A Technique on the Culture and Preservation of Marine Copepod Eggs

M. Kaviyarasan and P. Santhanam

Introduction

The search for the ideal copepod for marine fish larvae that can be cultured intensively is ongoing. Copepods are nutritionally suitable for marine fish larvae (Sargent et al. 1997; Stottrup 2000) and constitute a large percentage of the diet in the natural environment (Hunter 1981; Munk and Nielson 1994). Moreover copepods are way too higher in nutritional composition when compared to the traditional live feeds such as *Artemia* nauplii and rotifers. But it is difficult to culture copepod at sufficient densities to be economically efficient on a commercial scale, because they require high water volumes for cultivation in captivity (Esmaeili and Amiri 2011). Even though more than 12,000 species of copepods have been identified and classified (Humes 1994), a few species only are being cultured for the purpose of rearing fish larvae. Out of ten orders of copepods, only three orders, viz., Calanoida, Harpacticoida, and Cyclopoida, are being cultured widely around the world. Among these, the calanoid species receives much attention due to their abundance in pelagic waters and ease of culture in controlled environments. Apart from this, many calanoid copepods are capable of releasing free eggs unlike the cyclopoid and harpacticoid copepods which release their nauplii from the egg sacs itself. The production of diapause eggs has been recorded in many calanoid species during abnormal environmental conditions. These diapause eggs can undergo a long period of metabolic arrest until the environment turns to favorable conditions. This fact aids in the storage of diapause eggs for a longer period and could be used for culture when needed.

In order to meet the demand of hatcheries for large quantities of copepods at one time, mass-scale production techniques and viable methods for long-term cold storage of copepod eggs must be defined. Both subitaneous and diapause eggs have been investigated for storage and use in aquaculture. Diapause eggs are produced

M. Kaviyarasan · P. Santhanam (✉)
Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

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when environmental conditions signal the possibility of long-term changes; thus they have hardened shells. However, dormancy can be induced in subitaneous eggs if they are exposed to unfavorable environmental conditions, viz., salinity changes or low temperatures, etc. (Uye and Fleminger 1976). Such eggs can be preserved using different preservation techniques for many months and used as an inoculums for copepod culture. This chapter deals with some of the effective and proven methods of preservation of copepod eggs (Table 1).

Methods

The following are the criteria for selection of copepod species for egg storage:

- Dominant and cultivable species in lab scale
- Type of spawning (free spawner/egg carrying)
- High fecundity (eggs per female)
- Registered production of resting eggs
- Short generation time

Copepod Culture

Culture of calanoid copepods is quite simple, once proper environmental and food conditions are met. To start a culture, advanced copepodites and adult copepods are to be stocked at a ratio of 50 individuals per liter of filtered seawater with salinity of 30 psu and temperature around 25 ± 1 °C with a photoperiod of 12:12 h LD. The copepods should be fed with suitable microalgae at a rate of 25,000 cells/ml daily. Care should be taken in siphoning to remove detritus and sufficient water volume to allow addition of the new feed volume every day. Male calanoid copepods make use of their antennae to grasp the female for mating. So avoid rough handling (mostly vigorous aeration) which may result in damaging the antennae and similarly causes decline in the population. Adults will begin producing eggs in 9–12 days and release the eggs freely in the water column.

Egg Collection

The eggs can be collected by siphoning from the bottom of the container and rinsed through sieves with mesh size of 100 and 70 μm. After collecting the new eggs, they should be filtrated and washed thoroughly to remove all sorts of waste particles such as decomposed algae, feces, ciliates, nauplii, and exoskeletons of copepods. Before going for any further experiments on preservation of copepod eggs, the collected

Table 1 List of calanoid copepod species known to produce resting eggs (Marcus Engel 2005)

Species	Location	References
Acartia adriatica	Porto Cesareo, Lonic Sea, Italy	Belmonte (1997)
A. bifilosa	Southampton, Southampton Water, UK	Castro-Longoria (1999)
	StorfjÃ¤rden Baltic Sea, Finland	Katajisto et al. (1998)
A. californiensis	Yaquina Bay, Pacific Ocean, USA	Johnson (1980)
A. clausi	Aquaculture enclosures, W-Norway	Naess (1996)
	Aquaculture enclosures, N-Norway	Naess (1996)
	Pacific Ocean, CA, USA	Marcus (1990)
	Inland Sea of Japan, Japan	Uye et al. (1979)
	Mission Bay, Pacific Ocean, USA	Uye and Fleminger (1976)
A. erythraea	Inland Sea of Japan, Japan	Uye et al. (1979)
A. grani	Malaga Harbour, Mediterranean, Spain	Guerrero and Rodriguez (1998)
A. hudsonica	Pettaquamscutt Estuary, USA	Marcus et al. (1994)
	Narragansett Bay, Atlantic Ocean, USA	Sullivan and McManus (1986)
A. italica	Porto Cesareo, Lonic Sea, Italy	Belmonte (1997)
A. josephinae	Porto Cesareo, Lonic Sea, Italy	Belmonte and Puce (1994)
	Otranto, Adriatic Sea, Italy	Belmonte and Puce (1994)
A. latisetosa	Adriatic Sea, Italy	Belmonte (1992)
A. pacifica	Inland Sea of Japan, Japan	Uye (1985)
A. plumosa	Inland Sea of Japan, Japan	Uye (1985)
A. spinicauda	Xiamen, Taiwan Strait, China	Chen and Li, unpublished in: Marcus (1996)
A. steueri	Onagawa Bay, Pacific Ocean, Japan	Uye (1980)
A. teclae	Aquaculture enclosures, W-Norway	Naess (1996)

(continued)

Table 1 (continued)

Species	Location	References
A. tonsa	Tampa Bay, Gulf of Mexico, USA	Suderman and Marcus (2002)
	Southampton, Southampton Water, UK	Castro-Longoria (2001)
	StorfjÃ¤rden Baltic Sea, Finland	Katajisto et al. (1998)
	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Schlei Fjord, Baltic Sea, Germany	Madhupratap et al. (1996)
	Pacific Ocean, CA, USA	Marcus (1990)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
	Narragansett Bay, Atlantic Ocean, USA	Sullivan and McManus (1986)
	La Jolla, Pacific Ocean, USA	Uyeancl Fleminger (1976)
A. tsuensis	Inland Sea of Japan, Japan	Uye (1985)
Anomalacera pafersoni	Gulf of Naples, Mediterranean, Italy	Lanora and Santella (1991)
A. ornata	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
Boeckella hamata	Lake Waihola, South Island, NZ	Hall and Burns (2001)
Calanopia americana	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
C. thompsoni	Inland Sea of Japan, Japan	Uye et al. (1979)
Centropages abdominalis	Inland Sea of Japan, Japan	Uye et al. (1979)
C. furcatus	Alligator Harbor, Gulf of Mexico, USA	Marcus, 1989
C. hamatus	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Kiel Bay, Baltic Sea, Germany	Madhupratap et al. (1996)
	Aquaculture enclosures, W-Norway	Naess (1996)
	S-North Sea	Lindley (1990)
	Drogheda, Irish Sea, Ireland	Lindley (1990)
	Margate, English Channel, England	Lindley (1990)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
	White Sea, Russia	Perzova (1974)
C. *ponticus*	Black Sea	Sazhina (1968)

(continued)

Table 1 (continued)

Species	Location	References
C. *typicus*	S-North Sea	Lindley (1990)
C. *yamadai*	Inland Sea of Japan, Japan	Uye et al. (1979)
C. velificatus	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
Epilabidocera longipedata *E. amphitrites*	Pacific Ocean, CA, USA	Marcus (1990)
	Yaquina Bay, Pacific Ocean, USA	Johnson (1980)
Eurytemora americana	Pettaquamscutt Estuary, USA	Marcus et al. (1994)
E. affinis	Norrbyn, Baltic Sea, Sweden	Albertsson arid Leonardsson (2000)
	StorfjÃ¤rden Baltic Sea, Finland	Katajisto et al. (1998)
	Schlei Fjord, Baltic Sea, Germany	Madhupratap et al. (1996)
	Aquaculture enclosures, W-Norway	Naess (1996)
	Aquaculture enclosures, N-Norway	Naess (1996)
	Pettaquamscutt Estuary, USA	Marcus et al. (1994)
	Yaquina Bay, Pacific Ocean, USA	Johnson (1980)
E. pacifica	Onagawa Bay, Pacific Ocean, Japan	Uye (1985)
E. velox	Brackish water lake, SE-France	Champeau (1970)
Gippslandia estuarina	Hopkins River Estuary, Victoria, AUS	Newton and Mitchell (1999)
Gladioferens pectinatus	Lake Waihola, South Island, NZ	Hall and Burns (2001)
Labidocera aestiva	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
	Woods Hole, Atlantic Ocean, USA	Lawson and Grice (1976)
L. bipinnata	Inland Sea of Japan, Japan	Uye et al. (1979)
L. trispinosa	La Jolla, Pacific Ocean, USA	Uye (1985)
L. scotti	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
L. wollastoni	S-North Sea	Lindley (1990)
	Margate, English Channel, England	Lindley (1990)

(continued)

Table 1 (continued)

Species	Location	References
Pontella media	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Woods Hole, Atlantic Ocean, USA	Grice and Gibson (1977)
P. mediterranea	Gulf of Naples, Mediterranean, Italy	Santella and Ianora (1990)
	Cap Ferrat, Mediterranean, France	Grice and Gibson (1981)
	Black Sea	Sazhina (1968)
Sinocalanus tenellus	Fukuyama, Japan	Uye (1985)
Sulcanus conflictus	Hopkins River Estuary, Victoria, AUS	Newton and Mitchell (1999)
Temora longicornis	Menai Bridge, Irish Sea, UK	Castellani and Lucas (2003)
	Aquaculture enclosures, S-Norway	Naess (1996)
	S-North Sea	Lindley (1990)
	Margate, English Channel, England	Lindley (1990)
Tortanus derjugunii	Xiamen, Taiwan Strait, China	Chen and Li (1991)
T. discaudatus	Pacific Ocean, CA, USA	Marcus (1990)
T. forcipatus	Inland Sea of Japan, Japan	Kasahara et al. (1974)

eggs can be stored in normal saline water (30 psu) at 2 °C temperature for few days as *Acartia* non-diapause eggs can be stored for 2–6 weeks (Marcus and Wilcox 2007).

Counting of Eggs

A subsampling system is developed as following the method of Drillet (2010). After cleaning the eggs, they should be transferred into 500 ml seawater in a 500 ml plastic container. The container should be shaken for 15 s, and then after waiting for a few seconds to allow air bubbles to travel to the top of the container, the container should be reversed five times and a 10 ml subsample should be removed from the container by using a pipette. This should be repeated for five times, and the number of eggs in the subsamples can be counted. The average of the number of eggs in the subsamples can be calculated, and thereby the total number of eggs in the stock solution in the container could be estimated with a 10% deviation.

Cold Storage of Copepod Eggs

After collecting the eggs from the copepod culture vessels, the eggs should be cleaned and transferred into vials containing 1 μm filtered seawater adjusted to a salinity of 30 ppt. These vials should be stored in refrigerator at a constant temperature of 2–3 °C for predetermined time period (Hagemann 2011a). Before using the eggs for hatching experiments, the vials stored in refrigerator should be transferred to incubator to be heated to room temperature. Before the onset of incubation, a water exchange should be conducted in the vials to ensure that oxygen is present in the vials during the incubation, in case the oxygen had been depleted during the storage period. After the incubation, the eggs can be transferred from the vials to test tubes to carry out hatching experiments.

Cryopreservation of Copepod Eggs

Many experiments have shown that the copepod resting eggs can tolerate freezing down to −25 °C and that they are able to resist low temperatures (3–5 °C) for a longer period. The copepod resting eggs are generally obtained from sediments. They need to be cleaned properly prior to their use as inoculums. The copepod eggs collected from the culture vessels should be sieved through 150 μm and 70 μm sieves. For further cleaning of fraction-sized particles from copepod eggs, the eggs should be added to centrifuge tubes containing a 1:1 solution of sucrose and distilled water and centrifuged at 300 rpm for 5 min, and the supernatants should be washed through a double sieve of 100 μm and 70 μm. Then the copepod eggs should be immersed in the antibiotics such as kanamycin sulfate and oxytetracycline HCl (Drillet et al. 2007). The addition of antibiotics is to eliminate the microbial growth. Then the eggs should be washed with 1 μm filtered sterile seawater and transferred to storage vials and stored at determined temperature. In cryopreservation of copepod eggs, some of the most commonly used cryopreservants are methanol, glycerine, DMSO, ethylene glycol, and propylene glycol which are not toxic to the embryos. After preserving the eggs at determined temperature and time period, the eggs should be made ready for hatching by exposing the tubes containing eggs at room temperature for 10–20 min. Then the eggs should be carefully transferred to vials containing 30 ppt of filtered seawater. The eggs should be then allowed to hatch at room temperature (27 °C) with 12:12 h light-dark cycle with ambient light source.

Optimum Conditions for Preservation of Copepod Eggs

Salinity

The hypersaline water up to a concentration of 100 g/L has potential for short- and long-term storage of subitaneous *A. tonsa* eggs (Ohs et al. 2009). Andreas Hagemann (2011a, b) has also stated that hypersaline waters up to 50, 75, and 100 PSU resulted in 83, 85, and 76% of hatching success, respectively, when stored for up to 7.5 months. Holmstrup et al. (2006) observed that seawater with a salinity of 50‰ proved as a good cold storage media for periods up to 3 months. For short-term storage of copepod eggs, it is preferable to use 30‰ salinity, which could be effective for higher hatching rate.

Temperature

A decrease in the ambient temperature decreases the metabolic activities of an embryo. A study done by Drillet et al. (2005) showed that subitaneous *A. tonsa* eggs remained viable with a high hatching success for up to a period of 12 months of cold storage (2–3 °C) and that cold storage does not affect the reproductive capacity of the following generations. The developmental time from nauplii to adult copepods could increase slightly with the duration of cold storage (10 and 14 days for fresh and 12 months for cold stored eggs, respectively), most likely because the nauplii use up their energy reserves during cold storage and need to rebuild these after hatching before they can molt into the following naupliar stage.

Antibiotics

The application of antibiotics in copepod egg preservation has an important role as a disinfectant. Such antibiotics significantly increase the shelf life of stored eggs. However, exposure for a longer period or high concentrations of antibiotics would be lethal to the copepod eggs. The concentration of antibiotic should be adjusted depending on the period of cold storage planned. Drillet et al. (2007) have observed that antibiotics such as kanamycin sulfate and oxytetracycline HCl with glucose increased the shelf life of preserved eggs when compared to untreated eggs. He also stated that long storage periods with the use of antibiotics are likely to decrease the viability of the eggs and subsequent nauplii, which tend to become smaller after extended periods of cold storage.

Time Period

The duration of the storage period plays a significant role in copepod egg preservation as it directly affects the hatching rate of eggs when stored for a prolonged time (Drillet et al. 2011) in a study observed that the hatching success of copepod eggs increased during the first 5 months, from 0% to 83%, whereupon it decreased until no more hatching occurred after 12 months and also stated that eggs stored for more than 14 months showed no hatching. Many studies have suggested that for longer storage, fresh eggs could be more efficient than the older ones. After long storage periods, the eggs may not be able to hatch due to the lack of exploitable energy.

Cryopreservants

The recommended methods for cryopreservation of shrimp eggs may be similar for copepod eggs. The use of cryopreservants in cold storage of copepod eggs is very scarce. But cryopreservation of penaeid shrimp, *Penaeus japonicus*, embryos, naupliі, and zoea has been attempted by Gwo and Lin (1998) and Vuthiphandchai et al. (2005) and reported that methanol, glycerine, DMSO, ethylene glycol, and propylene glycol are shown not to be toxic to embryos, and the embryos remained viable following exposure to 0 °C. Ohs et al. (2009) observed that cryopreservants such as methanol, ethylene glycol, and glycerine at 5 M produced high viability of hatching in subitaneous eggs of *Acartia tonsa* at an exposure temperature of 4 °C. Propylene glycol and dimethyl sulfoxide produced high viability at 2 M at an exposure temperature of 4 °C.

Conclusion

Aquaculture of finfish and shellfish species has increased dramatically over the past few decades. However, many species generate larvae that are too small to be cultured using rotifer and *Artemia*-based hatchery technologies. After several decades of research, copepods are generally accepted to be of the appropriate size and nutritional value needed to support many of the challenging finfish and shellfish species through the critical first-feeding period. However, still the technology for copepods is at an early stage of development and requires significant advancement prior to widespread commercial implementation. In this state, an intensive culture and preservation of copepod resting eggs adopting suitable preservation techniques to use copepod resting eggs as an inoculum in hatcheries are greatly essential which ensure the sustainable aquaculture practices.

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