A precise comparison of developmental series of oocyte growth and oocyte maturation between real-oocytes and pseudo-oocytes in the coral *Galaxea fascicularis*

Ryota SUWA¹,²,* and Masaru NAKAMURA³,⁴

¹ Seto Marine Biological Laboratory, Field Science Education and Research Center, Kyoto University, 459 Shirahama, Wakayama 649–2211, Japan  
² Present address: Okinawa Institute of Science and Technology Graduate University, Tancha 1919–1, Onna-son, Okinawa, 904–0485 Japan  
³ Tropical Biosphere Research Center, University of the Ryukyu, Sesoko 3422, Motobu, Okinawa 905–0227, Japan  
⁴ Present address: General Research Center, Churashima Foundation, Ishikawa 888, Motobu-cho, Kunigami-gun, Okinawa 905–0206, Japan

* Corresponding author: Ryota Suwa  
E-mail: ryota@zenno.jp

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Abstract In this study, we histologically investigated the late stage of oogenesis, spermatogenesis, and pseudo-egg development processes of the pseudo-dioecious coral *Galaxea fascicularis* to form a base from which to elucidate the mechanisms of coral sexual reproduction. This coral has a large egg size and individual polyps, which are suitable for studying the mechanism of sexual reproduction. The histological analysis revealed that germinal vesicle breakdown of this coral begins 3 days before spawning in both female oocytes and male pseudo-oocytes. Spermatogonium, spermatocytes, spermatids, and sperm were observed in the male testicular lobules from 2 months to 1 day before spawning. The findings of our study might allow for the elucidation of the mechanisms of sexual reproduction in this species and facilitate the development of methods for its aquaculture.

Keywords *Galaxea fascicularis*, sexual reproduction, coral spawning, gametogenesis, Germinal vesicle breakdown

Introduction

Scleractinian corals include both hermaphroditic and gonochoric species. Each group of corals consists of broadcast spawners that males and females simultaneously release their gametes and brooders that develop larvae in their body. Most coral species are broadcast spawners and produce gametes every year (Harrison and Wallace 1990). *Galaxea fascicularis* (Linnaeus 1767) is a common coral in the Indo-Pacific Ocean and is found abundantly in a wide range of habitats (Veron 2000). This species is a broadcast spawner and is known to reproduce annually; it has been observed to spawn gametes 5 to 9 days after the full moon in summer in Okinawa, Japan (Heyward et al. 1987). The large egg size and individual polyps are suitable for elucidating the mechanism of sexual reproduction in this coral. Female colonies of this species have been proposed to spawn bundles of red eggs, whereas hermaphroditic individuals release bundles containing...
white eggs with sperm (Babcock et al. 1986, Heyward et al. 1987). This sexual pattern is known for gynodioecy, and gynodioecy in the scleractinian coral Porites astreoides has been reported in Jamaica (Chornesky and Peters 1987). The colonies of this species are either females or hermaphrodites. However, the sexual pattern of G. fascicularis is thought to be gonochoric, because the white eggs of this coral have been shown to be infertile by cross-fertilization experiments (Harrison 1988). The sex ratio of this coral is 1:1, and no sex change has been reported. Therefore, the sexual pattern and the infertile white eggs of this species are referred to as pseudo-gynodioecy and pseudo-eggs, respectively (Harrison 1988). Yolk proteins related to vitellogenesis have been detected in female eggs but not in pseudo-eggs (Hayakawa et al. 2005, Hayakawa et al. 2006). On the Great Barrier Reef, both eggs and pseudo-eggs of G. fascicularis are known to require 7 or 8 months to undergo gametogenesis. Spermatogenesis in morphologically hermaphroditic male colonies is initiated 1 to 2 months before spawning (Harrison 1985).

Galaxea fascicularis dominantly or predominantly inhabits the Ryukyu Archipelago, Japan. In this study, oogenesis, spermatogenesis, and pseudo-egg development at the late stage of G. fascicularis between 2 months to one day before spawning were investigated histologically to elucidate the late stage of coral gametogenesis and to develop techniques for predicting coral spawning.

Materials and Methods

Colonies of the coral Galaxea fascicularis were collected from southeast Sesoko Island, Okinawa, Japan (26°64′N, 127°87′E) in April 2008. The collected colonies were immediately transferred to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, and placed in outdoor tanks supplied with unfiltered natural seawater. The gametogenesis stage of this species was investigated between May and July 2008 by repeatedly sampling polyps from six tagged colonies. The sex of the gonochoric G. fascicularis colonies was determined by detecting whether sperm were present inside the polyps using a dissecting microscope (MZ12.5; Leica, Germany). Three male and two female colonies spawned on July 24 and one female colony spawned on June 26 and July 25, 2008. Three polyps (height, approx. 2–3 cm) were collected every month between May and July from each colony; newly formed small polyps were not collected. The female colony which spawned twice spawned only small number of eggs on June 26 and its gametogenesis were failed to be detected in the monthly sampling. In addition to the monthly sampling, Three male and two female colonies which spawned on July 24 were sampled 1 week before spawning and on a daily basis from 4 days before to 1 day after spawning. The female colony which spawned on July 25 were sampled 8 days before spawning and on a daily basis from 5 days before to 1 day after spawning. On the day of spawning, daily samples were collected 2 hours before the spawning. The daily sampling enabled us to monitor the late stage of gametogenesis. The sampled polyps were immersed in Bouin’s solution and left at room temperature for fixation and decalcification. After the polyps had decalcified, they were dehydrated in a series of ethanol and xylene and then embedded in paraffin. For histological analysis, 7-μm-thick sections were cut and stained with hematoxylin and eosin according to the standard histological protocols. Gonads and gametes were identified by obtaining photographs of the histological sections using an Olympus BX50 microscope equipped with an Olympus DP70 digital camera (Olympus Japan Co., Tokyo, Japan). The size of nuclei in oocytes/pseudo-oocytes was measured, and the size of gametes was determined from the images using the ImageJ 1.38 program (National Institutes of Health, USA).

Results and Discussion

Two months before spawning, female and morphologically hermaphroditic male colonies of Galaxea fascicularis had oocytes and pseudo-oocytes, respectively, in their gonads. The oocytes and pseudo-oocytes continued to enlarge until spawning, and their areas on histological sections reached their maximum before spawning (Table 1). Lipid droplets, which appear white in the photomicrograph, exhibited the polarized distribution in the oocytes of female colonies 1 month before spawning (Fig. 1a).
Germinal vesicle breakdown (GVBD) in the oocytes were observed with the samples which were collected 3 days before spawning, and the germinal vesicles were lost in the coral polyps which were collected 1 day before spawning (Fig. 1b). Translocation of the nucleolus toward the edge of the oocytes where higher densities of eosin-stained egg yolk were distributed was observed during the GVBD. The layer of follicular-like cells around the oocytes became thinner and was degraded and a polarized-distribution of lipid droplets was lost in the coral polyps which were collected 1 day before spawning (Fig. 1b), but not in the coral polyps of 2 days before spawning. Mature oocytes were formed after GVBD (Fig. 1c). In contrast, in male pseudo-oocytes, there was no polarity of lipid droplets distribution (Fig. 1d); however, fusions between lipid droplets had begun and were observed in pseudo-oocytes of collected samples 4 day before spawning. In pseudo-oocytes, GVBD was also observed at the same time as seen in female oocytes (Fig. 1e). The pseudo-eggs without germinal vesicles were observed in the male polyp of 1 day before spawning. The follicular-like cell layer around the pseudo-eggs was degraded and sperm were observed to be inside the pseudo-eggs in the coral polyps which were collected 2h before spawning, as revealed on histological sections (Fig. 1f). Female colonies spawned bundles of red eggs, whereas male colonies spawned bundles of pseudo-eggs and sperm at 20:00 to 21:00 h. A few small oocytes or pseudo-eggs with irregularly shaped sperm remained in the polyps 1 day after spawning. In addition to oogenesis, spermatogenesis was observed in the pseudo-oocytes of male colonies. At 2 months before spawning, male colonies had spermatogonia in their testicular lobules (Fig. 2a); mitosis of the spermatogonium was observed at this stage (Fig. 2b). Spermatocytes were noted 1 month before spawning (Fig. 2c), and spermatids along testicular with spermatocytes were observed in the lobules 1 week before spawning (Fig. 2d). Sperm and spermatids were observed 4 days before spawning (Fig. 2e). The testis was filled with sperm but no spermatids 1 day before spawning (Fig. 2f). These phenomena were observed in three males and two female colonies which spawned on July 24. One female colony which spawned on July 25 showed same phenomena one day later.

There were distinct morphological differences between female oogenesis and the maturation process of pseudo-eggs. First, polarity-based distribution of lipid droplets was observed in oocytes (Fig. 1a) but not in pseudo-eggs (Fig. 1d). Second, the polarity of lipid droplets distribution was lost after GVBD in oocytes (Fig. 1c), whereas droplets in the pseudo-eggs fused with each other and formed large droplets a few days before spawning (Fig. 1e, f).

The GVBD process in oocytes and pseudo-oocytes was elucidated for the first time in scleractinian corals by examining daily samples of coral polyps until spawning. The process and timing of GVBD were similar in oocytes and pseudo-eggs. The nuclear shift toward the edge of oocytes and pseudo-eggs started 3 days before spawning, and the germinal vesicle was lost 1 day before spawning (Fig 1b, e). This onset of GVBD may be regulated by chemical stimuli or signaling and related to the synchronous spawning of sperm (pseudo-eggs) and eggs. Gametogenesis and mass spawning of the soft coral Sinularia polydactyla are known to be regulated by sex

| Table 1 | The cross-section area of the oocytes and pseudo-oocytes measured from the histological sections. |
|---------|---------------------------------------------------------------------------------------------|
|         | 2 months before spawning | 1 month before spawning | 1 day before spawning |
| Female oocyte | 0.0110 ± 0.0016 | 0.0280 ± 0.0073 | 0.0564 ± 0.0081 |
| Male pseudo-oocyte | 0.0153 ± 0.0090 | 0.0191 ± 0.0184 | 0.0565 ± 0.0086 |

Mean (mm²) ± standard deviation (n=12)
Fig. 1  Histological sections of mature gonads of *Galaxea fascicularis*. a. Female oocytes (o) with germinal vesicles (gv) 1 month before spawning. b. Oocytes of the female colony during germinal vesicle breakdown, 1 day before spawning. c. Mature oocytes (Mo) in the female colony 2 hours before spawning. d. Pseudo-oocytes (Po) with germinal vesicles (Gv) and testicular lobules (Tl) of the male colony 1 month before spawning. e. Male pseudo-oocytes during germinal vesicle breakdown 1 day before spawning, showing fusion of lipid droplets. f. Pseudo-eggs (Pe) 2 hours before spawning with sperm (Sm). Scale bars are 100 μm.
hormones and pheromones (Slattery et al. 1999). The spermatogenesis process was also investigated, and the timing of each testicular developmental stage was elucidated. The testicular developmental stages overlapped at some sampling points, and one (Fig. 2a, c, f) or two (Fig. 2d, e) developmental stages coexisted. These results
provide the basic knowledge for understanding gametogenesis in *G. fascicularis*, and the timing of GVBD and spermatogenesis described in this study might be useful for accurately predicting the date of spawning in this coral species.

GVBD is known to be a morphological indicator of oocyte maturation (Hausen and Riebesell, 1991). A few studies have investigated the GVBD process in brooding coral species (e.g., Okubo et al. 2007; Rinkevich and Loya 1979); however, reports on the timing of its occurrence are lacking. Thus, to our knowledge, this is the first study to determine the precise timing of GVBD occurrence. The investigation of GVBD occurrence before spawning might allow us to predict the occurrence of coral spawning. However, since the preparation of histological sections takes several days, a rapid technique for the observation of GVBD need to be established. Sex determination in intact coral colonies or polyps has yet to be performed. Small oocytes or pseudo-eggs were detected in polyps of *G. fascicularis* 5 months before spawning, but their structures remained similar until female oocytes become red colored a few weeks before spawning (Harrison 1988). However, they can be distinguished from each other in histological sections because the ooplasm of a female oocyte is eosin positive, whereas pseudo-eggs are eosin negative. In male colonies, sperm were detected 2 months before spawning. The morphological differences described in this study might be useful for sex determination in *G. fascicularis* coral colonies.

It is suggested that the functional significance of pseudo-eggs in coral spawning is to lower the predation pressure on fertile eggs and make sperm buoyant, thereby increasing their fertilization efficiency on the sea surface (Harrison 1988). The lipid is shown in white in the histological sections, and thus pseudo-eggs may include more amount of lipid than oocytes.

Higher lipid content may give greater buoyancy to pseudo-eggs than female eggs and this may also increase their fertilization efficiency by spreading sperms on sea surface before female eggs arrive. For understanding this mechanism, a difference of the time required for egg and sperm (pseudo-egg) to arrive on sea surface should be elucidated. The “pseudo-dioecious” breeding system of *G. fascicularis* might have evolved from hermaphroditic sexual patterns, because the release of a few fertile red eggs along with pseudo-eggs has been reported in the Great Barrier Reef (Harrison 1988). Furthermore, *G. fascicularis* populations in Penghu Is., Taiwan have been reported to be composed of female and hermaphroditic colonies (Keshavmurthy et al. 2012). Functions associated with the fertilization of eggs, including the vitellogenesis-related female yolk protein production of *G. fascicularis* (Hayakawa et al. 2005), are thought to have been lost during the course of evolution. Investigation of the lost molecular functions of the male pseudo-eggs of *G. fascicularis* might enhance our understanding of the evolutionary history of reproduction. The presence of estradiol-17β (Atkinson and Atkinson 1999) and aromatase activity (Twan et al. 2006), and the recognition of immunoreactive gonadotropin-releasing hormone by antiserum against salmon GnRH (Twan et al. 2006) and of early germ cell development by an antibody to the germ cell marker gene *vasa* (Shikina et al. 2012), have been reported in the scleractinian coral *Euphyllia ancora*. However, unlike in other animals, the mechanism of sexual reproduction in corals has yet to be clarified. The detailed morphological characteristics and timing of gametogenesis in *G. fascicularis* elucidated in this study potentially provide useful information for further research on coral reproduction.

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