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EFFECT OF PRESERVATION PROCEDURES ON THE BODY SHAPE OF THE GOLDEN MOJARRA, *DIAPTERUS AUREOLUS* (ACTINOPTERYGII: PERCIFORMES: GERREIDAE), AND ITS REPERCUSSIONS IN A TAXONOMIC STUDY

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Background. In taxonomic studies of fish, the use of preserved samples has been a common practice. Under the framework of morphometrics, the effect of the preservation techniques on body shape is rarely taken into account. Changes during preservation can cause errors in the results, which may eventually lead to wrong conclusions. To explore the effect of a traditional preservation procedure, we quantified the changes in body shape and size of the golden mojarra, *Diapterus aureolus* (Jordan et Gilbert, 1882), using geometric morphometrics.
Materials and methods. Fish fresh samples were photographed and frozen for at least seven months. Then, they were set in 10% formaldehyde, and passed through a wash of tap water and submerged in 70% ethanol. After five months the samples were photographed again. The differences between the mean shapes of two data groups (fresh fish and fixed specimens) were calculated by using the Procrustes distance. Effects associated with preservation were visualized by comparing the mean shape from each group, using a thin plate spline. A canonical variate analysis was carried out to detect the degree of intra- and inter-specific variation with *D. brevirostris* as an outgroup. Also, matrices of correct classification, based on Mahalanobis distances, were obtained.
Results. Procrustes distance between the two data sets was statistically significant ($P < 0.001$), suggesting that the fixation process produces changes in body shape. Canonical Variate Analysis verified that there were significant differences among three groups (fresh *D. aureolus*, fixed *D. aureolus*, and *D. brevirostris*; $P < 0.001$ for both CV1 and CV2), which were correctly classified (98.9%). In spite of the significant differences detected between the fresh and fixed data sets, these were less than those found in the outgroup. The pooled data of all specimens belonging to *D. aureolus* (fresh and fixed) produced a definitive correct classification (100%) between *D. aureolus* and *D. brevirostris*, indicating that the differences caused by the preservation method is not enough to confound species and, consequently, taxonomic integrity was totally acceptable.
Conclusion. Our results show a change in size and shape that are consistent with changes obtained with this and other methods applied to other species: generally shapes change and sizes tend to shrink with preservation of specimens. This is probably caused by dehydration from freezing and thawing, exposure to ethanol, and dissolution of skeletal structures caused by formaldehyde. While this effect was clear, it was not enough to confuse species. Caution is advised when working with groups with a close relation (populations or not clearly-defined species). We recommend a similar exercise with a case study with these levels of divergence.

Keywords: geometric morphometrics, preservation methods, ethanol, formaldehyde, freezing, taxonomic bias

INTRODUCTION

Taxonomic, phylogenetic, or population studies of fishes usually require preserved specimens deposited in collections or museums, which are used to quantify variations in shape and size. This is clearly justified for convenience and lower costs of sampling. However a bias may be incorporated into the findings when preserved specimens are compared with fresh caught specimens.

Research efforts have focused on quantification of morphological effects caused by the preservation procedure, but mostly the studies involve crustaceans, amphibians, annelids, and fish larvae (Thibault-Botha and Bowen 2004,

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Gagliano et al. 2006, Costa-Paiva et al. 2007, Põllupüü 2007, Deichmann et al. 2009). Morphological variations associated with preservation of juvenile or adult fishes have rarely been reported. Changes have mainly been based on length or proportion measurements (Leslie and Moore 1986, Sagnes 1997, Smith and Walker 2003, Paradis et al. 2007, Florin and Lingman 2008, Wessels et al. 2010, Lee et al. 2012), or length–weight relations (Al-Hassan et al. 2000, Ogle 2009, Beamish et al. 2011). To our knowledge, few studies explore the effect of preservation by taking advantage of the benefits of geometric morphometrics (Berbel-Filho et al. 2013, Gaston et al. 2013, Martinez et al. 2013).

Previous studies of preserved fishes mainly focused on the analysis of some of the stages of the preservation process (Sagnes 1997, Ogle 2009, Wessels et al. 2010, Martinez et al. 2013) or else they compared the steps of the process in an independent way (Al-Hassan et al. 2000). A more effective approach for evaluating extreme changes in fishes should be implemented, using the combined application of the techniques for preservation of fishes stored in ichthyological collections. These may include freezing the sample, fixation with formaldehyde, and removal of formaldehyde with alcohol baths until it is finally stored in alcohol at 70%. Although it is known that there is an effect caused by preservation (Martinez et al. 2013), it is necessary to explore whether this could affect the conclusions in a given study.

In this study, geometric morphometrics was used to measure the changes in body shape derived from the preservation process in the golden mojarra, *Diapterus aureolus*. This approach permits analysis of morphological variation more comprehensively and provides a clearer view of deformation when thin plate splines are used (Bookstein 1989, Zelditch et al. 2004). It also permits a direct comparison with the results obtained by Martinez et al. (2013). Additionally, we carried out an exercise to explore potential deformation when making interspecific comparisons. Like Berbel-Filho et al. (2013), we quantified whether shape changes caused by preservation incorporate a bias in a given study.

MATERIALS AND METHODS

Thirty specimens of the golden mojarra, *Diapterus aureolus*, were collected off the coast of Nayarit, Mexico between December 2009 and March 2010. Fresh samples were photographed with a digital camera placed on a stand. The samples were frozen until October 2010; then, they were set for nine days in 10% formaldehyde. Afterwards, the formaldehyde was rinsed out with tap water and the specimens were passed through an ethanol wash and then submerged in 70% ethanol. The samples were stored until March 2011 and photographed again using the same equipment and procedure. Based on Sagnes (1997) and Al-Hassan et al. (2000), the fixation time was sufficient to record the maximum effect caused by the fixation process. A sample of short-beaked mojarra, *Diapterus brevirostris* (Sauvage, 1879) (n = 43), from the Ichthyological Collection (CI) of CICIMAR-IPN was used as an outgroup. The sample was collected between 1981 and 2002 off the coast of Baja California Sur, Mexico.

The photographs were used to generate the configurations of landmarks and semi-landmarks used in the analysis (Zelditch et al. 2004). The configuration consists of a constellation of 16 marks (12 landmarks and 4 semi-landmarks), representing the overall body shape of each fish (Fig. 1). All marks were obtained using the program TpsDig View 1.4* The standard length was estimated considering the tip of the snout (landmark 1) and the intersection of the lateral line and the caudal fin (landmark 9) using Tmorphgen 6 software**. This distance is calculated by taking as a reference a distance known between two marks given in a rule included in the photograph. The effects of rotation, scale, and position were eliminated by using the Generalized Procrustes method with the morphometrics software CoordGen6**.

The differences between the mean shapes of two data groups (fresh fish and fixed specimens) were calculated by using the Procrustes distance. The significance of the test was based on bootstrapping to determine whether observed F-value could have arisen by chance considering the distribution of bootstrapped F-values obtained from 900 permutations. This was done with the TwoGroup software*. The morphological modifications that are associated with preservation were visualized by comparing the mean shape from each group, using a thin plate spine.

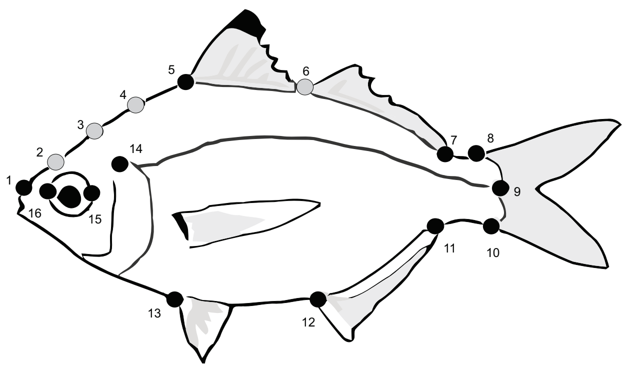

Fig. 1. Graphic representation of the marked configuration used to represent the shape of golden mojarra, *Diapterus aureolus*, for morphometric analysis; Gray dots represent semi-landmarks and black dots landmarks: 1 = tip of the snout, 5 = anterior insertion of the dorsal fin, 7 = posterior insertion of the dorsal fin, 8 = dorsal insertion of the caudal fin, 9 = intersection of lateral line and the caudal fin, 10 = ventral insertion of the caudal fin, 11 = posterior insertion of the anal fin, 12 = anterior insertion of the anal fin, 13 = anterior insertion of the pelvic fin, 14 = dorsal end of the operculum, 15 = posterior margin of the eye, 16 = anterior margin of the eye

* http://life.bio.sunysb.edu/morph

**http://www3.canisius.edu/~sheets/morphsoft.html

Collaterally, canonical variate analysis (CVA) was carried out to detect the degree of intra- and inter-specific variation with *Diapterus brevirostris* as an outgroup. This analysis was performed using the scores obtained from the first ten principal components (91% of variance explained), since we wanted to reduce the number of original variables considering the sample size in the smallest group (n = 30). This analysis was performed using the program CVAGen6m*. A matrix of correct classification, based on Mahalanobis distances, was obtained. Subsequently, these distances were used to generate a dendrogram based on the UPGMA algorithm, as implemented in the program STATISTICA®8.0 (StatSoft). Finally, all organisms belonging to *Diapterus aureolus* (fresh and fixed) were pooled and a new matrix of an inter-specific correct classification based on Mahalanobis distances was obtained from a previous similar statistical analysis.

Ethical issues. The fish species in this study are not listed in any section of the country (Norma Oficial Mexicana-NOM-094) or in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as endangered or subject to special protection. Specimens were treated with concern for reducing potential suffering.

RESULTS

The average length in the fresh samples of *Diapterus aureolus* was 8.7 cm (minimum 7.3 cm and maximum 9.3 cm) and the average length in preserved samples was 8.2 cm (about –5.7%) (minimum 6.9 cm and maximum 8.8 cm). The differences were not statistically significant (t = 1.256; DF = 58, P = 0.21).

Procrustes distance between the two data sets was statistically significant (F = 14.73, DF = 60, 1; P = 0.001), suggesting that the fixation process produces changes in body shape. Variations in the shape were mainly found on the ventral side, possibly as a consequence of the injection of formaldehyde into the abdominal cavity, which enlarged this area. The rest of the body was contracted, mainly in the anterior dorsal area and in the caudal peduncle, which showed a tendency to fold (Fig. 2).

CVA verifies that there were significant differences among three groups (fresh *Diapterus aureolus*, fixed *D. aureolus*, and *D. brevirostris*; Wilk's Lambda = 0.045, P < 0.001 for CV1 and 0.42, P < 0.001 for CV2) (Fig. 3), which were correctly classified (98.9%; Table 1). In spite of the significant differences detected between the fresh and fixed data sets, these were less than those found in the outgroup. CV1 explained 85.9% of the variance among groups and discriminated *D. aureolus* from *D. brevirostris*. The remaining 14.1% of the variance, associated with CV2, mainly explained the effect caused by the preservation procedure in *D. aureolus*, since it discriminated the fresh data set from the fixed data set (Fig. 3). Results from the dendrogram clearly supported a greater relation between data coming from *D. aureolus* specimens (Fig. 3). The pooled data of all specimens belonging to *D. aureolus* (fresh and fixed) produced a definitive correct classification (100%) of *D. aureolus* and *D. brevirostris* (Table 2), indicating that the differences caused by the preservation method are not enough to confuse species and, consequently, taxonomic integrity was totally acceptable.

DISCUSSION

Contractions in body shape (Fig. 2) were concordant with the reduction in body size. Although not statistically significant, the reduction in size was associated with the preservation process because the same sample was used in the analysis. Similarly, other studies have reported a reduction in size caused by preservation by means freezing, formaldehyde, and ethanol (Sagnes 1997, Al-Hassan et al. 2000). The reasons why the fixation process produces these contractions may be associated with of the following:

- Loss of water in tissues from freezing and thawing and exposure to ethanol (Ogle 2009) or
- Dissolution of skeletal structures caused by formaldehyde (Gagliano et al. 2006).
- Of these three stages, the formaldehyde produces the smallest shrinkage (Paradis et al. 2007).

Previous studies indicate that not all species react in the same way when subjected to a fixation technique (Ogle 2009, Martinez et al. 2013). Some authors have proposed inter-specific differences in the proportion of organic molecules, such as lipids and proteins in tissues, as a possible explanation for this effect (Sagnes 1997, Al-Hassan et al. 2000). An explanation consistent with previous findings is the inter-specific amount and proportion of red and white muscle (Martinez et al. 2013). The fact that shrinking was not homogeneous throughout the body may be explained by tissues not being arranged in the same way (Fig. 2).

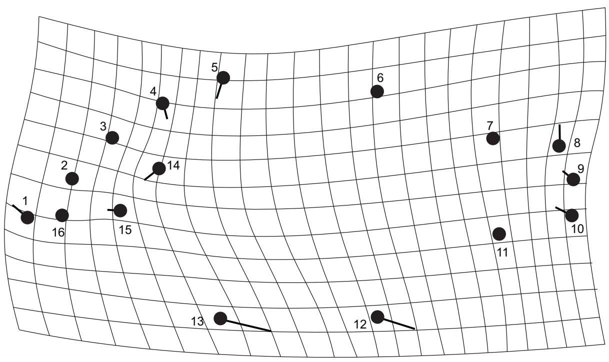

Fig. 2. Thin plate spline showing the body deformation recorded in fresh specimens of golden mojarra, *Diapterus aureolus*, when they passed through the fixation method; Black dots denote the average shape of fresh fishes and vector strength and direction of deformation toward the mean shape of the fixed specimens; Magnification = 3×

* See footnote on page 66.

Our findings indicate that preservation methods in fishes may affect body shape. Extreme caution when using morphometrics with preserved organisms has been suggested, like using bone structures examined by X-rays (Martinez et al. 2013). However, the intensity of this effect with respect to expected variance has not been evaluated.

The preservation effect in *Diapterus aureolus* was not sufficient to produce differences higher than those found at the species level, for example between *D. aureolus* and *D. brevirostris* (Table 2). These results should not necessarily be found when other taxonomic groups are studied, since the effect of formaldehyde can be different (Sagnes 1997, Al Hassan et al. 2000, Martinez et al. 2013). Similar attention should be considered in studies of intra-specific variation (population or stock analysis), since small differences caused by preservation could be confused with the natural small variation expected a priori. This could bias the conclusions. These considerations were also indicated by Berber-Filho et al. (2013) since they found significant changes in the geometric morphometric in individuals of the peacock

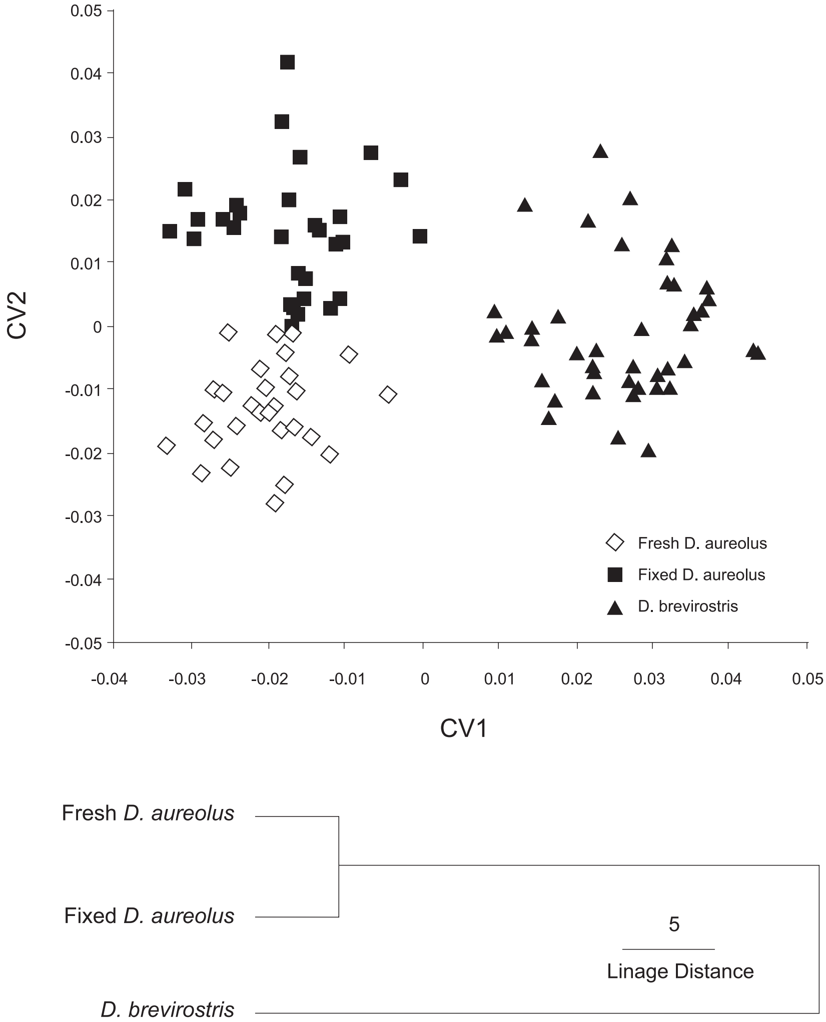

Fig. 3. Graphs generated from the scores of the two canonical variables generated from the CVA (above); Dendrogram constructed with the Mahalanobis distance using the UPGMA algorithm in addition to showing the affinities between the three groups used in the CVA (below)

Table 1

Correct assignment matrix showing the percentage of samples of *Diapterus* spp. correctly assigned to their group of origin

	Fresh DA	Fixed DA	DB
Fresh DA	100	0	0
Fixed DA	3.3	96.7	0
DB	0	0	100

DA = *Diapterus aureolus*, DB = *Diapterus brevirostris*.

Table 2

Correct assignment matrix generated from a CVA pooling fresh and fixed samples of golden mojarra, *Diapterus aureolus*

	Pooled *D. aureolus*	*D. brevirostris*
Pooled *D. aureolus*	100	0
D. brevirostris	0	100

bass, *Cichla kelberi* Kullander et Ferreira 2006, before and after freezing and alcohol preservation.

Considering that preservation methods can have an effect on changes in the shape of the fishes and, consequently, bias results, especially in studies at population level, we suggested using fresh samples as much as possible and simplifying the preservation method, for example by reducing the time devoted to freezing. Unfortunately, fresh fishes cannot always be collected, so the use of fishes found in collections or museums can sometimes be necessary. For this reason, the usefulness of creating an equation for determining the effect of preservation has been advocated (Simon 2013), but as Martinez et al. (2013) emphasize, this is relatively simple when working with weight and length because they are straightforward measures. Using either 2D or 3D morphometry to predict changes produced by preservation in different body parts is much more complicated because knowledge of tissue composition and arrangement in each species is not readily available. An alternative may be to create a correction factor that takes into account phylogeny, as closely related species tend to have similar body shapes. We would expect that the effect of preservation would act similarly in these species. One application of this idea is the similarity of deformation caused by preservation in *Eucinostomus argenteus* Baird et Girard, 1855, another species in the Gerreidae family (Martinez et al. 2013), and deformation in *Diapterus aureolus*, as reported in our study. Both species tended to contract, especially in the anterior dorsal area and the caudal peduncle, which has a tendency to fold. This pattern differs from those observed for *Pomadasys corvinaerformis* (Steindachner, 1868) (see Martinez et al. 2013).

The results of the presently reported study suggest that more experimentation should be carried out in order to support the use of preserved organisms in phylogenetic and population studies applying morphometric geometric analysis.

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