Protocol for quantitative shape analysis of deformities in early larval European seabass *Dicentrarchus labrax*

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This study established an optimized protocol for quantifying the shape variation of newly hatched larvae of European seabass *Dicentrarchus labrax*, focusing on the effect of fixatives and mounting on body shape from hatching until 14 days post hatching, while also minimizing the error introduced by handling. This assessment was performed based on both biometric and geometric shape data, with the latter relying on outline based elliptic Fourier analysis. The fixation and mounting effect on the total length and shape of newly hatched larvae of *D. labrax* was tested for four fixation treatments: (1) 8% formalin, (2) 70% ethanol, (3) 8% formalin for 48 h and then to 70% ethanol and (4) 3% phosphate-buffered glutaraldehyde. The analyses showed that no significant size and shape effect was observed on anaesthetized specimens 5 months post-fixation in glutaraldehyde, and that the glycerol mounting process of specimens fixed in glutaraldehyde provided the best results for further ontogenetic qualitative and quantitative analysis. The protocol proved to be sufficiently sensitive to even quantify and visualize subtle pre-fixation shape differences originating from a different egg batch.

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**INTRODUCTION**

Aquaculture increasingly focuses on improving larval fish quality, but in spite of the continuing progress in rearing methods, morphological abnormalities still pose a significant challenge in Mediterranean finfish aquaculture. Deformities, including reduction of the gill cover, pre-haemal lordosis and kyphosis, pugheadness, jaw deformities and swimbladder abnormalities, often appear in early stages of larval development (Koumoundouros, 2010) and affect the phenotype of the fish, their survival, growth rate, behaviour and finally the production cost and effectiveness of the hatcheries (Sfakianakis *et al.*, 2006). They can lead to fish that are not easily marketable, or even to mortalities at the larval stage or later. The first malformations in European seabass *Dicentrarchus labrax* (L. 1758), such as a twisted spinal cord

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and jaw abnormalities, can appear immediately after hatching (Barahona-Fernandes, 1982), and if they affect 10% or more of the newly hatched population, the discard of the entire batch is recommended (Moretti et al., 1999).

In hatcheries, detection of the early manifestations of abnormalities is crucial, as these can be signals of more pronounced deformities in later stages. Early embryonic and larval stages, however, generally pose few characteristics showing distinguishable variation, and therefore a powerful tool is required for its detection and quantification. Identification of deformities has mainly been done through the use of traditional biometric analysis, radiography and whole mount staining, most often based on estimations of erratic skeletal development by categorizing specimens as being normal, mildly distorted or severely distorted (e.g. based on the number of deformed or fused vertebrae). The limited discriminative power of these methods, however, becomes clear when dealing with early life stages of fishes that lack mineralized elements. Furthermore, traditional biometric analysis is not well suited in describing subtle variation in shape (Rohlf & Marcus, 1993). Methods of traditional morphometrics in fishes rely on measurements that are taken along the longest axis of the body, and thus fail to capture other aspects of shape (Bookstein et al., 1985).

In this study, metric data are complemented with shape data obtained from geometric morphometrics, which is a more accurate and objective method to evaluate shape variation (Adams et al., 2004).

Two data types are generally used to describe shape in geometric morphometrics: landmarks and outlines. The former involves the collection of two or three-dimensional co-ordinates of biologically definable landmarks, preferably anatomically homologous points (Smith, 1990), whereas the latter involves digitized points along an outline. Both approaches then involve a standardization to remove all non-shape variation, yielding a set of shape variables that allow a statistical testing of hypotheses, as well as a graphical representation of their variation. Studies on deformities of cultivated fishes have mainly focused on the use of the landmark method but in very early larval stages it cannot be applied safely due to the absence of discrete and homologous features to which landmarks can be assigned. Another disadvantage of the landmark method is the loss of useful information on shape variation in regions between landmarks (O’Higgins, 1997).

A procedure that overcomes these problems is the outline-based method, with elliptic Fourier descriptors being the most commonly used shape descriptors. It involves the decomposition of a shape contour into a sum of ellipses (Kuhl & Giardina, 1982), and can delineate any type of shape with a closed contour without the need for homologous points (Lestrel, 1997). This is particularly useful in very early larval stages similar to the ones of this study, where their position changes rapidly or they might be too few or even absent. Comparisons of the landmark and outline methods highlight the cases where the study of shape variability from the whole outline is advantageous (Loy et al., 2000; Russo et al., 2007; Marquez et al., 2010).

So far, the outline method has been uncommon in aquaculture research, with rare applications in adult specimens of *D. labrax* (Costa et al., 2013). Considering the rounded form of larval fishes, having their finfolds undifferentiated until the onset of the juvenile period (Balon, 1975), this approach can be sufficiently powerful to detect the early onset of deformities in cultured fishes, even before the first signs of skeletal differentiations have occurred. To achieve this, the first goal of this study was to establish a protocol for performing a detailed shape analysis on larval fish.
using outline-based geometric morphometrics, from the image acquisition up to the actual shape analysis.

The successful use of a detailed analysis of shape in any ichthyological study dealing with morphological changes or differences depends on the optimal reduction of possible artefacts caused in the preparatory steps: fixation and mounting. Fixation in shape analyses is crucial due to their labour-intensive nature which prevents them from being applied on live fishes. Consequently, the second goal of this study was to provide an optimal protocol for fixing the specimens, and mounting them for purposes of improved preservation and handling. The effects of different fixatives and preservatives on fishes are difficult to predict due to the involvement of several factors (Tucker & Chester, 1984; Sagnes, 1997), but they have been known to be able to influence the geometric morphometric analysis of fishes (Martinez et al., 2013). Shrinkage is usually observed, as are changes in total length (LT), mass (M), external appearance of the larval samples and axial curvatures (Fowler & Smith, 1983; Oozeki & Hirano, 1988; Jennings, 1991), and has important implications for studies on subtle shape changes in larval fishes. Another factor that should be taken into account is handling: there is an inherent variability in the distribution of fish larvae measurements due to individual investigator handling (Pepin et al., 1998), and the method involved causes shrinkage differences that could be interpreted as morphological differences (Theilacker, 1980). Shape changes might therefore be introduced, especially due to the fragility of the specimens. Furthermore, anaesthetization is a factor that should be examined as well, as it has been suggested by Parker (1963) and Theilacker (1980) that it contributes to the shrinkage of live larvae because MS-222 interferes with osmoregulation.

As far as is known, a detailed morphometric analysis on the effect of handling, anaesthetization, fixation and mounting on the shape of fish larvae is still lacking. This study tests the length and shape effects of four common fixation treatments (using formalin, ethanol, formalin followed by ethanol and glutaraldehyde), and tests a mounting technique on the specimens, analysing larvae of D. labrax from hatching until 14 days post hatching (dph). These procedures are tested and critically evaluated, and a protocol is suggested, yielding the lowest amount of artificially induced deformations. With this, it is hoped to provide a tool for future morphometric analyses during the early larval stages by establishing a protocol that can allow an accurate mathematical quantification of shape variation, while taking into account the detrimental shape effects of the aforementioned procedures, an issue which is frequently overlooked in morphometric studies.

**MATERIALS AND METHODS**

**FIXATION TREATMENTS, PROCEDURE AND SAMPLE SELECTION CRITERIA**

The shrinkage effect of four different fixation techniques was tested in two experiments where fertilized eggs of D. labrax obtained from the Ecloserie Marine de Gravelines hatchery in France were kept at a constant temperature of 16° C, range ±1° C and continuous blue light with an intensity of 50lx. The eggs were acclimatized immediately after arrival for 4h in UV-treated natural sea water, with a salinity of 36 and continuous aeration.

The larvae were stocked in 40 ml screw cap vials with filtered autoclaved sea water, at a density of 12 larvae per vial. They were reared until 14 dph, and fed with 35 axenic Artemia
Table I. Codes used for the experimental units according to fixation of *Dicentrarchus labrax* larvae, dph (days post hatching), time of fixation (5 months for all groups, 48 h in formalin for specimens of treatment FE before their transfer to ethanol for 5 months) and application of anaesthetic (all groups except G-na).

<table>
<thead>
<tr>
<th>Group code</th>
<th>Fixative</th>
<th>Anaesthetization</th>
<th>2 dph</th>
<th>7 dph</th>
<th>14 dph</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>8% Formalin</td>
<td>Yes</td>
<td>F2</td>
<td>F7</td>
<td>F14</td>
</tr>
<tr>
<td>E</td>
<td>70% Ethanol</td>
<td>Yes</td>
<td>E2</td>
<td>E7</td>
<td>E14</td>
</tr>
<tr>
<td>FE</td>
<td>8% Formalin for 48 h</td>
<td>Yes</td>
<td>FE2-48 h</td>
<td>FE7-48 h</td>
<td>FE14-48 h</td>
</tr>
<tr>
<td></td>
<td>70% Ethanol</td>
<td></td>
<td>FE2-48 h</td>
<td>FE7-48 h</td>
<td>FE14-48 h</td>
</tr>
<tr>
<td>G</td>
<td>3% Phosphate-buffered glutaraldehyde</td>
<td>No</td>
<td>G2</td>
<td>G7</td>
<td>G14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G2.na</td>
<td>G7.na</td>
<td>G14.na</td>
</tr>
</tbody>
</table>

*A. franciscana* per vial once every 2 days from 7 dph onwards. Axenic *A. franciscana* were prepared according to the method of Marques et al. (2004). The larvae of 7 and 14 dph were not fed on their sampling day. The remaining uneaten *A. franciscana* were not removed from the vials, nor was there any water exchange during the whole culture period. This method was chosen as a general protocol that can also be used in future studies of axenic eggs and larvae of *D. labrax*, in accordance with the procedure described by Dierckens et al. (2009). It was also used to permit sampling until 14 dph, which might have been hindered by mortalities if normal xenic water and xenic *A. franciscana* were used.

In the first experiment, the larvae were divided into three groups: the larvae of the formalin group (group F) were fixed in 8% unbuffered formalin in fresh water (pH 3.7), those of the ethanol group (group E) in 70% ethanol (pH 7.6) and those of group FE in 8% formalin for 48 h and then transferred to 70% ethanol. In the second experiment, larvae of the glutaraldehyde group (group G) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), prepared according to Glauert (1987), frozen immediately after preparation and kept at −20°C until the time of application. The fixation time was 5 months. For clarity, treatment groups are coded according to the fixative applied (F, E, FE and G) and 2, 7 and 14 dph. In the case of treatment FE, specimens in formalin before their transfer to ethanol were coded with “48 h”.

In the case of treatment G, three groups were fixed without prior anaesthetization (code “na”) to test the effect of anaesthetic on shape. Details of the groups are given in Table I.

In both experiments, at 2, 7 and 14 dph, a minimum of 30 specimens per fixative group were collected from their vials, anaesthetized with MS-222 and photographed with an Olympus Altra 20 digital camera mounted on an Olympus SZX9 microscope via AnalySIS GetIT software (www.olympus.com). Larvae were placed horizontally on glass slides facing to the right in a 100 μl seawater droplet, with their left and right palatoquadrate cartilages vertically aligned (Fig. 1). After taking their photograph, each larva was placed in an individually marked Eppendorf vial filled with 1.2 ml of fixative at room temperature (20°C), and photographed again after 5 months, with the same volume of 100 μl of fixative on top of each specimen. Group FE larvae were also photographed 48 h after fixation, immediately before their transfer to ethanol. The use of individually marked Eppendorf vials allowed evaluation of length and shape changes of each individual specimen in each step of the process.

For anaesthesia, 1.8–4.2 ml of an MS-222 solution of 1.2 g l⁻¹ was added to each stock vial, resulting in a slow stopping of activity within 10 min, after which heartbeat was observed to stop. Sudden overdoses were prevented, as preliminary tests showed that they may react violently to it, thus introducing deformation artefacts not related to the effect of the fixatives. Furthermore, in these preliminary tests, larvae had visibly unaffected body shapes when not anaesthetized prior to fixation. Therefore, the effect of the anaesthetic needed to be included in the analysis as a factor potentially influencing larval body shape. As such, in the second experiment, an extra group of a minimum of 35 larvae was fixed in phosphate-buffered glutaraldehyde at 2, 7 and 14 dph without prior anaesthetization. For these larvae, individual photographs were taken only post-fixation, but were sampled from
the same vials of the glutaraldehyde treatment, thus allowing a comparison between these two groups.

As specimens were fragile, a standardized procedure was established for the selection of larvae for further shape analyses. In order to maximize their explanatory power for studying deformities induced directly by the fixative, the following specimens were excluded: the ones that were not sedated properly, that were not completely submerged in the fixative and those that were asphyxiating or developing visible signs of stress and tissue contraction. Due to this, some specimens were severely bent after the end of the fixation period, which did not allow their positioning in a completely flat position, and hence the reliable acquisition of size and shape data. Based on these selection criteria, they were excluded from the analysis, as this protocol aims to examine subtle shape variations. The per cent per group are shown in Fig. 2. There was a highly significant effect of the fixative on the numbers of discarded specimens [analysis of variance (ANOVA), $F_{3,8} = 26.99$, $P < 0.001$]. The Tukey’s post hoc pair-wise comparisons indicated that they did not differ significantly between treatments F and FE, but treatments G were significantly higher than those of F and FE ($P < 0.01$ in both cases), and the non-anaesthetized specimens of treatments G were significantly fewer than those for all the other groups ($P < 0.001$ against G, $P < 0.05$ against F and FE). Group E larvae clearly showed a high degree of body and finfolding and bending, and were not included in further analyses.

**OPTICAL DISTORTION TESTING**

To avoid larvae becoming dehydrated, they were submerged in water while taking photographs. The $100\mu l$ drop spread around each specimen was the minimum in order to avoid optical distortions as a result of the curved surface of the water droplet. Optical aberrations

![Rejected specimens diagram](image-url)

**Fig. 2.** ( %) *Dicentrarchus labrax* larvae that did not meet the analysis selection criteria (☐, treatment F; □, treatment FE; ■, treatment G; ☐, G.na; see Table I). The numbers of discarded specimens out of all sampled larvae are included on top of each bar.
using the stereo microscope were avoided by using lenses corrected for spherical aberration, and a ×0.5 planar lens was chosen over ×1 and ×1.5 lenses due to its deeper focal range. The possibility of this droplet inducing an optical distortion was tested using photographs of a calibration glass slide with a 2 mm cross under the ×0.5 lens with magnifications of ×20, ×25 and ×28.5 (as used during the experiment). The cross was placed at five different positions within the photograph frame: bottom left, bottom right, centre, upper left and upper right. The photographs were taken with and without 100 μl of seawater, and the length of the horizontal and vertical lines of the cross was measured in each photograph. There was no significant effect of the application of the water droplet on the lengths of the cross lines in any magnification (for ×20: paired t-test, \( t_{18} = -0.13, P > 0.05, \text{s.d.} = 0.011 \text{ mm} \); for ×25: paired t-test, \( t_{18} = 0.2, P > 0.05, \text{s.d.} = 0.006 \text{ mm} \); for ×28.5: paired t-test, \( t_{18} = -1.38, P > 0.05, \text{s.d.} = 0.008 \text{ mm} \)), and therefore no optical distortion effect was induced. As such, all further analyses were based on data obtained with the application of the droplet.

**STAINING AND MOUNTING**

In order to establish a complete deformation study protocol that includes long-term storage of the larvae in a compact form for further qualitative and quantitative analyses, a number of different staining and mounting techniques were applied in order to determine whether they induce artefacts. Mounting media can be classified into two categories: organic and aqueous (Renshaw, 2007). For this study, an aqueous mounting medium (glycerol) and three organic [DPX by Sigma Aldrich (www.sigmaaldrich.com), toluene-based mounting medium by Richard Allan Scientific (www.thermoscientific.com) and transparent nail polish were used. To avoid fungal contamination, a few crystals of thymol were added to the glycerol solution.

Specimens to be mounted in aqueous media, such as glycerol, can be mounted straight from the aqueous phase with no dehydration or clearing (Renshaw, 2007). In the case of organic media, however, the specimens were placed for 10 s in 80% ethanol, and twice quickly in 96% ethanol prior to staining and mounting, following the method of Gurr (1962). This prevented the presence of a whitish colour that reduced the clarity of the specimens.

After their 5 month fixation, the specimens were washed with distilled water, and stained with toluidine blue and haematoxylin–eosin to increase the contrast between the larvae and the background, in order to improve the outline tracing for the shape analyses. The specimens were then mounted by being placed on a glass slide inside a pool of mounting medium within a paraffin ring, and sealed with a cover glass on top. For this, a modified version of the nematode mounting method of Maeneener and d’Herde (Hooper, 1986) was followed. One of the factors that was clearly introducing deformations in this method was the placement of the slide with the mounted larvae on top of a heated metal strip at 65°C for a few minutes, in order to melt the paraffin ring for attaching the cover glass. These distortion effects were also present when the temperature of the metal strip was raised gradually over a few minutes. Consequently, the use of the heated metal strip was omitted. Furthermore, the immediate addition of pure glycerol produced specimen distortion, and a series of consecutive glycerol baths was applied instead.

The modified procedure was then as follows. A 5.7 cm × 1.7 cm orthogonal metal frame was dipped into liquid paraffin kept at 68°C, and used to apply a wax ring on a glass slide. The paraffin was allowed to cool and dry for a few minutes. In this way, four consecutive wax rings were applied on top of each other in order to increase the height of the wax formation and hence to prevent the squashing of the larvae under the cover glass. The next steps were performed quickly in order to avoid any dehydration of the specimens caused by their prolonged exposure to air. A total of 3 ml of distilled water was added on top of each specimen in order to remove traces of fixative and excess water was removed by drying it very gently with the tip of an absorbent paper towel. This was repeated three times. Staining was then performed with a drop of toluidine blue or haematoxylin–eosin on top of each specimen (both staining methods gave equally good results).

In the case of glycerol, two different methods were applied: (1) specimens were placed in a series of four 30 min baths (distilled water, 25% glycerol, 50% glycerol and 75% glycerol). A maximum of three specimens per slide were picked up with a 400 μl micropipette, and placed parallel on the prepared slide. In order to avoid distorting the larvae, pipette tips with
a cut edge were used. Finally, a few drops of pure glycerol were added on top. (2) a few drops of pure glycerol were applied on top of a very thin dried layer of transparent nail polish over each of the three specimens. In the cases of DPX and Richard Allan Scientific mounting medium, these were applied directly on the three specimens without any intermediate baths. Finally, a fresh wax ring was applied to all preparations, and a cover glass was very gently placed on top of it. After a few minutes, the paraffin solidified and enclosed the specimens in a pool of the mounting medium. Finally, nail polish was applied around the wax ring to seal the slide.

SIZE ANALYSES

The $L_T$ of each larva was measured using ImageJ v1.46 (Abramoff et al., 2004), with an accuracy of 0.001 mm. The shrinkage ratio was calculated as $L_T$ at pre-fixation minus that at post-fixation, divided by pre-fixation $L_T$. Differences in $L_T$ and shrinkage ratios between the different treatments were examined using PAST 2.17b (Hammer et al., 2001). The normality of the distribution of the $L_T$ and shrinkage ratios of the larvae was tested with a Shapiro–Wilk’s test ($P_{\text{crit}} = 0.05$) and the homogeneity of group variances with Levene’s test ($P_{\text{crit}} = 0.05$). In the cases where the conditions of normality and homogeneity of variances were not met, the non-parametric Kruskal–Wallis test was performed, with Bonferroni-corrected $P$-levels for post hoc Mann–Whitney pair-wise comparisons. When the conditions were met, the one-way ANOVA test was applied to determine whether there were significant differences, followed by a Tukey’s post hoc test for pair-wise comparisons.

As the larvae of treatment G came from a different egg batch than those of F and FE, a test was performed for difference in pre-fixation larval $L_T$ at the onset of the treatments. There was a highly significant effect of the egg batch origin of the specimen groups on $L_T$ at all three ages (for 2 dph: ANOVA, $F_{2,75} = 23.87$, $P < 0.001$; for 7 dph: Kruskal–Wallis, $H_2 = 42.73$, $P < 0.001$; for 14 dph: Kruskal–Wallis, $H_2 = 20.17$, $P < 0.001$). Post hoc pair-wise comparisons indicated that specimen $L_T$ at all three ages was not significantly different between groups F and FE, but was highly significantly different between the G and both the F and FE groups ($P < 0.001$ in all cases). Consequently, the pre-fixation $L_T$ had to be taken into account in the next steps of the analysis, and for this purpose the individual shrinkage ratios of each specimen were used.

GEOMETRIC MORPHOMETRICS ANALYSIS OF SHAPE

To perform an outline-based morphometric analysis, the outlines of the larvae were traced manually on the images using Corel Draw 12 (Corel Corp.; www.corel.com). Specimens with widely opened mouths were traced according to the best approximation of their shape corresponding to their mouths closed, relying on the orientation of the upper jaw and their palatoquadrates. Outlines of larval profiles were traced in black on a white background and analysed using Shape 1.3 (Iwata & Ukai, 2002).

A total of 20 harmonics was used to describe the observed shape, with a normalization based on the first harmonic, involving a standardization for position, size and orientation of the specimens. According to Iwata & Ukai (2002), the use of 20 harmonics produces good results in most shapes, and is hence suitable to describe the simple shape of the larvae of $D.\ \text{labrax}$.

To reduce the number of variables, a principal component (PC) analysis (PCA) was performed on the elliptic Fourier coefficients using Shape. All further statistical analyses were performed using PAST 2.17b (Hammer et al., 2001). The analysis of between-group variation was done through a canonical variate analysis (CVA) and a multivariate analysis of variance (MANOVA) on the scores of all effective PCs, which Shape defines as the PCs with percentages of explained total variation $> 1/77$, with 77 being the total number of analysed coefficients. This allowed a reduction in the dimensionality of the dataset from 77 components to between six and nine, depending on the groups being pooled. Consequently, without losing relevant information, between 93.0 and 94.5% of the total variation was included in the datasets for further analyses.
Regarding the assumptions for MANOVA, multivariate normality was checked with Mardia’s multivariate skewness and kurtosis test, and the equivalence of covariance matrices with Box’s $M$ test. In the cases that were not met, a one-way non-parametric multivariate analysis of variance (NPMANOVA) was performed with 10,000 permutations. When significant differences were detected, a post hoc test was applied. In the case of MANOVA, Bonferroni-corrected Hotelling’s $T$-values were used. In the case of NPMANOVA, the Bonferroni-corrected $T$-values were calculated by permutation tests on distances, with these being the point-to-point Mahalanobis distances between pairs of individual multivariate observations (Anderson, 2001).

To test the significance of shape changes before and after fixation, and to determine whether each fixative has a different effect on shape, MANOVA or NPMANOVA tests were performed on the relevant PC scores of each separate age group, with prior anaesthetization. Similarly, in order to examine whether the fixatives induce different shape changes depending on the age of the specimens, the same tests were performed per fixative treatment. This included all 5 month fixation groups, and also the groups of 48 h fixation in the case of treatment FE, and the groups that were not anaesthetized in the case of treatment G. When significant differences were detected, the Mahalanobis distances between the centroids of the corresponding groups were calculated to examine which groups exhibited the largest differences.

In order to provide a percentage of specimens that were affected by the fixation, a PCA was performed on the Fourier coefficients of each fixation group per age class, followed by a CVA linear classification. The post-fixation specimens classified to belong in the pre-fixation group were identified as not affected by fixation. In the cases where some specimens of the pre-fixation group were classified as post-fixation, they were discounted, as they cannot be considered to represent an effect of the treatment.

Due to the fact that larvae for the glutaraldehyde treatment came from a different egg batch, a test similar to the one performed for the specimen $LT$ had to be performed in order to check whether the groups before fixation had significant differences in shape. There was a highly significant effect of the egg batch origin of the specimen groups on shape at all three ages (for 2 dph: NPMANOVA, $F_{2,76} = 5.68$, $P < 0.001$; for 7 dph: NPMANOVA, $F_{2,76} = 4.5$, $P < 0.001$; for 14 dph: NPMANOVA, $F_{2,83} = 3.69$, $P < 0.001$). Post hoc pairwise comparisons indicated that there were no significant differences between the F and FE groups at 2 dph and 7 dph ($P > 0.05$), but at both ages there were highly significant differences between G and both F and FE ($P < 0.001$ at both ages). On 14 dph, there were significant differences between all groups ($P < 0.05$ between F and FE and also between F and G, $P < 0.001$ between FE and G). Therefore, due to the fact that there were significant pre-fixation shape differences between the specimen groups, they had to be taken into account in the next steps of the analysis. They are visualized in Fig. 3 with a scatterplot of CVA on the PC shape scores of all age groups. At 2 dph, these differences are evident as the clusters of treatments F and FE clearly occupy a different morphospace than the cluster of G.

### RESULTS

**SIZE EFFECTS**

For treatment G, the pair-wise comparisons indicate that there was no significant shrinkage effect apart from the non-anaesthetized specimens of 2 dph (Kruskal–Wallis: $H_8 = 159.9$, $P < 0.001$; for 2 dph: $P_{na} < 0.05$). For treatments F (formalin) and FE (formalin to ethanol), according to the pair-wise comparisons in every age class a highly significant shrinkage effect was observed, including treatment FE at 48 h (treatment F: Kruskal–Wallis: $H_5 = 144.8$, $P < 0.001$; treatment FE: Kruskal–Wallis: $H_5 = 226.8$, $P < 0.001$). As far as the shrinkage ratios are concerned, a highly significant effect of the fixative was observed (Kruskal–Wallis: $H_8 = 208.5$, $P < 0.001$), and according to the pair-wise comparisons the ones of treatment F were always highly significantly lower ($P < 0.001$ in all cases) than
Fig. 3. Canonical variate analysis (CVA) scatterplot and group clusters of days post hatching (dph) (a) 2, (b) 7 and (c) 14 dph groups before fixation. The *Dicentrarchus labrax* larval shapes correspond to specimens that lie close to the group means, as indicated by the connecting line, with the label of each individual specimen also noted [― formalin group (F); ••••••• formalin and ethanol group (FE); — glutaraldehyde group (G); see Table I].

Fig. 4. Mean ± s.d. shrinkage ratios of all anaesthetized specimen groups of *Dicentrarchus labrax* on 2, 7 and 14 days post hatching (dph) after a 5 month fixation in formalin (group F), in formalin for 48 h followed by ethanol (group FE) and in glutaraldehyde (group G). The shrinkage ratios of the FE specimens after their initial 48 h fixation in ethanol are also provided. Mean 5 month shrinkages with the same lowercase letter are not significantly different (*P* > 0.05). The shrinkages of the glutaraldehyde groups were not significant.

the ones of treatment FE of the corresponding dph (Fig. 4). No pattern could be detected for age-dependent shrinkage in any fixative treatment.

Regarding the two fixation stages of treatment FE, there was a highly significant effect on shrinkage (Kruskal–Wallis: *H*$_{5}$ = 132.4, *P* < 0.001). The pair-wise comparisons indicated that in every age class the 5 month storage clearly induced a larger shrinkage than the initial 48 h fixation (*P* < 0.001 in all cases). This means that in addition to the initial shrinkage induced by the formalin during the first 48 h, a significantly additional shrinkage occurs during the 5 month storage in ethanol, with shrinkage levels almost doubling.

The comparison of the shrinkage ratios of group FE’s 48 h formalin fixation with group F’s 5 month formalin fixation shows that there was a highly significant effect of the duration of formalin fixation on shrinkage (Kruskal–Wallis: *H*$_{5}$ = 59.12, *P* < 0.001). According to the pair-wise comparisons, the shrinkages after 5 months were significantly higher than after 48 h, both in 7 and 14 dph (*P* < 0.001), but not in 2 dph (*P* > 0.05). In all three cases, however, the major formalin shrinkage had already occurred at 48 h: 6.6% after 48 h v. 7.09% after 5 months in the case of 2 dph, 5.89% v. 8.25% at 7 dph and 5.62% v. 8.3% at 14 dph.

SHAPE EFFECTS

There was a highly significant effect of the fixative on shape at all three ages (for 2 dph: NPMANOVA: *F*$_{5,153}$ = 7.54, *P* < 0.001; for 7 dph: NPMANOVA: *F*$_{5,153}$ = 6.98, *P* < 0.001; for 14 dph: NPMANOVA: *F*$_{5,153}$ = 5.2, *P* < 0.001). Post hoc pair-wise comparisons indicate that glutaraldehyde did not induce significant shape differences after the 5 month fixation of anaesthetized specimens in every age class, unlike the formalin and formalin to ethanol groups where shape changes were highly significant (*P* < 0.01 in every case). Additionally, in every age class, glutaraldehyde presented the lowest squared Mahalanobis distances between the corresponding groups before and after fixation, indicating that it exhibited the
smallest shape difference (squared Mahalanobis distances of G2: 1·53, F2: 5·29, FE2: 7·23; G7: 2·15, F7: 3·42, FE7: 11·83; G14: 1·15, FE14: 2·95, F14: 3·44). Glutaraldehyde also gave the lowest percentage of specimens classified as affected by fixation in all age classes (G2: 66·67%, FE2: 84%, F2: 88·46%; G7: 64·71%, F7: 85·71%, FE7: 96%; G14: 61·11%, F14: 76·19%, FE14: 86·67%).

On 2 dph, PC1 explained 38·55% of the total shape variation and PC2 26%, on 7 dph PC1 61·12% and PC2 13·84% and on 14 dph PC1 explained 65·36% and PC2 12·92% of the total shape variance. A pattern of an increase in the post-fixation variation represented by PC1 is evident in the fixed specimens of all treatments (Fig. 5), but to a lesser degree in the formalin to ethanol treatment (FE) of 7 and 14 dph. As far as changes in PC2 scores are concerned, there is no indication of an increase in post-fixation variation. Deformations are mostly present at the head and tail with positions that deviate from the horizontal axis of the body, as well as at the mid-body region. Furthermore, based on the squared Mahalanobis distances of the groups before and after fixation, there was no overall pattern of age-dependent shape changes in any treatment.

There was a highly significant effect of formalin fixation time on shape (NPMANOVA: $F_{8,254} = 11·78$, $P < 0·001$). According to the post hoc pair-wise comparisons, on 2 and 14 dph, the shape changes after 48 h were not significant. This suggests that the first fixation stage of 48 h in formalin does not induce a significant shape effect to the specimens. This was not the case, however, for 7 dph ($P < 0·01$), unlike glutaraldehyde that exhibited the best results in every age class.

There was a highly significant effect of anaesthesia on shape (NPMANOVA: $F_{8,231} = 9·95$, $P < 0·001$). The post hoc pair-wise comparisons indicated that the non-anaesthetized specimens fixed in glutaraldehyde on 2 and 7 dph presented highly significant shape differences ($P < 0·01$ in both cases). This suggests that omitting anaesthetization is detrimental. Such degree of shape change was not present in any group of properly anaesthetized specimens of the glutaraldehyde treatment.

In qualitative terms, glutaraldehyde also gave the best results regarding specimen condition; it did not induce visible tissue degrading or heavy folding at the margin of the finfolds. This was not the case for the ethanol fixation where these effects were frequently visible, and to a lesser degree in treatments F and FE. Fixed specimens of treatment FE had slightly better clarity than those of treatment F, which were more opaque. When the formalin fixation time was shorter, these effects were less evident; after 48 h in formalin and right before their transfer to ethanol, the majority of the specimens were in good shape with no apparent finfolding or tissue degradation.

MOUNTING

Deformation artefacts were induced in specimens from all treatment groups after the application of all mounting media. The mounted larvae remained fit for qualitative analysis, but unsuitable for further quantitative size and shape analyses. Dehydration of the fins and the head region, body folding, body damage and bending were sometimes visible (Fig. 6). This was not always the case, as demonstrated in Fig. 7. The use of DPX on specimens fixed in phosphate-buffered glutaraldehyde, and especially glycerol used according to procedure (1) (above), yielded very good results. These were not, however, consistent and a few specimens still exhibited small deformities or finfolding.
Fig. 5. Principal component 1 (PC1) and PC2 score ranges with means ± s.d. for Dicentrarchus labrax larval groups of (a) 2, (b) 7 and (c) 14 days post hatching (dph) before and after their 5 month fixation in formalin (F), formalin for 48 h followed by ethanol (FE) and glutaraldehyde (G) (■, PC1; □, PC2). The superimposed PC contours include the outlines of the mean and ±2 s.d. larvae shapes of each age group, reflecting the individual deformation effects of each group per day.
Before staining  |  After staining  |  End of mounting procedure
---|---|---
(a) | | |
(b) | | |
(c) | | |
(d) | | |

**Fig. 6.** Examples of the different mounting and staining methods applied. (a) FE2 *Dicentrachus labrax* larva stained with toluidine blue and mounted with DPX, (b) F14 larva stained with haematoxylin–eosin and mounted with glycerol over dried nail polish, (c) F2 larva stained with toluidine blue and mounted with toluene-based mounting medium by Richard Allan Scientific and (d) F2 larva treated in exactly the same way with (c), but mounted successfully with minimal changes in its condition (see Table I).

**DISCUSSION**

As shown by the results of ANOVA, NPMANOVA and the squared Mahalanobis distances, the 5 month glutaraldehyde treatment of anaesthetized specimens performed best for every age class, without inducing any significant size or shape changes. This treatment was followed in effectiveness by formalin and formalin for 48 h to ethanol, except for 14 dph where the latter induced smaller shape changes than

<table>
<thead>
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<th>Glycerol</th>
<th>DPX</th>
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<td>Sedated</td>
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<td>After fixation</td>
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<td>End of mounting</td>
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<td>13 Days later</td>
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**Fig. 7.** Two examples of *Dicentrachus labrax* larvae fixed in phosphate-buffered glutaraldehyde (group G2; see Table I) and mounted with glycerol and DPX. Neither specimens was stained.
the former. As opposed to glutaraldehyde, both of these treatments also demonstrated significant specimen shrinkage. One possible cause of this effect is the differences in osmolarity between the specimens and these two fixatives, which causes larvae to lose fluids passively through gills and other surfaces and shrink (Theilacker, 1980; Jennings, 1991). The formalin used in this study had an osmolarity of 1.0 Osm l\(^{-1}\), and ethanol 1.52 Osm l\(^{-1}\), which are high compared to blood osmolarity values between 0.305 and 0.376 Osm l\(^{-1}\) reported by authors for larger specimens of *D. labrax* of 24–100 g (Marino et al., 2001), and osmolalities between 0.4 and 0.55 Osm kg\(^{-1}\) for larvae between 0 and 27 dph at a salinity of 39.5 (Varsamos et al., 2001), which was close to 36 of the seawater used in this study. These values are similar, however, to the osmolarity of the phosphate-buffered glutaraldehyde fixative of treatment G (0.32 Osm l\(^{-1}\)), which may well explain its better performance.

Glutaraldehyde is a common fixative used for electron microscopy. It is usually more suitable than formalin due to the fact that it gives lower osmolarities while providing more available reactive groups for fixation (Fox et al., 1985). It has twice the potential of formalin to cross-link proteins, and acts more rapidly (Renshaw, 2007). Among aldehydes, it also presents the best preservation of tissue ultrastructure (Carson & Hladik, 2009). It did not induce significant size and shape changes after 5 months, which should be adequate for the time-consuming morphological analyses. Similarly to the findings of this study, it performed the best in terms of specimen preservation and shrinkage of yolk-sac larvae of the common snook *Centropomus undecimalis* (Bloch 1792), without any significant changes in mean notochord length after 3 months (De Leon et al., 1991). Oozeki & Hirano (1988) also reported that glutaraldehyde did not produce a significant difference on the \(L_T\) of larvae of the red seabream *Pagrus major* (Temminck & Schlegel 1843) at 3, 12, 18 and 25 dph over a 6 month fixation period, with the fixed specimens presenting a more natural appearance. This again concurs with the observations of this study: glutaraldehyde retained very well the clarity of the transparent bodies of the larvae, which was not the case for the other fixatives.

Standalone ethanol performed the worst, which might be explained by the fact that it does not form cross-links between proteins in tissues and has a poor tissue penetration (Renshaw, 2007). In some cases of long-term storage, ethanol may cause excessive tissue shrinkage due to dehydration effects (Carson & Hladik, 2009). This was also noticeable in the specimens of this study that were fixed for 5 months in ethanol, and also to a lesser degree where a fixation of 48 h in formalin was followed by a 5 month fixation in ethanol. In marine species such as the Northern anchovy *Engraulis mordax* Girard 1854, however, immediate fixation of larvae in ethanol has been shown to minimize the shrinkage effect (Methot & Kramer, 1979). Ethanol solutions of 70% (as used in this study) or 95% are sometimes chosen as preservatives in bone development studies in fishes ranging from the larval to the adult stage, due to the fact that they do not decalcify these structures. They also present the advantage of not restricting the use of tissues in subsequent genetic analysis, as formalin does (Giannella et al., 1997). Unfortunately, due to the inability to stabilize protein constituents, they frequently degrade fish larvae tissues (Gagliano et al., 2006).

Formalin induced significant shrinkage in larvae over both 48 h and 5 months. Oozeki & Hirano (1988) report that formalin fixation of fish larvae usually results in acute tissue shrinkage. The formalin solution used in this study was unbuffered,
instead of the common use of 4–10% formalin buffered to pH 7 with 0·1 M phosphate buffer in fish larvae research. Normally, formalin and glutaraldehyde demineralize skeletal structures, which can be avoided by buffering the solution to slow down the decalcification process (Hay, 1981). Buffer salts, however, increase the osmolality of the fixative solution, with the danger of raising it to extreme values (Fox et al., 1985). Long-term storage in a buffered acid fixative can also be problematic, as buffers may damage larvae or ultimately allow the solution to become acidic (Lavenberg et al., 1984; Leis & McGrouther, 1994). Furthermore, buffered formalin as a preservative destroys the stain uptake in cartilage, thus making it impossible to be stained (Pothoff, 1984). It is for these reasons that unbuffered formalin was used. This solution also becomes acidic (Carson & Hladik, 2009), as reflected in its pH which had a value of 3·7, but can produce larvae shrinkage not significantly different than buffered (Hay, 1982) or even less (Tucker & Chester, 1984). Another way to deal with the problems presented by buffered formalin is to transfer fixed specimens for long-term preservation in 70–95% ethanol, as tested in this study (treatment FE). The usage of ethanol after formalin fixation is also useful in cases where immunohistochemical stains are scheduled, as it stops cross-linking of tissue proteins, and is applied commonly in the post-fixation process to enhance the staining reaction (Carson & Hladik, 2009). It produces significant shape changes, however, in the adult silver moharra *Eucinostomus argenteus* Baird & Girard 1855 and the roughneck grunt *Haemulopsis corvinaeformis* (Steindachner 1868) (Martinez et al., 2013).

In larvae of *D. labrax*, only the first signs of ossification are starting to appear at the age of 14 dph (Rahman, 2008; Darias et al., 2010). At later stages, the more ossified specimens become less fragile, limiting shrinkage and deformations during fixation and mounting (Radtke & Waiwood, 1980; Tucker & Chester, 1984; Jennings, 1991; Mabee et al., 1998). Additionally, these effects may be limited at later stages due to the higher water content of smaller specimens, as reported for the Atlantic herring *Clupea harengus* L. 1758 and the European plaice *Pleuronectes platessa* L. 1758 (Ehrlich, 1974a, b), which explains the higher shrinkage due to greater water loss during fixation (Hay, 1982; Mabee et al., 1998).

In the glutaraldehyde treatment without prior anaesthetization, a highly significant lower number of specimens had to be discarded compared to treatments with anaesthetization. According to the Guidelines for the Use of Animals in Behavioural Research and Teaching (Anon, 2006), anaesthetics should be applied for ethical reasons, but in order to examine whether they hinder the size or shape analysis, their effect had to be studied and considered. As noted above, it has been suggested by Parker (1963) and Theilacker (1980) that MS-222 interferes with osmoregulation, and therefore can contribute to the shrinkage of live larvae. Additionally, in Methot & Kramer (1979), Theilacker (1980) and other comparative studies on the effect of fixatives such as by De Leon et al. (1991), it was not used. In this case, non-anaesthetized larvae fixed in glutaraldehyde displayed almost no visible changes in body posture. According to the protocol of this study, in the level of subtle distortions, this lack of anaesthetization did not produce the expected advantages as it introduced significant shape changes on 2 and 7 dph, unlike the anaesthetized glutaraldehyde groups where no significant shape changes were present. Therefore, anaesthetization is recommended.
One of the problems encountered in this study was finding the correct dosage of MS-222 for larvae of *D. labrax* at these very early stages. Poorly sedated larvae or larvae that distort due to an overdose can limit the usefulness for further analysis. Gradually applying the dosage can serve as a starting point, avoiding the shock effect of a quick death by overdose. There were high levels of discarded larvae in treatment G under anaesthetization (Fig. 2), and they might be attributed to a variety of reasons, such as insufficient anaesthetization during the experiment, additional larval stress induced by transportation and handling (as witnessed in this study) or to an effect of the fixative itself. This study does not allow discriminating between these random effects. When the specimen selection criteria are met, however, glutaraldehyde remains the recommended fixative for studies focusing on subtle shape differences in early stages of *D. labrax*.

The protocol proved to be sufficiently sensitive to even quantify and visualize shape differences originating from a different egg batch, as indicated by the lack of significant pre-fixation differences at 2 and 7 dph between groups F and FE that came from the same egg batch, and the presence of highly significant differences between G and both F and FE (Fig. 3). Furthermore, as indicated by the gradually increasing overlap of clusters of the different groups, the common shapes that start to appear as larvae grow suggest that observed differences during the early stages might gradually decrease. Similar ontogenetic convergences of shape as fish continue to develop into later juvenile stages have been demonstrated in *D. labrax* (Georgakopoulou et al., 2007).

Finally, the mounting procedure did induce some deformation artefacts, rendering it suitable for qualitative morphological studies, but not for morphometrics. Regarding the former, very good results were obtained with specimens fixed in phosphate-buffered glutaraldehyde that were mounted with glycerol using the described modified method of Maeseneer and d’Herde. The reason for the better results yielded by the consecutive glycerol baths instead of its immediate application can be the fact that glycerol is a hygroscopic agent that absorbs and replaces water in the specimens, penetrating the cells to the same extent as water, but at a slower rate than water release (Alemohammad & Knowles, 1974). Thus, it is assumed that the series of baths ensured a more gradual glycerol and water exchange.

The present analysis allowed the visualization and quantification of normal shape changes during the early life stages in *D. labrax*, and especially deformities resulting from common manipulations such as fixation and mounting. The broader applicability of this protocol is that it can be used to quantify deformities occurring in hatcheries at very early stages, thus allowing decisions and improvements to be made at this time point. Furthermore, it can prove useful not only in deformity studies of commercial fish species, but also for studying patterns of natural variation during their early life history, and in studies that aim to demonstrate an adverse effect attributable to any factor that has the potential to induce shape changes. As a final point, due to a certain degree of morphological similarity in the earliest fish developmental stages and the suitability of the outline analysis in cases of curved specimens where landmarks cannot be applied safely, it is highly likely that this protocol might be applicable to early stages of other marine or freshwater fish species, although this will have to be tested.

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