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Artificial Fertilization and Development through Hatching in the Oceanic Squids *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* (Cephalopoda: Ommastrephidae)

by

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Abstract. A technique for routinely obtaining hatchlings of oceanic squids via artificial fertilization is not available at present. This paper makes a major advance in that direction. A technique for artificial fertilization and rearing the resulting embryos through hatching for the oceanic ommastrephid squids *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* is presented that emphasizes the importance of egg hydration and methods of obtaining chorion expansion. Initial results comparing hatching time against rearing temperature show an expected decrease in time with increasing temperature.

INTRODUCTION

Experimental embryologists have artificially fertilized loliginid eggs for many years, but rearing the resulting embryos through hatching has been infrequent (Klein & Jaffe, 1984). Artificial fertilization of oceanic squids was first accomplished in *Todarodes pacificus* (Steenstrup, 1880) (Soeda, 1952, 1954) and has been repeated a number of times since (e.g., Hayashi, 1960). Except for the preliminary work of Arnold & O'Dor (1990) and the results of Sakurai & Ikeda (1994), artificially fertilized oceanic squid eggs have not been reared through hatching. In most cases, embryonic development stopped in the early developmental stages. For those embryos that survived the initial stages, death resulted from either failure of the chorion to expand, which constricted the embryo, or from bacterial infection.

Klein & Jaffe (1984) cultured fertilized embryos of *Loligo pealei* LeSueur, 1821 through hatching in petri dishes lined with agarose. Arnold & O'Dor (1990) provided more details concerning the Klein & Jaffe study: embryos were placed in depressions in the agarose; only partial lifting of the chorion occurred and only some embryos hatched. They concluded "the stimulus to chorionic swelling is, in part, due to general factors associated with being enclosed in a jelly-like substance rather than a specific factor in the egg jelly derived from the nidamental gland" (Arnold & O'Dor, 1990:22). Ikeda & Shimizaki (in press) have found, however, that nidamental gland jelly will not cause chorion expansion in *Todarodes pacificus*.

Arnold & O'Dor (1990) added lyophilized nidamental gland material to the water surrounding the eggs of *Sthenoteuthis oualaniensis* (Lesson, 1830) and *Abraliopsis* sp. to facilitate raising of the chorion. They did not mention, however, whether or not the chorion actually lifted in their embryos. Their photographs show that it did not lift in *Sthenoteuthis* and only partially lifted in *Abraliopsis*. Since *Abraliopsis* lacks nidamental glands, the partial lifting of the chorion in this species suggests that the presence of a gel alone may, in some species, be important in obtaining chorion expansion.

Ikeda et al. (1993b) surrounded embryos of the ommastrephid squid, *Todarodes pacificus*, with lyophilized oviducal gland. Their selection of the oviducal gland was based on the likelihood that jelly from this gland, rather

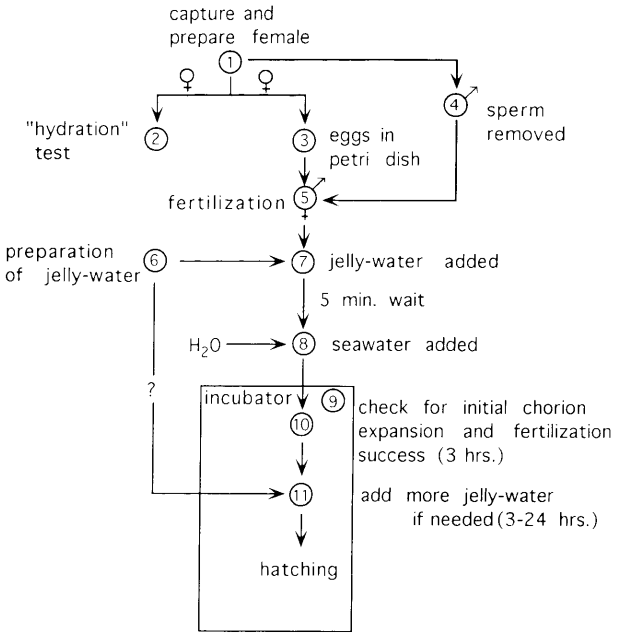

Figure 1

Schematic presentation of the protocol developed for artificial fertilization and rearing of embryos.

than the nidamental gland, first coats the eggs during spawning as suggested by Hamabe, 1962. They demonstrated that the initial phase of chorion expansion occurred only when the oviducal jelly was present. This approach, along with some innovations in general technique, enabled Sakurai & Ikeda (1994) to successfully hatch artificially fertilized eggs of this species.

Here we report on attempts to apply this latter technique, with modification, to two additional species of oceanic squids, the ommastrephids *Sthenoteuthis oualaniensis* and *Ommastrephes bartramii* (LeSueur, 1821). Our interest in hatching embryos of artificially fertilized oceanic squids is directed toward problems of maintaining, feeding, and rearing paralarval oceanic squids. Our present inability to study living paralarvae in the laboratory severely limits our knowledge of the biology of these early life stages. The first step in solving these problems is to obtain a large and consistent supply of hatchlings. At present, rearing artificially fertilized embryos seems the only feasible approach to this problem.

MATERIALS AND METHODS

Attempts at artificial fertilization were made during five cruises near Hawaii between 1992 and 1994. Three were with the FTS *Hokusei Maru* from Hokkaido University, one with the R/V *Moana Wave* from the University of Hawaii, and one with the R/V *Wecoma* from Oregon State University. Artificial fertilization experiments, always a second priority on these cruises, were limited by time and available resources.

To determine percent of successful fertilization, 20 eggs in each of two orthogonal transects across each dish containing eggs and sperm were evaluated for cleavage. These counts are conservative since some fertilized eggs were not recognized as having cleaved due to their orientation.

The most critical step in rearing squid embryos is to obtain chorion expansion. Chorion expansion occurs primarily in two stages. The first occurs 30 to 60 min (24°C) after fertilization and is a very distinct enlargement of the perivitelline space (e.g., see photographs in Ikeda et al., 1993a). The second occurs around 48 hr (24°C) after fertilization when the embryo begins to elongate. The second enlargement can occur even when the first enlargement did not, although the total expansion will be less than normal (Figure 5A, B). In such cases, however, hatching can still occur. When chorion expansion failed entirely, the increasingly compressed embryos continued to develop for up to 72 hr, but death followed as the embryo began to extrude through various breaks in the chorion. The timing of adding oviducal jelly to the embryos was not critical for final chorion expansion. Delays up to 24 hr still resulted in chorion expansion. Controls without oviducal jelly never developed chorion expansion.

Oviducal jelly was prepared from lyophilized glands, frozen glands, and fresh glands. All three preparations were successful in some cases, but treatment with the lyophilized glands resulted in more consistent chorion elevation and in a low rate of bacterial infection. Frozen and fresh preparations were made by thinly slicing the gland, then mashing the slices in a small amount of seawater with a toothpick. The lyophilized preparation depended on preparing oviducal powder in advance of the cruise. The lyophilized oviducal gland was ground with a mortar and pestle, then sifted through a filter with a 0.2 mm pore size to remove large pieces of tissue; it was then stored in a freezer until needed. Sterile techniques (where possible) and the use of freshly filtered seawater not older than about 2 or 3 days are important procedures to prevent infection of the embryos.

Attempts to develop a technique in a systematic manner were hampered by a number of factors (see Discussion), which were unknown at the time, and therefore uncontrolled. During the course of this study, the basic method or protocol evolved to the state presented below (Figure 1). Times indicated are for experiments done at 22°C.

(1) A freshly caught gravid female squid is placed on a pan, the mantle opened and the ink sac removed. Eggs are obtained by cutting through the wall of an oviduct and extracting with a plastic spoon.

(2) One batch of eggs is flooded with seawater as a control to determine how rapidly the eggs hydrate. If hydration takes longer than 10 minutes, the subsequent technique should be modified by a second addition of sperm after hydration has occurred.

(3) The eggs are placed in a small pile in a 50 mm diameter plastic petri dish. The eggs have not been exposed to seawater at this point. The chorion in the ommastrephids examined did not adhere to the petri dish as it does in *Loligo pealei* (Klein & Jaffe, 1984).

(4) Sperm are removed from spermathecae (seminal receptacles) on the buccal membrane of the female squid by gently squeezing the base of the saclike spermathecae with a pair of forceps. The extruded sperm are confined by mucous covering the buccal membrane. This allows the sperm within a mucous strand to be transferred to the eggs with a pair of forceps.

(5) The mucous strand containing the sperm is gently dragged over the surface of the eggs causing the sperm mass to become thinly spread over the eggs. Sufficient water is transferred with the mucous to cause sperm motility. Often a few drops of seawater are added at this stage, and the petri dish is covered and allowed to stand for a few minutes.

(6) About 20–30 mg of lyophilized oviducal gland powder is mixed with 30 mL of 0.4 μm filtered seawater and vigorously stirred until a barely detectable increase in viscosity of the water (jelly-water) is noted.

(7) The jelly-water is added to the eggs and sperm so that a thin layer of this fluid covers the eggs. The fluid causes the eggs to spread out across the dish in a single layer.

(8) About 5 min after step 7, filtered seawater is added to half fill the petri dish.

(9) The petri dish is placed in an incubator at 22°C. Heat sterilization of the incubator prior to use is an important precaution.

(10) After about 3 hr, the successfully fertilized eggs are usually in the second or third cleavage, and the chorion should exhibit initial expansion. As a result, they can be easily recognized under a dissecting microscope. Developing embryos with elevated chorions are then transferred to freshly filtered seawater in another petri dish at a density of 10–15 embryos per dish.

(11) Often jelly-water is added once or twice again before organogenesis begins to insure chorionic expansion. This should be done within 24 hr of fertilization and is important if initial chorionic expansion did not occur. Changing of the seawater medium during embryogenesis is unnecessary since oxygen concentration does not seem to be a limiting factor in these circumstances.

(12) Hatching occurs in about 3 to 4 days at 22°C depending on the species.

RESULTS

We provide here results of tests concerning several phases of the artificial fertilization and rearing protocol and some initial results on hatchlings. Sperm from spermatophores, spermatangia (discharged spermatophores) attached to the female, and spermathecae were tested. All three sources provided sperm that successfully fertilized eggs; however, sperm from spermathecae were most reliable. Sperm became motile only in seawater. In both *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis*, sperm in spermatangia or spermatophores were difficult to separate from the matrix in which they were embedded. As a result, only sperm along the fringe of the cut edges became active in the seawater. Sperm from spermathecae were much easier to disperse and thus easier to expose to seawater. We timed the survival of motile sperm; activity was greatly reduced after 15 min and completely stopped after 25 min (approximately 24°C).

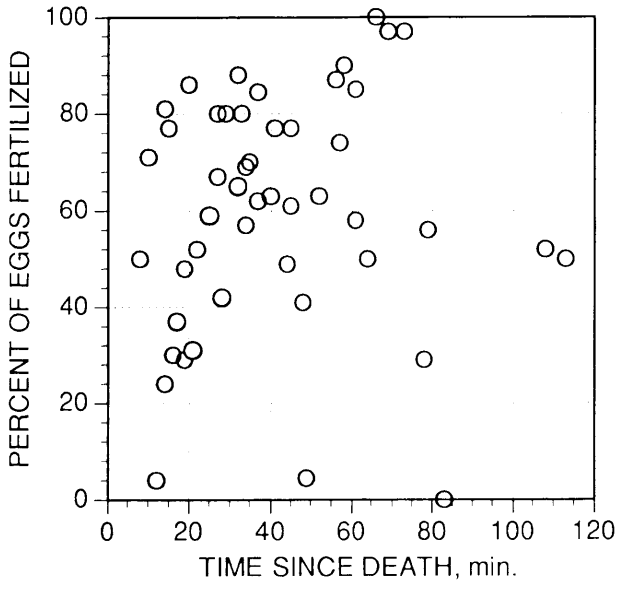

Figure 2

The effect of the time since death of the squid *Sthenoteuthis oualaniensis* on the ability of their eggs to be fertilized (fertilizability).

In 49 fertilization trials on *Sthenoteuthis oualaniensis*, where the percent of eggs fertilized was counted, a mean of 60.6% (24.8% standard deviation) of the eggs were successfully fertilized, and the median was 63% (range 0–100%).

Eggs removed from the oviducts of *Sthenoteuthis oualaniensis* did not have smooth rounded surfaces. Rather, they appeared like a slightly deflated ball with a number of indentations. When the eggs were placed in seawater, these indentations gradually disappeared. Since this process occurred in the presence of seawater, we term it "hydration," although the mechanism involved in the rounding of the chorion is unknown. Ten attempts were made to fertilize eggs that were not hydrated, and none could be fertilized. In the single *Ommastrephes bartramii* examined for hydration, over 90% of the eggs were hydrated after 2 min in seawater. While the fastest time to 90% hydration that we have measured in *S. oualaniensis* was also 2 min, hydration time was highly variable in this species. On one occasion, hydration of all eggs was incomplete after nearly

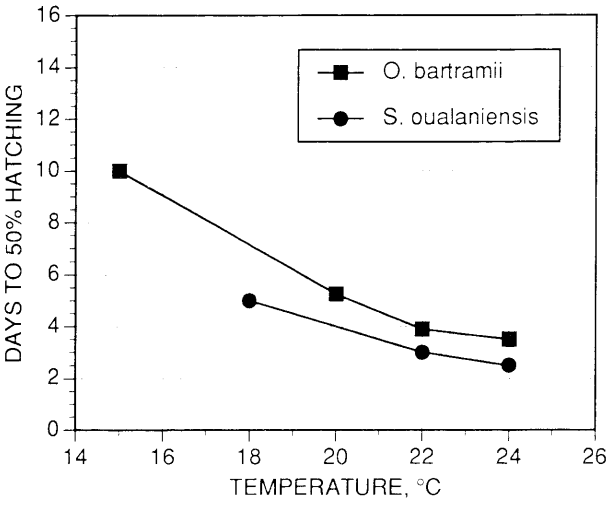

Figure 3

Time to 50% hatching of artificially fertilized eggs of *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* plotted against incubation temperature.

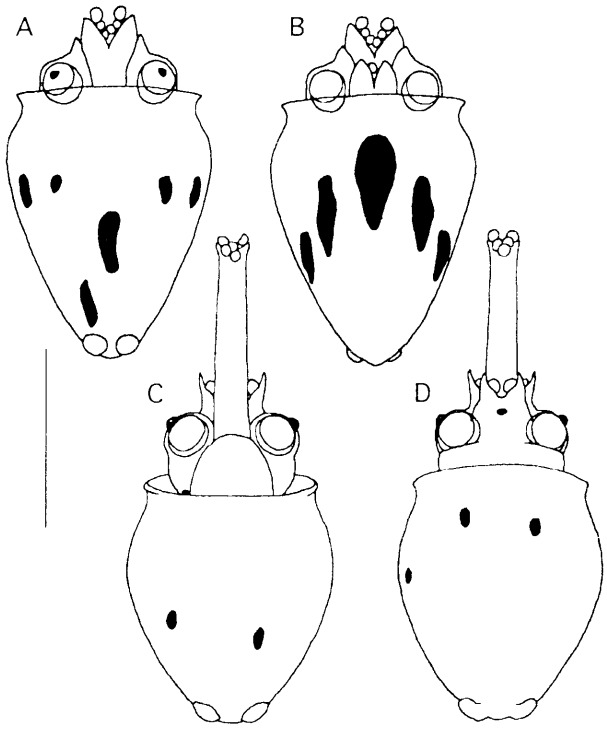

Figure 4

A, B. *Ommastrephes bartramii*. Ventral and dorsal views of a 2-day-old hatchling from an artificially fertilized egg showing chromatophore pattern. Although this is the same age as the hatchling in Figure 5F, G, it is not as advanced in development as the latter presumably due to premature hatching. Note the pair of enlarged proboscis suckers and short proboscis. C, D. *Sthenoteuthis oualaniensis*. Ventral and dorsal views of a 4-day-old hatchling from an artificially fertilized egg showing chromatophore pattern. Note equal sized proboscis suckers and length of the proboscis. Bar = 1 mm.

40 min, and a few had not hydrated after 9 hr in seawater. Most of these eggs did not fertilize (fertilization success was less than 5%). The eggs came from a 200 mm mantle length (ML) female whose oviducts were estimated to be one-third to one-half full. From another female of the same length but with oviducts about two-thirds full, less than 10% of the eggs hydrated in 10 min after seawater was added. Fertilization was not attempted in this case. We have examined hydration times in only five other *S. oualaniensis* females. Their hydration times in minutes (i.e., time taken to hydrate 90% of the eggs) were: 9, 2, 7, 15, and 4.

Eggs in the oviducts of dead female squid with open mantles remain viable for some time. We attempted to fertilize eggs from *Sthenoteuthis oualaniensis* up to nearly 2 hr past death and, within this time frame, we found no clear effect of the "age" of the eggs on their ability to be fertilized (Figure 2). In these cases, the mantle of the female squid was cut open immediately after death to allow free aeration of the oviducts. Peristaltic action of the oviducts continues for some time after death, which may also aid in continued aeration of the eggs. We have no data on whether or not developmental abnormalities might be related to the age of the eggs. As a general practice, however, fertilizations were performed within one hour of the death of the female.

We used the powder of lyophilized oviducal gland from the ommastrephids *Todarodes pacificus, Sthenoteuthis oualaniensis,* and *Ommastrephes bartramii* to stimulate chorion expansion among species. All nine possible combinations of eggs and powder between these three species (e.g., *T. pacificus* powder used to make jelly-water that is applied to eggs from *S. oualaniensis*) resulted in chorion expansion.

In spite of considerable variability in success rates, hundreds of embryos of *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* were hatched. Initial tests on the effect of temperature on the hatching time have been made with the objective of determining the optimum temperature range for development (Figure 3). Ultimately, such information can supply clues to the spawning depths and latitudes of the squids in nature. The time at which 50% of the embryos hatched was used as a measure of the hatching time. The offset between the two species may be due to the differences in their egg sizes. Most eggs of *O. bartramii* measured about 0.92 × 1.10 mm, although some eggs were much larger (1.25 × 1.10 mm). The larger eggs, which accounted for nearly half of the eggs in some females and virtually none in other females, could not be fertilized. In *S. oualaniensis,* eggs of only one size were present, and they measured 0.84 mm × 0.70 mm. Over the temperature range examined, the duration of embryonic development through hatching decreased with increasing temperature (Figure 3). Although there is an apparent change in the slope of the

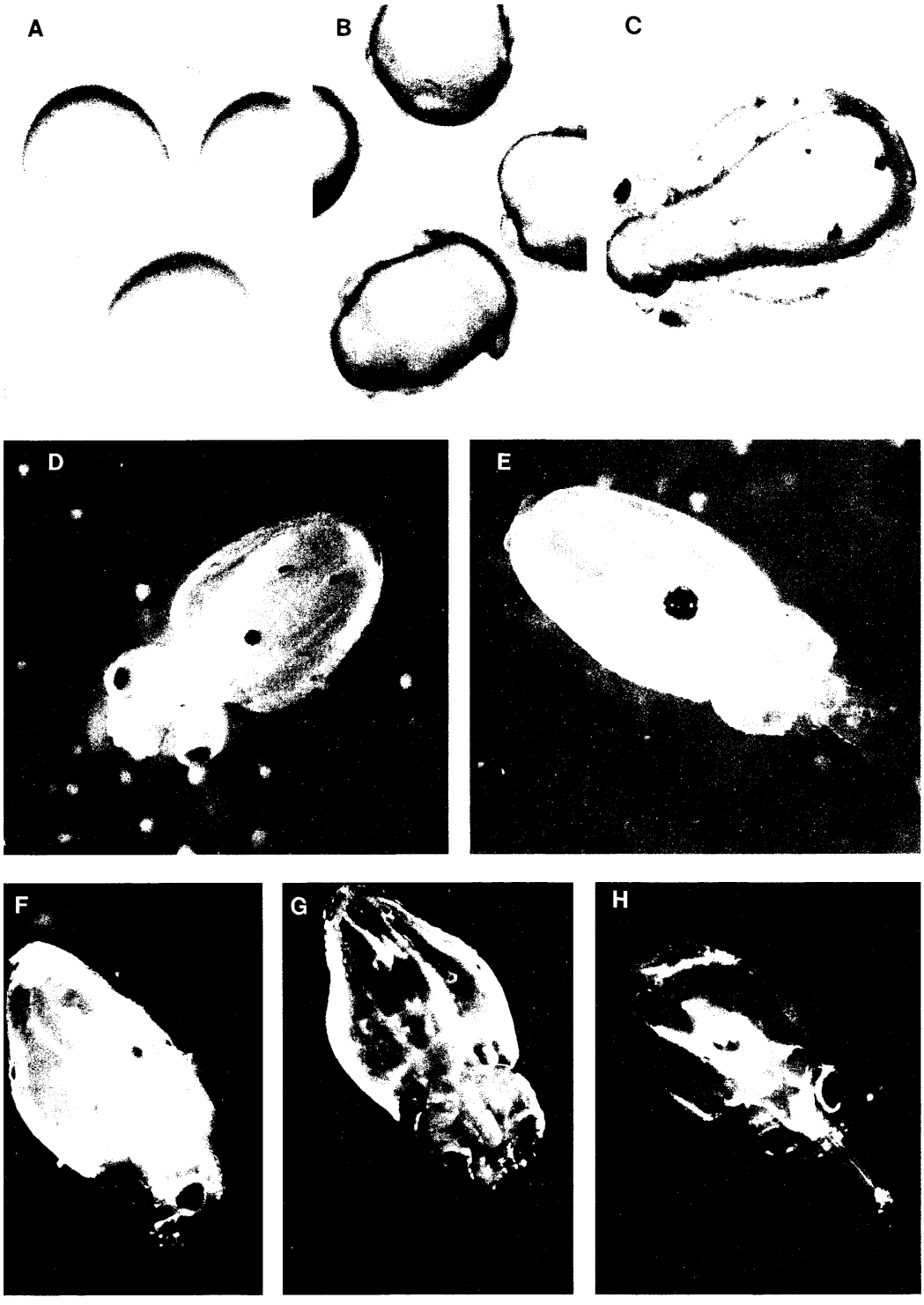

Figure 5

Ommastrephes bartramii embryos and hatchlings from artificially fertilized eggs. A. Approximately stage 14 of Arnold (1990). Shows initial chorion elevation. B. Approximately stage 20 of Arnold (1990). Shows only partial secondary expansion of chorion. C. Just prior to hatching with chorion only partially elevated. D. Just after hatching. Note large internal yolk sac. E. 1-day-old hatchling. F. 2-day-old hatchling. Side view showing slightly elongate proboscis. G. 2-day-old hatchling. Ventral view showing reduced internal yolk sac. H. 4-day-old hatchling. Ventral view showing elongate proboscis and absence of internal yolk.

curve for *O. bartramii* at 22°C, we could not determine the upper temperature optimum from this limited data.

Hatchlings have been kept alive up to 6 days in 250 ml of filtered seawater in glass bottles placed on a plankton rotator. Several attempts were made to feed 2- and 3-day-old hatchlings by adding a known number of small copepods of the genera *Oithona* and *Clausocalanus,* fish eggs, and squid eggs to the culture bottles. None of the potential prey were eaten. This lack of first feeding may reflect wrong prey items, inappropriate conditions for maintaining the paralarvae, or insufficient development of the hatchlings.

At hatching, the paralarvae have a very short proboscis and a large internal yolk supply (Figure 5C). By 4 days post hatching, the yolk sac is nearly gone, the proboscis has elongated greatly, and the eyes are more fully developed (Figure 5D–G). After 4 or 5 days at 22 to 24°C, the squid begins to deteriorate due to starvation. The 2-day-old hatchling of *Ommastrephes bartramii* exhibited the same chromatophore pattern on the mantle (Figure 4) as that previously described for very young (1.6 mm ML) paralarvae (Young & Hirota, 1990). No chromatophores, however, were present on the head of the hatchlings. The proboscis had enlarged lateral suckers (approximately 1.5 times the diameter of the medial suckers) as is characteristic for this species. In contrast, the 4-day-old *Sthenoteuthis oualaniensis* lacked the posteroventral chromatophores, although the suckers on the proboscis had their characteristic uniform size.

DISCUSSION

The fertilization technique we used placed sperm on the ova before the latter were capable of being fertilized due to lack of hydration. Because sperm motility decreases after approximately 15 min, the possibility exists that the sperm will become immobile before the ova are hydrated. Nevertheless, the technique generally resulted in good fertilization rates. Perhaps sperm from the center of the spread sperm mass become progressively activated as the mass dissipates, resulting in a constant new supply of sperm over a prolonged period. Presumably, timing mismatches between egg hydration time and sperm immobility in this step are responsible for some of the variability encountered. This potential problem can be avoided by hydration of the eggs first, followed by concentrating eggs at one side of a tilted petri dish, decanting off most of the seawater, then adding sperm. This method resulted in successful fertilization; however, because the eggs are already in seawater, they no longer provide the resistance needed to help spread the sperm mass, and the eggs are more difficult to coat with jelly. We preferred to risk a possible timing mismatch.

While the technique described here resulted in successful hatching of embryos, it still has not reached the level of refinement where it can be relied on for obtaining large numbers of hatchling squids on a regular basis. Improvement is still needed in the following areas:

(1) A more consistent method is needed for obtaining chorion expansion. The next step in this direction would be to prepare a range of oviducal jelly solutions of different viscosities and from the glands of different individual females. Since fertilization is not necessary for chorion expansion, the solutions could be quickly tested on batches of unfertilized eggs for initial chorion expansion. The solution producing the best result would then become the stock solution for subsequent fertilizations. The ultimate step in this direction, of course, would be to extract the specific chemicals that trigger chorion elevation from the oviducal gland so that standard solutions could be prepared.

(2) A good method is needed for preventing infection. Sterile technique where possible and low-density cultures help greatly, but insurance is needed from appropriate antibiotics that will not interfere with embryogenesis.

(3) We have noted variable, but sometimes frequent, occurrences of developmental abnormalities (e.g., inverted mantles) and occasional cessation of development during the early stages of embryogenesis. One possible cause of such problems could be the rolling of embryos in the petri dishes due to the ship's motion. In many cases, rolling is retarded by the jelly but it can be highly variable. One way to preventing rolling would be to position each embryo onto openings of pieces of nitex plankton netting placed on the bottom of the petri dishes.

(4) An effective method for releasing sperm from their matrix within spermatangia is needed. In general this would provide a larger supply of sperm for fertilizations. It would be especially useful for species that lack spermathecae.

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