Research Paper

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Reproductive Strategies in *Vibrio Harveyi* Infected *Litopenaeus vannamei* and its Feed Back on *Mucuna pruriens* Herbal Treatment

Zoology

KEYWORDS : *Litopenaeus vannamei, Mucuna pruriens* seed, *Vibrio harveyi,* Artemia enrichment, fecundity rate.

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ABSTRACT

To evaluate the effect of Mucuna pruriens (MP) seed enriched Artemia supplementation on reproductive performance in the spawners of Litopenaeus vannamei under laboratory conditions. The MP seed methanolic extracts enriched with Artemia at 100 ppm and 200 ppm fed to Vibrio harveyi infected male and female L.vannamei was conducted for 3 weeks. Reproductive assessment of V.harveyi infected male and female L.vannamei was achieved using assessing the male fecundity rate like sperm count, motility studies and female fecundity rate like egg size, egg count. The results are promising that normal male and female L.vannamei shrimps shows usual pattern of reproductive index whereas the V.harveyi infected shrimps showed drastic fecundity decline in terms of sperm and egg percentage. Shrimp on diet supplementation with MP enriched Artemia shows significant improvement in both male and female reproductive performance by providing vibriosis resistance. The result suggested that treated group proven the potential to improve the performance of fecundity rate in the spawners.

Introduction:

Aquaculture has been a main occupation for the coastal regions of India. In the last few decades it has gathered momentum, more and more scientific methods have been introduced and techniques refined to increase the production of the farm. Shrimp farming is regarded as the best economic activity in terms of returns to investment in areas of production, processing and exports and is referred as a high pay-off activity (Krishnan *et al*., 1999, 2000). The recent trend shows replacing from *P.monodon* to *L.vannamei* culture considerable increase in shrimp farming due to better growth rate of *L.vannamei* (Ravuru and Mude, 2014). Artemia is a genus of aquatic crustaceans (brine shrimp) is used as a food source for the commercial larvaculture of fish and crustaceans and by the aquarium trade (Treece, 2000; Van Stappen, 1996). Naessens et al (1997) reported that the Artemia biomass is a potential candidate for supplementation in maturation diets for *L.vannamei*. Vibriosis is a bacterial disease responsible for mortality of cultured shrimp worldwide (Lightner & Lewis, 1975; Chen et al., 2000). Vibrio harveyi have been described as the main pathogenic Vibrio species affecting penaeid shrimp (Lightner, 1996). Vibrio-related infections frequently occur in hatcheries, but epizootics also commonly occur in pond reared shrimp species. Outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within shrimp blood (Sizemore & Davis, 1985). The predominant quality problems faced by Indian seafood exports are microbiological, which includes bacterial load, aerobic plate count (APC) and the presence of pathogens like Salmonella spp., Fecal coliforms, and Vibrio spp., (Pandian et al. 2000). Huge amount of antibiotics is used to control infections in aquaculture which resulted in builtout of resistant strains. Futherover, the horizontal transfer of resistance determinants to human pathogens and the presence of antibiotic residues in aquaculture products for human consumption constitute important threats to public health (Defoirdt *et al.,* 2007). Therefore, new approach is required to control infection which will be novel environmental friendly antibacterial agents. *Mucuna pruriens* is found in the tropical regions and used for various purposes in traditional medicine in several countries. It is one of the most popular medicinal plants of India and is constituent of more than 200 indigenous drug formulations (Kavitha and Thangamani, 2014). The aim of this study is to assess the reproductive strategies of shrimp receiving MP enriched Artemia and their fecundity rate with enhanced immunity against vibriosis.

Materials and Methods:

Plant Extract:

The seeds of *M. pruriens* were collected from the local Ayurvedic shop in Pondicherry and the identity was confirmed by traditional and experts of plant botanist. Shade dried seeds were pulverized into fine powder using electric blender. Finely powdered *M. pruriens* seeds were subjected to methanol extraction (AR) (Merck, Inc). The concentrated *M. pruriens* seed extract was dried and stored at 4°C until use and this dry extract was subjected for enrichment assay.

Enrichment of *Artemia* for experiment diets:

The *Artemia* enriched with herbal extracts procedure was followed as described by Babu et al. (2008) with some modifications. The *Artemia franciscana* nauplii (Tuticorin, India) were acclimatized in normal seawater for 5 h. The dried Methanolic seed herbal extract of *Mucuna pruriens* were emulsified with water (Super-Selco, *Artemia* systems, SA, Baasrode, Belgium) and enriched at 100 ppm and 200 ppm concentration/ enrichment tank in *Artemia* biomass at the density of 5,000 *Artemia* /L seawater. After 4 h of enrichment, the enriched *Artemia* were rinsed in seawater of 32±2°C temperature and 30 ppt salinity followed by fresh water dip and frozen into 1 cm^3 blocks.

Experiment Animals:

Adult *L.vannamei* shrimp were obtained from local shrimp farms of Marakkanam (Near Pondicherry) and maintained in the wet laboratory for one month prior to experiment. The animals were kept in the cement quarantine tanks (500 L capacity) for two hours. Then they were disinfected with 200 ppm formalin (200 ml/1000 litre sea water) solution for removing attached parasites, bacteria and fungi. They were kept at a density of 0.2 shrimp per litre in 500-l tanks containing with salinity 35%, 25–28°C, 7.5–8.5 pH, and 4.5 mg/l minimum dissolved oxygen. The tanks were run on a recirculation system including sedimentation tank and biofilter. Approximately 20% of the water was changed per week and the sedimentation tank was siphoned once every 3–4 days. The water was taken from the sea through a sand filter, a 25 mm cartridge filter and an ultra violet sterilizer. The *L.vannamei* was fed twice daily with commercial shrimp pellets (CP-Aquaculture, India). Shrimp are infected experimentally by injection of *V.harveyi* suspension at 4.0 x 10^6 CFU/mL for two consecutive days through the ventral sinus of the cephalothorax were subjected to experimental purposes.

Experimental design:

Gravid spawners of *L.vannamei* (body weight of 150±20g) of 15 (n=15) individuals per group were used in the present study. The general condition of the shrimp, including feeding, swimming, moulting and other behaviour was monitored before sampling. Eight groups of spawners were stocked in spawning tank of 250 L capacity for 3 weeks. **Group I**. Normal male *L.vannamei*, **Group II**. Normal female *L.vannamei*, **Group III**. *V.harveyi* infected male *L.vannamei*, **Group IV.** *V.harveyi* infected female *L.vannamei*. **Group V.** *V.harveyi* infected male *L.vannamei* fed with *M.pruriens* (100 ppm) enriched *Artemia*, **Group VI**. *V.harveyi* infected male *L.vannamei* fed with *M.pruriens* (200 ppm) enriched *Artemia*. **Group VII.** *V.harveyi* infected female *L.vannamei* fed with *M.pruriens* (100 ppm) enriched *Artemia*. **Group VIII.** *V.harveyi* infected female *L.vannamei* fed with *M.pruriens* (200 ppm) enriched *Artemia*. During the feeding time, exchange of water has been stopped for one hour to avoid the wastage of Artemia and unfed food. The above mentioned water parameters were maintained during the experimental period.

Assessment of Female reproduction

Female gonadal index assessment:

Berried female shrimps ovary from all the experimental groups were dissected out, weighed and preserved in a 10% formalin solution for histological analysis. The remaining part of gonads was stored at –70 °C for biochemical analysis. The gonadosomatic index (GSI) was calculated as the weight of gonads to body weight as based on the methodology of De Vlaming *et al.* (1982). The GSI were calculated using the formula:

$$GSI = \frac{\text{Weight of the Gonad}}{\text{Wet weight of body}} \times 100$$

Data were calculated to 1 decimal place or rounded up or down to the nearest whole number.

Egg count and size measurement:

Ovaries were dissected out and cleaned with 0.9% normal saline. Oocytes were separated from one another and the ovarian membrane through a washing process (Bagenal & Braum 1978). The oocytes were flushed out of the ovarian membrane and into a 0.01 mm mesh sieve, which was held beneath the ovary. Oocytes collected in the sieve were again rinsed with fully-flowing tap water to help separate them from one another. After draining the water, oocytes were transferred to containers where they were preserved in 2% neutrally- buffered formalin. This formalin concentration was chosen because it was the lowest possible concentration that would ensure proper oocyte preservation and minimizing changes in oocyte size and appearance. Separated oocytes were preserved in 4% formalin for enumeration. Oocyte diameters were then measured, after a minimum sample size of 15 oocytes was determined using the iterative method described in Sokal & Rohlf (1981) (S=0.05, a=0.05; *P=0.90,* δ=0.06 mm). An ocular micrometer in a dissecting microscope was used to measure oocyte diameters to the nearest 0.038mm (l micrometer unit at a total magnification of 24x). Measurements were taken along the median axis of the oocyte, parallel to the horizontal micrometer gradations (Macer 1974, DeMartini & Fountain 1981).

Male fecundity assessment:

Spermatophore quality and biological material sampling

Spermatophore quality was measured by spermatophore weight, sperm count, morphology and color of spermatophores (presence of melanization) and spermatophore absence percentage. All parameters were measured at the beginning (day zero) with all shrimp upto the end of the experiment from each treatment. Spermatophores from each male were extruded using Electroejaculation technique at 7.82 volts (Soundarapandian *et al.*, 2013) and one was randomly selected and weighed to the nearest 0.001 g. This spermatophore was homogenized in 2 mL of calcium-free saline solution. Sperm counts were estimated by counting cells present in the resulting sperm–saline solution using hemacytometer under a light microscope according to the method described by Leung-Trujillo and Lawrence, (1987). Melanization and spermatophore absence percentages were checked by visual examination of the coxae of the fifth pereiopod pair and the extruded spermatophore.

Statistical analysis:

ANOVA analysis was used to identify significant differences among the mean values of each parameter (spermatophore weight, sperm count, GSI). When significant differences were found, ANOVA was followed by Tukey's post-hoc comparison test. All statistical analyses were executed at a level of significance of P > 0.05 using STATISTICA (version 7.0) software (StatSoft Inc. 2004, Tulsa, Oklahoma, USA).

Results:

Female GSI:

Ovaries with GSI index values up to 5.1% was recorded in *M.pruriens* seed 100 ppm enriched Artemia treated group where as 5.2% of GSI index value was observed in 200 ppm *M.pruriens* seed enriched Artemia treated group which shows significant match with GSI values of control group (Table.1). *V.harveyi* infected female group showed a decreasing trend in its GSI value (Fig.1).

Table.1. Gonadosomatic index (GSI) value of female L.vannamei

Groups	Normal female	V.harveyi infected female	M.pruriens 100 ppm treated female	M.pruriens 200 ppm treated female
GSI index (n=15)	5.2±0.2[a]	4.7±0.1[b]	5.1±0.2[c]	5.2±0.3[c]

S.E.D = a (p<0.05), b (p>0.05), c (p>0.001)

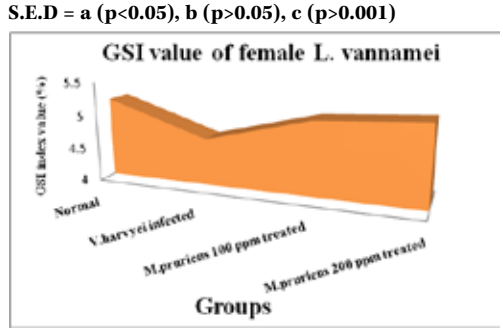

Fig.1. Gonadosomatic index (GSI) value of female L.vannamei

Egg count and size measurement:

Number of eggs and egg diameter seems to be normal in control groups. *V.harveyi* infected groups exhibited profound decrease in egg count with reduction in egg size (Table.2). The herbal extract of *M.pruriens* seed at 100 ppm and 200 ppm effectively returned the fecundity egg count and its size management in treated group (Fig.2 & 3).

Table. 2. Egg count and size measurement in female *L.vannamei*

Groups (n=15)	Normal female	V.harvyei infected female	M.pruriens 100 ppm treated female	M.pruriens 200 ppm treated female
No.of Eggs	190,401[b]	101,700[a]	171,100[b]	186,110[c]
Oocyte diameter (µm)	266±0.8[b]	260 ±1.5[a]	263±1.7[c]	265±1.4[c]

S.E.D = a (p<0.05), b (p>0.05), c (p>0.001)

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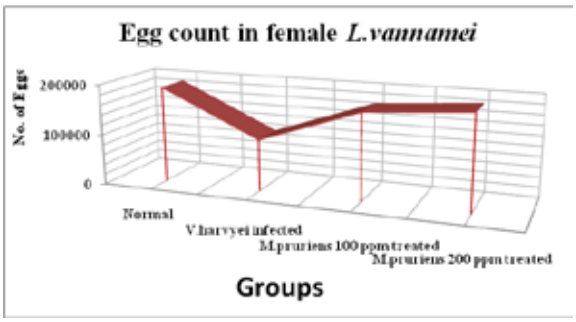

Fig. 2. Egg count in female *L.vannamei*

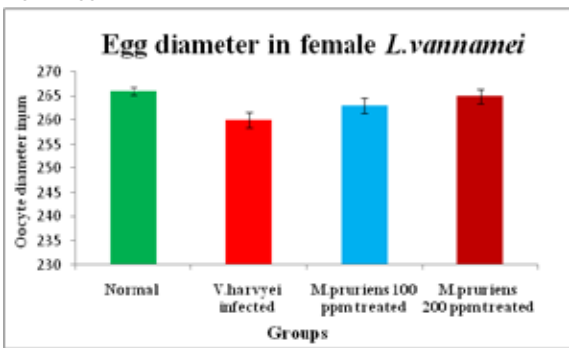

Fig. 3. Egg size measurement in *L.vannamei*

Male fecundity assessment:

Spermatophore quality and biological material sampling

The spermatophore quality data of males *L.vannamei* are summarized in Table.3. Spermatophore weight and sperm count were drastically lowered in *V.harveyi* infected groups. However, in all *M.pruriens* seed extract treated group, the means of spermatophore weight and sperm count were significantly increased at the end of the experimental period. Initially, none of the shrimp shows melanization or spermatophore absence effect in control and *M.pruriens* treated group (Fig.4 - 8). Highest percentages of melanization and spermatophore absence were observed in shrimp of *V.harveyi* infected groups. Above all the final survival rate of male *L.vannamei* was reduced by one fourth time in *V.harveyi* infected individuals and *M.pruriens* seed treated groups recovered the mortality rate.

Table. 3. Parameters of spermatophore quality and survival of the shrimp *L.vannamei* in four different experimental groups

Groups (n=15)	Normal Male	V.harvyei infected Male	M.pruriens 100 ppm treated Male	M.pruriens 200 ppm treated Male
Spermatophore weight (mg)				
Initial	22±0.3[a]	16.01±0.2[a]	19.02±0.3[b]	20.89±1.3[c]
Final	23±0.1[a]	14.02±0.1[a]	20.04±1.1[c]	21.06±1.4[c]
Sperm count (X 10^6)				
Initial	6.02±0.31[a]	3.21±0.22[a]	4.98±0.36[b]	5.15±0.43[b]
Final	5.92±0.21[a]	3..01±0.35[a]	5.12±0.47[b]	5.85±0.58[c]
Melanization (%)				
Initial	15.32[a]	12.51[a]	13.62[b]	14.62[c]
Final	14.89[a]	11.12[a]	13.89[c]	15.12[c]
Spermatophore absence (%)				
Initial	96.2[a]	76.21[a]	82.04[b]	90.41[c]
Final	95.89[a]	70.84[a]	86.21[b]	93.24[c]
Survival (%)v				
Final	95.21[a]	69.42[a]	84.51[b]	90.62[c]

Different superscript letters within rows indicate significant differences (P<0.05).

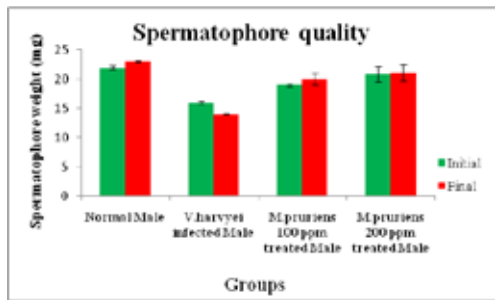

Fig.4. Spermatophore weight in experimental Male *L.vannamei*

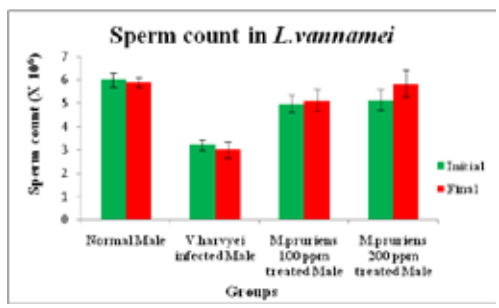

Fig.5. Sperm count in experimental Male *L.vannamei*

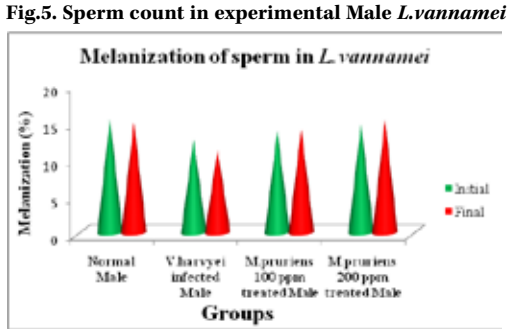

Fig.6. Spermatophore melanization in experimental Male *L.vannamei*

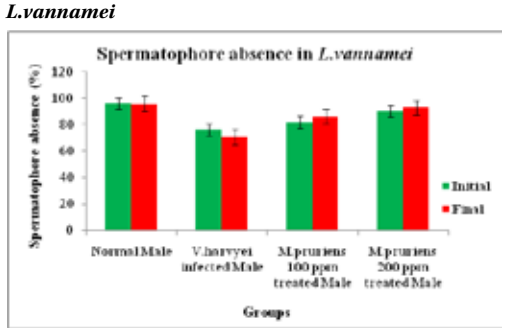

Fig.7. Spermatophore absence in experimental Male *L.vannamei*

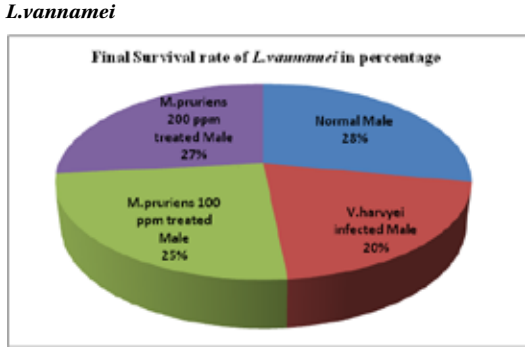

Fig.8. Survival rate in experimental male *L.vannamei*

Discussion:

In the present study, the herbal MP was selected on their proven antibacterial activity (Rajeshwar et al, 2005) as well as the non-hazardous nature of the phytochemicals to the environment (Citarasu *et al*., 2002). Application of the phytochemicals in aquaculture to overcome the drawbacks in the usage of chemical therapeutics is relatively new venture and the potential of the herbals with multifunctional active principles are promising (Sambhu and Jayaprakas, 2001; Sivaram *et al*., 2004). The crude combination of *Withania somnifera* (WS) and MP with other herbals has significantly influenced the offspring quality of the spent spawners (Babu and Marian, 2001).The methanolic extracts of the herb proved its significant influence over the various production parameters in shrimp hatchery industry than in its crude powder (Babu, 1999).The herbal extracts of both WS and MP have their swift positive influence over the reproductive performance and biochemical parameters in the spawners as well as offspring quality in the tiger shrimp *P. monodon* during the successive spawning (Babu et al, 2008).

A significant finding in the present study was that the female fecundity in terms of Gonad somatic index (GSI) was tentatively reduced in *V.harveyi* infected groups. On other hand *M.pruriens* herbal treated group exerted a positive influence on GSI index as compared to that of normal control groups. The total number of egg count and egg size in diameter was much affected in *V.harveyi* infected groups. All these parameters are reversed to normal in *M.pruriens* treated group at the concentration of 200 ppm level. The difference in the maximum reproductive output among crustacean species seems to be primarily due to differences in female body size; however, other biotic or abiotic factors, such as egg size, latitudinal and seasonal variation (Boddeke, 1982), and habitat adaptation (Mantelatto and Fransozo, 1997), may also influence reproductive output.

The male reproductive pattern and its changes in *V.harveyi* infected male shrimp were literally at alarming stage. The loss of spermatophore weight ($P< 0.05$) at the end of the experimental period has also been reported by Nakayama *et al*. (2008) in *F. paulensis* after 43 days. Some studies have attributed the loss of spermatophore quality to stress, degeneration of the digestive tract, and long time in captivity and/or nutrition due loss nutrient stores when wild animals are brought into captivity (Leung-Trujillo and Lawrence, 1987). Herbal extract treatment of *M.pruriens* at dose dependant concentration reflected a normal reproductive pattern in terms of spermatophore quality and its biological nature. Alfaro *et al*. (1993) reported that spermatophore and reproductive tract melanization may be the outcome of two different syndromes. Male Reproductive System Melanization (MRSM) is an infectious syndrome caused by microorganisms such as opportunistic bacteria, *Vibrio alginolyticus*, *Pseudomonas putrefaciens* and others. Male Reproductive Tract Degenerative Syndrome (MRTDS) is a stress believed to associated with the effects of captivity such as high temperatures (Pascual *et al*., 2003), unbalanced diet (Goimier *et al*., 2006) or simply lack of spermatophore ejaculation (Parnes *et al*., 2006). Complementary, Alfaro and Lozano, (1993) described a new condition: spermatophore deterioration, which generates new normal spermatophores after the complete deterioration of the melanized spermatophores.

Spermatophore absence to natural degeneration process (Alfaro and Lozano, 1993) and the fact that spermatophore degeneration is associated with the molt cycle (Heitzmann *et al*., 1993). Parnes *et al*. (2006) showed that spermatophores periodically disappeared from the terminal ampoules of males during 24 h premolt and then new spermatophores appeared after the exuviations. Spermatophore renewal is completed every two or three weeks (Pascual *et al*., 1998). However, this period may be decreased by improper captivity conditions like nutritional factors (Ceballos- Vázquez *et al*., 2004). At the end of the experiment, spermatophore reduction was only reported in *V.harveyi* infected groups. Nevertheless, the water quality for all treatments was considered ideal for the species (Peixoto *et al*., 2005), with temperatures between 24.5 °C to 29 °C and salinity of 33 to 35 ppt. Thus, bacterial infection of *V.harveyi* probably was important factor for aggrieved the spermatophore degeneration reported in the results of the present study.

Conclusion:

The present study documented that *V.harveyi* infected *L.vannamei* that received *M.pruriens* enriched Artemia showed enhanced of reproductive parameter in both male and female shrimp by improved resistance to vibriosis. The mortality rate of *V.harveyi* infected *L.vannamei* is due to decrease in fecundity aspect. As a concluding remark *M.pruriens* seed herbal extract has positive effect in reproduction performance in *L.vannamei*, which could be entrenched in the shrimp hatcheries for the successful management of the brood stocks.

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