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Cellular and Biochemical Changes in Early Embryonic Development of a Scleractinian Coral, *Fimbriaphyllia* (*Euphyllia*) *ancora*

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Knowledge of early life histories of scleractinian corals is essential for ecological studies (*e.g.*, larval dispersion and connectivity) and development of aquaculture techniques. The present study documents cellular and biochemical changes during early development of a scleractinian coral, *Fimbriaphyllia ancora* (Order Scleractinia, Family Euphyllidae). Observations of spawning revealed that *F. ancora* releases positively buoyant eggs. No fertilization membrane surrounded fertilized eggs, which developed into swimming planula larvae within 48 h after fertilization. Lipid content analysis showed that eggs are rich in wax esters, and that the wax ester concentration decreases significantly in planulae. Sugar content analysis revealed that the eggs are also rich in glycogen, and that the glycogen concentration increases as development progresses. Free glucose was not detected in samples that we analyzed. Moreover, a settlement assay showed that *F. ancora* planulae prefer to settle on dead coral debris, compared to other substrate materials, such as plastic, microscope slides, ceramics, and crustose coralline algae.

Key words: Scleractinian corals, Embryogenesis, Wax ester, Glycogen, *Fimbriaphyllia ancora*

BACKGROUND

Scleractinian corals are the keystone animals that create coral reef ecosystems, which support the highest levels of marine biodiversity on earth (Odum and Odum 1955). Knowledge of early life histories of scleractinians is essential to predict larval dispersion and genetic connectivity among populations (Graham et al. 2013). Moreover, interest in coral aquaculture has greatly increased worldwide, providing products/materials for research, environmental education programs, ornamental coral trading, and drug discovery (Epstein et al. 2003; Rinkevich 2008; Leal et al. 2014; Omori 2019). Better understanding of embryogenesis and behavioral characteristics of larvae is essential to further develop coral aquaculture.

Many scleractinian corals release positively buoyant eggs or egg-sperm bundles into the water column during spawning. Fertilized eggs generated near the ocean surface undergo embryogenesis while drifting with the current, and develop into free-swimming larvae called planulae within a few days. Thereafter, planulae settle on appropriate substrates, and finally, metamorphose into primary polyps to begin benthic

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life (Harrison and Wallace 1990). However, changes at the molecular and cellular levels that support these morphological transformations are little understood.

To better understand early life histories of scleractinian corals, the present study investigated cellular and biochemical changes during early development of the scleractinian coral, *Fimbriaphyllia ancora* (formerly called *Euphyllia ancora*) (Luzon et al. 2017). This species belongs to the family Euphyllidae and is one of the most widely distributed reef-building corals in the Indo-Pacific region (Luzon et al. 2017). As such, it is a useful experimental animal to study scleractinian reproductive biology (Shikina et al. 2012 2013 2020b; Chiu et al. 2020). A decrease in the level of a major yolk protein, vitellogenin (Vg), during early development has been reported in this species, and has been proposed as a possible energy source for embryonic development (Shikina et al. 2013).

In the present study, we first examined whether eggs of *F. ancora* are buoyant or non-buoyant by observing spawning behavior, and then described morphological and histological features of early development. Subsequently, we focused on lipid and sugar contents, and described changes in these metabolites during early development. Then we investigated substrate settlement preferences of *F. ancora* planulae.

MATERIALS AND METHODS

Sampling

Fragments of *F. ancora* colonies (~5 cm in length) were collected by scuba divers at Kenting National Park, in southern Taiwan, 1 week before the predicted spawning date, under a permit from the administrative office of the park. Coral fragments were transferred to Tungkang Biotechnology Research Center, in southern Taiwan, and maintained in outdoor fiber-reinforced plastic (FRP) tanks (250 L) under natural daylight (approximately 12.5L:11.5D) at 26–28°C. To record spawning, some fragments were maintained in aquaria (30 L) and monitored after dark from 6 to 9 pm. Fertilized eggs obtained in aquaria/FRP tanks were reared in 500-mL beakers with filter-sterilized seawater (FSW), and collected at different time points for subsequent analyses.

Histological analysis

Samples were fixed with 4% paraformaldehyde and 2% glutaraldehyde (Sigma-Aldrich, St. Louis, USA) in 100 mM HEPES buffer (pH 7.4). Fixed samples were dehydrated and embedded in paraplast (Thermo Fisher Scientific, Waltham, MA). Serial sections (4 μm) were prepared with a microtome (Thermo Shandon, Pittsburgh, USA), and rehydrated sections were stained with haematoxylin and eosin Y (Thermo Shandon, Pittsburgh, USA). Sections were observed and photographed with an Olympus IX71SF1 microscope (Tokyo, Japan).

Lipid extraction and analysis

Total lipids were extracted from released unfertilized eggs, early planulae (48 hpf) and planulae (168 hpf), according to the methodology of Bligh and Dyer (1959). High-performance thin-layer chromatography (HPTLC) was performed to analyze lipid contents, according to the methodology of Okubo et al. (2020). To determine lipid concentrations in samples, a standard lipid mixture containing wax esters (WEs), phospholipids, triglycerides, cholesterol, cholesterol-esters, and fatty acids (Sigma-Aldrich) was prepared, and spotted on plates (HPTLC Silica gel 60 F 254 plate; Merck, Darmstadt, Germany) at different concentrations (0.25, 0.5, 1, and 2 mg/mL). Developed plates were scanned, and bands were quantified using Image J64 software (National Institutes of Health, Bethesda, MD). Lipid contents in samples were determined from the standard curve of each standard lipid.

Determination of glycogen and glucose levels

Fifty unfertilized eggs, embryos, or planula larvae were homogenized in 0.6 M perchloric acid (PCA). Glycogen and glucose were measured enzymatically (Keppler and Decker 1986). To assay glycogen, pH of the homogenate was adjusted to 4.8 with 2 M KOH and incubated with amyloglucosidase (Sigma-Aldrich) in 1.0 M acetate buffer at pH 4.8 for 2 h at 40°C. After adding 0.6 M PCA, the medium was centrifuged at 10,000 g for 10 min at 4°C. After neutralization to pH 6.5–7.0 with 2 M KOH, supernatant was used to assay hydrolyzed glycogen as glucose. Neutralized supernatant was mixed with 0.3 M triethanolamine buffer (pH 7.5) containing 1 mM ATP, 1 mM NADP+ and 4 mM $MgCl_2$. After adding hexokinase/glucose 6-phosphate dehydrogenase (Sigma-Aldrich), glucose was measured by a standard method involving conversion of NADP+ to NADPH, determined as the rate of increase in absorbance at 340 nm using a Shimadzu UV-1700 spectrophotometer.

Settlement assay

Four types of substrates, *i.e.*, microscope glass (Thermo Shandon), crustose coralline algae (CCA) (*Hydrolithon* sp.), dead coral debris, and ceramic tile, were put in plastic culture dishes (9 cm in diameter, Jet Bio-Filtration, Guangzhou China) filled with 30 mL of filter-sterilized seawater (FSW). CCA were collected along the northern coast of Taiwan. Ceramic tile was purchased at a local market. Coral debris (a mixture of skeletons from various coral species) was purchased at a local aquarium shop. Ceramic tile and coral debris were washed at least 3 times with FSW and autoclaved before use. Plastic culture dishes filled with 30 mL of FSW without substrates were used as the control. Twenty planula larvae (at 64–128 hpf) were added to each dish, and maintained for 6 days at 24–26°C under a 12/12 h light-dark cycle. Larval settlement was determined under a microscope after 6 days of culture. These experiments were performed three times using larvae collected from different colonies.

Statistics

All data are shown as means ± standard errors (SE). Statistical differences between groups were determined using one-way ANOVA followed by Tukey's test with a statistical significance level of $p < 0.05$. Statistical Package for the Social Sciences (SPSS) was used for the analysis.

RESULTS

Fimbriaphyllia ancora releases positively buoyant eggs

At 7:30 pm, 25 May 2011, one day after the predicted spawning date, spawning of *F. ancora* was observed in aquaria (Fig. 1A). Dozens to hundreds of pinkish eggs were released through the mouths of *F. ancora* polyps, and slowly floated to the surface (Fig. 1B), demonstrating that the eggs are positively buoyant. Microscopic observation of eggs found that they had no symbiotic algae. Unfertilized eggs underwent cell death approximately 12 h after spawning.

Features and time course of early development

No fertilization membrane surrounded fertilized eggs (Fig. 2A and A'). The first cleavage was observed at ~1–2 hpf. The cleavage furrow initiated at the animal pole, and heart-shaped zygotes were observed. Cleavage was holoblastic, and embryos reached the 16–64-cell stage at 3–6 hpf (Fig. 2B and B', Table 1). Blastomeres divided in a more or less organized manner, and became

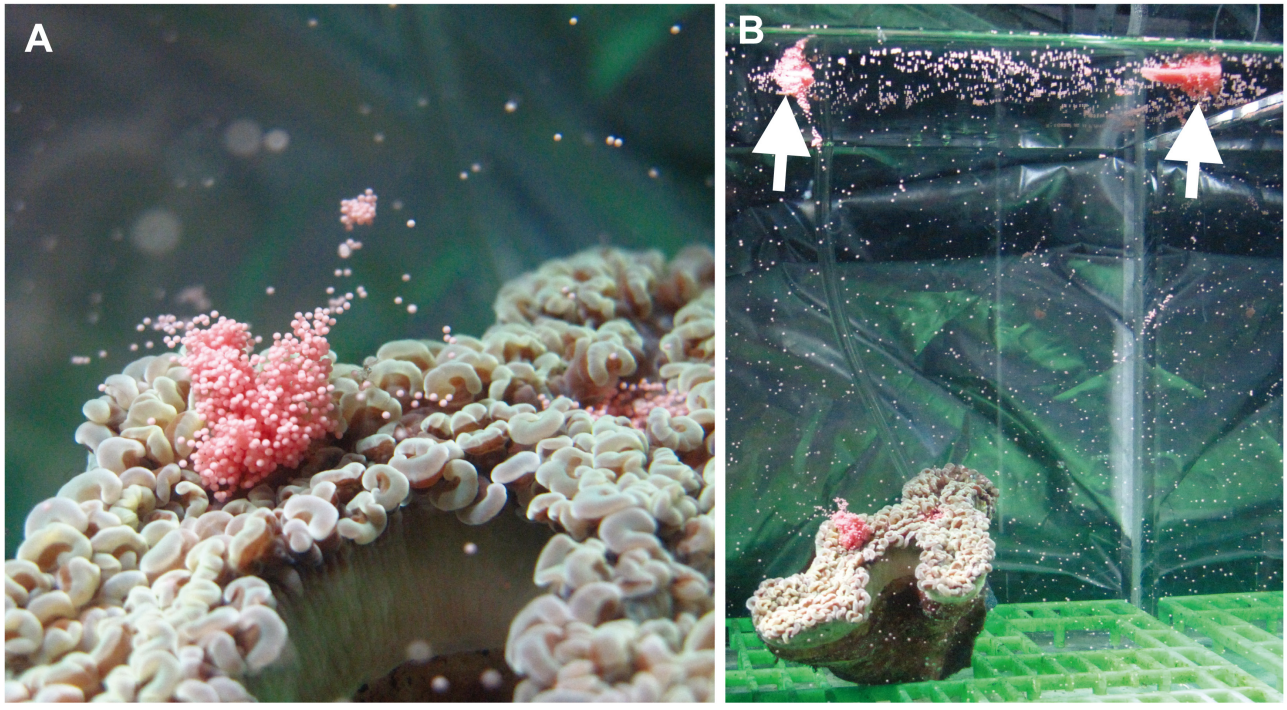

Fig. 1. *Fimbriaphyllia ancora* spawning in an aquarium. A: A female colony releasing pinkish eggs. B: Picture of the aquarium with spawning *F. ancora*. Note the released eggs floating at the surface (arrows).

flattened embryos (prawnchip stage) at 7–12 hpf (Fig. 2C and C', Table 1). After the bowl-shaped stage, spherical blastulae were formed at 14–16 hpf, and embryos were observed moving slowly, using cilia (Fig. 2D and D', Table 1). Initially lipid droplets in fertilized eggs were small and evenly distributed (Fig. 2A'), while those after the blastula stage were relatively large and distributed around the center (Fig. 2D').

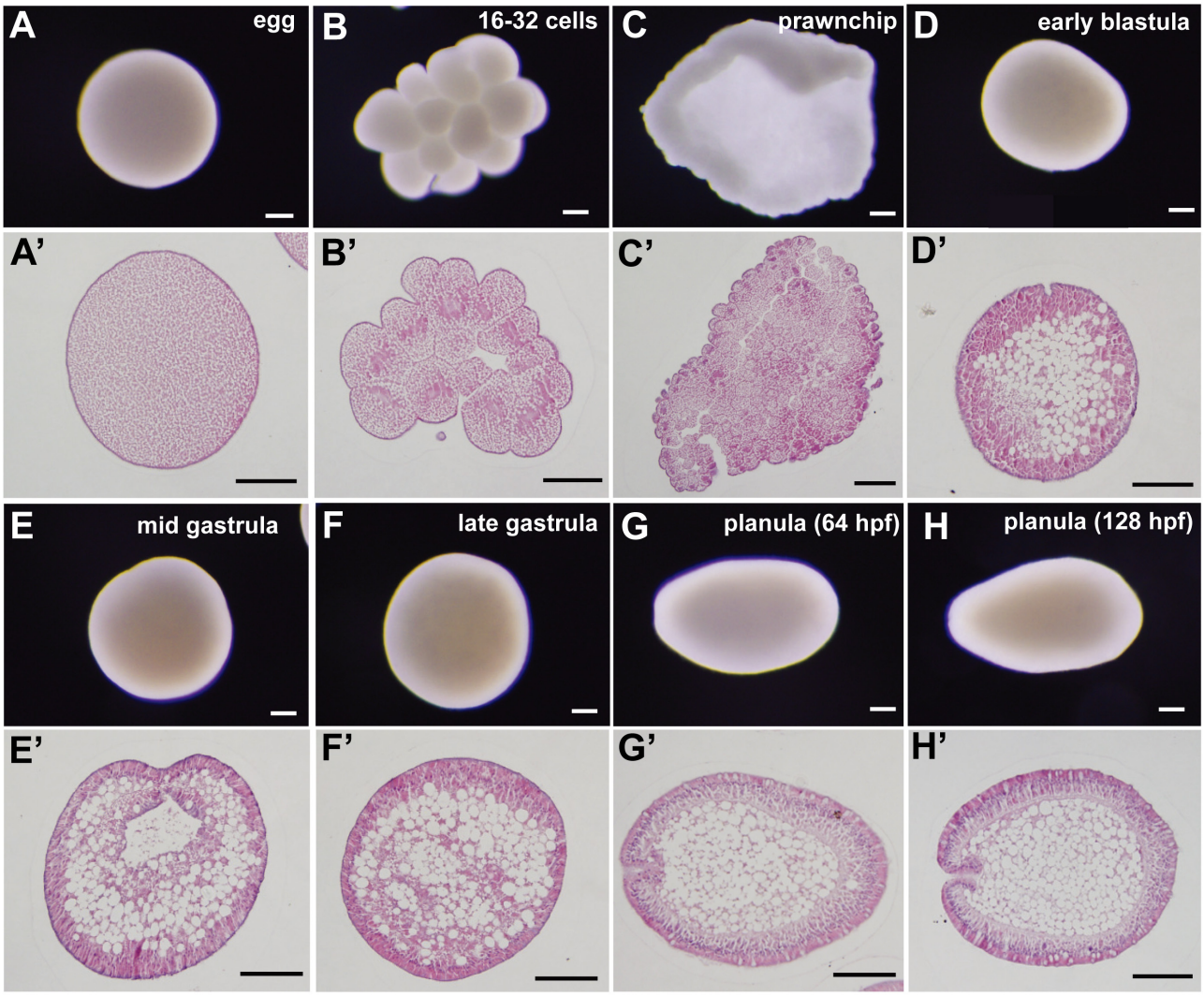

Fig. 2. Early development of *F. ancora*, as assessed microscopically and histologically. External and histological views of unfertilized eggs (A and A'), 16–32 cell stage (B and B'), prawnchip stage (C and C'), early blastula stage (D and D'), mid gastrula stage (E and E'), late gastrula stage (F and F'), planula at 64 hpf (G and G'), and planula at 128 hpf (H and H'). All scale bars = 100 μm.

Table 1. Summary of embryonic and larval development of *F. ancora*

Hour post fertilization (hpf)	Stage	Remarks
1-2 hpf	2 cells	Heart shaped
3-6 hpf	16-64 cells	Holoblastic cleavage
7-12 hpf	Prawnchip	Flatten (prawnchip) shaped
14-18 hpf	Blastula	Spherical shaped, Slow swimming
24-28 hpf	Early gastrula	Spherical shaped, Slow swimming, Blastopore invagination
32-36 hpf	Gastrula	Spherical shaped, Slow swimming, Formation of ectoderm and endoderm
48 hpf-	Planula	Pear shaped, Swimming, Formation of oral pore with pharynx, Mucus secretion

Invagination was observed histologically at 26 hpf (Fig. 2E and E', Table 1). Formation of ectoderm and endoderm commenced around 32 hpf, and mesoglea, a gelatinous extracellular matrix, began to appear between the two layers (Fig. 2F and F', Table 1). At this point, larvae were no longer at the surface of the beakers, but were drifting slowly in the middle or at the bottom. Swimming planulae were observed at ~48 hpf (Fig. 2G and H; Table 1), and oral pores and pharynxes were histologically confirmed (Fig. 2G' and H'). Mucus secretion was also observed at this stage. Larval settlement and metamorphosis on glass beakers or plastic culture plates was rarely observed during the entire course of observation. Some swimming planulae were maintained in glass beakers for more than 3 months.

Changes in lipid and sugar contents

Lipid component analysis by HPTLC demonstrated that eggs (0 hpf) are rich in WEs (Fig. 3A). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the major phospholipid components. Concentrations of those lipids, particularly WE in planulae at 48 hpf were significantly less than those of eggs. No further decrease of these lipid levels was observed in planulae from 48 hpf to 168 hpf (Fig. 3A). Sugar content analysis revealed that eggs are rich in glycogen, and that the glycogen concentration increased significantly during development (Fig. 3B). Free glucose was not detected in samples that we analyzed (Fig. 3B).

Settlement of *F. ancora* planulae

Planulae settled on coral debris and ceramic tile, but not on plastic dishes, microscope slides, or CCA (Fig. 4A). The highest settlement rate was observed on coral debris (17.5%, Fig. 4B), and some settled larvae metamorphosed into primary polyps with skeletons (Fig. 4C).

DISCUSSION

Eggs are specialized cells containing essential materials for embryonic development and survival. Previous studies have shown that buoyant coral eggs contain high levels of WEs (Arai et al. 1993; Harland et al. 1993; Harii et al. 2007; Norström and Sanddtröm 2010; Figueiredo et al. 2012; Okubo et al. 2020). WEs are fatty acids esterified to alcohols (Schots et al. 2020). In marine animals, WEs contain high levels of unsaturated fatty acids and alcohols (Kattner et al. 1996; Phleger 1998; Saito and Murata 1998; Patel et al. 2001) and are thought to function in buoyancy adjustment, energy storage, and insulation (Nevenzel 1970). Additionally, since levels of WEs decrease during embryogenesis and larval development in some corals (Harii et al. 2007; Figueiredo et al. 2012; Okubo et al. 2020), they are thought to be used as an energy source for embryogenesis and swimming of planulae larvae (Okubo et al. 2020). Our HPTLC analysis showed that *F. ancora* eggs are rich in WEs. In addition, levels of lipids, especially WEs, in *F. ancora* early planulae at

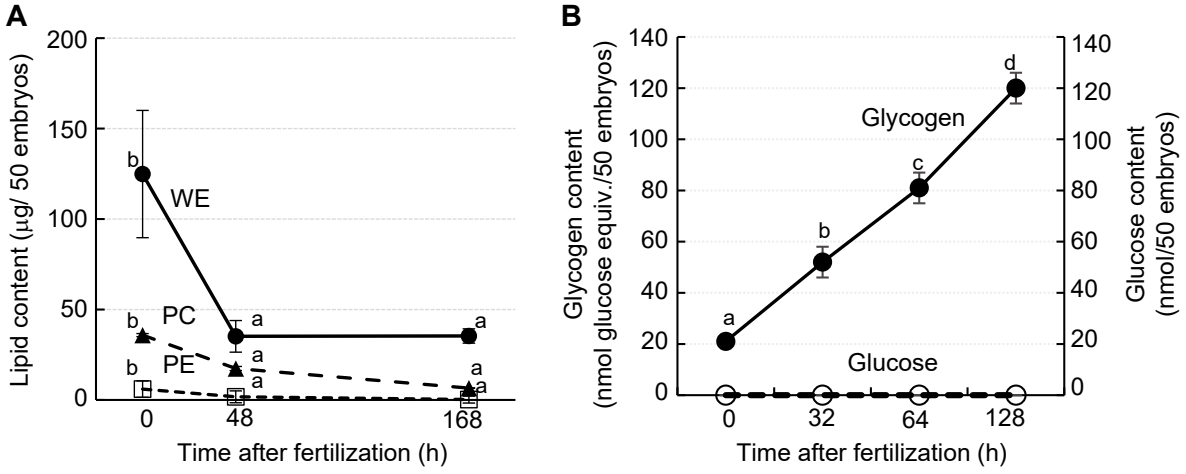

Fig. 3. Changes in lipid and sugar levels during early development of *F. ancora*. A: Changes in lipid levels. Lipids were extracted from released unfertilized eggs (0), early planulae at 48 hpf (48), and a planula at 168 hpf (168) and analyzed by high-performance thin-layer chromatography. WE, wax ester; PC, phosphatidylcholine; PE, phosphatidylethanolamine. B: Changes in glycogen and glucose levels. Sugars were extracted from released unfertilized eggs (0), gastrula at 32 hpf (32), planulae at 64 hpf (64), planulae at 128 hpf (128), and concentrations of glycogen and glucose were determined. Data are shown as the mean ± SE (n = 3 experiments). Groups with different letters are statistically different ($p < 0.05$).

48 hpf were significantly less than in unfertilized eggs. This pattern is somewhat similar to those reported in corals belonging to other families (Harii et al. 2007; Figueiredo et al. 2012; Okubo et al. 2020), suggesting that this biochemistry may be common to many corals that produce buoyant eggs. Since *F. ancora* embryos do not swim actively during this period, lipids may be consumed by embryonic metabolism. Indeed, increased oxygen consumption during gastrulation were reported in other corals (Okubo et al. 2010). In our previous study, we also showed a decrease in levels of polypeptides derived from a major yolk protein, Vg, during early embryonic development (32–64 hpf) of *F. ancora* (Shikina et al. 2013). Degradation of Vg likely supplies energy during early embryonic development of coral embryos, suggesting that *F. ancora* embryos have metabolic pathways that generate energy from both Vg and WEs.

Interestingly, we found an increase in glycogen concentration during early development of *F. ancora*. Glycogen is a branched polymer of glucose that acts as a fuel source in animal cells (Roach 2002; Roach et al. 2012). Glucose released from glycogen is oxidized to produce adenosine triphosphate (ATP) (Roach 2002; Roach et al. 2012). Our finding suggests that coral embryos and planulae use glycogen as an energy source to support motility, metamorphosis, and juvenile colony formation. Since *F. ancora* planulae are non-feeding and aposymbiotic, it is most likely that glycogen is synthesized de novo by larvae. There may be pathways to convert proteins and/or lipids into sugars and subsequently into glycogen. However, free glucose was below the detection limit in the present study, suggesting that newly synthesized glucose is immediately converted to glycogen in embryos/planulae. Since mucus secretion was observed in *F. ancora* planulae, another possibility is that newly synthesized glucose is immediately used for synthesis of major glycoproteins that compose mucus, such as mucin, and may be used for protection from pathogens, removal of foreign substances from the larval surface, etc. using mucus (Brown and Bythell 2005; Hadaidi et al. 2019). Genomic and transcriptomic studies will provide insight into this phenomenon in the future.

In the early life history of corals, settlement is an important transition from planktonic to benthic life. In the present study, we found that *F. ancora* planulae rarely settle on glass beakers or plastic dishes under our rearing conditions, and they continue to swim for several months. Similar behavior has also been reported in other coral planulae (Graham et al. 2013). Previous studies have shown that planulae settle at higher frequencies on some substrate materials (Lee et al. 2009) and colors (Mason et al. 2011). In addition, planulae sense other organisms on substrates, such as crustose coralline algae and/or associated biofilms, perceiving chemical cues from them before settling (Morse et al. 1988; Negri et al. 2001; Harrington et al. 2004; Webster et al. 2004).

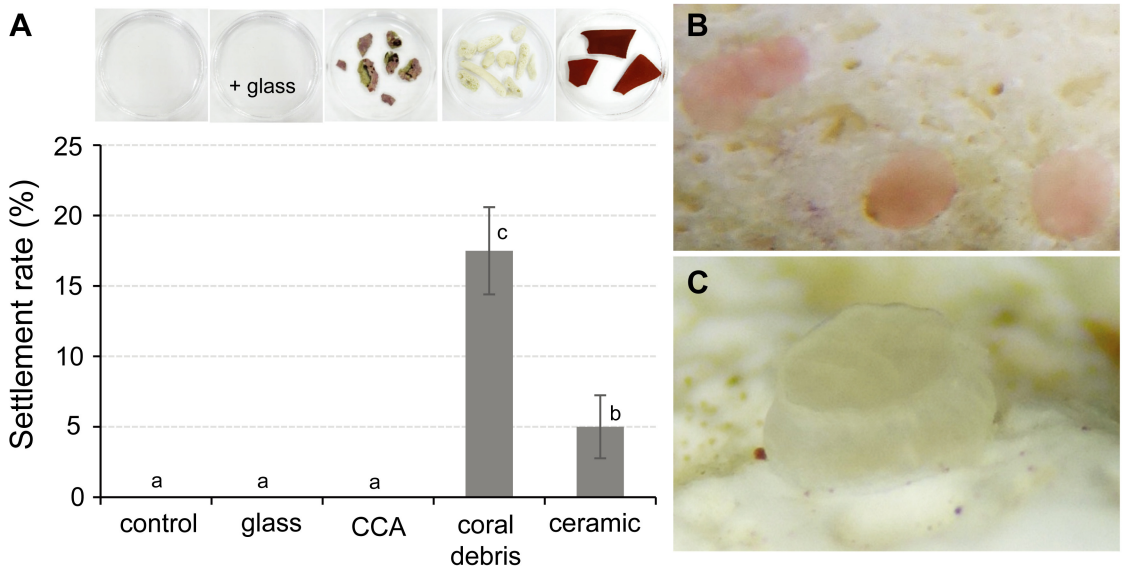

Fig. 4. Settlement of *F. ancora* planulae on different substrates. A: Results of settlement assay. Microscope slide (glass), crustose coralline algae, coral debris, or ceramic tile was added to plastic culture dishes (9 cm) filled with 30 mL of FSW. Then, 20 planulae (at 64–128 hpf) were added to each dish and maintained for 6 days. A dish with 30 mL of FSW, but no substrate was used as a control. The number of larvae settled on each dish surface or substrate was determined at 6 days of culture. Experiments were performed three times using planulae collected from different colonies. Data are shown as means ± SEs ($n = 3$ experiments). Groups with different letters are statistically different ($p < 0.05$). B: Micrograph of larvae settled on coral debris. C: Micrograph after metamorphosis.

Our settlement assay showed that *F. ancora* planulae prefer coral debris to other substrates, suggesting that they may prefer calcareous or porous substrates. The presence of coral debris may also signify suitable habitat. *F. ancora* planulae did not settle on the CCA that we prepared. This raises two possibilities. Either the prepared CCA species (*Hydrolithon* sp.) were not appropriate or else CCA and/or associated biofilms are repellent to *F. ancora* planulae. Further investigation is required to evaluate these two possibilities.

To the best of our knowledge, to date, embryogenesis of the family Euphyllidae has been examined only in *Galaxea fascicularis* (Okubo et al. 2013); thus, data from this study will facilitate taxonomy and embryological studies of scleractinian corals. For example, molecular phylogenetic studies using mitochondrial gene sequences suggest that the Order Scleractinia includes three major extant lineages, termed the "Basal", "Robust", and "Complex" clades (Romano and Palumdi 1996; Fukami et al. 2008; Kitahara et al. 2010). Okubo et al. discovered that there are obvious differences in embryogenesis between the Robust and Complex clades, though there is an exception (Okubo et al. 2013; Okubo 2016). During embryogenesis, species in the Robust clade have a blastocoel, while those in the Complex clade do not (Okubo et al. 2013). Thereafter, based on the presence or absence of a blastocoel and molecular phylogenetic data, two new suborders, Vacatina or Refertina, were established (Okubo 2016). The former includes corals in the Robust clade with an apparent blastocoel, while the latter includes corals in the Complex clade with no or little blastocoel. This study found that *F. ancora* has no blastocoel during embryogenesis. Additionally, this species has been classified in the Complex clade (Fukami et al. 2008). Thus, *F. ancora* belongs to the Suborder Refertina.

F. ancora is one of the most popular species in the marine ornamental trade (Bruckner 2001). Most *F. ancora* currently being traded have been collected in the wild or have been asexually propagated in captivity. Propagation of sexual reproductive techniques have not yet been established for this species. The present study offers the first early life history description of this species, and substrate preferences during settlement were demonstrated. This information will facilitate future establishment of a larval rearing method for this species. The next challenge will be to increase the settlement rate of planulae. Moreover, *F. ancora* is currently used as an experimental species to study reproductive biology and neurobiology of scleractinian corals. Molecular markers for detecting subpopulations of neurons and germline cells are available for this species (Shikina et al. 2012 2015 2020a). With these advantages, ontogeny of germline cells and/or neurons in scleractinians, which remain largely unexplored, can be investigated in future studies.

CONCLUSIONS

This study documented the process and time course of early development in *F. ancora*. Notably, changes in lipid levels and glycogen during early development were documented. Furthermore, substrates that encourage planulae to settle were identified, although settlement rates are still low. This study provides valuable information on the larval biology of scleractinian corals.

List of abbreviations

FRP, Fiber-reinforced plastic.
FSW, Filter-sterilized seawater.
WEs, Wax esters.
HPTLC, High-performance thin-layer chromatography.
PCA, Perchloric acid.
CCA, Crustose coralline algae.

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Availability of data and materials: All data are provided within this manuscript.

Consent for publication: Not applicable.

Ethics approval consent to participate: All experiments were carried out in accordance with principles and procedures approved by the Institutional Animal Care and Use Committee of National Taiwan Ocean University.

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