on LWR curves mark individually each specimen to allow paired measurements and aid with calculations of magnitude of change. Standardized methodology could vary for different groups. For example, we noted that transportation may damage the delicate tails of small gymnotiforme species, presenting another inconvenience when using preserved specimens for LWR studies. Given that existing evidence remains highly variable, and no consensus has been reached regarding the acceptability of using preserved fish individuals for biomass and condition factor estimates, our findings with previously unreported species represent new and significant evidence of the preservation effects in LWR studies. We also present a compilation of the varying results reported in the literature (Table S1) to complement previous literature reviews on the topic [18, 46]. We suggest that studies should report for how long the material used was preserved, and in what solvent was used to dilute HCOH, as this will give insight to possible anomalies in the results. Finally, it should be noted that in comparative studies like ours, other causes could explain some of the observed differences, such as the degree of specimen handling and the capture method [14, 15]. We recommend that future studies on the effect of preservation practices consider these factors, as well as perform measurements on tagged individuals to better evaluate individual differences in specimens.

5. Conclusions

Our study confirms that formalin fixation, although one of the best methods to preserve morphological structures, can produce shrinkage and/or weight gain or loss in fishes from the Western Amazon. Eight out of ten analyzed species showed weight losses after preservation, and two species exhibited both length and weight changes. Small freshwater fish species seem to be more prone to lose weight than length. In addition, fish size can also modulate the intensity of these changes, but body shape does not seem to be a significant factor for these smaller fishes. Measurements on fresh specimens should be encouraged for LWR studies. However, recognizing that this is not always feasible, studies using preserved specimens should consider the effects of fixation and preservation on fish body, length, and weight.

Data Availability

The data used to support the findings of this study are available from the authors upon request.

Additional Points

Highlights. (i) Fish specimens from ten species were measured fresh and after fixation in formaldehyde 10% and preservation in 70% ethanol. (ii) After preservation weight always varied while length diminished only for two species. (iii) Smaller individuals experience greater weight losses. (iv) Measurements on fresh specimens should be encouraged for LWR studies.

Ethical Approval

Ethical guidelines followed the Environmental Unified-Text-of-Secondary-Legislation of (Texto Unificado de Legislacion Secundaria de Medio Ambiente-TULSMA) regarding animal welfare laws and policies approved by the Ministry of Environment, Water, and Ecological Transition (Ministerio de Ambiente, Agua y Transicion Ecologica del Ecuador-MAATE) through the Authorization of Scientific Research No. MAE, 019-2018-IC-FAU-DNB/MAE.

Disclosure

The present study was part of "NUNA, Proyecto Descubre Napo." A preprint of the manuscript is available at https://papers.ssrn.com/sol3/papers.cfm?abstract_id=4342833.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Karla S. Barragán conceptualized the study, was involved in data curation, performed formal analysis, investigated the data, and wrote the original draft. Junior Chuctaya conceptualized the study, was involved in data curation, contributed to methodology, was responsible for software, investigated the data, supervised the study, and reviewed and edited the manuscript. José Vieira investigated the data. Daniel Escobar-Camacho supervised the study and reviewed and edited the manuscript. Andrea C. Encalada was responsible for resources, performed project administration, was involved in funding acquisition, supervised the study, and reviewed and edited the manuscript.

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Supplementary Materials

Table S1: literature review of reported changes in length, weight, body shape, and others associated with fixation and preservation in formaldehyde (CHCO) ethanol (EtOH) and other techniques. Standard length (SL). Table S2: sampling localities. Table S3: species analyzed in the present study. (*Supplementary Materials*)

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