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[Short Communication]

Rapid Oocyte Growth and Artificial Fertilization of the Larvaceans *Oikopleura dioica* and *Oikopleura longicauda*

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ABSTRACT—We describe here rapid gamete growth and artificial fertilization method of species of the larvaceans (appendicularians), *Oikopleura dioica* and *O. longicauda* (Family Oikopleuridae: Class Appendicularia: Subphylum Urochordata). In these species, oocytes grew very rapidly from about 40 μm in diameter to about 75 μm (*O. dioica*) and 110 μm (*O. longicauda*), respectively within a few hr. Moreover, cutting off the gonads at the last phase of the growth stage yielded matured gametes. The eggs and sperm obtained by the dissection of gonads could be fertilized when they were mixed together.

INTRODUCTION

The subphylum Urochordata (Tunicata) is, in general, subdivided into three major classes, Ascidiacea, Larvacea (= Appendicularia), and Thaliacea (including salps, doliolids and pyrosomes). Larvaceans comprise a group of diverse pelagic and planktonic species without exhibiting extensive morphogenic processes evident in some other tunicate species. Namely, while ascidians with indirect development and thaliaceans resorb their larval tail at the time of metamorphosis, larvaceans retain their tail throughout their entire life. These tails contain a notochord, a nerve cord, and muscle cells. Besides these features, larvaceans also develop other features of chordates; such as an endostyle and a pair of pharyngeal gill pores, although they lack the atrial cavity (e.g., Alldredge, 1976).

At present, our embryological understanding of larvaceans is very limited compared to other chordates, for example, ascidians (e.g., Satoh *et al*., 1996), vertebrates (e.g., Slack, 1991), or even cephalochordates (e.g., Holland *et al*., 1994), where information concerning their development is dramatically expanding. There are reports on the embryological development of two larvacean species, *Oikopleura dioica* and *O. longicauda* (Delsman, 1910, 1912; Galt, 1972; Fenaux, 1976; Fenaux and Gorsky, 1983). The latest illustration on their development is a review by Galt and Fenaux (1990).

To begin molecular developmental studies of larvaceans, it is necessary to establish the method to collect mature gametes and to inseminate artificially. Here we describe the rapid growth of oocytes, and an easy method of artificial insemination of two species of *Oikopleura*. First we observed that their oocytes grew in a few hr. When eggs and sperm were dissected at the last stage of the gamete growth and then mixed together, fertilization occurred at high frequency, and they developed into embryos, larvae, and swimming juveniles.

MATERIALS AND METHODS

Collection of *Oikopleura* was performed from October 1996 to April 1997 in Moroiso Bay, Misaki, Miura-peninsula, Kanagawa, Japan. In early morning, at about 6 to 8 o'clock, a plankton net (148 × 156 mesh/inch, aperture size 100 μm) was immersed into the ocean at about 15 m beneath the sea surface and then slowly pulled vertically to the surface. Samples were brought back to Misaki Marine Biological Station, University of Tokyo, and *Oikopleura dioica* and *O. longicauda* were isolated from the plankton sample. To identify these species, we consulted the description of Bückmann and Kapp (1975) and an illustration published by Shiga (1997). Thereafter, *Oikopleura* adults were individually placed into wells of a 24-well microtiter plate to prevent accidental fertilization. All culture procedures were performed at 18°C. Adults were cultured in Millipore-filtered seawater or boiled-filtered seawater (called together as filtered seawater, FSW) containing streptomycin (100 mg/L) and penicillin (10000 IU/L).

We observed embryos and larvae using a Nomarski differential interference contrast optics microscopy (Nikon OPTIPHOT, Tokyo, Japan), and adults were observed using a binocular microscope (SMZ-2T, Nikon, Tokyo, Japan). Living adults were sometimes anesthetized using menthol-containing (less than 50 μg/ml) seawater in order to take pictures. Washing the anesthetized adults several times with FSW allowed them to recover their swimming behaviour and gamete maturation.

To examine gonads histologically at various stages of growth, adult specimens were fixed individually using Bouin's fixative for 2 hr

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at room temperature. Before fixation, each adult was photographed using a binocular microscope to identify it and to show the appearance of the living gonad. After fixation, the specimens were dehydrated using an ethanol series, embedded in paraffin, cut into 5 μm sections, and stained using hematoxylin and eosin.

RESULTS AND DISCUSSION

Oikopleura dioica and *O. longicauda* were common in Moroiso Bay, Misaki. *O. longicauda* is a hermaphroditic species, and *O. dioica* is a dioecious larvacean (e.g., Alldredge, 1976).

Adults of *O. dioica* and *O. longicauda* were collected at various stages of maturation. Figure 1A shows the gonad which had enlarged in size completely, although the gametes were not yet matured. At that time, the ovary looked gray and the testis looked brown under the binocular (Fig. 2A, D, G). If the testis was cut out at that time, the dissected testis was very hard and swimming sperm were not released and appeared as a solid lump of cells. When the ovary was dissected, the ovarian component cells were very sticky with each other, and it was impossible to divide them individually.

Nevertheless, when we left the adults in each well of a microtiter plate under the room temperature (18°C), a part of them had matured gametes. In *O. longicauda*, at first, the testis looked brown and transparent, whereas the ovary looked gray. In a very short time, within a few hr, they changed drastically, as shown in Fig. 1. The color of the testis changed from brown to black and opaque. On the other hand, in the ovary, the oocytes obviously became larger with distinct boundaries. Figure 2 shows sections of the gonads stained using hematoxylin and eosin, which were fixed at the time corresponding to each growing stage. Firstly, oocyte growth became larger from about 40 μm in diameter to about 110 μm in *O. longicauda* (Fig. 2A-C), and from about 40 μm to about 75 μm in *O. dioica* (Fig. 2D-F). Secondly, accessary cells (usually regarded as nourishment cells, the diameter was about 20 μm), that filled almost all part of the gonad at first, became less and less in number as the oocyte growth progressed, and finally they disappeared completely (Fig. 2A-F). Any germinal vesicle-like structures were not observed at any stages of the oocyte growth (Fig. 2A-F). In the testes, the punctate staining with hematoxylin of sperm nuclei became more distinct (Fig. 2G-I)

At the last stage of maturation (Fig. 2C, F, I), after the adult was put on a glass slide with a drop of FSW, its gonad could be dissected using a tungsten needle, and the gametes

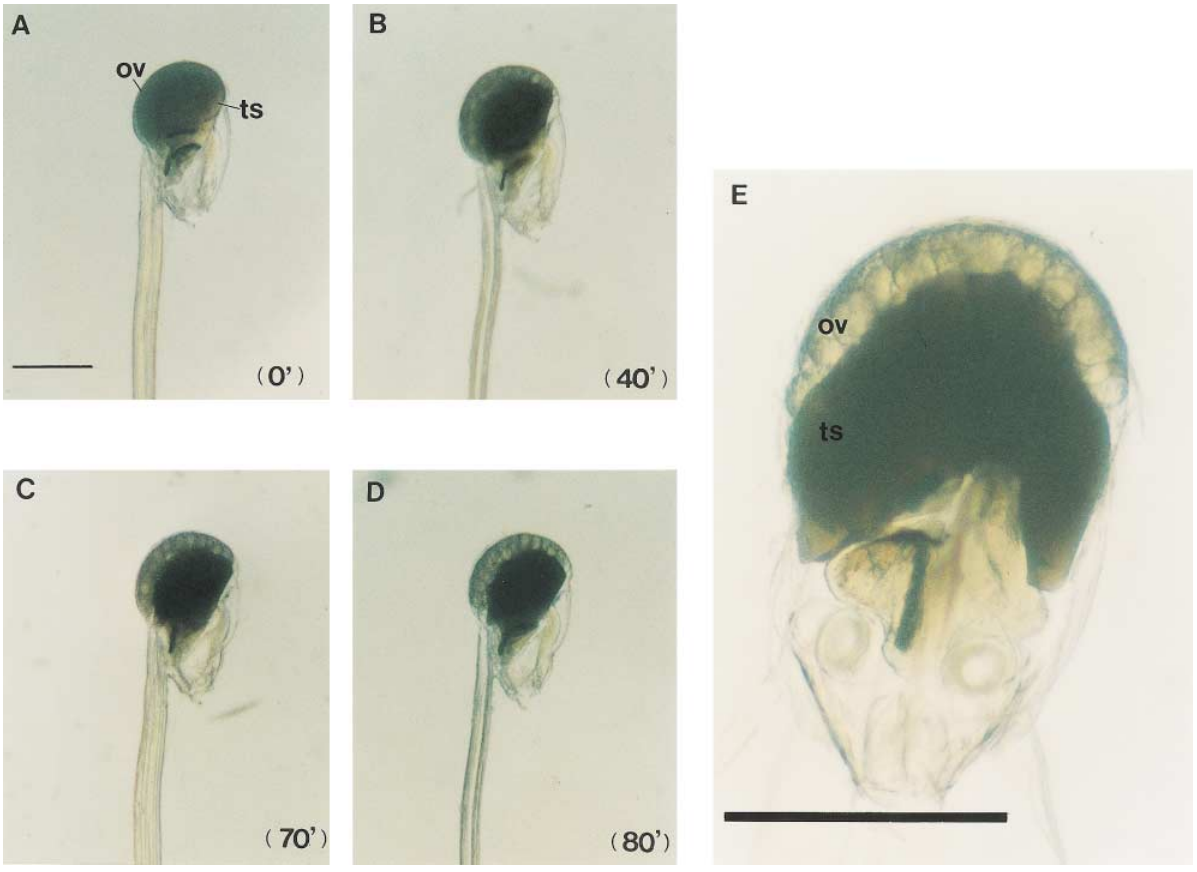

Fig. 1. Maturation of the gonad in *Oikopleura longicauda*. (**A**) Lateral view of an adult immediately after sorting of specimens, about 3 hr after collection from the ocean. Defining the time when this picture was taken as time zero, as indicated in a parenthesis. (**B-D**) Lateral view of the same adult, about 40 min (**B**), about 70 min (**C**), about 80 min (**D**). (**E**) Higher magnification of the trunk region of **D**. Oocytes in the ovary become larger and the color of the testis become darker, as the time passes. ov, ovary; ts, testis. Scale bars represent 500 μm for all photographs.

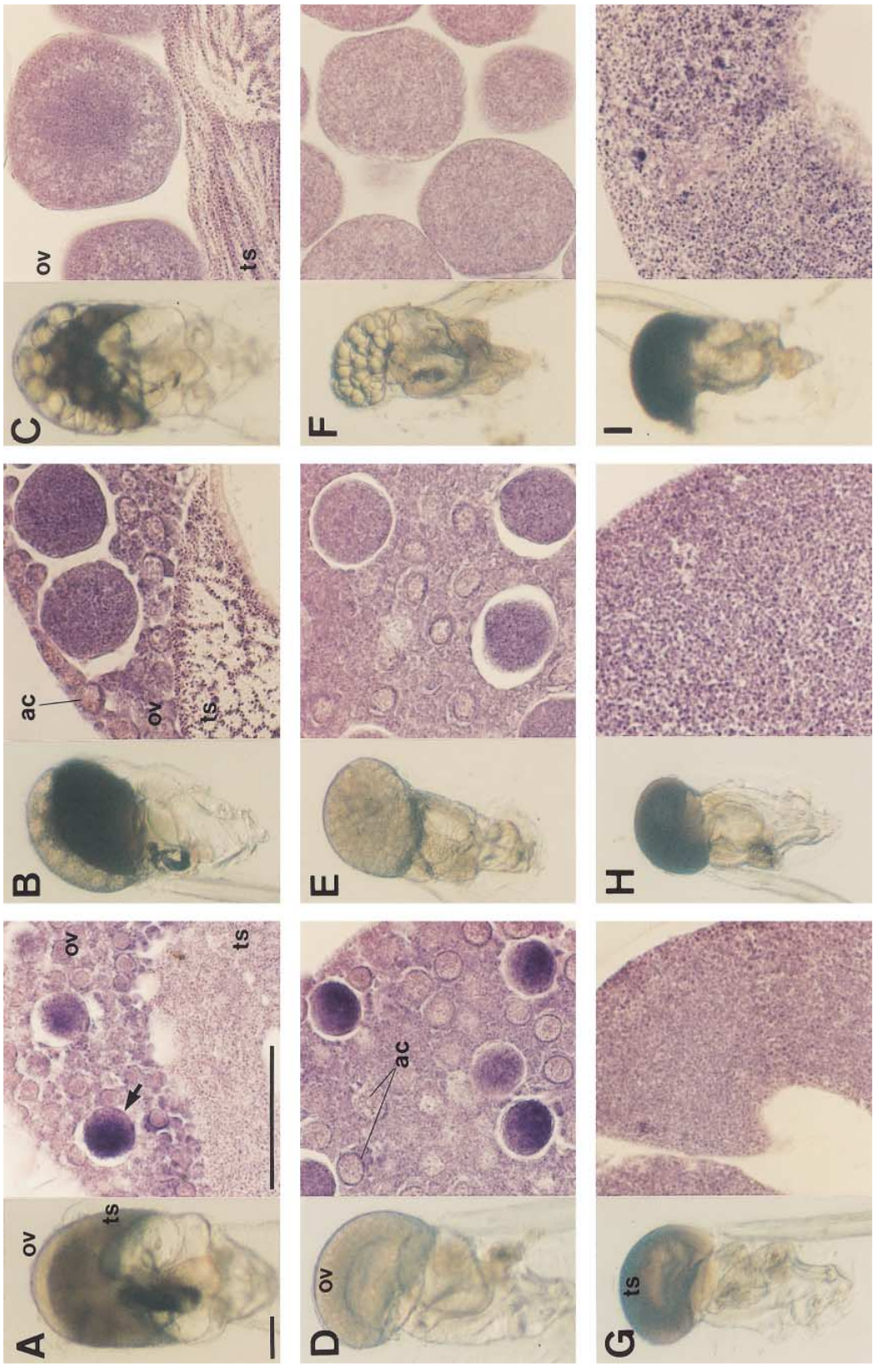

Fig. 2. Sections of the gonad of *Oikopleura dioica* and *O. longicauda*. Pictures of each adult were taken under the binocular microscope after fixation (left column of **A-I**). Thereafter each gonad was sectioned and observed (right column of **A-I**). (**A-C**) The gonad of *O. longicauda* at (**A**) early growing stage, (**B**) middle growing stage, and (**C**) late growing stage. One of the oocytes is indicated by an arrow. (**D-F**) Ovaries of *O. dioica* at (**D**) early growing stage, (**E**) middle growing stage, and (**F**) late growing stage. (**G-I**) Testes of *O. dioica* at (**G**) early maturation stage, (**H**) middle maturation stage, and (**I**) late maturation stage. ov, ovary; ts, testis; ac, accessory cells. Scale bars represent 100 µm for all photographs.

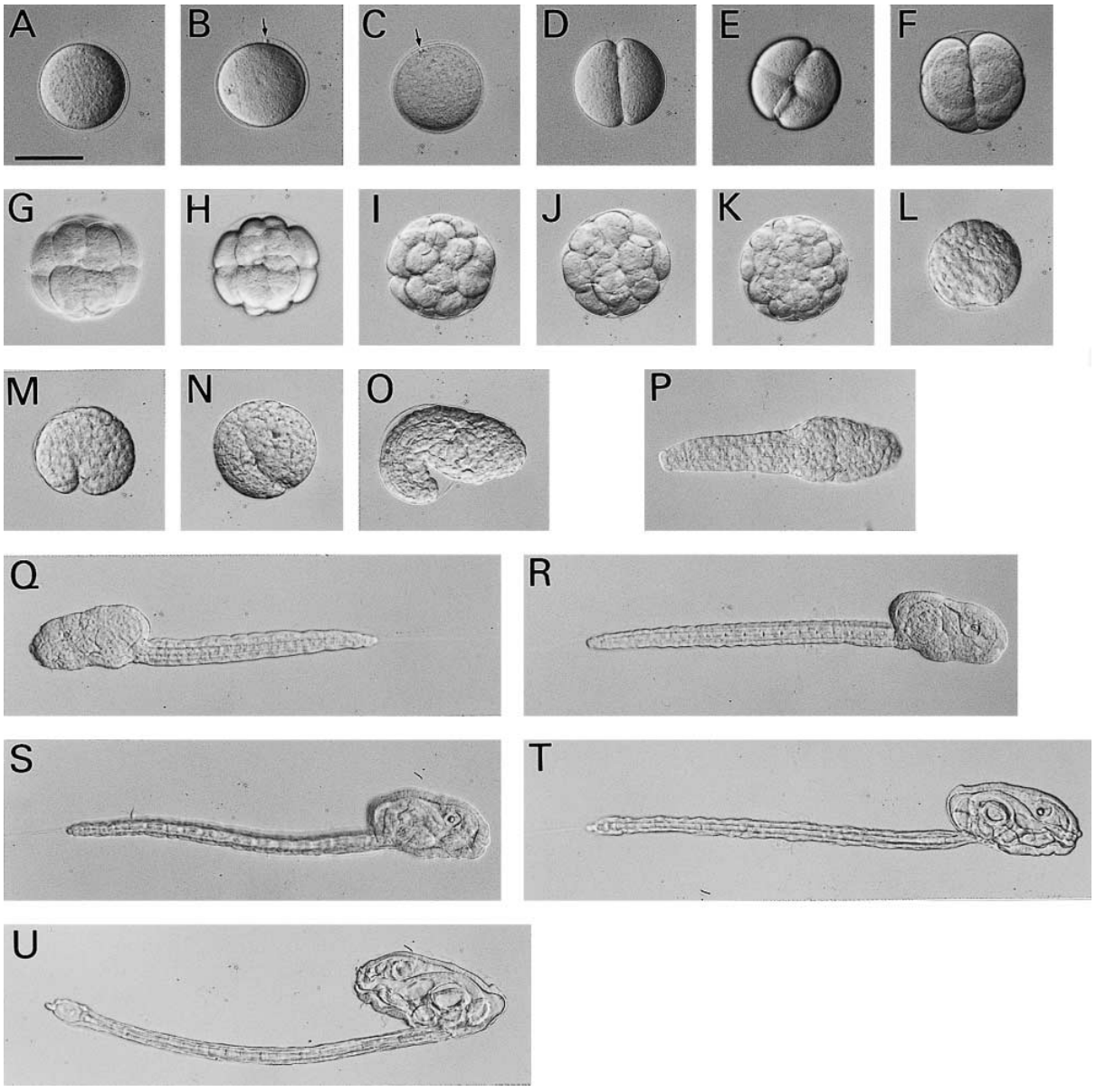

Fig. 3. Embryogenesis of *Oikopleura dioica* at 18°C. (**A**) A fertilized egg, immediately after fertilization. (**B**) An egg with first polar body (an arrow), 5 min after fertilization. (**C**) An egg with second polar body (arrow indicates polar bodies), 15 min after fertilization. (**D**) The 2-cell stage, about 25 min after insemination. (**E**) The 4-cell stage, about 35 min after insemination. (**F**) The 8-cell stage, about 50 min after insemination. (**G, H**) The 16-cell stage, viewed from the animal pole (**G**) and the vegetal pole (**H**), about 1.1 hr after insemination. (**I**) Blastula, about 1.5 hr after insemination. (**J**) Gastrula, about 1.8 hr after insemination. (**K**) An embryo at the stage between gastrulation and tailbud formation. The cells became smaller in size and increase in number. (**L**) The early tailbud stage, about 2.8 hr after insemination. (**M**) The middle tailbud stage, about 3.5 hr after insemination. (**N**) An embryo immediately before hatching, about 3.8 hr after insemination. (**O**) A hatching larva, about 4 hr after insemination. (**P**) A hatched larva, about 4 hr after insemination. (**Q**) A larva at about 6.5 hr after insemination, viewed from left side. (**R**) A larva at about 7.5 hr after insemination, viewed from right side. (**S**) A larva at about 9 hr after insemination. (**T**) A larva before metamorphosis at about 11.5 hr after insemination. (**U**) A juvenile after metamorphosis at about 15 hr after insemination. Scale bar represents 50 μm for all photographs.

spread out easily. Since *O. longicauda* is a hermaphrodite, the eggs and sperm were mixed each other in the drop after dissection of the gonad. However, when eggs were taken from a drop soon after the gonad dissection and then washed by FSW for several times, most of them could not be self-fertilized. Since *O. dioica* is dioecious, the eggs and sperm were separately obtained. If sperm were kept in tubes coated by 1% bovine serum albumin to prevent sticking of sperm to the wall of the tubes, and eggs in dishes after washed for several times by FSW containing the antimicrobials, at 12°C, then the gametes were fertilizable at least until 6 hr after dissection.

When eggs and sperm were mixed together, fertilization took place. The developmental stages are indicated in Fig. 3 (and see Galt and Fenaux, 1990). The fertilization occurred in high frequency as far as we got the fully matured gametes. We succeeded to inseminate more than 50 batches totally.

The condition of gametes was often easy to define; when the gametes from the dissected gonads were sticky or irregular in shape or size, they appeared to be immature and fail to develop normally.

There have been several descriptions of immatured oocytes in larvaceans including *O. dioica* and *O. longicauda* (Fenaux, 1963; Last, 1972). But they did not describe the rapid oocyte growth as described here. Galt (1972, 1987) and Holland *et al*. (1988) reported methods to inseminate *O. dioica* eggs. For example, Holland *et al*. (1988) utilized the cultivation system developed by Gorsky *et al*. (1986). They used naturally spawned eggs and sperm, or sperm which were prepared by putting matured males into seawater cooled to 4°C to rupture the testis wall. Their method is closer to the natural condition than ours, because we dissected the gonad. We waited for collected individuals to spawn naturally at first, but we often experienced that the adults died with fully matured eggs before they spawned, or the eggs themselves became over-matured. Therefore, we decided to cut out the gonad. For *O. longicauda*, if the adults were made spawn naturally, it was difficult, in reality, to separate the eggs from the sperm as early after the spawning as possible in order to prevent the self-fertilization. This time we chose a way to collect fresh specimens from the ocean instead of utilizing the culture system, and fully grown adults could be obtained almost everyday. In the vicinity of Misaki Marine Biological Station, we could collect species other than *O. dioica* and *O. longicauda*, for example *O. cophocerca* and *Fritillaria pellucida*. We succeeded in an artificial insemination of *O. cophocerca* using the same method as we described here. In *F. pellucida*, however, even if we dissected the ovary after confirming their full growth, we always found a large and remarkable germinal vesicle in each oocyte. When we left them in filtered seawater, for a while, the germinal vesicle broke down automatically (data not shown). However, the eggs were not activated by the motile sperm, and thus we did not succeed in artificial fertilization.

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