

Larval development of nypa palm worm *Namalycastis rhodochorde* (Polychaeta: Nereididae)

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Abstract. Junardi, Anggraeni T, Ridwan A, Yowono E. 2020. Larval development of nypa palm worm *Namalycastis rhodochorde* (Polychaeta: Nereididae). *Nusantara Bioscience* 12: 148-153. *Namalycastis* intensive culture is still facing problems in mass production due to limited information on reproduction, especially in fertilization and production of larvae. The present research was designated to find out optimum salinity for fertilization and the production of nypa palm worm larvae (*Namalycastis rhodochorde*). Gamete samples were collected using a capillary glass tube inserted into ventro-lateral part of a body segment of a mature worm. Artificial fertilization was done by mixing the sperms and oocytes in a fertilization dish containing sterilized seawater as the medium. The larval development was observed until the benthic phase larvae (3-setigers). Fertilization was performed in medium salinity of 7-21‰ and water temperature of 25-29°C. The cleavage and larva stage occurred respectively within 28.20 to 58.67 minutes and within 72 to 80 hours after fertilization. The fertilization and larval development of *N. rhodochorde* were highly influenced by the medium salinity and temperature.

Keywords: Fertilization, larval development, *Namalycastis rhodochorde*, Polychaeta, salinity

INTRODUCTION

The supply of polychaete worm has been suggested to alleviate the problem of feed for shrimp broodstock in the aquaculture industry. The worm contains nutrients essential to promote successful reproductive performance by improving the number of eggs per spawning, increased egg viability, and larval survival in shrimp broodstock (Babu 2013). The nutrient possessed by polychaete comprises essential amino acids, unsaturated fatty acids, and cholesterol, as well as chemoattractant properties (Chimsung 2014; Phoosamran et al. 2017). The nereid polychaetes, for example, *Nereis (Hediste) diversicolor* and *D. pinnaticirris*, are rich in unsaturated fatty acids such as arachidonic, eicosapentaenoic acids, docosapentaenoic and docosahexaenoic acids and has a balanced essential amino acid (Wang et al. 2019; Wang et al. 2020). Other nereids such as *P. cultrifera* (Retto et al. 2013) and *D. pinnaticirris* (Wibowo et al. 2020) contain essential amino acids dominated by chemoattractant for shrimp. In addition, *Perinereis* sp. possesses prostaglandin E₂ (PGE₂) and progesterone (P4) and 17- α hydroxyprogesterone (17 α -OHP4), which have an effect on vitellogenesis and maturity of shrimp gametes (Meunpol 2010). For that reason, the polychaete worm has been intensively used for marine shrimp broodstock maturation diets.

Nereidid polychaetes often occur as major components of the macrobenthic fauna in estuaries (Sato 2017; Ibrahim et al. 2019). Nypa palm worm (*Namalycastis rhodochorde*) is one of the Nereidid Polychaeta species that has long been used as fishing bait for fish and shrimp in West

Kalimantan, Indonesia (Junardi et al. 2014). The worm and other polychaetes, including Arenicolidae, Glyceridae, Nephtyidae, and Eunicidae, are harvested from its natural habitat for fishing bait (Carvalho et al. 2013; Saito et al. 2014). The massive collection of polychaete worm from its habitat for commercial purposes is considered non-sustainable for the environment, as it contributes to the depletion of natural resources (Pires et al. 2012). Unfortunately, scientific knowledge such as reproduction and larval development of *N. rhodochorde*, which could be applied in the worm mass culture and the maintenance of natural stock sustainability, is not considered available. Development and reproduction of *N. abiuma* were already studied (Junardi et al. 2013). Meanwhile, the study on reproduction, especially early embryonic development of *N. rhodochorde* has not been undertaken.

Temperature and salinity are key factors affecting the physiological and ecological responses of organisms, including polychaetes inhabiting estuaries and brackish waters (Daňko et al. 2020). For instance, uninterrupted embryonic and larval development of the polychaete worms *H. carunculata* does not occur at 22°C meanwhile, normal larval development takes place at 27°C (Toso et al. 2020). The nypa palm worm, *N. rhodochorde*, which inhabit estuary, encounters a highly dynamic physicochemical change in time and space scales. The worm ought to have an adaptive capacity to live in a wide range of temperature and salinity.

Epitokous phase of polychaete reproduction has become a reference for the study of fertilization, larval development, and the factors that influence it. However,

some species, such as *N. glandicineta* (Ibrahim et al. 2019), *N. abiuma* (Junardi et al. 2014), and *D. pinnaticirris* (Wibowo et al. 2020) do not have such epitokous phase that reproduction time cannot be predicted. The characteristics of larval development cannot readily be determined. Larval development and fertilization studies in polychaete have been done since the 1960s, and recent studies of larval development of *Mooreonuphis stigmatis* (Budaeva and Fauchald 2010), *N. abiuma* (Junardi et al. 2013), and *N. diversicolor* (Wang et al. 2020) were carried out. Since 1980s further study on polychaete embryonic development and artificial fertilization has been developed for providing scientific information applied in commercial polychaete aquaculture (Olive 1999). The present study is designated to find out the optimum salinity for fertilization and larval development of nypa palm worm, *N. rhodochorde* larvae.

MATERIALS AND METHODS

Collection of adult specimens

Nypa palm worms, *N. rhodochorde* were collected from the mangrove area of Kapuas estuarine in West Kalimantan, Indonesia on May-July 2012 at low spring tides. The worm was transported to the Zoology Laboratory, Tanjungpura University, Pontianak, West Kalimantan using a container filled with sediment taken from its natural habitat. As soon as its arrived at the laboratory (about 30 minutes), the specimen was then removed from the container by handpicking. The visual sorting was done to selectively seize mature worms from immature individuals. Selected mature worms were then placed in a separate container. Mature individuals were distinguished from the immatures based on body coloration. Female mature individuals showed reddish body color, while mature males showed greenish body color.

Collection of gametes

Gametes from each individual were drawn by inserting a capillary glass tube into the ventrolateral part of body segments, and the fresh gamete sample was observed under a light microscope. The matured egg was determined by measuring its diameter and identifying the appearance of lipid droplets in the ooplasm. Egg diameter was measured by eyepiece micrometer, and it is used as an indicator of fertilization success. The maturing egg diameter, in which lipid droplets have already undoubtedly appeared in ooplasm, ranged between 120-130 μ m. We used sperm samples taken from males with pale greenish ventral body color as an indication of maturity. Maturing eggs and spermatozoa with a 2:1 ratio, respectively, were then mixed in a dish containing a fertilization medium of sterilized filtered seawater.

Fertilization experiments

Fertilization was conducted in the laboratory within the medium of sterilized filtered seawater. Initially, the experiment was carried out in a series of salinity ranging from 1‰ to 33‰, i.e., 1-3‰ until 31-33‰ at room

temperature. The fertilization rate was calculated based on the percentage of the successfully fertilized egg to the total number of eggs in a sterilized glass petri dish (6 cm in diameter, eggs number in each dish = 30). The highest number of the fertilized egg and larval development among fertilization dishes containing medium with different salinities were ascertained and applied to determine the optimum salinity for artificial fertilization and growing the larvae. The required water salinity was obtained by mixing seawater and distilled water. Medium salinity was measured with a hand refractometer for salinity (Atago ATC-S/Mill-E, Japan). Optimum salinity was determined by designating the different salinity of seawater in the experimental dishes in which successful fertilization, cleavage, and larval development were found.

The fertilization process was observed under the Nikon Eclipse TE300 inverted microscope equipped with Nikon DXM 1200F camera. Successful fertilization was determined by the formation of the fertilization membrane and the initiation of egg cell division. The egg was then observed every 5 to 10 minutes. Unfertilized eggs were counted and then removed from the fertilization medium. The process of development comprises the cleavage stages of 2, 4, 8, 16, and 32 cells until the 3-setiger larval stage. Larval morphology and development were observed and described at each stage of development. Larval morphological characteristic refers to other larvae of Nereididae (Rouse 2000), *Hediste* (Sato 1999) and *Namanereis littoralis* (Ezhova 2011). Descriptive analysis was used to compare based on a photographic series of their development.

RESULTS AND DISCUSSION

Results

Description of larval development

Maturing eggs are indicated by the accumulation of lipid droplets in the center of ooplasm (Figure 1A), meanwhile fertilized eggs were indicated by perivitelline vesicle and gelatinous, adhesive outer membrane (Figure 1B). The fertilized eggs are surrounded by a fertilization membrane. An appearance of all developmental stages of nypa palm worm is presented in Figure 2.

Fertilization was accomplished during minutes 28.20 to 58.67 (n = 20) on average (Table 1). The characteristics of cell division were described by the spiral cleavage, holoblastic, meridional, and unequal (Figure 2A-D). The first cleavage occurred between 10 to 100 minutes after fertilization was initiated by the formation of unequal blastomeres.

Table 1. The cleavage of *Namalycastis rhodochorde* in a 7-21‰ salinity range

Salinity (‰)	Average time (minutes)	Number of experiments (n)
7-9	58.67	3
10-12	28.20	5
13-15	37.40	5
16-18	51.00	4
19-21	35.67	3

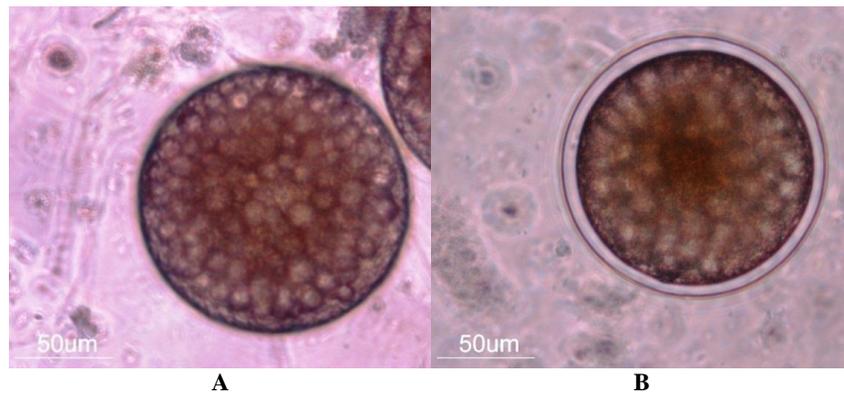


Figure 1. Eggs of *Namalycastis rhodochorde*. A. Unfertilized; B. Fertilized (zygote)

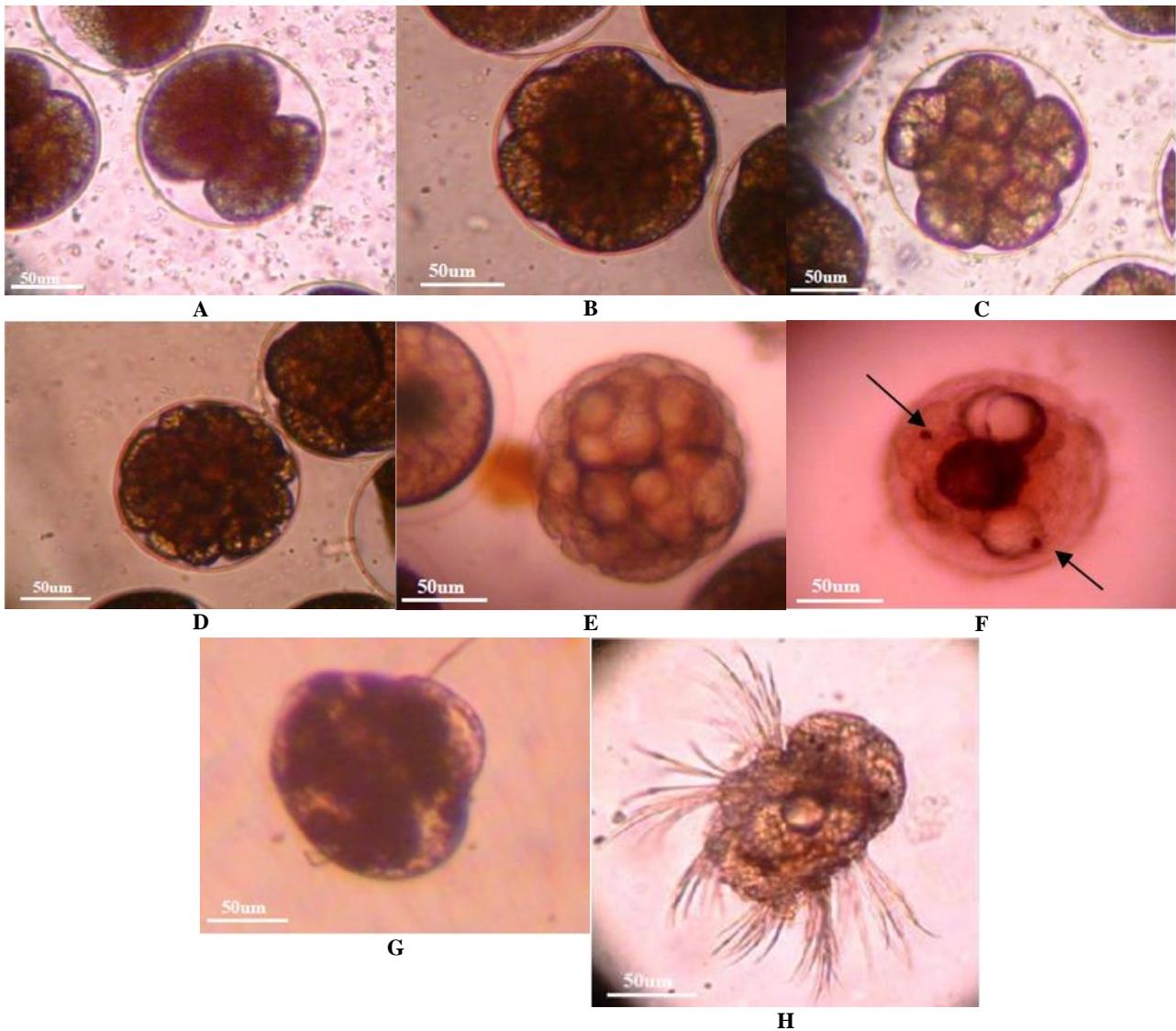


Figure 2. Stages of larval development of *Namalycastis rhodochorde*. A. 2-cell division (36 minutes); B. 4-cell division (42 minutes); C. 8-cell division (53 minutes); D. 16-cell division (120 minutes); E. 32-cell division (150 minutes, rotating larvae); F. trochophore larvae with two eyes (240 minutes, free-swimming larvae, black arrows indicated eyes); G. metatrochophore (22 hours); H. 3-setiger early nectochaeta (3 days)

Early trochophore larvae exhibited slow rotation driven by multi-ciliated equatorial prototroch (Figure 2E) and followed by the formation of two eyes (Figure 2F). The next stage was late trochophore (Figure 2G) and metatrochophore indicated by the development of chaetae and chaetigerous segment. The larvae were then supplemented by the development of chetigerous parapodia (chetiger) and antenna at the prostomium part of the larval body. Segmented larvae formation of the three first segments (3-setiger) or early nectochaeta (Figure 2H) was formed within three days after fertilization. The final stage of larval development is called nectochaeta, which takes six days. The morphological characteristic of this final stage is the formation of a pair of tentacular cirri and anal cirri in the part of the body.

Fertilization experiments

Free-swimming larvae have a high mortality rate when the salinity of the culture media is lower than 7‰ or higher than 21‰ (Figure 3). Optimum salinity for fertilization and larval development was ranged between 10-21‰. Based on this result, we conclude that larval survival is strongly influenced by water salinity. Survival of embryo until 3-chetiger larvae are only found in the salinity range of 10-18‰ (Figure 4). The highest survival was found in salinity 10-12‰ as much as 18%, followed by 13-15‰ (8%) and salinity 16-18‰ (6%).

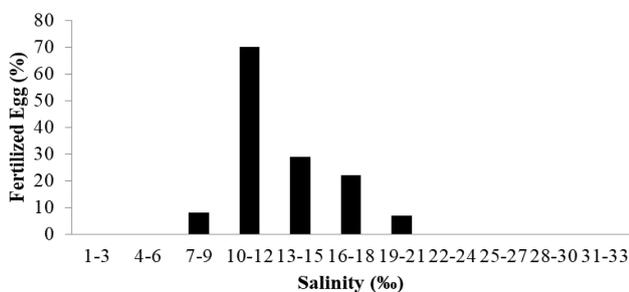


Figure 3. Percentage of eggs of *Namalycastis rhodochorde* fertilized with different salinity

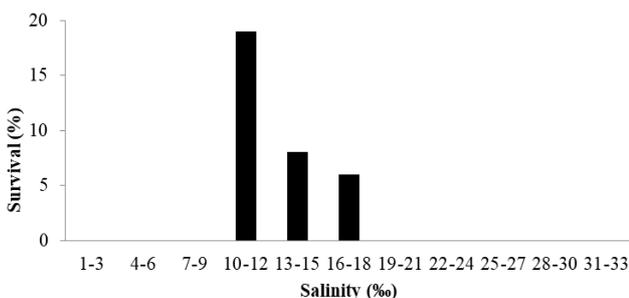


Figure 4. Percentage of 3-chetigers larvae of *Namalycastis rhodochorde* which were surviving on different salinity after three days

Discussion

In general, maturity of Nereid worm such as *Perinereis cultrifera* (Rettob et al. 2013), *N. glandicincta*, and *N. wilsonchani* (Lee and Glasby 2015; Ibrahim et al. 2019) was characterized by swarming epitoke and with or without changes of body color. Unlike other nereidid species such as *Neanthes glandicincta*, *Ceratonereis (Compositia) burmensis* (Lee and Glasby 2015), and *Neanthes glandicincta* (Ibrahim et al. 2019), the nypa palm worm does not form modified parapodia, chaeta, and eyes for spawning processes in the water (Junardi et al. 2014). Nypa palm worm maturity characteristic was much the same as *Nereis diversicolor* (Wang et al. 2020) and *D. aestuarina* (Jayachandran et al. 2015). The nypa palm worm maturity is characterized only by changes in body color and non-swarming epitoke. Matured male individuals were characterized by the changing of body color from pink to dark red with greenish, while the matured female individuals exhibited dark-red on the side part of the body (Junardi et al. 2014) while the females of mature *N. diversicolor* showed a yellowish, dark green color (Wang et al. 2020). Similar to *Nereis* sp. (Sahu et al. 2017) and the mature oocytes of *N. rhodochorde* were characterized by a spherical shape with a diameter ranged between 120-130µm. The oocyte shape of *N. rhodochorde* is different from that of *N. littoralis* egg, which is oval (Ezhova 2011). The size of matured eggs of *N. rhodochorde* is smaller than that of *P. cultrifera* (Rettob et al. 2013) and *Nereis* sp. (Sahu et al. 2017) that reach 240 µm and 325.82 µm in diameter, respectively.

Fertilized egg showed lipid droplets migration to the peripheral part of the egg. This characteristic is also found in other Nereidid species i.e., *P. dumerilii* (Fischer 2010). The cleavage of *N. rhodochorde* is also similar to *P. dumerilii* (only Nereididae) characterized by a series of asymmetric cell divisions (ACDs) that generates cells of different size and defined position within the early embryo (Nakama et al. 2017). After the 32-cell division, the next stage is a stereoblastula. The Prototrochopore indicated by slowly rotating, and then the early stage of trochophore larvae indicated by actively swimming in the water column and lipid droplets still exist in these stages. Lechitotropic larvae are short-period growing larvae with the yolk as a food source for metamorphosis. However, the characteristics of the nypa palm worm's larvae could be very different at the subfamily level. For example, late-stage *N. littoralis* larvae are coated inside a capsule rather than free-swimming larvae (Ezhova 2011).

The trochophore larvae of *N. rhodochorde* are also characterized by the enlargement of its diameter from 140 µm into 200 µm with body surrounding cilia (equatorial cilia) and two-eye development. Free-swimming larvae emerged on the second day after fertilization; it was gradually turned into the oval and ciliated body at the latero-dorsalis part. These free-swimming larvae have a high mortality rate when the culture media's salinity is lower than 7‰ or higher than 21‰. The low survival of the polychaete most probably is affected by low salinity

(Leung et al. 2013). Based on this result, we conclude that larval survival is strongly influenced by water salinity.

Many polychaetes species of family nereidid inhabit estuary ecosystems (Pederson et al. 2010; Saito et al. 2014; Linden et al. 2017) subjected to a high degree of variability in their environmental conditions, especially salinity variation due to freshwater and seawater inputs (Hernández-Alcántara et al. 2014). Such dynamic environmental conditions were presenting a number of problems for externally fertilizing species, and consequently, their fertilization success might occur in an optimum range of medium salinity. An extreme salinity level brings about osmotic stress associated with cell shrinkage or lysis. The mechanism for coping with osmotic stress is volume regulation (Hoffmann and Pedersen 2011) and such physiological response that facilitates the worm to tolerate a wide range of medium salinity. It has been reported that mature oocytes fertilization in Nereidid might occur in a wide range of medium salinity, but *Dendronereis aestuarina* fertilization occurs in a purely freshwater environment of Periyar River on the southwest coast of India (Jayachandran et al. 2015). In the present study, we found that optimum salinity for fertilization was ranged between 7-21‰ and 10-11‰ for larvae survival. It seems that the reproduction characteristic of *N. rhodochorde* is well fitted to fluctuating salinity in the estuarine environment. Similar to most estuarine polychaetes (Hernández-Alcántara et al. 2014), the nypa palm worm seems to be well adapted to salinity variations in its habitat. The range of salinity was also found to be convenient for nypa palm worm larval development. The optimum range of salinity was also found similar to that of subfamily level, *N. littoralis* (6-21‰) (Ezhova 2011). On the other hand, at family level, such as *Hediste diadroma* could adapt to higher salinity of 27.5-30‰, which is usually unfavorable for fertilization of other Nereidid species (Junardi et al. 2014).

The fertilization and larval development of *N. rhodochorde* is highly influenced by water salinity. In this study, salinity for fertilization and larval development before 3-chetigers ranged between 7-21‰. Meanwhile, the survival of *P. rullieri*, kept in salinity of 10‰ in first-month experiment, was 60% (Prevedelli and Vandini 1997). This salinity is lower than that of *Hediste japonica* larvae well developed in the range of 10-30‰ (Tosuji and Sato 2006) and salinity for development of *N. virens* in 22‰ (Ushakova and Sarantchova 2004). The difference in salinity depends on the species and locality for environmental adaptation. The salinity range for larval development in this study can be used for further studies on the favorable salinity for survival rate of the larvae.

Temperature is an important environmental factor affecting estuarine organisms' physiological responses (Daňko et al. 2020). The present study demonstrates that the water temperature of 25-29°C is favorable for nypa palm worm fertilization and larval development. This confirms previous findings that polychaete larval development does not occur at 22°C, but normal larval development occurs at 27°C (Toso et al. 2020). In fact, the temperature range in tropical estuaries is always reasonably

favorable because normally almost similar all year round, while in the temperate zone, the temperature drops during autumn and winter at the level unfavorable for reproduction and development. However, it is interesting to study the impact of the rising temperature due to global warming on Polychaetes reproduction and development from the tropical zone in the future.

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