

Staging egg development of marine copepods with DAPI and PicoGreen®

Marnie Jo Zirbel, Charles B. Miller, and Harold P. Batchelder

College of Oceanic and Atmospheric Sciences, Oregon State University, 104 Ocean Admin Bldg, Corvallis, Oregon, USA 97331

Abstract

Calanoid copepod eggs have a robust, chitinous outer chorion, which makes field-collected, formaldehyde-fixed eggs difficult to penetrate with stains or molecular probes. Egg development studies in copepods have involved physical, chemical, and enzymatic treatments to remove the chorion. We present an efficient, one-step method for staining copepod eggs with the fluorescent nucleic acid stains DAPI and PicoGreen®. Nuclei in treated eggs are clearly visible for examination and counting with compound and confocal microscopy, so that eggs can be rapidly classified with respect to developmental stage. The method is effective for eggs of *Calanus*, *Metridia*, and *Centropages*. Both stains were effective after 24-h exposure for eggs that were fixed from several days to 8 years. Early stages are distinguished by complete counts of nuclei. A blastula phase and gastrulation are distinctive. *Calanus pacificus* and *Calanus marshallae* embryos spend more than half the development period in the later 'gray ball' and limb bud stages. Stage classification by this method is useful for studies of copepod egg mortality in the field.

Copepod embryonic development has been little studied, perhaps because unreported attempts encountered difficulty in staining and visualizing the interior structure. That is because the chitinous chorion of the zygotes (termed 'eggs' hereafter) is only permeable to very small molecules, especially after preservation, making staining difficult. One old but detailed study of early prehatching embryogenesis in *Calanus* was carried out by Grobbsen (1881, who used the name *Cetochilus septentrionalis* Goodsir). Grobbsen looked at living eggs, following their development only to relatively early stages. His drawings are reproduced in Marshall and Orr (1955). Grobbsen then jumped to examinations of early nauplii. Marshall and Orr (1955) also show micrographs of whole eggs at several stages of development, apparently photographed alive, although no method is mentioned. Indeed, it is possible in live eggs to see developmental details, including

cell nuclei, using several modes of microscope illumination. Fuchs (1914) carried out a remarkable study of development in *Cyclops viridis* using stained sections, characterizing development as strongly deterministic and similar in that respect to other arthropods. Fuchs did not mention the chorion and attributed difficulties examining living eggs to the large quantities of yolk. Recent and valuable studies of early divisions in cyclopoids have given particular attention to the phenomenon of chromatin diminution (e.g., Wyngaard and Gregory 2001 and references therein). Those have been done mostly by squash techniques. A possibly similar phenomenon occurs in *Pseudocalanus* sp. (Robins and McLaren 1982).

We are working to evaluate the mortality rates of eggs in the field by developing life tables of pre-hatching embryonic stages. Large numbers of preserved eggs must be rapidly prepared and rapidly staged. This requires intensive sampling at several times during day and night, fixing the samples and sorting eggs for staining at a later time. Earlier techniques were both complicated to apply and not effective for eggs that had been fixed for months to years. Once a freely spawned copepod egg is released into seawater, the chorion is laid down in sequential layers from vesicles in its surface cytoplasm (Blades-Eckelbarger and Youngbluth 1984), including an inner layer of chitin. Among species producing both resting and subitaneous eggs, the chitin layer is thicker in the former, as observed in *Centropages* (Blades-Eckelbarger and Marcus 1992), *Pontella* (Santella and Ianora 1992), and Diaptomids (Champeau 1970; Hairston and Olds 1984; Cuoc et al. 1994; Dharani and Altaff

mjzirbel@coas.oregonstate.edu

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2004). However, a chitin layer appears to be a general feature, at least in the calanidae. Glands in the posterior prosome of calanoid females, the ducts from which are tributary to the oviducts, secrete *N*-acetylglucosamine glycoconjugated proteins (Barthélémy et al. 2001), presumably the origin of the surface vesicles producing chitin (polymerized *N*-acetylglucosamine) in the chorion. Other crustaceans also have relatively elaborate fertilization envelopes, but many of them do not include a chitin layer: *Carcinus maenus* (a crab, Goudeau 1984), *Homarus gammarus* (lobster, Talbot and Goudeau 1988), and *Sicyonia ingentis* (a shrimp, Pillai and Clark 1988). However, crustaceans that hatch at relatively advanced stages of development may lay down one or more chitin layers (five in *H. gammarus*, Goudeau et al. 1990), equivalent to the exoskeletons of early molts, between the embryo and the original fertilization envelope.

We have worked mostly with several species of *Calanus*. Hirose et al. (1992) have shown that the chorion in *Calanus sinicus* is a complex structure of three main layers: chitin innermost, an apparently crystalline matrix next that is most likely protein, and an amorphous outer cover folded into ridges over the whole surface. After fixation with formaldehyde, the chorion becomes progressively more robust, probably as the fixative creates cross-linkages within the aminopolysaccharide matrix of the chitin (Steedman 1976). We have found that only very small molecules will pass the chorion, presumably water, oxygen, carbon dioxide, and ammonia. Hypochlorite molecules are too large, making eggs of *Calanus* impervious to tissue corrosion by bleach.

Romano et al. (2003) devised an intricate method to successfully stain nuclei in fixed *Calanus helgolandicus* eggs using an apoptosis-specific probe. The probe penetrated the chorion (briefly fixed with formaldehyde) after treatments with chitinase enzyme, buffer solutions, and rinsing. The technique of Romano et al. (2003), using TUNEL staining, produced impressive results. They took well-defined confocal micrographs of nuclei in eggs damaged by diatom-derived aldehydes. Our project required a more rapid and streamlined staining method, since we needed to stain thousands of undamaged eggs for robust estimates of stage proportions in field populations. Buttino et al. (2004) have shown that the chorion can be removed from live *C. helgolandicus* eggs with chitinase, leaving a bare but viable embryo suitable for vital staining. They obtained good pictures suitable for nuclear counts. This is not applicable to the field-collected, fixed eggs that must be stained in our investigations.

Our goals were rapid classification of developmental stages of field-collected copepod eggs after fixation for periods of days to years and comparisons of eggs from our study site with even older, globally collected, archival samples. We also needed to assay eggs reared in the laboratory to observe durations of development stages. We sought in particular the capability to visualize and count cell nuclei, which suggested the use of fluorescent nucleic acid stains like 4',6-diamidino-2-phenylindole

hydrochloride (DAPI) and PicoGreen®. To count numbers of nuclei in each egg, nuclei must remain intact, which prohibited the use of corrosive or physically damaging procedures. The requirement was to get stain through the hardened chorion and stain the egg nuclei without destroying the egg.

Materials and procedures

Staining method—Successful nuclear staining is obtained by creating an osmotic imbalance between internal egg tissue and the external staining medium. We use de-ionized (DI) water as a staining vehicle, which stresses the egg chorion enough to allow fluorescent stain molecules into the egg and nuclei, without bursting the egg or compromising nuclei. The chitinous chorion is very resilient, resisting this full osmotic challenge.

Copepod eggs are fixed in sodium tetraborate-buffered 4% formaldehyde in seawater. They are transferred by drawn glass Pasteur pipettes into depression slides containing 100 μ L of 5 μ g DAPI (mL DI water^{-1}), then covered with a coverslip. Fewer than 30 eggs are transferred per depression, to minimize the volume of transfer medium (ca. 30 μ L), for a final concentration of ca. 3.8 μ g DAPI mL^{-1} . For staining with PicoGreen (Molecular Probes), eggs are transferred into 75 μ L DI water with 10 μ L PicoGreen original stock solution in depression slides with a coverslip. Slides are kept in the dark and refrigerated overnight. Eggs are examined using epifluorescence microscopy after 24 h.

Field collection—Eggs for field observations were collected in Dabob Bay, Washington, USA (47° 45'N, 122° 50'W) during spring and summer 2003 and 2004. Eggs of *Calanus pacificus*, *Calanus marshallae*, *Metridia pacifica*, and *Centropages abdominalis*, all of which are distinctive, were sorted from 73 μ m mesh, vertical, opening-closing, ring-net tows through six discrete sections of the water column, from 150 m to the surface. Eggs were also collected with MEGAPUMP, a pump-based sampling system with a nine-filter carousel and equipped with CTD and flowmeter in the stream beyond its filtering cones. It filters about 2 $\text{m}^3 \text{min}^{-1}$. This sampler will be described elsewhere. MEGAPUMP samples were collected with 73 μ m mesh, nylon-filtering cones. Preservation of plankton was with buffered 4% formaldehyde. Samples collected by pump in 1998 by the U.S. GLOBEC Georges Bank program were provided by Edward Durbin of University of Rhode Island, and samples taken in 1997 with vertical nets at Station M in the Norwegian Sea (Niehoff et al. 1999) were provided by Hans-Jürgen Hirche and Barbara Niehoff of Alfred-Wegener Institute, Bremerhaven. We sorted eggs of *Calanus finmarchicus* from both of these sets of formaldehyde-preserved samples.

Laboratory observations—For determining the durations of readily discerned embryonic stages (1-, 2-, 4-cell, and so on) live *Calanus pacificus* females were collected 20 km offshore near Newport, Oregon, USA from double net (bongo) tows and 1 m ring-net tows from 100 m to the surface. Clutches of eggs ($n = 72$ clutches) were harvested from females, and time

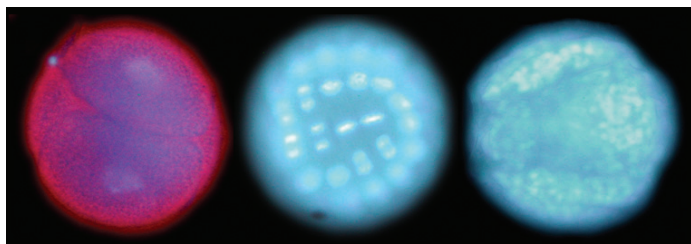


Fig. 1. Left to right: two cells, 64 cells, and limb bud stage *Metridia* sp. eggs. These eggs were field-collected, fixed, DAPI stained, and digitally imaged with an epifluorescence compound microscope. Some computer enhancement was applied to the 2-cell egg, but it is not required for routine laboratory evaluations. Eggs of *Metridia* sp. are approximately 160 μm in size.

of spawning was recorded with 2-h resolution. Eggs were incubated at 12°C, then preserved in buffered 5% formaldehyde at 2 h intervals until hatching. These were stained by the osmotic introduction of DAPI.

Microscopy—Compound microscopy was used for assessing stages of larger quantities of eggs during experiments. Eggs were fixed and DAPI stained as above, and viewed at 25 \times with UV excitation with a Zeiss epifluorescence compound microscope. Higher resolution images were made with a Zeiss laser confocal microscope (OSU Center for Gene Research & Biotechnology; Environmental & Health Sciences Center) with UV excitation at 405 nm for DAPI. For confocal images of PicoGreen-stained eggs, we used UV (405 nm) and argon laser excitation at 488 nm.

Assessment

The staining method is successful for eggs in all developmental stages and for field-collected samples as old as 8 years. Both DAPI and PicoGreen stains are effective in eggs after exposure for 24 h. Nuclei within eggs at all development stages are clearly visible with epifluorescence microscopy (Fig. 1). Eggs were classified into 11 stage categories: 1 cell, 2, 4, 8, 16, 32, 64, and 128 cells, blastula (cells arranged in many rows), gray ball (gastrulation and a period afterward), and limb bud. In 1-cell eggs, a bright spot of fluorescence is visible on the cell surface, presumably the polar body. One-celled eggs are uncommon in the field (< 2%), and some of the few examples could be unfertilized eggs with staining of the single female “pronucleus” (Ianora et al. 1999). In eight-cell and later stages, not all nuclei are visible at one focal plane (Fig. 1, middle image). The osmotic influx of the staining method does not change the shape of the eggs or burst the chorion. The detection limit for the DAPI concentration is ca. 3.5 μg DAPI mL^{-1} . At ca. 1.9 μg DAPI mL^{-1} , nuclear staining was patchy, and boundaries around nuclei were difficult to resolve.

Laser confocal microscopy revealed DAPI-stained nuclei in *C. pacificus* eggs at 461 nm emission, in addition to the external chitinous chorion (490 nm autofluorescence), visible with a longpass filter (Fig. 2). The PicoGreen-stained nuclei (523 nm emission) and intricately reticulated chorion (490 nm aut-

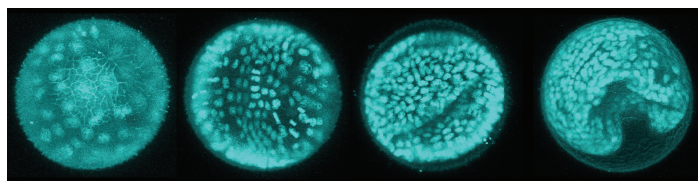


Fig. 2. Confocal images of 128-cell, blastula and late gastrula stages (left to right) of *Calanus pacificus*. The last shows the archenteron clearly. These eggs were reared in the laboratory and fixed before staining. The DAPI-stained nuclei (461 nm emission) within eggs and the external chitinous chorion (490 nm autofluorescence) are visible with a longpass filter. *Calanus pacificus* eggs are approximately 180 μm in size.

ofluorescence) were both visible with the longpass filter (Fig. 3). We could visualize optical slices through half the egg diameter with the confocal microscope.

The method successfully stains field-collected eggs of *Calanus pacificus*, *Calanus marshallae*, *Calanus finmarchicus*, *Metridia pacifica*, and *Centropages abdominalis*. Use of the stain in laboratory development rate studies was successful for both *C. pacificus* and *C. marshallae* and for *Centropages*. For eggs of *C. pacificus* and *C. marshallae*, development was examined at various intervals up to hatching for sets of ≥ 10 eggs from the same clutches. Throughout development, all eggs of any given clutch remained synchronous. Time to hatch in *C. pacificus* was 30 ± 2 h at 10–12°C. Embryos of *C. pacificus* and *C. marshallae* spent more than half of the development time in the later, gray ball and limb bud stages (Fig. 4). This result will be replaced soon by more precise data from new, still incomplete experiments.

The method works for eggs in archived samples that have been stored in seawater-formaldehyde solution for years. This allows comparisons between oceans and expands the reach of hypothesis testing. For example, we were able to compare development stage proportions of *Calanus pacificus* eggs at our current study site in Dabob Bay, Washington, to those of *C.*

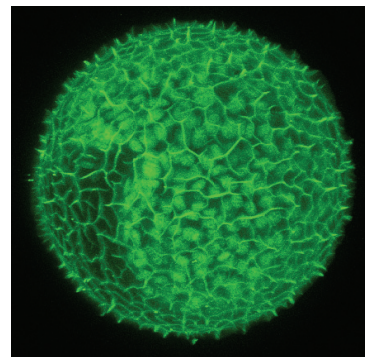


Fig. 3. PicoGreen in a *Calanus pacificus* egg. This egg was field-collected, fixed, stained, and imaged with confocal microscopy, with UV (405 nm) and argon laser excitation at 488 nm. The PicoGreen stained nuclei (523 nm emission) and intricately reticulated chorion (490 nm autofluorescence) are visible with the longpass filter.

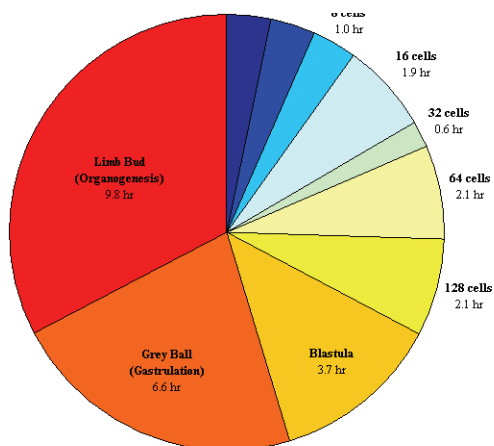


Fig. 4. Approximate observed development stage durations for *Calanus pacificus* eggs at 10-12°C, based on 72 clutches divided into groups of > 10 eggs and variously assigned to killing times.

finmarchicus eggs in 8-year-old Atlantic samples taken at Station M and Georges Bank (Fig. 5). Sharply defined nuclei are still present in eggs preserved in formaldehyde for 8 years. Results will be presented elsewhere.

Discussion

Staining by osmotic stretching of the chorion allows both field observations for copepod egg mortality assessment and laboratory observations for timing developmental stages. It enables rapid classification of developmental stages of several

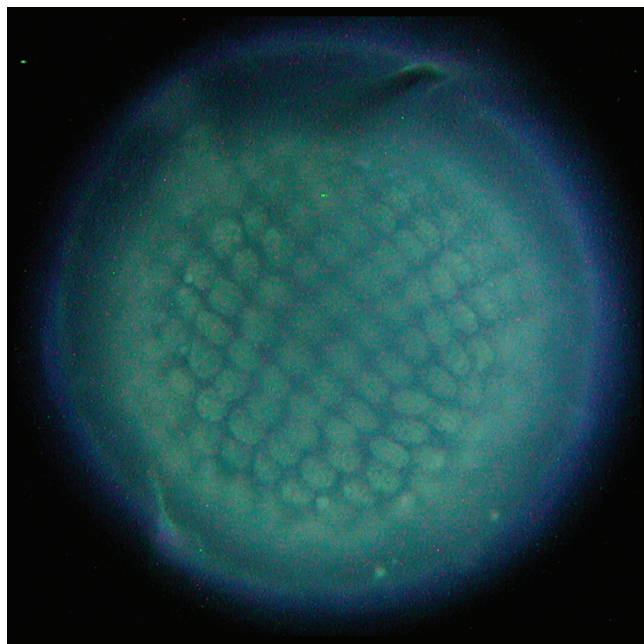


Fig. 5. Blastula stage *Calanus finmarchicus* egg collected and formaldehyde fixed 24 March 1998 on Georges Bank. The egg was sorted, stained, and examined with compound epifluorescence microscopy in February 2006 using the method described here. Contrast and brightness were adjusted for publication.

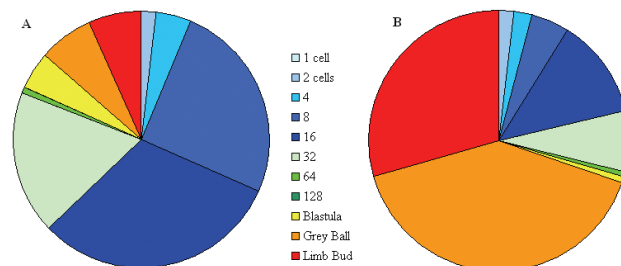


Fig. 6. Age proportions for eggs within 0-100 m of the water column in Dabob Bay, Washington, at 07:00 hour on (A) 23 April 2003 and (B) 24 April 2003. Eggs ($n > 200$ for each profile) were collected, sorted, stained, and assayed for development stage as described above.

species of fixed copepod eggs. The staining procedure involves minimal, simple steps. Egg nuclei can be visualized in 24 h; the time efficiency of the method is mainly limited by the speed at which the operator can sort eggs from field samples.

This method is an integral part of an ongoing field study of copepod egg mortality. For eggs of *Calanus* at temperatures cold enough ($\sim 12^\circ\text{C}$) that time to hatch is greater than 24 h, field samples collected in the post-dawn hours (e.g., 07:00) will include eggs spawned the previous night (eggs in early developmental stages) and eggs from the night before (eggs in late stages). High fractions of the latter imply low mortality, while low fractions imply high mortality. Proportions of stages through the water column of Dabob Bay from the surface to 100 m for two different sample dates (Fig. 6) show that *apparent* mortality can be high (very few eggs reaching late stages) or low (many eggs reaching late stages). Of course, these comparisons say nothing about the sources of mortality (e.g., predation, infertility, and toxic effects transferred from the mother's diet). Extensive results will be reported elsewhere.

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