Embryonic and larval development of the nudibranch *Phyllidiella nigra*

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Abstract. Nudibranchs *Phyllidiella nigra* (van Hasselt, 1824) were collected from Bidong Island, Terengganu, Malaysia. They were allowed to copulate and lay their egg masses under laboratory conditions. The egg masses were flat, coiled ribbons, spiraling in an anticlockwise direction. The mean egg size was 207.06±1.82 µm and the mean embryo size was 128.14±4.28 µm, after 24 hours from egg deposition. The embryo undergoes cell division, gastrula, blastula and trochophores stages, with the presence of velum and statocyst. The eggs hatched into planktonic veliger larvae on day 11, with the embryo size of 158.68±5.05 µm. The larval remain planktonic in a long pelagic period until day 44, without metamorphosis, with the size of 143.931 µm. *P. nigra* development is more similar to *Phyllidia varicosa* development and it grows relatively faster than what was presented in previous studies on phyllilididae. Therefore, the full development of *P. nigra* can bring new knowledge on the development of nudibranchs, and the information can be utilized to breed nudibranchs for the aquarium industry and for conservation purposes.

Key Words: hermaphrodite, egg masses, mollusc, phyllilididae.

Introduction. Nudibranchs are invertebrate organisms with naked gills and soft bodies from the phylum Mollusca. They are from the same class as snails and slugs (class Gasteropoda). The largest group from Opisthobranchia (marine slugs) is Nudibranchia, consisting of 190 genera and 60 families, with about 3000 species found worldwide (Wagele & Willan 2000; Karlsson 2001a). Nudibranch *Phyllidiella nigra* is a marine gastropod lacking a hard external shell, except during the larval stage. The shell will be lost after the larvae undergo metamorphosis.

Most species of Phyllidiidae can be found in the tropical Indo-Pacific region and a few species are also observed in the tropical Atlantic Ocean and in the Mediterranean. *P. nigra* is usually found on shallow coral reefs, at depths of about 1-6 m. The length size of *P. nigra* is ranging from 2.5 cm to 7.5 cm. The juvenile Opisthobranchs can grow rapidly to reach maturity, with a growth rate of more than 7% in length per day. They deposit egg masses repeatedly and die after one reproductive season because of energy loss (Wolf & Young 2012).

P. nigra presents a dark dorsal coloration with dark pink to red tubercles, evenly distributed and not clustered, over the dorsum. The bases of the tubercles are black and it body is entirely black, with rounded oral tentacles (Brunckhorst 1993).

Nudibranchs constantly glide over sediments, seaweeds, rocks, sponges, corals and other substrates, and develop colors and patterns very useful for effective camouflage (Marin et al 1997). It can live up to a year, but some species can only survive for a few weeks and reproduce quickly after finding a mate. Normally, nudibranchs will attach and lay egg strings to eelgrass (*Zostera*) and other algae, or whatever surface the nudibranch is feeding on. The eggs are encapsulated and are deposited in many different forms of gelatinous masses, such as broad bands, spirally coiled and attached to the substrate with a small, ribbon like edge. They will develop into planktonic (free swimming) larvae, which drift on ocean currents until they settle out as adults (Kasamesiri et al 2014).

All opisthobranchs are hermaphrodite and mate through mutual insemination. Hermaphrodites are organisms that possess both male and female sex organs and both individuals can donate and store sperm (Haase & Karlsson 2004). Multiple mating is common in many species of nudibranchs, which indicates that sperm competition may be as important in hermaphrodites as it is in gonochorists (Sekizawa et al 2013). The copulation in hermaphrodites involves an explosive and reciprocal eversion of the penises with an intromission that lasts only a few seconds, but can also last for many minutes or hours in the case of some nudibranch species (Rutowski 1983).

The hermaphrodism in nudibranch has advantages, one of them being that every mature individual of the same species encountered can be a potential mate. Therefore, it can increase the chances of fertilization. Moreover, each individual can lay eggs, thereby maximizing the reproductive effort (Thompson 1988; Karlsson 2001b).

The most important criteria for successful mating among nudibranchs are their body size. A pair of hermaphrodites must be significant in size (Bateman 1948; Karlsson 2001b). Furthermore, when the body size increases, the diameter of the egg mass produced is also bigger. A larger body size will increase the diameter of the egg mass and the number of eggs per capsule. The size also determines its sex allocation. It is because the smaller individuals usually invest more resources into male sexual products, whereas larger individuals would allocate more energy into eggs production. Hence, the reciprocal mating should be possible between similar sized individuals, while unilateral mating requires more frequent mating between individuals that are different in size. Moreover, when the pairs are different in sizes, the smaller partner will act as a male while the larger partner will receive the sperm, but may not reciprocate due to the low female reproductive potential of the smaller partner (Sprenger et al 2009).

Interest in the larval biology of nudibranch gastropods increased markedly after the publication of the paper of Thompson et al (1958) on the natural history and development of *Adalaria proxima.* The larvae of many nudibranch species have since been raised through metamorphosis in the laboratory and considerable progress has been made towards the understanding of the physiological, morphological, and behavioral aspects of the metamorphosis (Perron & Turner 1977). Most of planktotrophic larvae of opisthobranchia have a larval period ranging from a few days to months (Wolf & Young 2012). Most nudibranch species produce planktonic larvae that remain in their larval phase for periods of minutes to months or longer planktonic periods, sometimes dispersing over great distances before settling on suitable substrata. The appearance of eyespots and the propodium in the embryo stage, pigmented egg cytoplasm and shell pattern has been used to predict the development of nudibranch larvae (Kasamesiri et al 2014). The main aim of this study is to document and determine the eggs deposition, egg and larval development of *P. nigra* under laboratory conditions. The information will be useful for culturing phyllidiidae for aquarium or experimental purposes.

Material and Method

Nudibranch collection. Two pairs of adult specimens were collected from Southern South China Sea at Bidong Island, Terengganu, Malaysia (5.6212°N; 103.0616°E) in February 2016, by scuba divers. The samples were stored in aerated aquarium and transported to the lab for further observations (Figure 1). The size of samples was approximately 6 cm in length for both pairs.

Rearing the brood stock. Each pair of adult nudibranchs were maintained in an aquarium with the dimensions of 22x13x13 cm, at 29-33^{0}C (pair A and pair B). Filtered seawater (sieve size less than 50 μm) was used during the 60 days of the study period. Gentle aeration was provided all time and water parameters were maintained relatively constant, with pH 7.9-8.6 and salinity between 28-30 ppt. The sea water totally replaced once a week to maintain the quality. The specimens were feed with sponges attached to the substrate.

Figure 1. A pair of adult *Phyllidella nigra* in an aquarium.

Egg mass categorization. Each pair was observed regarding their egg laying behavior and the following larval description was conducted. The egg masses produced by nudibranch are divided into four categories: coiled ribbons, egg cords, ovoid or globular jelly masses and sac-like masses (Kasamesiri et al 2014).

Egg hatching. Once the nudibranch produced an egg mass, it was removed from the aquarium and placed into a new aquarium. The egg mass was handled with care and a 1-3 mm section was cut using scissors for a random observation of egg development. Sample were collected using a dropper and a compressed under cover slip to prevent movement. Observations were carried out using a calibrated microscope (Dino Eye) with a magnification of 40x and 100x to measure the embryos and egg capsules.

Larval development and rearing. Larval hatching time was recorded when the presence of first swimming larvae was observed. The swimming larvae were fed with *Isochrysis* sp. for the first five days (30 cells/larva) and continued with *Chaetoceros* sp. as they grew bigger, at 40 to 60 cells per larva. The nudibranch larval development was observed, monitored and measured daily using a calibrated microscope.

Data collection and analysis. The embryonic stage and the diameter of the egg and larva were recorded and measured using the DinoCapture 2.0 software. The mean values were recorded.

Results and Discussion. The egg masses of *P. nigra* are whitish in color and attached to the substrate made from dead coral with algae (Figure 2). The structure of egg masses is flat coiled ribbons and spirals in an anticlockwise direction. The coiling and rotation of the masses starts from the center of the eggs to the exterior, with triple layered rows. The process of egg masses deposition started with the nudibranchs moving their head from side to side to lay the eggs in parallel rows of strings. After that, they started to press the eggs into the substrate with their lips, lappets and feet. The detailed development of the eggs and larvae is presented in Table 1. During the second day the blastula stage could be seen clearly. On the third day the gastrula could be seen and the trocophore stage followed the next day. On day 6 the trocophores could be seen moving within the egg capsules and the prevelum was developed. The next day, the post-oral velum with smaller ciliation and the pair of statocysts appeared. It rotated slowly around the oral-aboral axis. On day 8 the velum with large cilia was observed, indicating that the trochophores started to develop into veliger larvae. On day 10, the veliger had developed complete digestive organs, such as the stomach and the digestive gland. The veligers started to break free from the egg mass and became free swimming larvae on day 11. The hatching continued for a few days until day 14 and the larvae were feed with microalgae. Some of them could be seen swimming and touching the bottom of the culture tank. The shell grew slowly and increased in size as they grew older. Starting from day 24 the shell size was constant until day 44, when they did not undergo metamorphosis.

The egg masses are oval capsules, each capsule containing a single egg with a mean size of 207.0622±1.82 μm. The mean embryo size was 128.1483±4.28 μm on the second day after egg deposition. Egg masses underwent blastula and gastrula stages, and the early trochophore stage was visible on days 4 and 5. The capsules and embryo increased in size from day 6-8, with a mean length from 197.71 to 206.02 μm, and 130.58 to 157.16 μm, respectively.

The shape of the shell is coiled with the presence of paired statocysts and unpigmented larvae. The larvae shell can be used to identify the type of nudibranch. Normally, dorididae larval shells are sinistrally coiled, with pigmented or unpigmented larvae. In this study the shell is roundish ovoid. Starting from day 14 to day 24 the size of shell increased slowly from 150.22 to 153.58 μm. The shell mean size slightly decreased from day 25 to day 44, to 143.931±8.94 μm, within the long pelagic period as veliger larvae. The shells were retained until day 44 and metamorphosis did not yet occur.

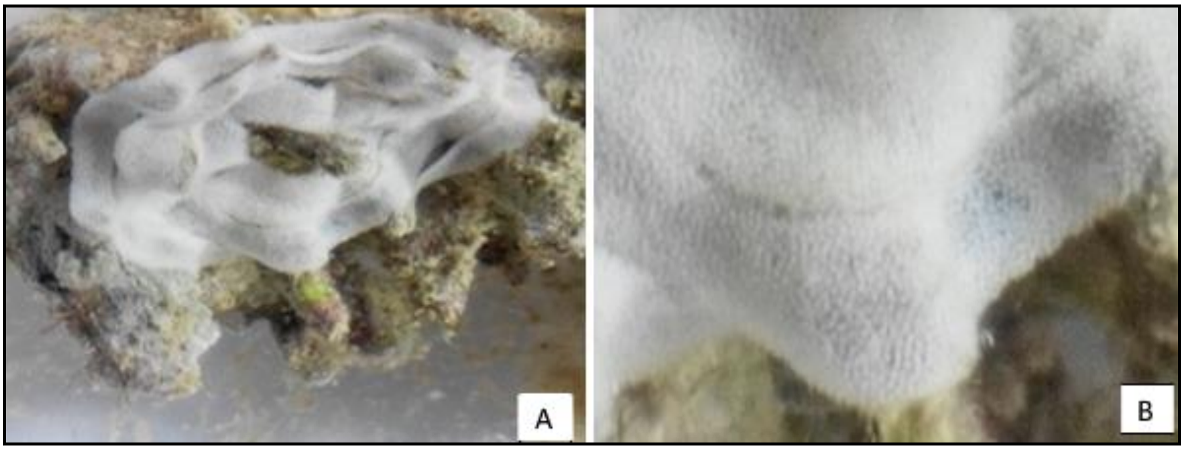

Figure 2. Egg masses of nudibranch. A - white egg masses in coiled ribbons; B - close-up of white egg masses.

Many marine invertebrates encase their eggs in gelatinous masses or egg capsules in order to protect the embryos from predators or various stress. Egg masses structure and composition among opisthobranch vary between genera and species (Table 2). Different families will show different egg characteristics. The *Chromodorididae* and *Dendrodorididae* for example, will have ribbon like egg masses, usually in the upright position, whilst the *Flabellinidae* will have more globular shaped egg masses. The different types of egg mass characteristics can represent different kind of nudibranchs families. This can be useful if we should find nudibranch eggs in the wild. For example, some nudibranchs such as *Doriprismatica atromarginata* and *Dendrodoris krusenternii* lay their egg masses in upright ribbons instead of flat coiled ribbons (Kasamesiri et al 2014). The egg masses of *P. nigra* are whitish in color, weaved like a coiled ribbon attached to the substrate. The egg color is similar with the substrate, helping to camouflage the eggs from potential danger. The eggs were laid flat by *P. nigra* in this study, the same as other *Phyllididae* studied previously (Kasamesiri et al 2014).

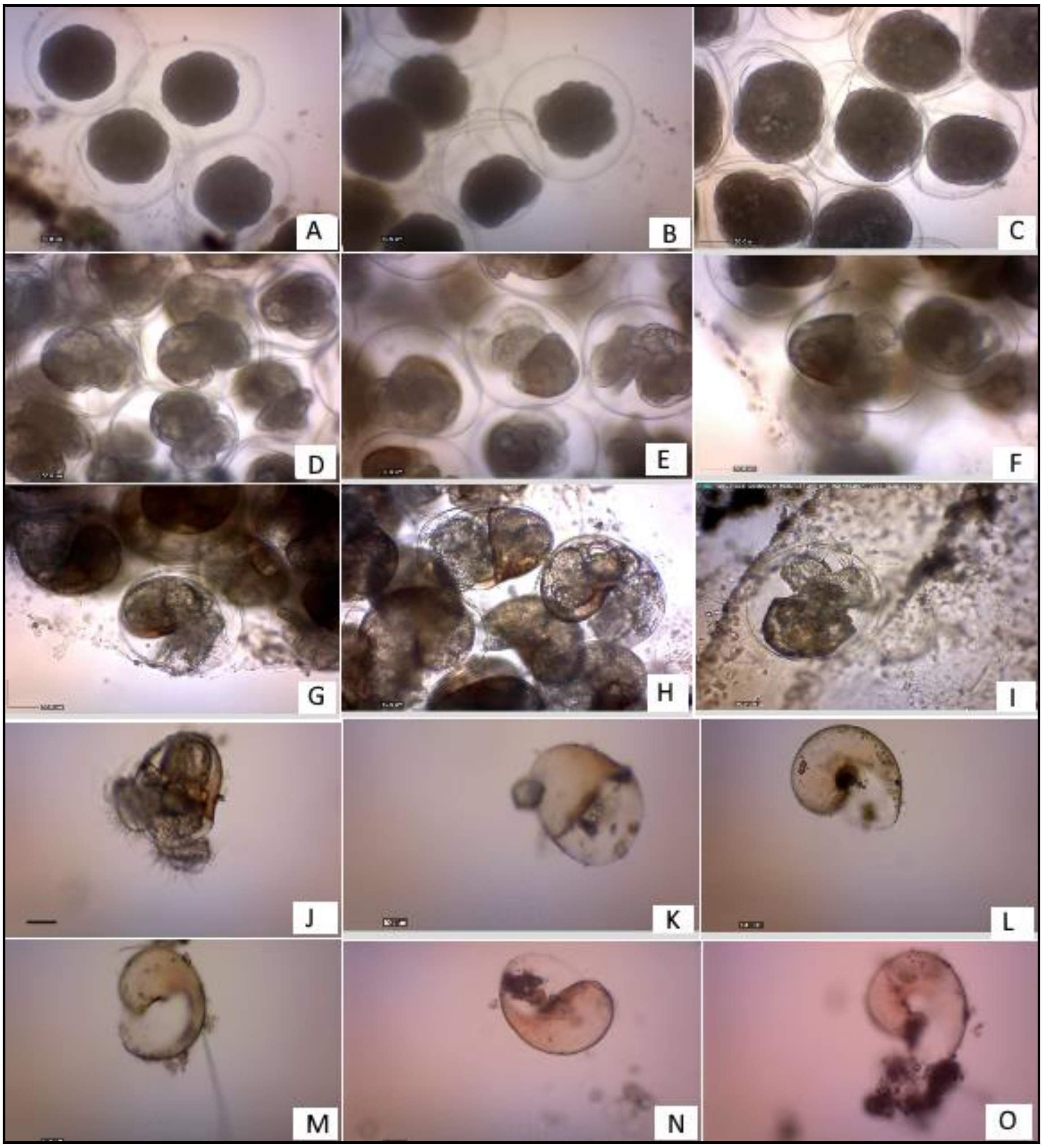

Figure 3. Embryonic and larval development of *Phyllidiella nigra* from Day 2 to Day 44 (scale bars indicate 50 µm). A - Day 2: blastula; B - Day 3: gastrulation can be seen in some of the embryos; C - Day 4: late blastula and gastrulation and early trochophore stage; D - Day 6: trochophores started moving within the egg capsules; precursors of the velum or prevelum are developed; E - Day 7: post-oral velum with smaller ciliation and pairing of statocysts; F - Day 8: the pre-oral velum is transformed into the velum with large cilia and trochophores start to develop into veliger larvae; G - Day 9: cilia attached to the velum of the larvae, nephrocyst (arrow) and shells are observable; H - Day 10: the veliger presents complete digestive organs; I - Day 11: portion of egg mass from which veliger larvae start to break out of; J - Day 14: hatched veliger with lack of eyes and unpigmented filled gut; K - Day 17: larvae swim near the bottom; L - Day 23: the shape of shell is sinisterly, roundish ovoid; M and N - Days 24-28: the shell growth is constant; O - Day 44: veliger larva close to metamorphosis.

Nudibranchs lay eggs in separate capsules that function as nutritional reserves, which are attached to each other in a long string. The embryonic development of nudibranchs can vary due to temperature and egg size (Lee et al 2014). For the *P.nigra* in this study, the embryonic development took 11 days, but for *P. rosans* it takes 17 days and for *Doriprismatica atromarginata* it takes 18 days (Kasamesiri et al 2014). Hatching occurred as early as day 11, at 28–32°C. The temperature was maintained in this range, because a slight increase by 2-4°C can decrease the hatching rate and survival rate of the larvae (Pedroso 2017). The mean size of the veliger larvae on day 10, one day before hatching, was 158.68±5.05 μm. Larvae were feed with *Isochrysis* sp. for the first five days and continued to be fed with *Chaetoceros*, as the food supply is an important factor for larvae development and survival during the pelagic stage (Paulson & Scheltema 1968). The shell pattern of nudibranch larvae can be categorized as Type 1 and Type 2 (Thompson 1961).

Table 2

Larvae development in the current study compared with other species (Kasamesiri et al 2014 with modifications)

Nudibranch species	*Phyllidiella nigra (Phyllidiidae - current study)*	*Phyllidia varicosa (Phyllidiidae)*	*Phyllidiella rosans (Phyllidiidae)*	*Doriprismatica atromarginata (Chromodorididae)*	*Dendrodoris krusenternii (Dendrorididae)*
Egg rotation	Anticlockwise	Anticlockwise	Anticlockwise	Anticlockwise	Anticlockwise
Cilia	Day 4	Day 5	Day 8	Day 4	Day 3
Statocyst	Day 7	Day 7	Day 10	Day 9	Day 5
Shell Pattern	Type 1	Type 1	Type 1	Type 1	Type 1
Egg mass color	White	yellow	Yellow	Yellow	White
Shape of egg mass	Flat ribbon	Flat ribbon	Flat ribbon	Upright ribbon	Upright ribbon
Total embryonic period	11 days	14 days	17 days	18 day	8 days
Uncleaved size±SD (μm)	158.68±6.91	93.50±10.84	134.00±0.36	178.00±1.85	113.57±6.27
Group range of uncleaved size/days	Large size 17-18 d	Small size 5-6 d	Large 17-18 d	Large 17-18 d	Medium 8-9 d

Note: group range of uncleaved sizes: small size (77.5-94.20 μm), medium size (100-122.70 μm), large size (134.00-178.00 μm).

During the embryonic development, the statocyst started to appear on day 7 and the larvae can be seen rotating slowly around the oral aboral axis. Development of statocyst is important, as it functions as a balance sensory receptor that aids in the orientation and balance of the organism during swimming. This will help the larvae after they hatch and become planktonic, which started on day 11 and continued for a few days until day 14. The total embryonic development period of *P. nigra* in this study was 14 days after the egg deposition. The sizes of the egg capsules are positively correlated with the size of the hatching veliger larvae and they also increase simultaneously with the embryonic development period (Lee et al 2014). Uncleaved sizes can be divided in three groups, which are small (77.5-94.2 μm), medium (100-122.7 μm) and large (134-178 μm) (Kasamesiri et al 2014). In Table 2, uncleaved sizes cpould be observed for the larvae that fully developed inside the egg capsule. *P. nigra* is in the large size group, together with other two species, *Phyllidiella rosans* and *Doriprismatica atromarginata*. However, *P. nigra* has a relatively short embryonic period of 11 days compared with other species from the large group. *Phyllidia varicosa*, from the small size group, has a period of embryonic development of 14 days. *Dendrodoris krusenternii*, from the medium sized group, has the shortest embryonic development period, of only 8 days. Based on the size group, the development of the larvae can be categorized into three main types, which are direct development for large size groups, lecithotrophic development for medium size groups and planktotrophy for small size groups.

Some nudibranch larvae still maintain their planktonic behavior even after discarding the shell and they can ingest microalgae as food. In fact, algal size and quality can affect the larval growth.

Conclusions. *Phyllidiella nigra* development is more similar with the development of *P. varicosa*, than with the one of *P. rosans*, even though *P. varicosa* is in the small size group. The appearance of cilia in *P. nigra* is observed at day 4. The appearance of the statocyst occurs at day 7. The appearance of statocyst is one of the clues that larvae are prepared to hatch and become planktonic. The total embryonic development of *P. nigra* takes 11 days, faster than those of *P. varicosa* and *P. rosans.* It seems that *P. nigra* grows relatively faster compared with other *Phylliediella* species.

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