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Reproductive Behavior and Embryonic Development of the Pharaoh Cuttlefish, *Sepia pharaonis* (Cephalopoda: Sepiidae)

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Mong-Fong Lee, Chun-Yen Lin, Chuan-Chin Chiao, and Chung-Cheng Lu (2016) The pharaoh cuttlefish, *Sepia pharaonis*, is one of the most important cephalopod fishery species in southeastern Asia. In the present study, we described their reproductive behavior and characterized their embryonic development. Sperm competition during mating was high in *S. pharaonis*; therefore, consort males always escorted their mates after pairing, although sneaker males were frequently observed. Their egg-laying behavior can be divided into three phases. Females first retracted and bent their arms into a fist-like posture to spawn eggs. They then extended their arms forward and used funnels to blow the spawning ground. Finally, they extended their arms again to deposit eggs onto appropriate substrata. Based on the characteristics of the embryos, a set of easily distinguished criteria was developed to define 30 stages of embryonic development. This classification scheme was consistent with that of *S. officinalis*. The present study provided an important basis for future investigations of the reproductive biology and aquaculture in the pharaoh cuttlefish, *S. pharaonis*.

Key words: Spawning behavior, Sperm competition, Embryonic development, Cuttlefish, *Sepia pharaonis*.

BACKGROUND

Cephalopods are fascinating and important among marine fauna in scientific research, such as neuroscience (Fiorito et al. 2014) and behavioral science (Boal 2006), or as fishery resources (Hunsicker et al. 2010). Global landings of cephalopods increased after the mid-20th century (Hunsicker et al. 2010), while the uncontrolled exploitation of the limited stock decreased their catch in recent years (Anderson et al. 2011). Due to the short life-spans and long developmental periods in most of this group of animals, understanding the characteristics of their reproductive behavior and embryonic development became a significant approach to maintain and conserve the resources and their artificial cultures in the future. Different mating tactics were reported in male cuttlefish, *Sepia officinalis* (Hanlon et al. 1999) and *Sepia apama* (Hall and Hanlon 2002), and in squid, *Doryteuthis pealeii* (Shashar and Hanlon 2013). The head-to-head position is a well-known mating behavior in Sepiidae (Hall and Hanlon 2002) and Loliginidae (Shashar and Hanlon 2013). There is little documentation regarding their egg-laying behaviors and the ways these delicate eggs were formed among cephalopods. In cephalopods, a variety of egg types, including

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single eggs, collective capsules, clusters or egg strings, were deposited (Boletzky 1998). After spawning, embryonic development simultaneously starts. Naef (1928) demonstrated sophisticated observations on the embryonic development of several species of cephalopods. Other studies of embryonic development in the Decapodiformes included the following species: *Sepia officinalis* (Lemaire 1970), *Euprymna scolopes* (Lee et al. 2009*)*, *Sepiella japonica* (Yamamoto 1982), *Loligo pealii (*Arnold 1965) and *Todarodes pacificus* (Watanabe et al. 1996). The ambient temperature when embryos incubated determines the development of cephalopods (Sakurai et al. 1996). Generally, 30 stages of embryonic development are recognized, except for 26 stages for *Todarodes pacificus* (Shashar and Hanlon 2013) and 40 stages for *Sepiella japonica* (Yamamoto 1982).

The pharaoh cuttlefish, *Sepia pharaonis*, is one of the most important fishery species of cephalopod and is widely distributed from the east Africa to the west Pacific Ocean (Anderson et al. 2010). In Penghu waters in the middle of the Taiwan Straits, *S. pharaonis* is present all year round. These animals can reproduce in captivity temporarily in a small aquarium. Unlike the black eggs of the European common cuttlefish *S. officinalis*, the eggs of *S. pharaonis* are white and translucent. We considered that it is much easier to observe the embryonic development through the egg capsules of *S. pharaonis in situ* than through those of *S. officinalis*. Nabhitabhata and Nilaphat (1999) reported that the developmental period was 9-25 days at 28°C in *S. pharaonis* in captivity. However, the detailed reproductive behavior and embryonic development of this popular species have not been documented to date.

In the present study, we reported the reproductive behavior, especially focusing on the spawning and egg laying behavior and the morphological characteristics of embryonic development of the pharaoh cuttlefish, *Sepia pharaonis*. We also proposed a set of easy criteria to distinguish the developmental stages with the naked eye.

MATERIALS AND METHODS

Animals

Mature *Sepia pharaonis* (*n* = 19, mantle length = 301.00 ± 12.00 mm; body weight = 2154.52 ± 239.09 g), caught by fisherman using angling from Penghu waters in the middle of the Taiwan Straits during February and April in 2014 and 2015, were reared and observed in this study. They included four females and three males caught in 2014 and five females and seven males in 2015. These animals were transported to the Department of Aquaculture, National Penghu University of Science and Technology and maintained in a 4 m-long tempered glass aquarium from February to April in 2014 and 2015. Two 2 × 1.2 × 1.2 m aquariums were connected by an oval opening, allowing cuttlefish to pass through. It was a semi-closed re-circulating seawater system, with seawater flowing through filters, a nitrifying bacterial bed and a protein skimmer. Natural seawater, with salinity ranging from 32-34 psu, was added into the system when cuttlefish ejected ink, and the flow rate was 3 L min-1 until clean. The water temperature was maintained under ambient conditions. The bottom substrata for each aquarium were coral reef stones and sands. These animals were fed with live crabs (*Etisus laevimanus* and *Thalamita* spp.) and frozen fishes (*Scomber japonicas* and *Etrumeus teres*).

Recording Reproductive Behavior

After the escorting behavior by the consort males were observed, the reproductive behavior was recorded by cameras (Coolpix P520, Nikon and TG-810, Olympus) angled outside the aquarium in different directions but close to the spawning grounds.

Eggs Deposition and Incubation and Observations of Embryonic Development

From March to May in 2014 and 2015, the eggs used for the observations of embryonic development were mainly from the paired cuttlefish maintained in the aquarium. Eggs collected from marine cages set up by fishermen for recreation fisheries at the Chieyuan waters in Penghu, Taiwan were also used. In total, twenty clusters of eggs were collected from the aquarium where mature *S. pharaonis* were pairing and depositing since early March to early April in each year. It took minutes for *S. pharaonis* to complete an individual egg-laying. The interval between the first egg and the last one laid may last for several hours or even days in the same egg clusters. Each egg cluster contained 300-600 eggs, and each egg was separated individually from the clusters and incubated in a plastic basket floating in the culture tanks. The

volume of each tank was 300 L. Naturally aerated seawater, with salinity ranging from 32-34 psu, was used to circulate through the system. The flow rate was 1.5 L min^{-1}. In Penghu, the temperature of the seawater varied from 18°C to 27°C from the beginning of March to the end of May. The hatching periods depend on the temperature of the seawater the eggs are incubated in. The time course of the development for each egg, even in the same egg clusters, is different because the eggs are laid singly, and the spawning periods may last for several days. It took 28-31 days to complete embryonic development at a water temperature between 18-21°C, while it took just 22-24 days between 22-25°C. During the period of cell cleavages, observations were made every 3-5 hours and once a day during organogenesis. Eight to ten eggs were randomly sampled each time and observed under a dissecting microscope during embryonic development. Before observation, the egg shells and mucosubstances were removed as much as possible to clearly see the embryo. The chorion of the yolk sac of *S. pharaonis* is extremely soft. Therefore, two to three plastic rings (6 mm in diameter and 7 mm in height) were used to help the fertilized ovum stand upright in order to observe the processes of embryonic development at the animal pole. The morphological characteristics of the cell cleavages and the organogenesis of the live embryos were recorded by camera (TG-810, Olympus), and images were taken directly from one of the eyepieces.

RESULTS

Mating Behavior and Sperm Competition

Mature male *Sepia pharaonis* showed obvious transverse and contrastive white stripes against dark brown skin on the dorsal mantle and arms. The females had thin white strips intermingled with roundish small grouped white spots on the pale brown skin of the dorsal mantle (Figs. 1A-C). These animals automatically paired within an hour after being released into the aquarium. Males sheltered the paired females with the advantage of body size (Fig. 2A). Paired males accompanied and guarded their mates all the time during spawning (Figs. 1A-D). We found multiple mates and sperm competition among 7 males and 5 females in captivity in 2015. Large lone males often boldly swam closely toward the paired mates to compete with the paired male (Figs. 2B-C). In contrast, small lone males waited around and sought to acquire opportunities to pair and mate with females (Fig. 2D). When the paired males proceeded to mate, they changed their escorting pattern (Fig. 1A) into the head-to-head position (Fig. 1B). Males hugged females with the first pair of arms, holding the head of the paired females while the second and third arms supported the arms of females. The mating lasted for 4-5 minutes. Afterwards, the paired females actively stopped mating, and the paired males then went back to their accompanying behavior.

Spawning, Fertilizing, Egg-encapsulating and Egg-laying Behavior

Mature ova ovulated from the ovary accumulated in the germinal sac and then arranged one by one in the oviduct (Fig. 3A). Female *S. pharaonis* aggregated to deposit eggs onto the spawning ground simultaneously (Fig. 3B). Individual eggs were adhered to coral reef stones (Fig. 3C) or other substrata (Fig. 1D) and stuck together at the bases of egg stalks into egg clusters (Fig. 3C). When the paired females began to lay eggs, they first bent their arms into a fist-like phase and maintained the posture temporarily for approximately 30 seconds to spawn (Fig. 1C). In the following phase, they extended their arms forward, ventilated hastily and blew the spawning ground and the previously laid eggs with their funnels. The final phase was egg deposition. Paired females extended their arms forward again to convey and deposit eggs surrounded by mucosubstances onto the appropriate substrata (Figs. 1D, 3C). Each intact egg capsule was translucent and approximately 29.08 ± 0.45 mm in length on the long axis and 17.67 ± 0.17 mm in length on the short axis (Fig. 3D). Paired females repeated the egg-spawning, egg-conveying and depositing phases until they finished egg-laying. During the intervals of egg-laying, the mating continued. We also found that *S. pharaonis* deposited eggs with open posterior ends opposite to egg stalks and not encapsulated completely (Fig. 3E). Most of the time, those incompletely encapsulated eggs would be attacked by small crustaceans or microorganisms in the aquarium (Fig. 3F).

Embryonic Development

Based on morphological characteristics observed during the embryonic development

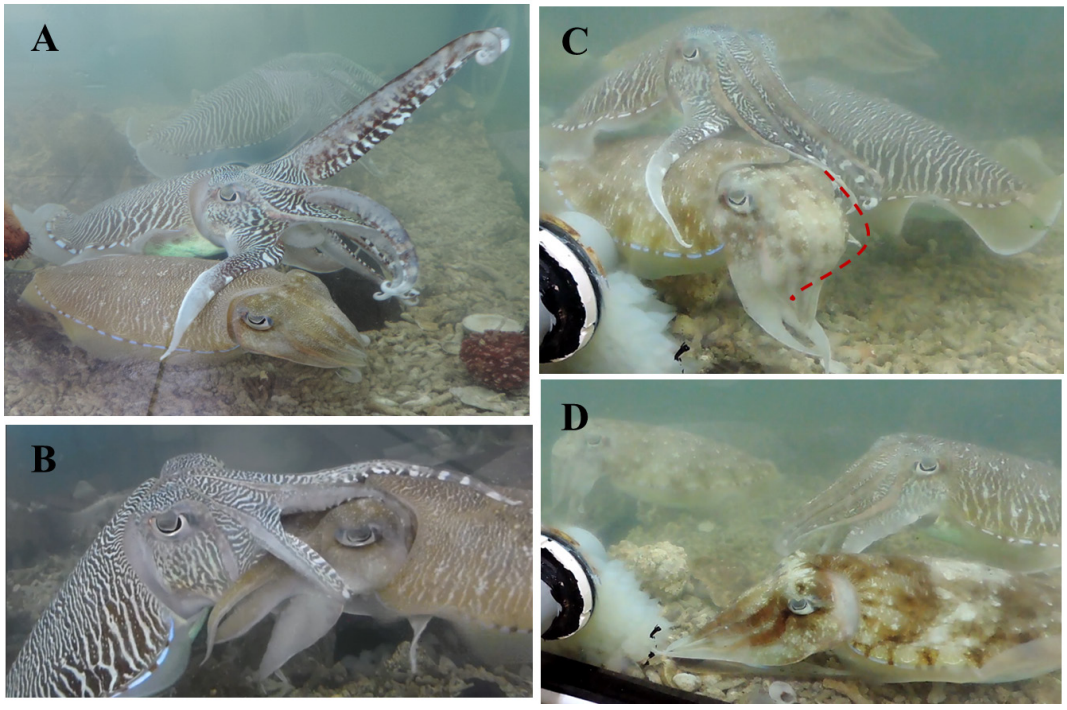


Fig. 1. Reproductive behavior of *Sepia pharaonis*. (A) Males, exhibiting a pattern with transverse and strongly contrasting white stripes against dark brown skin, escort females after pairing during spawning. A paired male raises the first pair of arms high to express antagonism to other males swimming nearby. (B) When mating, the male and the female change posture from an accompanying pattern to a head-to-head position. Then, the male holds the female with the first pair of arms on the head and the second and third pair of arms supporting the arms of the female. (C) A paired female bends its arms into a fist-like manner (indicated by red dash line) for approximately 30 seconds each time to form the chamber presumably resulting from the above posture for spawning. (D) A paired female extends its arms to convey and lay eggs. *Sepia pharaonis* repeats steps C and D until spawning is finished. The head-to-head mating behavior also occurs during the intervals of egg-laying.

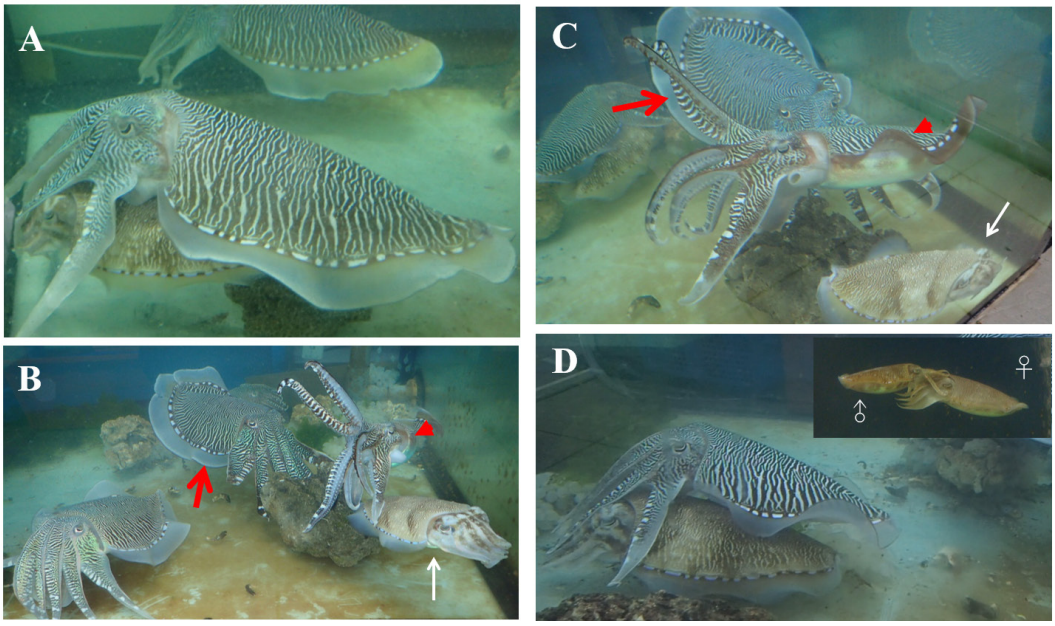


Fig. 2. Sperm competition of *Sepia pharaonis*. (A) A paired large male, with body size advantage, shelters a female. (B-C). A large lone male challenges the paired male and drives it away. Red arrow: large lone male; Red arrowhead: paired male; White arrow: paired female. (D) A small male gets an opportunity to pair and mate (inset) with a female.

of *S. pharaonis*, we described 30 stage scales for illustration (Table 1). Schematic profiles of the embryonic development of *S. pharaonis* are presented in figure 4.

Cleavage phase

The long and short axes of the fertilized ovum were approximately 6 mm and 5 mm, respectively (Fig. 3D). The animal pole was located at the sharp end of the fertilized ovum. Before the first cleavage, small satellite-like protrusions appeared at the central area of the animal pole (Stage 1, Fig. 5A). The first cleavage, vertically oriented, divided the germinal disc into two equal cells (Stage 2, Fig. 5B). The second cleavage divided unevenly and produced two large and two small cells (Stage 3, Fig. 5C). The third cleavage included two equal

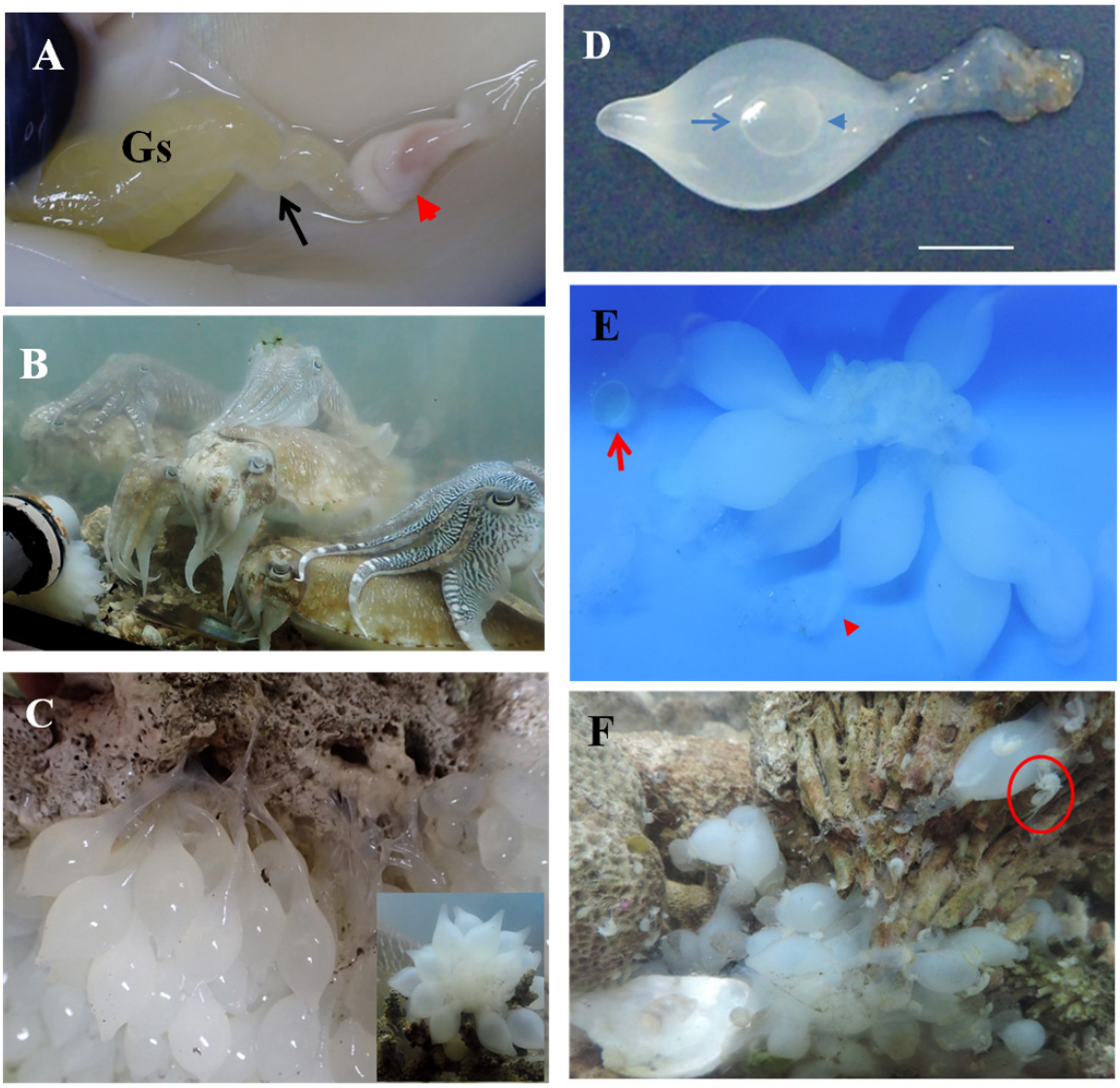


Fig. 3. Characteristics of ova and eggs reproduced by *Sepia pharaonis*. (A) Ovulated ova were accumulated in the germinal sac and arrayed in the oviduct. Arrow: oviduct; Red arrowhead: oviductal gland. Gs: germinal sac. (B) At the beginning of spawning, all females laid eggs simultaneously. (C) Individually pear-shaped eggs were deposited and attached on the coral reef stone. Intertwining egg stalks resulted from the dispersed current by females depositing eggs. Inset is a small egg cluster on the coral reef stone. (D) A successfully spawned egg capsule in which the fertilized ovum, approximately 6 mm in length on the long axis and 5 mm in length on the short axis, is encapsulated by mucosubstance secreted by the nidamental glands of the female and is translucent in appearance. The sharp end (arrow) of the fertilized ovum is the animal pole, and the blunt end is the vegetal pole (arrow head). Scale bar = 7 mm. (E) Unsuccessfully encapsulated eggs in which the posterior end opposite the egg stalk was open (arrowhead) and the ovum was exposed (arrow) were easily attacked by small crustaceans like amphipods (red circle in F) in the aquarium.

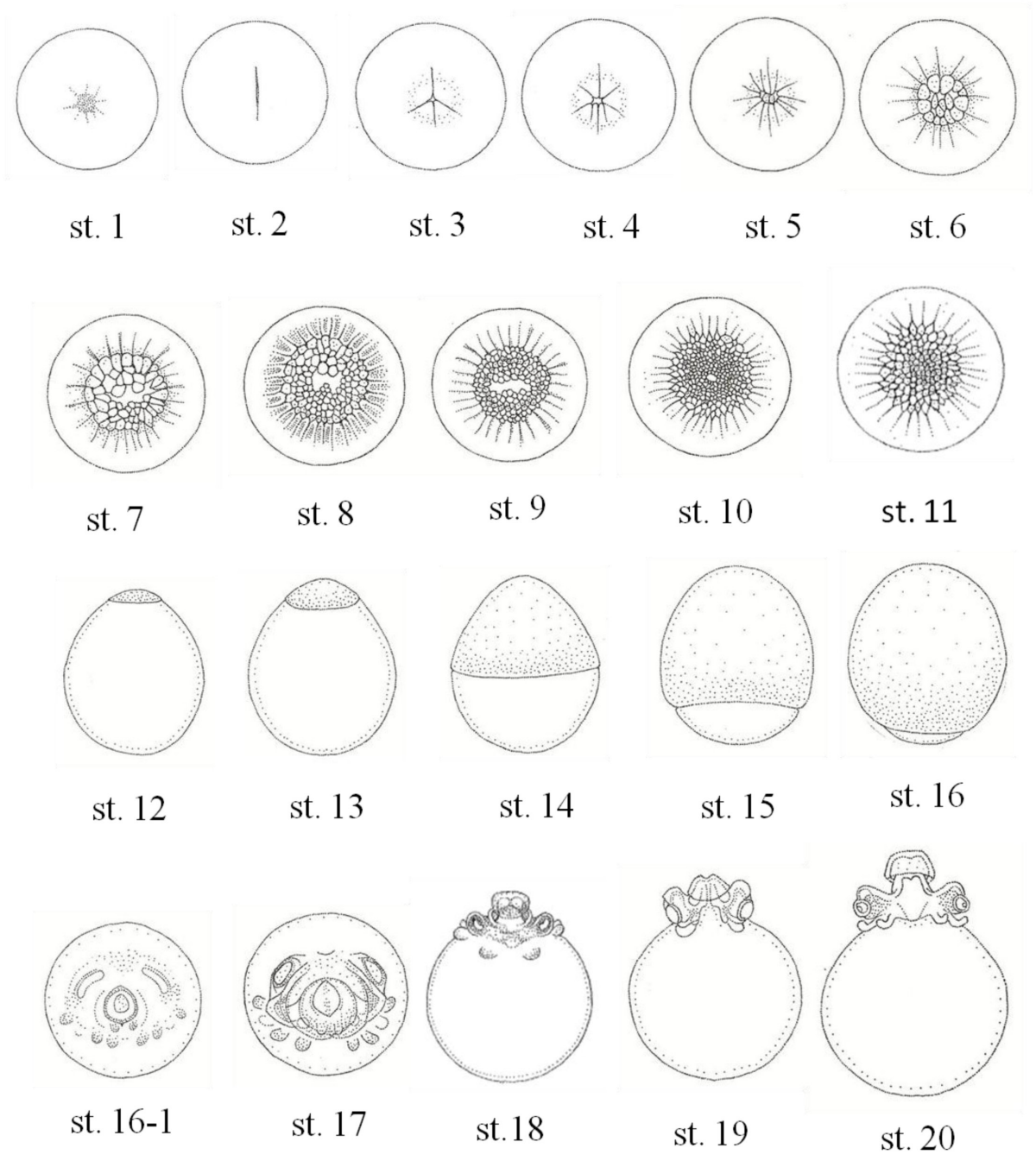
divisions of the two large cells into four equal cells and two unequal divisions of the two small cells, resulting in eight cells (Stage 4, Fig. 5D). The fourth cleavage produced 14 blastocones connecting to yolk cells and two blastomeres independent of the former (Stage 5, Fig. 5E). Then, the following cleavage produced more blastomeres than blastocones (Stage 6-10, Figs. 5F-I). There was an empty and clear zone in the center of the blastodisc from stage 7 to stage 9 (Figs. 5F-I).

Blastulation phase

Blastomeres continued to increase in numbers through the cleavage phase, and radial blastocones gradually disappeared (Stage 11, Fig. 5J) when the blastulation was complete and resulted in the formation of blastoderm.

Gastrulation phase

From this phase on, the phenomena of



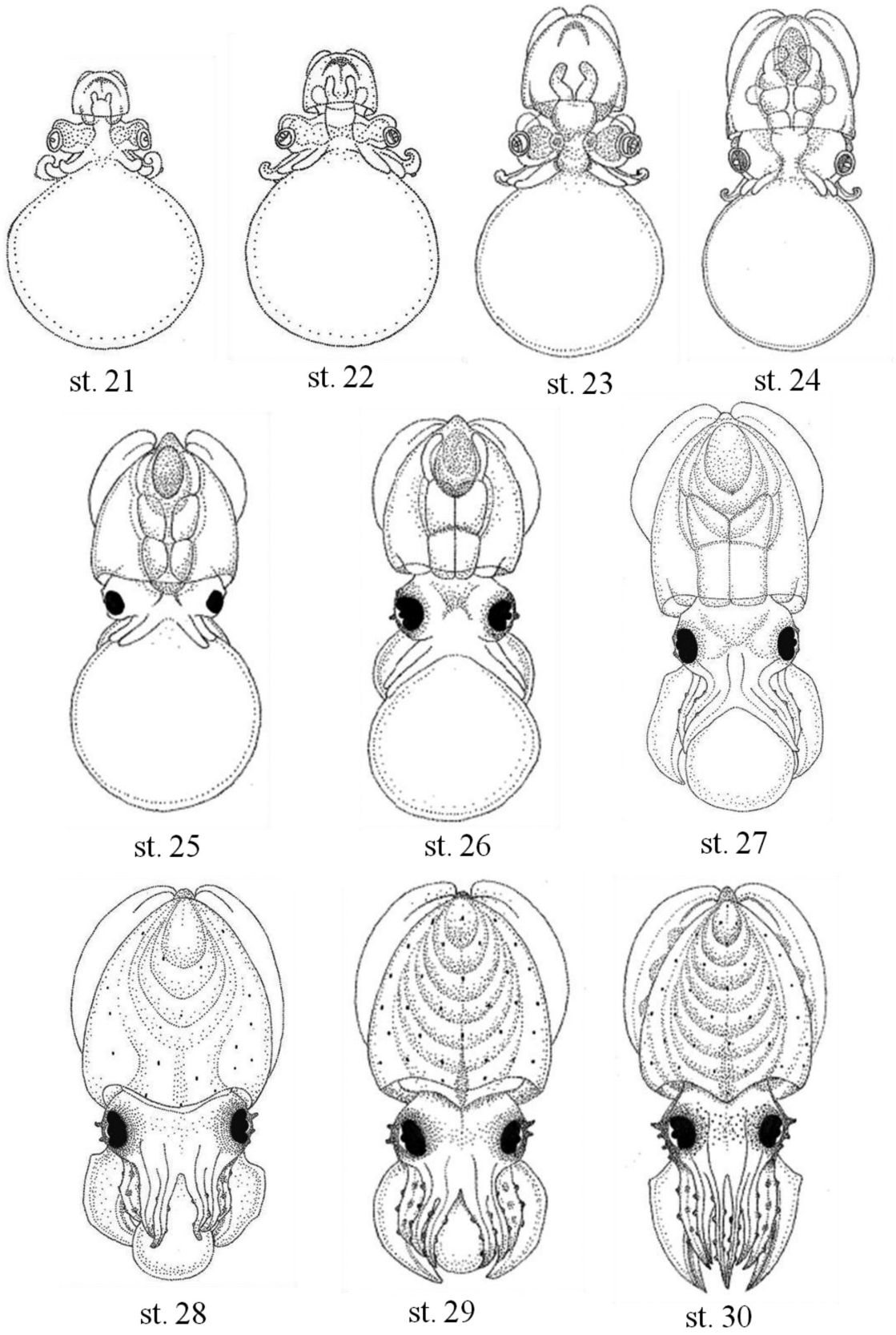


Fig. 4. Schematic profiles of embryonic development of *Sepia pharaonis.* Views from the animal pole of the embryos are shown from stages 1 to 17 and dorsal views from stages 18 to 30. st: stage.

Table 1. Morphological features of embryonic development of the pharaoh cuttlefish *Sepia pharaonis* at 18-25°C and natural running seawater conditions

Developmental stage	Morphological characteristics of embryos
Cleavage	
Stage 1	Granules are present at the outer margins of the vaguely stellar area (blastodisc) at the animal pole at the sharp side of the fertilized ovum. [~5 HPS]
Stage 2	(2 cells) The first cleavage occurs as a vertically oriented furrow incompletely dividing the blastodisc into right and left sides at the animal pole. [~10 HPS]
Stage 3	(4 cells) The second cleavage includes two uneven divisions. It tilts from the middle of the first furrow and produces two large anterior cells and two small posterior cells. [~12 HPS]
Stage 4	(8 cells) The third cleavage includes equal divisions of two larger anterior cells and unequal divisions of the two smaller posterior cells. The cleavage of the latter is parallel to the first furrow. [~14 HPS]
Stage 5	(16 cells) The fourth cleavage forms the first two independent blastomeres at the blastodisc. The cytoplasm of the other 14 cells keeps linking to yolk cells at the margins, resulting in the syncytial blastocones. [~15 HPS]
Stage 6	(32 cells) The fifth cleavage produces 14 blastomeres and 18 blastocones. The former divide unevenly but still appear as a bilateral symmetry. They are separated from the latter. [~16 HPS]
Stage 7	(greater than 64 cells) Since the sixth cleavage, it begins to produce more blastomeres than blastocones, and the bilateral symmetry is no longer seen. The latter surrounds the former and grows in a radial pattern. Anterior blastomeres are larger than those in the posterior region within the blastodisc. There is an empty and clear area, devoid of blastomeres, within the central region of the blastodisc (asterisk). [~18 HPS]
Stage 8	(more than 128 cells) Blastomeres continuously increase, and they vary in size. Blastocones are distinguishable by their radial syncytium. [~36 HPS]
Stage 9	(more than 500 cells) Blastomeres become homogenous, but the empty and clear region still exists. [~46 HPS]
Blastulation and Gastrulation	
Stage 10	The empty and clear areas nearly or totally disappear due to the continuous division of blastomeres. [~54 HPS]
Stage 11	The whole blastodisc looks like a sunflower contour with the homogenous blastomeres at the central circle and the radial blastocones at the margin. The rim of the blastodisc appears as a concentric ring showing light-shading. Radial blastocones arrange at intervals, reaching beyond the margin of blastodisc and connecting to yolk cells. [~82 HPS]
Stage 12	Radial blastocones gradually disappear and the blastoderm is complete. The blastoderm starts to expand and cover the surface of the yolk sac. [~112 HPS]
Stage13	The blastoderm expands and covers approximately 25% of the surface of the yolk sac. [~136 HPS]
Stage14	The blastoderm continuously expands continuously and covers approximately 50% of the surface of the yolk sac. [~198 HPS]
Stage15	The blastoderm continuously expands and covers 80-90% of the surface of the yolk sac. [~250 HPS]
Organogenesis	
Stage16	The blastoderm already covers more than 95% of the surface of the yolk sac. Rudiments of the shell sac, crescent primary optical sacs, and the crown of primordial arms and tentacles appear at the animal pole. [~372 HPS]
Stage17	Optic sacs, four pairs of discrete arms and two tentacles, statocysts and primordial gills, one pair of bilaterally primordial funnel are already visible at the animal pole. The positions of the mouth and anus can also be distinguished. [~384 HPS]
Stage18	The embryo protrudes from the animal pole. Individual arm anlagen are easily seen and the tentacles are straight. Fins anlagen are present at the posterior of the mantle. Cheek humps are present just behind the oval eye anlagen. [~408 HPS]
Stage19	Cheek humps grow outward significantly. The ends of the tentacle anlagen bulge. Two funnel anlagen meet together but do not seal completely. Eyes and projected cheek humps form the four-cornered head architecture. [~422 HPS]
Stage20	The embryo appears upright on the yolk sac. Lens anlagen appear at the center of the optical sacs. Four pairs of arms and one pair of tentacles are connected to the yolk sac. The embryo starts to accumulate yolk internally. The anterior funnel is sealed and elongated. [~432 HPS]

embryonic development could be seen easily by the naked eye. The blastoderm expanded and gradually covered the surface of the fertilized ovum from stage 12 to stage 16 (Figs. 6A-F).

Organogenesis

When the blastoderm covered the surface of the fertilized ovum near about 95%, the rudiments of the organs, including the shell sac, optic sac and arm crown, appeared at the animal pole (Stage 16, Fig. 6F). The adult body plan, including the orientation (Fig. 7A) and distribution of various tissues, was determined (Stage 17, Fig. 7B). The morphological changes visible during organogenesis contained the structures of eyes

Table 1. (Continued)

Developmental stage	Morphological characteristics of embryos
Stage21	The internal yolk sac develops toward the posterior and bifurcates so that a Y-shaped structure is seen clearly from the dorsal side of the embryonic body. It develops towards the anterior to connect the outer yolk sac. The fourth pair of arms is shorter than the bulged tentacles. The end portions of the tentacles are curved with sucker buds. The funnel pouch is complete. The iris forms and rod-shaped lenses are located within the iris. Masses of the optic lobe are located behind the eyes and look opaque. [~444 HPS]
Stage22	The bifurcated internal yolk sac continues to elongate toward the posterior. The optic lobes become enlarged. The retina becomes reddish. The fourth pair of arms elongates apparently longer than the bulged tentacles. [~456 HPS]
Stage23	The retina and the iris continue to deepen in pigmentation. The cornea proceeds to cover the eyes. The Y-shaped internal yolk sac becomes thicker at the anterior portion, and the fork portions grow inward. The first pair of arms becomes longer than the second ones. Fins grow laterally and reach half of the mantle. The first increment of the cuttlebone begins to accumulate. The synthesis of ink begins. [~480 HPS]
Stage24	The internal yolk sac differentiates into two thumb-like shapes, and its posterior end bend inward and meet together. The length of the mantle is approximately half that of the external yolk sac. Orange-colored chromatophores appear. The first increment of the cuttlebone is completed. The eyes appear fresh red. The inverted T-shaped Hoyle's organs appear at the margins between the mantle and fins. The fourth pair of arms becomes triangular due to the expansion at their bases. [~504 HPS]
Stage25	Two increments of cuttlebone form, and the internal yolk sac appears as an inverted shield with a can-shaped base and a triangle chief. The eyes are totally covered by the cornea and then become dark-red. Orange, red and black chromatophores distribute all over the skin. A solid and roughly round ink sac is present in the center of the ventral side. The mantle length is shorter than the long axis of the external yolk sac. [~552 HPS]
Stage26	Three to four increments of cuttlebone form. Internal yolk sac and single cheek projections are visible below the eyes. Vertical and short grooves (lateral lines) are present in the head region. The embryo is capable of ejecting ink when manipulated vigorously. The mantle length is approximately equal to the long axis of the external yolk sac. [~576 HPS]
Stage27	Lateral lines distribute on the head surface and along the dorsal side of the arms. The ends of arm IV reach beyond half of the yolk sac. Two to three small pigmented protrusions distribute along arms I to III. The mantle length is longer than the long axis of the external yolk sac. The W-shaped eyes are completed. [~600 HPS]
Stage28	Five to six increments of cuttlebone form. The rims of the frontal dorsal mantle become bow-shaped. Significantly sharp cheek projections, sometimes with one major and three to four minors, are present below the eyes. The embryo is capable of expressing chromatophore patterns. [~624 HPS]
Stage29	The diameter of the external yolk sac becomes 30% of the head width that it is nearly covered by arms. The arms reach or are even beyond the front end of the external yolk sac. The outer surface of the ink sac changes into a teardrop shape and shows a silver color when viewed from the ventral side of the embryo. [~648 HPS]
Stage30	The external yolk sac is nearly consumed so that it could not be seen from the dorsal side of the embryo. The embryo with eight increments of cuttlebone is going to hatch. [~672 HPS]

~HPS: approximate hours post spawning.

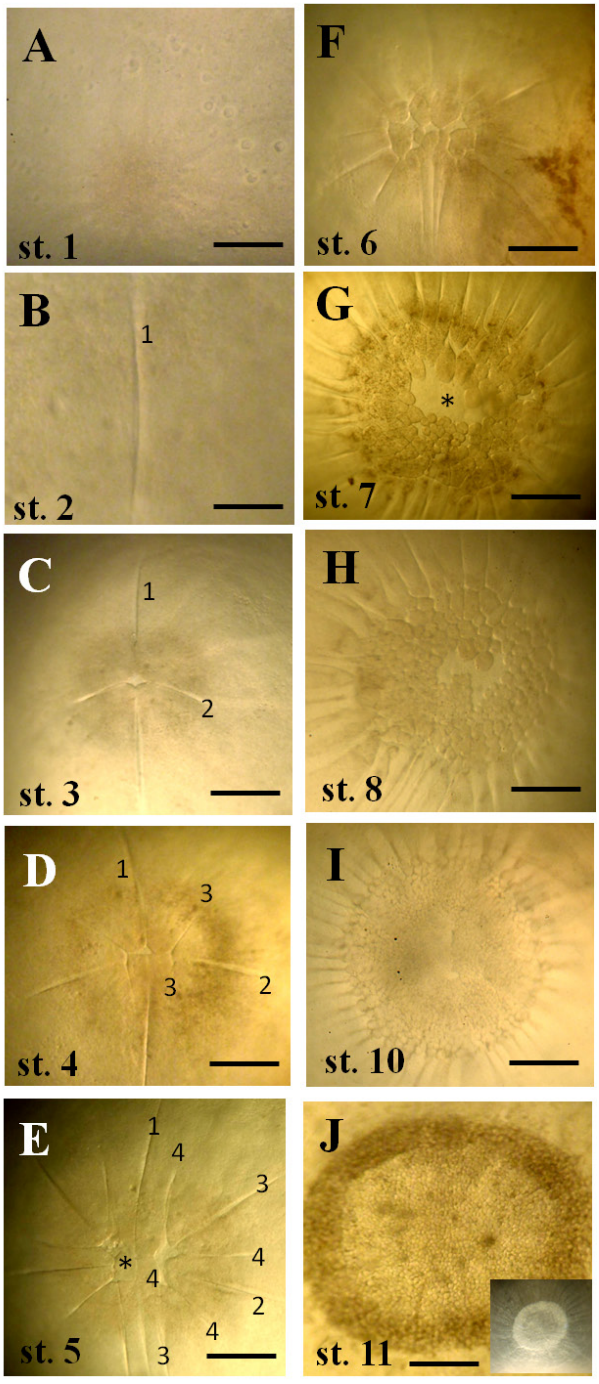


Fig. 5. Cell cleavages of embryonic development of *Sepia pharaonis*. The numerals beside the furrows indicate the successive order of cleavage, except those after the fifth step for simplicity. All of the images were taken from live embryos under the microscope, except that the inset of (J) (A-J) Morphological characteristics of embryos for stages 1-11 are illustrated in table 1. Asterisk in (E) and (G) represents the two independent blastomeres and a clear and empty zone, respectively. Inset in (J) is a direct view of a sunflower-shaped blastodisc with the homogenous blastomeres at central circle and the radial blastcones at margin at the animal pole of the embryo. st: stage. Scale bar = 300 nm.

(Stage 19-26, Figs. 8A-H), arms, funnel and mantle (Table 1, Fig. 9). Transparent, red-ringed and opaque eyes represented the different stages of eye development for lens and iris formation, retina development, and the cornea formation, respectively (Stage 20-26, Figs. 8B-H). Paired gills were first found at the central-ventral side and exposed outward from the mantle (Stage 17, Fig. 7B). They gradually grew laterally and were completely covered by the mantle (Stage 22, Fig. 9C). A unique pattern visible dorsally was the volume and morphological changes between the external and internal yolk sac (Stage 19-30, Figs. 9-10). The morphology of the internal yolk sac from the Y-shaped structure (Stage 21, Fig. 9B) through two thumb-shaped forms (Stage 23, Fig. 9D) finally looked like an inverted shield with a can-shaped

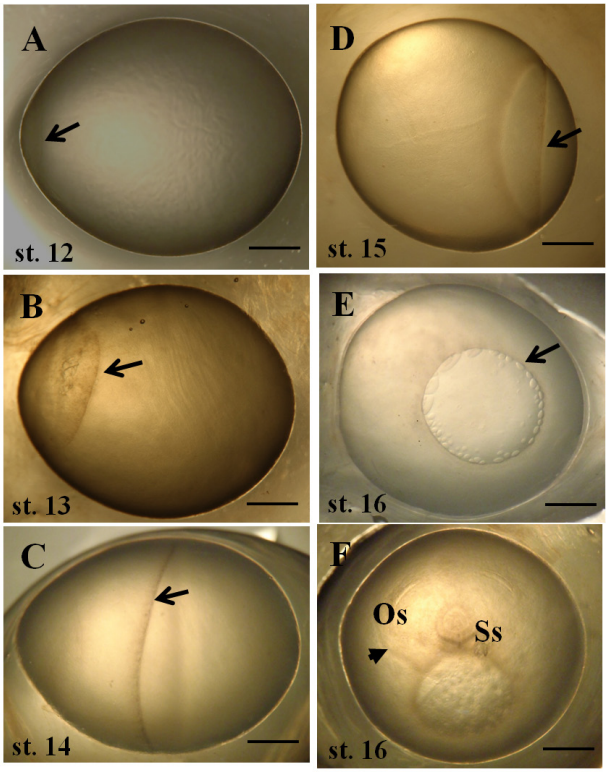


Fig. 6. Gastrulation of embryonic development of *Sepia pharaonis*. (A-E) Morphological characteristics of embryos for stages 12-16 are illustrated in table 1. (F) When gastrulation towards the vegetal pole is not yet complete, organogenesis has already started to proceed at the animal pole. Os: optic sac; Ss: shell sac; st: stage; Arrow: blastoderm; Arrowhead: arm crown. Scale bar = 1 mm.

base and a triangle chief (Stage 25, Fig. 9F). The increments of the cuttlebone accumulated one after the other and reached 8 increments when the embryos hatched (Stage 25-30, Figs. 10G-L). Individual orange chromatophores were first present at stage 24 and were followed by red and black ones. Embryos did not express skin patterns until stage 28 (Fig. 10J). The external yolk sac was consumed and it could not be seen from dorsal side of the embryo prior to hatching (Stage 30, Fig. 10 L).

DISCUSSION

We found that the reproductive seasons of *Sepia pharaonis* in Penghu waters started in mid-October to April of the following year (Lee, personal observation). The peak periods of reproduction were from March to mid-April. This also conformed to the reproductive characteristics of type II *S. pharaonis* described by Norman (2000). However, the molecular genetics data showed that at least five subclades were present among their distribution ranges (Anderson et al. 2010). In the present study, we described the reproductive behavior and embryonic development of *S. pharaonis*, for the first time. Although the body size advantages of males were dominant for acquiring opportunities to pair with females, small lone males were still able to pair and mate with females. Sperm competition behaviors were only observed in 2015, when the sex ratio was 7: 5 (male: female) in the aquarium. We suggested that the advantage of body size of male and male to female ratio both were the important factors to determine mating opportunities in captivity. In loliginid squids, several types of sperm competition were described (Shashar and Hanlon 2013), and they are more complicated than those of *Sepia officinalis* (Hanlon et al. 1999) and *Sepia apama* (Hall and Hanlon 2002). We did not find the sperm removal behavior described in the *Sepia esculenta* (Wada et al. 2005), and their behavioral sequence of mating was also different from our observations of *S. pharaonis*. Male *S. pharaonis* guarded their mates all the way from pairing to egg-laying and even until the end of the life of their mates. This was different from that of *S. officinalis*, which only temporarily guarded their mates (Hanlon et al. 1999) but was not clear in *S. apama*. There is no documentation to date about the mating system of

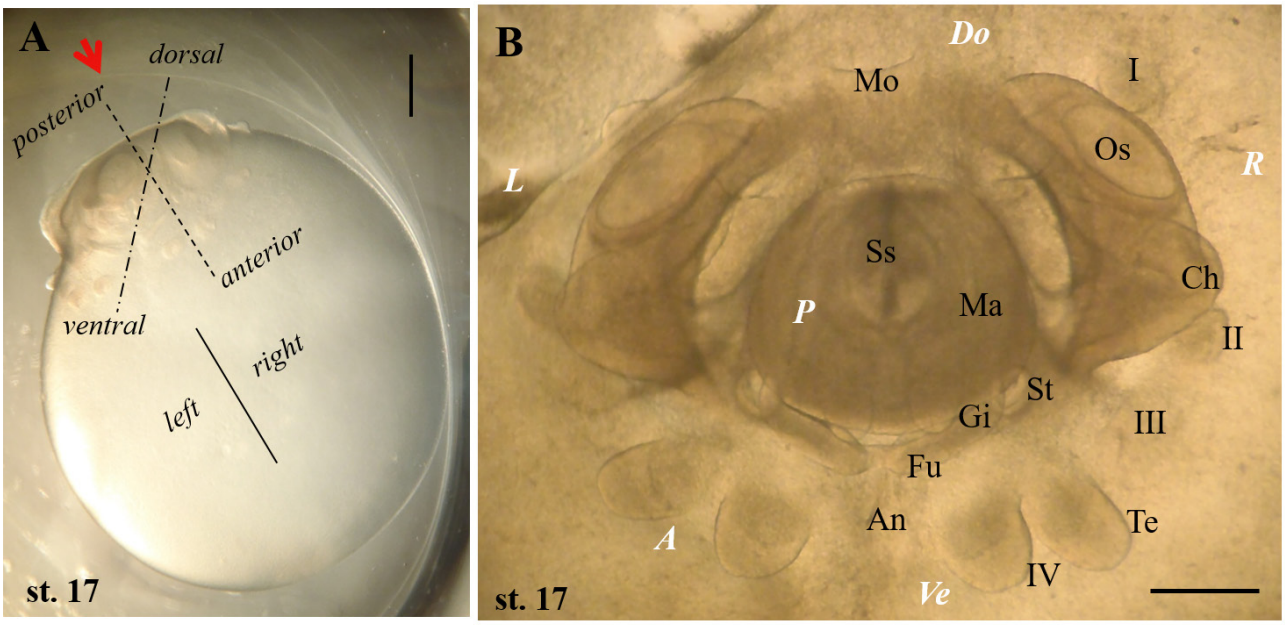


Fig. 7. Organogenesis of embryonic development of *Sepia pharaonis* illustrating the adult body plan. (A) Orientation of the embryo (st. 17) proceeding to organogenesis was already clear and determined. Scale bar: 2 mm. (B) Top view of the animal pole of the embryo in A (red arrow) depicts the upper portion is dorsal (Do) to the embryo and the lower portion is ventral (Ve). The perpendicular axis to the yolk sac facing our view is posterior (P) and the opposite side is anterior (A). I-IV: arm I-IV; A: anterior; An: anus; Ch: cheek pump; Do: dorsal; Os: optic sac; Fu: funnel; Gi: gill; L: left; Ma: mantle; Mo: mouth; P: posterior; R: right; Ss: shell sac; St: statocyst; st: stage; Te: tentacle. Scale bar = 400 nm.

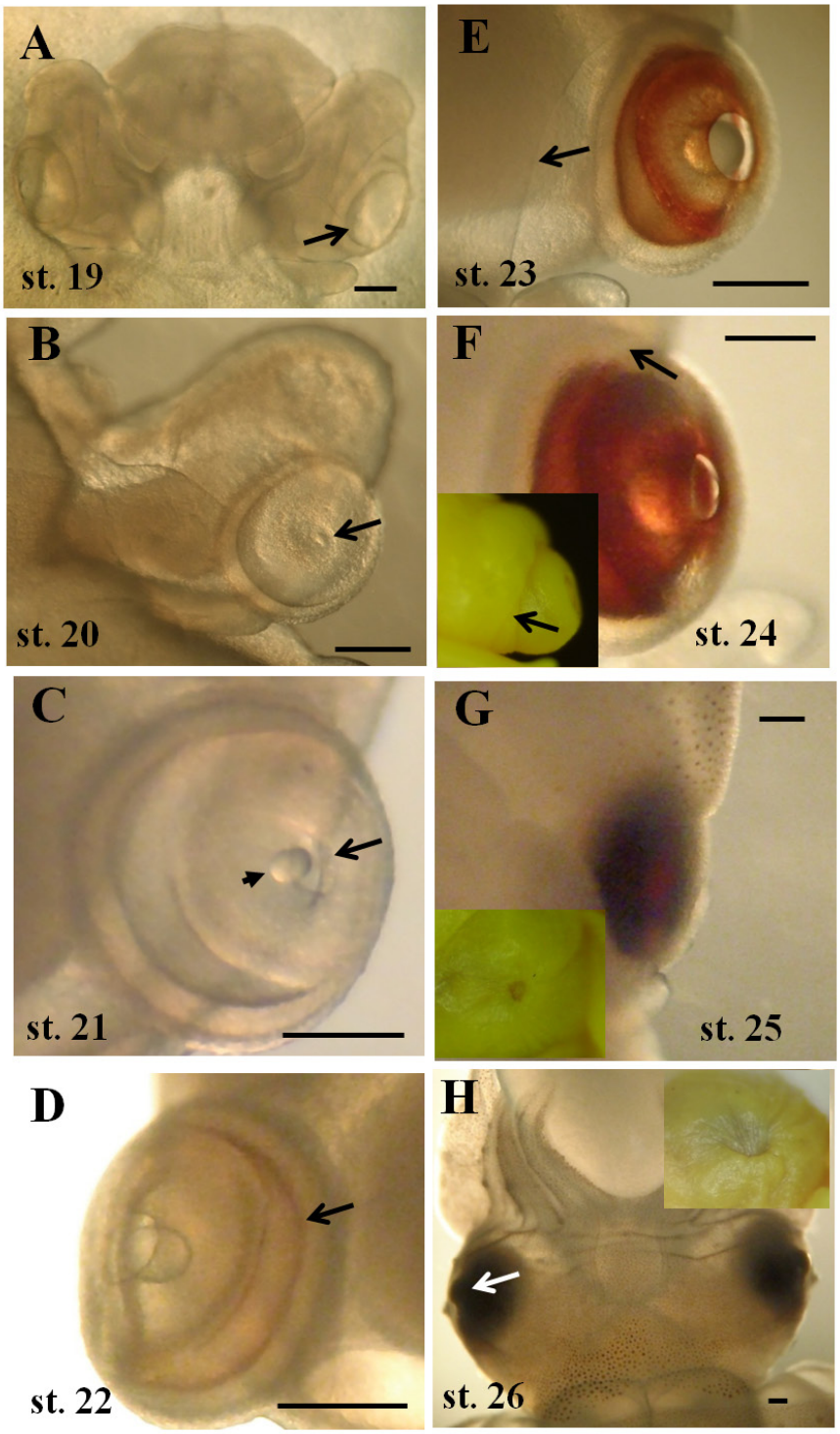


Fig. 8. Morphological characteristics of eye development of *Sepia pharaonis*. (A) Annular optic sac (arrow) is translucent (st. 19). (B) The iris diaphragm (arrow) in the center of the optic sac starts to develop (st. 20). (C) The bean-shaped lens (arrowhead) and the iris (arrow) form (st. 21). (D) A thinly pigmented annular ring (arrow) is present (st. 22). (E) Corneal tissues (arrow) proceed to cover eyes where pigmentation of the retina continues (st. 23). (F) Retina is intensely pigmented, and the cornea (arrow) develops to cover the eyes (st. 24). Inset is the close-up of the cornea layer of the embryo fixed in Bouin's solution. (G) The eyes are enclosed by cornea and become opaque and dark-red (st. 25). Inset is the close-up of the cornea layer of the embryo fixed in Bouin's solution. (H) Lens protrude out toward the orbit, and the eyes appear totally dark (st. 26). Inset is the close-up of the cornea structure of the embryo fixed in Bouin's solution. It is a preliminary form of the W-shaped eye structure. st: stage. Scale bar = 250 nm.

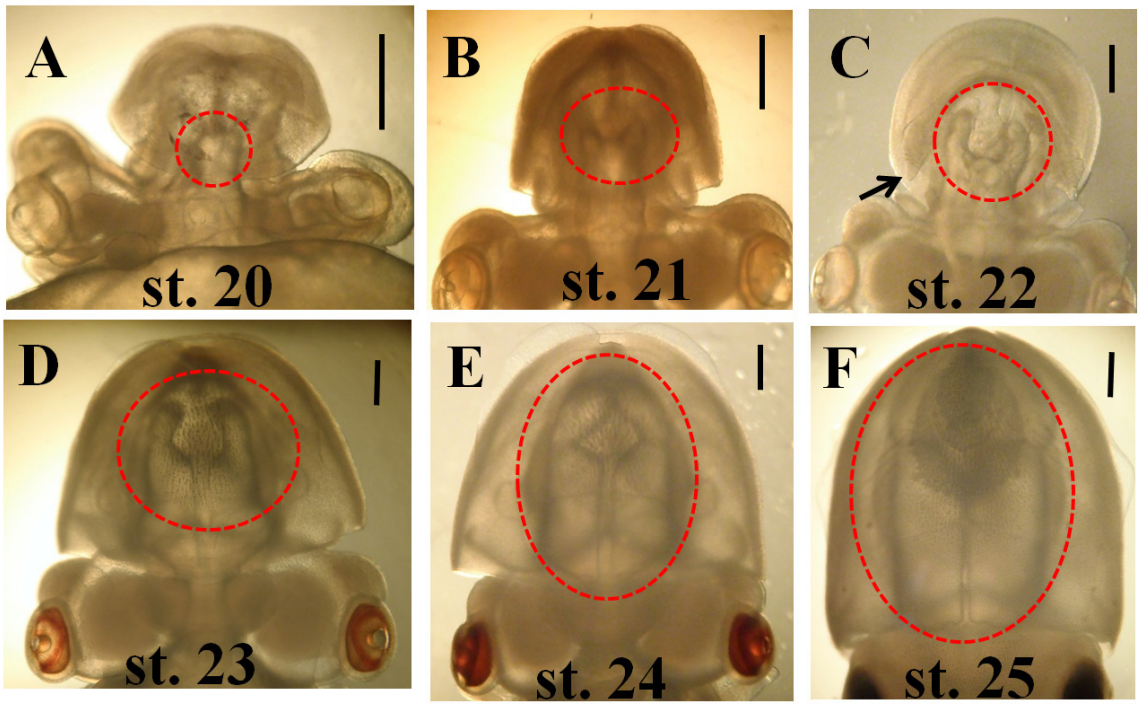


Fig. 9. Morphological changes of the internal yolk sac during late embryonic development of *Sepia pharaonis*. (A) The internal yolk sac of the embryo starts to develop at stage 20. (B) Two small Y-shaped buds protrude laterally from the back of the statocysts at stage 21. (C) Two apparent internal yolk pouches elongate backwards at stage 22. Arrow: gill. (D) Strong and enlarged internal yolk sac are like two thumb-shapes at stage 23. (E) Two laterally strong and enlarged internal yolk pouches meet together in the posterior at stage 24. (F) The internal yolk storage looks like an inverted shield with a can-shaped base and a triangle chief at stage 25. Increments of cuttlebone accumulate above the internal yolk storage. Red dashed circle: internal yolk sac. st: stage. Scale bar = 500 nm.

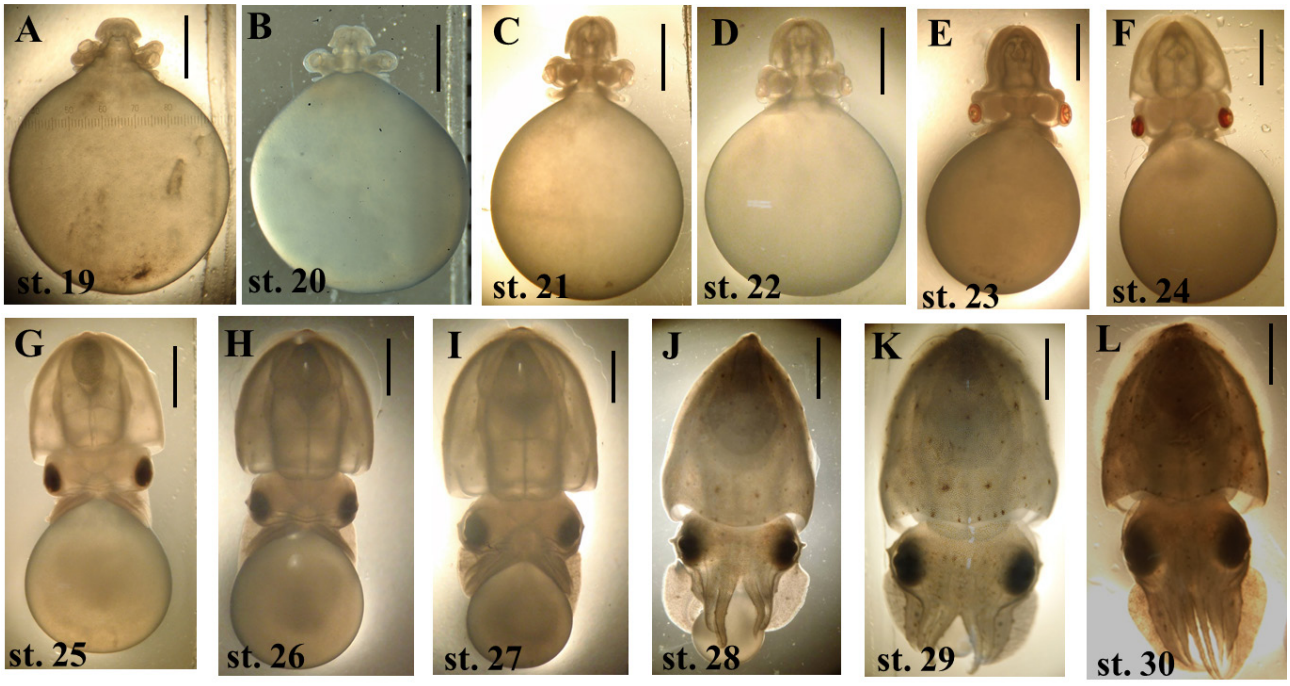


Fig. 10. Dorsal view of embryonic development of *Sepia pharaonis* (stages. 19-30). (A-E) Morphological characteristics of embryos for stages 19-30 are illustrated in Table 1. st: stage. Scale bar = 2 mm.

S. pharaonis in natural waters. The detailed feature of the mating strategy of *S. pharaonis* still needs to be studied further.

After spawning, mature ova were fertilized by sperm ejaculated from the spermatangia previously anchored or the sperm receptacles inside the buccal membranes of the female giant cuttlefish, *S. apama* (Naud et al. 2005). The fertilized eggs were then encapsulated by mucosubstances secreted by nidamental glands (Cornet et al. 2015). We inferred that *S. pharaonis* completed these processes within the chamber formed by bending their arms into a fist-like manner (Fig. 1C) for a very short time. A" humped appearance" in *S. apama* during egg deposition was described by Hall and Hanlon (2002). Unfortunately, as that image was taken from the frontal view, it was difficult to identify whether they were similar to our observations. During this period of time, the funnel of *S. pharaonis* appeared to stop ventilation temporarily. We inferred that the funnel may function first as a passage for ova and then for mucosubstances from the nidamental glands to encapsulate the eggs. After fertilization and encapsulation, eggs were deposited by the first and second pairs of arms, conveying them onto the appropriate substrata with the help of the funnel. Blowing currents towards the spawning ground and egg clusters by the funnel may help to clean the substrata, readjust the space for the coming egg and result in the twisting of the stalks of the eggs. All of these behaviors were repeated continuously until the female finished spawning. However, the mechanism that solidified and shaped the soft mucosubstance-shell into a small pear-shaped egg is still unknown. Kimura et al. (2004) reported that the solidified egg-shell layer and the interior gel-like portion of the egg-mass of squid, *Todarodes pacificus*, originated from water-soluble and insoluble mucin of the nidamental glands, respectively. Without parental protection, the eggs of squids and cuttlefishes were resistant to pathogens. Intact egg shells protected eggs from infections by microorganism or attack by small crustaceans. The antimicrobial activity resulted from the symbiotic bacteria in the accessory nidamental glands of squid (Barbieri et al. 1997) and the egg shell proteins produced from the nidamental glands of cuttlefish (Cornet et al. 2015).

We observed that *S. pharaonis* aggregated for spawning simultaneously at the beginning of reproduction. However, they did not always deposit eggs at the same place in the aquarium. Coral reef stones, nets, pipe holes and even the walls of the aquarium were the selected substrata for *S. pharaonis* to deposit eggs in captivity in our studies.

The embryonic development in cephalopods belonged to the bilateral cleavage pattern (Gilbert 2010). Very few species of cephalopods and their embryonic development have been thoroughly studied to date. One of the reasons may reside in the special traits of their eggs. In squids and cuttlefishes, mucosubstances secreted by nidamental glands served as an egg shell to protect the fertilized ova from infections (Cornet et al. 2015), but it also impeded observation of cell cleavage at the animal pole in the early stages of development. In particular, the chorion of the fertilized ova of *S. pharaonis* was very soft compared to that of *Sepioteuthis lessoniana* (Lee, personal observation). Therefore, it increased the difficulty of studying those early stages of embryonic development of this animal. In the present study, we used live embryos with their egg shells removed as much as possible and supported by artificial plastic rings to acquire the images from the animal pole of the ova under the dissecting microscope. The cell cleavage patterns of the pharaoh cuttlefish were similar to those of European common cuttlefish, *S. officinalis* (Lemaire 1970), but varied from those of the Hawaiian bobtail squid, *Euprymna scolopes* (Lee et al. 2009). Only two independent blastomeres formed in the embryos of *S. officinalis* and *S. pharaonis* after the fourth cleavage, while four independent blastomeres were present in the embryos of *E. scolopes*. Morphological changes of the internal yolk sac within the mantle of *S. pharaonis* embryos were revealed from the observation of live samples. The internal yolk reservoirs of cephalopods provided the nutrient sources for the embryos during development (Boletzky 2003) and even after hatching (Vidal et al. 2002). In addition, their shapes were also different among taxonomic groups in cephalopods, such as a glove-shaped pattern of *Sepiola* sp. (Boletzky 2003). Whether the varied reservoir volume of the internal yolk associates with the time of initiation for feeding after hatching needs to be studied further.

Significant changes in the ontogenetic development of the eyes were also observed. The cornea enclosed the eye of *S. pharaonis* at stage 25, while they occurred at stage 38 in *Sepiella japonica* (Yamamoto 1982), stage 28 in the *Sepia officinalis* (Lemaire 1970) and 2 days post-hatching in *Sepioteuthis australis* (Bozzano et al. 2009).

Whether the timing of the enclosure of the eyes was related to the habitat or feeding behavior of hatchlings still needs to be studied further.

CONCLUSIONS

This was the first detailed observation of the complete reproductive behavior, including sperm competition, egg-laying in captivity and embryonic development, of the pharaoh cuttlefish *Sepia pharaonis.* We also proposed an easy way to distinguish the developmental stages of *Sepia pharaonis* with the naked eye. The presence of radial lines at the animal pole occurred in stages 6-10. The presence of the sunflower-shaped blastodisc with the homogenous blastomeres at the central circle and the radial blastocones at the margin at the animal pole occurred in stages 11-12. The blastoderm covered the surface of the yolk sac approximately 25%, 50%, 80%, and 95% in stages 13, 14, 15 and 16, respectively. In stages 17-18, the embryos with organ anlagen protruded at the animal pole. In stages 19-20, the embryos appeared upright on the yolk sac, and the eyes were transparent. The presence of the pigmented retina (ring of orange color) occurred in stages 21-22. In stages 23-24, the embryos had fresh red eyes. In stages 25 and 26, the embryos had dark red eyes and the long axis of the external yolk sac was equal to the mantle length, respectively. In stages 27 and 28, the long axis of the external yolk sac was shorter than the mantle length and the presence of apparent chromatophore patterns on the dorsal surface of the mantle and the arms holding the external yolk sac was observed, respectively. In stage 29, the external yolk sac became 30% of the head width that it was observed between the first pair of arms, and in stage 30, the external yolk sac was not visible.

These data provided important information not only for research to further understand the reproductive strategy and embryonic development but also for aquaculture to improve practical manipulations in the rearing of the pharaoh cuttlefish.

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