Acute and Chronic Effects of Ammonia on Juvenile Cuttlefish, Sepia pharaonis: ACUTE AND CHRONIC EFFECT OF AMMONIA ON CUTTLEFISH

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Effect of Salinity on Embryonic Development of the Cuttlefish *Sepia pharaonis*

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Abstract

The effect of salinity on the embryonic development of *Sepia pharaonis* eggs was tested experimentally using *S. pharaonis* embryos at various developmental stages. The hatching rate, incubation period, hatching period, wet weight of hatchling cuttlefish, and yolk utilization efficiency ratio were quantified at salinities of 18, 21, 24, 27, 30, 33, and 36. These variables were significantly affected by salinity. The weight of the eggs first decreased and then increased during embryonic development. The range of suitable salinities for hatching of *S. pharaonis* eggs was 27–33, and the optimal salinity was 30.

The cuttlefish, *Sepia pharaonis*, is an economically important cephalopod in Southeast Asia. This species is a warm-ocean demersal cephalopod that inhabits waters 15–100 m deep. It is mainly distributed from the East China Sea to the Philippines, Vietnam, and other countries of Southeast Asia (Chen et al. 2009). Wild populations of *S. pharaonis* have declined dramatically in recent decades due to overfishing and environmental deterioration. Because of its rapid growth, good nutritional profile, and high market value, *S. pharaonis* is recognized as a potential species for commercial aquaculture (Gao et al. 2014). However, the success of hatching and breeding in cephalopods has been significantly affected by environmental factors such as water temperature, salinity, and pH (Lacoue-Labarthe et al. 2011; Vidal and Boletzky 2014; Caamal-Monsreal et al. 2016).

Most cephalopods are stenohaline, and salinity is one of the most important physicochemical factors to which marine embryos are exposed (Palmegiano and D'Apote 1983; D'Aniello et al. 1989; Fagundez and Robaina 1992; Sen 2005; Berger 2010; Vidal and Boletzky 2014). Salinities outside the range of 20–37 can be fatal to cephalopods (Vidal and Boletzky 2014). Peng et al. (2013) showed that it can be fatal to egg of *Sepia lycidas* if the salinity exceeds the range of 24–33. Therefore, salinity is a limiting factor in the distributions of most species (Vaz-Pires et al. 2004). Suitable water quality for egg incubation is a key factor in the success of artificial breeding (Cinti et al. 2004). Therefore, research on optimal water quality parameters for egg incubation is important for the development of a successful cephalopod aquaculture industry (Vidal et al. 2002a; Cinti et al. 2004; Lacoue-Labarthe et al. 2011; Vidal and Boletzky 2014; Caamal-Monsreal et al. 2016). When fertilized eggs are incubated within the optimum salinity range, the hatching rate can be increased, and the incubation time can be reduced (Cinti et al. 2004). The entire process of embryonic development, which involves a series of steps of cell differentiation and morphogenesis, is affected internally by gene expression and externally by environmental factors (Boletzky 2003; Cinti et al. 2004; Nabhitabhata et al. 2005). Salinity can influence the osmotic balance of embryos, and osmoregulation is mainly performed by the thin outer layer of protoplasm around the yolk (D'Aniello et al. 1989). Osmotic pressure is usually maintained at a relatively stable level within the appropriate salinity range for embryos; however, water salinity exceeding the embryo's tolerance will lead to dehydration, shrinkage, and a smaller yolk sac,

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and embryonic development will cease (Cinti et al. 2004). Thus, for breeding purposes, it is important to determine the salinity range that is suitable for embryonic development.

Nabhitabhata et al. (2001) incubated fertilized eggs of *Sepioteuthis lessoniana* at different salinities (range 12–44) at 29 C and observed hatching rates above 80% with salinities of 24–36‰. Jiang et al. (2010) showed that the suitable salinity range for the embryonic development of *Sepiella maindroni* is 20–33 (at 25 C) and that the optimal salinity range is 27–32. Peng et al. (2013) showed that the embryonic development of *S. lycidas* was significantly affected by salinity, the suitable salinity for hatching ranged from 27.0 to 33.0, and the optimum salinity was 30.0. Sen (2005) found that the optimal salinity range for egg incubation in *Loligo vulgaris* is 32–40. In *Sepia esculenta,* the optimum range of salinity for egg incubation is 24–36 (at 29 C) (Nabhitabhata et al. 2001). Therefore, different species of cephalopods exhibit different optimal ranges of salinity for egg incubation. This interspecific variability suggests that the optimal salinity range for egg incubation must be determined for each species. There is currently little information in relation to salinity for the pharaoh cuttlefish, *S. pharaonis*; therefore, a study focusing on determining the optimal salinity of egg incubation is necessary.

Water salinity influences the hatching rate and hatching time in cephalopods (Boletzky 1983; Cinti et al. 2004; Nabhitabhata et al. 2005). The weight and condition of hatchlings are also important reference indices in the study of the influence of salinity on the development of embryos. However, there is insufficient information available to determine whether water salinity influences the hatching period or the weight and health of hatchling cuttlefish, much less the yolk utilization or the development at various embryonic stages.

In this study, we simulated the salinities found in the wild (seawater salinity at the shelf and river mouths off Guangdong). We conducted experiments to investigate the effects of different salinities on embryos at different stages, including the effects on the hatching rate, incubation period, hatching period, wet weight of the hatchlings, and yolk utilization efficiency ratio. The objectives were (1) to determine whether water salinity affects the different embryonic stages and yolk utilization, as well as the weight and health of hatchling cuttlefish and (2) to determine the optimal salinity for the incubation of fertilized eggs from *S. pharaonis.*

Materials and Methods

Aquarium Tank and Pool System and Water Quality Management

Broodstock Rearing System. The broodstock were reared in an indoor cement pool (11.5 m length × 7.5 m width × 1.8 m depth) with smooth, black surfaces. The broodstock rearing system was open, and the seawater was sand filtered, ultraviolet (UV) irradiated, and aerated. The water flowed through an inlet pipe located at the left side of the pool and drained through an outlet pipe located in the bottom center of the pool, and all water movement was slow. Some smooth nylon net and nylon rope were used as collectors, and aeration was provided through air stones.

Egg Incubation System. The egg incubation tank system consisted of 21 individual blue test tanks (80 L, 0.50 m diameter × 0.40 m height) and 21 individual auxiliary tanks (150 L, 0.60 m diameter × 0.55 m height), referred to as a "tank line." The 21 individual test tanks were divided into seven groups (seven salinities × three tanks). The auxiliary tank is for storage of experimental water used in test tanks. Seawater entered the test tank through a pipe from the auxiliary tank (flow rate of 1 L/min). All the tanks had identical, smooth inner surfaces. The tanks in each line were maintained at the same salinity and temperature to provide experimental replicates. The temperature in each system was controlled using submersible titanium heaters. The test tank had an inlet, outlet, and aeration system. The seawater in the tank system for rearing eggs was completely replaced every day, using sand-filtered, aerated, UV-irradiated seawater.

Water Quality Measurements. Water salinity, pH, and dissolved oxygen were measured

using a YSI Pro Plus instrument (YSI, Yellow Springs, OH, USA). Ammonia was measured with prepackaged reagents (HACH, Loveland, CO, USA). Broodstock water quality was measured two times a day (0800 and 1730 h), whereas egg incubation water was measured three times a day (0730, 1130, and 1730 h).

Broodstock Collection and Rearing and Egg Collection

A total of 424 wild *S. pharaonis* individuals (207 males and 217 females) were collected with nets off the coast of Guangdong, China (110.37°E, 20.51°N, 21 C, and 29.5) on March 28, 2015. Broodstock were transported in six plastic buckets (1.50 m diameter × 1.25 m height) with the original seawater and aeration during transport. Total transport time was 5.5 h. Broodstock were reared in an indoor cement pool and were fed fresh fish three times per day to visible satiety (0730, 1230, and 1730 h). The seawater in the broodstock rearing system was 100% refreshed every day.

The broodstock started egg-laying activity 2.5 h after their arrival in the pool. The tested eggs were collected from all broodstock that laid eggs in the pool during a 30-min interval. Eggs at the same stage (cleavage) showing normal development and uniform size (mean weight, 3.18 ± 0.38 g) were selected as experimental subjects. Eggs were observed using a dissecting microscope with 10–40× magnification (SZX 7, Olympus, Japan), and *S. pharaonis* embryonic development was divided according to Jiang et al. (2010).

Egg Incubation and Experimental Design

To test the effects of salinity on embryonic development, salinity regimes of 18, 21, 24, 27, 30, 33, and 36 were chosen based on published information characterizing the seawater salinity at the shelf and river mouths off Guangdong (Wu et al. 2011).

A total of 1260 eggs were randomly allocated to 21 individual hatching tanks (80 L) housed indoors, at a stocking density of 60 eggs per tank. Each hatching tank held 40 L of seawater for egg incubation. The salinity was adjusted by adding freshwater or unrefined natural sea salt that was prepared and aerated before use (Sen 2005). To avoid rapid changes of seawater salinity, the eggs were first placed into each incubator at a salinity of 28, close to the salinity of the spawning site, and each incubator was then shifted to the next higher or lower salinity at 2-h intervals.

The eggs were weighed at 1200 h, and the embryonic stages were observed four times per day (0600, 1200, 1800, and 2400 h) until every embryo in each of the experimental groups had hatched. Before weighing, surface water of eggs was absorbed with absorbent paper. The eggs were weighed daily using a Sartorius BS 120S analytical balance (precision of 0.0001 g). The embryos were observed every 4 h on the first and second days of the experiment. Microscopic observations of samples of five embryos were performed using a dissecting microscope (SZX 7, Olympus,Tokyo, Japan), and the embryos were photographed with a digital camera (D90, Nikon, Tokyo, Japan) to determine the developmental stages. The tanks were thoroughly cleaned whenever needed, and embryo mortality was checked daily. The hatching rate, incubation period, hatching period, wet weight of the hatchling cuttlefish, and yolk utilization efficiency ratio were calculated at the end of the study.

To more conveniently and effectively observe the effects of salinity on embryonic development, 11 stages (oosperm, cleavage, blastula, gastrula, formation of the embryonic primordium, organogenesis, the red-bead stage, the occurrence of a heartbeat, endoskeleton formation, pigment formation, and hatching) were adopted during the embryonic development of *S. pharaonis* as observation points in this study (Fig. 1).

Calculations and Statistical Analysis

Peng et al. (2013) reported the effects of dissolved oxygen, hatching density, and salinity on the hatching rate, incubation period, hatching period, and wet weight of the hatchlings and on the yolk utilization efficiency ratio of

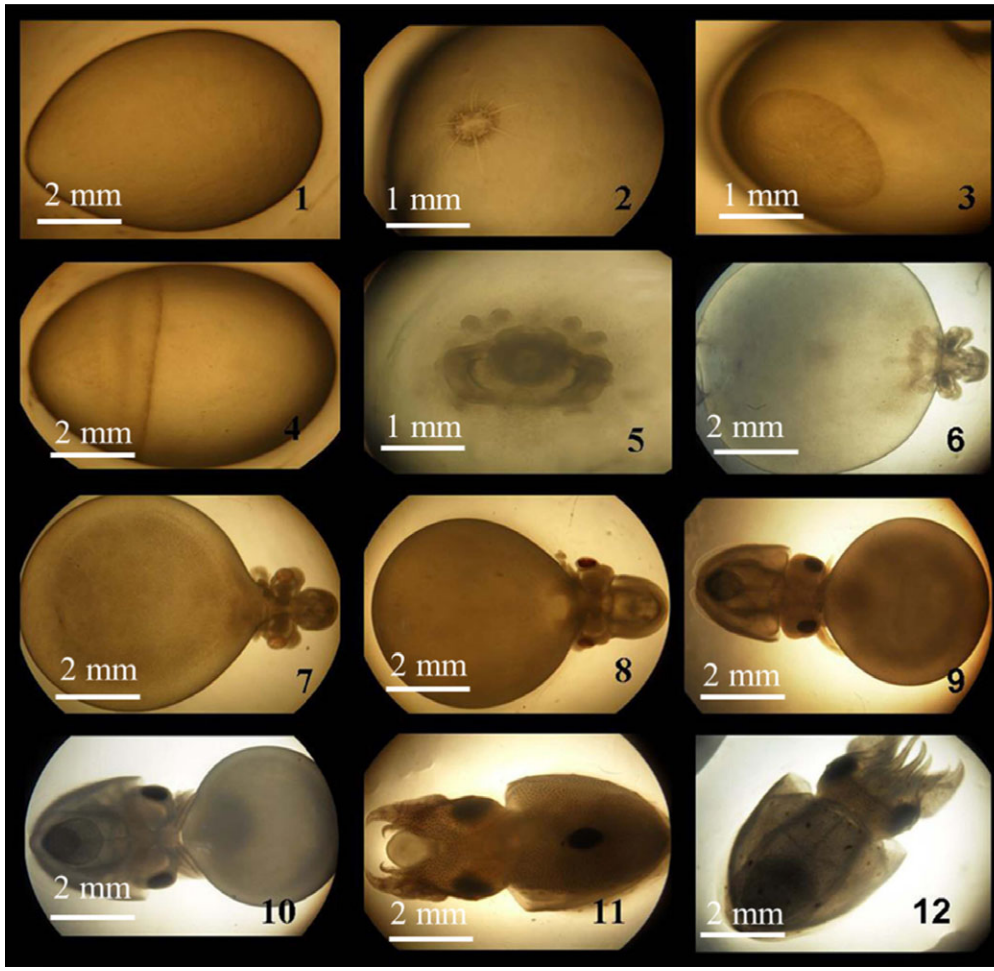

FIGURE 1. *The embryonic development of* Sepia pharaonis. *Explanation of Plates: 1, fertilied egg (oosperm); 2, cleavage; 3, blastula; 4, gastrula; 5, formation of embryonic primordium; 6, organogenesis; 7, red-bead; 8, heartbeat; 9, endoskeleton formation; 10, pigment formation; 11, hatching; 12, hatching cuttlefish. [Color figure can be viewed at wileyonlinelibrary.com].*

S. lycidas eggs. These parameters were calculated as follows (Peng et al. 2013): Incubation period: the incubation period was defined as the total time from the beginning of the experiment to the time when 50% of the normal embryos had hatched out. Hatching period: the hatching period was defined as the total time from the first hatching to the last. Hatching rate (%) = number of hatchlings/number of incubated eggs × 100. Yolk utilization efficiency ratio (%) = the number of hatchlings that did not still possess yolk when hatching/total number of hatchlings × 100.

The results are presented as the means ± SD. Group normality was initially evaluated using the Shapiro–Wilk test, and one-way ANOVA for significant differences was then performed using the software package SPSS 18.0 (SPSS, Chicago, IL, USA). The homogeneity of variances was checked using Bartlett's test. When necessary, post hoc analyses using the least significant difference (LSD) test were applied. The weight values for embryos and hatching cuttlefish were log-transformed to normalize their variance. Percentage data were arcsine-transformed to normalize their variance.

TABLE 1. *The water quality parameter in broodstock rearing and egg incubation systems during the experiment.*

	Broodstock rearing system	Egg incubation systems
Temperature (C)	22.00 ± 1.00	23.50 ± 0.50
Salinity	28.60 ± 1.00	–
Dissolved oxygen (mg/L)	8.07 ± 1.26	8.01 ± 1.23
pH	8.01 ± 0.11	8.00 ± 0.09
Ammonia nitrogen (mg/L)	0.08 ± 0.04	0.05 ± 0.02

Results

Water Quality

The safety requirements in the quality of the pool for broodstock rearing and tank for egg incubation water were observed during experiments. During the broodstock cultivation period, low light intensity (100–150 lx) with a photoperiod of 12 h light and 12 h dark, the seawater temperature was maintained at 22.0 ± 1.0 C, the pH at 8.01 ± 0.11, the salinity at 28.60 ± 1.0, the dissolved oxygen content at 8.07 ± 1.26 mg/L, and ammonia at 0.08 ± 0.04 mg/L (Table 1). During the experiments involving the incubation of eggs, the temperature, ammonia, pH, and dissolved oxygen content of the tank seawater were 23.5 ± 0.5 C, 0.05 ± 0.02 mg/L, 8.00 ± 0.09 and 8.01 ± 1.23 mg/L, respectively (Table 1). Eggs were maintained under a natural photoperiod via the window. The daily recorded data in each tank line showed that the salinity maintained reasonable stability for the course of the experiments, that is, ±0.1.

Effect of Seawater Salinity on the Development Time and Egg Weight

The development rate and hatching time varied widely depending on the seawater salinity during incubation (Table 2). Eggs were able to develop into cuttlefish juveniles at 24–33. In contrast, 100% mortality occurred at 18, 21, and 36, at which embryos ceased to develop at the gastrula, formation of embryonic primordium, and blastula stages, respectively. The time from cleavage to the formation of the embryonic primordium was 5.50–5.75 d at 27–33 but was approximately 8.5 and 6 d for eggs maintained at 21 and 24, respectively. Hatching occurred 28–30 d after the cleavage stage at 24, 24–27 d after the cleavage stage at 27, 25–26 d after the cleavage stage at 30, and 25–26 d after the cleavage stage at 33. These results regarding the hatching period and incubation period of eggs at different salinities indicated that the seawater salinity had no significant influence between 27 and 33 ($P > 0.05$). However, the hatching period and incubation period were longer at 24 than at 27–33 ($P < 0.05$). The shortest hatching period (25.67 d) and incubation period (5.33 d) were observed at 30 (Table 2).

The effects of the seawater salinity on the weight of eggs during incubation are shown in Figure 2. Salinity had a modest effect on egg weight, but it was not significant ($P > 0.05$). The weight of the egg first decreased and then increased with ongoing embryonic development at 24–33, with the minimum value occurring between the red-bead and occurrence-of-heartbeat stages. The average egg weight decreased from 3.0 g at the cleavage stage to 1.8 g by the occurrence-of-heartbeat stage, in a process that took approximately 15 d (at 23 C and 27–33).

Effect of Seawater Salinity on the Hatching Rate, Weight of Hatching Cuttlefish, and Yolk Utilization Efficiency Ratio

Based on the ANOVA results ($P < 0.05$), we conclude that the hatching rate of the eggs was significantly affected by the seawater salinity (Table 2). The hatching rate decreased with an increasing salinity within a certain range (salinity < 30). The hatching rate at 30–33 was > 80%.

As shown in Table 2, the maximum weight for hatching cuttlefish was obtained from eggs maintained at 30, and they were significantly larger than those maintained at 24, 27, or 33 ($P < 0.05$). The yolk utilization efficiency ratio first decreased and then increased with rising salinity. The highest yolk utilization efficiency ratio was observed at 30 (89.28%), followed by 27, 33, and 24 (78.42, 77.91, and 69.55%, respectively).

Discussion

Interestingly, our results showed that the *S. pharaonis* egg weight first decreased and then

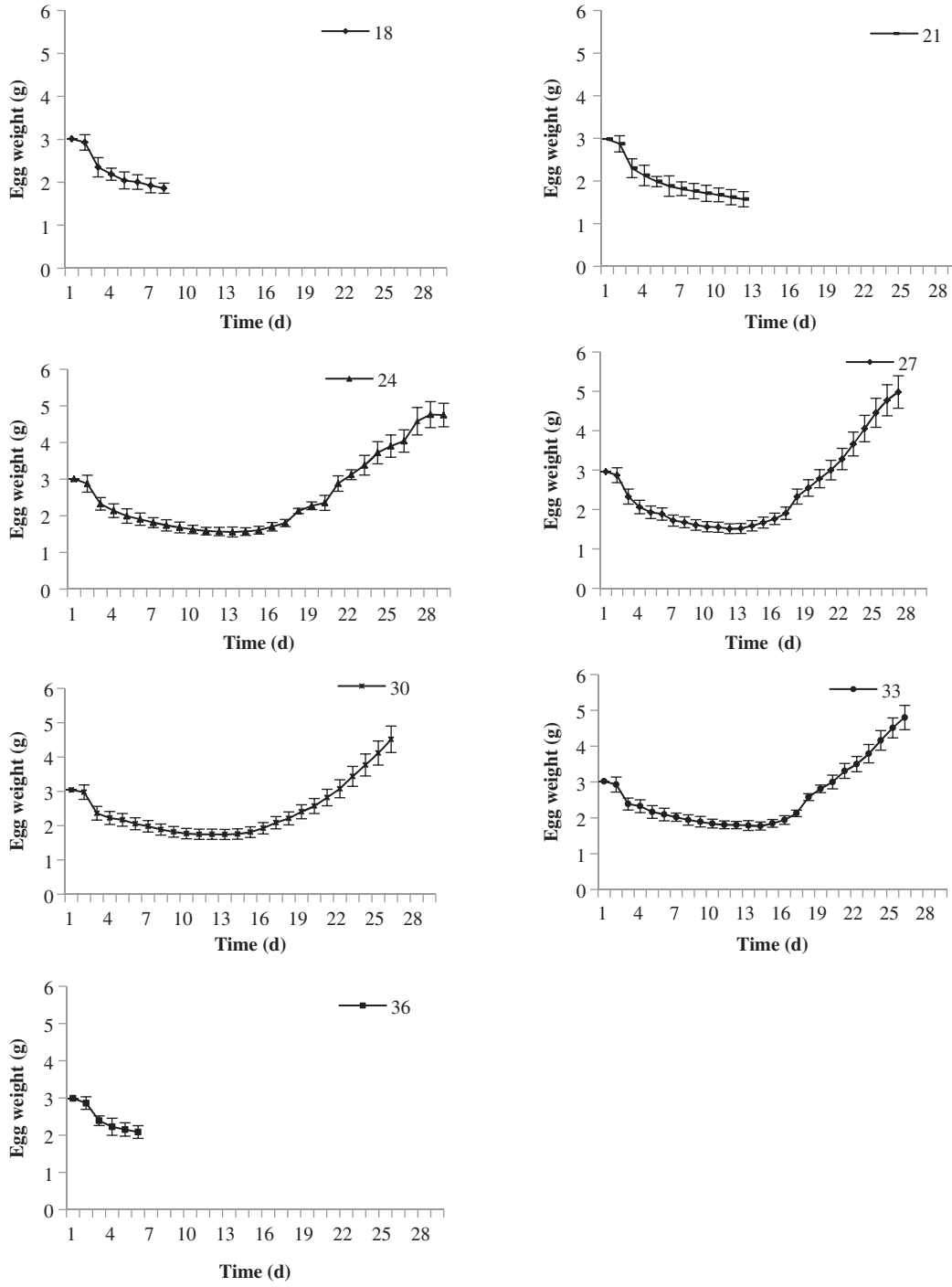

FIGURE 2. *The effect of the salinity on the weight of* Sepia pharaonis *embryo during embryonic development.*

TABLE 2. *Timing of the occurrence of developmental stages, mean hatching rate, hatching period, incubation period, mean weight of hatchling cuttlefish, and yolk utilization efficiency ratio of* Sepia pharaonis *egg at seven different seawater salinities. (The reported times are when 50% of the normally developing embryos reached that stage.)*[a]

	18	21	24	27	30	33	36
Blastula (d)	2.25–2.50	2.25–2.50	2.00	1.75	1.75	1.75	2.75–3.00
Gastrula (d)	5.25–5.50	5.00–5.25	3.50–3.75	3.25–3.50	3.50–3.75	3.25–3.50	–
Formation of embryonic primordium (d)	–	8.25–8.50	6.00–6.25	5.50–5.75	5.50–5.75	5.50–5.75	–
Organogenesis (d)	–	–	8.00–8.25	7.25–7.50	7.50–7.75	7.25–7.50	
Red-bead (d)	–	–	10.25–10.50	9.50–9.75	9.75–10.15	9.50–10.00	–
Heartbeat (d)	–	–	14.50–15.15.	13.25–13.75	13.50–14.25	13.25–13.75	–
Endoskeleton formation (d)	–	–	16.75–16.25	15.00–15.50	15.00–15.75	15.00–15.50	–
Pigment formation (d)	–	–	17.75–18.25	16.75–17.25	17.00–17.70	16.75–17.25	–
Hatching (d)	–	–	23.75–24.50	22.25–22.75	22.50–23.00	22.25–22.75	–
Breaking the membrane (d)	–	–	28–30	24–27	24–25	24–25	–
Hatching period (d)	–	–	6.67 ± 0.58^{a}	5.00 ± 1.00^{b}	5.33 ± 0.57^{b}	5.33 ± 0.57^{b}	–
Incubation period (d)	–	–	29.67 ± 0.57^{a}	26.00 ± 1.72^{b}	25.67 ± 0.57^{b}	26.00 ± 0.00^{b}	–
Hatching rate (%)	0	0	48.33 ± 7.63^{c}	80.00 ± 5.00^{b}	93.33 ± 2.89^{a}	81.67 ± 2.89^{b}	0
Weight of hatching cuttlefish (g)	–	–	0.223 ± 0.004^{c}	0.233 ± 0.004^{b}	0.247 ± 0.002^{a}	0.231 ± 0.003^{b}	–
Yolk utilization efficiency ratio (%)	–	–	69.55 ± 3.63^{c}	78.42 ± 3.01^{b}	89.28 ± 2.57^{a}	77.91 ± 3.27^{b}	–

[a]Different letters indicate significant differences between groups ($P < 0.05$). " – "means no data were obtained (the eggs have died).

increased with embryonic development at salinities 24–33. The lightest weight (1.8 g) occurred between the red-bead stage and heartbeat stages, and the heaviest (5.0 g) was observed just before hatching. The decrease in egg weight may have been due to the reduction in internal fluid and the consumption of the yolk sac during the early stages of embryonic development. Martins et al. (2010) showed that squid paralarvae do not appear to convert yolky matter into somatic tissue by catabolizing the internal yolk, which has high specific gravity, to fuel metabolism. However, the increase in weight may be due to the rapid absorption of water by the egg after organogenesis, and this may be related to the oxygen consumption during embryonic development. The probability of oxygen entering the egg rises with the increase in the surface area, which increases as weight increases. Peng et al. (2013) reported that the oxygen consumption rate of *S. lycidas* eggs increased significantly after the red-bead stage. Lei et al. (2013) reported that the volume and water content of the eggs of *S. esculenta* also tended to decrease in the early stages of embryonic development and then gradually increase after the organogenesis stage.

In our study, the embryo mortality occurred before organogenesis and eggs did not complete the development process at the lowest and highest salinities of 21 and 36, possibly because much lower or much higher seawater salinities lead to physiological and biochemical reactions that damage the embryos. Premature hatching is also observed at salinities close to the upper limit of tolerance for squid, whereas low salinities lead to abnormal development (Nabhitabhata et al. 2001; Sen 2005; Vidal and Boletzky 2014). Peng et al. (2013) showed that a higher or lower salinity can alter the permeability of the cell membranes of eggs, ultimately affecting embryonic development. Vidal and Boletzky (2014) reported that cephalopods are stenohaline, with salinities outside the range of 27–37 being potentially fatal. In neritic cephalopod eggs, 80% were observed to successfully complete embryonic development at 24–36

(Nabhitabhata et al. 2001). However, Sen (2005) indicated that normal development and hatching occur at 32–40 in *L. vulgaris* eggs; below this range, mortality is observed. These differences are species specific, with eggs of different species showing different preferences in incubation salinity. Based on our present results, the seawater salinity in areas used for *S. pharaonis* hatchling production and around the hatchery must be neither lower than 24 nor higher than 33.

On the other hand, changing salinity of spawning areas may cause fluctuations of *S. pharaonis* populations in the wild. The salinity range permitting the hatching of *S. pharaonis* eggs was 27–33, and the coastal (estuarine) waters of Guangdong can range from 18 to 36. Therefore, when planning open culture or maintenance systems for *S. pharaonis*, the potential for freshwater influx (such as from nearby rivers or in regions subject to heavy rain) and the variation of seawater salinity must be considered.

As the incubation period provides information about the developmental rate of eggs, the hatching period provides information about the consistency of that rate. This study showed that the shortest incubation period and hatching period occurred at 27–33. Outside this range, the rate of embryonic development and the synchrony of hatching decrease. Similar trends have been found in other cephalopod species, such as *L. vulgaris* (Sen 2005) and *S. maindroni* (Jiang et al. 2010). Therefore, a suitable salinity helps ensure the developmental rate and synchrony of eggs.

The hatching rate is an important reference index in the study of embryonic development for captive breeding. In this study, the hatching rate was significantly higher at 30–33‰ than at 24–27 and declined sharply in salinities below 30 or above 33. Sen (2005) found that the hatching rate of *L. vulgaris* eggs was highest at 32–38 and declined sharply when salinity fell below 32 or rose above 38. Jiang et al. (2010) observed high hatching rates at all suitable salinities and that *S. maindroni* did not hatch at salinities lower than 19. Paulij et al. (1990) found that the highest percentages of hatching in *S. officinalis* occurred at salinities above 29.8 and that no hatching occurred at salinities lower than 23.9. Thus, different species of cephalopods may be adapted to different salinities during embryonic development, but a suitable salinity range is generally necessary to ensure a high hatching rate.

The yolk plays an important metabolic role in embryonic development, storing and providing energy, transporting cellular secretions, and influencing postnatal development (Vidal et al. 2002b, 2005; Boyle et al. 2007). Environmental factors can significantly affect the utilization of the yolk during embryonic development (Bouchaud 1991; Vidal et al. 2002b; Boletzky 2003). Martins et al. (2010) found that yolk utilization rate in chokka squid paralarvae is affected by the environmental temperature and that the yolk is used at an exponential rate. Vidal et al. (2005) reported that yolk utilization is strongly influenced by temperature and that a suitable temperature increases yolk utilization ratios. The findings of this study, on the effects of water environmental parameters on the utilization of yolk in *S. pharaonis* eggs, concur with the findings of the above-mentioned studies. Our findings showed that the degree of consumption of yolk during embryonic development of *S. pharaonis* is affected by salinity.

The weight and condition of hatchlings are also important reference indices in the study of embryonic development and can be used to determine optimal incubation conditions for captive breeding. In this study, salinity affected the weight and condition of hatchling cuttlefish. Furthermore, all the maximum values were obtained from eggs incubated at 30, most likely because a suitable salinity increases yolk utilization efficiency ratio, and high yolk utilization efficiency ratio improves the condition of hatchling cuttlefish.

In conclusion, the results of this study indicate that the suitable range of salinities for egg hatching in *S. pharaonis* is 27–33 and that the optimum salinity is 30 based on the effects of salinity on the hatching rate, incubation period, hatching period, wet weight of hatchling cuttlefish, and yolk utilization efficiency ratio. The yolk reserve was completely exhausted upon hatching at the optimum hatching salinity. The yolk

utilization ratio at hatching is proportional to the weight of the hatchling cuttlefish. *S. pharaonis* is a stenohaline species, and aquaculturists should pay attention to changes in salinity and maintaining stable salinity during incubation. The seawater salinity around the suitable aquaculture areas of *S. pharaonis* must be between 27 and 33.

Acknowledgments

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