Benfotiamine counteracts the negative effects of a high dietary carbohydrate on growth and ammonia toxicity resistance in post larval *Penaeus monodon*

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**Key words:** thiamine utilization, dietary carbohydrate, *Penaeus monodon*, ammonia challenge

**Abstract**

The present study aimed to evaluate the effects of high carbohydrate diet in the absence of presence of dietary benfotiamine on growth and immune response against ammonia toxicity (50 mg NH₄Cl L⁻¹, average ambient temperature=27°C, pH 8.3) of post larval *Penaeus monodon*. A feeding trial involving three dietary treatments followed by an ammonia toxicity tests and a feed attractability test were conducted separately. During the feeding trial, shrimps were fed three experimental diets, namely, (1) a diet containing 15% starch (C), (2) a diet with 25% corn starch (high carbohydrate diet, HC) and (3) a diet containing both 25% starch supplemented with 0.2 g kg⁻¹ benfotiamine (HCB). At the termination of the feeding trial, the shrimps were subjected to ammonia toxicity test to determine the effects of the diets on the immune response of the post larval shrimps. Attractability tests using customized aquaria showed that the HCB diet elicited significantly the highest attractability even after 10 min of feed placement in the feeding chamber. In the feeding trial that lasted for 60 days, results showed that shrimps fed with diet containing HCB exhibited significantly the highest growth rate (*p*<0.05) among all groups of shrimps in terms of final average body weight (FABW), weight gain (WG), and specific growth rate (SGR); they also exhibited significantly the highest feed intake (*p*<0.05). Upon challenge of the shrimps with 50 mg L⁻¹ ammonia, shrimps fed with the HCB diet exhibited significantly the highest cumulative survival rate of 60% after 72 h while those fed with diet C and HC exhibited lower survival rates of 33% and 40%, respectively, and were not significantly different from each other. In conclusion, benfotiamine enhances attractability of the feed, growth performance and immune response against ammonia toxicity at the level of 0.2 g kg⁻¹.

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Introduction

In aquaculture, carbohydrates are the most economical dietary energy source due to their abundance, ready availability and relatively low cost. However, as an energy source, it cannot be fully and efficiently used by aquatic animals compared with poultry and mammals (Wilson and Poe, 1987). High dietary carbohydrate can cause decreased growth rate and other negative effects (Shiau et al., 1991b). Crustaceans have a limited ability to utilize carbohydrate and cannot adapt to high levels of dietary carbohydrate intake (Wang et al., 2016). Consumption of simple sugars in crustaceans can cause negative physiological effect since glucose has a high absorption rate across the digestive tract leading to prolonged hyperglycemia (Shiau et al., 1991; Rosas et al., 2001).

Regardless of the carbohydrate source, it is generally accepted that the carbohydrate requirement for crustaceans is 20–30% of the total diet, and increased carbohydrate intake can lead to slow growth, low immunity, and a high mortality rate (Wang et al., 2016). Shrimps are diabetic-like animals with high post prandial hemolymph levels of glucose even hours after consumption of a meal containing carbohydrates (Shiau et al., 1991; Rosas et al., 2001). This implies that shrimp needs an energy source, a special requirement linked to the build-up of the new exoskeleton after each molt, or a difficulty to handle the large load that the hemolymph glucose brings (Niu et al., 2012).

Thiamine (vitamin B1) plays a key role in the process of converting carbohydrates into energy (Balakumar et al. 2010). Benfotiamine (pro-vitamin B1) is a lipid-soluble thiamine derivative having much higher bioavailability in human than thiamine (Xie et al., 2014). It is commonly used for the treatment of type II diabetes to improve glucose homeostasis by enhancing glycolysis (Portari et al., 2013) as well as removing excess glycolytic intermediates and reducing hyperglycemic damage (Hammes et al., 2013). In mammals, pro-vitamin B1 stimulates glucose uptake by enhancing the glycolytic and glycogenetic capabilities, increasing mitochondrial glucose oxidation, promoting insulin synthesis, enhancing insulin-stimulated glucose disposal, and increasing the activity of GLUT-2, a glucose transporter (Seh-Hoon et al. 2009; Chung et al. 2014; Fraser et al. 2012). In aquatic animals, information regarding the beneficial effects of benfotiamine is scarce.

Studies on the effects of benfotiamine on the growth performance of shrimps have not been conducted so far. In fish such as the blunt snout bream Megalobrama amblycephala (Xu et al. 2017), it has been demonstrated that the fish fed a high carbohydrate diet supplemented with benfotiamine exhibited higher protein efficiency ratio, nitrogen efficiency ratio and energy retention efficiency. In addition, fish showed relatively high values of whole-body lipid contents, tissue glycogen and lipid contents. In our laboratory, the Nile tilapia fed high carbohydrates diet supplemented with benfotiamine showed significantly better growth than those fish fed high carbohydrate diet without benfotiamine (Lauzon et al. 2019). The present study aimed to evaluate the effects of high carbohydrate diet alone or supplemented with benfotiamine on the growth performance and immune response of the black tiger shrimp.

In addition, we also aimed to determine whether or not it is also an immune-enhancing feed additive. Ammonia and ammonium ion are two of the toxic components in the water medium in aquaculture media since these are produced by the shrimps themselves as a metabolic waste; they are the end products of protein utilization of aquatic animals. The toxicities that result from deteriorating water quality, such as that from ammonia stress, have lethal effects on juvenile shrimp and can increase their susceptibility to pathogens. The toxicity of ammonia plays an important role in the frequent high mortality occurrence during the early stage on shrimp farms (Lu et al., 2017). It is common knowledge that shrimps possess only the innate immune system and not the adaptive immune system; thus, the response to environmental stress such as an elevated ammonia/ammonium ion, or whether to a pathogenic microorganism will elicit similar mechanism of immune response from the shrimp. Thus, an additional objective of determining whether or not dietary benfotiamine enhances the immune response of the black tiger shrimp post larvae was done by challenging it with an elevated ammonia/ammonium level.
Materials and Methods

This study was conducted at the University of the Philippines Visayas-National Institute of Molecular Biology and Biotechnology (UPV-NIMBB), Miagao, Iloilo, Philippines.

Experimental Shrimps

Peneaus monodon (PL20) were purchased from a private hatchery in the nearby town (Guimbal, Iloilo). Prior to the feeding experiment, P. monodon post larvae were acclimatized to the experimental conditions and control diet for a week. During the feeding trial, apparent satiation was measured within the first three days of the week and was used as the basis for the feeding rate for the rest of the week, and the same cycle was observed until termination. After acclimatization, 270 P. monodon post larvae of similar size (average weight: 0.01g) were randomly distributed among nine indoor plastic containers (40 L volume) at 30 shrimps per container.

Experimental diet preparation

Three experimental diets were prepared for the first feeding trial namely: (1) control diet containing 15% corn starch; (2) 25% corn starch (high carbohydrate diet, HC); and (3) 25% corn starch (i.e. high carbohydrate diet, HCB) with benfotiamine (0.2 g kg⁻¹; Table 1). Dry ingredients were mixed first followed by the liquid components; the mixture was thoroughly blended manually with water until a soft dough was obtained. The mixture was steam-cooked and oven-dried at 60°C to a moisture content of 10% or less. An appropriate amount was allocated for proximate analysis. The dry feeds were stored at 4°C until use; they were ground and sieved to appropriate size before feeding.

Table 1 Composition of experimental diets to determine the effects of High Carbohydrates and High Carbohydrates supplemented with benfotiamine

| Feed ingredients                  | Control (g) | HC (g) | HCB (g) |
|-----------------------------------|-------------|--------|---------|
| Shrimp (acetes) meal              | 200.0       | 200.0  | 200.0   |
| Soybean meal                      | 200.0       | 200.0  | 200.0   |
| Corn starch                       | 130.0       | 200.0  | 200.0   |
| Cod liver oil                     | 40.0        | 40.0   | 40.0    |
| Carboxymethyl cellulose (CMC)     | 104.8       | 34.8   | 34.6    |
| Lecithin – Soy (70%)              | 5.0         | 5.0    | 5.0     |
| Trace mineral premix              | 10.0        | 10.0   | 10.0    |
| Vitamin premix                    | 10.0        | 10.0   | 10.0    |
| Peruvian Fishmeal                 | 300.0       | 300    | 300     |
| BHT                               | 0.2         | 0.2    | 0.2     |
| benfotiamine                      | 0.0         | 0.0    | 0.2     |
| TOTAL                             | 1000.0      | 1000.0 | 1000.0  |

Proximate Analysis

|                  | Control (%) | HC (%) | HCB (%) |
|------------------|-------------|--------|---------|
| Moisture         | 7.44        | 6.71   | 6.67    |
| Crude Protein    | 47.50       | 47.09  | 46.30   |
| Crude Lipid      | 8.31        | 9.80   | 9.43    |
| Crude Fiber      | 2.52        | 2.28   | 2.34    |
| Ash              | 13.18       | 14.75  | 14.13   |

HC : High carbohydrates; HCB : High carbohydrate supplemented with benfotiamine

Diet attractability test

Six separate attractability tests were conducted using rectangular glass tanks with multiple chambers (Suresh et al., 2011) (Figure 1). Each tank consisted of 3 major chambers (acclimatization chamber, middle chamber, and feeding chamber). The three chambers were separated with a removable glass shutter. The feeding chamber consisted of 5 sub-chambers (only 3 were used in the present experiment), each with a 6 x 5 cm access to the feeding chamber.
Figure 1 Schematic diagram of the glass tank used for the attractability test

Each glass tank (90 x 50 x 30 cm in length, width and height, respectively) consisted of an acclimatization chamber at one end and feeding chambers at the other end separated by a shutter glass that could be lifted to start the test. The tanks were set up in an unlit room that only received diffused fluorescent light, and all assessments were conducted at similar time of the day commencing at 0930 h. Diet attractability was performed on the three experimental diets in three simultaneous runs. Ten randomly selected shrimp was acclimatized for 1 h after which 2 g of each experimental diet were placed separately in each of the feeding chambers. Following the lifting of the glass shutter, the number of shrimps that would made it inside each of the feeding chambers was recorded. Diet attractability was expressed as percent of shrimp that were inside a particular feeding chamber containing a particular experimental diet after 1, 5, and 10 min after the shutter was lifted.

Feeding trials

The feeding trial was conducted in a closed recirculating system consisting of a physical filter (gravel and sand), a biological filter (sterile oyster shells), another physical filter (fiber filter) and a reservoir; the system contained recirculating saltwater (18-20 parts per thousand, ppt) flowing at a rate of 13.3 L h⁻¹, and provided with continuous aeration. This study was conducted under controlled laboratory conditions in which 270 healthy shrimp post larvae (0.01 g) was randomly distributed into 9 tanks. The shrimps were fed the experimental diets 4 times daily until termination. Since the feeding trial involved small and sensitive post larval shrimps, sampling was done every 30 days. At the initial stocking, 270 shrimps (ABW=0.01 g) were distributed into nine 50-L containers and a separate representative group of shrimps (about 100 shrimps) were weighed separately to obtain the initial weight. The following ranges of water quality parameters were observed during the feeding trial: temperature, 28-30°C; Salinity, 18-20 parts per thousand; pH - 7.8 – 8.2 parts per million (ppm); dissolved oxygen, 6.1 – 6.2 ppm; total ammonia-N, 0.0 – 0.1 ppm; and nitrite, 0.01 ppm.

Ammonia challenge test

Representative shrimps (10 shrimps per container) at the end of the feeding trial (Day 60) were used in the ammonia challenge test. The media containing ammonia (50 ppm or mg ammonia-N L⁻¹, average ambient temperature=27°C; average pH=8.3) was prepared by dissolving ammonium chloride (NH₄Cl) in sea water at 20 ppt salinity. This concentration of NH₄Cl was the result of a previous range-finding experiment done in our laboratory.
Total ammonia-N was measured using a commercially available water quality kit. Ten shrimps of *P. monodon* juveniles from each container in the feeding trial were placed into corresponding containers containing ammonia (i.e. 50 mg NH4Cl L\(^{-1}\) sea water at 20 ppt, pH 8.3 and average ambient temperature of 27°C). All tanks were provided with sufficient aeration and shrimps were fed with their corresponding diets at 6.5% body weight daily. Shrimp survival was monitored every 15 min during the first hour, every 30 min during the second hour, every hour after the 4th hour, every 4 h after the 16th hour, and every hour on the 24th hour. The shrimp considered dead when immobile and showed no response to touch with a glass rod. Cumulative survival was calculated as follows:

\[
\text{Survival (\%)} = 100 \times \frac{\text{Initial fish count} - \text{dead fish count}}{\text{Initial fish count}}
\]

**Response parameters**

During the experiment, Weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR) and survival rate (SR), were computed using the following formula:

- Weight Gain (WG, g) = Final Average Body Weight (FABW) – Initial Average Body Weight (IABW)

- Specific Growth Rate (SGR, % day\(^{-1}\)) = 100*(log of Final Weight (g) – log of Initial Weight (g))/No. of Days

- Feed Intake (FI, g) = sum of daily feed offered for 60 days (g)

- Feed Conversion Ratio (FCR) = FI (g)/WG (g)

- Survival Rate (%) = 100*Total no. of live Shrimp/Total no. of initial stock

**Statistical analyses**

Data (survival rate, weight gain, specific growth rate, and feed conversion ratio, survival rate and attractability test) were presented as mean ± standard error of the mean (SEM) and were tested for normality using Shapiro-Wilk test and Levene’s test for variance homogeneity. Log transformation was used for data that did not pass the two tests; those that passed the test were subjected to one-way Analysis of Variance (ANOVA) at 0.05 alpha using SPSS version 20.0. Percentage data were arc sine-transformed prior to the statistical analyses. Post hoc analyses were done using Duncan’s Multiple Range Test (DMRT) to rank the means.

**Results**

**Diet attractability test**

The HCB diet attracted significantly the highest percentage of shrimps even after 10 min throughout the test period while the C and HC group attracted lower percentages and were similar to each other at 5 min. However, at 10 min, the C diet attracted significantly the lowest percentage of shrimps.

| Table 2 Attractability of *P. monodon* to the experimental diets | Periodic percentage of shrimps ± SEM |
| --- | --- | --- |
| | 1 min | 5 min | 10 min |
| Control | 6.7±3.3\(^{bc}\) | 25.0 ±9.2\(^{a}\) | 30.0 ±0.0\(^{k}\) |
| HC | 3.3 ±3.3\(^{bc}\) | 21.7 ±4.8\(^{bc}\) | 20.0 ±0.0\(^{bc}\) |
| HCB | 30±0.0\(^{a}\) | 51.7 ±9.5\(^{a}\) | 43.0 ±3.3\(^{a}\) |

Superscripts indicate significant difference between diets at (p <0.05)

HC: high carbohydrate and HCB: high carbohydrate supplemented with benfotiamine

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Feeding trials

WG of shrimps fed the HCB diet was significantly the highest among the dietary treatments from 30 days to 60 days, followed by the HC group and C group in this order and were significantly different from each other (Figure 2 and Table 2).

![Figure 2](image-url) Periodic average body weight (ABW) of shrimp fed with control (C), high carbohydrates diet (HC) and high carbohydrates diet with benfotiamine (HCB). Letters represent significant difference of each treatment at each sampling time.

Growth performance and feed utilization at the termination of the feeding trial are summarized in Table 3. Shrimps fed the HCB diet exhibited the highest growth. FABW, WG, SGR values were significantly the highest in shrimps fed with the HCB diet, followed by the C and HC diets in this order, the latter two groups were significantly different from each other (p<0.05).

Shrimps fed with the HCB diet exhibited the best FCR (lowest value), followed by the HC groups and C in this order; the latter groups were significantly different from each other.

| Diets | IABW | FABW (g) | SGR (%) | FI (g) | FCR (g) | Surv. (%) |
|-------|------|----------|---------|--------|---------|-----------|
| Control | 0.01 | 0.23 ±0.003b | 5.44 ±0.03b | 0.22 ±0.003b | 1.8 ±0.00b | 99.99a |
| HC | 0.01 | 0.15 ±0.003c | 4.63 ±0.04c | 0.14 ±0.003c | 1.9 ±0.03a | 99.99a |
| HCB | 0.01 | 0.45 ±0.006a | 6.56 ±0.02a | 0.44 ±0.006a | 1.2 ±0.00c | 99.99a |

Superscripts indicate significant difference between diets at (p <0.05)
HC : high carbohydrate and HCB : high carbohydrate supplemented with benfotiamine

Ammonia challenge

Cumulative survival of shrimps in media containing 50 mg L⁻¹ NH₄Cl (dissolved in 20 ppt sea water, pH 8.3, ambient temperature of 27°C) is shown in Figure 3. At the end of the 72h in 50 mg L⁻¹ ammonia, the HCB group exhibited significantly the highest percentage of survival followed by those fed either the HC or C group; the latter two groups were not significantly different from each other (Figure 3 and Table 4).
Effects of dietary benfotiamine on P. monodon

Figure 3 Cumulative survival of shrimp during the 3 days (72h) 50 mg L⁻¹ ammonia challenge

Table 4. Survival of shrimp after 3 days (72h) 50 mg L⁻¹ ammonia challenge

| Treatments | Survival of shrimp (%) |
|------------|------------------------|
| Control    | 33.33 ± 3.33c          |
| HC         | 40.33 ± 5.78bc         |
| HCB        | 60.00 ± 5.78a          |

Superscripts indicate significant difference between diets at (p < 0.05)

Discussion

Carbohydrates are a source of cheap energy, but the ability of aquatic organisms, including shrimp to utilize it is limited. This is partly due to the lack of ability to digest and regulate plasma glucose concentrations (Wang et al., 2016). The low digestibility of carbohydrates associated with the availability of the enzyme α-amylase, whereas low concentrations of plasma glucose regulation allegedly caused by a deficiency of the hormone insulin (Zainuddin et al., 2018).

Result of the attractability test of the diet in the present study was in agreement with that of the Nile tilapia (Lauzon et al., 2019) in our previous study, in which high carbohydrate diet (HC) elicited lower attractability than did the control (C) diet containing low carbohydrate diet. In contrast, attractability was highest in shrimps fed with the high carbohydrate diet supplemented with benfotiamine (HCB). The attractant effect of (HCB) diet in the present study might be due to the inclusion of dietary benfotiamine; to our knowledge, no previous study has been done except that in our previous study on the Nile tilapia, to determine whether or not benfotiamine is a known attractant to shrimps or insects.

Thiamine is an essential cofactor in most organisms and is required at several stages of anabolic and catabolic intermediary metabolism, such as intracellular glucose metabolism, and is also a modulator of neuronal and neuro-muscular transmission (Beltramo et al., 2008). It can improve glucose homeostasis by blocking three major pathways associated with hyperglycemic damage (Hammes et al., 2003). Benfotiamine is a lipid-soluble thiamine precursor having much higher bioavailability than thiamine (Hammes et al 2003). In the present study, shrimp fed the high carbohydrate diet supplemented with benfotiamine (HCB) showed significantly higher growth performance than those fed with the control (C) and high carbohydrate diet with no benfotiamine supplementation (HC). The final average body weight gain (FABW); specific growth rate (SGR); weight gain (WG) and feed intake were significantly higher in (HCB) than the other
two diets. The feed conversion ratio was in opposite trend in which the HCB diet resulted in significantly lowest (i.e. most efficient) among other diets. These results showed that the high carbohydrate content was efficiently utilized by supplementing benfotiamine. Survival rate in the present study showed no significant difference among the three dietary groups. These results are in agreement with the previous work on the Nile tilapia that the addition of 20 mg kg\(^{-1}\) supplemental thiamin hydrochloride resulted in a significant growth enhancement (Lauzon et al., 2019). In the blunt snout sea bream, an increase in WG and SGR was observed as a result of feeding the fish with the diet supplemented with benfotiamine (Xu et al., 2018). Thiamine and its derivative benfotiamine protects the cells against oxidative stress, suggesting an antioxidant effect of thiamine (Sambon et al., 2019). In this study, shrimp fed the HCB showed significantly higher survival rate compared to either of the control and HC diet after a 50 mg L\(^{-1}\) ammonia challenge in 72 h. This result was in contrast to the previous study that aimed to determine the ammonia tolerance of *L. vannamei* in 72 h with a concentration of 32 mg L\(^{-1}\) (Lu et al., 2017). The previous study recorded a mortality rate of 98.6\% after the 72h exposure but with no supplementation of benfotiamine. The high survival rate of the present study was probably due to the benfotiamine inclusion in the high carbohydrate diet that enabled protection for the shrimp against oxidative stress. Ammonia in fish is known to induce oxidative injury (Sinha et al., 2015a; 2015b; Hegazi et al., 2010). It is apparent that benfotiamine might have protected the black tiger shrimp against oxidative stress by mitigating ammonia-induced oxidative injury.

In conclusion, incorporation of benfotiamine into the high carbohydrate diet of shrimp significantly enhanced growth performance; attractability of the diet and immune response.

**Acknowledgements**

The authors would like to thank the Department of Science and Technology- Accelerated Science and Technology Human Resource Development Program (DOST-ASTHRDP) for the scholarship and thesis grant; University of the Philippines Visayas – Office of the Vice Chancellor for Research and Extension (UPV-OVCRE) for the for the publication grant. The authors are also grateful to Ms. Apple Gray Deallo, Ms. Shellah Dee Canillo, Mr. Vicente Nim and Arly Nim for their technical assistance.

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