

An alternative method for the preservation of tropical fish larvae

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Preservation of newly hatched fish larvae in a 30% ethanol freshwater solution kept at -19°C allowed accurate measurement of morphology and otolith dimensions with minimal error due to shrinkage compared to other concentrations of ethanol or gluteraldehyde.

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Studies of larval processes often aim to explore a link between larval attributes associated with body condition or performance, food availability and larval abundance (Theilacker *et al.*, 1996; Bailey & Picquelle, 2002). Many of these studies involve the comparison of morphological traits, such as fish length and yolk-sac volume to otolith-derived characters, such as daily patterns of growth or fluctuating asymmetry, in an attempt to explore the mechanisms underlying population regulation (Hare & Cowen, 1994; Somarakis *et al.*, 1997; Groenkjaer & Schytte, 1999; Gronkjaer & Sand, 2003). To minimize *post mortem* degeneration, samples are usually preserved immediately upon capture. Aldehydes, such as formaldehyde solutions (formalin) and gluteraldehyde are effective for fixing and preserving body tissues for future examination, because they form stable cross-links with tissue molecules, thereby stopping metabolic processes (Kirkeby & Moe, 1968). They dissolve skeletal structures, however, including otoliths, preventing subsequent age and growth analyses. Because otolith breakdown is mainly due to the low pH often present in aldehyde solution (Steedman, 1976), the addition of a buffering agent such as simple borax or sodium carbonate can be used to keep the preserving solution nearly neutral or slightly basic, at least temporarily, and slow down the de-calcification process (Hay, 1981). If the solution is correctly buffered, it may be possible to use otoliths from specimens preserved in aldehydes (Suthers *et al.*, 1992; Kristoffersen & Gro Vea Salvanes, 1998). Preservation in buffered acid fixative such as formalin can be problematic, however, as many widely-used buffers damage larvae or ultimately allow the

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solution to become acid (Lavenberg *et al.*, 1984; Leis & McGrouther, 1994). Therefore, alcohol solutions such as 70 or 95% ethanol are most widely chosen as preservative for otoliths and other bones because they do not decalcify these structures. Unfortunately, they are unsuitable for stabilizing protein constituents and preventing degradation of the animal tissue. Further, both preservative methods cause significant and dissimilar shrinkage (Hay, 1982; Tucker & Chester, 1984; Kruse & Dalley, 1990; Hjörleifsson & Klein-Macphee, 1992; Kristoffersen & Gro Vea Salvanes, 1998; Fey, 1999), leading to biased estimates of trait distributions and their interrelationships if appropriate corrections are not made.

Ideally, the whole larval body should be preserved in a single solution suitable for the preservation of all traits, thereby minimizing correlative errors between different body measurements because of different preservation techniques. To address this issue, an alternative method was developed for the simultaneous preservation of body tissues and otoliths in individual larvae. First, a range of ethanol solutions were compared for efficiency in preserving larval body tissues and otoliths in the coral reef damselfish *Pomacentrus amboinensis* Bleeker. Secondly, to establish the quality of preservation of larval body tissues, the most effective ethanol solution was compared to 2.5% gluteraldehyde seawater solution (McCormick, 1998), which is routinely employed in electron microscopy (EM) examinations because it provides high quality of tissue preservation (Bowes & Cater, 1966). Finally, the extent of shrinkage of the most effective preservative solution was assessed over a 60 day period.

Newly hatched larvae originated from a natural breeding event in the lagoon at Lizard Island, on the northern Great Barrier Reef, Australia. A large clutch of *P. amboinensis* eggs, which had been spawned on artificial nesting substrata (McCormick, 1999) was collected prior to dusk on the night of hatching and placed in a well-aerated aquarium of flowing sea water at 29° C. At hatching, sub-samples of *c.* 100 hatched individuals were collected using a small hand net and a fine brush to avoid any damage directly associated to net abrasion (Theilacker, 1980), and immediately transferred into vials containing 10, 20, 30, 40 and 70% ethanol seawater solution, 10, 20, 30, 40 and 70% ethanol freshwater solution, and 2.5% gluteraldehyde seawater solution. All ethanol samples were stored in a freezer at -19° C, whereas the gluteraldehyde sample was stored in a fridge at 6° C. Twenty individuals were randomly selected from each preservation treatment and examined 3 months later. The quality of body tissue preservation, including standard length (L_S), yolk-sac and oil globule size was examined under a dissecting microscope, and otoliths examined under a compound microscope at $\times 40$ magnification. Individual traits were assigned to one of two distinct categories according to their state of preservation: easily measurable, all body tissue outlines clearly discernable (+), or not measurable (-). To test for significance between preserving solutions, the G test of independence was used.

Samples preserved in 10 and 20% ethanol diluted in both fresh water and sea water froze and were discarded because most body tissues had degenerated (in sea water) and otoliths were partially or completely dissolved (in fresh water). Preservation in 40 and 70% ethanol solution was found to be effective for otoliths but caused significant distortion of the body and ruptured the oil globule

making measurement of this trait impossible. The 30% ethanol solution in both fresh water and sea water was found to provide the best quality of preservation for all traits (G tests, $P > 0.05$), although the oil globule was measurable in a larger number of individuals preserved in 30% ethanol freshwater than the seawater solution ($G_{\text{adj}} = 6.641$, $P < 0.01$). The quality of body tissue preservation of larvae in the 30% ethanol freshwater solution was comparable to the preservation levels of individuals stored in the 2.5% gluteraldehyde seawater solution (G tests, $P > 0.05$; Fig. 1). As expected, however, gluteraldehyde completely dissolved all otoliths.

Having established that the 30% ethanol freshwater solution was the best for simultaneously preserving body tissues and otoliths, the extent of shrinkage in this solution was assessed over a 60 day period. To quantify the level of shrinkage, 10 live larvae originating from a second clutch were collected immediately after hatching and individually photographed under a dissecting microscope. Each individual larva was then transferred to a numbered vial containing 30% ethanol freshwater solution that had been kept at freezer temperature. All individual samples were stored at -19°C . The same 10 individuals were photographed after 1, 2, 3, 5, 10 and 60 days of preservation. All images of larvae were recorded against a scale bar and larval body dimensions (L_S , yolk-sac area and oil globule area) on these images were measured using image analysis software (Optimas 6.5). Analysis of these images showed that preservation in 30% ethanol freshwater solution caused no significant shrinkage for all body traits considered over the period examined (repeated measure ANOVAs, $P > 0.05$ for all traits; Fig. 2).

Using a cold 30% ethanol freshwater solution therefore is an easier, safer and more practical option to preserve larval samples than the traditional way of keeping part of the same sample in different solutions. The current experiment cannot distinguish between the effect of storage temperature and ethanol concentration, and whether this preservation technique may be appropriate for long-term storage (>3 months). It does conclusively show, however, that the method

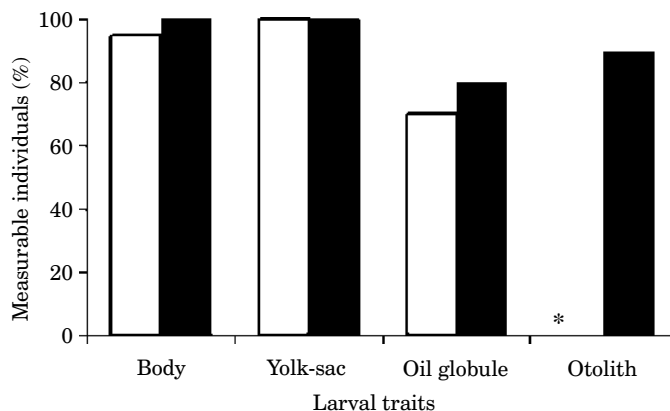


FIG. 1. Effect of 30% ethanol freshwater solution (■) and 2.5% gluteraldehyde seawater solution (□) on the preservation of larval traits of *Pomacentrus amboinensis*. *, significant difference ($P < 0.05$) in percentage of measurable individuals between the two treatments.

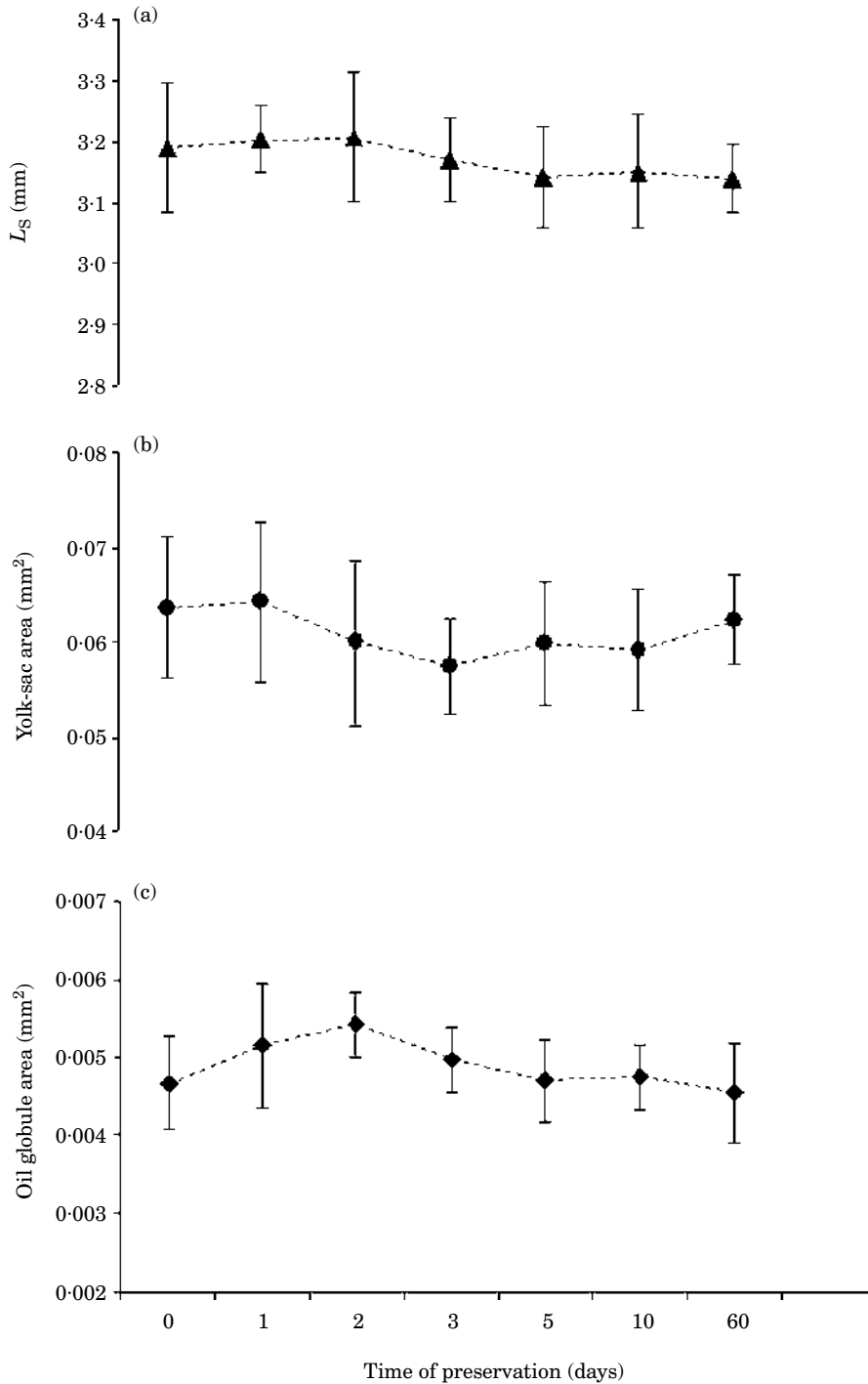


FIG. 2. Extent of shrinkage of (a) standard length (L_S), (b) yolk-sac area and (c) oil globule area of *Pomacentrus amboinensis* caused by preservation in 30% ethanol freshwater solution over a total of 60 days following hatching (day 0). Values are means \pm 95% CI.

presented here not only maintains the integrity of all tissue and skeletal structures within individual larvae, but it circumvents problems of underestimation or overestimation of traits distribution due to shrinkage (Dower *et al.*, 1997; Fey, 1999), a highly desirable characteristic particularly in field studies investigating early life-history traits and their influence on survival.

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