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Title: Relationship between Hatching Rate and the Outer Egg Membranes of the *In Vitro* Artificially Fertilized Eggs of the Japanese Mitten Crab *Eriocheir japonica*

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Abstract

This study shows that by removing the outer membranes of the *in vitro* artificially fertilized eggs of the Japanese mitten crab *Eriocheir japonica*, the normal zoea hatching rate of the eggs can be raised from 10% up to over 90%. This finding suggests that the normal zoea hatching rate is strongly influenced by the outer egg membranes of the *in vitro* artificially fertilized eggs. In addition, observations by transmission electron microscopy indicate that the outer egg membranes of the *in vitro* artificially fertilized eggs are 1.5 to 3 times thicker than those of the naturally spawned eggs. Discussion focuses on the role these thick outer egg membranes play in the normal zoea hatching rate of *in vitro* artificially fertilized eggs.

Keywords: Egg membrane; egg envelope; artificial fertilization; hatching rate; crab; decapod; *Eriocheir japonica*

Introduction

In aquaculture sciences, *in vitro* artificial fertilization is a fundamental technique that is essential for performing chromosome engineering, gene manipulations and so on

(Purdom, 1993). However, it has not been well established for crabs (brachurans). Success in *in vitro* artificial fertilization in crabs was first reported in the Chinese mitten crab *Eriocheir sinensis* by Lee and Yamazaki in 1989. Since then, no further attempts have been documented in other species of crabs. One difficult problem remains unresolved in Lee and Yamazaki's pioneer work, that is, the low normal zoea hatching rate of the *in vitro* artificially fertilized eggs, which was reported to be less than 20%. Even the use of germ-free artificial incubation failed to improve the hatching rate. The purpose of this study is to solve this particular problem by examining the role of the outer egg membrane, also called "fertilization membrane," "first egg membrane" and "outer investment coat" (Lee and Yamazaki 1989) in the normal zoea hatching rate of *in vitro* artificially fertilized eggs.

To avoid confusion of terminology, some explanation is given here on the use of the term "egg membrane" in this study. The term is widely used in the literature on decapods in biology and aquatic and fisheries sciences including two classic studies in decapods (Yonge, 1946; Cheung, 1966). Even though some researchers have suggested alternative terms such as "egg envelope" and "egg investment coat" (Goudeau and Lachaise, 1980a; Talbot, 1981; Fisher and Clark, 1983), there has been no serious discussion, from either a historical perspective or a logical one, about why the new terms are better than the term "egg membrane."

Although a number of studies have been reported on the egg membranes of decapods, most of them focused on their formation or origins (Cheung, 1966; Goudeau and Lachaise, 1980a, 1980b; Talbot, 1981; Goudeau and Becker, 1982; Goudeau and Lachaise, 1983; Goudeau, 1984; Goudeau, et al., 1991; Du, et al., 1995; Du, et al., 1997; Zhang, et al., 1998; Kang, et al., 2000a; Kang, et al., 2000b; Wang, et al., 2001; Palomino, et al., 2002; Saigusa, et al., 2002; Pongtippatee, et al., 2004; Ying, et al., 2005), their physiology (Yonge, 1946; Fisher, and Clark, 1983; Goudeau and Goudeau, 1981; Goudeau, et al., 1984; Charmantier and Aiken, 1987; Lynn and Clark, 1987; De Vries, and Forward, 1991; Lynn, et al., 1992; Lee, and Yamazaki, 1993; Glas, et al., 1995; Glas, et al., 1996; Goudeau and Goudeau, 2001; Goudeau and Goudeau, 2002; Rojas and Alfaro, 2007) and sperm-egg interaction (Goudeau, 1982; Lynn and Clark, 1983; Barros, et al., 1989; Rios and Barros, 1997; Kang, et al., 2000b; Kumar and Diwan, 2000; Chen, et al., 2001; Zhang, et al., 2001; Pongtippatee, et al., 2007). Nothing has been written on the relationship of hatching rate and the outer egg membranes of *in vitro* artificially fertilized eggs. The present study is totally different from previous

work in terms of purpose and significance.

Materials and Methods

This study used the Japanese mitten crab *E. japonica* as the experimental species for experiment. It is a freshwater crab of commercial value. The Japanese mitten crab inhabits the rivers of Japan and is closely related to the Chinese mitten crab *E. sinensis* in terms of taxonomy and morphology (Sakai, 1976; Peng, 1986; Dai, 1988; Li et al., 1993; Gao and Watanabe, 1998; Li and Li, 1999; Li and Zou, 1999; Xie et al., 1999; Zhao and Li, 1999; Zhao et al., 2002; Lee et al., 2004). Copulated adult females of the Japanese mitten crab *E. japonica* were collected from the estuary of the Shiodormari River in Hakodate, Hokkaido. They were maintained in 80% seawater (salinity: 27.6 ppt) in a well aerated, close circulation system at 20°C at the Faculty of Fisheries Sciences, Hokkaido University.

Artificial egg incubation experiment for the examination of the effects of the outer egg membrane

Female crabs were monitored 24 hours using an oviposition alarm system invented by the author (Lee, 2009). When the alarm system signalled that a female crab was beginning to oviposit, unfertilized ripe eggs were obtained directly from its ovary as soon as possible. *In vitro* artificial fertilization was then carried out using the methods described in early works by Lee and Yamazaki (1989; 1990). Following *in vitro* artificial fertilization, the eggs were rinsed three times with filtered (Millipore filter: 0.20 μ m) sea water (salinity: 27.6 ppt) and were artificially incubated at 20°C with filtered (Millipore filter: 0.20 μ m) sea water (salinity: 27.6 ppt) for 24 hours. As an experimental group, some of the *in vitro* artificially fertilized eggs were taken and their outer egg membranes were removed with surgery blades. Three replicates of 50 treated eggs were counted and artificially incubated in a 50ml sterile tissue culture flask filled with 30ml filtered (Millipore filter: 0.20 μ m) sea water (salinity: 27.6‰). The tissue culture flasks were placed in an orbital shaker (shaking speed: 30 rpm; orbit: horizontally reciprocating in a motion that resembles the number 8; amplitude: 2 cm) in order to keep the culture solution flowing continuously. As a control group, untreated *in vitro* artificially fertilized eggs were counted and artificially incubated in the same way as the experimental group.

The eggs of both groups were artificially incubated at 20°C until the tenth day after the first egg was hatched. The eggs and hatched zoeae were examined and counted under a stereoscopic microscope. At the same time, the external features and movements of the hatched zoeae were observed.

A *t*-test was conducted with the collected data to determine the significant difference between the experimental and control groups, focusing on their average normal zoea hatching rates, average abnormal zoea hatching rates, average rates of the eggs that were not hatched, and average rates of the eggs that died before hatch.

Transmission electron microscopy observations of the outer egg membrane and funiculus

In order to examine the thickness of the outer egg membranes of the *in vitro* artificially fertilized eggs and those of the naturally spawned eggs, and the funiculus of the naturally spawned eggs, transmission electron microscopy observations were carried out using the standard methods described in Hayat (1986). *In vitro* artificially fertilized eggs that were artificially incubated under the conditions described above were collected and fixed on the second and nineteenth days after fertilization. On the other hand, naturally spawned eggs that were attached to the non-plumose setae of the female crab with a funiculus after natural egg-laying were collected from the incubating female crab and were fixed on the second and nineteenth day after spawning. Following the fixation of the eggs, several cracks (holes or gaps) were made by pricking the eggs with a surgery blade for the penetration of chemicals to be used in the preparation of the transmission electron microscopy specimens. For comparison of the thickness of the egg membranes, all of the transverse sections were cut at the middle of the eggs.

Results

Artificial egg incubation experiment for the examination of the effects of the outer egg membrane

After 19 days of incubation, hatching was observed in both the experimental and

control groups. Within a week, almost all of the zoeae were hatched. Among them some zoeae were normal and some were abnormal. Abnormal zoeae refer to those that lacked normal dorsal and rostrum spines, the first and second maxillipeds, tail forks, and were unable to swim or predate. At the end of the incubation experiment, the average normal zoea hatching rates of the experimental and the control groups were 92% and 10.67%, respectively (Table 1). The average abnormal zoea hatching rate of the control group was 54.67% and that of the experimental group was 6.67% (Table 1). Statistical analysis showed that there was significant difference ($p < 0.01$) between the two groups in terms of average normal zoea hatching rate, average abnormal zoea hatching rate and average rate of the eggs that were not hatched (Table 1).

Transmission electron microscopy observations of the outer egg membrane and funiculus

Transmission electron microscopy observations showed that compared with the outer egg membranes of naturally spawned eggs, those of the *in vitro* artificially fertilized eggs were about 1.5 times thicker (Fig. 1) on the second day after fertilization and three times thicker (Fig. 2) on the nineteenth day after fertilization. On the one hand, the outer egg membranes of naturally spawned eggs on the nineteenth day after fertilization were found to be only 41% as thick as they were on the second day after fertilization (Figs. 1A and 2A). On the other hand, the outer egg membranes of the *in vitro* artificially fertilized eggs on the nineteenth day after fertilization were about 80% as thick as they were on the second day after fertilization (Figs. 1 and 2).

Transmission electron microscopy observations also suggested that the funiculus attached to the non-plumose seta of the incubating female crab was an extension of the outer egg membrane (Fig. 3).

Discussion

Based on the results of the artificial incubation experiment on *in vitro* artificially fertilized eggs with and without removal of the outer egg membranes, it is concluded that the existence of the outer egg membrane has an apparent negative effect on the normal zoea hatching rate. This effect is closely related to the thickness of the outer egg membranes of the *in vitro* artificially fertilized eggs in that they are found to be 1.5

to 3 times as thick as those of the naturally spawned eggs. The unusually thick outer egg membranes may decrease not only the exchanging rate of oxygen but also the discharging rate of the metabolic substance. In addition, the thick outer egg membranes may physically suppress the enlargement of the developing embryo and impede the ecdysis of the embryos. All of these lead to abnormal development of the zoeae.

Transmission electron microscopy observations suggested that the funiculi attached to the non-plumose setae of the incubating female crab were formed from the extension of the outer egg membranes of the naturally spawned eggs. The extension is considered to be the reason why on the second day after fertilization, the outer egg membranes of the naturally spawned eggs were found to be thinner compared with those of *the in vitro* artificially fertilized eggs.

Another question in relation to the thickness of the outer egg membrane is: Why were the outer egg membranes of the naturally spawned eggs on the nineteenth day after fertilization only about 41% as thick as they were on the second day after fertilization? The thinning of the outer egg membranes may be due to the fact that the eggs rubbed against each other during natural incubation while the female crab was pumping her own abdomen for aeration. The mechanical rubbing may have scraped the outer egg membranes and reduced their thickness during natural incubation. On the other hand, there was no such mechanical rubbing in the case of *in vitro* artificially fertilized eggs. The outer egg membranes on the nineteenth day after fertilization were 80% as thick as they were on the second day after fertilization. The slight change may be due to the enlargement of the growing embryos.

In the present study, two kinds of egg membranes were found in both naturally spawned eggs and *in vitro* artificially fertilized eggs on the second and nineteenth days after fertilization. They were an outer egg membrane and an inner egg membrane; the inner egg membrane was thicker than the outer egg membrane. Except for changes in the thickness of the outer egg membrane, no other apparent morphological changes were observed in the outer or inner egg membrane between the second day and nineteenth day after fertilization. Based on morphological comparison, the outer egg membrane and the inner egg membrane found in the present study closely resemble what are referred to as “envelope 1” and “envelope 2” in the crab *Carcinus maensa* (Goudeau and Lachaise, 1980a), the outermost egg membrane “E1” and thick middle egg membrane “E2” in the crab *Sesarma haematocheir* (Saigusa, *et al.*, 2002), and the

egg membrane “E1” and the egg membrane “E2” by Ying, *et al.* (2005).

Finally, it is necessary to point out that as removal of the outer egg membrane in this study was done with blades, it was not only time-consuming but also extremely exhausting. More efficient methods need to be developed for aquaculture in order to reduce labor and cost. For example, it is reported in penaeoidean eggs that the egg membranes can be removed easily with chemical and mechanical treatments (Lynn, *et al.*, 1993). This may give us some hints for finding a solution to the problem.

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Figure captions

Fig.1 Transmission electron microscopy transverse sections of the outer egg membrane on the second day after fertilization.

A: Specimen collected from naturally spawned eggs. B: Specimen collected from *in vitro* artificially fertilized eggs. Scale bar: $0.5\ \mu\text{ m}$.

eb=egg body; im=inner egg membrane; om=outer egg membrane.

Fig. 2 Transmission electron microscopy transverse sections of the outer egg membrane on the nineteenth day after fertilization.

A: Specimen collected from naturally spawned eggs. B: Specimen collected from *in vitro* artificially fertilized eggs. Scale bar: $0.5\ \mu\text{ m}$.

eb=egg body; im=inner egg membrane; om=outer egg membrane.

Fig. 3 Transmission electron microscopy transverse sections of naturally spawned eggs and the non-plumose seta on the second day after fertilization.

A: A funiculus was formed from the extension of the outer egg membrane. Scale bar: $0.5\ \mu\text{ m}$. B: A funiculus attached to a non-plumose seta of the incubating female crab. Scale bar: $5\ \mu\text{ m}$.

eb=egg body; fu=funiculus; im=inner egg membrane; mi=microorganism; ns=non-plumose seta; om=outer egg membrane.

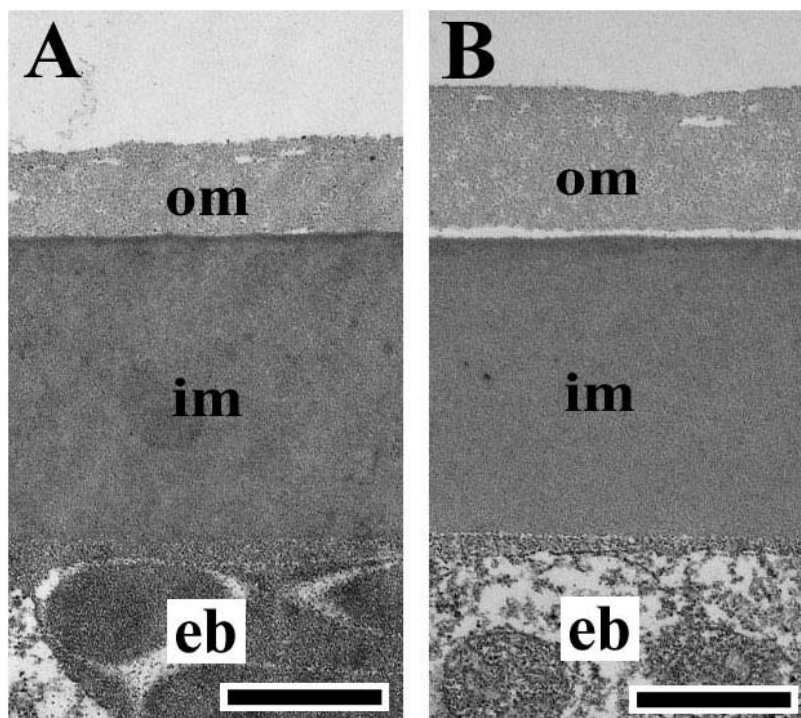


Figure 1

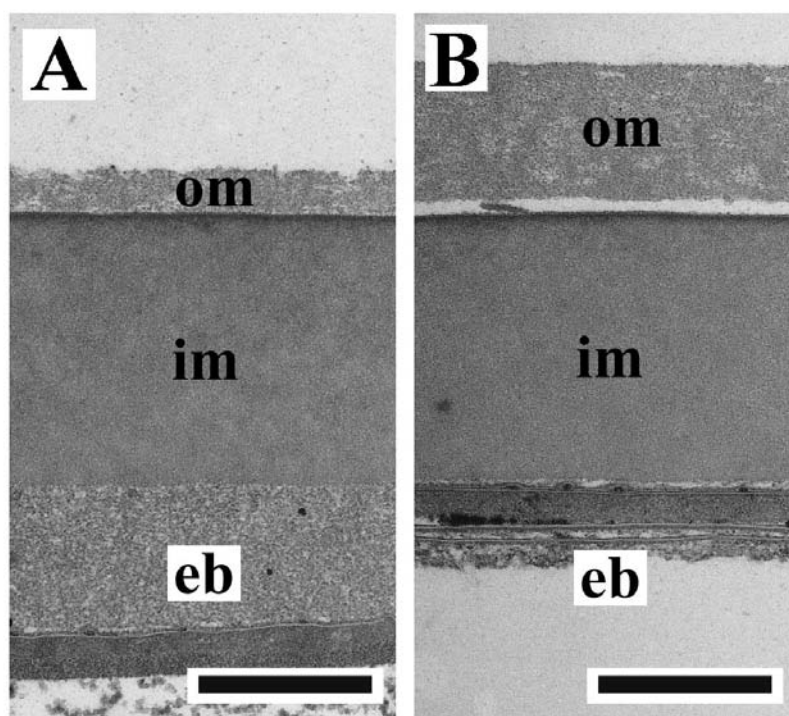


Figure 2

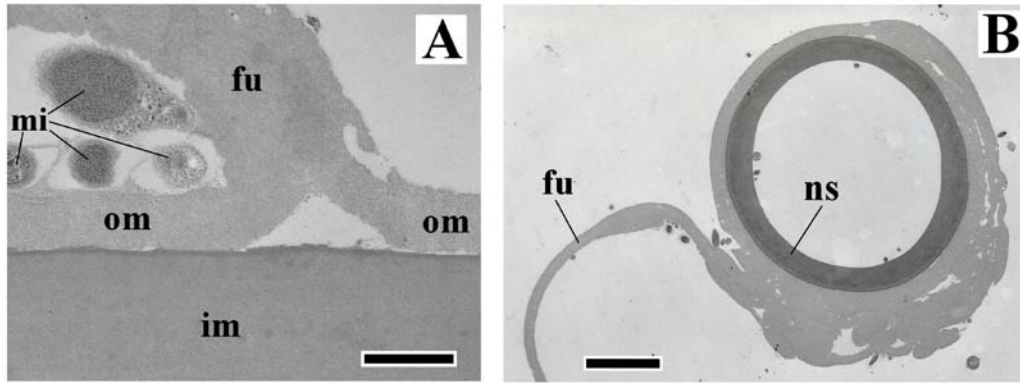


Figure 3

Table 1. Results of the artificial egg incubation experiment.

	Control groups	Experimental groups
Normal zoea hatching rate	10.67 ± 1.16	$92.00 \pm 6.00^{**}$
Abnormal zoea hatching rate	54.67 ± 2.31	$6.67 \pm 3.40^{**}$
Rate of the eggs unable to hatch	33.33 ± 2.31	$0.00 \pm 0.00^{**}$
Rate of the eggs dead before hatch	1.33 ± 1.16	1.33 ± 2.31
Total	100.00	100.00

Data represent mean (%) $\pm$ S.D.. Asterisks indicate significant differences in comparison with values of control groups ($^{**}P < 0.01$; t -test)