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Effects of Herbal Enriched Artemia Supplementation over the Reproductive Performance and Larval Quality in Spent Spawners of the Tiger Shrimp (*Penaeus monodon*)

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Abstract

The effects of methanolic extracts of two terrestrial herbs *Withania somnifera* (WS) and *Mucuna pruriens* (MP) enriched Artemia supplementation (5% of the spawner's body weight/day) over the reproductive performance, biochemical parameters and larval quality indices in the spent spawners of *Penaeus monodon* were investigated. Among the three groups, MP fed group registered higher values in the reproductive performance like fecundity and frequency of spawning than the control group ($P<0.05$). The offspring quality in terms of egg size, fertilization rate, cumulative metamorphosis, cumulative larval survival and reduced larval abnormalities were significantly influenced by the WS supplementation when compared to the control ($P<0.05$). Total Protein and lipid levels were significantly higher in the WS fed group values than the control ($P<0.05$). WS supplementation also significantly influenced the hatching percentage, reduced larval abnormalities, cumulative metamorphosis, cumulative larval survival, haemolymph protein and lipid level than the control group ($P<0.05$). Both MP and WS have the potential to improve the performance of fecundity and larval quality in multiple spawning during the forced reproduction in the tiger shrimp.

Key words: *Penaeus monodon*, Herbal enriched Artemia, Reproduction, larval quality, multiple spawning.

Introduction

At present, eyestalk ablation is the method commonly applied for the short-term induction of spawning cycles in shrimp (Browdy, 1992; Aquacop, 1983b), but the quality of the offspring was negatively affected in eye-stalk ablated females (Yano, 1995). Reproductive exhaustion in terms of forced reproduction during a short period of time might have an important effect in ablated shrimps, when compared to the natural spawns (Racotta *et al*., 2003). In *P. monodon*, also this phenomenon was well reported by several workers (Bread and Wickins, 1980; Hansford and Marsden, 1995; Marsden *et al.*, 1997). Consecutive spawning in short duration significantly affects both spawner's body biochemical parameters viz. total protein level, lipid in the haemolymph, triacylglycerides and cholesterol level in hepatopancreas (Teshima *et al*., 1985; Racotta *et al.*, 2003) and the quality of the offspring i.e., decreased hatching and survival of zoea (Marsden *et al*., 1997). Moreover, the hatchery operation of the tiger shrimp *P. monodon* depends on the wild caught breeders, where the availability of the gravid spawners is highly seasonal. Every hatchery has to spend more than 50% of their running capital for the purchase of the gravid spawners (Babu, 1999). An appropriate eco-friendly technology for the effective recycling of the spent spawners is woefully lacking for the successful hatchery management (Babu, 1999). In this regard, Babu and Marian (2001) have made an attempt to overcome the defects of forced reproduction, by co-feeding herbal products enriched Artemia to spent gravid spawners that has significantly improved the reproductive performance and larval quality 2.5 times more than the control group. Application of a mixed combination of the dry herbal powders of *Withania somnifera*, *Ferula asafetida* and *Mucuna pruriens* along with the appetizer *Piper longum* at the ratio of 4 : 3 : 2 : 1 improved the egg hatching percentage, stress tolerance and reduced larval abnormalities (Babu, 1999). The present study was aimed to characterize the individual influence of the two herbal extracts *Withania somnifera* and *Mucuna pruriens*, over the reproductive performance and larval quality during the successive spawns in *P. monodon.*

Materials and Methods

Preparation of the Hebal Extracts and Artemia Enrichment

Fresh roots of *Withania somnifera* (Solanaceae) (WS) and seeds of *Mucuna pruriens* (Fabaceae) (MP) purchased from the local market were shade dried and grounded into fine powder using teeth mill. The herbal powders were individually soaked in absolute methanol (1 : 2) for 24 h with mild agitation. The extracts were then centrifuged at 3500 rpm for 15 min and the supernatants were kept in room temperature until the solvent residue completely got evaporated. The dried extracts were carefully collected in screw capped tubes and stored at room temperature for

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further use.

The enrichment procedure of the herbal extracts in *Artemia* was followed as described by Naessens *et al*. (1997) and Immanuel *et al*. (2004) with slight modifications. The *Artemia franciscana* juveniles (Great Salt Lake, Utah) were acclimatized in normal seawater for 5 h. The dried herbal extract of WS and MP were separately emulsified with oil and water (Super-Selco, *Artemia* systems, SA, Baasrode, Belgium) and enriched at 100 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±2ºC temperature and 30 ppt salinity followed by fresh water dip and frozen into 1 cm^3 blocks. For the control, the *Artemia* was enriched only with water and oil without any herbal extracts.

Experimental Animals

Gravid spawners of *P. monodon* collected from the wild (body weight of 150±20g) were used for the present study. The animals were brought to the hatchery and kept in the white colour 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 and transferred to the individual spawning tank of 250 L capacity. The parameters such as salinity, dissolved oxygen, water temperature and pH were maintained at 35 ppt, > 5.5 mg L^{-1}, 32±0.5°C and 7.5±0.3, respectively for spawning. After the natural spawning was over, the spent animals were allowed into the maturation tank where the animals were kept in complete darkness. Unilateral eyestalk ablation was made to the spawners using aseptic procedure as described by Treece and Yates (1988). Another group of spent spawners obtained after the first natural spawning were also maintained in the same condition and treated as control. All spawners were fed with minced squid meat at the daily ration of 15% of their body weight. The spawn obtained after eyestalk ablation was considered as first spawning for this experiment.

Experiment

Three groups of spent-spawners in duplicate were stocked in 500 L round shaped experimental tanks, inner surface of which was coated with black epoxy paint. Among the first experimental group (WS group) (n = 18) received the 5% of WS enriched Super-selco frozen *Artemia* along with 10% squid meat at their body weight, the second group (MP group) (n = 18) was fed with 5% of MP enriched Super-selco frozen *Artemia* along with 10% squid meat at their body weight (Babu, 1999). The control group (C) (n = 18) was fed with 5% of *Artemia* enriched with Super-selco without the herbal principle and 15% of squid meat. During the feeding time, the water exchange was stopped for one hour to avoid the loss of *Artemia* and unfed food, faecal material were siphoned from the bottom of the tank.

The parameters such as fecundity (millions), frequency of spawning (days), ovary weight (g) were considered for the assessment of the reproductive performance. Egg size (µm), rate of fertilization (%), hatching (%), motility time of the larvae (seconds), abnormal larval development (%), cumulative metamorphosis (Nauplii to PL 15 in days), cumulative larval survival (Nauplii to PL 15 in %) were considered for the larval quality. The methodology for the observation of the above mentioned parameters were followed as described by Babu (1999). These data were recorded up to four successive spawnings.

Biochemical Analysis

The biochemical parameters recorded were total protein and lipid level in the haemolymph. Cholesterol and Triacylglycerides (TGA) in the hepatopancreas of the spawners were estimated after every spawning cycle. 1 ml of haemolymph sample was drawn from the sample spawners (2 spawners/group/cycle) using the anticoagulant (5% sodium oxalate) pre-washed 2 ml syringe and the collected haemolymph was centrifuged at 3000 rpm for 15 min to obtain the plasma. The supernatant plasma was stored at 4°C for further analysis. The

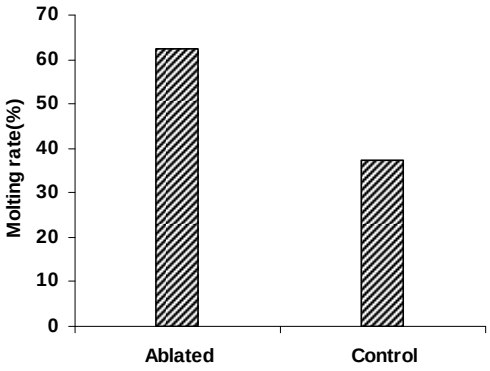

Figure 1. Comparison of molting rate in control group and ablated *A. leptodactylus.*

samples were carefully dissected to obtain hepatopancreas tissue and 1 g of tissue was homogenized with 3 ml of physiological saline (0.8% NaCl). The homogenate was then centrifuged at 3000 rpm and the supernatant was stored in buffer at 4°C until further analysis. The serum was subjected to determine the total protein (Bradford, 1976) and total lipids (Barnes and Blackstock, 1973) and hepatopancreas samples were analysed with enzymatic calorimetric assay to quantify the TGA (GPO-PAP, Merck) and Cholesterol (Chod-PAP, Merck).

Statistical Analysis

All data were compared for their significance of variance through one-way ANOVA at the significant level of 5%. The means were compared with Duncan's multiple range tests (Zar, 1985). The influence of the individual biochemical parameters in the herbal fed spawners over the cumulative larval survival was analyzed using Pearson's correlation co-efficient method. Statistical analyses were carried out separately for each spawning.

Results

Reproductive Performance

The results of the reproductive performance are presented in Table 1. The fecundity was significantly ($P<0.05$) higher in both experimental groups (WS and MP) than the control in all the successive spawning. But no significant difference of fecundity was noted between the herbal treatments during the first two spawning, whereas in the successive third and fourth spawning, MP supplementation significantly ($P<0.05$) improved than WS. Though in the treatment groups, the frequency of spawning that is the time between two subsequent spawning increased from first spawning to fourth spawning; but it was less when compared with the control. Among the treatment groups, MP supplemented group significantly ($P>0.05$) increased the frequency in the 1st and 4th spawning by reducing the time between two successive spawning. The ovary weight was also maximum in both the treatment and the results were significant ($P>0.05$) when compared with control.

Larval Indices

The overall results obtained for the larval quality indices during all four successive spawns were compared (Table 2). Egg size, fertilization percentage, larval motility time, abnormal larval development, cumulative metamorphosis and cumulative larval survival were significantly higher in WS group than control ($P<0.05$). The hatching percentage, larval abnormalities, metamorphosis and cumulative larval survival were also significantly improved by the *Mucuna pruriens* (MP) extracts than the control spawners ($P<0.05$). Among the two herbals, the size of the eggs and larval motility time were significantly higher in WS treated groups than the MP fed groups ($P<0.05$).

Biochemical Parameters

The supplementation of the herbal principles through *Artemia* was reflected in the protein level of the haemolymph of spawners (Figure 1 a) WS group showed significantly higher haemolymph protein levels like (244.34±1.4, 207.7±7.12, 213.32±4.71 and 182.41±4.00 mg/ml during 1st, 2nd, 3rd and 4th spawning, respectively than the control group ($P < 0.05$). But the MP supplementation significantly influenced the haemolymph protein level only up to third spawning ($P<0.05$).

The total lipid content of the haemolymph (Figure 1b) resulted by WS was noted to be higher in all four spawnings than the control group ($P<0.05$). The MP supplementation also improved the haemolymph lipid content in all four spawnings than control group ($P<0.05$).

The triacylglycerides (TGA) level influenced by the WS and MP group did not have any marked difference in the 1st and 2nd spawning when compared to the control but in the 3rd and 4th spawning, the treated animals developed more triacylglycerides than the control (Figure 2a).

Cholesterol level in the hepatopancreas was significantly improved (99, 98, 82 and 81 mg/ml) by WS supplementation than the control (81, 80, 75 and 65 mg/ml) in all four spawnings ($P<0.05$) (Figure 2b).

Apart from the variation in the results among the groups, the biochemical values were tested for the correlation with cumulative larval survival. There was a positive correlation in hepatopancreas lipid level in WS group in relation to the CLS ($R^2 = 0.9167$). This trend was also observed in the control group ($R^2 = 0.9729$). No correlations of the biochemical parameters with CLS were observed in MP group.

Discussion

A number of studies have been focused on the potential quick fixes through manipulation of nutrition and related supplements immediately prior to spawning in shrimps (Primavera, 1985). Reproductive exhaustion in relation to the poor larval quality in *P. monodon* was the key concern in the successful hatchery operations (Babu, 1999). Apparently, maternal conditions during the multiple spawning have the significant influence over the egg, larval and post larval quality in shrimps (Racotta *et al.*, 2003). Forced reproduction in terms of eye stalk ablation exhibits the critical decline in fecundity, larval quality and biochemical reserves and this was reported by several researchers in *P. monodon* (Beard and Wickins, 1980; Marsden *et al.*, 1997; Racotta *et*

Table 1. Reproductive performance of *Penaeus monodon* fed with Herbal extracts enriched Artemia

Parameters	Reproductive performance											
	1st Spawning			2nd Spawning			3rd Spawning			4th spawning		
	C	WS	MP	C	WS	MP	C	WS	MP	C	WS	MP
Fecundity (in million)	0.6±0.1[a]	0.9±0.1[b]	0.9±0.1[b]	0.5±0.1[a]	0.8±0.1[b]	0.8±0.4[b]	0.4±0.1[a]	0.6±0.2[b]	0.7±0.1[c]	0.3±0.2[a]	0.5±0.1[c]	0.4±0.1[b]
Frequency of spawning (days)	12.6±3.5[c]	7.5±3.3[b]	4.6±2.2[a]	15.3±2.2[b]	9.3±1.8[a]	8.5±5.6[a]	18.6±2.5[b]	11.5±2.4[a]	11.3±3.4[a]	29.7±12.6[c]	22.4±8.1[b]	17.1±6.7[a]
Ovary weight (gm)	6.9±1.2[a]	7.8±1.5[b]	7.8±1.6[b]	5.6±1.5[a]	6.4±1.6[b]	6.8±1.6[b]	3.8±1.5[a]	5.2±1.4[b]	4.2±2.1[b]	2.1±1.3[a]	4.2±1.0[b]	4.2±2.2[b]

Each value is a mean of three replications; Columns superscript with different alphabets are statistically significant (P<0.05)
Statistical analysis was conducted for each spawning separately

Table 2. Larval quality of *P. monodon* fed with Herbal extracts enriched Artemia

Parameters	Reproductive performance											
	1st Spawning			2nd Spawning			3rd Spawning			4th spawning		
	C	WS	MP	C	WS	MP	C	WS	MP	C	WS	MP
Egg size (µm)	210.2±5.7[a]	232.5±3[c]	217.5±6.1[b]	207.1±1.5[a]	231.4±6.3[b]	211.3±4.3[a]	205.7±4.0[a]	223.5±3.0[b]	208.2±4.0[a]	204.4±2.3[a]	217.3±11.5[b]	207.6±10.2[a]
% of Fertilization	72.4±2.7[a]	96.7±3.1[b]	92.4±3.2[b]	63.6±3.4[a]	91.6±3.4[c]	83.1±3.4[b]	51.6±3.6[a]	81.4±3.6[b]	74.6±2.4[c]	43.4±2.6[a]	78.2±8.4[c]	69.4±2.7[b]
% of hatching	73.4±2.6[a]	96.5±1.6[b]	92.6±2.7[b]	68.3±4.4[a]	90.5±2.8[b]	88.3±3.6[b]	62.8±4.9[b]	54.6±4.3[a]	84.3±3.4[c]	60.7±2.6[a]	78.2±3.6[c]	72.6±3.4[b]
Motility time of larvae (Sec.)	3.1±0.5[a]	10.4±1.2[c]	6.5±0.8[b]	2.6±0.5[a]	7.5±1.2[c]	5.6±0.6[b]	2.1±0.5[a]	7.2±1.0[c]	3.1±2.0[b]	1.2±0.5[a]	5.6±1.0[c]	2.1±0.4[b]
Larval abnormalities (%)	13.2±1.6[c]	1.5±0.5[a]	2.7±0.9[b]	15.6±2.7[c]	1.7±0.5[a]	3.7±0.4[b]	16.3±2.0[c]	3.6±0.5[a]	6.7±1.6[b]	18.2±2.4[c]	5.6±1.3[a]	7.3±1.5[b]
Metamorphosis (days)	15.4±1.6[b]	11.31±1.2[a]	12.4±1.2[a]	17.5±1.7[b]	12.3±0.9[a]	12.4±1.4[a]	17.3±2.1[a]	13.4±1.3[b]	13.8±0.5[b]	17.3±1.6[b]	12.4±1.4[a]	13.2±1.0[a]
Cumulative larval survival (%)	60.2±2.6[a]	82.8±2.4[b]	80.4±2.6[b]	57.2±2.6[a]	80.6±2.4[c]	71.3±3.6[b]	52.1±2.7[a]	76.6±3.4[c]	67.4±2.7[b]	43.4±2.8[a]	69.5±3.8[c]	58.8±4.4[b]

Each value is a mean of three replications; Columns superscript with different alphabets are statistically significant (P<0.05)
Statistical analysis was conducted for each spawning separately

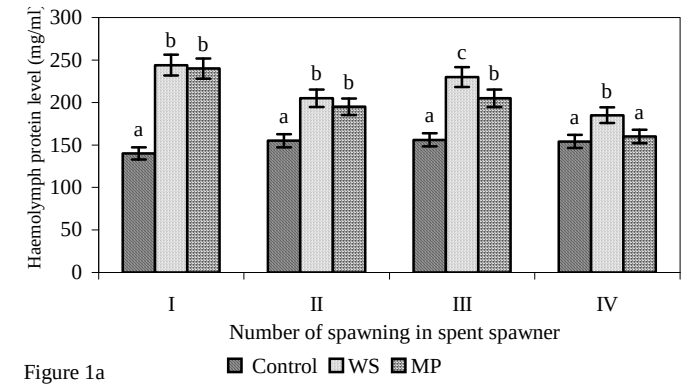

Figure 1a

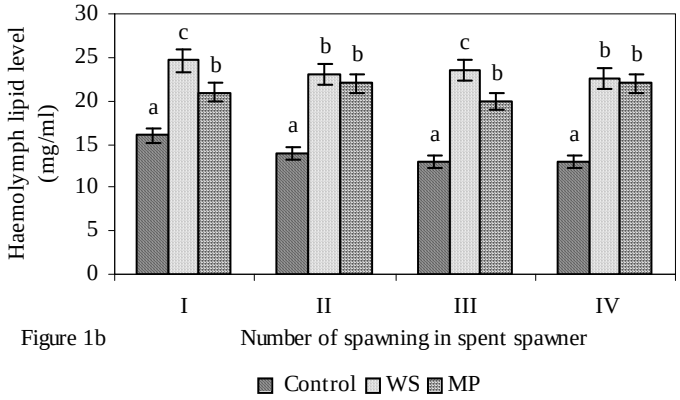

Figure 1b

Figure 1. Biochemical variables of the haemolymph during four successive spawns in the control and herbal enriched Artemia supplemented spent spawners (a) Total protein level (b) Total lipid level.

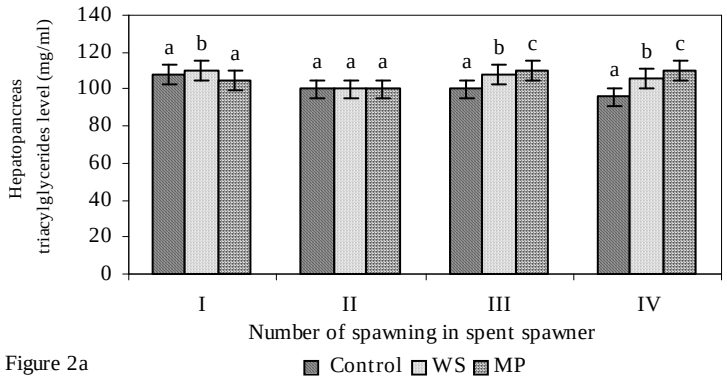

Figure 2a

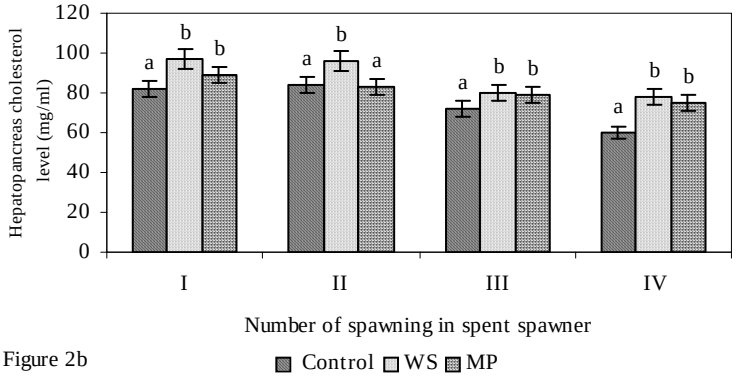

Figure 2b

Figure 2. Biochemical variables of the hepatopancreas during four successive spawns in the control and herbal enriched Artemia supplemented spent spawners (a) Trriacylglycerides (b) cholesterol.
Each value is a mean of three replicates: Error bar with different superscript alphabets are statistically significantly ($P<0.05$)

al., 2003). In the present study, the herbals were selected on their proven activity in this aspect as well as the non-hazardous nature of the phytochemicals to the environment (Citarasu *et al*., 2002). Application of the traditional medicines 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; Citarasu *et al*., 2002; Jian and Wu, 2003; Immanuel *et al*., 2003; Sivaram *et al*., 2004). In our previous study, crude combination of WS and MP with other herbals has significantly influenced the offspring quality of the spent spawners (Babu, 1999; Babu and Marian, 2001). This was confirmed in the present study. The observations of the decline in the quantity and quality of the spawners in the control group were similar to those reported in *P. monodon* (Hansford and Marsden, 1995) and *Penaeus vannamei* (Palacios *et al*., 1999; Palacios *et al*., 2003).

The supplementation of the enriched *Artemia* with a combination of five herbal products, *Hygrophila spinosa*, *Withania somnifera*, *Zingiber officinalis*, *Solanum trilobatum*, *Andrographis paniculata* and *Psoralea corylifolia* along with the cod liver oil to the postlarvae (PL1-30) of *P. monodon* has significantly influenced the stress tolerance, feed absorption, production and metabolism (Citarasu *et al*., 2002). Interestingly, there are significant improvements obtained in the larval quality in the herbal treatments (Citarasu *et al.*, 2003). It is worthy to note that even when the fecundity of the treatment groups did not significantly vary in successive spawning, but the overall larval qualities were found to change between the two treatments (WS and MP). Both experimental groups display significant increase in cumulative larval survival than the control group. It is not necessary that the higher fecundity could increase the quality larvae (Hansford and Marsden, 1995; Palacios *et al*., 1999). Apparently, biochemical reserves in the spawners positively regulate the quality of the offspring (Racotta *et al*., 2003). Level of the protein in haemolymph could be a predictive indicator for the multiple spawns (Aquacop, 1983a; Palacios *et al*., 2003). The protein levelling in the haemolymph of the both treatment groups, which resulted the spawning viability, was significantly higher than the control group. Also, among the treatment groups, WS fed spawners also had the higher protein values in haemolymph that positively regulated the larval quality. Moreover, a steady falling fecundity, long time duration between the consecutive spawns were reflected by the lipid level in hepatopancreas. The supplementation of the herbal principles successfully regulated the biochemical parameters during the spawns. *W. somnifera* exhibits the property of reducing lipid peroxidation and metabolism during the stress in higher vertebrates (Al-Qarawi *et al*., 2000). Also, this herb was confirmed for its vitellogenin stimulating activity and increasing the yolk related proteins level in *Artemia parthenogenetica* through western blotting (Dot-blot assay) studies (Grubh, 1998). 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). Moreover, *Withania somnifera* was evaluated for its proven multifunctional potential such as anti-stress, aphrodisiac, growth promoting and immunostimulative in shrimps as well as fin fish (Citarasu *et al*., 2002; Sivaram *et al*., 2004). In conclusion, the herbal extracts (both *W. somnifera* and *M. pruriens*) 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 which can be established in the shrimp hatcheries for the effective management of the brood stocks.

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