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Observation on the Embryonic Development of the Resting Eggs of Brine Shrimp Artemia using Artificial Decapsulation

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ABSTRACT

Brine shrimp Artemia *is an important model in biological and ecological studies. Unfortunately, the early development of the resting eggs ofArtemiacould not be easily observedbecause these resting eggs are covered with nontransparent shell. This study aims to investigate and describe the post-diapause development of* Artemia *through artificial decapsulation. Results showed that the outermost nontransparent shell of the resting eggs could be dissolved in hypochlorite solution. Thus, its embryonic development could be observed under a microscope. The embryonic resting eggs released from the maternal egg-capsules were in their blastula stage. Gastrulation occurred immediately after total rehydration under suitable hatching conditions. Three pairs of limb bud primordium started to differentiate during the limb bud stage. Two inner membrane naupliar stages, namely, naupliar and metanaupliar, were observed prior to hatching. The artificial decapsulation enabled the microscopic investigation of the postdiapause development of* Artemia.

Keywords: *early development;* Artemia; *resting egg; decapsulation; hypochlorite solution.*

INTRODUCTION

Artemia spp. (Crustacea, Branchiopoda, Anostraca), commonly known as brine shrimps, are small crustaceans that play an important role in saline waters worldwide. Brine shrimps are economically important in aquaculture as one of the major food sources for aquatic animals. *Artemia* nauplius hatched from the resting eggs is a suitable diet for the newly hatched larva of many economical species, whereas the adult *Artemia* is widely used as complete feed for animals, such as jellyfish and crustaceans (Bengtoson et al., 1991). In addition to its indispensable application in aquaculture, *Artemia* is also a historically popular biological model used in ecology, animal behavior, evolutionary ecology, and other fields (Ward Booth and Reiss, 1988; Abatzopoulos et al., 2002). The advantages of using *Artemia* in scientific research are as follows: (1) easy maintenance of a stable population in laboratory conditions, (2) available comprehensive background on the ecological and biological studies of *Artemia*, (3) requires simple food resources (e.g., unicellular algae), and (4) particular endpoints in experiments (i.e., hatching and reproduction Libralato et al., 2016; Kokkali et al., 2011).

Sexual reproduction and production of the resting eggs are adaptive strategies of animals to environmental stressors. Current studies reveal the sexual and parthenogenetic reproduction of different species with different evolutionary histories (Clark and Bowen, 1976; Bengton et al., 1991). Gonochoristic and parthenogenetic brine shrimps share the trait of producing nondiapause and diapause eggs (usually called resting eggs) (Abatzopoulos et al., 2002). Nondiapause eggs are small oosperms with thin shells. These eggs are either hatched until they reach the nauplius stage in an egg capsule or laid and hatched in the water. Resting eggs with relatively large size and thick shell can overcome cold weather and drought. Therefore, *Artemia* populations can thrive well in these conditions.

The commercialization, simple hatching, and incubation conditions (Lavens and Sorgeloos, 2000) of dehydrated resting eggs are conducive

to the study on phylogenesis of *Artemia*. However, the shell of the resting eggs is dense and nontransparent, causing difficulty in investigating its embryonic development. In this study, we investigate the early developmental stage of *Artemia* by shucking the shell using hypochlorite solution without affecting the embryo viability; this method adopted the protocol of Morris and Afzelius with some modifications (1967). The shell can be dissolved in hypochlorite solution, and the embryonic cuticle is hypochlorite resistant. The resting eggs were hatched in good condition and de-capsulated before investigation. The morphological changes during the early development of *Artemia* can be observed using the modified decapsulation method.

METHODS AND MATERIALS

Resting Eggs of *Artemia* and Hatching Condition

The resting eggs of *Artemia* sp. used in our study were obtained from Chengkou Salt works in Shang dong Province, China. This species is gonochoristic and can reproduce sexually. In accordance to previous experiments, we hatched the resting eggs in filtered seawater (30‰) at 25 °C at a light density of 30 µmol photons $m^{-2}s^{-1}$with 14 h/ 10 h light/dark cycle. These resting eggs were hatched in 500 ml beakers with 1000 eggs each. The hatching systems were run in triplicate.

Protocol to Shuck the Resting Eggs

Resting eggs of different *Artemia* species have different shell thicknesses. To determine the appropriate decapsulation time, we sampled the resting eggs at an interval of 1 min. The resting eggs were soaked in seawater for approximately 30 min to allow complete rehydration. Afterward, the resting eggs were removed from the decapsulation solution. Ten resting eggs were randomly sampled for1 min. The resting eggs were flushed with seawater immediately after sampling and were then observed under a light microscope. The decapsulation solution was prepared beforehand in a 50 ml centrifuge tube according to the following protocol: 0.26 g NaOH was dissolved in 16 ml of artificial seawater, and 10 ml of sodium hypochlorite solution with 10% active chlorine was added into the NaOH solution (Morris and Afzelius, 1967). The color of the shells gradually turned from dark brown to orange during de capsulation, indicating the complete dissolution of the dense outer shell. The decapsulation time was determined according to the results of microscopic inspection.

At each time point, we decapsulated the resting eggs of *Artemia* to microscopically inspect the developmental stages of *Artemia* embryos. Ten embryos were randomly selected from each beaker and were placed in the previously prepared decapsulation solution. After mixing the resting eggs in the decapsulation solution, the centrifuge tube was cooled under flushing water to avoid overheating. The decapsulated cysts were then filtered immediately and washed three times using artificial seawater.

Microscopic Observation and Measurement

Shucked *Artemia* resting eggs were investigated under an optical microscope (magnification of 200 ×, Nikon, Japan), and photographs were taken by the Imaging Formation System Nikon BR 4.40. The embryo size at each developmental stage and the newly hatched nauplius were also measured under this system.

RESULTS AND DISCUSSION

Using the decapsulation method, we investigated the post diapause development of *Artemia* sp. The decapsulation time was determined through sampling along a time gradient. The outermost, dense, nontransparent shell was dissolved in hypochlorite solution. The un-decapsulated resting eggs were dark and opaque (Fig. 1a), and no inclusion could be observed through the dense shell of the resting eggs. The shell started to dissolve immediately after being transferred into the hypochlorite solution (Fig. 1b). After 2 min of soaking, the remains of the shell contain edalveolates, and the dissolving shell exhibited an orange color (Fig. 1c). The shell was fragmented after being dissolved in hypochlorite solution for approximately 3 min (Fig. 1d). After 4 min, the shell was completely dissolved, and the homogeneous embryo was covered by the hypochlorite-tolerant embryonic cuticle (Fig. 1e). Although the embryonic cuticle could tolerate the hypochlorite solution, long-period soaking could still damage this cuticle. After soaking in hypochlorite solution for 8 min, the embryonic cuticle was destroyed, and the inclusion leaked out (Fig. 1f). Therefore, the decapsulation time should be within 4–8 min.

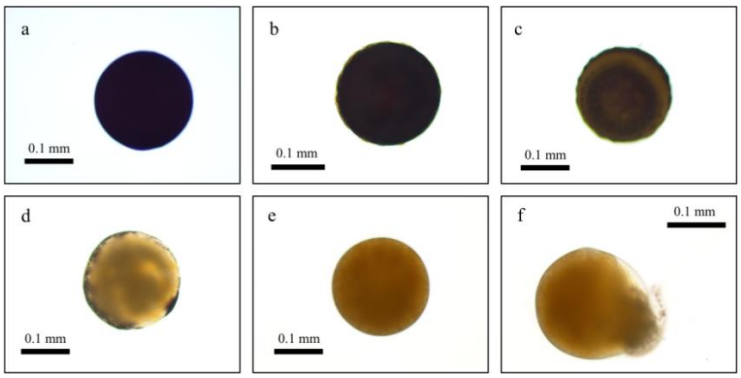

Figure1. *Microscopic photos for the decapsulation of the rehydrated resting eggs of* Artemia.

(a) Under capsulated resting eggs (control). (b–e) Resting eggs dissolved in hypochlorite solution for 1–4 min, separately. (f) Resting eggs dissolved in hypochlorite solution for 8 min.

In accordance to the previous experiment, we dissolved the resting eggs in hypochlorite solution for 5 min. Upon hatching in seawater, the red-blood-like resting eggs were immediately rehydrated and reverted back to their round shape (Figs. 2a and b). After 20 min, the resting eggs were completely rehydrated (Fig. 2c). The orange color indicated that the eggs were rich in yolk. The cleavage stage of *Artemia* embryonic development occurred in the maternal egg capsule (Benesch, 1969). *Artemia* showed complete egg cleavage with an equal distribution of yolk of blastomeres, and hollow spherical blastula formed at the 512-cell stage. Gastrulation occurred when the resting eggs were rehydrated after a diapause period. Thus, the embryonic development of resting eggs stopped during blastula stage when the resting eggs were laid out from the maternal egg capsule. After being totally rehydrated, the embryos started their gastrulation stage (Nakanishi, 1962).

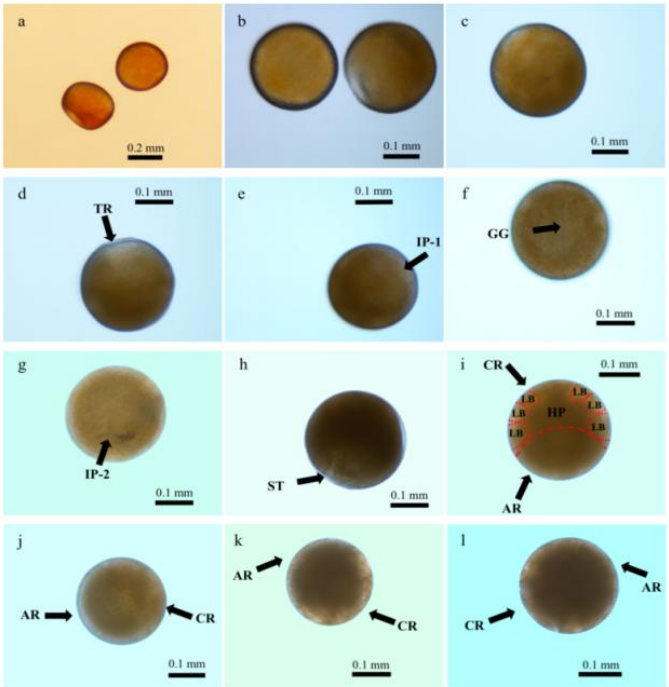

Figure2. *Microscopic photos of the early development of the resting eggs of* Artemia *decapsulated before observation.*

(a) *Dry resting eggs (0 min after hatching). (b) Resting eggs began to rehydrate (10 min). (c) Resting eggs totally rehydrated (20 min). (d–f) Early gastrula stage (2 h). (g–h) Late gastrula stage (4 h). (i–l) Limb bud stage (8 h).*

TR, transparent region; IP-1, first invagination pole; GG, gastrula groove; IP-2, second invagination pole; ST, stomodeum; CR, cephalotharax region; AR, abdomen region.

Gastrulation occurred for approximately 4 h after the embryos were rehydrated. After gastrulation, the external form of embryos began to change. The embryos can be divided into two regions, namely, cephalothorax and abdomen (Fig. 2i, the two regions were separated by short dashed lines). The abdomen in microscopic photos showed relatively darker color than the cephalothorax. This period is called the limb bud stage because of the limb bud primordium (Fig. 2i, inner dotted lines). The head primordium usually lies at the middle of the three pairs of limb buds in crustaceans. Cephalothorax, abdomen, and limb buds can be clearly differentiated from each other during the limb bud stage (Figs. 2i -l and Fig. 3a). During the limb bud stage that lasted for approximately 3–5 h, the *Artemia* embryos advanced into the early nauplius stage inside the egg membrane.

Nauplius and metanauplius are the two main stages of naupliar development that occur before hatching. Almost all crustaceans show naupliar stage in their early development (Müller et al., 2004; Addis et al., 2007). Species with intramembrane nauplius and three pairs of appendages but without body segment are called six-limb larvae. Different from those in the limb bud stage, the embryos at naupliar stage experience complex organ differentiation. A single eye spot was found in the forepart of the cephalothorax (Fig. 3c) at approximately 13 h to 14 h after hatching. According to a previous study (Benesch, 1969), cell mitoses were active in this stage, and the ectoderm cells arranged into muscles, mandible, ganglia, tritocerebrum, protocerebrum, and other organs. The abdomen started to constrict from the junction between the abdomen and cephalothorax (Figs. 3b–f).

The abdomen kept elongating during the metanaupliar stage (Figs. 3g–i). The second limb bud was differentiated into two parts, namely, endopodite and exopodite (Fig. 3h). Bristles could be observed in the terminal regions of appendages (Fig. 3h, red arrow indicated the bristle of the third appendage).

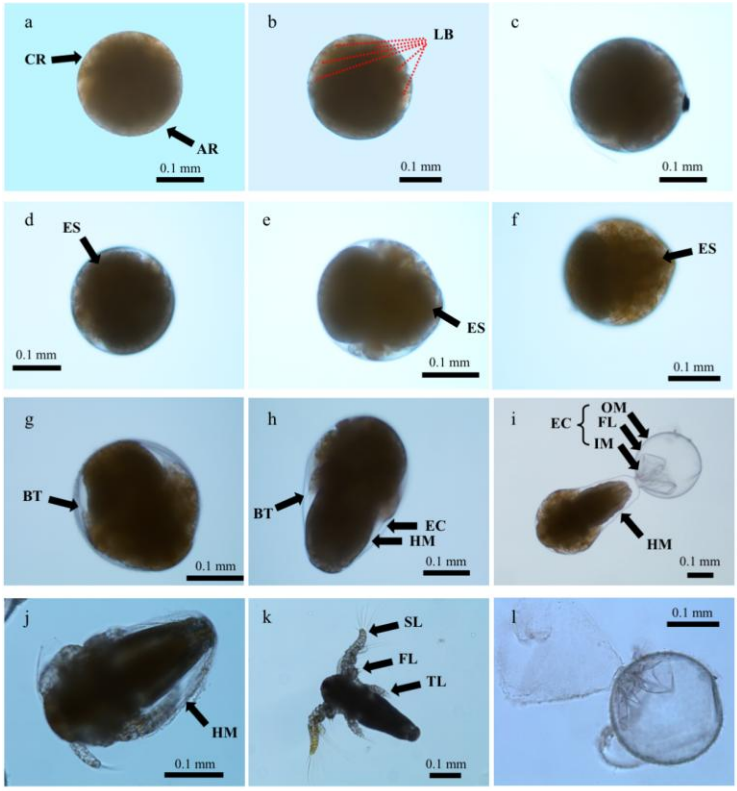

Figure3. *Microscopic photos of the early development of the resting eggs of* Artemia *decapsulated before observation.*

(a) Limb bud stage (9 h). (b–f) Naupliar stage (16 h). (g–i) Metanaupliar stage (22 h). (j) Nauplius was hatching out from the hatching membrane (23 h). (k) Free-swimming nauplius. (l) Embryonic membranes after hatching.

LB, limb bud; ES, eye spot; BT, bristle; EC, embryonic membrane; HM, hatching membrane; OM, outer cuticular membrane; FL, fibrous layer of the embryonic cuticle; IM, inner cuticular membrane; FL, first pair of limb buds; SL, second pair of limb buds; TL, third pair of limb buds.

In this stage, the nauplius could be seen rotating inside the embryonic membrane. In addition to the outer cuticular shell of resting eggs, the trilaminar embryonic membranes were observed as described by Morris and Afzelius (1967). Embryonic membranes generally contain four layers (Fig. 3i). The outermost membrane of the decapsulated resting eggs was the outer cuticular membrane (OM), which was thin and transparent (Wang and Sun, 2007). Clinging to the OM was the fibrous layer of the embryonic cuticle. When the larva emerged from the shell like an umbrella, a crumpled layer, called inner cuticular membrane, was found at the broken edge of the shell. This membrane was the innermost layer of the shell. These three layers formed the embryonic cuticle of resting eggs. Given that the embryonic cuticle was flexible, the elongated embryonic cuticle (Fig. 3) would revert back to its spherical shape after the larva emerged from the shell. The larva remained enclosed in a membrane, which was named hatching membrane (Figs. 3i and j). The movement of the inner-membrane larva was frequent, and the limbs continuously waved (see the Supplementary Section video: Inner-membrane nauplius). Approximately 3 h was required until the larva broke the hatching membrane. Afterward, the free-swimming nauplius hatched (Fig. 3k). The three pairs of limb buds could be clearly distinguished from each other. Different from the metanaupliar stage of other species in Branchiopoda, the inner-membrane metanauplius of *Artemia* did not exhibit differentiation of other trunk limbs (Kotov and Boikova). Hatching membranes were broken with one side attached to the embryonic cuticle. Each layer of the membranes could be clearly distinguished in Fig. 3l. The early inner-membrane development was terminated when the free-swimming nauplius hatched. The entire hatching occurred in less than 24 h in our hatching conditions.

In this study, early development could be observed after terminating the diapause of the resting eggs of *Artemia* through artificial decapsulation using hypochlorite solution. The embryonic development of the inner membrane of *Artemia* could be divided into six major stages, namely, cleavage, blastula, gastrula, limb bud, naupliar, and metanaupliar. The first two stages transpired inside the maternal egg-capsule, and the rest occurred in the water. Artificial decapsulation of the shell enabled the observation of embryonic development.

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