EFFECTS OF FORMALDEHYDE AND ETHANOL PRESERVATION ON BODY AND OTOLITHS OF *MAUROLICUS MUELLERI* AND *BENTHOSEMA GLACIALE*

JON BENT KRISTOFFERSEN & ANNE GRO VEA SALVANES

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First of its kind, this study examines effects of preservatives on otoliths as well as on fish size and weight. Effects of 200 days of preservation in 4 % seawater formaldehyde solution and 80 % ethanol were investigated for the two small, mesopelagic fishes, *Benthosema glaciale* (REINHARDT, 1837), and *Maurolicus muelleri* (GMELIN, 1789). The body weight loss was much higher in ethanol (37-39 %) than in formaldehyde (13-16 %). The decrease in standard length was small in both preservatives and for both species (0.8-3 %). The weight of the otoliths of *B. glaciale* was estimated to decrease by approximately 3 % in both formaldehyde and ethanol, while a radius change in one direction could not be demonstrated unambiguously. In contrast, there were no significant changes in the otoliths of *M. muelleri* in any of the preservatives, thus we can use otoliths from *M. muelleri* preserved for up to at least 200 days in correctly buffered formaldehyde. Growth rate and age can then easily be coupled with other life history parameters obtained from preserved fish.

Jon Bent Kristoffersen & Anne Gro Vea Salvanes, University of Bergen, Department of Fisheries and Marine Biology, Bergen High-Technology Centre, N-5020 Bergen, Norway.

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INTRODUCTION

When conducting studies using preserved fish one is often faced by a variety of storage methods, as freezing, formaldehyde, and ethanol. Different preservation methods lead to different changes in size and weight, and pigmentation and otoliths may break down. If available, conversion factors can correct for shrinkage and weight loss during storage (e.g. GJØSÆTER 1973; KRUSE & DALLEY 1990; FOX 1996). The amount of shrinkage generally depends on the osmolarity of the preservative (TUCKER & CHESTER 1984), thus seawater formaldehyde will lead to a greater shrinkage than freshwater formaldehyde. Formaldehyde preservation is advised against when otoliths must remain intact (GJØSÆTER & al. 1984; HÄRKÖNEN 1986; BROTHERS 1987), since otoliths are etched at the low pHs often present in formaldehyde solutions (STEEDMAN 1976b). However, addition of buffering agents can keep the pH high, at least temporarily, thus it may be possible to use otoliths from formaldehyde preserved specimens (SUTHERS & al. 1992). Ethanol is widely used as preservative in otolith studies, and will like formaldehyde cause changes in body size and weight (HJÖRLEIFSSON & KLEIN-MACPHEE 1992; QUIÑÓNEZ-VELÁZQUEZ & CHAUMILLON 1996). As fixative, ethanol is inferior to formaldehyde, and ethanol < 85 % is mildly acidic and may etch larval otoliths (RADTKE & WAIWOOD 1980).

Otoliths from formaldehyde preserved *Maurolicus muelleri* have previously been used by the authors, and identical age structures were found in formaldehyde preserved and frozen samples. Also, examination of samples stored for one year in formaldehyde has shown that a pH of 7.5-8 apparently renders the otoliths of *M. muelleri* unchanged, with both annual and daily increments as clear as in fresh otoliths. This study was thus done to provide correction factors for the shrinkage and body weight loss that occurs during preservation of *M. muelleri* and *Benthosema glaciale*, and to formally test the common notion that otoliths are destroyed by formaldehyde.

MATERIAL AND METHODS

Body size and weight

Samples of *B. glaciale* and *M. muelleri* were obtained from pelagic trawling in July 1996 in Herdlefjorden, western Norway, with a Harstad trawl. The fish were kept chilled in a refrigerator, from which small subsamples were taken for measurements within 12 hours after capture. A total number

of 160 individuals of each species were measured for total length (*TL*), standard length (*SL*), height (*H*) and width (*Wi*) immediately posterior to the opercula, to nearest 0.1 mm with a calliper. Using the formula for an ellipsoid, the volume of each fish was calculated as:

$$V = \frac{4}{3}\pi \cdot \frac{SL}{2} \cdot \frac{Wi}{2} \cdot \frac{H}{2}$$

Weight (*W*) was measured to the nearest 0.01 g after removing excess water using paper tissue. Each fish was transferred to individually labelled plastic vials (30 or 100 ml) containing 4 % formaldehyde mixed with seawater (salinity 30-34) and buffered with sodium borate resulting in a pH of 8.2, as measured at the end of the experiment. The resulting fish volume:preservative volume was 1:10 or less. The samples were stored in darkness at room temperature. At day 20, 80 specimens of each species were transferred to 80 % ethanol. Fishes were measured and weighed six times: before fixation, then at 10, 20, 50, 100 and 200 days after fixation.

Otolith size and weight after 200 days

Otolith size and weight relative to fish size and weight were compared for the fish stored in formaldehyde and ethanol with otoliths from frozen fish. At day 200 after fixation both sagittal otoliths were removed, rinsed in water and dried at room temperature for several days before weighing to nearest µg. Otoliths from 75 frozen *B. glaciale* and *M. muelleri,* all from the same station as the preserved fish, were extracted. The standard length of *B. glaciale* has previously been shown not to change during freezing (Gjøsæter 1981), we assume the same applies for *M. muelleri* (see however Armstrong & Stewart 1997). Body weight loss during freezing was corrected for from the difference between fresh and frozen fish in predicted body weight at standard length, applying regression lines of the type $W = aL^b$, where W is predicted weight, L is standard length, and a and b are constants. Otoliths with signs of calcite crystallisation (cf. Strong & al. 1986) were found to have a lower weight and size than normal and were excluded from the subsequent analyses. The otoliths were glued to glass slides with nail varnish leaving their upper (convex) surface dry, lightly polished, then covered with a drop of immersion oil to clear them and ease the identification of the primordium (centre of the otolith). The radius of both left and right otolith was measured with an ocular micrometer under 50 x magnification. F-tests were used to test for differences in otolith weight and radius relative to fish weight and length. The average radius and weight of the left and right otolith was used. The data were ln-transformed prior to analysis to improve normality. If an overall difference was found, pairwise testing was applied to each pair of groups. If no differences were found, a common slope was computed, and an F-test was applied to test for differences in intercept.

Otolith size and weight after 20 days

To test effects of different buffers and pHs, otoliths were weighed and measured before and after storage in three different formaldehyde solutions. A total number of 48 otoliths from frozen *B. glaciale* were rinsed in distilled water, dried at 60 °C for 24 hours, then weighed to nearest µg and measured with an ocular micrometer. The diameter of the longest axis was measured to get a precise measurement. 16 otoliths were placed in each of the three solutions: 4 % freshwater (distilled) formaldehyde buffered with a mixed sodium phosphate buffer to a pH of 7.4; 4 % freshwater formaldehyde saturated with borax resulting in a pH of 9.2, and 4 % seawater formaldehyde solution saturated with borax resulting in a pH of 8.8, respectively. After 20 days the otoliths were

Table 1. *M. muelleri* and *B. glaciale* after (A) 200 days of storage in formaldehyde, and (B) 20 days of storage in formaldehyde followed by 180 days in ethanol, with linear regressions predicting fresh standard length (SL, mm) and weight (W, g).

A

Measure	Regression	R^2	n
Weight *M. muelleri*	$W_{fresh} = 0.043 + 1.076 * W_{200d}$	0.995	72
Weight *B. glaciale*	$W_{fresh} = 0.039 + 1.097 * W_{200d}$	0.999	73
SL *M. muelleri*	$SL_{fresh} = 0.127 + 1.008 * SL_{200d}$	0.993	71
SL *B. glaciale*	$SL_{fresh} = -0.682 + 1.027 * SL_{200d}$	0.981	67

B

Measure	Regression	R^2	n
Weight *M. muelleri*	$W_{fresh} = 0.048 + 1.459 * W_{200d}$	0.992	77
Weight *B. glaciale*	$W_{fresh} = 0.074 + 1.430 * W_{200d}$	0.999	75
SL *M. muelleri*	$SL_{fresh} = 0.171 + 1.026 * SL_{200d}$	0.993	73
SL *B. glaciale*	$SL_{fresh} = -0.667 + 1.043 * SL_{200d}$	0.998	67

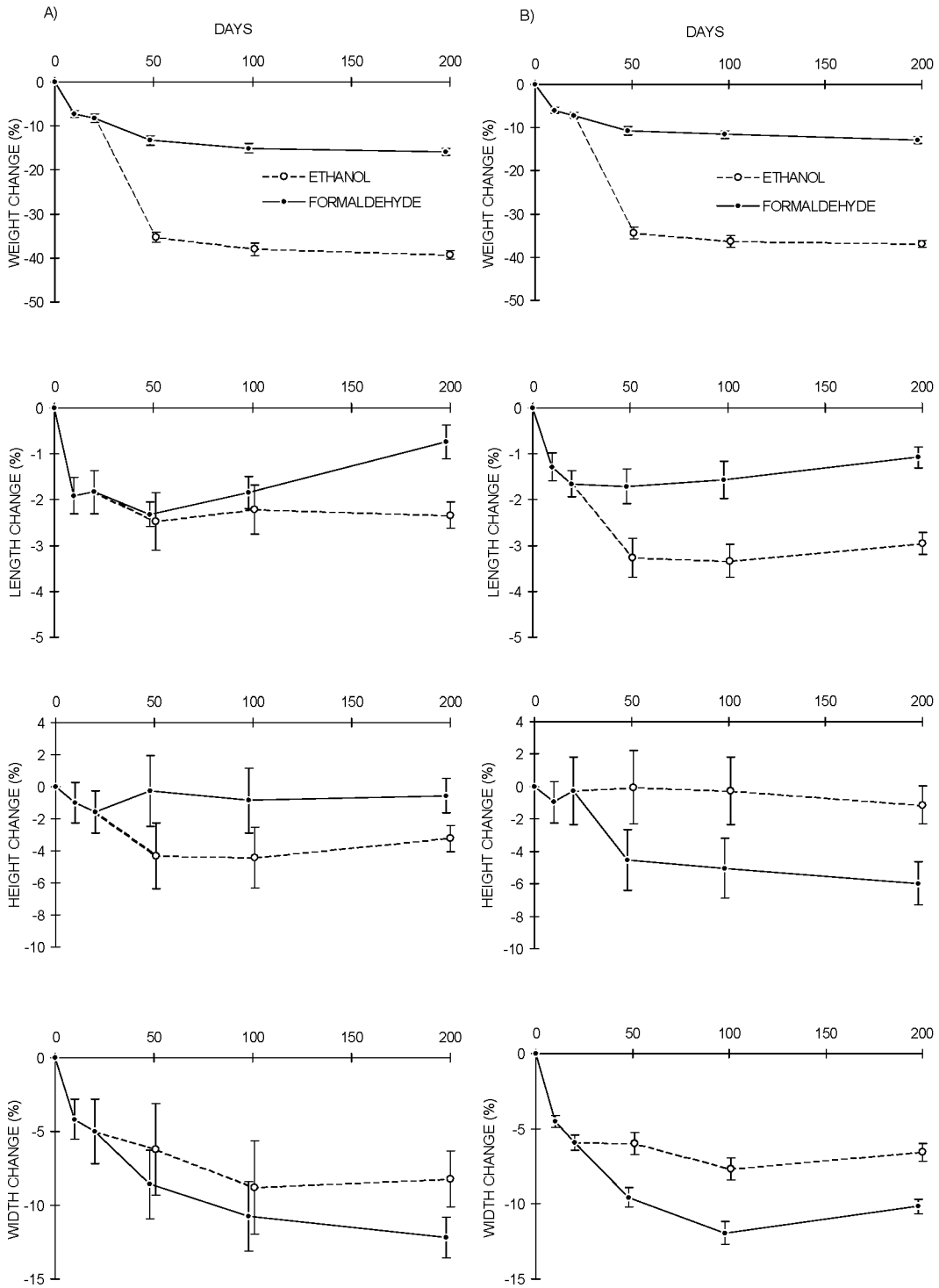

Fig. 1. Mean percentage change in weight, standard length, height, and width during storage in 4 % seawater formaldehyde and 80 % ethanol of *B. glaciale* (A) and *M. muelleri* (B). Vertical bars show 95 % confidence intervals.

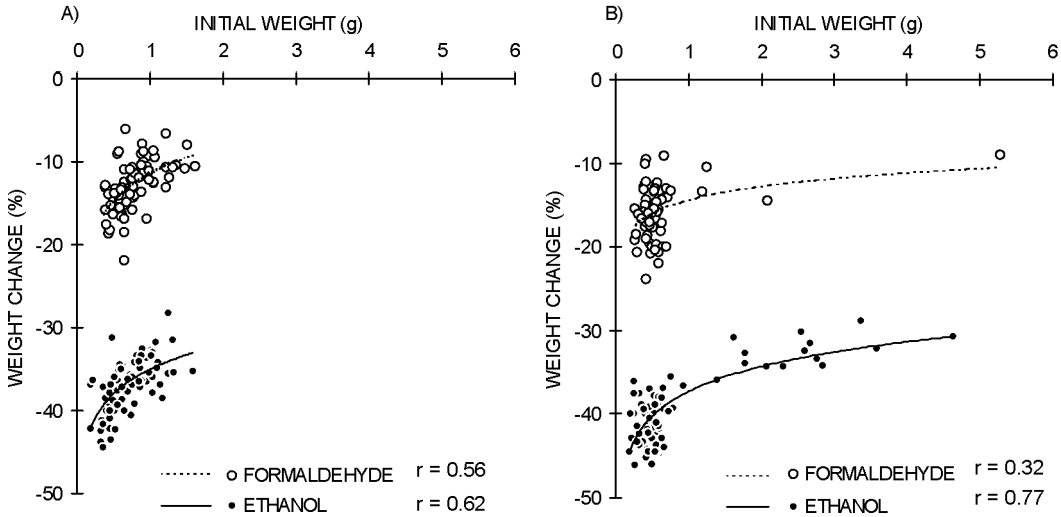

Fig. 2. Percentage body weight loss after 200 days of preservation of *M. muelleri* (A) and *B. glaciale* (B) relative to initial weight. Logarithmic trendlines are fit. All correlations are significant ($P < 0.002$).

removed from the solutions, again rinsed in distilled water, dried for 24 hours at 60 °C before weighing and measuring the diameter. T-tests were used to test for differences before and after storage.

In this paper we will use the terminology agreed upon at the Scientific Committee on Oceanographic Research (SCOR) Working Group 23 meeting of March 1968. The term formaldehyde should always be used. A 4 % solution of formaldehyde is made up of 1 part 40 % formaldehyde (as purchased) and 9 parts seawater or distilled water. This should not be referred to as 10 % formalin.

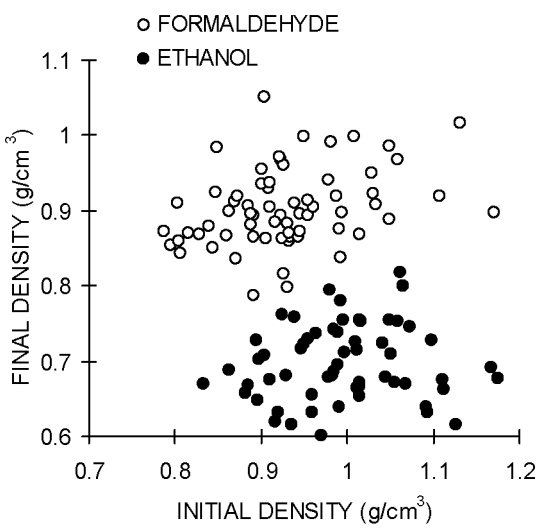

Fig. 3. Calculated density of the body of *B. glaciale* before and after 200 days of preservation.

RESULTS

Body size and weight

Formaldehyde. The decrease in body weight was initially rapid, and the majority of the weight reduction in both preservatives occurred during the first 50 days (Fig. 1). The mean weight loss after 200 days of preservation in formaldehyde was 12.9 % for *M. muelleri* and 15.9 % for *B. glaciale*. The standard length initially decreased, but after 50 days the length increased slightly. After 200 days the mean decrease in standard length was 1.1 % and 0.8 % for *M. muelleri* and *B. glaciale*, respectively. Table 1 presents regressions predicting fresh weight and length from preserved weight and length.

Ethanol. After the fish were transferred to ethanol, both length and particularly weight decreased, much more than in formaldehyde (Fig. 1). After 200 days, the mean weight loss for *M. muelleri* and *B. glaciale* was 36.9 % and 39.4 %, respectively. The mean decrease in standard length for *M. muelleri* and *B. glaciale* was 3 % and 2.3 %, respectively.

Measurements of height and width were found to have a high variance, but generally there seemed to be a decrease in the width in both formaldehyde and ethanol, in range of approximately 5 to 12 %. The decrease in height was less (0 to 6 %). Both species showed size dependent weight loss (Fig. 2), with larger fishes losing less weight relative to their initial weight. The density of *B. glaciale* was reduced during storage in ethanol, but not in formal-

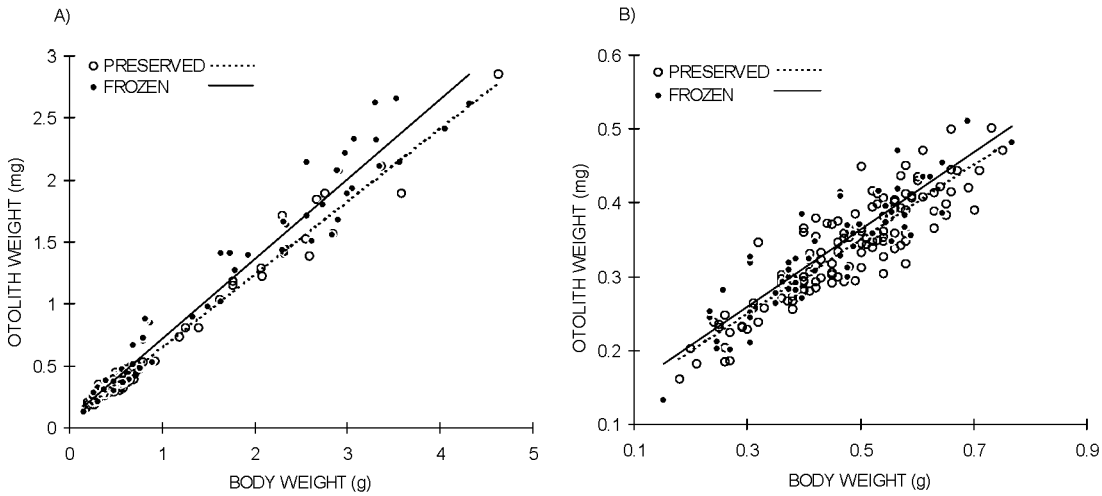

Fig. 4. Otolith weight versus body weight in *B. glaciale*, and predicted linear regressions for fish preserved in formaldehyde or ethanol (preserved), and frozen fish. A. All individuals. B. Individuals smaller than 40 mm SL only.

dehyde (Fig. 3). The same pattern was found for *M. muelleri*.

The weight of the frozen fish relative to their standard length was lower as compared to fresh individuals. In *B. glaciale* the estimated weight difference ranged from about 22 % lower weight after freezing of 30 mm SL individuals, to about 6 % lower in 70 mm individuals. In *M. muelleri,* the weight loss ranged from about 10 % in a 30 mm fish, to 2 % in a 50 mm fish. The weight of frozen fish was adjusted accordingly for subsequent analyses.

Otolith size and weight after 200 days

M. muelleri. In *M. muelleri,* there were no significant differences between frozen fish and formaldehyde or ethanol preserved fish in neither weight nor size of the otoliths ($P > 0.61$), although there was a slight tendency towards lower otolith weight in large fish in formaldehyde.

B. glaciale. The otoliths of *B. glaciale* lost weight during storage in both formaldehyde and ethanol (Fig. 4A, B). The weight of the otoliths were found not to be significantly different for the fish stored in ethanol and formaldehyde, respectively ($P > 0.09$). These two groups were therefore joined for comparison with otoliths from frozen fish. The otoliths from preserved fish were significantly lighter ($P < 0.05$) than otoliths from frozen fish. Comparing the difference in otolith weight predicted from body weight, the weight difference was about 10 % (Fig. 4A). A similar test was done excluding fishes above 40 mm SL (Fig. 4B), leaving a group most likely consisting of one year old fish, as estimated from both length-frequency and otolith readings. 90 % of the individuals belonged to this age group, and the data were evenly distributed in this size range, thus the difference obtained from this comparison may be more realistic. There was still a significantly lower otolith weight in preserved fish ($P < 0.012$), but on average only 4 % lower (Fig. 4B).

The otolith radius differed significantly between frozen fish and formaldehyde ($P = 0.013$), and between formaldehyde and ethanol ($P = 0.001$), but not between frozen and ethanol ($P = 0.057$). Because their slopes differed, the predicted regression lines (SL versus otolith radius) of frozen and formaldehyde preserved fish crossed at a fish length of about 30 mm. Thus, by using the difference in predicted otolith weight (from body weight), formaldehyde preserved fish smaller than 30 mm are predicted to have larger otoliths than frozen fish – about 5 % larger at 25 mm SL, whereas those above 30 mm will have smaller otoliths – about 5 % smaller at 45 mm SL.

The predicted otolith radius - otolith weight regressions did not differ between formaldehyde preserved and frozen fish ($P > 0.12$), but differed between these and ethanol preserved fish ($P < 0.012$), with a lower size-specific otolith weight in ethanol, i.e. the otolith density was lower after storage in ethanol.

Otolith size and weight after 20 days

Of the otoliths stored for 20 days in formaldehyde, the 4 % seawater solution had the least effect on the otoliths (Fig. 5); there was no significant difference neither in weight nor radius after storage ($P > 0.13$). The weight of

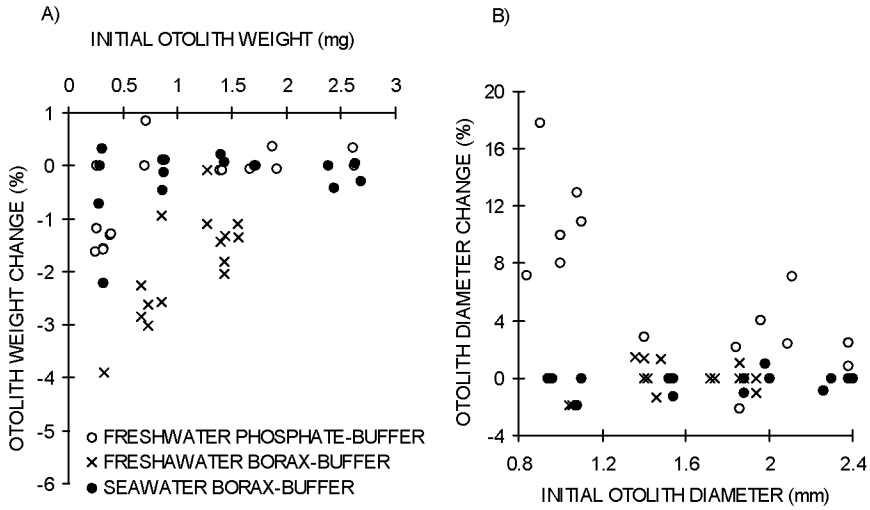

Fig. 5. Percentage change in *B. glaciale* otolith weight (A) and diameter (B) after 20 days in different formaldehyde solutions, relative to initial otolith weight and diameter.

the otoliths preserved in freshwater formaldehyde buffered with borax decreased slightly, but significantly (–2.1 %, $P < 0.01$), while their diameter was unchanged ($P > 0.99$). The weight of the otoliths preserved in phosphate buffered formaldehyde was the same after preservation ($P > 0.62$), while the diameter increased by an average of 6.7 % ($P < 0.01$). This was caused by deposits forming at the surface of the otoliths. Generally, the smallest otoliths had the greatest relative weight reduction. The smallest otoliths stored in phosphate buffered freshwater increased most in diameter. This was most likely caused by accretions forming at the surface of the otoliths at a constant rate.

DISCUSSION

Shrinkage and weight changes of fish body

So far, most studies on shrinkage have been carried out on fish larvae. The small size of the species in this study makes them vulnerable to shrinkage, as has been observed in other marine species (e.g. Hay 1982; Kruse & Dalley 1990). The small length decrease observed in formaldehyde preserved fish we believe mainly to be caused by the bending and distortion which occurs upon fixation, resulting in underestimated size. Thus the true length shrinkage in formaldehyde is likely to be close to zero. The increase in length observed after 50 days in formaldehyde may partially be an artefact caused by better flattening of the fish before measurement. Any measurement error could have been reduced by applying some sort of image analysis, see e.g. Sagnes (1995). The change in standard length was however marginal (less than 1 % between 20 and 200 days), thus little precision would have been gained for this variable. Gjøsæter (1973) reported a decrease in standard length of *B. glaciale* during formaldehyde storage of about 3.5 %, compared to 0.8 % in this study. No details were however given about the experimental procedure by Gjøsæter (1973).

Comparative studies on shrinkage in formaldehyde and ethanol are few. Some report a smaller shrinkage in ethanol (Theilacker 1980; Hjörleifsson & Klein-MacPhee 1992), others show a higher shrinkage in ethanol (Fowler & Smith 1983; Kruse & Dalley 1990; Fox 1996). Ethanol has been found to distort larvae and cause greater variability in shrinkage estimates compared with formaldehyde preservation (Fox 1996). The higher shrinkage in ethanol observed in our study may partly be caused by a genuine shrinkage, and partly by higher rigidity of the fish after preservation compared with fish preserved on formaldehyde, making it harder to flatten the fish. Workers who measure the length of fixated fish may encounter the same problems of measuring the correct length of bent fish, and the calculated regression lines may thus be regarded as functional equations estimating fresh length from 'bent lengths'. The weight measurements on the other hand were made with a high level of precision, and the regression equations will, under the given conditions, adequately predict fresh body weight from preserved weight.

Different processes caused weight loss in formaldehyde and ethanol, respectively. Both formaldehyde and ethanol will extract water from tissue. Formaldehyde will also dissolve glycogen, glucose, some phospholipids and inorganic salts (Steedman 1976a), while ethanol can

extract much of the lipids present in specimens (GLAUERT 1974), thus extraction of the lipids abundant in *B. glaciale* and *M. muelleri* (FALK-PETERSEN & al. 1986) accounts for their large reduction in weight and density in ethanol.

Otoliths

Looking for effects of preservatives on otoliths, one encounters some problems. Since the mere presence of tissue will act to lower the pH (STEEDMAN 1976a), a gradient can exist with a higher pH outside the fish than inside the tissues; the exact environment surrounding the otoliths within a fish is thus unknown. Also, this procedure relies on comparing predicted otolith size - fish size regressions of large samples since it is not the same otoliths that are measured in the different preservatives. If otoliths instead are placed directly into formaldehyde solutions, the environment is known, and the same otoliths can be measured before and after storage; however, the environment may be unrealistic. In this study we therefore used both approaches.

As there appears to be no literature on effects of preservation on otoliths, we turned to plankton studies. These have shown that degradation of zooplankton calcareous structures in formaldehyde solutions can happen with a pH below 7.5 or even as high as 8.2 (GRIFFITHS & al. 1976; STEEDMAN 1976b), which we believe also has the potential to dissolve the calcium carbonate in otoliths. A high pH on the other hand, may cause swelling with subsequent dissolution of proteins present in otoliths (DEGENS & al. 1969). The otoliths stored in freshwater borax formaldehyde had many cracks and crevices, possibly caused by proteins swelling at the high pH (9.2). Seawater in itself can have a dissolving effect on calcium carbonate, distilled water has therefore been recommended as dilutent for formaldehyde (STEEDMAN, 1976b). The reduction in otolith weight in *B. glaciale* after 200 days of preservation, estimated to be between 4 and 10 %, is thus unlikely to have been caused by inappropriate pH (8.2), but may have been caused by some dissolution of calcium carbonate by seawater. The radius may have changed too, but our results are inconclusive at this point, since, depending on fish size, an increase or decrease in radius was predicted. The density reduction of otoliths from *B. glaciale* stored in ethanol was possibly caused by a dissolution of small amounts of lipids present on or within the otolith.

In contrast to *B. glaciale,* the weight and radius of *M. muelleri* otoliths did not change significantly in either formaldehyde or ethanol, compared with a frozen sample. Otoliths from *M. muelleri* may be less vulnerable to formaldehyde; in addition, the size range of this species is much smaller than in *B. glaciale*. The smaller size range resulted in a higher scatter in otolith weight and size relative to fish weight and size for *M. muelleri*, consequently a higher within-group variability made it harder to demonstrate significant differences.

Conclusions

The length and weight of formaldehyde or ethanol preserved *B. glaciale* and *M. muelleri* can be converted to fresh length and weight with the provided equations. PARKER (1963) warns against the use of general correction factors on weight, since a large number of factors, such as species, size, length of storage, temperature and strength and buffering of the preservative, determines the actual weight changes. To quote STEEDMAN (1976a): 'The variables in this work are numerous and self-changing', hence before applying any conversion equations on weight, fixation and storage procedures must be properly standardised. Based on the small reduction of standard length in formaldehyde, we tentatively predict that formaldehyde shrinkage of adult specimens of other mesopelagic species will be small, and probably insignificant in species larger than *B. glaciale*.

The otoliths of formaldehyde preserved *B. glaciale* changed in weight, and possibly size, during storage, so their use in age and growth studies requires caution. The short-term experiment indicated that seawater formaldehyde buffered with borax had least effect, but freshwater formaldehyde may turn out to be better if the pH is controlled. The otoliths of *M. muelleri*, on the other hand, did not change significantly. We therefore suggest that if correctly buffered formaldehyde is used, it is possible to use otoliths from *M. muelleri* stored for up to a year in formaldehyde. Although we do not advocate a general use of formaldehyde preservation for otolith studies, there may be some associated advantages. Usage of otoliths from formaldehyde preserved fish means that growth rate and age easily can be coupled with other life history parameters as diet, gonad weight and fecundity, and at an individual level not previously attainable for *M. muelleri* and *B. glaciale*. We believe such a coupling can provide new insight into life history strategies chosen by individuals and populations of fish.

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