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REVIEW PAPER

Effects of preserving juvenile rainbow trout (*Oncorhynchus mykiss*) in formalin and alcohol on body length, mass, and condition factor estimates

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Abstract. Body length and weight are key parameters measured to provide information about the growth and condition of larval, juvenile, and adult fishes from both the wild and aquaculture. The goal of this study was to evaluate the effects of preservation in formalin and alcohol on juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum) standard body length (SL) and weight changes. Standard length (19.43–32.98 mm SL) decreased after 60 days of preservation by an average of 2.98% in 96% alcohol and 2.26% in 10% formalin. In contrast, fish body weight (0.094–0.63 g) decreased in alcohol (31.22%) but increased in formalin (11.65%). These percentage values can be used as correction factors, with the exception of fish body weight in alcohol since the size of individuals affected the magnitude of change. Accordingly, a correction formula accounting for fish weight must be applied (fresh weight (g) = -0.0363 + 1.667 × preserved length). Changes in fish length and body weight in different preservatives resulted in significant differences in the Fulton condition factor, with underestimation in alcohol (fresh F = 1.58; preserved F = 1.20) and overestimation in formalin (fresh F = 1.60; preserved F = 1.92).

Keywords: preservation, sample storage, shrinkage, size measurements

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Introduction

Body length and weight are key parameters measured in studying the early developmental stages of fishes. These parameters provide information about the growth and condition of individuals, which is important in a wide range of research, primarily in the fields of fish biology and ecology, including ontogenetic and evolutionary studies (Butler 1992, Campana and Jones 1992). Fish weight and body length can be used to determine the condition factor (Fulton's condition factor, K), which provides information about the condition of a given specimen, determining, to a large extent, the survival capacity of individuals or groups of fishes. Analysis of the basic parameters of the physical condition of fishes makes it possible to study population dynamics and responses to environmental changes, such as pollution or changes in food availability (Ricker 1975, Froese 2006, Nash et al. 2006). Ensuring the accuracy and correctness of measurements of the size of individuals is therefore important in research conducted both in the wild and under experimental and aquaculture conditions.

Fish body size is often measured several weeks or months after they are caught and preserved

(Ahlstrom 1976, Butler 1992). One of the most common methods of preserving fish samples, apart from freezing and storing in liquid nitrogen, is in formalin or alcohol (Rodríguez-Fernández et al. 2021) to prevent the biological decomposition of tissues (Steedman 1976, Fournie et al. 2000). The appropriate preservation method, i.e., the type of preservative and its concentration, depends on many factors, such as sample type (e.g., whole fish, selected organs, or tissue fragments), the developmental stage of the fish (larvae, fry, or adult fish), and the purpose of the study (e.g., morphometric measurements, biochemical analyses, otolith research, or genetic analyses) (Hay 1982, Fey 1999, Martinez et al. 2013, Gómez et al. 2014, Fey 2018). Other factors, such as the expected duration of storage time and the availability of equipment and chemicals, are also important. Each preservation method has both advantages and limitations, so it is important to consider the options carefully before preserving biological samples of fish.

A standard practice in research on the biology and ecology of early-stage fishes is the preservation of materials in formalin and alcohol. However, this method of preservation can affect the structure of proteins in tissues, causing changes in their physical characteristics, which in turn results in changes in the length and weight of individuals (Steedman 1976, Pepin et al. 1998, Fournie et al. 2000). Both formalin and alcohol can cause shrinkage and reduced length and weight in fishes (Butler 1992). However, in some studies, increases in body weight were observed during fish preservation in formalin (Karjalainen 1992, Treasurer 1992, Shields and Carlson 1996). Similarly, body length increase was observed after preservation in alcohol, but only for the preflexion stage of streaked prochilod, *Prochilodus lineatus* (Val.) larvae (Gómez et al. 2014).

Therefore, it is extremely important to account for the effects preservative fluids have on measurement results. This is the only way to obtain pre-preservation values that can be compared with other available data during analysis. Failure to apply appropriate corrections to the measurement of preserved materials may result in large inaccuracies in the results of analyses, e.g., during growth rate analysis (Fey 1999, Greszkiewicz and Fey 2018). An alternative to preserving samples for later body length estimates are photos or videos, which means that specific values can be read much later. This type of analysis can only be used under specific circumstances. When determining a coefficient or correction formula, it should be noted that shrinkage can vary not only with different preservatives but also for different fish species (Butler 1992), as shown for example by Fey (1999) for species of different body types and shapes, i.e., sprat, *Sprattus sprattus*; goby, *Pomatoscistus minutus*; and four-beard rockling, *Encheylopus cimbrius*. Therefore, correction coefficients must be introduced to account for such variability.

In this study, we present an analysis of the effect of fish preservation on body size measurements of juvenile rainbow trout, *Oncorhynchus mykiss*. Rainbow trout is recognized as an important species in many areas where it is introduced or occurs naturally, because of its economic and ecological importance (FAO 2009). It is also one of the most important farmed fish species in the world. Owing to its importance, this species has been the subject of numerous scientific studies of fish biology and aquaculture. An understanding of rainbow trout physiology, genetics, and ecology can contribute to a better understanding of the functioning of aquatic ecosystems and the development of aquaculture methods. Notably, trout is a model species in ecotoxicological studies. Currently, there is no information available in the literature concerning the consequences of preserving this species in formalin or alcohol during the early developmental stages. Thus, there are also no appropriate correction factors for the length and weight of the fish body after preservation.

The aim of this study was to determine the effects of preservation for 60 days in 96% ethyl alcohol and 10% formalin on fish length and weight measurements in juvenile rainbow trout (19–32 mm SL; 0.094–0.633 g body weight) and to present a formula for converting the values obtained after preservation to those before preservation. The consequences of

measurement errors on the results of Fulton's condition factor estimates were also determined.

Materials and Methods

Rainbow trout larvae were bred in the laboratory in an aquarium system (0.4 m^3 tank) with a closed freshwater circuit with water cooling devices and a biological filter. Fish eggs were obtained from commercial breeders (Dąbie, Poland). After mass hatching (approximately 34 days after fertilization) and yolk sac resorption (approximately 17 days after hatching), the fish were fed six times daily ad libitum with specialized complete feed for trout fry, and the amount of feed supplied was increased as the fish grew (Skretting, Perla Larva 5.0; Skretting, Nutra HP 0.5; Aller, Futura EX GR 0.5–1.0 mm; Aller, Performa 2 mm). The water temperature during both the incubation of eggs and the subsequent growth of larvae was 10 ± 0.7°C (mean ± SD).

To analyze the effects of preservatives on the shrinkage of fish of different sizes, samples for analysis were taken on two dates. The first sampling took place 24 days after mass hatching, with an average fish length of 23.84 mm and a weight of 0.20 g. The second sampling took place 35 days after mass hatching, with an average fish length of 29.05 mm and a weight of 0.44 g. Each time, 30 fish were taken for preservation in formalin, and 30 fish were taken for preservation in alcohol, resulting in a total of 60 fish in each preservative. Each fish was first measured and weighed and then placed in separate vials with the appropriate preservative: 96% alcohol or 10% formalin (4% formaldehyde solution). The vials were checked for tightness after the first month of preservation. Since the conditions during storage may affect the samples during preservation period, the storage-room environment should be routinely monitored (Simmons 1999). In our study the samples were stored in a darkened room with a constant temperature of 16 degrees. The conditions were maintained and monitored by a ventilation system with an ELP-HMI-Compact temperature sensor (Oleśnica, Poland). The standard length (SL) of the fish was measured to the nearest 0.01 mm with an image analysis system including a Nikon SMZ1270 microscope, a Nikon DS-Fi3 camera, and Nikon Nis Elements software v. 5.42.04 (Nikon Europe B.V., Amstelveen, The Netherlands). The weight of the fish was determined to the nearest 0.001 g with an Axis ACA520 balance (AXIS Sp. z o.o., Gdańsk, Poland). Before body weight was measured, each fish was placed on a paper towel for approximately 10 s to drain excess fluid. Measurements of each individual were repeated with the same methodology after 60 days of preservation. The initial SL length of the individuals analyzed ranged from 19.43 to 32.98 mm, and the body weight ranged from 0.094 to 0.633 g.

Based on the measurements taken, the relative body size change (%) in the fish was calculated. Fulton's condition factor was also calculated for each individual. $K = W \times L^{-3}$ where K = Fulton's condition factor, W = fish weight, and L = fish length. Student's t-test was used to assess the significance of differences in shrinkage between samples preserved in alcohol and those preserved in formalin. Prior to the analysis, the assumptions of data normality and homogeneity of variance were tested using Levene's test. In instances where the development of a correction formula was considered necessary instead of the application of a mean correction value, the formula was developed by fitting the optimal best-fit function within the generalized linear model (GLM). The optimal fit was determined through a process of residual observation and coefficient of determination (r_c) analysis. All calculations were performed in Statistica version 12.0 (StatSoft, Tulsa, Oklahoma).

Results

Changes in fish SL ranged from 0 to 11.28% in 96% alcohol (n = 120; mean = 2.98%, Fig. 1a) and from 0 to 12.34% in 10% formalin (n = 120; mean = 2.26%, Fig. 1b). Differences in fish shrinkage between the two preservatives, with greater shrinkage in alcohol, were statistically significant (t-test, $P < 0.05$). For

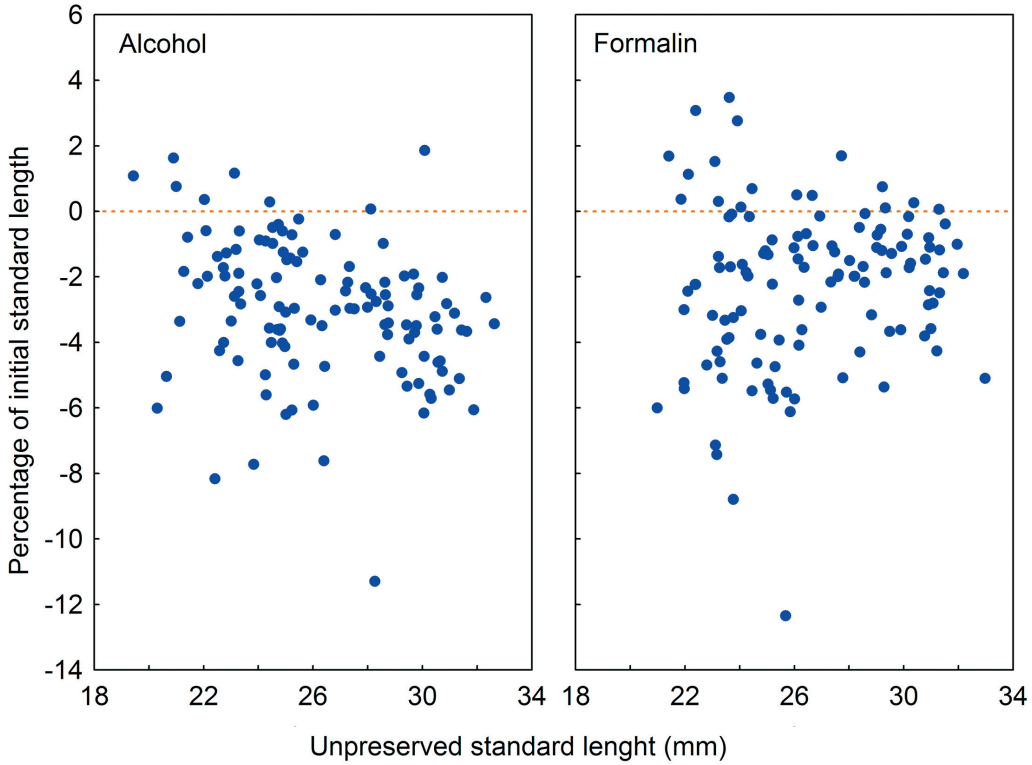

Figure 1. Changes in standard length of rainbow trout juveniles after 60 days of preservation in 96% alcohol (n = 120) and 10% formalin (n = 120).

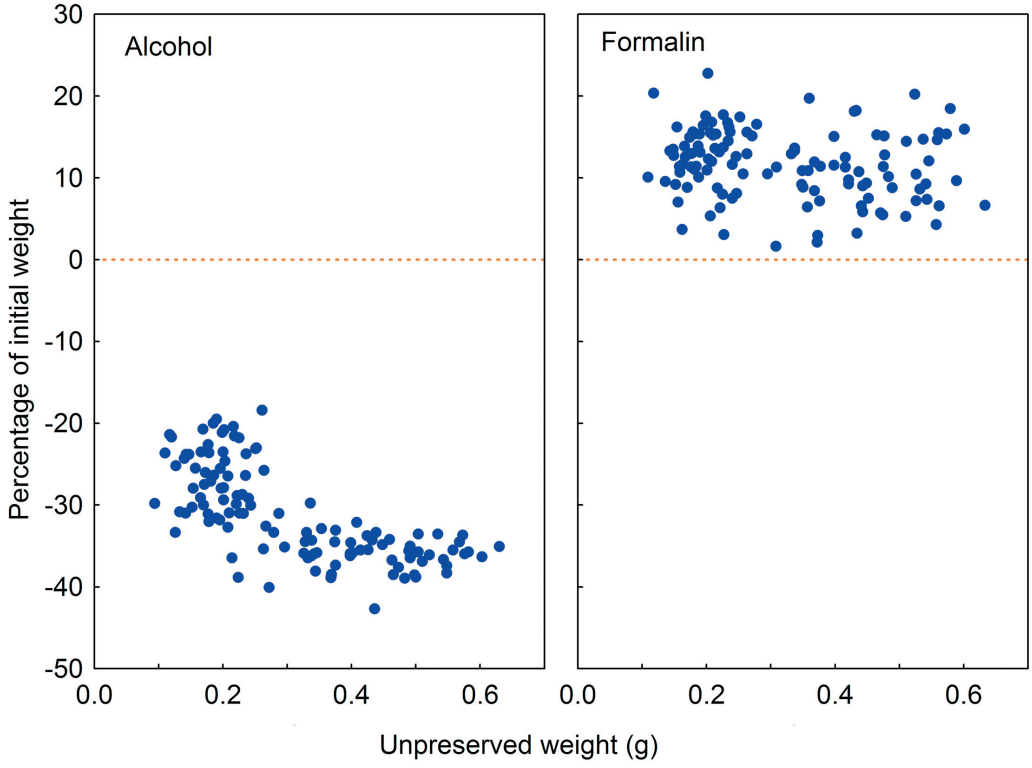

Figure 2. Changes in body weight of rainbow trout juveniles after 60 days of preservation in 96% alcohol (n = 120) or 10% formalin (n = 120).

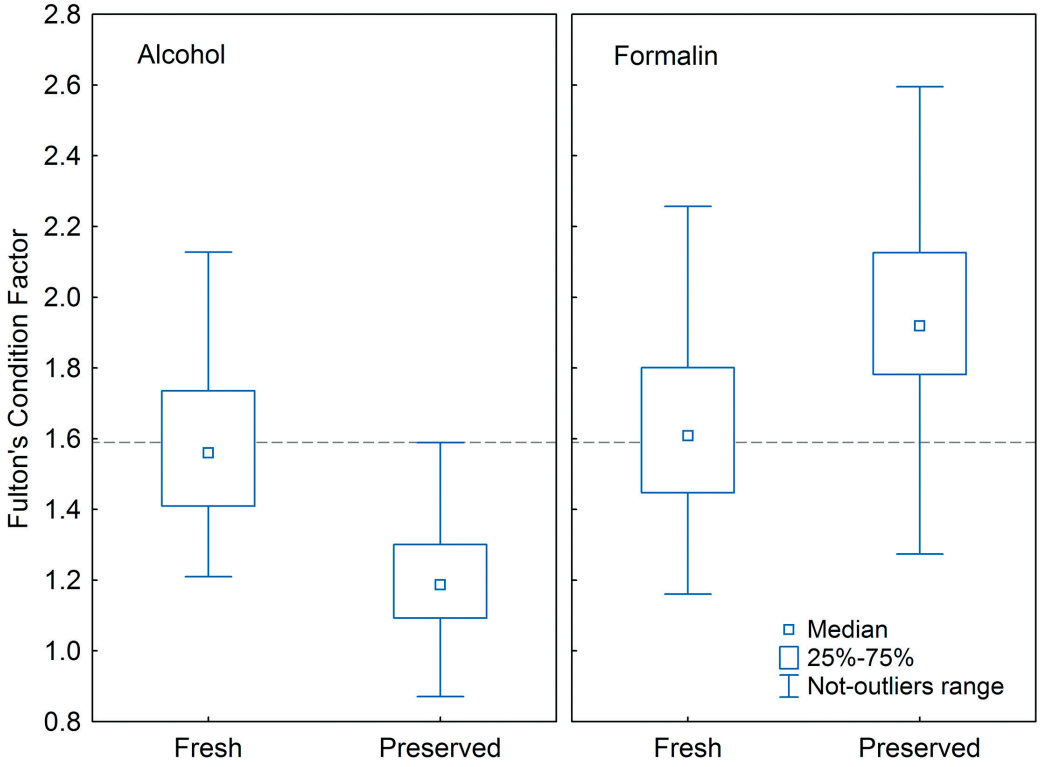

Figure 3. Fulton coefficients for juvenile rainbow trout before and after preservation in 96% alcohol (n = 120) and 10% formalin (n = 120).

body weight measurements, weight decreases in the 96% alcohol group ranged from 18.39 to 42.66% (n = 120; mean = 31.22%, Fig. 2a), whereas in the 10% formalin group, weight increases ranged from 0 to 22.77% (n = 120; mean = 11.65%, Fig. 2b). Differences in weight change between fish in the two preservatives were statistically significant (t-test, $P < 0.05$).

The degree of change in body length was not dependent on the size of the individual (matched linear regression function, $P > 0.05$). For body weight, formalin preservation produced the same degree of change regardless of the initial weight. In contrast, in alcohol, the amount of change depended on the size of the individuals preserved, with greater body weight reduction in larger individuals. Therefore, the percent body size change values can serve as correction factors, except for body weight in alcohol, in which case the size before preservation must be calculated using the following formula: fresh weight (g) = - 0.0363 + 1.667 preserved length (n = 60; r^2 = 0.985).

Relative to the values calculated for the same individuals before preservation, the condition coefficient F (Fig. 3) was underestimated for fish preserved in 96% alcohol (before F = 1.58; after F = 1.20) and overestimated for fish preserved in 10% formalin (before F = 1.60; after F = 1.92). The differences between pre- and post-preservation measurements were statistically significant for both alcohol and formalin (t-test, $P < 0.001$).

Discussion

The present study revealed that storing rainbow trout larvae (*O. mykiss*) in two common preservatives (96% alcohol and 10% formalin) resulted in changes in body length and weight, which affected the results of condition coefficient analyses. In alcohol, the shrinkage of fish relative to the initial length values (mean 2.98%, range 0–11%) was similar to the shrinkage values observed in studies for larvae and small fry of other fish species of similar size, for example, pike,

Esox lucius L. (Greszkiewicz and Fey 2018), largelip killifish, *Tlaloc labialis* (Günther) (Anzueto-Calvo et al. 2017), herring, *Clupea harengus* L. and smelt, *Osmerus eperlanus* (L.) (Fey 2002), Atlantic menhaden, *Brevoortia tyrannus* (Latrobe) (Fey and Hare 2005), and sockeye salmon, *Oncorhynchus nerka* (Walbaum) (Shields and Carlson 1996). Although, in contrast, while body length increase was observed for streaked prochilod (*P. lineatus*) larvae after preservation in alcohol, it was only for the preflexion stage (Gómez et al. 2014).

Similarly, weight loss in fish (mean 31.22%, range 18.39–42.66%) preserved in alcohol was similar to the results of studies conducted on silvery light fish and glacier lantern fish, *Benthosema glaciale* (Reinhardt) (Kristoffersen and Salvanes 1998), juvenile sockeye salmon (Shields and Carlson 1996), and larval and juvenile yellow perch, *Perca flavescens* (Mitchill) (Paradis et al. 2007). Unfortunately, there is no information available in the literature on the effects of preservatives on trout larvae and fry to make comparisons directly with studies on this species. This is significant considering the importance of trout as the subject of research in both environmental studies and aquaculture.

Formalin also had a significant effect on differences in fish standard length and weight measurements before and after preservation, with a mean length shrinkage of 2.26% and a range of 0–12.34% and a mean body weight increase of 11.65% and a range of 0–22.77%. These results are consistent with the literature data on the effects of formalin on larval and fry length (Butler 1992, Hjörleifsson and Klein-MacPhee 1992, Gómez et al. 2014, Greszkiewicz and Fey 2018, Rodriguez-Fernandez et al. 2021). On the other hand, with respect to the effects of formalin on the weights of preserved larvae and juveniles, literature reports are inconsistent. Examples can be found both for weight decreases (Kristoffersen and Salvanes 1998, Paradis et al. 2007, Anzueto-Calvo et al. 2017) and weight increases, which were observed in the present study. Results similar to ours were obtained by Shields and Carlson (1996) for juvenile sockeye salmon (7% increase), Karjalainen (1992) for larval vendace, *Coregonus albula* (L.) (17–21.5% increase), and Treasurer (1992) for perch, *Perca fluviatilis* L. (13.4–19.6% increase).

The discrepancy in the effects of formalin on the body weight of preserved fish may result from several factors related to differences in the type and distribution of tissues (Gill et al. 1982), water content in tissue (Ehrlich 1974), or changes in tissue cell structures over time (Love 1958). Formalin, which is a powerful disinfectant, leads to protein denaturation and cell structure stiffening (Steedman 1976). This process can cause weight gain via the binding of formalin to proteins and other cellular components, which increases the overall mass of fish tissues. This effect is specific to formalin and distinct from the effects of alcohol, which usually leads to tissue dehydration. On the other hand, a reduction in fish body weight during formalin preservation can occur from the opposite process, i.e., the removal of water from tissues (Steedman 1976). In addition, formaldehyde can cause the dissolution of glycogen, glucose, some phospholipids, and inorganic salts (Kristoffersen and Salvanes 1998). Interestingly, decreases in fish body weights in formalin were observed in individuals of different sizes: larval vendace 8–10 mm in size (weight loss of 17–21.5%) (Karjalainen 1992), larval yellow perch 10.8–18 mm in size (weight loss of 21.9% at 15 days; 23.2% at 8 months of preservation), juvenile yellow perch 39–56.5 mm in size (weight loss of 2.2% at 15 days; 3.9% at 8 months of preservation) (Paradis et al. 2007), and juvenile *T. labialis* 31–82 mm in size (weight loss of 5.78% at 30 days of preservation) (Anzueto-Calvo et al. 2017). In contrast, in studies in which an increase in the weight of preserved samples was observed, sockeye salmon juveniles analyzed had average lengths of 64 mm (weight increase of 7.51% at 16 days; 7.12% at 70 days of preservation) (Shields and Carlson 1996) and perch had a length of 37.8 mm (weight increase of 15.4–19.6%) and 54.9 mm (weight increase of 13.4–16.8%) (Treasurer 1992). Most probably the mechanism of body weight changes (increase or decrease) is therefore a combination of different factors, like fish size, body type, and species.

The magnitude of changes can be also affected by time. However, the greatest changes occur over a period of just several days (Fey 1999, Santos et al. 2009, König and Borcherding 2012, Rodríguez-Fernández 2021) or even several hours (Frimpong and Henebry 2012, Lee et al. 2012). Cod larvae and juveniles preserved for a period of up to three years did not show significant degrees of additional shrinkage (Fey 2012). Thus, the correction factor estimated in the present study approximately two months after preservation, allows it to be used for a wide range of samples if the samples are preserved for at least several days when most changes occur. Naturally, if feasible, the optimal solution is to measure fresh fishes prior to conservation in both experimental and field settings.

The results of the present study indicated that alcohol and formalin affect the length and weight of rainbow trout larvae differently, as reflected in the Fulton values calculated for the fish after preservation. Alcohol, which led to a reduction in fish weight and length, meant that the Fulton coefficient was underestimated. Formalin, on the other hand, caused fish length to shorten and simultaneously body weight to increase, causing overestimated Fulton coefficients. The effect of underestimating the fitness factor as a result of weight reduction after preservation was demonstrated previously by Anzueto-Calvo et al. (2017) for *T. labialis*. The results of this study have important implications for both environmental and aquaculture research practices.

In conclusion, the preservation and storage of rainbow trout larvae and fry in both ethyl alcohol and formalin significantly affected fish length and body weight. Formalin caused a reduction in length and weight gain, resulting in overestimated Fulton coefficients, whereas alcohol led to reductions in both body length and weight, causing underestimated condition factors. These results and the proposed correction coefficients for body length and weight can be used not only in environmental research but also in aquaculture. While the correction coefficients are indeed determined for specific species, they can also be applied to other species with comparable body types and shapes, as well those of a similar size. This is particularly the case with regard to the correction of body length, since additional factors such as the environment in which a given species occurs (freshwater or saltwater) may also be significant with respect to body weight. The correction factors presented can also be applied to materials preserved for considerably longer than the 60 days utilized in this study, potentially extending to numerous years.

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Declaration of Competing Interests. None

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