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Cyst development in the conchostracan shrimp, *Eulimnadia texana* (Crustacea: Spinicaudata)

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Abstract

The fertilized egg (or cyst) of branchiopods is a highly resistant stage in the life cycle of these aquatic crustaceans. Previous examinations of these cysts have determined that early embryonic development arrests at a late blastula stage, resulting in a small, crescent-shaped body within the egg shell of these shrimp. Herein, we examine the early development of these embryos by sectioning eggs in the ovotestis, brood chamber, and several time periods after exit from the brood chamber in the clam shrimp *Eulimnadia texana* Packard. The early sections find no evidence of internal fertilization in the ovotestis. Eggs in the ovotestis showed no signs of cell division, whereas eggs sectioned from the brood chamber were found to be undergoing early embryonic development. A number of empty egg shells and the lack of unfertilized eggs in the brood chamber suggested that egg yolks quickly degrade after egg extrusion from the ovotestis. Cysts that were allowed to develop for 24, 48, 72, and 96 h, 1 week and 1.5 years were sectioned, and embryonic development did not change after the 48 h time period. Thus, embryos appear to arrest development somewhere between 24 and 48 h after exiting the brood chamber.

Introduction

Most branchiopod crustaceans are typified by the production of very resistant, resting-stage eggs (or cysts). The cysts are integral to the life cycle of these shrimp because it allows for the drying (or freezing) of the pools in which these aquatic crustaceans inhabit, and then 'recolonization' of the pools when they refill. Nowhere is this more important than in the desert dwelling conchostracan branchiopods in the genus *Eulimnadia*. These 'clam shrimp' produce dozens to hundreds of cysts per day (Weeks et al., 1997), and then bury them within the top centimeter of soil (Knoll, 1995) at the bottom of the temporary pools in which they live (the deserts of the southwestern U.S.). The cysts then await a drying period, after which they will hatch upon rehydration, given the appropriate levels of temperature, light, and oxygen within the pool (Brendonck, 1996).

Recent genetic and histological evidence suggests that *Eulimnadia* populations consist of mixtures of males and self-compatible hermaphrodites (Sassaman & Weeks, 1993; Zucker et al., 1997). The exact mechanism of fertilization, whether by outcrossing or by self-fertilization, remains to be discovered. What is known is that the ovotestis of the hermaphrodites is comprised primarily of ovarian tissue, with a small, posterior portion devoted to testicular tissue (Zucker et al., 1997). Currently, it is unknown if fertilization is internal or external within the hermaphrodite's brood chamber (area between the body and the carapace in which the hermaphrodites retain their eggs for up to 24 h before burying them in the soil), although preliminary observations suggest that fertilization occurs in the brood chamber (Zucker et al., 2001).

In a thorough investigation of clam shrimp cysts, Belk (1987) examined the morphology of *Eulimnadia antlei* Mackin cysts and noted the following structures. The cyst is coated with a thick tertiary membrane,

which reduces embryo mortality due to mechanical damage and damaging sunlight (Belk, 1970). Beneath this membrane is an inelastic outer membrane, or 'embryonic cuticle 1' (EC1). When the cyst is rehydrated, the embryo forms a second, elastic membrane that directly surrounds the embryo ('hatching membrane' or EC2). The embryo itself is a crescent-shaped body that is arrested at the blastula stage (~4100 cells in *Artemia*; Nakanishi et al., 1962), and then develops into a prenauplius and then a nauplius within the first 24 h after rehydration (Belk, 1987). Upon rehydration, the tertiary membrane swells and ruptures, releasing the prenauplius larva surrounded by both EC1 and EC2 membranes. The prenauplius continues to develop, and after 1–3 h breaks through EC1. After an additional 0.5 – 2 h, the prenauplius completes its development into a nauplius, finally breaks free of EC2, and immediately begins swimming (Belk, 1987).

In the current study, we wish to expand on Belk's (1987) description of the development of *Eulimnadia* cysts. Some of the questions that remain are: (1) Is the presence of a crescent-shaped body within a cyst always indicative of a developing embryo, or can an unfertilized egg also appear with a crescent-shaped body (i.e. yolk)? (2) What is the period of development before the embryo arrests after the eggs have been fertilized? (3) Is there any evidence of embryo development within the ovotestis of a hermaphrodite (suggesting internal fertilization), or is cell division limited to the brood chamber? We address these questions using nucleic acid stains of sectioned whole *E. texana* and their cysts at different stages of development.

Materials and methods

Shrimp were reared and eggs were collected in the laboratory by hydrating soil containing cysts in 37 l aquaria under 'standard' rearing conditions (Sassaman & Weeks, 1993; Weeks et al., 1997). The soil was collected from two temporary ponds: one in New Mexico (JT4) located on the USDA experimental range within Doña Ana Co. (south-central New Mexico), and one site in Arizona (WAL; previously reported as 'Portal 1' by Sassaman, 1989) near Portal in Cochise County., near the base of the Chiricahua mountains. Two classes of eggs were studied in this experiment: eggs within a hermaphrodite (in the ovotestis and brood chamber) and eggs that were shed from a hermaphrodite's brood chamber, and then allowed to further develop for 24, 48, 72, and 96 h, 1 week, and 1.5 years after shedding. For the shed eggs, egg-bearing hermaphrodites were isolated from the rearing aquaria into 500 ml plastic cups and allowed to drop one clutch of eggs. Because these hermaphrodites were in rearing aquaria, the eggs could have been fertilized by a male or self fertilized.

For the sections of the eggs at different developmental stages, eggs were collected from a single day's production from 22 hermaphrodites from the two populations (6 from JT4 and 16 from WAL). Eggs were then placed in a glass vial with water for 24, 48, 72, and 96 h, and 1 week. Eggs for the 1.5 years treatment were only from WAL, and were from vials containing lifetime accumulations of eggs from several hermaphrodites. After the developmental period in the vials, eggs were fixed in formalin for 48 h prior to embedding in plastic. Eggs were rinsed with distilled water and then dehydrated through a graded series of alcohols before being embedded in plastic polymer (glycolmethacrylate). Thin sections (3–4 μm) were obtained from these embedded samples using a JB-4 microtome. The sections were then stained with 4,6-Diamidino-2-phenylindol (DAPI) and placed in a humid chamber in a 60 °C oven for 1 h.

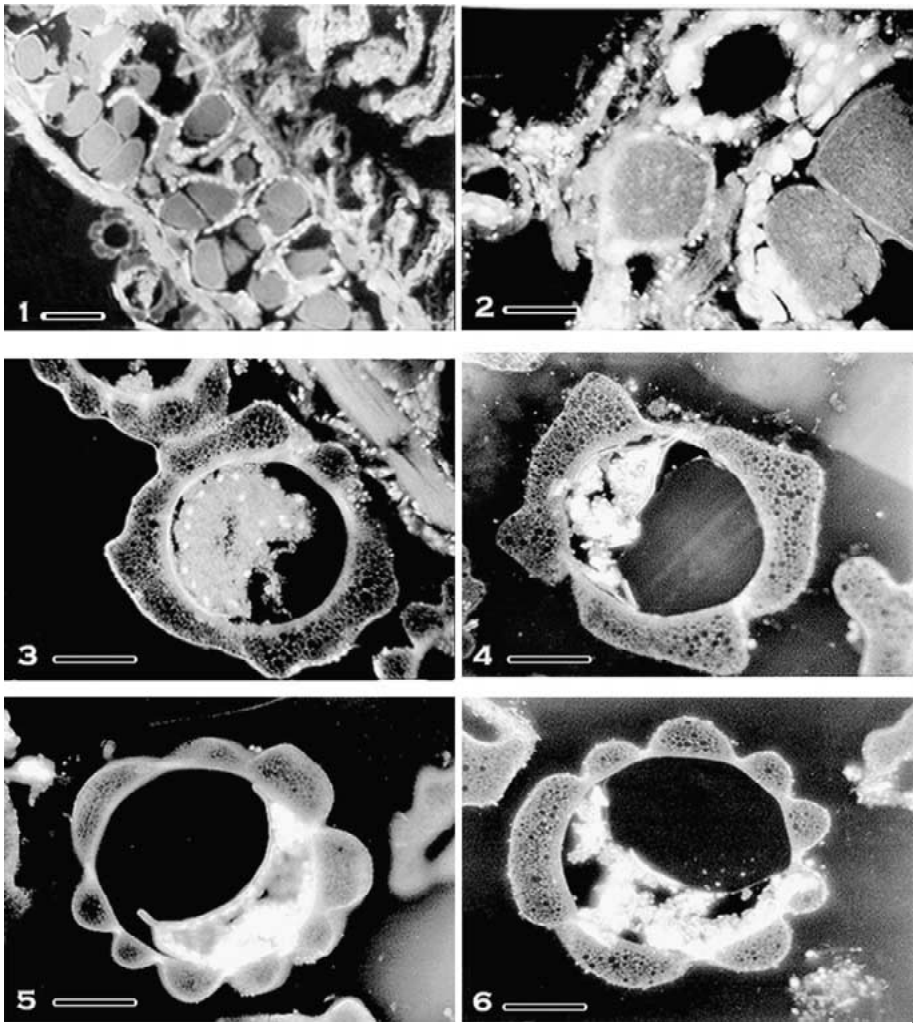
For cross sections of whole clam shrimp, 101 8-day old shrimp (50 from JT4 and 51 from WAL) were fixed in Bouin's fixative for 24 h. They were then put through 4 rinses of 75% ethanol and then processed and embedded in paraffin. Sagittal sections were cut at 6 μm. DAPI was then applied directly to the paraffin sections, and they were placed in a humid chamber in a 60 °C oven for 1 h.

For general staining of whole cysts, recently shed cysts were collected from rearing cups and placed on a slide with a few drops of DAPI solution and a cover slip. The slides were then placed in a humidity chamber in a 60 °C oven for approximately 2 h to allow the DAPI to infiltrate the eggs.

Observations of the eggs and cysts were made using a Zeiss standard microscope equipped with epifluorescence. Photographs were taken using a Nikon 35 mm camera loaded with Kodak 160 Ektachrome film and mounted on a Nikon UFX-II photo unit.

Results

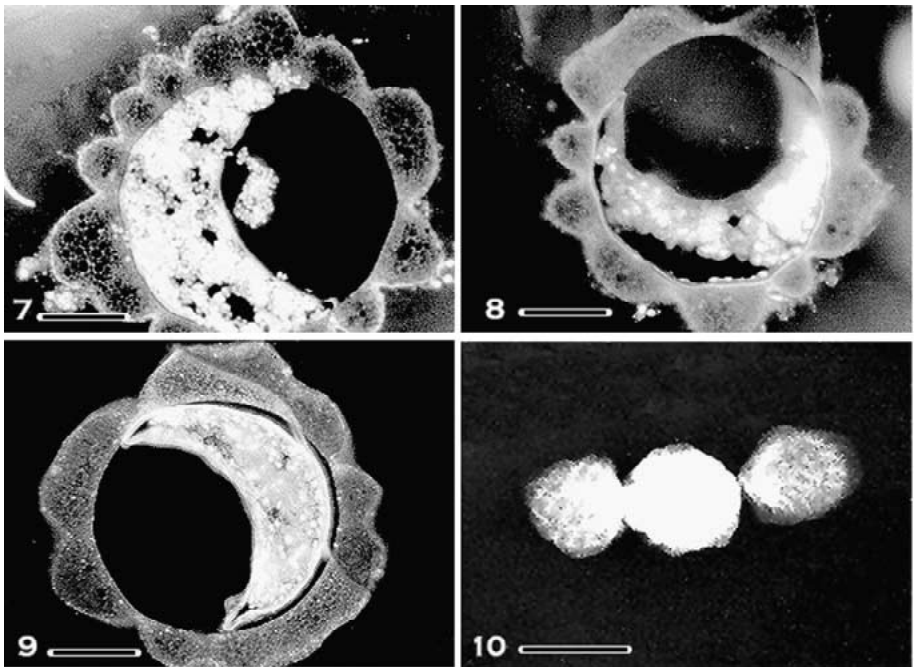
Yolked eggs within the ovotestis never showed signs of embryonic development (Fig. 1). Nurse cells surrounding the eggs were clearly nucleated, but none



Figures 1–6. (1) Lateral section of whole hermaphrodite showing yolking eggs in the ovotestis and shelled eggs in the brood chamber (scale bar = 200 μm). (2) Close-up of yolking eggs in the ovotestis (scale bar = 200 μm). (3) Cross section of developing cyst in the brood chamber (scale bar = 50 μm). (4) Cross section of developing cyst 24 h after release from the brood chamber (scale bar = 50 μm). (5) Cross section of developing cyst 48 h after release from the brood chamber (scale bar = 50 μm). (6) Cross section of developing cyst 72 h after release from the brood chamber (scale bar = 50 μm).

of the developing eggs showed any signs of the cell division indicative of early embryonic cleavage (Figs 1 and 2). Eggs within the brood chamber (from zero to 24 h of age; Fig. 3) showed early signs of development, with a ring of nuclei surrounding the outer portion of the embryo. At this stage, the embryos had not quite attained their 'crescent' shapes. Within 24 h after the eggs were dropped by the hermaphrodites, the embryos had developed the crescent shape, and were typified by numerous nuclei (Fig. 4). Since there were no significant changes in shape of the embryos or number of nuclei from the 24 h through 1.5 year cysts (Figs 5–9), it appears that the embryos reach their arrested stage of development within the first 24 h after being released from the brood chamber,

No cysts were found to contain crescent-shaped bodies that were not multinucleated. General DAPI stains of batches of eggs clearly revealed eggs with developing embryos (with crescent-shaped, brightly staining bodies) and empty eggs, which were presumably not fertilized (Fig. 10). Thus, it appears that egg yolks in unfertilized eggs quickly degrade, leaving empty egg shells (tertiary membranes).



Figures 7–10. (7) Cross section of developing cyst 96 h after release from the brood chamber (scale bar = 50 μm). (8) Cross section of developing cyst one week after release from the brood chamber (scale bar = 50 μm). (9) Cross section of developing cyst 1.5 years after release from the brood chamber (scale bar = 50 μm). (10) Three DAPI stained whole cysts freshly released from brood chamber. The two outer cysts contain no embryo, while the middle cyst has a distinctly crescent-shaped embryo (scale bar = 200 μm).

Discussion

To address the first of the three questions posed in this study, we conclude that the crescent-shaped body within the cyst tertiary membrane is truly indicative of a fertilized egg, as initially suggested by Belk (1987), and therefore it cannot be confused with unfertilized yolk. In the first 24 h after the cysts were released from the brood chamber (which could be between 36 and 48 h after emerging from the gonopore), cysts were either empty, or were found to contain crescent-shaped embryos with multiple nuclei. No egg after emerging from the gonopore was observed to contain enucleated yolk. Thus, the yolk within unfertilized eggs must degrade within the first 24–48 h after emerging from the ovotestis. This observation is consistent with another study (Hutchison, 1999) in which the tertiary membrane was dissolved away from one week old cysts (using a bleach solution; Belk, 1977). Two categories of cysts were found in this study: empty egg shells and cysts containing "opaque, almost black masses when viewed using the light microscope" (Hutchison, 1999). Therefore, one can calculate fertilization rates by simply comparing the proportion of cysts that are empty to those containing the crescent-shaped bodies (i.e. embryos).

The time series of cross sections of the cysts allows us to address the second question: when is embryonic development arrested in *E. texana*? The presence of nuclei in the embryos suggests that cell division is arrested within the first 24 h after the cysts are released from the brood chamber. All of the sections of developing embryos, beyond those from cysts in the brood chamber, showed similar patterns of multiple nuclei in the crescent-shaped embryos. In *Artemia salina*, embryological development arrests at the blastula stage, with ~4100 cells in the arrested embryo (Nakanishi et al., 1962). Interestingly, Nakanishi et al. (1962) did not find any increase in cell number between the arrested blastula stage and the naupliar stage after rehydration. The 4100 cells merely differentiate to produce the stage 0 nauplius, which then undergoes cell multiplication when the nauplius is free-swimming. Thus, it appears that embryological development arrests early in branchiopod development, quite possibly in the brood chamber before the fertilized eggs are released to the environment.

Finally, the sections of whole hermaphroditic shrimp allowed us to address the third question: is

there any evidence of embryonic development within the ovotestis of the hermaphrodites? The eggs in the ovotestis revealed a much different pattern from those in the brood chamber, or at any time after being released from the brood chamber. They clearly showed enucleated bodies with high yolk stores. None of these eggs showed any signs of early cell division, indicative of fertilization. Thus, there was no histological evidence for internal fertilization.

However, we cannot discount internal fertilization, since the lag time between fertilization and cell division may be great enough that such division cannot be detected until the eggs enter the brood chamber. Strenth (1977: 209) reported the presence of "small bodies attached in the area of the 10th or 11th pair of swimming appendages" on the 'females' (which we now know are actually hermaphrodites), which were 'yellow to orange in coloration and generally amorphous in shape.' These bodies stained positive for nucleic acid, and therefore Strenth believed them to be spermatophores. After the males deposit these spermatophores on the female, it is unclear from Strenth's description what occurs next. He states that the spermatophores "begin to move into the area just below and somewhat lateral to the posterior region of the digestive tract" (Strenth, 1977: 210). It is unclear whether 'into the area' suggests into the reproductive tract via the gonopore, or into the food groove between the phyllopod appendages, thus remaining exterior to the body. The former would suggest the potential for sperm storage, which has not been found in this species (Weeks et al., 2000). The latter would suggest that the spermatophores release sperm into the food groove, which is then combined with the eggs that are released from the gonopore. This alternate view is consistent with other behavioral observations of paired males and hermaphrodites, which suggest that males release sperm directly into the bivalved carapace rather than injecting sperm directly into the gonopore (Zucker et al., 2001). Additionally, *E. texana* males that are paired with hermaphrodites during egg extrusion have an eight-fold higher outcrossing rate than males paired before egg extrusion (Marquette et al., 2000), which also suggests that sperm fertilize eggs directly following egg extrusion.

Strenth (1977: 210) also suggested that once the spermatophores release their sperm, they leave behind "a structure which remains attached to the swimming appendage". He suggests that this structure can be used to identify outcrossed relative to selfed hermaphrodites. A similar mechanism for fertilization has been described in a related, gonochoric clam shrimp, *Leptestheria dahalacensis* (Scanabissi & Mondini, 2000): a viscous, sticky mass containing numerous sperm is 'glued' to the female's epipodites during mating. At some point, the spermatozoa are released from the spermatophore and somehow make it into the genital pore and the ovary (Scanabissi & Mondini, 2000). No evidence of such a structure has been observed in *E. texana*, neither in this study nor in a previous behavioral study (wherein mating behavior was filmed and viewed in slow motion, Zucker et al., 2001). Additionally Knoll (1995) did not report seeing any spermatophore-like structures in her studies. Thus, although there is precedence for spermatophores in clam shrimp, no one since Strenth has recognized them in *E. texana*.

All of these observations suggest that egg fertilization is external rather than internal. A problem with assuming egg fertilization is external is that the tertiary membrane of the egg is laid down in the lumen of the ovotestis (Zaffagnini, 1969; Zucker et al., 1997). Thus, if fertilization is not internal, sperm would need to pass through this egg shell to fertilize the eggs. Scanabissi & Mondini (2002) have apparently solved this quandary. They have evidence that in *Limnadia lenticularis*, sperm can penetrate the egg shell to fertilize the eggs after the egg shell has been laid down. In *L. lenticularis*, males are extremely rare and all eggs are self fertilized. Self sperm enters the egg shell in the ovotestis (Scanabissi & Mondini, 2002) but final fertilization does not occur until the eggs are in the brood chamber (Zaffagnini, 1969). Because of the lack of males, we have no evidence of external fertilization in this related clam shrimp.

In *Eulimnadia texana*, the circumstantial evidence suggests that male egg fertilization occurs immediately after egg extrusion or in the brood chamber. Future studies should concentrate on identification of spermatophores in males, and possibly marking male sperm and noting its location on or in mated hermaphrodites.

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