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〈Original article〉

Relationship between White Spot Symptom and Physiological Status of Two Penaeid Shrimps

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Abstract - Shrimps infected with WSSV (White Spot Syndrome Virus) generally exhibit white spots in their inner space of carapaces as an acute clinical sign. In an effort to identify the correlation between this acute clinical sign and the condition, the index factors (RNA/DNA concentration and ratio, trypsin activity) were analyzed. A total 580 farmed *Fenneropenaeus chinensis* and 130 *Lithopenaeus vannamei* were collected from western and southern fifteen outdoor ponds in Korea. The status of the white spot pathology was divided into four stages (stage 0, stage I, stage II, and stage III), in accordance with the clinical signs as to the size and area of white spots. A significant decrease in RNA concentration and RNA/DNA ratio for multi-infected fleshy prawn (WSSV and *vibrio* sp.) occurred during the stage III (the whole carapace is covered with a white spot). In particular, RNA/DNA ratio was significantly lower as 1.47 ± 0.04 than other groups. A similar trend was also found in the single infection (WSSV), but the decrease was less than the multi-infection. In the species comparison, both species were vulnerable to the multi-infection, but *L. vannamei* was more sensitive than *F. chinensis* (ANOVA, $p < 0.05$): A significant decrease in RNA concentration and RNA/DNA ratio was first found in stage II for the former species, while it was found in stage III for the latter species. Trypsin activity was also showed a similar tendency with nucleic acid variation. Multi-infected shrimp showed drastically decrease of trypsin activity. According to the results, clinical signs of the white spot under carapace have an only physiological effect on shrimp if they covered entirely with white spots.

Keywords : *Fenneropenaeus chinensis*, *Lithopenaeus vannamei*, RNA/DNA ratio, WSSV, *Vibrio* sp., trypsin activity

INTRODUCTION

Fleshy prawn, *Fenneropenaeus chinensis*, was a popular candidate for aquaculture in South Korea, with an annual production of 2,426 MT in 2004. However, production decreased rapidly to 16 MT in 2011 due to a severe outbreak of WSSV (White Spot Syndrome Virus). Since 2003, the whiteleg shrimp, *Litopenaeus vannamei*, has been imported into Korea from Hawaii, USA. First, about 100 tons of *L. vannamei* were produced in 2004 (Kim *et al*. 2006). The production of *L. vannamei* then increased rapidly, and it was 62.5% of total shrimp production in 2007 (Jang *et al*. 2007), reaching 99.4% at 2,844 tons in 2011 (MIFAFF 2012).

The main cause influencing shrimp mass mortality is the outbreak of disease caused by pathogens such as *Vibrio* sp. or a virus. These pathogens are present in the substrate and culturing water and can induce 100% mortality of the organisms (Lightner 1983; Nash *et al*. 1992). Virus-caused diseases result in an enormous loss in the shrimp industry around the world. There have been many studies on virus-based disease including those of White spot syndrome

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virus, hepatopancreas virus, taura virus, etc. (Durand *et al*. 1997; Lightner *et al*. 1998; Chen *et al*. 2000; Witteveldt *et al*. 2004). *Vibrio* spp. is by far the most numerous and widely reported major bacterial pathogen associated with shrimp disease, including *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, etc. (Lavilla-Pitoge *et al*. 1990).

Leu *et al*. (2007) suggested that WSSV infection strongly up-regulates the expression of cuticular proteins. Pathological studies have revealed that the cuticular epidermis is one of the main target tissues of WSSV (Chang *et al*. 1996). At the late stage of infection, it loses its cellular architecture and becomes necrotic. Two of the pathological characteristics caused by a WSSV infection are the loosening of the cuticle and appearance of white spots in the cuticular epidermis. From this, we can easily recognize white spots on the shrimp carapace in situ as the first external signs of WSSV infection. However, to date, no research has been published on whether the particular pattern of the white spots can indicate the disease status or show a correlation with the physiological status of the shrimp. To evaluate this correlation, the health of the shrimps was analyzed using the condition index in terms of the RNA/DNA ratio and trypsin activity. The RNA/DNA ratio has been used in phyto-zooplankton (Dortch *et al*. 1983; Wagner *et al*. 1998; Ikeda *et al*. 2007), fish larvae (Buckley 1984; Bulow 1987), adult fish (Thorpe *et al*. 1982; Chicharo *et al*. 1998), bivalve organisms (Grémare and Vétion 1994), cephalopods (Clarke *et al*. 1989), crustaceans (Lemos *et al*. 2002; Rosa and Nune 2004). Another factor that has been selected is the trypsin activity in the hepatopancreas and digestive tracks of the shrimp. Trypsin and chymotrypsin are responsible for more than 60% of total protein digestion in penaeid shrimp (Galgani *et al*. 1984). Trypsin activity is affected by the concentration of protein in food and by the starvation period of the shrimp (Muhlia-Almazan *et al*. 2003). Disease outbreak influences the feeding activity, and target animals exhibit less digestive ability.

The main subject of this study is to determine the physiological changes in shrimps correlated with the clinical signs of WSSV disease on the carapace and the condition indexes (RNA/DNA ratio, trypsin activity) of the two shrimp species. Moreover, the synergistic effect of multiple infections by two disease factors is also investigated in terms of shrimp physiology.

MATERIALS AND METHODS

1. Morphological measurement and disease detection

For this study, shrimps cultured in 13 hatcheries around the western and southern coast of Korea were collected from July 5 to 30 in 2006 and August 11 to 17 in 2011. The shrimp were 580 *F. chinensis* (body weight 0.3−12.4 g) and 130 *L. vannamei* (body weight 0.4−10.1 g). After photomicroscopical analysis, the shrimp were divided into 4 stages (stage 0, I, II and III) in terms of clinical signs (size and expanded area in carapace) in Table 1. Shrimp in stage 0 were tested again using PCR methods. Genomic DNA was extracted from two pleopods with a High Pure PCR Template Preparation kit (Roche co., Germany) and was used as a template. WSSV was proven via nested PCR (primer 146F1/146R1 and 146F2/146R2) provided in the OIE Diagnostic Manual (http://www.oie.int) and with the hepatopancreas showing any external symptoms of *Vibrio* sp. infection. The *Vibrio* sp. Infection was tested from a part of the hepatopancreas with culturing in TCBS medium.

2. RNA/DNA content and ratio

After measuring the weight and length, the 1st pleopods were examined to confirm condition of the shrimp, including the RNA/DNA index. The nucleic acid content was fluorometrically determined, as described by Clemmesen (1994) and Belchier *et al*. (2004). The tissue was freeze-dried (−50°C, 24 h), weighed to 5−6 mg, and homogenized

Table 1. Clinical signs and occurrence of white spots on the carapace according to the stage of white spot infection (author's own definition)

White spot stage	Clinical signs
O	No small white spots on the carapace. Diagnosed as non-infected by PCR test
I	Several small white spots on the carapace. Behavior (feeding and swimming) is normal.
II	White spots are embedded on half of the carapace area. The size of white spots is <0.5 mm or spots cover $<50\%$ of the carapace.
III	Presence of white spots 0.5−2.0 mm in size. The whole carapace is covered with white spots.

on ice with Tris-ethylenediaminetetraacetic acid (EDTA) buffer solution (400 μL) with a hand pestle (duration 60 sec). After centrifugation (10 min, 6000 rpm), the nucleic acids were extracted and purified from the homogenate, and a total of 200 μL (divided each 100 μL into microplate wells) was fluorometrically determined using ethidium bromide (EtBr, sigma) dye under excitation wavelengths of 340 nm and emission wavelengths of 595 nm with a fluorescence microplate reader (Fluoroskan Ascent FL; Thermo, Filderstadt, Germany). Then, the RNA was digested with RNase, and the remaining amount of DNA was determined using EtBr.

All data were statistically analyzed using SAS enterprise guide 4.1, USA (ANOVA, Duncan's multiple range test, $p<0.05$).

3. Trypsin analysis

Tryptic enzyme activity was measured in the mixed digestive organs (digestive gland, foregut, and midgut) of the shrimp with 0.2 mM Na-benzoyl-L-arginin-4-methylcoumarinyl-7-amid (Bachem, Bubendorf, Switzerland) and 0.5% dimethylsulfoxide (Merck, Whitehouse Station, NJ, USA) using the method by Ueberschaer (1993). The relative fluorescence was recorded five times every 1 min at excitation and emission wavelengths of 380 and 440 nm, respectively using a fluorescence microplate reader (Fluoroskan Ascent FL; Thermo, Filderstadt, Germany). The resulting tryptic enzyme activity was given as the amount of substrate hydrolyzed per time unit (nmol/mg/min).

RESULTS

1. Morphological parameters

The variation in the body weight to total length of *L. vannamei* and *F. chinensis* is illustrated in Fig. 1. The growth relation formula of *L. vannamei* was $Y=0.2559e^{0.3612x}$ ($r^2=0.849$) and that of *F. chinensis* was $Y=0.1630e^{0.3965x}$ ($r^2=0.668$). In the case of *F. chinensis*, they showed two distinct growth pattern of $Y=0.0937e^{0.4193x}$ ($r^2=0.913$) and $Y=0.0952e^{0.5272x}$ ($r^2=0.936$).

2. RNA/DNA contents and ratio of *F. chinensis*

In the case of non-*Vibrio* infected shrimps, the RNA concentrations for stages I and II were not significantly different. Only at stage III, the RNA concentration of the shrimp decreased significantly to 2.60±0.07 μg/mg. The DNA concentration varied from 1.22±0.02 μg/mg to 1.51±0.02 μg/mg independently from the appearance of white spots. The RNA/DNA ratio showed no statistical difference among stages I, II, and III (2.37±0.05, 2.23±0.04, and 2.40±0.04 μg/mg, respectively). However, the RNA/DNA ratio of the white spot at stage IV according to the clinical signs significantly decreased to 1.77±0.03 (Fig. 2, $p<0.05$). The highest RNA concentration that showed no white spot fir *F. chinensis* within *Vibrio* infected groups was 3.04±0.07 μg/mg (Fig. 3). However, it was not significantly different from the white spot stage I (2.71±0.06 μg/mg), stage II (2.66±0.04 μg/mg), and only stage III showed a

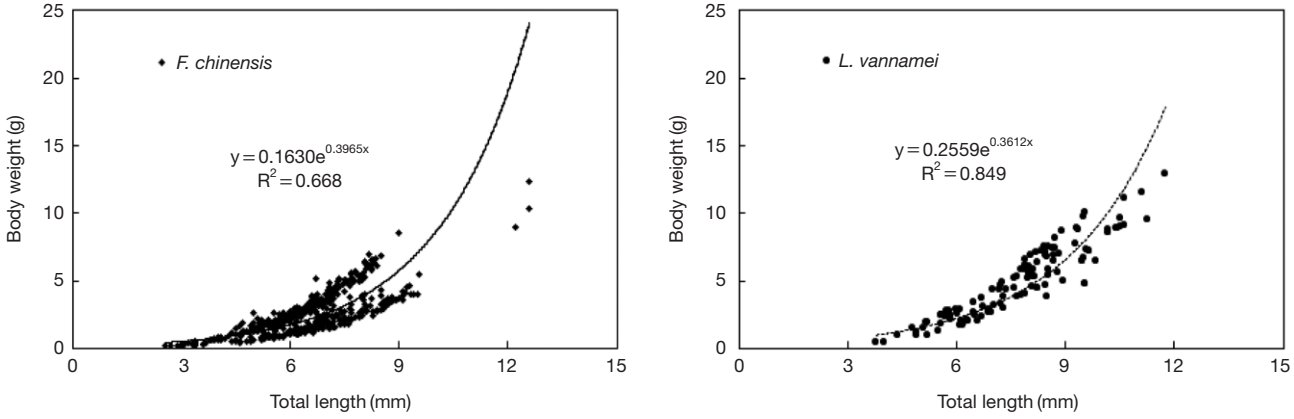

Fig. 1. The relationship between total length and body weight in *F. chinensis* (left) and *L. vannamei* (right).

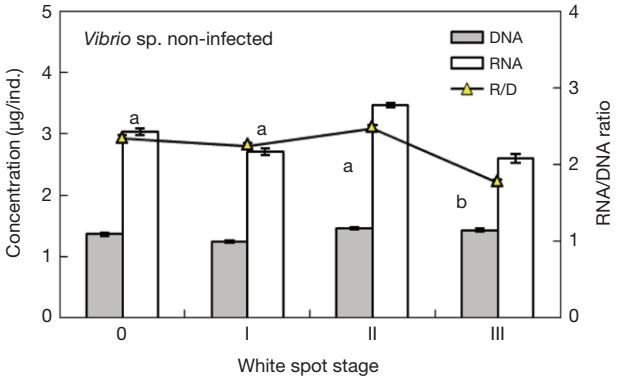

Fig. 2. RNA, DNA concentrations and their ratio depending on the white spot stages in non-infected *F. chinensis* by *Vibrio* sp. Error bar indicates the standard error (S.E.).

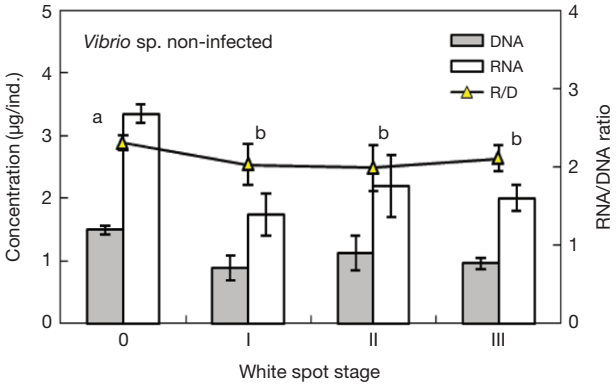

Fig. 4. RNA, DNA concentrations and their ratio depending on the white spot stages in non-infected *L. vannamei* by *Vibrio* sp. Error bar indicates the standard error (S.E.).

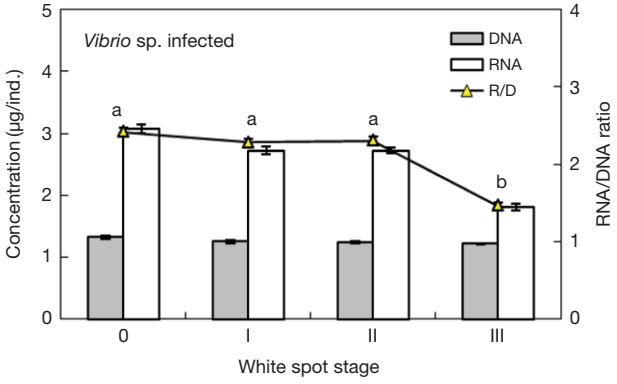

Fig. 3. RNA, DNA concentrations and their ratio depending on the white spot stages in infected *F. chinensis* by *Vibrio* sp. Error bar indicates the standard error (S.E.).

lowest value of RNA concentration of 1.82 ± 0.06 μg/mg. In general, the mean RNA concentration and RNA/DNA ratio decreased with the expansion of white spots, but it showed significant differences only from white spot stages II and III to stage 0 (t-test, $p < 0.05$). The DNA concentration varied from 1.22 ± 0.01 μg/mg to 1.35 ± 0.02 μg/mg without a correlation with the status of the white spots. The RNA/DNA ratio was also recorded as the highest value of 2.34 ± 0.06 in stage 0 and was significantly different with only the ratio of 1.47 ± 0.04 at stage III.

3. RNA/DNA contents and ratio of *L. vannamei*

In the non-*Vibrio* sp. detected group, the mean RNA concentration for white spot stage 0 in the whiteleg shrimp was 3.35 ± 0.15 μg/mg. The shrimp in stage I showed an RNA concentration of 3.00 ± 0.10 μg/mg, stage II of 3.03 ± 0.09 μg/mg and no specimen was observed in stage III. The DNA concentration of the shrimp in stage 0 was 1.50 ± 0.06 μg/mg, in stage I was 1.74 ± 0.23 μg/mg and in stage II was 1.71 ± 0.15 μg/mg. The RNA/DNA ratio of the healthy shrimp (no *Vibrio* and white spot detected stage 0) showed a significantly higher ratio of 2.31 ± 0.10 compared to the white spot stages of I (1.87 ± 0.23) and II (1.82 ± 0.18, $p <$ 0.05) in Fig. 4.

In the *Vibrio* sp. infected group, stage 0 and stage I shrimp showed an RNA concentration of 3.30 ± 0.14 μg/mg, 3.36 ± 0.49 μg/mg, respectively. However, the shrimp in stage II and III showed a significantly lower concentration of 2.25 ± 0.11 μg/mg and 1.52 ± 0.01 μg/mg ($p < 0.05$). The DNA concentration for stage 0, stage I, stage II and stage III were 1.62 ± 0.07 μg/mg, 1.46 ± 0.11 μg/mg, 1.69 ± 0.01 μg/mg, and 1.46 ± 0.01 μg/mg, respectively. The RNA/DNA ratio varied in the same pattern with a change in the RNA concentration at stage 0 (2.15 ± 0.13), stage I (2.45 ± 0.27), stage II (1.34 ± 0.10), and stage III (1.04 ± 0.01), respectively (Fig. 5, $p < 0.05$).

4. Trypsin activity of *F. chinensis*

The mean trypsin activity in hepatopancreas, fore- and mid-gut per mg unit of *F. chinensis* was analyzed in terms of the white spot and *Vibrio* sp. (Fig. 6). Stage 0 group for both *Vibrio* sp. infected or non-infected showed the highest trypsin activity of 1182.8 ± 45.5 nmol/mg/min and 1042.2 ± 60.2 nmol/mg/min. With the expansion of white spots divid-

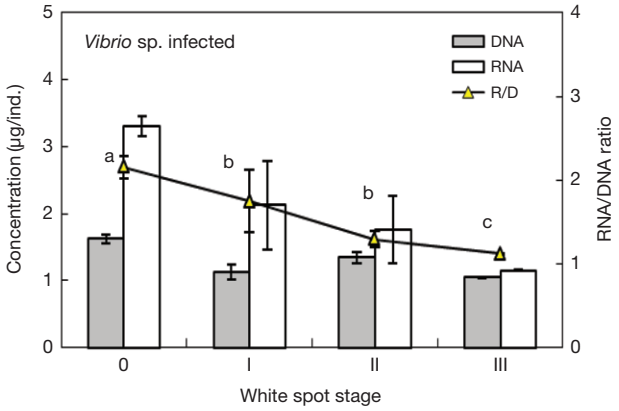

Fig. 5. RNA, DNA concentrations and their ratio depending on the white spot stages in infected *L. vannamei* by *Vibrio* sp. Error bar indicates the standard error (S.E.).

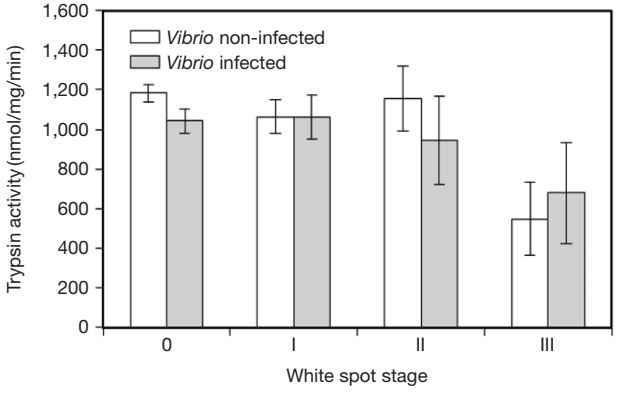

Fig. 6. Trypsin activity in hepatopancreas depending on the white spot stages in infected or non-infected *F. chinensis* by *Vibrio* sp. Error bar indicates the standard error (S.E.).

ed into stage I, stage II and stage III, the trypsin activity decreased to 1064.6 ± 83.5, 1155.9 ± 163.9 and 548.9 ± 182.5 nmol/mg/min in the groups without *Vibrio* infection. In the case of the *Vibrio* sp.-infected groups, the trypsin activity also decreased from 1042.2 ± 60.2 nmol/mg/min (stage 0) to 678.3 ± 257.0 nmol/mg/min (stage III) with the expansion of white spots.

5. Trypsin activity of the *L. vannamei*

The highest trypsin activity of the whiteleg shrimp was shown in stage 0 and that for the group without *Vibrio* sp. infection was 840.3 ± 40.9 nmol/mg/min. In this case, Stage I (688.6 ± 35.0 nmol/mg/min) and II (783.5 ± 128.4 nmol/mg/min) have no statistical difference in trypsin activity,

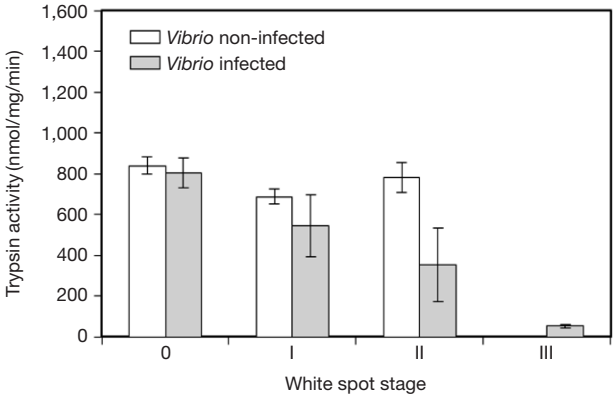

Fig. 7. Trypsin activity in hepatopancreas depending on the white spot stages in infected or non-infected *L. vannamei* by *Vibrio* sp. Error bar indicates the standard error (S.E.).

and the shrimp in stage III. However, the trypsin activity in shrimp infected with *Vibrio* sp. decreased rapidly to 545.6 ± 149.5 nmol/mg/min in stage II and 54.1 ± 8.5 nmol/mg/min in stage III (Fig. 7).

DISCUSSION

In this study, whiteleg shrimp and fleshy prawn showed different growth patterns even though they settled into outdoor ponds at the same time.

The relative weight to length ratio was 38.3 ± 18.2% (mean ± SD) for *F. chinensis* and 56.5 ± 23.0% for *L. vannamei*. The correlation between the length and weight of *F. chinensis* indicated two distinct different strait lines compare to *L. vannamei*, which showed only one pattern. This may be caused by an early infection at a juvenile stage or an imbalance in the culture condition, food, or water quality that strongly depend on the aquaculturist. However, no significant difference in this ratio was evident in the different white spot stages and *Vibrio*-infected or non-infected shrimps. Furthermore, *F. chinensis* shrimp with multiple infections of *Vibrio* sp. and at white spot stage III had the highest condition factor. In the 60-h WSSV inoculation experiment, moribund shrimp showed white spot stage III (Yoganandhan *et al*. 2003) because the white spots appear relatively soon after infection, but the condition factor does not change for some days thereafter.

The nucleic acid ratio (RNA/DNA) in shrimp was used

to interpret the health and growth condition of *L. vannamei* (Moss 1994). In this study, except for an acute clinical sign of stage III, the RNA concentrations of *F. chinensis* in stages I and II were not affected by the status of *Vibrio* sp. and were kept at a concentration between 2.6−3.4 μg/mg. The only statistical difference in the RNA concentration was recorded in multiple infections with *Vibrio* sp. in stage III to other stages. This means that early signals of the white spots cannot be used to interpret the physiological health of the shrimp. The RNA and RNA/DNA ratio decreased only when there were acute symptoms of white spot in the entire carapace (ANOVA, $p < 0.05$). However, the RNA/DNA ratio of *L. vannamei* significantly decreased ($p < 0.05$) in the WSSV infection stage I in both cases with and without *Vibrio* infection. With multiple infections at stage III, a statistical difference was shown compared to all other groups. The DNA concentration varied more in *L. vannamei* than in *F. chinensis*. However, there is not statistical difference between all stages of infection, which is supported by previous research showing that the DNA concentration in marine animals remained relatively stable under different extreme environmental conditions (Clemmesen *et al*. 1993; Moss 1994).

The mass mortality of the shrimp occurred not only with infection by WSSV but also due to interactions with the environment, namely when reaching a critical condition due to deterioration in water quality, stress-causing circumstances such as food depletion, a sudden change in temperature, osmoregulation, salinity, etc. (Lightner and Redman 1998; Briggs *et al*. 2004). The water quality of this study was suitable in terms of ammonia, nitrite, nitrate, temperature, and dissolved oxygen for shrimp aquaculture. *F. chinensis* and *L. vannamei* in this study showed no mass mortality and gradational death in outdoor ponds, even when their carapace was covered overall with white spots. Trypsin activities showed the same tendencies with variations in nucleic acid. Trypsin activity in the only WSSV infected groups showed no rapid decrease until stage II in both flesh prawn and whiteleg shrimps. However, the enzyme activity in the shrimp with multiple infections of *Vibrio* sp. drastically decreased. Therefore, *Vibrio* sp. had a more serious influence on digestion. The decrease in the RNA and RNA/DNA ratio in whiteleg shrimps appeared earlier than for fleshy prawn. No *L. vannamei* sample, *Vibrio* sp. non-infected and white spot stage III, were found during the experimental period. The effect of multiple infections by WSSV and *Vibrio* sp. was clear and significant in both fleshy prawn and whiteleg shrimps ($p < 0.05$). For these reasons, an early sign of white spots on the carapaces does not immediately indicate a decrease in the physiological conditions of the shrimp and cannot be used as an indicator to evaluate the health of the shrimp. Only when shrimps are covered entirely over their carapace with white spots, their physiological activity or condition will drastically decrease.

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