SHORT TERM EFFECTS OF ORAL PARTICULATE LEAD EXPOSURE IN QUAIL PLUS AN EVALUATION OF LEAD NITRATE EXPOSURE ON MAMMALIAN ANTIGEN PRESENTING CELLS, *IN VITRO*

by

RICHARD PETER KERR

(Under the Direction of Robert M. Gogal, Jr.)

ABSTRACT

Lead (Pb) is a well-known environmental contaminant of food and water. Previous rodent studies have shown that Pb exposure can alter blood physiology, induce neural and renal toxicity and affect nutrient absorption. In birds the primary route of exposure, with similar indications of toxicity, is by oral ingestion of Pb particles as grit or mistakenly as food. However, no data existed tracking the retention and clearance of these Pb pellets in the bird GI tract while linking toxicity and blood accumulated lead levels. Further, no data existed on the effects of oral Pb bullet fragment exposure in birds. To begin to address these gaps in the data, Northern Bobwhite quail were orally gavaged with spent lead shot (0, 1, 5 and 10 spent Pb shot/bird) or bullet fragments (0, 1, and 5 fragments/bird), radiographed and evaluated for changes in peripheral blood, neural, renal, and immune parameters. The majority of the pellets and fragments were voided within 7 to 14 days post exposure with no detection by radiograph by day 21. In the quail, Pb exposure resulted in severe weight loss, hemodynamic compromise, koilin degeneration, and at doses exceeding five 50 mg pellets, mortality from complications of emaciation. Pb inhibited δ-ALAD activity, a marker of heme synthesis, by as much as 90% with

doses as low as one 24 mg fragment after just one week of exposure. Initial functional immune evaluation in the quail was inconclusive, so we evaluated the effect of Pb at blood concentrations commonly seen *in vivo* on a model of antigen presenting cells (APCs), the RAW 267.4 cell line. Recent data had shown that APCs may play a major role in the upregulation of the adaptive immune response to antigen through altered surface expression of MHC and co-stimulatory molecules after exposure to Pb. In the present study, 2.5- 5.0 µM Pb dysregulated cellular metabolism in RAW 267.4 cells cultured for 17 hr resulting in enhanced autophagy, increased MIIC trafficking leading to increased surface expression of MHC-II and co-stimulatory proteins critical to T-helper type 2 activation suggesting an alternate mechanism of autoimmune induction, *in vitro.*

INDEX WORDS: Lead, Pb, Shot, Fragments, Metalic Pb, Avian, Toxicology, Immunology, Antigen Presentation, Major Histocompatability Complex Class II, MHC-II.

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SHORT TERM EFFECTS OF PARTICULATE LEAD EXPOSURE ON AVIAN WILDLIFE PLUS AN EVALUATION OF LEAD NITRATE EXPOSURE ON MAMMALIAN ANTIGEN PRESENTING CELLS, *IN VITRO*

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In Loving Memory of Catherine “Kate” Kerr, 1919 - 2002.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Lead (Pb) is a well-known heavy metal contaminant with world-wide exposure. Pb can adversely affect multiple organ systems and is retained in the body long after cessation of exposure. In spite of over 50 years of research and a history of more than 5,000 years of human use, critical gaps in the knowledge of Pb exposure still exist, in particular at the cellular and molecular level. The majority of the research in Pb has focused on its lethality, but it could be argued that the non-lethal effects are of equal or greater concern. Neuronal degeneration, hemodynamic compromise, and immune dysfunction are among the many pathologic disorders associated with Pb. It affects so many pathways that one must first consider these multiple effects when forming conclusions about the role Pb plays in disease.

Over the last several decades, there have been renewed concerns regarding the effects of Pb in both human and wildlife populations. Terrestrial birds are at particular risk as their habitat is frequently contaminated with particulate Pb similar in size to grit or food particles. Game birds such as the Northern Bobwhite, are raised on commercial farms and released on hunting preserves where their primary food source is seed. Birds require grit to facilitate seed digestion necessitating the oral collection of small particles in the crop with eventual passage into the ventriculus. Since hunting and shooting ranges are saturated with high quantities of Pb pellets and bullet fragments, birds in these regions can likely ingest these pellets. While there are reports on pellet exposure in quail, data are generally lacking with regards to controlled field studies, relating timed pellet exposure to changes in blood Pb concentration over time, and indexes these concentrations to toxic effects. This absence of Pb data limits an accurate assessment of risk to the general bird population and so warrants further investigation.

Lead

Pb was one of the first metals to be mined and smelted by early human civilizations and continues to be an important industrial material today. Presently, it is still found in many commercial products primarily because of its chemical and physical properties. Pb is a heavy, soft, blue-gray metal with an atomic weight of 207.19 amu, atomic number 82, and outer valance of +2 to +4 (Scott & Page, 1977; Laeter, 2003). It is easily malleable, resistant to corrosion and has a relatively low melting temperature of 327.5 °C. Common isotopes include four stable; Pb-204, Pb-206, and Pb-207, and Pb-208, and several radioactive isotopes, including Pb-202 with a half-life of 53,000 years (Sailor & Beard, 1993; Andersen, 2002). Pure metallic Pb is extremely rare in nature and is usually found associated with deposits rich in silver, zinc, copper or sulfur (Sangster & Scott, 1976; Goldhaber et al., 1995; Velasco, Pesquera, & Herrero, 1996; Klein, Brey, Durali-Müller, & Lahaye, 2010). The principle ore mined for lead, galena (PbS, and typically silver), can be found in deposits scattered throughout the globe, but are largely concentrated in North America, Asia and Australia (Foord & Shawe, 1989; Singer, 1995). Consequently, the United States, China, and Australia account for over half the Pb produced in the world today (Graedel & Cao, 2010).

Pb is commonly considered a post industrial revolution contaminant but based on historical accounts, human Pb exposure occurred much earlier. Early man extracted Pb from exposed galena ores using low temperature wood fires to crudely smelt the metal while discarding the chalky sulfur (Lessler, 1988). It's low melting point, relative abundance, and resistance to corrosion were noted by ancient civilizations including the Greeks and Romans (Véron, Goiran, Morhange, Marriner, & Empereur, 2006; Bode, Hauptmann, & Mezger, 2009). The word, lead, comes from the Anglo-Saxon "*lead*", the chemical symbol 'Pb' from the Latin

"*plumbum"*, formally the ancient Greek '*molubdos'* (*μόλυβδος)*, meaning malleable (Liddell, 1940; Laeter, 2003; Reenan, 2005). During the Greek era, Pb was smelted in furnaces to make pipes, goblets and many other metallic objects (Jerome O. Nriagu, 1996). Many early Greek tools contained consistent amounts of Pb indicating its use in copper alloys (Craddock, 1978). Later, the Greeks extracted silver from galena ores using higher temperature fires and soda ash to absorb the Pb in a process known as cupellation. The resulting Pb and silver was then traded by early Greeks across the Aegean (J.O. Nriagu, 1983).

Roman smelting and use of Pb was documented throughout early western history and astute observers during this period began to note it's toxicity. During the Julio-Claudian dynasty, Vitruvius wrote in *De Architectura* (~15 BC) that terracotta was preferred as a material for making pipes because Pb caused "pallid *colour*; for in casting lead, the fumes from it fixing on the different members ... destroy the *vigour* of the blood." (Retief & Cilliers, 2010). Despite this early warning, the use of Pb remained widespread as the risk was considered warranted due to the metal's versatility and use to make goblets, face applications, pigments, and food preservatives (Makra & Brimblecombe, 2004). Early Romans used Pb acetate (known as sugar of Pb) as a sweetener and used Pb alloy lined bronze cauldrons to produce *defrutum*, a concentrated sweet syrup made from boiled unfermented grapes (Al Saleh, 1994). It has been estimated that the resulting sweetener contained up to 1 gram Pb per liter, which was then diluted in wine or added to sauces for those who could afford them. Consequently, a substantial portion of Rome's elite citizenry and an estimated half the roman emperors were exposed to and experienced the effects of Pb (Reddy & Braun, 2010). In fact, the Emperor Nero was said to have been possibly driven insane by drinking sweetened wines, laughing and singing while Rome burned in 64 AD (Makra & Brimblecombe, 2004; Scullard, 2010).

Analysis of ice layers representing the period from about 500 BC to 300 AD, the height of the Roman Empire, show significantly raised Pb levels compared to background. These levels returned to baseline after the fall of the Roman Empire but steadily increased in the European middle ages into the early 19th century (S. Hong, Candelone, Patterson, & Boutron, 1994). During and after the Industrial Revolution, Pb concentrations rose exponentially in correlation with Pb emissions and hit their peak in the late 1970s (Bindler, Korsman, Renberg, & Hogberg, 2002).

Modern uses of lead also take into account its chemical properties. Pb was used as an anti-knock agent in gasoline in the early 20th century in the organic compound $(CH_3CH_2)_4Pb$ better known as Tetra-Ethyl-Lead (TEL). TEL reduces early fuel detonation inside the cylinder of a combustion engine resulting in higher compression ratios, a more powerful stroke, and greater efficiency (Jerome O. Nriagu, 1990). Pb compounds, particularly Pb-carbonates, were also used as a drying agents, pigments in house paints and in solder for electronics (Cao, Ma, Chen, Hardison Jr, & Harris, 2003). Legislation passed in 1970 created the Environmental Protection Agency (EPA) which was charged with regulating Pb emission and content in consumer goods (Jacobs et al., 2002). Between 1973 and 1985, the EPA reduced Pb in gasoline by 98% before eliminating it completely in 1986 (Golz, 1973; Lewis, 1985). The European Union has mandated the elimination of Pb from electronics goods (N. Lee, 1999). Collectively, these efforts contributed to a 7 fold decrease in atmospheric Pb, but have not totally eliminated the use of this metal in developed and underdeveloped countries (Hong, Candelone et al. 1994; Soto-Jimenez, Hibdon et al. 2005).

In the late 20th and early 21st centuries, Pb was used in the manufacturer of lead-acid energy storage batteries, various industrial pigments, rolled and cast metallic forms (such as the

keels of sailing ships), underwater cable sheeting, and as the primary component of the projectile in munitions (Mao, Dong, & Graedel, 2008). Although Pb-acid batteries comprise over 79% of the Pb in use in the US today, more than 93% of these batteries are recycled. Industrial pigments, underwater cables and cast metallic forms contain Pb that are termed "hibernating" or "in-use" stock and thus are not recognized as released into the environment (Mao, Cao, & Graedel, 2009). This leaves munitions and mine runoff as the most common end use forms of Pb dispersion (Wilson, 2006).

While efforts are under-way to replace Pb in ammunition with nanoparticles or alternative metals, the amount of metallic Pb in the environment is still substantial (Heath, 2000; Oltrogge, 2009). In 1999, it was estimated that over 60,000 metric tons of Pb shot and fragments were deposited annually on shooting ranges and hunting areas in the United States (Craig, Rimstidt, Bonnaffon, Collins, & Scanlon, 1999). This is on top of the estimated 3 million metric tons already accumulated. Particulate Pb is considerably less soluble in water than organic Pb making it more persistent in soils (X Cao, et al., 2003). While research is on-going to remediate contaminated soils using bio-surfactants, the cost is currently prohibitive (Juwarkar, Nair, Dubey, Singh, & Devotta, 2007). Estimates of the time required to convert particulate Pb into water soluble compounds, which would reduce the cost, range from 100 to 300 years indicating that particulate Pb will be an environmental contaminant for many centuries (Jørgensen & Willems, 1987; Mench, Didier, & Löffler, 1994; Huang, Chen, Berti, & Cunningham, 1997).

Routes of Oral Lead Exposure

For small mammals, birds, amphibians, and humans, the primary route of exposure is via oral ingestion (W. Stansley & D. E. Roscoe, 1996; Bennett, Kaufman, Koch, Sova, & Reimer, 2007; Bazar et al., 2010). Birds and small mammals are primarily at risk by orally ingesting Pb

contaminated food or mistaking particulate Pb as grit particles (Kendall et al., 1996; Darling & Thomas, 2005; Bennett, et al., 2007; Duggan & Dhawan, 2007). In amphibians and humans, contaminated water supplies downstream from contaminated soil, dust including Pb paint chips, and decaying organo-Pb are of particular concern (Hopkins, Mendonça, Rowe, & Congdon, 1998; Lanphear, Burgoon, Rust, Eberly, & Galke, 1998; Rice, Blackstone, Nixdorf, & Taylor, 1999; Ruby et al., 1999). The species most at risk on a given range depends, in part, on the pH of the soil on which the Pb was deposited because soil alkalinity and the resultant compounds influence the transport of Pb products through the soil column and eventually into water or food sources (K Murray et al., 1997; Sauvé, McBride, & Hendershot, 1997). In acidic soils, Pb bioavailability from Pb shot or fragments increases significantly and is transported in rain run-off into reservoirs and streams or down the soil column into aquifers contaminating water supplies (Knechtenhofer, Xifra, Scheinost, Flühler, & Kretzschmar, 2003; Genc & Ulupinar, 2010). Pb shot deposited in alkaline or slightly alkaline soils primarily form Carbon-Pb hydrocerussites $Pb_3(CO_3)_2(OH)_2$ and cerussites ($PbCO_3$) which are slightly less bioavailable forms (X. D. Cao, L. Q. Ma, M. Chen, D. W. Hardison, & W. G. Harris, 2003; Darling & Thomas, 2005). However, these compounds transport primarily across the surface soil where they are absorbed by grasses and other plants and consumed by local wildlife (Rooney, McLaren, & Cresswell, 1999; J. Yoon, Cao, Zhou, & Ma, 2006).

Lead Exposure in Birds

Birds can mistakenly ingest Pb pellets or fragments as food particles or grit to assist with the mechanical breakdown of food. (McConnell, 1967; Bennett, et al., 2007; Duggan & Dhawan, 2007). A soil and field study of upland and lowland managed game preserves estimated availability at 6,342 Pb pellets/ha and an ingestion rate of 0.3% in birds collected after

the hunting season (J. H. Schulz et al., 2002). However, in controlled exposure studies, the ingestion rates were calculated as high as 3.0% (Butler, Sage, Draycott, Carroll, & Potts, 2005). To help possibly explain this discrepancy in projected Pb exposure in birds, several researchers examined the potential influence of predatory species. In a study of 16 species of raptors, mortality associated with high liver concentrations of Pb were found to correlate with the ingestion of prey containing particulate Pb (Pain, Sears, & Newton, 1995). Predatory bird species such as hawks, eagles, condors, and vultures may also target weakened birds as prey leading to the accumulation of Pb in their tissues (Kendall, et al., 1996; Nam & Lee, 2009). While birds with Pb pellets lodged in their flight muscles have Pb concentrations higher than control, concentrations are typically localized whereas birds that ingest Pb have much higher body burdens (Custer, Franson, & Pattee, 1984; Scheuhammer, Perrault, Routhier, Braune, & Campbell, 1998; Spahn & Sherry, 1999). This suggests that the reported differences in the calculated ingestion rates may be directly associated with the amount of Pb contamination and level of predation within a given geographical region. Further, the ingestion of prey in high Pb contamination regions may result in a higher mortality in these predatory birds.

Lead Absorption and Distribution

Once ingested, particulate Pb enters the crop, ventriculus or stomach. Pb then dissolves in the low pH of these compartments. Pb ions in solution then transit to the small intestine and readily diffuse across the wall of the duodenum directly into the blood stream. Once in the blood, 99% of Pb ions are sequestered in the cellular fraction (Cake et al., 1998). In the bloodstream, Pb transits throughout the body but is primarily deposited in the liver, kidney and bone, and to a lesser extent in muscle and less vascular tissue.

In calcified tissues, Pb concentrations are highest in those currently undergoing growth or remodeling. During osteogenesis, significant Pb deposition into the bone matrix may take place as osteoblasts mistake Pb for Calcium (Rosen & Pounds, 1989). Type 1 collagen is the principle protein component of bone matrix, however, it has no mineral binding properties (Termine, Belcourt, Conn, & Kleinman, 1981; Eriksen et al., 1993). Another protein, osteocalcin, binds both collagen and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), the principle mineral in bone (Jundt, Berghäuser, Termine, & Schulz, 1987; Kikuchi, Itoh, Ichinose, Shinomiya, & Tanaka, 2001). Hydroxyapatite is indiscriminant in binding divalent metals and can bind calcium, magnesium, selenium, copper, zinc and Pb (Monteil-Rivera, Masset, Dumonceau, Fedoroff, & Jeanjean, 1999; Roy & Nishimoto, 2002; Prasad & Saxena, 2004). Of these, hydroxyapatite has an affinity for Pb 2 orders of magnitude higher than calcium and osteocalcin has a 40% higher affininity for Pb bound hydroxyapartite (Dowd, Rosen, Mints, & Gundberg, 2001). Although the crystal structure varies slightly from the calcium bound form, Pb bound hydroxyapartite is readily incorporated into the bone matrix (Bigi et al., 1989; Webster, Massa-Schlueter, Smith, & Slamovich, 2004; Milgram, Carrière, Malaval, & Gouget, 2008; Milgram, Carrière, Thiebault, Malaval, & Gouget, 2008). This deposition slows cartilaginous metabolism at growth plates and promotes bone remodeling resulting in stunted bone growth and lowered bone density (Escribano et al., 1997; Puzas, 2000).

Pb can also be found in tissues expressing high levels of metallothioneins. Metallothioneins are a class of proteins responsible for regulating concentrations of divalent metal ions and so serve to reduce metal-induced oxidative stress (Qian, Zheng, Ramos, & Tiffany-Castiglioni, 2005). They are expressed most significantly in the liver and kidney (Banerjee, Onosaka, & George Cherian, 1982). Pb binds metallothionein in cystine-rich metal

binding sites on the protein with greater affinity than other divalant metal ions except cadmium (Waalkes, Harvey, & Klaassen, 1984; Nielson, Atkin, & Winge, 1985). Pb also induces expression of metallothionein in the kidney, but unlike other xenobiotic metals, does not induce increased excretion in the urine (Heuchel et al., 1994; Qian, et al., 2005). This may explain the higher concentrations of Pb in the kidney during multiple metal exposures. Interestingly, expression returns to baseline 24 hr after cessation of exposure (Waalkes, et al., 1984; S. Yoon, Han, & Rana, 2008). While this mechanism has not been well characterized, this pattern of metallothionein expression may be a good indicator of metallic clearance.

Lead Elimination

Ingested Pb routes through the GI tract until it is either completely absorbed or excreted in the feces. After GI clearance, Pb blood (PbB) levels fall precipitously. Some absorbed Pb is filtered by the kidney and eliminated in the urine. However, urine Pb concentrations correlate best with plasma concentrations and Pb is 98-99% concentrated in the cellular fraction indicating that the kidney is an inefficient elimination route (Cake, et al., 1998; Satarug et al., 2004; Barbosa Jr, Tanus-Santos, Gerlach, & Parsons, 2005). Pb may also be eliminated in sebaceous, salivary, and milk secretions although these Pb concentrations correlate weakly with PbB (Omokhodion & Crockford, 1991; Dearth et al., 2002). In birds, relatively low concentrations of Pb may be deposited in feathers and eggshell (Burger, 1994). This leaves the feces as the primary route of absorbed Pb excretion mainly as remnant damaged red blood cells removed by the liver (Barbosa Jr, et al., 2005). Collectively, these routes of clearance effectively reduce PbB after exposure (Cooke, Andrews, & Johnson, 1990; Barbosa Jr, et al., 2005; Kerr et al., 2010).

Pb poses a particular threat, in part, because PbB levels remain higher than background even after GI clearance. Significant drops in Pb concentration are observed post exposure,

however Pb is deposited in several tissues that release the metal once PbB levels decline. After clearance, Pb may leach back into the blood released via osteoclastic reabsorption of bone containing Pb (Nilsson et al., 1991; Miyahara et al., 1994). Current estimates of Pb half-life in bone is greater than 20 years however, Pb has been documented in 70 year old patients who were exposed as juveniles (Patrick, 2006). These data would suggest that PbB concentrations may remain elevated above background for a significant portion of an individual's life.

Lead Hepatotoxicity

Of all the soft tissues that accumulate Pb, the liver retains the highest concentrations. Blood cells transiting through the liver exchange Pb in the small blood vessels allowing hepatocytes to concentrate the metal. However, Pb exposure interferes with heme synthesis and downregulates cytochrome P450 1A2 expression in hepatocytes (Banerjee, et al., 1982; Heuchel, et al., 1994; Jover, Lindberg, & Meyer, 1996; Tully et al., 2000). Reductions in heme availability decrease the efficacy of hemoproteins including catalase, peroxidase, and cytochromes (Jones & Masters, 1975; Cals, Mailliart, Brignon, Anglade, & Dumas, 1991; Keng, Privalle, Gilkeson, & Weinberg, 2000; Atamna, Liu, & Ames, 2001). In one study, the investigators observed that the reduction in P450 1A2 expression was at the mRNA level and linked to heme synthesis suppression suggesting that Pb interferes with these enzymes at several regulatory levels (Degawa, Matsuda, Arai, & Hashimoto, 1996). Thus, the decrease in concentration and efficacy of these enzymes would likely interfere with normal hepatic function including drug metabolism and zenobiotic detoxification (Patrick, 2006). As a result, Pb exposure may enhance the effects of toxic pro-drug components leading to further hepatic insult.

Pb's effect on the liver is not limited to enzyme dysregulation, as other changes have also been observed. Several studies have reported effects on hepatocytes and other resident liver

cells. For example, in rats exposed to 200 uM Pb for 10 weeks, histiocytic hemosiderin deposition and hepatocellular lipidosis were observed (Bruce A. Fowler, Kimmel, Woods, McConnell, & Grant, 1980; Patrick, 2006). Hemosiderin deposits are an indicator of hemodynamic compromise and red cell degradation, but do not necessarily indicate direct damage to the liver. However, lipidosis indicates direct hepatotoxicity and is associated with prolonged fasting, loss of body weight, and pancreatitis.

Lead Neurotoxicity

One of the most insidious effects of Pb exposure occurs in children. Juvenile Pb exposure has profound effects on early brain development with life-long consequences. Paint chips from old houses, contaminated dirt, and leaded toys are all sources of exposure in this at-risk population (Lepow et al., 1975). Pb impairs spatial learning, visual-spatial and visual-motor integration, adaptive behavioral learning, and cognitive development (Needleman et al., 1979; Bellinger, Leviton, Waternaux, Needleman, & Rabinowitz, 1987; Dietrich et al., 1987; Dietrich, Succop, Berger, Hammond, & Bornschein, 1991; Kuhlmann, McGlothan, & Guilarte, 1997). While the specific mechanisms that effect these changes are an ongoing area of research, much is already known about the effect of Pb on the individual neuron and on the developmental processes in rats. For example, Pb promotes apoptosis in newborn rat cerebellum, an area critical to motor control (Oberto, Marks, Evans, & Guidotti, 1996). Recent work has shown Alzheimer's disease-like pathology in adult monkeys that were exposed as infants (Wu & Basha, 2008). These monkeys presented similar pathology to experimentally exposed rats; amyloid β protein accumulation and increased mRNAs for the protein precursor and transcription factors (M. R. Basha et al., 2005). Unfortunately, the mechanisms linking amyloid protein deposition, microtubule and synaptic degradation, and neural apoptosis have yet to be fully elucidated,

however, the current hypothesis includes the disruption of calcium homeostasis. (Mattson, Rydel, Lieberburg, & Smith Swintosky, 1993; Khachaturian, 1994; Mattson & Chan, 2001). Amyloid proteins are thought to interfere with calcium concentrations and activate caspase-12, an endoplasmic reticulum (ER) stress signal (Mattson et al., 1992; Nakagawa et al., 2000). Caspase-12[1] is the upstream activator of caspase-3, an effector caspase of apoptosis (Kerbiriou, Teng, Benz, Trouvé, & Férec, 2009). Pb is known to interfere with calcium concentrations in rat neurons and promotes calcium deficiency within the ER (Minnema, Michaelson, & Cooper, 1988; Qian & Tiffany-Castiglioni, 2003). As caspase-12 activation is a response to the unfolded protein response[2] (UPR) in part due to ER calcium depletion observed in Alzheimer's Disease (AD) and Pb exposure, it is possible that Pb enhances amyloid β protein driven apoptosis in part through this mechanism (Szegezdi, Fitzgerald, & Samali, 2003).

Lead Hemotoxicity

Following transit and absorption through the GI tract, the circulatory system is the next system to encounter orally-ingested Pb. In mammals, chronic Pb exposure can lead to hypertension, erythrolysis, inhibitition of δ- aminolevulinic acid dehydratase (δ-ALAD), and hypoproteinemia (Victery, Vander, Shulak, Schoeps, & Julius, 1982; Bandhu, Dani, Garg, & Dhawan, 2006; Casado, Cecchini, Simao, Oliveira, & Cecehini, 2007; Vanparys et al., 2008). Lead exposure results in lower renin (angiotensinase) activity causing an accumulation of angiotensin II, a regulator of vascular pressure (Victery, et al., 1982; Vander, 1988; Iadecola & Gorelick, 2004). This accumulation results in vasoconstriction raising systolic pressure in rats and humans (Carmignani, Boscolo, Poma, & Volpe, 1999). Red blood cells (RBCs) exposed to

[1] Rodent specific caspase.
[2] Unfolded Protein Response: a protein pathway and genetic control response to the disruption of "normal" protein folding in the ER.

Pb, *in vitro,* experienced a Fenton reaction-enhanced lipid peroxidation resulting in hemolysis and hemosiderosis in liver and kidney (Casado, et al., 2007; Al-Mansour, Al-Otaibi, Alarifi, Ibrahim, & Jarrar, 2009; Kerr, et al., 2010). δ-ALAD, a known blood Pb biomarker, is the second enzyme in the heme synthesis pathway and is extremely sensitive to low PbB concentrations. Erythrocytic sequestration of Pb is, in part, due to the higher binding affinity of Pb to a cysteine-rich zinc binding site on δ-ALAD (Payne, ter Horst, & Godwin, 1999). Exposure to Pb causes a conformational shift in the enzyme disrupting porphobilinogen synthesis (Jarzęcki, 2007). The inhibition of heme synthesis and erythrolytic events may contribute to lethargy brought on by anemia and increases the accumulation of erythrocytic components by Kupffer cells in the liver (Parker & Picut, 2005).

Lead Immunotoxicology

Pb has a broad adverse effect on many cells of the immune system. It dysregulates T-helper cell differentiation, B-cell isotype switching, nitric oxide production in neutrophils, and eosinophilic infiltration. Pb exposure results in reduced resistance to bacterial and parasitic infection while some research has linked Pb exposurre to stomach, lung, bladder and kidney cancer (Lawrence, 1981; Fu & Boffetta, 1995; Steenland & Boffetta, 2000; Dietert & Piepenbrink, 2006; Fernandez-Cabezudo et al., 2007). In one study, Pb-exposed mice injected *IP* with LPS displayed TNF-alpha dependent hepatic necrosis. Interestingly, human peripheral mononuclear cells exposed to Pb and heat killed *Salmonella typhi,* a source of LPS, significantly increased TNF-alpha production within 24 hr, *in vitro*. This would indicate that an individual exposed to both Pb and a relatively mild bacterial infection simultaneously could suffer severe adverse health effects mediated by monocytic cell lineages.

Pb has also been shown to affect the adaptive arm of the immune system. Pb exposure enhances several Vβ T-cell populations, and significantly decreases at least one. CD4/Vβ9 TCR T-cells were significantly decreased compared to control in mice following 8 weeks of Pb exposure (Heo, Lee, & Lawrence, 1997). CD4/Vβ9 TCR T-cells react to type V collagen and are a predominantly Type 1 T-helper cell population (Haque et al., 2002). Pb interferes with INF-γ production arresting Th1 development, which may contribute to a reduction in CD4/Vβ9 TCR T-cells and an increase in Th2 autoimmune cells concurrent with an increase in serum IL-4 (Schorlemmer, Dickneite, Kanzy, & Enssle, 1995; Heo, et al., 1997; Heo et al., 2007). However, not all cells in the Th2 subset are enhanced by Pb exposure. In separate studies, it was discovered that two specific splenic autoreactive Th2 cell populations were enhanced and that Pb exposure also lead to an increase in autoreactive antibodies (Mudzinski, Rudofsky, Mitchell, & Lawrence, 1986; Heo, Lee, & Lawrence, 1998). Further, T-cells underwent significantly greater expansion when allowed direct contact with Pb exposed APCs and B-cell Ig heavy chain synthesis was enhanced by Pb only in the presence of T-cells, *in vitro* (Mudzinski, et al., 1986; McCabe Jr & Lawrence, 1990). This T-cell dependent auto-antibody production and the selective APC driven expansion of specific Th2 cell populations indicate that Pb affects the active immune system via changes in APCs leading to the attenuation of the interaction between T-cells and APCs suggesting an important role for APC surface protein expression.

MHC-II Expression

MHC-II is expressed constitutively on macrophages, dendritic cells and B-cells but can be induced to be expressed on other cell types (Muhlethaler-Mottet, Otten, Steimle, & Mach, 1997; Drozina, Kohoutek, Jabrane-Ferrat, & Peterlin, 2005). In any case, expression of the gene requires 5 transactivating factors (**Figure 1-1**); Regulatory Factor X number 5 (RFX-5), RFX-

associated protein (RFX-AP), RFX-associated ankyrin-containing protein (RFXANK), nuclear factor-Y (NF-Y), Class II Transcriptional Activator (CIITA) and several accessory proteins (Steimle et al., 1995; Currie, 1998; Masternak, Muhlethaler-Mottet, Villard, Peretti, & Reith, 2000). RFX-5, FRX-AP, and RFXANK bind together to form the RFX complex which binds the X region of the MHC-II promoter (Mach, Steimle, Martinez-Soria, & Reith, 1996). NF-Y binds the proximal downstream Y region and is stabilized by c-terminal bonding with RFX-5 (Villard et al., 2000). This complex recruits CIITA and cyclin-dependent kinases 7 and 9 (CDK7, CDK9) which then recruit and phosphorylate RNA polymerase II (Spilianakis et al., 2003). TATA binding proteins (TBPs) and TBP associated factors (TAFs) stabilize RNA polymerase II over the TATA box initiating transcription (Weis & Reinberg, 1997; Smale & Kadonaga, 2003).

Despite the large number of required proteins to transcribe the MHC-II mRNA transcript, regulation of MHC-II expression can be traced to a single master regulator. Cells void of CIITA lack MHC-II and blocking CIITA transcription prevents MHC-II expression (Kanazawa, Okamoto, & Peterlin, 2000). In CIITA knockouts, MHC-II expression is lost resulting in bare lymphocyte syndrome (BLS) (Boss, 1997). The RFX complex, NF-Y, TATA stabilizing proteins, and RNA polymerase II are all expressed ubiquitously in all cells (Muhlethaler-Mottet, et al., 1997). However, CIITA expression alone is insufficient to initiate MHC-II expression. In RFX knockouts, CIITA is not affected but MHC expression is lost (DeSandro, Nagarajan, & Boss, 1999). Therefore, CIITA is the master regulator of MHC-II transcription but requires the complete MHC-II enhanceosome[3] to function.

MHC-II Trafficking

Regulation of MHC-II trafficking is critical for proper antigen presentation in APCs. Mechanistically, newly expressed MHC-II accumulates on the inside surface membrane of intra-

[3] Enhanceosome: The constellation of proteins that work together to enhance the transcription of a gene product.

cellular vesicles in pre-activated APCs produced from the Golgi apparatus (Sadaka, Marloie-Provost, Soumelis, & Benaroch, 2009). Once these vesicles retain MHC-II they are called MHC-II positive compartments (MIICs) and accumulate enzymes necessary for antigen processing. While macrophages contain several MHC-II positive compartments including multivesicular bodies (MVBs) and multilamellar bodies (MLVs), in monocytes, MIICs are predominantly just one type, electron dense bodies (EDBs) (Bunbury, Potolicchio, Maitra, & Santambrogio, 2009).

In monocytes, EDB MIICs have only recently been characterized. EDBs have MHC-II and lysosome associated protein number 1 (LAMP-1) localized on their inner surface and depending on classification, a several vesicle associated membrane protein (VAMP) proteins on their outer surface (Y. Chen, Lang, & Wade, 2004; Bunbury, et al., 2009). VAMPs are a required component of SNAP (Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment Protein) Receptor (SNARE) complexes (**Figure 1-2**) and therefore are frequently called v-SNAREs (Haas, 2007).

V-SNAREs dock with target-SNAREs (t-SNAREs) to facilitate membrane fusion and are specific for each target membrane (Schiavo, Stenbeck, Rothman, & Sollner, 1997). In MIICs, the SNARE complex consist of the v-SNARE, and soluble N-ethylmaleimide-sensitive factor attachment protein number 25 (SNAP-25)/Syntaxin 4, the t-SNARE proteins on the plasma membrane (Fukuda et al., 2000). SNAREs are responsible for driving membrane fusion events and are regulated by calcium via calmodulin (CAM) and calmodulin 2 kinase (CAMKii) (J. Luzio et al., 2003). In a typical membrane fusion event, free calcium binds CAM which then binds CAMKii uncovering the active site (Nielander et al., 1995). Once bound, CAMKii then phosphorylates serine 61 of VAMP which may then dock with the SNAP-25/Syntaxin 4 complex

on the plasma membrane (Hirling & Scheller, 1996; Snyder, Kelly, & Woodbury, 2006). However, t/v SNARE docking is insufficient to mediate membrane fusion. Another protein, synapotagmin, located on the vesicle membrane and presenting two cytosol facing C2 membrane binding domains, is required. The C2 domains bind the plasma membrane rapidly but in a calcium dependent manner. While defining the precise role of synapotagmin is still being investigated, the prevailing theory suggests that it stabilizes the SNARE complex by binding both the vesicle and plasma membrane pulling the SNARE alpha helix components into alignment. Once complete, the SNARE complex catalyzes the fusion of the vesicle and plasma membranes by opening a pore in each membrane and favoring the conjoining of the membranes by maintaining a hydrophobic core. As the complex opens, the vesicle membrane becomes continuous with the plasma membrane. In this way, intra and trans membrane proteins on the inside of the vesicle are presented on the cell surface and the contents of the vesicle are secreted into the extracellular milieu.

Biologic importance of LAMP-1

Increased surface expression of LAMP-1 is an indicator of sustained exocytosis. However, LAMP-1 (CD107a) also has an important biologic function (Alberts, 2004). On the cell surface of natural killer (NK) cells and monocytes, LAMP-1 mediates adhesion to the vascular epithelium by associating with CD11b and CD18, the components of macrophage antigen 1 (MAC-1) (Kannan et al., 1996; Mordue & Sibley, 2003). MAC-1 mediates monocytic attachment and migration by binding intracellular adhesion molecules (ICAMs) on the surface of the epithelium (Meisel, Simcha, Shapiro MSc, Radnay PhD, & Neuman, 1998; D. LIU et al., 2009).

ICAM expression affects neutrophil and monocytic infiltration and may also play a role in activation of these cells. (Dougherty, Murdoch, & Hogg, 1988; Wang, Deng, Maier, Sun, & Yenari, 2002). MAC-1 is a pattern recognition receptor for LPS, mediates microglia reactivity, and is required for full CD14/TLR LPS induced IL-8 production (Dentener, Bazil, Von Asmuth, Ceska, & Buurman, 1993; Mukaida, Harada, & Matsushima, 1998; Perera et al., 2001; Pei et al., 2007). Increased plasma IL-8 is positively associated with increased surface expression of LAMP-1 on peripheral monocytes and with scores on Systemic Lupus Erythematosus (SLE) disease scales (Holcombe et al., 1994). IL-8 is a product of monocytes and is stored in vacuolated epithelial cells and performs a chemotactic role for neutrophils which infiltrate the glomeruli during SLE nephritis (Modi et al., 1990).

LAMP-1 expression is increased on epithelial cells with increased ICAM expression in part because epithelial cells recycle these surface markers via the exosomal pathway which shares elements of the LAMP/VAMP/SNAP-25 SNARE complex in monocytes (Advani et al., 1999; Lin, Almqvist, & Telemo, 2005; Proux-Gillardeaux, Raposo, Irinopoulou, & Galli, 2007). Therefore, increasing LAMP-mediated SNARE complex formation increases surface expression of ICAMs on epithelial cells, and MAC-1 on monocytes. MAC-1/ICAM ligation on monocytes induces IL-8 secretion. IL-8 and enhanced ICAM epithelial expression increases neutrophil infiltration during SLE progression. In this way, mechanisms that modulate LAMP-1 surface expression on multiple tissues may contribute to the severity of the SLE lesions.

Biologic Importance of LAMP-2

Lysosomal associated protein number 2 (LAMP-2), also known as CD107b, plays an important role in intracellular transport and is critical to exocytosis. Congenital LAMP-2 deficiency results in Dannon disease which is also known as glycogen storage disease Type IIb in humans and

mice. Dannon disease is indicated by early onset progressive skeletal muscle weakness, and in males, hypertrophic cardiomyopathy. The myopathy is characterized by clusters of vacuolated myocytes, an indicator of lysosomal storage disease (Nishino et al., 2000; Maron et al., 2009). Under electron microscopy, LAMP-2 deficient muscle cells contain numerous large vacuoles, dilated lysosomes, and accumulated autophagosome (E. L. Eskelinen, 2006). In platelets, increased cell surface expression of LAMP-2 is associated with degranulation events (Silverstein & Febbraio, 1992). And finally, in several cell types, increased LAMP-2 surface expression is associated with the membrane repair response (MRR), a calcium regulated response leading to the mobilization and fusion of endosomal compartments with the plasma membrane to facilitate resealing within seconds of a membrane tearing event (J. P. Luzio, Pryor, & Bright, 2007; Palm-Apergi, Lorents, Padari, Pooga, & Hällbrink, 2009; Palm-Apergi & Hällbrink, 2011). The importance and availability of direct measure of LAMP-2 make it an attractive endpoint to quantify exocytosis.

Apoptosis and Autophagy

There is much debate in the literature as to if and how Pb might induce apoptosis in cells. Generally, *in vivo* and *ex vivo* studies have demonstrated little evidence that Pb, at non-lethal doses, induces apoptosis in APCs. However, several biochemical studies using recombinant proteins have demonstrated a number of possible Pb targets which interact with the apoptotic pathway. Additionally, a recent microarray study concluded that Pb-induced changes in gene expression may be associated with apoptosis (Kasten-Jolly, Heo, & Lawrence, 2010).

In APCs, dysregulation of some apoptotic regulatory pathways in the cell appear to take place without leading to actual cell death. In bone marrow derived dendritic cells, Pb induced no more apoptosis than media alone but severely impaired cellular proliferation after 10 days of

exposure at 100 µM Pb (Gao, Mondal, & Lawrence, 2007). Human mononuclear cells exposed to up to 500 uM Pb did not show signs of apoptosis after 48 hr exposure (De la Fuente et al., 2002). In at least one case, it was reported that Pb enhanced apoptosis, but only in the presence of mediating factors. Apoptotic hepatocytes were present *ex vivo* after exposure for 48 hr at 10 mM (10,000 µM) Pb but only in Kupffer cell conditioned media. When incubated with Pb alone at this extraordinarily high concentration, 48% of isolated hepatocytes were necrotic (at 48 hr) but TUNEL analysis and propidium iodide staining indicated there were no signs of apoptosis (at 2, 4, 6, 8, 12, 24, or 48 hr) leading the authors to conclude that additional apoptotic factors from the Kupffer cells were required (Pagliara et al., 2003). These findings clearly suggest that, while cellular respiration is inhibited by Pb exposure, apoptotic signals are not being activated inferring an alternative pathway closely linked to apoptosis called autophagy.

Cells employ autophagy as a response to nutritional depravation to provide raw material for respiration/glycolysis, to intracellular infection, to load cytoplasmic epitopes into MHC-II compartments, or to maturation/activation signals requiring disposal of large cellular structures which are no longer needed (Nimmerjahn et al., 2003; Dengjel et al., 2005; Cecconi & Levine, 2008; Harris et al., 2009; Cuervo & Macian, 2011; Morris et al., 2011). However, there is emerging evidence that cells may also employ autophagy during severe cellular insult. The precise regulation of this process is an ongoing area of research but many proteins responsible for regulating apoptosis also regulate autophagy although their role may be different in each pathway (Walsh & Edinger, 2010). For example, members of the BCL-2 family of proteins modulate apoptosis and autophagy depending on the local protein environment and location within the cell (Levine, Sinha, & Kroemer, 2008; Wei, Sinha, & Levine, 2008). Another protein, p62, binds both LC3, a component of autophagosome membranes and caspase-8, an apoptotic

mediator (Moscat & Diaz-Meco, 2009). Cells deficient in LC3 (or ATG7) accumulate p62 and display increased rates of apoptosis although the exact mechanism is still debated (Komatsu et al., 2007; Korolchuk, Mansilla, Menzies, & Rubinsztein, 2009). It appears then that cells may have different avenues of response to cellular insult including apoptosis and autophagy.

Generally, autophagy comes in two forms; microautophagy, and macroautophagy. Microautophagy is the degradation of cytosolic proteins in LAMP-2 positive compartments that are chaperoned into autophagosomes by heat shock proteins (Hayes & Dice, 1996; Ryhänen et al., 2009; Witt, 2010). It is theorized that these proteins are too large or otherwise too complex to be degraded by the proteasome or regular protein degradation processes have been inhibited. Macroautophagy is the bulk degradation of cellular organelles and components (Yoshimori, 2004). Unlike microautophagy, organelles and cytosolic components are non-discriminately sequestered into LAMP-2 positive compartments called nascent autophagosomes (E.-L. Eskelinen et al., 2002; E. L. Eskelinen, 2006; Fortunato et al., 2009). These structures are distinct from other vacuoles in their early stages by having double membranes surrounding cytoplasm and organelles. In later stages, they possess irregular radiopaque intra-vesicular materials and remnants of the degraded second membrane. After degradation, late stage mature autophagosomes may become myelin figures or enter the endolytic pathway.

In APCs, there is evidence that autophagy plays a role in antigen loading. *In vitro*, targeting of recombinant influenza matrix protein 1 to the autophagosome-associated protein LC3 in dendritic cells led to strongly enhanced MHC class II presentation to CD4+ T cell clones (Schmid, Pypaert, & Munz, 2007). In thymic epithelial cells (TECs) genetic interference with autophagy lead to increased auto-reactive T-cell subset populations, severe colitis, and multi-organ inflammation (Nedjic, Aichinger, Emmerich, Mizushima, & Klein, 2008). The authors

concluded that TECs engaged in negative selection use autophagy as the source of self-antigen to generate a self-tolerant T-cell repertoire. Finally, dendritic cells use double membrane bound autophagic compartments as a means to constitutively deliver intracellular pathogenic antigens to MHC-II positive compartments in a process sometimes referred to as xenophagy (Levine & Deretic, 2007). Further, analysis of MHC-II loaded antigens indicated components of cytosolic and even nuclear origin delivered via autophagosomes (Menendez-Benito & Neefjes, 2007). These findings suggest that autophagy provides both endogenous and exogenous antigen inputs to MIIC compartments and dysregulation of this process can severely disbalance MHC-II antigen loading and T-cell homeostasis.

Rationale for studying Pb exposure in birds

Birds represent a rather large class of tetrapod vertebrate animals known to inhabit ecosystems throughout the globe, which by default make them biological sentinels to changes in the environment. A number of these ecosystems contain various types and concentrations of environmental contaminants, such as Pb. Presently, Pb exposure studies in birds have documented a number of different toxic effects. Red-tailed hawks chronically exposed to sub-lethal doses of lead over a 3 to 11 week period demonstrated decreased heme synthesis and a dose-dependent decrease in lymphocyte mitogenic response to concanavalin A (Redig et al., 1991). McConnell *et al*., examined Japanese quail that were force-fed No 6 and No 7 shot pellets. These investigators observed clinical signs of lead poisoning, reduced hatchability, and concluded that adult quail were more resistant to lead poisoning than young quail (McConnell, 1967). In another study, adult Japanese quail were force-fed 1 or 4 No. 9 (0.05g/pellet) lead pellets in a single exposure, whilst on a commercial growth diet. These birds were then challenged with either chucker partridge red blood cells (CRBCs) or Newcastle virus but had

lower PbB levels (0.15-0.3 ppm) than studies with significant findings had previously reported (Fair & Ricklefs, 2002). These quail had growth rates, antibody production, white blood cell counts, and scores on the dermal phytohemagglutinin (PHA) test similar to control. The only unique observation in this study was that the Pb-exposed birds had elevated circulating granulocytes. Interestingly, no pellets were recovered during necropsy or from fecal droppings and the PbB levels did not deviate from control after day 49. The authors proposed that the pellets dissolved by about day 32 and reported that PbB levels were approximately 5% of those observed in other studies. In another study involving higher lead concentrations (100-400 µg/ml) in the form of lead acetate in drinking water, Japanese quail, fed a corn meal diet, demonstrated the same elevated granulocyte profile in addition to depressed white blood cell count, macrophage count, and decreased PHA test scores, a measure of T cell response (Grasman & Scanlon, 1995). Collectively, these Pb exposure studies reveal a measured degree of variability in toxicity. Likely contributors to the variability reported in these studies may be due to differences within the bird species, husbandry and exposure conditions.

Despite this body of research, there is still limited data that faithfully replicates conditions experienced by game birds in the field. While there is concern for Pb levels in drinking water and earlier studies attempt to address this, the effects of oral Pb ingestion via the more common exposure route have not been well-characterized. Studies that did examine exposure via shot pellets failed to accurately track the absorption rate and clearance of orally administered lead. Further, it is currently not known if oral lead exposure from spent shot is comparable in toxicity to un-discharged lead, as a result of marked morphologic changes and fragmentation that occur subsequent to discharging and high-speed impacts. Other variables

that can affect lead toxicity outcome include bird diet, bird age and sex. Clearly, enough variables exist to warrant a more focused study of oral lead exposure in birds.

In this proposed body of work, we evaluated the physiologic effects of single and multiple spent Pb pellets and bullet fragments in Northern Bobwhite quail while comparing additional influences of bird sex over two time periods. We employed radiographs to determine the retention time of ventriculus-gavaged bullet fragments in the GI tract and link these data to bird lead levels. We included a series of tests to track bird health. Additionally, we collected and evaluated tissue samples from documented lead–sensitive organs. The anticipated outcome from this study was to generate data that will help provide a more accurate assessment of NOEL and LOEL following oral Pb administration in the Northern bobwhite.

Rationale for studying Pb exposure in antigen presenting cells, *in vitro*

Pb affects the immune system by dysregulating both innate and adaptive immune cells. Pb decreases pathogen resistance, exacerbates disease in autoimmune-prone mice, and induces ANA production in non-autoimmune-prone mice strains suggesting multiple immune cell involvement. Further, Pb exposure shifted differentiation of T-helper cells toward a Th2 profile and arrested IFN-γ production in Th1 cells. Similarly, dendritic cells experimentally exposed to theoretically lethal concentrations of Pb *in vitro* exhibit partially arrested development and increased MHC-II surface expression. Aberrant T-helper cell differentiation and MHC-II expression have been linked to the exacerbation of autoimmune disease but the underlying mechanisms are presently not well characterized. In this case, the primary route of Pb exposure results in elevated blood Pb concentrations, a site where dendritic cells and differentiated T-helper cells are relatively uncommon. The monocyte, a precursor to dendritic cells and macrophages, is a blood leukocyte and is capable of presenting antigen which can alter T-helper

cell differentiation. Currently, there is a debate between two mechanisms across the literature as to the source of self-antigen required to induce these changes; apoptosis and autophagy. The theory supporting apoptosis as the source of self-antigen suggests that Pb-induced erythrolysis increases the availability of circulating self-antigen that may be phagocytized, processed, and presented by APCs. On the other hand, dysregulated autophagy could disbalance antigen loading of MHC-II compartments leading to enhanced presentation of cytosolic antigens at potentially lower concentrations which would better align with current *in vivo* data. Therefore, we hypothesize that Pb increases surface expression of MHC-II and T-cell ligands and activates autophagy in APCs.

Initial evaluations of the effect of Pb on the immune system in the bobwhite were inconclusive, so to further elucidate the effect of Pb on the immune system, we exposed a well characterized model of APCs to concentrations of Pb similar to those observed *in vivo*. The focus of this study will be to evaluate the effect of sub-lethal acute doses of Pb on antigen presentation and elucidate the mechanism(s) involved in a well-established *in vitro* model of antigen presenting cells, the RAW 267.4 cell line. RAW 267.4 cells will be examined by flow cytometry for changes in surface expression of MHC-II, T-cell ligands (CD80, CD86, CD40), and exocytosis (LAMP-1, LAMP-2). Secreted β-hexosaminidase activity will be measured to confirm the rate of exocytosis and functional assays will be used to evaluate the metabolic state of Pb-treated cells. Further, both confocal and electron microscopy will be employed to evaluate the presence and degree of autophagy. Should the results of this study show that Pb exposure alters antigen loading while enhancing MHC-II expression, this could be a novel mechanism for promoting an increase in auto-reactive T cells, which has been reported in *in vivo* Pb studies.

Further, studies into these effects on cellular autophagy could shed new light on a potentially novel mechanism of autoimmue induction.

Figures

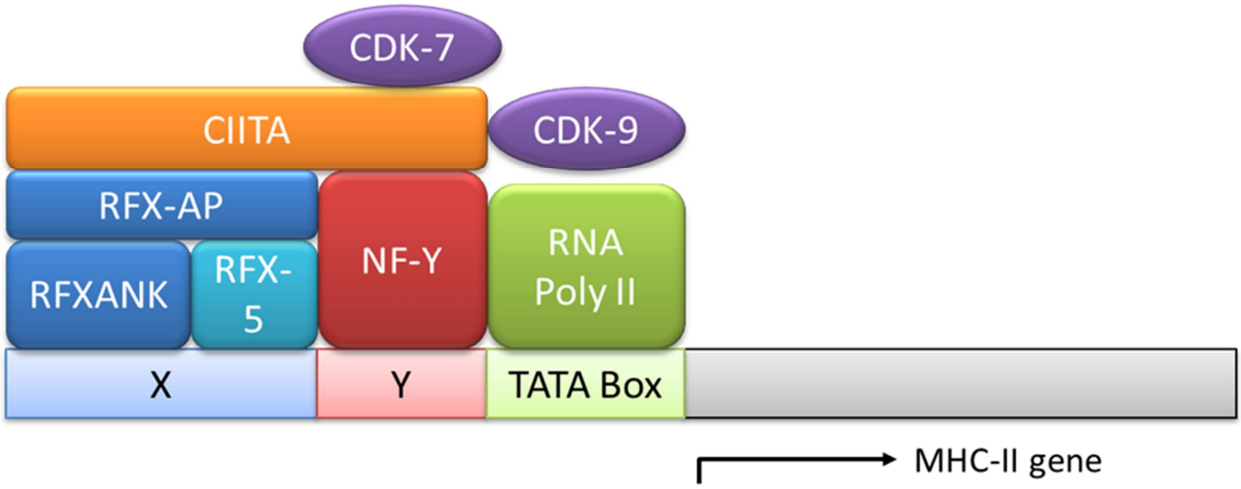


Figure 1-1: MHC class II Transactivating Factors

MHC-II requires 5 TFs for transcription; RFX-5, RFXANK, RFX-AP, NF-Y and CIITA. CIITA is the master regulator for MHC-II transcription.

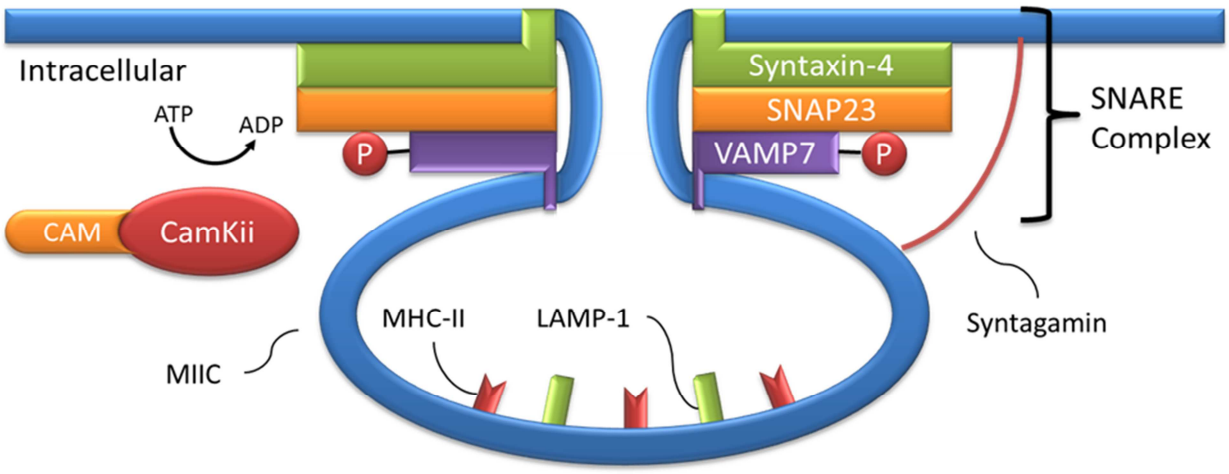


Figure 1-2: SNARE Complex Formation

SNARE Complexes dock intracellular vessicles with the plasma membrane modulating exocytosis. See text for details.

CHAPTER 2. ACUTE LEAD PELLET EXPOSURE IN NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*) ALTERED HEMATOLOGIC PROFILES, REDUCED WEIGHT GAIN, AND INCREASED MORTALITY[4]

[4]Kerr, R., Holladay, S., Jarrett, T., Selcer, B., Meldrum, B., Williams, S., Tannenbaum, L., Holladay, J., Williams, J., and Gogal, R. Accepted by *Environmental Toxicology and Chemistry*. Reprinted here with permission of publisher.

Abstract

Lead, a well-known worldwide environmental contaminant, adversely affects multiple organ systems across many species. Previous studies in rodents have shown that lead exposure can alter blood physiology, induce neural and renal toxicity and affect nutrient absorption. In avians the primary route of exposure, with similar indications of toxicity, is by oral ingestion of lead particles as grit. However, no data exist tracking the retention and clearance of these particles in the bird GI tract while linking toxicity and blood accumulated lead levels. In the present study, Northern Bobwhite quail were orally gavaged with spent lead shot (0, 1, 5 and 10 spent lead shot/bird), radiographed and evaluated for changes in peripheral blood, neural, renal, immune and reproductive parameters. The majority of the lead pellets were absorbed or excreted within 7 to 14 days post exposure with no detection by radiographs by 21 days. Blood lead levels were approximately one order of magnitude higher than those reported in previous studies. Feed consumption, body weight, blood packed cell volume (PCV), plasma protein concentration and δ-aminlevulic acid dehydrase (d-ALAD) activity were adversely affected by oral gavage with 5 or 10 lead pellets. Nerve and koilin degradation were also observed in the 10-pellet group. Birds receiving a single lead pellet also displayed depressed d-ALAD supporting impaired hematologic function while birds acutely exposed to either 5 or 10 pellets exhibited greater morbidity and mortality.

Keywords Lead (Pb), avian, blood, d-ALAD, radiography

Introduction

Lead (Pb) is a ubiquitous environmental contaminant largely based on its historical use in a variety of industrial manufacturing processes including munitions. Environmental Pb is frequently found in one of three major forms; dust particles deposited from the combustion of gasoline containing tetra-ethyl Pb (TEL), Pb-based paints, and bullet fragments or spent Pb pellets from expended ammunition (Mahaffey, 1995; Thomas, 1995; Craig, et al., 1999). TEL is of particular concern to human health as it may enter the food chain through contaminated foods and has been linked to elevated blood Pb levels (PbB) (Mielke & Reagan, 1998). While TEL may leach into the soil forming several organic Pb compounds, Pb in a larger solid form (i.e. spent Pb pellets and fragments) is slower to break down. As a result, these pellets and fragments can bind tightly to topsoil particles, yielding Pb soil concentrations 10-100 times greater than background (K. Murray et al., 1997; X. Cao, L. Q. Ma, M. Chen, D. W. Hardison, & W. G. Harris, 2003; Knechtenhofer, Xifra, Scheinost, Fluhler, & Kretzschmar, 2003). Although past legislation has made significant progress toward banning the manufacture and release of lead-containing products, large quantities of Pb contaminants still exist in the environment in many sites throughout the United States and thus, pose a significant health concern.

The health effects associated with Pb exposure are quite diverse. For example, the major clinical signs for all species associated with acute toxic Pb exposure include weakness and lethargy, anemia, emaciation, and death (Coburn, Metzler, & Treichler, 1951; Cook & Trainer, 1966; Kendall, et al., 1996). Sub-acute doses, although less visible clinically, can affect numerous physiologic pathways. For example, Pb can decrease the efficiency of δ-aminolevulinic acid dehydratase (δ-ALAD) in heme biosynthesis, interfere with *N*-methyl *D*-aspartate (NMDA) ionotrophic glutamate receptors in neural tissue, and alter the upregulation of

Nuclear Factor –κB (NK- κB), a potent immune transcription factor (B. A. Fowler, 1998; Ramesh, Manna, Aggarwal, & Jadhav, 1999; Gavazzo, Zanardi, Baranowska-Bosiacka, & Marchetti, 2008). As a result, the renal, neural, reproductive and immune systems are the organ systems commonly affected following Pb exposure. In addition, Pb can be sequestered in red blood cells (RBC), cartilage, and bone where it is released into circulation during periods of high bone turnover or cartilaginous repair (Eisler & Patuxent Wildlife Research Center., 1988; Silbergeld, 1991; B. A. Fowler, 1998; Carmouche et al., 2005b). Pb has also been shown to cause adducts and strand breaks in DNA as well as changes in gene promoter expression profiles. (Yuan & Tang, 1999; Tully, et al., 2000; Fracasso, Perbellini, Solda, Talamini, & Franceschetti, 2002; Palus et al., 2003). Thus, Pb exposure can adversely modulate a number of organ systems and physiologic pathways. The severity of these effects is a function of the route, quantity and duration of exposure.

Throughout the United States, federal, state and private shooting ranges and other areas where hunting is prevalent are prime sites where migratory or non-migratory birds have the potential to be exposed to Pb in the form of spent shot. Based on the vicinity and frequency of shooting activity, the amount of shot in these areas where birds may forage for food and grit particles can vary, thus impacting Pb ingestion and absorption (Beyer et al., 2005; Beyer, Gaston, Brazzle, O'Connell, & Audet, 2007; Ethier, Braune, Scheuhammer, & Bond, 2007; Jackson & Reddy, 2007; Rattner, Golden, Toschik, McGowan, & Custer, 2008; Buekers, Steen Redeker, & Smolders, 2009). In the 1990's, it was estimated that 80,000 tons of shot pellets and bullet fragments had been released annually during recreational shooting resulting in an estimated deposition of over 3 million metric tons of Pb on shooting ranges across the United States (Craig, et al., 1999). Field studies on select avian species near these locations suggest that

oral ingestion of Pb pellets is the predominant form of exposure. This would infer that birds mistakenly ingest Pb pellets as grit particles thus, presenting a unique hazard to avian wildlife. (McConnell, 1967; Bennett, et al., 2007; Duggan & Dhawan, 2007).

Although several studies have examined the effects of organically bound Pb using water or feed exposure routes in several species, critical data are lacking showing the effects of oral spent Pb pellet consumption as grit in birds (Coburn, et al., 1951; B. A. Fowler, 1998). The present work was initiated to determine the extent to which lead, mistakenly ingested as grit, is harmful to the health of the avian species, *Colinus virginianus*. This is the first study, to our knowledge, to employ radiographic imaging to track Pb grit retention and correlate it to blood Pb levels (PbB), hematologic, immunologic, and histologic parameters in assessing the toxicity of Pb in birds.

Methodology

Birds

Eighteen male and twenty female 16-week-old Northern Bobwhite quail were acquired from M&M Quail Farms (Goulds, FL) and maintained in individual quail cages fitted with an automatic watering system (Alternative Design, Siloam Springs, AR) at the Poultry Diagnostic and Research Center at the University of Georgia in Athens, GA. Birds were allowed 1-week acclimation with *ad libitum* access to a pelleted sporting bird conditioner (Southern States, Richmond, VA) and 2 additional weeks with access to a seed-based diet and fortified grit (Southern States). Quail were housed at 23.0 ± 5.0^{o}C, humidity of 45.0 ± 10.0%, on a 14/10 hr light/dark cycle. Bird health was assessed daily and feed consumption calculated weekly. All procedures were approved by the University of Georgia's Institutional Animal Care and Use

Committee (IACUC) and experiments were conducted in compliance with Good Laboratory Practice (GLP) standard operating procedures.

Test article

Spent Pb pellets (~ 50 mg/pellet, 4-6 mm) were provided by the U.S. Army Center for Health Promotion and Preventive Medicine from soil samples acquired from Fort Eustis, Virginia. Pellets were washed in 70% ethanol (Sigma, St. Louis, MO) to remove soil contaminants, washed 2x with distilled water, and allowed to air dry. Ten pellets were selected from the stock and submitted to the Diagnostic Toxicology Laboratory at the Virginia Maryland Regional College of Veterinary Medicine at Virginia Tech, Blacksburg, VA for Pb content analysis.

Exposure Paradigm

Birds ($n \geq 4$ per treatment) were individually selected at random and then orally gavaged with 0, 1, 5 or 10 pellets in 2 ml physiologic saline (Hospira, Lake Forest, IL) using rubber tubing and a 3 ml dosing syringe on Day 1. On day 2, Pb pellet content of the ventriculus and crop was confirmed in all gavaged quail by performing radiographs on each bird.

Radiography

Birds were radiographed weekly to determine the location and disposition of the Pb pellets. Each bird was placed in a passive restraint device and arranged in dorso-ventral (D-V) or lateral recumbency on a radiograph cassette (n=2 birds/cassette). AGFA Mammography cassettes with Mamoray screens and HORC-100 NIF film were used (AGFA-Gevaert N.V., Mortsel, Belgium). Radiographs were performed using a Transworld 625V X-ray machine (Transworld X-ray Corp, Charlotte, NC). A table-top technique was employed using a forty-inch focal-film distance exposed at 600mA, 1/40 second, and 56 kVp.

Blood Collection

On days 0, 7, 14, 21 and 28, blood was collected aseptically from the jugular vein of each bird with a 25-gauge needle and 1-ml syringe (Becton-Dickinson (BD), Franklin Lakes, NJ). After collection, an 18-gauge needle was attached to the syringe and the blood was transferred into a 4 ml depressurized, heparinized vacutainer tube (BD) and mixed by gentle inversion.

Pellet and blood Pb analysis

Heparinized whole blood samples (0.5 ml/bird) were frozen at -80 °C and shipped on dry ice, along with the Pb pellets, to the Diagnostic Toxicology Laboratory at the Virginia Maryland Regional College of Veterinary Medicine at Virginia Tech for Pb analysis as per the method described by Meldrum *et al.* 2003 (Meldrum & Ko, 2003). Briefly, the Pb pellets were digested in 20% perchloric acid/80% nitric acid solution by heating for 2 hr at 150 °C and evaluated on a atomic absorption spectrometer using a graphite furnace and expressed as %Pb. For each sample, 100 µl of blood was mixed with 900 µl of modifier solution (Triton-X, ammonium phosphate monobasic, nitric acid (Sigma)) and enumerated on an atomic absorption spectrometer by graphite tube atomization with the limits of detection at 25 ppb.

Red blood cell enumeration

For each sample, 1 µl heparinized whole blood was added to 1 ml physiologic saline and vortexed for 15 seconds. A 20 µl aliquot was added to a Nexcelom counting chamber and enumerated on an Auto T4 cell counter (Nexcelom Corp., Lawrence, MA). Results were expressed as billions of cells per ml ($1x10^9$ cells /ml).

Packed cell volume and total protein

A hematocrit capillary tube (Drummond Scientific, Broomall, PA) was filled to ¾ volume, sealed at the bottom and centrifuged in a hematocrit spinner (LW Scientific,

Lawrenceville, GA) for 5 min at 14,800 x g. The packed cell volume (PCV) was enumerated using a micro-capillary reader (International Equipment Company, Needham, MA) and expressed as a percent. The capillary tube was then cut above the RBC layer and approximately 30 µl of plasma were placed onto a refractometer (Reichert, Depew, NY) to determine the total protein, which was expressed as g/ 100ml.

Cytology

Approximately 5 µl whole heparinized blood were transferred to a microscope slide (Corning Inc, Corning, NY) and a blood smear made using standard technique. Resulting slides were allowed to air dry for at least 60 minutes before being fixed and stained using Wright-Giemsa stain in a Wescor 7150 Hematology Slide Stainer-Cytocentrifuge (ELI Tech Group, Logan, UT). Slides were coverslipped and a 5-point differential was performed with the following cell types enumerated; lymphocytes, monocytes, heterophils, eosinophils, and basophils (200 WBCs/ slide). Morphologic changes of the RBCs were also evaluated.

d-ALAD assay

Delta aminolevulinic-acid dehydratase (d-ALAD) activity was determined using an adaptation of the European standardized method as amended (Berlin & Schaller, 1974). Briefly, 50 µl heparinized whole blood from each bird were added to a separate 5.0 ml centrifuge tube (Fisher Scientific, Suwanee, GA) containing 1.45 ml molecular grade water (Sigma, St. Louis, MO) and vortexed for 15 sec. To each tube, 1 ml of 10 mM ALA solution (Sigma) was added, vortexed for 15 sec, and allowed to incubate for 60 min in the dark at 38 °C. One milliliter of trichloroacetic acid (TCA) stop solution (Sigma) was then added to each tube and vortexed for 15 sec. Tubes were then centrifuged for 10 min at 200 x g in a 5810R centrifuge (Eppendorf, Westbury, NY). Approximately 100 µl of the resulting supernatants were then added in

triplicate to clear, round bottom 96 well plates (Corning) and read in a Synergy 4 plate reader (Biotek, Winooski, VT) at 555 nm against a reagent blank. Results were expressed as nanomol of ALA used per min per ml RBCs.

Histopathology

Birds were euthanized via CO_2 gas and necropsied at the termination of the study or when displaying signs of significant morbidity or weight loss. Representative samples of kidney, liver, spleen, sciatic nerve, and reproductive organs were collected. These tissues were weighed, fixed in 10% buffered formalin, and trimmed for routine processing. Samples were then embedded in paraffin and sectioned at 4-5 microns. Slides were stained with routine hemotoxilyn and eosin and examined by light microscopy. Additional slides of sciatic nerves, sectioned at 7-8 microns, were stained with Luxol Fast Blue to determine if myelin degeneration had occurred. Slides were blindly scored from 0 (no degradation) to 5 (severe degradation) by a certified avian pathologist (SW).

Statistics

Data were compiled in Microsoft Excel (Microsoft, Redmond, WA) and statistics calculated in SAS. The Dunnetts test and Pearson coefficient statistical tests were performed where indicated and appropriate. Data were considered significant and described as different at the $p \leq 0.05$ level with $n \geq 3$.

Results

Feed consumption and body weight

Feed consumption and body weight were inversely correlated to quantity of Pb ingested. Birds gavaged with 5 or 10 pellets lost significant body weight after 2 weeks post exposure and were humanely euthanized. Saline control and 1 pellet dosed birds showed comparable trends

and were continually monitored to the study termination on day 28. The loss of body weight in 5 and 10 pellet groups correlated with reductions in feed consumption (**Figure 2-1, Figure 2-2)**. One pellet-dosed groups had a non-significant decreasing trend in feed consumption. Across sex, males generally ate less than females. One pellet-dosed females ate less feed than control while male 1 pellet birds ate numerically more than control.

Pb pellet retention and radiographs

Pellet content of the ventriculious and crop was confirmed with radiographs 24 hours post oral gavage (**Figure 2-3**) verifying that all birds received and retained the correct number of Pb pellets dosed. Analysis of radiographs conducted 1-week post oral exposure revealed that pellet retention, the number of pellets retained compared to the number of pellets dosed, decreased to 21%. By 2 weeks, pellet retention dropped to 7% (**Figure 2-4**). Bird groups gavaged with 10 pellets retained a larger percent of the initially gavaged pellets in the short term while birds gavaged with 1 pellet retained their individual pellets longer. Despite this large decline in retained pellets, 48% of the birds still retained ≥ 1 shot after 1 week (data not shown). The number of retained pellets decreased to 5% after two weeks and was not detectable by week three. The pellets in one male bird evaluated up to two weeks post exposure, visually appeared to decrease in size based on the radiographs, suggesting absorption from the ventriculus (**Figure 2-5**).

Pb pellet and Blood analysis

The mean percentage of Pb in the 10 pellets submitted for analysis was greater than 90% (data not shown). The PbBs levels showed a significant correlation by dose to the number of gavaged pellets for weeks 1 and 2 (Pierson's coefficient $r = 0.64$, $p \leq 0.01$; $r = 0.50$, $p \leq 0.01$, respectively). Blood Pb levels tended to drop over time regardless of initial dose, but tended to

fall more sharply in females rather than males (**Table 2-1**). Blood Pb levels dropped by as much as 60% from week to week.

δ-ALAD activity

d-ALAD activity was significantly reduced by oral exposure to Pb across all time points and doses of Pb (**Figure 2-6**). By week 3, the δ-ALAD activity in the 1-pellet birds began to increase. By week 4, female 1-pellet birds had regained approximately 25% of control δ-ALAD activity.

Blood parameters

Males dosed with 10 Pb pellets displayed significantly lower PCV levels compared to controls during week 1. By week 2, birds from both the 5 and 10 pellet dose groups had significantly lower PCV levels compared to controls. Total RBC numbers showed similar but non-significant trends (**Table 2-2**). Mean corpuscular volume (MCV) did not differ across dose, sex or time. Total protein levels were significantly lower in females dosed with 5 and 10 pellets during week 1.

Cytology

There were no significant differences in the morphology or percentages of leukocytes across treatments or time points. Microscopic observations of red blood cells showed severe lytic changes in many of the slides of the Pb shot-dosed birds but due to variability within the treatment groups these trends fell short of statistical significance.

Histopathology

Koilin degeneration in the ventriculus was observed in the 5 and 10-pellet birds groups. These lesions trended toward greater severity in the 10-pellet bird group reaching significance in the males (**Figure 2-7**). Likewise in the males, splenic hemosiderosis was observed to increase

in a dose dependent manner reaching significance in the 10-pellet group (**Figure 2-8**). The livers of the male 5 and 10 pellet groups had necrotic lesions, which was significant in the 10 pellet group. Analysis of the gonads showed decreased spermatogenesis in the male 5-pellet group, which was not observed in the 10-pellet group. Birds in the 1-pellet group had no significant lesions for all tissues evaluated (**Table 2-3**). Evaluation of the ovaries revealed no remarkable findings other than the females were in production (data not shown).

Discussion

Although numerous studies have examined the effects of Pb in rodents, data on Pb exposure in birds are limited. Field studies in various bird species are supportive of the role of Pb exposure and toxicity. However, the lack of a controlled environment in which to study these effects in the bird makes it difficult to correlate Pb toxicity changes to dose as well as address the potential risk of oral exposure to a single pellet. The focus of this work was to track the effects of oral exposure to Pb pellets at different doses on hematologic parameters, and to correlate these changes with radiographic verification of Pb shot retention, blood Pb levels, d-ALAD activity, and histopathology in bobwhite quail.

In the present study, a rapid decline in the health of the 5 and 10 Pb pellet birds was an observation not anticipated based on a review of the literature. Fair and Ricklefs et al., 2002 gavaged Japanese quail with 4 Pb shot (200 mg) and reported no significant mortality when compared to controls by 128 days, although they did not track pellet retention or clearance. In the current study, birds gavaged with 5 or 10 pellets displayed symptoms of Pb toxicity that manifested as decreased activity, decreased feed intake and weight loss, within the first 7 days. Birds that were gavaged with 1 pellet displayed no adverse clinical signs. In addition, there appeared to be no correlation between the number of pellets administered and the number of

pellets retained. This would imply that the oral ingestion, retention and clearance of Pb pellets were unique to each individual quail. Of note, a male bird in the 1 pellet group retained its gavaged pellet beyond 2 weeks. In each successive radiograph, the pellet appeared to reduce in size and as a result, this bird had elevated PbB concentration, lowered body weight and lowered feed consumption compared to other males in the same group, showing an apparent relationship between length of retention, absorption and toxicity.

Overall, blood Pb concentrations in the quail showed a correlation to the number of retained Pb pellets. In comparison to previous studies, these PbB concentrations were as much as 2 orders of magnitude higher than those values previously published in mammalian studies (Burger & Eichhorst, 2007). This suggests that Pb absorption via the pellet was significant, and that birds also appear to have the capacity to tolerate these higher Pb exposures. One possible explanation for the higher Pb tolerance is that birds possess a red blood cell that is nucleated making it more resistant to environmental assault, such as shifts in osmolarity (Gogal *et al.*, 1997). Another explanation could be that birds, like rodents, may possess a higher number of copies of the δ-ALAD gene. Previous research with mice has shown that strains with duplicate δ-ALAD genes sequestered almost twice the Pb in their blood and organs from Pb in drinking water for the same rate of exposure as mice with 1 copy (Burger & Eichhorst, 2007).

Based on the radiographs, quail appeared to either clear or absorb a significant portion of the ingested pellets between 1-14 days post gavage. Analysis of the PbB concentrations indicated a measureable amount of Pb was absorbed systemically. In the males, oral exposure of 5 pellets resulted in approximately a three-fold increase in PbB over a single pellet exposure while an oral exposure of 10 pellets resulted in an increase of three and one half-fold. These responses clearly demonstrate a lack of proportional increase in PbB concentration relative to the number of

gavaged Pb pellets. One explanation could be that the rate of clearance of the Pb pellets, as previously mentioned, is unique to each bird. Another possible explanation, based on the histopathology, was the observation of koilin degeneration in the ventriculous of birds exposed to 5 or 10 pellets, with the males having the more severe lesions. Degeneration to the ventricular lining can alter the absorption capacity in the avian GI tract, affecting Pb absorption, which would influence PbB concentration. A pattern of toxicity relative to PbB concentration was noted in other select tissues. Splenic hemosiderosis was observed in the 5 and 10 pellet groups suggestive of a Pb-induced hemolytic event (Ochial *et al.*,1993). As had been the trend, the male 5 and 10 pellet birds had the more severe lesions. This was further reflected in the liver with measureable necrosis in the male 5 and 10 pellet birds, which also had the higher mean PbB concentrations across sex.

Another important observation was that sensitivity to Pb exposure appeared to differ by sex. Based on the parameters measured, female quail appeared to be more tolerant to Pb than the males, with the exception of weight loss. A noteworthy observation related to the season in which the study was conducted was that the female quail were in active egg production. Physiologic weight loss during egg production is a common occurrence and thus could be a possible explanation for the greater weight loss in females (Gogal *et al.*, 2003). Of interest, females gavaged with either 5 or 10 pellets nonetheless showed less hematologic and microscopic changes compared to males in the same treatment. These females also had lower PbB concentrations and displayed a slight increase in d-ALAD activity post pellet clearance. These differences across sex would suggest that female quail possess the ability to either excrete or clear Pb at a higher rate than the males. In birds, Pb is removed from blood by the kidneys, through sequestration in bone, neural tissue, and red blood cells, and through deposition in

feathers and eggshells (Burger, 1994; Burger & Eichhorst, 2007). Since the present female quail were in production, it is likely that Pb deposition in the eggshells lowered total circulating Pb levels, contributing to the increased tolerance.

Blood Pb level and δ-ALAD activity are common assays for evaluating Pb toxicity. In the quail, δ-ALAD appeared to be a more sensitive and functional marker of Pb exposure. This enzyme is integral to the heme biosynthesis pathway and was quite sensitive to even low PbB concentrations. For example, one pellet exposure in quail was sufficient to depress δ-ALAD activity for the duration of the study, even after the PbB concentrations significantly declined from the initial peak concentration. Interestingly, 1 pellet exposure did not significantly affect any of the other parameters when compared to the controls of both sexes. Although tissues have no natural requirement for Pb, chemical properties of Pb can facilitate protein binding (i.e. δ-ALAD) to this metal. Further, biochemical pathways that employ zinc or calcium ions can be adversely affected by Pb exposure (Ochiai, Jin, Goryo, Tsuzuki, & Itakura, 1993; Gogal Jr et al., 2003). Thus even though there were no overt clinical signs of Pb toxicity from the oral ingestion of 1 pellet in quail, it is possible that other physiologic or biochemical pathways, that were not evaluated in this study, were affected.

Another noteworthy observation in this study post Pb exposure was the significant decrease in feed intake by the birds in the 5 and 10 pellet dose groups. It is possible that the Pb effect on body weight, PCV, red blood cell count, and plasma protein could, in part, be influenced by lowered caloric intake. Feed consumption rates of birds gavaged with 5 or 10 pellets were significantly lower than control birds throughout the study. This quantity of pellets was well below the average number of grit particles that were observed in the controls during gross examination of the ventriculus. Therefore, impaction or physical obstruction was not the

etiology for the decreased feed consumption. In the 1-pellet birds, decreasing non-significant trends in feed consumption and weight gain were also noted. In rodents, Pb (although not in shot pellet form) has been shown to adversely affects body weight gain (Hammond & Succop, 1995). Additionally, the authors in that study were able to demonstrate that body weight loss could be largely reversed with sufficient nutritional supplementation. Pb then, may influence caloric intake via two potential mechanisms; impairment of appetite through an unknown mechanism, and/or malabsorption via koilin degeneration in the quail. In another study with rats, the administration of growth hormone and thyroxine was ineffective at curbing the weight loss associated with lead. Also, subcutaneous administration of Pb in vehicle had a less significant, but still relevant negative effect on body weight indicating that currently known body weight control pathways were not affected and that the route of exposure is a factor (Hammond, Chernausek, Succop, Shukla, & Bornschein, 1989). In addition, insulin-like growth factors (IGFs) did not play a role in the Pb-associated suppression of appetite, and the reduction in body weight gain was almost completely explained by a lack of food intake and not by food aversion. Consequently, there appears to be a composite toxic effect on the avian GI by the spent PB shot that cannot be simply explained by systemic analysis of typical appetite markers or by the koilin degeneration found in our histological analysis. Future studies are suggested to further explore and understand this relationship.

To summarize, this is the first study to follow, with radiographic documentation of retention, the effects of acute oral lead exposure in upland birds and correlate it to Pb retention, blood Pb levels, hematologic parameters and histopathology. When orally ingested, bobwhite quail, while on a seed-based diet, eliminated or absorbed spent Pb pellets within 3 weeks. Further, the Pb transit rate through the GI tract was independent of the number of Pb pellets

ingested. Female quail appeared to tolerate higher concentrations of Pb, which was likely attributed, in part, to their egg laying status. Birds ingesting 5 or 10 pellets were unable to sustain either a neutral or positive weight gain, which was an adverse health effect. Microscopic analysis of the ventriculus showed koilin degeneration, an indicator of impaired ventricular function, in the 5 and 10 pellet birds with significance in the male 10 pellet group. Hemosiderosis, an indication of RBC lysis, was also observed in the spleens of the 5 and 10 pellet birds reaching significance in the male 10 pellet group. These male 5 and 10-pellet birds also had evidence of liver necrosis, which was significant in the male 10 Pb pellet group. Birds ingesting 1 Pb pellet had hematologic parameters similar to controls but no remarkable histopathology findings. The only evidence of Pb exposure, other than PbB levels, in the 1-pellet birds was the decreased δ-ALAD activity. This indicator of heme biosynthesis inhibition remained decreased, although it did start to rebound by day 21 post-exposure in female birds. Interestingly, throughout the study, 1-pellet birds appeared clinically normal, which warrants additional evaluation into the long-term impact of heme biosynthesis inhibition following Pb exposure in birds.

Acknowledgement

The authors would like to thank the U.S. Army Environmental Command and the Environmental & Natural Resources Division at Fort Eustis, VA for funding this project. The authors appreciate the assistance of Ms. Heather Bissette and Ms. Allison Watkins of the animal care office at the Poultry Diagnostic and Research Center, University of Georgia. The authors wish to also thank Ms. Barbara Wise of the Diagnostic Toxicology Lab at Virginia Tech.

Tables

Table 2-1 Shot Blood Pb Level (PbB)

Blood lead level µg/dl		Saline Mean ± SEM	1 pellet Mean ± SEM	5 pellet Mean ± SEM	10 pellet Mean ± SEM
Baseline	Male	1.25 ± 0.72	1.25 ± 0.56	1.25 ± 0.63	1.25 ± 0.56
	Female	1.25 ± 0.63	1.25 ± 0.63	1.25 ± 0.63	1.25 ± 0.63
Week 1	Male	1.25 ± 0.00	1347.28 ± 587.43	3884.53 ± 1173.82	**4131.58 ± 1332.57***
	Female	1.25 ± 0.00	**1720.25 ± 49.05***	**2157.10 ± 292.00***	—
Week 2	Male	2.96 ± 0.67	918.80 ± 561.70	—	—
	Female	4.87 ± 1.22	342.46 ± 192.54	—	—
Week 3	Male	1.25 ± 0.00	300.52 ± 168.26	—	—
	Female	1.25 ± 0.00	149.40 ± 95.77	—	—
Week 4	Male	1.25 ± 0.00	**90.64 ± 28.03***	—	—
	Female	1.25 ± 0.00	51.95 ± 23.24	—	—

nominal n=5, n ≥ 2 birds/ treatment/ sex (= p ≤ 0.05, Dunnet's test)*

Table 2-2 Shot Blood Parameters

Packed cell volume (PCV) **%**		Saline Mean ± SEM	1 pellet Mean ± SEM	5 pellet Mean ± SEM	10 pellet Mean ± SEM
Week 1	Male	40.00 ± 0.00	38.40 ± 2.09	34.25 ± 1.84	**30.00 ± 2.65***
	Female	33.00 ± 4.81	37.60 ± 1.81	28.75 ± 4.71	31.00 ± 3.46
Week 2	Male	44.25 ± 0.25	44.25 ± 1.75	**33.67 ± 3.18***	**36.50 ± 2.50***
	Female	37.00 ± 2.00	39.80 ± 2.89	36.33 ± 4.70	—
Week 3	Male	48.00 ± 1.15	46.60 ± 0.87	—	—
	Female	43.25 ± 1.70	42.25 ± 2.78	—	—
Week 4	Male	46.00 ± 2.04	47.80 ± 0.73	—	—
	Female	38.75 ± 1.25	38.75 ± 2.84	—	—
Red blood cell count (RBC) 1x10^6 cells/ml		Saline Mean ± SEM	1 pellet Mean ± SEM	5 pellet Mean ± SEM	10 pellet Mean ± SEM
Week 1	Male	2.87 ± 0.06	3.05 ± 0.27	2.69 ± 0.18	2.50 ± 0.40
	Female	3.19 ± 0.56	3.05 ± 0.42	1.96 ± 0.48	1.97 ± 0.45
Week 2	Male	4.04 ± 0.28	4.07 ± 0.34	3.31 ± 0.43	—
	Female	3.59 ± 0.18	3.68 ± 0.31	3.17 ± 0.62	—
Week 3	Male	2.86 ± 0.20	3.27 ± 0.24	—	—
	Female	2.50 ± 0.25	2.68 ± 0.58	—	—
Week 4	Male	2.55 ± 0.13	2.62 ± 0.05	—	—
	Female	2.49 ± 0.20	2.19 ± 0.14	—	—
Mean Corpuscular Vol. (MVC) fL		Saline Mean ± SEM	1 pellet Mean ± SEM	5 pellet Mean ± SEM	10 pellet Mean ± SEM
Week 1	Male	13.98 ± 0.32	12.81 ± 0.83	12.90 ± 1.15	13.90 ± 1.65
	Female	12.16 ± 1.37	13.30 ± 1.78	15.86 ± 1.64	17.00 ± 2.87
Week 2	Male	11.10 ± 0.74	11.15 ± 1.18	10.25 ± 0.36	—
	Female	10.43 ± 0.90	10.90 ± 0.58	10.85 ± 0.26	—
Week 3	Male	16.88 ± 0.87	14.55 ± 1.09	—	—
	Female	17.69 ± 1.45	17.76 ± 3.47	—	—
Week 4	Male	18.11 ± 0.37	18.28 ± 0.24	—	—
	Female	15.79 ± 0.99	17.77 ± 1.02	—	—

Total Protein g/100ml		Saline Mean ± SEM	1 pellet Mean ± SEM	5 pellet Mean ± SEM	10 pellet Mean ± SEM
Week 1	Male	5.00 ± 0.34	4.20 ± 0.80	4.20 ± 0.46	4.13 ± 0.32
	Female	4.90 ± 0.25	4.84 ± 0.10	**3.88 ± 0.06***	**3.93 ± 0.18***
Week 2	Male	4.90 ± 0.38	5.45 ± 0.12	4.93 ± 0.12	—
	Female	5.38 ± 0.41	5.46 ± 0.08	4.50 ± 0.89	—
Week 3	Male	4.97 ± 0.54	4.92 ± 0.24	—	—
	Female	5.58 ± 0.28	5.85 ± 0.36	—	—
Week 4	Male	4.93 ± 0.33	5.28 ± 0.21	—	—
	Female	5.85 ± 0.35	6.10 ± 0.39	—	—

n ≥ 2 birds/ treatment/ sex (= p ≤ 0.05, Dunnet's test)*

Table 2-3 Shot Microscope Scores

Histopathology Microscopic lesion score		Saline Mean± SEM	1 pellet Mean± SEM	5 pellet Mean± SEM	10 pellet Mean± SEM
Koilin degeneration	Male	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.25	**1.40 ± 0.60***
	Female	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.33	0.20 ± 0.20
Hemosiderin	Male	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.25	**0.60 ± 0.24***
	Female	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.17	0.10 ± 0.10
Liver necrosis	Male	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.25	**0.60 ± 0.24***
	Female	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Spermatogenesis (%)	Male	95.00 ± 0.00	78.00 ± 8.31	**37.50 ± 7.22***	83.00 ± 4.90

nominal n=5, n ≥ 3 birds/ treatment/ sex (= p ≤ 0.05, Dunnet's test)*

Figures

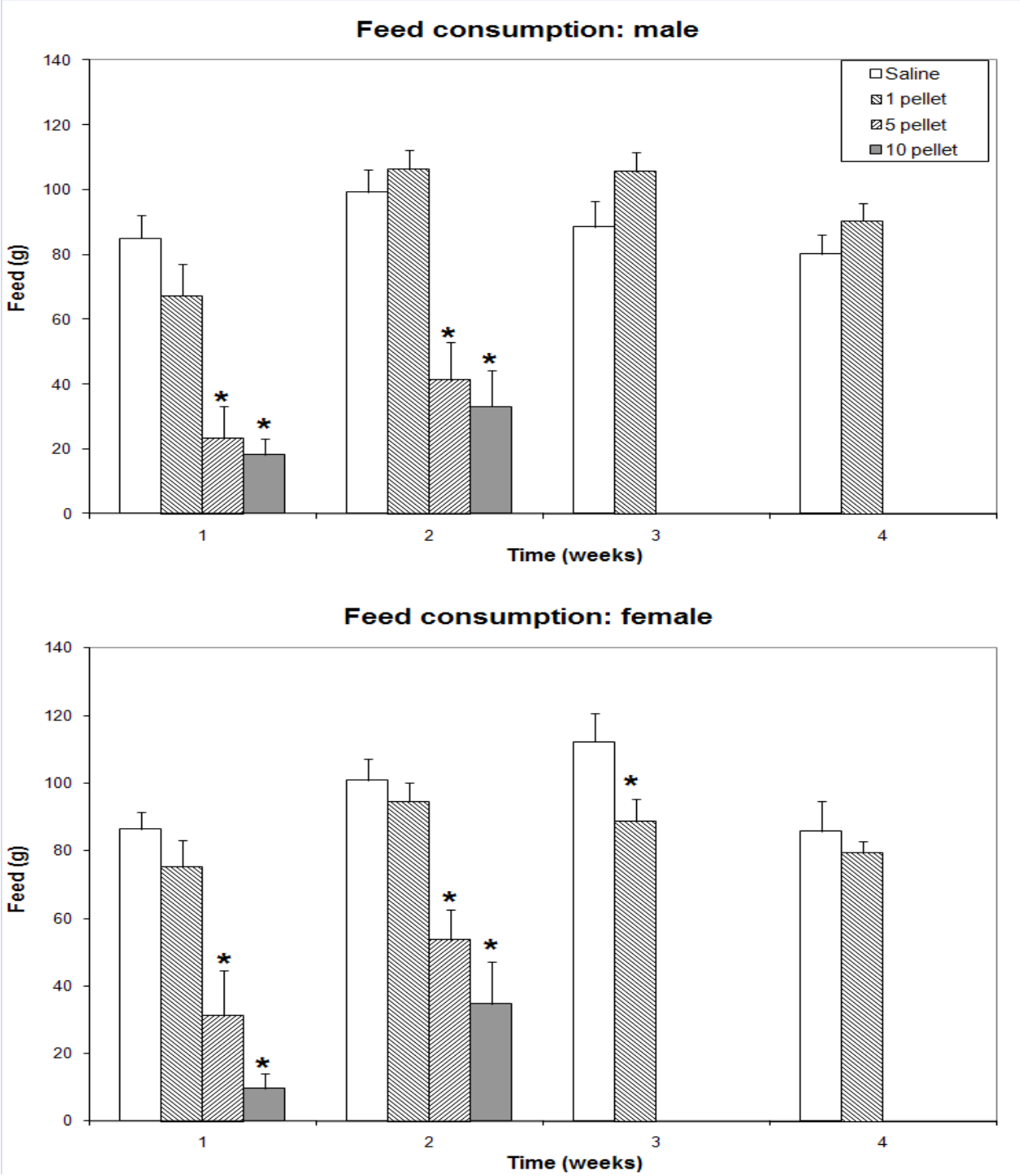


Figure 2-1: Feed consumption

Feed consumption was calculated weekly on a per bird basis with ad libitum access to feed and water. Male and female birds, gavaged 5 or 10 pellets, ate significantly less than controls or 1

pellet birds before humane euthanasia after week 2. Remaining 1 pellet and control birds were monitored for an additional 2 weeks. (*$p \leq 0.05$, Dunnett's test)

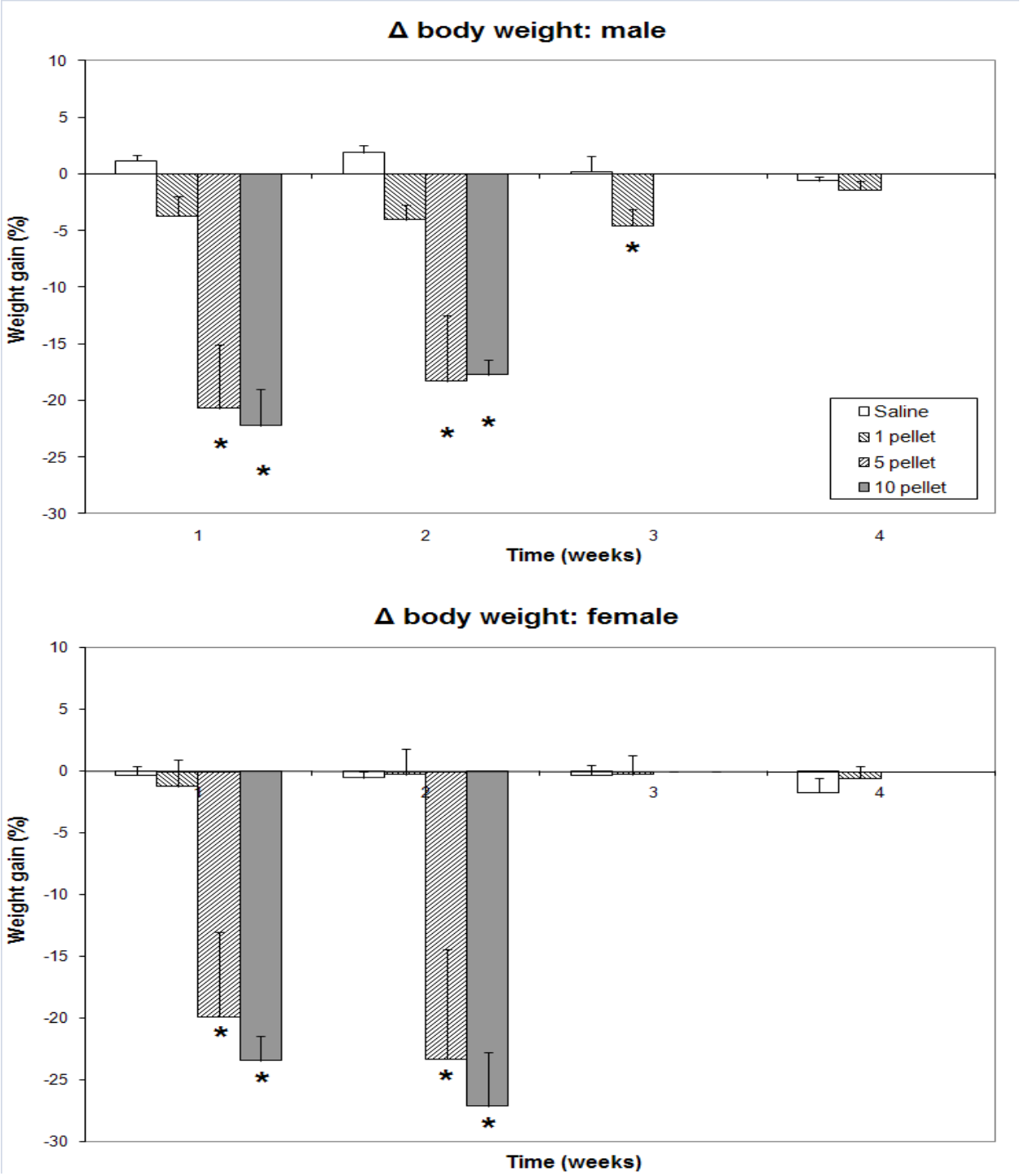


Figure 2-2: Body Weight

Body weight was recorded weekly for each bird. Though both male and female birds, gavaged with 5 or 10 pellets, lost significant weight, female birds began production during the study contributing to the greater weight loss seen in female birds. (*$p \leq 0.05$, Dunnett's test)

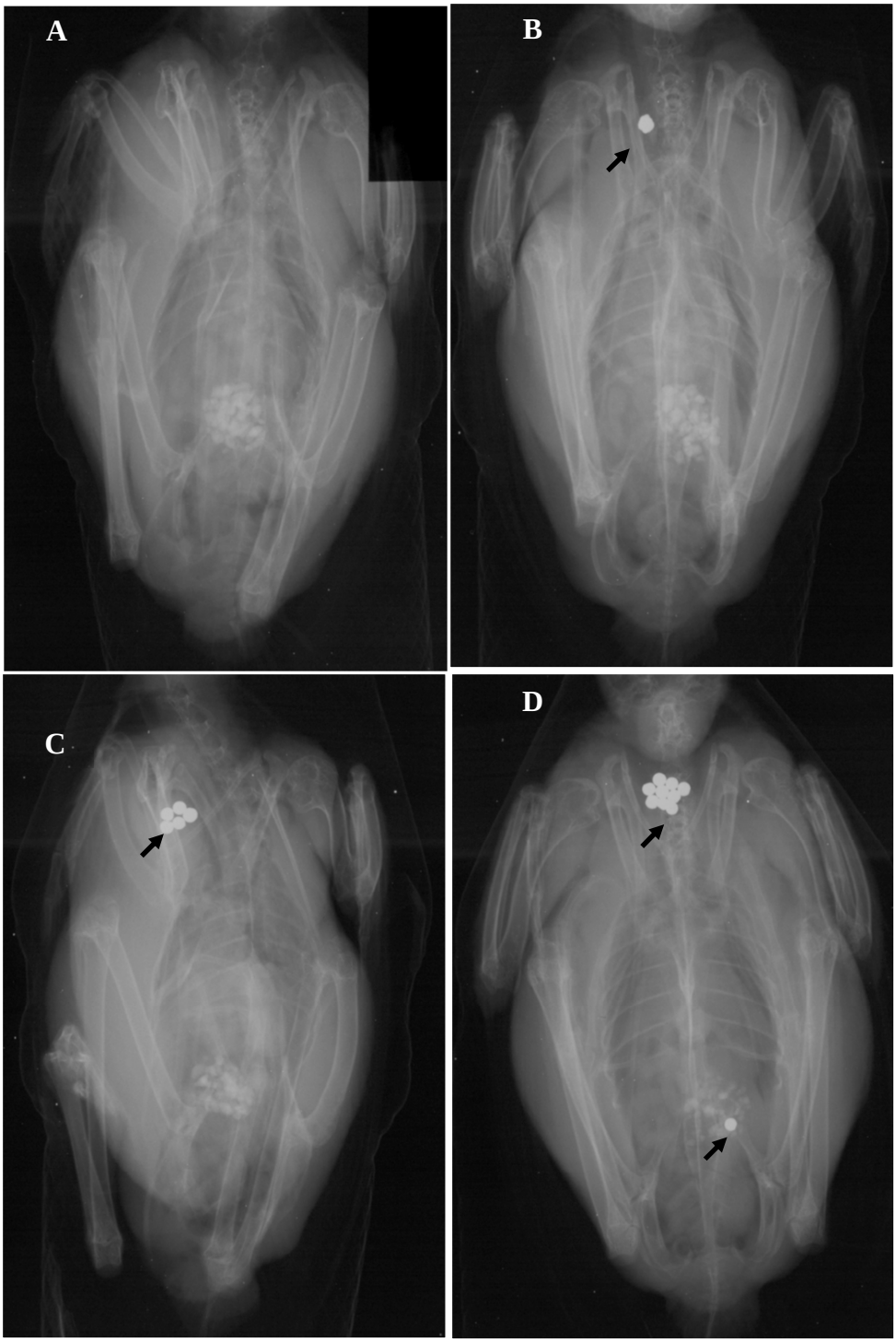


Figure 2-3: Day 1 Radiographs

Day 1 representative D-V radiographs of 16 week old quail dosed with 0, 1, 5, and 10 Pb pellets. Pb pellets completely block x-rays indicated by a densely white round shadow on the film.

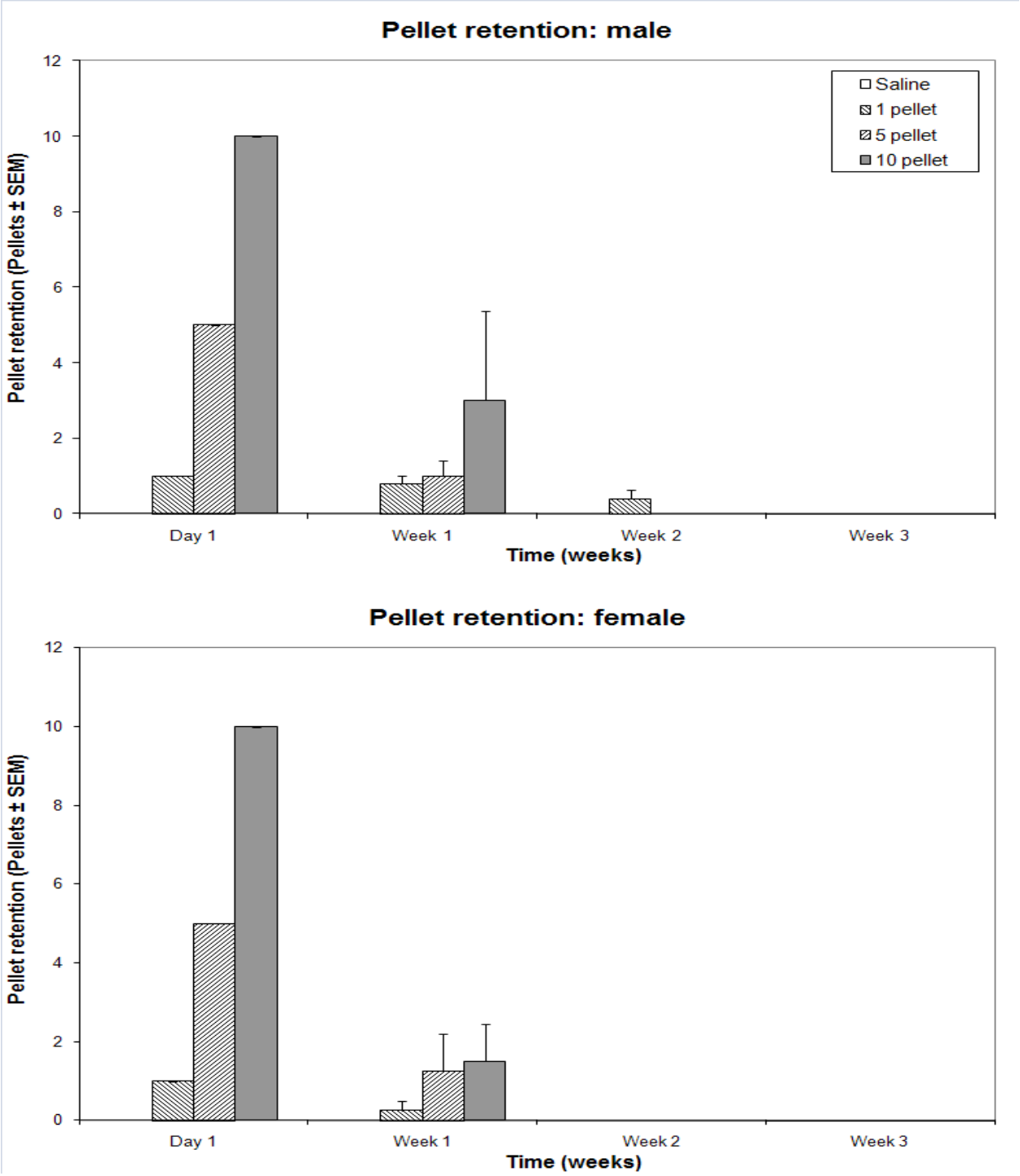
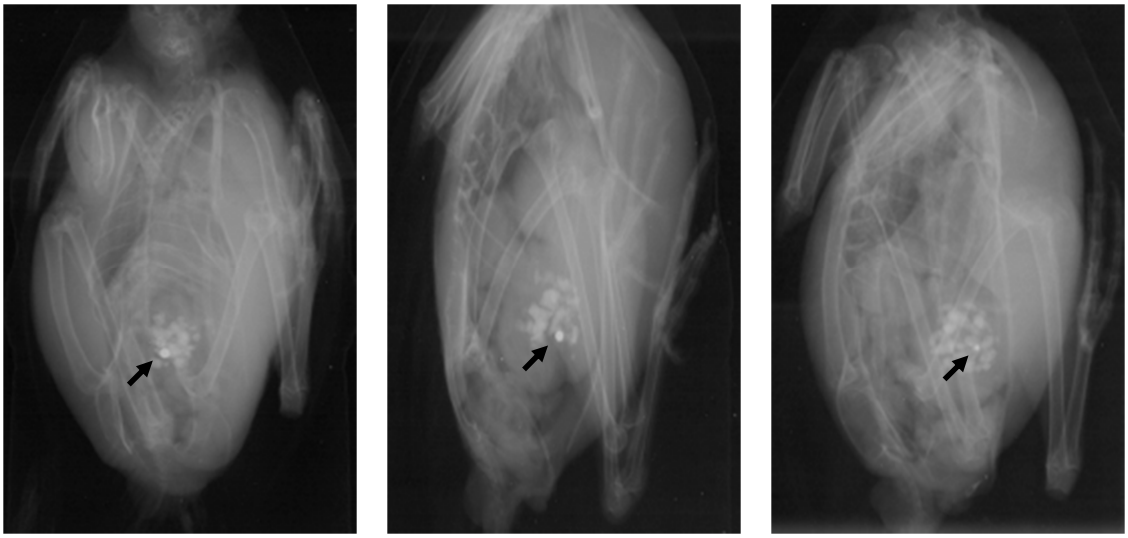


Figure 2-4: Pellet Retention

Retained pellet counts were recorded from radiographs in V-D and lateral poses. Most pellets were excreted or absorbed after 2 weeks while all pellets were eliminated by week 3.



Day 1 Day 7 Day 14

Figure 2-5: Representative Lateral Radiographs over 3 weeks

Representative lateral radiographs taken weekly of 16 week old adult quail dosed with one Pb pellet (indicated by arrows). Additional radio-dense material surrounding the pellet is grit present in the ventriculus.

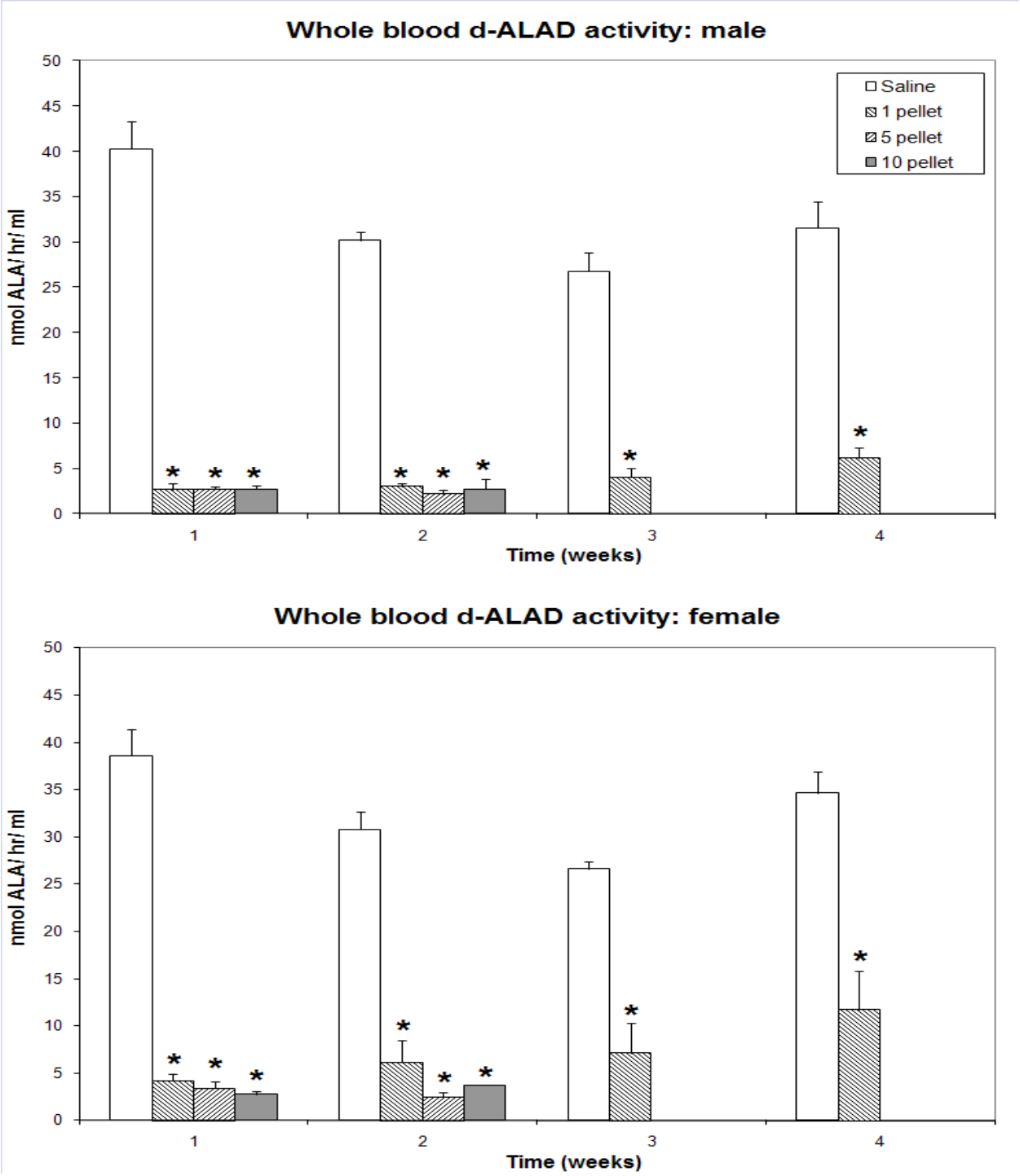


Figure 2-6: δ-ALAD Activity

Fifty microliter aliquots of whole blood were assayed for δ–ALAD activity. Control male and female birds ranged from 45 to 25 nmol ALA used per min/ ml blood while Pb treated birds (1, 5 and 10) ranged from less than 5 to 10. Interesting 1 pellet birds showed improvement after most shed their pellet in week 2. (*$p \leq 0.05$, Dunnett's test)

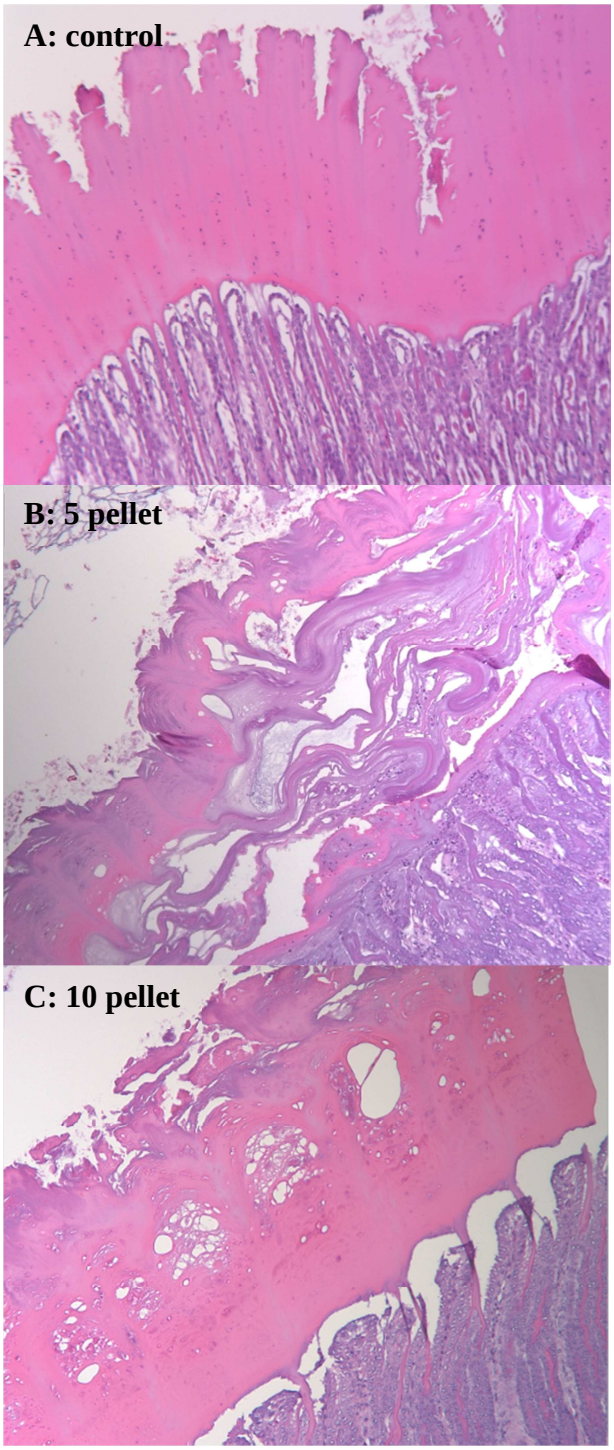


Figure 2-7: Ventricular Ulceration and Degeneration

Sections of ventricular tissue were fixed, stained with H&E and analyzed by a board certified avian pathologist. Five pellet (B), and 10 pellet (C) birds exhibited koilin degeneration but only the 10 pellet male group was statistically significant (100X H&E). Fig 2-7A is a representative control.

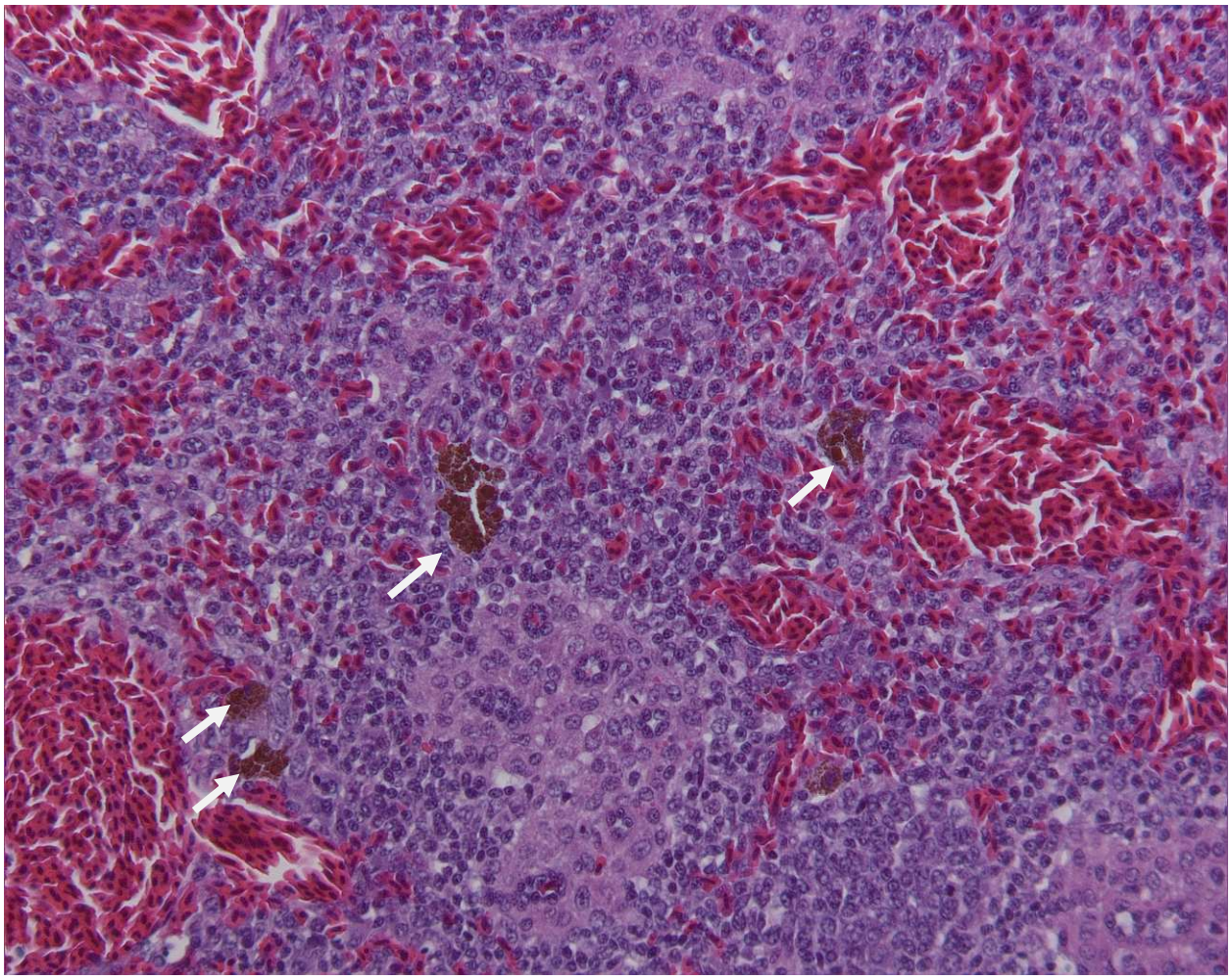


Figure 2-8: Splenic Multi-focal Hemosiderosis

Representative micrograph of splenic tissue with multifocal hemosiderin as indicated by arrows (200X, H&E).

CHAPTER 3. ORAL LEAD BULLET FRAGMENT EXPOSURE IN NORTHERN BOBWHITE QUAIL (*COLINUS VIRGINIANUS*)[5].

[5]Kerr, R., Holladay, S., Jarrett, T., Selcer, B., Meldrum, B., Williams, S., Tannenbaum, L., Holladay, J., and Gogal, R. Accepted by *Archives of Environmental Toxicology and Chemistry*. Reprinted here with permission of the publisher.

Abstract

Lead (Pb) is a worldwide environmental contaminant known to adversely affect multiple organ systems in both mammalian and avian species. In birds, the primary route of exposure is via oral ingestion of lead particles. Data are currently lacking in the retention and clearance of Pb bullet fragments in the bird's GI tract while linking toxicity with blood Pb levels. In the present study, Northern Bobwhite quail fed a seed-based diet were orally gavaged with Pb bullet fragments (0, 1 or 5 fragments/bird) and evaluated for rate of fragment clearance, and changes in peripheral blood, renal, immune and gastrointestinal parameters. Based on radiographs, the majority of the birds cleared or absorbed the fragments by 7 days with the exception of one 5-fragment bird, which took between 7-14 days. Blood Pb levels were higher in the males than females, which may be related to egg production in the females. In the males but not females, feed consumption, body weight gain, packed cell volume (PCV), plasma protein concentration, and δ-aminolevulinic acid dehydratase (δ-ALAD) activity were all adversely affected by 5 Pb fragments. Birds of both sexes that received a single Pb fragment displayed depressed δ–ALAD suggesting altered hematologic function while all birds dosed with 5 bullet fragments exhibited greater morbidity.

Introduction

Environmental Pb contamination is a recognized hazard to avian wildlife. In 1999, it was estimated that over three million metric tons of Pb shot and fragments were deposited annually on shooting ranges and hunting areas in the United States (Craig, et al., 1999). Oral ingestion is still the primary route of Pb exposure as birds mistake Pb pellets or fragments for either grit or food particles (McConnell, 1967; Bennett, et al., 2007; Duggan & Dhawan, 2007). Soil and field

studies of upland and lowland managed game preserves estimate availability at 6,342 Pb pellets/ha and an ingestion rate of 0.3% in birds collected after the hunting season (J. H. Schulz, et al., 2002). However in studies that controlled for predation, ingestion rates were calculated as high as 3.0% (Butler, et al., 2005). Further, predatory birds such as hawks, eagles, condors, and vultures may consume these Pb-exposed birds, accumulating Pb in their tissues and leading to increased mortality in these avian populations (Nam & Lee, 2009).

While Pb exposure affects many organ systems, hemotologic parameters appear to be particularly sensitive. In mammals, Pb exposure can lead to erythrolysis, inhibitition of δ-aminolevulinic acid dehydratase (δ-ALAD), and hypoproteinemia (Bandhu, et al., 2006; Casado, et al., 2007; Vanparys, et al., 2008). Red blood cells (RBCs) exposed to Pb *in vitro* show enhanced lipid peroxidation resulting in hemolysis and hemosiderosis in liver and kidney (Casado, et al., 2007; Al-Mansour, et al., 2009; Kerr, et al., 2010). δ-ALAD, a known blood Pb biomarker, is the second enzyme in the heme synthesis pathway and is extremely sensitive to low blood Pb concentrations (PbB). Erythrocytic sequestration of Pb is in part due to the higher binding affinity of Pb to a cysteine-rich zinc binding site on δ-ALAD (Payne, et al., 1999). Exposure to Pb causes a conformational shift in the enzyme disrupting porphobilinogen synthesis (Jarzęcki, 2007). This inhibition in heme synthesis and erythrolysis may contribute to lethargy brought on by anemia.

Northern Bobwhite quail (*Colinus virginianus*) is a representative avian wildlife species indigenous to areas where recreational hunting occurs. With a wide range spanning the mid-west, south, and Atlantic coastal United States, the Northern Bobwhite is also bred and released in hunting areas already contaminated with Pb (Rattner, et al., 2008; Buekers, Redeker, & Smolders, 2009). Other avian species such as rock and mourning doves have similar habitat

ranges and also had Pb detected in their blood and tissues (Kendall & Scanlon, 1982a, 1982b). In a study using Japanese quail *(Coturnix japonica)* a single oral exposure to a #4 Pb pellet (245 ± 13 mg Pb) resulted in readily detected Pb in blood, femur, liver and kidney after 6 weeks (Yamamoto et al., 1993). In contrast, Northern Bobwhite quail dosed with five round #9 Pb pellets (250 ± 25 mg Pb) showed several signs of acute toxicity, possibly due to larger surface area and increased absorption from multiple smaller pellets of about equivalent weight to a single #4 pellet (Kerr et al. 2010). Presently, there are no published studies tracking the effects from oral Pb bullet fragment exposure on fragment passage rate, blood Pb concentrations or other blood physiologic parameters. Irregular shaped fragments, like round pellets, are of concern because the bobwhite's seed based diet and grit requirement in the low pH ventriculus may contribute to the mechanical and chemical dissolution of ingested particulate lead. Intact Pb pellets are uniform in shape, size and weight whereas Pb fragments, due to their inherent lack of uniformity, have larger surface areas, and vary in size. These attributes may more easily facilitate fragment absorption compared to pellets. In the present single oral exposure Pb bullet fragment dose response study, we investigated fragment retention, dose-related effects on hematologic parameters, and tissue pathology in Northern Bobwhite quail over 2 weeks.

Methods and Materials

Animals.

Twenty-week old Northern bobwhite quail (n=17 male/n=16 feamle) were acquired from M&M Quail Farms (Goulds, FL) and maintained in individual quail cages fitted with an automatic watering system (Alternative Design, Siloam Springs, AR) at the Poultry Diagnostic and Research Center at the University of Georgia in Athens, GA. Birds were allowed 1 week acclimation with *ad libitum* access to a pelleted sporting bird conditioner (Southern States,

Richmond, VA) and 2 additional weeks with access to a seed based diet (Pennington Pride, white millet, milo, wheat, sunflower seed, calcium carbonate, vitamin A supplement, vitamin D3 supplement, potassium iodide, vegetable oil) with fortified grit (Purgarin, high calcium with phosphorus, magnesium, potassium and sulfur sources). During the trial, birds were housed on a 14/10 hr day/night cycle at 23 ± 5 °C. Body and feed weights were collected weekly and bird health assessed daily. During the acclimation period prior to the study, 14 of 16 female birds were in egg production. All procedures were approved by the University of Georgia's Institutional Animal Care and Use Committee and experiments were conducted in compliance with approved Good Laboratory Practice standard operating procedures.

Fragments.

Bullet fragments were provided by the U.S. Army Center for Health Promotion and Preventive Medicine from soil samples acquired from Fort Eustis, Virginia. Samples were washed in 70% Ethanol (Sigma, St. Louis, MO) to remove soil contaminants, and then washed 2x with deionized water, and allowed to air dry (**Figure 3-1**). Grit and fragments were then sorted by Pb content using a Alpha series XRF metal analyzer (Innov-X Systems, Woburn, MA). Fragments containing more than 175,000 ppm Pb were considered Pb fragments and remaining grit was discarded. Ten fragments were then selected at random and submitted to the Diagnostic Toxicology Laboratory at the Virginia Maryland Regional College of Veterinary Medicine at Virginia Tech, Blacksburg, VA for Pb content analysis.

Lead Exposure.

Birds ($n \geq 5$/treatment/sex) were randomly assigned to 0, 1 or 5 fragment treatment groups on day 0. Pb fragments were placed in 1 ml physiologic saline solution (Hospira, Lake Forest, IL) in a gavage tube fitted to a 3 ml dosing syringe.

Radiography.

Birds were radiographed 1, 7, and 14 days post gavage to determine the location and disposition of fragments according to the method of Kerr *et al*., 2010. Briefly, each bird was placed in a passive restraint device and arranged in a dorso-ventral (D-V) position on a radiograph cassette (2 birds/cassette). Mammory cassettes with HD S screens and HORC-100 NIF film were used (AGFA-Gevaert N.V., Mortsel, Belgium). Radiographs were performed with a Transworld 625V X-ray machine (Transworld X-ray Corp, Charlotte, NC). Table-top technique was employed using a forty-inch focal-film distance. Exposure factors were 600 mA, 1/40 second, and 56 kVp for all radiographs.

Fragment Pb content analysis.

Pb fragments were analyzed according to the method of Meldrum et al., 2003. Briefly, fragments were digested in a 5:1 solution of concentrated nitric acid and 70% perchloric acid, and then heated in a graphite furnace at 150 °C for 2 hr. Atomic absorption spectrophotometry was used to determine Pb concentrations, and results were expressed as % Pb. Recovery for standard samples with known Pb levels was greater than 90%.

Blood collection.

On day 0, 7, and 14, 1 ml whole blood was collected aseptically from the jugular vein of each bird with a 27-gauge needle and 1 ml syringe (Becton-Dickinson (BD), Franklin Lakes, NJ) and transferred to a depressurized 4 ml heparinized vacutainer (BD). Samples were then mixed by gently inversion and transferred to the immunotoxicology laboratory at the UGA College of Veterinary Medicine for further processing.

Blood Pb level analysis.

Blood samples were immediately frozen at -80 °C prior to submission to the Diagnostic Toxicology Laboratory at the Virginia Maryland Regional College of Veterinary Medicine at Virginia Tech for routine determination of blood Pb concentration (PbB). One hundred µl of blood were mixed with 900 µl of modifier solution (Triton-X, ammonium phosphate monobasic, nitric acid (Sigma)) and the absorbance measured on an atomic absorption spectrometer (Varian, Spectra AA Model 220FS, Walnut Creek, CA) by graphite tube atomization with the limits of detection at 25 ppb. Results were expressed as µg/dl.

Red blood cell enumeration.

For each sample, 1 µl heparinized whole blood was added to 1 ml physiologic saline and vortexed for 15 seconds. A 20 µl aliquot was added to a Nexcelom counting chamber and enumerated on an Auto T4 cell counter (Nexcelom Corp., Lawrence, MA). Results were expressed as billions of cells per ml ($1x10^9$ cells /ml).

Packed cell volume and total protein.

A hematocrit capillary tube (Drummond Scientific, Broomall, PA) was filled to approximately ¾ volume, sealed at the bottom and centrifuged in a hematocrit spinner (LW Scientific, Lawrenceville, GA) for 5 min at 14,800 x g. The packed cell volume (PCV) was measured using a micro-capillary reader (International Equipment Company, Needham, MA) and expressed as percent cell volume. The capillary tube was then cut above the RBC layer and approximately 30 µl plasma were placed onto a refractometer (Reichert, Depew, NY) to determine total protein, expressed as g/dl.

δ-ALAD assay.

Delta aminolevulinic-acid dehydratase (δ-ALAD) activity was determined using an adaptation of the European standardized method (Berlin & Schaller, 1974). Briefly, 50 µl heparinized whole blood from each bird were added to a separate 5.0 ml centrifuge tube (Fisher Scientific, Suwanee, GA) containing 1.45 ml molecular grade water (Sigma, St. Louis, MO) and vortexed for 5 sec. To each tube, 1 ml of 10 mM ALA solution (Sigma) was added, vortexed for 5 sec, and allowed to incubate for 60 min in the dark at 38 °C. One milliliter of trichloroacetic acid (TCA) stop solution (Sigma) was then added to each tube to stop the reaction and vortexed for 5 sec. Tubes were then centrifuged for 10 min at 200 x g in a 5810R centrifuge (Eppendorf, Westbury, NY). One hundred µl of the resulting supernatants were then added in triplicate to clear, round bottom 96 well plates (Corning) and read in a Synergy 4 plate reader (Biotek, Winooski, VT) at 555 nm against a reagent blank. Results were expressed as nanomol of ALA used per min per ml RBCs.

Histopathology.

Upon the conclusion of the study, birds were euthanized by CO_2 gas inhalation in accordance with AAALAC guidelines. Samples of lung, heart, ventriculus, liver, and kidney were fixed in 10% buffered formalin and trimmed for routine processing. Samples were embedded in paraffin wax and sectioned at 4-5 microns. Slides were then stained with routine hemotoxilyn and eosin. Additional slides of lung were prepared as above and stained with Perl's iron. Resulting slides were then examined via light microscopy by an avian pathologist and scored as showing 0 (nil) to 5 (severe) microscopic lesion.

Statistics.

Data were compiled in Microsoft Excel (Microsoft Inc. Redmond, WA). Statistical analysis was performed using JMP 8.0 software (SAS Institute, Cary, NC) and expressed as mean ± standard error of the mean (SEM). Feed consumption, delta body weight, PbB, PCV, red blood cell count, total serum protein and δ-ALAD activity were compared among sex and treatment across time by two-way repeated-measures analysis of variance (two-way RM ANOVA) with interaction. Means for each group were then compared using post hoc Tukeys HSD testing. Microscopic scores from H&E and Perl's iron stained tissue samples were evaluated using one-way ANOVA analysis followed by post hoc Dunnett's testing to compare groups to control. Least squares regression (LSR) was used to qualify relationships between PbB, feed consumption, bodyweight, and blood parameters. Data were considered significant with a double-sided $p \leq 0.05$.

Results

Fragment retention, radiography, and mortality.

Radiographic analysis confirmed the location and number of fragments in birds gavaged with Pb (Figure 3-2). No metal particles were observed in the GI tracts of male or female birds gavaged with 0 fragments during the study period. Male and female birds gavaged 1 fragment had cleared the fragment by day 7. Both sex ($p=0.02$, two-way RM ANOVA) and the number of fragments gavaged ($p<0.0001$, two-way RM ANOVA) had an significant effect on fragment retention with male birds gavaged with 5 fragments retaining more fragments on average across the study period ($p=0.034$, Tukey's HSD). Two of 6 female birds gavaged with 5 fragments died by day 7. The remaining 5 fragment female birds cleared their fragments by day 7. Two male birds gavaged with 5 fragments retained ≥ 3 fragments at day 7, and died prior to day 14. The

remaining three male birds retained an average of 2.7 ± 0.9 fragments at day 7. The fragments retained beyond day 7 by the surviving male 5 fragment birds were cleared by day 14.

Feed consumption.

Baseline feed consumption rates were similar for each treatment group prior to lead exposure, and 0 fragment gavaged birds did not differ throughout the trial (**Table 3-1**). Birds gavaged with one fragment were not different from baseline or birds gavaged with 0 fragments. Female birds gavaged 5 fragments ate similarly to baseline across the study. Sex and treatment affected feed consumption over time (*p=0.048*, two-way RM ANOVA) with the 5 fragment male birds eating significantly less than 1 fragment and control male groups (*p=0.001, p=0.004*, Tukeys HSD) at day 7. The surviving 5 fragment male group had a higher feed intake by day 14.

Body weight.

Female 0 fragment birds lost 6.4 % body weight by day 7 recovering to 4.1 % below baseline by day 14. Male 0 fragment birds gained weight at day 7 and 14. Body weights in male and female birds gavaged with 1 fragment were not different from 0 fragment birds at all time points during the trial. Male birds gavaged with 5 fragments weighed 17.8 % less than baseline at day 7, recovering to 14.7 % at day 14 (**Table 3-2**). Body weight of the male birds was adversely affected by Pb exposure in a dose dependant manner reaching significance in the 5 fragment male group at both day 7 and 14 (*p=0.0008, p=0.029*, Tukeys HSD).

Blood Pb level.

The PbB of all birds prior to gavage and all birds gavaged with 0 fragments at all time points was consistently lower than detection (less than 2.5 µg/dl). Female birds orally gavaged with Pb fragments had considerably lower PbBs than male birds, and within sex, PbB was dose dependant (*p<0.0001*, two-way RM ANOVA). The 1 fragment female bird PbB levels were

numerically elevated but not significant on day 7 and 14. The 5 fragment female group PbB levels were significantly increased on day 7 and 14 compared to controls although the PbB levels did decrease between day 7 and 14. PbBs of male 1 and 5 fragment birds were increased reaching significance in the 5 fragment group on day 7 and like the females declined by day 14 (**Table 3-3**).

δ-ALAD activity.

Baseline ALAD activity of all groups did not differ significantly prior to treatment. By day 7, mean ALAD activity of female birds gavaged with 1 fragment was numerically depressed by 29% compared to 0 fragment birds however this result was not significant ($p = 0.161$, Tukeys HSD). Female birds gavaged 5 fragments were depressed 92% at day 7 ($p = 0.003$, Tukeys HSD). At day 14, both female treatment groups' mean ALAD activitys were significantly inhibited. Mean ALAD activity of male birds gavaged with 1 or 5 fragment was depressed at day 7 by 72% and 94%, respectively ($p \leq 0.001$, Tukeys HSD). Both male treatment groups still significantly decreased at day 14 compared to 0 fragment birds (**Figure 3-3**).

Blood parameters.

The female 1 and 5 Pb fragment groups had PCV values, RBC counts and serum protein levels that were not significantly different from the 0 fragment females. The PCV, RBC count, and serum protein of male birds gavaged with 1 Pb fragment were comparable to 0 fragment males and baseline values, at day 7 and day 14. Male birds gavaged with 5 Pb fragments had decreased PCV and RBC counts at day 7 and 14 (Table 4). Total protein levels for these birds were depressed at day 7 compared to 0 fragment birds but began to increase by day 14 (**Table 3-4**). Mean corpuscular volume (MCV) did not differ across treatment, sex or time point during the trial (data not shown).

Histopathology.

In general, analysis of the major organs revealed no significant findings. There was some evidence of hepatic glycogen deposition and lipidosis in all the treatment groups, which was attributed to the seed diet. All the birds had some level of pulmonary hemosiderosis. A non-significant increasing trend in proximal tubular degeneration in the female kidneys was also observed. Moderate multifocal ventricular koilin degeneration was only noted in 2 male birds in the 5 fragment group and was absent in all the other treatment groups.

Discussion

Several studies have demonstrated that birds ingest Pb mistaking it as either food or grit (Kendall & Scanlon, 1982a; Kendall, et al., 1996; J. H. Schulz, et al., 2002; Fisher, Pain, & Thomas, 2006). The majority of these avian trials have focused on Pb shot (round pellets) in birds fed protein-supplemented granular diets (McConnell, 1967; Yamamoto, et al., 1993; John H. Schulz, Gao, Millspaugh, & Bermudez, 2009). While informative, these studies do not replicate the field conditions birds experience when seeds form their primary diet. Furthermore, birds are also likely to be exposed to irregular-shaped Pb fragments in addition to round shot pellets. Schultz et al. (2002) also suggested that field ingestion rates for Pb are likely understated and thus published data on bird morbidity may underestimate reality (J. H. Schulz, et al., 2002). These collective observations point to gaps in the literature regarding the effects of Pb bullet fragments in birds fed a seed-based diet.

In the present study, bobwhite quail gavaged with either 1 Pb fragment or vehicle saline (controls) experienced no mortality, while 30% of female and 40% of male birds gavaged with 5 fragments died or were euthanized due to poor health. These results differ from Japanese quail exposed to a single #4 round Pb shot pellet (245 ± 13 mg/pellet), which showed no bird death as

late as 6 weeks. The type of diet (seed vs. granular) fed to the Japanese quail was not defined and may have influenced the Pb retention and absorption (Yamamoto, et al., 1993; Kerr, et al., 2010). These results also differ from our recent Pb pellet study in which bobwhite quail were exposed to 0, 1, 5 or 10 Pb #9 round pellets (46 ± 3 mg/pellet) (Kerr et al. 2010). Of these, the 5 and 10 pellet birds all died or were euthanized within 14 days. The 5 pellet birds received about the same total weight of Pb (230 ± 15 mg) as the Japanese quail, however surface area for Pb absorption was larger which may have resulted in the observed increased toxicity.

Although the Pb fragments used in the present study were similar in size to the previous #9 Pb round pellets, the fragment density and thus concentration of Pb was approximately half that of a #9 pellet (24 ± 1.9 mg/fragment). Unlike the male birds gavaged with 5 round pellets, the present 5 fragment-dosed male birds began to recover from adverse signs by day 14, denoted by increased feed consumption and weight gain.

Pb fragment retention rates, as measured by radiography, were similar across all treatments in the female birds and in the 1 fragment male birds. The 5 fragment male birds had a longer Pb retention time that correlated with higher PbB levels. As expected, the female PbB levels were lower than the males and varied based on production status. Non-production female birds that were gavaged with 5 fragments had more than twice the PbB levels than the 5 fragment female birds in egg production (data not shown). This would suggest that Pb was being eliminated from the body into the eggs. This agrees with several studies in other bird species that have reported Pb deposition in eggshell (Pattee, 1984; Mora, 2003). Pb fragment retention rates also appeared to be shorter compared to round Pb pellet retention rates. This may relate in part to the unique characteristics of these fragments including a lower weight relative to size and increased irregular surface area.

The present 5 fragment males showed decreased blood PCV, RBC count, and δ-ALAD activity. Although this pattern was similar to our previous Pb pellet study that used round shot, the degree of response was different. The suppressed δ-ALAD activity and other toxicities were less dramatic with the fragments, which was likely due to the lower Pb concentration per fragment and the more rapid transit time of these fragments. With either round shot or Pb fragments, Pb exposure would predictably cause membrane peroxidation in mammalian RBCs supporting a hemolytic event (Casado, et al., 2007). The reduced PCV and δ -ALAD blood parameters were indicative of anemia in these birds and may have contributed to the higher mortality in these treatment groups.

The effects of Pb exposure on two important clinical parameters, bird body weight gain and plasma protein levels, appear to be influenced partly by the bird's specific diet (Yamamoto, et al., 1993; Fair & Ricklefs, 2002; Kerr, et al., 2010). In studies where bird weight loss was not observed, the birds were largely on a balanced protein-rich granular diet. However in the present study, the diet was of a commercial wild bird feed type consisting primarily of seeds and thus required grit. With this diet, we observed a number of noteworthy findings. On day 7, PbB levels had a significant inverse correlation with feed consumption (r= -0.68, p=0.0004, LSR), body weight gain (r=-.80, p<0.0001, LSR), and a weak inverse correlation with plasma protein (r= -.474, p=0.019, LSR). Additionally, there was a moderate but significant correlation between feed consumption and percent body weight gain (r= 0.6151, p=0.0008, LSR) suggesting that decreased caloric intake was at least in part linked to the decreased weight gain. Further, there was a moderate, but significant, inverse correlation between PbB levels and δ-ALAD activity (r= -.520, p=0.009, LSR). This was expected since Pb has been shown to inactivate δ-ALAD by preferentially binding with high affinity to the cystine rich metal binding sites, displacing Zn,

and causing a conformational shift in the enzyme's active site (Jaffe et al. 2001). Sequence identity analysis and 3D protein modeling have shown that each of these cystine molecules associate with a nearby lysine residue. Further, these lysine residues appear to play an important role in defining the structure of the metal binding site (Jaffe, Martins, Li, Kervinen, & Dunbrack, 2001). For example in one study, a δ-ALAD gene mutation at residue 59 resulted in asparginine being substituted for lysine which decreased RBC Pb sequestration and increased plasma Pb concentration (Montenegro, Barbosa, Sandrim, Gerlach, & Tanus-Santos, 2006). Of note, Leeming and Donaldson (1984) demonstrated that lysine supplementation ameliorates some of the weight loss in Pb exposed broiler chickens suggesting an important role of lysine in weight gain. This is consistent with the findings by Carew et al. (2005) who observed that deficiency in lysine reduces feed consumption and body weight. Therefore, while the loss in body weight in the Pb shot or fragment exposed birds can, in part, be attributed to insufficient caloric intake, it is likely that inadequate amino acid or protein availability due to Pb exposure may also be a contributing factor.

To summarize, data for Pb fragment retention time and toxicity are currently lacking in the literature, as compared to round Pb pellets. This is true even though Pb fragments from spent bullets make a significant contribution to environmental Pb contamination. We hypothesized that retention time of the irregular Pb fragments, which may more closely mimic typical irregular grit particles, may be greater than round shot of comparable size. However this proved to not be the case. The observed more rapid transit of Pb fragments through the birds would be expected to reduce toxicity due to reduced time for Pb mobilization and absorption. Alternately, the larger surface area of the fragments would be expected to increase Pb absorption. The present fragments were of similar size as the #9 Pb shot we previously studied (Kerr et al., 2010),

however were of about 50% the Pb content due to their origin from larger composite bullets. It remains unknown if irregular shaped Pb fragments of equal weight to round Pb shot would show greater toxicity due to increased absorption, lower toxicity due to more rapid transit time through wild bird species, or approximately equal toxicity due to a balance between these factors.

Tables

Table 3-1: Fragment Feed Consumption

		0 fragment			1 fragment			5 fragment		
(g)		Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
Baseline	Male	99.5	±	10.4	86.1	±	8.5	85.0	±	15.9
	Female	76.5	±	3.3	69.2	±	10.3	63.8	±	16.6
Day 7	Male	94.7	±	4.0	80.5	±	7.5	**35.8**	**±**	**16.9***
	Female	76.4	±	6.9	69.4	±	10.1	94.1	±	15.4
Day 14	Male	91.2	±	4.4	86.1	±	5.2	76.7	±	3.5
	Female	67.9	±	2.6	59.2	±	7.7	76.0	±	16.5

n = 5 birds/ treatment/ sex (= $p \leq 0.05$, Tukey's HSD)*

Table 3-2: Fragment Body Weight Change

		0 fragment			1 fragment			5 fragment		
(g)		Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
Day 7	Male	1.3	±	1.4	-5.2	±	2.2	**-32.2**	**±**	**8.6***
	Female	-11.4	±	1.6	-4.3	±	2.8	-21.8	±	6.5
Day 14	Male	3.1	±	1.3	-1.4	±	3.3	**-27.3**	**±**	**17.6***
	Female	-7.1	±	2.6	-4.5	±	2.6	-20.7	±	8.0

n = 5 birds/ treatment/ sex (= $p \leq 0.05$, Tukey's HSD)*

Table 3-3: Fragment Blood Pb Level (PbB)

		0 fragment			1 fragment			5 fragment		
(µg/dl)		Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
Baseline	Male	1	±	0	1	±	0	1	±	0
	Female	1	±	0	1	±	0	1	±	0
Day 7	Male	1	±	0	695	±	506	**3202**	**±**	**730***
	Female	1	±	0	12	±	11	**220**	**±**	**54***
Day 14	Male	1	±	0	88	±	41	898	±	784
	Female	1	±	0	21	±	14	**126**	**±**	**47***

n = 5 birds/ treatment/ sex (= $p \leq 0.05$, Tukey's HSD)*

Table 3-4: Fragment Blood Parameters

		0 fragment			1 fragment			5 fragment		
Packed Cell Volume		Mean	±	SEM	Mean	±	SEM	Mean	±	SEM
Baseline	Male	47.6	±	1.1	45.4	±	2.7	48.0	±	0.8
%	Female	34.6	±	1.1	35.2	±	2.9	38.2	±	0.6
Day 7	Male	47.0	±	0.7	45.8	±	1.2	**37.8**	**±**	**2.8***
	Female	32.0	±	2.4	34.8	±	2.5	37.3	±	2.6
Day 14	Male	47.8	±	0.7	46.6	±	1.0	**41.0**	**±**	**3.2***
	Female	34.0	±	0.7	34.4	±	1.6	36.0	±	1.5
RBC Count										
Baseline	Male	2.90	±	0.08	2.66	±	0.15	2.76	±	0.12
10^6 cells/µl	Female	2.01	±	0.15	2.19	±	0.06	2.41	±	0.18
Day 7	Male	2.59	±	0.07	2.63	±	0.17	**2.15**	**±**	**0.14***
	Female	2.14	±	0.12	2.00	±	0.19	2.25	±	0.06
Day 14	Male	2.91	±	0.09	2.90	±	0.12	**2.36**	**±**	**0.12***
	Female	2.27	±	0.05	2.22	±	0.08	2.40	±	0.17
Protein										
Baseline	Male	5.0	±	0.1	4.6	±	0.2	4.6	±	0.3
g/dL	Female	5.0	±	0.3	5.4	±	1.1	5.2	±	0.9
Day 7	Male	4.7	±	0.1	4.5	±	0.1	**3.5**	**±**	**0.2***
	Female	6.5	±	0.8	5.1	±	0.5	4.5	±	0.6
Day 14	Male	4.3	±	0.1	4.4	±	0.2	3.9	±	0.3
	Female	5.8	±	0.4	4.7	±	0.4	5.5	±	0.9

n = 5 birds/ treatment/ sex (= $p \leq 0.05$, Tukey's HSD)*

Table 3-5: Fragment Microscope Scores

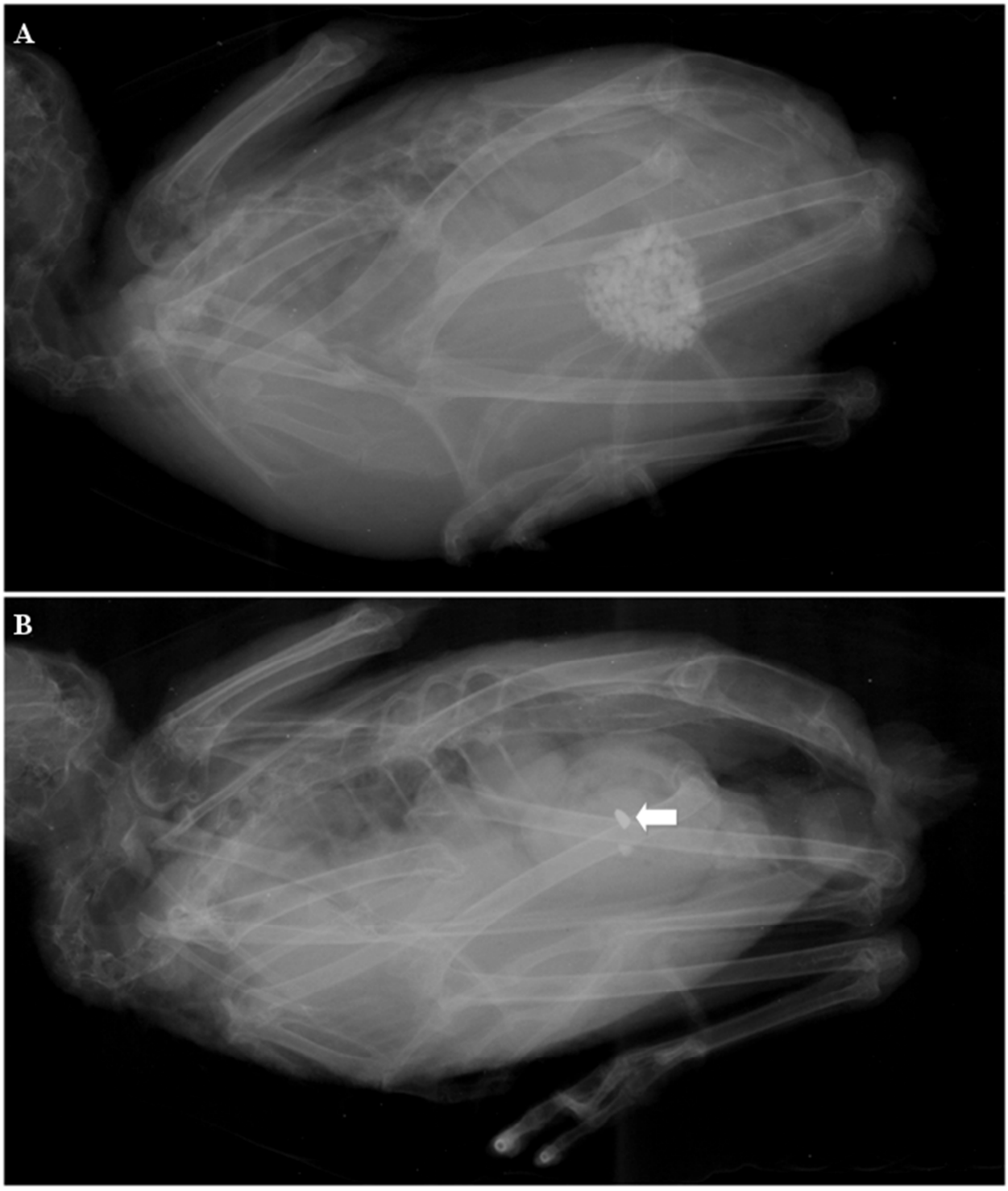
(Score)		0 fragment Mean ± SEM	1 fragment Mean ± SEM	5 fragment Mean ± SEM
Pulmonary hemosiderosis	Male	1.33 ± 0.33	1.57 ± 0.20	2.33 ± 1.33
	Female	3.00 ± 0.00	2.00 ± 0.45	2.00 ± 0.45
Hepatic glycogen deposition	Male	2.20 ± 0.92	2.50 ± 0.89	2.75 ± 0.95
	Female	0.20 ± 0.20	2.60 ± 1.12	3.00 ± 0.00
Hepatic lipidosis	Male	0.40 ± 0.24	0.17 ± 0.17	0.25 ± 0.25
	Female	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00
Proximal Tubular degeneration	Male	1.20 ± 0.73	2.57 ± 0.69	0.60 ± 0.60
	Female	0.50 ± 0.50	1.20 ± 0.73	2.20 ± 0.97
Ventricular koilin degeneration	Male	0.00 ± 0.00	0.00 ± 0.00	0.75 ± 0.75
	Female	0.00 ± 0.00	0.00 ± 0.00	**2.33 ± 1.20***

n = 5 birds/ treatment/ sex (= $p \leq 0.05$, Dunnet's test)*

Figures

Figure 3-1: Grit Particles

Grit particles including Pb containing fragments were recovered from a shooting range at Ft. Eustis, Virginia (A). Particles were washed in 70% Ethanol, and then 2x in DI. Samples were then sorted by Pb content using a Alpha series XRF metal analyzer. Fragments containing more than 175,000 ppm Pb were classified as Pb fragments (B) and the remaining grit discarded.


A
B

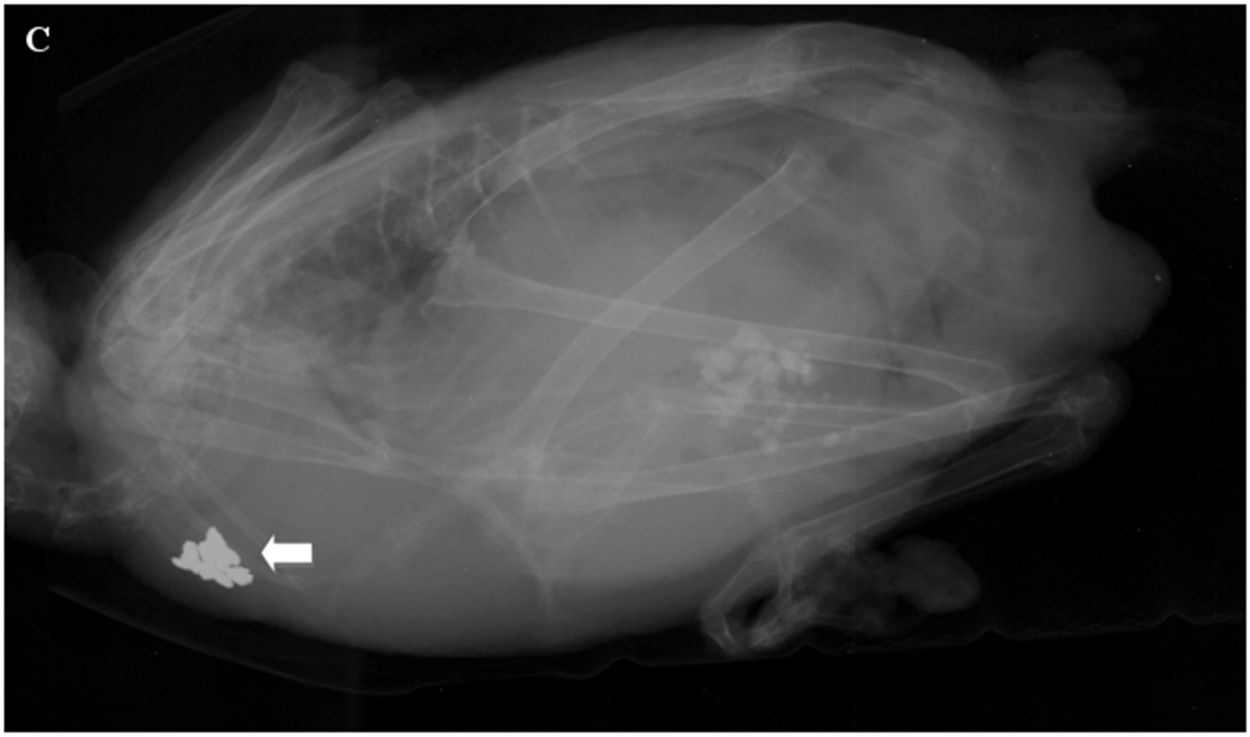


Figure 3-2: Fragment Radiographs

Radiographs from Day 1. The location and disposition of Pb Fragments was determined radiographically on day 1, 7, and 14. Birds were gavaged with 0 (A), 1 (B) and 5 (C) fragments. Pb fragments are indicated with white arrows.

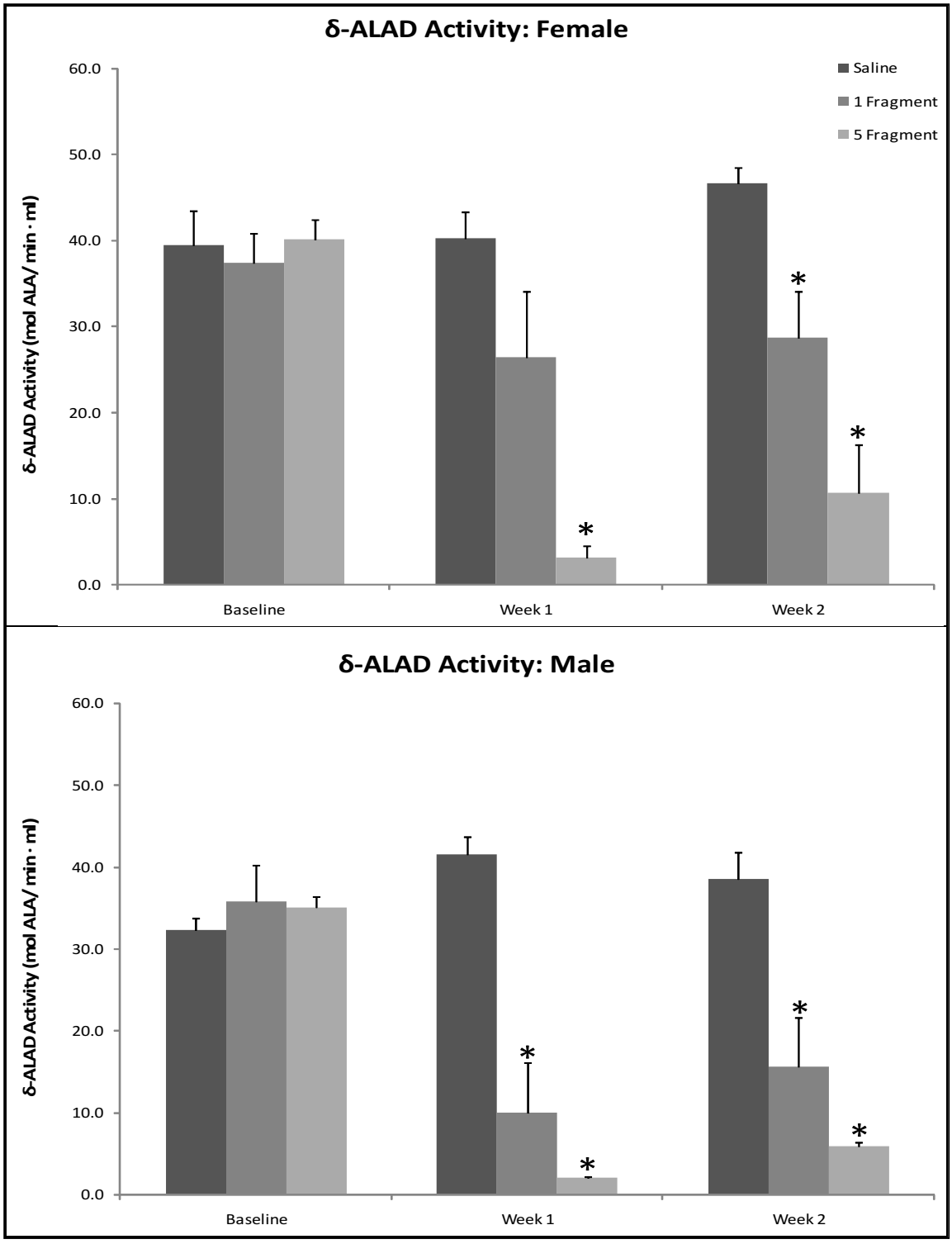


Figure 3-3: Fragment δ-ALAD Activity

Bird blood was assayed on day 0, 7, and 14 for δ-ALAD activity. Despite clearance of pellets, δ-ALAD remained depressed at Day 14.

CHAPTER 4. LEAD (Pb) INDUCES ABERRANT MAJOR HISTOCAMPATABILITY COMPLEX CLASS II (MHC-II) SURFACE EXPRESSION ON RAW 267.4 CELLS VIA DYSREGULATION OF MHC-II+ COMPARTMENT (MIIC) EXOCYTOSIS[6].

[6] Kerr, R., Krunkosky, T., Hurley, D., Cummings, B., and Gogal, R. To be submitted to *In Vitro Toxicology*.

Abstract

Aberrant major histocompatibility complex class II (MHC-II) surface expression on antigen presenting cells (APCs) is associated with dysregulated immune homeostasis. Environmental lead (Pb) is known to increase MHC-II surface expression on murine peritoneal macrophages *ex vivo* at concentrations exceeding 25 µM, however, there is little data examining the effect of immunopathologically relevant concentrations (2.5-5.0 µM) of Pb on MHC-II expression and no data on the possible mechanism involved. To address this deficit, we examined the effects of Pb on surface expression of MHC-II, and secondary T-cell activation markers (CD80, CD86, CD40) in the RAW 267.4 macrophage cell line. In addition, changes in cell viability, cellular metabolic activity, and β-hexosaminidase activity, a measure of lysosomal trafficking in cells cultured with and without Pb were evaluated. Electron and confocal microscopy were also performed to assess whether cell ultrastructure was affected. Pb induced a bi-phasic, dose dependent increase in surface MHC-II, CD86, LAMP-1, and LAMP-2 surface expression in exposed cells during one doubling cycle (17 hr). Although cell viability was unaffected by Pb, cellular metabolic activity was inhibited. β-hexosaminidase activity exhibited a bi-phasic response similar to the changes in surface expression of MHC-II. Electron microscopy revealed evidence of lipid vacuolization, macroautophagy and myelin figure formation in RAW 267.4 cells cultured with either Pb or LPS, a positive control for autophagy. Confocal microscopy with antibodies against LC3B showed a punctate pattern consistent with the presence of mature autophagosomes. Collectively, these data show that 2.5-5.0 µM Pb increased surface expression of MHC-II by inhibiting metabolic activity, inducing autophagy, and increasing MHC-II trafficking in RAW 267.4 cells, *in vitro.*

Introduction

Pb is a widely dispersed heavy metal contaminant. In an EPA study from the late 1990s, it was estimated that over 3 million metric tons of inorganic Pb had been dispersed across shooting ranges and hunting areas throughout the United States in the form of spent pellets and bullet fragments. Further, the EPA estimated that recreational and military shooting activity added an additional 80,000 tons each year (Craig, et al., 1999). Pb pellet densities in the top 3 inches of soil of up to 1.5 billion per acre have also been described at skeet shooting ranges (Stansley, Widjeskog, & Roscoe, 1992). Prior to legislation restricting its use, Pb was also an important component of gasoline. Tetra-ethyl lead (TEL), an anti-knock agent was the single biggest contributor to aerosolized Pb contamination until it was outlawed in 1978 (Davidson & Rabinowitz, 1992). Recent data show that despite this ban, TEL still exists as a major environmental contaminant especially near roadways where byproducts of gasoline combustion were deposited. Further, over the course of 13 years, approximately 17% of metallic Pb transformed into organic compounds contaminating soil and water sources. (Jørgensen & Willems, 1987; Woodard, Amarasiriwardena, Shrout, & Xing, 2007; Kadi, 2009; Magrisso, Belkin, & Erel, 2009).

While Pb exposure can occur via inhalation and topical application, the most common route is by oral ingestion. Terrestrial birds, such as the Northern bobwhite, ingest Pb pellets in hunting areas mistaking them for grit particles or food (McConnell, 1967; Bennett, et al., 2007; Duggan & Dhawan, 2007). Organic Pb compounds may leach into ground water supplies or get absorbed by plants then consumed by small mammals and amphibians resulting in elevated blood Pb levels in these species (Stansley, et al., 1992; W. Stansley & D. Roscoe, 1996; Wilde, Brigmon, Dunn, Heitkamp, & Dagnan, 2005; Trahan & Peterson, 2007; Robinson et al., 2008).

Upon ingestion, metallic and organic Pb readily dissolves in the stomach, in mammals, or gizzard, in birds, due to their low pH and is absorbed into the blood stream via the duodenum resulting in a rapid rise in Pb blood levels (Quarterman & Morrison, 2007; Martinez-Haro, Taggart, Green, & Mateo, 2009). Greater than 98% of the Pb absorbed by the blood is then sequestered in the cellular fraction (Cake, et al., 1998).

Pb affects several cells in the blood. In erythrocytes, Pb promotes phospholipid peroxidation and inhibits heme synthesis in birds and reptiles resulting in hemolytic anemia (Lawton & Donaldson, 1991; McFarland, 2006). Pb and LPS treatment stimulated B-cells to produce increased IgM, *in vitro*, (McCabe Jr & Lawrence, 1990). Chronic Pb exposure in humans and amphibians resulted in elevated serum IgA and IgE and depressed complement C3, IgG, and IgG response to antigen (Ewers, Stiller-Winkler, & Idel, 1982; Sun, Hu, Zhao, Li, & Cheng, 2003; Rosenberg, Fink, & Salibián, 2007). Further, peripheral blood T-cell populations shifted toward a Th2 profile with an increased Th2:Th1 cell ratio, depressed IFN-γ, and elevated IL-4 following oral chronic Pb exposure in mice (Heo, Parsons, & Lawrence, 1996; Boscolo et al., 1999; Heo, et al., 2007).

APCs affect T-helper function primarily through MHC-II antigen presentation and T-cell activation is positively associated with increased surface expression of MHC-II on APCs (Farrer, Hueber, & McCabe, 2005). MHC-II surface expression can be modulated in APCs by two distinct trafficking pathways; multi vascular body rearrangement in mature APCs and MHC-II positive compartment (MIIC) exocytosis in immature APCs (Farrer, et al., 2005). Prior to MIIC exocytosis, MHC-II is expressed in the endoplasmic reticulum and then packaged into lysosome associated protein number 1 (LAMP-1) positive compartments for entry into the endolytic pathway (Turley et al., 2000). β-hexosaminidase, a glycolipolytic enzyme, is targeted to MIICs

and is thought to aid with antigen preparation (Pierre, 2001; Dai et al., 2009). MIICs fuse with endosomal compartments where lysosomal enzymes degrade their contents and load MHC-II with antigen prior to fusion of the compartment with the plasma membrane. MIIC exocytosis, modulated by intracellular calcium concentrations, leads to increased LAMP-1 and MHC-II surface expression and the release of β-hexosaminidase into the extracellular milieu (Bunbury, et al., 2009).

Multiple studies suggest that Pb may mimic calcium by binding to a number of regulatory proteins and thereby modulate the calcium dependent pathways that regulate MIIC exocytosis (Bridges & Zalups, 2005). In a recent study, dendritic cells cultured with 100 μM Pb had a significant increase in surface MHC-II expression compared to vehicle-exposed control cells (Gao, et al., 2007). However concentrations at this high level, equivalent to a blood Pb concentration of 2,070 μg/dl, are rarely encountered *in vivo* and if so, would likely be fatal. In mice, blood Pb levels up to 450 μg/dl have been observed, but this is three times the maximum lethal dose for humans of 150 μg/dl (Carmouche et al., 2005a). In quail, blood Pb levels in excess of 1,500 μg/dl lead to severe emaciation and mortality (Kerr et al. 2010). Therefore, these studies underscore both the necessity and deficiency of data on the effect of immunopathologically relevant blood concentrations of Pb on MIIC exocytosis.

Methodology

Cells.

Raw 267.4 cells were acquired from ATCC (Manassas, VA). Cells were maintained in DMEM media supplemented with 10% Fetal Bovine Serum (FBS) (MediaTech, Herndon, VA) on T-75 cm^2 flasks (Corning, Corning, NY) at 37 °C, 5% CO_2, and 100% humidity. All cells assayed

were from the same passage sequence and data were representative of a minimum of 3 experiments.

Pb treatment and reagents.

All reagents were acquired from Sigma (St. Louis, MO) unless otherwise noted. Prior to treatment, cells were cultured to confluence over 48 hr, collected, enumerated in an T4 automated cell counter (Nexcelom, Lawrence, MA), and standardized at $2.0x10^6$ cells/ ml (Bunbury, et al., 2009). Aliquots of cells were then added to 24 well plates in 1 ml complete media containing pre-prepared 1 ml serial dilutions of $PbNO_3$, ionomycin or LPS, and incubated for 17 hr. Final concentrations of 0.0, 2.5, 5.0, and 10.0 μM $PbNO_3$, 30 μM ionomycin, and 100 μg/ml LPS were analyzed.

Cellular Viability.

Cellular viability was analyzed via staining with 7-amino-actinomycin D (7-AAD). Briefly, $0.2x10^6$ cells in 200 μl phosphate bufferd saline (PBS) in triplicate were incubated on ice in the dark for 30 min in 0.2 μg/ml 7-AAD as per the manufacturers protocol. Samples were then analyzed on a C6 Cytometer (Accuri, Ann Arbor, MI). Data were expressed as mean % unstained ± SEM.

Alamar Blue™ metabolic proliferation assay.

Two hundred microliters of complete media containing $0.2x10^6$ cells/well were added to a 96 well flat bottom microtiter plate and incubated for 17 hr. Twenty microliters Alamar Blue™ was added to each well and plates incubated for an additional 2 hr. Absorbance (O.D) was then measured in a Synergy 4 spectrophotometer (Biotek, Winooski, VT) at 570 nm and 600 nm. Data were expressed as mean delta (570-600nm) O.D ± SEM after blank subtraction.

Cell surface marker analysis.

Aliquots of cells at $0.2x10^6$ cells/ well in duplicate were fixed in 4% paraformaldahyde for 30 min and then blocked with unlabeled Abs against CD16/32 (BD Biosciences, San Jose, CA) for 60 min. Cells were then washed 3x in cold PBS. Saturating amounts (0.13 µg/well) of the following anti-mouse antibodies; MHC-II, LAMP-1, LAMP-2, CD80, CD86, CD40 and 7-AAD (BD Biosciences) were then added to each well in staining buffer (PBS, 0.1% BSA, 0.01% NaN_3) and incubated on ice in the dark for 30 min. For multicolor analysis, antibodies were added simultaneously. Samples were analyzed on a C6 Cytometer (Accuri, Ann Arbor, MI). Data were expressed as mean fluorescence ± SEM

β-hexosaminidase activity assay.

Supernatants were collected to measure secretory β-hexosaminidase from cells cultured in the presence or absence of Pb and LPS. The remaining cellular fraction was lysed in 0.5% Triton X-100/0.1 M citrate buffer, pH 5.0 and the supernatant collected to facilitate the comparison of secreted to total β-hexosaminidase activity. Twenty-five microliters of 2 mM 4-methylumbelliferyl *N*-acetyl-β-d-glucosaminide in 0.2 M sodium acetate buffer, pH 4.8, was incubated with an equal volume of supernatant or lysate at 37 °C for 1 hr in a water bath. The reaction was stopped with 100 µl of 0.1 M sodium carbonate buffer, pH 10.5, and the released 4-methylumbelliferone fluorescence was quantified in a Synergy 4 plate reader with excitation at 355 nm and emission at 460 nm. Data were expressed as mean percent fluorescence ± SEM.

Electron Microscopy.

Cells were fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.1M sodium cacodylate buffer (pH 7.0) for at least 1 hr. Cells were rinsed in 0.1 M sodium cacodylate buffer (pH 7.0) before enrobing in 3% purified agar. Cells were post-fixed in 1% osmium tetroxide in

0.1 M cacodylate buffer for 1 hr, rinsed in deionized water, dehydrated with increasing concentrations of ethanol, cleared in several changes of acetone and propylene oxide, and gradually infiltrated with Epon-Araldite resin (Sigma). Cells were embedded in flat molds (Ted Pella, Inc) and allowed to polymerize overnight in a 65 °C oven. Ultrathin sections (60–70 nm) were obtained using an Ultracut S ultramicrotome (Leica Microsystems, Buffalo Grove, IL). Sections were stained with uranyl acetate and lead citrate and examined using a JEM-1210 transmission electron microscope (JEOL USA, Inc) equipped with an XR41C Bottom-Mount CCD camera (Advanced Microscopy Techniques, Woburn, MA). Resulting images were then evaluated for changes in cell ultrastructure.

Confocal Microscopy.

Aliquots of cells at $0.2x10^6$ cells/ well were grown in 2 ml complete media on 24 well plates (Corning) containing borosilicate coverslips. After treatment, cells were fixed in 4% paraformaldahyde for at least 24 hr, permeabilized with 0.5% Triton X-100 for 1.5 hr at 23 ^oC, blocked with 10% goat serum at 4 °C for 10-12 hr, and incubated with saturating amounts of Anti-LC3B antibody (BD Biosciences) at 4 °C for 10-12 hr. Resulting coverslips were then washed 3x in blocking buffer and incubated with fluorescent-labeled secondary antibody for 30 min at 23 ^oC in a humidified chamber. Coverslips were then washed with PBS, fixed to slides with Slowfade containing DAPI and sealed. Slides were evaluated on a Zeiss confocal microscope (Zeiss). Results are from the entire z-stack.

Statistics.

Data were the result of 3 or more experiments with cultured cells from the same passage sequence. Data were tabulated and graphed in MS Excel 2007 (Microsoft, Redmond, WA) and analyzed in JMP 9.0 using ANOVA one-way analysis, Dunnett's post-hoc testing, and least

squares regression where indicated and appropriate. Results reported as different had an alpha level less than 0.05 ($\alpha \leq 0.05$).

Results

Cellular Viability.

RAW 267.4 cells were cultured in 0.0, 2.5, 5.0, and 10.0 μM $PbNO_3$, 30 μM ionomycin, and 100 μg/ml LPS for 17 hr then stained with 7-AAD and evaluated by flow cytometry. Cells cultured in complete media for 17 hr had a mean viability of 96.43 ± 0.69%. Cells cultured with different concentrations of Pb, 30 μM ionomycin, and 100 μg/ml LPS had a similar percentage of viable cells as the media-only controls (**Table 4-1**).

Cellular Metabolic Proliferation.

Raw cells had a baseline proliferative response of 0.94 ± 0.05 ODs. Cells cultured with LPS and ionomycin had a 19% and 17% decrease in proliferation, respectively. Cells cultured with Pb at 2.5, 5.0 and 10.0 μM yielded a 22 %, 35% and 30% decrease in proliferation, respectively. (**Figure 4-1**).

Cell Surface Marker Expression.

RAW 267.4 cells were cultured in media with and without Pb, Ionomycin, and LPS and evaluated for MHC-II, CD80, CD86, CD40, LAMP-1, and LAMP-2 surface expression. All cells expressed theses markers, however there were noticeable differences in mean fluorescent intensity which measures the relative number of binding sites on the cell surface. Pb treatment enhanced MHC-II, LAMP-1, LAMP-2, and CD86 surface expression after 17 hr culture (

Table 4-2). Ionomycin-treated cells displayed an increase in MHC-II, LAMP-1, and a significant decrease in CD86. LPS enhanced all the surface markers examined including CD40 which was unaffected by Pb or ionomycin treatment.

β-hexosaminidase assay.

Cells were cultured with treatments for 17 hr prior to assay. β-hexosaminidase activity was assessed in both the supernatant and cell lysate to calculate the percent secreted for each treatment. Secreted β-hexosaminidase was 6.54 ± 0.32 % of total cell activity in media without treatment. Pb at 2.5-5.0 µM, ionomycon, and LPS all significantly increased secreted β-hexosaminidase while 10 µM Pb had no effect (**Figure 4-2**).

Electron Microscopy.

Control (0.0 mM Pb-cultured) RAW 267.4 cells were characterized by an undulating plasma membrane forming several pseudopodia, granular cytoplasm, short strands of rough endoplasmic reticulum (ER), few ribosomes, a number of radiodense but variable form mitochondria, an irregular heterogeneous nucleus with 1 or more nucleoli, and a generally low number of semi-clear lytic vacuoles. Cells exposed to 2.5 to 5.0 µM Pb were visibly larger, displayed infrequent endoplasmic reticular distention 5-10 times larger than control, lipid vacuolization, the presence of autophagic sequestration membranes, mature autophagosomes, and late stage myelin figures, which is consistent with both cytoplasmic sequestration and mitophagy (**Table 4-3**). Although multiple stages of autophagy were observed in all the Pb-treated cell cultures, the 10 µM Pb-treated cells appeared to have fewer mature autophagosomes and myelin figures than the 2.5 or 5.0 µM Pb-treated cells. The 2.5 and 5.0 Pb-treated cells contained significantly greater numbers of mature autophagosomes than the 0.0 or 10 µM Pb-treated cells. LPS-treated cells, the positive control, had greater overall vacuolization and absolute counts of autophagocytotic markers than either the Pb or ionomycin-treated cells and were significantly increased compared to the media (0.0 µM Pb-treated) control.

Confocal Microscopy.

DAPI staining revealed regular round nuclei with 2 or more nucleoli under all treatments conditions and was consistent with the electron microscopy findings. Staining for LC3B, a component of autophagosomal membranes, revealed a dim diffuse pattern in the cytoplasm of cells in media alone with occasional localization. Cells exposed to Pb or LPS displayed a bright, punctate pattern indicating significant LC3 localization (**Figure 4-4**). Ionomycin-treated cells displayed both diffuse and punctate patterns both selectively and in the same cell.

Discussion

Several previous studies have demonstrated a potential link between Pb exposure and immune dysregulation. In immunocompetent mice, chronic Pb exposure results in increased susceptibility to Salmonella infection, a shift toward a Th2 bias, autoantibody production, and the expansion of several subsets of Vβ T-cells (McCabe & Lawrence, 1991; Fernandez-Cabezudo, et al., 2007). In congenital SLE-prone mice, Pb exacerbated glomerular lesions and accelerated mortality via early onset renal failure (Hudson, Cao, Kasten-Jolly, Kirkwood, & Lawrence, 2003). These collective results seem to indicate that Pb has the potential to adversely affect immune homeostasis leading to both immune suppression and increase susceptibility to infection, and enhanced hypersensitivity and the onset of autoimmunity. APCs have a prominent role in regulating this homeostasis through the selective presentation of antigen to, and activation/deactivation of, T-cells. In the periphery, dendritic cells engage tolerance by presenting self-antigen in the absence of secondary surface markers to cause self-reactive T-cell anergy (Bhandoola et al., 2002; Itano et al., 2003). Similarly, thymic epithelial cells (TECs) engaged in negative selection present self-antigen complexed to MHC-II with limited co-stimulatory molecules to induce auto-reactive T-cell apoptosis (Palmer, 2003). More

importantly, TECs use a process called autophagy to introduce self-antigen into the exosomal pathway (Nedjic, et al., 2008).

Autophagy is the degradation of cytoplasmic constituents including organelles and other complex, bulky components in a special purpose double membrane bound compartments called autophagosomes (Yoshimori, 2004). Autophagosomes enter the endolytic pathway to fuse with lysosomes, degrade their contents, and engage in exocytosis (Pfeffer, 2010). Generally, cells engage autophagy in response to nutritional deficiency, infection, or maturation signals, however, new emerging evidence suggests that cells may utilize autophagy as an alternative response to severe cellular insult that would otherwise lead to apoptosis. Many proteins responsible for regulating apoptosis also regulate autophagy although their role may be different for each pathway (Walsh & Edinger, 2010). For example, members of the BCL-2 family of proteins modulate apoptosis and autophagy depending on the local protein environment and location within the cell (Levine, et al., 2008; Wei, et al., 2008). Another protein, p62, binds both LC3, a component of autophagosome membranes, and caspase-8, an apoptotic mediator (Moscat & Diaz-Meco, 2009). Cells deficient in LC3 (or ATG7) accumulate p62 and display increased rates of apoptosis (Komatsu, et al., 2007; Korolchuk, et al., 2009).

In the present study, Pb decreased cellular metabolic activity but had no effect on viability in Raw 267.4 cells after 17 hr of exposure. These findings are consistent with other reports with higher concentrations and longer exposures in both human and murine models. Human mononuclear cells exposed to up to 500 μM Pb did not show signs of apoptosis after 48 hr exposure and in murine bone marrow derived dendritic cells, Pb induced no more apoptosis than media alone but severely impaired cellular proliferation after 10 days of exposure at 100 μM Pb (De la Fuente, et al., 2002; Gao, et al., 2007). Moreover, our findings coincided with

ultra-structural changes consistent with autophagy including cytoplasmic sequestration, enhanced quantities of mature autophagosomes, and LC3b localization. Further, 2.5-5.0 µM Pb increased MHC-II and LAMP-1 surface expression and relative β-hexosaminidase activity inferring an increase in MIIC trafficking (Bunbury, et al., 2009). Finally, Pb at these concentrations increased surface expression of the secondary surface ligand CD86, an important secondary activator ligand for Th2 cells. Surprisingly, 10 uM Pb, the highest concentration in this study, consistently failed to demonstrate significant increases in surface bound MHC-II or CD86 and had reduced indications of late stage autophagy. Since late-stage autophagy markers were unaffected while early stage (cytoplasmic sequestration) was enhanced, we can speculate that concentrations of 10 µM Pb may slow cellular metabolism so much that our 17 hr culture window was insufficient to demonstrate an effect or that 10 µM may be a threshold or plateau concentration since other reports indicate that concentrations as high as 100 µM increased MHC-II surface expression in mouse peritoneal macrophages, albeit, over a longer, 10 day exposure. These data suggest that APCs exposed to Pb can survive to potentially alter immune homeostasis through surface marker alterations brought on by metabolic dysregulation.

In summary, our results show that at Pb concentrations similar to blood concentrations observed during non-lethal exposure, autophagy was induced, MHC-II trafficking enhanced, and CD86 surface expression was increased. All else held equal, increased autophagy increases the number of autophagosomes containing endogenious antigens relative to the number of endosomes containing exogenious antigens in the endosomal pathway delivered to MIICs. This shifts the balance of antigenic inputs available to MHC-II toward endogenous sources. While these findings and the prior *in vivo* data indicate that this disbalancing increases self-antigen presentation leading to increased autoreactive T-cell activation, we cannot assume this is the case

as we did not measure self-antigen presentation directly. Nevertheless, these data show that Pb induces autophagy and then increase MHC-II presentation through increased MIIC trafficking in the presence of a crucial Th2 cell secondary activator ligand in APCs. Collectively, these findings suggest that even at low levels Pb has an adverse effect on immune homeostasis.

Tables

Table 4-1 RAW 267.4 Cell Viability

Viability *% Negative*	Media Mean ± SEM	Pb (2.5 µM) Mean ± SEM	Pb (5.0 µM) Mean ± SEM	Pb (10.0 µM) Mean ± SEM	Ionomycin (3.0 µM) Mean ± SEM	LPS (100ng/ml) Mean ± SEM
Viability (7-AAD negative)	96.43 ± 0.69	96.98 ± 1.02	96.83 ± 1.14	96.88 ± 1.14	96.93 ± 0.90	97.10 ± 0.59

*Mean n = 14 replicates/treatment, 3 or more experiments (*p ≤ 0.05, ANOVA & post-hoc Dunnet's test)*

Table 4-2: RAW 267.4 Cell Surface Marker Expression (MFI)

Surface Expression *Mean Fluorescent Intensity*	Media Mean±SEM	Pb (2.5 µM) Mean±SEM	Pb (5.0 µM) Mean±SEM	Pb (10.0 µM) Mean±SEM	Ionomycin (3.0 µM) Mean±SEM	LPS (100ng/ml) Mean±SEM
MHC-II	10,438 ± 701	**15,779 ± 1990***	**16,260 ± 1898***	14,981 ± 1417	**15,584 ± 1152***	**25,122 ± 1175***
LAMP-1	38,762 ± 1565	**57,725 ± 3787***	**55,414 ± 3989***	48,499 ± 6970	**62,306 ± 4693***	**116,239 ± 12251***
LAMP-2	19,359 ± 1277	**29,743 ± 2603***	**29,582 ± 1898***	**28,220 ± 1820***	26,748 ± 1763	**58,163 ± 3066***
CD80	1,167 ± 99	2,985 ± 253	3,171 ± 367	3,282 ± 231	2,242 ± 145	**23,802 ± 2211***
CD86	21,967 ± 121	**25,146 ± 891***	**25,850 ± 588***	**24,292 ± 338***	**15,622 ± 190***	**71,392 ± 879***
CD40	1,298 ± 77	1,536 ± 215	1,842 ± 435	2,422 ± 572	1,883 ± 453	**21,970 ± 508***
CD11b	84,703 ± 802	91,866 ± 1269	96,233 ± 819	96,245 ± 1476	68,738 ± 13653	**118,863 ± 5891***

*Mean n = 13 replicates/treatment, 3 or more experiments (*p ≤ 0.05, ANOVA & post-hoc Dunnet's test)*

Table 4-3: Autophagic Features

Autophagic Morphology *Mean count per cell*	Media Mean±SEM	Pb (2.5 µM) Mean±SEM	Pb (5.0 µM) Mean±SEM	Pb (10.0 µM) Mean±SEM	Ionomycin (3.0 µM) Mean±SEM	LPS (100ng/ml) Mean±SEM
Nacent Autophagosomes	0.38 ± 0.14	1.29 ± 0.30	1.22 ± 0.24	1.18 ± 0.25	0.45 ± 0.11	**1.83 ± 0.58***
Cytoplasmic Sequestration	0.85 ± 0.22	2.00 ± 0.47	**3.50 ± 0.65***	**2.59 ± 0.58***	1.95 ± 0.29	1.25 ± 0.28
Mature Autophagosomes	0.38 ± 0.18	**1.93 ± 0.41***	**2.39 ± 0.49***	1.06 ± 0.29	0.30 ± 0.11	**2.33 ± 0.31***
Mylin Figures	0.85 ± 0.46	1.64 ± 0.46	**2.67 ± 0.39***	1.71 ± 0.28	0.85 ± 0.13	**5.25 ± 0.59***
Mean Autophagic Markers	2.46 ± 0.71	**6.86 ± 0.81***	**9.78 ± 1.29***	**6.53 ± 0.69***	3.55 ± 0.38	**10.67 ± 0.63***
Lipidosis (Score)	2.38 ± 0.27	1.79 ± 0.15	2.39 ± 0.23	**1.65 ± 0.15***	**1.05 ± 0.05***	1.83 ± 0.21
Vaculization (Score)	2.31 ± 0.13	2.07 ± 0.13	2.72 ± 0.29	2.12 ± 0.17	1.90 ± 0.18	**3.25 ± 0.25***

*Mean n = 17 replicates/treatment, 3 or more experiments (*p ≤ 0.05, ANOVA & post-hoc Dunnet's test)*

Figures

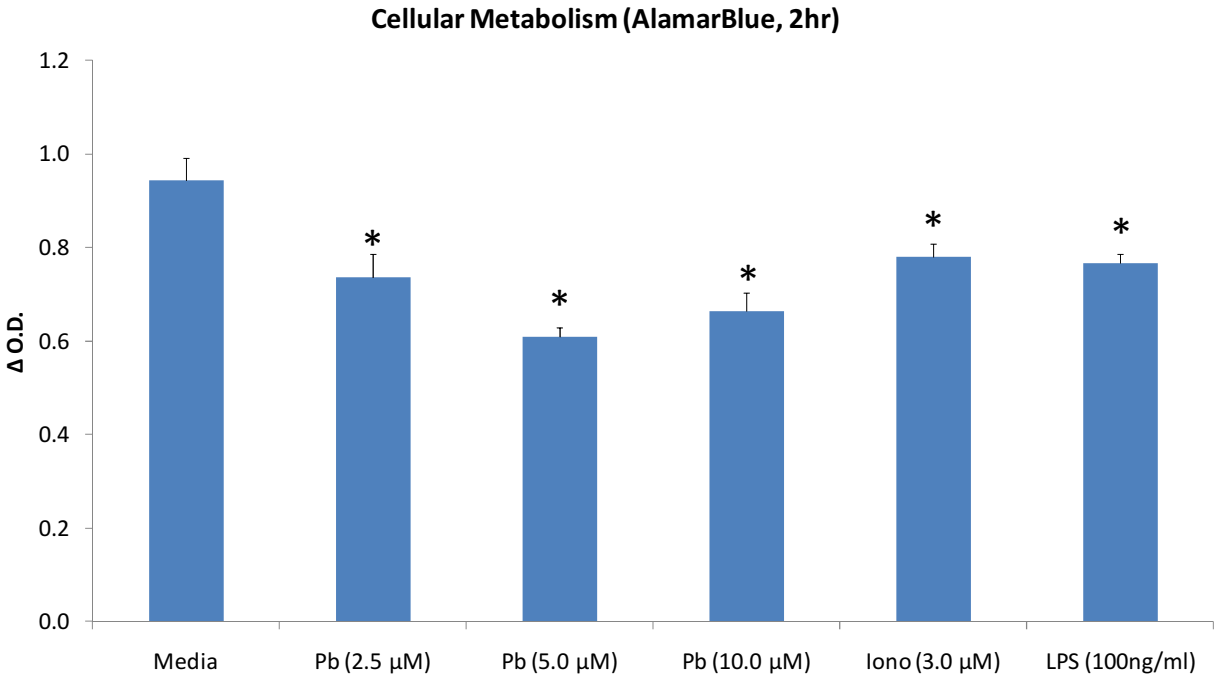


Figure 4-1: Cellular Metabolic Proliferation

Raw 267.4 cells were incubated in 200 µl complete media containing Pb, ionomycin or LPS at 2.0x105 cells/well for 17 hr culture followed by addition of AlamarBlue dye. Dye reduction was spectrophotometrically measured after an additional 2 hr culture and expressed as delta (Δ) OD. Metabolic proliferation was significantly inhibited by all the dose treatments of Pb, ionomycin and LPS. Mean *n=22* replicates, 3 or more experiments, **p≤0.05, ANOVA and post-hoc Dunnet's test.*

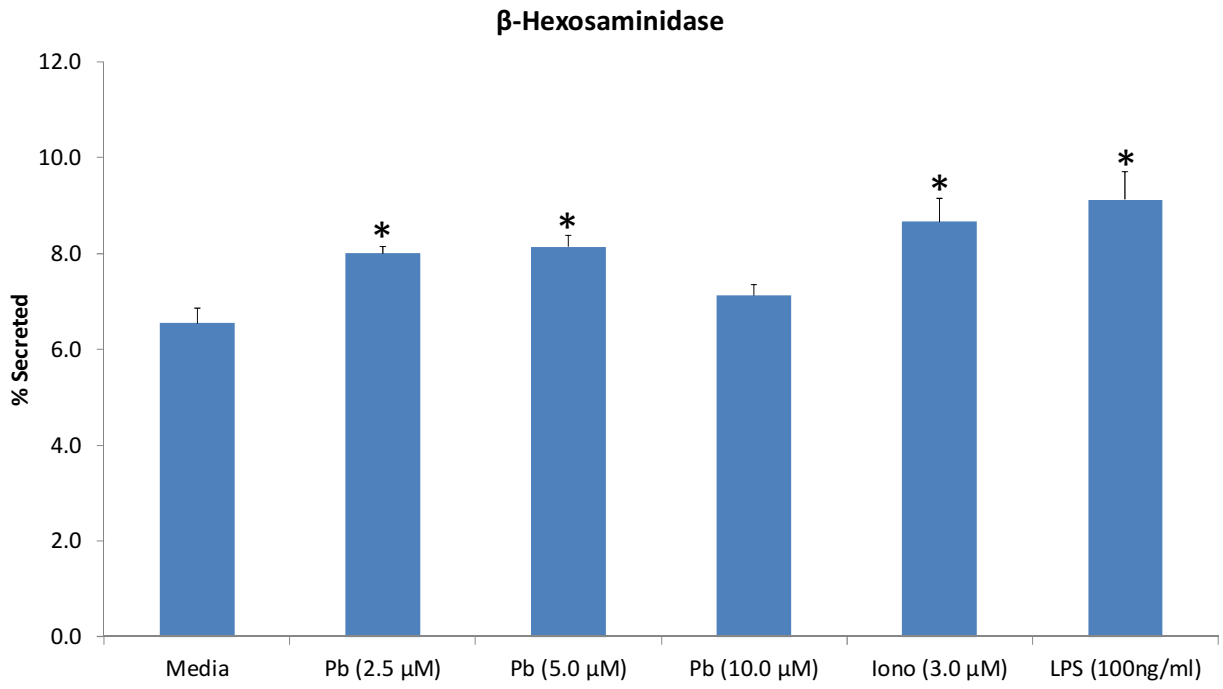


Figure 4-2: Secreted and cell lysate β-hexosaminidase Activity

Raw 267.4 cells were incubated in 200 µl complete media containing Pb, ionomycin or LPS at $2.0x10^5$ cells/well. After 17 hr, β-hexosaminidase activity were measured in supernatants and cell lysates and expressed as percent (%) secreted activity. 2.5-5.0 µM Pb, ionomycin and LPS all increased β-hexosaminidase activity while 10 µM Pb had no statistical effect compared to media alone. Mean *n=22* replicates, 3 or more experiments, **p≤0.05, ANOVA and post-hoc Dunnet's test.*

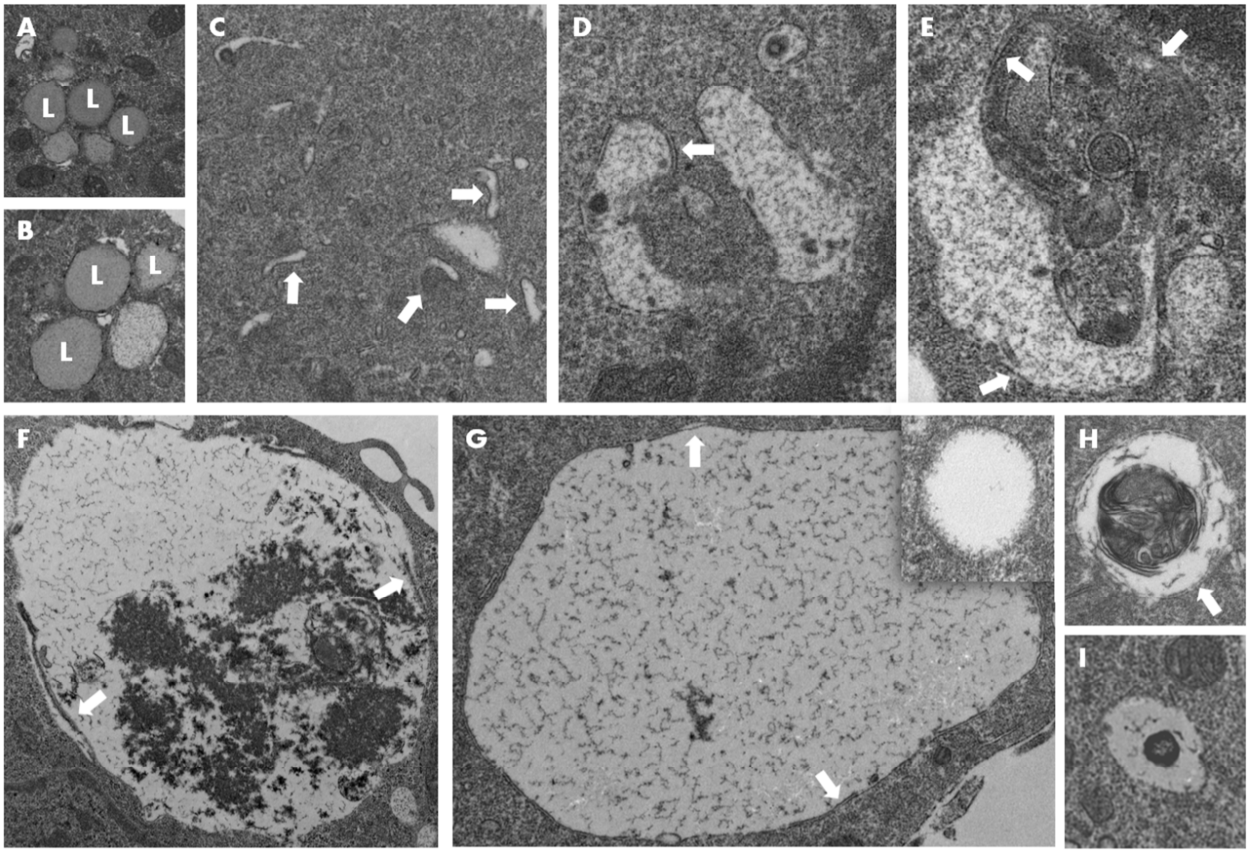


Figure 4-3: Autophagic Features

Lipid vacuolization (L in A&B). Nascent sequestering membranes (C). Cytoplasmic sequestration (D). Organellar sequestration (E). Mature (intermediate stage) autophagosome (F). Mature (late stage) autophagosome (G), normal vacuole (G insert). Early (H) and late (I) myelin figures. *Limiting membrane (arrows).

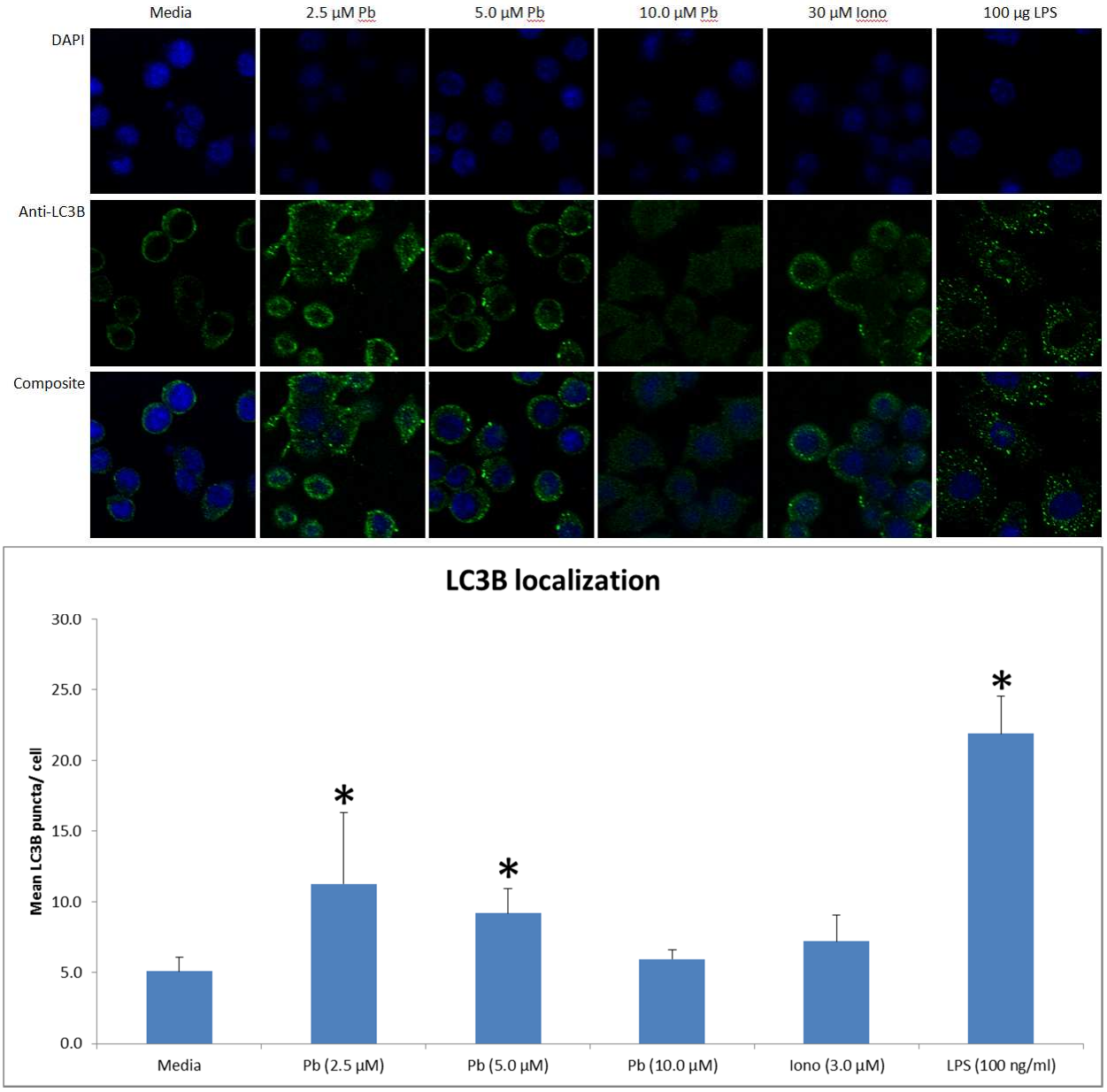


Figure 4-4: Confocal Images and LC3B localization

RAW 267.4 cells were exposed to Pb, LPS or ionomycin for 17 hr on coverslips, fixed and permeabilized, then stained with anti-LC3B fluorescent antibody, and affixed to 25x75mm slides. Confocal images were acquired, and LC3 puncta enumerated per cell (± SEM). The number of LC3B puncta per cell was increased with 2.5-5.0 µM Pb and LPS while cells cultured

with 10 µM Pb and ionomycin were comparable to media-alone. Mean *n=18* replicates, 3 or more experiments, **p≤0.05, ANOVA and post-hoc Dunnet's test.*

CONCLUSIONS

Pb continues to be a major environmental concern with a total estimated 3 million metric tons of shot and fragments deposited on shooting ranges across the United States. As further support to this concern, based on previous and ongoing research in humans, mammals, avians and reptiles, the maximum acceptable blood Pb concentration has declined in steps from 30 µg/dl in the 1970s to less than 10 µg/dl today. For wildlife, the wide-range in environmental availability of Pb, as well as the paucity of wildlife toxicity data indicate a critical need for controlled Pb exposure studies to refine these toxicity levels. The first phase of this dissertation explored the consequences of acute Pb exposure in a ground-foraging avian species, the Northern Bobwhite quail. This ecotoxicologic-based study tracked the effects of known ingested quantities of Pb on blood Pb concentration, pellet retention, weight changes, blood physiologic parameters, and pathology. This was then followed up by another study that assessed the effects of oral ingestion of bullet fragments using the same testing conditions as the previous Pb pellet study. In the final phase of this dissertation, the *in vitro* effect of Pb on MHC-II trafficking and cellular viability, phenotype and metabolism in the RAW 267.4 cell line was evaluated to suggest a novel mechanism of heavy metal-induced immune dysregulation.

Lead in the Northern Bobwhite

Acute, oral administration of Pb pellets and fragments resulted in adverse effects in cardiovascular, gastrointestinal, immune, renal and hepatic tissues of the Northern Bobwhite quail. Effects were dose dependent but physiologic changes were observed in all birds exposed to Pb; at all doses and at all timepoints, δ-ALAD was markedly inhibited. In most cases,

production and hemodynamic parameters were unaffected, however at doses exceeding 2 pellets or 5 fragments, feed consumption, body weight, PCV, and total serum protein, were reduced. Birds dosed with 5 or 10 pellets (49 mg/pellet) experienced acute morbidity. These birds either died prematurely or were humanely euthanized. Examination of the tissues of these birds showed multifocal koilin degeneration and ulceration, splenic multifocal moderate histiocytic hemosiderosis, and hepatic focal necrosis suggesting that the hematic and gastrointestinal systems are the most acutely effected.

A comparison between the effects of spent shot pellet and bullet fragment in quail yielded some interesting similarities and some unique differences. While a single fragment dose resulted in a similar effect to a single pellet dose, a dose of 5 fragments, the equivalent weight of 2.5 pellets, resulted in PbBs and hemotologic effects similar to 5 pellets in male birds. Retention times for pellets were also generally longer than for fragments and pellets were heavier and larger despite similar blood concentrations. These data suggest that that fragments were more readily absorbed than pellets. This may be due to the larger surface area to weight ratio in fragments or, potentially, a function of the absorption mechanics in the GI. For example, Pb may inhibit acid secretion, and since pellet digestion is affected by acidity, lower acid could lead to less Pb ions available to absorb creating a self-limiting feedback loop. This would also explain why graphs of pellet and fragment doses vs. PbBs generally appear more inversely logarithmic than linear although more data would need to be generated to draw a firm conclusion. Regardless, a NOAEL could not be determined from these data because significant δ-ALAD inhibition was observed at all doses. The LOAEL was determined to be one 24 mg fragment or one 49 mg pellet. The LD50 was approximately five 49 mg pellets however, since there was no observed mortality in the 5 Pb fragment group, we could not calculate a Pb fragment LD50.

These dose calculations are informative; however, dosage may not be the best indicator of morbidity. Interestingly, we observed across dosage groups that birds with similar PbBs also had similar degrees of morbidity. This would suggest that PbB is a more reliable indicator of expected toxic changes than number of particulates dosed and also conveniently bypasses the GI absorption mechanics question to provide a more direct measure of Pb that cells are likely to encounter once the Pb is absorbed and providing a basis to examine the effects of Pb *in vitro*.

Molecular Pb Dysregulation Mechanisms in APCs

Developmental Pb (0.1 mM) exposure in drinking water significantly affected transcription of 299 genes in the whole spleen of C57BL/6 mice (Kasten-Jolly, et al., 2010). Of the transcripts with the highest fold change relative to non-treated controls, Pb induced a significant increase in catabolic enzymes, transcription factors, regulatory proteins, and signal proteins related to cellular metabolism. Pb up-regulated pro cell survival (IRF2BP2, Tff2, EIF4G2, PLCe1, PAX7), and anti-apoptotic transcripts (BIRC2, BIRC3, BCL10) while simultaneously down-regulating pro-apoptotic transcripts (CAMK2N2, PSMB2, MAGE-2). Pb also decreased AKT1 and BCL2 mRNAs. AKT1 and BCL2 are associated with apoptosis but are also known to regulate autophagy, cellular metabolism, and mitochondrial function (X. Zhang et al., 2009). Pb modulated the mRNA of caspases 6 (2.84 fold decrease), 7 (1.5 fold increase), and 12 (2.12 fold increase) suggestive of both pro and anti-apoptotic responses and ER stress (Kerbiriou, et al., 2009). EIF2ak2 (PERK) (5.74 fold increase), also associated with ER stress, phosphorylates EIF2a leading to protein synthesis inhibition and is associated with low glucose G1 cell cycle arrest (P. Zhang et al., 2002). Changes in insulin (6.82 fold increase), Insulin-like growth factor-binding protein 7 (IGFBP7) (3.22 fold decrease), and phospholipase A2, group IB (PLA2G1B) (9.81 fold increase) are also consistent with glucose deficiency (Saltiel & Kahn,

2001; Labonté et al., 2006; Minghui, Yuqing, Linshan, & Hongjie, 2007; Wajapeyee, Serra, Zhu, Mahalingam, & Green, 2008; Scurr et al., 2010). Interestingly, Serologically defined colon cancer antigen 8 (SDCCAG8) has recently been shown as a suppressor of obesity in knockout mice and over-expression (6.12 fold increase) in C57BL/6 mice may be suggestive of nutritional deficiency (Scherag et al., 2010). Further, a 3.01 fold decrease in the proteasome subunit 2 (PSMB2) indicates that the ubiquitin protein degradation pathway may be compromised concurrent with a dramatic increase in transcripts of catabolic enzymes for lipids, complex sugars, and proteins including the top 12 most effected transcripts (Moffitt, Martin, & Walker, 2007). Functional analysis confirmed that Pb increased amylase (AMY2) activity and further experimentation has demonstrated that increased amylase expression and activity is a stress or nutritional depravation induced response (Rolland, Moore, & Sheen, 2002; Baena-González & Sheen, 2008; Sans, Crozier, & Williams, 2008; Kasten-Jolly, et al., 2010). Taken together, Pb-induced changes in mRNA expression potentiate the initiation of the unfolded protein response (UPR), alteration of glucose availability signaling, a functional response consistent with perceived nutritional depravation, and the down-regulation of pro-apoptotic markers.

Evidence of Pb-induced phenotypic changes resulting in the activation of the UPR has also been explored in the mouse model. Interestingly, extracellular Pb concentrations as low as 5 µM are capable of efficiently binding a signal protein inside the ER called GRP78 (aka; BiP, HSPA5, HSP70) (Qian, et al., 2005). Pb bound GRP78 disassociates from IRE-1, pancreatic ER eukaryotic translation initiation factor (eIF) 2α kinase (PERK), and activating transcription factor 6 (ATF6), and on the intra-ER membrane surface and then translocates to the cytosol forming aggregates (Qian, et al., 2005). Unbound ATF6 then binds coat protein complex (COPII), which forms vesicles for transport to the Golgi (X. Chen, Shen, & Prywes, 2002; Shen, Chen,

Hendershot, & Prywes, 2002; Schindler & Schekman, 2009). En-route to the Golgi, ATF-6 is sequentially cleaved by SP-1 and SP-2, membrane bound transcription factors to release a 390 AA transcription factor that translocates to the nucleus to enhance X-box binding protein 1 (XBP1) mediated transcription of ER chaperone proteins including GRP78 and GRP94 (aka; HSP90β1, ERp99, TA-3, Targ2, Tra-1, Tra1, endoplasmin, and gp96) and the cytokine IL-6 (K. Lee et al., 2002; M. Hong et al., 2004; Shen & Prywes, 2004; Martindale et al., 2006; Shi, Porter, Parameswaran, Bae, & Pestka, 2009). Cleaved ATF6 then translocates to the nucleus where it helps XBP1 promote the transcription of GRP94 (Martindale, et al., 2006). Sequentially, these GRPs and HSPs translocate to the ER and help fold proteins and the whole pathway is known as the unfolded protein response (UPR). Unchecked, the UPR can lead to apoptosis (Rao, Ellerby, & Bredesen, 2004). However, even though Pb may initiate the UPR, it also appears to interrupt the pathway downstream. SP-1 interacts with ATF-6 via a zinc finger binding domain which is inactivated via Pb binding (Zawia, Crumpton, Brydie, Reddy, & Razmiafshari, 2000; Atkins & Basha, 2003; M. Basha, 2003; Wu & Basha, 2008). SP-1 also uses it's zinc binding domain to bind to YY1 synergistically inducing transcription of several genes (Shrivastava & Calame, 1994). When compared to gene expression changes induced by Pb in the murine spleen, none of these YY1 induced genes were enhanced indicating that SP-1 is effectively inactivated (Kasten-Jolly, et al., 2010). This suggests that while Pb may initiate the UPR, it also interrupts the pathway before the nucleus gets the full signal.

Downstream, the initiation of the UPR and activation of XBP1, leads to enhanced GPR94 expression (Martindale, et al., 2006). GRP94 is both a component of autophagic membranes and has recently been implicated in autoimmune disease (Stetler et al., 2010). Transgenic mice with chronic surface expression of GRP94 (GP96) displayed significant DC activation, auto-reactive

T and B cells and spontaneous SLE-like lesions (B. Liu et al., 2003). Further, inhibition of GRP94 surface trafficking suppressed DC activation, reduced B220+ and MHC-II+ splenocytes, and prevented renal disease in the transgenic mouse (Han et al., 2010). Finally, GRP94 has been implicated in MHC-II antigen sequestration (Strawbridge & Blum, 2007). Taken together, these data suggest that changes in the ER may lead to increased MHC-II self-antigen loading facilitated by GRP94.

A New Model of Lead Enhanced Autoimmune Disease

Several *In vitro* studies have shown that antibody production can be driven by auto-reactive T-helper type 2 (Th2) cells and that these T-cells are activated by Pb exposed splenic APCs (McCabe & Lawrence, 1991; Heo, et al., 1997; Heo, et al., 1998; Gao, et al., 2007). In the present study, Pb induced autophagy and increased MHC-II and CD86 surface expression at low Pb concentrations indicating that Pb may bias antigenic inputs to MHC-II toward endogenous sources and then enhance the overall ability of APCs to both present to activate T-cells specific for those endogenous antigens. These data suggest a mechanism of how Pb may promote self-antigen presentation, ANA production, and ultimately SLE lesions in congenial SLE mice without inducing apoptosis. Intuitively, Pb may then exacerbate autoimmune disease at lower doses that previously thought and may even pre-dispose disease in non-prone species.

Summary

Presently, no known studies exist showing a mechanism through which non-lethal concentrations of Pb could cause APCs to induce auto-reactive T-cell clonal expansion. Since auto-reactive T-cell activation requires self-antigen presented in the context of MHC-II on APCs, I hypothesized that intracellular Pb increased MHC-II by enhancing MIIC trafficking and self-antigen presentation by disbalancing antigenic inputs to MHC-II. We found that Pb induced

autophagy in RAW 267.4 cells, which has been shown to increase delivery of endogenous antigens to MHC-II (Nedjic, et al., 2008). In the absence of intracellular infection, this would likely increase the amount of self-antigen loaded into MIICs and thus relatively increase the amount of self-antigen loaded MHC-II on the cell surface. In partial support of this hypothesis, we did observe a Pb-induced increase in MIIC trafficking denoted by enhanced MHC-II surface expression, an increase in LAMP-1 surface expression, and an increase in the amount of β-hexosaminidase secreted. Taken together, we predict that APCs present not just more MHC-II, but more self-antigen loaded MHC-II. Under optimal immune conditions, an increase in self-antigen loaded MHC-II in the absence of secondary activator ligands would lead to auto-reactive T-cells anergy. However in the presence of Pb, we observed an increase in CD86 surface expression, a crucial secondary T-cell activator ligand. These findings suggest that Pb-treated APCs could upregulate auto-reactive T-cells through increased self-antigen presentation and CD86 co-activation. Future studies evaluating the effects of Pb on APCs in the presence of T cells are needed to solidify this hypothesis, however, deciphering the mechanism associated with Pb-induced dysregulation of antigen processing and presentation could lead to the discovery of new therapeutic approaches aimed at preventing or depreciating this mode of autoimmune induction.

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