MATERNAL TRANSFER AND TISSUE DISTRIBUTION OF HMX IN QUAIL EGGS

by

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ABSTRACT

An efficient sample extraction and cleanup method was developed for determination of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in eggs. The procedure included solvent extraction of HMX from eggs followed by cleanup using florisil and styrene-divinyl benzene (SDB) cartridges. Chromatographic separation was achieved on a reverse phase (RP) C18 column, with a mobile phase containing 60% methanol + 40% 1.0 mM acetic acid aqueous solution. Overall recoveries from eggs containing 10, 50, 250 and 1000 ng/g of HMX were 84.0%, 88.0%, 90.6% and 87.4%. A method detection limit (MDL) of 0.15 ng/g was achieved.

Then we evaluated the use of the gas exchange rate as an indicator of chemical stress in avian embryos/eggs. Northern bobwhite quail (*Colinus virginianus*) were exposed to HMX via feed at concentrations of 0, 12.5, 50.0, and 125.0 mg/kg. Metabolic rates (oxygen consumptions) of incubated quail eggs were then measured via respirometry to examine potential effects of HMX exposure. Metabolic rate was examined on 5, 9, and 21 days of incubation. Next, concentrations of HMX in eggs were determined by liquid chromatography-mass spectrometry. Concentrations of HMX in eggs from the four dose groups were significantly different. Mean (± SE) concentrations of HMX in quail eggs were 1025 ± 77, 3610 ± 143 and 7021 ± 300 ng/g in the low, medium and high dose groups respectively. A significant difference in oxygen consumption rates was observed among eggs at the three developmental stages ($p<0.0001$), but not among the four dose groups ($p=0.14$). No evidence was observed for metabolic alterations of eggs associated with HMX exposure.

Maternal transfer of HMX from female northern bobwhite quail (*Colinus virginianus*) to their eggs and distribution of HMX in egg compartments were studied. Concentrations of HMX in the yolk, embryo and chorioallantoic membrane (CAM) were determined in eggs after being incubated for 5, 9 and 21 days. HMX was found in eggs from all treatment groups. HMX transfer to eggs was not different during the 30-day time course of the dosing. Concentrations of HMX in eggs were dose-, age- and compartment-dependent. At all embryo ages (5, 9 and 21-day of incubation), more than 97% HMX was distributed in the yolk and embryo, while less than 3% was in the CAM and albumin. HMX in the embryo increased rapidly from 9 to 21 days, which coincided with the normal transfer of yolk lipids to the embryo. Both concentration and mass of HMX found in the CAM was low, meaning the CAM could not be used for nonlethal assessment of HMX exposure. Bioconcentration factors (BCFs) of HMX in eggs were 0.09, 0.07 and 0.06, and maternal transfer coefficients (MTCs) were 0.030, 0.018 and 0.015 in the low, medium and high dose groups, respectively. BCF and MTC in the low dose were significantly higher than those in the medium and high doses.

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CHAPTER I

BACKGROUND AND LITERATURE REVIEW

1.1. Introduction of HMX

High melting explosive (HMX) is a man made nitramine with a molecular formula of $C_4H_8N_8O_8$ and a molecular weight of 296 g/mole. It is also known as octogen, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, cyclotetramethylenetetranitramine, or 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane. HMX is highly energetic and explodes violently at 534°F (ATSDR, 1997). Because of this property, HMX is widely used as an explosive in military applications, such as nuclear devices, plastic explosives, rocket fuels, and burster chargers (ATSDR, 1997).

HMX enters the environment through air, water, and soil because of extensive military applications. HMX released to air usually attaches to suspended particles. Its concentration in air is low due to its low vapor pressure and Henry's law constant. Reported vapor pressures ranged from 3.33×10^{-14} mm Hg (Burrows *et al*., 1989) to 2.4×10^{-8} mm Hg (Lyman *et al*., 1982). The Henry's law constant ranged from 2.6×10^{-15} atm·m^3/mole (Burrows *et al*., 1989) to 8.7×10^{-10} atm·m^3/mole (Meylan *et al*., 1991). Conversely, HMX is commonly found in water, soil, and sediments in locations near

munition sites. HMX has been identified in effluents from army ammunition plants. Concentrations of HMX downstream of the Holston Army Ammunition Plant, TN ranged from not detected to 4.4 mg/L (Small and Rosenblatt, 1974), which is close to HMX solubility (5 mg/L to 6.6 mg/L) in water (Burrows *et al*., 1989; USEPA, 1988). Concentrations of HMX in water samples of the Holston River ranged from 0.6-2.7 mg/L (Spanggord *et al.*, 1982). The concentration of HMX was 289 μg/L in load and pack wastewater following activated carbon treatment, and 1652 μg/L in wastewater from an old lagoon prior to treatment (Jenkins *et al*., 1986). In effluents from the Holston facility, HMX was detected at concentrations up to 3.36 mg/L (Talmage *et al*., 1999). HMX in groundwater also has been studied. In groundwater from a deep well in Canaan, NH, HMX was below the detection limit (26 μg/L), while RDX was 70 μg/L (Jenkins *et al*., 1986). In groundwater at the Milan Army Ammunition Plant, TN, concentrations of HMX ranged from 0.53 to 821 μg/L (USEPA, 1994). In groundwater collected at a former ammunition plant site at Elsnig, Saxony, Germany, concentrations of HMX ranged from 13 to 208 μg/L (Lewin *et al*., 1997). HMX concentrations were up to 4.2 mg/L in groundwater at the Louisiana Army Ammunition Plant site (Talmage *et al*., 1999).

HMX has been detected in munitions-contaminated soil from many U.S. military sites. The maximum reported concentration of HMX reached 5700 mg/kg in soil was

reported at army sites (Talmage *et al*., 1999). While HMX commonly occurred in soils at concentrations of several hundred mg/kg. Concentrations of HMX in soil from various army installations throughout the U.S. were reported as 1.1-115 μg/g (Walsh *et al*., 1992). HMX in soil from the U.S. Army munition depot near Umatilla, Oregon was 300 mg/kg (Funk *et al*., 1993). HMX was detected in contaminated soil from the Naval Surface Warfare Center in Crane, IN at an unspecified concentration (Grant *et al*., 1995). Concentrations of HMX were 39.8 and 36.3 mg/kg in two soil samples collected from the Louisiana Army Ammunition Plant site (Xue *et al*., 1995). Concentrations of HMX in contaminated soil collected at the Joliet Army Ammunition Plant in Joliet, IL ranged from 50-100 mg/kg (Boopathy and Manning, 1999). The concentrations of HMX in soil from the Wainwright firing range in Alberta, Canada, ranged from 25.3-50.7 mg/kg dry weight (Groom *et al*., 2002).

Plants accumulate HMX from soil into their tissues. HMX was detected (concentration not specified) in the blade tissue of rye-grass (*Lolium perenne*) grown in firing range soil (Groom *et al*., 2001). Concentrations of HMX in the agricultural crops alfalfa (*Medicago sativa*), bush bean (*Phaseolus vulgaris*), canola (*Brassica rapa*), wheat (*Triticum aestivum*), and perennial ryegrass (*Lolium perenne*) grown in contaminated soil were 289.3, 123.3, 223.5, 459.7 and 295.1 mg/kg. Estimated HMX uptake in these plants were 1.92, 1.81, 4.69, 7.20 and 4.38 mg/pot when grown in soil containing HMX at

100.9, 85.0, 90.5, 88.7 and 88.9 mg/pot, respectively (Groom *et al*., 2002). Uptake of HMX in unspecified species of lettuce, corn stover, and yellow nutsedge from soil were 43, 4.3, and 6.0 mg/kg, while HMX was not detected in tomatoes or corn kernels grown in these contaminated soils (Pennington and Brannon, 2002).

1.2. Environmental fate of HMX

HMX is released to the environment during its production and use. The vapor pressure and Henry's law constant of HMX is low (Burrows *et al*., 1989; Lyman *et al*., 1982; Meylan *et al*., 1991; ATSDR, 1997). Organic carbon partition coefficient (Koc) values of HMX range from 3.5 to 670 (Burrows *et al*., 1989; Monteil-Rivera *et al*., 2003; Spanggord *et al*., 1982). Octanol water partition coefficient (Kow) values of HMX range from 1.1 to 6.6 (Burrows *et al*., 1989; Monteil-Rivera *et al*., 2003; HSDB, 2005).

If released to air, HMX mainly exists in the particulate phase based on its low vapor pressure and Henry's law constant; particulate phase HMX will be removed from the atmosphere by wet and dry deposition (ATSDR, 1997).

If released to water, HMX is expected to adsorb to solids and sediments based upon a Koc of 670 (Spanggord *et al*., 1982). Volatilization of HMX from water surfaces is not significant based upon its low Henry's Law constant (ATSDR, 1997). Biodegradation of HMX in water is low. HMX was biodegraded slowly in anaerobic river water, with 50%

degradation after 50 days (Spanggord *et al*., 1982). No HMX was degraded after 28 days using a sludge enrichment culture from a sewage treatment plant (Boopathy *et al*., 1998). Photolysis is expected to be an important fate process for HMX in sunlit environments. Photolytic half-lives for HMX were 1.4, 1.7, and 70 days in pure water, Holston River water, and Louisiana Army Ammunition Plant lagoon water, respectively (Spanggord *et al*., 1983).

If released to soil, volatilization of HMX from dry soil surfaces is not significant based upon its low vapor pressure and Henry's Law constant. HMX is expected to have moderate to high mobility in soil based on Koc values. HMX was resistant to aerobic biodegradation based on 0% degradation in contaminated soil samples after 56 days (Grant *et al*., 1995). However, anaerobic biodegradation of HMX was significant based on 100% degradation after 20-22 days in tests using anaerobic sludge inocula with sulfate-reducing bacteria *Desulfovibrio* spp., and 77% after 28 days with methanogenic bacteria *Methanococcus* (Boopathy *et al*., 1998). In another study, 90% of HMX degraded after 10 days in tests using municipal anaerobic sludge cultures (unidentified microorganisms) (Hawari *et al*., 2001). Half-lives of HMX ranged from 133 to 2310 days in moist test soils from Fort Greely, Alaska, Yakima Training Center, Washington, and Camp Guernsey, Wyoming (Jenkins *et al*., 2003).

1.3. Toxicity of HMX

1.3.1 Mammals

1.3.1.1 Acute toxicity

Available data have shown that HMX has toxicological affects on laboratory mammals. There are several sources of median lethal dose (LD_{50}) values of HMX. In a U.S. Army study, acute oral LD_{50} values were 5500 and 6400 mg/kg for male and female rats, 1700 and 3200 mg/kg for male and female mice, and 100-250 mg/kg for rabbits, respectively (Cuthbert *et al*., 1985). Oral LD_{50}s for mice and guinea pigs were 1500 and 300 mg/kg, respectively. The intravenous LD_{50} for guinea pig was 28 mg/kg (Lewis and Sax, 1992, and an oral LD_{50} of 2300 mg/kg for rat was reported (Bingham *et al*., 2001).

Rats, mice and rabbits were given single oral dose of HMX in an acute toxicity study. In Fischer 334 (F334) rats following a single oral dose of 5447 mg/kg HMX, "reddening" of lungs, "white fluid" in gastrointestinal tracts, and "pale" kidneys were observed. Hyperkinesia and ataxia were exhibited in rats dosed with HMX. Situations were similar in B6C3F1 mice. Following a single oral dose of 1626 mg/kg HMX, "reddening" of lungs and "white fluid" in gastrointestinal tracts of mice were observed. Hyperkinesia and ataxia were observed in these mice as well. New Zealand white rabbits were orally dosed at 50, 100, 250, 429, 1000, and 2000 mg/kg HMX, 1 rabbit/dose.

Deaths were observed at doses of 250 mg/kg or more in males, and at all doses in females. “Reddening” of lungs, convulsions, hyperkinesia and mydriasis were observed at all doses (Cuthbert *et al*., 1985).

1.3.1.2 Fourteen-day subacute oral toxicity

In the study on F334 rats, 6 rats/sex/dose were exposed to diets containing HMX at target doses of 0, 333, 1000, 3000 and 9000 mg/kg/day for 14 days. The incidences of death were 0/6, 0/6, 0/6, 0/6 and 5/6 in males and 0/6, 0/6, 1/6, 1/6 and 6/6 in females. All HMX-treated rats displayed dose-dependent suppression of body weight gain except females in 333 mg/kg/day group. Rats receiving1000 mg/kg/day or more displayed hepatocyte hyperplasia and cytoplasmic eosinophilia in liver. A dose of 9000 mg/kg/day led to centrilobular degeneration in the liver, and congestion/ hemorrhage in the brain in male rats. Female rats receiving 1000 mg/kg/day and higher doses displayed congestion in the kidneys and lymphocyte depletion in the thymus and spleen (Greenhough *et al*., 1985a).

A similar study was conducted in B6C3F1 mice. Target dietary doses were 0, 100, 300, 900 and 2700 mg/kg/day in males, and 0, 320, 800, 2000, and 5000 mg/kg/day in females. The incidences of mortality were 0/6, 0/6, 5/6, 6/6, and 6/6 in males, and 0/6, 0/6, 2/6, 4/6, and 6/6 in females. Mice receiving HMX at 300 mg/kg/day and higher

doses displayed dose-related hepatocellular hyperplasia in the liver and lymphocyte depletion in the thymus and spleen (Greenhough *et al*., 1985b).

1.3.1.3 Thirteen-week subchronic toxicity

In the study on F344 rats, 20 rats/sex/dose received dietary HMX for 13 weeks at the following doses: 0, 50, 150, 450, 1350, and 4000 mg/kg/day for males and 0, 50, 115, 270, 620, and 1500 mg/kg/day for females. Three rats died during the study: a male in 150 mg/kg/day group died at week 9; a female in 1500 mg/kg/day group died at week 1; and a female in the control group died at week 13. No dose-dependent deaths were observed in this study. HMX-treated rats exhibited significant ($p<0.05$) dose-dependent reductions in body weight gain and food consumption in the early weeks of the study. By week 5, male and female rats in the highest dose groups continued to have significant reductions in weight gain which persisted throughout the study. Organ weight changes were observed in HMX-treated rats. Brain weights increased in females at 115 mg/kg/day or more, and brain-to-body weight ratios increased in males at 1350 and 4000 mg/kg/day. Liver-to-body weight ratios increased in females at 620 and 1500 mg/kg/day. Kidney-to-body weight ratios increased in females at 115 mg/kg/day or more. Adrenal-to-body weight ratios decreased in all dosed males, while spleen-to-body weight ratios decreased in all dosed females (Everett *et al*. 1985).

Hemoglobin, packed cell volume, and blood urea nitrogen decreased in female rats exposed to 1500 mg/kg/day HMX. Increased volume, decreased pH and crystal formation were observed in the urine of females at 1500 mg/kg/day, but no urinary effects were observed in males. There were sexual differences in target organ responses to HMX in rats. Significant liver changes including enlarged centrilobular cells, pale nuclei and dark cytoplasm, dilation of sinusoids, and necrosis were observed in males at 450, 1350, and 4000 mg/kg/day. High doses of 1500 mg/kg/day in females and 4000 mg/kg/day in males caused significant elevations in serum alkaline phosphatase activity, while no consistent changes in aspartate amino transferase, alanine amino transferase and lactate dehydrogenase activities. However, alkaline phosphatase activities in male controls were lower than normal at 12 weeks. Therefore, the importance of the increase is not clear. Focal tubular atrophy and dilatation were observed in female kidneys during exposure to 270 mg/kg/day and higher. These changes were related to increase of alkaline phosphatase activity and alteration of renal function (Everett *et al*. 1985).

A similar protocol was conducted in the B6C3F1 mice study. Doses were 0, 5, 12, 30, 75 and 200 mg/kg/day in males, and 0, 10, 30, 90, 250 and 750 mg/kg/day in females. Incidence of deaths were 13/20 in males at 200 mg/kg/day, 12/20 in females at 250 mg/kg/day, and 20/20 in females at 750 mg/kg/day. Results showed that the mortality was dose-related. No histopathological effects in liver and kidney were observed in male

mice exposed to 75 mg/kg/day and in female mice exposed to 90 mg/kg/day HMX. However, histopathological examinations were not performed in those mice that died during study, which limited data interpretation (Everett and Maddock, 1985).

1.3.2 Avian Species

There are few studies describing HMX toxicity in avian species. An approximate lethal dose (ALD) evaluation was conducted using northern bobwhite (*Colinus virginianus*). Sixteen birds were dosed by gavage at 8 doses ranging from 125 to 2125 mg/kg, 1 bird/sex/dose. One female in 187 mg/kg dose group died 6 days post exposure. Then a subsequent test was conducted using eight birds, at four doses of 3188, 4782, 7173 and 10760 mg/kg. One female in 7173 mg/kg group died on 12 days after exposure. So northern bobwhite quail could tolerate HMX concentrations as high as 10,760 mg/kg. After the ALD study, 64 quail were distributed to four dose groups (8 males and 8 females in each group) that receiving dietary exposure to HMX at 0, 100, 1000 and 10000 mg/kg feed for 28 days. Reductions in feed consumption, body weight and egg production were observed in the two high dose groups. These reductions might be correlated with food avoidance behavior caused by HMX dosing (Johnson *et al*., 2005a).

In a recent study, HMX was found readily and rapidly transferred from female northern bobwhite quail (*Colinus virginianus*) to eggs. Treatment-related reductions in

body weight of adults, food consumption and egg laying rates were observed, while no effects in chick survival and growth were found. Results of the study showed the reproductive toxicity of HMX to birds is low (Brunjes *et al*., 2007).

1.3.3 Amphibians and reptiles

There is no information on HMX toxicity in amphibian, and little information in reptilian species. Acute toxicity of HMX in adult male and female green anoles (*Anolis carolinensis*) was estimated using a standard up-down toxicity test, and ALD was determined to be >2000 mg/kg body weight. Accumulation of HMX into eggs incubated in contaminated soil was assessed at 0, 20, 200, and 2000 mg HMX/kg, and the accumulation appeared to be linear (Jones *et al*., 2005).

The toxicity of RDX, a structurally similar chemical to HMX, on red-backed salamanders (*Plethodon cinereus*) was studied. Salamanders (20/treatment) were exposed to 0, 10, 100, 1000, and 5000 mg RDX/kg soil for 28 days. Salamanders in 5000 mg/kg dose group exhibited signs of neuromuscular effects and significant weight loss relative to other groups, and one animal in this group died because of weight loss. No strong treatment-related histopathologic changes were found (Johnson *et al*., 2005b).

1.3.4 Aquatic species

Studies on aquatic species showed no adverse effects of HMX to algae, fishes and invertebrate species tested. LC_{50} values for bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) were >32 mg/L/24, 48, 96 hr; the LC50 for fathead minnow (*Pimephales promelas*) was 15 mg/L/96 hr; the LC50 for scud (*Gammarus fasciatus*) and water fleas (*Daphnia magna*) were >32 mg/L/24, 48 hr (Bentley *et al*., 1977). Notice that these concentrations of HMX are actually higher than the solubility of HMX in water, an indication that the toxicity of HMX on these species is low. Survival of the midge (*Chironomus tentans*) and the amphipod (*Hyalella azteca*) was unaffected after exposure to HMX at 400 mg/kg. However, enhanced midge growth was observed after exposure to sublethal concentrations of HMX (Steevens *et al*., 2002).

1.3.5 Terrestrial Invertebrates

The sublethal and chronic effects of HMX to earthworms (*Eisenia andrei*) has been assessed in both natural and artificial soils. Adult earthworm growth was reduced in artificial soils containing different HMX concentrations, although no mortality occurred even at the highest tested concentrations (Robidoux *et al*., 2001). Survival and growth of earthworms (*Eisenia andrei*) were not significantly reduced at concentrations of 711.0

mg/kg HMX, and reproduction parameters were significantly decreased by HMX >15.6 mg/kg (Robidoux *et al*., 2002). Survival and reproduction of earthworms (*Enchytraeus crypticus*) in a natural sandy loam soil amended with HMX were not affected (Kuperman *et al*., 2003). In another study, survival and reproduction of earthworms (*Eisenia fetida*) in a sandy loam soil were not affected by concentrations of HMX in soils (Simini *et al*., 2003).

1.4. Research Objectives

HMX has been commonly found in water and soils at military locations throughout the U.S. (ATSDR, 1997), and HMX has been found in terrestrial plant and invertebrate tissues (Groom *et al*., 2001, 2002; Pennington and Brannon, 2002; Robidoux *et al*., 2004). To our knowledge, birds have diverse foraging activities and occasional soil ingestion (Gionfriddo and Best, 1996). Birds living in contaminated sites are possibly exposed to HMX (Brunjes *et al*., 2007). The purpose of this research was to assess the utility of eggs as indicators of avian exposure to HMX. The scheme of the study was shown in Figure 1.1. There were several goals in this study.

In the first experiment, we developed a method to determine environmental relevant concentrations of HMX in eggs by liquid chromatography-mass spectrometry (LC-MS).

In the second experiment, northern bobwhite quail were exposed to HMX via food. Quail eggs were collected after avian exposure. We measured metabolic rates in ovo as a physiological indicator of chemical stress caused by HMX treatment. The main hypotheses in this experiment were: 1) HMX transfer from female quail to eggs is dose-dependent; 2) the metabolic rate of the developing egg is related to the amount of HMX deposited in the egg.

In the third experiment, we investigated concentrations of HMX in egg compartments-the yolk, embryo and chorioallantoic membrane (CAM). The partitioning of HMX among the egg compartments was discussed. Concentrations of HMX in eggs were calculated by data in egg compartments. Eggs laid on different days were compared to see if HMX accumulates during the dosing period. The main hypotheses in this experiment were: 1) HMX transfer to eggs is dose-, age- and compartment-dependent; 2) HMX transfer to eggs is affected by egg laying sequence.

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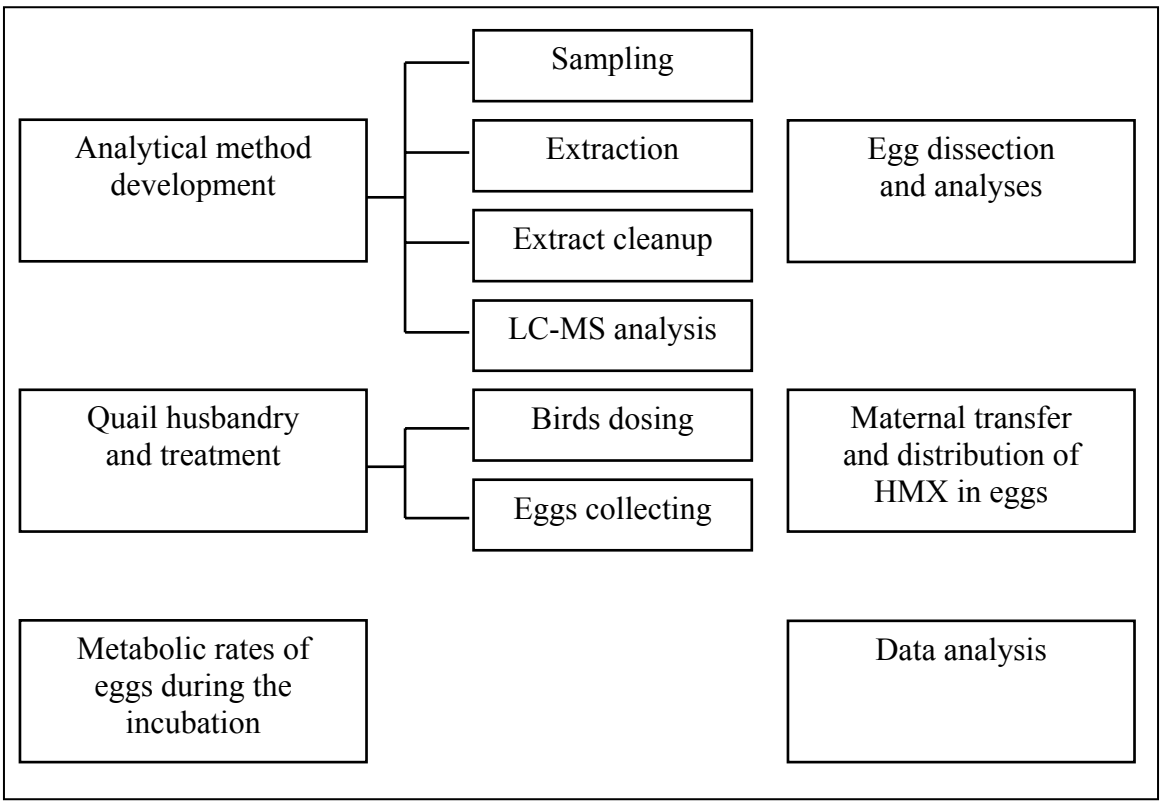

Figure 1.1. The scheme of the study of maternal transfer and distribution of HMX from northern bobwhite quail to their eggs. Modules are listed as the sequence of processing the study.

CHAPTER II

DEVELOPMENT OF AN EXTRACTION AND CLEANUP PROCEDURE FOR A LIQUID CHROMATOGRAPHIC-MASS SPECTROMETHIC METHOD TO ANALYZE OCTAHYDRO-1,3,5,7-TETRANITRO-1,3,5,7-TETRAZOCINE IN EGGS

Abstract

An efficient sample extraction and cleanup method was developed for determination of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in eggs. The procedure included solvent extraction of HMX from eggs followed by cleanup using florisil and styrene-divinyl benzene (SDB) cartridges. Homogenized egg aliquots were thoroughly mixed with 10 mL acetonitrile and extracted with ultrasonication for 1 h. Each sample was centrifuged and all liquid was collected for cleanup. After concentration by N_2 evaporation, each extract was cleaned by florisil and SDB cartridges to remove endogenous interfering compounds. Finally, each extract was filtered through a 0.2 μm PTFE membrane and stored for liquid chromatographic-mass spectrometric (LC-MS) analysis. Chromatographic separation was achieved with a reverse phase (RP) C18 column, with a mobile phase containing 60% methanol + 40% 1.0 mM acetic acid aqueous solution. Acetic acid was employed as mobile phase additive to form negatively

charged adduct ions $[M+CH_3COO]^-$, and m/z = 355 was quantified by selective ion monitoring (SIM). Overall recoveries from eggs containing 10, 50, 250, and 1000 ng/g of HMX were 84.0%, 88.0%, 90.6% and 87.4%. A method detection limit (MDL) of 0.15 ng/g was achieved.

2.1. Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Figure 2.1) is a highly energetic chemical that is widely used in nuclear devices, plastic explosives and rocket fuels (ATSDR, 1997). Because of its extensive military and civil application, HMX enters the environment via water, soil and air. HMX is relatively stable, and its half-life ranges from 133 to 2310 days in moist soil (Jenkins *et al*., 2003). Thus it is expected to persist in the environment. Plants, animals and other organisms living in areas contaminated with HMX have a high probability of accumulating HMX in their tissues (Groom *et al*., 2002), which suggests that HMX might cause toxicity in animals and humans.

HMX adversely affects laboratory mammals (rats, mice and rabbits) through inhalation, oral and dermal exposure, but the mechanism of HMX toxicity is still not well understood (ATSDR, 1997; Yinon, 1990; Talmage *et al*., 1999). There are few studies describing HMX toxicity to avian species. Recently Johnson *et al*. (2005) determined the approximate lethal dose (ALD) of HMX to northern bobwhite quail (*Colinus virginianus*) and found that quail tolerated up to 10,760 mg/kg (Johnson *et al*., 2005). Avian exposure to HMX at contaminated sites is probable because of their diverse foraging activities and soil ingestion (Gionfriddo and Best, 1996). Eggs have been widely used to assess wildlife exposure to various contaminants (Cobb and Wood, 1997; Rainwater *et al*., 2002; Bargar

et al., 2003). With this information in mind we designed and conducted a study to assess HMX transfer from adult quail into eggs and assess the possibility of using eggs as an indicator of avian exposure to HMX.

Sensitive analytical techniques are needed to accurately quantify concentrations of explosives in environmental and ecological risk assessments. Reverse phase high performance liquid chromatography (HPLC) is suitable for determining concentrations of thermally labile and non-volatile compounds. An HPLC method was introduced to determine concentrations of explosives when Bratin *et al*. (1981) developed a liquid chromatography (LC) method coupled with electrochemical detection (EC). Subsequently, more HPLC methods have been developed, and an ultraviolet (UV) detector has been most frequently used for identification (Bongiovanni *et al*., 1984; Bauer *et al*., 1986, 1990; Jenkins *et al*., 1989, 1992). HPLC-UV is recommended by the U.S. Environmental Protection Agency (USEPA) for determining nitroamine explosives (USEPA, 1998).

HPLC with selective detectors exhibits considerable sensitivity for detection of HMX. Fine *et al*. (1984) developed an LC system equipped with thermal energy analyzer (TEA) which could quantify HMX in explosive debris at a concentration of 420 ng/mL. Jenkins *et al*. (1992) reported a detection limit of 0.21 ng/mL for water samples analyzed by HPLC equipped with an UV detector. Monteil-Rivera *et al*. (2004) analyzed HMX by

solid phase extraction (SPE)/HPLC and solid phase micro extraction (SPME)/HPLC to achieve detection limits of 0.09 ng/mL and 7.0 ng/mL, respectively (by preconcentration).

Mass spectrometry (MS) is especially sensitive and highly selective compared to other common detection techniques. Voyksner *et al*. (1986) reported an analytical method for tracing HMX using thermospray interfaced with an LC-MS system. Berberich *et al*. (1988) expanded the method and provided a detection limit lower than 2.5 picograms. Casetta and Garofolo (1994) reported an LC-MS-MS method for analysis of HMX in the picogram range. Groom *et al*. (2002) used an electrospray ionization LC-MS method for HMX determination in plant tissue that allowed a detection limit of 50 ng/mL at mass scan range of 40-400 Da.

LC-MS has higher sensitivity and selectivity for detection of HMX than does HPLC-UV. However, its application is hampered due to the presence of numerous endogenous interferences. The egg matrix is difficult because it contains proteins, lipids, pigments and others compounds. It is widely reported that matrices significantly suppress or enhance MS signals (Buhrman *et al*., 1996; Matuszewski *et al*., 1998; Souverain *et al*., 2004). Thus efficient separation processes are needed when HMX trace analysis is to be conducted. The aim of this study was to develop an effective extraction and cleanup method for the detection of HMX in eggs by LC-MS.

2.2. Experimental section

2.2.1 Reagents and materials

A standard solution of HMX in acetonitrile (1000 μg/mL, 99.0% pure) was obtained from Supelco (Bellefonate, PA, USA). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultra-pure reagent water (>18 MΩ) was prepared by a Nanopure system (Barnstead Dubuque, Iowa, USA). Florisil and styrene-divinylbenzene (SDB) solid phase extraction (SPE) cartridges (bed weight 0.5 g, tube volume 3 mL) were purchased from Supelco (Bellafonte, PA, USA). PTFE membrane filters (0.2 μm) were obtained from Fisher (Pittsburg, PA, USA).

2.2.2 Analytical equipment

An LC system with autosampler and ion trap mass spectrometer (Finnigan, San Jose, CA, USA) was operated in negative-ion mode for analyte quantification. Chromatographic separation was achieved at room temperature using a Supelco reverse phase (RP) C18 column (4.6×25 mm, 5 μm packing). The effluent contained 60/40 (v/v) methanol/1.0 mM aqueous acetic acid flowing at a rate of 0.5 mL/min. Electrospray ionization (ESI) was employed with acetic acid as the mobile phase additive to form negatively charged adduct ions $[M+CH_3COO]^-$, and m/z = 355 was quantified by selective ion monitoring (SIM). Helium served as damping and collision gas, while

nitrogen served as sheath and auxiliary gas. The MS was operated with a heated capillary temperature of 140ºC and an ionization voltage of 3.5 kV.

An Agilent 1100 HPLC system with ultraviolet (UV) detector (Hewlett-Packard, Waldbrom, Germany) was also used to analyze samples, for comparison to LC-MS results. A same Supelco RP C18 column was used for separations. Column eluent was 1.0 mL/min acetonitrile/water (50/50, v/v). Absorbance was recorded at 254 nm.

2.2.3 Fortification procedure

Chicken eggs were purchased from a local supermarket and stored in the refrigerator. Eggs were thoroughly homogenized using a magnetic stirrer. About 1 g of homogenized egg slurry was weighed (weighed to 0.1 mg) for each sample and dispensed into polypropylene centrifuge tubes (VWR Scientific, West Chester, PA, USA). Each sample was spiked with 100 µL HMX stock standard. Four spiking levels of 10, 50, 250 and 1000 ng/g were used. Spikes were thoroughly mixed into egg slurries for 1 min using a Genie 2 vortex mixer (Scientific Industries, Bohemix, NY, USA). Blank eggs were similarly prepared by spiking with the same volume of acetonitrile. Tubes containing fortified and blank samples were sealed and then stored in a refrigerator at 4-10°C in the dark for no more than 2 days before extraction and analysis.

2.2.4 Extraction and cleanup procedure

2.2.4.1 Extraction of HMX from eggs

About 1 g of egg homogenate (weighed to 0.1 mg) was dispensed into 15 mL graduated centrifuge tubes. Then 2 mL acetonitrile was added. The sample was homogenized by vortexing until eggs were thoroughly mixed. More acetonitrile was added into the tube to produce a total volume of 10 mL. The tube was sealed and continuously vortexed for 1 min. Then each sample was extracted for 1 h with ultrasonication using Branson 5510 ultrasonic bath (Danbury, CT, USA) at room temperature. After standing for several minutes, samples were centrifuged by Beckman Allegra 6R centrifuge (Palo Alto, CA, USA) at 3000 rpm for 10 min. The supernatant was collected for evaporation to a volume <2 mL, which required an N-EVAP 111 (Organomation Associates Inc., Berlin, MA, USA) operated at room temperature. Extracts were then ready for cleanup and analysis.

2.2.4.2 Cleanup of extracts

Our previous studies showed that extracts contained many interfering compounds. In LC-MS analysis, these interferences significantly suppress the response to HMX. Therefore, extra cleanup was needed before LC-MS analysis. Thus, sample extracts were

eluted through florisil cartridges and then SDB cartridges to remove interfering compounds.

Cleanup of extracts by florisil was performed on a 24-port manifold (Supelco, Bellefonate, PA). First, the florisil cartridge was conditioned with 2×2 mL acetonitrile. The eluate was discarded. Then the entire extract was loaded on the cartridge without vacuum. Once loaded, analytes were eluted with 3×3 mL acetonitrile. This eluate was collected in 10 mL graduated centrifuge tubes. All of the eluate was collected and than evaporated with nitrogen to less than 2 mL.

A further cleanup was employed using an SDB cartridge that had been conditioned with 2×2 mL acetonitrile. Then the extract was loaded into the cartridge. When the entire extract had almost eluted, the cartridge was rinsed with 3×3 mL of acetonitrile. All eluate was collected and volumetrically adjusted to 10 mL with acetonitrile. A 1 mL aliquot was removed from the extract and diluted 1:1 (v/v) with water, then filtered through a 0.2 μm PTFE membrane, and stored in a PTFE-capped vial for instrumental analysis.

2.2.5 Calibration curve and method detection limit

HMX calibration standards were prepared at nine concentrations (0.1, 0.5, 1, 5, 10, 50, 100, 500 and 1000 ng/mL) in 1:1 (v/v) acetonitrile/water. Calibration curves exhibited curvature at high concentrations. Linearity was achieved from 0.1 to 100 ng/mL

with a determination coefficient (r^2) of 0.990. For a wider concentration range (0.1-1000 ng/mL), a quadratic model ($y=ax^2+bx+c$) provided a good determination coefficient more than 0.990. The instrument detection limit of HMX was reported as low as 0.031 ng/mL (Pan *et al*., 2006). And method detection limit (MDL) was determined by performing seven replicate egg samples spiked with 1 ng/mL HMX as procedure described by EPA (USEPA, 2004).

2.2.6 Stability of HMX in eggs

To evaluate the possible degradation of HMX in spiked eggs during storage, an inter-day comparison was made. Ten replicate egg samples were spiked with 1000 ng/mL HMX. Samples were randomly split into two groups (N=10; 5 for day 0, 5 for day 2). Group 1 was extracted immediately and Group 2 was extracted after 2 days storage in the refrigerator at 4-10ºC in the dark. Recoveries were compared among days using a Student's t-test to evaluate degradation of HMX in storage.

2.2.7 Method recovery

To estimate the recovery and precision of the method, replicate egg samples were fortified with HMX at four concentrations (10, 50, 250, 1000 ng/g) and processed according to the same extraction and cleanup procedures. Three groups of eggs were processed on three different days. Recoveries of HMX at each concentration were

calculated by comparing measured and nominal concentrations. All raw recovery data were processed by two-way analysis of variance (ANOVA) to estimate differences among concentrations and days (Zar, 1999).

2.3. Results and discussion

2.3.1 Stability of HMX in eggs

HMX stability in extracts was maintained for two days (Table 2.1). A two-tailed t-test showed similarity of data ($p=0.947$) from extracts experiencing different refrigerated storage.

2.3.2 Recoveries at different concentrations

For egg homogenates, a MDL of 0.15 ng/g was achieved in determination of HMX. Average recoveries of HMX for all days and spiked concentrations ranged from 77.9% to 95.7% with an overall recovery of 87.5±9.4% (Table 2.2). All recovery data were processed using a two-way analysis of variance (ANOVA) and there was no difference of recoveries among groups ($p=0.971$) and spiked concentrations ($p=0.204$).

2.3.3 Comparison of concentrations estimated by LC-MS and HPLC-UV

Twenty egg extracts containing 400 to 2000 ng/g were tested by both LC-MS and HPLC-UV to evaluate the comparability of these two analytical techniques (Figure 2.2). A favorable agreement was seen between the two methods with an average absolute

deviation between the two methods of 6.1% and a maximum deviation of 15%. Thus when high concentrations of HMX are present in eggs, either technique will produce acceptable data.

2.3.4 Cleanup of extracts

Endogenous interferences may suppress or enhance signals in LC-MS applications (see Introduction). We found that extract matrices significantly suppressed LC-MS responses to HMX. In concentrated extracts, the HMX signal could be totally overshadowed by background noise. Therefore, cleanup was required for analysis of HMX in eggs by LC-MS.

Before cleanup, even in blank egg matrix, there were some compounds that had high responses and coeluted with HMX (Figure 2.3A). Efficient cleanup processes are needed to reduce such interferences. Florisil, SDB and 0.2μm PTFE filter were used to effectively clean egg extracts. Florisil is a magnesium silicate and is often used to remove steroids, carbohydrates and other compounds. After florisil cleanup, the background of extract was significantly reduced (Figure 2.3B). Styrene divinylbenzene (SDB) has higher affinity to pigments and other interferences. After SDB cleanup, pigments were removed thereby producing a clearer extract with a decreased background signal (Figure 2.3C). PTFE filter was used for removing small particles in the extracts before LC-MS

analysis. Most endogenous interfering compounds were removed from extracts with cleanup. LC-MS background was significantly reduced, and the HMX signal was stabilized.

2.4. Conclusion

An efficient extraction and cleanup procedure was developed for determination of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) concentrations in eggs by LC-MS. The method provided high recovery, good accuracy (overall 87.5±9.4%) and a low detection limit of 0.15 ng/g. This method allows trace analysis of HMX in an important yet somewhat difficult to analyze egg matrix. Such trace analyses will also allow more reliable assessment of the avian exposure to HMX.

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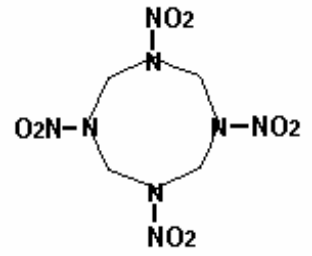

Figure 2.1. Chemical structure of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX).

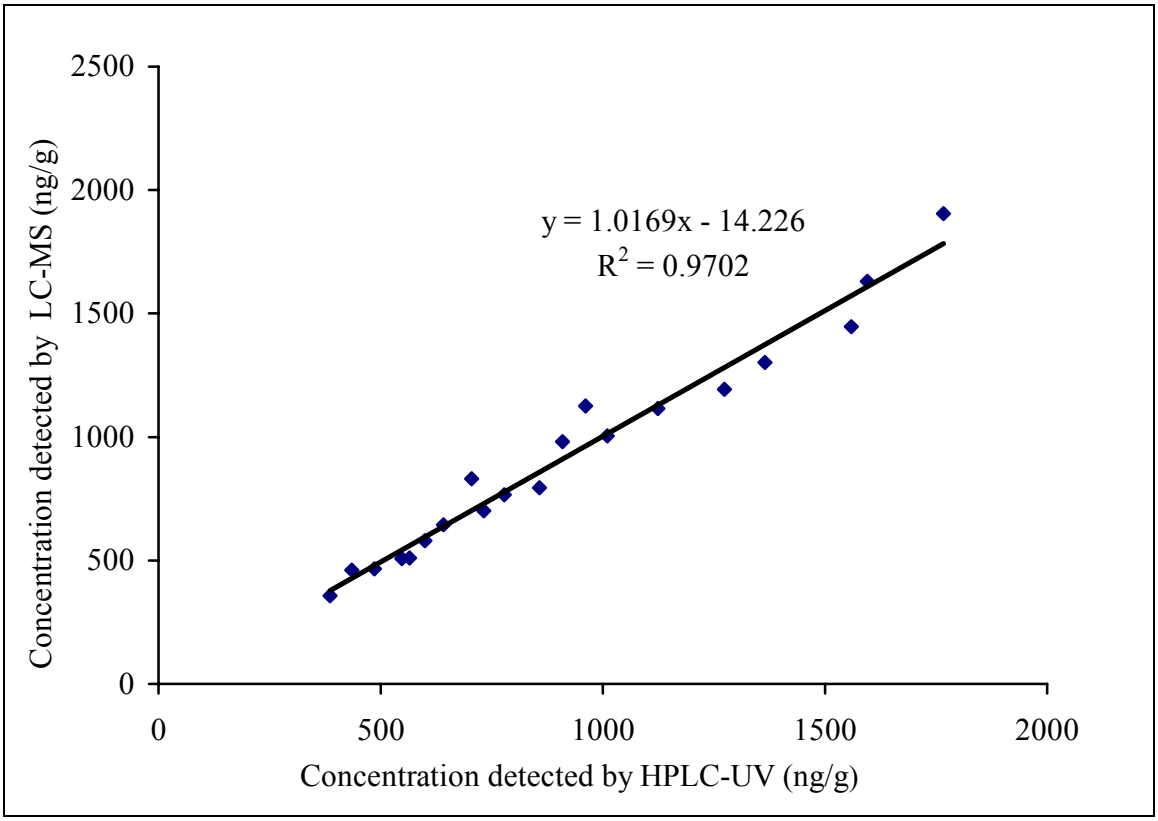

Figure 2.2. Correlation of HMX concentrations determined by LC-MS and HPLC-UV.

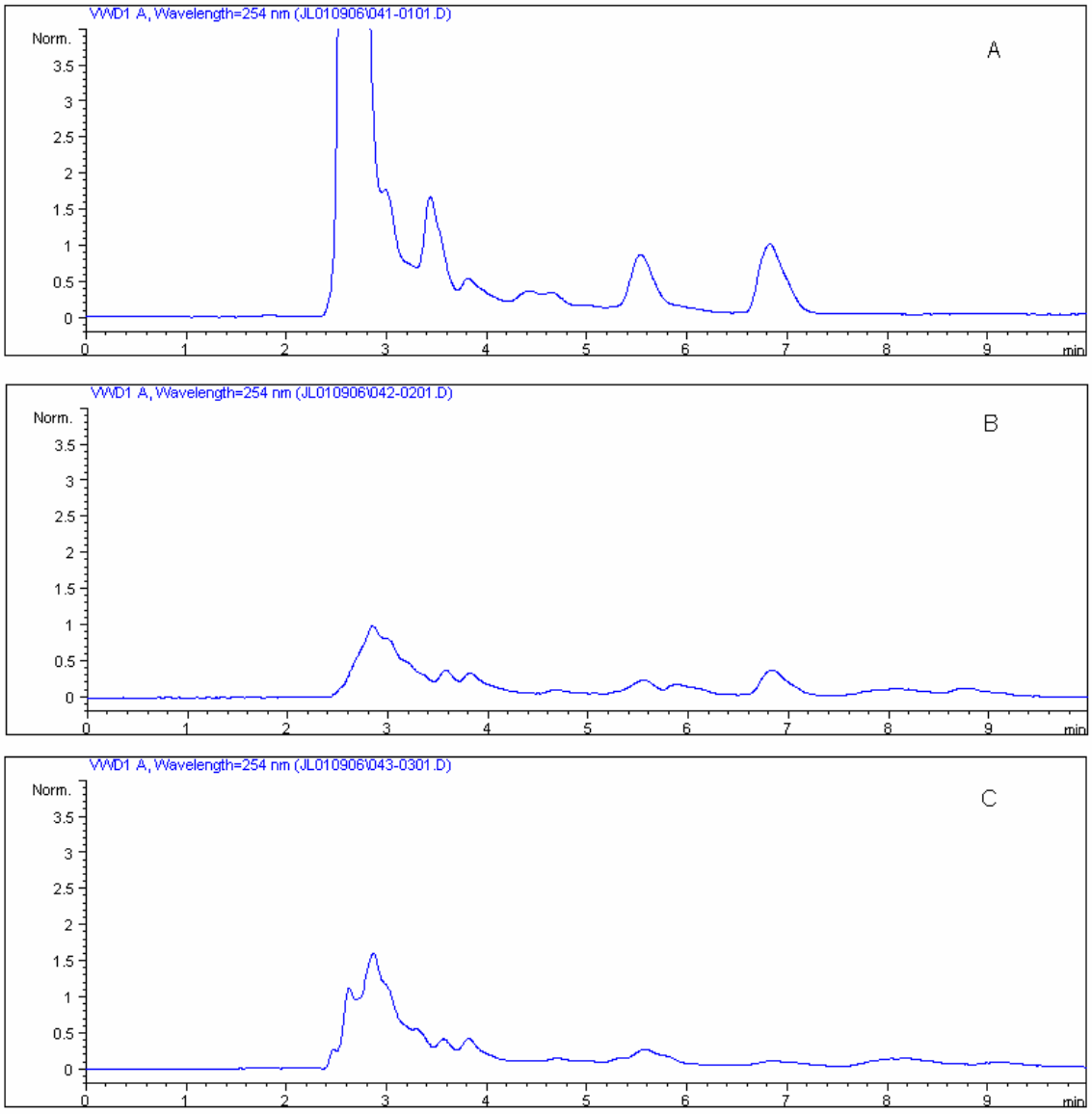

Figure 2.3. Representative HPLC-UV chromatograms of (A) blank egg without cleanup, (B) blank egg after florisil cleanup and (C) blank egg after florisil + SDB cleanup.

Table 2.1. Stability of HMX in spiked chicken egg aliquots.

Eggs	**Recovery (%)**	
	Day 0[a]	**Day 2[a]**
1	99.8	76.5
2	95.2	94.2
3	102.7	99.9
4	94.9	87.0
5	72.9	105.4
Overall	93.1±11.7[b]	92.6±11.3[b]

a - Number of days between spiking and extraction.

b - Mean ± standard deviation.

Table 2.2. Recoveries of HMX in fortified chicken eggs at four different concentrations.

Concentration spiked (ng/g)	**Recovery (%)**			
	Day 1	**Day 2**	**Day 3**	**Overall**
10	77.9±3.7(n=2)	84.9±7.3(n=2)	83.1±6.6(n=3)	84.0±9.3(n=7)
50	89.1±8.1(n=7)	91.9±4.6(n=7)	83.1±5.3(n=7)	88.0±7.4(n=21)
250	90.1±16.6(n=2)	90.6±10.6(n=2)	91.0±15.0(n=2)	90.6±12.5(n=6)
1000	80.8±9.9(n=7)	87.0±11.6(n=7)	95.7±13.1(n=6)	87.4±13.2(n=20)

CHAPTER III

EFFECTS OF HMX EXPOSURE UPON METABOLIC RATE OF NORTHERN BOBWHITE QUAIL (*COLINUS VIRGINIANUS*) IN OVO

Abstract

We evaluated the use of the gas exchange rate as an ecologically relevant indicator of chemical stress in avian embryos/eggs. Northern bobwhite quail (*Colinus virginianus*) were exposed to octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) via feed at concentrations of 0, 12.5, 50.0, and 125.0 mg/kg. Metabolic rates (oxygen consumption) of developing quail eggs were then measured via respirometry to examine potential effects of HMX exposure. Metabolic rates were examined on 5, 9, and 21 days of incubation. Next, concentrations of HMX in embryos/eggs were determined by liquid chromatography-mass spectrometry. Concentrations of HMX in eggs from the four dose groups were significantly different. Mean (± SE) concentrations of HMX in quail eggs were 1025 ± 77, 3610 ± 143 and 7021 ± 300 ng/g in the low, medium and high dose groups respectively. A significant difference in oxygen consumption rates was observed among eggs at the three developmental stages ($p<0.0001$), but not among the four dose groups ($p=0.18$). No evidence was observed for alterations of in ovo metabolic rates associated with HMX exposure.

3.1. Introduction

Physiological energetic methods have been used successfully to demonstrate the effects of chemicals and toxicants upon a variety of aquatic organisms in laboratory, mesocosm, and field studies (Widdows *et al*., 1991; Davison *et al*., 1992; Basha *et al*., 1984; Nordtug *et al*., 1991; Rowe *et al*., 1998, 2001; Hopkins *et al*., 1999). In contrast, physiological energetics has seldom been used when assessing the impact of environmental toxicants on endotherms such as northern bobwhite quail. Typically, exposure to chemical stressors disrupts metabolic homeostasis by activating compensatory mechanisms, thereby changing energy utilization (Forbes *et al*., 1998). Among avian species, chemical exposure may cause maternal excretion of contaminants into eggs during oogenesis (Bryan *et al*., 2003; Brunjes *et al*., 2007). Thus, the effects of toxicants deposited in eggs may be manifested as increased or decreased metabolic rates.

In this experiment, we measured gas exchange rates across eggshells to examine the effects of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX, $C_4H_8N_4(NO_2)_4$) in bobwhite quail embryos/eggs. HMX is an energetic nitramine compound that is widely used as an explosive (ATSDR, 1997). This contaminant has been detected in soil, plants, and biota at munitions manufacturing plants, military compounds, and military training grounds (Pennington and Brannon, 2002; Park *et al*., 2004). Photolytic half-lives of HMX were from 1.4 to 70 days in aqueous environments that receive direct sunlightsunlit

(ATSDR, 1997), and half-lives of HMX in soil and sediment ranged from 133 to 2310 days (Jenkins *et al*., 2003). HMX is thought to be relatively stable in water, soil, and sediments under ambient conditions (ATSDR, 1997).

In an acute toxicity test, adult northern bobwhite quail were not adversely affected by HMX concentration up to 10,760 mg/kg in feed (Johnson *et al*., 2005). However, a sub-chronic study showed that avoidance of food containing HMX caused a reduction in adult quail body mass and egg laying rates (Johnson *et al*., 2005; Brunjes *et al*., 2007). Also, HMX is readily deposited into fertilized quail eggs following the exposure of adult females (Brunjes *et al*., 2007). We hypothesized that HMX in the egg may alter the metabolic rate. Measures of metabolic rate could be an effective indicator of chemical-induced physiological stress on quail eggs. Oxygen consumption can actually be considered as a measure of embryonic life, and it has been widely used for measuring the metabolic rates and the physiological activities of avian embryos (Romanoff and Romanoff, 1967; Vleck and Vleck, 1987) as well as indicating adverse effects of contaminants (Van den Berg *et al*., 1994; Forbes *et al*., 1998). Therefore, the objective of this study was to determine if HMX deposited into fertilized quail eggs affected metabolic rates in ovo.

3.2. Materials and methods

3.2.1 Quail exposure and egg collection

Details of the experimental design and dosing methods are presented in Brunjes *et al.* (2007). Briefly, 30 pair of adult bobwhite quail were randomly distributed into four HMX dose groups (control, low, medium and high dose) and then dosed with HMX via dietary intake for continuous 30 days. HMX (Accurate Energetic Systems, McEwen, TN, USA) was dissolved in acetone, sprayed on commercial feed at nominal concentrations of 0, 12.5, 50, and 125 mg/kg, and then thoroughly mixed. Actual concentrations of HMX in control, low, medium, and high feed groups were determined by an Agilent 1100 high performance liquid chromatograph (HPLC) system with a UV detector (Hewlett-Packard, Waldbrom, Germany). Eggs were collected daily, weighed, and immediately moved into a Profi-I forced-air incubator (Lyon Electric Company, Chula Vista, CA, USA). Eggs were candled to determine fertility and viability, and all eggs unfertile or dead were removed from the experiments.

3.2.2 Respirometry

A Qubit 8-channel gas exchange system (Kingston, ON, Canada) was used to measure gas exchange rates of 93 quail eggs at different ages of incubation/development. The system was composed of a compressed air cylinder, flow controller, incubator, and

computer with Labview 6.0 software (National Instruments, Austin, TX, USA). Air (dry grade, 20.95% oxygen) flowed into an incubator, and then passed through a mass-flow controller and a gas multiplexer (G245 and G244, Qubit). Oxygen and carbon dioxide concentrations were quantified at the inlet and outlet of the chamber containing the egg. Data were imported into separate Microsoft Excel (Redmond, WA, USA) files for each sample. For further review of respirometry techniques used see Isanhart *et al*. (2005).

Quail egg gas exchange rates were determined by measuring oxygen consumption on days 5, 9, and 21 of incubation (± 1 day). Each egg was weighed (± 0.0001g) and placed in an experimental chamber (modified plastic chamber, volume of 60 mL) within a temperature-controlled (38.0 ± 1.0ºC) environmental chamber. Air flow rates were adjusted to 100 ± 1 mL/min (under standard temperature and barometric pressure conditions) for each chamber. The system was programmed to measure oxygen consumption automatically at 1 sec intervals for 15 min in each experimental chamber and then switch to the next chamber in the series.

Data files were separated for each egg so that oxygen consumption rates (μL/hr) could be evaluated by plotting the data in a line graph that displayed consumption trends over time. After a plateau of oxygen consumption was achieved, a mean oxygen exchange rate was quantified by averaging data collected for 120 sec (Figure 3.1). The interval was considered to be the most stable portion of the oxygen consumption trend

line. Oxygen exchange rate was divided by the egg mass to obtain oxygen consumption rate (μL/g/hr).

3.2.3 Analysis of HMX in eggs

After respirometry, eggs were removed from experimental chambers and immediately stored at -20 ºC until chemical analysis. Concentrations of HMX in eggs were determined using the method developed by Liu *et al*. (2007). Briefly, eggs were thawed and opened. All compartments of eggs were separated and wet weights of shell, yolk, embryo, and CAM (chorioallantoic membrane) were obtained (± 0.0001 g). Then the yolk, embryo, CAM and albumin of eggs were extracted with acetonitrile. After concentration by N-EVAP 111 nitrogen evaporator (Organomation Associates Inc., Berlin, MA, USA), extracts were passed through florisil and SDB cartridges (VWR Scientific, West Chester, PA, USA) to remove endogenous interfering compounds. In each batch of samples, at least two egg samples fortified with HMX standard solution were prepared to evaluate the recovery of HMX. A Thermo LCQ advantage HPLC system equipped with an ion trap mass spectrometer (Finnigan, San Jose, CA, USA) was used for HMX quantification. The analytical method provided an overall recovery rate of 87.5 ± 9.4% (mean ± standard deviation) and a detection limit of 0.15 ng/g (Liu *et al*., 2007). The sample quantitation limit was calculated by multipling the lowest reliable standard by the dilution factor for each batch of samples.

3.2.4 Statistical analysis

Measures of central tendency were expressed as mean ± standard error. All data were tested for normality and homogeneity of variances and transformed if required prior to statistical analysis. Feed consumption during acclimation and dosing was compared using paired t-test. Concentrations of HMX in eggs were tested using a two-way analysis of variance (ANOVA), with dose group and embryo age (number of days incubated) as factors. Fluctuations of HMX transfer to eggs during the 30-day dose period were tested using an analysis of covariance (ANCOVA) with the dose group as a factor and egg laying sequence (number of days after dosing began) as the covariate. A two-way ANOVA was conducted using oxygen consumption rates as dependent variant, with dose group and embryo age as two factors. Furthermore, an ANCOVA was conducted using oxygen consumption rate as dependent variant, with embryo age as a factor and HMX concentration as the covariate, because concentration of HMX reflected dose group. All statistical analyses were conducted using R 2.2.1 (www.r-project.org). The level of significance for all statistical tests was defined as $p<0.05$.

3.3. Results

3.3.1 Feed consumption and HMX intake

Quail feed for all dose groups was analyzed by HPLC-UV before use. Actual HMX concentrations in the feed of control, low, medium and high dose groups were 0, 12.3 ± 0.5, 52.5 ± 4.1, and 109.3 ± 7.2, respectively. Mean feed consumption by each pair of quail in the four groups was 88.5 ± 4.4, 92.2 ± 3.4, 91.2 ± 3.7, and 86.9 ± 3.0 g/kg/day (Figure 3.2). Paired t-test showed no significant differences in feed consumption between acclimation and dosing period (p=0.76, 0.46, and 0.23 for low, medium, and high groups, respectively). Mean HMX intake rates for each pair of quail in the four groups, calculated by multiplying feed consumption by HMX concentration in the food, were 0, 1.1 ± 0.1, 4.8 ± 0.2, and 9.5 ± 0.3 mg/kg/day, respectively.

3.3.2 HMX in eggs

HMX readily transferred into quail eggs in a dose-dependent manner. Mean (± SE) concentrations of HMX in eggs were 41 ± 6, 1025 ± 77, 3610 ± 143 and 7021 ± 300 ng/g in control, low, medium and high dose groups, respectively (Figure 3.3). Trace amounts of HMX were found in some control eggs. More than 70% (46 of 62) of control samples from the different egg compartments had concentrations that fell below both the inter-sample blank solvent response (10-30 ng/g) and the lowest standard of calibration (20-50

ng/g, considering the dilution factor). The occurrence of HMX in the remaining control eggs was likely due to contamination during extraction or instrumental analysis. We believe these facts indicate that more than 70% of the controls were in fact not detectable. Regardless, HMX concentrations in control eggs were orders of magnitude lower than the eggs in HMX-treated groups. These values from the instrumental output were used for statistics because they provided a distribution of values below the quantitation limit about 50-100 ng/g (considering the sample dilution factor). Variation of HMX concentrations in eggs among three embryo ages (5, 9, and 21 days incubated) was compared by a 2-way ANOVA, and the results showed significant differences among the four dose groups ($p<0.0001$), but no differences among three embryo ages ($p=0.11$).

3.3.3 Time course of HMX transfer from hens to eggs

ANCOVA showed significant differences among dose groups ($p<0.0001$), but not among the laying dates ($p=0.34$). Results indicated that HMX transfer from adult quail into their eggs did not change during the 30-day dose period of this study, and concentrations of HMX in eggs were temporally stable throughout the duration of dosing.

3.3.4 Oxygen consumption rates

Oxygen consumption rates of eggs increased according to the embryo age (days incubated). A two-way ANOVA showed significant differences in oxygen consumption rate among three embryo ages ($p<0.0001$), while no significant difference among the four dose groups ($p=0.18$). The ANCOVA also showed no significant differences among different HMX concentrations ($p=0.16$), indicating that oxygen consumption rates were not altered by the presence of HMX in eggs. Oxygen consumption by eggs in different dose groups and at different embryo ages is shown in Table 3.1. Since no significant difference was observed among dose groups, mean (± SE) oxygen consumption of 5-, 9- and 21-day old eggs was calculated to be 25.9 ± 1.4, 93.1 ± 3.2 and 636.6 ± 36.2 μL/g/hr.

3.4. Discussion

We have reported that HMX was readily deposited in quail eggs following exposure of breeding pairs (Brunjes *et al*., 2007). In this study, as expected, concentrations of HMX in eggs increased in a dose-dependent manner. From the 3rd day of exposure to the termination of the study, HMX transfer to eggs was temporally stable. Variation in HMX concentrations within each dose group was as expected, and it may relate to the oogenesis process. Although, the mechanism of HMX transfer in oogenesis is not known. Organochlorines (OCs) such as polychlorinated biphenyls (PCBs) were commonly found

in eggs of oviparous wildlife post adult exposure (Cobb *et al*., 2003). In eggs of glaucous gulls (*Larus hyperboreus*), OCs, PCBs and other contaminants were found, and maternal transfer favored low Kow and less persistent compounds, not the recalcitrant and higher-halogenated compounds (Verreault *et al*., 2006). In great tits (*Parus major*), PCB, PCDE and OC bioaccumulation in eggs were found related to contamination in mothers (Dauwe *et al*., 2006). The primary source of contaminants is assumed to be maternal transfer during oogenesis of the egg. Trace elements were found transported to the ovary by metal transport proteins (Richards *et al*., 1987; Unrine *et al*., 2006). During oogenesis, the diet of the maternal adult was the principal source of methylmercury in eggs (Hammerschmidt and Sandheinrich, 2005). Some birds produce more than one egg per day, so the amount of HMX may be distributed unevenly between eggs which develop simultaneously or in succession (Bryan *et al*., 2003). Fluctuations in daily feed consumption may affect the amount of HMX which is available for deposition into developing oocytes. Analytical methods may also contribute errors in determination of HMX.

Oxygen consumption of eggs increased about 4 times from 5 to 9 days, and then increased 7 times from 9 to 21 days, nearly an exponential trend (Figure 3.4). Similar trends in oxygen consumption were observed in studies using chicken and duck eggs,

where oxygen consumption rates of chicken and duck eggs increased exponentially during the first 80% part of incubation (Rahn *et al*., 1974; Hoyt and Rahn, 1980).

Some toxic chemicals adversely affect egg respiration. Van den Berg *et al*. (1994) measured oxygen consumption rates of cormorants eggs (*Phalacrocorax carbo*) exposed to PCBs, polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs). Cormorants exposed to high concentrations of PCBs produced eggs with higher oxygen consumption rates as compared to cormorants exposed to low concentrations of PCBs. They also found that birds from the PCBs, PCDDs and PCDFs contaminated colony showed increased cytochrome P450 and ethoxyresorufin-O-deethylation (EROD) activity (Van den Berg *et al*., 1994). Increased respiratory rates might have been related to increased cytochrome P450 and EROD activity. While Hoffman *et al*. (1998) found PCBs caused 90% mortality within 1 week of treatment in avian embryos exposed to 3.2 mg/kg. Therefore, sublethal doses of toxicants like PCBs may increase oxygen consumption rates, but may also totally eliminate oxygen consumption due to death of eggs at high doses.

With high concentrations of HMX deposited in eggs, toxic effects on egg metabolism seemed plausible. To our knowledge, no studies have described impacts of HMX on metabolic rates in any species. We initially expected to see an alteration in oxygen consumption rates of eggs produced by adult quail exposed to HMX as compared

to controls. However, in our study, there were no alterations in oxygen consumption rates among eggs containing different concentrations of HMX.

HMX had no effects on chick hatching and survival, and the reproductive toxicity on birds is low (Brunjes *et al*., 2007). This study supported that conclusion by showing that HMX did not affect metabolic rates of eggs. However, our findings can preclude neither metabolic effects on eggs nor the myriad of other sub-lethal effects at higher HMX concentrations that were untested during this study. According to toxicological principles, the toxicity of a chemical depends on the dose. Differences in the deposition of HMX may also produce different concentrations in a target organ (Klaassen *et al*., 2005). Given the information that quail tolerated HMX concentrations as high as 10,760 mg/kg (Johnson *et al*., 2005), higher dosing of HMX may alter egg metabolism, but a concentration as high as that seems not environmental relevant.

The mechanism of HMX producing toxicity in human and animals is unclear. One possible mechanism is that some mammalian enzyme systems process similar metabolic abilities as microorganisms thereby generating intermediates (ATSDR, 1997). Hepatic cytochrome P450 activities significantly correlated with PCB burdens in white leghorn chicken (*Gallus domesticus*) hatches (Bargar *et al*., 2003). Considering the relationship between increased enzyme activities and increased respiratory rates in one study on PCBs (Van den berg *et al*., 1994), cytochrome P450s such as EROD may play an important role

in the oxygen consumption. However, no studies have evaluated the relationship between the enzyme activity and exposure to HMX. Recently, RDX, a structurally similar compound of HMX, was found biodegraded in *Rhodococcus* strain DN22 through a process involving cytochrome P450 enzyme (Coleman *et al*., 2002). Furthermore, cytochrome P450 2B4 (from rabbit liver) was found the key enzyme responsible for RDX biotransformation by *Rhodococcus* sp. strain DN22 (Bhushan *et al*., 2003). In this study, concentrations of HMX in eggs did not change significantly after 5-, 9- and 21-day incubation, suggesting the transformation of HMX during the incubation might be low.

Further studies to describe the mechanism of toxicity of HMX are needed. Our research on physiological energetics may add to the understanding of the mechanisms by which toxicants affect embryonic development in the early life stages of avifauna. A better understanding of how chemical exposures alter embryonic metabolic function in ovo may lead to more ecologically relevant risk assessments.

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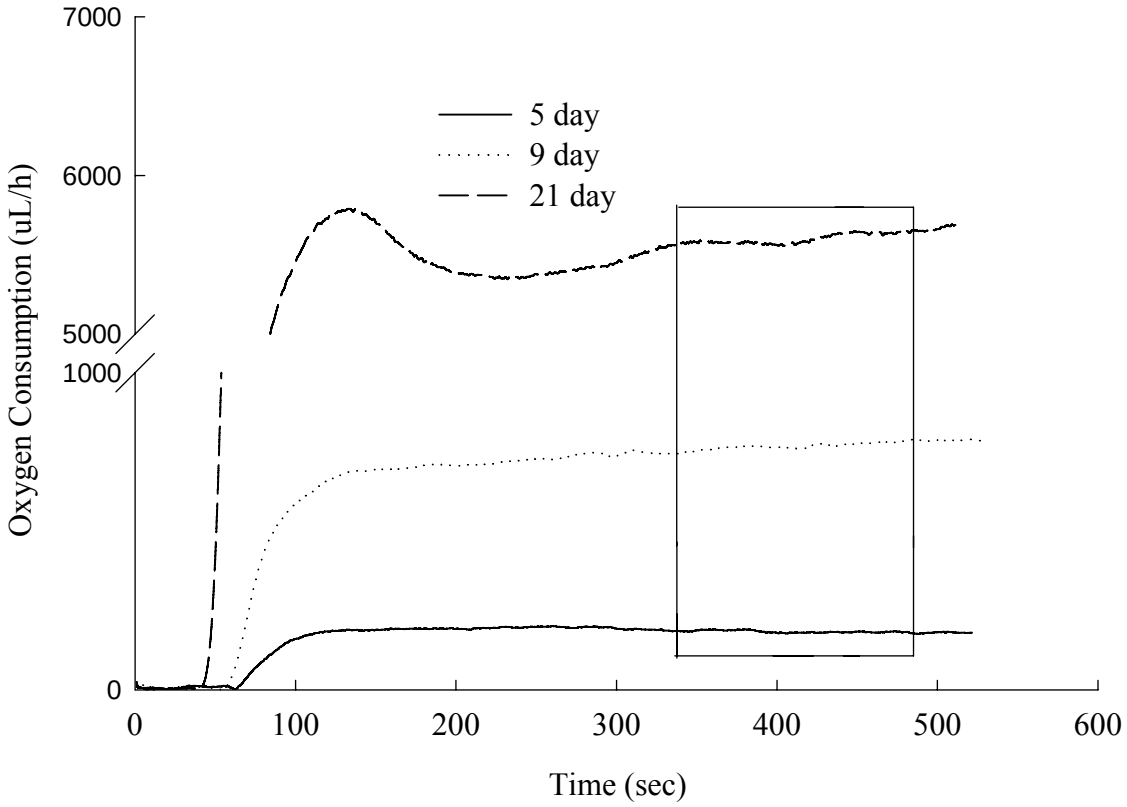

Figure 3.1. Representative graphs of oxygen consumption rates (μL/hr) by 5, 9, and 21 days old northern bobwhite quail eggs. The box represents a data collecting window of 120 s after achieving a plateau.

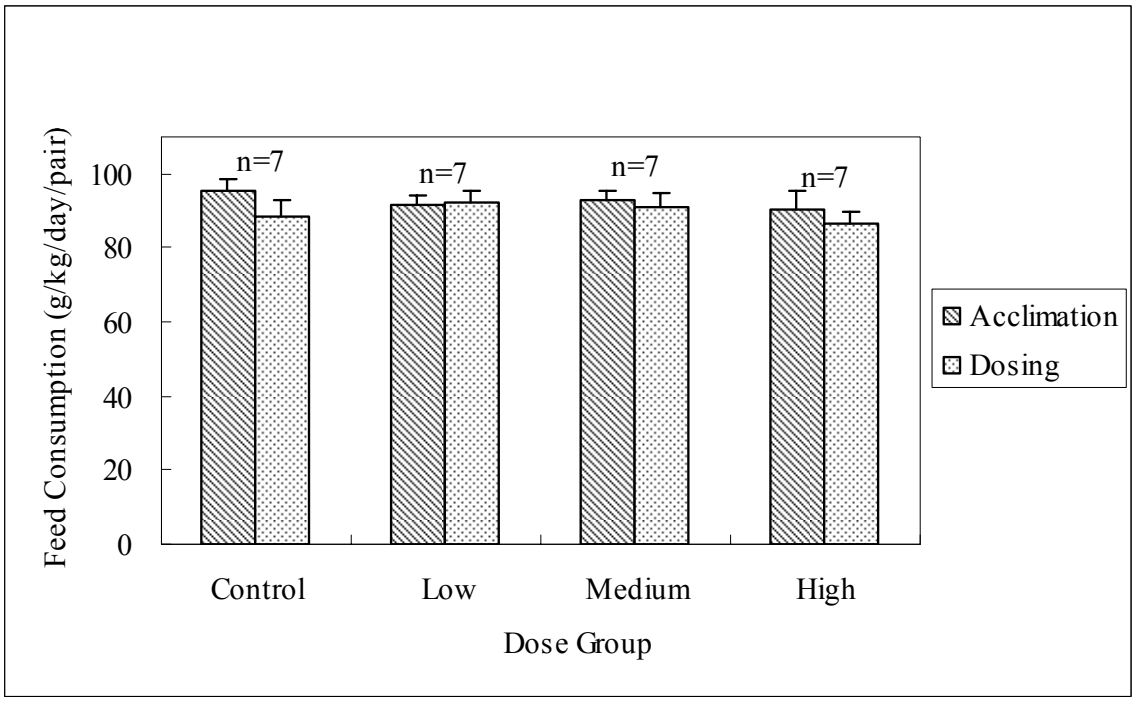

Figure 3.2. Mean (±SE) feed consumption by northern bobwhite quail in four dose groups during acclimation and 30-day dose period. Pair number of quail in each dose group is shown above each bar.

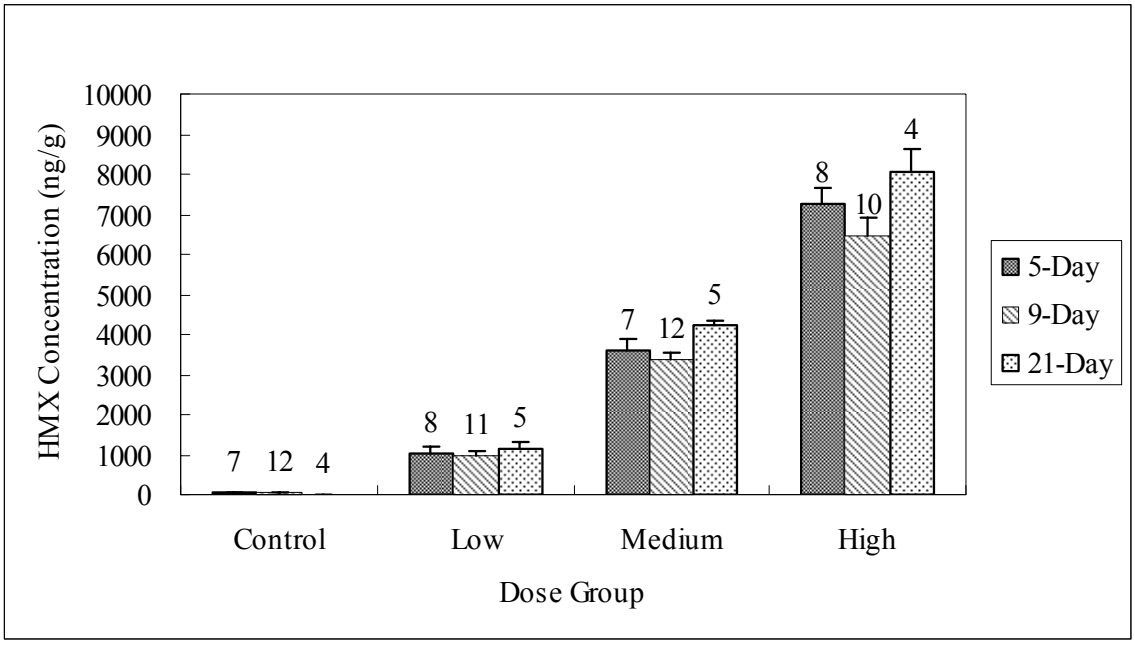

Figure 3.3. Mean (±SE) concentrations of HMX in northern bobwhite quail eggs in four dose groups on 5, 9, and 21 days of incubation. Number of eggs measured is shown above each bar.

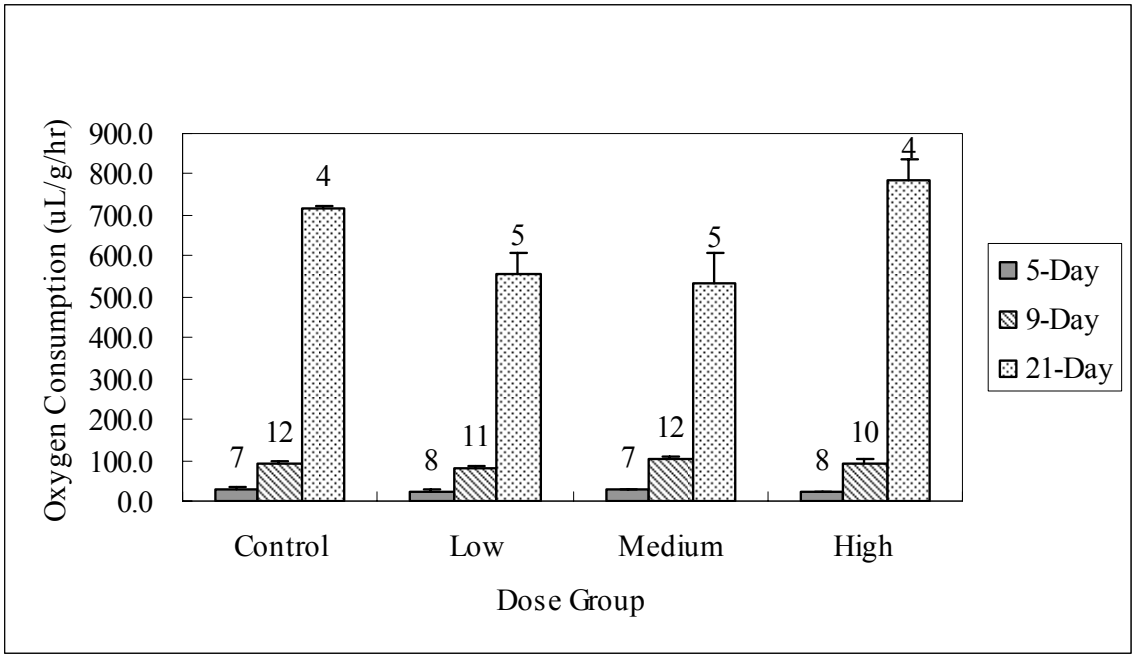

Figure 3.4. Mean(±SE) oxygen consumption rates of northern bobwhite quail eggs in the four dose groups on 5, 9, and 21 days of incubation. Number of eggs measured is shown above each bar.

Table 3.1. Mean (± SE) oxygen consumptions (μL/g/hr) of 5, 9, and 21-day incubated northern bobwhite quail eggs collected from four dose groups. Number of eggs measured is shown in each cell.

Dose Group	**Embryo Age (number of days incubated)**		
	5	**9**	**21**
Control	30.3 ± 3.2, n=7	92.9 ± 6.6, n=12	718.6 ± 34.7, n=4
Low	24.0 ± 2.3, n=8	81.3 ± 5.5, n=11	556.4 ± 49.0, n=5
Medium	27.5 ± 2.7, n=7	103.2 ± 4.2, n=12	533.7 ± 72.4, n=5
High	22.4 ± 2.6, n=8	94.3 ± 8.1, n=10	783.5 ± 53.8, n=4
Mean	25.9 ± 1.4, n=30	93.1 ± 3.2, n=45	636.6 ± 36.2, n=18

CHAPTER IV

MATERNAL TRANSFER AND TISSUE DISTRIBUTION OF HMX IN NORTHERN BOBWHITE QUAIL EGGS

Abstract

Maternal transfer of HMX from female northern bobwhite quail (*Colinus virginianus*) to their eggs and distribution of HMX in egg compartments were studied. Adult quail were dosed via feed containing HMX at four concentrations for continuous 30 days. Eggs produced by hens were incubated for 5, 9 and 21 days then dissected for analyses. Concentrations of HMX in the yolk, embryo and chorioallantoic membrane (CAM) were determined. HMX was found in eggs from all treatment groups. HMX transfer to eggs was not different during the 30-day time course of the dosing. Concentrations of HMX in eggs were dose-, age- and compartment-dependent. The yolk and embryo had much higher concentrations of HMX than the CAM. At all embryo ages (5-, 9- and 21-day of incubation), more than 97% of HMX was distributed in the yolk and embryo, while less than 3% was in the CAM and albumin. HMX in the embryo increased rapidly from 9 to 21 days, which coincided with the progress of accumulating lipids by the embryo. Both concentration and mass of HMX found in the CAM was low, indicating the CAM did not serve as a significant depot for HMX in the egg. Bioconcentration

factors (BCFs) of HMX in eggs were 0.084, 0.068 and 0.064, and maternal transfer coefficients (MTCs) were 0.030, 0.018 and 0.015 in the low, medium and high dose groups, respectively. BCF and MTC in the low dose were significantly higher than those in the medium and high doses, indicating portion of HMX excreted in eggs decreased in high doses.

4.1. Introduction

4.1.1 Maternal transfer of contaminants to eggs

Eggs have been widely used to monitor environmental pollutant exposure in avian, amphibian and fish species since females can transfer toxicants from their bodies to their eggs. Organohalogens are frequently studied. Maternal transfer rates of polychlorinated biphenyls (PCBs) ranged from 1% to 20% of avian body burdens, and the transfer was inversely related to congener chlorination (Bargar *et al*., 2001a). Uptake and distribution of bisphenol A (BPA) and tetrabromobisphenol A (TBBPA) in Japanese quail (*Coturnix japonica*) embryos showed that maternal transfer of BPA and TBBPA to the egg was low (Halldin *et al*., 2001). Concentrations of polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in eggs were several times higher than those in the frogs, indicating that maternal transfer reduced concentrations in tissues of females in this case (Kadokami *et al*., 2004). A study on p,p'-DDE, toxaphene, dieldrin and chlordane found that 0.6% of administered chemicals were maternally transferred to the eggs of American alligators (*Alligator mississippians*), and yolk burdens were predictive of maternal tissue burdens for certain tissues (Rauschenberger *et al*., 2004). Concentration of OCs (PCDDs, PCDFs and PCBs) in walleye (*Sander vitreus*) ova was found positively related to maternal age, size and lipid content in ova (Johnston *et al*., 2005). Maternal transfer of TCDDs caused the accumulation of 0.094-1.2 ng/g in eggs,

which was sufficient to induce toxicity (Heiden *et al*., 2005). Tributyltin (TBT) and PCBs were maternally transferred into eggs, causing early life-stage toxicity (Nakayama *et al*., 2005). In eggs of glaucous gulls (*Larus hyperboreus*) collected from the Norwegian Arctic, a suite of OCs, PCBs, PBBs and byproducts were found, and maternal transfer favored low Kow and less persistent compounds, not the recalcitrant and higher-halogenated compounds (Verreault *et al*., 2006). In great tits (*Parus major*), PCB, PCDE and OC bioaccumulation in eggs were found related to contamination in mothers (Dauwe *et al*., 2006).

Maternal transfer of mercury (Hg) and methylmercury (MeHg) has been investigated. Mercury in eggs is typically predictive of mercury risk to avian species (Wolfe *et al*., 1998). Concentrations of mercury (mainly MeHg) in different avian eggs ranged from 0.5 to 16 ng/g, and the reproductive toxicity of mercury included reduced productivity, decreased hatchability, and aberrant nesting behavior (Finley and Stendall, 1978; Heinz, 1979; Newton and Haas, 1988). Female walleye transferred MeHg from their body to their eggs, and MeHg burdens in eggs represented 0.2-2.1% of the total body burden (Johnston *et al*., 2001). A study on maternal transfer of MeHg to eggs of fathead minnows (*Pimephales promelas*) indicated the diet of the maternal adult during oogenesis, but not adult body burden, was the principal source of MeHg transferred to eggs (Hammerschmidt and Sandheinrich, 2005). Mercury accumulation in sea lamprey

(*Petromyzon marinus*) from the Connecticut River, USA was examined, and mercury in eggs (mean 84 ng/g wet weight) was correlated to concentrations in females and a high transfer rate was found (Drevnick *et al*., 2006).

Maternal transfer of trace elements has been studied. Maternal transfer of 18 elements to eggs in black-tailed gulls (*Larus crassirostris*) indicated large amounts of Sr, Ba, and Tl transferred to the eggs, while low amounts of V, Cd, Hg and Pb were transferred. Maternal transfer rates of trace elements ranged from 0.8% (Cd) to as much as 65% (Tl) of maternal body burden (Agusa *et al*., 2005). Selenium from dietary exposure to the oviparous lizard (*Sceloporus occidentalis*) was efficiently transferred from the mother to offsprings (Unrine *et al*., 2006). Maternal transfer of contaminants in eastern narrow-mouth toads (*Gastrophryne carolinensis*) collected near a coal-burning power plant was examined, and adult toads inhabiting the industrial area transferred significant quantities of selenium and strontium to their eggs (Hopkins *et al*., 2006).

4.1.2 Egg structure

Two major compartments in a freshly laid avian egg are the yolk and albumin. The yolk has the most fat-rich content in the egg, containing major nutritional proteins and lipids for the development of the embryo (Romanoff and Romanoff, 1967). The albumin also contains proteins and lipids, but it is primarily an aqueous compartment, containing

approximately 90% water, while the yolk contains 50% water (Freeman *et al*., 1974). During incubation, the yolk and albumin diminish in mass and are largely absorbed into the hatchling by the end of incubation. The embryo is too small to be seen without magnification during the early days of incubation, although it develops into the chick, the largest part before hatching (Romanoff, 1960).

A developing avian embryo has four extraembryonic membranes: yolk sac, amnion sac, chorion and allantois (Figure 4.1). The allantois extends to surround the embryo and eventually encloses the contents of the egg. The outer layer of the allantois fuses with the lining of the chorion to form the chorioallantoic membrane (CAM) (Romanoff, 1960). Primary functions of the CAM are respiration, waste storage, calcium and electrolyte transport (Romanoff, 1960; Simkiss, 1980).

4.1.3 Distribution of contaminants in eggs

Contaminant distribution in different compartments of the egg in many species has been investigated. The distribution of OCs between the yolk, embryo and CAM in piping Audouin's gulls (*Larus audouinii*) has been studied. More than 16 OCs were found in eggs, and PCBs and DDTs were the most abundant pollutants. The largest concentrations were generally found in yolk sacs, containing 48-50% by weight of PCBs and DDTs in eggs, followed by embryos and CAMs (Pastor *et al*., 1996). In another study on double-

crested cormorant chicks (*Phalacrocorax auritus*), embryos were decapitated and the yolk sac, liver, head, carcass and fecal sac (allantois) were analyzed separately; about 45-60% by weight of each OC (including 13 PCBs, DDE, oxychlordane, dieldrin, *et al*.) within eggs were found in yolk sacs (Custer *et al*., 1997). Distribution of veterinary drugs and feed additives in eggs showed neither lipid solubility nor pKa values could explain the distributions, and no factors could confirm the distribution of sulfonamides in egg white and yolk (Kan *et al*., 2000). A study of selenium distribution in egg compartments (yolk, albumen, shell, and shell membrane) was conducted on 8 different avian species. All avian species showed identical selenium concentrations in yolk-albumin complex. High concentrations of selenium occurred in ostrich shell and shell membrane, and incubation of fertile eggs decreased selenium content in eggshell (Golubkina *et al*., 2006).

The analysis of CAMs provides predictable estimates of organic pollutants in eggs and in maternal tissues, and has been used as a non-lethal method for evaluating contaminant exposure and effect in oviparous wildlife (Cobb *et al*., 2003). Chlorinated contaminant partitioning between the CAM and remaining egg contents in great blue herons (*Ardea herodias*) was found to be a good indicator of contaminant concentrations in whole eggs (Cobb *et al*., 1994, 1995). Eggs and CAMs were used to characterize American alligator (*Alligator mississippiensis*) exposure to PCBs in South Carolina.

Total PCB concentrations in CAM can be used to estimate PCB concentrations in eggs (Cobb *et al*., 1997; Bargar *et al*., 1999). PCB concentrations between the CAM and egg tissues in the loggerhead sea turtles (*Carretta carretta*) showed a high correlation (Cobb and Wood, 1997). A constant partitioning of PCBs between CAMs and eggs was found in white leghorn chicken (*Gallus domesticus*) eggs, and correlations of PCB concentrations between CAMs and hen livers suggested analysis of PCBs in CAMs could predict exposure among adult females (Bargar *et al*., 2001b). Good correlation was also achieved between mercury (MeHg) in CAMs and in mallard ducklings; the measurement of mercury in CAMs could apply to predict mercury in eggs or hatchlings in other species with mercury deposited in CAMs (Heinz and Hoffman, 2003).

Eggs have been widely accepted to assess wildlife exposure to various contaminants. However, there are few studies addressing maternal transfer and distribution of HMX in eggs. HMX was readily deposited into eggs following exposure of HMX to female quail (Brunjes *et al*., 2007). The purpose of this experiment was to assess the maternal transfer of HMX from female northern bobwhite quail to their eggs and the distribution of HMX in different compartments of the egg.

4.2. Materials and methods

4.2.1 Quail husbandry and treatment

Powdered HMX (Accurate Energetic Systems, McEwen, TN, USA) containing 20% water was dissolved in acetone, sprayed on commercial feed and then mixed thoroughly. Nominal concentrations of HMX in feed were 0, 12.5, 50 and 125 mg/kg for the four dose groups. Actual concentrations of HMX in feed were determined by an Agilent 1100 high performance liquid chromatography (HPLC) system with a UV detector (Hewlett-Packard, Waldbrom, Germany).

Thirty pair of quail were purchased from Stephenson Game Farm (Riverside, TX, USA). Birds were raised in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal care facility at Texas Tech University (Lubbock, TX, USA). Each pair of quail was housed in a 25.4 cm x 60.9 cm cage in a galvanized-steel battery breeding pen (30-section, Georgia Quail Farm, Savannah, GA, USA). Birds were uniquely identified by cage number. The laboratory temperature was maintained at 23.0 ± 2.0ºC. The relative humidity was maintained at 50.0 ± 5.0 % and the light cycle was 16:8 hr of light:dark. Birds were provided with distilled water and clean Bluebonnet feed *ad libitum* throughout the acclimation. The feed consumption was calculated every 3 days. The body weight of each bird was measured

weekly, and the egg production of each pair was recorded daily. During this period, birds were monitored daily, and any birds that appeared sick were excluded from the experiment. Two pairs of quail were removed from the experiment because one bird in each pair died. All pairs of quail began laying eggs before dosing. Pairs were randomly distributed into four HMX dose groups (control, low, medium and high dose) and dosed with HMX via feed for continuous 30 days (Brunjes *et al*., 2007).

Eggs were collected daily, weighed, and immediately moved into a Profi-I forced-air incubator (Lyon Electric Company, Chula Vista, CA, USA). The temperature in the incubator was maintained at 38.0 ± 2.0 ºC, and the relative humidity was maintained at 55.0 ± 5.0%. Eggs were candled to determine fertility and viability. Unfertilized and dead eggs were removed from subsequent experiments. On days 5, 9 and 21 of incubation (± 1 day allowed), eggs were moved from the incubator to the respirometer to determine metabolic rates (see Chapter 3), and then dissected for use in determining concentrations of HMX.

4.2.2 Analyses of HMX in eggs

Concentrations of HMX in eggs were determined in 30 eggs using the method developed in Chapter 2 (Liu *et al*., 2007). Briefly, eggs were opened and compartments of eggs were separated and wet weights of shell, yolk, embryo and CAM were measured

(± 0.0001 g). Albumin weight was calculated by subtracting weights of shell, yolk, embryo and CAM from the weight of the whole egg. Yolk, embryo, CAM and albumin were extracted with acetonitrile. After cleanup using florisil and SDB cartridges (VWR Scientific, West Chester, PA, USA), these samples were analyzed by a Thermo HPLC-MS system (Finnigan, San Jose, CA, USA) (Figure 4.2). The sample quantitation limit was calculated by multipling the lowest reliable standard multipled by the dilution factor.

4.2.3 Data calculations

HMX concentration in each egg was calculated based on concentrations of HMX in compartments of the egg, using the following equation, in which the contents of the egg in the equation include yolk, embryo, CAM and albumin.

$$[HMX]_{Egg} = \frac{\sum([HMX]_{Compartment} \times Weight_{Compartment})}{\sum Weight_{Compartment}} \qquad \text{(Eq 4.1)}$$

Bioconcentration factors (BCFs) are often used to describe the accumulation of chemicals in organisms. BCF is the ratio of chemical concentration in the organism to that in the surrounding environment. BCF values give a direct correlation between concentrations of HMX in feed and in quail eggs. In this study, The BCF was calculated using the following equation:

$$BCF = \frac{[HMX]_{Egg}}{[HMX]_{Feed}} \qquad \text{(Eq 4.2)}$$

Maternal transfer coefficients (MTCs) give the information that how much fraction of HMX intake by adult quail was transferred into eggs. The MTC was calculated using the following equation:

$$MTC = \frac{Weight_{Egg} \times [HMX]_{Egg} \times ELR}{IR \times Weight_{Hen}} \qquad \text{(Eq 4.3)}$$

Where: MTC = maternal transfer coefficient;

Weight $_{Egg}$ = weight of the yolk, embryo, CAM and albumin (g);

[HMX] $_{Egg}$ = concentration in the egg (mg/g);

ELR = egg laying rate of female quail (the hen) (day^{-1});

IR = intake rate of HMX by the hen (mg/kg/day);

Weight $_{Hen}$ = weight of the hen (kg).

4.2.4 Statistical analyses

Measures of data were expressed as mean ± standard error (SE). All data were tested for normality and homogeneity and transformed if required prior to statistics. HMX concentrations in egg compartments were analyzed using a 3-way analysis of variance (ANOVA), with dose group (control, low, medium and high), compartment (yolk, embryo, CAM) and embryo age (5-, 9- and 21-day of incubation) as factors. HMX transfer to eggs during the 30-day dose period was analyzed using an ANCOVA with

dose group as a factor and laying date (number of days after dosing began) as the covariate. All statistical analyses were conducted using R 2.2.1 (www.r-project.org). Significance was measured at $p<0.05$ for all statistical tests.

4.3. Results

4.3.1 Feed consumption, HMX intake and egg laying rate

Mean (± SE) concentrations of HMX in the feed of control, low, medium and high dose groups were 0, 12.3 ± 0.5, 52.5 ± 4.1 and 109.3 ± 7.2 mg/kg, respectively. Mean (± SE) feed consumption by each pair of quail was 88.5 ± 4.4, 92.2 ± 3.4, 91.2 ± 3.7, and 86.9 ± 3.0 g/kg/day in the four dose groups. No significant differences in feed consumption were found between acclimation and dose period ($p=0.76$, 0.46 and 0.23 in the low, medium and high dose groups). Mean HMX intake by each pair of quail was 0, 1.1 ± 0.1, 4.8 ± 0.2, and 9.5 ± 0.3 mg/kg/day in the four dose groups, respectively (See Chapter 3). Egg laying rates of quail were 0.88 ± 0.04, 0.93 ± 0.03, 0.70 ± 0.07, and 0.63 ± 0.07 egg/hen/day in the four dose groups, respectively. Significant reductions in egg laying rate were found in the medium and high dose groups ($p=0.002$).

4.3.2 Concentration of HMX in each compartment of the egg

Concentrations of HMX in the yolk, embryo and CAM were determined in all eggs, while concentrations of HMX in albumin were measured in eight eggs (1 control at 9-

day, 1 low at 5-day, 1 low at 9-day, 1 medium at 5-day, 1 medium at 9-day, 1 medium at 21-day, 1 high at 5-day and 1 high at 9-day). Concentrations of HMX in albumin and yolk were compared. The mean ratio was 0.011 ± 0.013 and the maximum ratio was 0.026. Therefore, the maximum HMX concentration in albumin, 2.6% of the HMX concentration in the yolk, was assigned to each egg (Figure 4.3).

The yolk had the highest concentration of HMX except for 21-day eggs in the low dose group, in which the embryo had a higher but similar concentration to that observed in the yolk ($p=0.31$). Occurrences of HMX in each compartment increased with dose group. A 3-way ANOVA showed significant differences among four dose groups ($p<0.0001$), three egg compartments ($p<0.0001$), and three embryo ages ($p<0.0001$), and significant interactions among all factors ($p<0.0001$). We noticed that the mass of egg compartments changes dramatically within three embryo ages, indicating the egg compartment are related to the embryo age. The covariance of egg compartments with embryo ages could explain significant interactions within the 3-way ANOVA.

4.3.3 Concentration of HMX in eggs

The HMX concentration in each egg was calculated using Equation 4.1. A 2-way ANOVA on HMX concentrations in eggs showed significant differences among dose groups ($p<0.0001$), while no significant differences were found among embryos at three

ages of incubation (p=0.13). Since HMX in eggs were not affected by the embryo age, mean (± SE) HMX concentrations in eggs of four dose groups were calculated as 39 ± 5, 1013 ± 59, 3626 ± 134, and 7021 ± 300 ng/g, respectively (Table 4.1). Trace amounts of HMX were found in control eggs. 58 of 79 control samples from different compartments had concentrations below blank solvent responses (10-30 ng/g) and the lowest standard of calibration (20-50 ng/g, considering the dilution factor). We believe these facts indicate that more than 70% of the controls were in fact not detectable. Any other occurrence of HMX in control egg was likely due to the contamination during extraction and instrumental analysis. Regardless, HMX concentrations in control eggs were orders of magnitude lower than the eggs in HMX-treated groups. These values from the instrumental output were used for statistics because they provided a distribution of values below the quantitation limit about 50-100 ng/g (considering the sample dilution factor).

4.3.4 Time course of HMX transfer from hens to eggs

The relationship between the HMX concentration in egg and the laying date (number of days after dosing began) was studied. All eggs in these experiments were collected from the 3rd to the 30th day of the dosing (Figure 4.4). Concentrations of HMX within each dose group were relatively stable according to the laying date. ANCOVA showed significant differences in HMX concentration among dose groups ($p<0.0001$), while no significant differences were observed among the egg laying dates (p=0.77).

Therefore, HMX did not accumulate during the dosing, and concentrations of HMX in eggs were temporally stable from the 3rd day to the termination of the study.

4.3.5 Bioconcentration factor of HMX in eggs

The regression between the concentration of HMX in egg and in feed (Figure 4.5) produces a trendline that is described by the equation:

$$[HMX]_{Egg} = 0.0636\ [HMX]_{Feed} + 0.1642 \qquad \text{(Eq 4.4)}$$

Both concentrations were changed to the same unit of scale. The slope of this graph was 0.0636, which represents the mean BCF value across all exposure groups. The BCF of HMX in each dose group was calculated using Equation 4.2. The BCF in the low dose group (0.084 ± 0.005) was significantly higher than the BCF in the medium (0.068 ± 0.003) and high (0.064 ± 0.003) dose groups (p=0.0026). While BCFs of the medium and high doses showed no difference (p=0.76).

4.3.6 Mass distribution of HMX during egg development

At different embryo ages (time after incubated), the mass of each egg compartment changed, sometimes dramatically. On day 5, the yolk and albumin mass proportions were both about 0.5, while the embryo and CAM together were less than 0.008. On day 9, mass proportion of the yolk and albumin were about 0.6 and 0.3, while the embryo and CAM were still less than 0.06. Then on day 21 of incubation, the embryo mass proportion

increased dramatically to about 0.6 and the albumin decreased to less than 0.1 (Figure 4.6). These dramatic changes on mass proportion of egg compartments could affect HMX distribution significantly.

On day 5 and day 9 of incubation, HMX was primarily distributed in the yolk (>0.97). On day 21, HMX was primarily in the yolk (0.45-0.65) and the embryo (0.35-0.55). HMX mass proportions in the other two compartments, CAM and albumin, were low (<0.03) at all three embryo ages (Figure 4.7).

4.3.7 Maternal transfer coefficient of HMX

Mean (±SE) of MTCs were 0.030 ± 0.002, 0.018 ± 0.001 and 0.015 ± 0.001 in the low, medium and high dose groups, respectively. MTC in the low dose group was significantly higher than the MTCs in medium and high dose groups ($p<0.0001$).

4.4. Discussion

Results showed that the transfer of HMX to eggs was dose-dependent, which follows the traditional view that higher doses yield more residues in tissues. Accurately determining concentrations of HMX in each compartment is important. In this study, we measured the wet weight of each compartment, so determinations of HMX concentrations in the embryo and CAM had higher relative deviations. This is especially true at early

embryo ages, because the mass of embryo and CAM were extremely small and any extraneous fluid contributed much error to the measurement.

Residues of contaminants in eggs come from the maternal blood (Richards *et al*., 1987; Unrine *et al*., 2006). In this study, HMX might be transported to the ovary by the circulatory system then deposited in the maturing oocyte. The partitioning of HMX between tissues and blood might follow a passive diffusion based on its physicochemical properties. Studies on PCBs showed distribution between tissues and plasma was affected by chemical structure, such as chlorination, planarity, polarity and position of chlorine atoms (Dickerson *et al*., 1994). A fat to plasma partition coefficient was used to evaluate excretion of PCBs into eggs, and inversely related to chlorination (Parham *et al*., 1997). Kow and solubility values of HMX are not high, and not enough to suggest the passive diffusion pathway. An active transport of chemicals is possible. For instance, trace elements (Zn, Cu and Fe) were found actively transported from the hen liver to the oocyte by metal transport proteins such as vitellogenin (Richards *et al*., 1987). Selenium was transported from liver to the ovary with vitellogenin or other proteins (Unrine *et al*., 2006). It is possible that HMX is associated with some proteins or lipids in circulation, and transported to the ovary and finally deposited in the egg. However, the lipid or protein contents of egg compartments are difficult to determine, especially in the small compartments. It is difficult to use lipid-corrected basis to evaluate the distribution of

HMX between egg compartments. Characterizations of these proteins and lipids may improve the understanding of maternal transfer of HMX in northern bobwhite quail.

In this study, BCF values of HMX in eggs were from 0.064 to 0.084, and maternal transfer coefficients of HMX were from 0.015 to 0.029. BCF and MTC values were found inversely proportional to doses. A previous study in rats and mice showed that most HMX was excreted in the feces in an unchanged form (70-85%), and a small fraction was found in the urine (3-4%) following a single oral dose of 500 mg/kg HMX; most of absorbed HMX dose (61%) was excreted in urine following a single intravenous dose of 2 mg/kg HMX (Cameron, 1986). Therefore, the maternal transfer of HMX to eggs might not be the main pathway of HMX excretion from hens after oral exposure. The absorption of HMX by the hen might decrease at high doses. Biotransformation of HMX in the liver could decrease HMX amount in the body of hens. Oogenesis could play an important role in the process because HMX in eggs comes from the hens. Any increase of HMX distribution in feces, bile, kidneys, lungs and other organs could also decrease maternal transfer of HMX to eggs. All of these processes together determine how much of HMX maternally transfers to eggs (Figure 4.8). However, we focused on HMX transfer to eggs in this study. HMX excreted to feces and other organs were not parts of this study design. The disposition of HMX in the whole body of the quail would provide more information on maternal transfer rates.

The distribution of contaminants in different egg compartments has been studied. Generally, lipophilic chemicals are thought more likely to deposit in the fat-rich yolk (Custer *et al*., 1997). However, studies in organic compounds have shown that the distribution of contaminants in eggs is more complicated. Partitioning of OCs between yolk and embryo indicated both simple diffusion and lipid-related transfer models were involved; partitioning of hydrophilic OCs (HCHs and low chlorinated PCBs) between the embryo and CAM followed simple diffusion, while hydrophobic OCs followed lipid-related transfer, but lipid distribution could not explain the relationship; when OCs in the rest of egg (yolk + embryo) and in the CAM were compared, a clear separation between polar and nonpolar compounds was found; and compounds with high solubility were more abundant in the CAM (Pastor *et al*., 1996). PCBs with low chlorine content, from diCBs to tetraCBs, were found more readily to partition in CAMs because they were more water soluble and were more readily metabolized by liver enzymes; DiCBs through tetraCBs had higher percentages of total PCBs in CAMs than in egg tissues, while pentaCBs through nonaCBs contributed more in egg tissues than in CAMs. (Cobb *et al*., 1997). Pharmacokinetic behavior and distribution of veterinary drugs in eggs were studied, and neither lipid-solubility nor pKa value could explain the distributions well (Kan *et al*., 2000). In a study of OCs in frogs, the maternal transfer of PCDDs and PCDFs with four or five chlorine atoms and coplanar PCBs followed a passive fugacity model

(Kadokami *et al*., 2004). Maternal transfer of organohalogens in glaucous gulls showed maternal transfer favored low Kow and less persistent compounds, not the recalcitrant and higher-halogenated compounds (Verreault *et al*., 2006). In summary, available information shows many factors could affect the distribution of a chemical in the egg, while interactions among these factors are complicated and no factor can determine the distribution absolutely.

In this study, most of HMX was in the yolk and embryo of the egg, while little HMX (<0.03) was in the CAM and albumin. We think that HMX is more likely in the fat-rich compartments of the egg, because the yolk and embryo have higher lipid portion than the CAM and albumin. On day 5 and 9, most of HMX mass (>0.97) was in the yolk; on day 21, 0.45-0.65 HMX was remained in the yolk; HMX mass in the embryo increased rapidly between day 9 and day 21 (from 0.01 to 0.35-0.55). This increase coincided with the process of accumulating lipid by the embryo. Studies of chick embryos showed that accumulation of lipid by the embryo is slow during the first 13 days of development, while the accumulation increased substantially between 15 and 21 days such that lipids were removed from the yolk at a rate of over 1 g/day during the last 2 days (Noble, 1987). Therefore, the correlation between the lipid content and the HMX mass in the embryo seems reasonable. It is possible that HMX distribution similarly transported in the egg and in the body of hens. It is unclear if this transport process

involves passive diffusion, facilitated diffusion, active transport, or some combination of these processes. Transport of HMX from the yolk to the embryo may be related to the blood circulation. At the beginning of incubation, HMX may move only by diffusion, because the circulatory system is not well formed. As incubation precedes, the circulatory system in the embryo, yolk sac and CAM develop to allow the transport of nutrients, oxygen and metabolic wastes (Romanoff, 1960). HMX in the yolk may also be transported to other compartments of the egg in this way. For instance, PCBs are supposed to be mobilized into the blood of the embryo and then into the CAM, causing transportation of PCBs to the CAM (Cobb *et al*., 1997).

We expected to find HMX in the CAM, because the fluid enclosed by the CAM is the place for waste storage (Romanoff, 1960). However, we did not find any evidence supporting the point that HMX concentrates in the CAM. The concentration of HMX in the CAM was much lower than that in the yolk, and the mass fraction of HMX in the CAM was less than 0.007 in all eggs. The excretion of HMX from the embryo to CAM is low. Results showed that HMX distributed differently than PCBs and other very nonpolar and lipophilic compounds.

HMX concentrations in the yolk, embryo and CAM in 21-day old quail eggs were compared (Figure 4.9), with three compartments of eggs fully developed. The slopes of HMX concentrations increased with the dose in the yolk > embryo > CAM. Therefore,

the distribution of HMX among egg compartments may not follow a simple passive diffusion pattern. Facilitated diffusion or active transportation related to lipid and protein contents in eggs seem more possible.

In conclusion, maternal transfer of HMX to eggs was dose-dependent. Partitioning of HMX in eggs seems to favor fat-rich compartments of the egg, such as the yolk and embryo, while little HMX is retained in the hydrophilic compartments, such as the albumin and CAM. Further studies on the mechanism are needed to explain the maternal transfer of HMX to eggs and the distribution among tissues therein.

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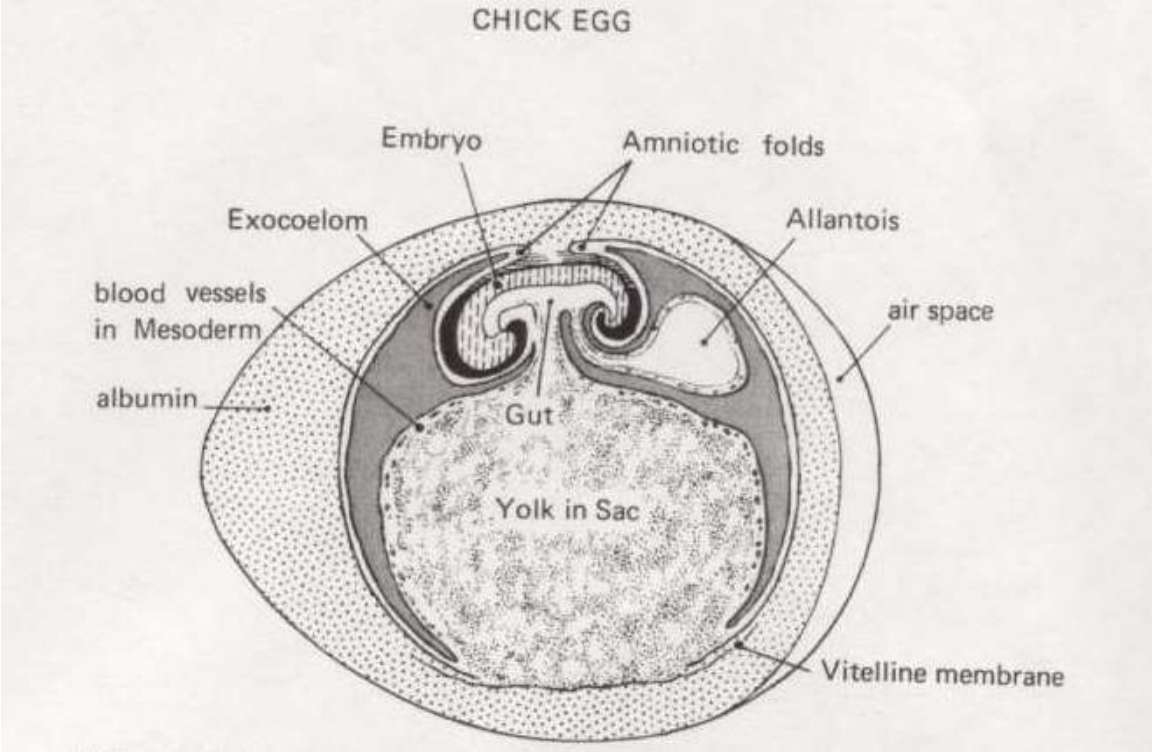

Figure 4.1. A representative stage in the development of the chick embryo (Ramsey, 1982, reproduced with the permission of Praeger Publishers, New York. Appendix B).

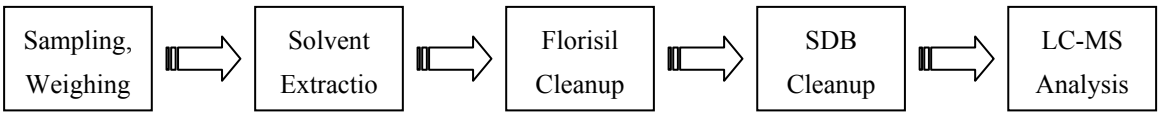

Figure 4.2. Sample preparation and analysis of HMX in northern bobwhite quail eggs.

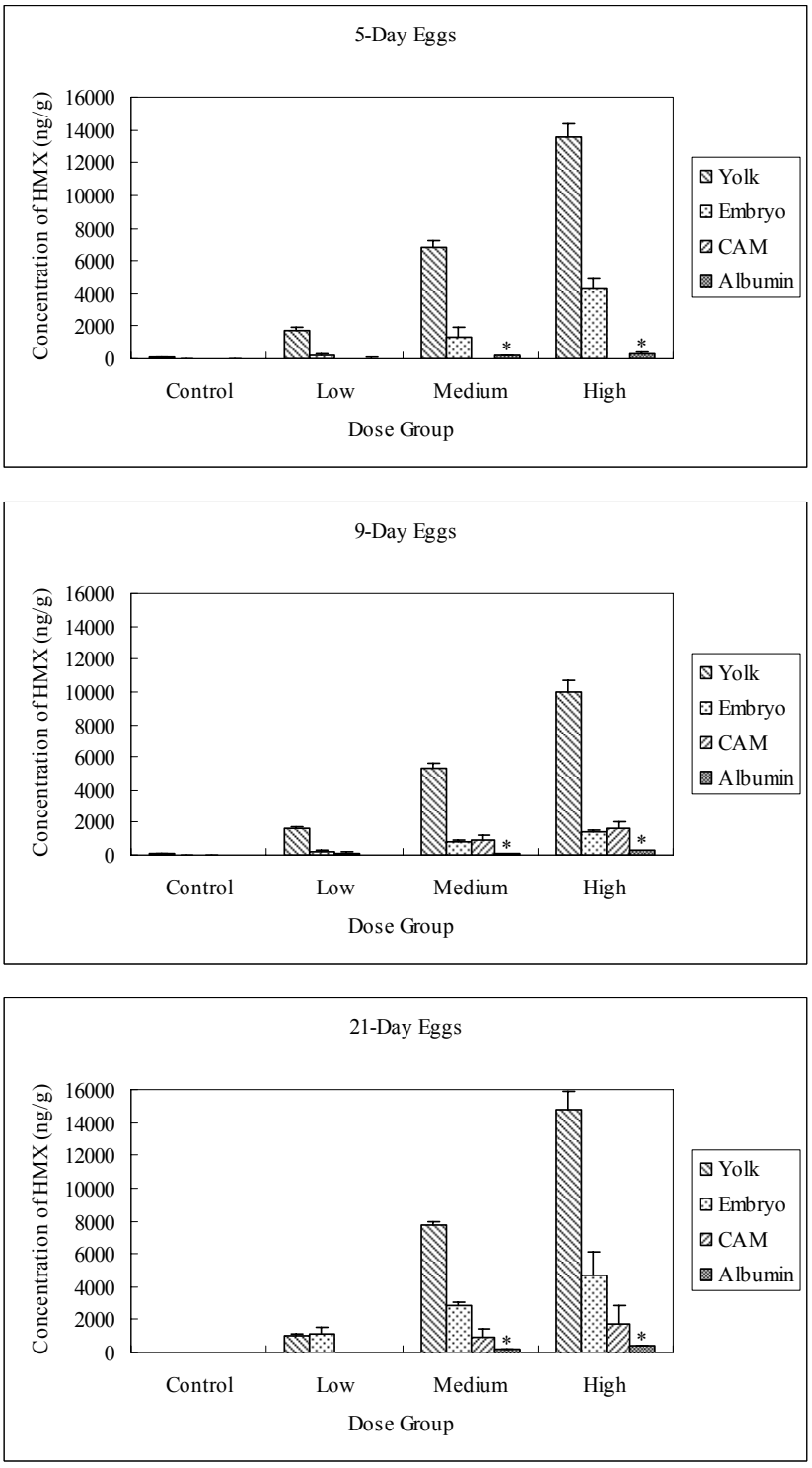

Figure 4.3. Mean (± SE) concentrations of HMX in each compartment of northern bobwhite quail eggs on 5, 9 and 21 days of incubation. Concentrations of HMX marked with "*" were estimated values.

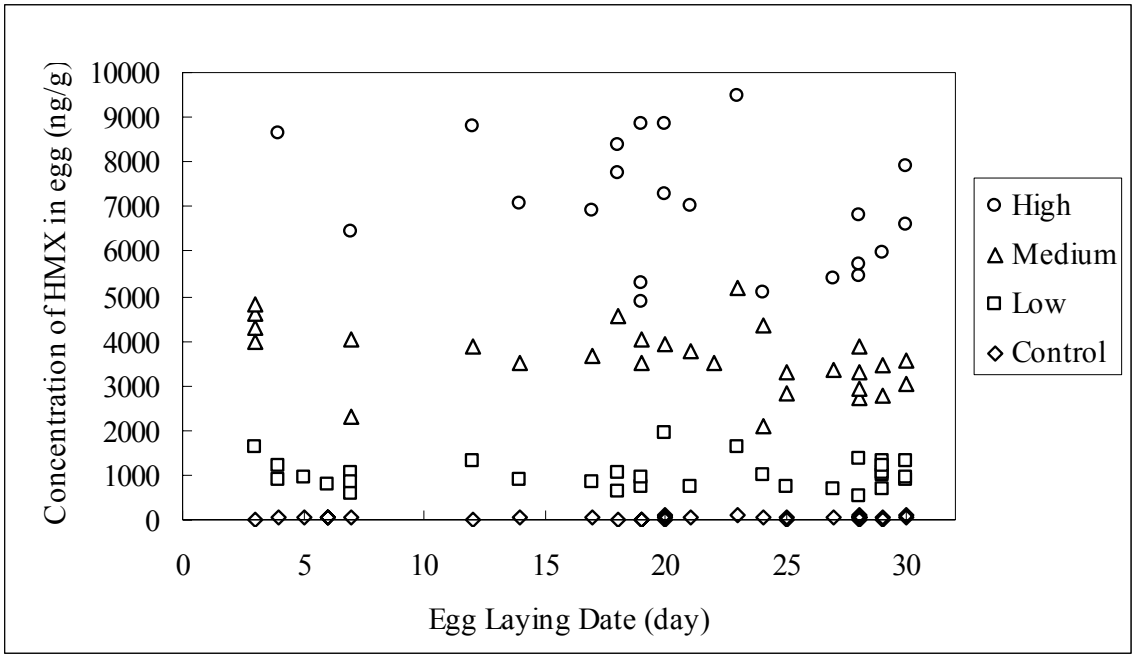

Figure 4.4. HMX concentration fluctuations over the egg laying sequence. Northern bobwhite quail eggs were collected from different days during the dosing period. Numbers of eggs from control, low, medium and high dose groups were 30, 31, 29 and 22 respectively.

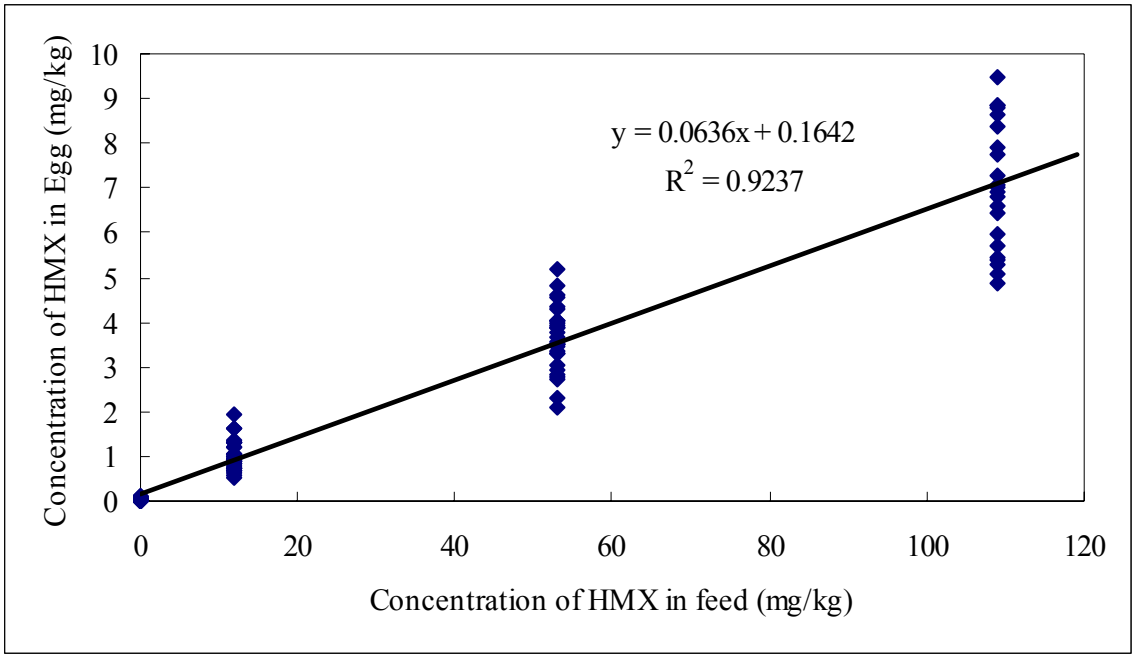

Figure 4.5. The relationship between concentrations of HMX in quail eggs and in feed. Slope in the graph shows the bioconcentration factor (BCF) of HMX in northern bobwhite quail eggs. Eggs from 12.5, 50 and 125 mg/kg dose groups were 31, 29 and 22, respectively.

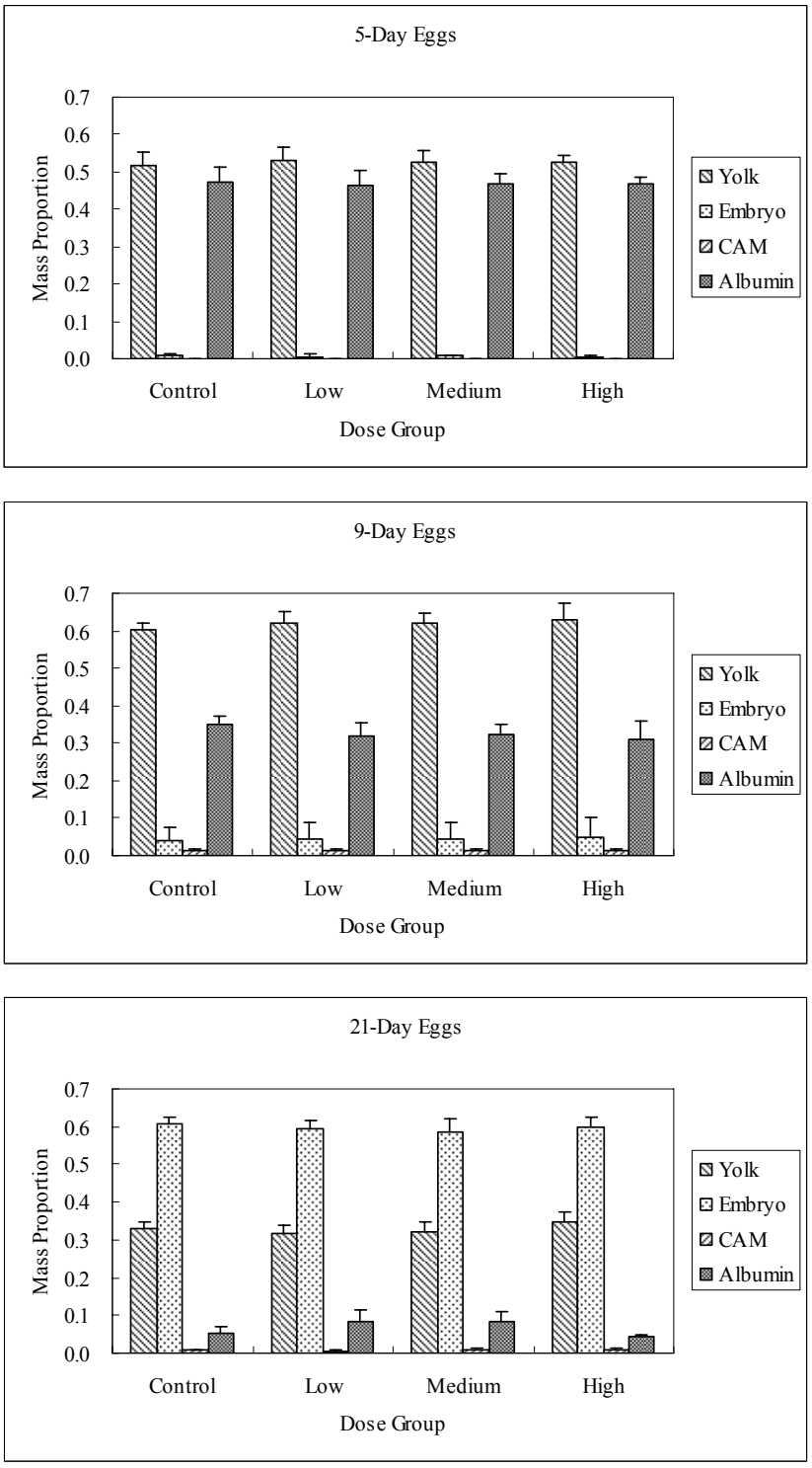

Figure 4.6. Mass proportions of northern bobwhite quail egg compartments during the incubation.

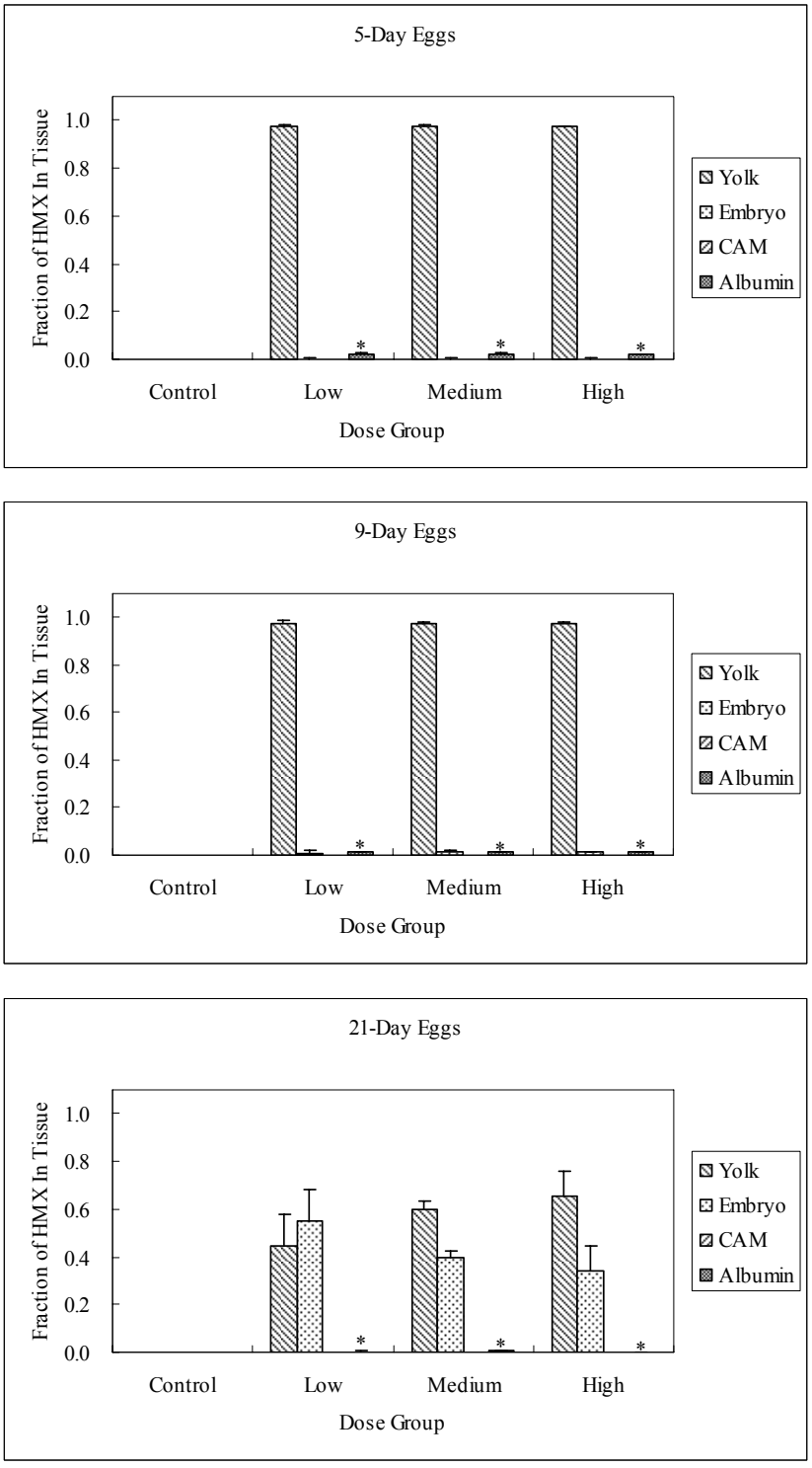

Figure 4.7. Fractions of HMX in northern bobwhite quail egg compartments. Values marked with "*" were estimated values.

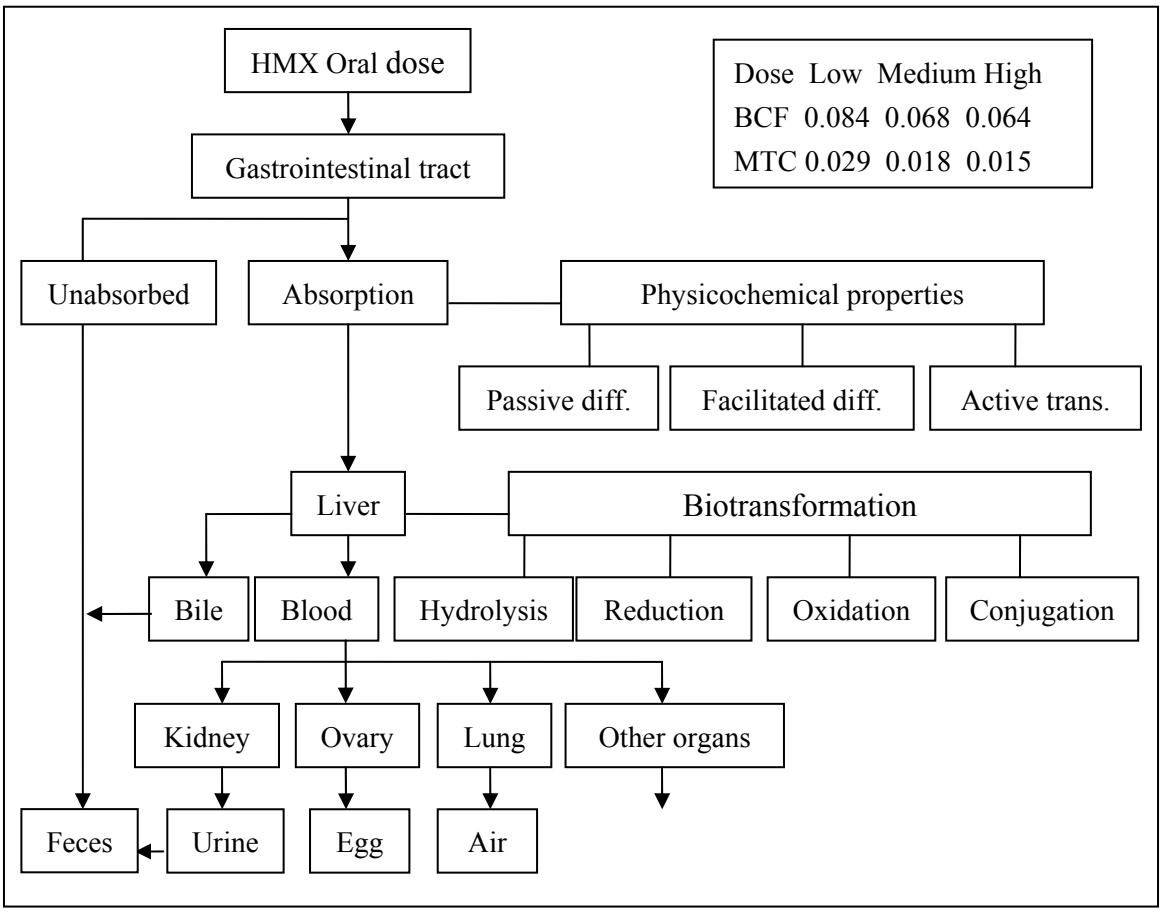

Figure 4.8. Possible disposition of HMX in female northern bobwhite quail through the oral dose.

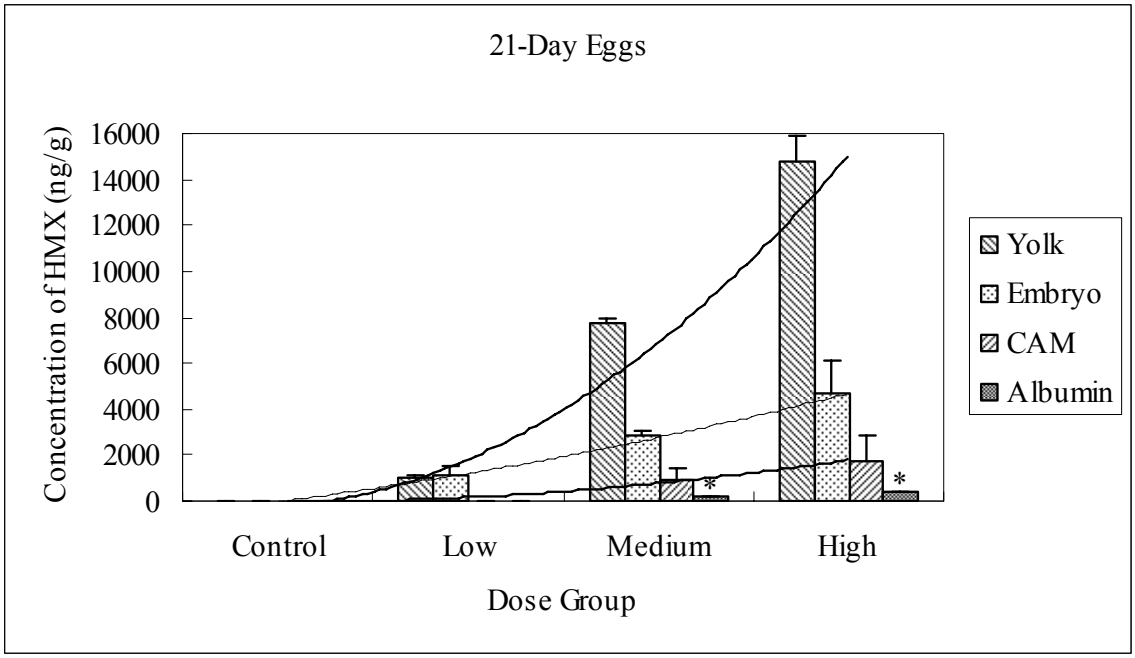

Figure 4.9. Comparison of HMX concentrations in the yolk, embryo and CAM in the 21-day old northern bobwhite quail eggs.

Table 4.1. Concentrations of HMX in northern bobwhite quail eggs at different embryo ages.

Embryo Age	Concentration of HMX (ng/g)			
	Control	**Low**	**Medium**	**High**
5	42±8 (n=11)*	939±110 (n=12)	3646±251 (n=10)	7237±436 (n=8)
9	51±7 (n=14)*	997±83 (n=13)	3361±194 (n=13)	6433±462 (n=10)
21	0±0 (n=5)*	1083±172 (n=6)	4166±120 (n=6)	8059±551 (n=4)
Mean	39±5 (n=30)*	991±62 (n=31)	3626±134 (n=29)	7021±300 (n=22)

* Values were used for statistic purpose because they provide a distribution of values below the quantitation limit.

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