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ORIGINAL PAPER

Is formalin fixation and ethanol preservation able to influence in geometric morphometric analysis? Fishes as a case study

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Abstract Geometric morphometric analysis has increased in the recent years, turning into a powerful tool to explore shape and size variation. Several biological studies use specimens that have been through some kind of preservation, mainly formalin preservation, commonly used in biological collections. This study analyzed the effect of preservation in shape on two fish species: *Eucinostomus argenteus* and *Pomadasys corvinaerformis*. Twenty-nine individuals of *E. argenteus* and twenty-five of *P. corvinaeformis* were collected, photographed twice, preserved in 10 % formalin for 1 week, and then transferred to 70 % ethanol for 83 days. We evaluated three levels of error: (1) error of landmark digitalization, (2) error of taking the picture and storage in JPEG format, and (3) the formalin and ethanol fixation error using Procrustes ANOVA, Discriminant Analysis, and Principal Component Analysis. Significant difference between treatments was observed on both species with Procrustes ANOVA and Discriminant Analysis. In addition, Principal Component Analysis showed a separation between groups of treatment on both species. These results represent the first evidence of preservation effects in studies of geometric morphometrics and show that according to the statistical test utilized, the fixation could affect the shape variations in different ways and could lead the researcher to false results or wrong conclusions. Other methods to explore the shape variation of organisms previously fixed should be tested in order to assess their influence in geometric mophrometric studies.

Keywords Procrustes ANOVA · Discriminant Analysis · Principal Component Analysis · Fishes · Fixation · Preservation

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Introduction

The geometric morphometric technique allows us to explore the geometric shape of a feature, describing it as a set of anatomical points known as landmarks (Bookstein 1991). Currently, this tool combined with the multivariate statistical analysis (Procustes ANOVA, Principal Component Analysis, multivariate regression, Discriminant Analysis, Canonical Variable Analysis, among others) has allowed the investigation into the variation in the biological shapes and the creation of a strong theoretical basis for morphological data analysis (Bookstein 1996; Dryden and Mardia 1998; Klingenberg 2011). Increasingly, geometric morphrometric studies combined with new statistical tools (which give a greater power of resolution), make this technique attractive for several research areas (Adams et al. 2004; Palaniswamy et al. 2010; Klingenberg 2011; Schunke et al. 2012 in press).

Several morphological studies use animals coming from biological collections, which normally pass through a preservation treatment of 10 % formalin fixation and then ethanol preservation (FE). Fixation by formalin is performed to prevent changes in tissues immediately subsequent do death, and the subsequent ethanol preservation

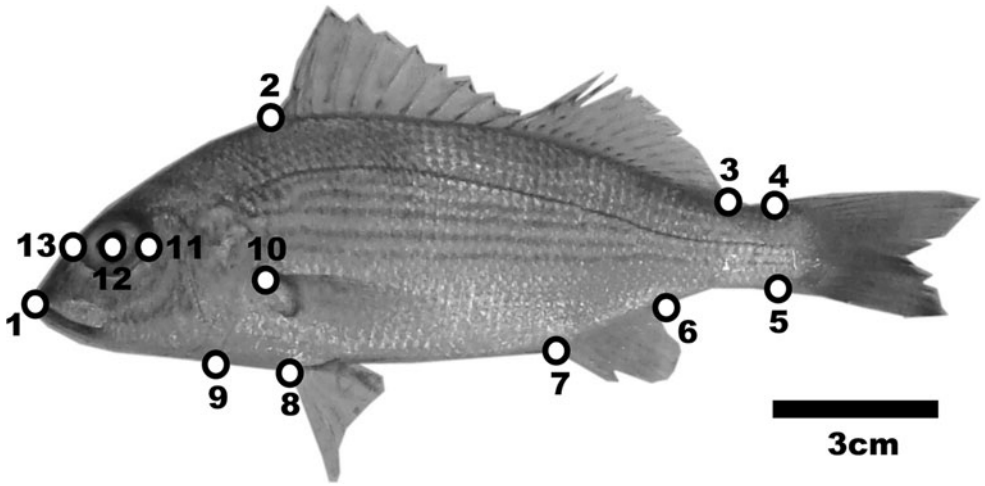

Fig. 1 Location of 13 the landmarks used for morphometric analyses, their definition and numbering: *1* Tip of the snout, *2* anterior insertion of the dorsal fin, *3* posterior insertion of the dorsal fin, *4* dorsal insertion of the caudal fin, *5* ventral insertion of the caudal fin, *6* posterior insertion of the anal fin, *7* anterior insertion of the anal fin, *8* anterior insertion of the pelvic fin, *9* opening of the operculum, *10* upper insertion of the pectoral fin, *11* posterior margin of the eye, *12* centre of eye, *13* anterior margin of the eye. Scale is 3 cm

is done to maintain the integrity of fixed specimens for long-term storage (Leslie and Moore 1986). Both substances, formalin and ethanol, have similar effects on body water and leave the specimen rigid. Furthermore, the oxidation caused by formol produces formic acid, which decreases the degree of calcium in bones, and eventually turns the subjects into soft tissue (Sturgess and Nicola 1975).

Changes in size and shape caused by the process of in formalin fixation and ethanol preservation have been shown using conventional morphometric analysis, and the intensity of these can be different according to the species (Butler 1992; Treasurer 1992; Fey 1999, 2012). However, the importance of these changes in shape for biological questions is still controversial (Shields and Carlson 1996; Nadeau et al. 2009).

Since the geometric morphometric analyses are able to detect small morphological changes, this kind of preservation could have some important effect when comparative studies are made. Thus, the present paper aims to quantify the effect of FE in morphology, as well as to determine whether there are different morphological variations between two different fish species.

Materials and methods

Tests of geometric morphometrics were done to explore the effect of FE fixation on the external shape of two species of fish. A total of twenty-nine adult individuals of *E. argenteus* Baid and Girard, 1855, and twenty-five of *Pomadasys corvinaeformis* (Steindachner 1868), caught in a Galinhos' beach (05°05′40″S 36°16′19″W), Rio Grande do Norte, Brazil, were measured. In order to avoid the capture photography error, individuals were photographed always by the same person, with a Sony h10, 8,1 megapixels digital camera, and the two-dimensional coordinates were taken by thirteen landmarks for each individual (Fig. 1), using TPSdig version 1.4 software (Rohlf 2004). Landmark alignment was made by the Procrustes superposition method of MorphoJ software (Klingenberg 2011).

In the present study, the Procrustes ANOVA test was made, to quantify error (Klingenberg and McIntyre 1998; Klingenberg et al. 2002), adapted for the study case. Three levels of error were analyzed (1) error of landmarks' digitalization, (2) error of taking the photograph and storage in JPEG format, and (3) FE fixation error. For these goals, were took two pictures of each individual, and each picture was duplicated. Later, the individuals were fixed in 10 % formalin, and later diluted in distiled water for 7 days. After this period, the individuals were transferred to 70 % ethanol, where they remained for 83 days. After the period in alcohol, the same individuals of both species were photographed two times, and each photograph was duplicated. This way, a total of 232 configurations for the 29 individuals of *E. argenteus* and 200 configurations for the 25 individual of *P. corvinaeformis* were analyzed (Fig. 2).

To evaluate if the effect of FE fixation could lead to errors of differentiations between groups, the Discriminant Analysis test was done. In order to know the intensity of the fixation effect, the T^2 statistical test and the permutation test (10,000 permutations) were used to compare the replications of each picture, both pictures of each individual per treatment, and all the pictures before and after fixation. Since the Discriminant Analysis maximizes differences between groups, we opted to do a more conservative test,

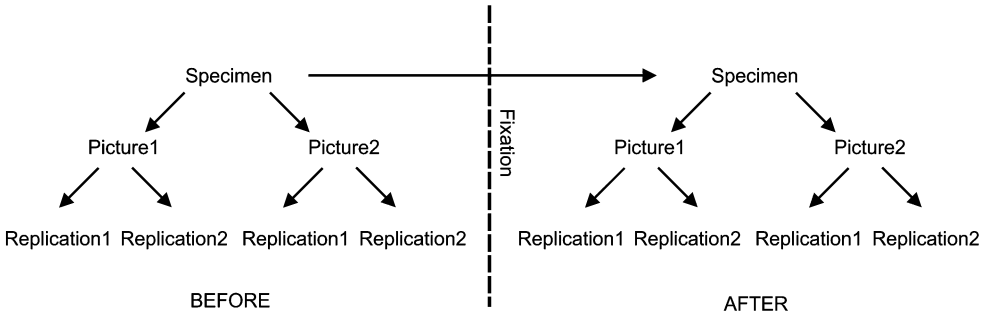

Fig. 2 Layout of quantification of the error in three levels: replication, picture, and fixation effect. Adapted from didactic material of Klingemberg Lab online course, 2011

Table 1 Procrustes ANOVA for the extern shape for both species. The shape variation of individuals before and after fixation (Ind*Trat). Sums of squares (SS) and mean squares (MS) are in dimensionless units of Procrustes distance

Specie	Effect	SS	MS	*df*	*F*	*p*
E. argenteus	Individual	0.26061728	0.00042308	616	4.52	<0.0001
	Tratament	0.03303345	0.0015015204	22	16.03	<0.0001
	Ind*Trat	0.05769575	9.36619E−005	616	1.64	<0.0001
	Picture	0.07303964	5.72411E−005	1276		
	Replication	0.04387554	1.71926E−005	2552		
P. corvinaeformis	Individual	0.13922141	0.0002636769	528	3.36	<0.0001
	Tratament	0.02645385	0.0012024479	22	15.34	<0.05
	Ind*Trat	0.04137523	7.83622E−005	528	2.21	<0.0001
	Picture	0.03906633	3.55148E−005	1,100		
	Replication	0.03005813	1.36628E−005	2,200		

the Principal Component Analysis, in which groups are not defined a priori, and allow us to have a global vision of the shape variation before and after the fixation. All the analyses were made with the MorphoJ software (Klingenberg 2011).

Results

For both species, the partitioned variation of the Procrustes ANOVA analysis showed that the variation of the specimens after the fixation was highly significant ($p < 0.0001$), being bigger than the sum of the landmarks' digitalization error and the photography error (Table 1).

With the Discriminant Analysis data, the differences between the replications (*E. argenteus*, $T^2 = 6.48$, $p = 0.99$; *P. corvinaeformis*, $T^2 = 5.29$ $p = 0.99$) and between the pictures were not significant (*E. argenteus*, $T^2 = 43.80$, $p = 0.50$; *P. corvinaeformis*, $T^2 = 19.50$ $p = 0.86$). However, significant differences were showed between the treatments before and after the fixation with FE (*E. argenteus*, $T^2 = 640.65$, $p < 0.0001$; *P. corvinaeformis*, $T^2 = 668.05$, $p < 0.0001$) (Fig. 3). The main modifications after the fixation, as seen on the wireframe graphic, were a contraction of the eye and a decrease in the body depth, for both species. Additionally, *E. argenteus* showed a profusion of the jaw apparatus (Fig. 4).

In the Principal Component Analysis, the first three principal components (PC) were responsible for 56 % of the shape variance in *E. argenteus*. The PC3 (13.06 %) was responsible for the separation of the subjects before and after fixation. For *P. corvinaeformis*, the first 3 principal components explained 51.84 % of the morphologic variation of the data, with PC1 (22.08 %) and PC3 (13.51 %) partly separating the specimens between treatments (Fig. 5).

Discussion

In our study, it was demonstrated that the fixation in FE has significant effects in the shape variation of the organisms. According to the objectives of the study, different statistical approaches are used in order to explore the effect of this fixation in different tests. The effect of fixation could have important implications on studies that seek to differentiate groups (sexual dimorphism, populations, ecotypes, species, etc.), as well as on those of shape variation analysis (allometry, relations between shape and environmental or biogeographical variables, reconstructions of evolutionary exchange, among others). This paper introduces the first evidence that this kind of preservation can affect the results and conclusions of geometric morphometric analysis used in biological research.

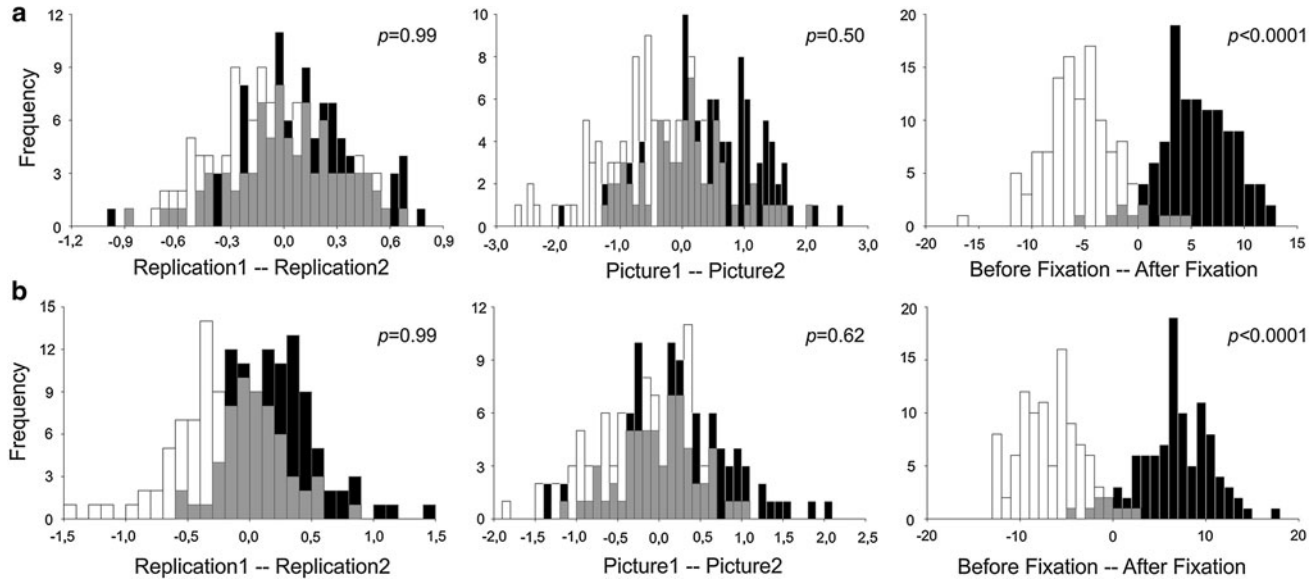

Fig. 3 The results obtained from the Discriminant Analysis for the three levels of error. **a** *E. argenteus.* **b** *P. corvinaeformis. White* Replication 1, Picture 1, Before Fixation. *Black* Replication 2, Picture 2, After fixation. *Grey* Overlapping regions

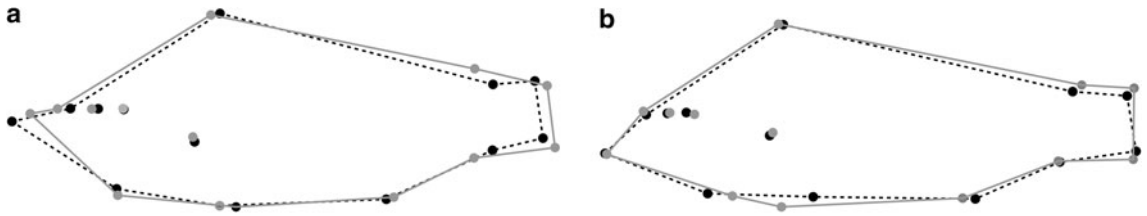

Fig. 4 Graphic of wireframe for both species. Continuous *grey line*: before fixation; *Dotted black line*: after fixation. **a** *E. argenteus* **b** *P. corvinaeformis*

The Procrustes ANOVA test is useful for estimating errors of measurement (Klingenberg and McIntyre 1998; Klingenberg et al. 2002). Through the mean squares (MS), it could be observed that the error caused by the fixation in FE was nearly five times bigger than the error caused by the landmarks' digitalization and twice that of the picture error for both species (Table 1). This results reveals that fixation incorporates an additional error in the analyses, which could be even bigger than the sum of picture error and digitalization error. It must be noted that this kind of test utilizes an extended approach of Goodall's F test, taking into account the amount of shape variation, but not the direction of variation (Klingenberg and McIntyre 1998).

Using the Discriminant Analysis, it was shown that when replications and pictures are compared, for both species, there were no significant differences (Fig. 3). There were some errors of landmarks digitalization and picture capture (normal errors). These errors are relatively small when compared to the variance of data (Klingenberg et al. 2002; Schmidt et al. 2010). In addition, they do not have a direction; on the contrary, they are distributed along the data. The absence of direction of these errors could lead the Discriminant Analysis not to detect differences between groups (Hair et al. 1998), having little meaning for biological research (nevertheless, it is important to quantify them). Comparing the individuals before and after the FE treatment using Discriminant Analysis, there were significant shape differences between treatments for both species ($p < 0.0001$) (Fig. 3). This result is very important in the biological context, since there are several studies that compare groups (sex, populations, species, ecotypes, etc.), using individuals from biological collections, mainly to fill some locations, sites, or specimens that are difficult to obtain. In these cases, when a geometric morphometric study is made with data of organisms that have been through FE fixation, and Discriminant Analysis or Canonical Variable analysis is used, the researcher could obtain wrong results (false positive) and be lead to the wrong conclusions, due to a morphological difference caused by the fixation, and not by biological factors.

Length and weight variations after the fixation had been reported in several studies with fishes. Different papers on conventional morphometrics have analyzed the influence

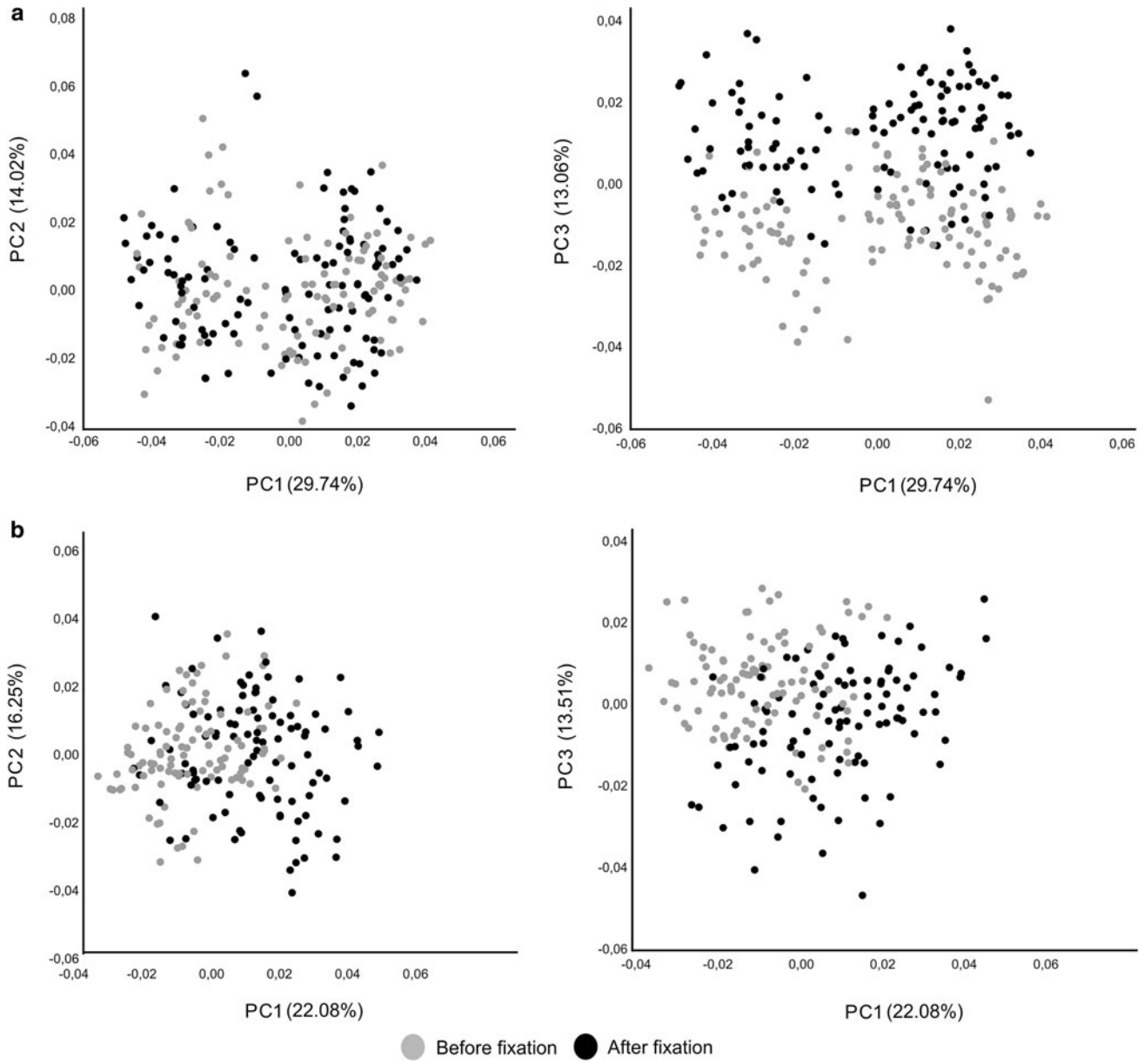

Fig. 5 Principal Component Analysis results. *Grey spot* before fixation; *black spot* after fixation **a** *E. argenteus* **b** *P. corvinaeformis*

of fixation effect at various stages in the ontogeny (larvae, juvenile, and adult). All studies show a reduction in length caused by the fixation in formol or ethanol (Leslie and Moore 1986; Fox 1996; Shields and Carlson 1996; Sagnes 1997; Fey 1999; Cunningham et al. 2000; Ajah and Nunoo 2003; Lee et al. 2012). Some of these studies propose correction equations of error for different methods of preservation in different species (for example, Smith and Walker 2003; Fey 2012). This task is more difficult for geometric morphometric approaches, since the shape is analyzed in a two-dimensional (even tridimensional, for some studies) space, instead of linear measurements of length and width.

The wireframe graphic showed different levels of variation intensity through the different landmarks for each species, and also different regions on the body shape vary with larger intensity (Fig. 4). These differentiated variations had already been demonstrated for some species of fish (Leslie and Moore 1986; Al-Hassan et al. 2000; Jawad et al. 2001). This could be explained by the differentiated genetic component present in these species, which influences the proportions of composition of white to red muscles in the tissues. Another possible explanation is that this change is due to the variation in the amount of water in each tissue, making it respond in different ways to different methods of preservation (Leslie and Moore 1986).

Principal Component Analysis is often used on geometric morphometric studies (Marcil et al. 2006; Ibañez et al. 2007; Cooper et al. 2010; Drake and Klingenberg 2010) because it can explore a large amount of variables combined, allowing to sum the shape variance in some variables, called principal components (Zelditch et al. 2004). In a Principal Component Analysis, the researcher does not define the groups a priori (unlike the Discriminant Analysis and the Canonical Variables Analysis) and does not maximize the differences between groups. The present study shows that the first three principal components are able to separate partly the individuals before and after the fixation for both species (Fig. 5). Often in studies of geometric morphometrics, the principal components of shape are used together with other variables (centroid size, geographical and/or climatic variables, time, phylogeny) trying to establish a possible relation among groups. According to our findings, fixation can modify the principal component values and these changes could underestimate or overestimate the relationships between variables (Schmidt et al. 2010).

Some geometric morphometric studies used X-ray, or cleared and stained individuals for studies of fishes and reptiles (Herrel et al. 2007; Landerhans et al. 2007; Sidlauskas 2008; Fontainer and Tobler 2009; Herczeg et al. 2010; among others). In view of the observed effect that fixation has on the external shape of organisms, we consider this method more appropriate for morphological biological research. Furthermore, some precaution should be taken to make conclusions out of these kinds of studies, mainly if the organisms used come from biological collections, or have gone through preservation in FE.

Additional geometric morphometric research should be made to evaluate other methods of conservation commonly used (e.g. ethanol, freezing) and their effect on the external morphology in different groups and at different stages of ontogeny.

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