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REPRODUCTIVE ASPECTS AND TOLERANCE TO TEMPERATURE AND SALINITY OF EGG OF PHARAOH CUTTLEFISH *Sepia pharaonis* Ehrenberg, 1831 FROM VIETNAM WATERS

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ABSTRACT: This paper reports sex ratio, gonad development, and fecundity of the pharaoh cuttlefish (*Sepia pharaonis* Ehrenberg, 1831) field collected specimens and tolerance of live eggs obtained from broodstock to temperature and salinity in tank conditions. The cuttlefish specimens collected from Vietnam waters showed an average ratio of male: female of 1:1.07. When reaching a mantle length of 81–90 mm, the cuttlefish became sexually mature. The fecundity was 52–1938 eggs depending on female cuttlefish size. The newly laid eggs from broodstock had an average size of 40 mm and weight of 1.8 g. After 14–20 days, the eggs hatched. The hatchlings had no metamorphosis, lived on the yolk for 3–5 days and then began feeding on provided foods. The embryo normally developed at temperature of 26–28ºC and salinity of 30–35‰, but with a hatching rate of lower than 50%.

Keywords: pharaoh cuttlefish, *Sepia pharaonis*, reproductive aspects, egg, tolerance, temperature, salinity, Vietnam

INTRODUCTION

Cephalopods represent a significant worldwide fishery resource which consisted of 13.6% cuttlefish in 2000 (Jereb and Roper 2005). Several studies on biological aspects of cuttlefish have been published (Oshima and Choe 1961; Nabhitabhata and Nilaphat 1999). According to Bui (2000), cuttlefish resource in Vietnam was estimated to be 66,900 tons. For decades, the exploitation of cuttlefish for seafood by Vietnamese fishermen has caused a severe decrease in annual catching yield indicating a depletion of this natural resource. Although, the Vietnamese government has issued regulations on catching and conservation, the management of this resource has not been effective because information on biology of these cuttlefish is still limited. In this paper, spawning characteristics of *Sepia pharaonis* maintained in tanks are reported. The results should be useful for conservation of this fishery resource.

MATERIALS AND METHODS

Sample collection

Specimens of pharaoh cuttlefish caught by fishermen from the wild were collected at fishing ports of Nha Trang twice a month from April 2003 to May 2004. A total of 687 individuals of pharaoh cuttlefish were collected. The specimens were washed with fresh water and mopped with blotting sheets to remove the external moisture before measuring biological indices.

Determination of sex ratio and gonad development

All 687 collected individuals were classified in 12 size classes: 18–50, 51–60, 61–70, 71–80, 81–90, 91–100, 101–110, 111–120, 121–130, 131–140, 141–150, 151–265 mm in length. Sex determination for all classes was done according to Dunning *et al*. (1994) and Nabhitabhata and Nilaphat (1999).

A total of 187 females which had been sexually distinguished were used for determining gonad stages. The stages of gonadal development were determined according to Mangold-Wirz (1963). The gonads were fixed in bouin solution and transferred to ethanol in preparation for serial sectioning. Tissue blocks were then embedded in paraffin; sections of 4-6 µm thickness were cut by microtome. Sections were stained with haematocylin and eosin; histological slides were observed with an optical microscope with magnifications of 40–400 times. Size at first sexual maturation was defined as the size at which at least 50% of the individuals of a size-group had its gonads developed at stages III and IV.

A total of 60 females from the collected specimens which had gonads developed at stage III so that the number of eggs could be counted were used for determining fecundity and the relative fecundities. The fecundity of a single individual (F) was the number of eggs counted (eggs). Three relative fecundities were calculated as follows:

Ftb (eggs/g) = F (egg nos.)/total body weight (g);

Fmb (eggs/g) = F (egg nos.)/mantle weight (g);

Fo (eggs/g) = F (egg nos.)/ovary weight (g);

where mantle weight (g) was the total body weight without viscerae, cuttlebone and head.

Broodstock maintenance and egg incubation

In preparation for experimental studies on eggs, 7 male and 7 female live cuttlefish of mantle length 130–152 mm were collected and maintained in concrete tanks according to the guidance in Nabhitabhata and Nilaphat (1999). In the concrete tanks of 3 m width, 10 m length and 0.5 m depth, water parameters were maintained as follows: temperature: 28–30ºC, salinity: 33–35‰ and pH: 7.8–8.5. Substrates such as a netting roll were placed in the water column for cuttlefish to attach their eggs. Seawater was rinsed through sand filters, aerated continuously and changed 100% every 5 days. The clusters of eggs laid by these females were maintained in tanks to observe the embryonic development. The body weight of the females after their spawning was 228.6–342.1 g.

Experiment on the effect of salinity on the eggs

Newly spawned eggs separated from a cluster were incubated indoor in 100-L plastic tanks at a density of 50 eggs /tank. The seawater in these tanks was aerated continuously. Seven different salinities (15, 20, 25, 30, 35, 40, and 45‰) were used in the experiments. Each treatment was triplicated. Almost all the water in the tank was changed every 5 days. To obtain a certain assigned salinity level, seawater of 35‰ was diluted by adding freshwater for lower salinity levels, and for higher salinity levels by adding brine water of over 200‰ salinity from salt fields. During the experiment, the salinity of each treatment increased due to evaporation; which was controlled by adding freshwater. Salinity was measured and checked every day at 1700 during the experiment with a salinity meter (Extech Ec170 Hanna). The temperature and pH were recorded daily at 0600 and 1400 by Extech Ec170 Hanna and Milwaukee MW 101 Emin, respectively.

Experiment was continued until hatching was completed and the hatching rate was determined by the following equation:

Hatching rate (%) = Total number of hatched eggs / Total number of eggs x 100.

Experiment on the effect of temperature on the eggs

Newly spawned eggs from a cluster were collected and incubated indoor in 100-L plastic tanks at a density of 50 eggs/tank. The seawater used for the experiment had salinity of 35‰, pH of 7.8–8.2. The water in experiment tanks was aerated continuously. Five temperature intervals were applied: 18–20; 22–24; 26–28; 30–32; 34–36ºC. The water temperature in each treatment was maintained by a heat controller (W3230 AC110–220V) with a precision of 0.5ºC. Each treatment was triplicated. Almost all water in tanks was changed every 5 days. The temperature of new seawater was adjusted to the correct interval before supplying to each experimental tank. During the experiment, water pH and salinity were checked daily at 0600 as outlined above.

The experiment was continued until hatching was completed and the hatching rate was determined as described above.

RESULTS

Sex ratio and gonad development

A total of 275 males, 342 females and 70 specimens of undeterminable sex were identified. The ratio of *Sepia pharaonis* males to females varied with month of the year and with length size class. The average ratio was 1:1.07. The lowest proportion of males found in May and August 2003, was 1:1.6 (38.6% of males and 61.4% of females) and the highest was 1:1 in April 2004.

The sex ratio in a mantle size group fluctuated irregularly, males being more abundant than females in the size groups of 71–80, 101–110, 111–120 and 121–130 mm, but females most abundant in other size groups (Table 1).

This study showed that female pharaoh cuttlefish matured sexually at the time they reached a mantle length of 81–90 mm, and body weight of 54.7–76.5 g. In this class, 51.43% (34.29+17.14%) of the collected specimens had ovaries containing eggs in stage III and IV (Table 2).

Table 1: Sex ratio (male: female) of pharaoh cuttlefish collected from fishing ports, classified by size of mantle length (mm).

Size class (mm)	Male: female ratio
51–60	1: 1.06
61–70	1: 1.34
71–80	1: 0.66
81–90	1: 1.89
91–100	1: 1.16
101–110	1: 0.50
111–120	1: 0.60
121–130	1: 0.54
131–140	1: 1.71
141–150	1: 1.81
151–265	1: 1.15

The fecundity of cultured cuttlefish increased with their body size (Table 3). The mantle size group of 61–70 mm (immature) had the lowest number of eggs/ind. ranging from 52 to 221 eggs. The highest fecundity was in the large size group from 151 mm with 397–1938 eggs/ind. The relative fecundity in the small size group was higher than the largest size group: the highest was 4.06 eggs/g body weight; 5.5 eggs/g mantle weight and 90.85 eggs/g gonad weight (size group of 61–70 mm) and the lowest of 1.77 eggs/g body weight; 1.67 eggs/g mantle weight and 31.14 eggs/g gonad weight (size group of above 150 mm). The average fecundity was 477.53 ± 89.58 eggs/g body weight; 2.77 ± 0.42 eggs/g mantle weight and 62.58 ± 7.54 eggs/g ovary weight.

Mating and spawning in captivity

The pharaoh cuttlefish were unisexual and had external fertilization. Reproductive activities occurred when male and female matched and paired, and began copulating. The male hovered above the female, caressing her dorsum with his arms in a parallel position. Their heads were in the same direction. The male grasped female arms by his arms and twisted to a head-to-head position for copulation. The male's hectocotylus was the modified left fourth arm. The spermatophores were transferred by hectocotylus and attached to the seminal receptacle at the buccal region of the female. This process lasted about 5–20 minutes.

Before spawning, the female swam finding a substrate to which her egg capsules would be attached, while the male continued escorting her. The female began to lay eggs on the substrate one by one. The female used arms to hold each egg capsule and attach them to the substrate forming a cluster of eggs. After spawning, the male and female swam backwards and around the place where they attached their egg capsules. Sometimes they flushed the cluster of eggs by the water jet from their funnels. Each female could spawn from 1–5 times in a period of 20–25 days.

Table 2: Proportion of maturity stages of collected female pharaoh cuttlefish specimens (%) by their size class (mantle length: mm).

Size class (mm)	Nos. of cuttlefish	Stage II (%)	Stage III (%)	Stage IV (%)	Total proportion of determined stage (%)
51–60	30	30.00	0.00	0.00	30.00
61–70	32	18.75	12.50	0.00	31.25
71–80	30	16.67	33.33	0.00	50.00
81–90	35	11.34	34.29	17.14	62.86
91–100	25	8.00	44.00	48.00	100.00
101–265	35	0.00	71.70	28.30	100.00

Table 3: The relative fecundity (eggs/g) of collected pharaoh cuttlefish from Khanh Hoa province. The average in brackets.

Size class (mm)	F (egg nos.)	Nos. of cuttlefish	Relative fecundity (eggs/g)		
			Ftb	Fmb	Fo
61–70	52–221	4	1.57–7.54	1.93–10.05	64.55–115.71
	(139.89)		(4.06)	(5.50)	(90.85)
71–90	146–418	22	2.58–9.08	3.16–12.23	65.36–123.03
	(286.36)		(3.95)	(5.16)	(86.03)
91–110	89–441	14	1.03–3.75	1.31–5.04	24.01–91.11
	(293.67)		(2.69)	(3.57)	(62.26)
111–130	358–959	6	1.16–3.39	1.64–4.69	19.43–64.36
	(657.83)		(2.13)	(2.93)	(42.12)
131–150	385–1269	7	0.95–2.86	1.37–4.01	24.84–79.31
	(692.07)		(1.99)	(2.77)	(47.90)
151–265	397–1938	7	0.36–2.57	0.52–3.78	10.16–76.00
	(865.63)		(1.77)	(1.67)	(31.14)
Average	477.53		2.77	3.72	62.58
Std	89.58		0.42	0.54	7.54

Embryonic development

The newly laid egg capsules were opaque white, almost uniform, oval shape with a stalk. They were stuck together by stalks, which were attached to the substrate. The embryos were round or oblong. The egg capsules turned larger, became more transparent and fragile in concert with the embryonic development and the more locomotory activity of the cuttlefish at near hatching.

The whole length of newly laid egg capsule, including the stalk, was 15–65 mm (average of 40 mm); length of egg capsule was 6–28 mm (average of 22 mm); width was 7–17 mm (average of 13 mm), and weight was 0.4–3.2 g (average of 1.8 g) (n= 32). The egg capsule size and weight increased over time and reached maximum just before hatching. At near hatching, the capsule was transparent so the cuttlefish was visible inside. The average length of egg capsule at near hatching was 24 mm, and the average weight was 2.7 g.

After 14–20 days at water temperature of 28–30ºC, the eggs hatched. Cuttlefish eggs were rich in yolk, the yolk mass of embryo comprised internal and external sacs, (providing energy for growth), which gradually decreased in size during the development. On day 5, ocular cups and mantle developed. On day 6–7, eyes and arms were observed. On day 8–10, ink-sac and cuttlebone were observed. The chromatophores could be observed on the surface of the dorsal and ventral mantle (dots of light brown color). On day 11–13, the arms developed longer, the eyes were black, the mantle was larger and milky colored, most of the organs were developed. On day 20, the eggs hatched. The newly hatched cuttlefish had an average total length of 12 mm, an average mantle length of 6 mm, and an average weight of 0.1 g. The hatchling lived on the yolk for 3–5 days. Then they started feeding on live foods available in the rearing tank.

The effect of temperature on egg hatching

During the experiment, the water salinity and pH were 35.2–36.6‰ and 7.7–8.2 respectively. Eggs developed and hatched at the temperatures between 22 and 32ºC (Table 4). In this range, the higher the temperature, the faster the development and the shorter the incubation period. At a temperature of 26–28ºC, the embryos normally developed, achieved a higher hatching rate. At temperature of 30–32ºC the embryos developed faster but the abnormality rate was higher. Experimental results showed that the optimum temperature for pharaoh cuttlefish egg incubation was 26–28ºC.

The effect of salinity on egg hatching

During the experiment, the water temperature and pH were 28.2–29.6ºC and 7.8–8.2 respectively. Salinity did not affect the incubation period, but obviously affected the hatching rate (Table 5). In the salinity range of 25–40‰, the embryonic period was not different and lasted 13–14 days. At a salinity of 20 or 45 ‰, the embryos died after 3 days. The hatching rate was only 6 and 12% at the salinity of 25 and 40 ‰, respectively. The embryos normally developed at salinity of 30 and 35 ‰, achieving hatching rates of 28 and 40%, respectively.

Table 4: Hatching of eggs and embryonic period (day) at different temperature (numbers in brackets indicate time till death).

Temperature (ºC)	Hatching rate (%)	Embryonic period (day)	Conditions of embryos
18–20	0.0	(10)	Development relatively slow and death after 10 days
22–24	5.5 ± 0.2	23.4 ± 1.6	Normal development
26–28	20.3 ± 2.8	20.1 ± 2.2	Normal development
30–32	15.5 ± 1.3	13.0 ± 1.2	Normal development with abnormalities in some embryos
34–36	0.0	(2)	Abnormal development and death after 2 days

Table 5: Hatching of eggs (%) and embryonic period (day) at different salinities (numbers in brackets indicate time till death).

Salinity (‰)	Hatching rate (%)	Embryonic period (day)	Conditions of embryos
15	0.0	(3)	A milky white spot occurred in the egg capsule and the embryo decay after 3 days
20	0.0	(4)	A milky white spot occurred in the egg capsule and the embryo decay after 4 days
25	6.2 ± 1.7	13.1 ± 0.1	Normal development, but yolks of some embryos were crumbled and decay
30	28.2 ± 1.4	13.4 ± 0.3	Normal development
35	40.3 ± 2.5	13.5 ± 0.20	Normal development
40	12.2 ± 1.4	14.2 ± 1.0	Normal development, but yolks of some embryos were crumbled and decay
45	0.0	(3)	The egg capsule turned milky white inside and the embryo decay after 3 days

DISCUSSION

Reproductive aspects

For pharaoh cuttlefish, in this study, the average ratio of males to females was 1:1.07, they were sexually mature and started spawning at the mantle length of 99–100 mm and their fecundity was 52–1938 eggs. These results corresponded to those previously reported (Nabhitabhata and Nilaphat 1999). The pharaoh cuttlefish were sexually mature after 90 days and mating was observed from that time. Spawning occurred at the age of 110 days and one female laid from 50 to 3000 eggs. The average life span was 149.4 days due to mortality of both sexes after spawning. Largest final size was 162.0 mm mantle length and 368.48 g body weight (Nabhitabhata and Nilaphat 1999).

Tolerance of eggs to temperature

In this study, eggs of pharaoh cuttlefish could hatch at temperatures of 22-32°C achieving a maximum hatching rate of 20.3% and embryonic period of 13.0-23.4 days. In an investigation on *Sepia officinalis* L. the hatching rate was 50% and embryonic period was up to 30 days. Temperature was found to affect only the incubation time (Palmegiano and D'Apote 1983). The incubation period of eggs from cuttlefish was 14.3 days at 28°C (Nabhitabhata and Nilaphat 1999). The observed results indicate the lower temperature caused longer embryonic periods. The low hatching rate and longer embryonic period in the present study, even at optimal temperature, indicates that some unknown factor of the laboratory culture conditions was sub-optimal.

Tolerance of eggs to salinity

In this study the embryos of cuttlefish could hatch at a salinity range of 25–40‰ and better hatching was observed at salinity of 30 and 35‰ achieving the hatching rate of 28 and 40%, respectively. This observation is similar to those reported that optimum ranges of salinity was 21.8–36.6‰ for bigfin squids, *Sepioteuthis lessoniana*, and 22.5–37.5‰ for pharaoh cuttlefish. However, eggs of these two species did not hatch at 40‰ (Nabhitabhata *et al*. 2001). Oshima and Choe (1961) reported 75% hatching of cuttlefish and bigfin squids in a salinity range of 14.3–16.3‰. The difference in optimum salinity was probably caused by the difference in rearing condition, for example the temperature of each experimental unit was not controlled. This might also explain the different hatchability of eggs at 40‰ salinity level. The hatching rate of cuttlefish eggs in this study was less than 50% while it was 75% reported by Oshima and Choe (1961) and up to 100% by Nabhitabhata and Nilaphat (1999). The low quality of gametes from unhealthy broodstock, or some unknown factor of the laboratory maintenance conditions might be the reasons for low hatching rate in this study.

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