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Quantitative Investigations of Hatching in Brine Shrimp Cysts

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Introduction

Hatching brine shrimp (*Artemia*) cysts in salt water is a popular demonstration in introductory biology classes. However, despite the fact that *Artemia* cysts are readily available and inexpensive, they are rarely utilized for controlled and quantitative investigation in student labs (Ward-Booth and Reiss, 1988). Presumably, this is because of presumed or perceived difficulties in handling and counting cysts. Indeed, the cysts are light-weight, tiny (about 0.25 mm diameter), and very susceptible to mechanical damage (crushing). Furthermore, it is difficult to follow the developmental progress of loose collections of cysts, especially if there is any slight movement of water. Lastly, if an overabundance of cysts is placed together in an unaerated container to observe mass hatching, the typical result is rapid and premature “crashing” of the newly hatched brine shrimp population.



Novel protocols described here circumvent all these difficulties by (1) permitting easy handling of cysts and precise quantification of their hatching success, (2) enabling clear viewing of development and hatching in individual cysts, and (3) promoting conservative use of cysts as well as prolonged survival of hatched larvae in the absence of aeration.

These new protocols use small numbers of dry cysts (actually encysted, gastrula-stage embryos) that are literally painted onto an adhesive patch for precise counting and subsequent viewing under immersion. Importantly, subsequent development and hatching are unimpaired by adhesion to the patch, and cysts may be easily observed at low-power within a single focal plane. Daily counts of emerged nauplius larvae yield reliable hatching curves for experimental and control groups. A myriad of open-ended investigations are now possible using environmentally relevant variables (*i.e.*, heat, light, cold, salinity, radiation, oxygenation, re-hydration, or pollutant exposure). It is difficult to imagine a simpler, cheaper, and more reliable method for engaging students in inquiry-based, quantitative investigations involving multiple experimental variables.

Background

Cryptobiosis is a state of ‘suspended animation’ induced by environmental adversity. An example is the cryptobiotic state of arrested embryonic development in the North American brine shrimp, *Artemia franciscana*, a species that resides in the Great Salt Lake and similar saline environments (Dennis, 1996; Eriksen and Belk, 1999). Prior to release by the female, developing *Artemia* embryos are encysted in a protective capsule. During the short time that fertilized eggs are retained in the brood sac, egg development proceeds rapidly through cleavage and blastula stages. Eggs are then deposited in the environment where they remain encysted, with embryonic development arrested at the early gastrula stage. At this stage, there are about 4,000 highly organized cells in the embryo, but no organs are discernible. These encysted embryos are capable of long-term survival even when subjected to environmental extremes, such as prolonged dehydration or sub-freezing temperatures. Cysts in a cryptobiotic state remain viable for many years or even decades.

When dried cysts are exposed to more favorable conditions (rehydration), eggs swell and embryonic development rapidly resumes. Hatching of nauplius-stage larvae typically occurs 1-2 days after hydration at room temperature. See **Appendix A** for additional details related to *Artemia* development and life history.

Materials

- 10 cm-diameter, disposable, plastic Petri dishes
- 1-2 liters of ASW = artificial sea water [*Recommendations*: Mix ASW at a concentration = 36 g sea salt/liter of spring water. About 20 ml of ASW will be used per Petri dish. Avoid exposing brine shrimp to chlorinated water which is toxic.]
- double-stick tape [*Recommendation*: Due to excellent water resistance properties, use only 3M Scotch brand double-stick tape.]
- marking pen
- small, watercolor-type paint brush with soft, camel's hair bristles [*Recommendation*: Brush bristles size should be about 1 mm wide and 6 mm long]
- dried brine shrimp cysts [*Recommendations*: There are many commercial sources of brine shrimp cysts. (e.g., see: www.aqualink.com/marine/z-atemia.html). To insure freshness and viability, purchase cysts in a small quantity from a source that frequently 'turns over' their stock. To prolong shelf-life, store cysts in a capped container in a cool, dry place such as a refrigerator.]
- scissors
- small forceps
- small-bore plastic pipets [www.eeob.iastate.edu/faculty/DrewesC/htdocs/toolbox-III.htm]
- hand-held metal paper punch [*Recommendation*: It is important that the punch will easily and cleanly punch a round hole in the acetate sheet; see Method A.]
- clear acetate transparency sheets [*Recommendation*: Due to potential toxicity, avoid using transparency material that has a rough, emulsion-like coating on one side. The transparency material should be perfectly clear. If cysts will be painted onto photocopied grid (Method B), make sure the transparency material is compatible with your photocopy machine.]
- dissecting microscope [*Recommendation*: Although viewing under a dissecting microscope is highly recommended, it is sometimes possible to view cysts with a compound microscope equipped with low-power or scanning objectives.]
- microscope illumination [*Recommendation*: It is critical not to expose hatching cysts or larval shrimp to over-heating. Sub-stage illuminators built into many dissecting microscopes may cause rapid over-heating of viewed specimens. In such cases, reduce heat transfer by placing an inverted, empty Petri dish as a 'spacer' between the microscope stage and specimen dish.]
- food for larval and adult *Artemia* [*Recommendation*: Dry brine shrimp food (*Artemia* Food Special Blend; catalog #BS-16; 1.0 lb/\$29) supports growth from nauplius to adult and is sold by *Aquatic Ecosystems* (www.aquaticceco.com). This company also sells *Spirulina* powdered algae that supports growth from 1-week-old larvae to adult (powdered *Spirulina* Algae Feast, catalog #: SP1; 1 lb/\$25). Algae may be fed to other phytophagous aquatic invertebrates, such as snails, daphnids, aquatic oligochaetes, etc.]
- photocopies of **Appendix B** and **Appendix C**; clear transparencies of **Appendix D**.

Preparations and Procedures

- (1) Make sure your hands and the paint brush are clean and completely dry.
- (2) Use the scissors to carefully trim the extreme tips of the paint brush bristles so that the bristles are squared off at the end.
- (3) Select Method A or Method B (below) for affixing cysts to a sticky surface for viewing.

Method A – Cysts attached to a sticky circle

- (4a) Use a forceps to affix a 2 cm length of double-stick tape to the bottom of a plastic Petri dish. Avoid making fingerprints on the tape.
- (5a) Use a scissors to cut out a 2 cm x 4 cm rectangular strip of clear acetate transparency. Then, use the paper punch to punch a round hole in the center of the transparency strip.
- (6a) Carefully place the punched strip over the tape strip in the bottom of the dish (see **Figure 1A**). Use the blunt end of a paintbrush or forceps to gently press against the transparency strip, thus securing it to the tape.
- (7a) Next, very carefully touch just the bristled tip of the paint brush into the container of dried brine shrimp cysts. Touch the cysts so lightly with the tip of the bristles that only a few cysts attach to the bristles. If too many cysts attach, then gently tap the bristles against the lip of the container so that some of the cysts fall back into the container. Then, gently “paint” the cysts that are attached to the bristles onto the sticky circle in the bottom of the Petri dish. Brush gently back and forth to make sure the cysts are secured to the tape. If necessary, repeat this procedure until a total of about 20-40 cysts are stuck to the tape within the grid area (see **Figure 1B**).
- (8a) Now, grasp the Petri dish in your fingers and invert it so that the sticky circle faces the floor. Then, use the finger and thumb on your other hand to gently flick the bottom of the Petri dish. The idea is to dislodge and discard any cysts that are not securely stuck to the sticky circle. Thus, your count of cysts within the circle should be an accurate count for the entire dish contents. *WHEN DOING THIS, BE CAREFUL NOT TO DIRECTLY TOUCH OR PRESS ON THE CYSTS BECAUSE THEY ARE VERY FRAGILE!*
- (9a) Under a dissecting microscope, count the number of cysts that are stuck to the circle. Draw a map of the distribution of cysts in the circle. (Refer to circular templates in **Appendix B**).
- (10a) Proceed to step 11.

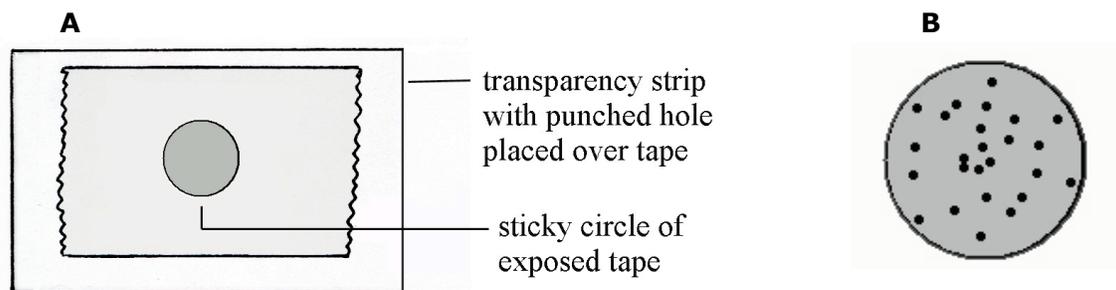


Figure 1. Panel A shows a sticky circle created by laying a punched transparency strip over double-stick tape stuck to the bottom of a Petri dish. Panel B shows 25 brine shrimp cysts stuck to the sticky circle.

Method B – Cysts attached to sticky grid

- (4a) Use scissors to cut out a 2 cm x 5 cm transparency strip with a 10 mm x 10 mm grid.
- (5a) Obtain a 2 cm long strip of double-stick tape. Make sure to handle the tape by the edge with a forceps so you do not get fingerprints on the tape. Carefully lay the strip of tape directly over the grid pattern, as shown in **Figure 2A**. Then, use the blunt (non-brushy) end of the paintbrush to trace around the edge of the tape with gentle pressure so that the tape is securely attached to the transparency strip.
- (6a) Now, using the bristled-end of the paintbrush, very lightly touch just the tip of the brush into the container of dried brine shrimp cysts. A small number of cysts should adhere to the brush. Then, carefully and gently “paint” these cysts onto the sticky surface covering the grid. Brush gently back and forth to make sure the cysts are secured to the tape. Repeat this procedure until a total of about 50-100 cysts are stuck to the sticky grid (see **Figure 2**). Try to achieve a fairly uniform distribution of cysts over the grid area. Do not worry if a few cysts are stuck outside the grid area, though they should still be counted.

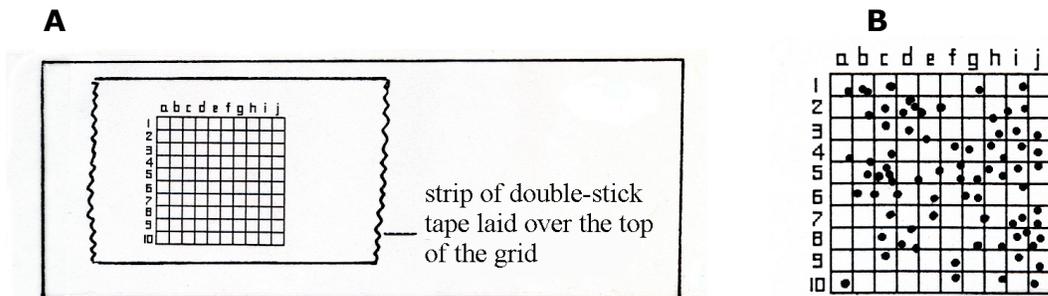
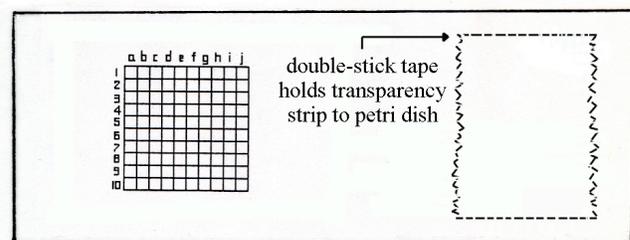


Figure 2. Panel A shows a strip of double-stick tape placed over the grid on a transparency strip. Panel B shows 77 cysts ‘painted’ onto the sticky surface of the grid.

- (7a) Next, while grasping the edge of the strip in your fingers, use the thumb and a finger on your other hand to gently flick the edge of transparency strip. The idea is to dislodge and discard any cysts which are not securely stuck to the tape. *WHEN HANDLING THE STRIP, BE CAREFUL NOT TO GRASP OR PRESS ON THE CYSTS BECAUSE THEY ARE VERY FRAGILE!*
- (8a) Attach a short piece of double-stick tape to the bottom of the dish. Then, with the sticky grid facing upward, position the unmarked end of the transparency strip over the tape in the dish (see **Figure 3**). Press down on the strip to secure it to the dish, taking special care not to touch or press on the cysts or grid at the other end of the strip.

Figure 3. A short strip of double-stick tape (placed on the underside of the transparency strip) anchors the transparency strip to the bottom of the Petri dish.



- (9a) Under a dissecting microscope, count the number of cysts stuck to the grid and surrounding tape. Map the distribution of cysts in the grid using the template in **Appendix C**.
- (10a) Proceed to step 11.
- (11a) If a particular dish is designated as an experimental treatment group in which *dry cysts will be exposed to some environmental extreme* (e.g., freezing, microwave irradiation, heating, etc), then that treatment should be done now, before starting the next step.
- (12a) Next, fill the Petri dish about half-full of artificial sea water (about 20 ml), making sure the sticky surface with attached cysts is fully immersed. Cover and label the container. Then, place it in continuous room light at room temperature.
- (13a) If possible, inspect and make sketches of the cysts at 12, 18, and 24 hours after immersion begins. Each day for the next four days, continue to inspect and make close-up sketches of cysts.
- (14a) Each day for the first four days, use a small-bore pipet to carefully remove and count all newly hatched nauplius larvae. After four days, few if any brine shrimp should be hatching.
- (15a) Complete a table of results (**Appendix B** or **Appendix C**) for each group of cysts. Then use graph paper to plot a hatching curve for each group. The graph should show the *daily cumulative percentage of hatched nauplius larvae*. The vertical coordinate should represent hatching success (i.e., percent of cysts that hatched). A maximum of 100% hatching success would correspond to hatching of all cysts originally placed in the dish. The horizontal coordinate shows time increments: day 0, day 1, day 2, day 3, day 4, etc.

Questions and Follow-up Investigations

- Q1:** Do cysts change in shape or size following immersion and hydration? Describe any changes in shape and size. When and how do such changes occur?
- Q2:** Describe and discuss the full sequence of observable events that precede release of nauplius larvae from the cysts.
- Q3:** Describe and discuss day-to-day patterns and trends in hatching, as evidenced by hatching curves for control and any experimental groups.
- Q4:** Will brine shrimp cysts that fail to hatch after an initial hydration successfully hatch after a second hydration? Design and perform experiments that would test this question. For example, hydrate and hatch a batch of cysts that are attached to tape as outlined in Method A or B. Then, when hatching ceases after 4-5 days, determine the final hatching percent (presumably a value <100%) and pour off all excess water. Let the tape along with attached cysts completely dry out for 1-2 days. Then, use salt water to re-hydrate remaining cysts in the dish and look for the emergence of any hatched larvae. What are some possible outcomes and interpretations of this experiment? Any emergence of nauplius larvae after a second hydration is a significant result. Explain the biological and/or ecological significance of such a result.
- Q5:** Investigate the effect of exposing dry cysts to extreme environmental treatments such as freezing, microwave irradiation, UV irradiation, bright light, brief exposure to steam heat, complete darkness, etc.

- Q6:** Will brine shrimp cysts hatch if they are confined and crowded into a small space? To address this question, prepare two separate transparency-grids, each with a known number of cysts attached, as described in Method B. Use additional pieces of double-stick tape to attach corners of the strips to the bottom of two separate Petri dishes. However, one strip should have cysts facing up and the other should have cysts facing downward and nearly touching the bottom of the Petri dish. Be very careful not to apply direct pressure to cysts since they are fragile and may easily rupture. Add salt water to both dishes. If necessary, use a forceps to gently lift the edge of the inverted grid to release any trapped air. Compare the developmental progress of the two groups, especially noting when and how many cysts crack open, as well as when and how many nauplius larvae emerge. Explain your results in relation to differences in a key environmental variable that could retard development and hatching in one dish and enhance them in the other dish.
- Q7:** What effect do salinity variations have on hatching success? [*Recommendation:* Use multiple concentrations representing large steps in salt concentrations, such as 300%, 100%, 30%, 10%, 3%, 1%, 0%, etc.]
- Q8:** Will cysts hatch if continuously kept immersed at 5 C° (= typical refrigerator temperature) for one week? If the cysts do not hatch, will they subsequently begin delayed hatching after they are transferred from refrigeration to room temperature?
- Q9:** Can you care for and feed a small group of brine shrimp so that they reach adult stage and sexual maturity? Note that it is easily possible for brine shrimp to grow from nauplius larvae to adult stages while remaining at low density in a shallow, unaerated container of ASW. A suggested food that supports growth from hatched nauplius to adult is Dry Brine Shrimp Feed (Cat. #E-16; 1.0 lb/\$25) sold by *Aquatic Eco-Systems, Inc.* (<http://www.aquaticceco.com/>). This food is exceptionally good for sustaining growth. An alternative food source is yeast, fed during the first week after hatching, followed by *Spirulina* powdered algae powder (Aquatic Ecosystems catalog #SP-1; www.aquaticceco.com). Powdered *Spirulina* is an excellent food source for a wide variety of freshwater and marine invertebrates including brine shrimp, aquatic oligochaetes, snails, fairy shrimp, tadpole shrimp, daphnia, copepods, etc. Dispense the powder in *tiny quantities*. A small, capped dispenser may be made by using a poster pin to punch a hole in the tapered tip of a capped centrifuge tube. The poster pin serves to cap the hole. Fill the tube with powdered food and dispense it through the open pin hole by shaking the tube over the water surface in “salt shaker fashion.” Stir the water lightly. Re-feed each day or two, as needed. Do not overfeed!]
- Q10:** Does the presence of low concentrations of environmental toxicants or pharmacologically active chemicals in the salt water affect the timing or success of hatching? Before selecting any toxicant and designing toxicological experiments, students consult the article entitled: “*Biological Smoke Detectors*” (Drewes, 2003). This article gives suggestions for selecting chemicals and using safe procedures.
- Q11:** Do brine shrimp exhibit phototaxis orientation throughout all life stages (see Bradley and Forward, 1984)? In which stage(s) is phototaxis most pronounced? What is the spectral sensitivity of phototaxis behavior?

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- Williams, T.A. (1994) A model of rowing propulsion and the ontogeny of locomotion in *Artemia* larvae, Biological Bulletin, 187:164-173.

Web Resources on *Artemia*

- www.britishecologicalsociety.org/articles/education/resources/curriculum/brineshrimp/ (114 pp)
- www.fao.org/DOCREP/003/W3732E/w3732e0m.htm (excellent background)
- www.eeob.iastate.edu/faculty/DrewesC/htdocs/Artemph.jpg (images of development)
- www.eeob.iastate.edu/faculty/DrewesC/htdocs/ARTEMIA.PDF (background)
- www.eeob.iastate.edu/faculty/DrewesC/htdocs/artemia-web.ppt (instructional slides)
- www.eeob.iastate.edu/faculty/DrewesC/htdocs/Artemia-KIT.JPG (commercial source)
- <http://allserv.rug.ac.be/aquaculture/general/general2.htm> (Belgian *Artemia* research center)

Acknowledgements

I am most grateful to the many high school biology teachers who I have been privileged to work with for more than a decade in summer residential workshops at Iowa Lakeside Laboratory. These teachers provided me with inspiration and support in developing this student lab investigation. I have previously presented and shared versions of this investigation at numerous workshop venues, including NABT, KATS, WisTEP, BTANJ, and ISTA.

Notes about Intellectual Property

I have developed a version of this investigation as a commercial kit that is currently sold by Flinn Scientific. Nevertheless, I retain full rights to copy and freely share all ideas and materials herein for non-profit purposes. In addition, I am pleased to grant all teachers and students permission to copy and use these materials as they wish, for any non-profit educational or research purposes.

Appendix A. *Artemia franciscana*

Phylum: Arthropoda

Subphylum: Crustacea

Class: Branchiopoda (includes fairy shrimp, brine shrimp, daphnia, clam shrimp, tadpole shrimp)

Order: Anostraca (brine shrimp and fairy shrimp)

Genus species: *Artemia franciscana* (= the North American version of *Artemia salina*)

[Note: The species commonly referred to as “*Artemia salina*” in older research and educational literature, in fact, consists of several closely related species or subspecies. One of these species, *Artemia franciscana*, is the main North American species (Eriksen and Belk, 1999).]

Reproduction. Typically, sexes are separate and adults are sexually dimorphic. Males have large graspers (modified second antennae) which easily distinguish them from females. In some species and populations of *Artemia* (for example, parts of Europe), males may be rare and females reproduce by parthenogenesis.

During mating, males deposit sperm in the female ovisac where eggs are fertilized and encapsulated. Eggs are then deposited and stored in a brood sac near the posterior end of the thorax (Figure 4M). Once fertilized, eggs quickly undergo cleavage and development through the gastrula stage (Figure 4A-E). After one or a few days, eggs are then released by the female (oviposition). Multiple batches of eggs may be released at intervals of every few days by the same female.

Two types of eggs may be laid -- (1) thin-shelled “summer eggs” that continue developing and hatch quickly, or (2) thick-shelled, brown “winter eggs” in which development is arrested at about early gastrula stage. While encysted, such “winter eggs” may completely dry out while remaining in metabolically inactive state (termed anabiosis) for up to 10 or more years. In this state, cysts can withstand severe environmental conditions. For example, *Artemia* cysts may remain viable after heating to 80 °C for 1 hr, cooling to -190 °C for 24 hrs, or reducing air pressure to 0.000001 mm mercury for 6 months!

Embryology. Cleavage of developing eggs is total and yolk is equally distributed among blastomeres (Kaestner, 1970; Anderson, 1973; Schram, 1986). While in the female brood sac, egg development passes rapidly through cleavage and blastula stages (Figure 4A-C). Eggs are then deposited in the environment where they may remain encysted, with embryonic development arrested at about early gastrula stage (Figure 4D-C). At this time, there are about 4,000 cells in the embryo and these are highly organized, but no organs are discernible (Benesch, 1969).

When encysted eggs are exposed to more favorable conditions (rehydration), the eggs swell and rapid development of the embryo resumes, resulting in completion of the nauplius stage (Figure 4F-G). Hatching occurs in about 1-2 days, depending on temperature. For the first few hours, the nauplius stays within a hatching membrane that is attached to the cyst capsule. This is also called the “umbrella stage” in which development of the nauplius stage is completed.

At hatching, the nauplius larva (= instar #1) emerges as a free-swimming stage (Figure 4H). This stage is about 0.4-0.5 mm in length and brownish-orange in color, due to the presence of yolk material. In a sense, the body of the nauplius larva consists mainly of a head. It has three pairs of “head” appendages -- a pair of small first antennae (antennules), a pair of well-developed second antennae, and a pair of mandibles. There is a large lip-like structure (labrum) covering a ventral mouth. A nauplius eye is present but it is not easily distinguished at this stage.

The posterior end of the nauplius consists of the future trunk. Initially, it is short, undifferentiated, and unsegmented (Figure 4H). The nauplius larva does not have a complete digestive tract and does not immediately feed. It relies on stored yolk as an energy source. Depending on temperature, it swims weakly for about 12-20 hrs and then molts into the metanauplius larva (= second instar).

Artemia Development from Egg to Adult Stages

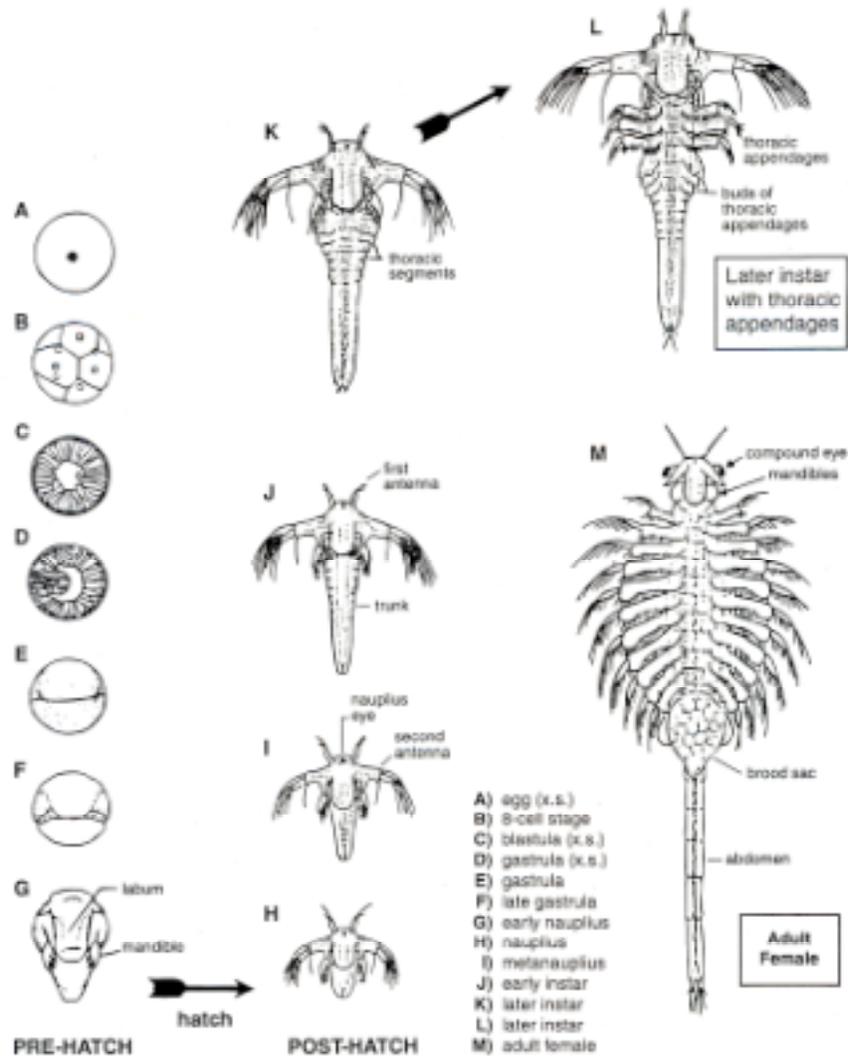


Figure 4. Diagram of Artemia development and life cycle.

Larval stages and growth. Larval development of *Artemia* has been described in detail by several authors (see references). Although basic interpretations of development are similar, there are differences between authors regarding the numbering of molts and the naming of various instar stages.

The metanauplius larva is translucent in color and about 0.6 mm in length (Figure 4I). Its trunk region is noticeably longer, and this region continues to lengthen and differentiate through the next series of molts. The metanauplius swims vigorously using its second antennae which are now better developed. At this stage, it starts filter-feeding. Its food consists mainly of microalgae, bacteria, and detritus.

The next three stages (each terminated by a molt) are also classified as metanauplius stages. Examples are shown in Figure 4J-K. Some developmental trends during these later metanauplius stages include more developed mouthpart appendages (maxillules and maxillae) and a longer thoracic region, with some definition of thoracic segments.

Next, there are seven postnaupliar stages -- one example is shown in Figure 4L. During these stages, the antennae begin to undergo a reduction in size and paired thoracic appendages begin forming. With each stage, these appendages become more numerous, larger, and functional. In addition, the compound eyes become more fully developed, the labrum is reduced in size, and abdominal segments become defined (Anderson, 1967).

Then, there are a series of five postlarval stages (not illustrated) involving further reduction in the antennae, multiplication of ommatidial facets in the compound eyes, lengthening of the eyestalks, and formation of sexual organs. Completion of the 17th molt marks the end of post-embryonic development and the beginning of the final adult stage (Figure 4M). [Note that some authors recognize only 14-15 molts, rather than 17.]

Brine shrimp grow extremely rapidly. The adult stage is reached about three weeks after hatching. At adult size, biomass is about 500 times more than the nauplius biomass. Adults may live up to about 4 months.

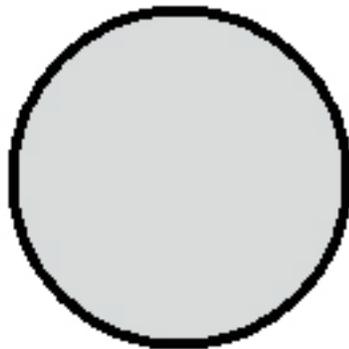
Adults. Adult body size is variable, but typically it is about 8 mm in length. The anterior part of the body is not covered by a shield or carapace. The head has a pair of compound eyes at the end of stalks. Head appendages include a short pair of first antennae (also called antennules), a pair of second antennae, mandibles, and paired maxillules and maxillae -- the latter are greatly reduced in size. In males, the second antennae are enlarged and modified as claspers -- in females, they are short and thickened.

The body has 20 trunk segments (some authors say 19). The first 11 trunk segments are classified as thoracic segments and bear paired, paddle-like appendages, also called phyllopod. Posterior to the thorax, there are 7 abdominal segments that bear no appendages. The last body segment bears a pair of long tail filaments.

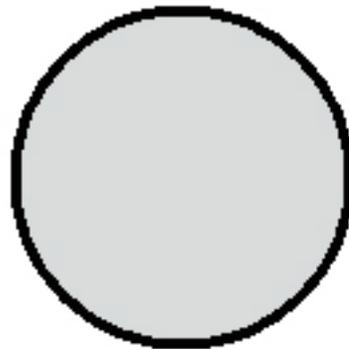
Thoracic appendages are used for swimming and the animal swims ventral side up (Williams, 1994). During swimming, appendages move in a rhythmic and wave-like pattern, at a frequency of about 5-10 waves per second. Although difficult to see with the naked eye, each wave of movement actually starts out in posterior segments and then, rapidly and sequentially, progresses into more anterior segments. During the "power stroke" of each cycle of movement, the paddle-like appendages push water in a rearward direction, thus smoothly propelling the animal forward. Importantly, such water currents also function in food gathering, as well as in respiration, since thoracic appendages also have gills.

Appendix B. Templates for mapping initial distribution of cysts on sticky circle.

The accompanying table is for daily recording of the numbers of hatched nauplius larvae.



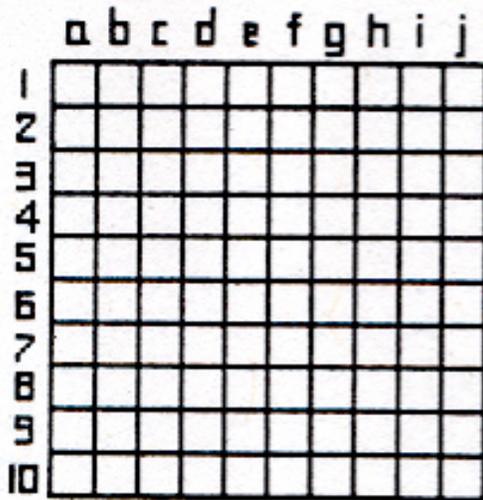
Control group dish



Experimental group

| Treatment Group (control or experimental) _____ Initial number of cysts in dish _____ | | | |
|--|---|---|--|
| | # of newly hatched nauplius larvae removed | cumulative # of hatched nauplius larvae (day1 + day2 + day3...) | cumulative hatching percentage (cumulative number of hatched larvae / initial number of cysts X 100) |
| Day 0 start | | | |
| Day 1 (24 h) | | | |
| Day 2 (48 h) | | | |
| Day 3 (72 h) | | | |
| Day 4 (96 h) | | | |
| | | | |

Appendix C. Templates for mapping initial distribution of cysts on sticky grid and accompanying table for daily recording of the numbers of hatched nauplius larvae.



| Treatment Group (control or experimental) _____ | | | |
|---|--|---|--|
| Initial number of cysts in dish _____ | | | |
| | # of newly hatched nauplius larvae removed | cumulative # of hatched nauplius larvae (day1 + day2 + day3...) | cumulative hatching percentage (cumulative number of hatched larvae / initial number of cysts X 100) |
| Day 0 start | | | |
| Day 1 (24 h) | | | |
| Day 2 (48 h) | | | |
| Day 3 (72 h) | | | |
| Day 4 (96 h) | | | |
| | | | |

Appendix D. Mastercopy for making grid transparencies.

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