IXATION of larval fishes in formalin is most commonly used and has long been recommended for standard ichthyoplankton surveys (Ahlstrom, 1976; Smith and Richardson, 1977) as well as for diet studies (Bowen, 1983). Furthermore, until publication of the Ahlstrom Symposium Volume, Ontogeny and Systematics of Fishes (Moser et al., 1984; especially see chapter from Lavenberg et al., 1984), it was believed that 4% unbuffered formalin (or buffered formalin) is the most appropriate long-term preservation solution for larval fishes and eggs primarily because it supposedly prevents melanistic pigment loss and specimen shrinkage, whereas other chromatophores, like xanthophores and erythrophores, fade after fixation either in formalin or ethanol (see Baldwin, 2013).

We herewith comment on the advantages of larval fish collections that have been fixed in 95% ethanol: 1) specimens clear and double stain rapidly and brilliantly after ethanol fixation; 2) otoliths are preserved (Brothers, 1987); and 3) high-quality DNA is available from the tissues (Baldwin and Johnson, 2014). High-proof ethanol fixation of larval fishes has become more common as molecular studies and otolith microanalyses have become increasingly important in larval fish studies. Accordingly, for several years we have been experimenting with clearing and double staining larval and juvenile fishes (following the protocols of Dingerkus and Uhler, 1977; Potthoff, 1984; and Taylor and van Dyke, 1985) that have been fixed in 95% ethanol and never exposed to formalin. For several reasons, we wanted to establish a clearing and staining method that avoids the usage of formalin, as it is toxic, a human carcinogen, and many researchers are sensitive or allergic to its fumes. The practical disadvantage of formalin for long-term preservation of fish eggs and larvae emanates from temporal changes in pH (Lavenberg et al., 1984; Leis and McGruther, 1994); therefore, buffers are essential to prevent a fluctuating pH. In a pH of less than 6.4, the process of decalcification begins, which results in a mineral loss in bone structures and otoliths (Brothers, 1984, 1987; Lavenberg et al., 1984) and, as a consequence, reduces or may even prevent staining for either bone or cartilage using the methods of Dingerkus and Uhler (1977), Potthoff (1984), or Taylor and van Dyke (1985). On the other hand, a pH over 7.0 initiates the clearing of tissues that results in translucent specimens and complete dissolution of melanin. To our knowledge, the single disadvantage of a high-proof ethanol fixation is the alleged loss of pigments and a significant and dissimilar shrinkage of larval specimens due to dehydration, although results seem to be species-specific (e.g., Hay, 1982; Tucker and Chester, 1984; Kruse and Dalley, 1990; Hjörleifsson and Klein-MacPhee, 1992; DiStefano et al., 1994; Kristoffersen and Gro Vea Salvanes, 1998; Fey, 1999; Cunningham et al., 2000; Smith and Walker, 2003; Gagliano et al., 2006). On a quantitative basis, we do not see this as a serious problem, as detailed morphometric data can be gathered from photographs of fresh material and has gradually and understandably fallen out of favor in larval fishes from ichthyoplankton surveys.

Taylor and van Dyke (1985) noted that clearing and double staining specimens that have been fixed and preserved in ethanol is unreliable due to possible disintegration. However, they did not experiment with the fixation of specimens in high-proof ethanol; instead they used specimens from older collections that had been fixed in formalin and stored in ethanol or that they assumed were fixed in ethanol because they were collected prior to use of formaldehyde and had the appearance of alcohol fixation (e.g., some collected by the USS Albatross in the late 1800s; Taylor, 1967; Taylor and Van Dyke, 1985). The quality of fixation of this old collection material as well as the proof of ethanol is unknown, and low-proof ethanol as well as possible shortcomings in the fixation might explain the disintegration of specimens during the clearing and staining process.

In a direct comparison of larval and juvenile cleared and stained specimens that have been fixed in three different solutions (70% ethanol, 95% ethanol, 4% buffered formalin) with and without a preceding freezing step at –20°C, we comment on the advantages of high-proof ethanol fixation of ichthyoplankton collections and refute the notion that ethanol-fixed specimens are unreliable for clearing and double staining.

MATERIALS AND METHODS

We have cleared and double stained numerous larval and juvenile fish specimens (representing a variety of taxa) that
were fixed in 95% ethanol and have consistently obtained excellent results (Fig. 1). For this paper, we experimented with the direct fixation of several larval and juvenile taxa in 70% ethanol, 95% ethanol, and 4% buffered formalin. We further compared clearing and staining results of specimens with and without a preceding freezing step (−20°C) before fixation in the aforementioned three different solutions.

The fixation process in 70%, 95% ethanol, and 4% buffered formalin takes 3–7 days depending on the size of the specimen, which can then be transferred to a 70% ethanol solution for storage and to avoid further dehydration. The latter step is only recommended if tissue samples for DNA extraction and otoliths have already been removed from the specimen. All fixatives (70%, 95% ethanol, 4% buffered formalin, and −20°C freezing) produced acceptable cleared and stained specimens, but there are notable differences among them. Below we describe our modified clearing and staining method and the major differences we observed:

1. Dehydration in absolute ethanol for 24 hours.
2. Cartilage staining with Alcian blue for at least 12 hours.
3. Neutralization in a 0.5% KOH solution or in 70% ethanol. Usually the neutralization in KOH is the best and fastest way to wash out the residual acidity in the tissue, but we noticed that for specimens that have been frozen, the use of KOH is unpredictable. Good results have been obtained (see Fig. 1), but several specimens disintegrated (Fig. 2D), possibly due to textural changes like cell membrane rupture caused by ice crystals during a slow freezing process. We also accept that the disintegrating specimens may not have been properly frozen (e.g., time difference between catch and freezing) or that the freezing process might have been interrupted. However, the use of KOH might result in the destruction of the previously frozen specimen and can be avoided by neutralizing it in 70% ethanol.
4. Bleaching pigments in a 0.5% KOH solution with several drops of 3% H2O2. However, the bleaching process in KOH has to be monitored carefully since the chemical reaction and the resulting mechanical force can damage the specimens. If KOH needs to be avoided, specimens can be bleached in a 65% sodium borate solution and several drops of 3% H2O2 under strong UV light. Ethanol-fixed specimens bleach much slower in both solutions than the formalin-fixed specimens, and some pigment might even remain.
5. Clearing specimens in a 65% sodium borate solution with trypsin. The specimens fixed in ethanol clear much faster in the trypsin solution (depending on the size of the specimen, 10 minutes to several hours) than the formalin-fixed specimens (at least 1 hour to several days). Therefore, specimens fixed in ethanol need to be watched carefully. Fortunately, less trypsin is needed.
6. Alizarin staining in 0.5% KOH for several hours or overnight depending on the size of the specimen or alternatively in 70% ethanol. In the latter, the staining process takes less time (possibly 1–4 hours depending on the size of the specimen, but never overnight).
7. Washing specimens in 0.5% KOH or alternatively in 70% ethanol and final placement in 70% glycerin.

RESULTS

Fixation.—For clearing and staining larval and juvenile specimens, the best results can be obtained if live specimens that have been anesthetized with clove oil are directly fixed in 95% ethanol. Another option is freezing specimens in seawater (Carole Baldwin, pers. comm., 2015) and fixation in 95% ethanol afterwards. The latter method produces specimens that react unreliably during the clearing and staining process, and in most of our experiments they disintegrated especially if KOH was used during one of the clearing and staining steps (possible problems with this method are detailed in the Materials and Methods section). Figure 1 shows the good results we were able to obtain with specimens that were initially dry frozen, thawed in water, and subsequently placed for three days into 95% ethanol before they were cleared and double stained.

Larval and juvenile specimens that have been fixed in 70% and 95% ethanol clear and stain rapidly and brilliantly (Fig. 3). The difference between the two fixatives is that the tissues of the specimens fixed in 70% ethanol do not hold up that well. The formalin-fixed specimens are denser than the ethanol-fixed specimens and took seven days longer to clear, whereas bleaching of melanistic pigment took two days longer in the ethanol-fixed specimens (Fig. 2).

Detailed differences we observed.—The cartilage of the gill arches stained much better in the ethanol-fixed specimens (Fig. 3D, E, G, H) than in the formalin-fixed specimen (Fig. 3F, I). The intermuscular bones stained better in the ethanol-fixed specimens (Fig. 3J, K) than in the formalin-fixed specimen (Fig. 3L). There are no significant differences regarding the oral jaws, suspensorium, opercular bones (Fig. 3A–C), the dorsal fin (Fig. 3M–O), anal fin, and paired fins. The superficial chevrons of cartilage associated with the distal tip of each anterior epicentral bone in Alosa aestivalis stained better in the formalin-fixed specimens (Fig. 2C).

DISCUSSION

Many large and valuable ichthyoplankton collections have been stored in formalin for decades, and due to their vast size, it is often impossible to provide the curatorial expenses to monitor the pH levels or even transfer them to ethanol. In any case, whenever formalin is used as the basis for long-term storage, there will be problems maintaining a pH between 6.3 and 7.0. As a consequence, Lavenberg et al. (1984) recommended, for the first time, 70% ethanol as the final preservation fluid for ichthyoplankton, and many curators of larval fish collections now follow this protocol. For osteological studies, the duration in the initial formalin fixative is critical to successful staining of cartilage and bone. Additionally, formalin decalcifies otoliths and degrades DNA. Fixation of ichthyoplankton collections in 95% ethanol provides specimens that are significantly more scientifically valuable for anatomical, life history, developmental, and molecular research. For histological sections it is necessary to have specimens fixed in formalin. In larger specimens, formalin fixed tissues hold up better if used for histology. However, larval and juvenile teleosts can be post-fixed in formalin if the initial fixative has been ethanol (Katja Felbel, pers. comm., 2016). To the best of our knowledge, the only notable disadvantages for the specimens are possible shrinkage and the alleged loss of pigments.

It is believed that pigments will be better preserved in formalin than in ethanol solutions, possibly due to the fact that formalin was recommended as fixative in several protocols for the fixation and preservation of ichthyoplankton (e.g., Ahlstrom, 1976; Smith and Richardson, 1977; Leis and McGrouther, 1994). Taylor (1981) noted that there is no easy
way to preserve all types of pigments, as some are water soluble and others alcohol soluble. For best pigment retention, Taylor (1981) suggested avoiding alkaline fixatives and preservatives, noting that the solution should have a pH between 6.3 and 7.0. Using tap water for dilution of ethanol or formalin might account for some pigment loss depending on the water hardness and the correlated alkaline activity of the water (Taylor, 1981; Simmons, 2014). Taylor (1981) also suggested that oxidation causes melanistic pigment loss; the essential components for oxidation are energy (light, heat), water, and oxygen, the latter of which are both present in ethanol solutions. Much pigment bleaching takes place in water (thus it is not recommended to wash specimens in water when transferring them from formalin into ethanol) and,
most importantly, in diluted ethanol (Taylor, 1981). Melanistic pigments are retained well in specimens we fixed in 95% ethanol, but we are unable to comment on a long-term effect.

With growing ichthyoplankton collections in which specimens are fixed in 95% ethanol, we find the advantages of omitting formalin from the fixation and storage process of such collections on many levels convincing, given that the subsequent research is not dependent on length and weight data. Cleared and stained ethanol-fixed specimens have a brilliant quality regarding cartilage and bone staining as well as transparency of the muscle tissue. We reject previous notions that ethanol-fixed specimens *per se* disintegrate during clearing and staining. We only observed specimen disintegration in some cases when specimens have been frozen before fixation. Such specimens react more unpredictably to chemicals and seem to be more fragile, so that KOH should be replaced by other more gentle solutions (see above). By following this protocol, nicely cleared and stained specimens can still be obtained. We also noted that melanistic pigment was difficult (and in some cases impossible) to bleach in the ethanol-fixed specimens. The maceration of muscle tissue takes less time in all stages, and therefore each step requires fewer chemicals and needs to be watched carefully.

**MATERIAL EXAMINED**

Institutional abbreviations follow Sabaj P´erez (2014).

**Clupeiformes**

*Alosa aestivalis*: MNHN 2014 2997, 42.8 mm SL, 3 sp., fixed in 70% EtOH; MNHN 2014 2998, 40.4–43.5 mm SL, 3 sp., fixed in 95% EtOH; MNHN 2014 2999, 45.3 mm SL, fixed in 4% buffered formalin; VIMS 33001, 44.6 mm SL, frozen at −20°C with a following fixation in 95% ethanol. Scale bars = 5 mm.

**Syngnathoidei**

Syngnathidae sp.: MNHN 2014 2980, 42 mm SL, 1 sp., frozen and fixed in 95% EtOH.

**Gobiodei**

*Pomatoschistus minutus*: MNHN 2014 2992, 18.7 mm SL, 1 sp., frozen and fixed in 70% EtOH; MNHN 2014 2993, 24.8 mm SL, 1 sp., frozen and fixed in 4% buffered formalin;
MNHN 2014 2994, 14.5 mm SL, 1 sp., frozen and fixed in 95% EtOH, CS without KOH; MNHN 2014 2995, 22.0–47.0 mm SL, 3 sp., frozen and fixed in 95% EtOH; MNHN 2014 2996, 19.0 mm SL, 1 sp., frozen and fixed in 95% EtOH.

Pleuronectiformes

*Platichthys flesus*: MNHN 2014 2984, 22.0 mm SL, 1 sp., frozen and fixed in 95% EtOH; MNHN 2014 2985, 16.0–21.0 mm SL, 3 sp., frozen and fixed in 95% EtOH.

*Pleuronectes platessa*: MNHN 2014 2986, 27.7 mm SL, 1 sp., frozen and fixed in 95% EtOH; MNHN 2014 2987, 29.9 mm SL, frozen and fixed in 70% EtOH; MNHN 2014 2988, 28.0–41.0 mm SL, 3 sp., frozen and fixed in 95% EtOH; MNHN 2014 2989, 29.2 mm SL, frozen and fixed in 4% buffered formalin.

*Solea solea*: MNHN 2014 2981, 27.2–47.9 mm SL, 4 sp., frozen and fixed in 95% EtOH; MNHN 2014 2982, 46.9 mm SL, 2 sp., frozen and fixed in 95% EtOH; MNHN 2014 2983, 27.5–31.3 mm SL, 2 sp., frozen and fixed in 95% EtOH; MNHN 2014 2990, 31.3 mm SL, 1 sp., frozen and fixed in 95% EtOH, CS without KOH; MNHN 2014 2991, 22.0 mm SL, 1 sp., frozen and fixed in 95% EtOH, CS without KOH.

ACKNOWLEDGMENTS

For discussion, opinions, and inspiration, we are grateful to C. Baldwin, A. Ohler, R. Britz, G. Arratia, and K. Conway. We thank A. Rhea, B. Watkins, P. McGrath (VIMS), C. Gallut (UPMC), and E. Luchetti (MNHN) for collecting and providing material, and K. Felbel (Friedrich Schiller University, Jena) for comments and her insights into the histology techniques for larval and juvenile teleosts.

LITERATURE CITED


Johnson (eds.). American Fisheries Society, Bethesda, Maryland.


