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Effect of ingestion and waterborne routes under different shrimp densities on white spot syndrome virus susceptibility in three commercially important penaeid shrimps

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ABSTRACT

A study was undertaken to evaluate some factors that are important for better management of the white spot syndrome virus (WSSV). We examined the likelihood of that the mud crab (*Scylla serrata*) is a potential carrier of WSSV in shrimp farms and then compared susceptibility of three shrimp species to WSSV, and finally tested the effects of shrimp stocking density and transmission routes on the intensity of WSSV infection under experimental conditions. Mud crabs were collected from the crab fattening unit of Pichavaram mangroves area, southeast coast of India. The infectivity test was conducted on *Penaeus monodon*, *P. indicus*, *Litopenaeus vannamei* under three different shrimp densities and two different routes of infection. Each treatment was executed in triplicate of 100 L tanks. The results showed that *S. serrata* is a carrier of WSSV. Among three species of shrimps, *P. indicus* was most susceptible and *L. vannamei* was most resistant in term of cumulative mortality. Ingestion route was more effective than waterborne route on WSSV outbreaks. Cumulative mortality of shrimps due to WSSV infection increased with increasing shrimp density. The result of this study is important to improve management especially to minimize the WSSV infection in the shrimp farms.

1. Introduction

Shrimp enjoy high consumer preference and market price in many countries of the world (Asaduzzaman et al., 2010). Due to this popularity, world aquacultural production of shrimp is increasing every year (FAO, 2011). Among the various kind of shrimps, *Penaeus monodon* (Fabricius, 1798), *Penaeus indicus* (Edwards, 1837), *Litopenaeus vannamei* (Boone, 1931) are very important because they are widely used as stock aquaculture species in many countries. In 2011, these three species together contributed around 75% of total shrimp production in the world (FAO, 2011; Rajkumar et al., 2013). However, disease is an inherent part of most aquaculture practices and shrimp farming is no exception (Rahman and Verdegem, 2007; Gopalakrishnan et al., 2014). Presently, disease has caused unsustainability of the shrimp industry in many countries of the world. With respect to loss in production by various disease agents, more than half of the loss is attributed to viral diseases. Among these the white spot syndrome virus (WSSV) is the most important viral diseases in shrimp (Lightner, 1996). The WSSV spreads very rapidly in the culture and also in the wild. Therefore, the WSSV is considered as the most serious threat to shrimp farms across all shrimp producing nations of the world (Lightner, 1996). The WSSV has a wide range of hosts, including 40 species of crustaceans of which some commercially important marine crabs (Corbel et al., 2001; Gopalakrishnan et al., 2011; Lightner et al., 1998). The clinical signs of this disease include white spots in the inner surface of the carapace (Gopalakrishnan et al., 2011) and lethargic movement by infected crabs. Among the many different carriers, mud crabs (*Scylla serrata* (Forsskal, 1775)) are generally highly tolerant to WSSV and remain infected for long periods of time without signs and symptom of disease. Therefore, mud crabs are assumed to pose dangerous threat to shrimp farms. Published information regarding the likely risks to shrimp aquaculture from mud crabs is, however, very limited. Among three commonly cultured shrimp species: *P. monodon, P. indicus, L. vannamei*, the latter is anecdotally assumed to be more resistant to WSSV than the other two. How-

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ever, there is no published evidence to support this assumption. Apart from inter–specific variability in resistance to WSSV, shrimp holding density in the culture system is another important factor in the intensity and severity of disease outbreak. In aquaculture practise, animal density is a crucial factor that affects all states of physiology including feeding (Rahman et al., 2009; Rahman et al., 2010, 2008a,b), behaviour (Rahman et al., 2008c; Rahman and Meyer, 2009; Rahman and Verdegem, 2010), growth and disease susceptibility of the cultured animal (Khatune-Jannat et al., 2012; Rahman et al., 2008d). Unfortunately, research on the effects of shrimp stocking density on the intensity and severity of WSSV outbreaks are lacking. There are two potential sources whereby WSSV can be transmitted among shrimps. These are direct transmission whereby, one shrimp eats an infected individual (ingestion route) or the indirect transmission from an infected to uninfected shrimp of the virus through the water (waterborne route). To date there is no knowledge of which of the two routes is the most rapid for outbreaks of WSSV on shrimp farms. Based on the above issues, the objectives of the study were to (1) provide evidence that the mud crab is a potential carrier of WSSV in shrimp farms, (2) compare susceptibility to WSSV among *P. monodon, P. indicus* and *L. vannamei*, and (3) investigate the effects of shrimp stocking density and the route of infection on the intensity and severity of WSSV infection under experimental condition.

2. Materials and methods

The WSSV infected *S. serrata* were collected from a small crab culture farm located at M.G.R. Thittu, Cuddalore district, Tamil Nadu, southeast coast of India (Lat. 11° 28′ 13 N; Long. 79° 46′ 41 E). In the farm, a total of 108 crabs were cultured in 12 polyvinyl chloride (PVC) cages, each of which had 9 (0.3 × 0.3 m) chambers. The stocking density was one crab/chamber, whereby crabs ranged between 510 and 740 g. These were collected from wild catch around Pichavaram mangroves and on rare occasions from ponds and the live export rejected crabs. Crabs were fed twice a day at the rate of 10% of their total body weight as wet feed. These feeds were trash fish (consisting of both fin– and shell–fish) from trawl landing centres in and around fattening areas. The moribund crabs were tested for WSSV using the commercial nested polymerase chain reaction (PCR) method (Applied Biosystems Inc., Foster City, CA, USA).

The infectivity experiment was conducted on *P. monodon, P. indicus, L.vannamei* (average individual weight 5 g) under three different densities: 5, 10, and 20 shrimps/tank. Two different routes of infection (ingestion and waterborne) were also compared under the same experimental conditions. For the ingestion route of infection test, each species of shrimp was reared at the rate of 5, 10 and 20 individuals per tank of 100 L with sufficient aeration. On day one all shrimps were fed with WSSV infected (moribund) mud crab flesh of chelate legs containing WSSV at libitum. From day two onwards the shrimps were fed only with dry commercial pellet feed containing 25% crude protein. A similar experimental setting was applied for testing waterborne route (control) test except on Day two shrimps were fed only with beef liver. Each treatment had three replicates. The water born infection trial was performed using the filtrate of the epidermis from live WSSV infected *Scylla serrata.* The epidermis from infected crab was removed and homogenised in brackish water at 4 oC at a ratio 1:9. Followed by centrifuged at 8510 × *g* for 5 min, the supernatant fluid was filtered through 0.45 μm membrane and diluted 500 times with brackish water. The equal volume of the dilution was used for the experiment. For both ingestion and waterborne routes, feeding was withheld for 24 h before starting the experiment. Water salinity and temperature were maintained constant for both control and experiment throughout the study period 29 days. Mortality was recorded daily and dead shrimps were removed after being counted. The moribund and dead shrimps were tested for WSSV using same method (PCR) of testing WSSV in the moribund crabs. After PCR analysis, virus–infected crab and shrimps tissues were further analysed electron microscopy. For this process, crab and shrimps tissues were fixed in 3% glutaraldehyde (pH 7.2), washed in phosphate buffer (pH 7.2), post–fixed in 1% osmium tetroxide and washed in buffer, dehydrated through an ascending series of graded alcohol from 50% to 100% cleared in propylene oxide. The gills were further infiltrated by propylene oxide and embedded in epoxy resin. The embedded mould was kept in an incubator (Technico Laboratories Product Private Limited, Chennai, Tamil Nadu, India) at 60 °C for 48 h, and allowed to cool. Semi–thin sections of 1 Mm were cut using an ultra–microtome (Leica ultra– cut UCT, Leica Ltd., Germany) with a glass knife and stained with 1% toludine blue. Ultrathin sections were taken on a copper grid and stained with Uranyl acetate and Reynold's solution. The sections were examined using a transmission electron microscope (TEM) (Phillips model 201–C, Phillips Electron Instruments, Mahwah, NJ, USA). For scanning electron microscopy (SEM), the crab carapace and the gill were fixed immediately in 2.5% glutaraldehyde in 0.2 M phosphate buffer at a pH of 7.2 (JEOL JSM–5610LV SEM, JEOL Ltd., Tokyo, Japan). The samples were post–fixed with 1% osmium tetroxide in the same buffer, dehydrated through a graded series of ethanol and critical point dried.

3. Results

Of the 108 crabs, 12 were dead on the 17th day of stocking and all other crabs were sluggish. White spots were observed on the inner carapace of the moribund crab shells (Fig. 1A). All of the moribund crabs shows positive for WSSV after PCR analysis and the WSSV infection was recorded in the gill nucleus (Fig. 1B). Gill chocked crab gill lamella were characterised by epithelial thickening and intracellular necrosis (Fig. 1C). The viral particles were also present in the cytoplasm of the crab gill cells (Fig. 1D). White spots were observed on the carapace of the experimentally WSSV infected *P. monodon* (Fig. 1E). The microscopical view white spot (ameboidal) of the *P. monodon* is very clear (Fig. 1F). The electron dense viral particles (10–170 nm in length) are clear in the gill of experimentally infected *P. monodon* gill cell (Fig. 1G).

Among the three species of shrimps, mortality rate was the fastest in *P. indicus* (Fig. 2A and B), followed by *P. monodon* (Fig. 2C and D) and *L. vannamei* (Fig. 2E and F). The ingestion route was more effective than waterborne route of infection for cumulative shrimp mortality. For this species, the 100% mortality was observed on the 23rd, 20th and 15th day of culture period in tanks with 5, 10, 20 individuals, respectively for the ingestion mode of infection (Fig. 2A). For the waterborne route, the 100% mortality was observed on the 26th, 23rd and 20th day of culture period in tanks with 5, 10 and 20 shrimps, respectively (Fig. 2B). For the *L. vannamei,* maximum mortalities of 75%, 73% and 67% were observed on the 15th, 17th, 19th day of culture period in tanks with 20, 10 and 5 shrimps, respectively for the ingestion route of infection (Fig. 2E). For the waterborne route, maximum mortalities of 78%, 77% and 69% occurred on the 19th, 22nd and 24th day of culture period, in tanks with 20, 10 and 5 shrimps, respectively (Fig. 2F).

Mortality of all species of shrimp was greatly affected by shrimp density for both routes infection, with mortality rates was increasing with increasing shrimp density. For all species of shrimps (*P. monodon, P. indicus* and *L. vannamei*) and all routes of infections (ingestion and waterborne route), mortality rates were highest in 20, followed by 10, then 5 shrimps/tank.

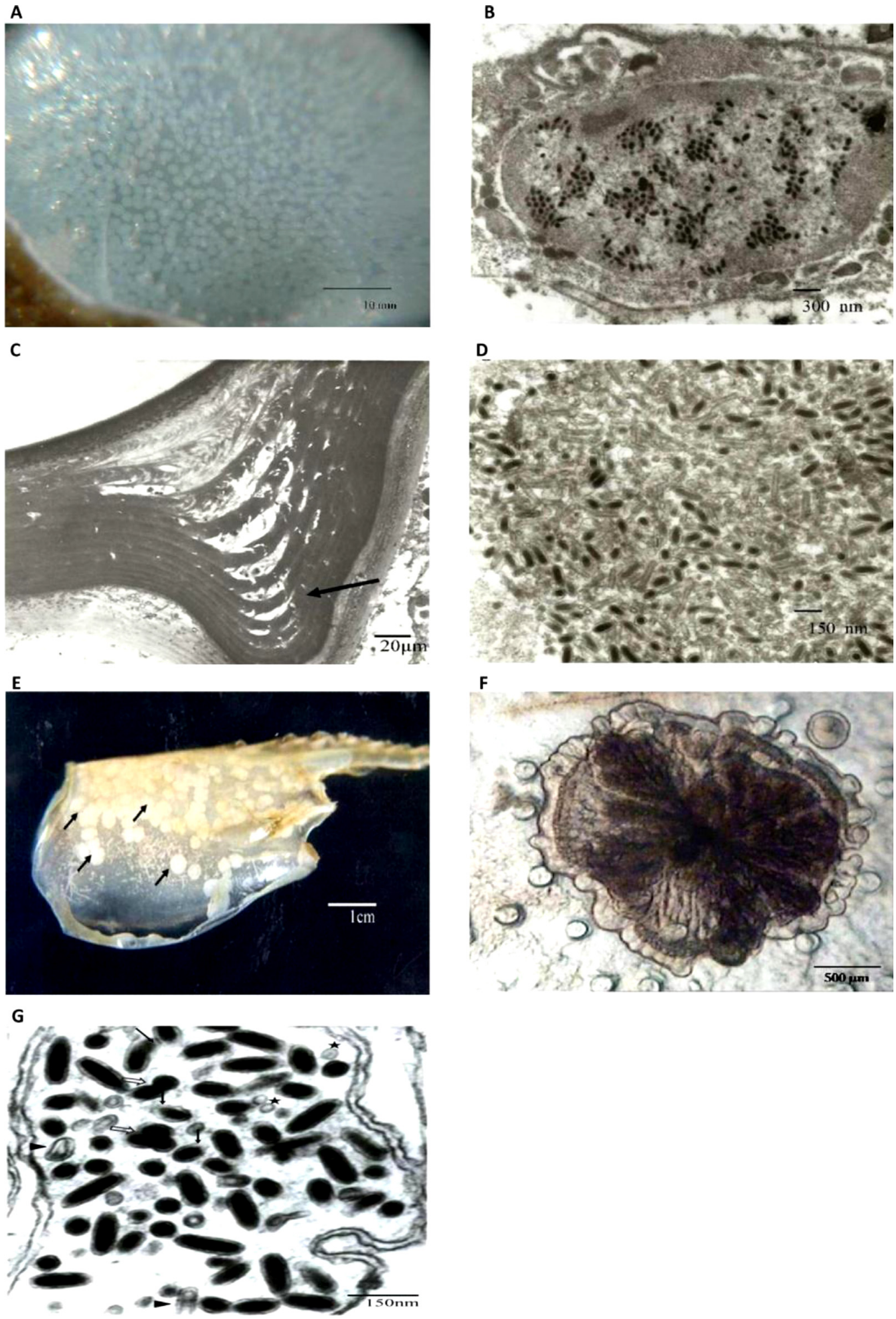

Fig.1. White spot syndrome virus (WSSV) on Scylla *serrata* (Forsskal, 1775) (A) light photo micrograph of *S. serrata* of inner side carapace surface with white spots; (B) transmission electron micrograph of viral particles in the nucleus of the gill cell of *S. serrata*; (C) scanning electron micrograph of gill lamellae thickening (arrow) bacterial fouling in the white spot syndrome virus infected moribund crab; (D) transmission electron micrograph of viral particles in the cytoplasm; (E) *Penaeus monodon* Fabricius, 1798 carapace with white spots; (F) light micrograph of WSSV on *P. monodon* showing peripheral ring and melonized dots in the inner side; (G) transmission electron micrograph of viral particle from carapace of WSSV infected *P. monodon*, virus particle envelope are still open at one end (arrow), virus particle without nucleolus (stars), large virus particle with partially developed nucleolus (arrow head) and virus particle division (blank arrow).

4. Discussion

Among various pathogens that impact on shrimp health, WSSV is the most dangerous. According to Lightner et al. (1998), 100% shrimp mortality can occur within 7–10 days of WSSV infection. However, different species of marine crabs such as *Calappa lophos* (Herbst, 1782), *Portunus sanguinolentus* (Herbst, 1783), *P. pelagicus* (Linnaeus, 1758), *Charybdis* sp., *Helice tridens* (De Haan, 1835) and

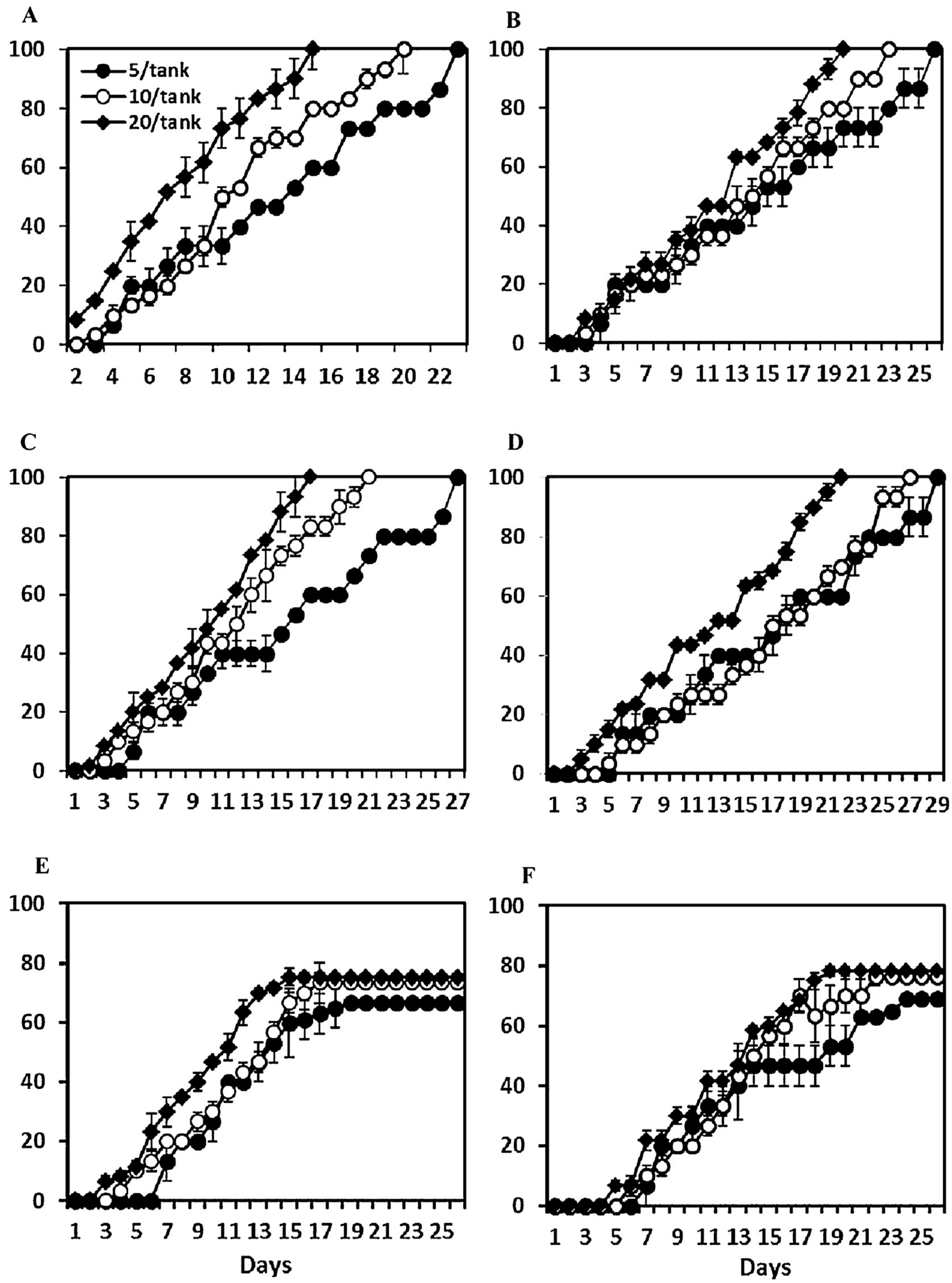

Fig. 2. Cumulative mortality of Penaeus *indicus* (H. Milne Edwards, 1837) through ingestion routes (A), *Penaeus indicus* through water borne (B), *Penaeus monodon* Fabricius, 1798 through ingestion routes (C), *Penaeus monodon* through water borne (D), *Litopenaeus vannamei* (Boone, 1931) through ingestion route (E) and *Litopenaeus vannamei* through water borne (F).

S. tranquebarica (Fabricius, 1798) are the main carriers of WWSV in shrimp farms (Corbel et al., 2001; Lightner et al., 1998). Recently Gopalakrishnan et al. (2011) detected WSSV in the mangrove crab *S. tranquebarica*. This study confirms another new carrier (*S. serrata*) of WSSV, which was confirmed by polymerase chain reaction (PCR) method. We detected rod–shaped viral particles in the nucleus of gill cells ranging in the size between 180 and 300 Mm in lengths and 30–50 Mm in width. The viral particle sizes observed agree with Gopalakrishnan et al. (2011), who reported similar shape and size of viral particle in the nucleus of gill cells of the *S. tranquebarica*.

Besides reporting *S. serrata* as a new carrier of the WSSV, this study also describes some important factors that affect the likelihood of epidemics in cultured and wild shrimp. Understanding such actors is important for the optimal management of cultured shrimp, particularly to minimize the WSSV infection and transmission. In this study, mortality throughWSSV infection was tested on three commercially important penaeid shrimps *P.* m*onodon, P. indicus* and *L. vannamei.* Among these, *P. indicus* was most susceptible and *L. vannamei* was most resistant in terms of cumulative mortality. A maximum 78% mortality was observed for *L. vannamei* while in the case of *P. indicus* and *P. monodon* all individuals were dead within 25 and 29 days, respectively. This finding in a way concurs with Rahman (2007) and Soto and Lotz (2001). According to Rahman (2007), *L. vannamei* is a pathogen resistant shrimp compare to many other shrimp. Soto and Lotz (2001) compared the cumulative mortality *L. vannamei* and *L. setiferus* (Linnaeus, 1767) through WSSV infection and observed that *L. vannamei* was more disease resistant than *L. setiferus*. It is already well understood that that WSSV can be horizontally transmitted via both ingestion and waterborne routes (Chou et al., 1998). Most of the shrimps are cannibalistic in nature and dead shrimps are often eaten by live shrimps. The dead shrimp can be super infected with WSSV which can then be transmitted to live shrimps after feeding on infected tissues. In this study, the effectiveness of two routes of infections was tested. The observed faster mortality through the ingestion route than through waterborne route potentially a higher concentration of viral particles in the WSSV infected crab muscle compared to the ambient levels in the water. The slower transmission through water could additionally be related to the number of steps to transfer particles across the vagarious mediums. Generally, viral particles are suspended in water, attached to microalgae, or carried by zooplankton (Esparza-Leal et al., 2009). Therefore, more time is needed to transfer viral particles through waterborne route than through ingestion route. However, the results of faster mortality through the ingestion route than through waterborne route agree with Gopalakrishnan et al. (2011), who observed faster mortality through the ingestion mode than through the waterborne route in *P. monodon* suggesting that it is more likely that there are higher concentrations in muscle compared to water and thus higher likelihood of transmission.

This study also provides experimental evidences that shrimp stocking density has a strong influence on cumulative mortality following WSSV infection. It was clearly observed that the shrimp cumulative mortality increased with increasing density. This could be best explained by the likelihood of increased stress levels in shrimps that are stocked at higher densities compared to those stocked at lower densities. Although stress was not measured in this study, it is a commonly accepted theory that animal stress levels are positively associated with their stocking density in farming systems. It is also known that stress levels in animals have a strong influence on outbreaks of diseases and the transmission and impacts of these outbreaks. There is no previous study comparing the effects of similar shrimp density on their cumulative mortality. However, our finding in part agree with Wu et al. (2001), who observed higher cumulative mortality in higher shrimp (*Penaeus japonicus* (Spence Bate, 1888)) density. According to Lightner and Redman (1998), shrimp become more susceptible to pathogens when they are crowded and environmental conditions are stressful.

5. Conclusion

Scylla serrata is a new carrier of WSSV. Among *P. monodon, P. indicus* and *L. vannamei*, *P. indicus* was most susceptible and *L. vannamei* was most resistant in term of cumulative mortality. Ingestion route was more effective than waterborne route on WSSV outbreaks. Cumulative mortality of the shrimps due to WSSV infection increases with increasing shrimp density. These results have important management implications to minimize the WSSV infections in shrimp farms.

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