

**EXAMINATION OF DIFFERENT PRESERVATIVES FOR *TODARODES PACIFICUS* PARALARVAE FIXED WITH BORAX-BUFFERED FORMALIN-SEAWATER SOLUTION**

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**ABSTRACT:** The effects of different preservatives on 36 *Todarodes pacificus* paralarvae were examined. After fixation in a solution of borax-buffered 2–4% formaldehyde in seawater, each dorsal mantle length was measured and dorsal chromatophores were photographed. The paralarvae were divided into the following six different preservatives; 1) 5% phosphate-buffered formalin, 2) 5% borax-buffered formalin, 3) 5% hexamine-buffered formalin, 4) 99% ethanol, 5) 70% ethanol, and 6) 40% isopropanol. After 3, 6, 9 and 12 months, each specimen was measured and photographed. In preservatives 3 to 6, pigment in the chromatophores remained for almost 12 months. However, specimens in preservatives 1 and 2 had considerably translucent mantles after 3 months. In addition, specimens in group 5 were remarkably shrunk (20%) after 3 months and in 6 were slightly shrunk after 12 months. In groups 1 to 4, certain changes in mantle length were undetected as the month elapsed. The results show that 5% hexamine-buffered formalin and 99% ethanol may be good preservatives.

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**INTRODUCTION**

Chromatophore pattern is an important character for identification keys in paralarval squids (Young and Harman, 1985) and has sometimes been used in identifying species in the family Ommastrephidae (*e.g.* Sato and Sawada, 1974; Harman and Young, 1985; Young and Hirota, 1990; Wormuth *et al.*, 1992; Wakabayashi *et al.*, 2002).

The Japan Sea National Fisheries Research Institute (JSNFRI) has conducted fall surveys to determine distribution of the paralarvae of the ommastrephid squid *Todarodes pacificus* (Goto *et al.*, 2002). Samples collected by plankton nets were immediately fixed with borax (sodium borate)-buffered 2–4% formaldehyde in seawater. After paralarval squids were sorted under tap water in the laboratory, they were transferred to screw vials filled with phosphate-buffered formalin as a preservative. Although the chromatophores of many specimens remained initially, they faded away afterwards and the specimens became almost transparent. The chromatophores remaining on the paralarvae corresponded to brown chromatophores reported by Young and Hirota (1990). The preservative used was changed to borax-buffered

formalin in 1999 and it seemed to the author that the chromatophores of specimens became invisible more quickly than before.

The purposes of this study, therefore, are to examine the effects of different preservatives on fixed specimens from the viewpoint of chromatophore pigment longevity, the size change of the specimens, and to determine the best preservative.

**MATERIALS AND METHODS**

In the vicinity of the Oki islands in the southwest Sea of Japan, samples were collected by 8 oblique tows of an 80 cm diameter plankton net with a 0.500 mm mesh from 75 m to the surface on 4 November 2000. They were immediately fixed in a solution of borax-buffered approximately 2% formaldehyde in seawater. From the samples collected, 43 paralarvae of *T. pacificus* were sorted out under tap water on 29 March 2001. After their dorsal mantle lengths were measured to the nearest 0.1 mm using a binocular dissecting microscope with an ocular micrometer, 36 specimens were chosen with similar mantle lengths. After the dorsal chromatophore pattern

was photographed, the specimens were divided into 6 groups and each group was placed in one of the following different preservatives; 1) 5% phosphate-buffered formalin, 2) 5% borax-buffered formalin, 3) 5% hexamine-buffered formalin, 4) 99% ethanol, 5) 70% ethanol and 6) 40% isopropanol. The diluent used in each of the preservatives was pure water. The 5% phosphate-buffered formalin used in this study was made according to Markle (1984) prior to 1998. Borax-buffered formalin is the supernatant fluid in borax supersaturated formalin (Steedman, 1976). The 5% borax-buffered formalin used in this study was made in 2000. Hexamine-buffered formalin was made at the ratio of one litre formalin to 20 g hexamine (Tohoku National Fisheries Research Institute, 1994) before the experiment. Each paralarva was put into a 2 ml screw vial and stored in a dark area of the laboratory. After 3, 6, 9 and 12 months, the photography and mantle length measurements were done individually. In addition, the pH of the formalin preservative in each vial was measured using a pH meter.

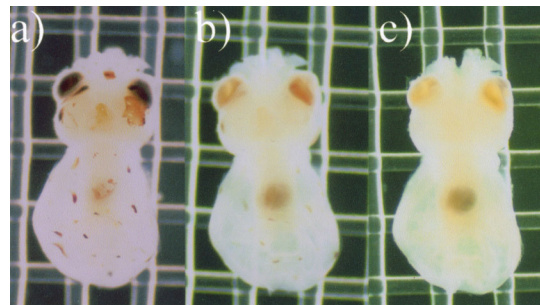
In order to examine the stability of the mantle lengths during those 12 months, length data, except for the specimens with distorted mantles, were preliminarily subjected to a repeated analysis of variance for each of the six preservatives (see Fox, 1996). Significance was accepted at the  $p=0.05$  level.

## RESULTS

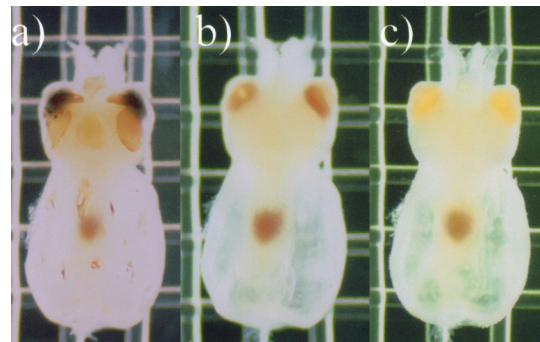
### Visual chromatophores

Photographs of the specimens preserved in the formalin and alcohol solutions are shown in Figs. 1 and 2, respectively. Mantle chromatophores preserved in phosphate-buffered formalin after 3 months were slightly visible and then showed considerable transparency after 12 months (Fig. 1-I). The specimens preserved in the borax-buffered formalin had translucent mantles after 3 months (Fig. 1-II). On the other hand, chromatophores of the specimens in the hexamine-buffered formalin were visible for nearly 12 months (Fig. 1-III). In all three alcohol preservatives, mantle chromatophores were also observed during those 12 months (Fig. 2).

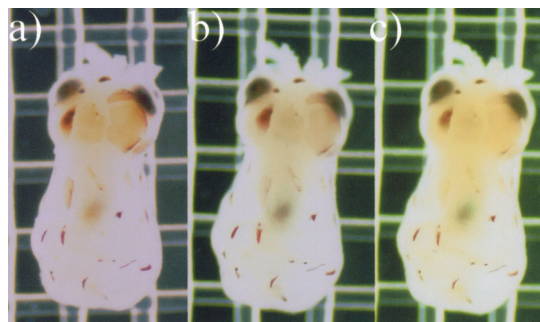
#### I. 5% phosphate-buffered formalin



#### II. 5% borax-buffered formalin



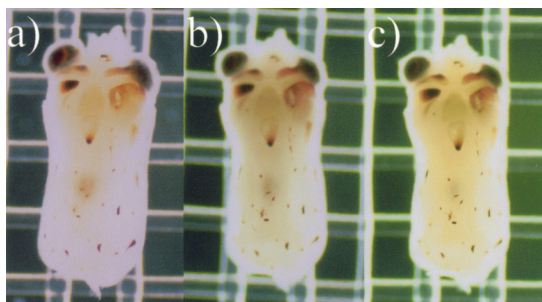
#### III. 5% hexamine-buffered formalin



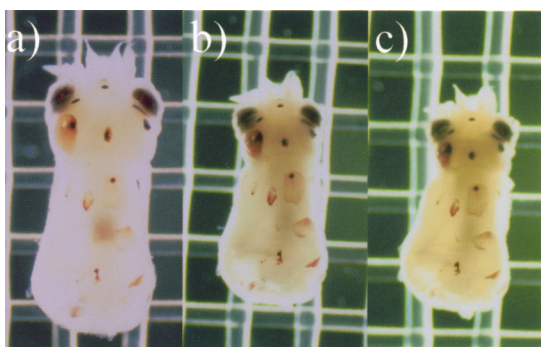
**Figure 1.** Photographs of the dorsal side of specimens preserved in formalin solutions at the time of capture (month 0) (a), 3 (b) and 12 (c) months after the start of the experiment. Dorsal mantle length at month 0: I. 2.2 mm, II. 2.7 mm, III. 2.5 mm.

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I. 99% ethanol



II. 70% ethanol



III. 40% isopropanol



**Figure 2.** Photographs of the dorsal side of specimens preserved in alcohol solutions at the time of capture (month 0) (a), 3 (b) and 12 (c) months after the start of the experiment. Dorsal mantle length at month 0: I. 2.8 mm, II. 2.6 mm, III. 3.3 mm.

### Change in mantle length

The relationships between percentage of initial mantle length and time in preservative are shown in Fig. 3 in formalin, Fig. 4 in alcohol. In the three formalin preservatives and 99% ethanol preservative (Figs 3 and 4-I), changes of the mantle lengths were not statistically significant during the experiment ( $F=2.43$  in phosphate,  $F=0.28$  in borax,  $F=0.71$  in hexamine when  $F_{0.05}=3.26$ , and  $F=1.85$  in 99% ethanol when  $F_{0.05}=3.01$ ). In the 70% ethanol (Fig. 4-II) time in preservative was a significant factor ( $F=42.34$ ,  $P<0.001$ ). However, changes in lengths after 3 months were not statistically significant ( $F=1.20$  when  $F_{0.05}=5.19$ ), meaning the serious shrinkage (ca 20%) occurred within the first 3 months. Moreover, time in the 40% isopropanol preservative (Fig. 4-III) was a significant factor ( $F=6.21$ ,  $P<0.01$ ). Tukey's HSD test indicated that differences of the mean length between 0 to 3 months and 12 months were statistically significant ( $q=6.21$  and  $5.17$ , respectively, when  $q_{0.05}=4.51$ ), suggesting the specimens gradually shrank over the 12 month period.

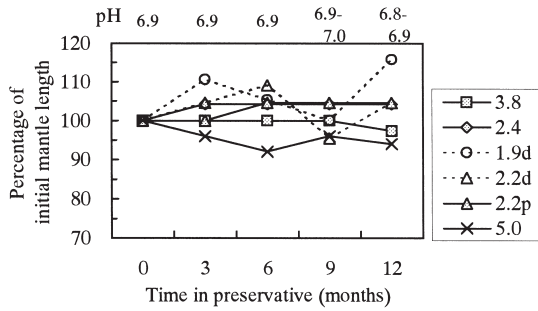
The pH ranges of formalin preservatives at each time were also indicated on the top of each graph in Fig. 3. The phosphate-buffered formalin neutralized during the course of the experiment and demonstrated small fluctuations (Fig. 3-I). In the borax-buffered formalin, the solution was alkaline with a pH of approximately 9.0 (Fig. 3-II). The pH of the hexamine-buffered formalin was a little higher than that of the phosphate-buffered formalin with a pH of 7.1–7.8 (Fig. 3-III).

### DISCUSSION

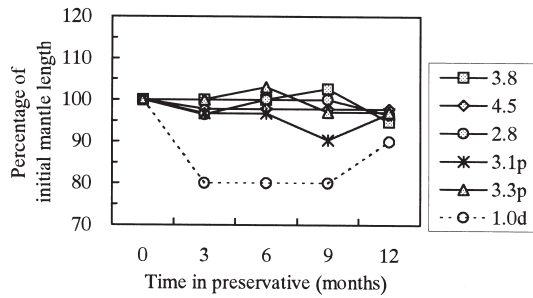
The results are summarized in Table 1. From the viewpoint of the maintenance of chromatophores and the mantle change, 5% hexamine-buffered formalin and 99% ethanol are suggested to be good preservatives.

The three kinds of formalin solution used in this study were separately made (see Materials and Methods), suggesting that each formalin was diverse and their quality was varied (Steedman, 1976). It is possible that this led to different

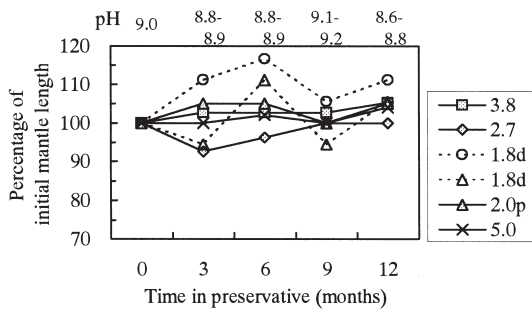
I. 5% phosphate-buffered formalin



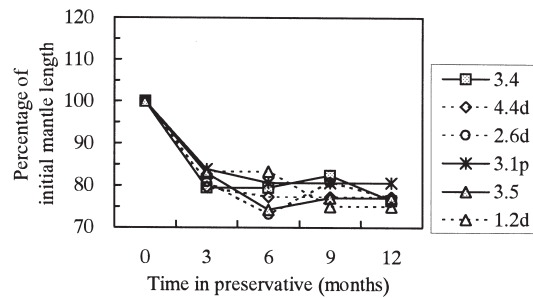
I. 99% ethanol



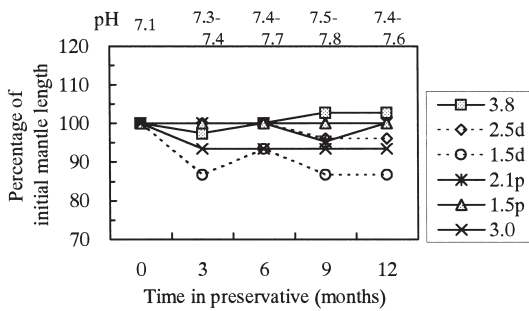
II. 5% borax-buffered formalin



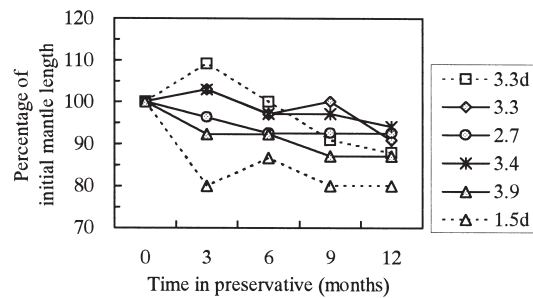
II. 70% ethanol



III. 5% hexamine-buffered formalin



III. 40% isopropanol



**Figure 3.** Relationships between percentage of initial mantle length and time in preservative consisting of 5% formalin solution. Numerals beside the legends indicate dorsal mantle length (mm) in the beginning of the experiment. d: specimens with distorted mantle, p: specimens with the head withdrawn into the mantle cavity.

**Figure 4.** Relationships between percentage of initial mantle length and time in preservative consisting of alcohol solution. Numerals beside the legends indicate dorsal mantle length (mm) in the beginning of the experiment. d: specimens with distorted mantle, p: specimens with the head withdrawn into the mantle cavity.

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Table 1. Summary of this study

Preservative	Chromatophores	Mantle	pH
<b>5% buffered formalin</b>			
Phosphate	invisible	unchanged	6.8–7.0
Borax	invisible	unchanged	8.6–9.2
Hexamine	visible	unchanged	7.1–7.8
<b>Alcohol</b>			
99% ethanol	visible	unchanged	-
70% ethanol	visible	shrunk	-
40% isopropanol	visible	shrunk slightly	-

reactions among these formalin solutions to specimens. Therefore, an additional experiment using specimens collected in 2001 started in the spring of 2002 where these three kinds of 5% buffered formalin were made from the same formalin. For 6 months, the chromatophore appearance had the same results (Goto, unpublished data).

Since hexamine may adversely affect the preservation of some zooplankton species (*e.g.* crustaceans), it has not been recommended as a reagent of buffered formalin (Steedman, 1976; Omori and Ikeda, 1992). Hexamine-buffered formalin certainly should not be used as a fixative of net samples containing various zooplankton species. Hexamine-buffered formalin solution might possibly be used as a preservative of certain taxon like paralarval squid in this study.

The phosphate-buffered formalin was reported to be a good preservative for ichthyoplankton (Markle, 1984; Lavenberg, *et al.*, 1984). This is why JSNFRRI used this preservative from 1992 to 1998. Specimens in the preservative were not as transparent when they were observed the next spring after sampling. At JSNFRRI when the preservative was changed to borax-buffered formalin in 1999, the author noted that the chromatophore pigment became invisible earlier than in the phosphate-buffered formalin. In this study, mantle chromatophores after 3 months were slightly visible in the phosphate-buffered formalin while already invisible in the borax-buffered formalin (Fig. 1). Phosphate-buffered formalin might delay the loss of paralarval squid pigment.

Specimens just after sorted out from plankton net samples had visible chromatophores. Borax-buffered formalin therefore would certainly be a good fixative of net samples including paralarval squid (*e.g.* Vecchione, 1978; Omori and Ikeda, 1992). Taylor (1977) pointed out that fish larvae fixed in borax-buffered formalin with distilled water diluent showed the greatest transparency. Sea-water as a diluent might be better, however further examination is needed.

High pH may lead to the loss of pigment in specimens of fish larvae (Taylor, 1977; Tucker and Chester, 1984). Borax-buffered formalin indicated the highest pH in this experiment (Fig. 3; Table 1). While high pH might account for mantle transparency, the pigment of specimens in phosphate-buffered formalin was almost lost in spite of neutrality in the preservative. In addition to pH, constituents of the pigment might relate to the transparency. It is important to understand the chemical reactions between pigment and preservatives / fixatives (Lavenberg, *et al.*, 1984). Loss of pigment is not only due to preservatives but also specimens' condition during capture and / or fixation (Markle, 1984; Harman and Young, 1985).

Shrinkage occurring during the time from capture to fixation has been reported in fish larvae (*e.g.* Cunningham *et al.*, 2000). This study demonstrated that shrinkage would occur in even fixed specimens when the specimens were preserved in 70% ethanol or 40% isopropanol. On the other hand, shrinkage in 99% ethanol did not occur. Shrinkage of a specimen in ethanol probably occurs based on the dehydration caused by the hydrophilic alcohol. When a higher

alcohol concentration is used as a preservative, greater shrinkage of a specimen would be expected to occur. The reason why the result was quite different is unknown. Since the number of individuals examined is not enough, further experiments for these preservatives are required. Even if this event is validated, experiments should be carried out on individual species because degree of shrinkage is considered to be species-specific (Jennings, 1991).

This study suggests that in the three kinds of formalin solution, 5% hexamine-buffered formalin may be the best preservative for *T. pacificus* paralarvae fixed with borax-buffered formalin-seawater solution. Further studies should be conducted based on the following questions, 1)

how long are paralarval pigments in this preservative retained?, 2) are there any other neutralizing reagents (Steedman, 1976; Tucker and Chester, 1984) for preserving squid paralarvae in formalin solution?

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