REPRODUCTIVE AND DEVELOPMENTAL LEAD TOXICITY IN ROLLER PIGEONS AND AN INVESTIGATION OF LEAD NITRATE IMPACT ON INFLAMMATORY RESPONSE AND CALCIUM SIGNALING IN A MAMMALIAN MACROPHAGE CELL LINE

by

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(Under the Direction of Robert M Gogal Jr)

ABSTRACT

The heavy metal lead (Pb) is a persistent environmental pollutant that does not breakdown over time like other chemicals. Acute and chronic exposure to Pb can cause severe toxicities in numerous organ systems. Avian species are susceptible to exposure from numerous forms of Pb in the environment including spent Pb ammunition. While it is known that Pb can cause multi-organ toxicities in the developing birds, there are limited data demonstrating the role of maternal Pb in the developing offspring. Hen roller pigeons were exposed to a single Pb pellet commonly deposited in the environment through recreational shooting and allowed to mate. Egg production and size, hatchling initial weight and final weight, blood analysis, and organ collection were all conducted to determine maternal Pb impact on F1 development. These data showed that eggs and progeny of the first clutch were significantly smaller than unexposed hens. Further histologic analysis indicated that Pb significantly altered the development of the thymus and spleen in progeny months following exposure. These data demonstrate that

developmental exposure to Pb can cause immune suppression in avian species. Given these data and previous knowledge, the pro-inflammatory response of macrophages exposed to Pb was investigated using the RAW 264.7 cell line and an environmentally relevant concentration of Pb (5μM $Pb(NO_3)_2$). These cells were induced towards a pro-inflammatory state using a bacterial antigen and select cytokine alone or in combination. Western blot data showed that initial exposure to Pb altered cytoplasmic protein signaling in calcium pathways as well as key pro-inflammatory pathways. Functional assays showed that cellular proliferation, nitric oxide production, and cytokine expression were significantly altered in the Pb exposed cells. These data offer a new mechanism for macrophage pro-inflammatory inhibition by Pb. These data also suggest a potential mechanism for macrophage further driving Pb induced Th2 cells towards anti-inflammatory functional response resulting in the Th2 immune profiles presented in chronic Pb exposure.

INDEX WORDS: Lead, Ammunition, Toxicity, Avian, Reproductive, Deposition, Calcium, Macrophage, Inflammation, Protein Signaling

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DEDICATION

I dedicate this dissertation to my grandmother Carolyn Jowers Williams who loved to brag about all her grandchildren

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CHAPTER 1

INTRODUCTION

BRIEF REVIEW OF PB IN THE UNITED STATES

Lead (Pb) is a heavy metal toxicant that's use in industry and consumer products has plagued much of the world since the middle of the twentieth century. The three major industry-related sources that were implicated in Pb entering the environment are Pb gasoline emissions, Pb-based paint on exterior of structures, and primary sources such as Pb smelters and mines (Battelle and EPA 1998). Pb gasoline (tetraethyl Pb) was used in the United States during much of the 20th century and is classically recognized as one of the leading causes of Pb toxicity during this time. The publication *Contaminated and Natural Pb Environments of Man* (1965) resulted in the gradual removal of Pb from consumer products, particularly fuel. Tetraethyl Pb is extremely toxic to the nervous system of children and severe toxicities and developmental issues were seen for years, even after the removal of Pb from fuel due to early childhood exposure (Needleman 2000).

Pb-based paints were also phased out during the same time as tetraethyl Pb in the United States. The consumption of Pb paint chips by children significantly increased blood Pb levels and caused numerous chronic toxicities (McElvaine et al. 1992). To try and limit subsequent exposures, in 1977 the United States Consumer Product Safety Commission (USCPSC) lowered the allowable concentration of Pb in paint from 5000 ppm to 600 ppm (0.06%)(USCPSC 1977). The issue of exposure remained though in

painted homes and buildings built prior to 1977. A study conducted from 1998-2000 showed that within the United States 24 million households still had significant Pb-based paint hazards (Jacobs et al. 2002). Renovation of these homes could lead to Pb-based paint entering the surrounding environment through dust and debris. A variety of different techniques have been used to prevent Pb from leaching into the environment during renovations, however complete prevention has not yet been achieved, with up to 6g of Pb per square foot per hour depositing as dust (Jacobs et al. 2013). These findings suggest that potential exposure to Pb-based paints is still a concern in the United States.

The predominant pathways for Pb exposure in human and animals are consumption and inhalation, with very little research examining the role of dermal absorption (ATSDR 2010). It was previously described that young children consume Pb-based paint chips in older homes and buildings (McElvaine et al. 1992). However, everyday food products can also contain trace amounts of Pb therefore in 2006 the FDA placed an action level on food or candy commonly consumed by children containing 0.5 ppm Pb or higher (USFDA 2006). The general population is most commonly exposed to Pb via inhalation through Pb dust particles originating from Pb-based paints in homes and buildings built prior to 1978 (Jacobs et al. 2002; Jacobs et al. 2013). The individuals most at risk of Pb exposure through inhalation are those exposed to it via occupation. Pb smelters and secondary processing facilities have increased concentrations of Pb and other heavy metals in the air, chronically exposing workers to low levels of these toxicants (Felix et al. 2013). Residential areas surrounding these metal industries are also at risk from exposure to Pb via inhalation, placing large concern on children living nearby (Roels et al. 1980; Richmond-Bryant et al. 2014).

RATIONALE FOR REVIEW OF PB TOXICITY IN AVIAN SPECIES

The United States Center for Disease Control (CDC) and Environmental Protection Agency (EPA) have made Pb and human health information available to the general public for decades, therefore the threat of Pb gasoline, Pb pipes and paint, and Pb industrial facilities causing neurologic toxicity is widely known. The extent of environmental Pb pollution and the impact on wildlife is an area of research that is predominantly known and discussed amongst the scientific community studying Pb. Therefore this dissertation set to search the literature for environmental Pb contamination and the global impact on avian wildlife.

Avian species are effective models for research given they inhabit a wide variety of ecosystems throughout the world with numerous species occupying multiple ecosystems in rural and residential areas. Birds are also susceptible to Pb exposure through multiple sources. Given these facts, avian species are unique organisms in that they offer global perspective on Pb wildlife toxicity. The results of this review will be to present the historical and most current research in avian Pb toxicity. The different sources of Pb in avian exposures will be explored in both terrestrial and aquatic species. The multiple organ system toxicities will be thoroughly described including nervous, hepatic, renal, reproductive, and immune systems as well as the developmental toxicities in avian hatchlings. Current diagnostic and treatment methods for Pb exposure will be presented with case study reports from veterinarians encountering birds where Pb exposure was not initially known. Lastly, regulations for preventing further Pb from entering the environment through the unique sources described in this review and remediation techniques for removing Pb currently polluting the environment will be explored.

RATIONALE FOR AVIAN REPRODUCTIVE RESEARCH

As stated previously chapter 2 of this dissertation is a published literature review of the current avian toxicity research; therefore the rationale for the manuscript on avian reproductive and developmental toxicity (Chapter 3) will be brief as to not duplicate data presented in the next chapter. Previous research has shown that northern bobwhite quail (*Colinus virginianus*) and domestic roller pigeons orally gavaged with a single Pb pellet from a shotgun shell had significantly increased blood-Pb levels and suffered from hematologic toxicities well after the Pb pellet had been excreted (Kerr et al 2010; Holladay et al. 2012). It has also been reported in human cases that deposited Pb in soft tissue and the skeletal system is mobilized into maternal circulation during pregnancy, years after initial exposure occured (Gulson et al. 1997; Gulson et al. 2003; Riess and Halm 2007). Given that multiple avian species present with severe hematologic toxicities following a single oral Pb pellet exposure, a single pellet exposure in hens may result in reproductive and developmental toxicities in F1 progeny. Mobilization of Pb during pregnancy in mammalian species also suggests that hens may deposit Pb in subsequent clutches weeks or months following exposure.

In this dissertation, we obtained known breeding pairs of roller pigeons and orally gavaged the hens of each pair with a single 45mg Pb pellet, 95mg Pb pellet, or dH_2O (control). The pairs were housed individually at the University of Georgia Poultry Diagnostic and Research Center, Athens, GA and allowed to breed for two consecutive clutches. A clutch is defined, as a single egg laying period, and the clutch size is the number of eggs in that period. The hens were radiographed to ensure the pellets were retained and blood was collected one-week post exposure to determine blood-Pb levels.

The number of eggs produced by each hen was tracked throughout the study. Eggs were weighed and measured for size comparison and candled to determine fertilization. Hatchlings were briefly weighed approximately one-week post hatch and then remained with the hen and cock bird until the weaning period (28 days post hatch), where upon blood was collected for blood-Pb levels and hematologic toxicity. Hatchlings were then euthanized and the spleen, liver, kidney, sciatic nerve, thymus, and brain were collected for histologic analysis.

The expected results from the hen radiographs and the blood analysis were to confirm to toxicities reported in previous research. The anticipated hatchling results from the first clutch are that Pb elicits negative effects on egg production, hatchability of the egg, and developmental toxicities presented by decreased body weight and organ development. The second clutch results will occur weeks following exposure, therefore the anticipated results from the second clutch are that developmental toxicities will still be present, however less severe than the first clutch hatchlings. If these results are generated, then it will be the first data demonstrating that a single Pb pellet exposure in hen roller pigeons decreases production of eggs and F1 generation offspring that are physically, phenotypically, and clinically comparable to offspring from unexposed hens.

RATIONALE FOR INVESTIGATING PB MODULATION OF CALCIUM SIGNALING IN THE MACROPHAGE, *IN VITRO*

It has been well documented that Pb is an immunotoxicant that alters immune profiles *in vivo*. The most severe cases of Pb-induced immune toxicity involve those chronically exposed to Pb in paint and pipes. Industry workers chronically exposed to Pb had increased serum antibody levels of IgA and IgE (Ewers et al. 1982). These two

antibodies are associated with an anti-inflammatory Th2 immune profile. A shift towards a Th2 immune profile in humans has been linked with the increase of autoimmunity leading to disease such as systemic lupus erythematosus (Ramanujam and Davidson 2008). Another study saw depressed concentrations of the antibody IgG in humans chronically exposed to Pb (Sun et al. 2003). This could also lead to an increase in the Th2 profile, as IgG is the antibody commonly associated with Th1. These collective data suggest that Pb exposure can shift the immune profile towards a Th2 response, limiting the ability of the immune system to respond appropriately to certain pathogen exposures and also, increasing the potential for the development of autoimmune disease.

The current exposure model in avian species in this dissertation results in increased blood-Pb levels. Circulation of Pb through the blood can potentiate exposure to peripheral blood leukocytes as well as the secondary immune organs such as the spleen and lymph nodes. The monocyte is a circulating phagocytic cell of the innate immune system that communicates with cells of the adaptive immune system through antigen presentation. It has previously been documented that Pb negatively impacts monocyte/macrophage inflammatory response through disregulation of cytokine production (Krocova et al. 2000), phagocytosis (Knowles et al. 1997), reactive nitrogen species production (Tian and Lawrence 1997), and MHC II expression for antigen presentation (Kerr et al. 2013). Given the crucial role of the monocyte/macrophage in linking the innate and adaptive immune response, functional alterations induced by Pb could significantly impact the immune system *in vivo*.

One key pathway required for monocyte/macrophage function is calcium signaling in the cytoplasm of the cell (Wright et al. 1985). Pb and calcium are both

divalent cations and it has previously been shown that Pb has the ability to mimic calcium in multiple cell types and suppress protein function and signaling within the cell (Wiemman et al. 1999; Kirberger et al. 2008). The cellular mechanism of Pb toxicity in the macrophage has not previously been linked to calcium signaling within the cell. Therefore, the research presented in this dissertation set to investigate the function of the monocyte/macrophage in the presence of Pb and the possible impacts on calcium signaling within the cell.

The established *in vitro* RAW 264.7 macrophage cell line was the cell line chosen for this dissertation research. The cells were stimulated towards a proinflammatory response and exposed to a previously determined environmentally relevant concentration of Pb. Cellular viability and proliferation were evaluated to determine if Pb decreased the viability of the cells or impedes the ability of the cells to grow and divide. Protein signaling in inflammatory and calcium/calmodulin pathways were evaluated through western blot to determine Pb affect on signal transduction in each individual pathway. Finally phagocytosis, reactive nitrogen species, and cytokine assays were conducted to determine the functional response of the cells in the presence of Pb.

The anticipated results for this study are that Pb will inhibit calcium signaling within the macrophage and significantly decrease pro-inflammatory activation. First it is predicted that cellular proliferation will be significantly decreased by Pb while cellular viability will remain high. It is expected that signal transduction in the calcium/calmodulin pathway will be significantly inhibited by Pb. If this occurs, then cross talk between the calcium/calmodulin pathway and the inflammatory pathways will results in decreased nuclear translocation of transcription factors in these pathways. This

would result in altered functionality of the cells evident by decreased phagocytosis, production of reactive nitrogen species, and altered expression of pro-inflammatory and anti-inflammatory cytokines. These results would demonstrate that calcium signaling in macrophages is a target of environmentally relevant concentrations of Pb.

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CHAPTER 2

ENVIRONMENTAL PB AND WILD BIRDS- A REVIEW[1]

[1] Williams RJ, Holladay SD, Williams SM, and Gogal RM Jr. 2017. *Reviews of Environmental Contamination and Toxicology*. pp 1-24.
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ABSTRACT

Pb is a persistent inorganic environmental pollutant that affects humans and animals worldwide. Avian species are especially susceptible to Pb exposure through consumption of Pb ammunition, Pb fishing tackle, and other contaminated food sources such as aquatic species ingesting Pb contaminated sediments in mining areas. Even with government regulation on the use of Pb ammunition in many countries, including the United States, terrestrial, aquatic, predatory, and scavenger avian species are still at risk of exposure to potentially lethal concentrations of Pb. The toxicities seen in these avian species include increased oxidative stress and decreased anti-oxidant enzymes in hepatic and renal tissue. The avian immune system is also a target of Pb, and displays a number of altered functions suggestive of immune suppression, however studies in wildlife and laboratory species remain too limited for definitive statements with regard to population risk. In contrast, Pb clearly inhibits reproductive capabilities in adult birds, and alters growth and development of hatchlings. Environmental remediation for Pb removal, which would lower toxic exposure in wildlife, presently is a monumental and prohibitively expensive effort. Wildlife exposure will therefore continue in contaminated areas, necessitating development of new remediation practices. These plans should aim toward limiting more widespread or heavier contamination of wildlife habitats. This paper reviews presently available information of Pb toxicity in wild bird species, and suggests continued monitoring and reduction strategies to reduce Pb exposure for at-risk avian populations.

INTRODUCTION

Pb composition, classification, and use

Elemental Pb has an atomic mass of 207.2 g and density 11.34 g/cm^3. It is classified as a heavy metal by virtue of its high molecular weight and ~~a~~ density ≥ 5.0 g/cm^3 (Tchounwou et al. 2012). As with other heavy metals, Pb is considered to be a trace element and in the United States under natural conditions its mean environmental concentration in soil is 16 ppm. (Shacklette and Boerngen 1984). Pure Pb does not naturally exist in the environment, but rather it is found as a component of mineral ores. The most abundant of these ores is galena, which contain other elements that predominantly include silver (Dube 2006).

The first human documented use of Pb dates back to the Roman empire, after which Pb use was minimal until societies became more industrialized (Milton 1988: Nriagu 1983). Pb use then dramatically increased such that, in 2015, more than 385 thousand metric tons of Pb were mined in the United States with an additional 1.12 million tons produced through recycling of post-consumer scrap (USGS 2016). These data verify that vast amounts of Pb continue to be refined and used by consumers in the United States, and are then extended by even higher production in other countries such as China (USGS 2016). Based on the millions of tons of Pb mined in past and present years, it is not surprising that Pb is viewed as a persistent global environmental threat. Further, while some environmental toxicants degrade over time, Pb as a heavy metal element is relatively inert in its natural form.

Distribution and biomarkers of Pb toxicity

Systemic distribution of Pb following exposure via inhalation or consumption occurs primarily through the circulatory system. Based largely on mammalian studies, it has been estimated that 30% of consumed Pb and 50% of inhaled Pb is absorbed into circulation (Agrawal 2012). Once in circulation, ~5% of total Pb remains in blood while up to 95% distributes to other tissues, and is then slowly excreted from all these compartments through urine or feces (Agrawal 2012). Pb deposition in bone is highly persistent, occurs via the formation of a Pb-phosphate complex, and can account for up to 95% of the total body burden from sub-chronic to chronic exposures (Agrawal 2012). The half-life of Pb in the blood of mammals has been estimated as ~30 days while Pb deposited in bone has a half-life of 20-30 years, with risk of Pb re-entering circulation and causing toxicities long after the initial exposure (Rabinowitz 1991). While limited research exists on the half-life of Pb in the blood of avian species, Fry and Maurer determined that the half-life of Pb in blood of California condors was approximately 13 days.

Both blood and urine samples are commonly collected to determine the level of exposure to diverse toxicants as well as to help determine the severity of the systemic health effects to these toxic contaminants (Strimbu and Tavel 2010). The enzyme, delta aminolevulinic-acid dehydratase (δ-ALAD), is extremely sensitive to Pb exposure, and decreased activity of this enzyme in blood cells is the classic biomarker of Pb exposure (Berlin and Schaller 1974). δ-ALAD is the second of eight enzymes involved in heme synthesis; therefore, inhibition of this enzyme by Pb significantly hinders an organism's ability to produce heme (Figure 2.I). Another biomarker associated with Pb toxicity is

urine aminolevulinic acid (ALA). ALA is a substrate of δ-ALAD, so inhibition of the enzyme yields an increase in blood ALA concentrations. The increased ALA is filtered by the kidneys and is secreted into the urine. Both of these biomarkers are routinely used for assessing Pb toxicity in human, domestic and wildlife species as well as tracking responses to interventional treatment.

Cellular Mechanism of Pb toxicity

All animal species depend on sodium, calcium, potassium, iron, and zinc for proper cellular function and survival. Pb, as a divalent cation, competes for ionic binding sites in proteins and thereby prevents essential divalent cations such as zinc and calcium from binding (Godwin 2001). This mechanism of competition and ion displacement by Pb has been coined "*ionic mimicry*" (Kirberger and Yang 2008). The biomarker protein for Pb exposure, δ-ALAD, requires a single zinc ion for proper enzyme structure and function (Godwin 2001). The binding site for zinc in δ-ALAD contains three cysteine residues, to which Pb binds with moderate affinity, slightly modifying the protein structure and inhibiting ALA from binding the active site (Godwin 2001).

Another class of high affinity receptors for Pb is the calcium dependent proteins such as calmodulin (Kirberger and Yang 2008). Calmodulin is a cytoplasmic protein expressed in nearly all cells of the body, which initiates a signal cascade for protein transcription following calcium binding at its four active sites (Wiemann 1999; Wayman et al. 2011). Pb competes with calcium for calmodulin's four binding sites but also can bind at various points along the structure of the protein in an action known as "*opportunistic binding*" (Kirberger et al. 2013). The binding of Pb to calmodulin initiates improper folding of the protein, resulting in inhibition of many cellular functions

including prevention of signal transduction to the nucleus (Kirberger et al. 2013). Depending on a cell's level of dependence on calcium, the degree of Pb's toxicity can thereby vary. This in part explains why some organ systems (i.e. nervous system) are more sensitive to Pb exposure than other systems.

PB SOURCES AND AVIAN SPECIES

Pb ammunition and avian species

Avian wildlife encounter Pb as a direct result of the human defense and hunting-related activities. In particular, foraging avian species often consume particulate Pb in the form of spent shotgun pellets or bullet fragments, confusing them for grit or food (Mateo et al. 2007; McConnel 1967; Schulz et al. 2002). It has been estimated during the 20th century alone that approximately 3 million metric tons of Pb were deposited in the form of spent ammunition in the United States (Craig et al. 1999). According to a 1999 estimate, approximately 60,000 additional tons of Pb are deposited in high concentrations each year in the top few inches of soil at private and military shooting ranges as well as recreational hunting sites throughout the United States and other countries (Craig et al. 1999). According to Pain (1991) Pb pellet distribution in Camargue, France (Rhone Delta) was as high as two million pellets/hectare and over a two-year period studying settlement of the pellets, concluding that the half-life of the pellets in the top 6 cm of soil was 46 years. Therefore, not only are certain avian species at risk of exposure to this unique form of Pb, but they are also in a unique situation of elevated risk for multiple Pb pellet or fragment exposures in these select sites.

Pb exposures from sources other than Pb ammunition

Although Pb ammunition is a predominant source of Pb pollution in the environment, wildlife species are also exposed to Pb from other sources. Cai and Calisí (2016) examined 825 blood Pb level records from visibly ill feral pigeons in different neighborhoods of New York City collected during 2011-2015, a city where Pb ammunition would likely be a minimal source of environmental Pb. Their data showed that the area of the city where each pigeon was found did not influence the blood-Pb levels however, the blood-Pb levels in the pigeons were higher in summer months compared to any other time of the year (Cai and Calisí 2016). The most interesting finding was that increased blood-Pb levels in pigeons during summer months positively correlated with the blood-Pb levels >10 μg/dL of children, suggesting that continued monitoring of feral pigeons in New York City for Pb could help determine the risk for further human and wildlife exposure as well as guide efforts to decrease it (Cai and Calisí 2016).

Common sources of Pb contamination for avian species are Pb tackle and fishing weights. Sidor et al. (2003) analyzed 522 common loons (*Gavia immer*) found dead in New England from 1987-2000. The authors determined that 50% of the adult loons had died from Pb toxicity induced by consumption of Pb fishing tackle (Sidor et al. 2003). A similar case study examined three loons found dead in the Northeastern United States. Two of the three birds had fishing line sections with Pb weights still attached in their venrticulus, while the third loon had a fishing line with no weights (Locke 1982). All three loons had significantly increased Pb concentrations in the liver and death was attributed to Pb toxicosis (Locke 1982). A similar study in Canada investigated the death

of six loons found on the shore of a lake (Daoust et al. 1998). The authors reported Pb fragments from sinkers in the ventriculus of 4 of the 6 loons and all six had significantly increased Pb concentrations in the kidneys that were comparable to known Pb poisoning concentrations (Daoust et al. 1998). Other wild aquatic avian species have been found with Pb fishing tackle in their ventriculus including brown pelicans (*Pelecanus occidentalis*), Trumpeter swan (*Cygnus buccinator*), and a number of duck species (Locke et al. 1982; USEPA 1994; Franson et al. 2003).

Another source that contributes to heavy metal environmental pollution including Pb is mining operations. In 2013 Beyer et al. examined the songbird populations in Missouri near a Pb mining operation. It was already documented that the Pb mining had yielded highly increased Pb concentrations in the soil surrounding the facility. The rationale for the study was that bird populations consume earthworms living in the contaminated soil. The researchers showed that the songbirds in this region had Pb levels increased by a factor of 8, 13, and 23 in the blood, liver, and kidney respectively, as compared with songbirds in uncontaminated regions of Missouri (Beyer et al. 2013). Further, the δ-ALAD activity in these birds was decreased by approximately 50-80% (Beyer et al. 2013). Based on these results, the authors concluded that the health of bird populations living in areas near the mines were at high exposure risk to the Pb and other heavy metals being extracted. As with mining operations, industrial smelting releases numerous metals into the environment through emissions. In Finland, liver accumulation of heavy metals including Pb in passerine birds was reduced by 58-95% following reduction of atmospheric emissions from the country's largest nonferrous smelter (Berglund et al. 2012). Mallards given a commercial pellet diet containing 24% sediment

from the contaminated Coeur d'Alene River Basin in Idaho (3400 μg Pb /g of sediment) had increased breast muscle atrophy, viscous bile, and increase in nuclear inclusion bodies in renal tubular cells (Heinz et al. 1999).

Another study attempted to link bird exposure to Pb by way of natural Pb concentrations in the environment rather than anthropogenic sources. The authors used the Alaskan Tundra swan as the model for their experiment based on their migratory patterns in relation to Pb contaminated sites (Ely and Franson 2014). Previously, it had been shown that, because of their winter migration patterns, Alaskan tundra swans are exposed to Pb in the warmer climates where Pb is prevalent due to human use (Ely and Franson 2014). However, during the summer months these swan populations migrate to northern areas of Alaska where there is very limited human influence. The researchers showed that during the summer months, the swan populations had on average <0.2 mg/mL blood Pb levels compared to winter months where birds had Pb levels ranging from 0.2-0.5 mg/mL (Ely and Franson 2014). These results strongly suggest that the Pb levels seen in avian tundra swan populations are less likely to be attributed to natural occurrences in their environment. Whooper swans wintering in Great Britain with blood-Pb levels ≥4400 mg/mL had significantly decreased winter body conditions, showing that sublethal levels of Pb had a very detrimental impact on the health of the swans during winter months (Newth et al. 2016). These data coupled with the data from the swans in Alaska suggest that migratory birds exposed to anthropogenic Pb during summer and winter months could experience adverse health consequences, even at sublethal doses of Pb.

Pb and Terrestrial Avian Species

Much of the earlier studies addressing potential Pb toxicity in birds were focused on aquatic species, however terrestrial species are also at risk. Kerr et al. 2010 reported that northern bobwhite quail orally gavaged with five Pb pellets (~45 mg/pellet) survived the acute exposure resulted in blood Pb levels that would be lethal to most mammalian species. Reasons for which avian species are more tolerant to higher Pb are not currently known, however may be linked to rapid ability to replenish their RBC and the fact that avian RBCs are nucleated. Kerr et al. (2010) suggested that, while these adult birds appear capable of surviving acute and possible chronic very high levels of Pb, the long-term impact of such environmental Pb exposure on avian reproduction and development remains unclear.

More recently, Holladay et al. (2012) orally gavaged roller pigeons with 1, 2, or 3 Pb pellets (45 mg/pellet). The Roller pigeons showed a higher tolerance to Pb as compared to the Northern Bobwhite quail (Kerr et al. 2010), with no outward clinical signs at blood-Pb levels up to 80 times higher than the control pigeons. The Roller pigeon has a higher tolerance to Pb at these concentrations likely in part, due to its larger body mass compared to the bobwhite quail. Another noteworthy finding from this study was that Pb pellet retention within the digestive tract varied between the two species. In the pigeons, mean pellet retention in the gut extended four weeks into the study whereas in the quail pellet mean retention time was less than two weeks (Holladay et al. 2012). A prolonged retention time of Pb pellets by some species would presumably result in greater deposition of mobilized Pb into the tissues of the birds and therefore pose a different risk of toxicity. Although not yet considered, prolonged retention time of Pb

pellets in some avian species might also result in increased risk of Pb toxicity in predator or scavenger species of those birds. . Another factor that could influence the variation in Pb toxicity observed among avian species is the rate of uptake of Pb through the ventriculous and distal digestive track. Kerr et al. (2010) working with quail and Holladay et al. (2012) with roller pigeons recorded different retention times of the Pb pellets between the two species, but did not assess whether the pellets were broken down, absorbed or excreted. The observed increased mortality of the quail at the same dose of Pb compared to the pigeon could be explained by the quail having a higher uptake of Pb and therefore would have a higher acute toxicity.

A study by Mateo et al. (2001) in Spain found that approximately 11% (12/109 eagles) of the Spanish Imperial eagle population, listed as an endangered species, had Pb pellets in their digestive tract. The study also reported that the Pb concentration in these eagles and the number of eagles containing Pb increased during waterfowl hunting season (Mateo et al. 2001). These data suggest that waterfowl exposed to Pb via consumption, or possibly non-lethal birdshot injury, are consumed by the eagles and thus act as a vector for Pb exposure up the trophic levels. These data are not unique to the Spanish Imperial Eagle. A study by Cruz-Martinez et al. (2012) showed increased Pb levels in the bald eagle in the United States from secondary exposure. These authors analyzed more than 1200 eagles in Minnesota, and found that greater than 25% of the birds had elevated Pb levels that were temporally associated with consuming of deer killed by hunters (Cruz-Martinez et al. 2012). Just as with the eagles of Spain, maximally increased Pb levels were seen during hunting season and linked to Pb ammunition. In addition, these researchers reported that the eagles had elevated copper concentrations (Cruz-Martinez et

al. 2012). Pb ammunition is generally coated with a solid copper jacket, therefore the co-presence of Pb and copper further supported indirect Pb exposure through consumption of deer killed by hunters. Another case of Pb exposure through scavenging was reported in the Iberian Peninsula where three lethargic Griffon vultures (*Gyps fulvus*) were found in the wild and died within 24 hours of being transported to a clinic (Dvm et al. 2016). All three vultures were shown to have blood-Pb levels ranging from 969-1384 μg/dL, liver-Pb levels ranging from 309-1077 μg/dL dry weight, and one vulture was necropsied and had nine Pb pellets in its stomach (Dvm et al. 2016).

The most publicized and likely most recognized case of secondary exposure of avian species to Pb involves the California condor (*Gymnogyps californianus)*. This high trophic predator has been on the endangered list since the 1980's when populations dwindled to 22 birds, but through intensive human management rebounded to approximately 400 birds by 2010 (Snyder and Snyder 2000; Walters et al. 2010). A recent survey by the United States Fish and Wildlife Service (USFWS) in 2015 reported the overall population to be at 435 (265 in the wild and 167 captive). A survey study of the California condors between 1997 and 2010 showed that approximately 50-88% of free flying condors had blood Pb levels exceeding the 100 ng/mL maximum safe level set in place by conservationists (Finkelstein et al. 2012). Approximately 20% of these same birds had blood Pb levels ≥450 ng/mL, which is the threshold set by the Center of Disease Control and Prevention for requiring immediate therapy in children (Finkelstein et al. 2012). In an attempt to alleviate the burden of Pb exposure on the California condor and other wildlife, the California state government passed a law in 2013 banning the purchase and use of Pb ammunition for hunting by 2019 (California Department of Fish

and Wildlife 2017). A recent study examining the risk of Pb exposure in condors reintroduced in California monitored blood-Pb levels of these birds from 1997-2011 (Kelly et al. 2014). Even with the regulations on Pb ammunition during waterfowl hunting season prior to the complete ban in 2013 the authors showed that the blood-Pb levels of the reintroduced birds were significantly higher than those of birds still in captivity and the blood-Pb levels increased with the age of the condor (Kelly et al. 2014). While this action prevents new Pb from being deposited into soil in the form of spent ammunition, it does not impact previous deposited Pb. The long-term outcome of this ban and the effect it will have on California condor won't be known for many years, again, due to the longevity of pre-existing Pb in the environment.

Pb and Aquatic Avian Species

Aquatic avian species such as waterfowl are susceptible to ingestion of spent Pb ammunition much like other foraging avian species. One such case of Pb exposure in aquatic birds occurred within a wildlife management area (WMA) in Kansas in 1979. Workers at the WMA found a total of 79 deceased Canadian geese plus 10 additional geese that were too lethargic to fly (Howard and Penumarthy 1979). Seventeen of the deceased geese were necropsied and were found to have an average of 13 Pb pellets deposited in the crop and ventriculus, high Pb concentrations in the kidney and liver, and other clinical signs of Pb toxicity (Howard and Penumarthy 1979). In 2011-2012 in Argentina, 415 ducks (hunter killed and live captured) were analyzed for Pb exposure (Ferreyra et al. 2014). While only 10% of the ducks had Pb pellets in the digestive tracts, all 415 ducks had detectable bone-Pb levels (Ferreyra et al. 2014). Although the bone-Pb

levels were not representative of Pb toxicity, the presence of Pb in the bone indicates that all birds were exposed to environmental Pb.

Chronic Pb exposure in waterfowl throughout much of the twentieth century coupled with increased Pb deposition in bodies of water during waterfowl hunting season led to a nationwide ban on Pb ammunition for waterfowl in 1991. This was done to help prevent further environmental contamination and loss of waterfowl populations from Pb toxicity, however exposure still occurs through ingestion of spent Pb ammunition legally used in recreational shooting sites and for hunting of other wildlife. Romano et al. (2016) examined wetland areas and rice field for Pb pellet contamination and found a varying degrees of pollution with areas having anywhere from 5.5-141 pellets/square meter. It was determined that in the areas with higher Pb contamination, the water was slightly acidic indicating oxidation of Pb and readily mobilization of Pb into the waterways (Romano et al. 2016). California was one of the first states to implement replacing of Pb pellets with steel pellets. Nonetheless, a study conducted one year after the removal of Pb shot in California found that approximately 20% of the mallards captured at two separate wildlife refuges had elevated blood-Pb levels (Mauser et al. 1990). Ten of the captured mallards died from what appeared to be Pb toxicity and were found to have as few as one Pb pellet in the ventriculus; while one bird had 52 Pb pellets in its ventriculus at time of death (Mauser et al. 1990). One study conducted in 2011-2012 in Spain on the Ebro delta showed that even though Pb shot had been banned since 2003 (thus 8-9 years earlier), approximately 16% of the mallard duck population had ingested Pb in the form of spent shot (a.Vallverdu-Coll et al. 2015). These findings are comparable to an Argentinian study where, of the 415 Mallard ducks sampled in 2012, 10.4% had ingested Pb in the

form of spent ammunition at the time of analysis (Ferreyra et al. 2014). Pb was detected in 100% of the bones analyzed from these same Mallards, and in 60% of the livers (Ferreyra et al. 2014).

A study in Poland that evaluated both Mallards and Coots collected by hunters during 2006-2008, provided evidence of Pb toxicity in both species (Binkowski et al. 2013). Histological analysis showed inflammatory and leukocytic infiltrative lesions consistent with Pb toxicity in the birds, with the liver and kidney most affected (Binkowski et al. 2013). These data again show that Pb exposure in aquatic birds across diverse locations remains a critical problem. As might be expected, additional studies are showing that Pb toxicity is not a problem limited to waterfowl, but also can affect other aquatic avian species, such as marsh and coastal birds. For example, the black-necked stilt is a bird primarily found in the marshes of Texas. A recent conducted in the Upper Texas Coast found that almost 80% of the test population had blood Pb levels exceeding 20 μg/dL (Riecke et al. 2015). Although these blood Pb levels were low compared to other studies, they could still potentially have long-term adverse outcomes on bird population health (Riecke et al. 2015).

Although growing focus is being given to health effects of Pb in avian wildlife species, the co-presence of other environmental contaminants must be considered to truly estimate risk to different bird population health. Two recent studies reported increased levels of mercury, Pb, arsenic, cadmium, chromium, and other metals in two geographically different locations in the United States (Burger et al 2014; St. Clair et al. 2015). The avian species analyzed in these experiments were the Semipalmated sandpipers in Delaware Bay and the Pacific Dunlin on the west coast (Burger et al 2014;

St. Clair et al. 2015). These studies show that not just environmental Pb contamination, but heavy metal contamination in general remains a nation-wide issue.

AVIAN PB TOXICITIES

Organ System Toxicity

Pb is a toxicant that has the ability to affect multiple organs and organ systems in numerous species, including avian species. The classic toxicity seen in all animal models is the depression of delta-aminolevulinic acid dehydratase (δ-ALAD). This hematologic toxicity has been documented for decades and is so specific to Pb that it is routinely used as a biomarker to verify Pb exposure (Pain 1989; Binkowski and Sawicka-Kapusta 2014). Martinez-Haro et al. (2011) showed that mallards and coots with blood-Pb levels as little as 6μg/dL had decreased δ-ALAD activity. Pb in the bloodstream has two fates, excretion or deposition into tissue.

The liver is the largest soft tissue store of Pb in the body and is therefore commonly analyzed in avian Pb toxicity cases and studies (Aloupi et al. 2017; Behmke et al. 2017). Mateo et al. (2003) reported that mallards exposed to dietary Pb, 2 g/kg, for a period of 3 weeks had increased lipid peroxidation in the blood, liver, and bile. There was also a negative correlation with lipid peroxidation and glutathione peroxidase activity in the liver (Mateo et al. 2003). A similar study using Japanese quail showed that a single exposure to Pb shot via oral gavage resulted in significantly reduced glutathione activity coupled with increased lipid peroxidation (Osickova et al. 2014). These results have been shown in diverse avian species studied including vultures, pied flycatcher (*Ficedula hypoleuca*), Canada geese (*Branta canadenisis*) and many more (Mateo and Hoffman 2001; Berglund et al. 2007; Behmke et al. 2017).

The renal system is another target of Pb toxicity. Numerous laboratory studies have used acid-fast staining to show inclusion bodies in the nuclei of renal cells after relatively high levels of Pb exposure (Locke et al. 1966; Kelly et al. 1998). It has also been shown that subchronic to chronic exposures to Pb at lower doses can also result in these inclusion bodies. For instance, a team of researchers in the United Kingdom investigated an urban population of pigeons (*Columba livia*) and found that the majority of birds necropsied had significantly increased nuclear inclusion bodies, all of which contained Pb (Johnson et al. 1982). Further, it was determined that the Pb within the kidneys was trialkyl Pb, most commonly formed from the exhaust of vehicles using fuel with Pb additives (Johnson et al. 1982). Another group of researchers verified that kidneys from mallards exposed to Pb via contaminated sediment not only contained these classic inclusion bodies, but also had decreased glutathione activity and increased oxidative stress in renal cells (Hoffman et al. 2006). Kidney toxicosis from Pb was seen in red-crown cranes (*Grus japonensis*) in northern China indicating Pb exposure is a serious threat to the endangered species (Luo et al. 2016).

Pb exposure, under select conditions, has also been found to alter immune function of avian species and can increase susceptibility to infection. Birds do not have lymph nodes like mammals and therefore the spleen is more critical to the integrity of the immune system. Ferreyra et al. (2015) reported that wild mallards from wetlands in Argentina with known high Pb contamination had significantly lower spleen/bodyweight ratios compared to mallards from other areas. These same birds showed a negative correlation between bone-Pb concentrations and spleen size. Another study monitored a wild population of great tit (*Parus major*) nestlings in a known metal contaminated site,

for four innate immune markers: agglutination, lysis, haptoglobin concentrations, and nitric oxide concentrations (Vermeulen et al. 2015). These researchers showed that among birds displaying the greatest signs of Pb toxicity, the innate immune functional marker most altered was the capacity to induce cell lysis. A similar study evaluated the transfer of maternal Pb to eggs and offspring, including characterizing Pb-induced alterations to the developing immune system (b.Vallverdu-Coll et al. 2015). These researchers collected wild mallard eggs from known Pb contaminated areas and measured eggshell Pb concentrations and duckling blood Pb levels to determine exposure. They then showed a negative correlation with blood Pb levels and the T cell mediated immune response measured with the PHA skin test. After hatching, the F_1 ducklings also had decreased antioxidant activity and increased reactive oxygen species with increased blood Pb levels (b.Vallverdu-Coll et al. 2015). Dietary Pb exposure in adult chickens revealed that after 30 and 60 day exposure, peripheral blood lymphocytes had an increase in mRNA expression of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-a), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS)(Sun et al. 2016). This would suggest that the circulating lymphocytes were being activated by Pb in the blood. The implications from this research may be particularly critical for future Pb research in avian species, because it would imply that maternal transfer of Pb to offspring in the wild may adversely impact the developing immune system of the avian offspring. This in turn may suggest, in areas where Pb contamination is prevalent, that entire populations could be at elevated risk for disease susceptibility. Another team of researchers examined the role of dietary Pb exposure and gene expression of HSP (27, 40, 60, 70, and 90) and inflammatory factors iNOS, tumor necrosis factor-α TNF-α, and

COX-2 in the cartilage of chickens (Zheng et al. 2016). The gene expression of all the HSP and inflammatory factors were increased through Pb exposure, suggesting Pb was causing an inflammatory response in the cartilage and providing evidence that HSP could be used as a biomarker of Pb induced damage (Zheng et al. 2016).

As with mammalian species, avian species are susceptible to neurotoxic effects induced by Pb. Burger and Gochfeld (1994) injected 1-2 day old Herring Gulls intraperitoneally with 100 mg/kg Pb acetate and observed behavior, activity, feeding habits, and general health. Approximately 65% of the Pb injected birds survived to day 14 compared to 96% of the control birds; of the surviving Pb injected birds, significant neurologic deficits were observed through increased stumbling when walking, decreased begging for food, and decreased accuracy when pecking the parent birds bills for feeding (Burger and Gochfeld 1994). Douglas-Stroebel et al. (2004) reported that mallard ducklings given feed containing 24% sediment from the Coeur d'Alene River Basin, a known contaminated river basin with Pb levels reaching 3449 μg/g, had brain-Pb levels 485 ppb. These ducklings were also reported to have lower brain weights and increased oxidized glutathione as compared to the control ducklings (Douglas-Stroebel et al. 2004). The ducklings in the 24% river basin sediment treatment group also had behavioral changes including decreased time swimming and issues with balance and mobility (Douglas-Stroebel et al. 2005). American kestrel (*Falco sparverius*) nestlings exposed to 25, 125, and 625 mg/kg metallic Pb in corn oil had decreased brain RNA to protein ratios and brain δ-ALAD, while brain monoamine oxidase and ATPase were not significantly different (Hoffman et al. 1985). These biochemical changes were more severe in the

nestlings compared to adults or young precocial birds exposed to Pb (Hoffman et al. 1985).

Reproductive Toxicity

Another area of research that has gained increased attention in the past few decades is Pb-induced reproductive toxicity in avian species. It has previously been shown that hens from multiple species, exposed to Pb in the environment or laboratory settings, deposit Pb into the eggshells and egg contents during lay periods (Finley et al. 1976; Burger 1994; Trampel et al. 2003; Burger and Gochfeld 2004; Tsipoura et al. 2011). Vallverdu-Coll (b.2015) showed that developmental exposure to Pb in red-legged partridges caused sex specific and seasonal changes. In particular, the male birds showed increased coloration during spring months, while females showed increased humoral antioxidant activity. The researchers concluded that the males most likely increased coloration to increase chances of breeding while the females increased antioxidant levels to defend against reactive oxygen species commonly produced by Pb.

Veit et al. (1983) orally gavaged ringed turtledoves with four Pb pellets (110 mg/pellet) and examined testicular changes. The birds treated with Pb had significantly lower testes weight and, histologically, displayed significant degeneration suggesting suppressed reproductive capabilities (Veit et al. 1983). Another study examining Pb toxicity showed that male red-legged partridges exposed to Pb shot had altered sperm quality and decreased motility (Vallverdu-Coll et al. 2016). An increase in heat shock proteins (HSP) has been correlated with Pb induced cell damage in avian testis. Huang et al. (2017) showed that selenium administered with Pb in the water of Hyline chickens prevented testicular damage and decreased mRNA expression of HSP70. The researchers

concluded that HSP70 could be a biomarker of testicular damage for Pb in the avian model (Huang et al. 2017).

Vallverdu-Coll (a.2015) reported that mallard hatchlings from the Ebro Delta of Spain with blood-Pb concentrations greater than 180 ng/mL died within one-week post hatch. These hatchlings were collected as eggs and were not exposed to Pb in the laboratory setting, therefore these blood-Pb levels were a result of maternal deposition. Another study conducted by the same group evaluated red-legged partridge (*Alectoris* rufa) hens gavaged with three #6 Pb pellets (~109 mg/pellet), and showed a decrease in hatchability of eggs laid (Vallverdu-Coll et al. 2016).

Ruiz et al. (2016) examined dietary Pb exposure on great tit nestlings and the role of vitamins A, D3, K, and E, which are essential for growth and development. The researchers concluded that the nestlings retained higher concentrations of plasma vitamin A in an attempt to counter the stress induced by Pb during growth and development (Ruiz et al. 2016). Sediment contaminated with Pb is another dietary exposure in avian species (Beyer et al. 2000; Hoffman et al. a.2000). Hoffman et al. (a.2000) fed day old mallard ducklings feed mixed with 0, 12, and 24% sediment diet (3449 μg Pb/g sediment) from the Coeur d'Alene River basin. At 6 weeks-of-age, the ducklings in the 24% diet had liver and kidney Pb concentrations of 7.92 and 7.97 ppm respectively and a 40% reduction of hepatic glutathione and nuclear inclusion bodies in renal cells (a. Hoffman 2000). These results were duplicated in Canada geese receiving an identical diet with an additional group receiving a 48% diet (b. Hoffman et al. 2000). The geese receiving the 48% diet had liver and kidney Pb concentrations of 6.57 and 14.93 ppm respectively with one gosling suffering from subacute renal tubular nephrosis (b. Hoffman et al. 2000). The

collective available results therefore suggest that both hens and cocks of avian species are susceptible to gender specific reproductive toxicities after exposure to Pb. Further, maternal Pb can be deposited in the eggs, with resulting effects on the development and survival of the hatchlings.

One thing to note is that hatchlings of altricial species such as passerines and raptors are more sensitive to Pb exposure than hatchlings of precocial species since they are less physiologically advanced at hatching and thus, totally dependent on parents for feeding similar to mammalian neonates. Thus, this may constitute one of the most sensitive periods of the avian life cycle with respect to Pb and other environmental contaminants (Hoffman et al. 2003).

DIAGNOSTIC AND THERAPEUTIC PROTOCOLS FOR BIRDS WITH ELEVATED BLOOD PB LEVELS

The least invasive technique for diagnosing Pb exposure in avian species is radiographic imaging. Radiographs can be performed in at risk avian species such as the condor to determine if radio-dense Pb is present in the crop or stomach (Redig and Arent 2008). In clinical avian cases where orally-consumed particulate Pb is detected, it can be physically removed from the crop and/or stomach, and if Pb is located more distally in the digestive tract then treatment to induce excretion is employed (Redig and Arent 2008). The caveat with radiographs is that if the pellet has already been metabolized or excreted, then it will not be visible and diagnosis of Pb toxicity can only be determined through blood analysis.

The current and most effective treatment for elevated blood Pb levels is chelation therapy. Chelation therapy is used in human medicine to treat a number of issues

including metal toxicities such as Pb, arsenic, and mercury. Selection of an effective chelator requires that the chemical meet certain criteria. These include the chemical's ability to cross biologic barriers to reach metals, effective binding to metals and formation of stable compounds, and upon binding, rendering the metal nontoxic and increasing the excretion from the body (Jones 1994). The common chemicals used for chelation therapy include calcium disodium ethylenediaminetetraacetic acid (calcium EDTA), dimercaptosuccinic acid (DMSA), and dimercaprol (Garcia and Snodgrass 2012). These chemicals are injected directly into the blood stream of exposed individuals and bind Pb to prevent further deposition into tissues and bone, and facilitate metal excretion (Garcia and Snodgrass 2012). Treatment generally involves a combination of the chemicals with dimercaprol being administered first and calcium EDTA following (Garcia and Snodgrass 2012). Chelation therapy is a multiple step process given over a number of days to ensure that blood Pb levels decrease to normal; follow up is also required because chelation therapy does not remove deposited Pb from the body. Thus, deposited Pb from tissue or bone can reenter the blood stream and cause further toxicities necessitating additional treatment (Garcia and Snodgrass 2012).

Chelation therapy has been used to treat birds with high blood Pb levels. Monitored free flying California condors and other endangered avian species with blood Pb levels of 450 ng/mL have been treated using chelation therapy (Finkelstein et al. 2012). The blood Pb level threshold of 450 ng/mL in birds is equivalent to blood Pb level of 45 μg/dL in humans, the value set as an action level for treatment in children (CDC 2012). The fact that condors have reached blood Pb levels that require chelation therapy shows how dire the situation is for these highly-endangered species. The most common

chelator used in avian species has been EDTA. Use of EDTA in humans and other mammals has been shown to be toxic in some cases where increased concentrations of EDTA caused nephrosis and central nervous system toxicity however none of these toxicities have been recorded in avian species, including when using higher concentrations of EDTA over prolonged periods of time (Redig and Arent 2008; Samour and Naldo 2002).

Diagnosing Pb poisoning in wild birds can be challenging, with delivery of test results for blood Pb levels usually requiring a number of days to be returned. One case in Minnesota involved a wild Golden eagle, which lacked the ability to fly or properly perch (Shimmel and Snell 1999). Initially the clinicians diagnosed the eagle as having been exposed to a cholinesterase inhibitor insecticide due to its behavior and leg paralysis and immediately initiated treatment; it wasn't until after four days post-treatment during which the bird showed no clinical improvement, that the clinicians began intramuscular EDTA treatment for Pb poisoning (Shimmel and Snell 1999). Within 24 hr post-EDTA treatment, the eagle started to regain toe movement, however, it wasn't until after six weeks of intramuscular and oral EDTA treatment that blood Pb levels returned to reference intervals (Shimmel and Snell 1999). The original misdiagnosis was due to Pb not being visible in radiographs, which is a common hurdle clinicians face.

Another case in New Zealand involved an Australian harrier found on the side of the road and delivered to an avian rescue center, with the belief that the bird had been hit by a car (Nijman 2016). Just as with the case of the above-described Golden eagle, no Pb was apparent in the radiographs, however the harrier was lethargic and blood-Pb levels were 0.65mg/L, representing the upper limit of the Pbcare Analyzer used by the attending

veterinarian (Nijman 2016). Following immediate intramuscular administration of CaEDTA and days of oral chelation therapy, blood-Pb levels returned to reference levels and the harrier regained energy and mobility (Nijman 2016). The challenge in both cases was that the Pb was systemic and therefore not visualized as particles in the radiographs. The extended treatment using EDTA demonstrates the difficulty in removing sequestered Pb from birds as well as the amount of human effort and time required to treat a bird suffering from Pb toxicity. Although the cost for treating these birds was not provided, the treatment duration would suggest it to be considerable. Further, these cases show, that even with a thorough medical workup, it is difficult to determine when and how often an individual bird may have been exposed to Pb.

A bird's nutritional status is also an important factor that can contribute to Pb reentering the blood via the tissues. Mobilization of Pb and other elements from bone can occur when nutrient demand is high and dietary intake is inadequate. This may have been a contributing factor in the above case involving the clinically ill Golden eagle whose blood Pb levels never dropped below 0.08 ppm following four weeks of EDTA treatment. The eagle had a recorded dietary intake deficiency accompanying the infection (Shimmel and Snell 1999). Thus, this form of semi-chronic or chronic Pb exposure, as a result of Pb mobilization from the tissues, could be an important additional health hazard to avian species such as the endangered condors. Heinz et al. (1999) demonstrated in adult mallards that a total corn diet, which is a nutritionally inferior diet (Jordan 1968), increased the uptake or storage of Pb from contaminated mining sediment in the liver. Pb contaminated sediment ingestion in mallard ducklings generally affected more variables in combination with a less optimal diet with respect to hematological, hepatic, and renal

effects (Hoffman et al., 2000a). Also, Scheuhammer (1997) reported that the level of calcium (0.3% vs. 3.0%) in the diet had a significant effect on Pb accumulation in liver and kidney of birds, with greater Pb accumulation in the tissues of birds with low dietary calcium. Pb toxicity associated with ingested Pb shot has also been shown to be greater in birds on a diet consisting primarily of corn, possibly due to corn's low total protein content and deficiencies in certain amino acids, calcium, phosphorus, and zinc, as well as an increased abrasion of Pb pellets in the gizzard when corn is present (Jordan 1968; Finley and Dieter 1978; Carlson and Nielsen 1985).

ENVIRONMENTAL REMOVAL AND REMEDIATION

As previously stated, a major source of environmental Pb is particulate from spent ammunition concentrated at outdoor shooting ranges, managed hunting areas, and military bases. This particulate Pb is found in the top 1-3 inches of soil leaving foraging avian species and other animals at extremely high potential risk for consumption (Pain 1991). In an effort to prevent spent ammunition from contaminating the environment and poisoning local wildlife, the Environmental Protection Agency (EPA) published a guideline in 2005 titled *Best Management Practices for Pb at Outdoor Shooting Ranges*, which explains a series of management protocols for more sustainable and safe management of spent Pb.

The first management technique recommended by the EPA was to create a containment area for the spent ammunition using "earthen backstops" or other traps to isolate the Pb in a manageable area (EPA 2005). This approach was effective for target shooting, however ranges that offer sport clay shooting do not utilize earthen traps, thus, the pellet dispersal (i.e. hundreds of Pb pellets) is over an open field. To prevent the

pellets from breaking down and leaching Pb into the environment, the EPA has recommended monitoring the pH of the soil and keeping it at a more neutral pH (EPA 2005). Acidic environments break down the Pb pellets and mobilize them into the soil or water nearby, thus increasing the potential exposure of more plant and animal species (EPA 2005). Adding phosphate to the soil prevents additional leaching of Pb due to complexing of phosphate and Pb to form a stable compound, decreasing the mobilization of Pb (EPA 2005). At target and sport clay shooting ranges the most efficient technique for Pb management is removal and recycling. Range owners can remove spent Pb ammunition through raking and sifting as well as other manual techniques (EPA 2005). This is beneficial not only because it removes Pb from the environment, but also because the spent ammunition can be washed and reused, essentially lowering the amount of Pb needed for future ammunition production. At larger target ranges and sport clay ranges, outside third-party companies that specialize in Pb ammunition removal can be hired (EPA 2005). Companies such as these remove the top several inches of soil and remove the Pb using machinery prior to spreading the soil back on the range (EPA 2005). Again, in cases such as this, the Pb can be recycled for the range manager or taken elsewhere by the company to be used. The final management technique issued by the EPA is keeping detailed logs of all shooting activity on the range (EPA 2005). Records of shooting activity allow for a more accurate estimate of how much Pb has been deposited on the range compared to how much is reclaimed through recycling techniques.

In the case of California condor Pb poisoning, outdoor shooting ranges were not the source of Pb. Instead the California condors most likely consumed Pb ammunition deposited in muscle tissue of game birds and mammals (Finkelstein et al. 2012). Hunting

is a nation-wide activity in the United States and the lands where hunting occurs are generally not managed like outdoor shooting ranges. Instead, deposited ammunition is predominantly left in the environment causing local contamination, and animals wounded or killed and not found leave carnivores and scavengers such as the California condor at risk of exposure. The most effective way to prevent Pb from entering the environment through recreational hunting is to commercially remove Pb as a component of ammunition. California has already begun the process of removing Pb ammunition from use in all hunting, which will be completed in 2019, however the remaining 49 states only require non-Pb ammunition for waterfowl hunting. Until all 50 states require non-Pb ammunition for hunting, wildlife will be at ongoing, likely increasing, risk of exposure to Pb in the form of spent ammunition.

CONCLUSION

The physical characteristics and abundance of Pb have made this heavy metal a useful component of building materials, cooking utensils, weaponry, plumbing, fuel additives, and many more products throughout Roman Empire and post-Roman Empire human history. The same characteristics that have attributed to the anthropologic use of Pb contribute to Pb being a persistent environmental toxicant (Battelle and EPA 1998; Dube 2006). As technology has advanced, the uses of Pb have only grown, further compounding the potential of environmental pollution (USGS 2016). Over the last century, the multiple organ system toxicities induced by Pb have been recognized and studied to quantify the level of exposure that will cause no detrimental effect, however the overall outcome of this research has shown that there is no safe level of Pb exposure.

The major effort in Pb research has focused on human exposure and toxicity. This effort was driven by studies during the latter half of the twentieth century, which showed that Pb leached into the environment from automobile emissions and that children could be exposed to Pb via paint chips (McElvaine et al. 1992; Needleman 2000; Bridbord and Hansen 2009). Based on these past studies and the strict laws enacted, human Pb exposure has been greatly reduced and reports of recent human Pb exposure incidents such as the Flint Michigan incident (Craft-Blacksheare et al. 2017; Sadler et al. 2017) are increasingly rare. Currently, the greatest threat of Pb exposure is from the environment and the animals and plants that inhabit it (Table 2.1).

Although California has taken noteworthy steps towards protecting wildlife species, in particular the California condor, additional efforts are needed at the national level. It is currently unknown how many populations of different avian species across the United States are at risk of harm from Pb exposure, or how diverse additional environmental factors may influence that risk. In addition, there are very little data on the effects of sub-clinical Pb exposure on the long-term, trans-generational population health of avian species. This is an area that warrants further investigation. If the protection of all species is a goal at the state and federal level, then the removal of Pb from ammunition is necessary. Further, more aggressive environmental remediation at sites where Pb ammunition has been used in high quantities is crucial to prevent the leaching and spread of Pb into surrounding areas. The breakdown of these bullets into smaller fragments increases the potential for consumption by animal species, especially avian species but also increases the potential for soil, water, and plant contamination, compounding the ability for Pb to enter higher trophic levels.

SUMMARY

This review summarizes historic and recent reports of Pb toxicity in avian wildlife species. These reports show that both aquatic and terrestrial birds are at continued risk of Pb exposure from sources that include spent Pb ammunition, Pb fishing weights, and industry-related contaminated Pb sediments. Avian exposure to such Pb at sublethal doses can cause detectable toxicities in multiple organ systems, with harmful reproductive and developmental effects being of increased recent concern. Diagnosis and treatment of Pb toxicity is costly and generally reserved for only the most endangered of species, particularly California condors and several eagle species. Minimizing further exposures to these and other birds will most effectively be achieved by blocking entry of new Pb into the environment, through measures such as California's ban on Pb ammunition by 2019. Remediation techniques to remove existing Pb from the environment will also help prevent exposure of wildlife, however in most cases will be prohibitively expensive.

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Table 2.1: List of avian species and exposure model of references listed in this dissertation

Species	Latin Name	Exposure Model	Reference
Northern pintail	*Anas acuta*	Environmental	Mateo et al. 2007
Greylag goose	*Anser anser*	Environmental	Mateo et al. 2007
Greater flamingo	*Phoenicopterus ruber*	Environmental	Mateo et al. 2007
Glossy ibis	*Plegadis falcinellus*	Environmental	Mateo et al. 2007
Mourning doves	*Zenaida macroura*	Environmental	Schulz et al. 2002
Northern bobwhite	*Colinus virginianus*	Oral gavage 1, 5, or 10 45 mg pellets	Kerr et al. 2010
Roller pigeon	*Columba livia*	Oral gavage 1,2, or 3 45mg pellets	Holladay et al. 2012
Spanish emperial eagle	*Aquila adalberti*	Environmental	Mateo et al. 2007
Red kite	*Milvus milvus*	Environmental	Mateo et al. 2007
Bald eagle	*Haliaeetus leucocephalus*	Environmental	Cruz-Martinez et al. 2012
Griffon vultures	*Gyps fulvus*	Environmental	Dvm et al. 2016
California condor	*Gymnogyps californianus*	Environmental	Walters et al. 2010
Canada geese	*Branta canadensis*	Environmental	Howard and Penumarthy 1979
Whistling duck	*Dendrocygna bicolor*	Environmental	Ferreyra et al. 2014
White-faced tree duck	*Dendrocygna viduata*	Environmental	Ferreyra et al. 2014
Black-bellied whistling-duck	*Dendrocygna autumnalis*	Environmental	Ferreyra et al. 2014
Rosy-billed pochard	*Netta peposaca*	Environmental	Ferreyra et al. 2014
Brazilian duck	*Amazonetta brasiliensis*	Environmental	Ferreyra et al. 2014
Mallard duck	*Anas platyrhynchos*	Environmental	Vallverdu-Coll et al.

			2015
Coot	*Fulic ralidae*	Environmental	Binkowski at al. 2013
Black-necked stilt	*Himantopus mexicanus*	Environmental	Riecke et al. 2015
Semipalmated sandpiper	*Calidris pusilla*	Environmental	Burger et al. 2014
Pacific dunlin	*Calidris alpina pacifica*	Environmental	St Clair et al. 2015
Black duck	*Anas rubripes*	Environmental	Pain 1989
Common pochard	*Aythya ferina*	Environmental	Martinez-Haro et al. 2011
Black vultures	*Coragyps atratus*	Environmental	Behmke et al. 2017
Turkey vultures	*Cathartes aura*	Environmental	Behmke et al. 2017
Pied flycatcher	*Ficedula hypoleuca*	Environmental	Berglund et al. 2007
Red-crowned cranes	*Grus japonensis*	Environmental	Luo et al. 2016
Great tit	*Parus major*	Environmental	Vermeulen et al. 2015
Red-legged partridge	*Alectoris rufa*	1 or 3 109 mg pellet oral gavage	Vallverdu-Coll et al. 2015
Broiler chicken	*Gallus gallus domesticus*	0.5 mg/kg or 350 mg/kg dietary Pb acetate	Sun et al. 2016
Ringed turtle dove	*Streptopelia risoria*	4 100mg Pb pellet oral gavage	Veit et al. 1983
Common loon	*Gavia immer*	Environmental	Sidor et al. 2003
Brown pelican	*Pelecanus occidentalis*	Environmental	Franson et al. 2003
Double-crested cormorants	*Phalacrocorax auritus*	Environmental	Franson et al. 2003
Black-crowned night herons	*Nycticorax nycticorax*	Environmental	Franson et al. 2003
Northern cardinal	*Cardinalis cardinalis*	Environmental	Beyer et al. 2013
American robin	*Turdus migratorius*	Environmental	Beyer et al. 2013
Tundra swan	*Cygnus columbianus*	Environmental	Berglund et al. 2012

Whooper swan	*Cygnus cygnus*	Environmental	Newth et al. 2016
Golden eagle	*Aquila chrysaetos*	Environmental	Redig and Arent 2008
Saker falcon	*Falco cherrug*	Environmental	Samour et al. 2002
Peregrine falcon	*Falco peregrinus*	Environmental	Samour et al. 2002
Lanner falcon	*Falco biarmicus*	Environmental	Samour et al. 2002
Swamp harrier	*Circus approximans*	Environmental	Nijman 2016

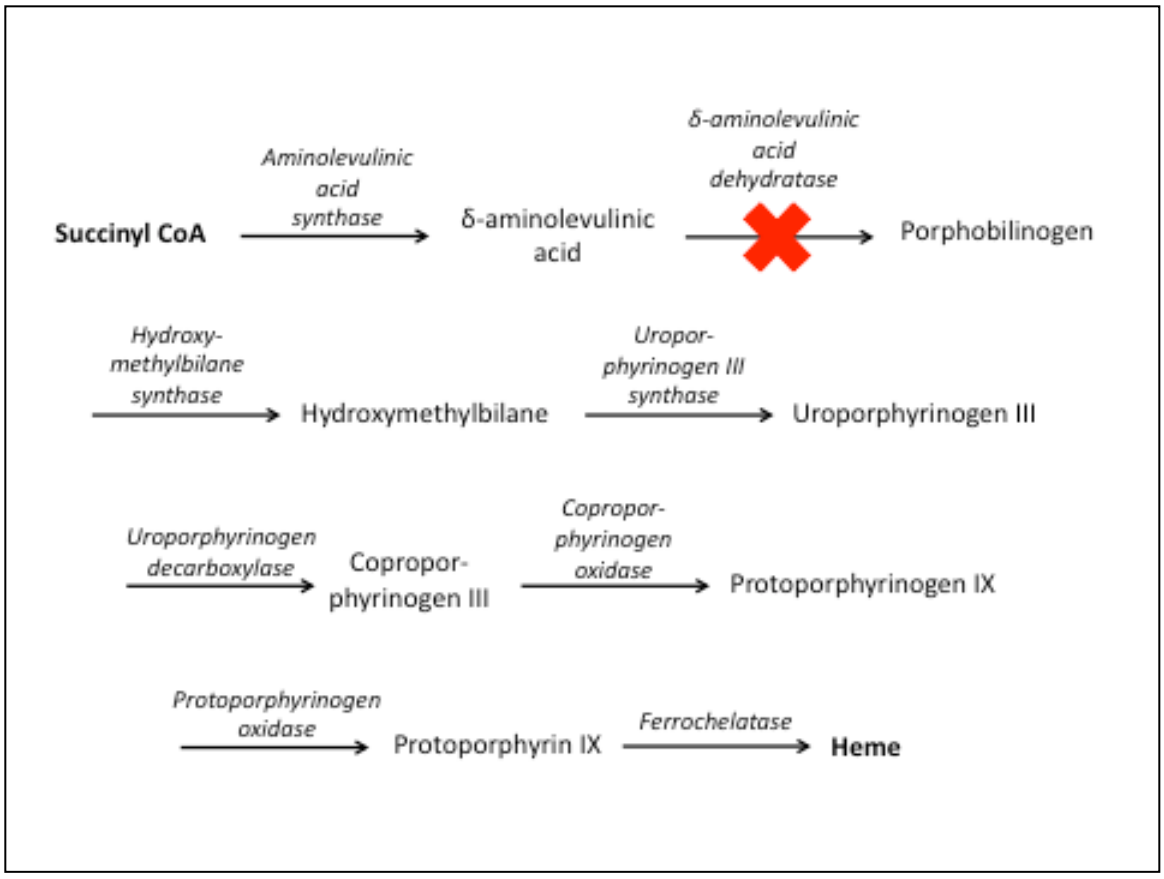

Figure 2.1: Inhibition of Heme Synthesis by Pb

The red X indicates the inhibition of heme synthesis through Pb binding and suppressing δ-aminolevulinic-acid dehydratase.

CHAPTER 3

INGESTION OF A SINGLE 2.3 MM PB PELLET BY LAYING ROLLER PIGEON HENS REDUCES EGG SIZE AND ADVERSELY AFFECTS F1 GENERATION HATCHLINGS[2]

[2] Williams RJ, Tannenbaum LV, Williams SM, Holladay SD, Tuckfield RC, Sharm A, Humphrey DJ, Gogal RM Jr. 2017. *Archives of Environmental Contamination and Toxicology*. 73(4): 513-524.
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ABSTRACT

Many aquatic and terrestrial avian species inadvertently ingest lead (Pb) in the form of spent or fragmented ammunition, mistaking it for food or grit. Previous studies in our laboratory have shown that ingestion of even a single 45 mg pellet can significantly increase blood-Pb levels and significantly inhibit the enzyme delta aminolevulinic-acid dehydratase (δ-ALAD) for a period of greater than four weeks. In the current study, proven breeder pairs of domestic Roller pigeons were housed in individual cages. The hens were orally gavaged with dH_2O vehicle, a single #9 Pb pellet (2.0 mm/45 mg), or a single #7.5 Pb pellet (2.3 mm/95 mg), placed back with the cock bird and allowed to mate for two consecutive clutches. The eggs were monitored for fertilization, shell damage, egg weight and length during the 16-18 day incubation period. Hatchlings remained with the hen and cock through the weaning period (28-35 days post hatch) and were monitored for weight, development, and mortality. Weanling blood was collected for blood-Pb levels, δ-ALAD activity, red blood cell counts, total protein, and packed cell volume. Following euthanasia, weanling liver, spleen, kidney, sciatic nerve, thymus, and brain were collected for histopathology. Egg weight and length were significantly decreased in the #7.5 Pb pellet treatment group for the first clutch, and hatchling weight 7 days post hatch was also significantly less in the #7.5 Pb pellet treatment group during the first clutch. Histopathologic analysis showed increased lesions in liver, kidney, spleen, and thymus of the Pb treatment weanlings, during both the first and second clutch compared to the non Pb-treated weanlings. These data suggest that maternal consumption of a single 95 mg Pb pellet can adversely impact egg size and hatchling organ development.

INTRODUCTION

Lead (Pb) toxicity is an area of research that has regained attention in recent years, due to cases such as the citywide contamination of Flint Michigan drinking water in 2014 through 2015 (Hanna-Attisha et al. 2014). The United States has issued increasingly stringent regulations over the last four decades on inclusion of Pb in consumer products, due to the discovery of potentially severe toxicity in children (Needleman 2000). While much of the Pb entering the environment has been reduced with the removal of Pb from paints, pipes, and gasoline, Pb ammunition still poses a serious threat to a number of species. Annually >12 million Americans participate in outdoor target shooting and it was estimated in the late 1990's that approximately 3 million metric tons of spent ammunition from shooting ranges and recreational hunting grounds were deposited in the soil during the twentieth century alone (Craig et al. 1999; Peddicord and LaKind 2000). European data similarly showed that wetland areas can contain >100 Pb pellets per square meter in the top 20 cm of soil (Mateo 2009). Of the species potentially exposed to Pb in the form of spent ammunition, >120 avian species, both terrestrial and aquatic, are at heightened risk since these species are known to mistakenly ingest spent ammunition and bullet fragments as a food source or grit (Bennett et al. 2007; Haig et al. 2014; Pain et al. 2009).

Environmental exposure to high levels of Pb or multiple exposures to low levels of Pb can be lethal to aquatic and terrestrial avian species (Taggart et al. 2008; Kerr et al. 2011). Furthermore, environmental concentrations to which many species are exposed can cause a number of sub-lethal toxic effects in diverse organ systems. A highly-sensitive biomarker of Pb toxicity is suppression of δ-aminolevulinic acid dehydratase (δ-

ALAD) activity, an enzyme involved in heme synthesis. Such suppression can cause anemia in mammalian species including humans. Recent studies have shown that δ-ALAD activity is severely depressed following oral exposure to a single 45 mg Pb pellet in two separate avian species, the northern bobwhite quail and the Roller pigeon (Kerr et al. 2011; Holladay et al. 2012). A study conducted in Argentina showed that free ranging ducks exposed to environmental Pb had similar depression of δ-ALAD activity as well as significantly increased bone-Pb concentrations, the latter indicating prolonged exposure (Ferreyra et al. 2015). These same researchers found that ducks with higher levels of Pb in their bones also had significantly reduced spleen size, suggesting that the immune system could be compromised in these birds. Regarding possible immunosuppression in birds exposed to Pb, a study in the red-legged partridge showed significantly reduced T cell proliferation in birds sub-lethally dosed with Pb shot pellets (Vallverdu-Coll and Lopez-Antia et al. 2015).

Another study investigating the influence seasonal changes might contribute to Pb induced immune changes in red-legged partridges found that while Pb increased the T cell PHA response in fall and spring, the T cell independent humoral response was decreased in the autumn, indicating that both the cell mediated and humoral immune responses are targets for Pb (Vallverdu-Coll and Ortiz-Santaliestra et al. 2015). Interestingly, the researchers showed that during Spring, oxidative stress was increased in both male and female birds, however the response was sex dependent (Vallverdu-Coll and Ortiz-Santaliestra et al. 2015). These data were replicated in mallard ducks from the Ebro Delta (Spain) by the same team of researchers that showed environmentally relevant

concentrations of Pb caused sex dependent changes in antioxidant ability and oxidative stress, particularly during mating season (Vallverdu-Coll and Mougeot et al. 2015).

Pb is particularly toxic during development in mammalian species. Limited studies have examined, however, potential developmental toxicity of Pb in avian species. It has previously been shown that maternal Pb is deposited into the egg-shells during the laying period of both terrestrial and aquatic avian species (Burger and Gochfeld 2004; Tsipoura et al. 2011). Vallverdu-Coll and Lopez-Antia et al. (2015) collected mallard eggs from wild nests in the Ebro Delta of Spain, where Pb contamination from spent ammunition is high. Birds with blood-Pb levels of >100 ng/mL were found, and had significantly smaller bodies, increased deposition of Pb in liver and bone, increased Pb concentrations in their respective eggshells, and all failed to survive past post-hatch day seven. Another study evaluating Pb influence on avian reproduction showed that red-legged partridge (*Alectoris* rufa) hens gavaged with three #6 Pb pellets (~109mg/pellet) had a reduction in hatching rate (Vallverdu-Coll et al. 2016). Hatchability was also decreased in mourning doves (*Zenaida macroura*) when hens were exposed to a signle #8 Pb pellet (~70mg)(Buerger et al. 1986). Results from these studies indicate that maternal transfer of Pb into the developing bird can significantly impact hatchability, growth, and survivability in multiple avian species of different ecosystems.

The aim of the present study was to determine the extent of maternal transfer of known amounts of gavaged Pb to the offspring, and evaluate growth and development of the hatchlings through the weaning period. A previous study conducted in our laboratory showed the dosing of adult roller pigeons with a single 45 mg pellet significantly increased blood-Pb levels to approximately 20x of control, four weeks after exposure,

while suppressing δ-ALAD throughout the four-week study duration (Holladay et al. 2012). Based on these results, our hypothesis for the current study was that exposure of laying Roller pigeon hens to even a single Pb pellet would negatively impact reproductive outcome and growth in F1 progeny.

MATERIALS AND METHODS

Animals

Eighteen breeder pairs of Roller pigeons approximately three years of age were obtained from a private breeder (Color Pigeons, Kinston North Carolina, USA) and were randomly assigned to individual cages (one male and one female/cage) in a standard flock house at the University of Georgia Poultry Diagnostic Research Center (UGA PDRC, Athens Georgia, USA). The birds were maintained at approximately 23°C on a 24hr light cycle to increase probability of reproduction. Birds were given a diet consisting of a mixture of 30% laying pellet, 30% whole wheat, and 40% whole corn. Water was supplied *ad libitum* via two-gallon jugs. The University of Georgia's Institutional Animal Care and Use Committee approved all animal procedures performed before study initiation, and experiments were conducted in compliance with Good Laboratory Practice (GLP) standard operating procedures.

Pb Pellet Preparation

Number 9 (spherical, 2mm, 45-50mg) and number 7.5 (spherical, 3mm, 90-95mg) Pb shot pellets were supplied by the U.S. Army Institute of Public Health. All pellets were rinsed with dH_2O to remove any debris from the surface and then weighed to confirm weight.

Pb Pellet Administration

The day of arrival, all birds were weighed and randomly assigned to one of the three treatment groups. The hens were orally gavaged in the crop using rubber tubing attached to a 2mL syringe. Control hens received approximately 1mL of dH_2O while treatment birds received a single #9 or #7.5 Pb pellet in 1mL of dH_2O. Following gavage birds were briefly restrained to ensure the pellet was not regurgitated.

Radiographs

Birds were radiographed immediately after pellet administration to document the presence and location of Pb pellets in the gastrointestinal tract. Direct digital radiography was performed in the PDRC flock house using a portable X-ray unit (MinXray-HF8015+dlp UltraLight) and Canon radiography image detector plate (EDR3 MKII Sensor Panel, Manufacture date: June 2007). The X-ray tube was mounted on a tripod and the focal film distance was 28 inches. The birds were placed in a conical plastic restraint device and radiographs were obtained in left-right lateral projections (50 kVp, 10mAs – 15mA, 0.7s). A single radiograph was obtained for each bird. Appropriate radiation safety procedures were observed for personnel with respect to time, shielding and distance.

Breeding

Following the radiographs, the breeding pairs received minimal human interaction to limit unnecessary stress and increase the likelihood of successful breeding. The focus was on examining the effects of a single acute maternal Pb exposure prior to breeding on two consecutive clutches (one-two eggs/clutch). In this article, eggs from the first clutch

from each treatment group were referred to as the “first clutch” and eggs from the second clutch from each treatment group were referred to as the “second clutch”.

Eggs

The cages were checked daily for the presence of new eggs. Each egg was briefly removed from the cage, visually inspected for cracks or imperfections, and dated. The weight in grams (g) and length/width in centimeters (cm) were recorded for all fertilized eggs. All recoverable eggshells were gathered following a hatch, broken egg or unhatched egg, and stored at 4°C for Pb analysis.

Hatchlings

Hatchlings were monitored daily for behavioral or physical changes. Body weight was recorded in grams seven days post-hatch and at weaning (28-35 days post hatch) just prior to euthanasia. All hatchlings remained in the cages with the hen and cock until euthanasia.

Blood Collection

Blood (~1.5 mL) was collected aseptically from the wing vein (V. cutanea ulnaris) using a 27-gauge needle attached to a 3mL syringe. Immediately following the collection, the needle was removed to prevent hemolysis and the blood was transferred to a heparinized Vacutainer tube and gently inverted to ensure mixing of the blood and heparin.

Delta-aminolevulinic acid dehydratase

Whole blood aliquots stored at -80°C were used for Delta-aminolevulinic acid dehydratase (δ-ALAD) activity. δ-ALAD activity was analyzed using the European

Standardized method (Berlin and Schaller 1974), and results were expressed as nanomoles of aminolevulinic-acid/min/mL red blood cells.

Blood-Pb analysis

Blood-Pb levels were analyzed by the Plasma Chem Lab of the Center for Applied Isotope Studies at the University of Georgia (Athens, Ga). One hundred fifty micoliters of whole blood were transferred to Savillex digestion beakers and incubated overnight with 300 μl of trace metal grade HNO_3. Following overnight incubation, the whole blood solution was heated to 90°C for 3 hrs. Next 300 μl of trace metal grade H_2O_2 were added and heated to 90°C for 1 additional hour. Samples containing precipitate were treated with another 150 μl of HNO_3 and incubated at 90°C for 1hr. Samples were then transferred to 15 mL conical tubes and diluted with 15% trace metal grade HNO_3 to 5 mL total volume and analyzed using ICP-MS on a Plasma Quad VG PQ Excell. The machine was calibrated using certified Pb standard. The standards ranged from 0.98-500 ppb with a "blank" that read zero. All samples were analyzed quantitatively and fell within the measurable range. The dilutions were factored in to give the final concentrations shown (Table 3.1).

Eggshell and feather Pb concentration analysis

Eggshells and weanling feathers were sent to Lawrence Tannenbaum at the US Army Institute of Public Health (Aberdeen Proving Ground, Maryland). The eggshells were ground using a mortar and pestle to better analyze the samples while the feathers were left whole. For a Pb reference, a new #7.5 Pb pellet was scanned and produced a reading of 4545 ± 55 mg/kg. A commercial egg was tested for a negative control and got

a reading of <12 mg/kg. All samples were analyzed using a handheld DELTA Element XRF analyzer (Olympus Corporation). Results were expressed as parts per million (ppm).

Histopathology

Weanlings were euthanized between days 28-35 using CO_2 asphyxiation, and necropsied. The tissues collected included the spleen, liver, kidney, thymus, sciatic nerve, and brain. All tissues were embedded in paraffin, sectioned, and stained using hematoxylin and eosin. A portion of the liver and one kidney were collected and stored at -80°C for further analysis if necessary.

Statistics

All data except the histopathology data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). Significance among the groups was determined using Tukey-Kramer multiple comparisons one-way ANOVA. The histopathology data were analyzed using a two-sample test of the equivalence of binomial proportions. The data were run in Graphpad Prism 6 using the chi-square contingency table to test the hypothesis that the proportion 1 (p1) of pigeon with lesions under a Pb treatment group is no different than the proportion 2 (p2) of pigeons with lesions in the control group. This is represented by the null hypothesis:

H_0: p1 = p2

Versus the alternative hypothesis that these two proportions are different:

H_α: p1 ≠ p2

Significance for all statistics was determined by a *p* value less than or equal to 0.05 ($p \leq 0.05$).

RESULTS

Radiographs

All twelve hens gavaged with a Pb pellet, which was visible as a metallic opacity (bright white) in the digital radiographs evaluated by a board certified radiologist (co-author, A. Sharma), showed placement primarily in the crop (radiographs not shown).

Hen blood-Pb levels and δ-ALAD activity

The blood collected from the hens seven days post gavage was analyzed for Pb levels and δ-ALAD activity. The #9 pellet treatment hens had elevated blood-Pb levels, however not significant while the #7.5 treatment hens had significantly increased blood-Pb levels (p=0.036) (Table 3.1). Similarly, the δ-ALAD activity of both the #9 treatment hens and the #7.5 treatment hens was significantly decreased compared to the control hens (Table 3.1).

Egg production

The eggs were monitored daily through the incubation period (day 16-18 post lay) for cracks/breaks and were candled to determine if fertilization occurred. The eggs of the first clutch were laid 1-6 weeks post Pb exposure (Table 3.2**)**. The eggs of the second clutch were laid 7-16 weeks post Pb exposure. There were no significant differences among the treatment groups for egg production, egg fertilization, or number of eggs that hatched throughout the study. The total number of eggs laid for the control, #9 treatment group, and #7.5 treatment group in the first clutch were 16, 11 and 14 respectively. Of the eggs laid in the first clutch, 8, 6, and 7 eggs of control, #9 treatment group, and #7.5 treatment group were fertilized and 6, 5, and 5 respectively hatched. The total number of eggs laid for the control, #9 treatment group, and #7.5 treatment group in the second

clutch were 10, 12, and 11 respectively. Comparative to the first clutch, 6, 5, and 6 eggs of the control, #9 treatment, and #7.5 treatment groups were fertilized and 4, 4, and 5 eggs respectively hatched. These data did not yield any significant differences among treatment groups nor clutches.

Egg parameters

All fertilized eggs were weighed plus length and width measured to determine if Pb altered the dimensions of the egg. The egg data from the first clutch showed a significant decrease in the weight and length of the eggs from hens in the #7.5 treatment group (Table 3.3). The width of the eggs produced by the #7.5 treatment hens was numerically but not significantly decreased compared to control. The second clutch egg weight and dimensions showed no significant differences among the treatment groups (Table 3.4).

Eggshell and feather Pb level analysis

The eggshells and feathers were analyzed with a handheld DELTA Element XRF analyzer for Pb concentrations. There were no significant differences among the treatment groups for either eggshells or feathers (data not shown). The values for all samples analyzed were <20 ppm ranging to <12 ppm in all three treatment groups.

Hatchling and Survivability

The hatchlings were checked daily for any physical signs of toxicity including lethargy, loss of appetite (pronounced keel indicating empty crop), impaired movement, and mortality. During the study there were no recorded signs of behavioral differences among treatment groups that would indicate toxicity however there were differences in survivability among the treatment groups (Table 3.5). The control group had 100%

survivability throughout the study while both the Pb treatment groups had mortality during both the first and second clutch.

Hatchling week 1 and final weights

Hatchlings were weighed one week post-hatch to record initial weights. The one-week delay was employed to ensure that handling the hatchling would not cause injury or prevent the hen or cock from brooding. Weights were taken again just prior to necropsy to record the final weight of the offspring and measure the weight gain over the weaning period. The first clutch hatchlings from hens that received a single #7.5 Pb pellet were significantly smaller (p=0.037) than the first hatchlings from the control hens (Table 3.6). The #7.5 treatment weanlings were numerically but not significantly smaller than the control weanlings at necropsy. There were no significant differences among treatment groups during the second clutch.

Weanling Blood Parameters

The blood collected from the weanlings was used to determine total blood cell counts, packed cell volume, and total protein. No significant differences were seen among the treatment groups for either clutch. The control weanling values in the first clutch for total blood cell counts, packed cell volume, and total protein were 2.81($x10^9$/mL) ± 0.32, 32.08 ± 2.33, and 4.88 (g/100mL) ± 0.51 respectively. The second clutch data were comparable to the first clutch.

Weanling δ-ALAD activity

Weanling blood samples were analyzed for δ-ALAD activity. The samples were initially frozen after collection and all samples were run at the same time. The first clutch δ-ALAD activity for control, #9 pellet treatment, and #7.5 pellet treatment weanlings

were 85.0 ± 9.5, 91.4 ± 7.7, and 94.5 ± 6.8 nanomoles of aminolevulinic-acid/min/mL red blood cells respectively. There were no significant differences among the treatment groups in either clutch for weanling δ-ALAD activity.

Weanling Blood-Pb levels

Weanling blood samples were analyzed for blood-Pb levels to determine the deposition of Pb from the hen to the weanling (Table 3.7). There were no significant differences among the experimental groups for the first or second clutch. All samples analyzed in each treatment group ranged from 3.14-5.36 ppb.

Weanling Histology

Weanling spleen, liver, kidney, thymus, sciatic nerve, and brain were all collected for histological analysis. The tissues were analyzed for lesions including necrosis, inclusion bodies, and other standard indicators of toxicity. Severity of lesions was scored by a board certified pathologist (co-author S. Williams) who was blind as to treatment group. The first clutch histology results showed that there were no lesions in the organs of the control weanlings (Table 3.8). However, the #9 treatment and #7.5 treatment weanlings showed that 22.8% of the organs had lesions present. These lesions included severe degeneration of hepatocytes due to glycogen storage in the cytoplasm, severe lymphoid depletion of the cortical regions of the thymus, immature spleen development, lymphoplasmacytic infiltrates in the interstitium of the kidney, and moderate to severe extramedullary granulopoesis of the kidney. The second clutch had results similar to the first clutch (Table 3.9). There was one control weanling that had mild lymphoid depletion however the cause was not determined and the degree of depletion was not thought to be biologically relevant. The #9 treatment and #7.5 treatment weanlings again showed that

22.8% of the organs analyzed had lesions present. These included the same lesions of the liver, kidney, and thymus seen from the first clutch. There were no splenic lesions in the second clutch and only mild depletion of hepatocytes seen in two of the livers of the Pb treated weanlings. One weanling in the #7.5 treatment group showed severe depletion of the cortical regions of the thymus (Figure 3.1).

The lesion data from the two clutches were initially combined and analyzed using a chi-square test. The presence of lesions in the combined Pb treatment groups was significantly increased compared to the control group (z= 3.2303, degrees of freedom= 1, p=0.0012). Each clutch was then analyzed separately using the chi-square test. Lesions in the first clutch were significantly increased in the Pb treatment groups (z=2.7963, degrees of freedom=1, p=0.0052), and showed a near-significant trend toward increase in the second clutch (z=2.9654. degrees of freedom=1, p=0.0851).

DISCUSSION

The radiographs showed retention of the pellet in the crop on day one, however the hen data verified that the pellets moved into the ventriculus and was broken down for absorption. Consistent with previous studies in our laboratory (Kerr et al. 2011: Holladay et al. 2012), blood Pb concentrations were dramatically increased in pigeon hens that had been gavaged with a single pellet. These concentrations were approximately 150-fold above the low ppb background (control) with the #9 pellet and approximately 430-fold above control with the #7.5 pellet, and accompanied by respective blood δ-ALAD activity decreases of approximately 43% and 38%. These data suggest absorption from the gut for systemic mobilization, and suggests the potential for developmental toxicity as Pb is eliminated from laying hens into eggshell or shell contents.

Reproductive toxicity due to Pb exposure has been observed in multiple mammalian species including humans (Pant et al. 2003; Vigeh et al 2011). The present oral exposure of birds to a single Pb shot did not affect number of eggs laid by the hens or hatch rate of those eggs. The first eggs laid by hens dosed with the #7.5 shot were, however, significantly lighter at 83% of control weight. These eggs were also modestly diminished in length and width, with both of these dimensions being 92% of control. Interestingly, Vallverdu-Coll et al. (2016) saw the opposite effect in red-legged partridges where hens gavaged with one #6 Pb pellet produced heavier eggs and hatchlings than control birds. Second clutch eggs from #7.5 shot birds were non-significantly lighter at 93% of control weight. Hen weight data taken throughout the study (data not shown) showed no differences in weight among the hens of each treatment group, therefore the smaller egg size in the #7.5 shot birds couldn't be attributed to smaller hen size. These results suggest developmental toxicity due to Pb shot ingestion that affects egg size, and that predominates in the first clutch, which presumably experienced greater levels of Pb downloaded from the hen.

Previous studies in our laboratory showed significantly elevated blood Pb levels in pigeons for six weeks post exposure to a single Pb shoot (Holladay et al., 2012). Pb levels in these birds remained non-significantly elevated for an additional two weeks. The second clutch in the current study occurred approximately seven weeks post exposure, with results that again suggest blood Pb levels had dropped sufficiently to not affect egg weight, size, or hatch rate. Blood Pb levels were not followed in the present birds, however, to minimize stress that may impact reproduction.

Similar to egg size and weight, post hatchling survival of birds from Pb treated hens appeared to be compromised. All ten of the control birds survived to weaning, whereas 3 of 10 (30%) first clutch birds from Pb exposed hens did not survive to weaning and 2 of 13 (15%) second clutch birds from Pb exposed hens did not survive. In all cases except for the hatchling lost in the #9 treatment group of the second clutch, the hatchlings died within the first ten days post hatch. It was observed that the hen and cock that had ceased brooding the hatchlings that died within the first ten days, such that cause of death was ruled as starvation and exposure. Ceasing of parental care by the hens could suggest behavior changes due to Pb exposure, however, the cocks received no Pb exposure and showed the same change in brooding. Previous studies have shown that inorganic Pb exposure in pigeons can result in behavioral changes however these studies were sub-chronic and chronic exposures and did not examine reproductive behavior (Barthalmus et al. 1977; Anders et al. 1982). Given that the exposure in the present study was a single exposure then it may suggest that the behavioral of the hen and cock bird was parental recognition of hatchlings that were failing to thrive, and therefore not worth the expenditure of energy required for continued brooding. If such is the case, these results would again suggest developmental toxicity leading to increased hatchling mortality, as a consequence of hen exposure to a single Pb shot.

Vallverdu-Coll and Lopez-Antia et al. (2015) recently found that mallard duck hatchlings with blood-Pb concentrations greater than 180 ng/mL died within the first seven days of life, supporting a relationship between hatchling Pb levels and survivability. First and second clutch birds from the present Pb-exposed hens, similar to the eggs from which they hatched, either tended to be of lower weight or were of

significantly lower weight. The present #9 pellet hatchling that survived to post-brooding but then also died, was found to have a crop full of whole seeds and cracked corn indicating that it was consuming food on its own. This bird also presented at necropsy with an enlarged heart and liver and ascites of the abdominal and thoracic cavities. These collective observations again suggest developmental toxicity in offspring caused by excessive levels of Pb in the hens.

Post-weaning blood collected from Pb-exposed hatchlings, just prior to necropsy, showed no significant differences between treatment groups. δ-ALAD activity in these birds was also not different between treatments, suggesting Pb had been at least largely cleared from the blood by 4-5 weeks after hatching. The weanling total blood cell counts, packed cell volume, and total protein were also not altered by Pb. This would suggest that Pb had been cleared from the body, however Kendall and Scanlon (1981) showed that ringed turtle dove (*Streptopelia risoria*) hatchlings exposed to Pb through drinking water and maternal transfer had significantly increased Pb concentrations in bones, liver, and feathers, while the packed cell volume was not affected. This would infer that the Pb in the ringed turtle dove exited the bone into circulation and deposited in the tissues. Therefore, it is possible in the present study that when Pb transferred from the hen to the weanling it could have been cleared from the body or was deposited into the tissues resulting in low blood Pb levels. The half-life of Pb in mammalian blood is about 25 days due to excretion and depositing of Pb into the bones and soft tissues such as the liver (Griffin et al. 1975; Rabinowitz et al. 1976). Pb levels were not determined in these tissues, however, lesions were found in the liver, spleen, thymus, brain, and kidney of 5 of 7 hatchlings from Pb-exposed hens, and no hatchlings from control hens. Liver lesions

typically included formation of large clear vacuoles within the cytoplasm of hepatocytes, and in one weanling were associated with liver hepatocyte degeneration. Binkowski et al. (2013) saw similar degeneration of the liver in free-living adult mallards exposed to Pb. Further, it was previously reported that Pb increases oxidative stress within hepatocytes in multiple avian species, resulting in lipid peroxidation leading to necrosis (Mateo et al. 2003; Wang et al. 2016). Additional lesions in hatchlings from Pb-exposed hens included a much smaller spleen in a bird that also showed severe lymphoid depletion in the cortical regions of the thymus. A second hatchling showed severe thymic atrophy (Figure 3.1) in the absence of changes in the spleen, again suggesting potential for T cell mediated immune suppression from the developmental Pb exposure. Previous studies have also shown Pb-induced thymic changes in developing chickens including depression in DTH (delayed type hypersensitivity), significant decrease in IFN-γ production, and decrease in nitric oxide production (Lee et al. 2001; Lee and Dietert 2003). Renal lesions were noted in both Pb treatment groups but not control birds, in the form of lymphoplasmacytic infiltrates causing interstitial nephritis. Intranuclear inclusion bodies are a hallmark of Pb toxicity in birds and have been seen in a wide variety of avian species (Locke et al. 1966; Beyer et al. 1988). No inclusion bodies were seen in the kidneys in the current study, however inclusion bodies in weanlings have not been observed in any Pb exposure reproductive study. Earlier studies have shown a wide degree of histologic changes due to Pb toxicity in many of the tissues examined in this study (Cory-Slechta et al. 1979; Anders et al. 1982). These studies were conducted in adult pigeons in sub-chronic to chronic exposures. The severity of the lesions was greater in these studies and Pb concentrations were measurable in the tissue but given that the Pb

exposure was daily over a 1-3 month period, this would be expected (Cory-Slechta et al. 1979; Anders et al. 1982). The tissue lesions in the present study indicate that the single pellet exposure in adult hens may not cause toxicity to the hen, but maternal transfer to the developing offspring may cause developmental toxicity leading to the liver, kidney, and thymic lesions.

CONCLUSION

The present experiments were conducted because levels of Pb we previously detected in the blood of birds orally dosed with Pb shot (Holladay et al. 2012), suggested clear potential for developmental toxicity in F1 or subsequent offspring of laying hens. Such developmental toxicity was verified in the form of reduced hatchling size and weight, decreased survivability to weaning, and lesions in diverse organs that might correlate with compromised immune and other systems. The single Pb shot that caused these effects were small, at 2.0 mm diameter for #9 shot and 2.3 mm diameter for #7.5 shot. This compares to the 4.5 mm diameter of the familiar and much larger BB pellet commonly used in air guns, and suggests relative ease with which these particles may be inadvertently consumed during foraging. These results suggest the potential for adverse health effects in wild bird species as a consequence of foraging in Pb shot contaminated areas. Questions are also raised about possible increased risk of mortality at the flock or population level of such birds, should additional stressors such as introduced pathogens or unusually cold winter seasons combine with the Pb shot consumption.

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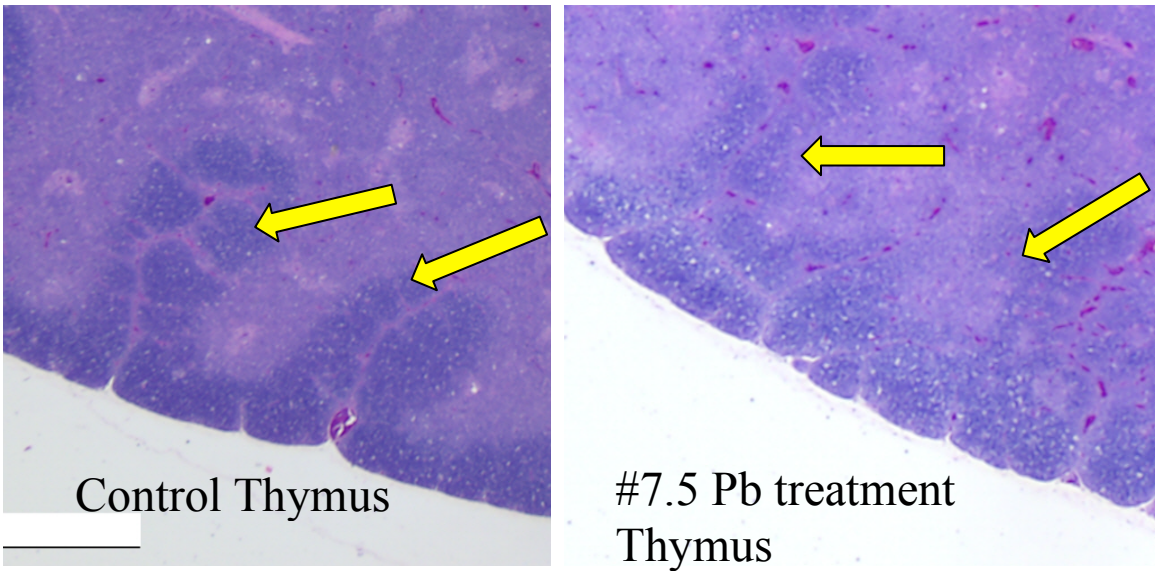

Figure 3.1: Thymic Cortical Degeneration in #7.5 Pb treated weanling

The image on the left shows a healthy thymus in a control weanling. The arrows indicate well defined cortical regions. The arrows in the image on the right show cortical degeneration in a #7.5 Pb pellet treated weanling.

Table 3.1: 7-day post exposure hen blood-Pb levels and δ-ALAD Activity

Table 1 shows the mean blood-Pb levels and δ-ALAD activity from blood collected 7-days post exposure. *$p \leq 0.05$, **$p \leq 0.005$, Tukey Kramer's One Way ANOVA.

	Control (Mean ± SEM, ppb) n=5	#9 Pellet (Mean ± SEM, ppb) n=4	#7.5 Pellet (Mean ± SEM, ppb) n=5
Blood-Pb Levels (ppb)	8.3 ± 2.15 (Range: 2.8 - 15.4)	1237 ± 538 (Range: 650 – 2311)	3549 ± 1311 * (Range: 850 – 7980)
δ-ALAD Activity	87.5 ± 11.0	37.5 ± 2.5 **	33.6 ± 3.3 **

Table 3.2: First clutch egg and hatchling production

Control	Eggs Laid	Fertilized Eggs	Eggs Hatch
Hen 1	4	0	0
Hen 2	3	3	2
Hen 3	1	1	1
Hen 4	2	0	0
Hen 5	2	2	1
Hen 6	4	2	2
Total	**16**	**8**	**6**
#9 Pellet (45mg)			
Hen 1	3	0	0
Hen 2	2	2	2
Hen 3	0	0	0
Hen 4	2	0	0
Hen 5	2	2	1
Hen 6	2	2	2
Total	**11**	**6**	**5**
#7.5 Pellet (95mg)			
Hen 1	0	0	0
Hen 2	3	0	0
Hen 3	1	1	1
Hen 4	4	3	2
Hen 5	5	3	2
Hen 6	1	0	0
Total	**14**	**7**	**5**

Table 3.3: First clutch egg weight, length and width

*$p \leq 0.05$, **$p \leq 0.005$, Tukey Kramer's One Way ANOVA.

Treatment	Weight (g) (Mean ± SEM)	Length (cm) (Mean ± SEM)	Width (cm) (Mean ± SEM)
Control (n=8)	17.9 ± 0.26	3.8 ± 0.03	2.5 ± 0.04
#9 Pellet (n=6)	18.1 ± 0.46	4.0 ± 0.06	2.6 ± 0.04
#7.5 Pellet (n=7)	14.8 ± 1.10**	3.5 ± 0.11*	2.3 ± 0.10

Table 3.4: Second clutch egg weight, length and width

*$p \leq 0.05$, Tukey Kramer's One Way ANOVA.

Treatment	Weight (g) Mean ± SEM	Length (cm) Mean ± SEM	Width (cm) Mean ± SEM
Control (n=6)	18.3 ± 0.4	3.9 ± 0.1	2.6 ± 0.02
#9 Pellet (n=5)	18.1 ± 0.7	4.1 ± 0.1	2.6 ± 0.04
#7.5 Pellet (n=6)	17.1 ± 1.3	3.7 ± 0.2	2.6 ± 0.13

Table 3.5: First and second clutch survivability

	Treatment	Number of Hatchlings Per Treatment	Number of Hatchlings Survive to Weaning Period	Survival to Weaning Period (%)
First Clutch	Control	6	6	100
	#9 Pellet	5	4	80
	#7.5 Pellet	5	3	60
Second Clutch	Control	4	4	100
	#9 Pellet	4	3	75
	#7.5 Pellet	5	4	80

Table 3.6: First and second clutch week one and final weights

*$p \leq 0.05$, Tukey Kramer's One Way ANOVA.

	Treatment	Week 1 Weight (g) (Mean ± SEM)	Final Weight (g) (Mean ± SEM)
First Clutch	Control (n=6)	155.3 ± 10.7	261.5 ± 7.6
	#9 Pellet (n=4)	124.7 ± 5.8	265.4 ± 19.4
	#7.5 Pellet (n=3)	112.3 ± 9.3*	240.2 ± 24.2
Second Clutch	Control (n=4)	155.3 ± 4.7	271.4 ± 8.7
	#9 Pellet (n=3)	121.2 ± 10.5	227.6 ± 34.0
	#7.5 Pellet (n=4)	132.2 ± 12.6	268.3 ± 22.0

Table 3.7: Weanling blood-Pb levels

*$p \leq 0.05$, Tukey Kramer's One Way ANOVA.

Treatment	First Clutch Weanling Blood-Pb Levels (ppb) (Mean ± SEM)	Second Clutch Weanling Blood-Pb levels (ppb) (Mean ± SEM)
Control	4.92 ± 1.21	3.14 ± 0.38
#9 Pellet	3.62 ± 0.40	5.36 ± 2.07
#7.5 Pellet	3.86 ± 0.32	4.07 ± 0.81

Table 3.8: First clutch histology results

The "+" indicates if lesions were present in the organs while the "-" indicates that no lesions were seen. +: mild lesions; ++: moderate lesions; +++: moderate to severe lesions.

Treatment	Spleen	Liver	Kidney	Thymus	Brain
Control	-	-	-	-	-
Control	-	-	-	-	-
Control	-	-	-	-	-
Control	-	-	-	-	-
Control	-	-	-	-	-
Control	-	-	-	-	-
#9 Pellet	-	+++	-	-	-
#9 Pellet	++	-	-	+++	-
#9 Pellet	-	+	+	-	-
#9 Pellet	-	-	-	-	+
#7.5 Pellet	-	-	++	+++	-
#7.5 Pellet	-	-	-	-	-
#7.5 Pellet	-	-	-	-	-

Table 3.9: Second clutch histology results

The "+" indicates if lesions were present in the organs while the "-" indicates that no lesions were seen. +: mild lesions; ++: moderate lesions; +++: moderate to severe lesions.

Treatment	Spleen	Liver	Kidney	Thymus
Control	-	-	-	-
Control	-	-	-	-
Control	-	-	-	-
Control	-	-	-	-
Control	-	-	-	-
Control	-	-	-	+
#9 Pellet	-	+	+	-
#9 Pellet	-	+	+++	-
#9 Pellet	-	-	-	-
#7.5 Pellet	-	-	+	+
#7.5 Pellet	-	-	-	+++
#7.5 Pellet	-	-	-	-
#7.5 Pellet	-	-	+	-

CHAPTER 4

LEAD DISRUPTION OF INTRACELLULAR PROTEIN SIGNALING AND SUPPRESSION OF PRO-INFLAMMATORY ACTIVATION IN TLR4 AND IFNR STIMULATED RAW 264.7 CELLS[3]

[3] Williams RJ, Karpuzoglu E, Connell H, Hurley JD, Holladay SD, and Gogal RM Jr. To be submitted to *Toxicology In Vitro*

ABSTRACT

Lead (Pb) is a persistent environmental pollutant that has been shown to modulate the immune system of exposed individuals. There are many ions with similar structures as Pb that are required for normal cellular function. Pb has been shown previously to compete for protein binding sites with calcium and inhibit signaling pathways within the cell. The current study assessed whether the calcium/calmodulin pathway is a principal target of environmentally relevant Pb during pro-inflammatory activation in the RAW 264.7 macrophage cell line. RAW cells were cultured with 5μM Pb nitrate, LPS, IFN-γ, or LPS/IFN-γ for 12, 24, and 48hr. Intracellular protein signaling and multiple functional endpoints were investigated to determine Pb effects on macrophage function. Western blot analysis revealed that Pb initially modulated nuclear localization of NF-κB p.65 and cytoplasmic phosphorylation of CaMKIV and increased phosphorylation of STAT1 beta at 24hr. Macrophage proliferation was significantly decreased at 12hr in the presence of Pb and NO was significantly reduced at 12 and 24hr. The cytokine results from cells cultured with Pb for 12, 24, and 48hr showed that Pb altered cytokine expression unique to the activation stimuli. Collectively these results indicate that the macrophage pro-inflammatory response is significantly altered by Pb. Further, activation of CaMKIV could be a mechanism of autophagy previously reported in this exposure model.

INTRODUCTION

Pb is a persistent inorganic environmental pollutant that affects humans and animal species worldwide. The United States has set forth many regulations over the past fifty years to decrease future Pb polluting the environment. These include the cessation of Pb being used in consumer products such as pipes and fuels (US EPA 1973; US EPA 1984; Rabin 2008; Bridbord and Hanson 2009), the closure of all primary Pb smelters (Schmidt 2010; US EPA 2010), and limiting the use of Pb ammunition during specific hunting seasons (U.S. Fish and Wildlife Service 1985). Despite these regulations, human Pb exposure continues to exist with the most recent 2014 Flint Michigan water crisis where the entire city was exposed to drinking water with Pb levels as high as 700 μg/mL (Champney 2016; Pieper et al. 2017).

Pb is a divalent cation that has no known biological function in an organism, yet upon entry, has the ability to compete with essential divalent cations in the body for binding sites. Pb has previously been shown to compete with calcium and bind calmodulin at the four EF calcium-binding sites and through opportunistic binding along the central helix of the protein (Kirberger et al. 2008; Kirberger et al. 2013). Calmodulin is a ubiquitously expressed protein located in the cytoplasm of cells that selectively binds calcium. Once calcium binding occurs, calmodulin activates downstream proteins in the cascade such as kinases that further relay the cellular signal (Soderling1999). One class of proteins that is a target for calmodulin is the calcium/calmodulin dependent protein kinases (CaMK) (Wayman et al. 2011). The proteins within this class include CaMKK, CaMKI, CaMKIV, and CaMKII, which are involved in regulation of a vast array of cellular functions (Wayman et al. 2011). The binding of Pb to calmodulin causes

conformational changes, which has the potential to inhibit activation of downstream kinases and thus limit the response to cellular stimuli (Kirberger et al. 2013).

Calcium is an important messenger in all cells of the immune system and is required for proper macrophage activation (Wright et al. 1985; Tintinger et al. 2005; Fracchia and Walsh 2013). Numerous studies have shown that calcium signaling is involved in the macrophage's pro-inflammatory processes and a decrease of free calcium or inhibition of calcium signaling suppresses M1 activation in macrophages (Wright et al. 1985; Raddassi et al. 1994; Murakami et al. 2012; Diler et al. 2014). Proinflammatory cytokine production of IL-6 and TNF-α were suppressed in RAW 264.7 cells when CaMKII was inhibited with KN62 (Liu et al. 2008). A similar study using differentiated THP-1 cells primed with platelet-activating factor and treated with LPS for inflammatory response showed that inhibition of CaMKII significantly decreased NFkB and AP-1 activation and TNF-α production (Cuschieri et al. 2005). The authors also demonstrated that inhibition of CaMKIV produced similar results as CaMKII, but subsequently increased IL-10 production, a potent anti-inflammatory cytokine (Cuschieri et al. 2005; Couper et al. 2008). The ability of macrophages harvested from CaMKII knockout mice to phagocytize *E.coli* was significantly decreased suggesting the CaMKII has an inhibitory role in the macrophage's ability to rid the body of foreign pathogens (Racioppi et al. 2012).

Pb has been shown to negatively impact macrophage function and it's ability to respond to inflammatory stimuli. One key component of macrophage function is migration to site of infection. Studies have shown that *in vivo* exposure of Swiss albino mice injected with 10 mg/kg Pb acetate intraperitoneal for 15 days had splenic

macrophages with significantly decreased chemotaxis (Bishayi and Sengupta 2006). Knowles and Donaldson (1997) reported decreased phagocytosis of sheep erythrocytes by peritoneal macrophages isolated from turkeys exposed to 100 ppm Pb acetate. These results suggest that Pb suppresses migration to and clearance of foreign pathogens. Nitric oxide (NO) production is an innate defense mechanism utilized by macrophages and is a pro-inflammatory response to pathogens (MacMicking et al. 1997). Secreted NO from macrophages binds and disrupts the extracellular membrane of pathogens and can lead to destruction of pathogens. (MacMicking et al. 1997; Aktan 2004). It was shown that 0.625 μM Pb chloride significantly reduced NO production in isolated murine splenic macrophages treated with 500 and 1000 u/mL IFN-γ and TNF-α respectively (Tian and Lawrence 1995). NO production was significantly decreased *in vitro* in RAW 264.7 macrophage cells by Pb acetate when stimulated with LPS (Mishra et al. 2006). Recently Kerr et al. (2013) demonstrated that environmentally relevant concentrations of Pb nitrate had no affect on viability but did induce autophagy in RAW 264.7 cells. This suggests that even without activation, the homeostatic functions of the macrophage are perturbed by Pb exposure and induces catabolism of internal components for cell survival.

The negative impact of Pb mimicking calcium and other cations throughout the body have been well documented. Given that Pb suppresses proinflammatory responses in multiple species and alters cellular response, it would suggest that calcium signaling in cells of the innate and adaptive immune system is directly affected by Pb. The goal of the current experiment was to determine if an environmentally relevant concentration of Pb would suppress mouse peritoneal macrophage response to M1 stimuli. The macrophage was chosen because of its key role in the innate pro-inflammatory response and as a

mediator of the adaptive immune response system through antigen presentation. Downregulation of macrophage M1 activation would impair innate and adaptive immune response, therefore limiting an organism's ability to respond effectively and appropriately to pathogen challenge.

MATERIALS AND METHODS

Cells and Treatment

The RAW 264.7 macrophage/monocyte cell line was purchased from ATCC (Manassas, Virginia). The cells were cultured in TPP (Trasadingen, Switzerland) T75 flasks using Dubelcos Modified Eagle Medium containing 10% FBS and 1% Penstrep. All cells were cultured at 37°C at 5% CO_2 under constant humidity. Throughout the study the cells were subjected to 8 different culture treatments. The treatments were media, 100ng/mL LPS, 10ng/mL IFN-γ, 100ng/mL LPS and 10ng/mL IFN-γ, media with 5μM $Pb(NO_3)_2$, 5μM $Pb(NO_3)_2$ and 100ng/mL LPS, 5μM $Pb(NO_3)_2$ and 10ng/mL IFN-γ, and 5μM $Pb(NO_3)_2$ and 100ng/mL LPS 10ng/mL IFN-γ. The LPS and IFN-γ were purchased from SOANDSO, catalog numbers 5856 and 3939 respectively. The $Pb(NO_3)_2$ was purchased from Abcam (Cambridge, MA, USA). All treatments were prepared prior to use and stored at -80°C.

Cellular Proliferation

Cells were seeded in 96 well tissue culture treated plates (Corning, Corning, NY) at $1x10^5$ cells/well. The cells were cultured with the previously mentioned treatment conditions and at final volumes of 200μl/well and incubated for 12, 24, and 48hr. The cells were cultured with 20μl Alamar Blue for the final four hours of the incubation periods to determine cellular proliferation (Saker et al. 2001). Following incubation,

plates were read at 450 absorbance using a Synergy 4 Biotek (Winooski, VT, USA) plate reader.

Cellular Viability

Viability was determined using Trypan Blue. Cells were seeded at $1x10^6$ cells/well in 6 well tissue culture plates with the previously made treatments. Following incubation of 12, 24, or 48hr, the cells were collected into individual 15mL conical tubes and centrifuged at 130xg. The cells were resuspended in 3mL complete media and a small aliquot of 25µl were removed and placed in a microcentrifuge tube. Next 25µl of trypan blue were added to the tube and thoroughly mixed. Lastly 20µl of the cell/Trypan blue mixture were added to a Nexcelom (Lawrence, MA, USA) chamber slide and the cells were enumerated and viability determined using a Nexcelom Cellometer Auto T4 cell counter.

Griess Assay

Supernanents from the cells cultured for viability, phagocytosis, and DHR were collected in microcentrifuge tubes and frozen at -80°C for later use. A small aliquot of 100µl were used immediately for Griess assay. Fifty microliters of each cell supernanent were pipetted into a well of a 96 well nontissue culture plate. Pre-prepared sulfonamide (50 µl /well) was added to each well followed by 50µl of previously made naph. The plate was incubated at 23°C for 5 minutes to allow for full color change. Following the incubation, the plate was read at 550 absorbance in a Biotek Synergy 4 plate reader. Units were expressed as microgram/microliter.

Phagocytosis and Cytoplasmic Reactive Oxygen Species

Phagocytosis activity was determined using heat killed *Escherichia coli* (*E. coli*). *E. coli* was stained with 100µM propidium iodide (PI; catalog no. P4170, Sigma-Aldrich, St. Louis, MO). The labeling of bacteria was performed as established by Hasui et al. (1989), with certain modifications (Batista et al. 2015).

Cytoplasmic free radical production was assessed using Dihydrorhodamine 123 (DH-R123). A 10µm tock solution of DH-R123 was prepared by dissolving lyophilized powder in tissue culture grade DMSO. The solution was mixed gently and stored in 50µl aliquots at -20C. One 50µl aliquot of stock solution was further diluted in 5ml of each medium to be tested, yielding 10^{-4} M working solution.

The cells collected for the viability measurements were used for the phagocytosis and DH-R123 assay. Following the enumeration, the cells were diluted in each tube to $2x10^{6}$/mL. A 100µl aliquot of the cells were then transferred to a 5mL microcentrifuge tube. Next 50µl of *E. coli* tagged with PI were added to each tube followed by 5µl of DHR-123. The final volume in the microcentrifuge tubes was brought to 500µl by adding 345µl of complete media and the tubes were placed in the incubator at 37°C and allowed to incubate for 30 min. Following the incubation, 2mL of ice cold 3mM EDTA were added to each tube to stop phagocytosis. The tubes were then centrifuged at 400g. The supernanent was discarded and the cells were suspended with 1mL PBS with 0.5% BSA and centrifuged again at 400g. Following the final centrification, the supernatants were discarded and the cells were resuspended in 300µl PBS with 0.5% BSA and placed on ice. The cells were analyzed on an Accuri flow cytometer () and results were expressed as percent cells positive for PI (phagocytosis) and FITC (free radical formation).

RAW cell cytokine expression

A customized cytokine UPLEX kit was purchased from Meso Scale Discovery (Rockville, MD, USA) to quantify the concentrations of IL-1β, IL-6, TNF-α, IL-12p.70, IL-10, IL-13, and VEGF-α in supernatants. These cytokines represent a partial population of the M1 and M2 cytokines produced by macrophages. The M1 cytokines were IL-1B, IL-6, IL-12p.70, and TNF-α while the M2 were IL-10, IL-13, and VEGF-α (Martinez and Gordon 2014). The kit protocol provided by the company was followed for completing the assay. The samples were analyzed on the Meso Scale Discovery MESO QuickPLex SQ 120 and the data were generated as picograms/mL.

Western blot analysis

Cells were seeded at $1x10^7$ cells in T75 flasks with the study treatments and incubated at 37°C with 5% CO_2 and constant humidity for 4 or 24hrs. Cytoplasmic and nuclear protein expression was determined using western blotting (Karpuzoglu et al. 2009; Karpuzoglu et al. 2011) using antibodies specific for: iNOS (Santa Cruz), phosphorylated STAT1 (Santa Cruz), STAT1 (Santa Cruz), phosphorylated CaMKII (Cell Signal), CaMKII (Santa Cruz), phosphorylated CaMKIV (Cell Signal), CaMKIV (Santa Cruz), phosphorylated CREB (Cell signal), CREB (Cell Signal), NF-κB p.65 (Santa Cruz), and β-actin (Abcam) with secondary antibodies (Santa Cruz Biotechnology). All cytoplasmic and nuclear samples were processed under the same conditions and normalized with quantitative Bradford assay (data not shown) before loading to the gel, which reflects equivalent β-actin levels in Western blots. Blots were visualized with Kodak Image Station 4000R using ECL Plus Substrate and Pierce Super Signal. Figure 4.1 is a diagram of the three protein pathways investigated in this study.

Statistics

All data was analyzed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). Unpaired student t-test with a Welch's correction for unequal standard deviations was used to determine the statistical differences between non-Pb and respective Pb groups. Significance was determined by a p value of ≤ 0.05 and is denoted with * throughout the manuscript.

RESULTS

Pb significantly alters RAW cell function without pro-inflammatory stimuli

Cellular viability was not affected by Pb exposure alone at 12, 24, or 48hr indicating that the concentration of Pb used in this study was not overtly toxic to the macrophages (data not shown). However, Pb induced a nonsignificant decrease in macrophage proliferation over the three time points compared to cells in media alone. Culture with Pb alone caused a significant decrease of 15.5% (p=0.05) in OD compared to media alone (Figure 4.2). These data suggest that Pb decreases metabolic function of the macrophage while leaving viability unaltered. Western blot data showed that Pb alone did not significantly alter transcription factor activation in naïve macrophages at 4 or 24hrs. These data indicate that Pb alone does not activate NF-κB p.65 or STAT1 in RAW cells. Further Pb alone did not significantly alter phagocytosis whereas cytoplasmic free radical levels were numerically but not significantly increased. Cytokine production was unremarkable in P-treated cells across all timepoints when compared to cells in media alone (Table 4.1).

Pb significantly suppresses early NF-κB p.65 translocation and functional M1 response in RAW cells stimulated with LPS

LPS is a conserved cell membrane component of gram-negative bacteria that induces a strong pro-inflammatory response in macrophages. LPS cultured with Pb did not affect cell viability, but did numerically lower metabolic proliferation response reaching significance at 48hr (Figure 4.4). At 4hr, Pb significantly decreased nuclear translocation of NF-κB p.65 in cells cultured with LPS (Figure 4.5). By 24hr, the nuclear translocation of NF-κB p.65 was comparable between LPS treatments (data not shown). These data suggest that the concentration of Pb nitrate used in this study negatively regulates TLR4 signaling during initial response, but the macrophages were able to overcome the inhibition. Another explanation could be associated with the concentration of LPS (100 ng/mL) used in this study. This dose was chosen because it is known to induce a strong M1 response in macrophages (Kerr et al. 2013), however it is not a biologically relevant dose. Circulating LPS in humans generally occurs through disease such as inflammatory bowel diseases and levels >1ng/mL are potentially lethal (Brocke-Unte et al. 1988; Dardiner et al. 1995; Guo et al. 2013), therefore at a dose within this range, inhibition of nuclear translocation of NF-κB p.65 could be greater in Pb treated macrophages and may not be overcome so quickly. The decreased translocation of NF-κB p.65 at 4hr positively correlated with significantly decreased production of TNF-α at 12hr in LPS-stimulated macrophages cultured with Pb (Table 4.2). Interestingly, TNF-α production was still significantly decreased at 24hr even when nuclear NF-κB p.65 levels had returned to control levels (Table 4.3). This would suggest that transcription of TNF-α was not occurring at the same rate as LPS-stimulated RAW cells without Pb. At 48hr,

TNF-α production was numerically decreased, but not significantly. Again, this would appear to suggest that the RAW cells were overcoming early PB-induced inhibition of LPS-induced M1 activation.

Phagocytosis and cytoplasmic free radical production in LPS-cultured cells were not altered by Pb at any of the time points investigated in this study. However, LPS did induce nitric oxide production in RAW cells at 12 (Figure 4.10), 24 (Figure 4.11), and 48hrs (Figure 4.12) however, Pb significantly suppressed NO production at all three time points. These results suggest that although Pb does appear to affect pathogen uptake into the macrophage, it does appear to inhibit the destruction and clearance.

Pb differentially alters M1 activation of RAW cells cultured with IFN-γ and 24hr STAT1 activation

At 12hr of incubation, Pb significantly decreased metabolic proliferation in IFN-γ –cultured RAW cells. However at 24 hr, Pb IFN-γ-stimulated Raw cells in the presence of Pb were numerically increased reaching significance at 48hr (Figure 4.4). As before, viability was comparable among the treatment groups at all three time points (data not shown).

Analysis of the IFN-γ/STAT1 pathway indicated that RAW cells cultured with IFN-γ for 4hr had increased cytoplasmic phosphorylation of STAT1 alpha in cells treated with and without Pb (data not shown). Nuclear expression of phosphorylated STAT1 alpha was comparable among Pb-treated and untreated cells inferring that at 4hr Pb had no inhibitory effect on STAT1. Interestingly, phosphorylation of STAT1 beta was significantly increased in the cytoplasm of cells treated with Pb at 24hr, while phosphorylated STAT1 alpha (Figure 4.6) and nuclear phosphorylated STAT1 alpha and

beta remained comparable to the control group. This could indicate that phosphorylation of STAT1 beta was beginning to occur at approximately 24hr incubation and translocation of the protein had not yet occurred.

Functionally, pathogen destruction and clearance in IFN-γ -stimulated RAW cells was significantly suppressed by Pb exposure for up to 24hr. At 12hrs, phagocytosis and NO production was significantly decreased by Pb. At 24hr, phagocytosis was comparable to the non-Pb group, but NO production was still significantly suppressed. NO is produced by iNOS in the cytoplasm of macrophages and iNOS is transcribed by STAT1. Cytoplasmic expression of iNOS was altered by Pb at 4 and 24hr in the RAW cells, therefore function of the protein at 12 and 24hr was inhibited by Pb. By 48hr, phagocytosis and NO production were comparable between Pb and non-Pb IFN-γ groups. This indicates that Pb significantly suppressed the functional responses of pathogen clearance in RAW cells stimulated by IFN-γ.

Pb suppresses M1 activation in RAW cells stimulated with LPS/IFN-γ alternatively than RAW cells stimulated with LPS or IFN-γ alone

Stimulation with LPS or IFN-γ alone was investigated to determine whether Pb alters select M1 activation pathways in macrophages however *in vivo,* the macrophage will receive multiple stimuli during the activation against a pathogen and cytokine interaction. Therefore, the co-stimulation with LPS and IFN-γ was chosen to simulate an *in vivo* pro-inflammatory exposure in the RAW 264.7 cells. Interestingly, LPS/IFN-γ stimulation of RAW cells in the presence of Pb induced some similar responses compared to the individual receptor-stimulated pathways and yet did have some functional changes unique to this culture method.

As with the other individual cultures, 12hr metabolic proliferation was significantly decreased by Pb in cells cultured with LPS/IFN-γ. However, comparable to cells cultured with IFN-γ alone, LPS/IFN-γ stimulated RAW cells cultured with Pb induced significantly increased metabolic proliferation at 24hr which remained elevated at 48hr, however not significant.

Western blot data showed that at 4hr, activation and nuclear translocation of STAT1 was comparable to cells cultured with IFN-γ alone. The increased phosphorylation of STAT1 beta was not seen in LPS/IFN-γ RAW cells cultured with Pb at 24hr therefore, cytoplasmic activation of STAT1 was not altered by Pb when the RAW cells were simultaneously activated through the TLR4 and IFNγR pathways. Also, comparable to 4hr and 24hr LPS-stimulated RAW cells, nuclear translocation of NF-κB p.65 had a decreasing trend in the presence of Pb, but this inhibition was overcome by 24hr. Although decreased nuclear translocation of NF-κB p.65 was present in the RAW cells at 4hr, the Pb associated altered cytokine production, observed in LPS-stimulated RAWS cells at 12hr, was not observed in cells cultured with LPS/IFN-γ. This could be associated with a strong M1 activation mediated by LPS/IFN-γ co-stimulation.

RAW 264.7 cells cultured with LPS/IFN-γ are known to induce increase transcription of iNOS and high production of NO in the supernatant. Western blot data showed strong iNOS expression at 4 and 24hr in cells cultured with LPS/IFN-γ and it was noted that Pb did not alter this expression. At 12hr, NO production was significantly decreased by Pb however by 24hr the production was comparable to untreated control cells. This indicates that LPS/IFN-γ stimulated macrophages were able to overcome NO suppression faster than the cells cultured with each of these stimuli individually.

Phagocytosis was significantly decreased in RAW cells cultured with Pb LPS/IFN-γ at 12, 24, and 48hr (Table 4.5). This was not seen in any other treatment group over the three time periods and 12hr Pb IFN-γ was the only sample time phagocytosis was significantly decreased. Phagocytosis was increased in cells cultured with LPS/IFN-γ compared to the cells stimulated with either stimuli individually indicating that duel stimulation greatly enhances phagocytic activity in macrophages. The significant decrease in Pb cells at all three time points demonstrates a functional suppression that the macrophage was not able to overcome. This indicates Pb induces a significant suppression of M1 functional activation and would suggest decreased pathogen clearance *in vivo*.

Pb does not inhibit calcium/calmodulin dependent kinase or CREB activation in unstimulated or M1 stimulated RAW cells

Calcium signaling via the calcium/calmodulin pathway was not inhibited by Pb in the RAW 264.7 cells. Western blot data at 4hr demonstrated that cytoplasmic calmodulin expression was increased in RAW cells when stimulated with LPS or IFN-γ (Figure 4.7). Pb did not significantly alter the expression of calmodulin. The same trend was observed at 24hr (data not shown). These data did not support or refute inhibition of calmodulin by Pb, therefore the downstream kinases and transcription factor were investigated.

Activation of CaMKIV through phosphorylation was detected in all treatment groups at 4 and 24hr. Phosphorylation of CaMKIV has been shown to be calcium/calmodulin dependent (Soderling 1999; Swulius and Waxham 2008). It requires activated calmodulin interacting with CaMKIV, which changes CaMKIV confirmation relieving what is known as autoinhibition and allowing CaMK kinase to phosphorylate

CaMKIV at tyrosine 197 (Krebs 1998). Once activated, phosphorylated CaMKIV can function independent of calmodulin and activate other cytoplasmic proteins or translocate into the nucleus (Krebs 1998; Soderling 1999). Western blot analysis indicated that M1 stimulation of the macrophage increased phosphorylation of cytoplasmic CaMKIV compared to unstimulated cells. These data suggest that cells culture with LPS or LPS/IFN-γ had the strongest expression of cytoplasmic CaMKIV and that the addition of Pb did not alter this expression (Figure 4.8). However, significantly increased phosphorylation of CaMKIV was seen at 4hr in cells cultured with Pb alone and with Pb cultured IFN-γ-stimulated RAW cells compared to untreated cells. At 24hr, cells cultured with Pb alone had significantly higher expression of phosphorylated cytoplasmic CaMKIV compared to untreated controls (Figure 4.9). These data suggest that Pb alone sustains prolonged calcium signaling in the RAW cells resulting in increased cytoplasmic activation of CaMKIV. Nuclear phosphorylated CaMKIV was only detected after 24hr and was increased by LPS and IFN-γ, but was not significantly altered by Pb.

Cytoplasmic CaMKII expression was increased when RAW cells were activated with LPS or IFN-γ. Interestingly, total cytoplasmic expression of CaMKII was not altered by Pb throughout the 48hr culture. RAW cell cytoplasmic expression of CaMKII was detectable at 4 and 24hr, however phosphorylated CaMKII was not detected at either time point. Still, this does not definitively indicate that CaMKII was not activated in the RAW cells. Phosphorylation of CaMKII serves as an intermediate in signal transduction, therefore phosphorylation and subsequent dephosphorylation through signal transduction could be almost simultaneous. This could be one explanation why phosphorylated CaMKII was not detected in this study.

CREB is a transcription factor downstream from the CaM kinases and is activated through phosphorylation. As was observed with the other proteins in the calcium/calmodulin protein cascade, cytoplasmic expression and phosphorylation was increased when RAW cells were stimulated with LPS or IFN-γ (Figure 4.7). This supports that the activation of CREB and its role as a transcription factor in pro-inflammatory activation. In the present study, the addition of Pb did not significantly alter the cytoplasmic or nuclear expression of activated CREB in any cell treatment. These data, combined with the previously presented calcium signaling data suggests that Pb does not inhibit protein signaling in the calcium/calmodulin pathway or activation of CREB during pro-inflammatory activation of RAW 264.7 cells.

DISCUSSION

Industry workers chronically exposed to Pb have been reported to have increased serum antibody levels of IgA and IgE (Ewers et al. 1982). These two antibodies are associated with an anti-inflammatory Th2 immune profile. Similarly, BALB/c mice subcutaneously exposed to Pb had significantly increased plasma IL-4 and IgE levels and significantly decreased IFN-γ, indicating a shift towards Th2 immune profile (Heo et al. 1996). An increased Th2 immune profile in humans has also been linked with the increase of autoimmunity leading to disease such as systemic lupus erythematosus (Ramanujam and Davidson 2008). Another study reported depressed IgG2 concentrations in humans chronically exposed to Pb (Sun et al. 2003). This could also Pb to an increase in the Th2 profile, as IgG is the antibody commonly associated with Th1. Further, Mishra et al. (2003) reported a significant decrease in T cell proliferation following phytohaemagglutinin (PHA) in three-wheeler drivers, battery workers, and silver jewelry

makers exposed to Pb as compared to unexposed volunteers. The PHA test invokes a Th1 response and decrease in proliferation seen in the study indicates immune suppression and shift towards Th2.

Raw cell proliferation measured via the Alamar Blue™ assay showed high metabolic activity within the cells. There was a numeric decrease in proliferation in all treatment groups with cells cultured with Pb for 12hr (Figure 1a). These results infer that at 12hr of incubation, Pb induces some level of metabolic proliferation inhibition of the macrophage even in the presence of M1 stimuli. All cells were at >90% viability (data not shown), therefore the decrease in metabolic proliferation did not appear to be associated with cell death. Pro-inflammatory activation in macrophages involves differentiation of the cell that requires high metabolic activity. The metabolic decrease at 12hr suggests that Pb suppresses initial pro-inflammatory response in RAW 264.7 cells.

Nuclear translocation of NF-κB p.65 was significantly decreased at 4hr in cells cultured with Pb+LPS and Pb+ LPS/ IFN-γ. This corresponded with the significant decrease in TNF-α expression in RAW cells at 12 and 24hr. Cross talk between the TLR4 pathway and CREB has been investigated previously and the conclusions differ among studies. In one study, induced increases in cytoplasmic cyclic adenosine monophosphate activated the protein kinase a (PKA)-CREB resulting in increased IL-10 production and decreased TNF-α production in LPS-stimulated RAW 264.7 cells (Avni et al. 2010). The authors theroizied that CREB bound to the IL-10 CRE promoter region on DNA, which inhibited NF-κB binding the TNF-α promoter region (Avni et al. 2010). Interestingly, a CRE promoter site is also associated with TNF-α transcription and mutation of the site results in loss of transcription of TNF-α in LPS-stimulated RAW 264.7 cells (O'Donnell

and Taffet 2002). Another study evaluated the role of CREB in RAW 264.7 cells during bacterial activation. Roach et al. (2005) reported that CREB activation and TNF-α production was significantly higher in RAW 264.7 cells infected with the Gram positive *Mycobacterium smegmatis* compared to *Mycobacterium avum*. The activation of CREB in these bacterial exposures appeared to be more dependent on PKA and not CaMKII (Roach et al. 2005). These data suggest that CREB is essential in regulating TNF-α production via LPS activation in macrophages, however the regulatory role seems to be dependent upon the signaling pathway activating CREB.

A potential mechanism explaining the decreased NF-κB p.65 at 4hr in the current study is that Pb may directly interact with the NF-κB pathway. As stated earlier, zinc is a trace element crucial in mammalian physiology. Previous studies have shown that zinc is critical in the control of reactive oxygen species and inflammatory responses plus it directly impacts the NF-κB pathway by suppressing translocation of NF-κB into the nucleus (Ho et al. 2004; Mariani et al. 2008; Foster and Samman 2012; Jarosz et al. 2017). One suggested mechanism in the macrophage is the inhibition of IKKβ upstream of NF-κB through zinc binding (Liu et al. 2013). A study investigating the role of the divalent cation cadmium in macrophage response to LPS saw a significant dose dependent (0.1-100 μM cadmium) decrease in NF-κB p.65 translocation into the nucleus and cytokine expression of IL-6, IL-8, and TNF-α (Cox et al. 2016). Therefore, another explanation for the decrease in NF-κB p.65 translocation could be Pb directly impacting the NF-κB pathway via inhibition of IKKβ.

Phosphorylation of STAT1 via IFN-γ is a strong inducer of M1 activation in macrophages (Ramana et al. 2000). It has been demonstrated in the macrophage *in vivo*

and *in vitro* using RAW 264.7 cells that full activation of STAT1 allowing it to function as a transcription factor not only requires phosphorylation at tyrosine 701 via the IFN-γ receptor and JAK signaling but also requires secondary phosphorylation at serine 727 (Rhee et al. 2003; Varinou et al 2003; Barnholt 2009). One proposed mechanism of STAT1 serine 727 phosphorylation is activation of CaMKII via calcium flux due to IFN-γ binding the IFN-γ receptor, and CaMKII phosphorylating STAT1 at serine 727 (Nair et al. 2002). Since, in the current study, nuclear localization of phosphorylated STAT1 and protein expression of target genes such as iNOS were not significantly altered by Pb, then phosphorylation of serine 727 in STAT1 potentially was unaffected. As stated earlier phosphorylated CaMKII was not detected in the cytoplasm of the RAW cells at 4hr however, CaMKII could have been activated and subsequently phosphorylated STAT1 at 727 prior to detection. This would suggest that calcium signaling via activation of CaMKII and phosphorylation of STAT1 at 727 was not significantly inhibited by Pb in RAW 264.7 cells.

The 4hr Western blot data indicated that only STAT1 alpha was phosphorylated while at 24hr, STAT1 alpha and beta were both phosphorylated. These variants of STAT1 have been shown to have distinctly different roles in pro-inflammatory activation. Phosphorylation of STAT1 alpha is considered the active form of the protein allowing it to serve as a transcription factor, while phosphorylation of STAT1 beta has been shown to inhibit function of STAT1 as a transcription factor (Gao et al. 1998; Baran-Marszak et al. 2004). In the current study, Pb-treated cells had significantly increased phosphorylation of STAT1 beta at 24hr when stimulated with IFN-γ. Subsequently, M2 cytokine expression had an increased trend in these same treatment groups at 24hr and

48hr. Phosphorylation of STAT1 beta could be the mechanism shifting these cells towards M2 cytokine expression, indicating a potential shift from M1 activation towards M2.

In the present study, Pb caused a significant decrease in NO production in stimulated macrophages at 12 (Figure 2a) and 24 hr (Figure 2b). Western blot showed that Pb did not alter iNOS expression in the macrophages. These results agree with those previously reported by Tian and Lawrence (1996). In that study, isolated murine splenic macrophages and RAW cell lysates were stimulated with LPS and IFN-γ and treated with 20 μM and 10 μM Pb chloride, respectively. Both the current and previously reported findings indicate that Pb suppression of NO production in macrophages appears to involve direct or indirect interference with iNOS function in the cytoplasm of the cells. One indirect mechanism could be inhibition of iNOS binding calmodulin in the cytoplasm. Macrophage NO production by iNOS is dependent upon iNOS binding the N-terminal binding domain of calmodulin and forming a tight complex in the cytoplasm (Nussler and Biliar 1994; Stevens-Truss and Marletta 1995; Alderton et al. 2001; Spratt et al. 2006). Kirberger et al. (2013) determined that Pb has an 8x higher affinity than calcium to bind the N-terminal binding domain of calmodulin, resulting in confirmation changes of the N-terminal domain and rigidity of the protein. Therefore, the decrease in NO production in the present study could be explained by Pb binding and inhibiting calmodulin in the cytoplasm of the cell and preventing the iNOS/calmodulin complex from being formed.

Macrophage derived NO has many roles in the immune system therefore NO suppression by Pb, in this study, could result in numerous negative outcomes *in vivo*. One

immediate outcome would be impaired macrophage NO destruction of encountered pathogens, potentially leaving an organism susceptible to infection (MacMicking et al. 1997; Aktan 2004; Dorpinghaus et al. 2016). However, the downstream consequences of decreased NO production could have much greater impact on the immune system. The macrophage links the innate and adaptive immune systems through antigen presentation and cell surface protein interaction with T helper (Th) cells. Another important role of NO in the immune system is as a paracrine signaling molecule that has been shown to suppress proliferation of Th cells stimulated by the Th cell mitogen Concanavalin A (Con A)(Albina 1991; Bingisser et al. 1998; Sato et al. 2007). A proposed mechanism for this is NO inhibiting phosphorylation of the transcription factor STAT 5 in the cytoplasm of Th cells (Bingisser et al 1998; Sato 2007). Activation of STAT 5 is a key component of naïve Th cell differentiation (Owen and Farrar 2017). Therefore, NO is a key regulatory molecule involved in Th cell differentiation and proliferation during macrophage/Th cell interaction. It has previously been reported that Pb induces T cell production of the Th2 cytokine IL-4 while inhibiting production of IFN-γ and proliferation of Th2 T cells (Heo et al. 1996; Miller et al. 1998). Given these previous findings, an Pb-exposed individual challenged by a pathogen could have an increase in IL-4 production shifting naïve Th cells towards Th2 cells encountering activated macrophages producing NO at significantly decreased concentrations. This would result in IL-4 primed Th cells undergoing activation with decreased NO regulated proliferation via macrophage interaction. This mechanism could possibly explain the Th2 immune profiles of individuals chronically exposed to Pb via occupational or residential settings.

Macrophage phagocytic activity differences induced by Pb has been previously investigated with no definitive conclusion. De Guise et al. (2000) reported that isolated bovine macrophages following *in vitro* 10 μm Pb exposure showed no significant difference in phagocytosis of latex beads compared to control. An *in vivo* study of Sprague-Dawley rats exposed to Pb via drinking water showed no changes in phagocytic activity, however another set of rats exposed to Pb through intravenous injection had significantly reduced phagocytic activity of Kupffer cells (Trejo et al. 1972). However, sheep exposed to 100-ppm Pb acetate-trihydrate via feed had 50% reduction in phagocytic activity of peritoneal macrophages compared to controls (Knowles et al. 1997). The results of the current study showed that LPS/IFN-γ stimulated RAW phagocytic activity was significantly decreased by Pb at 12, 24, and 48hr. The Pb LPS/IFN-γ RAW cells also had significantly decreased NO production at 12hr, suggesting pathogen destruction is markedly decreased in Pb exposed RAW cells under these conditions inferring increased susceptibility to pathogen infection *in vivo*.

All TLR4 and IFNγR-mediated treatments showed cytoplasmic phosphorylation of CaMKIV at 4hr when Pb was present in the media alone or with IFN-γ being significantly increased compared to the treatments without Pb. At 24hr, the stimulated macrophages had comparable cytoplasmic expression of CaMKIV compared to Pb-exposed stimulated macrophages, however cells cultured with Pb alone had significantly higher concentrations of activated CaMKIV compared to media alone. The role of CaMKIV and its function in cell signaling been investigated in numerous studies and is dependent on cell type. This protein was shown to induce expression of the Bcl-2 (anti-apoptotic protein) in dendritic cells and was critical for cell survival during LPS toxicity

(Illario et al. 2008). It was also found that nuclear translocation of CaMKIV suppressed IL-2 transcription in systemic lupus erythematosus T cells (Juang et al. 2005). In RAW cells, it was reported that nuclear CaMKIV was responsible for shuttling high mobility group box 1 (HMBG1), a proinflammatory cytokine, into the cytoplasm of the cell for expression (Zhang et al. 2008). Unfortunately, HMBG1 was not investigated in the current study therefore no conclusions can be made regarding HMBG1.

In a recent study, Kerr et al. (2013) reported that RAW 264.7 cells treated with 2.5 and 5 μM Pb nitrate induced autophagy after 17hrs of incubation. Autophagy is a process that cells can induce to regulate cellular function during times of nutrient deficiency or environmental stress by metabolizing cytoplasmic proteins and organelles (Klionsky 2005; Mizushima and Komatsu 2011; Filomeni et al. 2015). While induction of autophagy is generally accepted as damaging, it has been shown to have proactive effects in liver disease, preventing further damage (Rautou et al. 2010). Evankovich et al. (2012) reported that induction of autophagy in hepatocytes of CaMKIV KO mice was significantly suppressed during ischemic-reperfusion injury resulting in increased liver damage. The CaMKIV KO mice also had significantly lower cellular expression of the protein LC3. Cultured hepatocytes also had markedly depressed LC3 expression when CaMKIV or the CaMKIV activator CaMK kinase were inhibited. LC3 localizes on the autophagosome in the cytoplasm of cells and is a biomarker of autophagy (Kabeya et al. 2000; Tanida et al. 2005). These data suggest that expression and activation of CaMKIV is key in the induction of autophagy. Additionally a recent study showed that activated CaMKIV was required for isolated murine macrophages and RAW 264.7 cells to induce autophagy (Zhang et al. 2014). As with the previously described hepatocytes, CaMKIV

KO *in vivo* and inhibition *in* vitro significantly decreased LC3 expression in the murine macrophages and RAW 264.7 cells (Zhang et al. 2014). Therefore, the induction of autophagy, previously seen in RAW cells treated under the same conditions as the current study, could be regulated by the activation of cytoplasmic CaMKIV presented in this study.

CONCLUSION

The current study attempted to show a causative link between inhibition of calcium signaling and a shift towards anti-inflammatory M2 activation in macrophages. Nuclear translocation of NFkB p.65 was also significantly suppressed by Pb in cells stimulated with LPS. This manifested as significantly decreased TNF-α cytokine expression in the cells at 12hr, indicating some suppression of M1 activation via TLR-4. Likewise, increased cytoplasmic phosphorylation of STAT1 beta at 24hr in cells treated with Pb and IFN-γ was observed, indicating an inhibitory effect of STAT1 as a transcription factor. These results demonstrate that Pb interrupted signal transduction in the cells at different times points, which resulted in different levels of cytokine expression and cellular response. *In vivo* exposure could result in greater macrophage inhibition due to Th2 cell cytokine signaling from Pb exposed T cells. The suppression of NO production and phagocytosis in stimulated RAW cells demonstrates a significant decrease in pathogen clearance, and critical component of M1 activation Surprisingly, Pb appeared to cause significant activation of CaMKIV in multiple treatment groups at 4hr, which contradicted the hypothesis that Pb would inhibit calcium signaling and the activation of the downstream kinases. It is unclear if this is associated with an M2 shift in macrophages, because there are limited data on the role of CaMKIV in this respect.

However, this is interesting when compared with Kerr et al. (2013) where Pb initiated autophagy in RAW cells cultured under the same Pb conditions and the role of CaMKIV in autophagy induction in murine macrophages (Zhang et al. 2014). Further research will have to be conducted to determine the role of CaMKIV in M2 activation of macrophages and how this correlates with autophagy.

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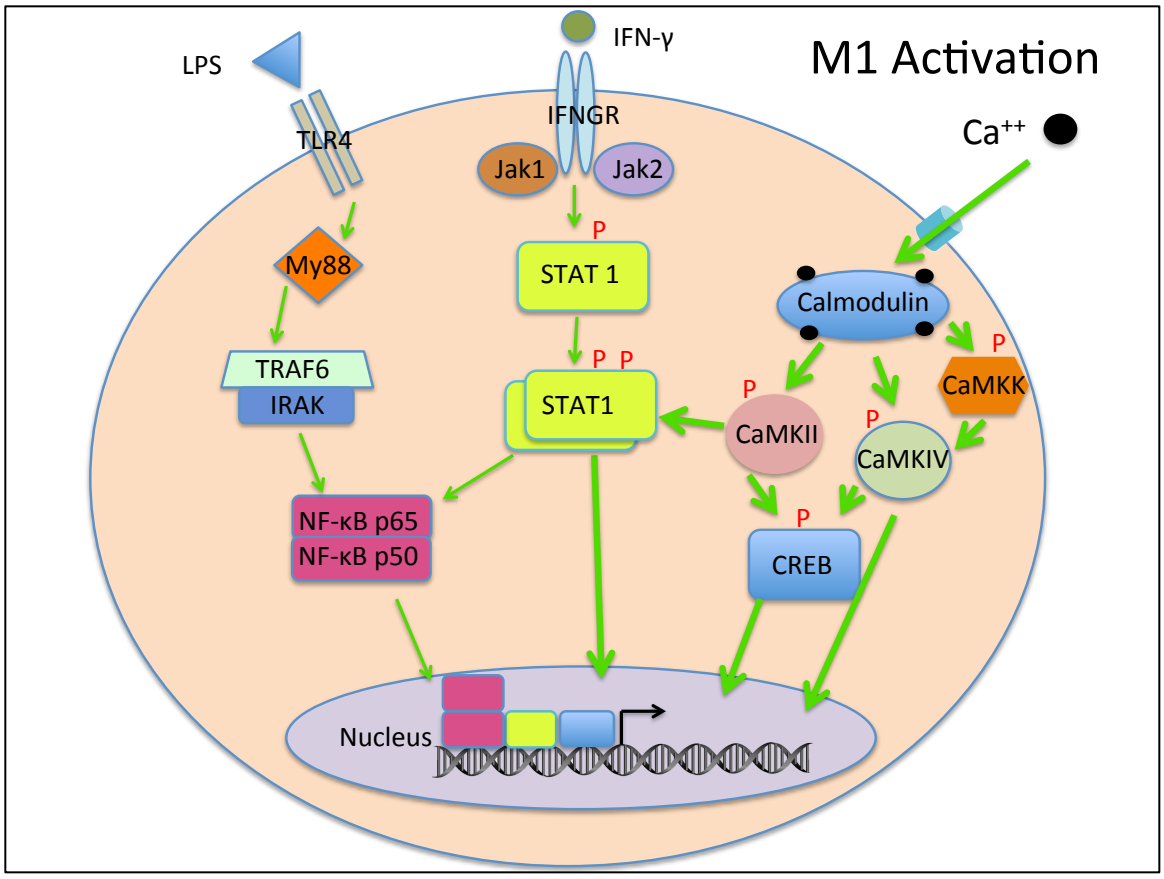
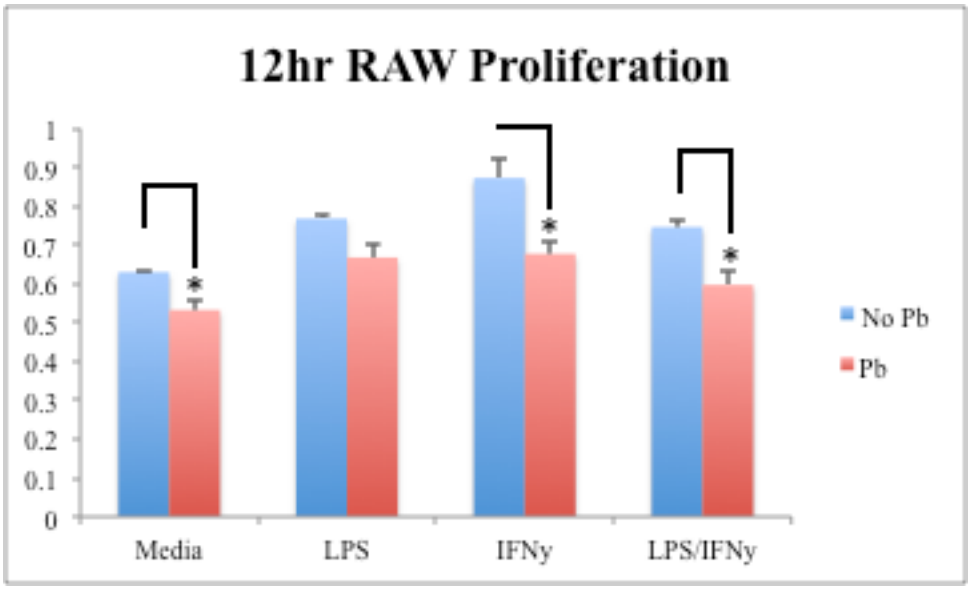


Figure 4.1: The protein signaling cascades investigated in this dissertation



Figues 4.2: Twelve hour RAW cell proliferation

Cellular proliferation was significantly decreased in cells cultured with Pb, Pb IFN-y and Pb LPS/IFN-y. Significance is denoted with an * ($p \leq 0.05$).

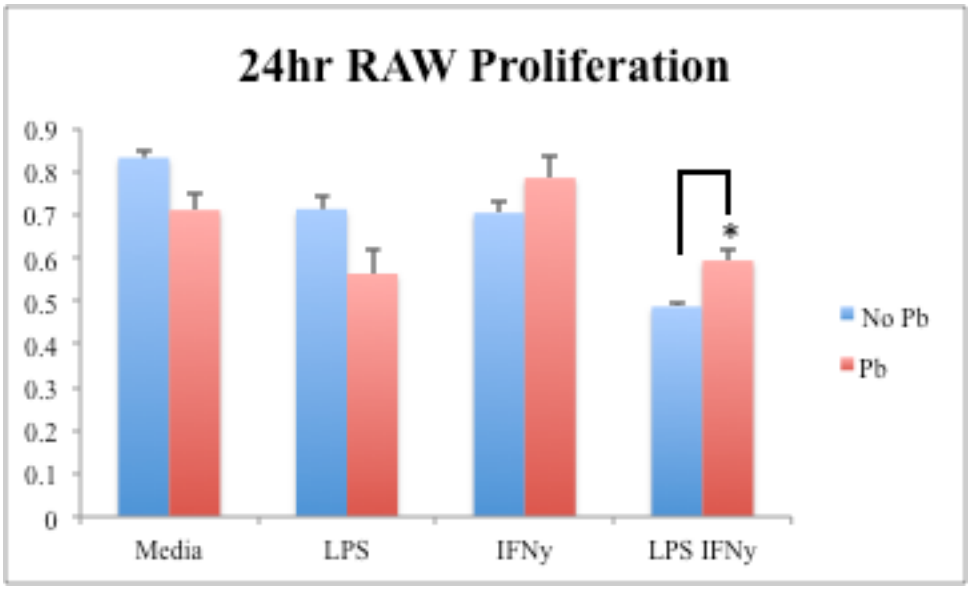


Figure 4.3: Twenty-four hour RAW cell proliferation

Cellular proliferation was significantly increased in cells cultured with Pb LPS/IFN-y. Significance is denoted with an * ($p \leq 0.05$).

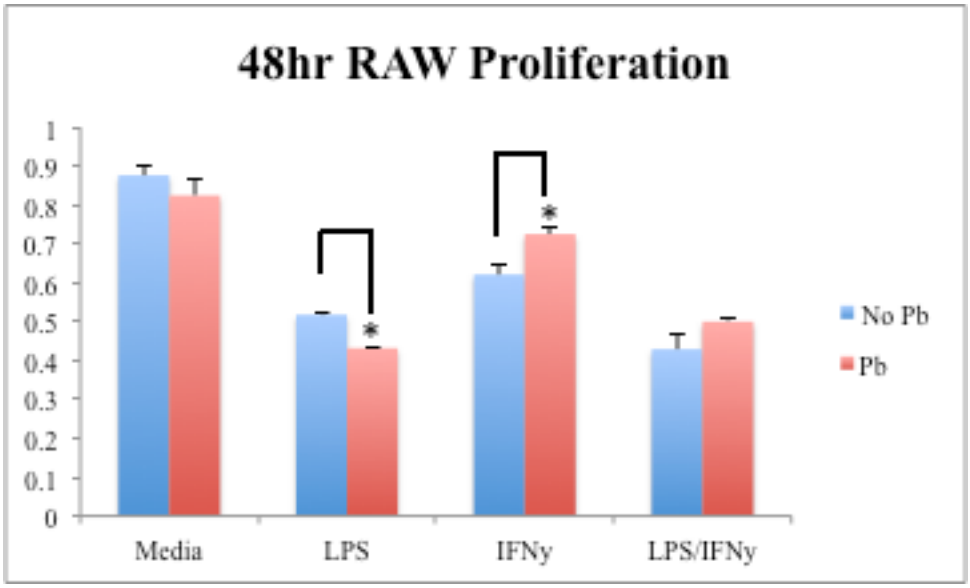


Figure 4.4: Forty-eight hour RAW cell proliferation

Cellular proliferation was significantly altered by Pb. Significance is denoted with an * ($p \leq 0.05$).

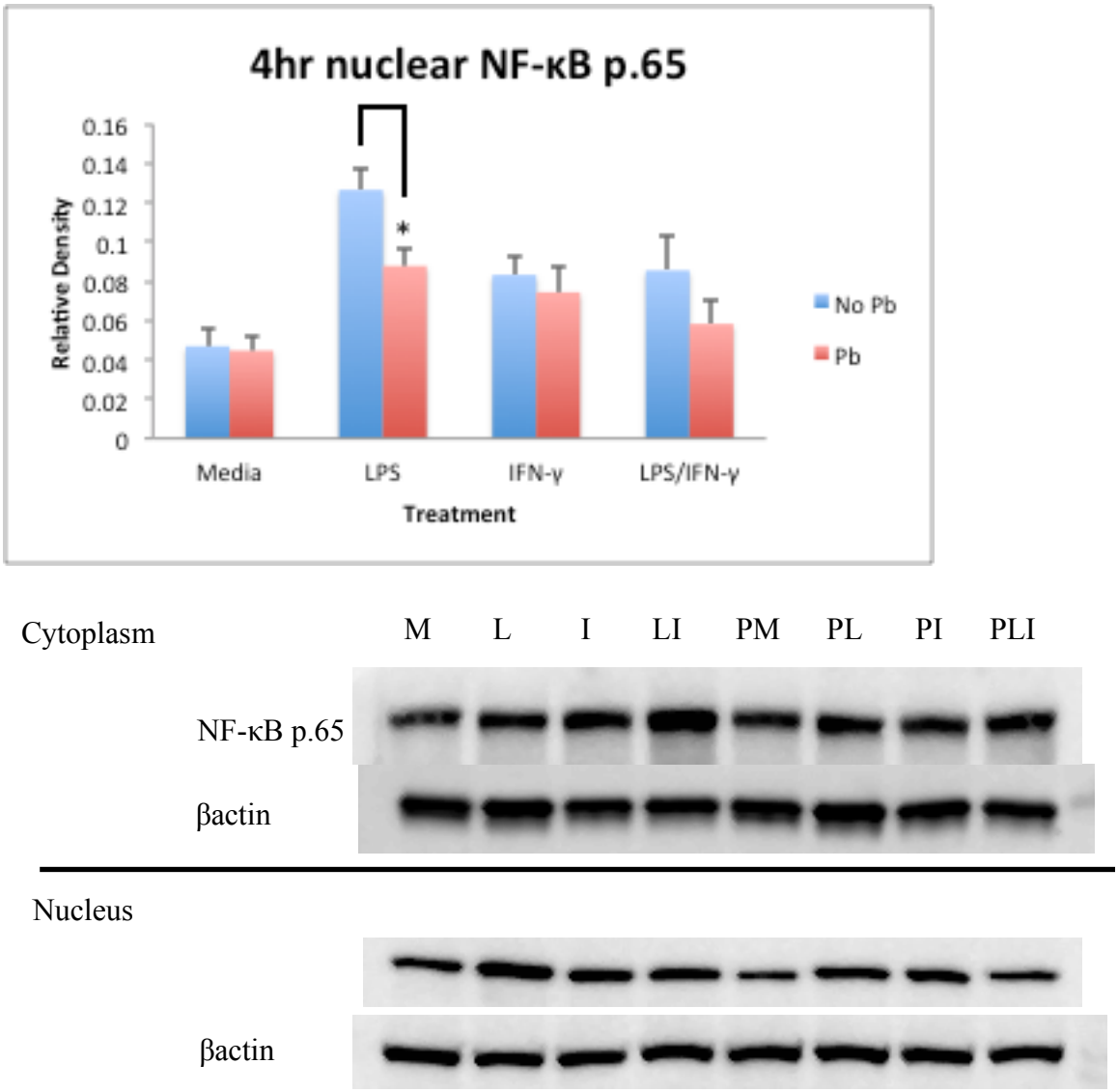
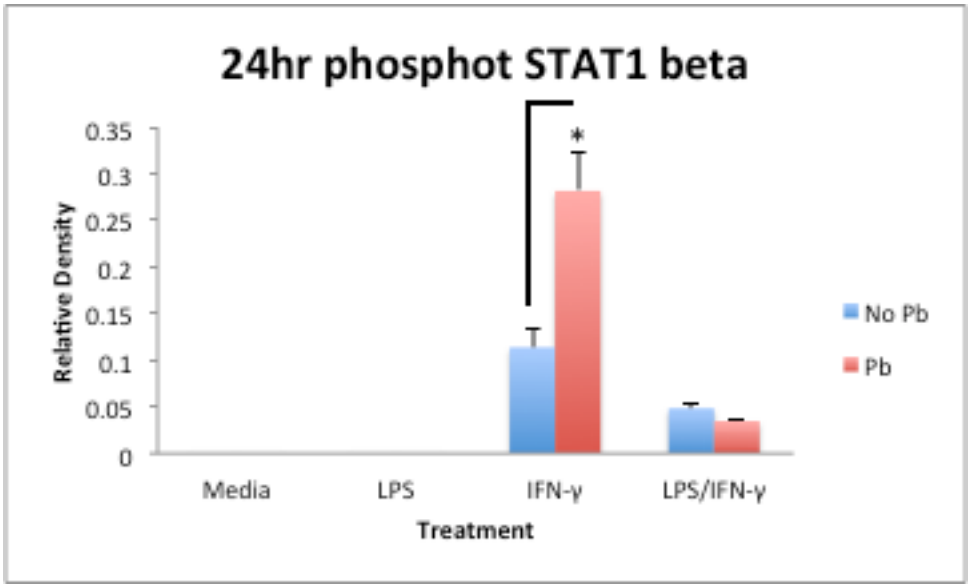


Figure 4.5: Four hour nuclear expression of NF-κB p.65 in RAW cells

The culture descriptions for western blot are as follows: M= Media, L= LPS, I= IFN-y, LI= LPS/IFN-y, PM= Pb Media, PL= Pb LPS, PI= Pb IFN-y, and PLI= Pb LPS/IFN-y. Significance is denoted with an * ($p \leq 0.05$).



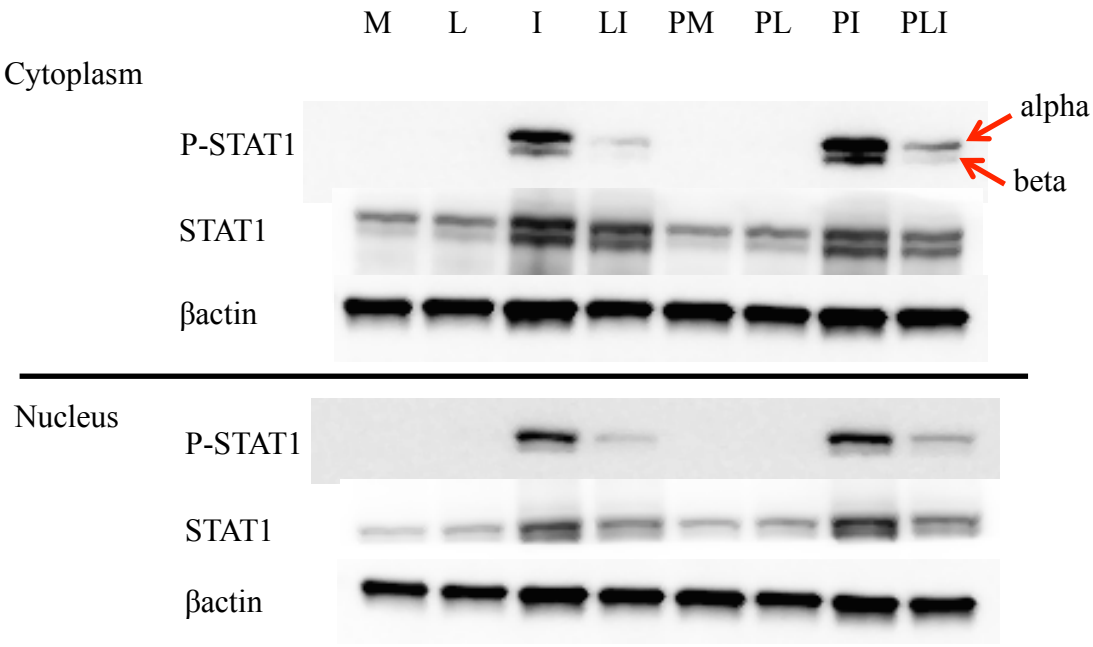


Figure 4.6: Twenty-four hour phosphorylated STAT1 in RAW cells

The culture descriptions for western blot are as follows: M= Media, L= LPS, I= IFN-y, LI= LPS/IFN-y, PM= Pb Media, PL= Pb LPS, PI= Pb IFN-y, and PLI= Pb LPS/IFN-y. Significance is denoted with an * ($p \leq 0.05$).

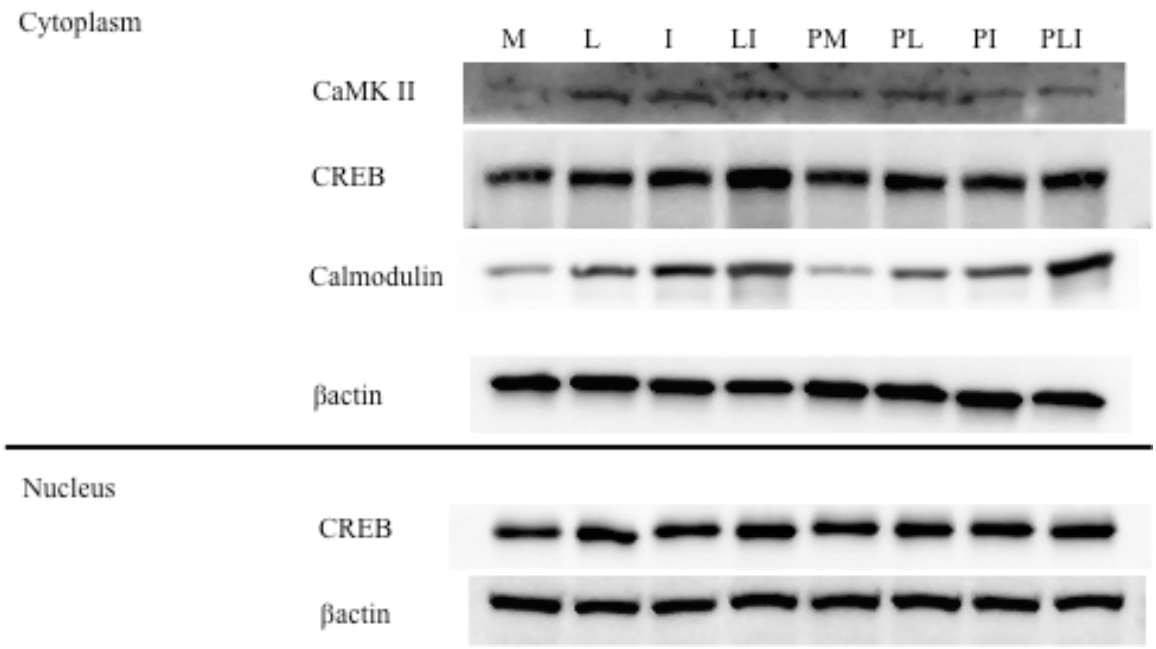


FIGURE 4.7: Four hour cytoplasmic and nuclear expression of CaMKII, CREB, and calmodulin in RAW cells

The culture descriptions for western blot are as follows: M= Media, L= LPS, I= IFN-y, LI= LPS/IFN-y, PM= Pb Media, PL= Pb LPS, PI= Pb IFN-y, and PLI= Pb LPS/IFN-y

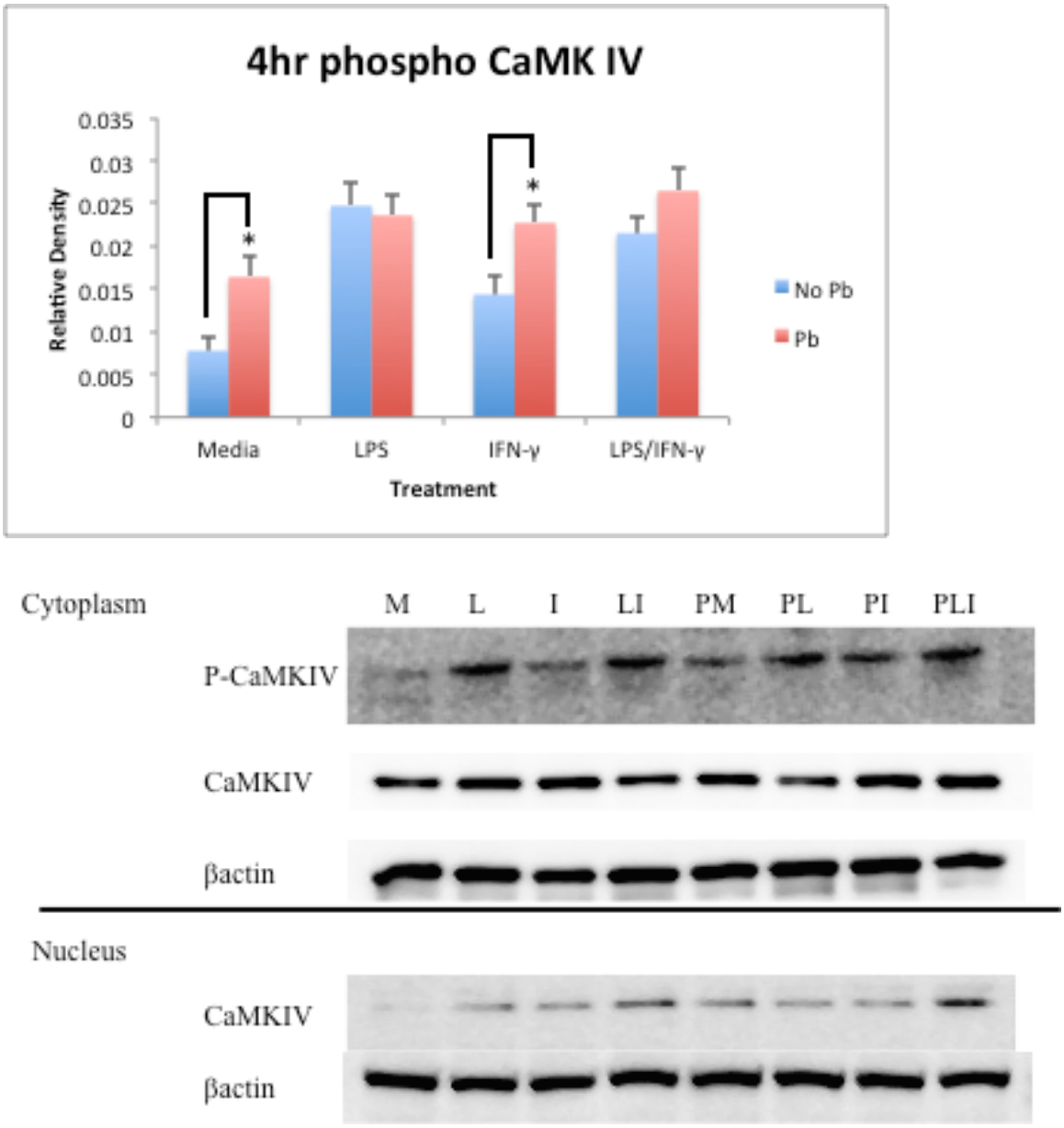


Figure 4.8: Four hour cytoplasmic phosphorylated CaMKIV in RAW cells.

The culture descriptions for western blot are as follows: M= Media, L= LPS, I= IFN-y, LI= LPS/IFN-y, PM= Pb Media, PL= Pb LPS, PI= Pb IFN-y, and PLI= Pb LPS/IFN-y. Significance is denoted with an * ($p \leq 0.05$).

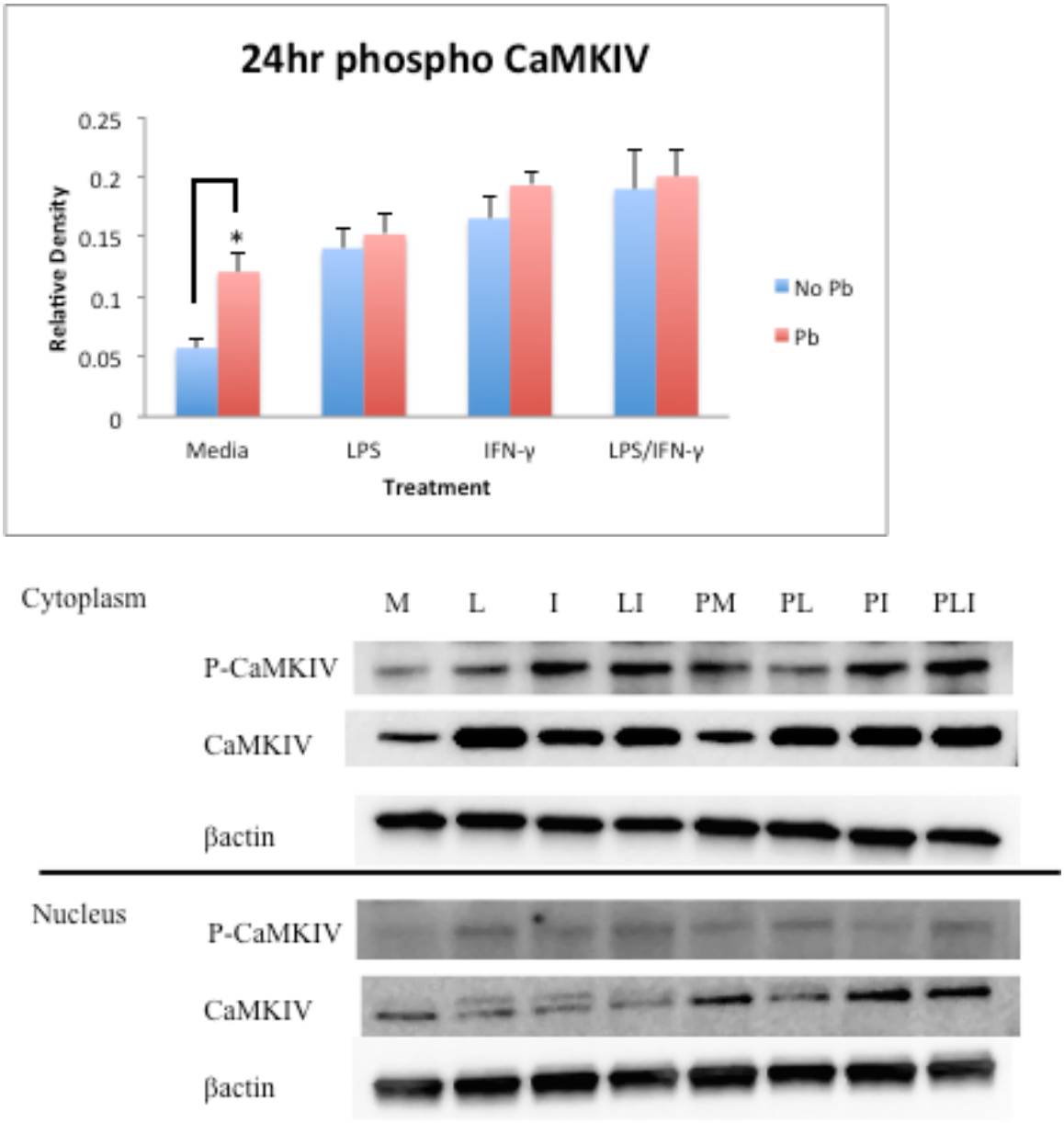


Figure 4.9: Twenty-four hour cytoplasmic phosphorylated CaMKIV in RAW cells. The culture descriptions for western blot are as follows: M= Media, L= LPS, I= IFN-y, LI= LPS/IFN-y, PM= Pb Media, PL= Pb LPS, PI= Pb IFN-y, and PLI= Pb LPS/IFN-y. Significance is denoted with an * ($p \leq 0.05$).

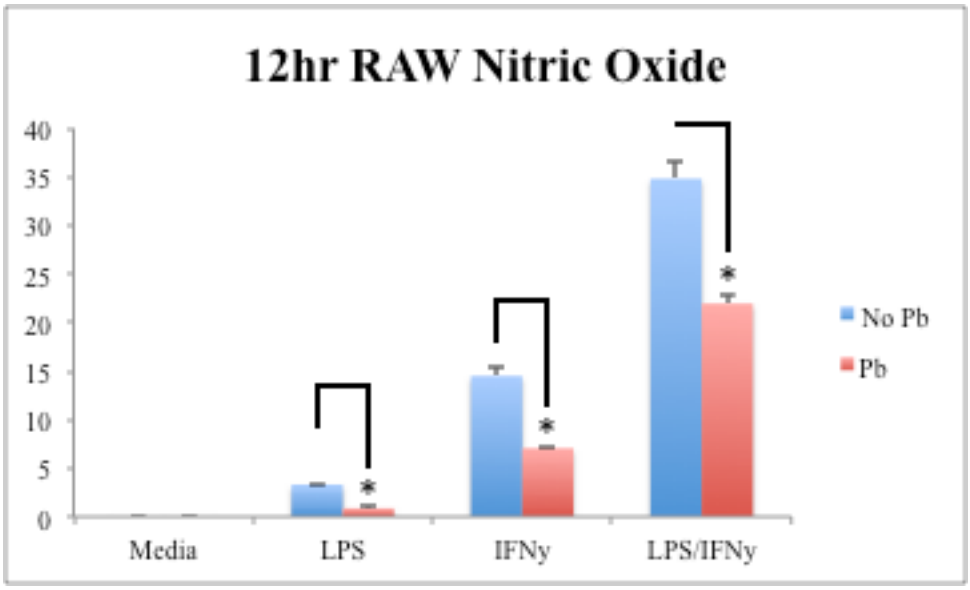


Figure 4.10: Twelve hour nitric oxide production in RAW cells

Nitric oxide production was significantly inhibited by Pb in all detectable culture groups. Significance is denoted with an * ($p \leq 0.05$).

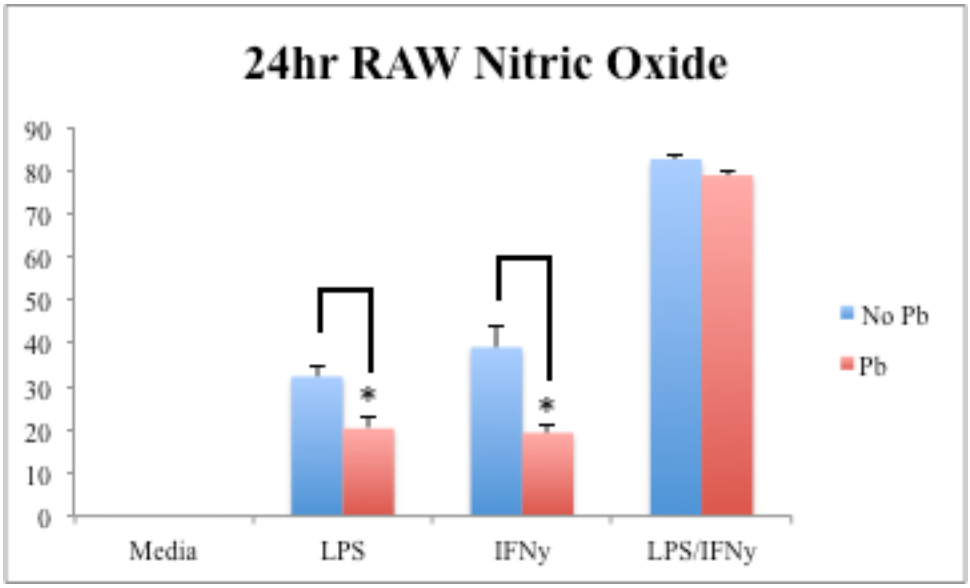


Figure 4.11: Twenty-four hour nitric oxide production in RAW cells

Nitric oxide production was significantly inhibited by Pb in cells cultured with LPS or IFN-y. Significance is denoted with an * ($p \leq 0.05$).

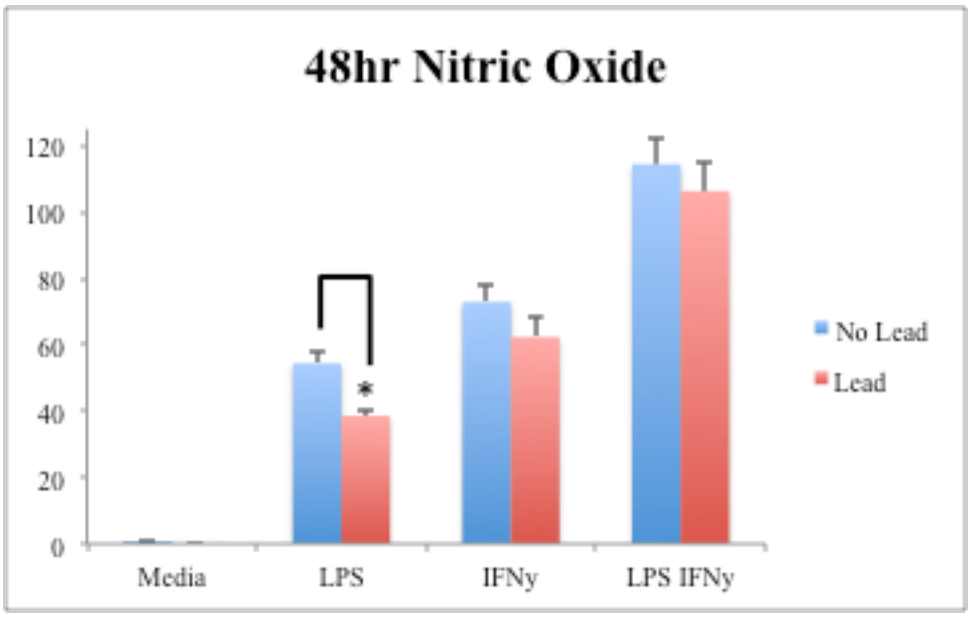


Figure 4.12: Forty-eight hour nitric oxide production in RAW cells

Pb significantly decreased NO production in cells cultured with LPS Significance is denoted with an * ($p \leq 0.05$).

Table 4.1: Unstimulated RAW cell cytokine expression

Cells shaded with light green indicate Pb increasing cytokine expression ≥10% compared to non-Pb cells. Cells shaded with orange indicate Pb decreasing cytokine expression ≥10% compared to non-Pb cells. All values represent picograms/mL in supernatant. Significance is denoted with an * ($p \leq 0.05$).

Media	12hr (Mean ± SEM)	24hr (Mean ± SEM)	48hr (Mean ± SEM)
IL-1β	1.27 ± 0.62	2.12 ± 0.07	13.5 ± 4.58
Pb	1.56 ± 0.25	1.35 ± 0.19	8.75 ± 0.68
IL-6	140 ± 36.2	589 ± 33.2	11100 ± 2530
Pb	172 ± 22.3	694 ± 162	10000 ± 1860
TNF-a	1280 ± 132	1210 ± 138	2910 ± 206
Pb	1450 ± 255	1120 ± 36.8	2780 ± 65.0
IL-12p.70	4.90 ± 1.50	10.5 ± 0.86	27.9 ± 1.59
Pb	9.95 ± 0.63	7.81 ± 1.05	27.4 ± 2.40
IL-10	0.32 ± 0.00	0.32 ± 0.00	2.19 ± 0.73
Pb	0.32 ± 0.00	0.32 ± 0.00	3.40 ± 0.90
IL-13	0.92 ± 0.00	2.75 ± 0.96	14.9 ± 3.00
Pb	1.54 ± 0.62	1.39 ± 0.47	15.6 ± 3.85
VEGF	1.91 ± 0.83	14.6 ± 1.24	79.9 ± 6.54
Pb	4.73 ± 0.85	15.8 ± 1.43	67.8 ± 8.20

Table 4.2: LPS stimulated RAW cell cytokine expression

Cells shaded with light green indicate Pb increasing cytokine expression ≥10% compared to non-Pb cells. Cells shaded with orange indicate Pb decreasing cytokine expression ≥10% compared to non-Pb cells. All values represent picograms/mL in supernatant. Significance is denoted with an * ($p \leq 0.05$).

LPS	12hr (Mean ± SEM)	24hr (Mean ± SEM)	48hr (Mean ± SEM)
IL-1β	10.3 ± 1.07	75.1 ± 15.9	75.4 ± 6.80
Pb	14.4 ± 1.18	41.0 ± 5.22	71.1 ± 1.88
IL-6	70700 ± 747	73100 ± 454	73800 ± 326
Pb	70200 ± 774	70400 ± 647*	74000 ± 316
TNF-a	10700 ± 293	11000 ± 269	9680 ± 708
Pb	8840 ± 424*	9410 ± 238*	8710 ± 665
IL-12p.70	93.2 ± 3.76	109 ± 12.6	146 ± 12.0
Pb	113 ± 3.39*	120 ± 1.33	150 ± 4.64
IL-10	11.8 ± 0.51	13.4 ± 1.44	17.0 ± 0.88
Pb	14.8 ± 0.71*	11.8 ± 1.57	20.7 ± 0.90*
IL-13	73.7 ± 9.92	78.4 ± 6.09	90.6 ± 2.26
Pb	81.7 ± 5.61	72.0 ± 4.32	94.3 ± 3.15
VEGF	8.98 ± 0.73	31.3 ± 2.58	78.3 ± 5.06
Pb	14.5 ± 2.01	37.1 ± 0.73	120 ± 8.87*

Table 4.3: IFN-γ stimulated RAW cell cytokine expression

Cells shaded with light green indicate Pb increasing cytokine expression ≥10% compared to non-Pb cells. Cells shaded with orange indicate Pb decreasing cytokine expression ≥10% compared to non-Pb cells. All values represent picograms/mL in supernatant. Significance is denoted with an * ($p \leq 0.05$).

IFN-γ	12hr (Mean ± SEM)	24hr (Mean ± SEM)	48hr (Mean ± SEM)
IL-1β	5.09 ± 1.34	6.27 ± 0.84	14.4 ± 2.16
Pb	3.60 ± 0.21	9.05 ± 3.06	19.5 ± 4.65
IL-6	2090 ± 220	4450 ± 705	26000 ± 3630
Pb	1340 ± 130	2490 ± 353	21500 ± 3900
TNF-a	3100 ± 216	3920 ± 186	5430 ± 704
Pb	2300 ± 221	3200 ± 169*	5730 ± 399
IL-12p.70	12.0 ± 3.27	21.6 ± 0.36	29.5 ± 1.27
Pb	19.9 ± 0.75	27.7 ± 1.51*	39.7 ± 1.65**
IL-10	0.64 ± 0.32	0.53 ± 0.20	3.49 ± 0.39
Pb	0.63 ± 0.16	3.44 ± 0.62*	6.00 ± 0.44*
IL-13	3.92 ± 1.51	8.51 ± 2.96	22.3 ± 3.71
Pb	3.86 ± 2.31	15.5 ± 6.45	25.5 ± 1.65
VEGF	1.56 ± 0.72	8.00 ± 0.50	41.3 ± 2.38
Pb	2.77 ± 0.73	11.1 ± 0.38	37.1 ± 2.57

Table 4.4: LPS/IFN-γ stimulated RAW cell cytokine expression

Cells shaded with light green indicate Pb increasing cytokine expression ≥10% compared to non-Pb cells. Cells shaded with orange indicate Pb decreasing cytokine expression ≥10% compared to non-Pb cells. All values represent picograms/mL in supernatant. Significance is denoted with an * ($p \leq 0.05$).

LPS/IFN-γ	12hr (Mean ± SEM)	24hr (Mean ± SEM)	48hr (Mean ± SEM)
IL-1β	53.2 ± 7.09	93.8 ± 19.5	84.5 ± 4.35
Pb	54.5 ± 5.83	99.3 ± 19.2	105 ± 21.1
IL-6	72800 ± 514	74100 ± 117	74400 ± 836
Pb	74000 ± 407	75400 ± 496	75800 ± 891
TNF-a	10500 ± 625	10800 ± 325	11100 ± 705
Pb	10800 ± 599	11300 ± 548	12100 ± 275
IL-12p.70	161 ± 6.13	166 ± 8.19	162 ± 6.69
Pb	176 ± 8.65	198 ± 8.64	190 ± 5.93*
IL-10	19.0 ± 0.85	18.9 ± 0.36	27.6 ± 5.25
Pb	19.5 ± 1.56	23.3 ± 1.06*	25.0 ± 1.56
IL-13	98.9 ± 4.88	97.6 ± 1.61	103 ± 3.07
Pb	101 ± 4.24	109 ± 1.50**	116 ± 2.90*
VEGF	15.1 ± 2.09	54.4 ± 3.09	246 ± 11.6
Pb	13.9 ± 1.70	64.4 ± 4.08**	292 ± 4.36*

Table 4.5: RAW cell phagocytosis and cytoplasmic free radical production.

Significance is denoted with an * ($p \leq 0.05$).

	Media	LPS	IFNy	LPS/IFNy
12hr	(percent ± SEM)	(percent ± SEM)	(percent ± SEM)	(percent ± SEM)
Free radicals	85.4 ± 0.64	76.4 ± 0.92	83.1 ± 2.00	68.2 ± 2.76
Pb	84.3 ± 2.10	74.1 ± 1.97	84.7 ± 2.24	77.5 ± 1.72*
Phagocytosis	19.2 ± 1.60	29.8 ± 1.35	30.1 ± 1.26	46.4 ± 0.84
Pb	21.8 ± 2.13	28.1 ± 1.85	21.1 ± 0.92*	38.7 ± 1.25*
24hr				
Free radicals	87.9 ± 2.45	86.0 ± 1.41	84.7 ± 1.42	84.0 ± 1.03
Pb	88.7 ± 2.26	89.3 ± 0.34	83.0 ± 3.48	81.9 ± 0.82
Phagocytosis	11.6 ± 1.88	23.4 ± 0.96	25.5 ± 1.78	37.3 ± 0.75
Pb	14.4 ± 1.58	22.7 ± 0.99	20.6 ± 1.69	28.1 + 1.01*
48hr				
Free radicals	77.0 ± 5.99	83.8 ± 2.31	86.1 ± 2.09	85.1 ± 1.76
Pb	85.3 ± 4.03	85.2 ± 2.50	89.2 ± 1.24	85.2 ± 3.54
Phagocytosis	7.87 ± 0.52	13.8 ± 1.75	11.2 ± 0.88	40.5 + 1.69
Pb	8.70 + 0.44	11.2 ± 0.70	10.1 ± 0.82	21.5 ± 1.17*

CHAPTER 5

SUMMARY AND CONCLUSIONS

The United States government has recognized the health risks of Pb contamination in the environment since the middle of the twentieth century and has implemented ever-changing regulations to treat current exposures and prevent future exposures. As current research has continued to come out, the lowest observable effect level (LOEL) of Pb is changed every few years. As of 2012, the CDC has set the blood-Pb levels of 5 μg/dL as the level in children where immediate public safety response is required (CDC 2017). Prior to 2012, blood-Pb levels in children less than 10 μg/dL did not have to be reported to the parents, as the CDC did not recognize these as levels of concern. In the wake of the events that occurred in Flint Michigan over the past three years, public recognition of the risk of Pb exposure has risen to levels not seen since the end of the twentieth century. However, much of the current concern is focused on human exposure from sources such as Pb in pipes comparable to what occurred in Flint Michigan. The goals of this dissertation were to assess the current environmental pollution of Pb and demonstrate that a single exposure to Pb could have significant health risks in wildlife. The first part of this dissertation presented the multiple sources of exposure to Pb in avian species and the wide range of organ system toxicities that occur. This was followed by a manuscript that demonstrated that a single pellet exposure in hen roller pigeons significantly decreased the growth and development of F1 progeny, with primary and secondary lymphoid organs being a target of Pb. The last phase of this

dissertation presented data showing Pb significantly impacted pro-inflammatory activation in the RAW 264.7 macrophage cell line by disrupting calcium and protein signaling in the cytoplasm of the cells.

PB EXPOSURE AND AVIAN TOXICITIES

The manuscript *Environmental Lead and Wild Birds- A Review* revealed that Pb entering the environment from sources such as recreational hunting or fishing and from industrial facilities is still a critical problem worldwide. There are dozens of avian species, both aquatic and terrestrial, that are at continued risk of Pb exposure. The research shows that Pb induces toxicity in multiple organ systems of birds including the nervous, renal, hepatic, reproductive, and immune systems. Pb in the form of spent ammunition is one key source of avian exposure. According to the U.S. Fish and Wildlife Service (USFWS) there were 13.2 million United States citizens that participated in legal hunting in 2011 (USFWS 2015). The ban of Pb ammunition during waterfowl hunting season helped to prevent future contamination in wetland areas, however it did not protect all ecosystems and species. The California condor is an endangered species extremely susceptible to Pb toxicity and is monitored under the same blood-Pb levels as children (Finkelstein et al. 2012). The condor is a scavenger species, therefore exposure typically occurs when injured game animals are consumed and Pb pellets and bullet fragments are ingested. Waterfowl and condors inhabit vastly different ecosystems and would not regularly encounter one another; therefore the Pb ban during waterfowl season has little to no effect on current condor exposures. This evidence clearly demonstrates the need for revised regulations on Pb ammunition.

MATERNAL DEPOSITION OF PB IN ROLLER PIGEONS

The hen blood-Pb levels and δ-ALAD activity data following a single pellet exposure verifies the results published previously in multiple species (Kerr et al. 2010; Holladay et al. 2012). Visually, hen health was not affected by Pb exposure evidenced by no loss of appetite, ability to ambulate and perch, and avoidance of handling by the researchers. These results were desired because the intent of the study was to determine the affects on egg production and development of progeny. A hen suffering from overt Pb toxicity would not actively reproduce. Surprisingly, egg production and hatchability were not affected by Pb exposure at either pellet dose. The first clutch occurred 1-6 weeks post exposure. Holladay et al. (2012) reported that single pellet exposure in roller pigeons resulted in pellet retention in birds at 4 weeks post exposure and blood-Pb levels of ~40 ppb. Therefore it was anticipated that the Pb body burden on hens would decrease egg production or hatchability. The hens gavaged with a #7.5 Pb pellet did however produce much lighter and smaller dimension eggs than unexposed hens. The average hen body weights across the three treatment groups were numerically similar therefore it was concluded that the change in egg size was a result of Pb. The results in the second clutch showed no differences among the treatment groups, further suggesting the results of the first clutch were products of the increased concentration of Pb in hens.

There was a dose dependent trend decrease in hatchling weight one-week post hatch in the hatchlings of the first clutch, with hatchlings from #7.5 hens weighing significantly less than hatchlings of unexposed hens. This was not evident in hatchlings from the second clutch. This data suggests a negative correlation with higher blood-Pb levels in hens and offspring body weight at hatch. Body weights at weaning were not

significantly different across treatment groups therefore it appears hatchling Pb exposure through maternal deposition at these Pb concentrations does not negatively impact hatching growth.

Weanling blood-Pb levels and δ-ALAD activity from Pb exposed hens were comparable to control weanlings. It was anticipated that weanlings from the first clutch would have hematologic results similar to hen data presented in this dissertation. Limited data exists for the half-life of circulating Pb in avian species, however a previous report states that the half-life of Pb in the blood of California condors is 13 days (Fry and Mauer 2003). The concentration of maternally deposited Pb was not determined in this study, but given the potential short half-life of Pb in the blood, Pb could have been excreted or deposited in other tissues in hatchlings by the 28-35 day weaning.

Histology results indicate that Pb significantly altered organ development of multiple organs. Of these, the degeneration of the thymus and splenic changes could potentially suppress immune function. The thymus is a primary lymphoid organ that produces the T cells of the adaptive immune system. Some of the potential consequences of Pb degeneration in this dissertation could be the weanling immune systems ability to recognize and combat virally infected cells or the production of autoreactive T cells. Birds do not have lymphnodes like mammals therefore the spleen is the only secondary lymphoid organ. Secondary lymphoid organs are sites for immune cell maturation and differentiation. The splenic changes reported in this dissertation further suggest that maternal Pb significantly decreased immune system development in F1 progeny potentially causing lifelong immune suppression.

PB DECREASES M1 RESPONSE IN RAW 264.7 CELL THROUGH MODULATION OF CYTOPLASMIC PROTEIN SIGNALING

Proliferation of cells at 12hrs cultured with Pb alone was significantly less than cells cultured in media as well. This indicates that Pb suppresses homeostatic function in RAW cells prior to activation. Stimulation via LPS or IFN-y induces M1 pro-inflammatory activation in macrophages, which results in a number of phenotypic changes associated with proliferation. The data showed that Pb significantly decreased proliferation in RAW cells cultured with LPS and IFN-y. Pb was not causing cell death proven by viability, demonstrating that initial pro-inflammatory response to stimuli was significantly suppressed by Pb without causing cell death.

Pb has been shown to mimic calcium in normal cellular function (Kirberger et al. 2008), therefore it was hypothesized that depression of M1 activation in macrophages by Pb was due to inhibition of calcium signaling in the cytoplasm of the cell. Cytoplasmic calmodulin expression was not altered by exposure to Pb in any treatment group. It is known that Pb binds calmodulin and induces conformational changes (Kirberger et al. 2013), however this is not observed in Western blot therefore expression was not expected to be altered by Pb. Cytoplasmic phosphorylated CaMK II was not detected in the RAW cells at 4 or 24hrs but cytoplasmic expression of the protein was shown with Western blot. The 4hr phosphorylated STAT1 data showed that nuclear phosphor-STAT1 was comparable between cells cultured with IFN-y and Pb/IFN-y indicating equal translocation between the groups. It is know that activated CaMK II phosphorylates STAT1 at serine 727 and this is required for full activation of STAT1 and translocation to the nucleus (Nair et al. 2002; Barnholt et al. 2009). Given this and the translocation of

STAT1 in this dissertation, it would suggest that activation of CaMK II via calmodulin was not inhibited by Pb.

At 24hrs, Pb cells stimulated with IFN-y had significantly increased phosphorylated STAT1 beta compared to non-Pb cells. STAT1 beta is a negative inhibitor of STAT1 alpha and decreases the protein's function as a transcription factor. This indicates Pb potentially suppressing M1 activation in IFN-y stimulated macrophages. Cytokine expression in these cells at 24 and 48hrs showed a trend increase in the M2 cytokines examined in this dissertation. M1 and M2 activation negatively regulate one another, as one activation is induced, the other is suppressed (Martinez and Gordon 2014). As STAT1 beta suppresses the M1 IFN-y pathway, potential activation of the M2 transcription factor STAT6 may have been occurring in the cells. This could offer an explanation for the cytokine production in Pb treated cells as 24 and 48hrs.

Unlike STAT1, NF-kB p.65 saw early inhibition of nuclear translocation in cells cultured with Pb and LPS. This inhibition was overcome at 24hrs indicated by equal expression in Pb and non-Pb cells in Western blot. This corresponded to significantly decreased TNF-a cytokine expression in the supernatant of these cells. TNF-a is a strong M1 cytokine produced by macrophages and the subsequent decrease in Pb treated cells suggests suppression of M1 function. One possible mechanism for this inhibition of NF-kB translocation is Pb directly inhibiting the pathway. It has been shown that other divalent cations such as cadmium and zinc can inhibit release of NF-kB by binding IkkB and preventing protein function (Liu et al. 2013). Therefore Pb possibly functions in the same manner.

Nitric oxide production in stimulated cells was significantly inhibited by Pb at 12 and 24hrs. This suppression would be associated with decreased pathogen destruction and paracrine signaling with other cells of the immune system. The protein iNOS produces NO in the macrophage (Nussler and Biliar 1993) and data in this dissertation showed that cytoplasmic expression of iNOS was not altered by Pb, indicating that protein function and not transcription is the target of Pb. One possible mechanism of this is Pb binding calmodulin at the N terminal binding sites and preventing calmodulin from binding iNOS in the cytoplasm. The iNOS/calmodulin complex is required for NO production in the macrophage, therefore this could explain the data reported.

The role of the macrophage in the immune system is pathogen clearance and antigen presentation to adaptive immune cells. The data showed that Pb significantly inhibited NO production in cells cultured with Pb LPS/IFN-y at 12 hrs. This in itself would impact pathogen clearance by these macrophages. It was also shown that the same treatment in RAW cells significantly decreased phagocytosis at 12, 24, and 48hrs. These data demonstrate that Pb significantly suppressed pathogen destruction and clearance in RAW cells stimulated with LPS/IFN-y.

The increased cytoplasmic activation of CaMKIV by Pb in this dissertation was not expected. Given that calmodulin is known to be a target of Pb, it was expected that downstream kinases would not be activated. The role of CaMKIV in M1 and M2 activation is not completely known; therefore the suppression of M1 activation in this dissertation cannot be tied directly to CaMKIV. It has been shown that CaMKIV is a key signaling protein in autophagy induction in cells, including the RAW 264.7 macrophage (Zhang et al. 2014). Kerr et al. (2013) demonstrated that RAW cells cultured with the

same concentration of Pb as this dissertation induced autophagy. The increased cytoplasmic activation of CaMKIV in Pb cells in the current study could be associated with induction of autophagy rather than shifts in macrophage activation.

CONCLUSIONS

The Pb pellets administered to the hens in this dissertation are labeled as #9 and #7.5 Pb pellets. These numbers denote the size of the pellet and are the numbers labeled on shotgun shells that describe the difference between shells. These pellet sizes are two very common sizes used in bird hunting for species such as dove, quail, pheasant, and crow, all of which the use of Pb ammunition is legal. A shotgun shell for either of these pellet sizes would contain hundreds of pellets that when fired, contaminate a large surface area of ground. The regulations on bird hunting in most states including Georgia allow for three shotgun shells to be fired by one hunter at a single time, therefore potential exists for greater than 1000 Pb pellets deposited in a particular area (Georgia Department of Natural Resources 2017). This demonstrates why frequently hunted areas can have Pb pellet distribution exceeding a million Pb pellets in the top centimeters of soil per hectare of land (Pain 1991). Given this data, it is evident that foraging avian species such as the roller pigeon potentially could consume multiple Pb pellets at a single time. Therefore, the developmental toxicity data presented in this dissertation represents the best possible outcome for avian exposure in the wild.

The *in vitro* study in the RAW 264.7 cells showed that Pb suppresses signal transduction and functional responses in macrophages during M1 activation through LPS and stimulation. The original hypothesis was that Pb would suppress calcium signaling by inhibiting calmodulin and preventing activation of downstream kinases and

transcription factors. The data actually contradicted the hypothesis and showed that kinase activation and activation of CREB was present in cells cultured with Pb. The data presented in this study suggests that Pb induces autophagy in RAW 264.7 cells through increase cytoplasmic activation of CaMKIV.

FUTURE DIRECTIONS

The data presented in Chapter 3 of this dissertation demonstrated that some weanlings from Pb exposed Roller pigeon hens had lesions on the thymus. The thymus is the primary lymphoid organ for T cells and is the site of T cell differentiation. The thymic degradation seen could adversely impact circulating T cell number and function in the weanlings. One simple test that could be conducted in future studies is the phytohemagglutinin (PHA) skin test. The PHA skin test measures functional cell-mediated immunity and would demonstrate if T cell function is suppressed in these weanlings (Bonforte et al. 1971).

Another test that could be conducted is vaccination of the weanlings with a common avian vaccine followed by an ELISA measuring total IgY antibodies specific for the epitopes of the vaccine. Essentially, by conducting a vaccine challenge study, one could evaluate Pb's effect on antibody production and by evaluating the isotypes indirectly assess T cell function.. B cells are the lymphocytes that produce antibodies, but isotype switching and production of IgY requires B cell interaction with activated T cells also specific for the antigen. Downstream, cells of the innate immune system such as macrophages must encounter the pathogen epitopes or determinants of the vaccine, process it, and present antigens to T cells of the adaptive immune system. Therefore, if thymic degradation in the weanlings resulted in nonfunctional T cells, T cells with

specific T cell receptors for the presented antigen may not be produced. T cell activation may also be hindered in these weanlings as well. If either scenario was present, T cell activation of B cells and induction of isotype switching to IgY would not occur and antibody titers would reflect this.

Another future direction would be to isolate macrophages from Pb-exposed roller pigeons and then assess the ability of the macrophage to respond to M1 stimuli such as LPS or IFN-γ. This research can be conducted in macrophages isolated from the weanlings presented in this dissertation or the Pb exposed hens. To my knowledge, Pb modulation of avian macrophage function has not been previously investigated. Functional endpoints such as viability, phagocytosis, nitric oxide production, and cytokine expression could be investigated to evaluate the changes Pb may elicit on avian macrophages. Kerr et al. (2013) showed that the RAW cells cultured with 5μM Pb had altered cell surface expression of CD80, CD86, and MHCII. These proteins are associated with antigen presentation and activation of T cells of the adaptive immune system. Assessing these proteins in avian macrophages would assess the ability of these cells to communicate with cells of the adaptive immune system. This could also play a role in the above proposed scenario with vaccination of the weanlings in the #7.5 Pb-treated hens. If macrophage function or protein expression are altered, this would likely affect the macrophage's ability to activate T cells during a vaccine challenge. Downstream, this could also result in decreased production of antigen specific IgY from activated B cells.

There are many directions that can be explored to investigate the mechanism of calcium signaling inhibition in mammalian macrophages. The data generated in this study

suggest that phagocytosis of heat killed *E. coli* was only significantly decreased in cells treated with Pb and stimulated with the combination of LPS and IFN-γ. Another important point is that is has previously been shown that phagocytosis of killed bacteria is not nearly as efficient in macrophages as phagocytosis of live bacteria (DeChatelet et al. 1974; Perun et al. 2017). Therefore, the data generated in this study, while biologically important, does not represent the complete phagocytic ability of these cells and does not simulate an *in vivo* exposure when an individual is exposed to live bacteria. It is possible that the lack of phagocytic ability in the other treatment groups in this dissertation were impacted by the heat killed bacteria. Future studies could investigate phagocytosis of multiple strains of killed and live bacteria to further determine if Pb's modulatory role on phagocytosis is influenced type of bacteria present or the viability of the bacteria.

Another area to consider evaluating further is culture environment. The culture methods in this dissertation do not fully represent a typical *in vivo* set of exposure conditions. RAW cells were activated with LPS or IFN-y and exposed to Pb simultaneously. An *in vivo* model would generally occur with Pb exposure first followed by potential immune challenge and activation. Therefore, a future study could determine RAW cell pro-inflammatory activation following culture with Pb. The potential design could be cell culture with Pb for a specified period of time such as 12 and 24hr followed by addition of LPS or IFN-y and continued culture for up to 24hr. Signal transduction and functional endpoints could be investigated using the same techniques as this dissertation. Given that Pb alone alters functional response of macrophages and induces autophagy (Kerr et al. 2013), this proposed future study could present further data supporting M1

suppression in RAW cells and correlate the *in vivo* Th2 immune profile shift observed in chronically Pb-exposed individuals.

Co-culturing RAW 264.7 cells with a comparable murine T helper cell line or isolated murine T cells would also be a good future direction that would determine Pb modulation of activation and communication between these cells. Biologically, macrophage activation and antigen presentation to T cells is required for adaptive immune response. It is already known that T cells are a target or Pb toxicity both *in vitro* and *in vivo* (Ewers et al. 1982; Mishra et al. 2003) and the data presented in this dissertation suggest that the macrophage is susceptible to Pb toxicity during pro-inflammatory activation. Therefore, co-culturing RAW cells and T cells with Pb would simulate an *in vivo* model for cell interaction. One model could be treatment of both cell lines with Pb and RAW cells stimulated towards pro-inflammatory activation with LPS. This would represent the RAW cell activation and how subsequent T cell activation occurred. A second model would be to again treat both cell lines with Pb but stimulate T cells with Concanavalin A (ConA), a known T cell mitogen (Palacios R. 1982). This would help assess how Pb modulates T cell activation and how this activation alters macrophage response (RAW cells).

The research in this dissertation used the RAW 264.7 as a macrophage/monocyte cell line that is well established in research. This cell line was isolated from male BALB/c mice and is a peritoneal cell that expresses phenotypic characteristics of a monocyte and macrophage and has a strong M1 response when stimulated with LPS or IFN-γ (Yagnik et al. 2000). Future work could be done to evaluate the culture techniques used in this dissertation on other macrophage/monocyte cell lines. One of which could be

the J774A.1 macrophage/monocyte cell line. This cell line expresses many of the same phenotypic characteristics as the RAW cells and has a strong M1 response but the J774A.1 was isolated from female BALB/c mice (Chamberlain et al. 2008). Given that certain toxicants can be sex specific, this cell line would evaluate if Pb modulation of macrophage activation has differential results based on sex.

This dissertation focused on Pb modulation of pro-inflammatory activation in the RAW cells. One other future area of study would be to evaluate how Pb affects anti-inflammatory activation in RAW cells. Given that Pb shifts immune profiles towards Th2 anti-inflammation, Pb may actually induce a strong M2 response on RAW cells when stimulated with the T cell cytokine IL-4 (He et al. 2011). Another area to investigate would be to use a macrophage/monocyte cell line that is known to produce a strong M2 response. One such cell line is the murine IC-21 macrophage cell line. This is another well-established cell line and has been shown to have a robust M2 response and when stimulated biases towards anti-inflammation (Yagnik et al. 2000). It is possible the Pb could induce a strong M2 response in these cell lines and the use of IC-21 would allow for investigation of protein signaling in the cells.

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