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Substances for the Initiation of Sperm Motility in Egg-Jelly of the Japanese Newt, *Cynops pyrrhogaster*

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ABSTRACT—The initiation of sperm motility is regulated by certain factors, including a change of osmolality or ion concentration. The cue for the initiation of sperm motility is unique to species and suits the environment in which the fertilization occurs.

In the newt, *Cynops pyrrhogaster*, eggs are fertilized in the cloaca of the female with sperm stored in the sperm reservoir. In this study, we investigated possible factors initiating sperm motility in this unique environment. Sperm of *C. pyrrhogaster* could be initiated to move by a decrease of osmolality. However, eggs were fertilized with dry sperm and developed to four-cell stage embryos without immersion in solution. They continued to develop normally to the tail-bud stage when placed in Steinberg’s salt solution after fertilization. These results indicate that sperm motility was initiated without the change of osmolality around sperm. In egg-jelly extract, the activity for the initiation of sperm motility was strong and heat stable, but disappeared by proteinase treatment. ICP luminescence analysis revealed that sodium, potassium, calcium and magnesium ions were major cations in egg-jelly. The monovalent cations showed the activity for the initiation of sperm motility in high concentration at high pH, and this activity becomes stronger by the addition of calcium ion. However, the reconstructed ionic solution that was prepared according to the concentrations of these four ions and pH in egg-jelly did not show the activity as strong as egg-jelly extract. These results suggest that the proteinacious factor and cations in egg-jelly are significant to regulate the initiation of sperm motility in *C. pyrrhogaster*.

INTRODUCTION

Sperm possess a vigorous motility that assist in their fertilization of eggs. While in the vas deferens, they acquire the capacity to move, and they begin such movement after they are spawned or ejaculated (Morisawa and Morisawa, 1990; Morisawa, 1994). Several factors have been reported to regulate the initiation of sperm motility. In salmonids, the K⁺ concentration around sperm results in the initiation of sperm motility upon spawning (Morisawa, 1994; Morisawa and Suzuki, 1980). In mammals, the concentration of HCO₃⁻ or Ca²⁺ in the seminal plasma regulates sperm motility (Okamura, et al., 1985; Morisawa, 1994). The cue for the initiation of sperm motility is unique to each species and thought to accord with the environment in which fertilization occurs.

Among amphibians, low osmolality has been shown to initiate sperm to move both in anura (Inoda and Morisawa, 1987) and urodele (Hardy and Dent, 1986). However, the fertilization environments of urodele and anura are quite different. In the newt, *Cynops pyrrhogaster*, a female picks up the spermatophore that emerges from the cloaca of the male. Sperm in it was stored in the spermatheca of the cloaca of the female (Tsutsui, 1931). Therefore, fertilization is achieved in the cloaca of the female. It has been reported that a female can spawn fertilized eggs on her own as late as 190 days after picking up the spermatophore (Tsutsui, 1931). This indicates that sperm motility is re-initiated during the fertilization process. At that time, because no change in osmolality occurs around sperm, other factors must initiate sperm motility.

The egg-jelly is the first point of contact between sperm and an egg, and is thus important for fertilization in many species. In fertilization of anura, for example, the egg-jelly acts as a barrier to sperm penetration, and divalent cations within the jelly encourage the sperm-egg interactions and sperm motility (Katagiri, 1987).

In the present study, we investigated the role of egg-jelly in the initiation of sperm motility in the newt, *C. pyrrhogaster*. The factors for the initiation of sperm motility were also investigated in egg-jelly.

MATERIALS AND METHODS

Gametes

Matured newts, *Cynops pyrrhogaster*, were collected in Yamagata, Japan.

Ovulation was induced by two injections of gonadotropin (HCG; Teikoku Zoki Inc., Tokyo, Japan) at a dose of 100 IU daily. Mature eggs were surgically removed from the uterus. To obtain dry sperm,
Jellies were independently collected from the mature eggs obtained in any solution. Each jelly was placed on the electrode of the pH meter (pH boy-2, Sindengen Inc., Japan) to measure its surface pH.

Preparation of reconstructed ionic solution
Reconstructed ionic solution (RIS) of egg-jelly was prepared according to the concentration of four major ions (Table 3). It contained 20 mM NaCl, 2.66 mM KCl, 5.06 mM CaCl₂, 0.39 mM MgCl₂, and 10 mM Tris-HCl, and was adjusted to pH 8.5.

RESULTS

Effect of osmolality on sperm motility
Decrease of osmolality around a sperm results in the initiation of motility in amphibian sperm as well as in the sperm of other species (Morisawa, 1994). To examine the effect of osmolality in C. pyrrhogaster, sperm motility was observed in the solutions of 10, 120 and 240 mOsm/kg of NaCl, mannitol or Steinberg’s salt solution. In 240 and 120 mOsm/kg solutions, no or faint motility was observed (Table 1). No significant difference in sperm motility was seen among the solutions of any of the three types. However, vigorous motility was observed in distilled water or 10 mOsm/kg solutions. These results indicate that sperm motility of C. pyrrhogaster is induced by a decrease of osmolality.

Table 1. Effect of osmolality on sperm motility in C. pyrrhogaster

<table>
<thead>
<tr>
<th>Osmolarity (mOsm/kg)</th>
<th>Test solution</th>
<th>Time after the Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 min.</td>
</tr>
<tr>
<td>240</td>
<td>NaCl (pH 5.4)</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>2&gt;ST</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Mannitol (pH 5.4)</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>ST</td>
<td>±</td>
</tr>
<tr>
<td>120</td>
<td>NaCl (pH 5.6)</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1/10&gt;ST</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>DW (pH 7.0)</td>
<td>+++</td>
</tr>
<tr>
<td>0</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Dry sperm was added in each solution and sperm motility was observed in 1, 3 and 5 min. ST indicates Steinberg’s salt solution. Motile sperm were judged by the waving movement in the undulating membrane or flagellum. +++: All sperm were motile. ++: More than 50% of sperm were motile. +: Less than 50% of sperm were motile. ±: A few sperm were motile. –: No sperm was motile.

Insemination of the egg with dry sperm
The mature eggs were inseminated with dry sperm in the absence of solution. Ten min after the insemination, sperm entry points were observed in 44.3% of eggs (Figs. 1B, 2). At 30 min, the percentage had increased to 90.2%, and the eggs had developed to the four-cell stage in the moist chamber.
Sperm Motility Initiated in Egg-Jelly

Fig. 1. Development of *C. pyrrhogaster* without immersion in any solutions. Eggs were inseminated with dry sperm and cultured in a moist chamber. **A**: An unfertilized egg. **B**: An egg 30 minutes after the insemination. Sperm entry points were seen on the surface. **C**: A 4-cell stage embryo. A view from the animal hemisphere is shown. Bar=0.5 mm.

Fig. 2. The percentage of eggs with sperm entry points of *C. pyrrhogaster* after insemination with dry sperm. Eggs were cultured in a moist chamber and the number of eggs with sperm entry points was counted with binoculars.

within 1 day (Fig. 1C). After the transfer into water, embryos developed normally to the tail-bud stage (Fig. 3). These results indicate that sperm can fertilize eggs in the absence of a change of osmolality.

The observation that dry sperm penetrated directly into egg-jelly suggested that sperm migrated forward by the waving of the undulating membrane in egg-jelly when dry sperm were directly inseminated (Fig. 4). The tail of motile sperm was straight, and the migration speed was 16 to 32 \( \mu \text{m/sec} \) (Table 2).

**Effect of egg-jelly extract on sperm motility**

During fertilization, the first contact between sperm and egg occurs in the egg-jelly. To examine the effect of egg-jelly on sperm motility, dry sperm was treated with egg-jelly extract. 52.3% of total sperm were motile in 1 min (Figs. 5, 6A). The percentage had rapidly increased to 98.5% at 3 min and remained high at 10 min. In Steinberg’s salt solution, 3.52% of total sperm were motile in 1 min, and the percentage did not increase further. This result indicates that jelly extract contains the substances responsible for initiation of sperm motility.

To characterize these substances, jelly extract was boiled for 30 min. 63.0% of total sperm were motile in 1 min (Fig. 6B). The percentage had increased to 98.1% at 3 min and remained high at 10 min. This result indicates that the substances were heat-stable. When jelly extract was treated with trypsin, 2.9% of total sperm were motile in 1 min (Fig. 6B). The percentage did not increase further. In jelly extract prepared in the same manner except for trypsin treatment, 50% of total sperm were motile in 1 min and most sperm were motile by 3 min (Fig. 6B). These results suggest that a proteinacious substance was involved in the initiation of sperm motility. 
molecule is significant for the initiation of sperm motility.

**Estimation of ions in egg-jelly**

Ions in egg-jelly were identified by ICP luminescence analysis. An assay was made for 26 kinds of ions, and sodium, potassium, magnesium, calcium, barium and strontium ions were detected. The former four were major ions, while the latter two were present in very low numbers. In order to estimate the concentrations of these four major cations in egg-jelly, eggs were collected from 11 females and the ion concentrations were independently measured. The concentrations of sodium, potassium, magnesium and calcium ions were approximately 23.83, 2.66, 5.06 and 0.39 mM (Table 3).

**Effect of ions on sperm motility**

Sperm of *C. pyrrhogaster* is inseminated with eggs in the cloaca of the female, where the osmolality around sperm is thought to be similar to that of body fluid and does not change during the process of fertilization. It has been reported that the osmolality is about 200 mOsm/kg in the red spotted newt (Hardy and Dent, 1986). In the present study, dry sperm were treated with solutions containing 100 mM of monovalent ions or 67.5 mM of divalent ions. In sodium-ion solution at pH 7.8, 5.6% of total sperm were motile in 1 min. The percentage had gradually increased to 54.7% by 10 min. In potassium-ion solution, 27.1% of total sperm were motile in 1 min, and the percentage had rapidly increased to 94.1% by 3 min (Fig. 7A). When dry sperm were treated with the sodium-ion solution at pH 8.5, 19.3% of total sperm were motile in 1 min, and the percentage had rapidly increased to 80.1% by 3 min. In potassium-ion solution, 83.8% of total sperm were motile as early as 1 min (Fig. 7B). No sperm was actually motile for 10 min in the solutions of calcium or magnesium ions (Fig. 7), or in those of barium or strontium ions (data not shown) at either pH 7.8 or 8.5. These results suggest that both sodium and potassium ions may independently initiate sperm motility without a change of osmolality.

Next, the dependency on the concentration and pH was examined in these two monovalent cations. In sodium-ion solution, the activity for the initiation of sperm motility was not detected in 1 to 60 mM NaCl at pH 7.8 (Fig. 8A). At pH 8.5, when dry sperm were treated with 60 mM NaCl, 17.8% of total sperm were motile by 3 min (Figs. 7B, 8B). The percentage gradually increased to 80.7% at 10 min. In potassium-ion solution, when dry sperm were treated with 60 mM KCl at pH 7.8, 15.9% of total sperm were motile in 3 min (Fig. 8C), and the percentage gradually increased to 53.5% at 10 min. At pH 8.5, when dry sperm were treated with 60 mM KCl, 18.7% of total sperm were motile in 1 min. This percentage had rapidly increased to 87.2% at 3 min and remained high at 10 min (Fig. 8D). To estimate the effect of chloride ion on sperm motility, choline chloride was used. No sperm was actually motile for 10 min in the solutions of 60 mM choline chloride at pH 7.8 or 8.5 (data not shown). These results indicate that the activity for the initiation of sperm motility was

**Table 2. Speed of motile sperm in egg jelly of *C. pyrrhogaster***

<table>
<thead>
<tr>
<th>Exp.</th>
<th>no. of sperm</th>
<th>Average speed (µm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43</td>
<td>17.52±5.76</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>32.90±9.44</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>16.41±5.62</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>18.66±6.15</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>28.12±5.83</td>
</tr>
</tbody>
</table>
Fig. 5. Observation of sperm of *C. pyrrhogaster* in solution by phase contrast microscope. **A**: Immotile sperm. The undulating membrane was clearly observed (arrow heads). **B**: Motile sperm. The undulating membrane was moving too vigorously to be detected. Arrows indicate the head of sperm. Bar=25 µm.

**Table 3.** Cation concentrations in egg-jelly of *Cynops pyrrhogaster* (mM)

<table>
<thead>
<tr>
<th>experiment</th>
<th>no. of specimen</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>58.81±7.48</td>
<td>7.75±5.58</td>
<td>6.64±0.35</td>
<td>0.19±0.06</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>22.30±1.14</td>
<td>1.36±0.16</td>
<td>5.01±0.57</td>
<td>0.60±0.04</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1.58±0.10</td>
<td>1.87±0.05</td>
<td>4.09±0.02</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>total</td>
<td>11</td>
<td>23.83±6.09</td>
<td>2.66±1.07</td>
<td>5.06±0.40</td>
<td>0.39±0.07</td>
</tr>
</tbody>
</table>

Sixty-one to eighty-six of the mature eggs were obtained from the uterine of each female of *C. pyrrhogaster*. Egg-jellies were independently collected and baked for 10 hr at 70°C. They were dissolved in deionized water of about 7.1 µl/egg, that is the approximate volume of egg-jelly calculated from the diameter. The concentration of each cation was measured by ICP luminescence analysis.
induced only in solutions of sodium or potassium ion at concentrations of greater than 60 mM. Furthermore, this activity was enhanced by high pH.

Calcium ion solution did not, by itself, induce the initiation of sperm motility (Fig. 7). However, when dry sperm were treated with the solution of 60 mM NaCl and 6 mM CaCl\(_2\) at pH 8.5, 85.2\% of total sperm were motile by 3 min (Fig. 9A). The percentage remained high at 10 min. In the solution of 60 mM KCl and 6 mM CaCl\(_2\) at pH 8.5, 52.9\% of total sperm were motile in 1 min (Fig. 9B). This percentage had rapidly increased to 91.8\% by 3 min and remained high at 10 min. These results indicate that, although calcium ion did not in itself have the ability to initiate sperm motility, it strengthened the activity caused by sodium or potassium ion.

**pH in egg-jelly**

Egg-jellies without the immersion of any solution were used. Five mature eggs were collected from the uterus of each of 10 females and the pH of fifty egg-jellies were measured. The mean pH of the egg-jellies was 8.5±0.02.

**Effect of reconstructed ionic solution on sperm motility**

Reconstructed ionic solution (RIS) was prepared according to the concentration of sodium, potassium, calcium and magnesium ions (Table 3) and pH in egg-jelly. When dry sperm was treated with RIS, 20.3\% of total sperm were motile by 1 min (Fig. 10). However, this percentage had not increased at 10 min. The activity was quite low compared to that of jelly extract, suggesting that these ions cannot initiate sperm motility in egg-jelly by themselves.

**DISCUSSION**

Motility is essential to a sperm’s reaching and fertilizing an egg. Factors affecting sperm motility are species specific and are suited to the physiological environment in fertilization. A change of osmolality or of the ion concentration triggers the initiation of sperm motility in lower vertebrates (Morisawa, 1994). In amphibians, it has also been reported that a change of osmolality initiates sperm motility (Inoda and Morisawa, 1987; Hardy and Dent, 1986). However, fertilization is achieved in the cloaca of the female in most urodeles, while the osmolality of the body fluid around the sperm is kept high during fertilization (Horonowski and Armstrong, 1977; Hardy and Dent, 1986; Inoda and Morisawa, 1987). Other factors must be involved in the initiation of sperm motility during fertilization of urodeles.

In this study, sperm were initiated to move in the solution at low osmolality (Table 1). This result indicates that a decrease of osmolality around sperm can initiate sperm motility in *C. pyrrhogaster* as in other amphibians. It has reported that the osmolalities of blood fluid and spermatid fluid were 208 mOsm/kg and 110 mOsm/kg, respectively, in the red spotted newt (Hardy and Dent, 1986). However, sperm were not initiated to move in the solution at either osmolality. This suggests that osmolality is not the main factor regulating sperm motility at the time of insemination in the cloaca.

When the mature egg was inseminated with dry sperm without immersion in any solution, sperm entry points appeared on the surface within 10 min (Figs. 1, 2). Eggs of newt are physiologically polyspermy and several sperm entry points can be seen on the surface of eggs during fertilization; in amphibians, sperm entry points have been detected within 15 min.
after insemination (Chabonneau et al., 1983; Elinson, 1986; Katagiri, 1987; Iwao, 1989; Grandin and Chabonneau, 1992). This result indicates that egg-jelly of *C. pyrrhogaster* contains factors that initiate sperm motility. However, the 4-cell stage embryo did not continue to develop to the tail-bud stage unless immersed in solution. Thus, in the newt, spawning of the fertilized eggs in water is necessary not for fertilization but for subsequent development in the newt.

Egg-jelly is known to regulate sperm motility in some species (Morisawa, 1994). In amphibians, it has been reported that sperm became motile after contacting the egg-jelly in *Discoglossus pictus* (Campanella and Gabbiani, 1979). This findings is similar to those of the present study. In *D. pictus*, fertilization was achieved in water, and a decrease of osmolarity around sperm initiated motility (Talevi, 1989). Thus, the activity of egg-jelly in *D. pictus* may affect the activation of sperm motility in fertilization.

**Substances for the initiation of sperm motility in egg-jelly**

The present study demonstrated that the substances for the initiation of sperm motility exist in the egg-jelly of *C. pyrrhogaster* (Figs. 4, 5). One of the substances was stable under heat treatment and sensitive to trypsin digestion (Fig. 6), suggesting that a proteinaceous factor is involved in the initiation of sperm motility. Non-ionic factors for the regulation of sperm motility have been reported in the sea urchin (Suzuki...
Fig. 9. Co-effect of monovalent cation with calcium ion on sperm motility in *C. pyrrhogaster*. Dry sperm was added in the solutions of NaCl (A) or KCl (B) containing 6 mM CaCl$_2$ with 10 mM Tris-HCl at pH 8.5, and sperm motility was observed with a phase contrast microscope. The concentration of monovalent cation in each test solution was 60 mM (solid circle), 30 mM (open circle), 10 mM (solid square) or 1 mM (open square). Most sperm were motile in three minutes after the treatment of 60 mM NaCl, 6 mM CaCl$_2$ (A) or KCl, 6 mM CaCl$_2$ (B).

Fig. 10. Effect of reconstructed ionic solution on sperm motility in *C. pyrrhogaster*. Reconstructed ionic solution (RIS) was prepared according to the ion concentration in egg-jelly shown in Table 3. Dry sperm was added in RIS, and sperm motility was observed with a phase contrast microscope. In RIS (open circle), some sperm were motile in one minute but the percentage of motile sperm remained low at ten minutes. Open and solid squares indicate JE and ST.

et al., 1981; Garbers et al., 1982; Suzuki, 1990), the Horse-shoe crab (Clapper and Brown, 1980a, b; Clapper and Epel, 1982a, b), Ciona (Yoshida et al., 1993; Yoshida et al., 1994) and the Herring (Morisawa et al., 1992; Oda et al., 1994; Pillai et al., 1993). We recently identified a molecule of 50 kDa in molecular weight in jelly extract as an active substance for the initiation of sperm motility (Mizuno et al., submitted).

Sodium or potassium ion independently showed the ability to initiate sperm motility (Fig. 7). In salmonids, a decrease in the concentration of potassium ion has been shown to initiate sperm motility (Morisawa, 1994; Morisawa and Suzuki, 1980). This is unlikely to be the case in the newt, *C. pyrrhogaster*, because the decrease in ion concentration does not occur in time for the fertilization. In this study, the activity for the initiation of sperm motility was low in both the monovalent cations compared to the jelly extract (Figs. 6A, 7A, C). Furthermore, the activity was actually detected in the sodium and potassium ion solutions of more than 60 mM in concentration (Fig. 8), while the concentration of sodium and potassium ions in egg-jelly was approximately 23.28 mM and 2.66 mM respectively (Table 3). These results indicate that although monovalent cations have the ability to initiate sperm motility, they cannot act as the main factor to induce sperm motility during egg insemination. It has been reported that external potassium ion maintains or activates sperm motility in some species (Morisawa, 1983; Mita and Yasumasu, 1984; Kobayashi, 1993). Monovalent cations may therefore cooperate with other substances in the initiation of sperm motility in egg-jelly.

Calcium ion is known to be essential for sperm motility. In many species, the influx of calcium ion into sperm is necessary for the initiation and the activation of sperm motility (Morisawa, 1994). In this study, 5.06 mM of calcium ion was detected in egg-jelly (Table 3). Although no activity for the initiation of sperm motility was observed by calcium ion alone (Fig. 7), 6 mM calcium ion strengthened the activity for the...
initiation of sperm motility of sodium or potassium ion (Fig. 9). These results suggest that calcium ion may co-operate with monovalent cations to induce sperm motility in egg-jelly of *C. pyrrhogaster*.

The activity for the initiation of sperm motility was stronger in the solutions of monovalent cations at pH 8.5 than pH 7.8 (Fig. 7), while it was not observed in the solutions of sodium ion at 240 mOsm/kg or 120 mOsm/kg (Table 1). The pH of those solutions was below 7.0. These results suggest that sperm motility is sensitive to higher pH, and are thus incompatible with a previous report that the external pH does not affect the initiation of sperm motility in *Notophthalmus viridescens* (Hardy and Dent, 1986). However, these previous experiments investigated pH change over a range of pH 6.5 to 7.4, while in the present study, a decrease in osmolality, rather than a change in pH, was thought to affect the initiation of sperm motility at the low pH range. The egg-jelly of *C. pyrrhogaster* was at pH 8.5, indicating that egg-jelly is in a suitable pH range for sperm to initiate motility.

In reconstructed ionic solution that was prepared according to the concentration of four major cations and pH in egg-jelly, the activity for the initiation of sperm motility was detected but was weak compared to that in jelly extract (Fig. 10). This result suggests that these ions are effective but not sufficient for the initiation of sperm motility in egg-jelly; this would in turn suggest that the proteinaceous factor in jelly extract and the cations in egg-jelly may cooperate to initiate sperm motility in egg-jelly of *C. pyrrhogaster* during fertilization.

In *C. pyrrhogaster*, a female picks up a spermatophore and the sperm in it were stored in the sperm reservoirs (Tsutsui, 1931). Fertilization is achieved in the cloaca of the female with the stored sperm. Several regulating factors are involved in the initiation of sperm motility. One acts as the sperm moves to spermatheca in the cloaca of the female. At that time, sperm may be initiated to move probably by the decrease of osmolality, because the spermatophore is immersed in water. Others act without the change of osmolality as the sperm touches the egg-jelly. In order to inseminate sperm with an egg, sperm must be transported to the egg surface from spermatheca. Probably, the myoepithelial cells around spermatheca contract and push the sperm out to the eggs (Dent, 1970).

In this study, motile sperm migrated in egg-jelly at a speed of 16 to 32 µm/sec (Fig. 4, Table 2). It has been reported that it takes 5 minutes for the female of *C. pyrrhogaster* to spawn an egg (Street, 1940). This is enough time for motile sperm to reach the vitelline envelope and achieve fertilization before spawning, since the egg-jelly of *C. pyrrhogaster* is approximately 500 µm thick. Therefore, *C. pyrrhogaster* is suitable for the investigation of not only the initiation of sperm motility but the fertilization process in egg-jelly of amphibians.

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