Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi: 10.12980/JCLM.2.2014JCLM-2014-0080

Embryonic development of nudibranch species (Mollusca: Opisthobranchia) in the Gulf of Thailand

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PEER REVIEW

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Comments

The aim and results of this study are interesting to malacologists, marine invertebrate biologists and researchers working on developmental biology in general. It is a wide aspect research, on several species and considering different aspects of its reproductive biology. The considerations may be applied for culturing and commercial regulatory policies for native specimens exploitation.
(Details on Page 938)

ABSTRACT

Objective: To find the possible way to predict the mode of embryonic development of nudibranchs, focusing on egg mass characteristics, embryonic development, and shell patterns of the veligers.

Methods: Eight species of nudibranch were collected in the eastern part of the Gulf of Thailand. The specimens were allowed to copulate and lay their egg masses under laboratory conditions. Embryonic development was monitored under a light microscope with a digital camera every day until hatching.

Results: Most of the species of nudibranch collected had a single larva in each egg capsule that developed, except for *Jorunna funebris*, which had 1-4 larvae in each egg capsule. All the specimens had the same pattern of cell division and hatching into the water column during the veliger form. However, the species developed at different rates in each stage.

Conclusions: All species in the current study had planktotrophic development except *Doriprismatica atromarginata*, which showed lecithotrophic development. Based on embryonic development among the nudibranchs that showed planktotrophic development, *Jorunna funebris* appeared to be the most advantageous species for culture development with regard to utilization and conservation in the future.

KEYWORDS

Gulf of Thailand, Embryonic development, Nudibranch, Embryo, Veliger

1. Introduction

Nudibranchs are known as one of the most attractive marine animals. Because of their attractive colors, many of them are offered for sale in pet shops. Some nudibranch species have secondary metabolites which can be used as an antitumor, antimicrobial and inhibitor for barnacle larval settling[1-3]. Several researchers in various regions have attempted to culture nudibranch in aquarium settings, with varying degrees of success. Information about embryonic development is important to improve nudibranch culture. Over the last few decades, research into nudibranch embryonic development has been carried out in many areas of the world[4-10]. Embryonic development modalities

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Foundation Project: Partly funded by the Center of Advanced Studies in Tropical Natural Resources, KU Institute for Advanced Studies, Kasetsart University and Faculty of Technology, Mahasarakham University.

Article history:
Received 26 Jun 2014
Received in revised form 2 Jul, 2nd revised form 7 Jul, 3rd revised form 12 Jul 2014
Accepted 20 Jul 2014
Available online 7 Oct 2014

and dietary factors can have an effect on the distribution of larvae in their habitat[11,12]. Extensive documentation (as the appearance of eyespots and the propodium; of embryo size; pigmented egg cytoplasm and shell pattern) has been used to predict the development mode of nudibranch larvae[11-14]. Since embryonic development is closely correlated with embryo size, this development can be classified into three groups based on embryo (uncleavage stage) size: 1) direct development, where the embryos are usually large (>200 μm) and the larvae completed the development within the egg capsule (including an appearance of eyespots and the propodium) and after hatching immediately settle on the sea bottom; 2) lecithotrophic larvae, which normally have medium sized embryos (uncleavage stage) (110-250 μm) and spend a few days in a veliger stage before metamorphosing to the juvenile stage; and 3) planktotrophic larvae, which generally have small embryos (30-170 μm) and a longer planktonic period than lecithotrophic larvae and thus have a larger dispersal potential[15]. In addition, shell patterns have been used for the prediction of the developmental mode. Thompson classified shell patterns into two groups (Type 1 and Type 2), with the veliger shells in Type 1 being coiled and exhibiting a growth line, while Type 2 typically have egg-shaped, inflated shells[16]. Goddard concluded that Type 2 shells hatch at larger embryo sizes than Type 1[11].

Both the developmental mode and the egg mass patterns were associated with the survival likelihood of larvae proxies, such as the variation of oxygen concentrations within each egg mass pattern[17,18]. The different egg mass patterns in each species are controlled by evolutionary processes. Klussmann-Kolb and Wägele reported that egg masses of opisthobranchs could be divided into three groups: a broad band, spirally coiled and attached to the substrate with a small, ribbon-like edge[19]. However, the categorization of the egg mass in nudibranchs is still unclear. Wilson classified the egg mass patterns of Chromodorididae into three groups that had the potential to reflect the phylogeny[20]. However, very little is known about comparisons of egg mass patterns between suborders and families.

Sixty species of nudibranchs are found in Thai waters[21], with most of them occurring around coral reefs. However, there appears to be limited knowledge of nudibranch biology and/or ecology in Thailand. Nudibranch species in the Gulf of Thailand are now threatened by coastal pollution and tourist activities, such as scuba diving. Moreover, nudibranch specimens, including *Jorunna funebris* (*J. funebris*) and *Phyllidia varicosa* (*P. varicosa*) are caught from coral reefs and sold at aquarium stalls in Sunday markets in the northern part of Bangkok, Thailand. *J. funebris* is widely distributed along the coastline of Thailand[21]. Since the nudibranch population is very low, it is quite hard to find two mature adults of the same species in natural waters. At the sites explored in the current study, even though several years were spent seeking as many nudibranch species as possibile for this research, only eight nudibranch species were represented by at least two mature adults. The specimens were identified as *J. funebris*, *Goniobranchus fidelis* (*G. fidelis*), *Doriprismatica atromarginata* (*D. atromarginata*), *Hypselodoris tryoni* (*H. tryoni*), *Dendrodoris krusensternii* (*D. krusensternii*), *P. varicosa*, *Phyllidiella rosans* (*P. rosans*) and *Flabellina rubrolineata* (*F. rubrolineata*). The aim of the current study is to find the best possible way to predict the mode of embryonic development of nudibranch, focusing on egg mass characteristics, embryonic development features, and a detailed description of the shell patterns of the veliger larvae of the eight nudibranch species mentioned above. The knowledge obtained from this research may help to support improvements in nudibranch culture for future utilization and conservation.

2. Materials and methods

2.1. Nudibranch collection

The eight nudibranch species used for the experiment were collected from depths between 3 to 10 m on coral reefs on the eastern coast of the Gulf of Thailand, near Krok Island, Pattaya (12°56′33.42″ N; 100°47′32.48″ E) and near the Chang Islands (11°57′58.59″ N; 102°29′29.79″ E and 11°57′38.11″ N; 102°30′49.74″ E); both locations are shown in Figure 1.

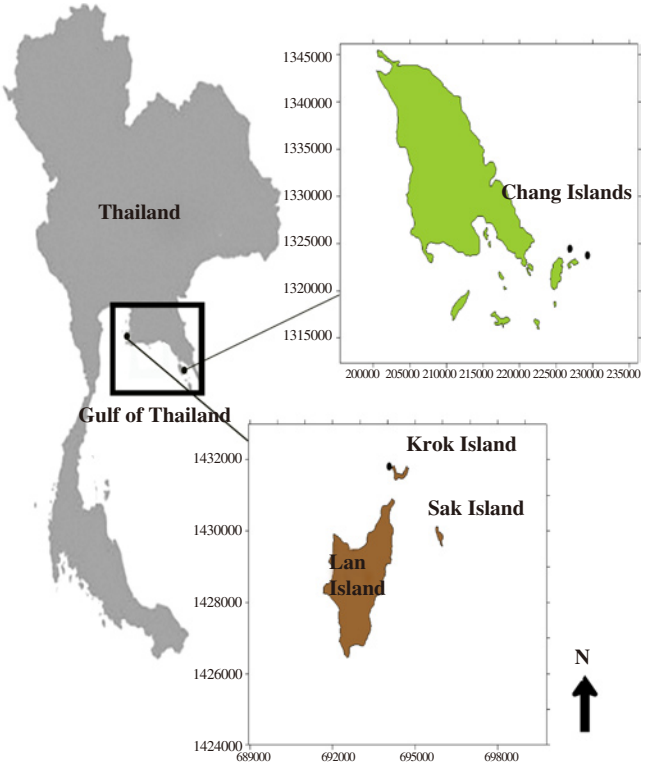

Figure 1. Map of sampling locations.

The Chang Islands Marine National Park comprises 52 islands; the current study focused on Monai and Monok Islands, because these sites had a high diversity and density of nudibranch species. Krok Island is approximately 8 km offshore. All the study sites were on flat reefs that were comprised of massive hard corals (mainly from the genus *Porites*), table corals and staghorn corals (genus *Acropora*) and other sessile animals, such as sponges, sea anemones and macroalgae. The samples were obtained by scuba diving during the dry seasons (December to April) of 2003, 2004, 2006 and 2008 (Table 1). Collected broodstock nudibranch were identified according to the World Register of Marine Species

as *J. funebris* (Kelaart, 1858) in the Suborder Euctenidiacea, Family Discodoridiidae, *G. fidelis* (Kelaart, 1858) in the Suborder Euctenidiacea, Family Chromodorididae, *D. atromarginata* (Cuvier, 1804) in the Suborder Euctenidiacea, Family Chromodorididae, *H. tryoni* (Garrett, 1873) in the Suborder Euctenidiacea, Family Chromodorididae, *D. krusensternii* (Gray, 1850) in the Suborder Euctenidiacea, Family Dendrodoridiidae, *P. varicosa* (Lamarck, 1801) in the Suborder Euctenidiacea, Family Phyllidiidae, *P. rosans* (van Hasselt, 1824) in the Suborder Euctenidiacea, Family Phyllidiidae and *F. rubrolineata* (O'Donoghue, 1929) in the Suborder Dexiarchia, Family Flabellinidae. *G. fidelis* , *D. atromarginata*, *P. varicosa* and *P. rosans* were collected near the Chang Islands, while *H. tryoni*, *D. krusensternii* and *F. rubrolineata* were collected near Krok Island. *J. funebris* was found at all study sites, but was collected near Krok Island only, because this site had a higher density of *J. funebris* than Monai and Monak Islands. Although some species, such as *G. fidelis* and *D. krusensternii*, had very low densities, at least two adult specimens of each were collected.

2.2. Rearing of broodstock and embryos

The adult nudibranchs samples were separated according to their species (Table 2) and 2 to 10 individuals were released per tank (61 cm×41 cm×46 cm) with aerated seawater and conditions of: temperature 24-26 °C, salinity 30-32 psu, dissolved oxygen 7.5-7.6 mg/L and pH 8.3-8.8, at the Department of Marine Science, Kasetsart University. The seawater in all broodstock aquaria was filtered through a typical wet-dry filter that included a mass of Bio-Balls, kept constantly damp, with room above for a particulate filter pad. The available substrate for oviposition was live rock of the aquarium inner walls. The environmental conditions in the tanks were monitored twice weekly by a Multi-Parameter Water Quality Sonde (YSI 6600, YSI Environmental-USA) to adjust the salinity to compensate for evaporation of seawater. To avoid any effects from chemical substances released from the blue sponge (used for feeding the adult individuals)[22], the individuals were starved until the spawning had finished. As soon as possible after spawning (within 3 h), egg masses were removed to 250 mL Erlenmeyer flasks containing sterilized seawater (natural seawater from the study site was autoclaved at 121.0 °C for at least 15 min before use in an incubation chamber). The egg masses were maintained in an incubation chamber at 25.0-26.0 °C, salinity 32 psu with a cycle of 12 h light and 12 h darkness. The seawater in each culture was changed every 2 days throughout the experiment, until hatching. After hatching, veligers were transferred to small tanks (41 cm×20 cm×25 cm) under the same environmental conditions as the broodstock tanks. The flasks containing the veligers were placed in the tanks and the veligers were released under water to prevent mortality of the veligers from air-water interface entrapment. The density of veligers was kept between 300 and 450 individuals per 100 mL. The salinity in the veliger tanks was adjusted every 2 days to about 32.0 psu.

2.3. Egg mass categorization

The egg masses were observed on the first day in order to categorize them by morphological descriptors, such as shape, color, rotation form and extra-capsular yolk, according to a scheme introduced by Hurst[23], Klussmann-

Table 1
Details of sample collection.

Species	Location	Collection date	Depth (m)	Temperature (°C)	Salinity (psu)	No. of specimens
J. funebris	Krok Island	12/2006	3-6	29.0	31.9	8
	Krok Island	01/2008	3-6	-	-	10
G. fidelis	Chang Islands	12/2003	3-10	28.5	-	2
D. atromarginata	Chang Islands	02/2004	3-10	27.4	31.7	2
	Chang Islands	03/2004	3-10	29.7	32.0	2
H. tryoni	Krok Island	12/2006	3-6	29.0	31.9	2
D. krusensternii	Krok Island	12/2006	3-6	29.0	31.9	2
P. varicosa	Chang Islands	12/2003	3-10	28.5	-	2
	Chang Islands	04/2004	3-10	32.3	31.6	2
P. rosans	Chang Islands	12/2003	3-10	28.5	-	2
	Chang Islands	04/2004	3-10	32.3	31.6	2
F. rubrolineata	Krok Island	12/2006	3-6	29.0	31.9	2

Table 2
Summary of the data collected from samples of the eight nudibranchs.

Species	No. of adults	No. of egg masses	Timing of oviposition (d)	Embryonic period (d) (No. of samples)	Color of egg mass	Shape	Rotation form of egg mass	Uncleaved size±SD (μm, n=10)	Veliger shell length±SD (μm, n=10)
J. funebris	18	6	2-3	7-8 (480)	White	Upright ribbon	Anticlockwise	122.73±7.54	170.00±10.54
G. fidelis	2	1	8	9 (90)	Orange	Upright ribbon	Anticlockwise	100.00±0.33	192.00±4.51
D. atromarginata	4	3	1-2	17-18 (510)	Yellow	Upright ribbon	Anticlockwise	178.00±1.85	273.00±6.18
H. tryoni	2	1	6	6 (60)	Red	Upright ribbon	Anticlockwise	94.16±5.48	162.50±5.00
D. krusensternii	2	1	2	8 (80)	White	Upright ribbon	Anticlockwise	113.57±6.27	218.33±4.08
P. varicosa	4	1	7	14 (140)	Yellow	Flat ribbon	Anticlockwise	93.50±10.84	161.00±10.37
P. rosans	4	1	13	17 (170)	Yellow	Flat ribbon	Anticlockwise	134.00±0.36	177.00±2.07
F. rubrolineata	2	1	1	5 (50)	Orange	Spiral	-	77.50±5.40	126.67±5.77

Timing oviposition:Approximate timing of oviposition with regard to collection; Embryonic period: Duration of egg capsule development.

Kolb and Wägele[19], and Wilson[20]. Hurst suggested four categories of egg masses (coiled ribbons, egg cords, ovoid or globular jelly masses and sac-like masses)[23]. In addition, egg masses of the Family Chromodorididae can be classified into three types: Type A is laid flat on the substratum; Type B is laid upright and may slope inwards; and Type C is laid upright, slopes outwards and may be crenulated[20].

2.4. Embryonic development observation

Embryonic development was daily monitored under a light microscope (Olympus CX41, USA) with a digital camera (Olympus DP12, USA). In addition, every day until hatching, embryo and egg capsule diameters were measured for a random sample of at least 10 embryos and egg capsules per egg mass, using an ocular micrometer, and embryonic development was monitored at the same time. The developmental stages were identified as uncleaved eggs, first division of cleavage, 4-cell stage, 8-cell stage, morula, blastula, gastrula, trocophora, pediveliger, pre-hatching and hatching, as proposed by Chia and Koss[24], and Buckland-Nicks *et al*[25]. The appearance of characteristics of larval structure, such as the velum, statocyst, left and right digestive diverticula, eyespots and propodium was recorded. The shell patterns were categorized according to Thompson[16]. The uncleavaged size and shell size at hatching stage were used to describe mode of development according to Goddard[11], and Thompson[15].

2.5. Data analysis

To examine the relationship between embryos and egg capsules diameter for some species a linear regression analysis was used.

3. Results

Egg mass characteristics, intracapsular development and shell patterns are explained in detail for each species below and summarized in Tables 2 and 3.

3.1. *J. funebris*

Adults were found at both study sites with range size 2-6.5 cm, along with their diet (the blue sponge, *Xestospongia* sp.). *J. funebris* under natural conditions usually lay their egg masses on colonies of blue sponge or on dead coral or rock. Under laboratory conditions (Figure 2A), they laid their egg masses on live rock; if no live rocks were placed in the aquarium, the broodstock laid egg masses attached to the aquarium walls with an anticlockwise rotation. The egg masses were white, ribbon-like, upright and sloped outwards with the substratum. The free edge of the ribbon was slightly longer than the attached edge, causing undulations along the ribbon. Extra-capsular yolk was not present. The length (mean±SD, n=10) of uncleaved eggs was (122.73 ±7.54) μm and each egg capsule contained one to four embryos (6 egg masses) (Figure 2B). The embryonic developmental period was 8 d (480 embryos) at 24-26 °C. The cleavage stage finished on the first day and the blastula and gastrula stages began on the second day. On the third day, the embryo ciliated movements appeared and the trochophore moved around inside the egg capsule. The larval shell, velum, velar cilia and statocyst appeared on Day 4 (Figure 2C), with the shell pattern of the early veligers being Type 1 (Figure 2D), according to Thompson[16]. On the sixth day, early veligers had developed their digestive organs completely. The veligers hatched on Days 7-8 and the shell length (mean±SD, n=10) on the first day after hatching was (170.00±10.54) μm.

Table 3
Embryonic period and mode of development in current study compared to other species from temperate region.

Species		Embryonic period (d)	Mode of development	Uncleaved diameter (μm)	Temperature (°C)	Reference
Order Notaspidea	*Pleurobranchaea maculata*	8	Planktotrophic	100.91±2.18	14	[31]
Order Nudibranchia	*Doridella steinbergae*	7.5-8	Planktotrophic	75-85	12-15	[32]
	J. funebris	8	Planktotrophic	122.73	24-26	Current study
	Rostanga pulchra	15-16	Planktotrophic	80	10-15	[24]
	G. fidelis	9	Planktotrophic	100	24-26	Current study
	D. atromarginata	17-18	Lecithotrophic	178	24-26	Current study
	H. tryoni	6	Planktotrophic	94.16	24-26	Current study
	Dendrodoris behrensi	38	Ametamorphic	181-187	16-19	[29]
	D. krusensternii	8	Planktotrophic	113.57	25-26	Current study
	Doriopsilla albopunctata	18-19	Planktotrophic	108	14-15	[11]
	P. varicosa	14	Planktotrophic	93.5	24-26	Current study
	P. rosans	17	Planktotrophic	134	24-26	Current study
	F. rubrolineata	5	Planktotrophic	77.5	24-26	Current study
	Berghia verrucicornis	11-12	Lecithotrophic	< 150	23.9±1.3	[30]

There was no evidence of eyespots or a propodium on any newly hatched veliger.

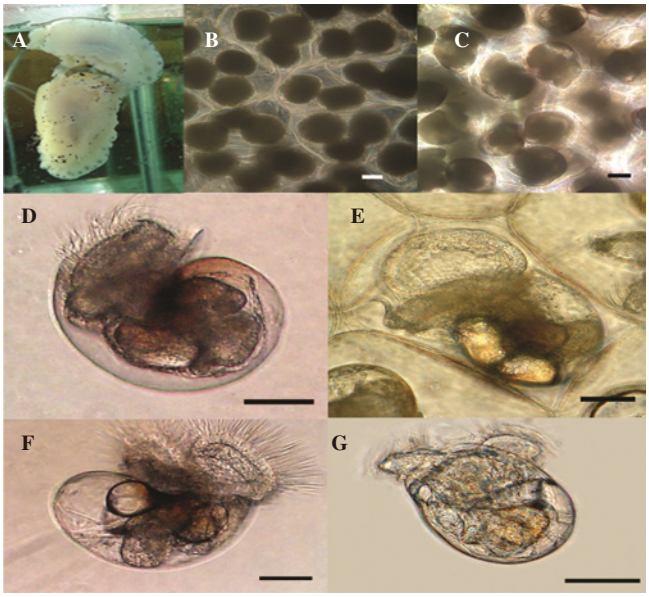

Figure 2. Reproduction and larval development of nudibranchs.
A: Mating of *J. funebris*; B: 2-3 larvae in single egg capsule of *J. funebris*, young gastrula stage, 2 d after oviposition; C: Embryonic veliger of *J. funebris* in egg capsule; D: Hatched veliger of *J. funebris*; E: Veliger in egg capsule of *D. krusensternii* (7 d old); F: Hatched veliger of *D. krusensternii* (8 d old); G: Veliger of *F. rubrolineata*; Scale bars=50 μm.

3.2. G. fidelis

G. fidelis is a low density species in the Gulf of Thailand. Only two adults were found at the reef edge between Monai and Monok Islands, where the current velocity was very high. The adult size was between 1.5 and 2.0 cm. This nudibranch laid a single egg mass on the aquarium wall with an anticlockwise rotation aspect. The egg mass was orange in color and upright with substratum. The extra-capsular yolk was orange and the zygotes were pale yellow. The length (mean± SD, n=10) of the uncleaved eggs was (100.00±0.33) μm and each egg capsule contained a single embryo (1 egg mass). The capsule and embryo diameter relationship (n=25, R^2=0.811 7) is shown in Figure 3 and the following equation:

y=0.930 8x+35.592

Where, y is an egg capsule diameter (μm), x is an embryo length (μm).

The embryonic developmental period was 9 d (90 embryos) at 24-26 °C. The cleavage stage was completed on the third day. The blastula and gastrula stages started on the fourth day. Formation of a digestive gland had already developed by Day 8 and hatching occurred on Day 9. The shell pattern of veligers was Type 1[16]. The shell length (mean±SD, n=10) on the first day after hatching was (192.00±4.51) μm. Eyespots of the newly hatched veliger were found but propodium was not found.

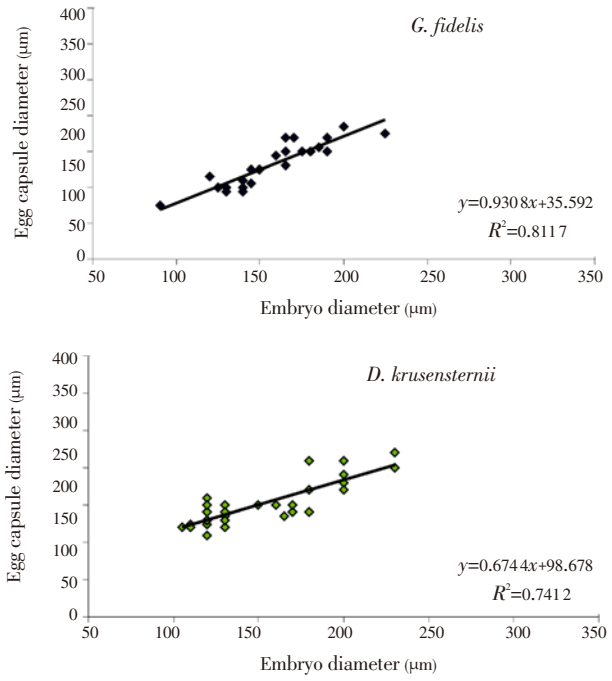

Figure 3. The relationship between embryo and the egg capsule diameter of *G. fidelis* and *D. krusensternii*.

3.3. D. atromarginata

A couple of adults were found in February and March 2004 near the Chang Islands (Table 1). Three egg masses were observed for 1-2 d after the adults were caught. The egg masses were upright, ribbon-like and had an anticlockwise rotation feature. The free edge was crenulated and curved; the zygotes were creamy in color and each egg capsule contained a single embryo. Extra-capsular yolk was not observed. The diameter (mean± SD, n=10) of the uncleaved eggs was (178.00±1.85) μm. The cleavage stage lasted up to 3 d after spawning. The blastula and gastrula stages were observed for a short period on the fourth day, thereafter the embryos developed cilia and began to move around inside the capsule and remained in this stage until the end of Day 9. The velum and shell were observed during Days 10-12 and the digestive gland and kidney was observed 13 d after spawning. The veligers hatched out on Day 18. The total embryonic developmental period was 17-18 d after spawning (510 embryos) at 24-26 °C. The shell pattern of the veligers belonged to Type 1[16]. The shell length (mean ±SD, n=10) on the first day after hatching was (273.00±6.18) μm. There was no evidence of eyespots or a propodium on any newly hatched veliger.

3.4. H. tryoni

Only two adults were found near Krok Island in December 2006 (Table 1). A single egg mass was observed for 6 d after adults were released into the aquarium. The egg mass was ribbon-like, had an anticlockwise rotation

and was laid upright, with one edge attached to live rock in the tank. The free edge was not crenulated or curved. The zygotes were red and extra-capsular yolk was not apparent. Each egg capsule contained a single embryo. The diameter (mean±SD, n=10) of uncleaved embryos was (94.16±5.48) μm. The embryonic development in this species was very fast compared to the other species in this study as the blastula and gastrula stages were completed by the end of Day 3. On the fourth day, embryos began to move, using their cilia. On the fifth day, the velum, shell, statocyst and digestive gland had formed in embryos. The total embryonic developmental period was 6 d after spawning (60 embryos) at 24-26 °C. The shell pattern of the veligers belonged to Type 1[16]. The shell length (mean±SD, n=10) on the first day after hatching was (162.50±5.00) μm. There was no evidence of eyespots or a propodium on any newly hatched veliger.

3.5. D. krusensternii

Two adults of *D. krusensternii* were found near Krok Island in December 2006 (Table 1). A single egg mass was observed for 2 d after the adults were released into the aquarium. The egg mass was creamy in color, ribbon shaped, with an anticlockwise rotation; spawning was upright, with the free edge being crenulated or curved. The zygotes were creamy in color and extra-capsular yolk was not apparent. Each egg capsule contained a single embryo. The diameter (mean ±SD, n=10) of uncleaved embryos was (113.57±6.27) μm. The capsule and embryo diameter relationship (n=45, R^2=0.741 2) was estimated as shown in Figure 3 and the following equation:

y=0.674 4x+98.678

Where, y is an egg capsule diameter (μm), x is an embryo length (μm).

The cleavage stage lasted up to 2 d after spawning. The blastula and gastrula stages were observed on Day 3 and then they began to form cilia. The embryo was able to move around inside the capsule on the fourth day. On the fifth day, each embryo formed its velum, shell, statocyst and a digestive gland on the same day. The digestive gland and stomach continued to develop until the end of Day 7 (Figure 2E). The total embryonic developmental period was 8 d after spawning (80 embryos) at 25-26 °C. The shell pattern of the veligers belonged to Type 1[16] (Figure 2F). The shell length (mean±SD, n=10) on the first day after hatching was (218.33± 4.08) μm. There was no evidence of eyespots or a propodium on any newly hatched veliger.

3.6. P. varicosa

Four adults of *P. varicosa* were found near the Chang Islands in December 2003 and April 2004 (Table 1). Egg masses were observed for 7 d after adults were released into the aquarium. Egg masses were yellow, with an anticlockwise rotation and were laid flat on the substratum. The zygotes were yellow and extra-capsular yolk was not apparent. Each egg capsule contained a single embryo. The diameter (mean±SD, n=10) of uncleaved embryos was (93.50± 10.84) μm. Cell division in the cleavage stage was completed 2 d after spawning. The blastula and gastrula stages were noticed from Day 3 until the end of Day 5. On the sixth day, embryos began to move inside the egg capsule by using their cilia. On Day 7, embryos formed their velum, shell and statocyst. The digestive gland and stomach were developed on Day 10 and remained at this stage for 2 d before hatching. The total embryonic developmental period was 14 d after spawning (140 embryos) at 24-26 °C. The shell pattern of the veligers belonged to Type 1[16]. The shell length (mean±SD, n=10) on the first day after hatching was (161.00±10.37) μm. There was no evidence of eyespots or a propodium on any newly hatched veliger.

3.7. P. rosans

P. rosans is a common species among the Phyllidid nudibranchs found in the Gulf of Thailand during the current study. Four adults of this species were collected near the Chang Islands in December 2003 and 2004 (Table 1). The broodstock laid only one egg mass that was attached to the aquarium screen for 3 d after releasing into the aquarium. The egg mass was yellow, had an anticlockwise rotation and was laid flat on the aquarium screen. Extra-capsular yolk was not observed. The length (mean±SD, n=10) of uncleaved embryos was (134.00±0.36) μm and each egg capsule contained a single embryo (1 egg mass). The embryonic developmental period was 17 d at 24-26 °C (170 embryos). The cleavage stage extended to 2 d and then the blastula and gastrula stages appeared from Day 3 until the end of Day 7. A cilia band around the embryo was observed on Day 8 and remained at this stage until the end of Day 9. On Day 10, velum, velar cilia, shell and statocyst were produced and continued to develop until the end of Day 13. The digestive gland and stomach were observed on Day 15. The veligers were hatched on Day 17. The shell pattern of the veligers belonged to Type 1[16]. The shell length (mean± SD, n=10) on the first day after hatching was (177.00±2.07) μm. There was no evidence of eyespots or a propodium on any newly hatched veliger.

3.8. F. rubrolineata

Only two adults of *F. rubrolineata* were found near Krok Island during December 2006 and only a single egg mass was attached at the branch of the hydroids. The spiral egg mass was a transparent cylinder and zygotes were orange in color. Extra-capsular yolk was not observed and each egg capsule contained a single embryo. The diameter (mean±SD, n=10) of the uncleaved embryos was (77.50±5.40) μm. Cell division during the cleavage stage occurred rapidly. The blastula and gastrula stages were noticed on the first day. On the second day, the embryos moved around inside the egg capsule using their cilia. Velum, shell and statocyst were developed on Day 3 and the digestive gland and stomach began to form on Day 4. The veligers were hatched out on the fifth day. The total embryonic developmental period was 5 d after spawning (50 embryos), at 24-26 °C. The shell pattern of the veligers belonged to Type 1[16](Figure 2G). The shell length on the first day after hatching was (126.67±5.77) μm (mean±SD, n=10). There was no evidence of eyespots or a propodium on any newly hatched veliger.

Changes in embryo diameter (μm) of eight nudibranchs during the embryonic period and embryo length and shell length at hatching are concluded in Figures 4 and 5.

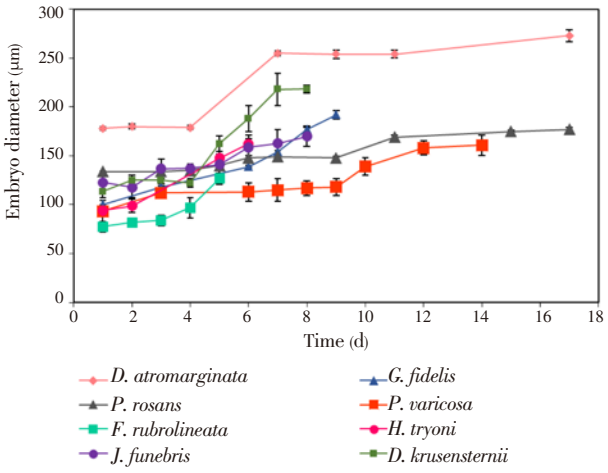

Figure 4. Changes in embryo diameter of eight nudibranchs during the embryonic period.
The vertical bars represent the standard deviation.

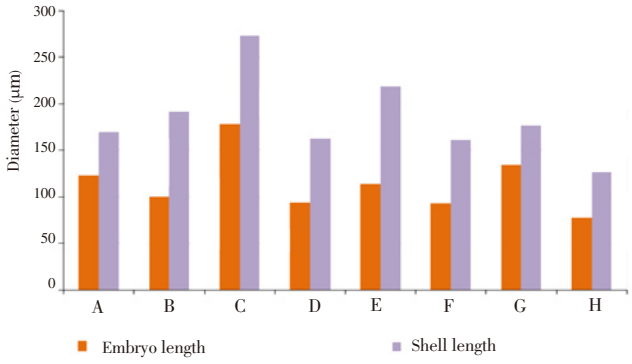

Figure 5. Embryo length and shell length at hatching for eight species of nudibranchs.
A: *J. funebris*; B: *G. fidelis*; C: *D. atromarginata*; D: *H. tryoni*; E: *D. krusensternii*; F: *P. varicosa*; G: *P. rosans*; H: *F. rubrolineata*.

4. Discussion

The three egg mass patterns of the eight species of nudibranch may be classified into three groups, according to their characteristics. The egg masses of *J. funebris*, *G. fidelis*, *D. atromarginata*, *H. tryoni* and *D. krusensternii* belonged to the group with upright, ribbon egg masses, which were similar to the other dorid nudibranchs. Wilson suggested that ribbon and upright egg masses are present in more than 50 species of chromodorid nudibranchs[20]. This suggests that more highly derived genera would lay more complex egg masses; thus the egg masses can reflect the potential of any phylogenetic influence. According to the phylogeny of the Chromodorididae proposed by Rudman[26], *Glossodoris* and *Chromodoris* are typically considered in the mid-region of evolution within the family and *Risbecia* is the crown group of the lineage. All these genera show that egg masses have an upright ribbon pattern and may also slope outwards or be crenulated. The structure of the egg mass has been said to reflect the degree of anatomical complexity of the reproductive system[27].

The Phyllidiidae (represented in the current study by *P. varicosa* and *P. rosans*) laid flat-ribbon egg masses on the substratum similar to the genus *Cadlinella* and were considered in the lower group of lineage of the Chromodorididae[26]. The advantage of this egg mass pattern could be camouflage, as it matches closely the color of the substratum. Usually the substrate is rock or dead coral that has been covered by sponges or other epibiotic lifeform. This mechanism may help nudibranch individuals to hide from predators.

In the current study, intra-capsular yolk was found only in *G. fidelis*. Wilson observed that extra-capsular yolk was present only in the upright egg masses of the Indo-Pacific *Chromodoris* species, while in other regions, some species of the same genus did not incorporate extra-capsular yolk[20]. These differences may reflect environmental, rather than phylogenetic influences[24].

Each egg capsule of all species in the current study contained one larva, except for *J. funebris*, which had 1-4 larvae inside each egg capsule, while *Madrella sanguinea* was reported to have a single fertilized egg per capsule[13].

In general, the egg capsule is composed of an outer membrane layer and an albuminous layer[19], with the capsule expanding simultaneously with the growth of the embryo. In the current study, *G. fidelis* and *D. krusensternii* demonstrated a good relationship between egg capsule and embryo diameter, with R^2 values of 0.8117 and 0.7412, respectively. This was similar to *Rostanga pulchra*, where the capsule was reported to enlarge progressively[24].

The uncleaved eggs can be classified into three groups: 1) small size (77.5-94.2 μm), taking 5-6 d to develop inside the egg capsule; 2) medium size (100.0-122.7 μm), taking 8-9 d to develop inside the egg capsule; and 3) large size (134-178 μm), taking 17-18 d to develop inside the egg capsule. However, an exception was noted for *P. varicosa*, which belonged to the small-size group but took more than 12 d for embryo development, because blastulation, gastrulation and the formation of the visceral organ took longer. This phenomenon may be a specific characteristic of the Phyllidiidae. In the case of *Dendronotus frondosus*, gastrulation was reported to be completed within 120 h at 15 °C after spawning longer than the current study (24-96 h), except for the two species of Phyllidiidae, which completed gastrulation within 120-168 h after spawning[28].

In the current study, the uncleaved egg of *J. funebris* could be classified as medium sized (122.7 μm) and required 8 d for embryonic development inside the egg capsule, whereas Page reported that the uncleaved embryo size of *Madrella sanguinea* was 74.5 μm and required 6.5 d for embryonic development inside the egg capsule[13]. The embryonic development mode of this species was planktotrophic development under water temperature ranged from 17-18 °C. This phenomenon indicated that the uncleaved embryo size varied somewhat for prediction of the mode of development of nudibranchs.

Overall, the data demonstrated that it was possible to predict the mode of development of the eight species of nudibranchs,

using the integrated knowledge of the shell pattern, uncleaved size, embryonic period and the appearance of eyespots and the propodium. According to Thompson[16], the shell patterns of all species in the current study are Type 1 shells, with the shell coiled and exhibiting a growth line. In contrast, Type 2 shells generally grow little after hatching, but the body grows during larval development to fill the shell. Goddard reported that the shell sizes at hatching of nudibranchs with planktotrophic and lecithotrophic development were 100-291 μm and 219-360 μm, respectively[11]; therefore, all of the species in the current study have planktotrophic development except *D. atromarginata* (shell size=273 μm), which may have lecithotrophic development. Predictions based upon the appearance of eyespots and the propodium, as proposed by Page[13], were not helpful in the current study, because none of the species studied showed a propodium before hatching, and only *G. fidelis* developed eyespots before hatching. More suitable criteria are needed to predict the exact mode of development of the latter species.

Based on the data of uncleaved size[15], *D. atromarginata*, *J. funebris*, *D. krusensternii* and *P. rosans* overlapped between planktotrophic and lecithotrophic development, although their shells were all described as Type 1[16]. It appears that more information is needed to predict nudibranch embryonic development. In the cases of *J. funebris* and *P. rosans*, the embryos could enlarge only 43 μm and 47.3 μm in length (38.5% and 32.1%), respectively. Thus, information about the enlargement of the embryo inside the egg capsule can help to confirm that the development mode of these nudibranchs is planktotrophic.

Goddard reported that 54% of the Family Dendrodorididae had planktotrophic development[29]. These nudibranchs usually spend less than 10 d in the embryonic period, similar to *D. krusensternii* in the current study. *D. krusensternii* had small-medium sized embryos (113.6 μm) and the embryonic period was only 8 d. However, if the enlargement of the embryo inside the egg capsule of *D. krusensternii* is considered, this suggests to be lecithotrophic development because the embryo of this nudibranch can enlarge itself about 105 μm in length (92.2%). This demonstrates that the enlargement efficiency of *D. krusensternii* in the embryonic stage is higher than that of the other species in the current study. This phenomenon is similar to the case of *Berghia verucicornis* where the shell pattern of the veligers belongs to Type 1 with an uncleaved length of 150 μm and the embryo can enlarge inside the egg capsule by about 110 μm, but it also has lecithotrophic development[30].

For identifying the mode of development, by setting the first priority criteria to be the appearance of eyespots and the propodium, it follows that all eight species of nudibranchs of the current study have planktotrophic development. If the shell size at hatching is considered, *D. atromarginata* may have lecithotrophic development.

In conclusion, it is suggested that all species in the current study have planktotrophic development except for *D. atromarginata* which may have lecithotrophic development, since this species has uncleaved and hatching sizes that are close to those of lecithotrophic species. Among the planktotrophic development species, *J. funebris* had more larvae inside the egg capsule compared to the other species; the embryonic period was not as long and it was widely distributed in Thai waters[21], which suggested it could be the first priority species for culture development with regard to any utilization and conservation aspects in the near future.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

A part of this research was also funded by the Center of Advanced Studies in Tropical Natural Resources, KU Institute for Advanced Studies, Kasetsart University and Mahasarakham University. The authors gratefully acknowledge the assistance of Associate Professors Jiraphat Aujjimangkul and Jantra Srisomwong. The authors express special thanks to the staff of the Marine Environmental Laboratory and the Fishery Resources Laboratory, Faculty of Fisheries, Kasetsart University, for ensuring the success of the field operations.

Comments

Background

Information about embryonic development is important to improve nudibranch culture. Over the last few decades, research on nudibranch embryonic development has been carried out in many areas of the world. Embryonic development modalities and dietary factors can have an effect on the distribution of larvae in their habitat. Extensive documentation (as the appearance of eyespots and the propodium, embryo size, pigmented egg cytoplasm and shell pattern) has been used to predict the development mode of nudibranch larvae. The knowledge obtained from this research may help to support improvements in nudibranch culture for future utilization and conservation.

Research frontiers

The current study explores the possible way to predict the mode of embryonic development of nudibranch, focusing on egg mass characteristics and embryonic development features, and including a detailed description of the shell patterns of the veliger larvae of the eight nudibranch species. The knowledge obtained from this research may help to support improvements in nudibranch culture for future utilization and conservation.

Related reports

It is an original work, and no other similar work on these species have been published before.

Innovations and breakthroughs

The methods are adequate for the study aims, and the results are new for science.

Applications

The knowledge on this manuscript is interesting for malacologists, marine invertebrate biologists and researchers working on developmental biology in general. Applicability may be achieved for commercial interest related to the studied species.

Peer review

The aim and results of this study are interesting to malacologists, marine invertebrate biologists and researchers working on developmental biology in general. It is a wide aspect research, on several species and considering different aspects of its reproductive biology. The considerations may be applied for culturing and commercial regulatory policies for native specimens exploitation.

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