Iranian Journal of Fisheries Sciences 19(6) 3098-3111 2020
DOI: 10.22092/ijfs.2020.122939

Spawning and larval rearing of red sea urchin *Salmacis bicolor* (L. Agassiz and Desor, 1846;Echinodermata: Echinoidea)

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Received: February 2019 Accepted: May 2019

Abstract

Gonads of sea urchin attract consumers due to its high nutritional value than any other seafood delicacies. Aquaculturists are also very keen on developing larval culture methods for large-scale cultivation. The present investigation systematically examined the larval rearing, development, survival and growth rate of *Salmacis bicolor* fed with various microalgal diets under laboratory condition. Fertilization rate was estimated as 95%. The blastula and gastrula stages attained at 8.25 h and 23.10 h post-fertilization. The 4 - armed pluteus larvae were formed with two well - developed post-oral arms at 44.20 h following post-fertilization. The 8 - armed pluteus attained at 9 days post fertilization. The competent larva with complete rudiment growth was developed on 25th days post - fertilization. Monodiet algal feed - *Chaetoceros calcitrans* and *Dunaliella salina* resulted medium (50.6 ± 2.7%) and low survival rate (36.8 ± 1.7%) of *S. bicolor* larvae. However, combination algal feed – *Isochrysis galbana* and *Chaetoceros calcitrans* has promoted high survival rate (68.3 ± 2.5%) which was significantly different between the mono and combination diet. From the observations of the study, combination diet could be adopted as an effective feed measure to promote the production of nutritionally valuable roes of *S. bicolor*.

Keywords: Sea urchin, *Salmacis bicolor,* Spawning, Embryo, Larvae, Microalgae

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Introduction

Echinoids aquaculture is being carried out as a commercial activity to harvest the gonads or roe which is a delicacy in Asian and Mediterranean countries. A sea urchins roe is being consumed by humans since pre-historic times in many countries around the world and are considered a high nutritional valuable food source (Chen, 2013). High exploitation rate has endangered this species in many countries that in turn created interest towards development of sea urchin larval cultivation and rearing techniques (Kelly *et al*., 2000; Bustos and Olave, 2001). Development of sea urchin aquaculture has greatly advanced by the enhancement of ranching techniques, which directly targets gonadal growth and reproduction by the nutritional enhancement.

Aquaculture of sea urchins have focused on larval feeding (George *et al*., 2004; Liu *et al*., 2007), culture methods for both larva and adults (Kelly *et al*., 2000). Adaption of the recirculation system for larval growth and development in *Paracentrotus lividus* progressing from fertilization to harvest method was first developed by Le Gall and Bucaille (1989). The system provides features beginning from spawning, larval and algal cultures, and a separate rearing system for the animal settlement and development of mature adults for harvesting.

Aiyar (1935) has initially documented the anatomy of the sea urchin *S. bicolor* and reported the early development and metamorphosis of the tropical echinoid from Chennai Coast. Saravanan *et al*. (2017) have studied the temporal variation of gonadal maturity of *S. virgulata and Temnopleurus toreumaticus* from Mandapam region of Indian South-East Coast. Moreover, Rahman *et al*. (2012) reported that spawning, embryonic and larval development of the tropical sea urchin, *Salmacis sphaeroides* from Malaysian coast. Earlier investigations on the influence of dietary treatments on larval survival and development of various echinoids species were reported (Meidel *et al*., 1999; Kelly *et al*., 2000; Jimmy *et al*., 2003; Liu *et al*., 2007).

Sea urchin releases large quantities of eggs and sperm into the water for fertilization and the larva of this organism play an important role in the marine food chain (Mishra *et al*., 2012). Spawning and larval life span can be determined by the timing of larval settlement.

Twenty species of edible sea urchins are widely consumed globally. Indian coastal waters include over 150 species of sea urchins of which 14 have been found to be edible (James, 1985; Kaliaperumal and James, 1993; Venkataraman and Wafer, 2005). Edible sea urchins are selected based on three criteria: i) accessibility of the species, ii) palatability, and iii) historical and culture practices needed to be taken into consideration (Lawrence, 2007).

Phytoplanktons are the major food source of echinoderm larvae. Culturing practices of phytoplanktons support massive production of sea urchin larvae (Sakai *et al*., 2004). Combinational diet of *I. galbana* and *C. gracilis* were found to be useful in the mass production of competent larvae of *Loxechinus albus* (Carcamo, 2004). Combination of various algal diet of *Cricosphaera elongata*, *Pleurochrysis carterae* and *Dunaliella tertiolecta* as well as *Pleurocrysis elongata* and *D. tertiolecta* resulted in the successful growth of *P. lividus* and *P miliaris* larvae (Kelly *et al*., 2000; Stefano*et al*., 2012).

S. bicolor, a species of echinoid belonging to the family Temnopleuroidae, is a regular echinoid. Morphological features include 0.5 to 4 cm spines in length with white and red color bands with long tube feet; circular test, dense covering of short spine, and spine around peristome markedly flattened; spine, red around base with attractive yellow banding towards distal ends. *S. bicolor* is a relatively well-known tropical Indo - West Pacific species (Clark and Row, 1971; Richmond, 1997). Pearce (2010) reported a scientific research aim to bridge the gap between supply and demand of aquaculture products. Caboni *et al*. (2013) reported sea urchin adults excellent quality gonads could be available for the market throughout the whole year, while juveniles bred in controlled conditions could be employed in restocking activities.

In this paper, we made first report on the effect of various dietary microalgae on growth, larval development and survival of sea urchin larvae *S. bicolor* from Tamil Nadu, South India. It also provides a possible solution to improve production output of commercial hatchery. To this end, we compared sea urchin larvae growth performance when fed different live microalgae that are easy to culture and readily accepted by the larvae. Furthermore, this work will help to further understanding the physiological requirements of echinoplutei and enable optimization of *S. bicolor* larval rearing protocols to increase hatchery profitability. The present study aimed at the influence of micro algal strains as monodiet: *C. Calcitrans*, *D. salina* and combination diet: *C. calcitrans* and *I. galbana* towards the morphological development and larval survival rate of *S. bicolor* grown under laboratory conditions.

Materials and methods

Geographical location

The animal was collected from Chennai, East Coast of Tamil Nadu state, India (Lat. 13° 8'43.86’N; Long. 80°21'42.43’E) (Fig. 1). The region covers shallow subtidal and continental slope area with typically covered muddy bottoms and rock materials.

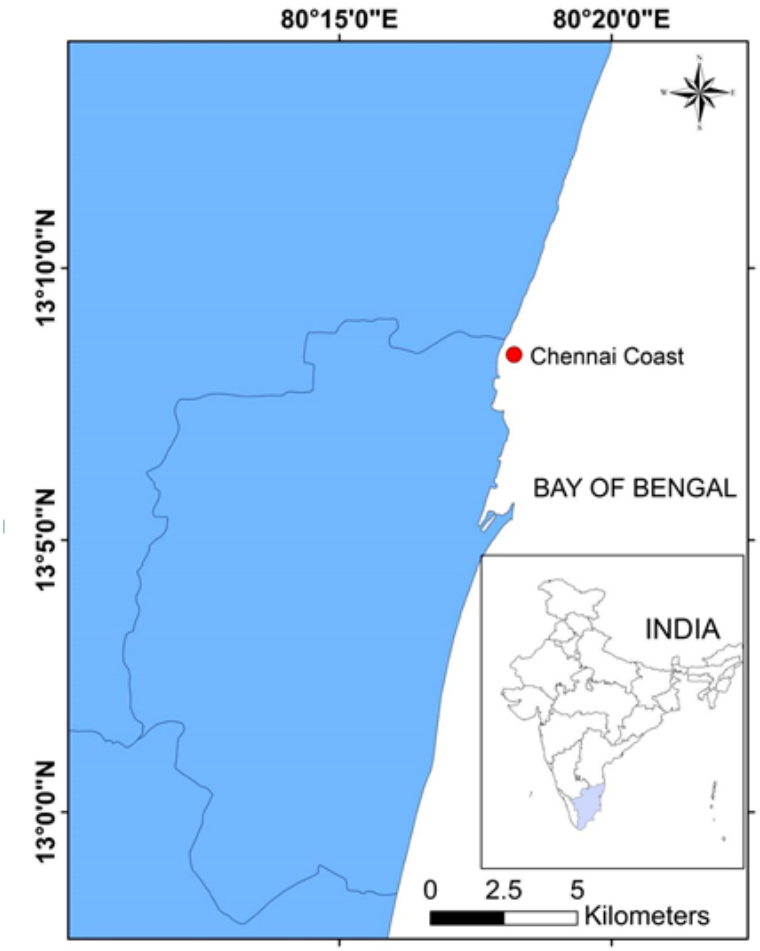

Figure 1: Location of study sites, South East Coast of India. The map shows the collection site of *S. bicolor* from Chennai Coast, Tamil Nadu.

Live samples of *S. bicolor* were collected by using the fishing net from a depth of 15-20 m from Chennai Coast and transferred to the Marine Biotechnology lab in aerated plastic containers.

Animal maintenance

For maintenance in aquaria, UV-sterilized 5-µm filtered seawater stocked in closed glass aquarium tanks of 10 L capacity with ambient temperature (28 ± 2°C) was used. The animals were fed *ad libitum* with green macroalgae: *Chaetomorpha antennina*, *Ulva rigida* and *Enteromorpha* sp. The experimental study was carried out for duration of 25 days.

Larval rearing

After 2 to 4 days of acclimatization, Adult sea urchins were selected for induced spawning according to their test diameter size (63 ± 3.4 mm) and weight (54 ± 1.6 g). Adult sea urchins were induced spawning by injection with 0.5 M KCl into the haemocoel via the peristomal membrane (Fig. 2a). The injected animals (9 males and 6 females) were transffered to individual

vessels contained 5-µm filtered seawater. After 2-3 min, the animals started releasing the eggs and spermatozoa into the water column in plastic bowls and extruded gametes were collected by pipette. Totally, 15 individual culture jars were maintained. Eggs were washed twice with 5-µm filtered seawater and high quality gametes were selected for fertilization. The suspension of eggs was fertilized with 2 ml volume of sperm which was added in the water column and after gamete fusion, the fertilization rate was estimated to be 95%. Hatching rate was determined to be (88.5±2.3%). Embryos and larvae were maintained in the 1 L glass beaker with water temperature: 26–28˚C, salinity: 30–33 ppt; light density: 100–1000 lux without aeration. Initially, the count of embryos was maintained greater than 6 per ml followed by less than 1 per ml in the two arm stage. Microalgae cultures were grown in 150 L polyethylene bags in UV – sterilized filtered seawater enriched with Walne's medium. Microalgae were harvested in the exponential growth phase and fed to the larvae during 3rd day. Larval survival rate was assessed volumetrically every three days interval. When the larvae reached the 2-arm stage, mono algal feed – *C. calcitrans* and *D. salina*, as well as 50% mixture of combinational feed *I. galbana* and *C. calcitrans*, were provided separately with the concentration of 500 cells/ml^{-1}. When the larvae reached the 6 – arm stage, increased microalgal strains concentration of 1500 cells ml^{-1} were provided separately, according to the method described by Liu *et al*. (2007) and Stefano *et al*. (2012).

All treatments were carried out in triplicates. Water was exchanged between 2-3 days intervals and the larvae were monitored for the development of new arms or other structures and morphological features such as lengths of the antero-lateral, postoral, somatic, posterodorsal and posterolateral arms. The timing was determined based on the morphological changes in the larval development. After the first week, larval cultures were stirred with 50 rpm agitator paddle system similar to the method described by Strathmann (1987).

All the developmental stages of embryos and larvae were observed under a phase contrast microscope (Olympus CX41). All morphometric measurements of embryo and larvae at different stages were monitored after fixation with 4% formalin solution prior to microscopic examination.

Statistical analysis

Tukey's test and one-way ANOVA was performed to identify the significant difference among the dietary treatments (Graph pad prism 7.0 software package). Results with $p<0.05$ were considered statistically significant. The experiment was carried in triplicates and expressed in mean ± SD.

Results

The mean diameter of the unfertilized eggs of *S. bicolor* (90.12- 96.45 µm) and fertilized egg (96.58 ± 2.38 µm) was recorded. The fully developed eggs are transparent, spherical in shape and light green in color (Fig. 2b). The vitelline membrane of the egg was raised after 20-40 sec of sperm entry and the fertilization membrane began to form (Fig. 2c). The fertilized egg with formation of fertilized membrane was found within 10 mins after the insemination (Fig. 2d). The 2-cell, 4-cell, 8-cell, 16-cell and 32-cell stages were achieved in 15 min, 30 min, 01.15 h, 02.25 h and 03.20 h, respectively (Figs. 2e - i).

After 04.30 h post-fertilization, occurred without micromere division and the embryos formed morula stage (Fig. 2j). In blastula, cells in one side slightly more columnar than those on the other side. This pole is blastoporal end (Fig. 2k). The vegetal plate thickened and cilia were formed on the perimeter 08.25 h after fertilization, before hatching stage. The gastrulation commencement was indicated by a few of the cells from those pole pushing inwards. This grow forwards rapidly, almost to the anterior end and this process was completed in about 23.10 h (Fig. 2l).

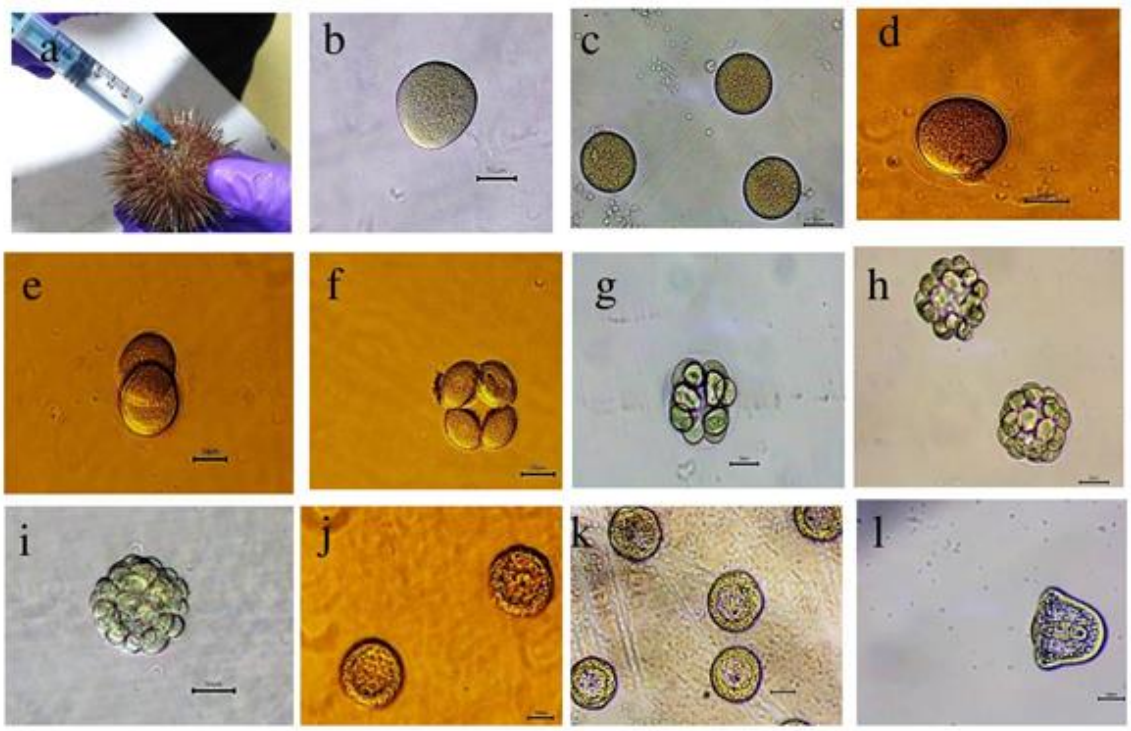

Figure 2: Induced spawning of *S. bicolor* by using 0.5 M KCl solution (a), Egg (b), Fertilization egg (c), Fertilized eggs with complete formation of fertilization membrane (d), 2 - cell stage (e), 4 – cell stage (f), 8 - cell stage (g), 16 - cell stage (h), 32 - cell stage (i), Morula stage (j), Blastula stage (k), Gastrula stage (l). Scale bar = 50 µM.

The prism stage was observed 29.30 h after fertilization (Fig. 3a). The two - arm pluteus stage was observed 36.40 h after fertilization (Fig. 3b). At this stage, opening of the mouth was observed. The larvae has become humped with a presence of esophagus, stomach and a short intestine. Muscles of the esophagus began to contract; the stomach grew in diameter, while its epithelium became thinner. The 4 - arm pluteus larvae was observed 44.20 h after fertilization with two well - developed postoral arms (Fig. 3c). In this stage, larvae attained (423.76 µm - 526. 17 µm) length. The well-defined opening in the lower half of the larvae represented the anus.

The 6 - arm pluteus larval stage was formed on 4th day after fertilization (Fig. 3d). At this stage, postoral arm further elongated and anterolateral arms were supported by well-formed skeletal rods. The 8 - arm pluteus larval stage was formed on the 9th day after fertilization. In this stage, postero-dorsal arms formed clearly as shown and grew rapidly (Fig. 3e). No additional arms developed after 8 - arm pluteus larval stage. At this stage, larva attained a maximum length of (830.43 µm - 890.43 µm) after 10th day of fertilization. On 13th day, rudiment of dorsal arch and two anterior coeloms has formed followed by complete development of the larval skeleton, arch, and formation of first pedicellaria stage. During 15th day , the pluteus larvae possess three pairs of well-developed arms and kept swimming constantly with arms directly upwards. At this stage, competent larva with complete rudiment growth was recorded (Fig. 3f). On 15th day, larva fully matured with hydrocoel ring and grew on amnion rudiment. On 19th day, old larvae form ear - shaped postero-lateral process supported by branches from the post-transverse rod which developed well and became prominent. The left side commences to bulge out considerably, the stomach being pushed to the right. Sometimes variation seems to be more pronounced in the post-oral and postero-dorsal arms. Yellow pigment bodies are found in considerable numbers in relation to the postero-dorsal and post-oral arms (Fig. 3g). From 18th to 21st day, larvae shown the rapid growth of echinus rudiment with enlargement of mouth and active larvae started feeding voraciously (Fig. 3h and 3i). On 25th day, competent larvae with complete rudiment growth were settled down. At this stage, larval tissues accumulated on the aboral surface of the rudiment forming a globoid structure.

Significant difference in larval survival was identified among the diet treatments (p<0.05). During 2-5th day of post fertilization, there was no significant difference (p<0.05) in the larval survival between the dietary treatments. However, significant differences (p<0.05) in the growth of competent larvae was identified from 11th to 25th days of post fertilization between the mono and combination diets.

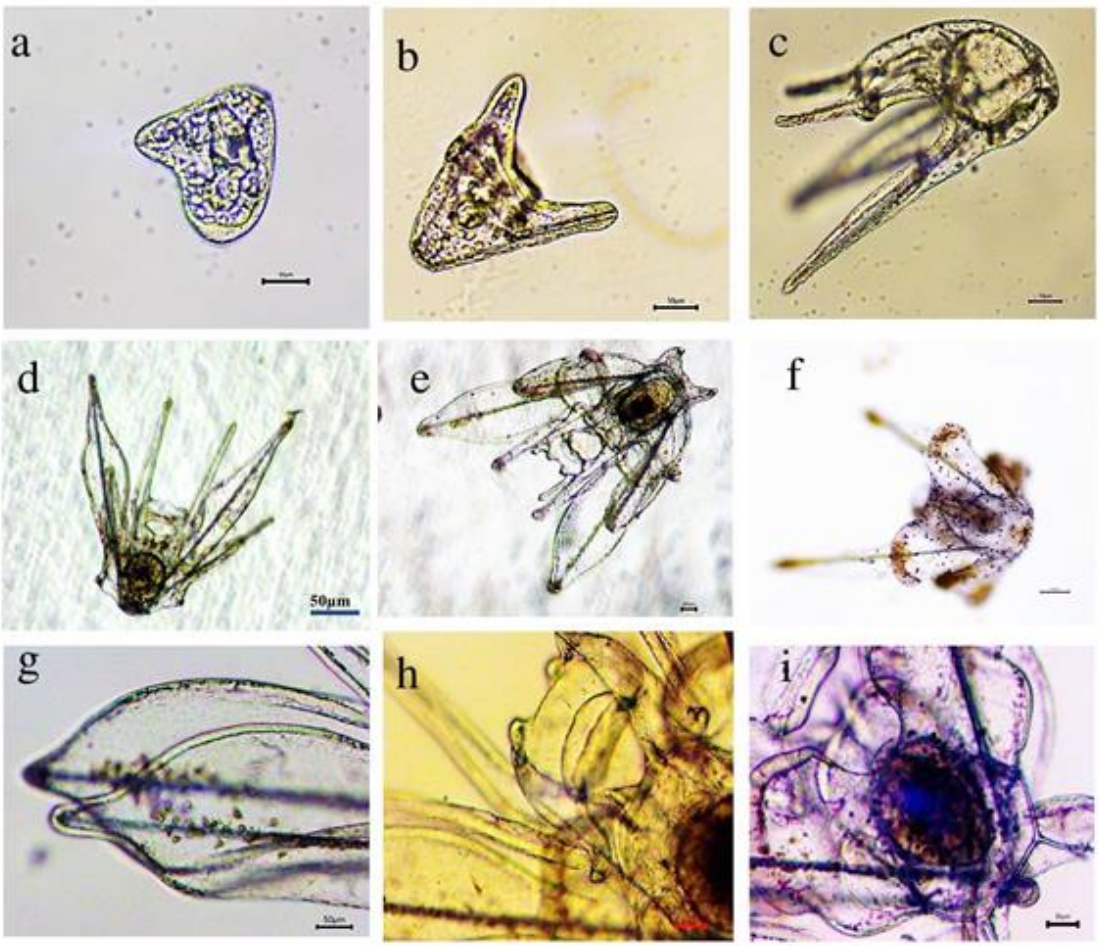

Figure 3: Larval developmental stages of *S. bicolor.* Prism stage (a), 2 - arm pluteus larvae (b), 4 - arm pluteus (c), 6 – arm pluteus (d), 8 – arm pluteus (e), Competent larvae with complete rudiment growth development (f), Yellow pigment tips of left postoral arm (g), Rapid growth of echinus rudiment (h), Enlargement of mouth and started feeding voraciously (i). Scale bar = 50 µM.

Overall statistical analysis of the dietary response showed the highest survival rate of the larvae with significant difference (p<0.05) in combinational diet of *I. galbana* and *C. calcitrans* (68. ± 2.5%) (Fig. 4). In contrast,variations in the larval survival rate were observed with significant difference (p<0.05) in the monodiet. Moderate level was observed in larvae fed with *C. calcitrans* (50.6 ± 2.7%) and low level with *D. salina* (36.8 ± 1.7%) at competent stage, respectively. The statistical significance firmly supports the promotional influence of the combination diet.

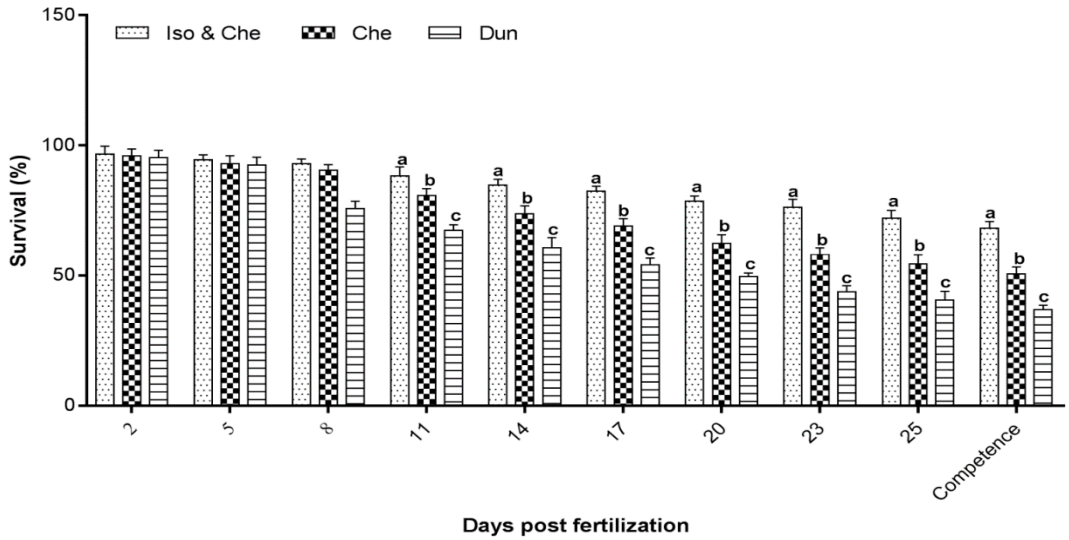

Figure 4: Survival rate of *S. bicolor* larvae fed combination diet of *I. galbana* and *C. calcitrans* and monodiet of *C. calcitrans* and *D. salina.* Data are expressed as mean ± SD (*n*=3). Letters above bars indicate the results of Tukey's test with different letters showing significant (*P<0.05*) difference between the three algal diets treatments. (Error bars shows standard deviation).

Discussion

The results of the present study systematically documented the embroyolgy, larval development, survivability and various microalgae diets which has influenced faster competency in the larval development. Preliminary study on larval development and metamorphosis of the same species was reported in which the growth parameters - optimal lab condition and survivial rate was not reported (Aiyar, 1935). However, our study was much concerned and varied from the earlier study in several aspects such as larval rearing protocol, growth response and survival rate of the larvae under the influence of different microalgal feeds and also the study provides better microscopic observations of the larvae captured at different phases. The results of this study will be useful for better understanding of the larvae culture. From the above observations, combination diet - *I. galbana* and *C. calcitrans* could be adopted as an effective feed measure to promote the production of nutritionally valuable roes of *S. bicolor*. This could serve as attractive nutritional source because when food is abundant with high quality, growth is directed toward non-ephemeral larval structures which are carried through metamorphosis level.

Early stages of embryology and larval development have been reported for a number of bathyal and abyssal invertebrates (Young *et al*., 1993; Mortensen, 1921). Competency of *S bicolor* was attained after 25 days. Similar observations in *S. sphaeroides* larvae (complete rudiment growth after 35 days) (Rahman *et al*., 2012).

Cleavage and development of embryo and larvae of *S. bicolor* were similar to those reported in various echinoids species - *Clypeaster rosaceus* and *S sphaeroides* (Emlet, 1986; Rahman *et al*., 2012). Mortensen (1921) described feeding larval forms of echinoids which generally showed clear morphological similarity to the temnopleurids family such as *Temnopleurus toreuinaticus*, *T. sculptum* and *Mespilia globulus*. McEdward (1984) reported that morphometric changes in sea urchin larvae *Dendraster excentricus* undergo during the course of larval development have important functional consequences as the shape is related to feeding capability and metabolic activity.

On the 13th day, posterior coelom of *S. bicolor* larvae extended backward but the two layers are in contact throughout their extent. At this stage, madreporic vesicle well developed. Similar phenomena in *Echinus microtuberculatus* and *E. miliaris* was observed (Metschnikoff, 1869; Murti, 1932). *S. bicolor* larvae found yellow pigmented presented on postero-dorsal and post-oral arms tips clearly. Also, *Stomopneustes variolaris* larvae have found deep red pigmented tips on posterolateral arms (Richard, 2009). The body rods of *S. bicolor* larvae attained at 2.5 days age were similar to the findings in *T. torematicus* and *S. virgulata* larvae in 29 hours old age (Mortensen, 1921; Tennent, 1929). *S. bicolor* anterior ciliated epaulettes began to appear on the 12th day, complete separation from the main band has taken place by the end of 15th day. All arms were broad at the base and could be seen to open and shut. Mortensen (1921) also reported similarity at this stage on the 15th day in *Mesopilia globulus.* Microalgal genera - *Tetraselmis, Isochrysis,* and *Chaetoceros* include species preferred for feeding marine invertebrates in mass cultures (Duerr *et al*.,1998; Brown, 2002). *S. bicolor* larvae fed with *D. salina* possess low survival rate (36.7 ± 3.2%) that failed to reach competence and this could be due to the low nutritional value of this species. An increased survival rate of *S. bicolor* (68.3 ± 2.5 %) was observed the significant difference in the combination diet of *I. galbana* and *C. calcitrans*. Survival up to 82.3 ± 9.0% was achieved in *Loxechinus albus* by providing a combined diet of *C. calcitrans* and *I. galbana* (Carcamo *et al*., 2015). Stefano *et al*. (2012) reported that survival rate at the competence of *P. lividus* larvae (82.8 ± 10.6%) was achieved in *D. tertiolecta* diet. Bustos *et al*. (1992) suggested that larvae of *L. albus* may be fed exclusively on *Chaetoceros* species as a monodiet, as in the production of larvae of *Strongylocentrotus intermedius* and *S. nudus* and *Glyptocidaris crenularis* (Hagen, 1996; Chang and Wang, 2004; Sakai *et al*., 2004). The apparently high nutritional value of *C. calcitrans* may be attributable to high levels of 20:5n-3 and 20:4n-6 PUFA (Delaporte *et al*., 2003).

Rearing of *S. bicolor* larvae in lab conditions was enhanced with a combination diet of *C. calcitrans* and *I. galbana* to increase its survival rate . The present data clearly show increased production output and a shorter production cycle when combination diet of *C. calcitrans* and *I. galbana* is used as larval diet providing production cost reduction and increased revenue thus increasing hatchery profitability. *S. bicolor* is an ideal candidate species for aquaculture. The findings would immensely be helpful towards the development of induced breeding, commercial larval production for achieving high aquaculture potential of Temnopleuridae sea urchins under captive rearing condition. In future, development of sea urchin aquaculture will rely on positive interactions of commercial fisheries and aquaculturists towards mutual developmental activity for growth management and commercial production of larvae. As the market demand for sea urchin roes has been continuously increasing all over the globe, commercial culturing activities to enhance productivity needs to be addressed. The present report will be highly beneficial to understand the biological and environmental parameters required for successful culturing of other echinoids species.

Acknowledgments

We gratefully acknowledge the support provided by Ministry of Earth Science, Government of India, by funding the Research Grant Number: MoES/36/OOIS/Extra/39/2014. We would like to thank the management of Sathyabama Institute of Science and Technology for providing necessary laboratory facilities.

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