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Individual and Combined Effects of Melamine and Cyanuric Acid in Young Pekin Ducks

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Abstract: An experiment was conducted with Pekin ducks to determine the toxicity of melamine (MEL) and cyanuric acid (CYA) in ducks fed treatments from day 3 to day 21 of age. Two hundred and twenty three three-day-old male ducks were assigned to one of 10 treatment groups. Treatments included: (1) a basal diet (BD) containing no MEL or CYA; (2) BD+0.5% MEL; (3) BD+1.0% MEL; (4) BD+1.5% MEL; (5) BD+0.5% CYA; (6) BD+1.0% CYA; (7) BD+1.5% CYA; (8) BD+0.5% MEL+0.5% CYA; (9) BD+1.0% MEL+1.0% CYA and (10) BD+1.5% MEL+1.5% CYA. Control and treatments fed MEL alone or MEL+CYA were fed to 5 pens of 5 ducks each. Treatments fed CYA alone were fed to 4 pens of 4 ducks each. Compared to controls, birds fed ≥ 1.0% MEL had lower (P<0.05) feed intake (FI) and body weight gain (BWG), heavier (P<0.05) relative kidney weights and higher (P<0.05) mortality. No mortality was observed in birds fed CYA alone or MEL+CYA combinations. No differences in FI, BWG, relative kidney weights or mortality were noted among controls and ducks fed CYA alone or MEL+CYA combinations. Melamine crystals were only observed in the bile of ducks fed≥1.0% MEL alone. Renal histopathology included mild dilation of the embryonal nephrons and collecting tubules. Eosinophilic to basophilic casts, some containing spherical eosinophilic crystals were also present in the embryonal nephrons and collecting tubules. Histopathology results suggested that ≥1.00 % MEL in the diet of ducks could cause severe renal pathology and mortality due to renal failure. The renal pathology observed in ducks was similar to that seen in other poultry species fed toxic concentrations of MEL. CYA alone up to 1.5% of the diet was not toxic to ducks and CYA reduced the toxicity of MEL when the two compounds were fed in combination.

Key words: Melamine, cyanuric acid, kidney, crystal, pekin duck

INTRODUCTION

Melamine ($C_3H_6N_6$; MEL) is a white, crystalline powder (OSHA, 2006) with a wide variety of industrial applications including use in the manufacturing of plastics, adhesives, laminates, paints, flame retardants, textile finishes and fertilizers (Hilts and Pelletier, 2009). Melamine is 66% nitrogen by mass and if added to any matrix will artificially increase the protein content of the matrix when the Kjeldahl method is used for protein analysis (Yang *et al*., 2009). Therefore, MEL has been added to feed ingredients to increase their apparent crude protein value and subsequently their monetary value (WHO, 2008). Cyanuric acid (CYA) along with ammeline and ammelide are structural analogues of MEL (Tyan *et al*., 2009; WHO, 2008) and all belong to the class of chemicals known as *s-triazines* (Wackett *et al*., 2002). Hydrolysis or amination of one *s-triazine* can result in the production of another *s-triazine* (Baynes and Riviere, 2010).

There are several possible mechanisms by which MEL and related compounds can be found in biological systems simultaneously. Conversion of MEL to CYA can occur during the manufacturing of MEL resulting in a final product that is not 100% pure MEL (Dobson *et al*., 2008). Jutzi *et al*. (1982) reported that bacteria can convert MEL to CYA. However, Filigenzi *et al*. (2007) stated that no conversion of MEL to related compounds is known to occur in mammals. Baynes and Riviere (2010), however, suggest that bacterial degradation of MEL could possibly take place in the gastrointestinal tract.

In 2007, the deaths of cats and dogs related to renal failure were documented across North America and South Africa (Hilts and Pelletier, 2009). It was later determined that wheat gluten, imported from China and incorporated into pet food, had been contaminated with MEL and CYA (Hilts and Pelletier, 2009). Brown *et al*. (2007) examined six dogs and ten cats that consumed contaminated pet food and observed that all had elevated blood urea nitrogen (BUN) and creatinine levels as well as polarizable crystals present in the distal tubules and collecting ducts of their kidneys.

Toxicity in mammals occurs because of the ability of MEL and CYA to self-assemble and form a hydrogen-

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bonded bimolecular network (Perdigao *et al*., 2006). Self-assembly can lead to the formation of insoluble compounds that can precipitate in the kidneys leading to renal failure (Seffernick *et al*., 2010). Dobson *et al*. (2008) and Tolleson (2009) described possible routes of absorption of MEL and related compounds and the potential for precipitation of the insoluble compounds which can lead to renal failure.

Following reports of contamination of pet food in 2007, it was determined that waste material containing MEL and CYA acid from pet food manufacturing was incorporated into swine, poultry and aquaculture feeds (Buur *et al*., 2008; Karbiwnyk *et al*., 2009; USDA, 2007). Several studies were then conducted to determine the effects of MEL and CYA, alone or in combination on animal health and possible residue levels in meat destined for human consumption (Brand, 2011; Reimschuessel *et al*., 2008; Stine *et al*., 2011; Landers *et al*., 2012).

Brand (2011) conducted several experiments to determine the effects of dietary MEL and CYA, alone or in combination, in broilers and poults fed dietary treatments from hatch to 21 days of age and reported that MEL alone significantly depressed growth performance of young broilers and poults when it was included in the diet at ≥1.0 and ≥1.5%, respectively. Inclusion of up to 3.0% CYA in diets of young broilers and poults did not affect growth performance or kidney function (Brand, 2011). The inclusion of CYA in diets that contained MEL (0.5, 1.0 and 1.5% of each compound) reduced the negative effects of MEL in poults (Brand, 2011). However, in young broilers the combination of MEL and CYA resulted in poorer growth performance and production of crystals in the kidneys as compared to either compound alone (Brand, 2011).

Previous research on young Pekin ducks demonstrated that feeding ≥1.0 % MEL reduced feed intake and body weight gain and increased relative kidney weights. (Landers *et al*., 2012). Data also indicated that ≥1.5% melamine in the diet could cause renal pathology and mortality due to renal failure, similar to that observed in other poultry species (Landers *et al*., 2012). High-performance liquid chromatography analysis of tissues collected from ducks fed graded levels of MEL revealed that ≥0.25 and ≥0.75% MEL resulted in increased MEL residue levels in the kidney and breast muscle, respectively (Landers *et al*., 2012).

The objective of the current study was to determine the effects of feeding MEL and CYA, alone or in combination, to Pekin ducks from day 3 to 21 days of age. Dietary treatments included MEL and CYA individually at 0.5, 1.0 and 1.5% of the diet and combinations of 0.5, 1.0 and 1.5% of both compounds.

MATERIALS AND METHODS

The animal care and use protocol was reviewed and approved by the University of Missouri-Columbia Animal Care and Use Committee (ACUC).

Diet preparation: A basal diet (Table 1) was formulated to meet or exceed all requirements of young Pekin ducks as suggested by the National Research Council (NRC, 1994). Ten dietary treatments were prepared by adding MEL or CYA purchased from Fisher Scientific to the basal diet. MEL and CYA were substituted for sand to obtain the desired dietary concentrations: (1) Control basal diet (BD) containing no MEL or CYA); (2) BD+0.50% MEL; (3) BD+1.00% MEL; (4) BD+1.50% MEL; (5) BD+0.50% CYA; (6) BD+1.00% CYA; (7) BD+1.50% CYA; (8) BD+0.50% MEL and 0.50% CYA; (9) BD+1.00% MEL and 1.00% CYA and (10) BD+1.50% MEL and 1.50% CYA.

Table 1: Ingredients and nutrient composition of basal diet

Ingredient	Composition (%)
Corn	58.14
Soybean meal	35.04
Dicalcium phosphate	1.46
Limestone	0.51
Soybean oil	1.06
Salt	0.33
Vitamin/mineral mix[1]	0.35
DL-methionine	0.11
Sand	3.00
Total	100.00
Nutrient composition (calculated)	
Crude protein (%)	22.00
Metabolizable energy (kcal/Kg)	2.900
Lysine (%)	1.19
Methionine (%)	0.44
Methionine+cysteine (%)	0.80
Threonine (%)	0.82
Calcium (%)	0.65
None phytate phosphorus (%)	0.40

[1]Supplied per kilogram of feed: manganese, 140 mg; zinc, 140 mg; iron, 70 mg; copper, 15.75 mg; iodine, 2.1 mg; selenium, 0.21 mg; Vitamin A, 10,780 IU; vitamin D3, 3,850 ICU; vitamin E, 23.1 IU; vitamin B12, 15.4 μg; vitamin K, 1.16 mg; riboflavin, 9.24 mg; thiamin, 1.54 mg; pantothenic acid, 9.24 mg; niacin, 38.5 mg; pyridoxine, 1.92 mg; folic acid, 0.96 mg; choline, 539 mg; biotin, 46.2 μg

Birds, management and response variables: Two hundred and twenty three day-old male Pekin ducks were purchased from a commercial hatchery, weighed, leg banded and assigned to pens in stainless steel batteries. A completely randomized design was used. Controls and dietary treatments fed diets containing MEL alone or MEL+CYA were fed to five replicates pens of five ducks each. Dietary treatments containing CYA alone were fed to four replicate pens of four ducks each. Ducks were housed in an environmentally controlled room and placed on a 24 h constant light schedule. Feed and water were supplied for *ad libitum* consumption from day 3-21. Ducks were observed daily and mortality was recorded as it occurred. All ducks that died before day 21 were weighed and sent to the Avian Pathology Laboratory at the University of Missouri's (Columbia, MO) Veterinary Medical Diagnostic Laboratory for necropsy.

On day 21, ducks and feed were weighed and average body weight gain, average feed intake and feed conversion were calculated. All ducks were then anesthetized with carbon dioxide and blood samples were collected via cardiac puncture from ten ducks per treatment. Blood samples were centrifuged (Sorvall, RC 3 B plus) at 1, 400 x g for 30 minutes at 7°C and serum was separated and frozen until analyzed. Serum samples were analyzed for glucose (GLU), albumin (ALB), total protein (TP), globulin (GLOB), calcium (Ca), asparate transaminase (AST), gamma glutamyl-transerase (GGT) and uric acid (UA) at the University of Missouri Veterinary Clinical Laboratory (Columbia, MO). Following blood collection, ducks were euthanized by cervical dislocation. The liver and kidneys were removed from three ducks per pen and weighed. Relative liver and kidney weights were calculated by dividing organ weight by body weight. Sections of liver and kidney from six ducks per treatment were fixed in 10% neutral buffered formalin for histopathological evaluation. Samples of liver, kidney, breast muscle and bile were collected from all treatments and frozen for analysis of MEL concentrations.

Melamine analysis: Melamine extraction from tissue and bile samples was based on the method used by Brand *et al.* (2012) and involved high-performance liquid chromatography (HPLC) with UV detection. For kidney and muscle, 10 mL of water:acetonitrile (1:2) was added to 1 g of tissue and the tissues homogenized (Bio Homogenizer, model M133/1281-0, Biospec Products Inc. Bartlesville OK 74005) for 30 sec in a 50 mL conical centrifuge tube. The homogenized sample was then centrifuged for 5 min at 1,000 rpm (Dynac II centrifuge) and the supernatant was transferred to microcentrifuge tubes and further centrifuged for 5 min at 10,000 rpm (Spectrafuge 16M). The supernatant was extracted and passed through a MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the filtered supernatant was diluted (1:1) with buffer solution (BUFF; 1.924 g citric acid and 2.34 g of octanesulfonate dissolved in 1 L of distilled water, pH adjusted to 3 using NaOH) before HPLC analysis was performed.

For bile, extraction involved adding 200 µL of bile to 1,800 µL of water:acetonitrile (1:2), vortexing and transferring the samples to microcentrifuge tubes and centrifuging for 5 min at 10,000 rpm (Spectrafuge 16M). The supernatant was collected and passed through a MycoSep® 224 AflaZon column (Romer Labs, 2011). Finally, 500 µL of the eluant was diluted (1:1) with BUFF before HPLC analysis was performed.

A Hitachi Model L-7100 pump with a Hitachi Model L-7485 UV detector, Hitachi Model L-7200 autosampler with Hitachi D-7000 data acquisition interface and ConcertChrom software on a microcomputer were used for HPLC analysis. A HyperClone (Phenomenex) C_{18} column (100 x 4.60 mm) was used with a mobile phase consisting of BUFF:acetonitrile (ACN; 87:13) at a flow rate of 1 mL/min and a MEL retention time of 6 min. UV detection was at 240 nm.

A primary standard of consisting of a 2,000 ppm MEL solution was diluted with BUFF:ACN (1:1) to prepare standards of 1, 5, 10 and 20 ppm MEL. MEL standards were ran before and after each set of samples and used to calculate a standard curve. The area under the peak was used to calculate individual MEL concentrations in samples from the standard curve.

Statistical analysis: Data were analyzed using the general linear model procedures of Statistical Analysis Software (SAS, 2006). Pen was the experimental unit for all response variables except for serum chemistry where bird was the experimental unit. Variables that showed significant differences in the ANOVA were compared using Fisher's protected least significant difference procedure (SAS, 2006). Statistical significance was accepted at a P-value of $\leq$0.05. An arcsine transformation was applied to percent mortality data before statistical analysis was performed. Data for MEL residue levels in tissues were ranked based on a method developed by Conover and Iman (1981), before analysis.

RESULTS

Performance and mortality: The effects of MEL and or CYA on body weight gain, feed intake, feed conversion and mortality are summarized in Table 2. Inclusion of MEL at 0.50% of the diet did not negatively affect body weight gain (BWG), feed intake (FI), or percent mortality. However, ducks fed $\geq$ 1.00% MEL had lower (P<0.05) BWG and FI and higher (P<0.05) percent mortality than control ducks. Birds fed 1.50 % MEL also had poorer (P<0.05) feed conversion than that of control birds.

The addition of up to 1.50% CYA to the basal diet did not negatively affect body weight gain, feed intake, feed conversion, or mortality. The addition of CYA to diets containing MEL, in a 1:1 ratio of CYA to MEL, prevented the reduction in growth performance and increased mortality caused by MEL alone. As a result, there were no differences in BWG, FI, feed conversion, or percent mortality among controls and birds fed combinations (up to 1.50 % of each compound) of MEL and CYA.

Organ weights: The effects of MEL, CYA and combinations of MEL+CYA on organ weights of young Pekin ducks are summarized in Table 2. There was no effect of MEL or CYA, alone or in combination, on relative liver weights of ducks. Ducks fed $\geq$1.0% MEL had heavier (P<0.05) relative kidney weights than controls. Relative kidney weights of ducks fed up to 1.5% CYA and ducks fed combinations of CYA and MEL were similar to those of control ducks.

Table 2: Individual and combined effects of melamine and cyanuric acid on performance and organ weights of Pekin ducks

Treatment[1]		Response variables[2]					
Melamine (%)	Cyanuric acid (%)	BWG (g)	FI (g)	F:G (g:g)	Mortality[3] (%)	Liver[4] (%)	Kidney[4] (%)
0.00	0.00	897^{ab}	1,857^{a}	2.07^{bc}	0^{c}	3.74	1.11^{c}
0.50	0.00	857^{ab}	1,783^{a}	2.09^{bc}	4^{bc}	4.01	1.26^{bc}
1.00	0.00	660^{c}	1,361^{b}	2.25^{b}	16^{b}	3.89	1.55^{b}
1.50	0.00	447^{d}	868^{c}	2.49^{a}	44^{a}	3.97	2.22^{a}
0.00	0.50	923^{a}	1,897^{a}	2.05^{c}	0^{c}	3.94	1.12^{c}
0.00	1.00	880^{ab}	1,759^{a}	2.00^{c}	0^{c}	4.06	1.11^{c}
0.00	1.50	870^{ab}	1,826^{a}	2.11^{bc}	0^{c}	3.90	1.04^{c}
0.50	0.50	871^{ab}	1,820^{a}	2.09^{bc}	0^{c}	4.02	1.14^{c}
1.00	1.00	839^{ab}	1,721^{a}	2.05^{c}	0^{c}	3.91	1.16^{c}
1.50	1.50	829^{b}	1,701^{a}	2.05^{c}	0^{c}	3.79	1.08^{c}
	S.E.M.	28	68	0.06	0.1	0.19	0.12

[1]Treatments were the addition of melamine and or cyanuric acid in percent indicated, to the basal diet. Diets were fed from day 3 to 21. [2]Data are means of five replicate pens with five ducks per pen for control and treatments fed melamine alone or melamine + cyanuric acid. Data are means of four replicate pens with four ducks per pen for treatments fed cyanuric acid alone.
[3]Means are percent of mortality that occurred out of the total number of ducks in each treatment. Statistical analysis was performed on transformed data (arcsine).
[4]Relative organ weights expressed as a percentage of body weight.
[a-d]Means within a column with no common superscript are different (P<0.05).
BWG = Body weight gain; FI = Feed intake; F:G = Feed to gain.

Table 3: Individual and combined effects of melamine and cyanuric acid on serum chemistry of Pekin ducks

Treatment[1]		Response variables[2]							
Melamine (%)	Cyanuric acid (%)	GLU (mg/dL)	ALB (g/dL)	TP (g/dL)	GLOB (g/dL)	Ca (mg/dL)	AST (U/L)	GGT (U/L)	UA (mg/dL)
0.00	0.00	223	1.31^{c}	3.18^{cd}	1.87^{cd}	11.69	62.7	3.6	5.75^{c}
0.50	0.00	188	1.38^{bc}	3.39^{bc}	2.01^{bc}	12.14	21.1	3.9	7.95^{c}
1.00	0.00	223	1.50^{ab}	3.68^{ab}	2.18^{b}	11.63	55.2	4.1	15.56^{b}
1.50	0.00	349	1.55^{a}	3.96^{a}	2.41^{a}	12.34	94.2	3.6	25.48^{a}
0.00	0.50	291	1.28^{c}	3.17^{cd}	1.89^{cd}	11.14	59.5	3.4	8.38^{c}
0.00	1.00	254	1.26^{c}	2.98^{d}	1.72^{d}	12.03	59.2	2.8	6.11^{c}
0.00	1.50	208	1.28^{c}	3.05^{d}	1.77^{d}	11.79	34.7	3.3	4.83^{c}
0.50	0.50	222	1.34^{c}	3.22^{cd}	1.88^{cd}	11.88	59.9	3.1	5.45^{c}
1.00	1.00	271	1.27^{c}	3.07^{d}	1.80^{d}	11.69	17.7	3.9	4.70^{c}
1.50	1.50	269	1.25^{c}	3.03^{d}	1.78^{d}	11.78	24.4	3.4	6.13^{c}
	S.E.M.	38	0.05	0.11	0.06	0.41	25.95	0.3	2.35

[1]Treatments were the addition of melamine and or cyanuric acid in percent indicated, to the basal diet. Diets were fed from day 3-21. [2]Data are means of ten ducks per treatment.
[a-d]Means within a column with no common superscript are different (P<0.05).
GLU = Glucose; ALB = Albumin; TP = Total protein; GLOB = Globulin; Ca = Calcium; AST = Asparate transaminase; GGT = Gamma glutamyltranserase; UA = Uric acid

Serum chemistry: Table 3 shows the effects of MEL and CYA, alone or in combination, on the serum chemistry of young Pekin ducks. Serum levels of GLU, Ca, AST and GGT were not affected by dietary treatments. Serum ALB, TP, GLOB and UA were elevated (P<0.05) above control levels in ducks fed ≥1.00% MEL in the diet. Serum levels of ALB, TP, GLOB and UA of birds fed up to 1.5% CYA were similar to levels of control ducks. Similarly, serum concentrations of ALB, TP, GLOB and UA of birds fed combinations of MEL and CYA were similar to those of control ducks.

Tissue residues: The effects of MEL and CYA, alone or in combination, on residue levels of MEL in the kidneys, breast muscle and bile of the ducks are summarized in Table 4. Inclusion of MEL at ≥0.50% of the diet increased (P<0.05) MEL concentrations in kidneys and breast muscle compared to control ducks. No MEL was detected in the kidneys or breast muscle of ducks fed up to 1.50% CYA. Ducks fed combinations of MEL+CYA had lower (P<0.05) MEL concentrations in their kidneys and breast muscle compared to ducks fed ≥1.00% MEL alone. No MEL was detected in the kidneys or breast muscle of ducks fed the highest combination of MEL+CYA (1.50% of each compound). Ducks fed lower concentrations of MEL+CYA (≤1.00% of each compound) had higher (P<0.05) MEL concentrations in kidneys than ducks fed 1.50% MEL+1.50% CYA but approximately three and half fold lower concentrations than that of ducks fed 0.50% MEL alone.

Due to logistical problems during termination, bile from birds in each treatment was pooled. Therefore,

Table 4: Residue levels of melamine in kidney, breast muscle and bile of Pekin ducks

Treatment[1]		Response variables		
M (%)	CYA (%)	K[2] (ppm)	BM[2] (ppm)	B[3] (ppm)
0.00	0.00	Nd[c]	ND[d]	ND
0.50	0.00	46[b]	32[b]	92
1.00	0.00	179[a]	93[ab]	490
1.50	0.00	238[a]	155[a]	568
0.00	0.50	Nd[c]	ND[d]	ND
0.00	1.00	Nd[c]	ND[d]	ND
0.00	1.50	Nd[c]	ND[d]	ND
0.50	0.50	13[b]	11[c]	23
1.00	1.00	12[b]	ND[d]	30
1.50	1.50	Nd[c]	ND[d]	24
	S.E.M.	2.1	2.1	-

M = Melamine, CYA = Cyanuric acid, K = kidney, BM = Breast muscle, B = Bile

[1]Treatments were the addition of melamine and or cyanuric acid, in percent indicated to the basal diet. Diets were fed from day 3 to 21.

[2]Data are means of four replicate pens, with one duck per pen. Statistical analysis was performed on transformed data (ranked data).

[3]Bile from all replicates was pooled and data are means of duplicate HPLC analysis per treatment. Statistical analysis not performed due to lack of replicates.

[a-d]Means within a column with no common superscript are different ($P<0.05$).

ND = none detected.

statistical analysis could not be performed on the bile. Means presented in Table 4 are an average of duplicate HPLC analysis performed on bile samples taken from each treatment. No MEL was detected in the bile of birds fed up to 1.50% CYA. Melamine concentrations in the bile increased as more MEL was included in the diet, ranging from 92 ppm in ducks fed 0.50% MEL alone to 568 ppm in ducks fed 1.50% MEL alone. Ducks fed combinations of MEL+CYA had MEL concentrations in the bile ranging from 23-30 ppm, these bile MEL concentrations are 4-24 fold lower than bile MEL concentrations observed in ducks fed MEL alone.

Gross pathology-mortality: The effects of dietary treatments on mortality are summarized in Table 2. Only diets that included MEL alone (0.50, 1.00 and 1.50%) incurred mortality at 4, 16 and 44%, respectively. Eleven of the thirteen ducks that were examined had pale and enlarged kidneys. Eight of the thirteen ducks had white crystals present in the bile during gross examination. Figure 1 shows crystals present in the bile of a duck fed 1.50% MEL. Crystals from the bile of early mortality ducks were spherical and brown in appearance.

Bile analysis: Microscopic examination of pooled bile specimens collected during termination revealed isolated spherical crystals present in the bile of ducks fed 1.00% MEL and numerous brown small spherical crystals, with some aggregates of spherical crystals present in the bile of ducks fed 1.50% MEL. Bile from all other groups appeared clear and without crystals when viewed microscopically. Figure 2 and 3 show crystals in the bile of ducks fed 1.00 and 1.50% MEL.

Histopathology: Histopathology of liver sections from all treatment groups was unremarkable. Ducks fed 1.00 and 1.50% MEL were the only treatments to have kidneys that showed abnormalities during histopathological examination. Four kidneys sections from the 1.00% MEL treatment group and five kidneys from the 1.50% MEL treatment group had mild dilation of the embryonal nephrons and collecting tubules. Eosinophilic to basophilic casts, some containing spherical eosinophilic crystals were also present in the embryonal nephrons and collecting tubules (Fig. 4). One kidney from the 1.50% MEL treatment had moderate dilation of the embryonal nephrons and collecting tubules with eosinophilic to basophilic casts, some with spherical eosinophilic crystals, present. Affected nephrons were often surrounded by a mild heterophilic infiltration. On rare occasions, casts or crystals, similar to the ones previously described, were found in the interstitial spaces and associated with mild multifocal hetrophil infiltration (Fig. 5).

DISCUSSION

In the current study, significant mortality was only observed in ducks fed ≥1.00% MEL. In a previous study, 15% mortality was observed in ducks fed 1.50% MEL (Landers *et al*., 2012). This mortality rate is lower than the 44 % mortality that occurred in ducks fed 1.50% MEL in the current study. Fifty six percent mortality was reported in young poults fed 1.50% MEL for 21 days (Brand, 2011). However, Brand (2011) reported no treatment related mortality when feeding up to 1.50% MEL to young broilers for 21 days. Lu *et al*. (2009) reported no visible signs of ill health, mortality, or changes in the behavior of broilers fed graded levels of MEL up to 0.1% of the diet for 42 days. Mortalities from the current study were MEL related with ducks having pale and enlarged kidneys. Pale and enlarged kidneys as a result of MEL ingestion has been reported in poults fed ≥1.00% MEL (Brand, 2011), in broilers fed ≥2.00% MEL (Brand *et al*., 2012) and in ducks fed ≥1.50% MEL (Landers *et al*., 2012). No mortality occurred in ducks fed up to 1.50% CYA alone in the current study. Brand (2011) also reported no mortality in young broilers and poults fed up to 3.00% CYA, suggesting that CYA is not toxic to young ducks, broilers or poults at these dietary concentrations.

Decreases in performance (body weight gain, feed intake, feed conversion) are in agreement with results from a previous study where body weight gain and feed intake were decreased in ducks fed ≥1.00% MEL and feed conversion increased in ducks fed ≥1.50% MEL (Landers *et al*., 2012). The reduction in body weight gain

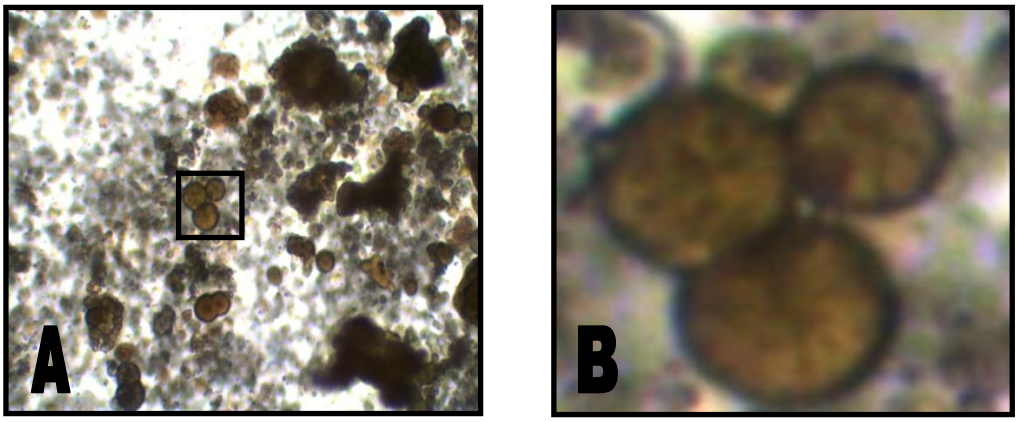

Fig. 1(a-b): Crystals in the bile of early mortality ducks fed 1.50% melamine. Photo 'A' was taken at 100 X magnification.
The expanded view shows three round crystals that appear brown in appearance. Photo 'B' is an expanded view of three crystals that are shown in photo 'A'

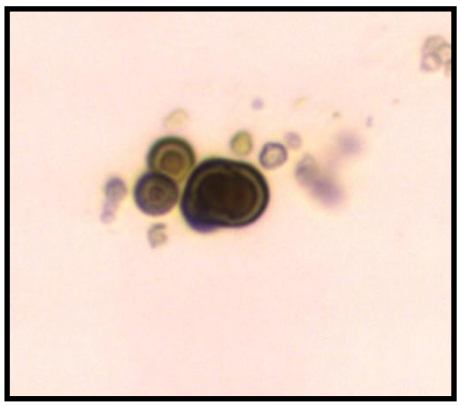

Fig. 2: Crystals in the bile of ducks fed 1.00% melamine and survived to termination. Photo taken at 200 X magnification, shows round, brown crystals in the bile

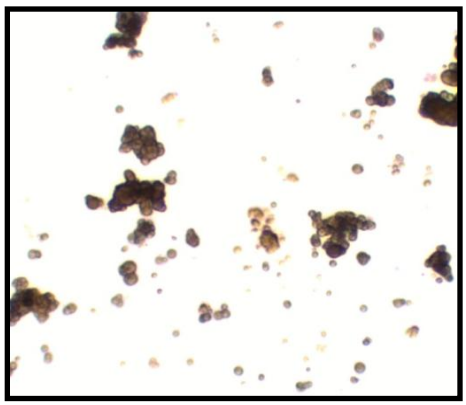

Fig. 3: Crystals in the bile of ducks fed 1.50% melamine that survived to termination. Photo taken at 100 X magnification

is consistent with research in broilers and poults that consumed ≥1.00 and ≥1.50% MEL, respectively (Brand, 2011). Feed intake was also reduced in young broilers fed ≥1.00% MEL for 21 days (Brand, 2011). Results of the current study are consistent with results of Lu *et al.* (2009), who observed no negative effects on weight gain, feed intake, or feed conversions of broilers fed up to 0.1% MEL for 42 consecutive days.

Inclusion of up to 1.50% CYA in diets of ducks had no negative impact on weight gain, feed intake, or feed conversion. Similarly, inclusion of up to 3.00% CYA in the diets of young broilers and poults did not negatively impact weight gain, feed intake, or feed conversion (Brand, 2011). Similar results were also observed in ducks fed combinations of MEL+CYA (up to 1.50% of each compound) with weight gain, feed intake and feed conversion being similar to control ducks. These results are similar to those reported in poults, where the inclusion of CYA in a diet that contained MEL (up to 1.50% of each compound) reduced the negative effects of MEL with respect to body weight gain and feed intake (Brand, 2011). In contrast to the findings in the current study and the findings in poults by Brand (2011), broilers that consumed a combination of MEL and CYA (up to 1.50% of each compound) had poorer performance (feed intake and body weight gain) than broilers fed MEL (up to 1.50%) or CYA (up to 1.50%) alone (Brand, 2011).

Relative liver weights were unaffected by dietary treatments in the current study. However, relative kidney weights were increased in ducks fed ≥1.00% MEL. Similar results are reported in earlier research (Landers *et al.*, 2012), where feeding ≥1.00% MEL to ducks

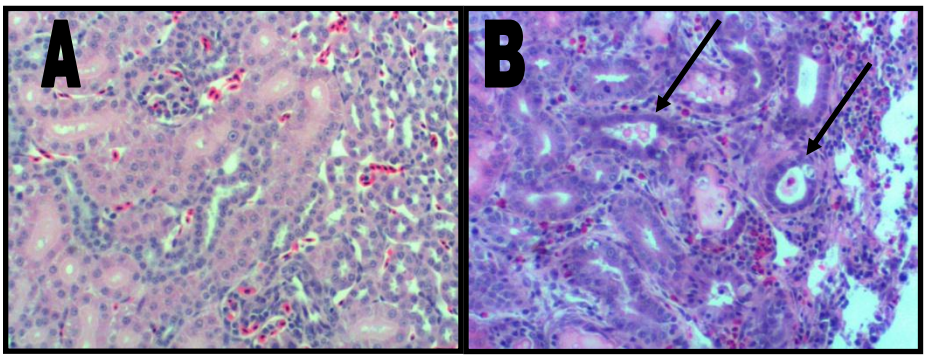

Fig. 4(a-b): Kidney sections from a control duck and one fed 1.50% melamine. Photo 'A' is a kidney section from a control duck viewed at 100 X magnification. Photo 'B' is a kidney section from a duck fed 1.50% MEL and viewed at 100 X magnification. Note dilated collecting tubules with casts present in photo 'B'

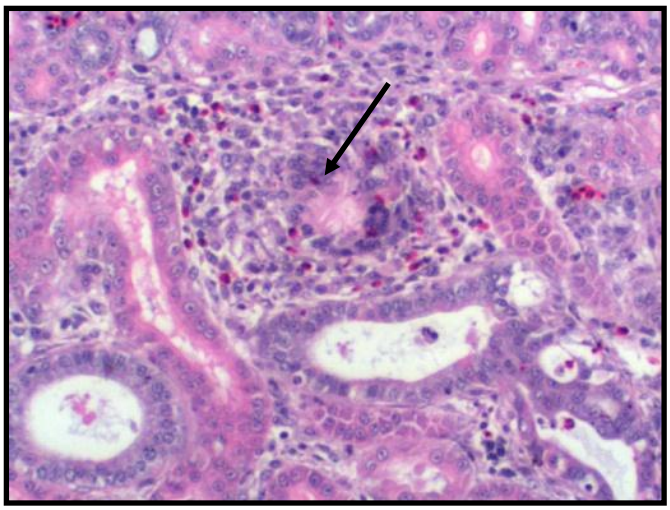

Fig. 5: Kidney section from a duck fed 1.50% melamine. Viewed at 100 X magnification. Casts and crystals are present in the interstitial space.

increased relative kidney weights above that of controls. Increased relative kidney weight has also been observed in studies with broilers fed ≥1.50% MEL and poults fed ≥1.00% MEL (Brand, 2011). Relative kidney weights of ducks fed CYA alone or MEL+CYA were similar to that of control ducks. Brand (2011) reported similar finding in poults, where feeding a combination of MEL and CYA (up to 1.50% of each compound) did not result in an increase in relative kidney weights. However, the ability of CYA to reduce the negative effects of MEL on relative kidney weights that was observed in the current study and in poults (Brand, 2011), was not observed in young broilers (Brand, 2011).

Results indicate that feeding ducks up to 1.50% CYA did not affect growth performance, mortality, or relative liver and kidneys weight. The addition of ≥1.00% MEL caused reductions in feed intake, body weight gain, percent mortality and increased relative kidney weight. However, when up to 1.50% CYA was added to diets containing MEL (1:1 ratio of CYA to MEL), CYA reduced the negative effects of MEL on feed intake, body weight gain, percent mortality and relative kidney weights. This ability of CYA to reduce the negative effects of MEL is in agreement with research in poults fed similar dietary concentrations (Brand, 2011). However, these data are in contrast to findings in broilers fed similar dietary concentrations where the combination of MEL and CYA was more toxic than MEL alone (Brand, 2011) . Data from the current experiment are also in contrast to a previous study with pigs (Stine *et al*., 2011) where the combination of MEL and CYA (100 mg/kg BW/day, of each compound) caused a greater reduction in gain than MEL (200 mg/kg BW/day) alone.

Elevated levels of ALB, TP and GLOB observed in ducks fed ≥1.00% MEL in the current study could be the result of dehydration. It has been reported that serum

concentrations of ALB, TP and GLOB can all be elevated during periods of dehydration (Cornell, 2010a; Nicoll *et al*., 2012). In the current study, the diuretic effect of MEL which has been reported in dogs (Lipschitz and Stockey, 1945), could have caused dehydration in the ducks.

The increase in UA seen in ducks fed ≥1.00% MEL could have been caused by decreased renal function. Increased levels of UA are used to diagnose renal failure with increased levels occurring when more than 70% of kidney function is lost (Cornell, 2010b). Ducks that consumed 1.50% MEL had UA levels that were 4.4 times higher than levels in control animals. Increases in UA levels have also been reported in mature laying ducks fed ≥50 ppm MEL for 21 days (Gao *et al*., 2010) and young Pekin ducks fed ≥1.00% MEL for 21 days (Landers *et al*., 2012). Gross pathological and histopathological observations that the kidneys of ducks fed ≥1.00 % MEL appeared pale and enlarged with mild dilation of the embryonal nephrons and collecting tubules would be consistent with decreased renal function leading to increased serum concentrations of UA.

The fact that serum concentrations of GLU, ALB, TP, GLOB, Ca, AST, GGT and UA in ducks fed CYA alone (≤1.50%) or combinations of MEL+CYA (≤1.50% of each compound) were not increased above that of control ducks suggest that renal function was not affected in ducks fed these dietary treatments. The gross appearance of the kidneys and the absence of histopathological changes in the kidneys of these birds at termination would tend to support this conclusion.

The presence of MEL residues in the tissues of ducks fed MEL in the current study is consistent with previous reports. Landers *et al*. (2012) fed graded levels of MEL (0.25-2.25%) to young Pekin ducks and observed higher concentrations of MEL in kidney followed by breast muscle. Yan *et al*. (2009) fed graded levels of MEL (2 to 1000 mg/kg feed) to ducks from hatch to day 42 and found that MEL concentrations in tissues increased linearly with levels of MEL higher than 100 mg/kg. At high dietary concentrations of MEL (500 and 1000 mg/kg), the kidneys had the highest concentration of MEL followed by the liver and breast meat (Yan *et al*., 2009). Higher concentrations of MEL in kidneys compared to muscle have also been reported in broilers (Brand *et al*., 2012; Lu *et al*., 2009; Ding *et al*., 2012), poults (Brand, 2011) and layers (Bai *et al*., 2010) fed MEL. Higher MEL concentrations in the kidney compared to breast muscle could be partially explained by reports of MEL precipitation in the kidney. Precipitation of MEL and CA complexes in the kidney probably occur because of increased concentrations of the compounds as they move down the osmotic gradient (Dobson *et al*., 2008). Therefore, it is reasonable to assume that MEL concentrations in the kidney would be greater than that in the muscle, due to the compound becoming more concentrated by the function of the kidneys. Another possible explanation for the difference in MEL concentrations between the muscle and kidney could be the relative mass of the two tissues. The greater mass of muscle tissue could have contributed to a dilution effect resulting in a lower MEL concentration.

In the current study, bile had a much higher concentration of MEL than the kidney. Higher concentrations of MEL in bile compared to other tissues have been observed in previous studies with ducks (Landers *et al*., 2012), broilers (Brand *et al*., 2012) and turkey poults (Brand, 2011). These data suggest that bile is a route of MEL excretion in these avian species. However, it remains to be determined if biliary excretion occurs only at high concentrations, such as those used in the current study, or also at lower dietary concentrations of MEL.

In the current study, ducks fed combinations of MEL+CYA (≤1.50% of each compound, in a 1:1 ratio) had lower MEL concentrations in the kidney, breast muscle and bile than ducks fed ≤1.50% MEL. Reimschuessel *et al*. (2008) observed similar results in fish, noting that fish that were fed MEL or CYA alone had higher residue levels of the compounds as compared to fish that were administered both compounds simultaneously. Reimschuessel *et al*. (2008) went on to state that the decrease in muscle residues levels was due to the MEL-CYA complex precipitating out in the gastrointestinal tract and kidneys, thus decreasing its bioavailability. The results of the current study support this hypothesis since ducks fed combinations of MEL+CYA (≤1.50% of each compound) had lower muscle MEL concentrations compared to ducks fed ≤1.50% MEL. Ducks fed combinations of MEL+CYA (≤1.50 % of each compound) also had lower kidney MEL concentrations than ducks fed MEL (≤1.50%). It is possible that most of the precipitation of the MEL-CYA complex occurs in the gastrointestinal tract, thus not allowing MEL to be absorbed.

With no lesions or pathologic changes observed in the liver section of ducks from all treatments it appears that MEL, CYA, or MEL+CYA at the dietary inclusion levels used in the current study does not cause hepatic damage. Histopathology results also indicate that CYA, up to 1.50% of the diet and fed for 18 consecutive days does not damage the kidney of young Pekin ducks. In contrast, the addition of ≥1.00% MEL does cause renal lesions that are compatible with mild MEL toxicity. Renal damage seen in ducks fed ≥1.00% MEL contributed to or directly caused the decreased growth performance and changes in blood serum chemistry observed in the current experiment. Similar results with respect to gross pathology and histopathology of the kidney have been reported in a previous study with ducks (Landers *et al*., 2012) and in studies with poults (Brand, 2011) and broilers fed MEL (Brand *et al*., 2012). However, addition of CYA to the diet contaminated with MEL protected the

kidney against the damaging effects of MEL. This ability of CYA to alleviate the negative effects of MEL has been observed in poults fed similar dietary levels of MEL and CYA (Brand, 2011). However, the combination of MEL and CYA was more toxic than MEL alone when fed to broilers at similar dietary levels used in the current study (Brand, 2011). As indicated previously, it is possible that MEL-CYA complexes formed by feeding the two compounds simultaneously may have precipitated in the gastrointestinal tract reducing the amount of MEL absorbed resulting in reduced toxicity. Differences in gastrointestinal characteristics (pH, residence time etc.) between ducks and poults compared to chicks may have contributed to the reduced toxicity of MEL when it is ingested in combination with CYA.

Microscopic examination of the bile revealed the presence of MEL crystals in the bile of ducks fed ≥1.00% MEL. Crystals were not observed in the bile of ducks fed 0.50% MEL, up to 1.50% CYA and combinations of MEL+CYA (up to 1.50% of each compound). Figure 1 and 3 show the contrast in the concentrations of MEL crystals present in the bile of a duck that died during the study (Fig. 1) to one that survived to termination (Fig. 3); both ducks were fed 1.50% MEL. The greater concentration of crystals in the bile of the early mortality duck suggests that this duck might have been more efficient in absorbing MEL or less efficient in excreting MEL. Differences in sensitivities to MEL was reported by Brand (2011) who observed that a high number of poults died (due to renal failure) within the first half of the experiment while others, consuming the same concentration of MEL in their diet, survived until termination. Others have reported that birds fed MEL for 42 days had lower tissue MEL concentrations than birds that consumed MEL for 28 days (Lu *et al*., 2009). Lu *et al*. (2009) hypothesized that as birds age they develop a greater capacity to clear MEL from their body. Another hypothesis is that as these ducks age, tissue concentrations are lower since the ducks have increased in size, thus diluting the MEL that is deposited in their tissues.

Conclusion: With the observed changes in performance, serum chemistry values and gross and microscopic appearance of the kidneys, the study indicates that ducks fed ≥1.00% MEL had decreased renal function leading to decreased growth and in some cases eventually death. It is also evident that addition of up to 1.50% CYA was not toxic to young Pekin ducks. These data also indicate that inclusion of CYA in diets that contain MEL (in a one to one ratio of MEL to CYA) reduces the negative effect of MEL in young Pekin ducks. It appears this decrease in toxicity of MEL due to CYA is from the precipitation of the MEL-CYA complex in the gastrointestinal tract, thus decreasing the absorption of MEL as compared to ducks fed MEL alone (Reimschuessel *et al*., 2008). It also appears that young ducks can eliminate MEL via their bile, a process that has been suggested to occur in other avian species (Brand, 2011).

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