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Title: A method for reducing the thickness of the outer egg membrane of the Japanese mitten crab *Eriocheir japonica* to improve the normal zoeal larvae hatching rate of *in vitro* artificial fertilized eggs

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Abstract

This study was an attempt to prove that the thickness of the outer egg membrane is the key factor that contributes to the normal zoeal larvae hatching rate of the *in vitro* artificially fertilized eggs of the Japanese mitten crab *Eriocheir japonica* by artificially stretching and thinning the outer egg membrane with the help of water surface tension. The results showed that the artificially thinned outer egg membrane became six times thinner. In addition, the normal zoeal larvae hatching rate of the *in vitro* artificially fertilized eggs rose from 10% to over 66% after the thinning treatment. Discussion focuses on the mechanism of this thinning phenomenon, the role the thinned outer egg membrane plays in the increase of the normal zoeal larvae hatching rate of the *in vitro* artificially fertilized eggs and the utilization of this particular outer egg membrane

thinning method.

Introduction

In aquaculture sciences, *in vitro* artificial fertilization is essential for performing various techniques such as chromosome engineering, gene manipulations (Purdom, 1993). As far as crabs (brachurans) are concerned, the technique has not been well established. The first success in *in vitro* artificial fertilization in crabs was reported in 1989 by Lee and Yamazaki in the Chinese mitten crab *Eriocheir sinensis.* The normal zoeal larvae hatching rate of the *in vitro* artificially fertilized eggs, however, was less than 20%.

In the author's recent study (Lee, 2009b), it was found that the normal zoeal larvae hatching rate of the *in vitro* artificially fertilized eggs in the Japanese mitten crab *Eriocheir japonica* rose from 10% up to over 90% after the outer membrane of the *in vitro* artificially fertilized eggs was removed. The finding strongly suggested that there is a close relationship between the normal zoeal larvae hatching rate and the outer egg membrane in *in vitro* artificially fertilized eggs. In addition, transmission electron microscopy observations indicated that the outer egg membrane of the *in vitro* artificially fertilized egg was 1.5 to 3 times thicker than that of the naturally spawned egg, suggesting that the abnormal thickness of the outer egg membrane of the *in vitro* artificially fertilized egg might have a negative effect on the normal zoeal larvae hatching rate. I hypothesized that this unusually thick outer egg membrane may contribute to a decrease in not only the exchanging rate of oxygen but also the clearance rate of the metabolic waste. In addition, the thick membrane may physically suppress the enlargement of the developing embryo and impede the ecdysis of the embryos. All of these result in abnormal development of the zoeae (Lee, 2009b). Furthermore, I pointed out that the removal of the outer egg membrane was not only time-consuming but also extremely exhausting.

The purpose of this study was two-fold: 1) to prove the hypothesis proposed by Lee (2009b) that the thickness of the outer egg membrane is a key factor that contributes to the normal zoeal larvae hatching rate of *in vitro* artificially fertilized eggs, and 2) to introduce a simple and easy method for improving the normal zoeal larvae hatching rate of *in vitro* artificial fertilized eggs by reducing the thickness of the outer egg membrane.

Materials and methods

The experimental species used for this study was the Japanese mitten crab *E. japonica*, a freshwater crab of commercial value. This 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℃ at the Faculty of Fisheries Sciences, Hokkaido University.

To reduce the thickness of the outer egg membrane of *the in vitro artificially fertilized egg by water surface tension*

Female crabs were monitored 24 hours using a self-made oviposition alarm system (Lee, 2009a). When the alarm system signaled 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 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).

For the thinning of the outer egg membrane, part of the rinsed fertilized eggs were immediately placed at the bottom of the sterile plastic Petri dishes filled with filtered (Millipore filter: 0.20 μ m) seawater (salinity: 27.6‰) (Fig.1A). The seawater was then removed by pipette until part of the outer egg membrane was exposed to air (Fig.1B). And then filtered (Millipore filter: 0.20 μ m) seawater (salinity: 27.6‰) was added once again by pipette. With this treatment, the eggs floated on the water surface and part of the outer egg membrane was exposed to air and stretched by the water surface tension of the seawater (salinity: 27.6 ppt). As a result, part of the outer egg membrane became thin (Fig.1C and D).

Egg incubation trial for the examination of the effect of the stretched outer egg membrane

After the *in vitro* artificially fertilized eggs had floated overnight, they were brought down to the bottom of the tissue culture flask by pipette. For the experimental groups, three replicates of 50 floating treated eggs were counted and artificially incubated in 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. For the control groups, the untreated *in vitro* artificially fertilized eggs were counted and artificially incubated in the same way as the experimental groups.

The eggs in both the experimental and control groups were artificially incubated at 20℃ until 10 days elapsed after the beginning of hatch. The eggs and hatched zoeae were checked and counted every day under the stereoscopic microscope. In addition, the external features and movement of the hatched zoeae were observed.

T-test was conducted in the statistical analysis of the results of artificial incubation in the experimental and control groups, i.e., the average normal zoeal hatching rate, average abnormal zoeal hatching rate, average rate of the eggs that were unable to hatch, and average rate of the eggs that died before hatch.

To examine the diameter of the outer egg membrane exposed to air

After the *in vitro* artificial fertilized eggs were floated on the surface of the filtered (Millipore filter: 0.20 μ m) sea water (salinity: 27.6 ppt), twenty of them were collected at 20min, 30min, 40min, 1hr, 1.5hr, and 24hr. The outer egg membranes exposed to air were photomicrographed with light microscopy for measurement of their diameters.

To examine the thickness of the outer egg membrane of the floating egg

In order to examine the thickness of the outer egg membranes, transmission electron microscopy observations were carried out using the standard methods described in Hayat (1986). *In vitro* artificially fertilized eggs that had floated on filtered (Millipore filter: 0.20 μ m) sea water (salinity: 27.6‰) overnight were collected and fixed.

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 outer egg membranes, all of the transverse sections were cut at the middle of the eggs.

Results

Eggs Incubation trial for the examination of the effect of the thinned 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 spins, the first and second maxilipeds, 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 66.67% 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 29.33% (Table 1). Statistical analysis showed that there was significant difference between the two groups in terms of average normal zoea hatching rate ($p<0.001$), average abnormal zoea hatching rate ($p<0.001$) and average rate of the eggs that were unable to hatch ($p<0.01$) (Table 1).

Changes of the diameter of the outer egg membrane exposed to air

The diameter of the outer egg membrane exposed to air extended rapidly during the first hour after floating and then began to stop at 1.5 hours after floating (Table 2). Figure 2 shows the feature of a floating egg 24 hours after the egg floating treatment. The diameter of the stretched outer egg membrane exposed to air grew up to more than 700 μm. When the floating eggs were brought down to the bottom of the dish, the shape of the stretched outer egg membrane became distorted (Fig. 3).

Transmission electron microscopy observations of the outer egg membrane of the floating egg

Transmission electron microscopy observations showed that the thickness of the outer egg membranes exposed to air were about six times thinner than the outer egg membranes submerged in seawater (Fig. 4). There was, however, no remarkable difference in the structure between these two kinds of outer egg membranes.

Figure 5 shows that the outer egg membrane was torn into two layers by the water surface tension at the joint region of the water surface.

Discussion

Based on the results of transmission electron microscopy observations and diameter measurement, it is concluded that 1) the water surface tension stretched the outer egg membrane exposed to air and 2) it caused the outer egg membrane exposed to air to become six times thinner than that of the outer egg membrane submerged in seawater about one hour after floating on water surface. On the other hand, the outer egg membrane submerged in seawater showed no difference in thickness compared with the outer egg membrane of the control groups reported in Lee (2009b). This suggests that water surface tension was not able to stretch the outer egg membrane submerged in seawater. These results must be related to the intensity of the water surface tension, the initial area of the outer egg membrane exposed to air, and the flexibility of the outer egg membrane. Based on the data in Table 2, it is likely that the outer egg membrane hardened within about one hour after floating on water surface. As for the flexibility of the outer egg membrane, it has been found that the outer egg membrane of the *in vitro* fertilized egg of the Japanese mitten crab *E. japonica* can extend itself after fertilization due to the density of the incubated eggs (Lee and Yamazaki, 1993). Moreover, during the formation of the outer egg membrane of the penaeid shrimp *Sicyonia ingentis*, there is evidence for the presence of a chitin-like, or similarly linked, carbohydrate component in the outer egg membrane (Glas, et al., 1996), and there is also evidence for the presence of an oxidase in the assembly of the outer egg membrane (Glas, et al., 1995). These two substances may be very germane to the change of the flexibility of the outer egg membrane after fertilization. Further research into the physical and chemical characteristics with respect to the flexibility of the outer egg membrane of the Japanese mitten crab *Eriocheir japonica* after fertilization is needed.

At the joint region of the water surface, the outer egg membrane was torn into two layers. This phenomenon may be related to the formation of the outer egg membrane. In Chinese mitten crab *E. sinensis,* it is reported that the outer egg membrane is formed by two layers of membrane before oviposition. After fertilization, these two layers of membrane fuse together and form a single layer of egg membrane (Ying and Yang, 2005), i.e., what is referred to as the outer egg membrane throughout this paper. It is possible that the two layers of membrane observed in this study were separated around the joint region of the seawater by water surface tension before they fuse together.

The results of the eggs incubation trial indicate that the normal hatching rate of the experimental groups is apparently higher than that of the control groups. This provides evidence to prove the hypothesis proposed by Lee (2009b) that the thickness of the outer egg membrane of the *in vitro* fertilized egg is the key factor that affects the normal hatching rate. The thinned outer egg membrane may increase not only the exchanging rate of oxygen but also the discharging rate of the metabolic substance. All of these contribute greatly to the increase of normal hatching rate.

In this study, the floating treatment allowed us to treat thousands of eggs in a few minutes and increased the normal hatching rate from 10.69% to 66.67%, whereas that of the *in vitro* artificially fertilized eggs with the outer egg membrane removal treatment described in (Lee, 2009b) was 92.00%. This suggests that the thickness reduction area of the outer egg membrane may not be sufficient to guarantee a high normal hatching rate. In order to obtain a higher normal hatching rate, a new method for inducing a larger thickness reduction area is needed. Alternatively, we can invent a new method for high normal hatching rate by combining the floating treatment and the removal treatment. This study demonstrates that the outer egg membrane can be easily stretched with the floating treatment. The extended outer egg membrane can be utilized to devise a simpler method for removing the outer egg membrane. For example, we can fix an egg by sucking its extended outer egg membrane into a small hole at first and then remove the outer egg membrane with mechanical force.

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Figure captions

Fig. 1 Schematic illustration of the treatment for thinning the outer egg membrane. A: Rinsed fertilized eggs were brought down to the bottom of the dish. B: Seawater (salinity: 27.6‰) was removed until part of the outer egg membrane was exposed to air.

C: Seawater (salinity: 27.6‰) was added until the eggs floated on the water surface. D: The outer egg membrane exposed to air was stretched and became thin as a result of the water surface tension of the seawater (salinity: 27.6‰). b = bottom of the dish; j = joint region of the water surface and the outer egg membrane; oma = outer egg membrane exposed to air; oms = outer egg membrane submerged in seawater; ws = water surface.

Fig. 2 The feature of the stretched outer egg membrane exposed to air on the second day after the egg floating treatment. j = joint region of the water surface and the outer egg membrane. Scale bar: 300 μm.

Fig. 3 The shape of the stretched outer egg membrane was distorted when the floating eggs were brought down to the bottom of the dish. Scale bar: 300 μm

Fig. 4 Transmission electron microscopy transverse sections of the outer egg membrane of a floating egg on the second day after the floating treatment. A: The outer egg membrane exposed to air. B: The outer egg membrane submerged in seawater (salinity: 27.6‰). Scale bar: 0.5 μm.

Fig. 5 Transmission electron microscopy transverse section of the outer egg membrane of a floating egg near the joint region of the water surface. At the joint region of the water surface, the outer egg membrane was torn into two layers. oma = outer egg membrane exposed to air; oms = outer egg membrane submerged in seawater. Circle indicates the joint region that stretched by the water surface tension. Star indicates the internal side of the outer egg membrane.

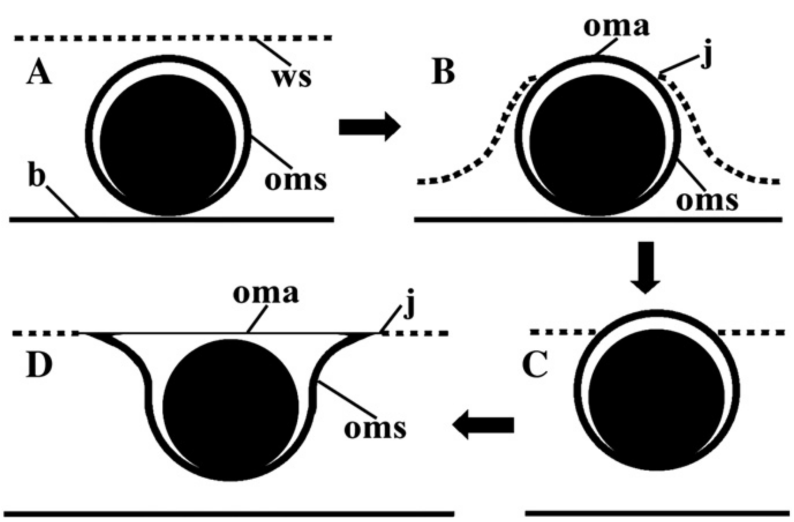

Figure 1

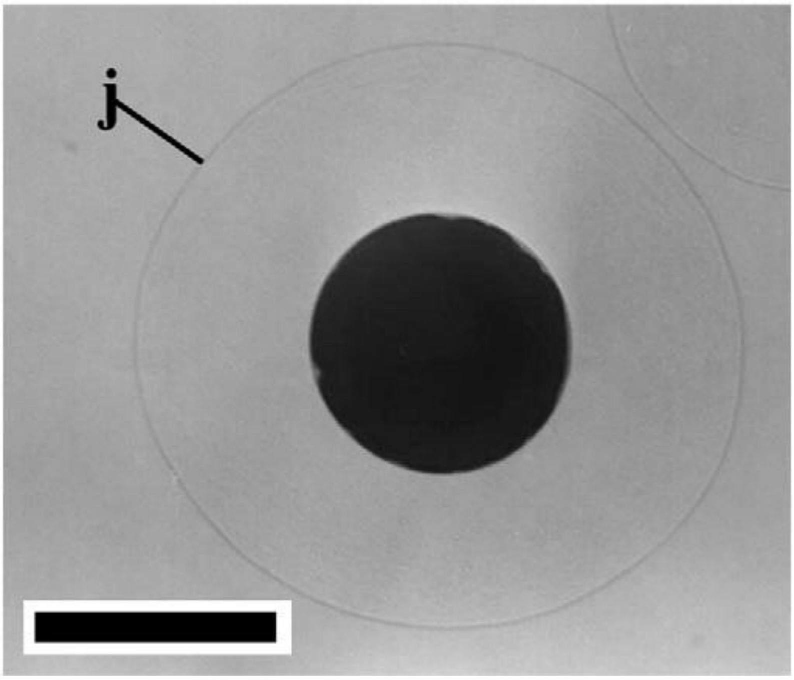

Figure 2

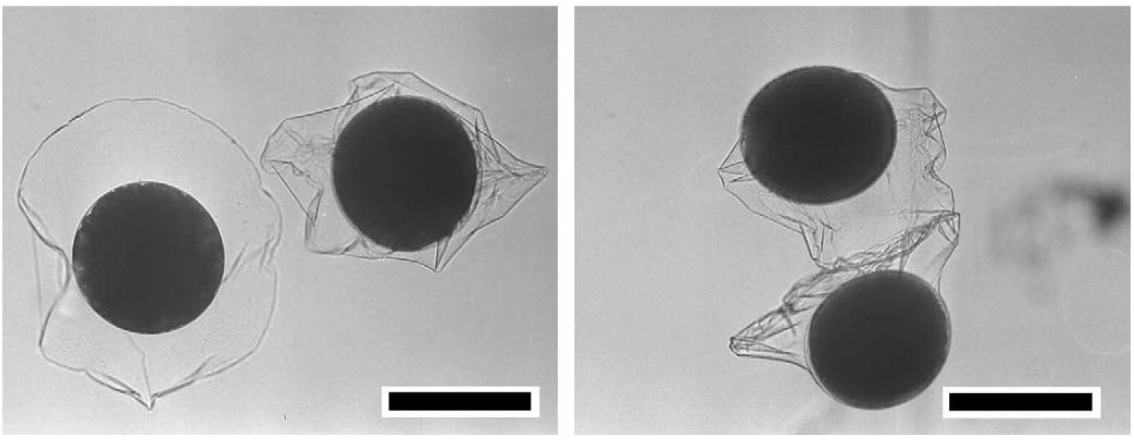

Figure 3

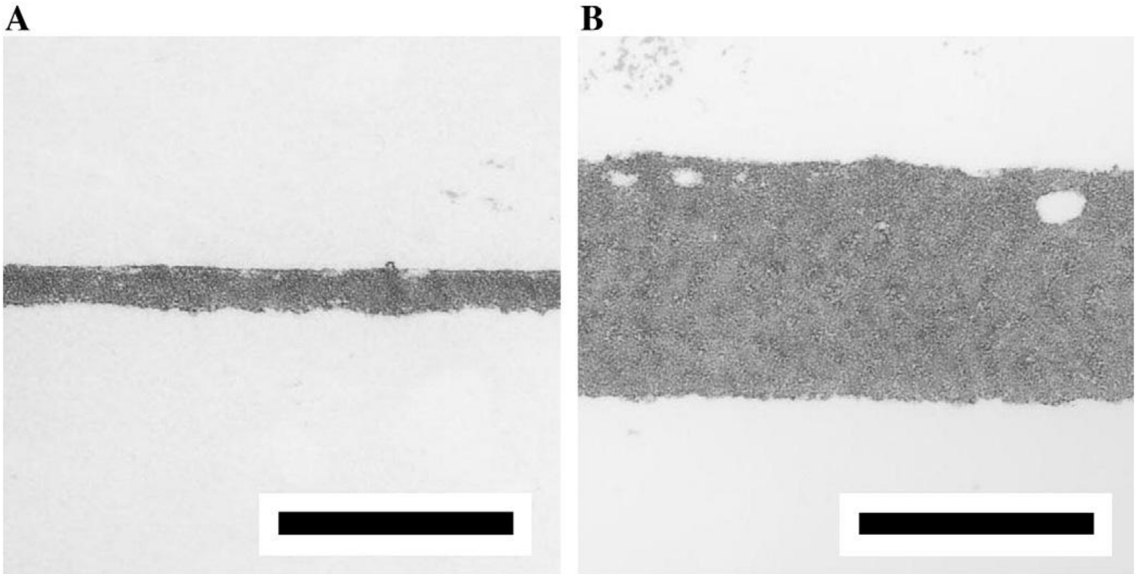

Figure 4

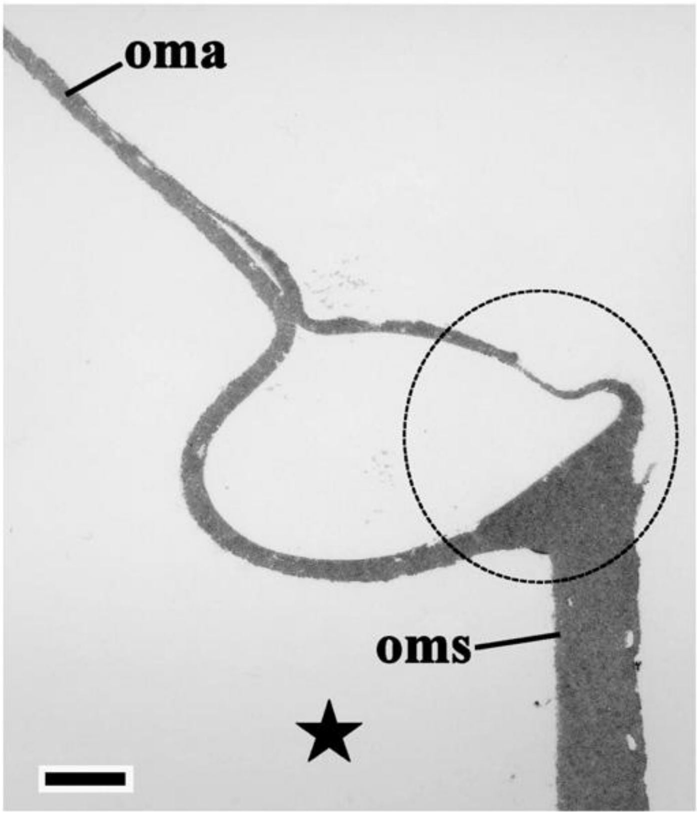

Figure 5

Table 1. Results of the incubation trial in the experimental groups and control groups.

	Control groups	Experimental groups
Normal zoea hatching rate	10.67 ± 1.16	66.67 ± 2.31***
Abnormal zoea hatching rate	54.67 ± 2.31	29.33 ± 3.06***
Rate of the eggs unable to hatch	33.33 ± 2.31	1.33 ± 2.31**
Rate of the eggs dead before hatch	1.33 ± 1.16	2.67 ± 3.06
Total	100.00	100.00

Data represent mean (%) ± S.D.. Asterisks indicate significant differences in comparison with values of control groups(** $P<0.01$; *** $P<0.001$; t-test)

Table 2. Changes of the diameters of the outer egg membranes exposed to air after floating.

Time elapsed after floating	20min	30min	1hr	1.5hr	24hr
Diameter(μm)	400.25 ± 22.38	456.30 ± 22.39	669.45 ± 36.92	689.70 ± 33.34	677.85 ± 56.69

Data represent mean (%) ± S.D.