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Optimization of Culture Conditions for rearing of Barnacle Nauplii Amphibalanus amphitrite

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ABSTRACT

Amphibalanus amphitrite is a ship-fouling species encountered in many water bodies of world. Inspite of its fouling characteristic, these organisms are used in laboratory for many studies like toxic test of chemicals, heavy metals and for antifouling research. Larval culture conditions for barnacle has to be optimized. The effects of temperature, photoperiod, diet and salinity on the growth and survival of *A. amphitrite* larvae was studied. Effect of temperature played a role on development time and survival of the nauplii. At 24˚C the time utilized for the larvae to reach the cyprid stage was longer when compared with that of 28˚C, and above 28°C there was an increase in mortality over 90%.Mid-range temperatures (25 – 28˚C) increased total survival (20–35%). Diatom diets of *Thalasiosirra subtilis*, *Endomoneis paludosa* and green algae *Isochrysis galbana* did not have significant effect on specific growth rate, but the proportion of high-quality healthy cyprids was greatly higher with mixed diet. The development time taken for the larvae to reach the cyprid stage differs depending upon the availability of light (2500 lux). Photoperiod did not play a role on the survival, but specific growth rate was higher at 24:0 and 16:8 L:D. The release and survival of the nauplius depends upon the ranges of salinity (25 and 30 psu). The survival and growth were got using rearing temperatures of 25 – 27°C, daily feeding with *T. subtilis, E. paludosa, I.galbana* and a photoperiod of 24:0 L:D along with salinity of 30 psu.

KEYWORDS: Barnacle larvae, Nauplii, Cyprids, Ambient level, A. amphitrite

1. INTRODUCTION

In the marine environment all natural or man-made surfaces are prone to biofouling of a wide variety of micro and macro-organisms [7], which causes serious problems to maritime industries and vessel hulls, by increasing degradation of submerged materials and reducing performance. Due to the use of toxic antifouling agents and increased energy consumption, major environmental and economic consequences has been reported [9]. Therefore, studies have been made regarding larval development and the transition from planktonic to sessile life stages of biofouling species, in order to understand the fouling and figuring out a new way to prevent it. *Amphibalanus amphitrite* is one of the most common macrofouling species, however its larval development and larval cycle characteristics are under explored. This is often seen on shady rock surfaces of the subtidal and intertidal zones, and on artificial marine structures, such as aquaculture nets and piping, ship hulls etc. The current study was aimed to describe *A. amphitrite*

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larval development and examining the characteristics of the different larval stages. Laboratory culture of *A. amphitrite* is done globally to understand their biology, life cycle as well as mechanism of settlement which is essential in order to prevent marine fouling [1]. *A. amphitrite* larvae can be cultured individually [8] or in groups [10,15]. Individual culture usually carried out in multi-well plates, where very small number of naupliar instars are inoculated and cultured till the cyprid stage [2]. In group or batch culture, a large number of naupliar instars were cultured together in a larger volume container. Individual culture usually carried out in multi-well plates,with a small number of larvae; growth and survival can be followed individually; and cultures can be maintained easier in the experimental period.

2. MATERIALS AND METHODS

Collection and maintenance of nauplii

Barnacle nauplii collected from the Ennore coastal waters (Lat. 13.232190; Long.80.330068) by horizontal towing of plankton net (90µ mesh size) were transferred into polypropylene bottles and transported to laboratory. The nauplii *Amphibalanus amphitrite* was identified using the morphological identification characteristics described by Lang, (1979) [12]. The nauplii were maintained in a glass aquarium tank, feed twice a day with the diatom species namely, *T. subtilis, E. paludosa,* and *I. galbana* as food sources.

Experimental Design

The barnacles life cycle has six free-swimming larval stages, non- feeding cyprid stage and adult stage sedentary within a calcareous shell. For culturing larvae supply of seawater, facility for temperature control, salinity, light, food and for rearing larvae right from nauplius stage to young adults is essential. Temperature plays a vital role in the development of the larvae. The quantitative effects of temperature on larval size and rate of development were observed in *A. amphitrite*. In this study, stage II nauplii separated into two populations were maintained at 22˚C and 28˚C with same conditions of light and food. At regular intervals the samples in the populations were seen. As successive larval stages appeared, the lengths and widths of 10 to 15 individuals were recorded. Salinity played an important role in both the survival and release of nauplius from the adult. The salinity was maintained between 30 ± 3psu in a controlled condition. This was found to be an appropriate level for the survival and release of nauplius. The barnacle larvae and adults were maintained at different salinity levels and their survival rate and development have been recorded. Light plays a vital role in the rearing of barnacles. In the dark, the larvae were found to be defective in development. The significance of the availability of food is uncertain in different conditions, since the diatoms multiply under the light conditions and not in dark. It was examined that constant overhead light produces optimum conditions for uniform rearing throughout the experiments. Feed and its concentration were found to be a vital factor in the growth rate and survival of barnacle larvae during development. Three diatom species namely,

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T. subtilis, *E. paludosa*, and *I. galbana* were used as food sources. Abnormal development of the barnacle larvae variation could be caused by variation in food culture has been recorded.

Measurement of Physico-chemical parameters

Light intensity, temperature and salinity were maintained throughout the growth experiment. The temperature was measured twice a day using a glass mercury thermometer with 0.1°C resolution in the experimental chamber. Refractometer was used to measure the salinity (Make: ATAGO, Serial No. 0144294). The light intensity was measured by placing a photo receptor cell of a digital lux meter (Make: Lutron, Model: LX-101) on the aquarium tank.

Morphologial studies

Sampling of nauplii was performed every day with fixation in saline formaldehyde to be further analysed on an inverted microscope (Nikon Eclipse TS100). Different morphometric traits were measured in all naupliar stages: total body length, shield width, shield length, length of the fronto-lateral horn and the length till theend of the abdominal process, from the anterior margin of the shield to the beginning of the bifurcate ramus. Length of the Shield stages I to III was measured accordingly our perspective from where shield should form since there is no shield delineation in this stages. Many Photographs were taken of the whole nauplii in high resolution specifically of the anatomical features, such as naupliar eye, labrum, filaments, thoracico-abdominal process, antennules, antennae and mandibles (Nikon Imaging Software - NIS-Elements) to help to completely analyze the different stages and to compare with the scientific drawing.

3. RESULTS

Effect of physico-chemical parameters for Barnacle

Temperature

Effect on development time and effect on survival of larvae depends upon temperature under controlled conditions. At different temperatures (24˚C and 28˚C) the time taken by the barnacle larvae (Stage II) to develop to the cyprid stage was recorded. At 24˚C the time taken for the larvae to reach the cyprid stage was longer when compared with that of 28˚C. (Table 1). High or complete mortality of the larvae was observed at high temperatures, which exceeds 28˚C in controlled conditions with survival increasing at a lower temperature (Table 2). However, comparison of the development and survival rates at both the temperatures would appear to be valid, as the conditions of seawater, light and added food were the same at both temperatures.

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Table 1 Number of days taken for the barnacle larvae (stage II) to develop into cyprid at different temperatures (24˚C and 28˚C)

Experiments	24 ˚C	28 ˚C
1	14	6
2	12	7
3	15	8
4	13	7
5	12	6

Table 2 Survival of the barnacle larvae (stage II) till cyprid stage at different temperatures (24˚C and 28˚C)

Experiments	Number of larvae	24˚C	28˚C
1	10	8	10
2	11	7	10
3	10	7	9
4	11	8	10
5	12	9	11

Salinity

The release and survival of the nauplius depend upon the ranges of salinity. At two different salinity ranges (25 and 30psu), the frequency for the release of nauplius from the adult barnacle was observed to be different. The release of the nauplius and its survival rate is found to be less in low salinity when compared to that of high salinity, under the controlled condition (Table 3 & 4).

Table 3 Frequency (per week) of the release of barnacle nauplii at two different salinity ranges

Experiments	25 psu	30 psu
1	1	3
2	2	3
3	2	4
4	1	3
5	1	4

Table 4 Survival of the barnacle nauplii (Stage II) at two different salinity ranges

Experiments	Number of larvae	25 psu	30 psu
1	10	6	10
2	12	7	11
3	10	5	10

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4	11	5	11
5	11	6	10

Light

The effect on larval development was seen under dark and light conditions. The time taken for development of the larvae to the cyprid stage depends upon the availability of light. Under the dark condition, the development rate of the cyprid was found to be longer (Table 5).

Table 5 Developmental time taken (Days) for the larvae (stage II) to reach the cyprid stage under dark and light source

Experiments	Under dark condition	Under Light (2500 lux)
1	12	7
2	15	6
3	12	6
4	16	7
5	16	6

Feed

The growth rate and survival of the larvae mainly depend upon the feed available for them. Mixed diatoms serve as an effective feed for the Balanus larvae and adults, under controlled conditions as of temperature between 28 ± 2, salinity 30 ± 2 and light period of 12h: 12h.

Development and Morphological characteristics of Barnacle larvae (*Amphibalanus amphitrite*)

Barnacle's life cycle consists of two larval forms, first form, the nauplius which undergoes a series of molts results in six different developmental naupliar stages [11]. Each stage is sequentially larger in size and its appendages bear number bristles or setae than the prior. The last naupliar stage molts into the non-feeding cyprid stage, which finds a suitable place for attachment and undergoes final metamorphosis into a juvenile sessile barnacle.

The dorsal surface of the barnacle nauplius is covered with a triangular or shield-shaped cephalic structure known as the carapace. Pair of prominent front-lateral horns on either side of the anterior end of the cephalic shield and a prominent naupliar eye present at the center of the anterior end along with three pairs of biramous appendages and two prominent posterior

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structures, the dorsal thoracic spine and the ventral furcal ramus were present. The cyprid larva is oblong, bivalved and has six pairs of thoracic appendages and a pair of compound eyes.

Table 6 The average developmental periods of the six larval and one cyprid stages of *A. amphitrite* under controlled condition

Developmental stages	Developmental stages in hours
Stage I to stage II	4 to 6
Stage II to stage III	70 to 80
Stage III to stage IV	35 to 72
Stage IV to stage V	24 to 36
Stage V to stage VI	60 to 96
Stage VI to cyprid stage	120 to 144

Table 7 Size of laboratory-reared *A. amphitrite* larvae in controlled condition

Stages	Carapace width (µm)			Carapace length (µm)			Total Length (µm)	
	Mean ± S.D	Range		Mean ± S.D	Range		Mean ± S.D	Range
I	177 ± 11	170 - 185					265 ± 7	260 - 270
II	202 ± 9	195 - 210					332 ± 4	315 - 350
III	242 ± 6	220 - 265					365 ± 6	340 - 390
IV	275 ± 11	255 - 325		280 ± 11	250 - 310		408 ± 11	385- 430
V	377 ± 5	360 - 395		373 ± 14	360 - 385		503 ± 13	485 - 520
VI	462 ± 3	435 - 490		452 ± 12	430 - 475		550 ± 5	525 -575
CYPRID	405 ± 8	390 - 420					465 ± 9	440 - 490

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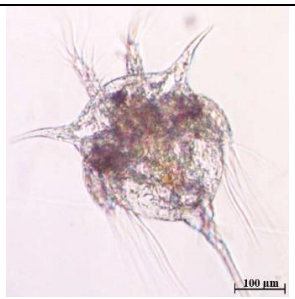
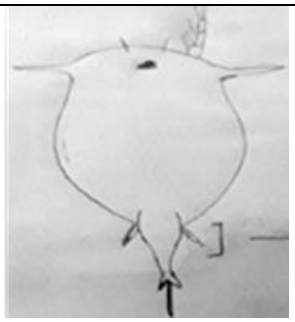
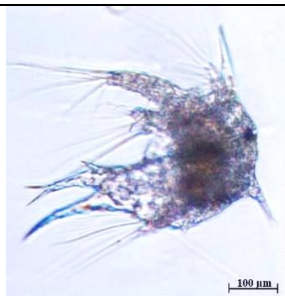
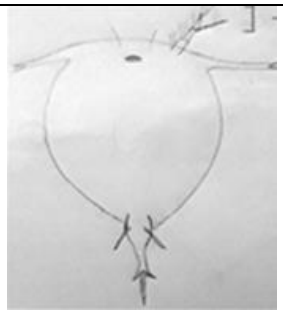
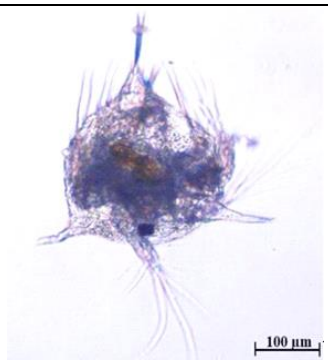
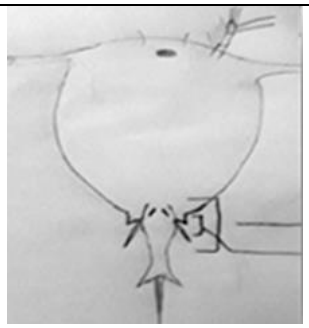
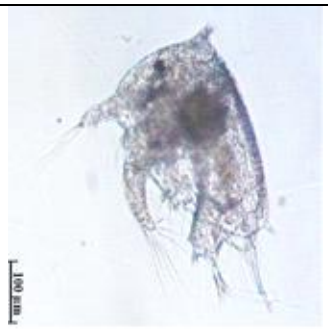
Table 8 Nauplius Larval stages can be identified based on the basis of the antennules setation and the presence of abdominal spines

STAGES	MORPHOLOGICAL CHARACTERISTICS	DIAGRAM	IMAGES
I	Pearshaped Presence of folded fronto-lateral horn		
II	Presence of pointed fronto-lateral horn Tip of each horns are unsplited One pair of abdominal spines on the antennules.		
III	Split fronto-lateral horn tips 1 preaxial seta on the antennule		
IV	2 preaxial setae on the antennule Two pairs of abdominal spines Carapace modification		

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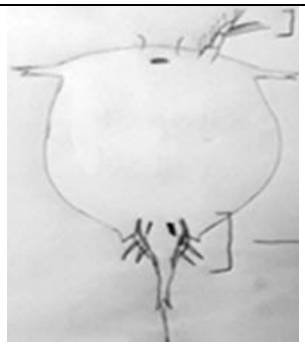
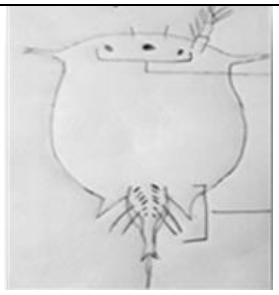
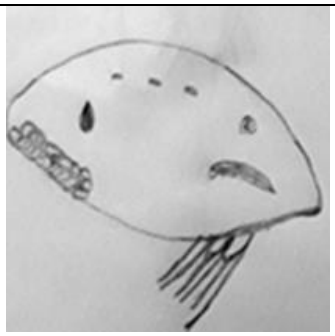
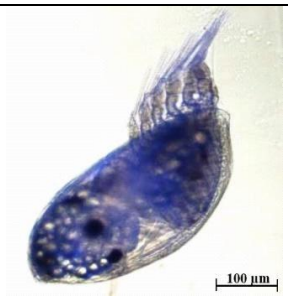
V	3 preaxial setae on the antennule Three pairs of abdominal spines		
VI	Presence of compound eyes. Six pairs of abdominal spines		
CYPRID	Six pairs of thoracic appendages, these appendages are modified into cirri of adult which aids in feeding. Presence of compound eye.		

4. DISCUSSION

Optimization of *A. amphitrite* plays a vital role in continuous rearing of larvae in laboratory condition which could be used to improve the techniques of evaluating toxins. A better understanding is sought for controlling mechanisms in metamorphosis and growth of barnacles, and to investigate the possibilities of exploiting these biological control mechanisms in an antifouling system. The life cycle of barnacle has six free-swimming larval stages. An adult sedentary stage is also within a calcareous shell. The first naupliar stage is produced by the egg in the shell and five further stages are developed. Each one is larger and complex than the previous one. Phase VI nauplius converts the cypris into the young adult barnacle and undergoes the final metamorphosis after a period of time. In order to understand the growth and metamorphosis in barnacle larvae, a number of experiments have been carried out by adapting different laboratory culture techniques.

A rearing technique was developed in order to provide continuous supply for larvae and young adult barnacles. The growth rate of young adults in the laboratory is dependent on whether they are kept in static or flowing seawater. Though barnacles under static conditions were fed with

diatoms at a concentration of 5×10^5 cells/ml, their growth rates were less than those in flowing seawater. A greater variety of planktonic species available as food in flowing seawater may explain the faster growth, even though larvae will develop satisfactorily on a unialgal supplementary food. Hence, the type and concentration of algal food appear to be a critical factor in the rearing of cirripede larvae. The food supply has to be increased to achieve the efficient production of barnacle larvae. In the present study, the best growth rate was observed with the larvae fed with haptophyte microalgae *I. galbana* followed by mixed diatoms of *T. subtilis* and *E. paludosa*. Other workers [16] have all reported successful development of a variety of barnacle species using *S. costatum* and it appears to have a wide application as a food in the rearing of larvae of the Balanidae.

Earlier workers used phytoplankton species for this purpose, but *Phaeodactylum tricornutum, Cyclotel Ianana* and *Skeletonema costatum* formed the prevalent diatoms. Barker, (1976) [3] reared cyprids of the boreal species *Balanus balanoides* with diatoms as food. The survival rate of the larvae was reported to be maximum. *C. columna* was reared to the cyprid stage with the flagellate *Isochrysis galbana. C. brunnea* to nauplius VI was on a diet of equal amounts of the flagellates *Isochrysis* and *Dunaliella primolecta.* Metamorphosis to the cyprid stage did not occur until the diatom *Skeletonema* was supplied to the sixth stage nauplii; thus, diatoms may be important in the diet of later stage larvae of this species. In cultures with *Skeletonema* as the only food source, development did not proceed past nauplius III. Brine shrimp *Artemia sp*. and microalgae such as *Chaetoceros sp.* or *Skeletonema sp.* are usually used as substitute feed for the brood stock and the specified microalgae are used for the rearing of larvae. *Elminius modestus* cyprids appeared after 6 days at 22°C. When *Skeletonema costatum* is fed the development peried are less than 15 to 20 days at 19°C to 22°C than using *Phaeodactylum tricornutum.* A critical factor in rearing techniques is the food source by Corner and Cowey, (1968) [4].

Duration of larval development of *Amphibalanus amphitrite* from naupli I to cyprid was recorded to be 5-6 days, 80% of larvae survived up to cyprid stage. The optimum temperature was recorded to be 28˚C ± 2 with salinity 30 psu. The larvae of *A. amphitrite* released at the stage of nauplius I and are identified by its uniformly arched frontal border with the single median eye. The average body length of larval stages were recorded as 270 µ, 350 µ, 390µ, 460 µ, 500 µ, 575 µ and 620 µ for nauplius-I, II, III, IV, V, VI and cyprid stage respectively. Maximum mortality of larvae was recorded in the 3rd and 4th day of culture during nauplii IV and V. Desai and Anil, (2005) reported similar observations suggesting that nauplius IV and nauplius V are vulnerable phases in the development of *B. amphitrite* in laboratory conditions [6]. Costlow and Bookhout, (1957) observed maximum mortality of the *Balanus eburneus* larvae reared under laboratory conditions during nauplius V stage and suggested bacterial infection as the chief cause [5].

Patro, (2012) reviewed and compared the biometry of laboratory reared larvae of *A. amphitrite* from different geographical locations such as Mumbai, Kerala, South Carolina, North Carolina, Brazil, Australia and Japan. The data suggests that though there is a general agreement on the description of different larval stages, considerable variation in size does exist between different geographical locations. The body length of larval stages of *A. amphitrite* from South Andaman differed from previous observations mentioned from other geographical areas. The variation in size of the same species from different geographical locations may be due to the environmental conditions of a particular geographical area which affect the growth of the adult barnacles and size attained by the nauplii.

The early description of laboratory culture of *Balanus trigonus* in laboratory condition demonstrates that that the percentage of larvae survivorship is maximum at temperature of 28˚C and salinity of 34psu. Libralato *et al.,* 2016 tested the effect of salinity on *B. amphitrite* larvae culturing under a controlled condition at 25±1˚C, food concentration of $2x10^5$ cells/ml (*Chaetoceros calcitrans*) and found that the survival of larvae is highest (66%) during low salinity (12 psu) and lowest (14 psu) during high salinity (36psu) condition [13]. Gossrlin and Qian, (1997), proposed that *B. amphitrite larval survivorship* and development duration has impact on food concentration in laboratory conditions . The study suggests that at maximum food concentration (2×10^5 cells/ml) the percentage of larval survivorship is highest and reduces with decrease in food concentration. Nasrolahi, (2007) cultured the larvae of *Balanus improvisus* under laboratory conditions at different food concentrations and found that the larval settlement is maximum at high food concentration (2×10^5 cells/ml) and minimum during low food concentration ($0.5x10^5$ cells/ml) [14].

5. CONCLUSION

The study of growth characteristics of marine barnacle is essential for various applications. There are approximately a dozen species of commercially interesting barnacles worldwide, some of which have been cultured on a semi-industrial scale, as they are consumable. Globally for antifouling research, barnacle larvae are reared in the laboratory. As these organisms are very sensitive, produce a number of nauplii at a time and have the high breeding capability, this organism is considered for toxic studies in many laboratories.

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