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綠島(台灣)星野黑皮海綿(*Terpios hoshinota*)之有性生殖研究及野外族群幼生排放之觀察

Sexual reproduction of the black sponge, *Terpios hoshinota*, and *in situ* observation of its larval release in Green Island, Taiwan

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中文摘要

在 2006 年台灣珊瑚總體檢時，發現了以往未曾被注意到的現象：在綠島及蘭嶼，一種黑色的海綿覆蓋了多種石珊瑚，鑑定結果發現，是一種有藍菌共生的海綿 *Terpios hoshinota*（星野黑皮海綿），其擁有生長快速的特性，且能覆蓋跨種類的石珊瑚，嚴重降低當地石珊瑚的覆蓋率及生物多樣性。雖然早在 1973 年就有星野黑皮海綿在關島大爆發的報導及初步的研究，但直到現在，對於其族群量遽增的原因及其族群動態仍不了解。此篇研究針對在綠島的星野黑皮海綿族群，以標記特定群體(colony)進行密集採樣(2011 年 6 月 16 日到 8 月 17 日，2-3 天採樣一次)，再以組織切片染色法探討其配子發育及胚胎發育；並每 2-3 天觀察紀錄 25 個群體幼生排放的情況。

組織切片的結果顯示：(1)星野黑皮海綿的精發育時間很短，小於 8 天，精子的釋放有半月周期存在，時間與滿月及新月的時間相同，(2)卵(< 40 μm)及早期胚胎在各個時間點都有被觀察到，顯示卵持續被製造出來，(3)成熟胚胎(length: ~400 μm, width 285 μm)出現在滿月及新月附近，且在組織切片中發現成熟胚胎的時間點與野外觀察到的幼生排放時間點相符合；可能是受到精子排放的半月週期影響，導致胚胎成熟及排放的時間近似半月週期。

從組織切片的結果，推測星野黑皮海綿的胚胎發育過程可分為三個階段：(1)在一個由扁平細胞(pinacocytes)包圍的腔室中，由成體提供的共生藍菌及海綿細胞逐漸累積；早期胚胎並非位於此腔室中，而是在此腔室之外進行卵裂，(2)卵裂進行到某個階段後，胚胎進入扁平細胞包圍的腔室中，繼續分裂增生，(3) 最後胚胎細胞包圍在整團細胞(大部分是藍菌)的最外層，完成胚胎發育。此種胚胎發育過程相當獨特，未曾有研究指出類似的胚胎發育過程，將來可對星野黑皮海綿的胚胎做更進一步的研究，提供更多關於海綿胚胎發育的資訊。在成體海綿體內共生的藍菌也出現在胚胎的中央空腔內及幼生體內，顯示星野黑皮海綿在胚胎發育過程中獲得和親代相同的共生菌。

星野黑皮海綿的幼生排放後會附著於海綿表面，在野外可直接用肉眼觀察，經過兩個月的野外觀察(每兩到三天觀察一次)，發現在六月中、七月初、七月中及八月中有大規模的幼生釋放，此四次幼生釋放事件為整個公館港的族群同時進

行，一次釋放持續約八天，中間日為釋放的高峰；每株海綿排放幼生的數量及面積差異很大，但同一個群體的幼生是同時釋放，亦即幼生排放事件在單一群體內及整個族群都是同時發生;其中七月十五日在 25 株海綿中有 24 株排放幼生(96%)。

關鍵字：星野黑皮海綿、綠島、有性生殖、配子發育、胚胎發育、幼生釋放、共生菌

ABSTRACT

Outbreaks of the black cyanobacteriosponge, *Terpios hoshinota* have been reported in Green Island and Orchid Island, Taiwan since 2006. Because *T. hoshinota* grows fast and has the ability to kill and overgrow scleractinian corals, the outbreak of *T. hoshinota* results in serious decrease of coral coverage and biodiversity. Although the outbreak of *T. hoshinota* has also been reported in Guam and Ryukyu Archipelago (Japan) since 1970s, little is known about its life history and ecology of *T. hoshinota*, which are essential to understand its outbreak mechanisms and population dynamics after the outbreak. Accordingly, in this study, some reproductive information of *T. hoshinota* (gametogenesis, embryogenesis and larval release pattern) was examined in Green Island, Taiwan. Intensive sampling and field observation of 2-3 day intervals were performed on 15 tagged colonies as well as haphazardly chosen colonies for 2 months (June 16-August 17) in 2011.

Histological observations revealed some patterns in sexual reproduction of *T. hoshinota*: (1) spermatogenesis is very short (< 8 days); sperm was released on a semi-lunar cycle around the full moon and new moon, (2) oocytes (< 40 μm) and early embryos were always presented, and (3) mature embryos (length: ~400 μm, width 285 μm) were presented only around the full moon and new moon. The semi-lunar cycle of embryo maturation is also coincided well with larval release timing observed in the field (around the full moon and new moon). One larval release event continued for ~8 days. The semi-lunar cycle of embryo maturation and larval release may be determined by the semi-lunar cycle of sperm-release. Embryogenesis of *T. hoshinota* was unique and never been reported: (1) embryo at morula stage developed outside the embryo-nursing chamber, which consisted of a single layer of pinacocytes and contained numerous cyanobacteria and maternal sponge cells transferred gradually from adult, (2) and then embryo penetrated into the nursing chamber and underwent blastulation, (3) finally, embryonic cells migrated to the periphery of the nursing chamber, packed all of the cyanobacteria and maternal sponge cells inside, and formed a compact single outer layer to become a mature embryo. Symbiotic cyanobacteria were not found in oocyte, but numerously in central cavity of mature embryo and larvae, suggesting vertical transmission of the symbionts from mother sponges to larvae during embryogenesis.

Key words: *Terpios hoshinota,* Green Island, sexual reproduction, gametogenesis, embryogenesis, larval release, symbiotic cyanobacteria

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Introduction

Sponges

The sponges are the most ancient animals on earth which presented 580 million years ago. There are more than 8000 described species of sponges (Maldonado & Riesgo, 2008). The sponges can be divided into two subphyla: Symplasma (class Hexactinellida), characterized by the presence of syncytia, and Cellularia (class Calcarea and class Demospongiae), characterized by having discrete cells (Maldonado & Bergquist, 2002). In class Calcarea, their spicules are made of calcium carbonate; in class Demospongiae and subphylum Symplasma, their skeletons are made of silicon dioxide spicules and collagenous spongin.

Sponges are sessile, suspension feeders that consume bacteria and other food particles (Wilkinson et al., 1984), thus they are thought to be important to keep water clean in coral reef waters. The shapes of solitary sponges are like a cup or a cylinder, while the shapes of colonial sponges are encrusting or massive. The sponge body can be subdivided into two general areas: the ectosome and the choanosome. The ectosome encompasses the peripheral regions of the sponge. The outermost layer of tissue, exopinacoderm, is composed of thin, flattened pinacocytes and encloses the subdermal spaces. The choanosome, which encompasses the inner regions of the sponge, includes choanocyte chambers, complex aquiferous canals and mesohyl (Smith, 1988). With many small openings (ostia) and one (or more in colonial sponge) big osculum, water flows through the complex canals inside a sponge efficiently by Bernoulli's principle (Barnes et al., 2001).

Although without true tissues and organs, cells with different morphologies and functions were found in sponges (Maldonado & Riesgo, 2008). Choanocytes have a single flagellum which is surrounded by a collar of microvilli. The flagellum of choanocytes beats and improves water flow. A group of choanocytes form the choano chamber, the basic unit for sponge to obtain food particles. Archaeocytes are highly motile and totipotent amoebocytes, which can de-differentiate and transform into other types of sponge cells. Pinacocytes are flat cells which form the outer layer of sponge.

Sponges are good materials to study cell aggregation because they are the simplest multicellular organisms and their cells can be easily dissociated into single cells which rapidly aggregation into reconstructed functional organisms (Muller WEG & Muller I, 1980; Muller WEG, 2003). In *Geodia cydonium*, the model system of cell aggregation was established from laboratory experiments by Muller. A series of macromolecules, like aggregation factor, aggregation receptor and anti-aggregation receptor etc., are known which are involved in the control of aggregation and separation of sponge cells. The mechanisms are controlled by an assembly of interacting macromolecules: first, located on the cell surface, second, spanning the cell membrane as a bridge between receptor proteins on neighboring cells and third associated with sub-membraneous structures. When dissociated single cells from different species cultured together, they can undergo species-specific reaggregation under certain condition (John et al., 1971). The experimental analysis of cell aggregation can apply insights to understand specific mechanisms of cell movement, coordinate cell division and cell-cell interactions which ultimately result in differential gene expression.

Bacteria, cyanobacteria, dinoflagellate, yeast and even archaea have been found in sponges, intracellularly or, mostly, intercellularly, and some of them are thought to be specific symbionts and have important roles in primary production, nitrogen fixation

and providing antimicrobial substances (Arillo et al., 1993; Corredor et al., 1988; Fieseler et al., 2001; Maldonado, 2007; Muller, 2003; Schmitt et al., 2007)Symbionts can be obtained from ambient seawater (in *Petrosia ficiformis*, Maldonado, 2007) or transferred from parents to offspring (Maldonado, 2007; Schmitt et al., 2007; Usher et al., 2001) by different mechanisms: in *Chondrilla nucula* and *Chondrilla australiensis*, bacteria and cyanobacteria were collected (engulfed) by nurse cells (somatic cells around oocytes or embryos), and then transported and transferred to the oocytes; in *Corticium candelabrum*, intercellular bacteria spread into the division furrows of early embryos and proliferated in the central cavity of larvae.

Sexual reproduction of sponges

Gametogenesis

Without predetermined germ line, gametogenesis of sponges, including oogenesis and spermatogenesis, take place from different types of somatic cells (Maldonado & Riesgo, 2008; Usher et al., 2004): in some calcareous species, the choanocytes left the choanocyte chamber, entered adjacent mesohyl and differentiated into oogonium-like cells; in *Ascandra minchini* (calcareous sponge), oogonia were suggested deriving from pinacocytes; In many demosponges, amoeboid cells were suggested to form the oogonia, while spermatogonia were reported to be derived from choanocytes in most members of Demospongiae and Calcarea; in *Chondrilla australiensis* (demosponge), both oogonia and spermatogonia developed from choanocytes. Gametes of male and female may occur form different individuals (gonochoristic, in *Chondrosia australiensis* and *Chondrosia reniformis*) or both occur in one individual (hermaphrodite, in *Corticium candelabrum*) (Table 1).

In spermatogenesis, a group of spermatogonia enveloped by a single layer of flat sponge cells and/or a thin collagen layer forms the spermatic cyst. In spermatic cysts, spermatogonia undergo mitosis to form primary spermatocytes, and then primary spermatocytes undergo meiosis into secondary spermatocytes and then spermatids (haploid), after modification, spermatids become mature spermatozoa that can really fertilize oocytes (Giese AC & Pearse JS, 1991). Structures of spermatozoa are diverse in sponges. In *Halichondria pancicea*, there are no acrosomal vesicles and rootlet structure (basal part of flagellum) in its spermatozoa; in *Asbestoplum occidentalis*, there are proacrosomal vesicles but no rootlet; in *Corticium candelabrum*, both acrosomal vesicles and flagellum presented (reviewed in Maldonado & Riesgo, 2008). The forms of sperm-maturation are also diverse in sponges. All sperms in the same spermatic cyst mature synchronously in *Crambe crambe* but form a maturation gradient from peripheral toward the center of the cyst in a homosclerophoride demosponge *Corticium candelabrum* (Maldonado & Riesgo, 2008).

In oogenesis, oogonia become primary oocytes, which are similar to somatic cells but slightly larger and have a bigger nucleus. Early-stage oocytes are usually amoeboid cells and may emit pseudopodia or microvilli to capture symbiotic bacteria (Riesgo & Maldonado, 2009). As oogenesis progresses, the size of oocytes increases, and the oocytes become rounder. After the oocytes reach a certain size, they start to accumulate yolk bodies (termed vitellogenesis). Yolk bodies can be produced by oocyte itself (autosynthesis) or/and by transferred from other sponge cells (heterosynthesis). Somatic cells that help the development of oocytes or embryos are called “nurse cells” in some articles. Nurse cells can engulf and digest symbionts into yolk bodies (or sometimes, the digest is still processing when the food vesicles were already transferred into oocytes) and transfer the food vesicle to the oocyte, or the nurse cell totally engulfed by the

oocyte (Giese AC & Pearse JS, 1991; Maldonado & Riesgo, 2008; Maldonado & Riesgo, 2009; Riesgo & Maldonado, 2009). Mature oocytes in many sponge species are enveloped by somatic cells (pinacocytes), collagen fibers or sponging (Maldonado & Riesgo, 2008).

How much time it takes for gametogenesis are also diverse in sponges (Table 1). The time needed for spermatogenesis ranges from 2 weeks to 2 months; and the time needed for oogenesis ranges from 4 weeks to 8 months.

Embryogenesis

Morphogenesis is the mechanism responsible for creation of body plan during embryonic development, metamorphosis, asexual reproduction and regeneration (Ereskovsky, 2007). Formation of multilayer embryos in Metazoa is achieved either by movements of cell sheets (epithelial morphogenesis) or by the migration of individual cells (mesenchymal morphogenesis) (Ereskovsky, 2007). With the associated extra cellular molecules and adhesive junctions, the formation of a continuous polarized cell sheet is one of the most basic forms of multicellular organization, and the epithelial blastoderm is the starting point for morphogenesis during the development of a wide range of Metazoa (Ereskovsky et al., 2012). In contrast, the mesenchyme is composed of more motile, loosely associated cells without cellular junctions. Mesenchymal cells are clearly designed to move with front-to-back polarity that positions leading-edge filopodia that pull cells forward (Ereskovsky et al., 2012).

Until 1994, only 3 larval types were recognized in sponges: parenchymellae (solid larvae), amphiblastulae (hollow larvae with differentiation between macromeres and micromeres) and coeloblastulae (hollow larvae with no differentiation between macromeres and micromeres) (Maldonado & Bergquist, 2002). With the application of

electron microscopy, larval structures and complete development from egg to juvenile were investigated at ultra-structure level in some species (reviewed in Ereskovsky, 2007). According to increasing information, additional larval types were suggested (Fig. 1, Maldonado & Bergquist, 2002) and more details were reviewed by Maldonado (Maldonado, 2006).

Cleavage is the first stages of embryogenesis in Metazoa (Ereskovsky & Boury-Esnault., 2002). There are four cleavage patterns in sponges: (1) incurvational cleavage in Calcaronea (Calcarea), (2) polyaxial cleavage in Halisarcida (Demospongiae), (3) radial cleavage in Demospongiae and Hexactinellida, and (4) chaotic cleavage in Homoscleromorpha (Fig. 2, Ereskovsky, 2007). Second stage is blastulation. In blastulation of sponges, almost all types of cell movements that characteristic of Eumetazoa are accompanied, and reviewed by Ereskovsky in Fig. 2 (Ereskovsky, 2007): cell delamination in *Oopsacas minuta* (Demospongiae), morula delamination in Haplosclerida (Demospongiae), invagination, unipolar and multipolar ingression in *Halisarca dujardini* (Demospongiae). And some unique morphogenesis, not found in other multicellular animals, have described in sponges: multipolar egression in Homoscleromorpha, polarized delamination in Poecilosclerida (Demospongiae) and Halichondrida (Demospongiae), excurvation in Calcaronea (Calcarea), formation of blastula (pseudoblastula) by means of ingression of maternal cells into the embryo in *Chondrosia reniformis* (Demospongiae) and unipolar proliferation in *Vaceletia crypta*. The presence of the third stage, gastrulation, is still argued under different definitions of the word “gastrulation” in sponges: cell sheet of epithelial layer moved to form endodermic layer (gut) or movement and reorganization of embryonic cells (Ereskovsky & Dondua, 2006).

Most of morphogenetic movements require that a subset of cells detach from their neighbors and acquire properties allowing them to migrate to new position (Ereskovsky, 2007). Integrin, the trans-membrane protein, may be the key molecules during early animal development and are found in some adult sponges (*Ophlitaspongia tenuis*, *Microciona prolifera*, *Geodia cydonium* and *Suberites domuncula*), but if integrin involved in embryogenesis or not in sponge remains unknown. Though cell-aggregation associated proteins were isolated in some species of sponges, there is not a single work demonstrating the relationship or mechanism of cellular adhesiveness in sponge embryonic development (Ereskovsky, 2007).

Terpios hoshinota

Since the outbreak of *Terpios hoshinota* (Suberitidae, Hadromerida, Demospongiae) reported in Guam in 1973, this encrusting black sponge arouse attention for its rapid growth accompanied with the ability to over grow most scleractinian coral genera (Bryan, 1973). From the experiments performed by Bryan, growth rate of *T. hoshinota* on *Porites lutea* was 23 mm per month, and this sponge grew faster on living corals than on non-living substrata, so the possibility of *T. hoshinota* consuming coral tissue as nutrients was suggested; when a small fragment of *T. hoshinota* was put on a *Fungia Echinata*, the toxicity of *T. hoshinota* was obvious that caused retraction of the tentacles of coral immediately and death of coral tissue under and around the sponge fragment within 24 hours.

According to the finding of Bryan that *T. hoshinota* grew faster on living corals than non-living substrata, further experiments to test the growth rate of *T. hoshinota* on different substrata were designed by Plucer-Rosario (Plucer-Rosario, 1987): 4 substrata, including living coral and 3 different kinds of non-living substrata (plexiglass plates,

reef rocks and clean coral skeletons, which were air-blasted to remove the living coral tissue), were prepared for *T. hoshinota* to grow; in the result, *T. hoshinota* grew faster on clean coral skeletons than on living coral; and in another experiment, growth rate of *T. hoshinota* on 21 forked branches of *Acropora formosa* were prepared by air-blasting one of the two prongs, and a fragment of *T. hoshinota* was transplanted onto the base of each fork; in the result, growth rate on cleaned prong was significantly higher than on the prong with living coral tissue, suggesting that *T. hoshinota* may utilize space of coral skeleton as a substratum rather than the nutrients form corals.

In 1993, outbreaks of *T. hoshinota* were reported in Ryukyu Archipelago (Rutzler & Muzik, 1993) and this sponge was described as a new species. Microscope preparations showed an unusually high number of unicellular cyanobacteria present throughout the mesohyl of the sponge.

Outbreaks of *T. hoshinota* took place at Green Island and Orchid Island, Taiwan, in 2006 (Liao et al., 2007), which aroused highly attention about the health of coral ecosystem in Taiwan. Distribution and growth rate of *T. hoshinota* in Green Island and Orchid Island were investigated (Lin, 2009). The average growth rate of *T. hoshinota* at Green Island was 0.18 cm per day. Different from the observations in Guam, the distributed depth of *T. hoshinota* in Taiwan was much shallower (mainly presented in 2-5 m). From the result of light-blocked growth experiment, this sponge cannot grow normally when shaded. This may result in the symbiotic relationship with cyanobacteria. When additional nutrients were supplied, growth rate of *T. hoshinota* did not increase.

With limited ecological information of *T. hoshinota*, prediction of the population dynamics is impossible. To understand one key point of its life history, sexual reproduction pattern of *T. hoshinota* were investigated from monthly sampling in Green Island (方, 2010) and Okinawa, Japan (Hirose & Murakami, 2011); and intensive

sampling within 1 month (every 2 d in July-August, 2009) were performed (方, 2010). Gametes (oocytes and sperms) of *T. hoshinota* were both found in samples from Green Island and Okinawa, and the presence of embryos was found almost year-round at Green Island, but sexual reproduction pattern in one year remains unclear since the presence of oocytes, sperms and embryos were not related to the others. And from the results of intensive sampling within one month in summer, the traits of sexual reproduction remains confusing--the relationship between the presence of gametes and embryos remains unknown (方, 2010).

The possible problems that caused the failure to find pattern of sexual reproduction in *T. hoshinota* may be: (1) the limited areas examined by histological observation, and/or (2) asynchronous in sexual reproduction among colonies. To solve the first possible problem, observation areas should be increased but it would cost more time to process the histological sections and for microscopic observation. To solve the second possible problem, we can take samples from the same tagged colonies continuously to chase the development of gametes and embryos from each colony. However, it was also reported that sampling on the same colony may damage and reduce the reproductive activity of sponges (Mercurio et al., 2007). But in the case of *Chondrilla australiensis*, an encrusting sponge with huge colony size, intensive sampling of twice a week for 5 years on the same colonies did not reduce their reproductive activity (Usher et al., 2004). From the results in intensive sampling to reveal the sexual reproduction pattern of *T. hoshinota* in Green Island, gametogenesis and embryogenesis in *T. hoshinota* may be very short, occur in several weeks. To investigate the reproductive cycle, it is necessary to sample at short intervals.

In November 2010, larval release of *T. hoshinota* happened to be observed for the first time from a fragment that was collected at Green Island and kept alive in the laboratory (Wang et al., 2012). Those brown, oval and entirely ciliated larvae were negatively buoyant with limited ability to swim. Size of larvae was ~650 μm long and ~350 μm wide. Ectoderm of larvae consisted of columnar cells with cilia. The inner part of a larva was occupied by spherical cyanobacterial and amoeboid sponge cells. Like the findings in adult *T. hoshinota* from Hirose (Hirose & Murakami, 2011), dividing cyanobacteria and cyanobacteria which engulfed by amoeboid sponge cells were also found in larvae (Wang et al., 2012). Cyanobacteria with disintegrated thylakoid membranes within phagosome in the sponge cells indicated that sponge cells inside the larva can digest cyanobacteria as nutrients.

Soon after the discovery of *T. hoshinota* larvae, *in situ* larval release of *T. hoshinota* was also observed in Green Island in March 2011 (Y. Nozawa, personal observation). In the observation, many dark brown oval-shaped larvae of *T. hoshinota* (~600 μm in length) were seen on surfaces of several *T. hoshinota* colonies. Larvae were slightly negatively buoyant and dispersed gradually from the colony surface by occasional water movements. Larvae of *T. hoshinota* were observed several times sporadically in Green Island since then, and the larval release appeared to have a general season (April-September) and a pattern in relation to lunar phase with its peak around new moon or full moon.

In sponges, sexual reproductive activities studied only by means of histology are often underestimated because of the following reasons (Mercurio et al., 2007): (1) examination areas we can deal with in histological studies are very limited. (2) It is possible that areas with no gamete or embryo happened to be sampled in a large sponge colony that was indeed reproductively active. (3) Samples were taken at limited

occasions in the reproduction season. Therefore, incorporating *in situ* observation of larval release, we can complement the histological information, and also broaden the investigation on reproductive activities of *T. hoshinota* on scales of whole colony and population. Accordingly, a simple *in situ* observation scheme was designed in this study to investigate the timing and pattern of larvae release of *T. hoshinota* in Green Island.

Study objective

To improve ecological information of *T. hoshinota*, (1) histological studies were performed to reveal the sexual reproduction pattern, gametogenesis and embryogenesis of *T. hoshinota*; (2) presence of larvae on surfaces of *T. hoshinota* were monitored at population level to reveal the pattern of larval release; (3) larvae were collected to do some preliminary investigations on their settlement behavior for the first 6 days after release in a laboratory.

Materials and methods

Histological studies on sexual reproduction of *Terpios hoshinota*

Specimens of *T. hoshinota* (Fig. 3) were collected every 2-3 days for two months (June 18 to August 17 in 2011) from 15 tagged colonies of middle to large sizes (the maximum length of > 30 cm) at Gong-guan harbor (22°44'36"N; 121°29'26"E), Green Island, Taiwan (Fig. 4). The sampling was made by scuba diving using a hammer and a chisel. Samples were fixed in 4% formaldehyde in sea water for more than one week. After that they were preserved in 70% ethanol and decalcified by 10% formic acid for 4-24 h (depend on the thickness of coral skeleton, on which *T. hoshinota* colonies usually grow). In a laboratory, the samples were dehydrated through a graded ethanol series (70%, 90%, 95%, 99%, 99.5%), embedded in paraffin, and cut to 6 μm-thick sections. After deparaffining with xylene, tissue sections were stained with Hematoxylin-Eosin (Hematoxylin and eosin staining (H&E stain) is one of the most commonly used techniques in histology. Basophilic components (i.e. nuclei) are attained blue or purple by hematoxylin while eosinophilic structures such as cytoplasm and collagen are stained by eosin to show pink-orange.), and observed under a light microscope (Olympus BX51) equipped with a digital CCD camera (Olympus, DP72).

In situ observation of *Terpios hoshinota* larvae

To understand the release pattern of *T. hoshinota* larvae in Green Island, the same 15 tagged colonies used in histological studies were monitored for the presence or absence of larvae on their surfaces every 2-3 days for 2 months (June 18 to August 17, 2011) as well as in haphazardly chosen colonies at each examination (n = ~10 colonies

per examination). Survey was done using scuba diving at approximately 10-11 a.m. in the morning.

When larvae were present, the numbers of larvae were recorded at 4 separate areas of the colony surface using a small quadrate (8 cm × 5 cm) (i.e., a total survey area of 160 cm^2). For counting larvae *in situ*, the following categories were used in each quadrate: (a) many larvae (more than 50 larvae), (b) moderate number of larvae (10-50 larvae), (c) few larvae (less than 10 larvae), and (d) no larva. The larval numbers of 4 areas were summed up using the following representative number of each category and used for the analysis: 50 larvae for many larvae, 30 larvae for moderate number of larvae, 10 larvae for few larvae and 0 for no larva.

In order to understand the diurnal pattern of larval release, the additional survey was done on the 15 tagged colonies in the afternoon around 4 p.m. on June 18, 20, 22, 27, 29 and July 1. For the depth influence on larval release, additional survey at deeper water (~10 m) was also made on July 15. Seven *T. hoshinota* colonies were found at ~10 m and examined on the presence and amount of larvae using the same method described above.

Settlement experiment of *Terpios hoshinota* larvae

Larvae of *T. hoshinota* were collected from several colonies using a plastic pipette on August 12, 13, and 14. Larvae collected from different days were used separately for 3 repeated experiments independently. Larvae collected from different colonies were kept in a different plastic tube (Falcon) and transferred to the laboratory within 2 hours. In the laboratory, larvae were washed with 5-μm-filtered seawater several times, and put into plastic, transparent tissue-culture-plates (Falcon) with 6 wells (as 6 replications) by pipetting. Each well contained about 10 larvae, each from different mother colonies.

The number of larvae used in the settlement experiments depended on how many larvae were available on each of the days.

Larvae were maintained in a growth chamber during the experimental period. Two culture conditions were used for the experiments; control (26°C, 12 h light/ 12 h dark) and shaded (26°C, 24 h dark). During the experimental period, seawater was changed every 12 h with fresh 5-μm-filtered seawater. Larvae were observed under a stereo microscope every 8 to 12 hours, and larval settlement pattern was recorded by photographing settled larvae.

Results

Gametogenesis of *Terpios hoshinota*

Sperm, oocytes and embryos were observed in *T. hoshinota* at Green Island (Fig. 5, Fig. 6, and Fig. 7). Spermatic cysts (Sc.) and embryos were often found in the same sample (Fig. 5a), suggesting that *T. hoshinota* were hermaphrodite. In one Sc., all sperms were at the same development stage of spermatogenesis (Fig. 5b), suggesting the synchronous development in one Sc. However, spermatogenesis in different spermatic cysts at the same colony was asynchronous (Fig. 5). Sizes of spermatic cysts varied greatly. Large spermatic cysts filled with primary spermatocytes and small spermatic cysts with spermatids were also found, suggesting that the size of spermatic cyst does not related to the stage of spermatogenesis.

Because of the difficulty to distinguish early oocyte form non-reproductive sponge cells, the oocytes dealt with in this thesis are the oocytes in later stages that could be distinguished from non-reproductive sponge cells easily. The definitions of oocyte in the thesis are: (1) single cell larger than peripheral sponge cells, (2) with a clear nucleus (larger than the nucleus in peripheral sponge cells) with vivid nucleolus, and (3) enclosed in a vacuole that is slightly larger than an oocyte itself. The size of oocytes (under the definition described above) range from ~15 to 40 μm (Fig. 6). One fertilizing oocyte surrounded by sperms (Fig 6, lower-right) was observed with its size ~25 μm, so this size was considered as the size of mature oocyte.

The presences of sperm and oocytes were summarized in Fig. 8 with the presences of mature embryos as a reference. Oocytes of *T. hoshinota* presented continuously from mid-June to mid-August, 2011, suggesting that *T. hoshinota* produce oocytes continuously. There were four peak periods for the presence of sperm: around full moon

in June, new moon between June and July, full moon in July and new moon between July and August. This data supports a semi-lunar cycle of spermatogenesis of *T. hoshinota*. The presence of spermatic cysts that filled with spermatids is marked especial as a reference for the termination of spermatogenesis. Because sperm-producing event continued more than 8 days were not observed, the spermatogenesis in *T. hoshinota* may be shorter than 8 days. Mature embryos were found on June 16-20, July 1, July 11-15 and August 10-12 around the full moon and new moon. Although oocytes were produced continuously, mature embryos occurred with a semi-lunar cycle pattern.

Embryogenesis of *T. hoshinota*

Embryos at different stages of embryogenesis were observed (Fig. 9) and were sorted into 4 categories defined as: (1) *early embryo* (morula stage, Figs. 9a-c): a solid ball consists of 4 to more than 128 embryonic cells formed from cleavage of a zygote. (2) "*Cell mass"* (Figs. 9d-f): a mass of cells consists of huge amount of cyanobacteria and some sponge cells transferred from mother sponge, located in a space which is surrounded by a layer of pinacocytes (embryo-nursing chamber). The cell mass is incorporated into an embryo at the later embryogenic stage. (3) *The combination stage of an embryo and the "cell mass"* (Figs. 9g-i): a stage of embryogenesis that an embryo and the "cell mass" were seen combined in the same nursing chamber. (4) *Mature embryo* (Figs. 9 j-l): embryos consist of the cell mass inside surrounded by a single layer of columnar embryonic cells.

Early embryos from 8-cell stage to ~128-cell stage were observed (Fig. 9a-c). To speak of, early embryos of various cell stages (8-cell stage to ~128-cell stage) were also observed on the same day (June 22) in the sample from colony # 11. The size of early

embryos (at morula stage) was ~20 μm, which was similar to the size of predicted mature oocyte (Fig. 6, lower-right).

Enclosed in a nursing chamber, the “cell mass” consisted of numerous cyanobacteria and some maternal sponge cells that were transferred from mother sponge (Fig. 10). Size of embryo-nursing chamber (Figs. 9d-f) was similar to mature embryos (Figs. 9j-l). The amount of cyanobacteria and sponge cells in the cell mass varied greatly (Figs. 9d-f).

In some cases, one blue cell ball consisted of many tiny sponge cells was observed in the nursing chamber, located near or inside the “cell mass” (Figs. 9g-i, 11). Because of the morphological traits of the blue cell ball, i.e., many extraordinary small cells with clear nucleus and limited volume of cytoplasm, it was thought to be the early embryo. The cases that the embryo presented in nursing chamber may be a stage that embryo and the “cell mass” combined together. The early embryo may keep developing inside the nursing chamber after entering it so that the size of early embryos inside the nursing chamber was larger than those observed outside.

Mature embryos measured ~400 x 285 μm, oval-shaped and were formed by a single layer of columnar sponges cells enclosed the cell mass consisted of cyanobacteria and maternal sponge cells (Figs. 9j-l, 12). Embryonic cells that formed the outer layer were very close to each other and formed a compact layer (Fig. 12).

There appeared to be at least two kinds of sponge cells in the “cell mass”: amoeboid sponge cells with normal size and another kind of rounder and larger sponge cells (Figs. 10, 13a, b). Figs. 13c and d show that many tiny sponge cells located in the periphery of the nursing chamber. This may be a stage of embryogenesis that the embryonic cells moved to the periphery of the “cell mass” and started to package all the

cell mass inside the chamber to form the mature embryo.

To understand the pattern of embryogenesis of *T. hoshinota*, the different stages of embryogenesis were sorted into three categories and shown separately (Fig. 14): (1) early embryos (did not locate in the nursing chamber), (2) the "cell masses" or unripe embryos that were not surrounded by single-layered sponge cells and, (3) mature embryos. The cases that the embryo presented inside the "cell mass" (the combination stage) were marked especially as a special stage in embryogenesis in the figure. Early embryos were found throughout the observation period. In all colonies (except colony # 1), the presence of the "cell mass" continued for several days, suggested that the development from "cell mass" to mature embryo may take several days. Mature embryos were found in comparatively limited period of time, around full moon in June, new moon between June and July, full moon in July and full moon in August, suggesting the synchronous maturation of embryos. The combination stage was observed several times in every colony, but the relationship between this stage and maturation was not clear. It may be due to the methodological difficulty of histological studies in which reproductive productions were underestimated and some critical information could be lost easily. But a possible relationship still can be guessed from some cases. In the colony # 8, the combination stage found on July 1 and 4 were 12-14 days earlier than mature embryos found on July 15. In the colony # 11, the combination stage found on June 18 was 13 days before mature embryo presented on July 1. In the colony # 12, the combination stage found on July 25 was 16 days before mature embryo presented on August 10. And in the colony # 2, although the combination stage presented on June 27, 29 and July 4, and the mature embryos presented on July 11 and 15, a possible relationship between the timings of combination stage and mature stage may be: 13-15 days between the combination stage presented on June 27 and 29 and the

mature stage presented on July 11; and 11 days between the combination stage presented on July 4 and the mature stage presented on July 15. As a summary, it may take 11 to 16 days for the development from the combination stage to form a mature embryo.

Larval release pattern of *T. hoshinota*

Larvae were released not from the oscula (the central of star mark) but from entire sponge surfaces (Fig. 15). Larvae appeared to be extruded from the inside gradually, and many larvae were seen still half-embedded on the surface (difficult to separate the half-embedded larva from the sponge surface using pipette). Larvae that had been extruded and presented on sponge surfaces were negatively buoyant and still attached to the parent surface. When water current occurred, larvae were swept away from the colony surface.

On most colonies, larvae were seen patchy on the *T. hoshinota* surface, and the patch size with larvae varied on each colony (Fig. 16). While very large colonies (tagged colony numbers 5 and 9, > 2m in diameter) had only few patchy areas with larvae, one colony with medium size (tagged colony number 8) released its larvae from the whole sponge surface in every observed larvae release events.

Results of *in situ* larvae release observation of *T. hoshinota* for 2 months (mid-June to mid-August in 2011) are summarized in Fig. 17. The larval release events of *T. hoshinota* appeared to be synchronized among the colonies (Fig. 17, left figure). Massive larval release was observed 4 times during the 2-month observation period: 3 times around the full moon and once around the new moon. The largest release event was observed on July 15 (full moon), with 24 of 25 examined colonies (96%) releasing larvae.

More detailed data on the amount of larvae released by each tagged colony are presented in Fig. 17 (right figure). The pattern of synchronous larval release with four peak periods is shown clearer in this figure. The duration of each peak larval release event appeared to continue 6-12 days. Eight of the 15 colonies (tag numbers 1, 5, 6, 7, 8, 10, 12, 13) participated in all of the 4 massive larvae release events, 5 of the 15 colonies (tag numbers 2, 4, 9, 11, 14) participated in 3 of 4 massive release events, the colony of tag number 15 released larvae twice on the full moon of July and near the full moon of August, and the colony of tag number 3 released its larvae only once on the full moon of July. The amount of larvae released from each colony varied on each release event, but most colonies released the largest amount of larvae on July 15 in the 2-month observation period.

Comparing the results in histological study for 5 tagged colonies and the results of *in situ* larval release observation (Fig. 18), presence of mature embryos accompanied larval release, that is, timing of embryo maturation agreed with larval release. On the other hand, larval release did not always accompany the finding of mature embryos by histology, suggesting the underestimation of histological studies.

The possible difference in larval release at morning (10-11 a.m.) and afternoon (~4 p.m.) was examined on the 15 tagged colonies on 6 different days (June 18, 20, 22, 27, 29, and July 1) (Fig. 19). Although percentages of colonies with larvae looked slightly lower in the afternoon, no significant difference was detected between the two observation periods (Wilcoxon matched pairs test, $p=0.074$).

In comparisons between 2 depth environments (~3m vs. ~10m), 24 of 25 *T. hoshinota* colonies had larvae at 3 m on July 15, whereas only 2 of 7 colonies had larvae at 10 m (Table 2 Comparison of frequency of *Terpios hoshinota* colonies with larvae at the depths of 3 m and 10 m.). The proportions of the two groups were

significantly different (Fisher's exact test; $p < 0.001$). The low number of *T. hoshinota* colonies examined at ~10 m was due to the lower density of *T. hoshinota* colonies at deeper water in Green Island.

Larval settlement experiment

In larvae settlement experiments, the larvae were negatively buoyant and sank to the bottom of the wells of tissue culture plates soon after transferred (Fig. 20). Some larvae started settling immediately, some larvae crawled at the bottom, and most larvae did not move (Fig. 21). Larvae were dark brown, with many symbiotic cyanobacteria, and round to sesame shape. When sands and stones were supplied, larvae could have settled on the materials, besides plastic surfaces of the tissue culture plates (Fig. 22).

Results of 3 settlement experiments of larvae of *T. hoshinota* were shown in Fig. 23. In general, larval settlement rates increased slowly with time. The high variations of larval settlement rates indicated high variation of settlement time among larvae. However, most larvae (ca. 60-80%) had settled by 4^{th}-5^{th} day of the experiments. In comparison of dark and control conditions, settlement rates appeared to be slightly higher in dark condition in the 1^{st} experiment on larvae from August 12. Larvae from August 13 (2^{nd} experiment) and August 14 (3^{rd} experiment) did not appear to be different in settlement rates between the 2 experimental conditions. Statistical analyses indicated no significant difference between the 2 experimental conditions in all of the experiments (Two way repeated measures ANOVA, 1^{st} experiment: $F(1, 10)=4.079$, $p=0.071$, 2^{nd} experiment: $F(1, 10)=0.031$, $p=0.864$, 3^{rd} experiment: $F(1, 10)=0.506$, $p=0.493$)

Photographic images of larvae and settlers were shown in

Fig. 24. Settlers became very flat and thin, attached to the bottom of tissue culture plates.

Settlers grew with time, and after about 4 days, cannels for water filtering system formed (Fig. 24b). There were many cases that larvae settled together or very close to each other (Figs. 24c-f). In some cases, the contacting settlers fused and grew well. In other cases, the fused settlers grew well in the beginning, but later a barrier formed between them or they stop increasing size and started to degrade.

Discussion

Gametogenesis and embryogenesis of *Terpios hoshinota*

The size of oocytes in *T. hoshinota* (~15-40 μm) was comparatively smaller than other sponge species (Table 1). This result is similar to the previous study in Green Island (方, 2010), but different from the result in Okinawa: one presumable oocyte of *T. hoshinota* was >100 μm in diameter (Hirose & Murakami, 2011). Because only one presumable oocyte was reported in Okinawa, from this limited data, whether the oocyte of *T. hoshinota* in Okinawa is larger than that in Green Island is uncertain.

Semi-lunar cycle of sperm-release and the short spermatogenesis (< 8 days) were found in *T. hoshinota,* whereas oocytes were produced continuously throughout the 2 month study period. Some cases on the influence of lunar phase as a trigger to the spawning of sponges and corals were reviewed by Usher (Usher et al., 2004). In *T. hoshinota*, the sperm-release was highly synchronized to the lunar phases around the full moon and new moon. In the influence of lunar phase, tidal change (changes of water pressure) and lunar illumination need to be considered. To exam which factor is important as a trigger of sperm release in *T. hoshinota*, it is necessary to keep *T. hoshinota* colonies under controlled conditions to test the hypothesis.

Combining the semi-lunar cycle of sperm-release (Fig. 8) and the predicted period of embryogenesis (11-16 days, Fig. 14), possible patterns of sexual reproduction of *T. hoshinota* may be: oocytes are produced all the time, sperms are produced bimonthly, after released from adult colonies, it takes time for sperm to be recaptured by sponge colonies and fertilize oocytes because early embryos were found not only after the sperm-release but continuously. It may be very short for a fertilized oocyte to develop to 128-cell stage because embryos at 8-cell stage to 128-cell stage were found in the same

sample (# 11 on June 22). Embryogenesis may have taken 11-16 days (Fig. 14) or slightly longer after zygote starting the cleavage (maybe few days after sperm-release), resulting in the semi-lunar cycle pattern observed in larvae release. Given nutrients and energy as limiting factors, the absence of larval release on new moon in August may due to the greatest larval release event on mid-July, which used up the energy of sponge, the highest peak of sperm production and oocyte production could also be observed on new moon in early-July which may cost large amount of energy.

In sponges, various ways to obtain specific symbionts have been reported (Schmitt et al., 2007; Usher et al., 2001). In *T. hoshinota,* symbiotic cyanobacteria were not found in the oocytes nor in the early embryos before 128-cell stage, but in the central cavity of mature embryos, suggested that symbionts were transmitted vertically (i.e. directly from adult, not from ambient sea water) in this sponge.

Histological observations of the present study revealed that: (1) size of embryos at morula stage that were found outside the nursing chamber was very small, (2) the "cell mass" in nursing chambers contained different amount of cyanobacteria and sponge cells, (3) the blue cell ball, the embryo-like cell mass, were only found occasionally inside the nursing chamber, (4) the mature embryo finally enclosed huge amount of cyanobacteria and mother sponge cells. A possible embryogenesis process in *T. hoshinota* was summarized in Fig. 25: A fertilized oocyte (zygote) started to cleavage, which increasing its cell number but not its volume much (Fig. 25A). At the same time, a space (embryo-nursing chamber) surrounded by a single layer of pinacocytes formed. Cyanobacteria and maternal sponge cells accumulated in the space gradually (Fig. 25B). The early embryo (at morula stage) entered the nursing chamber and kept growing to a certain size (blastulation) (Fig. 25C). After that, by unknown process, the embryonic cells move toward the periphery of the cell mass and formed a compact single layer

which packed all cells inside (Fig. 25D). This embryogenesis in *T. hoshinota* is very unique that has never been reported in other sponges and even in metazoan. One similar embryogenesis case which finally formed a special larva "pseudoblastula" was reported in *Chondrosia reniformis* (Demosponge) and reviewed by Ereskovsky (Fig. 26, Ereskovsky & Dondua, 2006). At the stage of ~32 cells, large numbers of maternal cells that have surrounded the cleaving embryo start to penetrate into the morula-like embryo. As a result, embryonic cells are finally ousted to the periphery and arrange in a single layer, forming the coeloblastula. A dense internal cell mass in the embryo consists of maternal cells. Contrary to the formation of pseudoblastula, in which the maternal cells penetrated into embryo, the embryogenesis in *T. hoshinota* is more likely that the embryonic cells move outward and enclosed the huge amount of maternal cells and cyanobacteria (Figs. 13c, d).

Embryogenesis of sponges is very diverse and some of them were not found in other group of Metazoa (Ereskovsky & Dondua, 2006). Many unique ways of cell movement in embryogenesis have been reported. Although more than 8000 species were described in Porifera, embryo developments were only studied in ~100 species and reviewed by Ereskovsky, Leys and Maldonado (Ereskovsky & Dondua, 2006; Leys & Ereskovsky, 2006; Maldonado, 2006). Larval type of Subertitidae (the family of *T. hoshinota*) is unknown (Maldonado, 2006). However, larval types of some other families in Hadromerida (the order of *T. hoshinota*) were reported: clavablastula in Clionidae, Polymastiidae and Tethyidae and direct development (absent of larva) in Stylocordylidae. The morphology of clavablastula is a big central cavity that filled with cells which occurring form mother sponge or embryo itself, and a single layer of cubic or columnar cells forming the outer layer (Maldonado & Bergquist, 2002). Larva of *T. hoshinota* is morphologically similar to clavablastula, but the period needed for

embryogenesis seems longer and the size of larvae is larger in *T. hoshinota* (Table 3). Embryogenesis in *T. hoshinota* (early embryo develop outside a nursing chamber, and then penetrate the nursing chamber and enclose all cells inside the chamber) is never reported, but the idea that embryonic cells segregate and mix with maternal cells is similar to embryogenesis of *Chondrasia reniformis* (Chondrosida, Demospongiae, Ereskovsky & Dondua, 2006). In *Chondrasia reniformis*, maternal cells penetrate into the embryo (Fig. 26), but in *T. hoshinota*, because of the significant difference between the amount of cells inside the embryo, it is more likely that embryonic cells move outward to enclose all of the cells inside the nursing chamber. If the predicted embryogenesis of *T. hoshinota* in this MSc thesis is true, it will be a new finding of embryogenesis in Metazoa, and this may suggest more variety of embryogenesis in Porifera that never be discovered. Using genetic information and fluoresce *in situ* staining techniques, sponge cells originated from embryo or mother sponge could become distinguishable, and the location of cells from different origin could provide further information about embryogenesis in *T. hoshinota.*

Pattern of larval release

Before the start of this study, we have no idea if the intensive sampling of the same colonies would reduce the reproductive activity of *T. hoshinota* or not. From the result of *in situ* larval release observation (Fig. 17), percentage of colonies that released larvae were not decreased on sampled (tagged) colonies than haphazardly chosen colonies for the 2 months study period, suggesting that intensive sampling did not reduce the reproductive activity of *T. hoshinota* in the 2 months. It may be a useful method (taking samples from the same colonies intensively) for investigating sponges with short reproductive cycle and big colony size to examine their reproductive patterns.

Larval release of *T. hoshinota* was not randomly or continuously but highly synchronously with 4 peak periods in the 2 month observation period (3 times around full moon and once around new moon). Tough timing of larval release seemed to be synchronous to the full moon and the new moon, the timing were not so accurate. On the other hand, the timing of sperm release were highly synchronous to the full moon and new moon, so the pattern of semi-lunar cycle of larval release may be determined by the timing of sperm-release and embryogenesis period that may be 11-16 days.

Although no significant difference was observed in larval release at two different times of a day in the present study, the data may not be sufficient to deny the possibility of diurnal pattern of larvae release in *T. hoshinot*a. To examine the diurnal timing of larval release, it may be a better way to keep *T. hoshinot*a adults in a tank in the lab and observe its larvae release pattern for days.

The number of colonies with larvae in the shallow water was significantly higher than that in the deeper waters (~10 m) on July 15. However, it should be noted that one of the two colonies with larvae at 10 m depth had very high number of larvae (> 1000 larvae on the colony with its diameter < 1 m). The difference in the percentage of colonies with larvae between 3 m and 10 m (96% and 29%) may suggest that: (1) a difference in reproductive activities of *T. hoshinot*a between the shallow and deep waters, and (2) a difference in the frequency of larval release between the depths: i.e., deeper colonies may have lower frequency of larval release. With the symbiotic cyanobacteria that inhabit in *T. hoshinota* , colonies in shallower waters may be able to gain more energy from photosynthetic activities of cyanobacteria than colonies in deeper water. Hence, if nutrient condition and/or health state of *T. hoshinot*a colonies were not the same between the 2 depths, the number of larvae they can produce may naturally be different.

Larval settlement

In the 4-5 day settlement experiments, the settlement rate increased slowly (Fig. 23), suggesting that *T. hoshinota* larvae may have a dispersal period of several days. However, because of their negative buoyancy and limited swimming ability, dispersal distance of *T. hoshinota* larvae may be very short. Although larvae could settle on sand (Fig. 22), they were torn easily when the sand were moved, so it may not be common for *T. hoshinota* larvae to settle and survive on sand in natural substrata. In the *in situ* observation, several larvae surrounded by mucus-like substance were sometimes observed on adult surfaces. Those larvae were removed together by strong water current. For larvae of *T. hoshinota*, long-distance dispersal may depend on this kind of random and passive events, rather than their own ability of locomotion.

From the TEM photos of larvae, the sponge cells inside the larvae of *T. hoshinota* were suggested to have the ability to engulf symbiotic cyanobacteria (Wang et al., 2012). This phenomenon was also found in somatic cells inside adult sponge of *T. hoshinota* (Hirose & Murakami, 2011). Cases of oocytes engulfing symbiotic bacteria as food to produce yolk inclusions are not uncommon. In *Halisarca dujardini* (Demospongiae), developing oocytes engulf symbiotic bacteria, and there were two fates of those engulfed bacteria: a part of the bacteria were digested as food and the other part remains undigested in membrane-bound vacuoles within the cytoplasm of the oocytes (Ereskovsky & Gonobobleva, 2005). In *Axinella damicornis* (Demospongiae), developing oocytes stretch out numerous microvilli toward bacteria, and digested bacteria were also found inside the oocytes (Riesgo & Maldonado, 2009). On the other hand, somatic cells of sponges that engulfed and digested symbionts were also reported. In *Raspaciona aculeate* (Demospongiae), somatic cells engulfed symbiotic bacteria and

transported them to the oocytes by the forms of lipid granules and heterogeneous yolk bodies (Riesgo & Maldonado, 2009). In the case of *T. hoshinota*, both somatic cells and cells inside the embryos seemed able to engulf symbiotic cyanobacteria as food, but for symbionts transmission, it is another mechanism mentioned above.

The amount of larvae that could have been collected decreased on August 14, indicating the end of the larval release event in the mid-August. The results of settlement rates from the larvae of August 14 were somewhat different from those on larvae of August 12 and 13. Nearly half of the larvae collected on August 14 settled immediately when they were put into the tissue culture plates. The mean settlement rates archived nearly 40% just within the 1st hour. After 40 hours, settlement rates did not increase much, indicating that the larvae that haven't settled may have lost the ability to settle. The difference between larvae collected on August 14 and previous two days may be the time after being released, i.e. the age of larvae. Because nearly the end of larval release event, larvae collected on August 14 may possibly be released in previous days but haven't been removed by water currents and were still attached to the adult surface and collected. When put into the tissue culture plates, the larvae may have been already more aged, so that they may have settled quickly and some of them may have already lost their ability to metamorphosis.

Because of huge amount of symbiotic cyanobacteria, higher settlement rates on control group rather than on dark group were expected. But in the results, settlement rates on dark group were not lower than that of control group, indicating that light was not the limiting factor for larvae to settle.

Larvae that settled together or settled in very close places and fused later were often observed in the present study. If the settlers fused successfully, this new chimera has more materials to construct a new sponge colony in the beginning, and may take

advantage from growing faster and higher probability to survive. But there were also examples that the chimera failed to grow well. In some cases, a barrier was formed between two fused settlers (

Fig. 24d). In another cases (often observed when more than two larvae settled very closely), settlers grew and fused together at the beginning, but after 1 or 2 days, the colony size didn't increase anymore, and the chimera started to degrade (

Fig. 24e, f).

The possibility for larvae to fuse may depend on the similarity of genotypes. After contacting, they recognize each other and decide whether they accept or reject each other. Although sponge cells locate in mesohyl separately and with seldom cases of cell junctions (mainly found in the outer layer of larvae, reviewed in Ereskovsky, 2007), the ability of them to communicate and recognize each other were reported (Muller. 2003). In adult *T. hoshinota*, fragments from different colonies have been transplanted and put together to examine their allo-recognition responses (方, 2010). In the results, fusion and rejection responses occurred in the adult *T. hoshinota*. The rejection behaviors in adult *T. hoshinota* and larvae also provide an evidence for sexual reproduction of *T. hoshinota* in Green Island.

The outbreak of *T. hoshinota* took place in several distant locations mainly in Taiwan, Okinawa (Japan) and Guam since 1970s. This may due to the dispersal of *T. hoshinota* by sexual or asexual reproductive products. Because of the negative buoyancy and poor swimming ability of the larvae, the probability of disperse via sexually produced larvae to the distant location may be low. And given the amazing amount of symbiotic cyanobacteria harbored in adult sponges and larvae, the success of dispersal and establishment of *T. hoshinota* populations may strongly relate to those symbionts.

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TABLE 1. **Larval type and development mode (viviparous vs. oviparous) in the major taxonomic categories of Porifera**

Taxon	*Larval type*
Subphylum Symplasma	
Class Hexactinellida	
Subclass Amphidiscophora	
Order Amphidiscosida	?
Subclass Hexasterophora	
Order Hexactinosida	Trichimella (viviparous)
Order Lychniscosida	?
Order Lyssacinosida	Trichimella (viviparous)
Subphylum Cellularia	
Class Calcarea	
Subclass Calcinea	
Order Clathrinida	Calciblastula (viviparous)
Order Murrayonida	Calciblastula ? (viviparous)
Subclass Calcaronea	
Order Leucosoleniida	Amphiblastula (viviparous)
Order Lithonida	Amphiblastula (viviparous)
Class Demospongiae	
Subclass Homoscleromorpha	
Order Homosclerophorida	Cinctoblastula (viviparous)
Subclass Tetractinomorpha	
Superorder Tetractinellida	
Order Astrophorida	? (oviparous)
**Alectona* and *Thoosa*	Hoplitomella
Order Spirophorida	Direct development
Superorder Clavaxinellida	
Order Chondrosida	Clavablastula (oviparous)
Order Hadromerida	Clavablastula (oviparous)
Order Axinellida	Clavablastula (oviparous)
Order Agelasida	Clavablastula? (oviparous)
Subclass Ceractinomorpha	
Order Verticillitida	Parenchymella-like? (viviparous)
(i.e., living sphinctozoans)	
Order Halichondrida	Parenchymella (viviparous)
Order Poecilosclerida	Parenchymella (viviparous)
Family Latrunculiidae*	Parenchymella-like? (viviparous)
Family Stylocordylidae*	Parenchymella-like? (viviparous)
Order Haplosclerida	Parenchymella (viviparous)
Order Petrosiida	Parenchymella-like? (oviparous)
Order Dendroceratida	Parenchymella-like (viviparous)
Order Dictyoceratida	Parenchymella-like (viviparous)
Order Verongida	? (oviparous)
Order Halisarcida	Dispherula (viviparous)

/hich the larva remains elusive.
c reallocations proposed here based on developmental information.

硬海綿目

Fig. 1 Larval types in sponges (Maldonado & Bergquist, 2002). The order Hadromerida is marked especially because *Terpios hoshinota* belongs to this order.

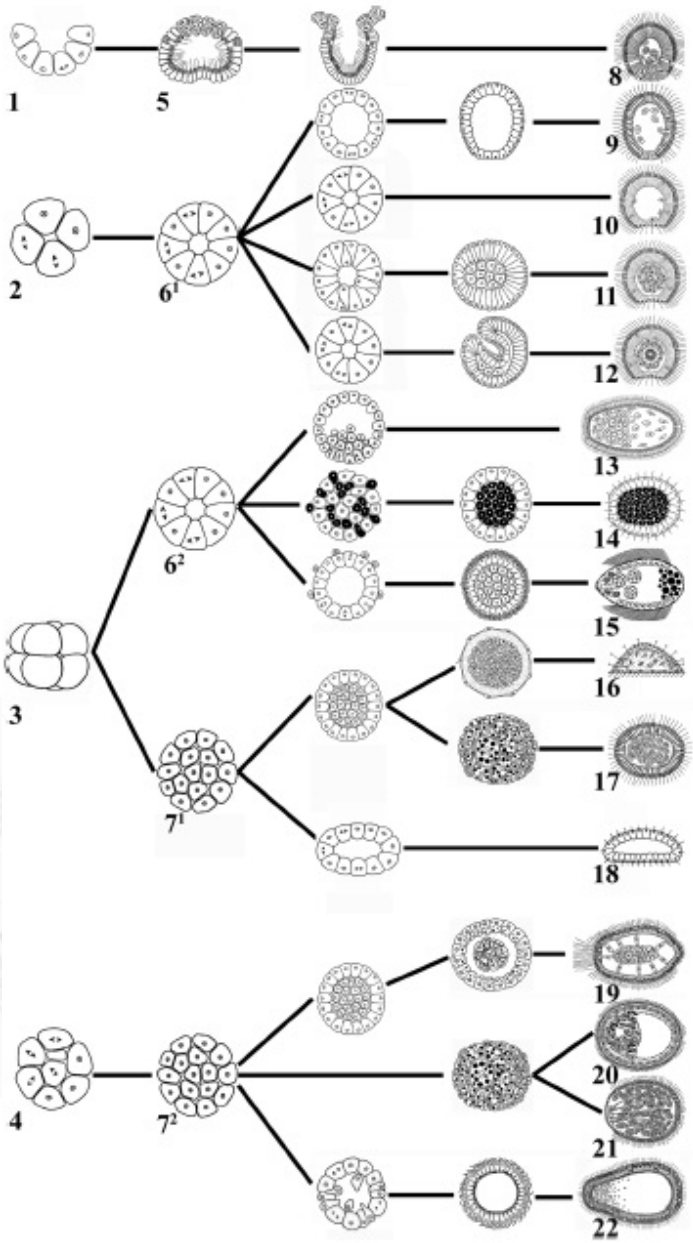
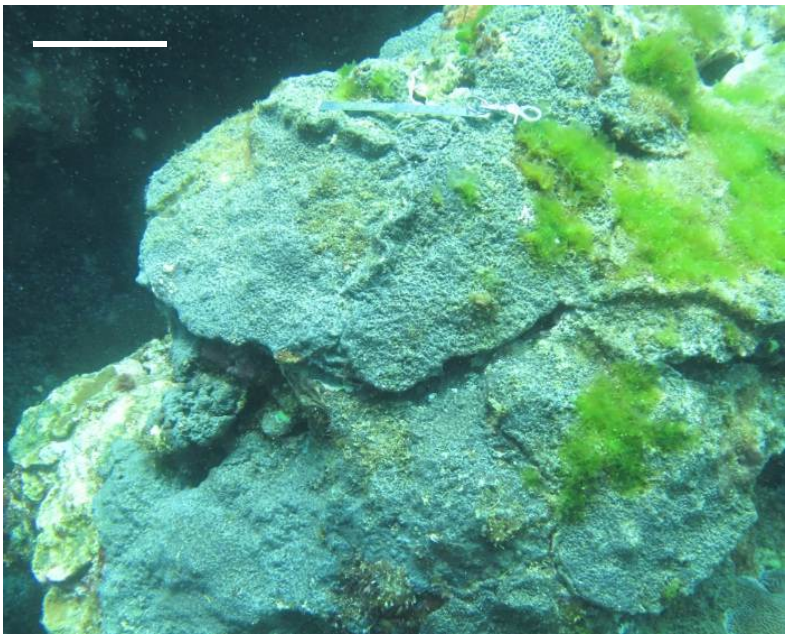


Fig. 11: Diagram of sponges cleavage and morphogenesis, leading to the larvae. *1–4* - Cleavage patterns in sponges: incurvational (*1*), polyaxial (*2*), radial (*3*), and chaotic (*4*). Three main form of sponges blastula: stomoblastula (*5*), coeloblastula (*6*), and stereoblastula (*7*). Different larval types of sponges: amphiblastula of Calcaronea (Calcarea) (*8*); calciblastula of Calcinea (Calcarea) (*9*); coeloblastula (*10*), parenchymella (11), and disphaerula (*12*) of *Halisarca* (Halisarcida); parenchymella of *Vaceletia crypta* (Verticillitida) (*13*); pseudoblastula of *Chondrosia reniformis* (Chondrosida) (*14*); trichimella of *Oopsacas minuta* (Hexactinellida) (*15*); juvenile of *Tetilla* under direct development (*16*); parenchymella of *Tethya aurantium* (Pallas, 1766; Hadromerida) (*17*); coeloblastula of *Polymastia robusta* Bowerbank, 1866 (Hadromerida) (*18*); parenchymella of Dictyoceratida (*19*); parenchymella of freshwater Haplosclerida (*20*); parenchymella of Poecilosclerida (*21*); cinctoblastula of Homoscleromorpha (*22*).

Fig. 2 Embryogenesis in sponges (Ereskovsky, 2007). Embryogenesis is very diverse in sponges. There are four cleavage patterns: (1) incurvational cleavage in Calcaronea (Calcarea), (2) polyaxial cleavage in Halisarcida (Demospongiae), (3) radial cleavage in Hexactinellida and most Demospongiae, and (4) chaotic cleavage in Homoscleromorpha (Demospongiae), and leading to several types of blastula in sponges.

(a)



(b)

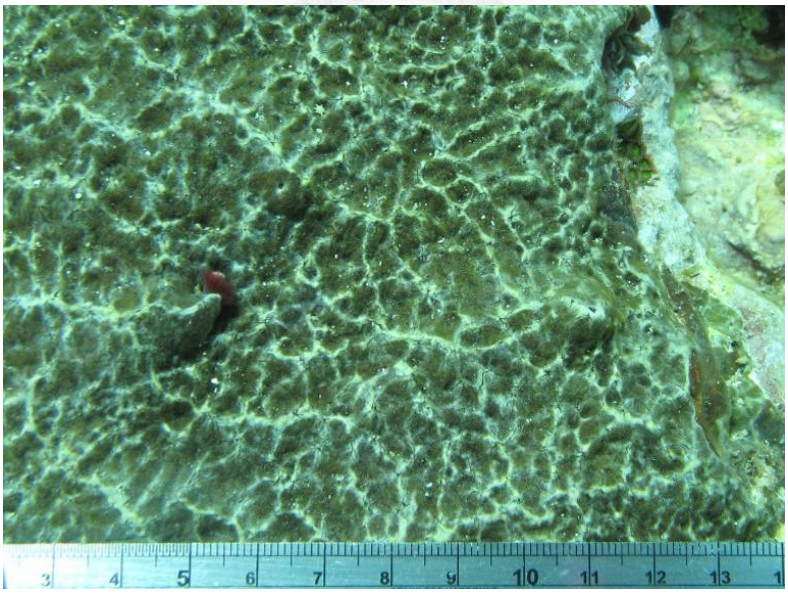


Fig. 3 (a) A large *Terpios hoshinota* colony in Gong-guan habor, Green Island, Taiwan (scale = 15 cm). (b) Close up photo of *T. hoshinota*. This sponge is very thin (thickness < 1 mm), encrusting and colonial with small (~ 3 mm) oscula at the center of radiaiton superficial exhalant networks. Surface stucture of *T. hoshinota* usually presented the surface struture of coral skeleton where the sponge grew on.

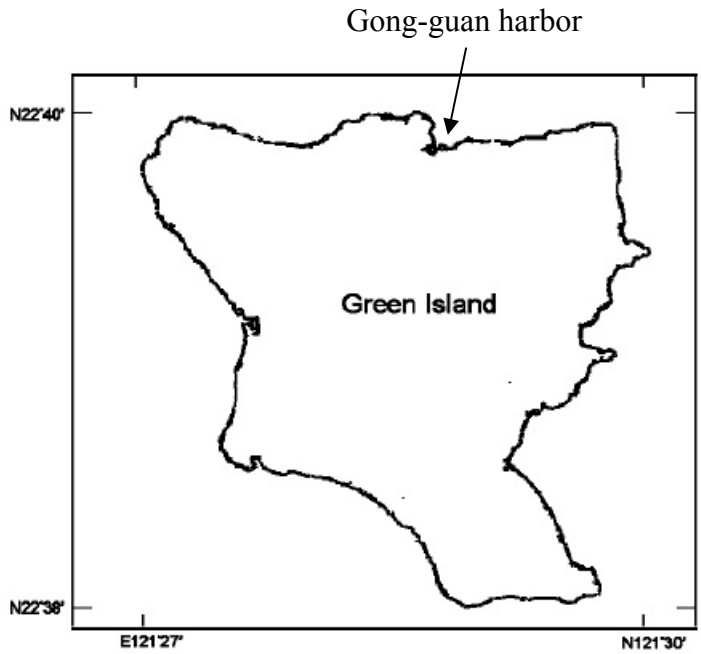


Fig. 4 Study site: Gong-guan harbor (22°44’36”N; 121°29’26”E), north of Green Island, Taiwan.

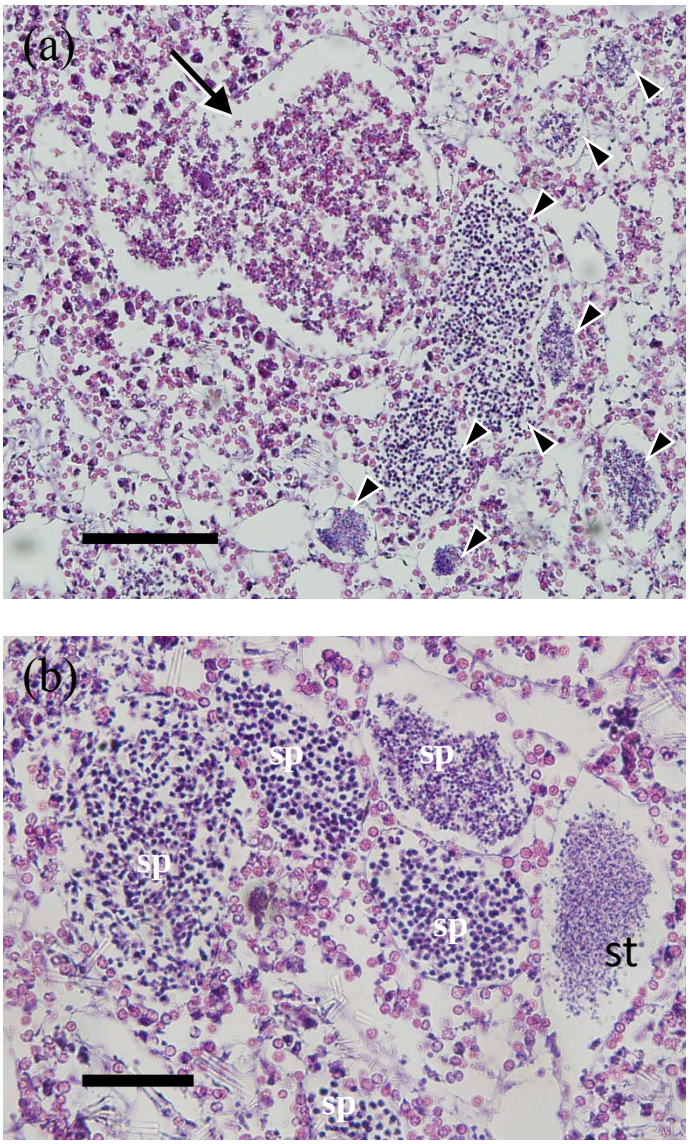


Fig. 5 Spermatic cysts of *Terpios hoshinota*. (a) Spermatic cysts coexisted with the embryo. (b) Spermatic cysts in various maturation stages. Spermatogenesis is synchronous in one spermatic cyst but asynchronous between different spermatic cysts in one specimen. Arrowheads, spermatic cysts; arrow, embryo; sp, spermatogonia or spermatocytes; st, spermatids. Scale bars: a = 100 μm, b = 50 μm.

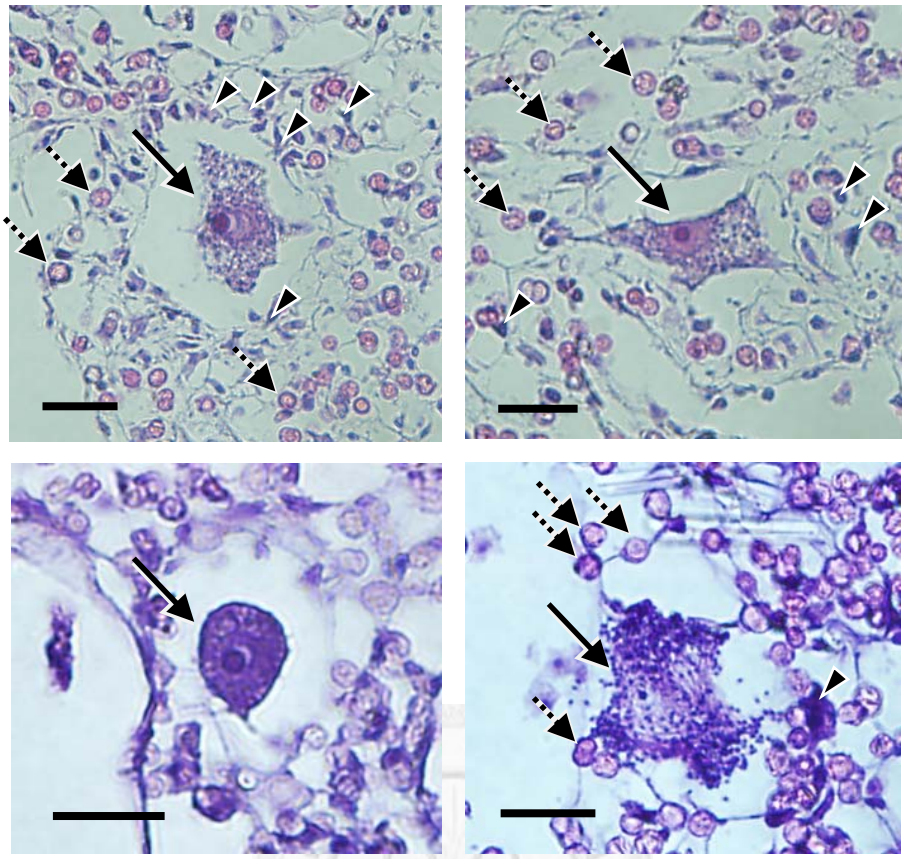


Fig. 6 Oocytes and a fertilizing oocyte surrounded by sperm (lower right) of *Terpios hoshinota*. Arrows, oocytes; arrowheads, sponge cells, dotted arrows, cyanobacteria. Scale bars = 20 μm.

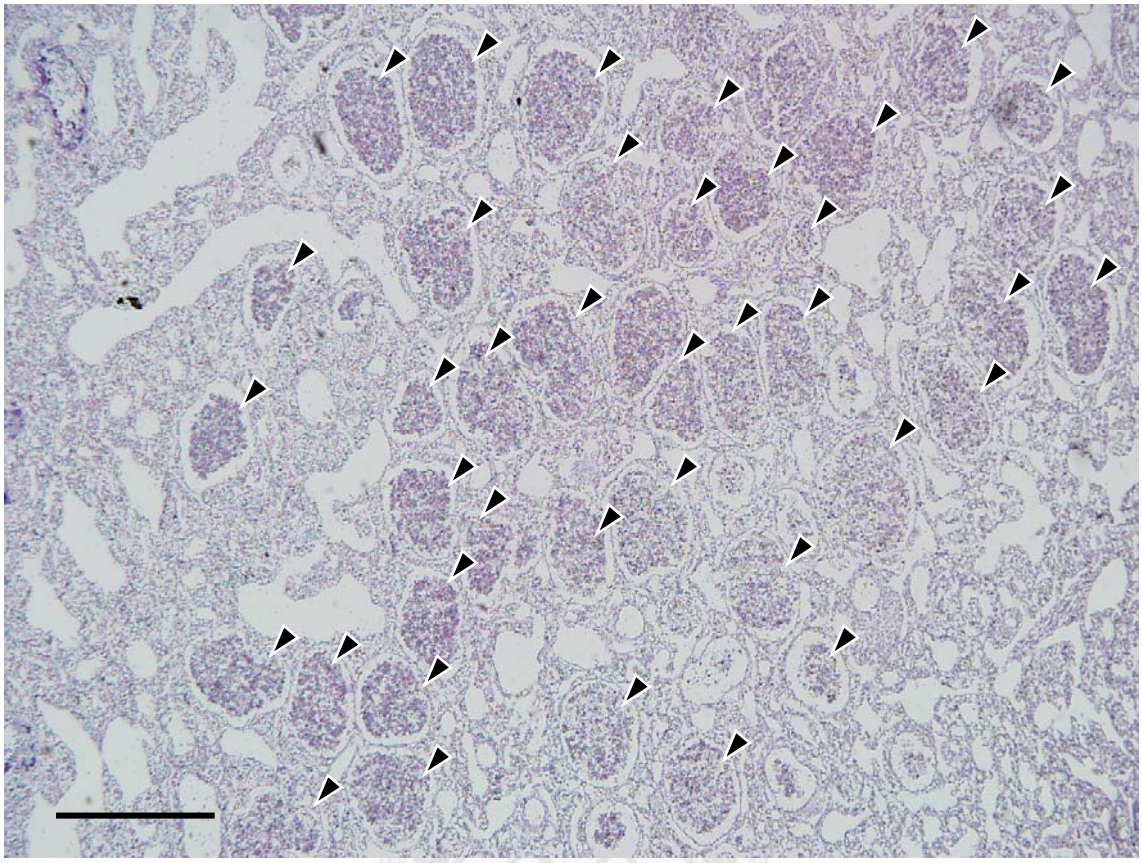


Fig. 7 Many embryos in *Terpios hoshinota*. Arrowheads, embryos. Scale bar = 0.5 mm.

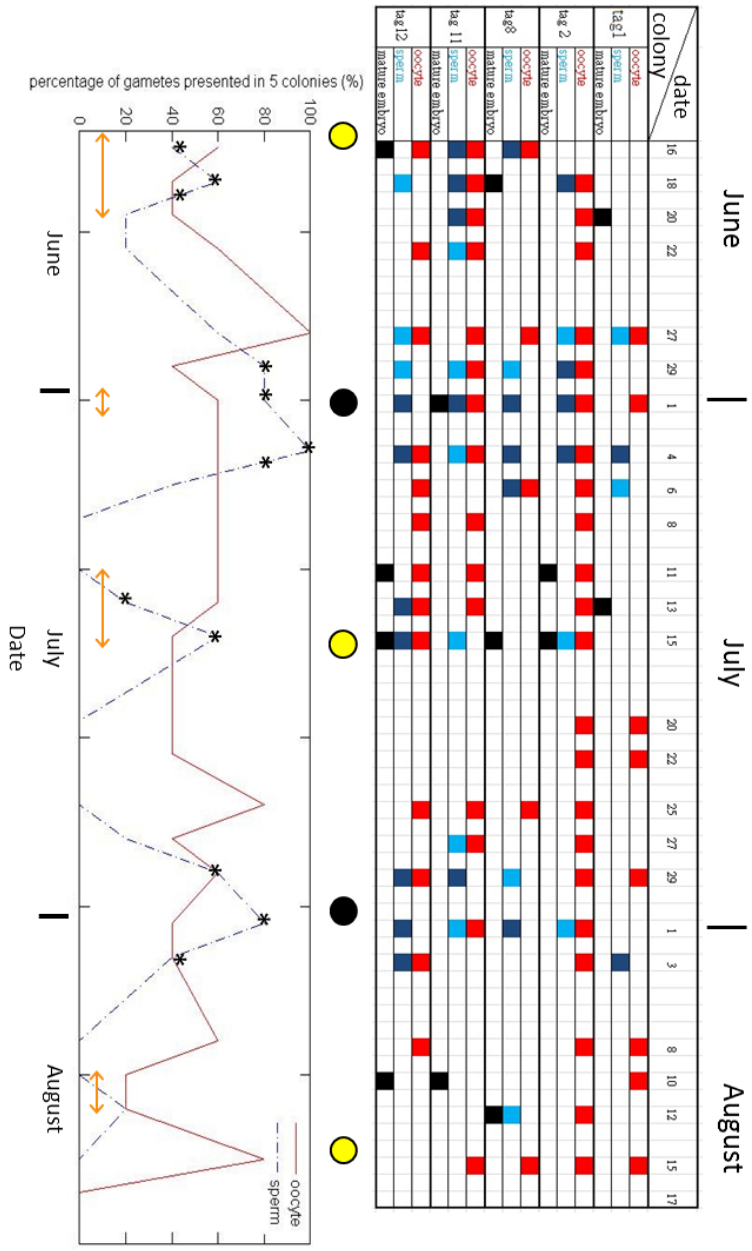


Fig. 8 Presence of gametes (oocytes and sperm) and mature embryos of *Terpios hoshinota* in mid-June to mid-August in 2011. Yellow and black circles denote the full moon and the new moon, respectively. *Left figure*: percentage of gametes presented in 5 colonies, solid line for oocytes and dotted line for spermatic cysts, star marks: presence of spermatids, double-headed lines: presence of mature embryo. *Right figure*: the presence of gametes (oocytes and sperm) and mature embryos in each tagged colony. Dates in the figure show the examination dates with colored cells for the results. Red for oocytes, light blue for spermatic cysts, darker blue: spermatids in spermatic cysts, black: mature embryos.

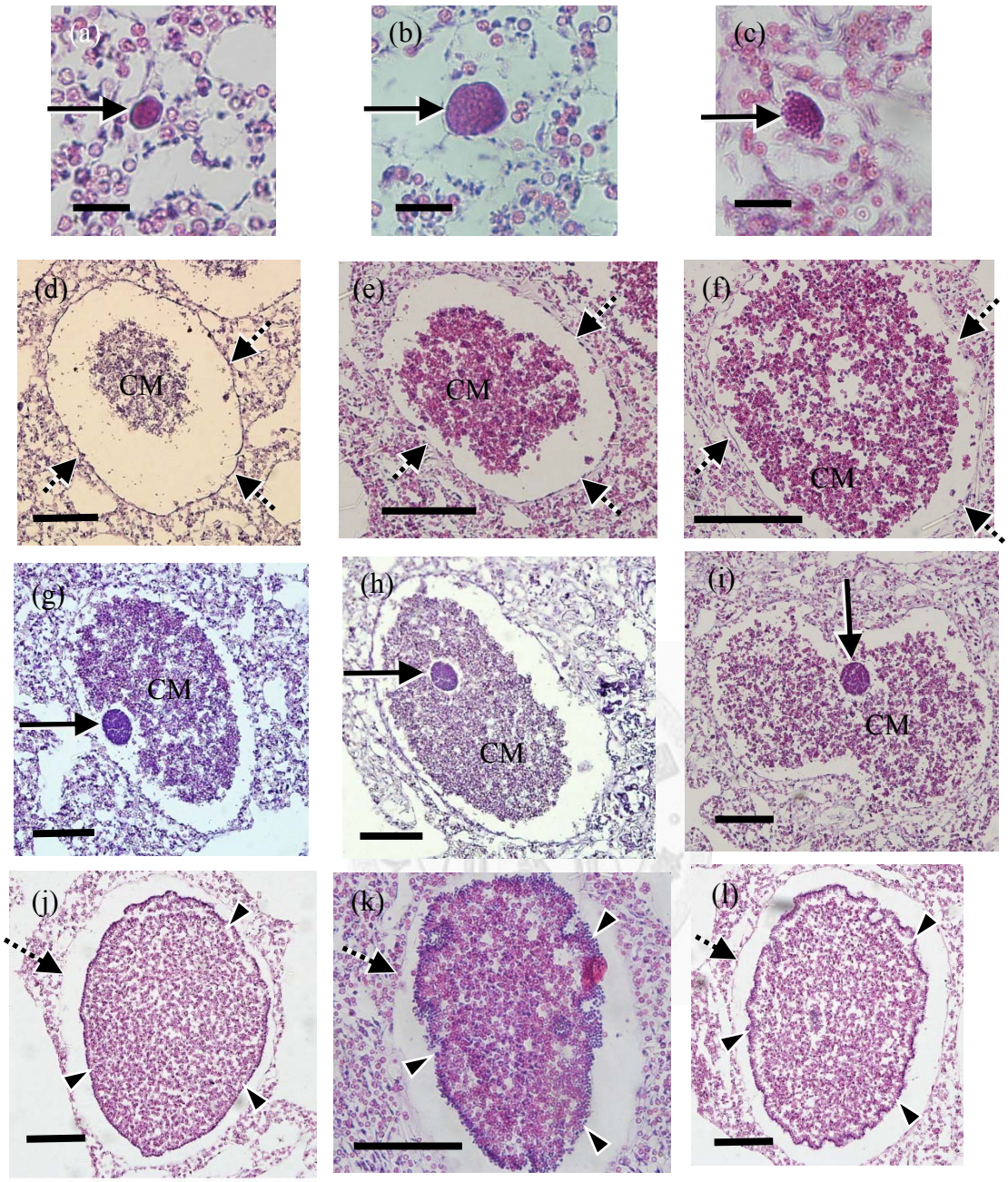


Fig. 9 Different stages of embryogenesis in *Terpios hoshinota*. Early embryos (morula) at ~8-cell stage (a), ~32 to 64-cell stage (b), and ~128-cell stage (c). Embryo-nursing chambers contained the "cell mass" which consisted of many symbiotic cyanobacteria and some maternal sponge cells that were transferred from adult (d-f). Embryos at blastula stage presented in embryo-nursing chamber (g-i). Mature embryos with a compact single layer of columnar sponge cells formed the outer layer and enclosed the "cell mass" (j-l). CM, "cell mass". Arrows, embryos at morula and early blastula stage;

dotted arrowheads, the edge of embryo-nursing chamber; arrowheads, outer layer of mature embryos. Scale bars: a-c = 20 μm, d-l = 100 μm.

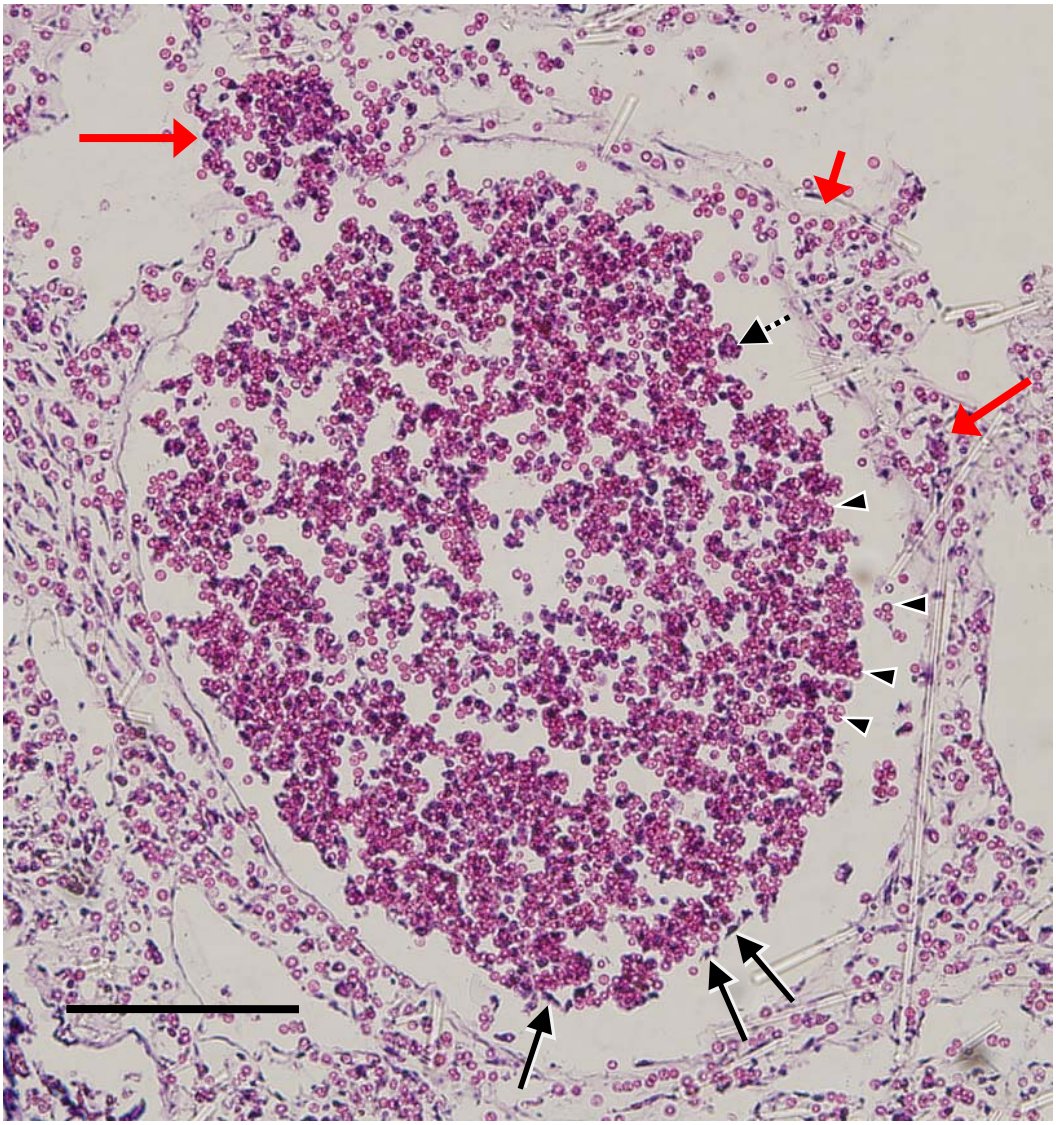


Fig. 10 Embryo-nursing chamber enclosed the “cell mass”, a zoom-in figure from fig. 9, f. Cyanobacteria and sponge cells were transferred from adult sponge (red arrows). Black arrows, sponge cells; arrowheads, cyanobacteria; dotted arrow, extraordinary large sponge cell. Scale bar = 100 μm.

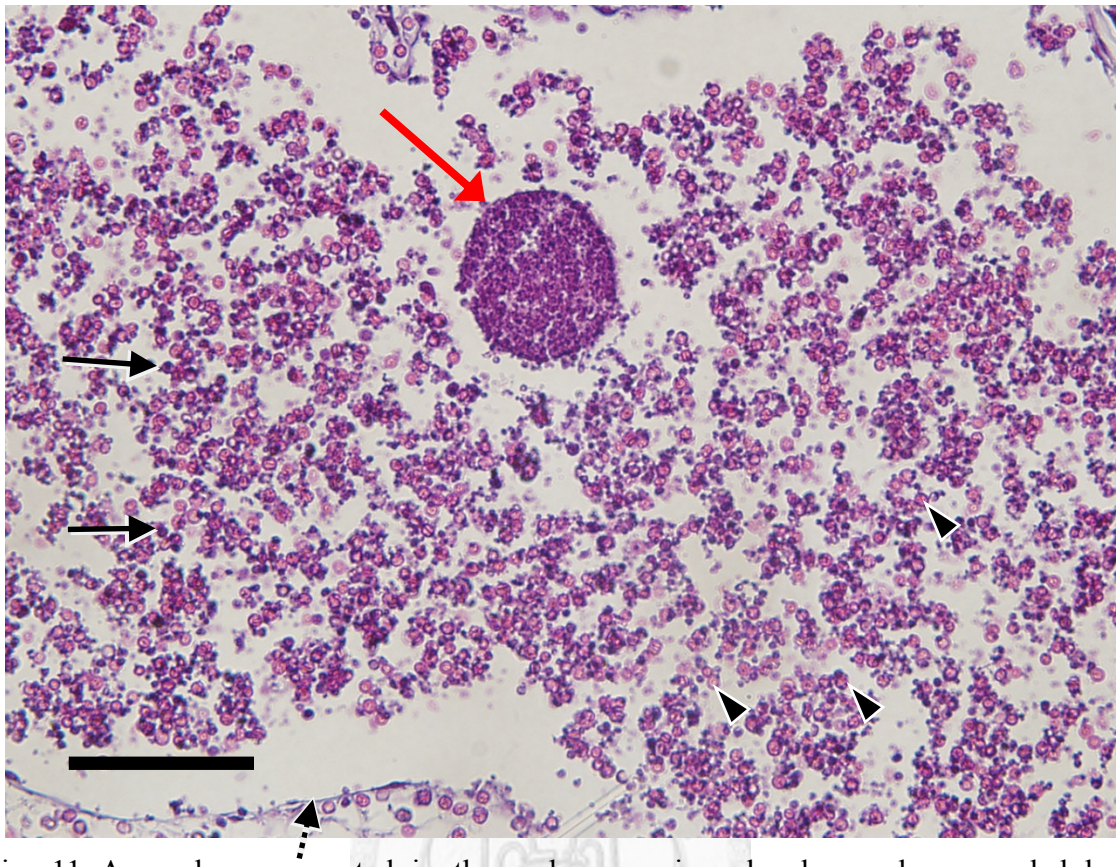


Fig. 11 An embryo presented in the embryo-nursing chamber and surrounded by cyanobacteria and maternal sponge cells, a zoom-in figure of fig. 9, i. Size of embryo was obviously larger than oocyte, suggesting the blastulation at this stage. Red arrow, embryo at blastula stage; black arrows, maternal sponge cells; arrowheads, cyanobacteria; dotted arrow, edge of embryo-nursing chamber. Scale bar = 100 μm.

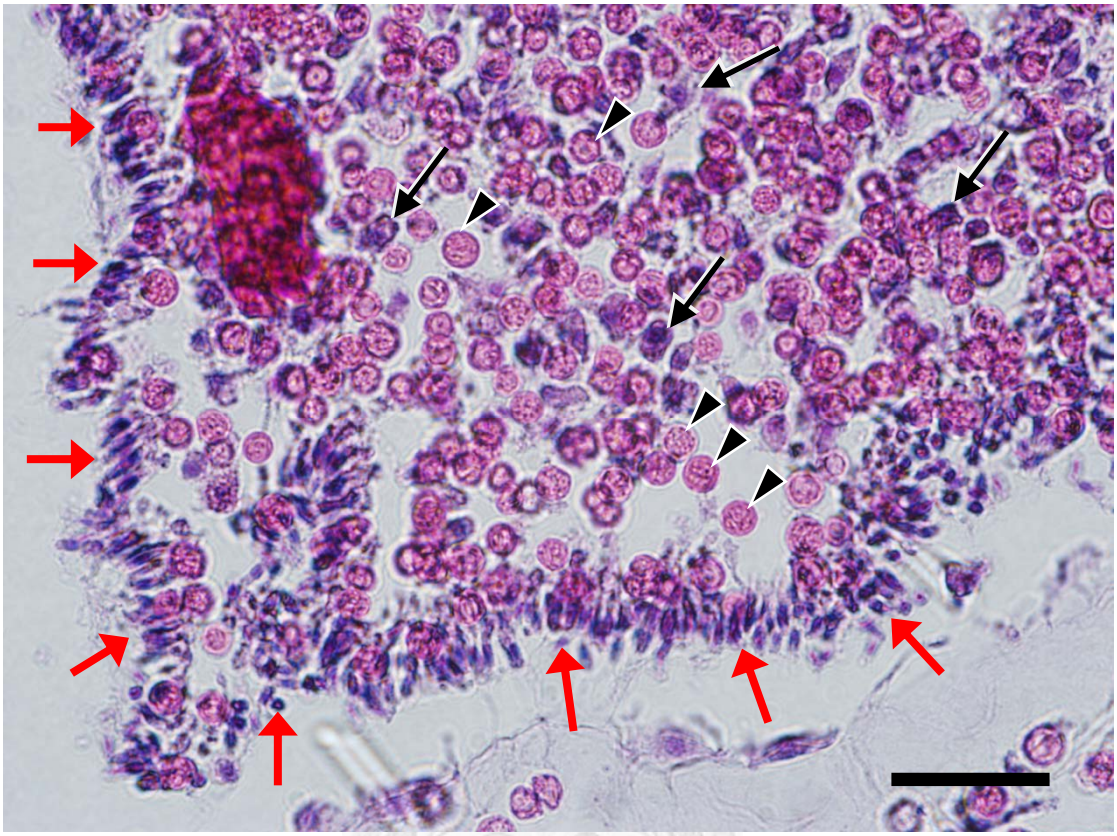


Fig. 12 Columnar embryonic cells (red arrows) enclose the “cell mass” which consisted of cyanobacteria (arrowheads) and maternal sponge cells (black arrows) to form a mature embryo. Scale bar = 20 μm.

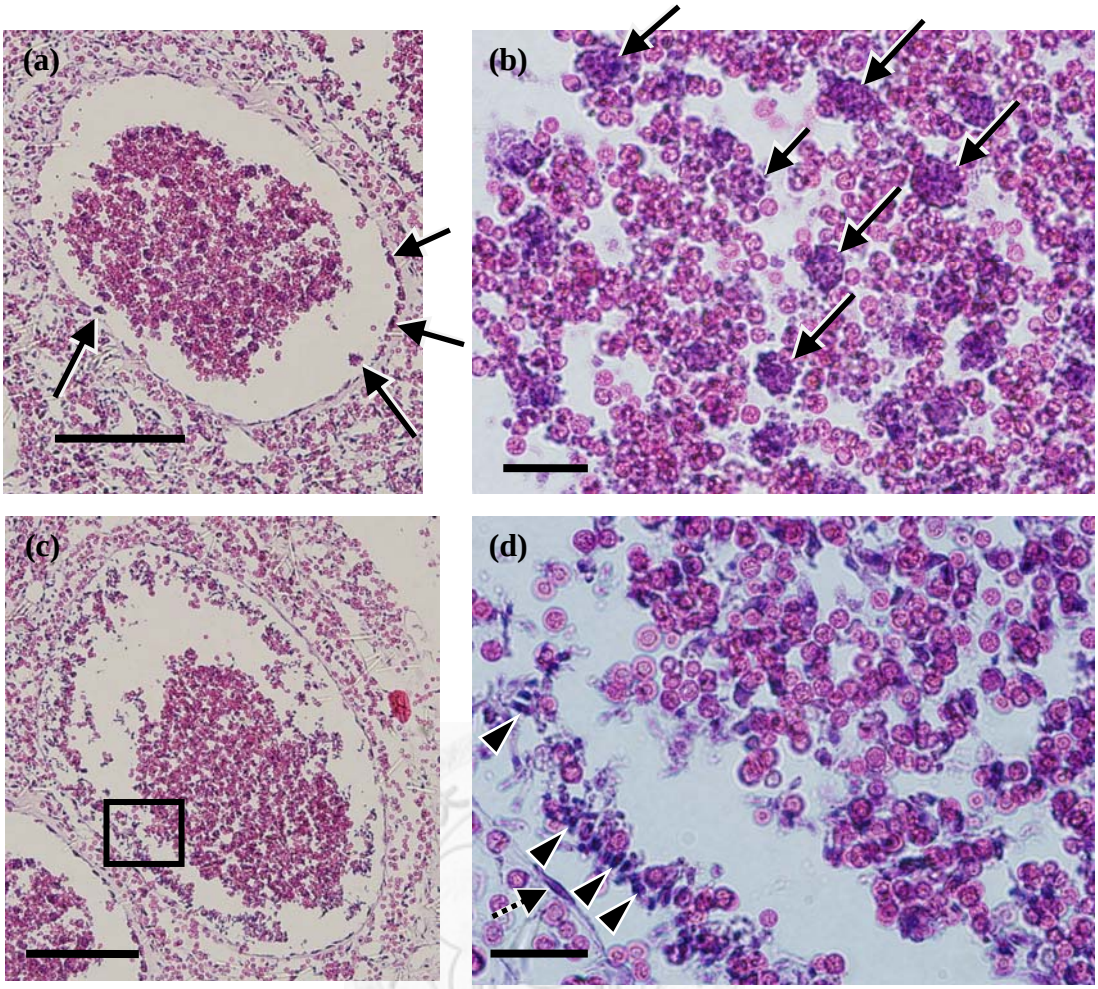


Fig. 13 Some stages in embryogenesis. (a) Some extraordinary large sponge cells (arrows) penetrated the pinacocyte layer of nursing chamber and accumulated in the nursing chamber. (b) A zoom-in figure of (a). (c) Embryonic cells presented near the periphery of nursing chamber. (d) A zoom-in figure of (c), tiny embryonic cells (arrowheads) with a vivid nucleus gathered near the periphery of nursing chamber. Dotted arrow, pinacocyte of nursing chamber. Scale bars: a, c = 100 μm, b, d = 20 μm.

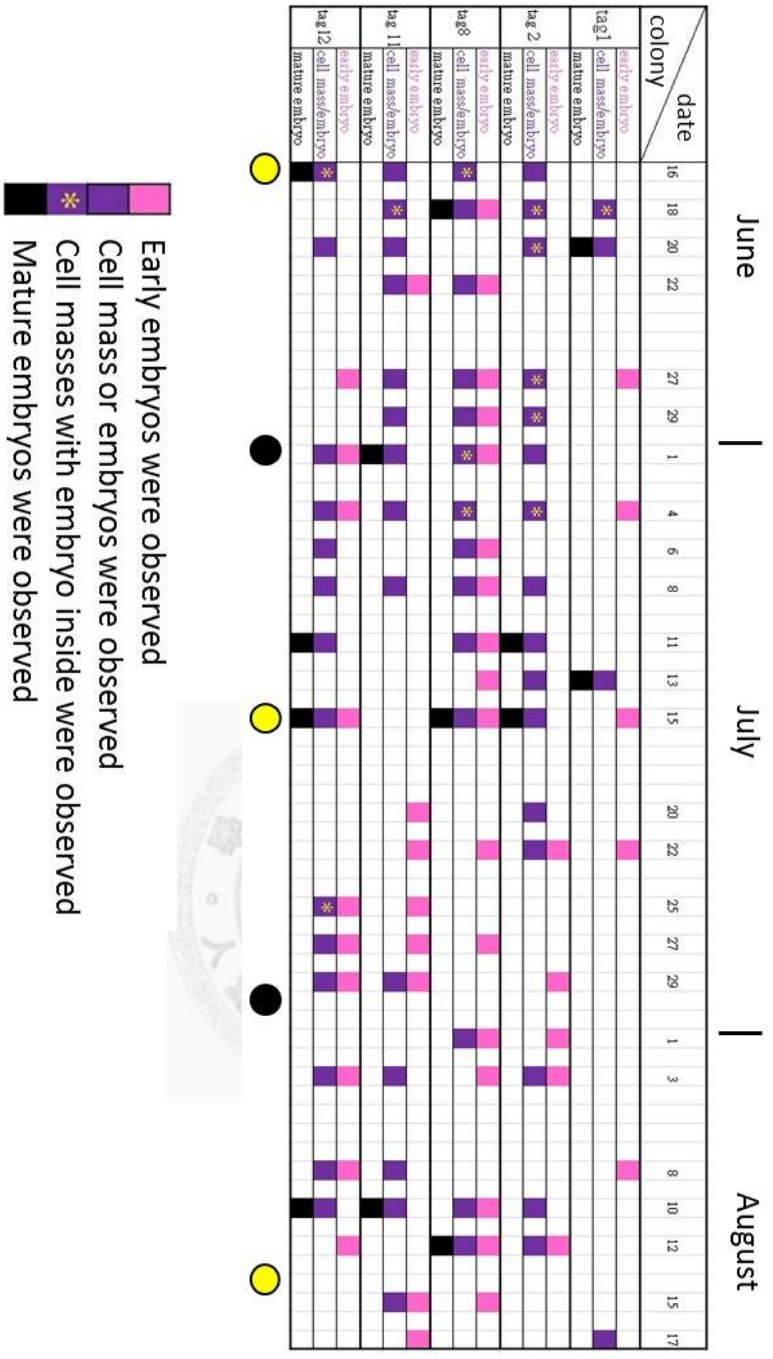


Fig. 14 Presence of the “cell mass” and embryos at different embryogenic stages in 5 *Terpios hoshinota* colonies at Green Island during the 2 month study period. Yellow and black circles denote the full moon and the new moon, respectively. Star marks: the dates when the embryo and the “cell mass” presented in one nursing chamber.

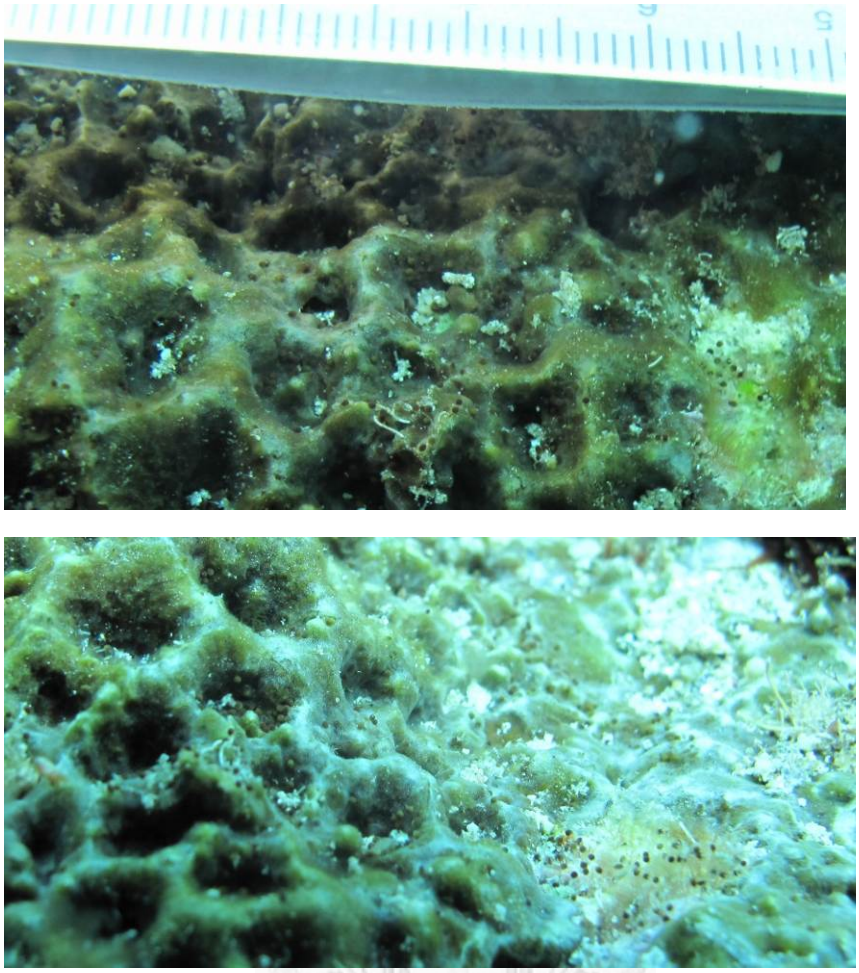


Fig. 15 *Terpios hoshinota* larvae on adult colony surfaces.

(a)

(b)

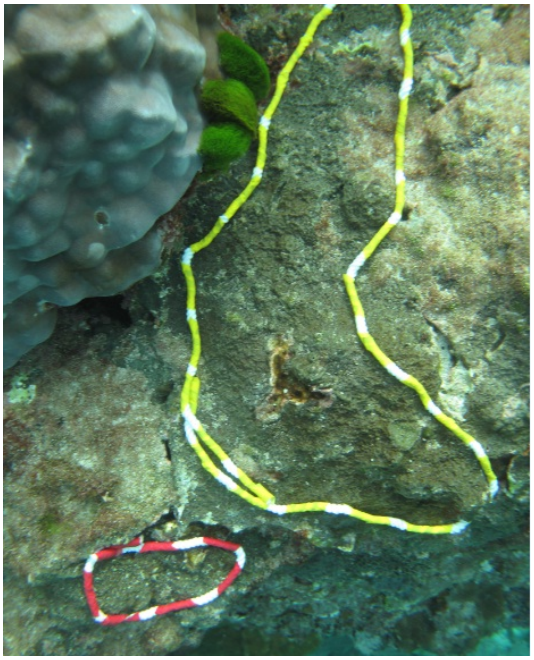


Fig. 16 Larval releasing areas on different colonies of *Terpios hoshinota*. Areas enclosed with color ropes (red and yellow) represented the areas with larvae. (a) Larvae presented on only a few areas of this colony. (b) The area with larvae was almost all the surface of this colony (yellow line).

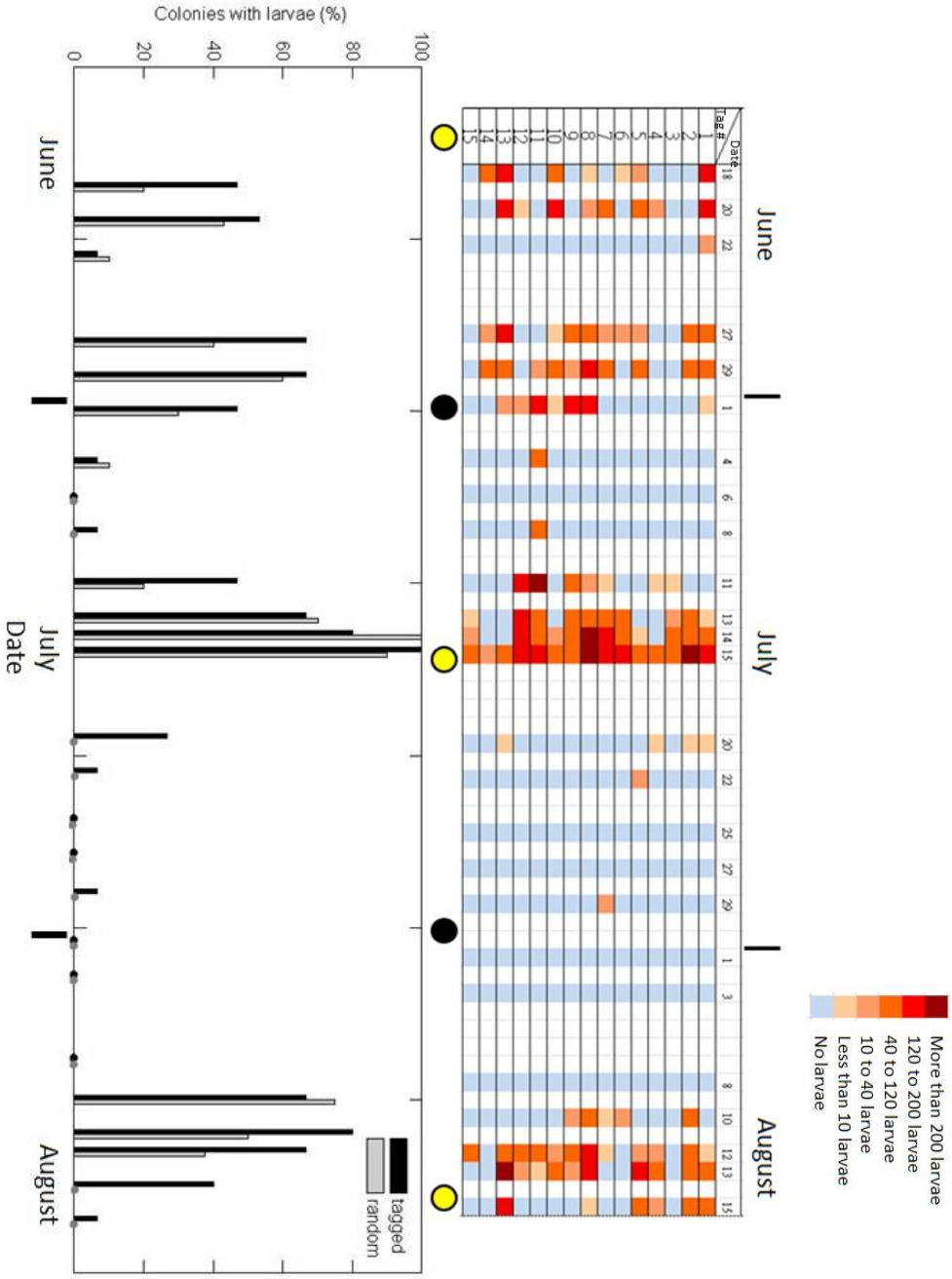


Fig. 17 *In situ* larval release patterns of *Terpios hoshinota* in Green Island for 2 months from mid-June to mid-August, 2011. Yellow and black circles denote the full moon and the new moon, respectively. *Left figure*: frequency of colonies with larvae. Black bars for the 15 tagged colonies and gray bars for 10 haphazardly chosen colonies at each examination. *Right figures*: The estimated amount of larvae in 160 cm^2 area on each tagged colony. Dates in the figure show the examination dates with colored cells for the results.

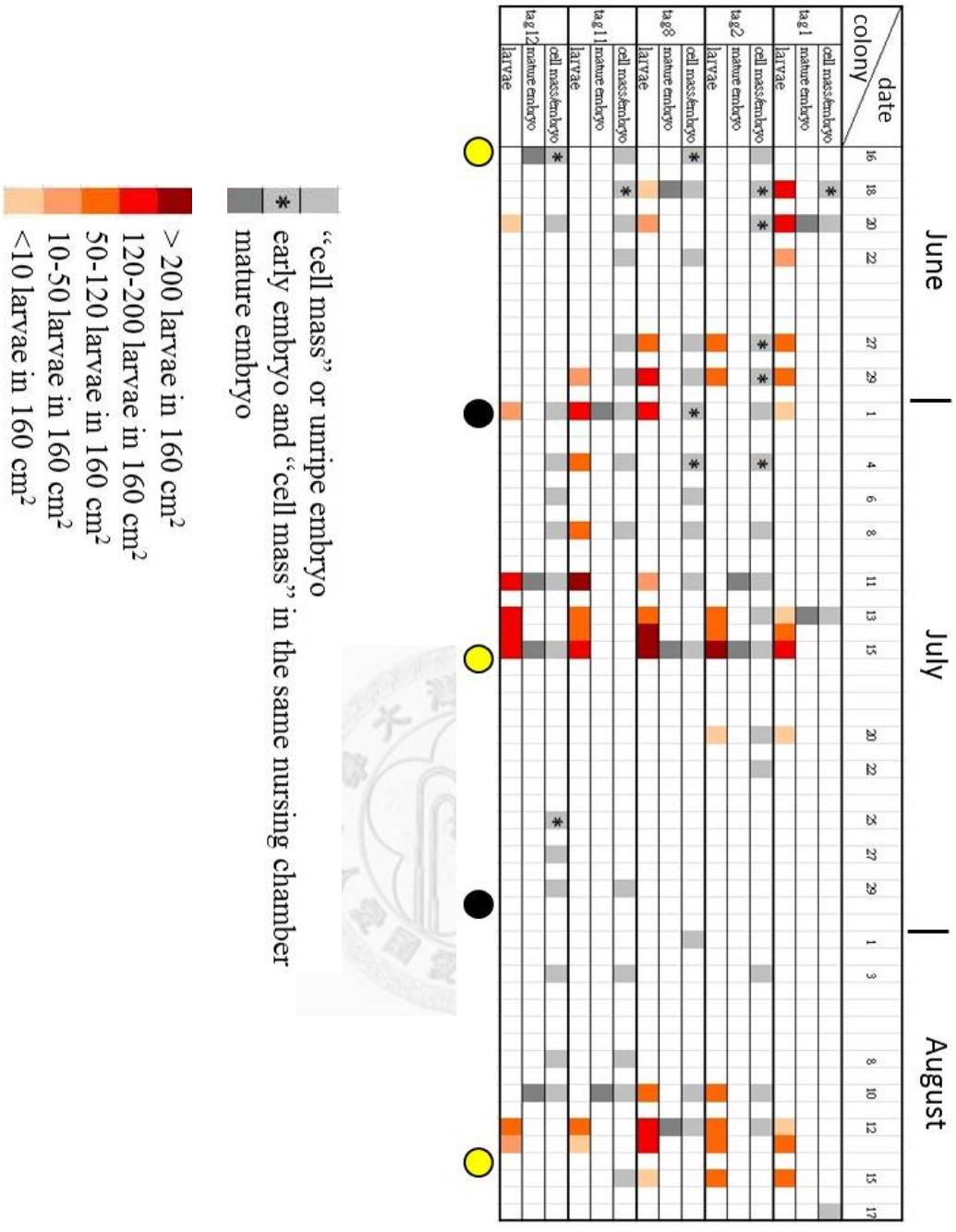


Fig. 18 Presence of embryos and larvae in 5 tagged *Terpios hoshinota* colonies in Green Island for 2 months from mid-June to mid-August, 2011. Yellow and black circles denote the full moon and the new moon, respectively.. Presence of mature embryos and unripe embryos were marked separately as: "cell mass", corporation of early embryo and "cell mass" and mature embryos. Amounts of larvae were marked by different colors.

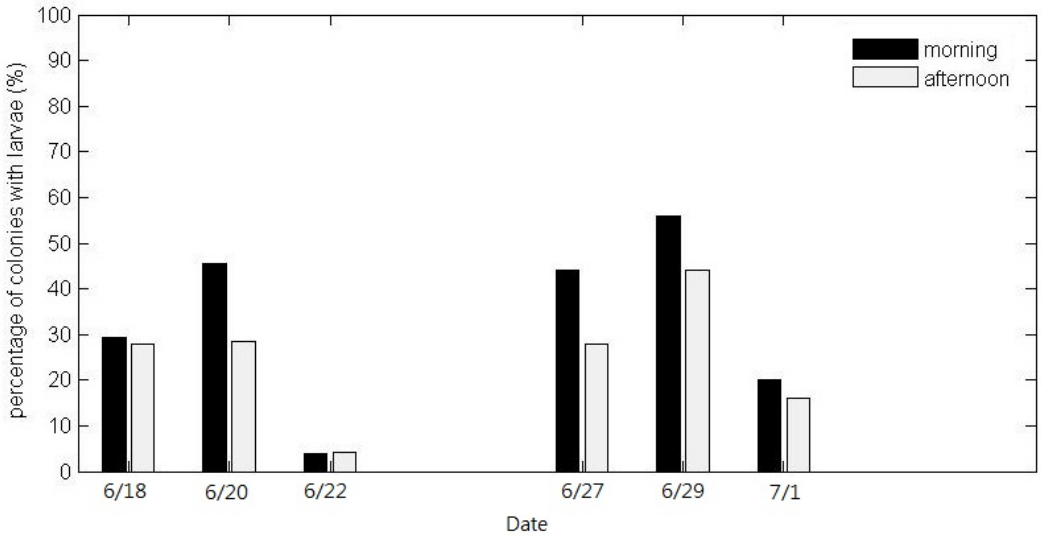


Fig. 19 Comparison of larval release between the morning (black bars, 10-11 a.m.) and the evening (gray bars, ~4 p.m.). Frequencies of the 15 tagged colonies with larvae are shown.

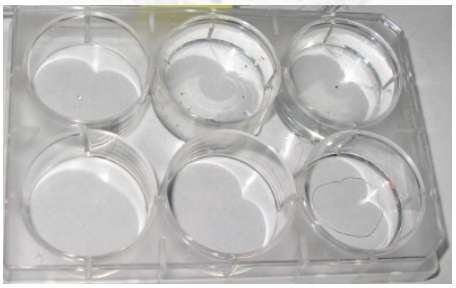


Fig. 20 Tissue culture plates used in the settlement experiments for larvae of *Terpios hoshinota*.

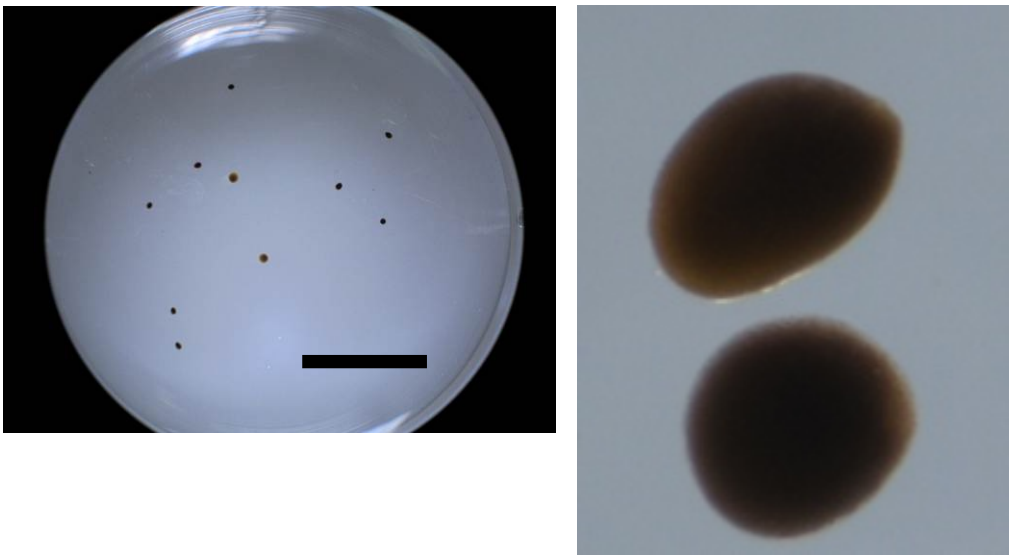


Fig. 21 (a) Larvae of *Terpios hoshinota* in a well of the tissue culture plate. Some larvae have been settled. Scale bar = 1 cm. (b) Larvae of *T. hoshinota*, dark brown and round to sesame shape (~600 μm in diameter).

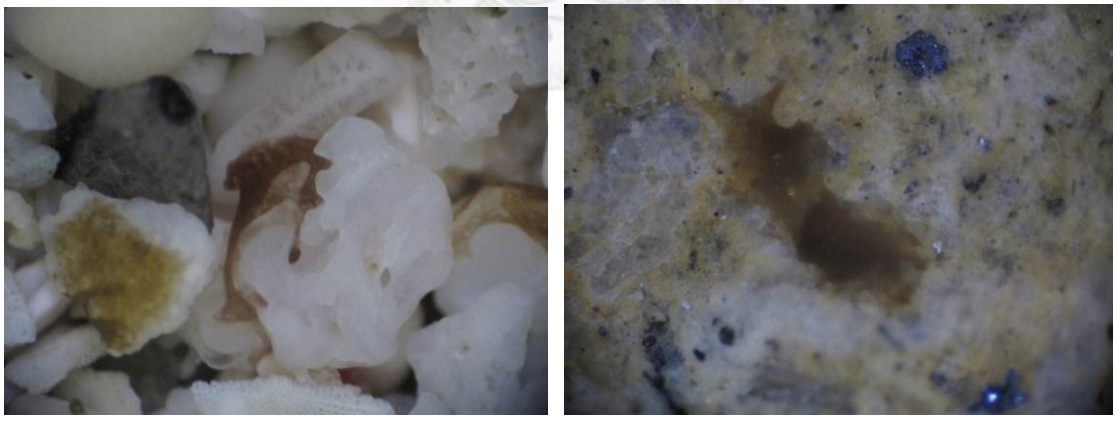
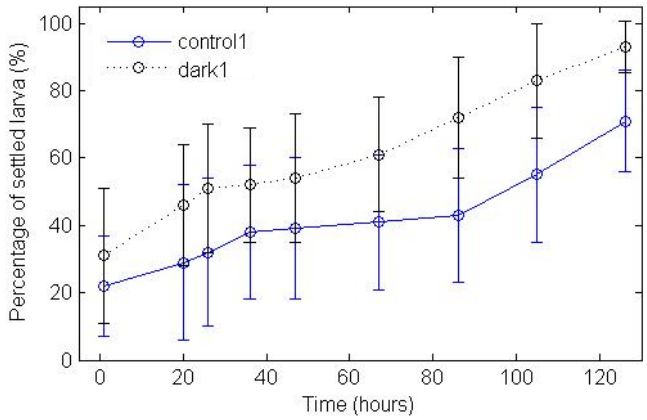
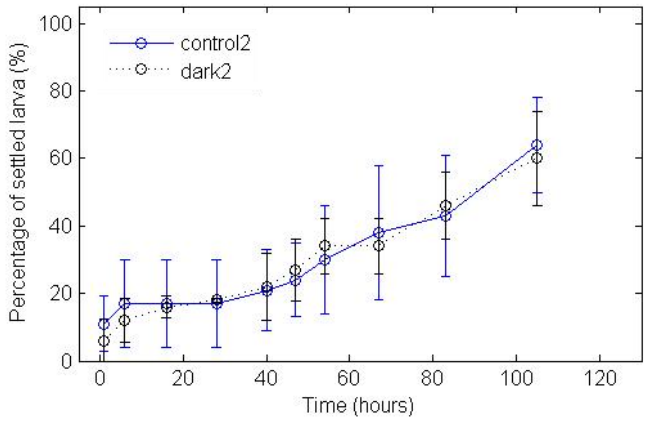


Fig. 22 *Terpios hoshinota* larvae settling on sand (left) and stone (right).



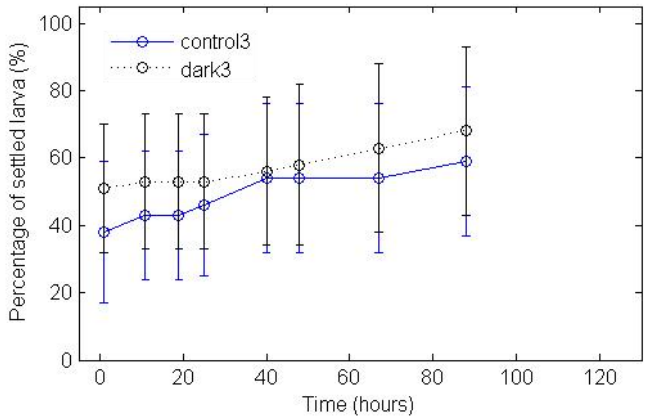
August 12

Sample size: 10 larvae well^{-1}



August 13

Sample size: 12 larvae well^{-1}



August 14

Sample size: 6 larvae well^{-1}

Fig. 23 Settlement patterns of *Terpios hoshinota* larvae collected on August 12th, 13th and 14th, 2011. There were 6 replicated wells for each light condition on each day. X axis: time after the start of the experiment. Solid line: control condition (12 h light/ 12 h dark). Dotted line: dark condition: (24 h dark).

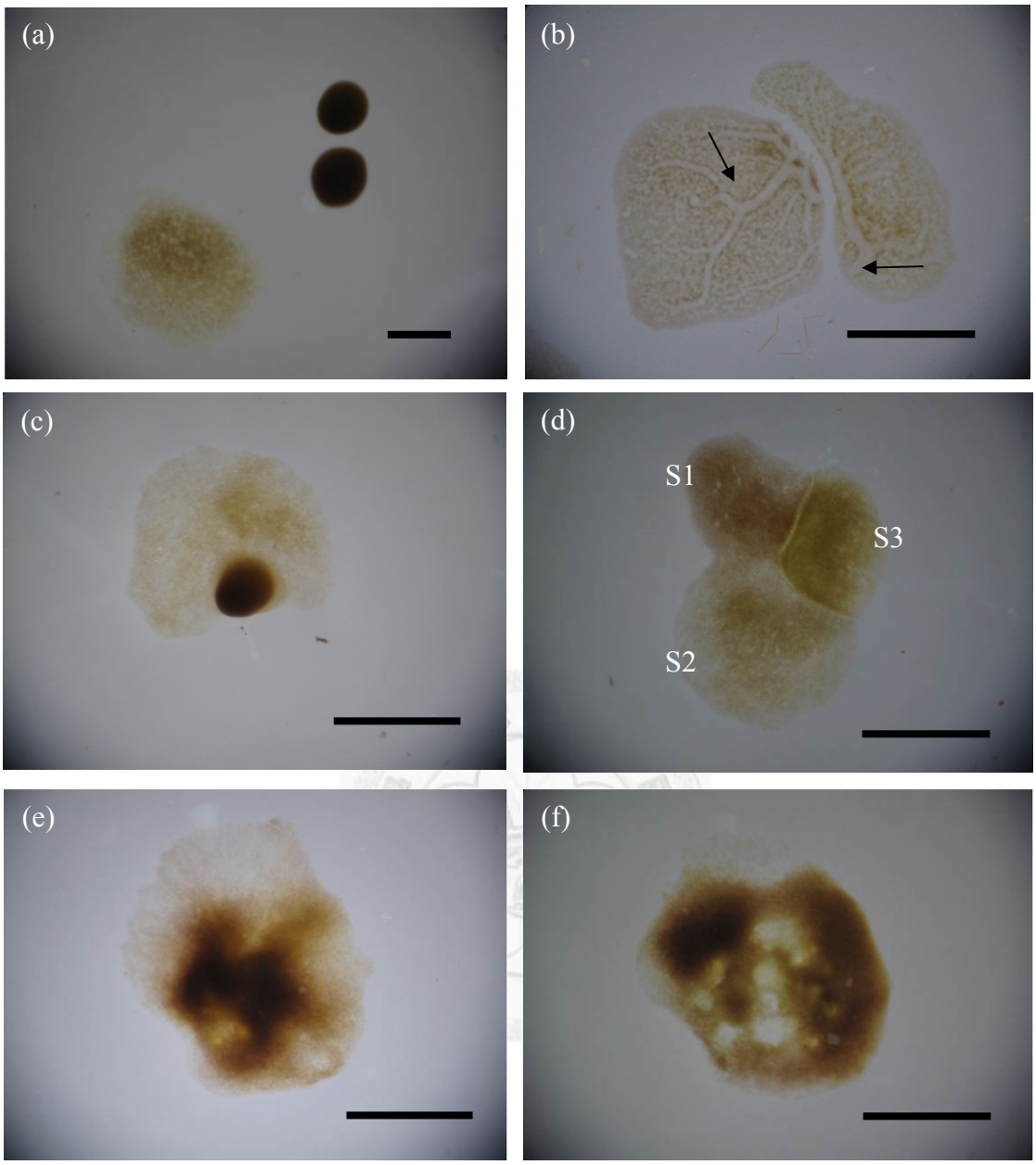


Fig. 24 Photographic images of *Terpios hoshinota* larvae and settlers. (a) Two larvae and one settler. (b) After 106 hours cultured, cannels of water filtering system have been formed. (c) One larva was going to settle near a settler. (d) Three settlers named S1, S2 and S3 here. S1 and S2 fused together with no barrier. Barriers formed between S1/S3 and S2/S3. (e, f) 6 larvae that fused in the beginning (e), but after a while, the chimera stopped growing and started to degrade (f). Cultured time: a = 14 h, b = 106 h, c &d = 20 h, e = 13 h, f = 38 h. Scale bars = 0.5 mm in (a) and 1 mm for others.

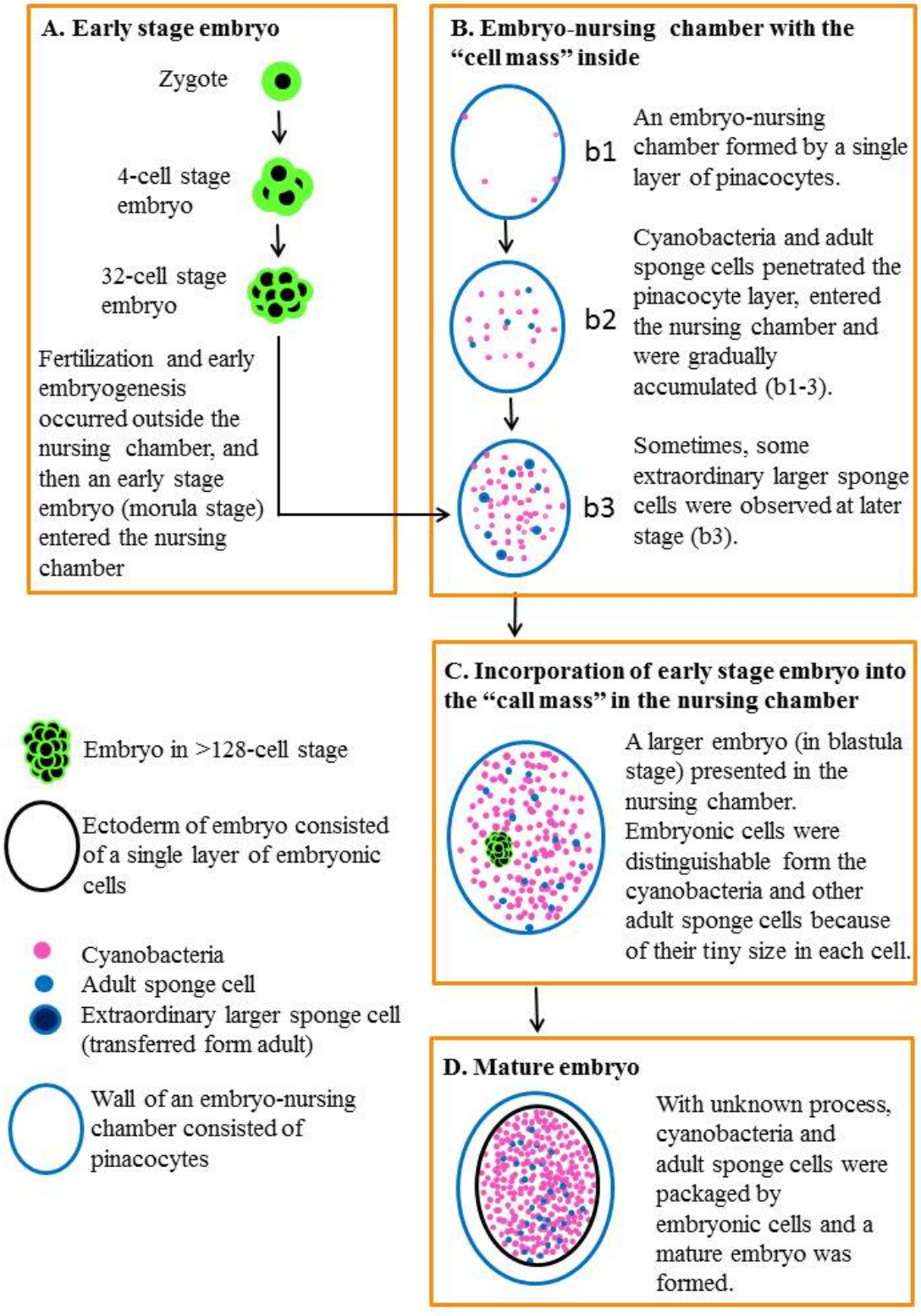


Fig. 25 A possible embryogenesis process of *Terpios hoshinota*. A: A zygote undergoes cleavage to form early embryo (morula); the number of cell increase in the early embryo

but its volume does not increase. B: A chamber surrounded by pinacocytes forms (b1). Cyanobacteria and maternal sponge cells start to be accumulated in this chamber gradually (b1-b3). The mass of all the cells inside the nursing chamber is termed the "cell mass". There is no embryonic cell in the nursing chamber at this stage. Sometimes, some larger sponge cells present in the nursing chamber (b3). C: Early embryo enters the nursing chamber. The whole cell mass with the early embryo inside the nursing chamber is considered as an embryo now. The embryogenic process from this stage to the end of embryogenesis may take 11-16 days. D: Finally, the embryo develops to form a mature embryo with huge amount of cyanobacteria and some sponge cells enclosed by a single outer layer consisted of columnar embryonic cells.

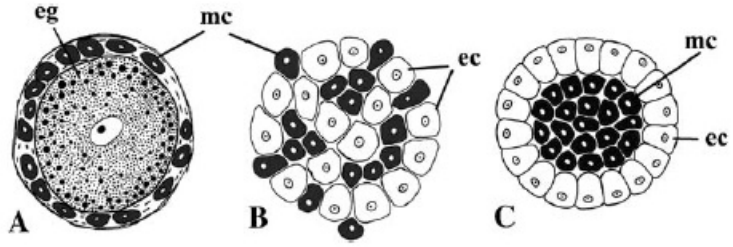


Fig. 4. Morphogenesis associated with penetration of maternal cells into the morula in *Chondrosia reniformis* (the class Demospongiae). (A) egg (eg) surrounded by maternal cells (mc); (B) penetration of maternal cells (mc) into the morula; (C) pseudoblastula with embryonic cells (ec) at the periphery and maternal cells (mc) inside.

Fig. 26 Diagram of embryogenesis of *Chondrosia reniformis* demonstrated how somatic cells entered the embryo (Ereskovsky & Dondua, 2006).

species	size of oocyte (micrometer)		g/h	o/v	time needed for gametogenesis		Reference
	early oocyte	mature oocyte			oogenesis	spermato-genesis	
Chondrosia australiensis	20	55	g	o	4 w	2 w	Usher et al., 2004
Chondrosia reniformis	35	65	g	o	2-3 m	<1m	reviewed in Riesgo & Maldonado, 2008
Corticium candelabrum	15	125-175	h	v			reviewed in Riesgo & Maldonado, 2008
Petrosia ficiformis	30	200	g	o	7 m	2.5 w	Maldonado & Riesgo, 2009
Axinella damicornis	23	150	g	o	7-8 m		Riesgo & Maldonado, 2009
Raspaciona aculeata	50	160-180	g	o	3-5 m	1-2 m	Riesgo & Maldonado, 2009

Table 1 Size of oocytes and time needed for gametogenesis in sponges. g, gonochoristic; h, hermaphrodite; o, oviparous; v, viviparous.

Date	Depth	Total number of colonies observed	Number of colonies with larvae	Percentage
2011/07/15	3 m	25	24	96%
	10 m	7	2	29%

Table 2 Comparison of frequency of *Terpios hoshinota* colonies with larvae at the depths of 3 m and 10 m.

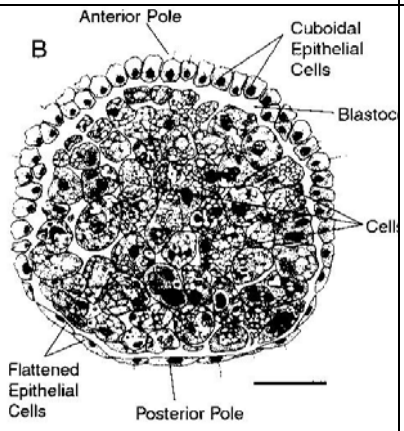
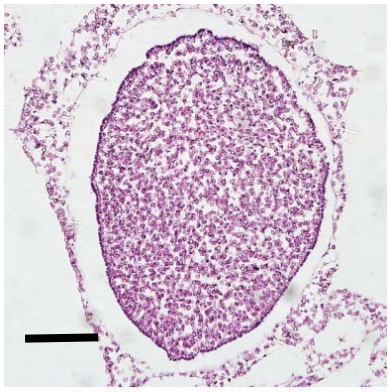
Larval type	*Clavablastula*	*Embryo of Terpios hoshinota*
Morphology		
Developing time	3h-3d	Longer
Size (diameter)	~250 μm	~600 μm
Entirely ciliated	Yes	Yes (Wang et al., 2012)
Epithelial layer	Cubical single layer	Columnar or cubical single layer
Size of blastocoel	Large	Large
Origin of cells in blastocoel	Derived from mother sponge and/or embryo itself	Symbiotic cyanobacteria and somatic cells transferred from mother sponge

Table 3 Characteristics of Clavablastula and embryo of *Terpios hoshinota.* Scale bars: clavablastula = 20 μm, embryo of *Terpios hoshinota* = 100 μm.