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Effects of Dietary Nucleotides on the Immune Response and Growth of Juvenile Pacific White Shrimp *Litopenaeus vannamei* (Boone, 1931)

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Abstract

Effects of dietary supplementation of nucleotides on immune response, survival against white spot syndrome virus (WSSV) challenge infection and growth of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) juveniles were evaluated. The basal diet for Pacific white shrimp supplemented with nucleotide formula (Vannagen; Chemoforma, Augst, Switzerland) at 0%, 0.2%, 0.4% and 0.6% kg^{-1} of feed were tested. Survival of shrimp was in the range of 92-97% and was not significantly different among treatments. However, specific growth rate (SGR), feed conversion efficiency (FCE) and protein productive value (PPV) were significantly better in shrimps fed the nucleotide-supplemented diet than those fed the control diet after a 60-day feeding trial. Survival of test animals upon challenge with WSSV infection was significantly higher in the nucleotide group than in the control group. Immune response indices i.e. total haemocyte count (THC), respiratory burst activity and phenoloxidase activity were significantly enhanced in shrimp fed diets containing nucleotides as compared to those fed diets with no supplementation. At the levels tested, 0.2% nucleotide in the diet was the optimum. These results indicate that dietary supplementation of nucleotides resulted in beneficial effects in improving growth, feed utilisation, protein utilisation and accelerate shrimp immune response against WSSV infection.

Introduction

Aquaculture continues to be the fastest growing animal food-producing sector and is making an important contribution to shellfish production which is a high value activity worldwide. Shrimp farming for instance constitutes an important source of revenue and employment in many developing countries. However, infectious diseases have affected the profitability of the shrimp industry (Rodriguez and Le Moullac, 2000).

The white spot syndrome virus (WSSV), one of the most devastating viral pathogen, has already become established in the local marine environment and in wild populations of shrimps in the Philippines (de la Peña et al. 2007). This virus has emerged as the most serious threat to commercial shrimp farming. Traditional control strategies for the disease are being employed such as the use of antibiotics and chemical disinfectants. These are no longer recommended due to the emergence of pathogen-resistance and to the growing concerns over environmental impact and wildlife protection (Rocha-Montero et al. 2006). In keeping with developments in aquaculture, shrimp immunology has become a key element in establishing strategies for the

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control of diseases in shrimp aquaculture (Bachere, 2000). Shrimp farmers have begun to examine the potential use of immunostimulants as a more environmental friendly approach to disease management (Raa, 1996; Smith et al. 2003).

For years, nucleotides were not considered essential nutrients for use in any dietary programmes. It was thought that all organisms can supply sufficient amounts of nucleotides to meet their physiological demands via *de novo* synthesis and a so- called "salvage pathway", which means recycling of nucleotides from dead cells (Hoffmann, 2007). For a healthy animal or human, the constant supply of nucleotides is very well regulated and maintained in response to occasional stress. During times of extraordinary stress, such as rapid growth, reproduction, environmental change, combating disease and recovery from injury, trillions of additional nucleotides must be readily available for cell proliferation (Hoffmann, 2008). It has been proposed that because the *de novo* synthesis and salvage of nucleotides is a metabolically costly process, an additional source of exogenous nucleotides in the diet may optimise the functions of rapidly dividing tissues, particularly when growth is rapid and during stress or health challenges (Burrells et al. 2001a).

The present study aims to examine the effects of dietary administration of nucleotides to the immune response of juvenile Pacific white shrimp (*Litopenaeus. vannamei* (Boone, 1931), Penaeidae) and to the shrimp's resistance against WSSV. Additionally, it aims to test the effects of nucleotides on the growth and survival of *L. vannamei*.

Materials and Methods

Experimental animals and acclimation period

Healthy, *L. vannamei* post larvae (PL-25) were obtained from Jamandre Hatcheries, Inc, maintained and reared indoor in 1 ton-capacity fibreglass tanks at the Brackish Water Aquaculture Center, University of the Philippines Visayas, Leganes, Iloilo. The shrimps were acclimated for 3 weeks, continuous aeration was provided and shrimps were maintained with formulated shrimp diet (control diet, without nucleotides) (Table 1) before the growth trial (final average body weight (ABW) of 0.2-0.3 g). Prior to the experiment, the shrimps were randomly selected and screened by polymerase chain reaction (PCR) for WSSV infection. PCR assays were conducted at Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC-AQD), Tigbauan, Iloilo, Philippines.

Table 1. Composition of Test Diets

Ingredients	**0% Nucleotide (Control)**	**0.2% Nucleotide**	**0.4%Nucleotide**	**0.6%Nucleotide**
Tuna Fish Meal	46.00	46.00	46.00	46.00
Squid Meal	8.00	8.00	8.00	8.00
Soybean Meal	21.00	21.00	21.00	21.00
Cellulose	5.00	4.80	4.60	4.40
Vit. and Min. Mix[a]	1.00	1.00	1.00	1.00
BHT (Butylated Hydroxy Toluene)	0.02	0.02	0.02	0.02
Lecithin	0.50	0.50	0.50	0.50
Danish Fish Oil	4.00	4.00	4.00	4.00
Starch	15.00	15.00	15.00	15.00
Nucleotides	0.00	0.20	0.40	0.60
TOTAL	**100.00**	**100.00**	**100.00**	**100.00**

[a] Vitamin-mineral mix (g· 100 g^{-1} diet): 4,000,000 IU Vitamin A; 300,000 IU Vitamin D2; 1,000 IU Vitamin E; 0.04 Vitamin B1; 0.12 Vitamin B2; 0.12 Vitamin B3; 2.50 Vitamin C; 0.06 Folic Acid; 0.60 Niacin; 1.00 Calcium Pantothenate; 2.00 Biotin; 1.00 Choline Chloride; 1.20 Iron; 0.12 Copper; 0.04 Iodine; 0.50 Manganese; 0.60 Zinc; 0.002 Cobalt; 0.002 Selenium

Growth trial: rearing conditions

Water quality was maintained by chlorinating the culture water with 100 ppm of sodium hypochlorite and dechlorinated by vigorous aeration for 3 days. Water was recirculated from the reservoir to the aquaria with biological and mechanical filters (like fibre fill, sand, pebbles and charcoal). Water quality parameters viz., salinity (25-27 $^{0}/_{00}$), temperature (24-28 °C), pH (7.8-8.0) and dissolved oxygen (6.6 ppm) were recorded daily, and NH_3–N and NO_2–N weekly, and maintained at optimal levels. Each experimental unit was provided with adequate aeration; 40% water change was done in each aquarium daily and 100% water change was done in the reservoir every 5-7 days to maintain good water quality. During the experiment biosecurity measures were cautiously followed.

Growth trial: experimental design

Following acclimation, shrimps were randomly divided into 16 aquaria of 80 L capacity with 15 shrimps to each aquarium at a density of 1 individual·L^{-1}. These constituted three dietary treatments and a control, all in four replicates, in a completely randomised design.

Ingredients of the basal ration were purchased and prepared at SEAFDEC-AQD following the formulation of Li et al. (2007) with modification which contained 42.3% crude protein, 7.9% crude fat, 15.3% ash and 4% moisture. The formulated basal diet (Table 1) for Pacific white shrimp was supplemented with nucleotide formula (Vannagen; Chemoforma, Augst, Switzerland) at 0%, 0.2%, 0.4% and 0.6% kg^{-1} of feed. The product used had 44% purity based on UV-spectrophotometric analysis. Each diet was fed to the shrimps of each treatment group at 10% body weight four times daily at 8:00, 12:00, 16:00 and 20:00 hr for 60 days. Shrimps were weighed every 10 days and the daily feed allocation was adjusted accordingly. Growth was measured as specific growth rate (SGR), feed conversion efficiency (FCE) and protein productive value (PPV). In addition, survival during the growth trial was determined.

Immune response and disease resistance trial

After the 60-day growth trial the immune response and disease resistance trial followed. Same batch of shrimps obtained from Jamandre Hatcheries, Inc. were used except that the shrimps were reared and maintained under laboratory conditions until ABW of 4-6 g was reached and were used in this experiment. The rearing conditions and experimental design were the same as that of the growth trial except that the shrimps were fed to satiation only up to 14 days with 0%, 0.2%, 0.4% and 0.6% dietary nucleotides. Shrimps were then subjected to WSSV infection challenge and immune analysis.

WSSV infection challenge

The test animals (30 shrimps·$treatment^{-1}$) in the WSSV infection challenge were placed in 30 L plastic containers that were situated inside the challenge room. The set-up was a static water system but adequate aeration was provided. White spot syndrome virus infected shrimp was obtained from SEAFDEC-AQD and was activated through passage by feeding the infected tissues (shrimp flesh) to a batch of uninfected shrimps; transmittance of the virus was done three times. The test animals were then challenged through oral administration i.e., by feeding infected tissue at the rate of 1 g·$shrimp^{-1}$ (Joseph and Philip, 2007). Infected shrimp tissues were minced thoroughly and were fed at least four times at 8:00, 12:00, 16:00 and 20:00 hr in 1 day to make sure that all test animals were infected. A blank control (unchallenged control) was also carried out. Survival was monitored daily; the dead animals were removed promptly. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace as well as PCR assay. Using a primer for WSSV, electrophoresis of the PCR products revealed if it is positive or negative with WSSV. If it is positive heavy bands could be seen, absence of WSSV in shrimps did not show bands at all.

Extraction of haemolymph

Anticoagulant for haemolymph extraction was prepared by adding 10 mM EDTA-Na_2 salt to a salt solution (450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3, 850 $mOsm \cdot kg^{-1}$, (Hernandez-Lopez et al. 1996). Haemolymph was collected from the base of the pleopod at the first abdominal segment near the genital pore, using a 1 mL syringe with 26 gauge hypodermic needle rinsed thoroughly with pre-cooled anticoagulant. Haemolymph was collected from shrimps in each treatment for total haemocyte count (THC), superoxide anion assay and phenoloxidase assay.

Immune assays

Total haemocyte count

A drop of anticoagulant-haemolymph mixture was placed on a Neubaeur's haemocytometer. Haemocytes were counted and the values expressed as $THC \cdot mL^{-1}$ haemolymph.

Superoxide anion (NBT reduction) assay

Respiratory burst activity of haemocytes was quantified using reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production. The assay was conducted as described by Muñoz et al. (2000) with modification. One hundred microlitre of the sample was placed in each well of a microtitre plate and was incubated at room temperature for 2 hr. The supernatant was discarded and was replaced with 50 μL MHBSS (Modified Hank's Balanced Salt Solution) medium. One hundred microlitre NBT-PMA (Nitroblue tetrazolium-phorbol myristate acetate) solution was added to each well and was incubated for 30 min. Supernatants were removed and the haemocytes were fixed by adding 200 μL absolute methanol for 10 min and washed twice with 70% methanol, then dried. The formazan deposits were solubilised by addition of 120 μL of 2M KOH and 140 μL of DMSO (dimethyl sufoxide) in each well. Intensity of the turquoise blue colour was measured at 620 nm in a microplate reader and the activity expressed as optical density 100 μL^{-1} haemolymph. Blank control reactions were performed using 120 μL of KOH and 140 μL of DMSO.

Phenoloxidase activity assay

Phenoloxidase (PO) activity was assayed spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA), according to the method described by Hernandez-Lopez et al. (1996) with modification. Anticoagulant-free haemolymph was placed in sterile 1.5 mL microcentrifuge tubes. The haemolymph was subjected to a freeze-thaw cycle five times to induce cell lysis and degranulation. Samples were vortexed, centrifuged at 13,000 x *g* for 15 min at 4 °C and the haemocyte supernatant collected. For PO measurement, 25 μL haemocyte supernatant was placed in 96-well microtitre plates and incubated for 30 min with 25 μL of 0.1% trypsin in SSS (Shrimp Salt Solution). Then 25 μL of 0.3% L- DOPA (L-3, 4-dihydroxyphenylalanine) was added and incubated for 10 min. Optical density was measured

at 490 nm using a microplate reader. Enzyme activity was expressed as the change in absorbance $min^{-1}.100\ \mu L^{-1}$ haemolymph (Joseph and Philip, 2007). One enzyme activity was equivalent to the increase of 0.001 in absorbance.

Statistical analysis

Statistical analyses were carried out using the software SPSS 16.0. Data obtained from the growth trial, survival rate following a WSSV challenge test and immune assays were analysed by one way analysis (ANOVA) and Duncan's multiple comparisons of means. All probability values were set at significance level of 0.05.

Results

Growth and Survival

Gain in biomass of shrimps fed with nucleotide-supplemented diet was significantly higher than those fed the control diet (Fig. 1). The survival of shrimp was in the range of 92-97% and was not significantly different among treatments (Fig. 2).

Feed Conversion Efficiency

During the 60-day feeding trial, the nucleotide-supplemented diets were substantially better utilised by the test animals. There was a significant difference in the feed conversion efficiency (FCE) between the control group and the nucleotide fed group (Fig. 3).

Protein Productive Value

Protein deposition rates were significantly higher in shrimps fed the nucleotide-supplemented diets than those fed the control diet (Fig. 4).

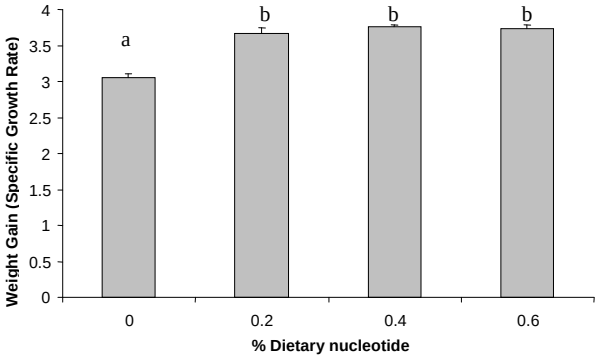


Fig 1. Weight gain (Specific Growth Rate) of *L. vannamei*.

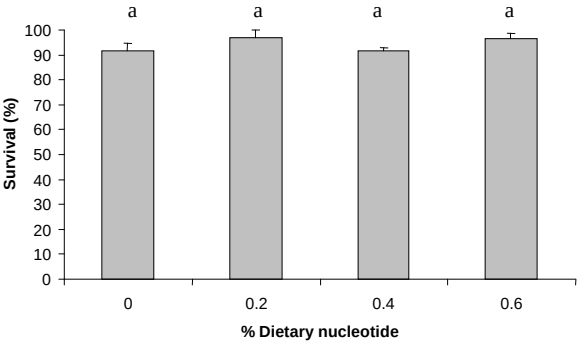


Fig 2. Survival (%) of shrimp *L. vannamei*.

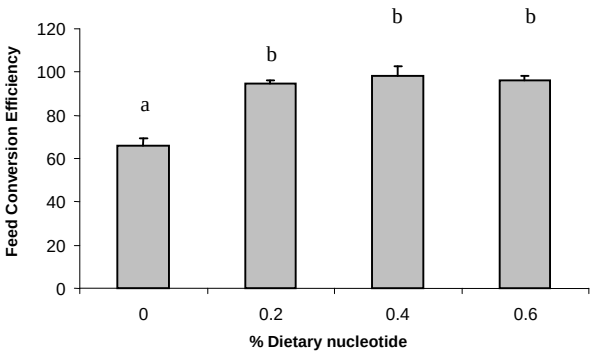


Fig 3. Feed Conversion Efficiency of *L. vannamei*.

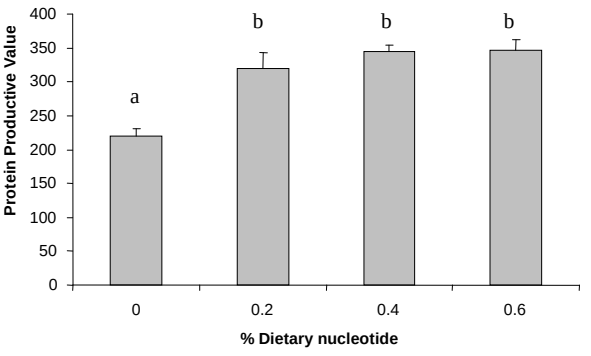


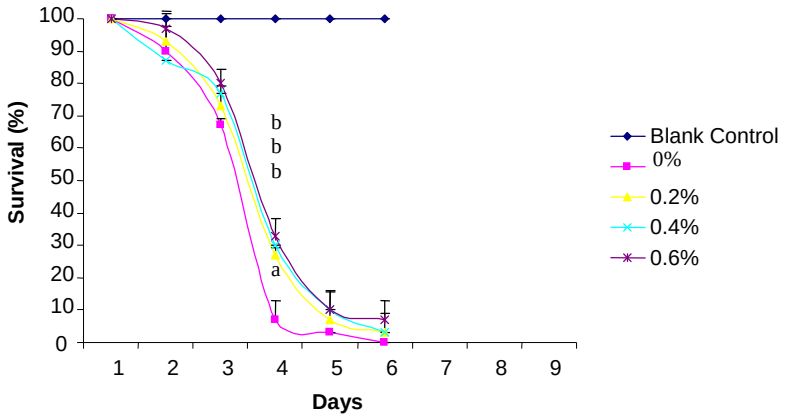
Fig 4. Protein Productive Value of *L. vannamei*.

WSSV challenge

The survival of shrimps fed the control and test diets are shown in Fig. 5. The unchallenged shrimps (blank control) showed 100% survival. Percentage survival rates of *L. vannamei* fed nucleotide-supplemented diets were significantly higher during the 4th day of post challenge than that of the shrimps fed the control diet. The WSSV infection challenge lasted for 9 days. Least survival rate was observed in shrimps fed diet devoid of nucleotide which succumbed to death (100%) within the 6th day of challenge. First step amplification (982 bp product) and nested PCR (570 bp product) diagnosis revealed that all mortalities were caused by WSSV infection (Fig. 6).

Total haemocyte count (THC)

Significantly lower THC was observed in shrimps fed the control diet (Fig. 7). Shrimps fed 0.6% nucleotide exhibited the highest count. However, there was no significant difference in the THCs of shrimps fed the nucleotide-supplemented diets.



	Day 1	Day 2	Day 3	Day 4
Blank Control	**100**	**100**	**100**	**100**
Control Diet (0% Nucleotide)	**100**	**90**	**67**	**7**
0.2% Nucleotide	**100**	**93**	**73**	**27**
0.4% Nucleotide	**100**	**87**	**77**	**30**
0.6% Nucleotide	**100**	**97**	**80**	**33**

Fig 5. Survival (%) of shrimp *L. vannamei* fed diets containing different concentrations of nucleotides after challenged with WSSV.

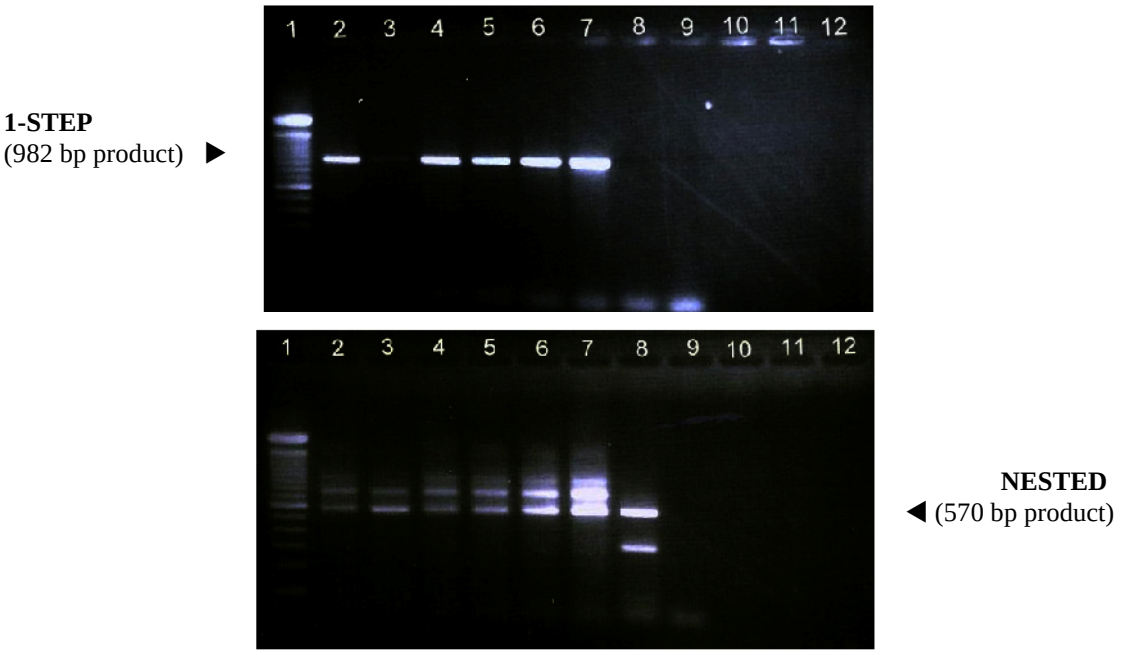


Fig 6. Agarose gel electrophoresis of PCR amplification products of WSSV. Lanes (1) DNA marker; (2) positive control; (3) pooled gill samples from 0% treatment; (4) pooled gill samples from 0.2% treatment;(5) pooled gill samples from 0.4% treatment; (6) pooled gill samples from 0.6% treatment; (9) negative control.

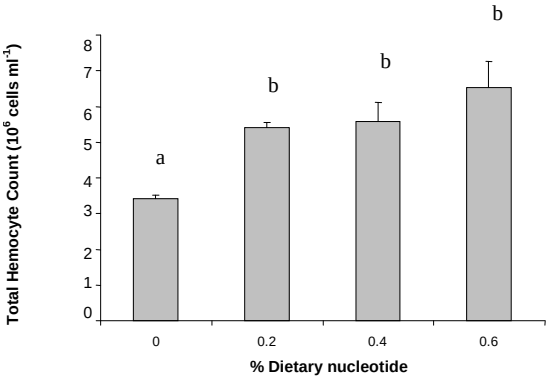


Fig. 7. Total Haemocyte Count of *L. vannamei*.

Superoxide anion assay

Respiratory burst responses of shrimps fed the nucleotide-supplemented diets were significantly higher than those fed the control diet (Fig. 8). However, the nucleotide fed groups were not significantly different from each other.

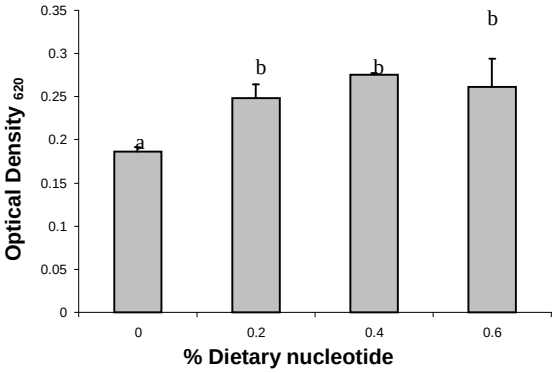


Fig. 8. Respiratory burst activity of *L. vannamei*.

Phenoloxidase activity assay

Shrimps fed nucleotide diet containing 0.6% and 0.4% nucleotides showed a PO concentration significantly higher than that of the nucleotide-free group (Fig. 9).

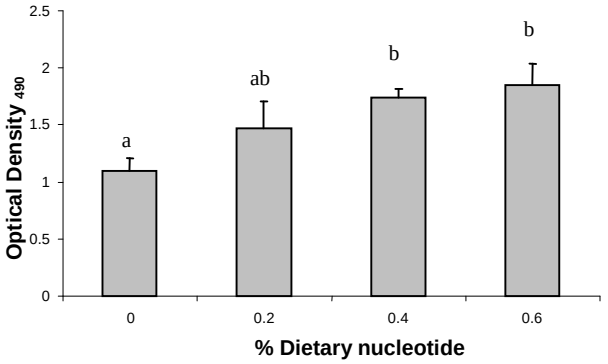


Fig 9. Phenoloxidase (PO) values of *L. vannamei*.

Discussion

Dietary administration of nucleotides to juvenile Pacific white shrimp *L. vannamei* enhanced their growth, immune response and survival against WSSV in the present study. Enhancements in weight gain with dietary administration of nucleotides have been reported with both Pacific white shrimp (Li et al. 2007) and *Penaeus monodon* Fabricius 1798 (Hertrampf and Mishra, 2006). Dietary nucleotides have been reported to enhance growth of several fish species

including Atlantic salmon *Salmon salar* Linnaeus, 1758 (Burrells et al. 2001a), largemouth bass *Micropterus salmoides* (Lacepède, 1802) (Kubitza et al. 1997) and rainbow trout *Oncorhyncus mykiss* (Walbaum, 1792) (Adámek et al. 1996).

Though, most cell types can synthesise nucleotides from purines and pyrimidines, the *de novo* synthesis and salvage of nucleotides is a metabolically costly process. Additional source of exogenous nucleotides in the diet may optimise the functions of rapidly dividing tissues, particularly when growth is rapid (Burrels et al. 2001a). The present study showed that nucleotides at 0.6%, 0.4% and 0.2% resulted in significantly better SGR, FCE and PPV than that of the control with 0.2% being the optimum after a 60-day growth trial. The inclusion of nucleotide in the diet in the present study likely supported high rate of cell replication during shrimp growth.

Survival after challenge with certain pathogens is usually considered as a measure of disease resistance. In the present study, challenge test clearly showed that nucleotide-supplemented diets significantly enhanced protection and survival of juvenile shrimp against WSSV infection as compared with shrimps fed diets devoid of nucleotides. Factors in the present study such as the virulence of WSSV infected tissue used, the number of individuals tested, the water system and volume of container used, might have affected the percentage survival of the nucleotide-treated shrimps during post challenge infection. In the present study, a static water system and smaller volume of experimental containers were used, a factor that might have contributed to the rapid progress of infection including that of the nucleotide-supplemented group since susceptibility of shrimps to disease could be greatly affected by lower dissolved oxygen levels. In addition, it has been established that gills, stomach, cuticular epithelium, haematopoietic tissue and lymphoid organ are major target organs of WSSV replication (Phuoc et al. 2008). Stress in relation to lower dissolved oxygen levels might render the shrimp more susceptible to the viral infection. Shrimps fed diet devoid of nucleotide in this study succumbed to death (100%) within the 6th day of challenge as compared to nucleotide-treated diets where the survival was extended until the ninth day. Despite this, the study still demonstrated that the nucleotide-supplemented diets significantly enhanced protection and slightly prolonged survival of shrimps against WSSV.

Dietary supplementation of nucleotides has shown positive effects on immune responses and disease resistance of carp *Cyprinus carpio* Linnaeus, 1758 against *Aeromonas hydrophila* (Sakai et al. 2001), *O. mykiss* against *Vibrio anguillarium*, *S. salar* against infectious salmon anaemia (ISA) virus, coho salmon *Oncorhynchus kisutch* (Walbaum, 1792) against *Piscirickettsia salmonis* and Atlantic salmon against sea lice *Lepeophtheirus salmonis* (Krøyer, 1837) (Burrells et al. 2001b). Most studies focus on farmed fish, information is very limited for economically important marine invertebrates, especially shrimps, which represent one of the most profitable aquaculture enterprises (Li et al. 2007). The present study revealed that the administration of dietary nucleotides significantly increased survival of juvenile Pacific white shrimp against WSSV infection as supported by an elevated immune response i.e. PO activity, respiratory burst activity and THC.

The protective effects of dietary nucleotides in the present study were likely due to the accelerated response of the immune system because the proliferation of cells involved could have been facilitated. Immune indices such as total haemocyte count, respiratory burst activity and PO activity were used as indicators of the protective effects of exogenous nucleotides in the present study. Elevated immune responses including phagocytic, respiratory burst, serum complement and lysozyme activities have been reported in carp (Sakai et al. 2001), higher stimulation indices of both "B" and "T" lymphocytes in rainbow trout (Leonardi et al. 2003) and specific antibody production in Atlantic salmon (Burrells et al. 2001a).

Enhanced THC and respiratory burst activity observed in shrimp fed diets supplemented with nucleotides in the present study were comparable to results reported earlier for Pacific white shrimp fed β-glucan and nucleotides (Murthy et al. 2009). In addition, PO activity was also significantly increased in shrimp fed with nucleotides as compared to those with no supplementation.

Conclusion

In summary, the present study showed that among the levels of nucleotide tested, 0.2% incorporation in the diet was the optimum. Nonetheless, all concentrations of nucleotide-supplemented diet significantly increased survival of juvenile Pacific white shrimp upon challenge with WSSV infection. Immunological indices such as THC, respiratory burst activity and PO activity were all enhanced in shrimp fed the nucleotide-supplemented diet. Moreover, specific growth rate, feed conversion efficiency and shrimp protein increment were shown to be significantly improved in the nucleotide-fed group. The results indicate that nucleotides had the capacity to improve growth, feed utilisation, protein utilisation and accelerate shrimp immune response of *L. vannamei* against WSSV.

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References

Adamek, Z., J. Hemackova, J. Kouril, R. Vachta and I. Stibranyiova. 1996. Effect of Ascogen probiotics supplementation on farming success in rainbow trout (*Onchorhyncus mykiss*) and wells (*Silurus glais*) under conditions of intensive culture. Krmiva (Zagreb) 38, 11-20. Cited by Li, P. and D.M. III Gatlin. 2006. Nucleotide nutrition in fish: Current knowledge and future applications. Aquaculture 251:141- 152.

Bachere, E. 2000. Shrimp immunity and disease control. Aquaculture 191:3-11.

Burrells, C., P.D. Williams and P.F. Forno. 2001a. Dietary nucleotides: a novel supplement in fish feeds 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon *(Salmo salar* L.). Aquaculture 199:171- 184.

Burrells, C., P.D. Williams and P.F. Forno. 2001b. Dietary nucleotides: a novel supplement in fish feeds 1. Effects on resistance to disease in salmonids. Aquaculture 199:159- 169.

de la Peña, L.D., C.R. Lavilla-Pitogo, C.R. Villar, M.G. Paner, C.D. Sombito and G.C. Capulos. 2007. Prevalence of white spot syndrome virus (WSSV) in wild shrimp *Penaeus monodon* in the Philippines. Diseases of Aquatic Organisms 77:175-179.

Hernandez-Lopez, J., T. Gollas-Galvan and F. Vargas-Albores. 1996. Activation of the Prophenoloxidase system of the Brown Shrimp (*Penaeus californiensis* Holmes). Comparative Biochemistry and Physiology 113C: 61-66.

Hertrampf, J.W. and S.K. Mishra. 2006. Benefits of nucleotides on shrimp farming. Feed Tech 10 (9):27-30.

Hoffmann, K. 2007. The use of nucleotides in animal feed. Feed Mix 15 (4):25-27.

Hoffmann, K. 2008. Nucleotides-essential nutrients if fish farming. INFOFISH International *JUNE ISSUE* 8-11.

Joseph, A. and R. Philip. 2007. Acute salinity stress alters the haemolymph metabolic profile of *Penaeus monodon* and reduces immunocompetence to white spot syndrome virus infection. Aquaculture 272:87-97.

Kubitza, F., L.L. Lovshin and R.T. Lovell. 1997. Identification of feed enhancers for juvenile largemouth bass *Micropterus salmoides*. Aquaculture 148:191-200.

Leonardi, M., A.M. Sandino and A. Klempau. 2003. Effect of a nucleotide enriched diet on the immune system, plasma cortisol levels and resistance to infectious pancreatic necrosis (IPN) in juvenile rainbow trout (*Oncorhynchus mykiss*). Bulletin of the European Association of Fish Pathologists 23:52–59.

Li, P., A.L. Lawrence, F.L. Castille and D.M. Gatlin III. 2007. Preliminary evaluation of a purified nucleotide mixture as a dietary supplement for Pacific white shrimp *Litopenaeus vannamei* (Boone). Aquaculture Research 38:887-890.

Muñoz, M., R. Cedeño, J. Rodriguez, W.P.W. van der Knaap, E. Mialhe and E. Bachere. 2000. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp *Penaeus vannamei*. Aquaculture 191:89-107.

Murthy, H.S., P. Li, A.L. Lawrence and D.M. Gatlin III. 2009. Dietary β-glucan and nucleotide effects on growth and immune responses of Pacific white shrimp *Litopenaeus vannamei*. Journal of Applied Aquaculture 21:160-168.

Phuoc, L.H., M. Corteel, H.J. Nauwynck, M.B. Pensaert, V. Alday-Sanz, W. Van den Broeck, P. Sorgeloos and P. Bossier. 2008. Increased susceptibility of white spot syndrome virus-infected *Litopenaeus vannamei* to *Vibrio campbellii.* Environmental Microbiology 10:2718-2727.

Raa, J. 1996. The use of immunostimulatory substances in fish and shellfish farming. Reviews in Fisheries Science 4:229-288.

Rocha-Montero, A., D. McIntosh, R. Sanchez-Merino and I. Flores. 2006. Immunostimulation of white shrimp (*Litopenaeus vannamei*) following dietary administration of Ergosan. Journal of Invertebrate Pathology 91:188-194.

Rodriguez, J. and G. Le Moullac. 2000. State of the art of immunological tools and health control of penaeid shrimp. Aquaculture 191:109-119.

Sakai, M., K. Taniguchi, K. Mamoto, H. Ogawa and M. Tabata. 2001. Immunostimulant effects of nucleotide isolated from yeast RNA on carp, *Cyprinus carpio* L. *Journal of Fish Diseases 24: 433-438.*

Smith, V.J., J.H. Brown and C. Hauton. 2003. Immunostimulation in crustaceans: does it really protect against infection? Fish and Shellfish Immunology 15:71-90.

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