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Original Article

The impact of *Enterocytozoon hepatopenaei* (Ehp) on *penaeus vannamei* shrimp seed growth in a nursery and grow out system in andhra pradesh, south east coast of India

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ABSTRACT: In the Asia-Pacific region, shrimp cultivation is the most lucrative and profitable aquaculture industry, and recent developments in India have threatened the industry's success due to microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) associated with retarded growth without other clinical signs, causing significant economic losses to the shrimp industry. In the present study in the nursery Mild EHP was noticed before shifting to the grow-out pond. After shifting to the grow-out pond, very poor growth was noticed in all the ponds, and also heavy size variations were noted. In V5 pond growth was stented due to EHP, and in pond V1 reverse growth was noticed due to size variation. Overall in all ponds, very poor weekly gains of .6 to .9 grams were recorded. In the present study due to slow growth with size variation Pond V4 shrimp were harvested at 136 counts and Pond V2 was harvested at 120 counts. The remaining ponds harvested 133 and 136 counts. So the present study confirms that sometimes the EHP-infected seeds show negative results PCR lab. But after stocking to nursery or growing out EHP infection can expose the middle of the culture also. So better before shifting the seeds to the grow-out pond to confirm through microscopic analysis as well as PCR lab results as free from EHP**.**

Key word: *Enterocytozoon hepatopenaei*, Microsporidian parasite, *Penaeus vannamei*, Nursery culture, and shrimp culture.

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1. INTRODUCTION

The remarkable revival and growth in shrimp culture in India was brought about by the introduction of the non-native species Penaeus vannamei in 2009, nearly replacing the native species Penaeus monodon, the culture of which failed due to disease problems mainly by White Spot Syndrome Virus (WSSV). The white leg shrimp, *Penaeus vannamei*, is an economically significant species representing 52.9% of total shellfish aquaculture production (FAO, 2020).

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Even though production has been steadily increasing in recent years, shrimp aquaculture is under constant challenge to the emergence and spread of new diseases such as hepatopancreatic microsporidiosis (HPM) caused by an emergent pathogen *Enterocytozoon hepatopenaei* (EHP).

Recently several disease syndromes such as running mortality syndrome (RMS), white feces syndrome (WFS) / white gut syndrome, and growth retardation have been negatively impacting shrimp aquaculture in India (Otta *et al.*, 2016). Shrimp farms in Asia are now facing a threat from a microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) causing significant losses to aquaculture.

Enterocytozoon hepatopenaei belongs to microsporidia, an obligate, intracellular parasite related to fungi (Wittner and Weiss, 1999). Stentiford *et al.,* (2013) reported that microsporidians were causing infection in many aquatic animals.

Enterocytozoon hepatopenaei was first reported from *Penaeus monodon* in Thailand (2009) and subsequently from China, Indonesia, Thailand, Malaysia, Vietnam, and India (Joshi *et al.,* 2014; Rajendran *et al.,* 2016; Thitamadee *et al.,* 2016).

Although *Enterocytozoon hepatopenaei* has not been associated with mortality, recent studies clearly indicate that *Enterocytozoon hepatopenaei* (EHP) is associated with severe growth retardation in *P. vannamei* and *P. monodon* (Tangprasittipap *et al.,* 2013; Tang *et al.,* 2016, 2017 and Giridharan and Uma, 2017).

In India, the first report of incidence of EHP was in 2016 from Andhra Pradesh, one of the south-eastern states of India, where shrimp culture is a major livelihood to the farmers. The economic loss in shrimp production due to EHP appears to be significant. Hence the present study was carried out to investigate the impact of the microsporidian parasite; *Enterocytozoon hepatopenaei* affected *Penaeus vannamei* shrimp seed growth in a nursery and grow out system in Singarakonda, Prakasam district of Andhra Pradesh.

2. METHODOLOGY

The present study was carried out in JC aqua sea foods (September to November 2021), Binginapalli, Singarakonda, Andhra, Pradesh. Six nursery tanks were selected and the each tank capacity is 270 MT water with the stocking density of 2.7pcs/litre (Fig. 1&2)

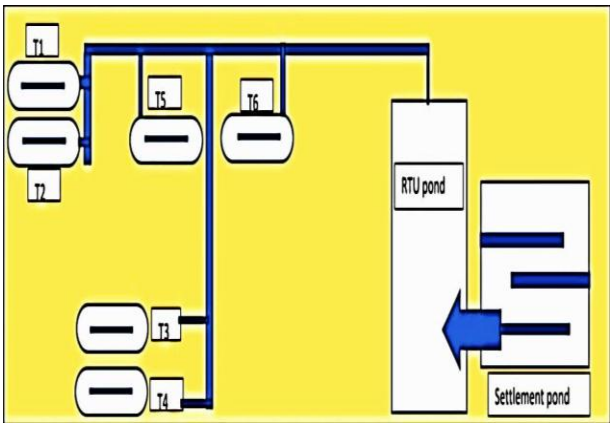
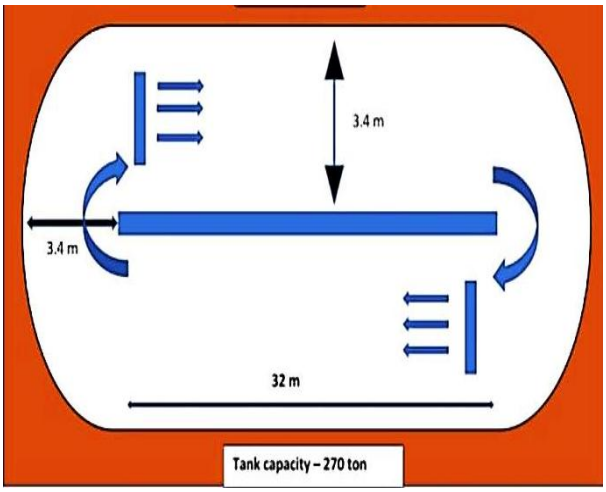


Fig: 1. shows the Nursery layout



F**ig. 2.** shows Shape of the tank is Rectangular Elliptical

Table 1: shows the Nursery Area Details**:**

Sedimentation pond	1×2000 m2	2000 m2
Reservoir or RTU	1× 8000 m2	8000 m2
Nursery tank	6× 270 T	1620 T

2.1. Reservoir and Water System:

28 ppt bore water was used in entire culture period. In this sedimentation tank water treated with PAC (Poly Aluminium Chloride), Chlorine (30 ppm) and Virkon (disinfectant). Treated water passed through Zic-Zac way to Sediment the big molecular particles and end of this section way connected with Reservoir or Ready to Use pond to Store the water. Once the water ready to use for nursery tank, simultaneously nursery tank preparation was carried out through following methods (Fig. 3).

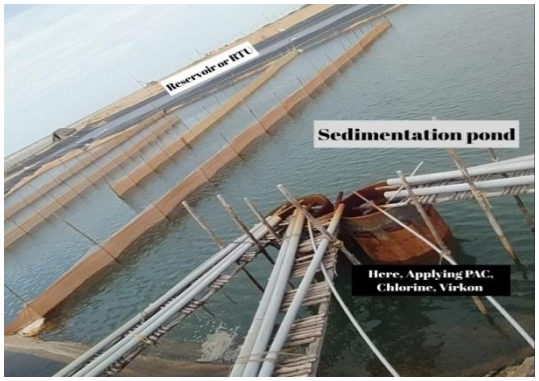


Fig: 3 show the reservoir pond (sedimentation and RTU)

2.2. Nursery Tank Washing:

- Acid washing: HCl acid used to clean the lab of tank side and bottom.
- Chlorine washing: After acid wash, next day washed with 30 ppm Chlorine.
- Kmno4 washing: 20-50 ppm potassium Permanganate over sprayed that tank side, bottom and drain pipe. After proper cleaning of the tank, water filled from the ready to use pond. All the aeration system was checked properly (Fig. 4& 5).

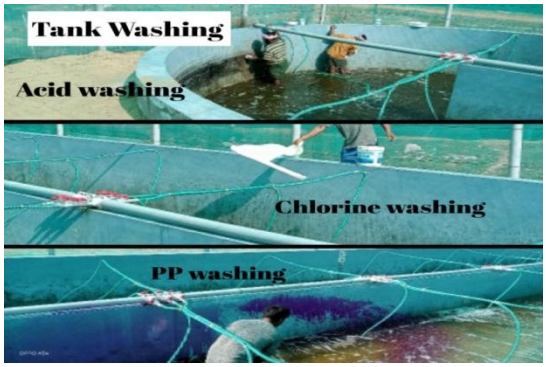


Fig: 4 shows the Nursery cleaning methods

Fig 5. Shows the filling of nursery tank

All the tanks were initially filled with a water depth of 0.7 m, and Probiotics and Minerals were applied. After stocking, the next day onwards 10cm increased and third-day water exchange started slowly.

Table 2: Nursery Stocking Details

Hatchery	CP, Pondy
Tank capacity	270 T / tank
Stocking Density	1 million / tank 3.7 pcs / litre

The *Penaeus vannamei* Specific Pathogen Free (SPF) seeds (post-larval stage 10, that had been acclimated to a salinity level of 28 ppt and confirmed negative for the white spot syndrome virus (WSSV) and *Enterocytozoon hepatopenaei* (EHP) by the polymerase chain reaction) were purchased from CP Aquaculture India Private Ltd, hatchery at Marakanam, Pondicherry. The morphological, microbial analysis and PCR test was done to confirm that the seeds are free from viral and bacterial diseases.

The seeds were transported in oxygenated double-layered polythene bags with crushed ice packs between the inner and outer covers of the bag to maintain optimum temperature (23C to 25C) in turn to keep less stress to the shrimps and the entire set up was packed in a

carton. The seeds were brought to the farm site. After acclimatization (adjusting the temperature, pH, and salinity) without any stress the seeds were released into the nursery. As per the CP feeding chart, the initial feed was given in the format of wet powder. Daily increment per lac seed 100 g for first five days, then 200 g next five days, from the 11th day onwards 300 g, 400g. Here in Nursery both Turbo and CP Tiger (9001&2) feeds are used. Feed supplements are essential to produce good quality seeds. The everyday first meal is C-150 -5g (vit c) and the last meal is max - mineral (50g/kg). In middle meals Gut probiotics and Superbiotic (beneficial bacteria) were mixed with feed, and the binder was used. 0.2 – 0.3 g growth was calculated during the first sampling on the 15th day.

On the 20th day of sampling 0.4 - 0.5 g growth was calculated. Every three days once the shrimp sample will be allowed to check for microbial analysis as well as EHP analysis (Sathish Kumar *et al.,* 2022). On the 25th day, shrimp reached 8 grams. It's ready for the shift to grow out of ponds. In the culture pond (grow out pond), Pond preparation methods, bio-secured method, and water culture techniques followed as per (Gunalan *et al.,* 2011).

2.3. **Seed shifting:**

Through Sintex tank (1000 litre capacity) seed were shifted to grow out ponds (in the tank blower and Oxygen cylinder was used). Initially starting biomass per tank 30 kgs.

2.4.**Grow out Feed Management:**

After seed shifting to grow out pond feed ration 20 kg feed/day/lac seeds / 1 g size and 15 kg feed/day/lac seeds / 0.5 g size. First month completely blind feeding given 1g / kg feed given in check tray up to 10g. At harvest, biomass, survival rate, Average daily growth (ADG), and FCR were calculated (Gunalan et al., 2011; Fourooghi and Kamali 2010; Afsharnasab *et al.* 2008; Salehi 2007; Kumuran *et al.* 2003) using the following formula: Survival rate = total harvested shrimp weight (g) / (shrimp average weight at harvest [g] × number of PLs stocked)

Estimated biomass (g) = average weight (g) × number of stocking × survival rate (%)

FCR = consumed feed (kg)/total harvested shrimp weight (kg)

ADG = Total weight gained by the shrimps / Total days of culture

The water quality parameters like salinity, pH, temperature, dissolved oxygen and light transparency were measured by using hand Refractometer, pH pen, thermometer, and dissolved oxygen meter and secchi disc, respectively.

3. RESULTS

In the nursery tanks 28 ppt average salinity was recorded. The average pH range 7.8 to 8, total alkalinity 170 ppm, total hardness 5800ppm, minerals calcium 360 ppm and magnesium 1321 ppm was recorded. In all the tanks absence of ammonia and nitrite was noted (Table: 3). All the water quality parameters checked daily in the laboratory. Before transfer to grow out pond, the shrimp were checked for microbial analysis and EHP (Baumann and Schubert, 1984; Lightner 1996). There is mild percentage of EHP noticed. The seed growth was monitored keenly.

Table: 3 Average nursery water quality parameters:

Parameters	RTU or Reservoir
pH	7.8 - 8
Salinity (ppt)	28
Total Alkalinity (ppm)	170
Ammonia (ppm)	0
Nitrite (ppm)	0
Transparency (cm)	60
Total Hardness (ppm)	5800
Calcium (ppm)	360
Magnesium (ppm)	1321

The first seed shifting was started at 31 days, pond V4 was stocked with 6 lakhs of seeds,

pond V5 was stocked with 5.6 lakhs of seeds, followed by Pond V1 stocked 5 lakhs, V2 stocked with 5.1 lakhs and V3 stocked with 5.8 lakhs seeds. In all the ponds stocking density varied from 63 to 83 pcs/m^2. The shrimp's body weight ranged from 1 g to 1.3g (Table: 4).

Table 4. Pond wise seed shifting details:

Pond No	V1	V2	V3	V4	V5
Area (Ha)	0.8	0.8	0.7	0.81	0.83
Nursery DOC	35	36	37	31	34
ABW (g)	1.2	1.2	1.2	1	1.3
Biomass (kgs)	600	612	696	600	728
No of Seeds(mill)	0.5	0.51	0.58	0.6	0.56
Density (pcs /m^2)	63	64	83	74	67

In the grow-out ponds, pH ranged from 7.6 to 8.1, salinity was recorded at 27 ppt as a minimum in pond V4, and maximum salinity of 35 ppt was recorded in pond V1. The total alkalinity range was recorded from 210 ppm to 220 ppm. The minimum range of Calcium of 320 ppm was recorded in Pond V4 and a maximum of 400 ppm was recorded in Pond V1. The magnesium level in the water ranged from 1229 ppm to 1346 ppm. The total hardness minimum level was recorded at 5400 ppm in pond V5 and a maximum of 6000 ppm was recorded in pond V1. In V3 and V5 ponds .1 ppm ammonia was recorded. In the V5 pond, .05 ppm, and V1 pond.04 ppm ammonia was recorded. There is an ammonia problem in the V2 pond .only in the V3 pond 0.05 pm nitrite was recorded and the other pond does not have a problem with nitrite level (Table 5).

Table: 5 shows the average water quality parameters in grow out ponds:

Parameters	V1	V2	V3	V4	**V5**
pH	7.7	8.1	7.8	7.6	**7.8**
Salinity (ppt)	35	30	28	27	**30**
Carbonates (ppm)	0	0	0	12	**0**
Bicarbonates (ppm)	220	210	218	203	**212**
Total Alkalinity (ppm)	220	210	218	215	**215**
Calcium (ppm)	400	360	340	320	**340**
Magnesium (ppm)	1360	1346	1326	1258	**1229**
Total Hardness (ppm)	6000	5900	5800	5500	**5400**
Ammonia (ppm)	0.04	0	0.1	0.05	**0.1**
Nitrite (ppm)	0	0	0.05	0	**0**

After shifting the seed to grow out pond the shrimp were monitored closely. In the first sampling, it's the growth was not showing good significant results. The shrimp samples were collected from all the ponds and EHP was checked. The positive results show the shrimp were fully infected with EHP. At the same time we could see the very poor growth in all the ponds and also heavy size variations noted (Fig: 6& 7 and Table 6). In V5 pond growth was stented due to EHP, and in pond V1 reverse growth was noticed due to size variation. Overall in all ponds very poor weekly gains 0.6 to 0.9 grams were recorded.

Table 6. Sampling Details for all the ponds

V1		V2		V3		V4		V5	
DOC	*ABW*	DOC	*ABW*	DOC	*ABW*	DOC	*ABW*	DOC	*ABW*
25	4.3	24	4.2	23	4.4	15	3.7	26	4.8
32	5.2	31	5	30	5.2	22	4.6	33	6
39	7.1	38	6.36	37	6.6	29	5.4	40	7.1
46	7.8	45	7.7	44	6.6	36	6.9	47	8
54	7.5	57	8.2	55	7.5	48	7.3	54	8

3.1.EHP confirmation results:

The seeds were collected and sent to the laboratory. The animal gills were pressed gently and checked under a digital microscope. The shrimp gill structure of normal shrimp and the affected shrimp photograph show the hepatopancreas tubule with contraction.

At the same time the PCR results also co-insides with the microscopic results (Kumar *et al.*, 2022) (Fig 8, 9& 10).

Fig: 6 and 7. shows the size variation and poor growth

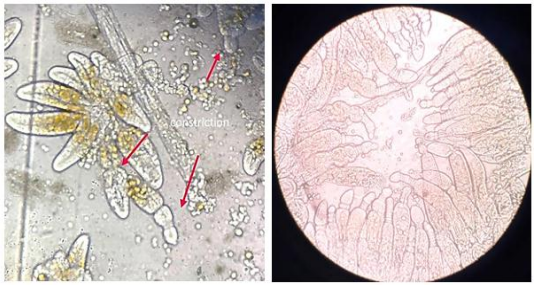


Fig 8 & 9. shows the hepatopancreas tubule with contriction (EHP)

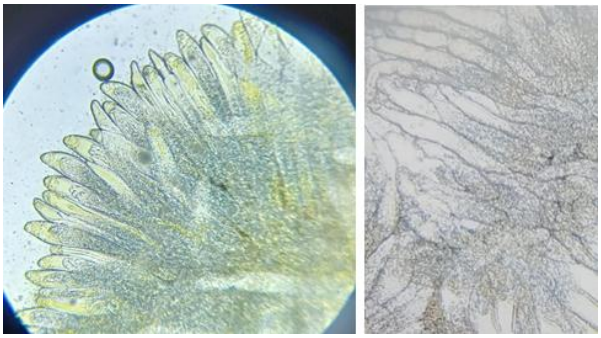


Fig: 9 & 10. Show the normal shrimp hepatopancreas tubule

From nursery 1 to 1.3-gram size shrimp seeds were shifted to grow out ponds. Due to sever EHP problems very poor growth and size variations are recorded. So immediately harvest was fixed. Pond V4 shrimp were harvested at 136 counts and pond V2 were harvested at 120 counts. The remaining ponds harvested 133 and 136 counts. The average daily gain of .13 to .15 was recorded. The maximum feed conversion ratio of 2.2 was recorded in pond V3 and the 1.8 lowest FCR was recorded in pond V1. The maximum 81.3 % survival was recorded in the pond V1 and maximum production 3500 kg shrimps harvested in the pond V5 (Table :7).

Table: 7. Harvesting Details

Pond No	V1	V2	V3	V4	V5
Area (Ha)	0.8	0.8	0.7	0.81	0.83
Stocking (mill)	0.5	0.51	0.58	0.6	0.56
Density (pcs/l)	63	64	83	74	67
Nursery ABW (g)	1.2	1.2	1.2	1	1.3
Biomass (kgs)	600	612	696	600	728
Nursery DOC	35	36	37	31	34
Growout DOC	54	57	55	48	54
COUNT	133	120	133	136	125
ABW (g)	7.5	8.2	7.5	7.3	8
ADG (g)	0.13	0.14	0.13	0.15	0.14
TAF (kgs)	5624	6198	7268	4835	5789
T. Biomass (kgs)	3049.1	3000	3200	3000	3500
FCR	1.8	2	2.2	1.6	1.6
Survival (%)	81.3	71.7	73.5	68.4	78
Productivity(kgs/Ha)	3811	3750	4571	3703	4216

4. DISCUSSION

Hepatopancreatic microsporidiosis (HPM) is an emergent threat to sustainable shrimp farming worldwide. Our understanding of host–pathogen interactions in HPM will allow the development of strategies to mitigate the risk of disease outbreaks in shrimp farming (López‐Carvallo *et al.*, 2022).

Recently, growth retardation/slow growth due to EHP has become a matter of concern in shrimp farms across the world. It has now become one of the most serious emerging pathogens, leading to significant economic losses in the shrimp culture industry. However, there was no published data on the incidence of EHP in India until recently Rajendran *et al.* (2016) reported the first incidence of EHP from shrimp farms in Andhra Pradesh and Tamil Nadu and estimated the overall incidence of EHP to be 96.50% (Kummari *et al.*, 2018). During this study, the incidence of EHP infection was identified by PCR as reported by (Rajendran *et al.*, 2016; Tang *et al.*, 2016; Kummari *et al.*, 2018).

The damage caused to the hepatopancreas affects the metabolic activities and growth of the shrimp, accounting for the retarded growth observed in shrimp ponds with high EHP incidence. Tangprasittipap *et al.*, (2013) studied EHP and its causal relationship with White Faeces Syndrome (WFS).

They concluded that EHP is not the cause of WFS in *P. vannamei* culture. Later, Tang *et al.*, (2015) observed densely packed spores of the microsporidian EHP and relatively fewer numbers of rod-shaped bacteria within the white feces and stated that EHP is a cause of

WFS in *P. vannamei*. But in the present study, only size variation with slow growth was recorded without any white feces.

Rajendran et al., (2016) studied the incidence of EHP in Andhra Pradesh, India, and reported an incidence of 39.7% in ponds without White Faeces Syndrome (WFS) and a very high incidence (96.4%) in the ponds which experienced WFS. In the present study, there is no white feces disease problem in the nursery as well as grow-out ponds.

Salachan *et al.,* (2017) stated that EHP can be directly transmitted to other shrimps via water. Kummari *et al.,* (2018) reported the aquatic macrofauna screened for EHP by nested PCR was positive, indicating that the shrimps, polychaetes, crabs, and non-penaeids may act as carriers of EHP. Similar studies conducted by Tang *et al.,* (2015) and Chiyansuvata *et al.,* (2015) revealed that Artemia and Grapsidae family crabs were infected by EHP. In the present study, live feeds are not used, even though the shrimp got affected by EHP. The first report for the slow growth in shrimps was reported by Anderson *et al.* (1989) at Malaysia in Penaeus monodon. And later, similar slow growth syndrome was reported by Hudson *et al.* (2001) in *Penaeus japonicus*. More evidence were obtained by knowing that retarded giant or black tiger shrimp *P. monodon* was reported with microsporidian infestation in hepatopancreas from Thailand in 2004. India EHP is also reported from shrimps showing slow growth (Sriurairatana *et al.,* 2014) and very recently *P. vannamei* farm in India (Rajendran *et al.,* 2016; Raveendra *et al.,* 2018).

In the present study due to slow growth with size variation Pond V4 shrimp harvested at 136 counts and in pond V2 harvested at 120 counts. Remaining ponds with 133 and 136 counts. Probiotics are known to improve water quality in aquaculture ponds because the bacteria in it also participate in the absorption of organic nutrients (Moriarty, 1997). Okomoda *et al.,* (2022) reported from the ammonia level was very low due to the application of probiotics in the culture ponds. In the present study also very less ammonia level was recorded in the grow-out ponds as well as in the nursery system .

The hepatopancreas is the main target organ of EHP infection, and the life stages of EHP are mainly present in the tubule epithelial cells of the hepatopancreas (Chaijarasphong *et al.,* 2021).

In crustaceans, the hepatopancreas is an important immune organ for the synthesis of steroid hormones as well as certain biosynthetic steps (Swevers *et al.,* 1991).

It is also an important organ for the absorption and storage of nutrients and is essential for molting activities (Zamal and Ollevier, 1995). In the present study also hepatopancreas tubules clearly show the EHP infection.

5. CONCLUSION

The present study concludes that sometimes the EHP-infected seeds show negative results PCR lab. But after stocking to nursery or growing out EHP infection can expose the middle of the culture also. So better before shifting the seeds to the grow-out pond confirm through microscopic analysis as well as PCR lab results as free from EHP.

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