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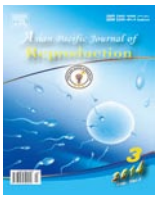

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Influence of rearing water temperature on induced gonadal development and spawning behaviour of tropical green mussel, *Perna viridis*

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ABSTRACT

Objective: To standardize the technique of induced breeding and spawning of green mussel *Perna viridis* (*P. viridis*), in captivity. **Methods:** In Experiment-A, the temperature was increased at a rate of 2 ℃/5 days interval. In Experiment-B, a rise of 3 ℃/5 days was practiced, whereas in Experiment C and D, respectively 4 and 5 ℃ was increased in 5 days interval. The temperature was maintained constant at 20 ℃ in the Control. **Results:** The increase in temperature showed a progressive effect on the gonadal development of mussels. The gonads ripped at 30 to 32 ℃ in all the experimental tanks, irrespective of the difference in temperature hike. Complete spawning in *P. viridis* was achieved by gradually raising the temperature from 20 to 35 ℃ at a rate of 3 or 4 ℃/5 days. **Conclusion:** According to the present study temperature induced spawning method is very simple and cost effective and can accelerate the production of mussel seeds in hatchery units and further stock improvement through genetic manipulation.

1. Introduction

Molluscs are the second largest aquaculture product produced (24%) worldwide[1] and mussels form the most representative mollusc in the world bivalve market. Mussels are commercially valuable species, easy to cultivate or collect in coastal areas. Consumption of these bivalve molluscs provides an inexpensive source of protein with high biological value, essential minerals and vitamins[2].

Mussels, because of their fast growth rate, are well suited for commercial culture in sub tidal biotopes. The mussels do not need additional feed, as they are filter feeders primarily utilizing phytoplankton; require only a continuous supply of high productive saline water to grow. It also seems to be relatively free from mass mortality due to diseases that often affect other molluscs[3]. Commercial cultivation of marine mussel *Perna* [*Perna viridis* (*P. viridis*) , *Perna perna* (*P. perna*) and *Perna canaliculus* (*P. canaliculus*)] is extensively carried out in several countries[4].The tropical and subtropical marine mussel *Perna viridis* (*P. viridis*) achieves marketable size relatively within a short culture period of about 6 months[5,6] , conferring the potential advantage of *P. viridis* as a perfect candidate for culture purpose[7]. Nearly two hundred and eighty two thousand tonnes of *P. viridis* is produced worldwide per year through culture[1].

The availability of seed mussels is fundamental for the success of culturing activities[8]. At present, seed

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requirements for mussel farming are met mainly from the wild[9]. Since the natural mussel beds are restricted to a few rocky patches, only a fraction of the larval population gets a chance to settle on these beds and the rest suffers mortality due to various reasons. However, the seed supply from these grounds cannot meet the requirements for expansion of mussel culture as an industry. Therefore it is necessary to organize a system of seed production for commercial use. Artificial seed production has the advantage of assured seed supply and stock improvement through genetic manipulation [10]. During the last three decades there has been a growing interest in the world for the production of seed of cultivable molluscs through hatchery system[11].

The reproductive 'strategy' of mussels is its high fecundity, small eggs, external fertilization and pelagic larva that feeds on the phytoplankton[12]. Green mussels are classified both as dioecious and hermaphroditic in different regions and different habitats, but hermaphroditism is rarely reported[13]. Sexual reproduction occurs when gametes are released into the water column where fertilization takes place. Its life cycle exhibits a long larval dispersal period and a sedentary adulthood[14]. The external environment plays an important role in the sexual behaviour of bivalve molluscs. In natural conditions, the environment acts on organism and the sex cell processes in a complex manner, although various factors have different and independent significance. Especially important for poikilothermal organisms is the role of temperature, which is considered as the signal and initiating factor of reproductive activity in bivalve molluscs. The reproductive cycle of mussels are synchronised with the annual temperature cycles of water[15]. Hence in the present study a varying degree of temperature increase was practiced to standardize the technique of induced breeding and spawning of tropical green mussel *P. viridis*, in captivity. The effects of temperature on the gonadal maturity, its efficiency in induce spawning *P. viridis* and the appropriate temperature regime for the same was intended to be studied.

2. Materials and methods

P. viridis adults were collected from the natural mussel beds at Narakkal (10°043′N.76. 218 E), Central Kerala, India, and brought to the laboratory, provided with hatchery facilities. The shells of the mussels were thoroughly scrubbed and rinsed to remove epifaunal (fouling) organisms and sediment, and then placed in a well aerated cement tank (1 500 litre volume) containing filtered sea water having, temperature of 20 ℃, 33 ppt salinity and 13-15 mg/L dissolved oxygen. The mussels were acclimatized to the hatchery conditions by maintaining them in the tank for 5 days. The mussels could feed on the organisms in the sea water as well as the commercial algal paste (a mixture of the small flagellates: *Tahitian isochrysis* (*T. isochrysis*), *Isochrysis galbana* (*I. galbana*), *Pavlova lutheri* (*P. lutheri*), and *Nannochloropsis oculata* (*N. oculata*) - sizes ranging from 2 to 5 μm), supplemented at a rate of 0.2 g / individual/day (The paste has an average of 6 billion cells per gram). Mature *P. viridis* individuals having 40-50 mm shell length were selected for the experiment and were transferred to the designated tanks.

Four pattern of temperature increase was designed. Experiment-A, the temperature was increased at a rate of 2 ℃ in every 5 day interval. In Experiment-B, a rise of 3 ℃/5 days was practiced, whereas in Experiment C and D, respectively 4 and 5 ℃ was increased in 5 days interval. The temperature was maintained constant at 20 ℃ in the Control (Table 1). Triplicates were maintained for all the experiments and control. The temperature was increased and controlled in the tanks using 500 watt, submersible aquarium water heaters. 50 adults of *P. viridis* were introduced to each tank (35 litre volume). Temperature was adjusted to 20 ℃ in all the tanks for the first 5 days, after which the temperature variation as per the designations was practiced. Mild aeration was provided in all the tanks. Water was exchanged in 5 days interval. Supplemental feeding was continued, as done during the acclimatization period (Table 1).

Table 1
The temperature (℃) in the experimental and control tanks during the days of experiment

Experiment	Initial	5th day onwards	10th day onwards	15th day onwards	20th da0y onwards	25th day onwards
Control	20 ℃	20 ℃	20 ℃	20 ℃	20 ℃	20 ℃
A	20 ℃	22 ℃	24 ℃	26 ℃	28 ℃	30 ℃
B	20 ℃	23 ℃	26 ℃	29 ℃	32 ℃	35 ℃
C	20 ℃	24 ℃	28 ℃	32 ℃	36 ℃	40 ℃
D	20 ℃	25 ℃	30 ℃	35 ℃	40 ℃	45 ℃

Determination of reproductive stage for mussels is based on the maturation stages of mussel gonads, which are located in the mantle[16]. Histological analysis was done to study the reproductive stage of mussels and to calculate the Gonad Index, for which five animals were sacrificed from each tank from the initial 5th day (before the raise of temperature) to the final 30th day, at an interval of 5 days. Mussels were preserved whole, and a dorso-ventral slice was taken after fixation[17]. Tissue slices were embedded in paraffin, sectioned, and stained in hematoxylin and eosin. Stained sections were examined microscopically to determine the stage of gonadal development. The stage in the gametogenic cycle was assigned based on the maturity of the follicles and gametes. Gonad Index was determined based on the formula [18, 19].

Gonad Index (GI) = [Number in each reproductive stage ×

Numerical ranking of that stage × 100] / Number of animals in the sample

In histological analysis a semi-quantitative numerical assignment was used to rank the reproductive stage. The ranks were allotted as follows: 0 - resting, 1 - immature and/or spent, 2 - developing and/ or spawning, 3 - ripe and / or redeveloping. Gonad index of the sample may vary from 0 (all mussels resting) to 300 (all mussels ripe or redeveloping) [20, 21]. Mortality rate was also noted.

Values of each parameter measured and are expressed as the arithmetic Mean ± standard Deviation (SD). One way ANOVA was performed to analyze the significance in the difference of GI between the experiments and control using SPSS- 13.00 programme for windows.

3. Results

During the study the ripening and maturation of mussel gonads was clearly evidenced in all the experiments. As the individuals ripened, the gonadal tissue seemed to proliferate throughout the mantle region and the mantle thickness increased progressively. The mantle turned creamy to milky white colour in males, while it was orange to brick red colour in females. Histological examination of the gonads revealed the occurrence of following stages in the gametogenic cycle of *P. viridis*.

Stage1 Developing/ redeveloping gonad, characterized by:

a)Immature gonads, characterized by a thin, transparent and colourless mantle; hardly distinguishable sexes, follicles distinguishable as small opaque areas, gametogenesis initiated.

b)Developing gonads, specimens with relatively thick mantle. Male and female gonads are distinguishable, creamy white in case of males and orange in case of females. Follicles larger and denser; gametogenesis still in progress. (Figure 1a)

Stage 2 Ripe gonad: Mantle much thicker than in stage 1; male mantle milky white in colour and female bright orange/ brick red in colour, entirely packed with follicles and little connective tissue seen between follicles(Figure 1 b).

Stage 3 Spawning gonad: gonad tissues become dull, appearance of empty spaces along with full follicles; the follicles are about one third full of ripe gametes (Figure 1c).

Stage 4 Spent/resting gonad: Specimens that have completed spawning; mantle semi-transparent; follicle wall ruptured and few signs of residual gametes (Figure 1 d).

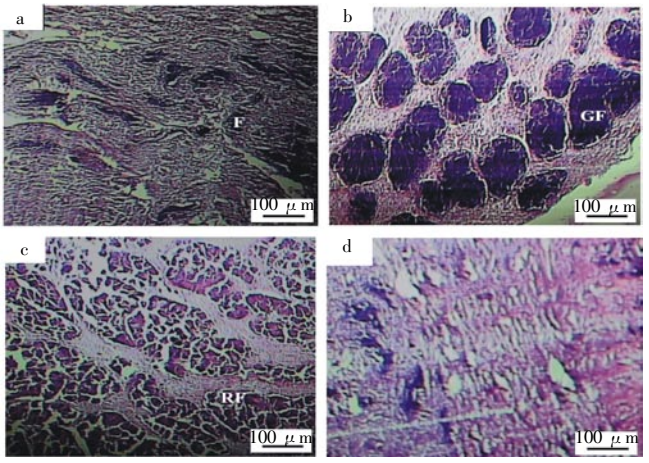

Figure 1. Micrographs showing the various stages of gonadal development of female green mussel, *P. viridis*.
a. Developing gonad (F- follicles), b. Ripe female gonad (GF- gravid follicles), c. Spawning gonad (RF-ruptured follicles), and d. Spent gonad with some residual gametes.

The GI of Experiment A sequentially increased from 10th day onwards to final 30th day. The initial GI of Experiment B was (46.67±11.55), from which it increased and dropped to (40.00±20.00) on the final analysis. The animals in Experiment B showed mass spawning on 25th day afternoon, just after the increase of temperature gradually from 32 to 35 ℃. The highest GI value in Experiment C was attained on 20th day, showing almost all individuals have become fully ripe. Massive spawning occurred on the 21st day. GI of Experiment D steadily increased up to a temperature of 30 ℃. The individuals spawned by 16th day evening, a day after the temperature was raised to 35 ℃. The GI showed a decrease in the 20th day and further increase of temperature to 40 ℃ caused the mass mortality of mussels. GI showed a gradual increase in the Control (Table 2). No spawning was observed in the Control and Experiment A tanks. Abnormal gonadal

Table 2

Mean ± standard deviation of the Gonad Index of the green mussel, *P. viridis* during the days of histological analysis.

Day of analysis	Control	Experiment-A	Experiment-B	Experiment-C	Experiment-D
5th day	46.67±11.55	40.00±20.00	46.67±11.55	46.67±11.55	40.00±20.00
10th day	53.33±30.55	53.33±11.55	66.67±11.55	100.00±20.00	120.00±20.00
15th day	53.33±30.55	80.00±20.00	140.00±20.00	173.33±30.55	233.33±41.63
20th day	80.00±20.00	133.33±11.55	180.00±34.64	293.33±11.55	140.00±52.92
25th day	86.66±11.55	140.00±20.00	280.00±20.00	40.00±20.00	0.00
30th day	100.00±60.00	193.33±11.55	140.00±20.00	0.00	0.00

development was observed in mussels in Experiment C and D respectively on 25th and 20th days, where the temperature was above 35 ℃. This is often characterized by unusual development of gametes at the base of the follicles. Analysis of variance showed that the difference in GI between the control and experiments was significant at 1% level on all days except the initial day (Table 3).

Table 3
The One way ANOVA of Gonad Index between the control and experiments.

Days of analysis	Variance ratio F-value	Significance
5th day	0.000	1.000 (Not significant)
10th day	6.733	0.007 (Significant)
15th day	17.773	0.000 (Significant)
20th day	20.529	0.000 (Significant)
25th day	117.850	0.000 (Significant)
30th day	26.694	0.000 (Significant)

It is obvious from the study that the Gonad Index of the mussels shows a steady increase with the increase in temperature in all the experimental tanks and further it collapsed either due to spawning or intolerance to extreme temperature. The increasing trend of GI was common to all experiments but varied according to the degree of temperature hike. It was more or less highest at 30 to 32 ℃ in all experiments and spawned when the temperature was further increased. No considerable mortality was observed up to 30 ℃, after which 21% mortality was observed at 32 ℃ and further increase of temperature from 36 ℃ lead to the 100% mortality in all the experimental tanks (Figure 2).

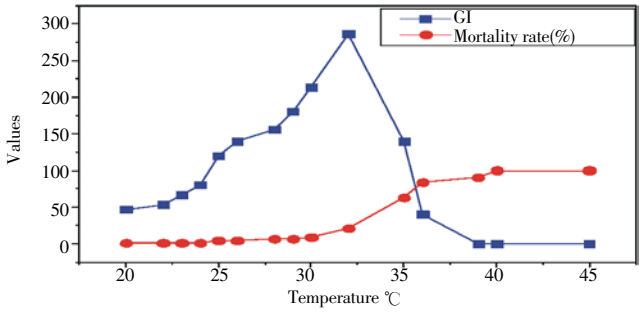

Figure 2. Average Gonad Index and mean percentage mortality of green mussel, *P. viridis* at different temperatures.

4. Discussion

The timing and duration of the reproductive cycle of mussels are believed to be controlled by an interaction of environmental and endogenous factors[22-24]. Increase in temperature showed a progressive effect on the gonadal development of *P. viridis* individuals and resulted in the rise of GI in the present study. The GI was more or less 46.66 at 20 ℃ and increased up to 286.6 at 32 ℃. In all the experimental tanks, despite the varying degree of temperature hike more or less the gonads of the mussels ripped by 30 to 32 ℃. An increase of temperature accelerates gamete development and reduces the duration of the sexual cycle. In artificial conditions, an increase of water temperature from mid-winter to summer temperatures results in the initiation of gametogenesis and gamete formation long before these events takes place in the natural environment[25]. Temperature stimulation results in gonad growth and a rise of the gonad index and can considerably change the cellular composition of testis and ovary. The gonads gain weight because of different endogenous processes; proliferation, growth and differentiation of sex cells and growth of follicles in size and number. The temperature could influence the course of oocyte development, the number of differentiating oocytes (thence the gonad size) and the onset of spawning in bivalves[22, 23, 26].

The increase of temperature further from 32 ℃ in the present study led to the massive spawning process leading to the decrease in GI. The degree of gonad maturation is of great importance for the initiation of spawning. Previous studies on gonadal conditions of *P. viridis* indicate that spawning is largely influenced by temperature, salinity and food availability[6, 26]. The extension of gonad wall results in strong excitation of the exteroreceptors and the subsequent generation of excitatory stimuli from the foci of nervous centres. In bivalve molluscs, the dopaminergic and serotoninergic neurons of central nervous system and serotonergic gonad fibres participate in spawning induction[27]. Almost all the individuals in each tank spawned simultaneously; probably the presence of spawning individuals stimulates other ripe individuals to spawn. The molluscs have specific chemoreceptors, recognizing the sexual products of its species, thereby synchronizing spawning to a degree[14]. Further increase of temperature from 35 ℃ does not induce any other non-spawned individuals to spawn, and rather leads to the deformation of gonads and mass mortality. Oocyte degeneration and resorption in molluscs can be brought about by extremes of temperature and desiccation[28]. Green mussels in the Indo-Pacific region experience an average annual water temperature range between 12 and 32 ℃[7], with an optimal range between 26-32 ℃[29]. *P.viridis* tolerates a temperature

of 15-32.5 ℃ without much problem. The species could survive at a temperature of 39 ℃ for only about 200 min[30].

A gradual rise of temperature at a rate of 3 or 4 ℃ from 20 ℃ to 35 ℃ works effectively in conditioning the *P. viridis* for spawning. This in fact leads to the ripening of the gonads in a normal manner, still faster than in natural conditions. Consistent to the present findings, spawning has been reported in *P. viridis* by raising the temperature from 26.5-28.0 ℃ to 32-35 ℃[30]. *P. viridis* has also been induced spawned by temperature shock, by repeated changes in water temperature from 18-24 ℃, at every 2-4 hrs[31]. This seeks a lot of manual work and the number of cool and warm cycles required for induce spawning depends on the state of maturity of the gametes and the readiness of the adults to spawn[32]. The scheme of temperature variation practiced, the degree of gonad development and the time period required to obtain the spawn are obscure in the previous reports[30].

Complete spawning of mature mussels was achieved in the present study within 20 to 25 days by gradually raising the temperature respectively at a rate of 3 or 4 ℃/ 5 days from 20 to 35 ℃. The percentage mortality rate during this process is negligible, which varied between 1%-9%. This method can accelerate the production of mussel seeds in hatchery units, and promises its availability for culture purpose throughout the year.

The green mussel fishery of the South west Coast of India has unique features which contribute to the sustainability of the fishery. The increased demand for green mussel in recent years has led to increased effort and exploitation of the green mussels[33]. The periodical increase of temperature has shown to stimulate the gonad development and subsequent spawning in green mussel, *P. viridis*. This technique of raising the temperature at the rate of 3-4 ℃ to 35 ℃ seems to be a sustainable one, as it never harms not only the animal but also the environment and ensures incessant supply of seed according to the demand. As being a simple and economically feasible technique, farmers and laymen could easily practice it, with a little technical expertise.

Conflict of interest statement

We declare that the present work has no conflict of interest.

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