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RESEARCH ARTICLE

Artificial activation of mature unfertilized eggs in the malaria vector mosquito, *Anopheles stephensi* (Diptera, Culicidae)

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SUMMARY

In the past decade, many transgenic lines of mosquitoes have been generated and analyzed, whereas the maintenance of a large number of transgenic lines requires a great deal of effort and cost. *In vitro* fertilization by an injection of cryopreserved sperm into eggs has been proven to be effective for the maintenance of strains in mammals. The technique of artificial egg activation is a prerequisite for the establishment of *in vitro* fertilization by sperm injection. We demonstrated that artificial egg activation is feasible in the malaria vector mosquito, *Anopheles stephensi* (Diptera, Culicidae). Nearly 100% of eggs dissected from virgin females immersed in distilled water darkened, similar to normally oviposited fertilized eggs. It was revealed by the cytological examination of chromosomes that meiotic arrest was relieved in these eggs approximately 20 min after incubation in water. Biochemical examinations revealed that MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated protein kinase) and MEK (MAPK/ERK kinase) were dephosphorylated similar to that in fertilized eggs. These results indicate that dissected unfertilized eggs were activated in distilled water and started development. Injection of distilled water into body cavity of the virgin blood-fed females also induced activation of a portion of eggs in the ovaries. The technique of artificial egg activation is expected to contribute to the success of *in vitro* fertilization in *A. stephensi*.

Key words: egg activation, meiosis, *in vitro* fertilization, insect, mosquito, *Anopheles stephensi*.

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INTRODUCTION

Some anopheline mosquito species transmit the malaria parasite to the host with their saliva *via* blood feeding. Recently, transgenesis has been applied to mosquitoes with an aim to control malaria and analyze mosquito–parasite interactions. The expression of transgenes in some tissues is controlled using tissue-specific promoters. Several effector molecules against the malaria parasite have been expressed in the midgut, hemolymph and salivary glands of mosquitoes (Ito et al., 2002; Kokoza et al., 2010; Dong et al., 2011; Isaacs et al., 2011; Isaacs et al., 2012; Sumitani et al., 2013). Moreover, vaccine candidate molecules have been shown to be expressed in salivary glands (Matsuoka et al., 2010; Yamamoto et al., 2010; Yamamoto et al., 2012). The stable maintenance of a large number of transgenic lines requires a large effort and cost. Therefore, development of efficient methods for maintenance and storage of transgenic lines is desired. In *Aedes aegypti*, the vector of dengue, yellow fever and other diseases, oviposited eggs can be stored under dried conditions for many months, and these eggs then hatch when immersed in water (Kliewer, 1961). In the model species, *Drosophila melanogaster*, methods for the cryopreservation of embryos have been developed (Mazur et al., 1992). Furthermore, when cryopreserved and thawed larval gonads were transplanted into other larvae, a small fraction of the eclosed adults carried donor gonads that were capable of producing normal gametes in *D. melanogaster* and *Bombyx mori* (Brüschweiler and Gehring, 1973; Shinbo, 1989). None of these techniques has been established in anopheline mosquitoes.

Artificial fertilization, such as intracytoplasmic sperm injection (ICSI) using cryopreserved sperm, is one of the most general methods for the storage of strains in mammals (Yanagimachi, 2012). The cryopreservation of sperm is a very simple and efficient method that is an alternative to the cryopreservation of larval gonads or embryos. In insects, ICSI has been established in the sawfly *Athalia rosae* (Hymenoptera) (Sawa and Oishi, 1989b). Moreover, in this species, transgenic strains have been successfully stored and recovered by artificial fertilization using cryopreserved sperm (Hatakeyama and Sumitani, 2005). Therefore, the method for artificial fertilization by microinjection of sperm into unfertilized eggs is likely to be developed in other insect species including mosquitoes. As the technique of microinjection of a plasmid vector into embryos to produce transgenic lines has been established in mosquitoes (Catteruccia et al., 2000), the injection of sperm into eggs seems to be practicable.

What would be the prerequisite for success of artificial fertilization? It is the method to artificially activate unfertilized eggs. In insect species, egg activation has been shown to be independent of fertilization (Doane, 1960; Horner and Wolfner, 2008b). Parthenogenetic insect species develop normally without fertilization (Suomalainen et al., 1987). Moreover, eggs are activated in the oviduct before fertilization in *D. melanogaster* (Hatsumi et al., 2001; Heifetz et al., 2001). *Athalia rosae*, in which *in vitro* fertilization has been shown to be successful, is a parthenogenetic insect in which nearly 100% of unfertilized eggs dissected from female ovaries can be activated artificially without fertilization by simply immersing

them in water (Sawa and Oishi, 1989a). An indicator for whether an egg is developing would be required for evaluation of egg activation. In some aquatic insect species that lay eggs in water, such as mosquitoes, dragonflies and mayflies, coloration of the egg is often an index of normal development (Corbet, 1962; Clements, 1992; Watanabe et al., 1999; Tojo et al., 2006). Therefore, coloration of eggs could be one of the visible indices of egg activation. The phosphorylation status of MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) and MEK (MAPK/ERK kinase) in the Mos/MEK/MAPK pathway could also be used as one of the biochemical indexes of egg activation. The mature oocytes of most animals before fertilization are arrested at specific stages of meiosis, and MEK and MAPK are phosphorylated. Once meiosis resumes after fertilization, MEK and MAPK are dephosphorylated (Nishiyama et al., 2010).

In the present study, we demonstrated the feasibility of artificial activation of mature unfertilized eggs dissected from virgin females by immersing them in distilled water, which represents a step towards *in vitro* fertilization using cryopreserved sperm in the malaria vector mosquito, *Anopheles stephensi* Liston 1901.

MATERIALS AND METHODS

Ethics statement

All animal procedures were approved by the Animal Ethics Committee of Jichi Medical University, Japan.

Animals

The *A. stephensi* mosquito strain SDA500 was maintained at Jichi Medical University according to previously described methods (Yamamoto et al., 2012). Female ICR strain mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan) and were used at 8 weeks of age.

Four- to seven-day-old virgin female adults were fed the blood of mice, and unfertilized eggs were dissected from virgin females 4 to 7 days after blood feeding. Eggs were dissected and observed under a stereomicroscope (MZ16F, Leica, Wetzlar, Germany).

Cytological examination of chromosomes in eggs

Eggs were fixed in methanol at 4°C. The fixative solution was replaced with a series of 75%, 50%, 30% methanol in phosphate-buffered saline (PBS; pH 7.4) and finally with PBS. Fixed eggs were then stained with 4′,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 μg ml^{-1} in PBS for 10 min, and washed twice with PBS. Stained samples were mounted on a slide glass with 30% glycerol in PBS and were observed under a BX51 fluorescent microscope equipped with a DP71 digital camera (Olympus, Tokyo, Japan) and BIOREVO fluorescent microscope (KEYENCE, Osaka, Japan).

To remove the exochorion and endochorion of darkened eggs, the procedures described in Goltsev et al. (Goltsev et al., 2004) were slightly modified and applied as follows. Darkened eggs or embryos were incubated in 25% antiformin for 2 min and were then washed in distilled water three times. Treated eggs were replaced and shaken in a glass vial with a 1:1 mixture of heptane (Nacalai Tesque, Kyoto, Japan) and 4% paraformaldehyde (Nacalai Tesque) for 30 min. Next, the paraformaldehyde phase was removed and replaced with an equal volume of distilled water twice, and eggs were shaken for 30 min. Thereafter, the water phase was removed and replaced with 95°C distilled water, and vials were incubated for 30 s. The hot water phase was removed and replaced with ice-cold distilled water. Vials were incubated on ice for 15 min. Subsequently, the water phase and heptane phase were removed, and fresh heptane was added into the vials. An equal volume of methanol was quickly added to the vials. Vials were vigorously shaken for 1 min, and were then incubated for 15 min at room temperature. The heptane phase was removed and the methanol phase was replaced with fresh methanol three times. Eggs were incubated overnight in methanol at 4°C. Methanol was replaced with a series of 75%, 50%, 30% methanol in PBS and finally with PBS. Ruptured endochorions were removed with fine tungsten needles in PBS. Eggs were used for DAPI staining.

Immunoblotting

Mouse anti-phosphorylated MAPK antibody (p-ERK, sc-7383, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-MAPK monoclonal antibody (p42 MAPK, 3A7 mAb, #9107, Cell Signaling, Danvers, MA, USA), rabbit anti-phosphorylated MEK monoclonal antibody (phospho-MEK1/2, 41G9 mAb, #9154, Cell Signaling), rabbit anti-MEK monoclonal antibody (MEK1/2, D1A5 mAb, #8727, Cell Signaling) and rabbit anti-alpha-tubulin monoclonal antibody (11H10 mAb, #2125, Cell Signaling) were used as primary antibodies.

Groups of 40 eggs were homogenized by a plastic homogenizer with 40 μl of sample buffer (Nacalai Tesque) containing 5% 2-mercaptoethanol, and were then boiled at 95°C for 3 min. Five microliters of each sample (equivalent to five eggs) was separated on a 10% SDS-PAGE gel, and was transferred to a Hybond^{TM} ECL Membrane (GE Healthcare UK, Buckinghamshire, UK). Membranes were blocked with T-TBS (20 mmol l^{-1} Tris-HCl, 137 mmol l^{-1} NaCl, pH 7.4, 0.05% Tween-20) containing 5% skimmed milk (Megmilk Snow Brand, Tokyo, Japan). Membranes were incubated with primary antibodies. Polypeptides recognized by primary antibodies were detected with either horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG or HRP-conjugated donkey anti-rabbit IgG (GE Healthcare UK). The detection of HRP-labeled antibodies was performed by exposing membranes on a Hyperfilm-ECL using ECL Western Blotting Detection Reagents or ECL Prime Western Blotting Detection Reagents (all GE Healthcare UK) according to the supplier's protocol.

Injection

Injection of distilled water or 0.2 mol l^{-1} NaCl solution into adult females was performed using a micromanipulator (YOU-1, Narishige, Tokyo, Japan) and a microinjector IM-11 (Narishige) under a stereomicroscope. A fine glass micropipette was made by pulling a 1–5 μl calibrated pipet (Drummond, Broomall, CA, USA) with the capillary puller PC-10 (Narishige), and grinding the tip with the EG-44 grinder (Narishige). A volume of 280 nl of distilled water or 0.2 mol l^{-1} NaCl was injected into the lateral hemocoel in the abdomen or thorax of 10- to 14-day-old virgin adult females 4 to 7 days after blood feeding. Injected adult mosquitoes and ovaries were observed under an SZX7 stereomicroscope equipped with a DP73 digital camera (Olympus, Tokyo, Japan).

RESULTS

Darkening of unfertilized eggs by immersing in water

In the parthenogenetic sawfly *A. rosae*, nearly 100% eggs dissected from adult females were activated by immersing them in hypotonic solution or distilled water, and these eggs developed to haploid male adults (Sawa and Oishi, 1989a). We applied this method to *A. stephensi* mosquitoes. Eggs dissected from adult virgin females in a saline solution (0.15 mol l^{-1} NaCl) were immersed in distilled water for 20 min and replaced on filter paper soaked with distilled water in a small Petri dish. In many mosquito species, including anopheline

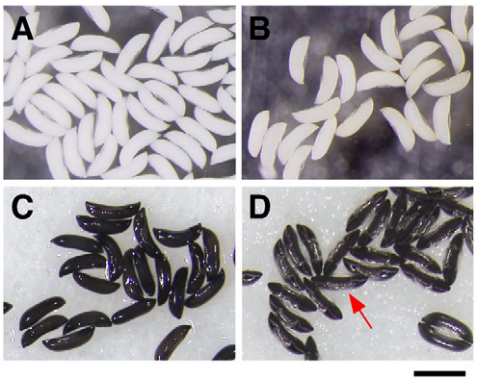

Fig. 1. Results of egg darkening by immersing *Anopheles stephensi* eggs in distilled water. (A) Unfertilized eggs immediately after dissection from virgin females in 0.15 mol l^{-1} NaCl. (B) Unfertilized eggs that were dissected from virgin females and incubated in 0.15 mol l^{-1} NaCl for 4 h. (C) Darkened unfertilized eggs, which were dissected from virgin females and incubated in distilled water for 4 h. (D) Normal fertilized eggs 4 h after being laid. Air-filled expansion of the exochorion was observed (arrow). Scale bar, 500 μm.

species, the inner layer of the chorion of fertilized eggs, termed the endochorion, darkens when they are oviposited in water (Clements, 1992). The endochorion of most water-immersed eggs was black in color (darkened) within 4 h, similar to normally oviposited eggs (Fig. 1). In contrast, only a very small fraction of eggs incubated in 0.15 mol l^{-1} NaCl for 4 h darkened in color (Fig. 1). A notable difference between oviposited and water-immersed darkened eggs was the presence of floats. The outer layer of the chorion, termed the exochorion, was filled with air and expanded in the lateral region of oviposited eggs (Clements, 1992), while the exochorion was not expanded in water-immersed eggs (Fig. 1). Larvae hatched from nearly all darkened oviposited eggs, whereas no larvae hatched from darkened eggs treated with distilled water.

In another experiment, 3.5% of eggs incubated in 0.15 mol l^{-1} NaCl for 12 h were darkened (Table 1). To examine the possible effect of NaCl concentrations on egg activation, eggs were incubated in various concentrations of NaCl solution. The darkening of eggs did not occur at concentrations higher than 0.2 mol l^{-1} NaCl (Table 1). This result suggested that the darkening (activation) of eggs occurred in a sodium-concentration-dependent manner.

We then examined the effects of the duration of immersion in distilled water and 0.2 mol l^{-1} NaCl on egg activation. Eggs dissected in 0.2 mol l^{-1} NaCl were immersed in distilled water for 5, 10 and 20 min, and were returned to 0.2 mol l^{-1} NaCl. Most eggs immersed in distilled water for more than 10 min darkened after transfer to 0.2 mol l^{-1} NaCl (Table 2). This result suggests that egg activation takes at least 10 min, and high NaCl concentrations do not interfere with the darkening of the endochorion once eggs have been activated. Next, dissected unfertilized eggs were first immersed in 0.2 mol l^{-1} NaCl for various durations and then eggs were transferred to distilled water. More than 80% of eggs darkened after the 0.2 mol l^{-1} NaCl incubation for 20 min, and the ratio of activated eggs was significantly reduced after 0.2 mol l^{-1} NaCl incubation for more than 40 min (Table 3). Approximately 70% and 50% of eggs that failed to be activated after incubation in 0.2 mol l^{-1} NaCl for 60 and 40 min, respectively, ruptured and leaked yolk. These results indicate that eggs dissected from females could be maintained in a healthy state in 0.2 mol l^{-1} NaCl for up to 20 min.

Table 1. Darkening of unfertilized *Anopheles stephensi* eggs by immersion in distilled water or NaCl solution

Solution	No. eggs treated	No. eggs darkened after 12 h (%)
Distilled water[a] experiment 1	313	308 (98.4)
Distilled water[a] experiment 2	440	438 (99.5)
Distilled water[b]	464	453 (97.6)
0.1 mol l^{-1} NaCl[b]	536	526 (98.1)
0.15 mol l^{-1} NaCl[b]	536	19 (3.5)
0.2 mol l^{-1} NaCl[b]	655	0
0.3 mol l^{-1} NaCl[b]	724	0

[a]Eggs were dissected in 0.15 mol l^{-1} NaCl, immersed in distilled water for 20 min, and incubated on filter paper soaked with distilled water.
[b]Eggs were dissected and incubated in each solution.

Table 2. Darkening of unfertilized *A. stephensi* eggs in 0.2 mol l^{-1} NaCl after incubation in distilled water

Duration of immersion of eggs in distilled water (min)[a]	No. eggs treated	No. eggs darkened after 12 h (%)
20	400	398 (99.5)
10	343	338 (98.5)
5	326	188 (57.7)

[a]Eggs dissected in 0.2 mol l^{-1} NaCl were immersed in distilled water for each period, and then returned to 0.2 mol l^{-1} NaCl.

Resumption of meiosis in unfertilized eggs immersed in water

Meiotic chromosomes in eggs immersed in distilled water were examined by DAPI staining to evaluate whether darkened eggs begin developing. The meiotic cell cycle in insect eggs is generally arrested at metaphase of meiotic division I (meta-I arrest) until fertilization (Nishiyama et al., 2010). In *A. stephensi*, meiosis of mature unfertilized eggs was arrested at metaphase of the first meiotic division (meta-I), similar to other insects (Fig. 2A). Meiotic meta-I arrest was relieved upon treatment with distilled water. The separation of homologous chromosomes occurring at approximately 10 min after treatment with distilled water indicated that the meiotic cell cycle progressed to anaphase I (Fig. 2A). The meiotic cell cycle entered the second meiotic division at approximately 40 min and it completed 80 min after treatment with distilled water. One female pronucleus migrated inside the egg and three polar body nuclei migrated toward the periphery approximately 120 min after treatment with distilled water (Fig. 2A). In contrast, meiosis remained arrested at meta-I in eggs 2 h after incubation in 0.2 mol l^{-1} NaCl (Fig. 2A). Oviposited fertilized eggs reached the blastoderm stage 4 h after oviposition. Dissected mature unfertilized eggs darkened 4 h after incubation with distilled water; nevertheless, mitotic divisions did not occur in these water-immersed eggs (Fig. 2B). In these eggs, a

Table 3. Darkening of unfertilized *A. stephensi* eggs in distilled water after incubation in 0.2 mol l^{-1} NaCl

Duration of immersion of eggs in 0.2 mol l^{-1} NaCl (min)[a]	No. eggs treated	No. eggs darkened after 12 h (%)
20	122	103 (84.4)
40	151	12 (7.9)
60	157	10 (6.4)

[a]Eggs dissected and immersed in 0.2 mol l^{-1} NaCl were transferred to distilled water.

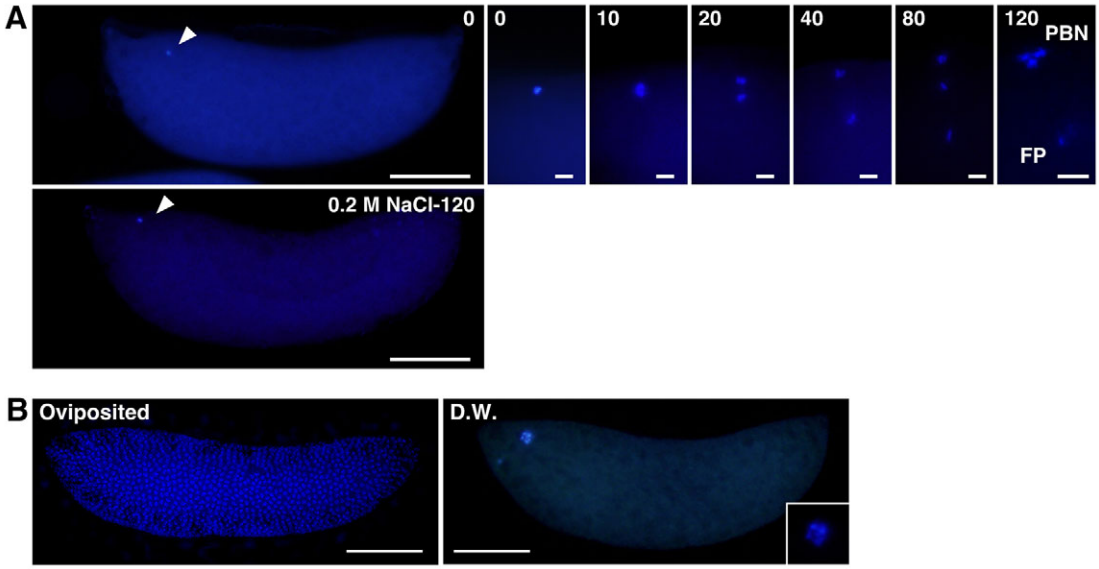

Fig. 2. Progression of meiosis in unfertilized eggs of *A. stephensi* incubated in distilled water. Nuclei were stained with DAPI. (A) The egg nucleus was located beneath the dorsal surface at the anterior end (arrowhead). The meiotic cell cycle was arrested at metaphase of meiotic division I (meta-I arrest) in mature unfertilized eggs (0 min). Meta-I arrest was relived upon immersing eggs in distilled water (10 min). Meiosis progressed to anaphase I of meiotic division I (20 min), metaphase of meiotic division II (40 min), anaphase I of meiotic division II (80 min) and pronucleus formation (120 min). Meiosis is not resumed in eggs incubated in 0.2 mol l^{-1} NaCl for 120 min. FP, female pronucleus; PBN, polar body nuclei. Scale bars, whole egg panels: 100 µm; other panels: 10 µm. (B) Embryo 4 h after egg laying (blastoderm stage) and an unfertilized egg, which was dissected from a virgin female and incubated in distilled water (DW) for 4 h. Inset is a magnified image of the chromosome mass structure. Scale bars, 100 µm.

condensed chromosome mass resembling a 'rosette-like structure' or 'chromosome bouquet' derived from polar body nuclei in *D. melanogaster* and *A. rosae* (Campos-Ortega and Hartenstein, 1985; Foe et al., 1993; Page and Orr-Weaver, 1997; Yamamoto et al., 2008) was observed, indicating that meiosis was completed but further mitotic division did not occur.

Dephosphorylation of MEK and MAPK in unfertilized eggs immersed in water

Next, we examined the phosphorylation status of MEK and MAPK as a biochemical index of egg activation. MEK and MAPK are phosphorylated in the mature oocytes of animals before fertilization. After fertilization or egg activation, MEK and MAPK are dephosphorylated. MEK and MAPK of mature unfertilized eggs, in which meiosis arrests at meta-I, were phosphorylated in *A. stephensi*. MEK and MAPK were rapidly dephosphorylated within 10–20 min of eggs being immersed in distilled water and remained dephosphorylated thereafter (Fig. 3). In contrast, MEK and MAPK remained phosphorylated for at least 60 min in eggs incubated in 0.2 mol l^{-1} NaCl that were not activated. It was proven biochemically that mature unfertilized eggs immersed in distilled water were activated in *A. stephensi*. Taking these results together, we concluded that the mature unfertilized eggs of *A. stephensi* could be artificially activated by simply immersing them in distilled water, and darkening

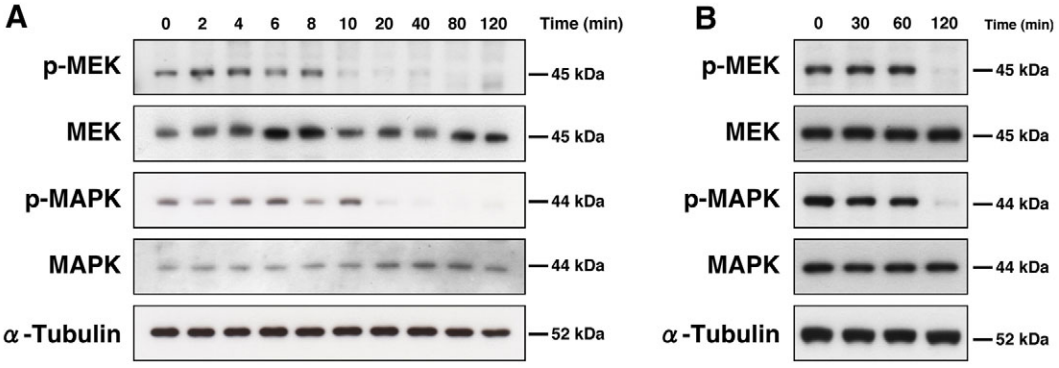

Fig. 3. Status of MEK and MAPK phosphorylation in eggs of *A. stephensi* by immunoblotting. (A) Dephosphorylation of MEK and MAPK in unfertilized eggs incubated in distilled water. Anti-phosphorylated MEK antibody (p-MEK), anti-MEK antibody (MEK), anti-phosphorylated MAPK antibody (p-MAPK) and anti-MAPK antibody (MAPK) were used as primary antibodies. Dephosphorylation of MEK and MAPK began 10 and 20 min, respectively, after incubation in distilled water. Anti-alpha-tubulin antibody (α-tubulin) was used as a loading control. Numbers above panels indicate incubation times in distilled water. (B) Phosphorylation of MEK and MAPK in unfertilized eggs kept in 0.2 mol l^{-1} NaCl. Phosphorylation of both MEK and MAPK was maintained until 120 min after incubation in 0.2 mol l^{-1} NaCl. Numbers above panels indicate incubation times in 0.2 mol l^{-1} NaCl. Molecular mass of each peptide is indicated on right.

of the endochorion was a useful visible index of the occurrence of egg activation.

Artificial egg activation *in vivo*

Finally, we injected distilled water into the hemocoel of blood-fed females that contained mature eggs. In more than 70% of females (N=53) that received injection with distilled water, a portion of the eggs in ovary located near the site of injection darkened (Fig. 4A,B), In contrast, no eggs darkened in the ovary of adult females (N=31) that received injection of 0.2 mol l^{-1} NaCl. We confirmed that MEK and MAPK were dephosphorylated in the darkened eggs 2 h after injection with distilled water (Fig. 4C). These results indicate that eggs could be artificially activated even *in vivo* by injection of distilled water into the hemocoel.

DISCUSSION

Establishment of the methods of artificial egg activation is essential for *in vitro* fertilization to succeed. In this study, we demonstrated that meiotic arrest at metaphase of meiosis I (meta-I) in mature unfertilized eggs dissected from virgin females of *A. stephensi* could be relieved and meiosis could complete by simply immersing these eggs in distilled water. The endochorion of nearly all eggs incubated in distilled water darkened, similar to that of normally oviposited fertilized eggs. In these activated eggs, the endochorion darkened in color unexceptionally. In *A. aegypti*, egg chorion darkening is caused by chorion protein cross-linking and melanization, and these processes are initiated after egg oviposition (Li, 1994; Li and Li, 2006). The *Drosophila* eggshell, which consists of a chorion and vitelline membrane, is changed in protein cross-linking by enzymatic activation upon egg activation without fertilization (Horner and Wolfner, 2008b). Therefore, egg darkening in mosquitoes is considered to be an index of the occurrence of egg activation. In a dragonfly and mayfly, unfertilized eggs can be activated by immersing them in water (Tojo and Machida, 1998; Watanabe et al., 1999). Egg activation by immersion in water may be a relatively common phenomenon in aquatic insect species. The occurrence of egg activation in *A. stephensi* depends on the concentration of NaCl, i.e. the change in the osmolality. It has been shown in some insects that the osmolality of egg circumference contributes to egg activation (Sawa and Oishi, 1989a; Horner and Wolfner, 2008a). In *A. rosae*, a hypotonic solution has been shown to stimulate egg activation (Sawa and Oishi, 1989a). Moreover, a relationship between egg activation and hypo-osmotic pressure has been demonstrated in *D. melanogaster* (Horner and Wolfner, 2008b). In the mosquito *A. stephensi*, the osmotic pressure of the hemolymph of adult females was shown to be maintained in ~0.2 to 0.3 mol l^{-1} NaCl equivalent (Mack and Vanderberg, 1978). This agrees with our results, in which unfertilized eggs were never activated in NaCl solutions higher than 0.2 mol l^{-1} in concentration. In other words, the hyper-osmotic pressure of the female hemolymph will prevent accidental parthenogenetic development of haploid eggs in the ovary. The results of egg activation *in vivo* by injection of distilled water into the adult hemocoel support this hypothesis. The eggs in the ovary located close to the site of injection with distilled water could be activated. A volume of 280 nl of distilled water per individual was injected into the hemocoel of blood-fed females that have approximately 190 nl of hemolymph (Mack et al., 1979). We assume that the effect of injected water to reduce osmotic pressure for egg activation is restricted to the site of injection. Similarly, in the parthenogenetic sawfly *A. rosae*, in which *in vitro* egg activation by immersing eggs in water has been shown (Sawa and Oishi, 1989a), a portion of eggs could also be activated *in vivo* by injection of distilled water into the hemocoel, and embryogenesis completes in ovaries (M.H., unpublished results). Hyper-osmotic pressure of hemolymph may contribute to prevent accidental egg activation *in vivo* in some insects. In the present study, egg activation occurred in an extremely hypotonic solution (distilled water) compared with 0.15 mol l^{-1} NaCl equivalent. Therefore, hypo-osmotic pressure is one of the triggers of artificial egg activation in *A. stephensi*.

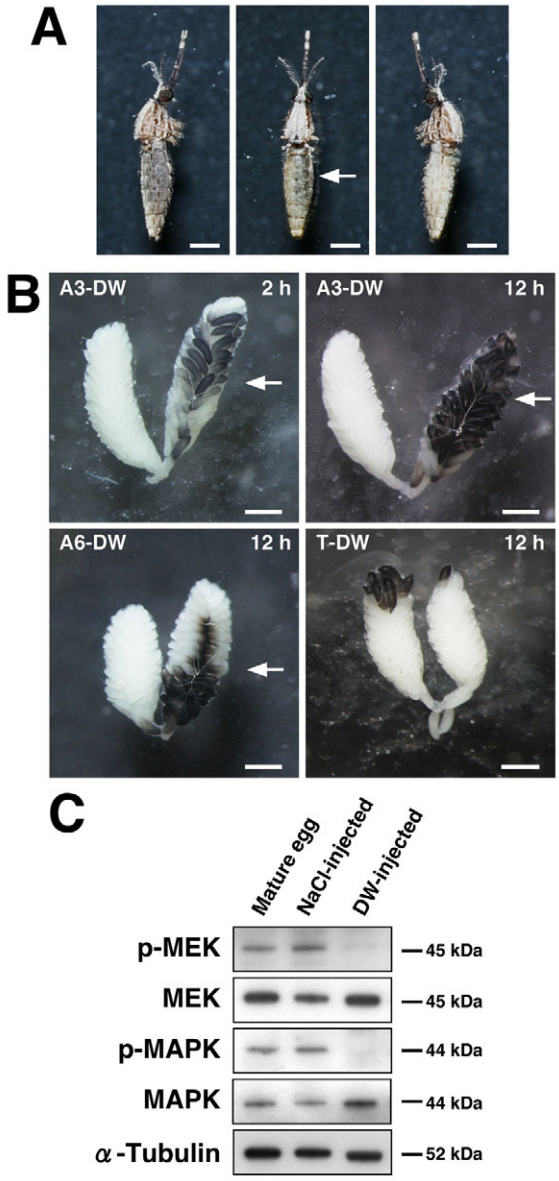

Fig. 4. *In vivo* egg activation by injection of distilled water (DW) into blood-fed female *A. stephensi*. (A) A female 12 h after injection of water into the lateral hemocoel in the third abdominal segment. Left: lateral view from the side of injection. Darkened eggs could be seen through abdominal cuticle. Middle: dorsal view. Arrow indicates the site of injection. Right: lateral view from the opposite side of injection. Scale bars, 1 mm. (B) A pair of ovaries dissected from females that received injection of water into the third abdominal segment (A3), the sixth abdominal segment (A6) and the thorax (T). Females were dissected at 2 or 12 h after injection. Scale bars, 500 μm. (C) MEK and MAPK were dephosphorylated in darkened eggs dissected from females 2 h after injection of distilled water into the third abdominal segment. Phosphorylation of MEK and MAPK were maintained in eggs from females 2 h after injection of 0.2 mol l^{-1} NaCl solution.

Although more than 60% of eggs immersed in distilled water for 5 min were activated, 10 min is the minimum time required to ensure that nearly all eggs were activated. However, these apparently activated eggs did not complete embryogenesis. This may be largely due to the haploidy of eggs because fusion of the female pronucleus does not take place with the male pronucleus or the polar body nuclei. Alternatively, these artificially activated eggs would unable to undertake mitosis because they did not receive a centriole from sperm.

MEK and MAPK were dephosphorylated in eggs when meiosis was resumed by egg activation. The phosphorylation status of the MEK/MAPK pathway is one of the indicators of egg meiotic arrest and its resumption occurs following egg activation. Our present results indicated that the resumption of meiosis was linked to the MEK/MAPK pathway in *A. stephensi*, similar to other organisms examined so far (Tachibana et al., 2000; Kondoh et al., 2006; Sensui et al., 2012). In most vertebrates, MEK and MAPK are phosphorylated by the presence of Mos – a key component of cytostatic factor – in mature oocytes, and meiosis is arrested at meiotic metaphase II before fertilization. Once meiosis resumes after fertilization, degradation of Mos occurs, and MEK and MAPK are dephosphorylated (Nishiyama et al., 2010). The MEK/MAPK pathway is also involved in meiotic meta-I arrest in *A. rosae* (Yamamoto et al., 2008). In *A. rosae* eggs, MEK and MAPK are phosphorylated due to the presence of Mos before fertilization, and are dephosphorylated upon egg activation (Yamamoto et al., 2008). Although Mos is not essential in meiotic meta-I arrest in *D. melanogaster*, the phosphorylation status of MEK and MAPK reflects meta-I arrest and its release (Ivanovska et al., 2004; Sackton et al., 2007; Von Stetina and Orr-Weaver, 2011). Therefore, the phosphorylation status of MEK and MAPK is a useful marker for egg activation in insects. Although MAPK was dephosphorylated in *A. stephensi* eggs incubated in 0.2 mol l^{-1} NaCl for 120 min, meiosis was not resumed in these eggs (Fig. 2A). It is presumed that this is due to the death of eggs or deterioration of egg quality, but not the consequence of proper egg activation. Because egg activation hardly occurs in eggs immersed in distilled water after incubation in 0.2 mol l^{-1} NaCl for more than 40 min, dissected eggs may be stored in a healthy state for up to 20 min only.

We established a technique of egg activation as a step towards the development of artificial fertilization by sperm injection in the eggs of *A. stephensi*. The technique of microinjection to embryos has been established for producing transgenic strains in *A. stephensi* (Catteruccia et al., 2000). As long as sperm are injected directly into eggs, similar to ICSI performed in mammals, the quality of sperm does not seem to be an issue. A spermatid can be used for ICSI in mammals (Yanagimachi, 2012). Elongated spermatids can be used for ICSI in *A. rosae* as well as mammals (Hatakeyama et al., 2000). Recently, freeze-dried sperm was successful in fertilizing eggs by ICSI in rats (Kaneko and Serikawa, 2012). The long-term preservation of freeze-dried sperm has been shown to result in normal fertility in rats (Kaneko and Serikawa, 2012). Furthermore, sperm dissected from freeze-thawed male adults can be used for ICSI in *A. rosae* (Hatakeyama et al., 1994; Hatakeyama and Sumitani, 2005). Therefore, the establishment of *in vitro* fertilization by sperm injection in *A. stephensi* by combining the methods for microinjection and artificial egg activation is not implausible.

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AUTHOR CONTRIBUTIONS

D.S.Y. conceived and designed the research, and performed all experiments; D.S.Y., M.H. and H.M. analyzed and discussed the data; D.S.Y. and M.H. wrote and revised the manuscript.

COMPETING INTERESTS

No competing interests declared.

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