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ORIGINAL RESEARCH

Dietary exposure to melamine and cyanuric acid induced growth reduction, oxidative stress and pathological changes of hepatopancreas in Pacific white shrimp

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Abstract This study examined the effects of dietary melamine (MEL) and cyanuric acid (CYA) singly and in combination on growth, nutrient utilization, immunological responses, oxidative stress, and histological changes in Pacific white shrimp. Seven experimental isonitrogenous (35%) and isolipidic (8%) diets were formulated, namely diet 1 (a control diet without MEL and CYA); diets 2–5 (with MEL and CYA at 2.5 + 2.5, 5 + 5, 7.5 + 7.5 and 10 + 10 g kg^{-1} diet); diet 6 (with only MEL at 10 g kg^{-1} diet) and diet 7 (with CYA alone at 10 g kg^{-1} diet). The shrimp with initial body weight 2.37 ± 0.02 g were fed with these diets for 10 weeks. The results indicate that all the diets with MEL and CYA singly or in combination had adverse effects on growth and nutrient utilization relative to the control diet ($p < 0.05$). Total protease and trypsin activities were significantly lowered by all diets containing MEL ($p < 0.05$). Haemolymph parameters, including total hemocyte count, phenoloxidase (PO) activity, respiratory burst, and lysozyme activity, were significantly decreased ($p < 0.05$) in shrimp receiving MEL alone (10 g kg^{-1} diet) and at high combination dosages (10 + 10 g kg^{-1} diet). Moreover, MEL and CYA induced oxidative stress, damaged hepatopancreas, decreased antioxidant responses, increased lipid peroxidation, and caused abnormality of hepatocytes.

Keywords Pacific white shrimp · Melamine · Cyanuric acid · Growth · Histology · Oxidative stress

Introduction

Feed contamination is a worldwide major current concern with regard to consumer health, economic losses, and safety and reliability of food products (Maule et al. 2007; Pettersson 2012; O'Keefe and Campabadal 2015). In general, melamine (MEL) and its derivatives including cyanuric acid (CYA), ammeline (AMN), and ammelide (AMD), are commonly used in thermoplastic materials, such as dishware, laminating resins, coating materials, and fire retardants (Casu et al. 1997; Roviello et al. 2015). Moreover, the US Food and Drug Administration (2007) reported illegal adulteration of animal feed with MEL and CYA in many countries.

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MEL is a nitrogenous material with 66% nitrogen by mass. Therefore, adding MEL in a foodstuff increases the total nitrogen content typically determined by the Kjeldahl method to estimate the protein content, so MEL is also known as a "fake protein" (US-FDA 2007; Reimschuessel et al. 2008). For this reason, protein-rich ingredients are the major targets for MEL adulteration that has been documented in many feedstuffs, such as fishmeal, gluten, soybean meal, squid meal, and shrimp meal, and contamination of pets and farm animals including fish and shrimp has also been reported (Ehling et al. 2007; WHO 2009). Adverse effects of MEL and CYA have been reported in different fish species; these included growth retardation, behavioral abnormalities, immunosuppression, hepatotoxicity and nephrotoxicity (Reimschuessel et al. 2010; Xue et al. 2011; Pirarat et al. 2012; Pacini et al. 2013; Phromkunthong et al. 2013, 2015a, b; Mahardika et al. 2017). Thus, contamination of feed with MEL and CYA is one of the critical concerns in aquatic industry. To date, due to its toxic effects and other potential health effects impacting consumers, many countries have incorporated MEL and its structural analogs into a list of food additives and substances prohibited in animal feeds. In Europe, the maximum permitted levels of MEL in feed are strictly set as 2.5 mg kg^{-1} (European Commission 2013). In 2016, the Department of Livestock Development, Thailand has banned dried squid-derived meal imported from Vietnam after laboratory tests indicated the presence of CYA (Byrne 2016), and there is evidence that these chemical substances are still being used illegally in some parts of the world.

The culturing of Pacific white shrimp (*Litopenaeus vannamei*) is gaining importance in the food sector, with the increasing trend worldwide since 2004 (World Bank 2013). This species has great global demand due to good taste and high nutritional value (FAO 2016). For producing high-quality shrimp, the feed used in culturing is a key factor. In recent years, shrimp feed development has focused on the suitability of protein sources and the reduction of production costs without negative effects on growth and health status of the shrimp (Amaya et al. 2007; Carvalho et al. 2016). Shrimp feed needs to have a high protein content to stimulate growth and feed utilization, so it is a suitable target for adulteration by MEL and its derivatives (Karbiwnyk et al. 2010). In fact, there are a few reports of adulteration of shrimp feed by MEL and its derivatives. Lightner et al. (2009) reported diagnostic results on black tiger shrimp, *Penaeus monodon,* and Pacific white shrimp from India and Indonesia. Golden to greenish-brown needle-like crystals were present in the antennal glands, a likely adverse effect from MEL and CYA poisoning. In contrast, JAVMA (2007) reported that MEL has been intentionally added into shrimp feeds in the USA, to improve water stability. However, after an incident was caused by use of MEL in 2007, its use as a pellet binder was discontinued.

A few recent reports have mentioned the toxicity of MEL and CYA to crustaceans, especially the penaeid shrimp (Lightner et al. 2009; Karbiwnyk et al. 2010). Hence, this present study investigated the effects of MEL and CYA supplementation, singly or in combination, in practical feed for Pacific white shrimp. The effects on growth, nutrient utilization, oxidative stress, immunological responses and histopathological changes were evaluated. This study improves the understanding of shrimp responses to MEL and CYA exposures.

Materials and methods

Experimental conditions and setup

This study was conducted at Kidchakan Supamattaya Aquatic Animal Health Research Center (KS-AAHRC), Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. Sea water with 30‰ salinity was transported from a commercial shrimp farm located in Songkhla Province, Thailand, stocked in a 7 m^3 concrete pond with continuous aeration, and was diluted with dechlorinated freshwater to 15‰ brackish water that was used for the entire experiment. The water quality parameters monitored for suitability to a marine shrimp culture included DO, alkalinity, and pH (Thai Agricultural Standards TAS 7401-2009 2009). The salinity was determined using a handheld refractometer (ATAGO, Tokyo, Japan), while alkalinity was controlled to a sodium bicarbonate titration level (120 mg L^{-1}) and pH 7.8–8.2. The feeding trial was conducted in 200 L fiberglass tanks. Each tank was equipped with an air stone and water draining system, and was covered with a plastic tray to prevent escape of the shrimp.

Test shrimp and feeding

A completely random design (CRD) was used in this study. Pacific white shrimp, *L. vannamei* (0.5–1.0 g average weight) were acclimated in a 3 m^3 indoor concrete pond with 15‰ brackish water and fed with the control feed (diet 1 without MEL or CYA) four times daily for 3 weeks. At the initiation of feeding experiment, 18 shrimp with an average body weight of 2.37 ± 0.02g were randomly released into 35 fiberglass tanks (200 L). Each tank was then randomly assigned to one of five replicates of the seven dietary treatments. The shrimp were fed to apparent satiation four times daily, for 10 weeks. The care and handling of the white shrimp were in accordance with the guidelines of the Canadian Council on Animal Care (CCAC 2005) and with Thai Agricultural Standards TAS 7401-2009 (2009).

Diet preparation

Seven isonitrogenous (35% crude protein) and isolipidic (8% crude lipid) laboratory-made diets were formulated to meet the nutritional requirements of Pacific white shrimp. Major protein ingredients used in the diets were provided from Charoen Pokphand Foods PCL, Songkhla, Thailand. MEL with 99.5% purity was supplied by Chang Chun Petrochemical Co. Ltd., Taiwan. CYA (98% purity) was purchased from Sigma-Aldrich (MO, USA). The experimental diets are detailed in Table 1. Diet 1 had no added MEL or CYA; MEL and CYA were incorporated (g kg^{-1} diet) at levels 2.5 + 2.5 (diet 2), 5 + 5 (diet 3), 7.5 + 7.5 (diet 4), 10 + 10 (diet 5), 10 + 0 (diet 6), and 0 + 10 (diet 7), respectively. The diets were processed at the KS-AAHRC using Hobart mixer A-200 (OH, USA). All the dry raw ingredients were finely ground, passed

Table 1 Ingredients and formulations of the experimental diets

Ingredient (g kg^{-1} diet)	Diet (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5 + 2.5)	Diet 3 (5 + 5)	Diet 4 (7.5 + 7.5)	Diet 5 (10 + 10)	Diet 6 (10 + 0)	Diet 7 (0 + 10)
Fish meal	200	200	200	200	200	200	200
De-hulled soybean meal	250	250	250	250	250	250	250
Squid liver meal	30	30	30	30	30	30	30
Corn gluten	50	50	50	50	50	50	50
Canola meal	28.5	28.5	28.5	28.5	28.5	28.5	28.5
Wheat flour	320	320	320	320	320	320	320
Wheat gluten	30	30	30	30	30	30	30
Fish oil	15	15	15	15	15	15	15
Soybean oil	1	1	1	1	1	1	1
Lecithin	20	20	20	20	20	20	20
Vitamin and mineral premix[a]	2	2	2	2	2	2	2
Di-calcium phosphate	22	22	22	22	22	22	22
Choline chloride	1	1	1	1	1	1	1
Inosital	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Melamine (MEL)[b]	0	2.5	5	7.5	10	10	0
Cyanuric acid (CYA)[c]	0	2.5	5	7.5	10	0	10
Microcrystalline cellulose	30	25	20	15	10	20	20

[a]Vitamin and mineral premixed (unit kg^{-1} feed): retinal (A) 7000 IU; cholecalciferol (D3) 3000 IU; tocopherol (E) 1500 mg; menadione sodium bisulfite (K3) 30 mg; thiamine (B1) 25 mg; riboflavin (B2) 20 mg; pyridoxine (B6) 25 mg; cobalamin (B12) 0.02 mg; niacin 100 mg; pantotenic acid 80 mg; ascorbic acid (C) 200 mg; biotin 1 mg; folic acid 10 mg; Cu 25 mg; Fe 30 mg; Mn 30 mg; I 1 mg; Co 0.2 mg; Zn 100 mg; Se 0.35 mg

[b]Melamine: Chang Chun Petrochemical Co., Ltd., Taipei Taiwan (purity 99.5%)

[c]Cyanuric acid (98%): Sigma, Aldrich Co., Ltd.

through a 30-mesh sieve, and weighed. After mixing for 15 min, lipid sources (fish oil, soybean oil, and lecithin) and distilled water (400 mL kg^{-1} feed) were added with continuous mixing. The homogenous mixture was pelleted (3-mm diameter) and dried to below 10% moisture. The dry pellets were then packed in low-density polyethylene bags and stored in a freezer at – 20 °C. The experimental diets were analyzed for proximal composition according to the standard methods of AOAC (1995). The proximal compositions are presented in Table 2. MEL and CYA content in the diets were determined by LC–MS/MS using a modified method of US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky 2008), as described in our previous publication (Phromkunthong et al. 2013, 2015a).

Growth performance

In the beginning and at the end of the dietary treatment trial, the shrimp in each tank were counted and bulk weighed after immersing in low-temperature water (22 °C) to reduce their physical activity. The mean final weight, number of shrimp, and feed intake were determined for each tank to calculate growth parameters, feed utilization and survival according to Kim et al. (2011) as follows:

Weight gain (g) = final body weight (g) – initial body weight (g).
Average daily gain (ADG, g $shrimp^{-1}$ day^{-1}) = [final body weight (g) – initial body weight (g)]/days.
Specific growth rate (SGR, % day^{-1}) = [(ln final body weight (g) – ln initial body weight (g))/days] $\times 100$.
Feed conversion ratio (FCR) = dry feed intake (g)/weight gain (g).
Feed intake (FI, g $shrimp^{-1}$ day^{-1}) = [feed consumed (g)/number of shrimp]/days.
Survival (%) = 100 $\times$ (final count of shrimp)/(initial count of shrimp).

Chemical composition analyzes

At the beginning of the experiment, 20 shrimp were scarified and they were stored at – 20 °C until analysis of the initial proximate composition. At the end of the feeding trial, 3 shrimp from each tank (15 shrimp per treatment group) were collected to analyze the whole body composition. Nitrogen, crude lipid, moisture and ash content in the whole body were determined following the procedures of AOAC (1995). Briefly, dry matter was determined by drying the samples in a hot air oven (Memmert UF110, Germany) at 105 °C until constant weight. Nitrogen content (N $\times$ 6.25) was determined by the Kjeldahl method using Kjeltec protein analyzer (Kjeltec™ 8100, FOSS, Tecator, Sweden). Crude lipid was determined by methylene chloride extraction using Soxhlet method (Soxtec™ 8000, China). Ash was measured after combustion in a muffle furnace (Gallenkamp Box Furnace, UK) at 550 °C for 6 h.

Hemolymph analysis and immunological response

Hemolymph from individual shrimp (n = 10/test group) was collected without the use of anticoagulants in week 10, after the final weights of the shrimp in all treatment groups were recorded. Haemolymph was withdrawn from the base of the 3rd walking leg of each shrimp, using a 1 mL syringe with 25 G (25 gage) needle, and then immediately transferred into 1.7 mL sterile micro-centrifuge tubes. Soluble protein concentration was determined by the method of Bradford (1976). Hemolymph parameters related to immune functions were analyzed by the methods described below.

Total hemocyte count (THC)

THC was determined by the method previously described by Supamattaya et al. (2005). Briefly, 50 µL of haemolymph was diluted with 450 µL pre-cooled (4 °C) trypan blue solution (0.5% trypan blue in 2.6% NaCl) and gently mixed. Hemocytes were counted using a haemocytometer (Bright-Line™, NY, USA) under a compound microscope (Olympus CH30, Tokyo, Japan) and recorded as the number of cells (total hemocytes mL^{-1}).

Table 2 Proximate composition of the experimental diets (g kg^{-1} as dry matter basis, analyses of three batches of diet) given as mean ± SD

Ingredient (g kg^{-1} diet)	Diet (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5 + 2.5)	Diet 3 (5 + 5)	Diet 4 (7.5 + 7.5)	Diet 5 (10 + 10)	Diet 6 (10 + 0)	Diet 7 (0 + 10)
Nitrogen content (N × 6.25)	367.97 ± 1.46	381.12 ± 2.12	399.78 ± 0.75	415.51 ± 2.32	437.01 ± 1.38	411.53 ± 1.15	387.67 ± 0.91
Crude lipid	77.71 ± 1.33	78.24 ± 2.12	75.95 ± 1.47	77.70 ± 0.82	78.47 ± 0.13	73.90 ± 1.75	75.43 ± 1.34
Crude ash	65.17 ± 1.43	63.90 ± 0.52	70.24 ± 2.11	67.88 ± 2.70	63.09 ± 4.01	65.81 ± 1.29	67.90 ± 1.52
Gross energy (MJ kg^{-1} diet)	15.86	15.86	15.86	15.86	15.86	15.86	15.86
MEL content[a]	0	2.3	4.5	7.3	9.7	10.1	0
CYA content[a]	0	2.2	4.2	7.1	9.5	0	9.6

[a]LC–MS/MS using a modified method of US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky 2008)

Phenoloxidase (PO) activity

PO activity was determined according to the method of Liu et al. (2004) with minor modifications. Briefly, 100 µL of haemolymph was transferred into sterile micro-centrifuge tube containing 100 µL cacodylate (CAC) buffer (10 mM sodium cacodylate, 0.45 M NaCl, 10 mM $CaCl_2$, 0.26 M $MgCl_2$) at pH 7.4 and flushed with liquid nitrogen. The glaced mixture was broken down with a sterile plastic pestle, and centrifuged (Avanti^{TM}30, Beckman Coulter, CA, USA) at 12,879×*g* for 10 min at 4 °C. The supernatant (25 µL) was collected and placed in a well of a flat-bottomed 96-well plate. An enzyme reaction was started by adding 25 µL trypsin (Sigma-Aldrich, Buchs, Switzerland) (1 mg mL^{-1} in CAC buffer) and the plate was incubated at 25 °C for 2 min. Finally, 150 µl of CAC buffer and 50 µl of L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich, Shanghai, China) (3 mg mL^{-1} in CAC buffer) were mixed. The optical density at 490 nm was recorded in kinetic mode every minute for a total of 30 min using a microplate spectrophotometer (PowerWave$_x$, Bio-Tek Instruments, VT, USA). One unit of PO activity was defined as increasing the absorbance by 0.001 min^{-1}, and the results are reported as unit min^{-1} mg $protein^{-1}$.

Respiratory burst activity

Study of respiratory burst activity of the haemocytes was performed following the method as described by Hsu and Chen (2007). Briefly, 100 µL hemolymph was incubated with nitro-blue tetrazolium (NBT, Sigma-Aldrich, MO, USA) solution (0.3% in 1.5% NaCl). The mixture was removed and the pellet was fixed with 100% methanol, and washed three times with 100 µL 70% methanol. After air drying, formazan was dissolved by the addition of 120 µL 2 M KOH and 140 µL dimethyl sulfoxide (DMSO). The optical density at 630 nm was measured using a microplate reader, and is expressed as NBT reduction in 100 µL haemolymph.

Lysozyme

Lysozyme activity was observed through enzyme activity and bacterial target clearance during incubation. The suspension of 0.02% lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, MO, USA) in 50 mM phosphate buffer saline at pH 6.2 was used as the substrate. Lysozyme activity was measured following the method described by Shen et al. (2010). Briefly, 25 µL of diluted hemolymph was mixed with 175 µL of the substrate in a flat-bottomed 96-well plate. The reaction was carried out at room temperature, and the absorbance at 490 nm was measured in kinetic mode every minute for a total of 10 min, using a microplate reader. One unit of lysozyme activity was defined as reducing the absorbance by 0.001 per min (Ellis 1990), and results are reported in unit mL^{-1}.

Antioxidant enzyme analysis and lipid peroxidation assays

Antioxidant enzyme activities of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were determined at end of the feeding study. At the same time with hemolymph collection, the gill and the hepatopancreas of the same individual were dissected rapidly in an ice-cooled petri dish, and were then transferred to cryogenic vials (Corning®, Mexico City, Mexico) that were immediately stored in liquid nitrogen. Prior to analysis, the target tissue was individually homogenized (1:2, 1:4 w/v for gill and hepatopancreas tissues, respectively) in ice-cold extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, pH 7.5, 20% protease inhibitor, Amresco), and was then centrifuged at 12,879×*g* for 15 min at 4 °C. The supernatant was transferred into a new 1.7 mL micro-centrifuge tube for enzyme activities assay. Protein content in enzyme extract was measured by the method of Bradford (1976) using bovine serum albumin (Sigma-Aldrich, MO, USA) as the protein standard.

CAT activity was determined based on formaldehyde production from reacting the sample with hydrogen peroxide (H_2O_2). Formaldehyde concentration was determined from colored complexes it forms with Purpald® (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, Sigma-Aldrich, WI, USA), and was measured at 540 nm (Trasviña-Arenas et al. 2013). The CAT activity was calculated using a standard curve calibrated with known formaldehyde concentrations (r^2 = 0.9993) and the results are expressed in unit min^{-1} mg $protein^{-1}$.

GPx and SOD activities were measured using assay kits (Cayman Chemical Company Inc., MI, USA). These enzyme activities were determined according to the assay protocols. Specific activities of the enzymes are expressed in unit min^{-1} mg $protein^{-1}$.

Ten shrimp per treatment group were euthanized by immersion in cold water until no movement of their appendages was observed. The shrimp were wiped to remove excess water and then individually weighed. The hepatopancreas was immediately dissected and weighed to determine the hepato-somatic index (HSI) as follows:

HSI =100 × [hepatopancreas weight (g)/shrimp body weight (g)].

Lipid peroxidation (LPO) in the hepatopancreas was determined as thiobarbituric acid reactive substances (TBARS) level, based on the reaction of 2-thiobarbituric acid (TBA) with malondialdehyde (MDA). Measurements of LPO were conducted according to the method of Senphan and Benjakul (2012). Tissue samples (~ 0.3 g) (n = 10/feed group) were blended and mixed with TBA working solution (0.375% (w/v) TBA (Sigma-Aldrich, Steinheim, Germany), 15% (w/v) trichloroacetic acid (TCA) and 0.25 M HCl). The homogenized samples were heated in a 95 °C shaking water bath for 10 min, and were then cooled with running tap water, and centrifuged at 4800×g for 10 min at 4 °C. The supernatant was measured by a microplate reader at 532 nm. MDA concentration in each sample was calculated from an eight-point standard curve (r^2 = 0.9997) for 1,1,3,3-tetramethoxypropane (TEP) (Sigma-Aldrich, Steinheim, Germany) calibration standards, and the results are expressed in nmols MDA per gram of wet weight tissue.

Digestive enzyme analysis

The activities of digestive enzymes in shrimp hepatopancreas were determined at the termination of feeding study. The hepatopancreas (n = 10/treatment) was dissected and collected, weighed and homogenized with 50 mM Tris–HCl. The homogenized mixture was then centrifuged at 12,879×g for 15 min at 4 °C. For the different enzyme analyzes, the supernatant was aliquoted in triplicate into cryogenic vials, to determine total protease, trypsin, and amylase. The vials were kept at – 70 °C until analysis.

Total proteinase activity was estimated as suggested by Ávila-Villa et al. (2012). Briefly, 20 µL of sample extract was added in a test tube, then 500 µL of 2% azocasein (Sigma-Aldrich, MO, USA) and 240 µL of 50 mM Tris–HCl buffer (pH 7.5) were added and mixed well in a vortex shaker. The mixture was incubated at room temperature for 30 min. The reaction was stopped by adding 500 µL of 20% TCA and then the sample was centrifuged at 6500×g for 5 min. The supernatant was collected and measured in a microplate reader at 450 nm. The activity is expressed in unit mg $protein^{-1}$. One unit of protease activity was defined as the amount of enzyme that catalyzes the release of the azo dye, causing the change in absorbance at 450 nm of 0.001 per minute under assay conditions.

Trypsin activity was estimated by the method described by García-Carreño and Haard (1994) with some modification. Benzoil-Arg-p-nitroanilide (BAPNA) (Sigma-Aldrich, Tokyo, Japan) was used as the substrate. The analysis was started by mixing 30 µL of the sample extract with 320 µL of 0.1 mM BAPNA. After incubating for 20 min at 37 °C, the reaction was stopped by adding 50 µL of 20% TCA, and the product was then centrifuged at 6500×g for 5 min. The absorbance was read in a microplate reader at 410 nm. Trypsin activity was estimated as [(Abs410nm/min × volume (mL) of reaction mixture × dilution factor)/(8800 × volume (mL) of enzyme ×mg protein of the sample extract)], where the constant 8800 is the extinction coefficient of p-nitroanilide. The activity is expressed in unit mg $protein^{-1}$. One unit of trypsin activity was defined as 1 µmol of p-nitroanilide released per minute under the specified conditions.

Amylase activity was determined with the 3,5-dinitrosalicylic acid (DNS) method (Rick and Stegbauer 1974) using 1% (w/v) starch solution as substrate. First, 100 µL of the sample extract was placed in a test tube and then 100 µL of substrate and 1.8 mL phosphate buffer (0.1 M, pH 7) were added and mixed thoroughly. The mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 2 mL DNS (Sigma-Aldrich, Bangalore, India), and then the sample was heated in boiling water for 5 min. After cooling with running tap water, the reaction mixture was diluted with distilled water and measured by microplate reader at 540 nm. Amylase activity was calculated from a standard curve for maltose. One unit of enzyme activity was defined as 1 mg of maltose liberated in 1 min at 37 °C.

Histological examination

At the end of the feeding trial, ten shrimp per treatment group were euthanized by immersion in cold water. Shrimp heads were collected, cut in half lengthwise, and then immediately preserved in Davidson's fixative (Lightner et al. 2009). After fixation, samples were embedded in cassette molds and were processed overnight by automatic tissue processors (Leica, Nussloch, Germany). The samples were then embedded into paraffin blocks and cut to 3-μm thickness with a sliding microtome (R. Jung AG, Heidelberg, Germany). The sections were stained with haematoxylin and eosin (H&E) (Bancroft 1967; Humason 1979). The stained sections were examined under a light microscope (Olympus CH30, Olympus Corporation, Tokyo, Japan), and then a selected area in the stained section was imaged with an Olympus DP71 digital camera and software (Olympus Corporation, Tokyo, Japan).

Statistical analysis

The data are presented as mean ± standard deviation (SD). The statistical analyzes used SPSS version 11.5 for Windows. One-way analysis of variance (ANOVA) was used for comparison between groups, and the means were compared with LSD post hoc method. Differences were considered statistically significant at $p < 0.05$.

Results

Growth performance and feed efficiency

Survival rate of shrimp fed the control diet was significantly higher than that of shrimp fed the MEL alone diet ($p < 0.05$), but these were not significantly different from the other treatments ($p > 0.05$) (Table 3. Furthermore, the diets including MEL or CYA (singly or in combination) did not cause any obvious external abnormality. However, the FCR was significantly higher ($p < 0.05$) in shrimp fed the diets 2–7 compared to those fed the control diet (Table 3). Shrimp receiving high doses of MEL (alone or with CYA, diets 4–6) had significantly ($p < 0.05$) lower feed consumption than that of shrimp fed the control diet. The growth performances are shown in Table 3. The diets 2–7 gave significantly ($p < 0.05$) lower final body weight, ADG, and SGR than the control treatment.

Proximate composition of whole shrimp body

The proximal composition of the whole shrimp bodies is presented in Table 4. At the end of feeding trial, shrimp fed diets supplemented with MEL and CYA, either singly or combined at various dose levels, had significant ($p < 0.05$) effects on the whole body composition, including dry matter, crude protein, and crude lipid, deviating from the control group. It was noted that protein in the shrimp carcass was significantly ($p < 0.05$) elevated, whereas lipid content was significantly ($p < 0.05$) reduced by the actual dietary treatments. However, crude ash was not significantly ($p > 0.05$) different between the treatments.

Hemolymph analysis and immunological response

Hemolymph analyzes for the different treatment groups are presented in Table 5. Relative to the control group, the total hemocyte counts (THC) of shrimp receiving MEL were significantly decreased ($p < 0.05$), but no significant ($p > 0.05$) difference was caused by CYA alone in the diet. PO activity in the shrimp fed combined doses of MEL and CYA increased from the 5 + 5 to the 10 + 10 g kg^{-1} diet group, and MEL dosing alone at 10 g kg^{-1} diet gave significantly ($p < 0.05$) lower PO than the control diet. However, no significant ($p > 0.05$) effect was found in shrimp fed 2.5 + 2.5 g kg^{-1} diet or with CYA dosing alone at 10 g kg^{-1} diet. Respiratory burst activity in shrimp fed the control diet was found significantly ($p < 0.05$) higher than with the actual treatments. Significantly ($p < 0.05$) reduced lysozyme activity was found in the shrimp fed 10 + 10 g kg^{-1} diet combined dose of MEL and CYA, or MEL alone at 10 g kg^{-1} diet.

Table 3 Growth parameters and nutrient utilization of Pacific white shrimp fed experimental diets for 10 weeks

Parameter	Diet (MEL + CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5 + 2.5)	Diet 3 (5 + 5)	Diet 4 (7.5 + 7.5)	Diet 5 (10 + 10)	Diet 6 (10 + 0)	Diet 7 (0 + 10)
Survival (% cumulative)							
Week 10	95.00 ± 2.89^{b}	88.33 ± 6.38^{ab}	87.92 ± 3.70^{ab}	90.67 ± 3.65^{ab}	90.67 ± 5.96^{ab}	84.67 ± 2.98^{a}	90.00 ± 3.33^{ab}
Average body weight (g shrimp^{-1})							
Week 0	2.37 ± 0.02^{a}	2.37 ± 0.02^{a}	2.37 ± 0.02^{a}	2.37 ± 0.01^{a}	2.37 ± 0.01^{a}	2.37 ± 0.02^{a}	2.37 ± 0.02^{a}
Week 10	13.65 ± 0.43^{d}	11.62 ± 0.34^{b}	11.69 ± 0.05^{b}	11.33 ± 0.68^{b}	11.38 ± 0.29^{b}	10.65 ± 0.43^{a}	12.44 ± 0.12^{c}
Average daily gain (g shrimp^{-1} day^{-1})							
Week 10	0.15 ± 0.01^{d}	0.13 ± 0.00^{b}	0.13 ± 0.01^{b}	0.12 ± 0.01^{ab}	0.12 ± 0.00^{ab}	0.11 ± 0.01^{a}	0.14 ± 0.00^{c}
Specific growth rate (% day^{-1})							
Week 10	2.46 ± 0.07^{d}	2.27 ± 0.05^{b}	2.31 ± 0.08^{b}	2.23 ± 0.09^{ab}	2.24 ± 0.04^{ab}	2.18 ± 0.09^{a}	2.37 ± 0.00^{c}
Feed conversion ratio							
Week 10	1.31 ± 0.04^{a}	1.54 ± 0.05^{b}	1.41 ± 0.03^{b}	1.43 ± 0.03^{b}	1.43 ± 0.05^{b}	1.58 ± 0.15^{b}	1.48 ± 0.07^{b}
Feed intake (% BW day^{-1})							
Week 4	7.48 ± 0.40^{b}	6.68 ± 0.99^{b}	6.64 ± 0.52^{b}	5.12 ± 0.79^{a}	4.47 ± 0.89^{a}	4.58 ± 0.92^{a}	6.74 ± 0.74^{b}
Week 8	3.45 ± 0.26^{c}	3.27 ± 0.30^{bc}	3.05 ± 0.51^{abc}	2.82 ± 0.08^{ab}	2.64 ± 0.46^{a}	2.82 ± 0.49^{ab}	3.30 ± 0.21^{bc}
Week 10	2.57 ± 0.25^{b}	2.52 ± 0.29^{b}	2.48 ± 0.17^{ab}	2.10 ± 0.09^{a}	2.08 ± 0.47^{a}	2.07 ± 0.42^{a}	2.55 ± 0.17^{b}

Mean ± SD from five replicates. Means within a row having different superscripts were significantly different ($p < 0.05$)

Table 4 Whole body chemical composition of Pacific white shrimp fed experimental diets for 10 weeks

Parameter	Diet (MEL + CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5 + 2.5)	Diet 3 (5 + 5)	Diet 4 (7.5 + 7.5)	Diet 5 (10 + 10)	Diet 6 (10 + 0)	Diet 7 (0 + 10)
Whole body composition (g kg^{-1} wet weight basis) (mean ± SD; n = 3 shrimp from each of the 5 replicate tanks)							
Dry matter	245.33 ± 0.21^{f}	248.70 ± 0.92^{g}	239.30 ± 0.95^{e}	231.23 ± 0.21^{c}	224.70 ± 0.30^{b}	221.83 ± 0.85^{a}	235.47 ± 0.55^{d}
Crude protein (nitrogen content × 6.25)	181.78 ± 0.19^{a}	202.75 ± 0.70^{d}	203.63 ± 0.50^{d}	195.25 ± 1.18^{c}	195.45 ± 0.45^{c}	192.08 ± 1.07^{b}	193.40 ± 0.89^{b}
Crude lipid	23.31 ± 0.46^{e}	17.91 ± 0.40^{d}	17.48 ± 0.18^{cd}	15.20 ± 0.28^{b}	13.93 ± 0.45^{a}	13.38 ± 0.20^{a}	17.12 ± 0.58^{c}
Crude ash	23.50 ± 0.40^{a}	23.41 ± 0.36^{a}	23.38 ± 0.56^{a}	23.66 ± 1.26^{a}	24.13 ± 0.42^{a}	23.37 ± 0.39^{a}	23.66 ± 0.74^{a}

Mean ± SD from five replicates. Means within a row having different superscripts were significantly different ($p < 0.05$)

Table 5 Blood parameters of Pacific white shrimp fed experimental diets for 10 weeks

Parameter	Diet (MEL + CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5 + 2.5)	Diet 3 (5 + 5)	Diet 4 (7.5 + 7.5)	Diet 5 (10 + 10)	Diet 6 (10 + 0)	Diet 7 (0 + 10)
Blood parameters							
Total hemocyte counts (THC, $\times 10^7$ cells mL^{-1})	3.92 ± 0.44^{e}	2.83 ± 0.57^{bc}	3.11 ± 0.59^{cd}	2.37 ± 0.42^{ab}	2.30 ± 0.39^{a}	2.92 ± 0.58^{c}	3.58 ± 0.37^{de}
Phenoloxidase (PO) activity (unit min^{-1} mg $protein^{-1}$)	132.69 ± 10.99^{c}	129.26 ± 23.58^{bc}	105.78 ± 23.56^{b}	81.74 ± 14.20^{a}	80.56 ± 29.08^{a}	78.76 ± 13.65^{a}	115.59 ± 26.75^{bc}
Respiratory burst activity (OD 630 nm)	0.063 ± 0.008^{b}	0.055 ± 0.007^{a}	0.054 ± 0.004^{a}	0.055 ± 0.006^{a}	0.055 ± 0.002^{a}	0.054 ± 0.005^{a}	0.054 ± 0.003^{a}
Lysozyme activity (unit mL^{-1})	16.75 ± 3.44^{b}	16.50 ± 3.45^{b}	16.59 ± 3.58^{b}	13.41 ± 4.05^{ab}	12.17 ± 2.71^{a}	12.67 ± 2.25^{a}	16.33 ± 4.52^{b}

Mean $\pm$ SD from ten individual shrimp. Means within a row having different superscripts were significantly different ($p < 0.05$)

Antioxidant enzyme activities

The antioxidant enzyme activities are presented in Table 6. Catalase (CAT) activity in both hepatopancreas and gill of shrimp treated with MEL+CYA doses of 5 + 5 or 10 + 10 g kg^{-1} diet, or with MEL alone at 10 g kg^{-1} diet, were significantly ($p < 0.05$) lower than with the control diet. Glutathione peroxidase (GPx) activity in hepatopancreas was significantly ($p < 0.05$) lower in shrimp fed MEL+CYA at 7.5 + 7.5 or 10 + 10 g kg^{-1} diet, or with MEL alone at 10 g kg^{-1} diet compared to those fed the control diet, whereas the GPx activity in gill was significantly ($p < 0.05$) affected only in the shrimp fed 10 + 10 g kg^{-1} diet combined dose of MEL+CYA or the MEL at 10 g kg^{-1} diet. Superoxide dismutase (SOD) activities in both hepatopancreas and gill were significantly ($p < 0.05$) lower in shrimp fed the MEL+CYA dose at 10 + 10 g kg^{-1} diet, or MEL alone at 10 g kg^{-1} diet.

Lipid peroxidation (LPO) in the hepatopancreas was significantly ($p < 0.05$) increased in the shrimp that were fed the combination MEL+CYA, increasing from 7.5 + 7.5 to 10 + 10 g kg^{-1} diet dose level, or MEL alone at 10 g kg^{-1} diet (Table 6). The hepato-somatic index (HSI) in shrimp receiving combined dose that increased from the 7.5 + 7.5 to the 10 + 10 g kg^{-1} diet dose level, giving a significantly ($p < 0.05$) lower index than the control diet (Table 6).

Digestive enzyme activities

Digestive enzyme activities in hepatopancreas are presented in Table 6. Compared to the control group (diet 1), total protease and trypsin activities were significantly lower ($p < 0.05$) in the shrimp fed combined MEL+CYA doses or the CYA at 10 g kg^{-1} diet. Furthermore, the shrimp fed combined MEL+CYA doses from 7.5 + 7.5 to 10 + 10 g kg^{-1} diet had significantly ($p < 0.05$) reduced amylase activity. Total protease, trypsin and amylase were affected by MEL alone at 10 g kg^{-1} diet ($p < 0.05$).

Histopathological changes

Histopathological alterations in the hepatopancreas of shrimp after 10-week dietary treatments containing MEL and CYA singly or in combination are presented in Fig. 1. The normal cells of hepatopancreas, tubular morphology and numbers of B-(blister-like) cells, F-(fibrillar) cells, and R-(resorptive/absorptive) cells were found in the control group (Fig. 1a). Severe atrophic changes, degenerative hepatopancreatic tubules, and lacking B-, F- and R-cells were detected in shrimp fed combined MEL+CYA doses from 2.5 + 2.5 to 10 + 10 g kg^{-1} diet (Fig. 1b–d). With MEL or CYA singly, degenerated and shrunken hepatopancreatic tubules were observed (Fig. 1e–f).

Discussion

Since the pet food crisis in 2007, MEL and its analogs including CYA have been illegally used to elevate the apparent total protein content, thereby adulterating certain protein-rich feed ingredients (Brown et al. 2007; Puschner et al. 2007; Stine et al. 2011). There have been reports of MEL and related analogs in feed ingredients and in animal feeds for swine, poultry, fish, and shrimp (Andersen et al. 2008; Stine et al. 2012). Negative effects from such adulteration in animals have been reported, having as symptoms nephrotoxicity, growth retardation, discoloration, and immune suppression (Baynes et al. 2008; Reimschuessel et al. 2008, 2010; Xue et al. 2011; Phromkunthong et al. 2013, 2015a, b). However, data relating to the toxicity in penaeid shrimp of MEL or CYA exposures is limited. In this study, the toxicity of MEL and CYA to Pacific white shrimp was examined over 10 weeks of laboratory conditions. The results from this study demonstrate depressed growth and negative effects by dietary MEL-CYA. In the present study, combined MEL+CYA from 2.5 + 2.5 to 10 + 10 g kg^{-1} diet had negative effects on shrimp growth and feed conversion ratio. As previously reported, growth depression is a clinical sign of the toxicity of combined MEL and CYA that has been reported in mice (Lv et al. 2013) and in some farmed fish species, such as red tilapia (Phromkunthong et al. 2013, 2015a) and Asian sea bass (Phromkunthong et al. 2015b). In addition, the combination of MEL and CYA is more toxic than exposure to either chemical separately (Karbiwnyk et al. 2010). However, in this

Table 6 Antioxidant enzyme activities, lipid peroxidation in hepatopancreas and digestive enzyme activities of Pacific white shrimp fed experimental diets for 10 weeks

Parameter	Target organs	Diet (MEL + CYA, g kg^{-1} diet)						
		Diet 1 (Control)	Diet 2 (2.5 + 2.5)	Diet 3 (5 + 5)	Diet 4 (7.5 + 7.5)	Diet 5 (10 + 10)	Diet 6 (10 + 0)	Diet 7 (0:10)
Antioxidant enzyme activities (Unit mg protein^{-1})								
CAT	Hepatopancreas	189.17 ± 25.83^{b}	159.50 ± 37.82^{ab}	136.61 ± 26.32^{a}	135.61 ± 34.76^{a}	138.82 ± 35.91^{a}	136.82 ± 44.89^{a}	165.59 ± 28.16^{ab}
	Gill	72.69 ± 11.49^{b}	60.86 ± 20.80^{ab}	48.65 ± 11.68^{a}	49.30 ± 19.88^{a}	44.43 ± 13.79^{a}	46.60 ± 9.20^{a}	61.23 ± 16.53^{ab}
GPx	Hepatopancreas	5237.67 ± 421.88^{d}	4927.92 ± 615.59^{cd}	4719.16 ± 416.49^{bcd}	4656.57 ± 339.32^{bc}	4105.59 ± 500.56^{a}	4193.15 ± 538.86^{ab}	4864.21 ± 518.49^{cd}
	Gill	684.63 ± 101.97^{b}	564.66 ± 142.52^{ab}	593.87 ± 103.82^{ab}	555.10 ± 159.37^{ab}	512.00 ± 65.45^{a}	544.14 ± 119.54^{a}	589.13 ± 80.61^{ab}
SOD	Hepatopancreas	5.58 ± 1.14^{c}	4.85 ± 0.36^{c}	4.13 ± 0.85^{bc}	4.44 ± 1.33^{bc}	3.30 ± 0.98^{ab}	2.18 ± 0.59^{a}	4.39 ± 0.91^{bc}
	Gill	6.75 ± 0.35^{b}	6.02 ± 0.87^{ab}	5.89 ± 0.769^{ab}	5.77 ± 0.86^{ab}	5.59 ± 0.69^{a}	5.56 ± 0.37^{a}	6.30 ± 0.40^{ab}
Lipid peroxidation (LPO) in hepatopancreas (nmol g^{-1} wet weight tissue) and hepato-somatic index (HSI)(%)								
LPO	Hepatopancreas	9.86 ± 2.53^{a}	11.47 ± 2.08^{ab}	11.59 ± 2.74^{ab}	13.03 ± 2.51^{b}	13.10 ± 2.75^{b}	13.04 ± 2.64^{b}	9.95 ± 2.69^{a}
HSI	Hepatopancreas	6.14 ± 0.66^{b}	6.24 ± 1.40^{b}	5.57 ± 0.64^{ab}	5.21 ± 0.90^{a}	5.20 ± 0.88^{a}	5.41 ± 0.48^{ab}	6.00 ± 1.24^{ab}
Digestive enzyme activities (unit mg protein^{-1})								
Total protease	Hepatopancreas	0.160 ± 0.026^{b}	0.128 ± 0.037^{a}	0.107 ± 0.026^{a}	0.110 ± 0.024^{a}	0.120 ± 0.016^{a}	0.123 ± 0.021^{a}	0.137 ± 0.031^{a}
Trypsin	Hepatopancreas	0.176 ± 0.018^{b}	0.137 ± 0.026^{a}	0.131 ± 0.043^{a}	0.124 ± 0.021^{a}	0.131 ± 0.020^{a}	0.128 ± 0.023^{a}	0.148 ± 0.034^{a}
Amylase	Hepatopancreas	23.25 ± 2.68^{b}	22.85 ± 5.00^{b}	20.34 ± 4.52^{ab}	18.35 ± 3.49^{a}	19.11 ± 2.57^{a}	16.53 ± 3.56^{a}	23.49 ± 4.69^{b}

Mean ± SD from ten individual shrimp. Means within a row having different superscripts were significantly different ($p < 0.05$)

CAT catalase, *GPx* glutathione peroxidase, *SOD* superoxide dismutase

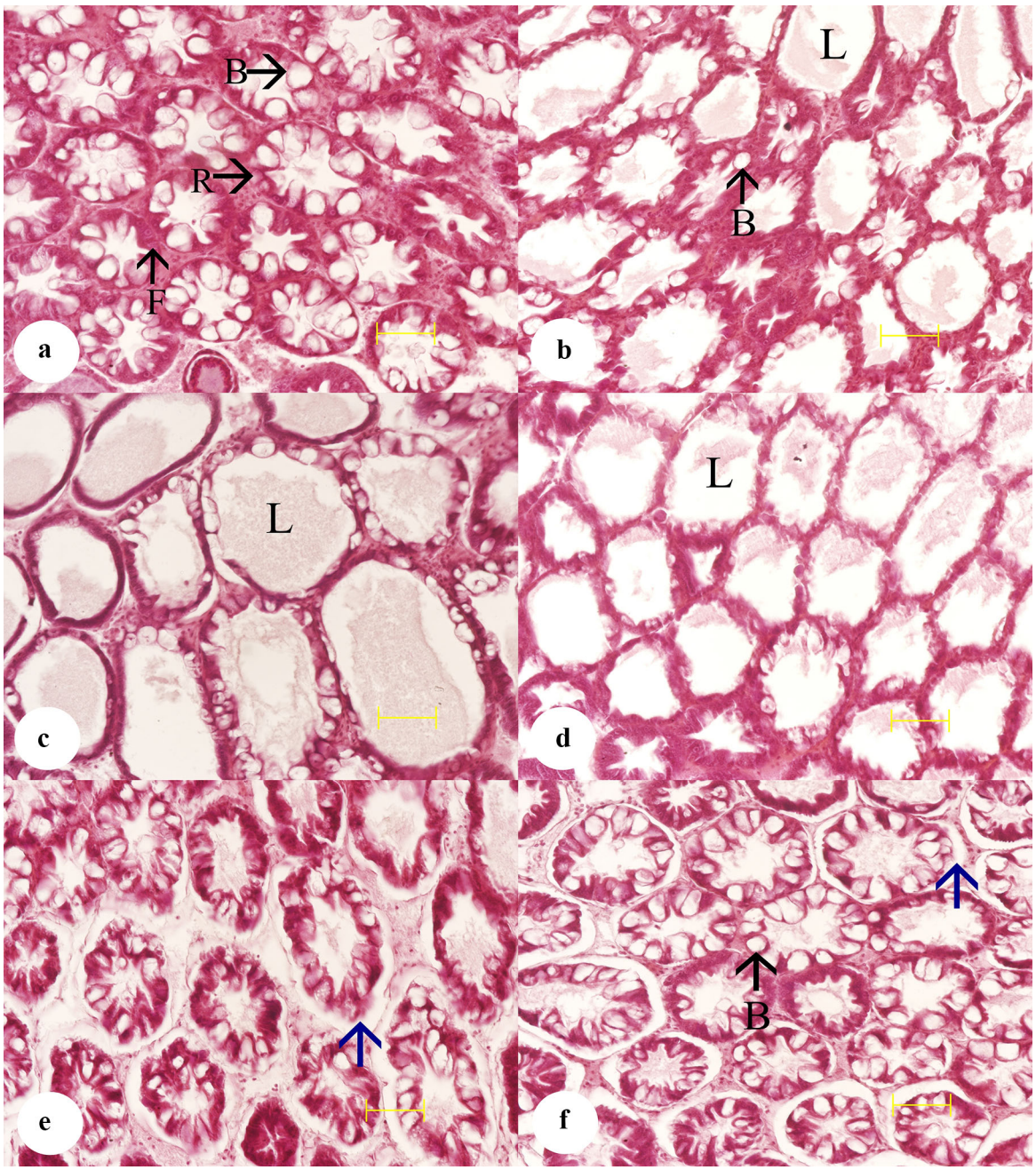

Fig.1 Photomicrographs of haematoxylin and eosin (H&E) stained sections of hepatopancreas from Pacific white shrimp fed diets containing various levels of MEL and/or CYA for 10 weeks. **a** The sections of hepatopancreatic tubules of shrimp fed the control diet showing normal structure of R ("restzellen") cells, F ("fibrillenzellen" or fibrous) cells and B ("blasenzenllen") cells (magnification × 200; scale bar 100 μm); **b** atrophic changes and degenerated tubules were observed in shrimp fed a combination MEL+CYA at 2.5 + 2.5 g kg^{-1} diet (L = lumen, magnification × 200; scale bar 100 μm); **c** shrimp receiving MEL+CYA at 5 + 5 g kg^{-1} diet showed severe atrophic changes of hepatopancreatic tubule (L = lumen, magnification × 200; scale bar 100 μm); **d** shrimp receiving MEL+CYA at 10 + 10 g kg^{-1} diet showed degenerative changes in the hepatopancreas (L = lumen, magnification × 200; scale bar 100 μm); degenerated and shrunken hepatopancreatic tubules (blue arrow) were observed in shrimp receiving MEL alone (10 + 0 g kg^{-1} diet) (**e**) or CYA alone (0 + 10 g kg^{-1} diet) (**f**) (magnification × 200; scale bar 100 μm)

study, the growth performance of shrimp receiving MEL alone at 10 g kg^{-1} diet was significantly lower than in the control group. In another experimental study, the growth performance and feed utilization of black tiger shrimp (*Penaeus monodon*) were not significantly affected by dietary MEL doses ranging from 0.005 to 0.5 g

kg^{-1} diet for 11 weeks (Samranrat et al. 2011). Therefore, the safe dose level for Pacific white shrimp should not exceed 0.5 g kg^{-1} diet. Furthermore, the results in this current study show that shrimp exposure to dietary CYA decreased growth. Data on adulteration by CYA alone in shrimp feed is limited. In our previous study, Phromkunthong et al. (2015b) reported no significant difference in the growth of Asian seabass between receiving CYA alone and control. That the reported results tend to differ qualitatively suggests that sensitivity to these chemicals strongly depends on the species.

In fish and higher animals, the toxic effects of MEL and CYA singly or in combination are directly related to kidney pathology and functions (Dorne et al. 2013). One possible reason for the growth depression and poor feed utilization is directly linked to the dysfunction of hepatopancreas, which is an important organ for several metabolic functions, including nutrient digestion, absorption, and storage, in crustaceans (Burgos-Hernández et al. 2005; Zhao et al. 2017). Activities of the digestive enzymes are indicative of the feeding habits, diet composition, digestive functions, and nutrient utilization, which directly relate to shrimp growth (Li et al. 2008a; Zokaeifar et al. 2012; Duan et al. 2017). The function of digestive enzymes in shrimp can be influenced by diet composition (Córdova-Murueta and García-Carreño 2002; Song et al. 2017). In the present study, the reductions of digestive enzyme activities (especially of protease) were marked in shrimp receiving MEL alone or in combination with CYA. However, prior reports on effects of MEL+CYA on the digestive enzymes in shrimp are limited. In some cases, exposure to toxic substances (e.g., heavy metals, pesticides and toxins) can adversely affect the digestive function (Li et al. 2008b; Chiodi Boudet et al. 2015). Moreover, our study found that shrimp dosed combined MEL+CYA up to 7.5 + 7.5 g kg^{-1} diet or MEL alone had significantly decreased enzyme activities, indicating poor nutrient absorption and storage. Similar to our results, Seebaugh et al. (2011) reported a significant decrease in digestive enzyme activities of shrimp that was related to reduced food ingestion.

Furthermore, hepatopancreas is an important organ for the chemical detoxification of shrimp, and is sensitive to stress responses (Bautista et al. 1994; Yu et al. 2016). Exposure to chronic stress elevates the generation of reactive oxygen species (ROS) leading to an imbalance between ROS and antioxidant enzyme activities, which results in cellular damage from oxidative stress (Yang et al. 2010; An et al. 2015; Lee et al. 2016). In this study, the activities of CAT, GPx and SOD were significantly decreased in both gill and hepatopancreas of shrimp fed the highest combination level of MEL-CYA (10:10 g kg^{-1} diet) or MEL alone (10 g kg^{-1} diet). This suggests that the main toxic effects of MEL and CYA take place via oxidative stress, resulting in cellular damage and dysfunction of the related detoxification organs in shrimp, which adversely affects growth (Li et al. 2008a). Evidence of oxidative stress induced by the combination of MEL and CYA in diet has been reported by Lv et al. (2013), who found that the activity of SOD in mouse kidney was decreased after 13-week exposure to a mixture of MEL and CYA. In addition, You et al. (2012) found significantly decreased total antioxidant capacity (T-AOC) and SOD activity in mouse testes after a mixture of MEL and CYA was orally administered for 28 days.

Malondialdehyde (MDA) is among the main lipid peroxidation indicators in cells and tissues, and increased MDA levels indicate elevated oxidative stress (Zenteno-Savín et al. 2006; Liu et al. 2011; Chiodi Boudet et al. 2015; Liang et al. 2016). In this study, our results showed that the MDA content in hepatopancreas of shrimp fed combined MEL+CYA up to 7.5 + 7.5 g kg^{-1} diet, or MEL alone, was significantly elevated from that with the control diet. This indicates oxidative damage induced by these chemicals. A similar result was also reported by Lee et al. (2016), who found elevated lipid peroxidation in mouse kidney induced by co-exposure to MEL and CYA, which also caused apoptotic changes in the renal tubular cells and suppressed antioxidant enzyme activities. In melamine-exposed animals, An et al. (2015) reported increased MDA levels in the hippocampus after oral administration of MEL to male Wistar rats (dosage 300 mg kg^{-1} animal day^{-1} for 28 days). Moreover, there are several reports on significantly increased MDA level by stress in many aquatic animals. Liang et al. (2016) reported that MDA levels in hepatopancreas significantly increased after exposure to ammonia for 96 h.

The hepato-somatic index (HSI) is an important parameter indicative of hepatopancreas conditions, as well as of glycogen reserve. In the present study, HSI was significantly decreased in shrimp fed MEL alone or in combination with CYA at 7.5 + 7.5 or 10 + 10 g kg^{-1} diet, relative to the control group. This suggests that long-term diet supplementation with MEL and CYA to shrimp in their feed caused extra energy expenditures in detoxification. Moreover, reduced energy storage may have been induced indirectly through anorexia caused by MEL or CYA (Khalil et al. 2017).

Hemolymph is an indicator of shrimp performance, health status, and rearing conditions, and is closely related to the innate immune system (Li et al. 2008a; Chen et al. 2015; Qiu et al. 2016). In crustaceans, THC and PO system play important roles in the immune system, especially in shrimp (Sritunyalucksana and

Söderhäll 2000; Li et al. 2008a; Wanlem et al. 2011). In the present study, the immune parameters in shrimp fed diets supplemented with MEL alone or in combination with CYA at the highest dose (10 + 10 g kg^{-1} diet) had decreased THC and PO activities. Moreover, Yin et al. (2016) reported that MEL alone or its combination with CYA appeared to be toxic to the immune system of mice.

The results from this study indicate that crude protein content in shrimp treated with a combination of MEL and CYA, or either of these singly, was significantly increased as compared to control after 10 weeks of feeding. In general, the protein content in the whole body of an animal, including fish and shrimp, is determined by the dietary protein levels and their quality, digestibility, and feeding (Halver and Hardy 2002; Kureshy and Davis 2002). The increased protein retention suggests improved protein utilization positively affecting growth performance (Glencross et al. 2007). In our current study, in contrast to growth, increased protein content was recorded in shrimp treated with MEL and CYA singly or in combination. These results agree with previous reports regarding red tilapia (Phromkunthong et al. 2013, 2015a) and Asian seabass (Phromkunthong et al. 2015b), indicating that the protein content in the whole body and in various parts of fish, such as filet or viscera, directly increased in response to dietary MEL+CYA. Therefore, the increased protein content of shrimp whole body in the MEL+CYA treated group may be attributed to the accumulation of nitrogen from these substances. Karbiwnyk et al. (2010) reported accumulation of CYA in edible tissues at 0.767 and 0.406 mg kg^{-1} in shrimp fed with 1666 or 3333 mg kg^{-1} diet CYA (approximately 55 and 124 mg kg^{-1} bodyweight) in their diet, respectively. However, the behavior of these substances in a shrimp body is not well-understood; the pharmacokinetics/dynamics need to be further studied.

Histological analysis of hepatopancreas has been used as a practical means for assessing the nutritional condition and toxic substance accumulation in the shrimp (Qiu et al. 2016). According to our current study, MEL and CYA directly affect the hepatopancreas. In fish, the presence of melamine–cyanurate crystals in kidney is the main pathological sign of chronic exposure to these compounds, and leads to renal nephropathy (Reimschuessel et al. 2010; Pirarat et al. 2012; Phromkunthong et al. 2015a, b). Previously, in Thailand, "water sac syndrome" or "big head syndrome" have been claimed to be pathological signs in shrimp exposed to MEL and CYA contamination (Limsuwan et al. 2009; WHO 2009). Similar results have been reported by Lightner et al. (2009), who found that shrimp fed MEL and CYA in their diets could have insoluble crystals in the antennal glands, obstructing and impairing the excretory system and causing swollen cephalothorax. Moreover, the results of our current study indicate severe histopathological alterations including severe atrophic changes and necrosis in hepatopancreas of shrimp exposed to MEL and CYA for 10 weeks.

Conclusion

This study investigated the responses of Pacific white shrimp, *L. vannamei,* to MEL and CYA exposures either singly or in combination to understand the mechanisms of toxicity. Results of the present study indicate that growth performance and feed utilization were negatively affected by MEL and CYA exposure, with reductions in mean weight gain, average daily gain, specific growth rate and an increase in the feed conversion ratio. Exposure to these substances also damaged the cells of hepatopancreas, leading to the generation of oxidative stress, increased lipid peroxidation and decreased antioxidant and immune responses, which demonstrate that the hepatopancreas of shrimp is sensitive to MEL and CYA. In summary, our results provide some basic information for a toxicological risk assessment of MEL and CYA in the context of shrimp farming. It is noted that further studies should assess the accumulation of these substances in the edible part of shrimp to address public concerns of health risks to consumers.

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