


Ingestion of a Single 2.3 mm Lead Pellet by Laying Roller Pigeon Hens Reduces Egg Size and Adversely Affects F1 Generation Hatchlings

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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 4 weeks. In the current study, proven breeder pairs of domestic Roller pigeons were housed in individual cages. The hens were orally gavaged with dH₂O 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- to 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 also was 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-treated weanlings, during both the first and second clutch compared with 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.

Lead (Pb) toxicity is an area of research that has gained 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. 2016). The United States has issued increasingly stringent regulations over the past four decades on inclusion of Pb in consumer products, due to the discovery of potentially severe toxicity in children (Needleman 2000). Whereas 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 1990s 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 (Matero 2009). Of the species potentially exposed to Pb in the form of spent ammunition, >120 avian species, both terrestrial

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and aquatic, are at heightened risk, because these species are known to ingest spent ammunition and bullet fragments mistakenly 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 sublethal 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 indicated prolonged exposure (Ferreira et al. 2014). 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 sublethally dosed with Pb shot pellets (Vallverdu-Coll et al. 2015a).

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 et al. 2015c). Interestingly, the researchers showed that during the spring, oxidative stress was increased in both male and female birds; however, the response was sex-dependent (Vallverdu-Coll et al. 2015c). 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 et al. 2015b).

Lead is particularly toxic during development in mammalian species. Limited studies have examined 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; Tsipora

et al. 2011). Vallverdú-Coll et al. (2015a) 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 7. Another study evaluating Pb influence on avian reproduction showed that red-legged partridge (*Alectoris rufa*) hens gavaged with three #6 Pb pellets (~109 mg/pellet) had a reduction in hatching rate (Vallverdú-Coll et al. 2016). Hatchability also was decreased in mourning doves (*Zenaidura macroura*) when hens were exposed to a single #8 Pb pellet (~70 mg) (Burger 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 purpose of the present study was to determine the extent of maternal transfer of known amounts of gavaged Pb to the offspring and to 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 20× of control, 4 weeks after exposure, while suppressing δ -ALAD throughout the 4-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 3 years of age were obtained from a private breeder (Color Pigeons, Kinston, NC) and were randomly assigned to individual cages (1 male and 1 female/cage) in a standard flock house at the University of Georgia Poultry Diagnostic Research Center (UGA PDRC, Athens, GA). The birds were maintained at approximately 23 °C on a 24-h 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 2-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, 2 mm, 45–50 mg) and number 7.5 (spherical, 3 mm, 90–95 mg) Pb shot pellets were supplied by the U.S. Army Institute of Public Health. All pellets were rinsed with dH₂O to remove any debris from the surface and then weighed to confirm weight.

Pb Pellet Administration

On 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 2-mL syringe. Control hens received approximately 1 mL of dH₂O, and treatment birds received a single #9 or #7.5 Pb pellet in 1 mL of dH₂O. 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, 10–15 mA, 0.7 s). 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 before breeding on two consecutive clutches (1–2 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.

Hatchlings

Hatchlings were monitored daily for behavioral or physical changes. Body weight was recorded in grams 7 days post-hatch and at weaning (28–35 days post hatch) just before 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 3-mL syringe. Immediately after 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). A total of 150 μ l of whole blood were transferred to Savillex digestion beakers and incubated overnight with 300 μ l of trace metal grade HNO₃. Following overnight incubation, the whole blood solution was heated to 90 °C for 3 h. Next, 300 μ l of trace metal grade H₂O₂ were added and heated to 90 °C for 1 additional hour. Samples containing precipitate were treated with another 150 μ l of HNO₃ and incubated at 90°C for 1 h. Samples were then transferred to 15-mL conical tubes and diluted with 15% trace metal grade HNO₃ to 5-mL total volume and analyzed using ICP-MS on a Plasma Quad VG PQ Excell. The machine was calibrated using certified lead standard. The standards ranged from 0.98 to 500 ppb with a “blank” that read zero. All samples were analyzed quantitatively and fell within the measurable range. The dilutions were factored into give the final concentrations shown (Table 1).

Table 1 7-day post exposure hen blood-Pb levels and δ -ALAD activity

	Control (mean \pm SEM, ppb) $n = 5$	#9 Pellet (mean \pm SEM, ppb) $n = 4$	#7.5 Pellet (mean \pm SEM, ppb) $n = 5$
Blood-Pb levels (ppb)	8.3 \pm 2.15 (range 2.8–15.4)	1237 \pm 538 (range 650–2311)	3549 \pm 1311 * (range 850–7980)
δ -ALAD activity	87.5 \pm 11.0	37.5 \pm 2.5 **	33.6 \pm 3.3 **

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

Histopathology

Weanlings were euthanized between days 28–35 using CO₂ 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 (p_1) of pigeon with lesions under a Pb treatment group is no different than the proportion 2 (p_2) of pigeons with lesions in the control group. This is represented by the null hypothesis:

$$H_0 : p_1 = p_2$$

Versus the alternative hypothesis that these two proportions are different:

$$H_a : p_1 \neq p_2$$

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

Results

Radiographs

All 12 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

Blood collected from the hens 7 days post gavage was analyzed for Pb levels and δ -ALAD activity. The #9 pellet treatment hens had elevated blood-Pb levels, although not significant, whereas the #7.5 treatment hens had significantly increased blood-Pb levels ($p = 0.036$; Table 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 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. 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 2). The width of the eggs produced by the #7.5 treatment hens was numerically but

Table 2 First clutch egg weight, length and width

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

* $p \leq 0.05$; ** $p \leq 0.005$, Tukey–Kramer’s One Way ANOVA

not significantly decreased compared with control. The second clutch egg weight and dimensions showed no significant differences among the treatment groups (Table 3).

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 4). The control group had 100% survivability throughout the study, whereas both the Pb treatment groups had mortality during both the first and second clutch.

Hatchling Week 1 and Final Weights

Hatchlings were weighed 1 week post-hatch to record initial weights. The 1-week delay was used to ensure that handling the hatchling would not cause injury or prevent the hen or cock from brooding. Weights were taken again just before 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 5). 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.

Table 3 Second clutch egg weight, length and width

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

* $p \leq 0.05$, Tukey–Kramer’s One Way ANOVA

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 $2.81(\times 10^9/\text{mL}) \pm 0.32$, 32.08 ± 2.33 , and $4.88 (\text{g}/100 \text{mL}) \pm 0.51$ 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 nmol 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. 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 to 5.36 ppb.

Table 4 First and second hatch 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 5 First and second hatch week one and final weights

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

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

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. 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 granulopoiesis of the kidney. The second clutch had results similar to the first clutch. 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 (Fig. 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 with the control group ($z = 3.2303$, $df = 1$, $p = 0.0012$). Each clutch was then analyzed separately using the Chi square test. Lesions in the first clutch were significantly increased in the Pb treatment groups ($z = 2.7963$, $df = 1$, $p = 0.0052$), and showed a near-significant trend toward increase in the second clutch ($z = 2.9654$, $df = 1$, $p = 0.0851$).

Discussion

The radiographs showed retention of the pellet in the crop on day 1; 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 a decrease in blood δ -ALAD activity of approximately 43 and 38%, respectively. These data

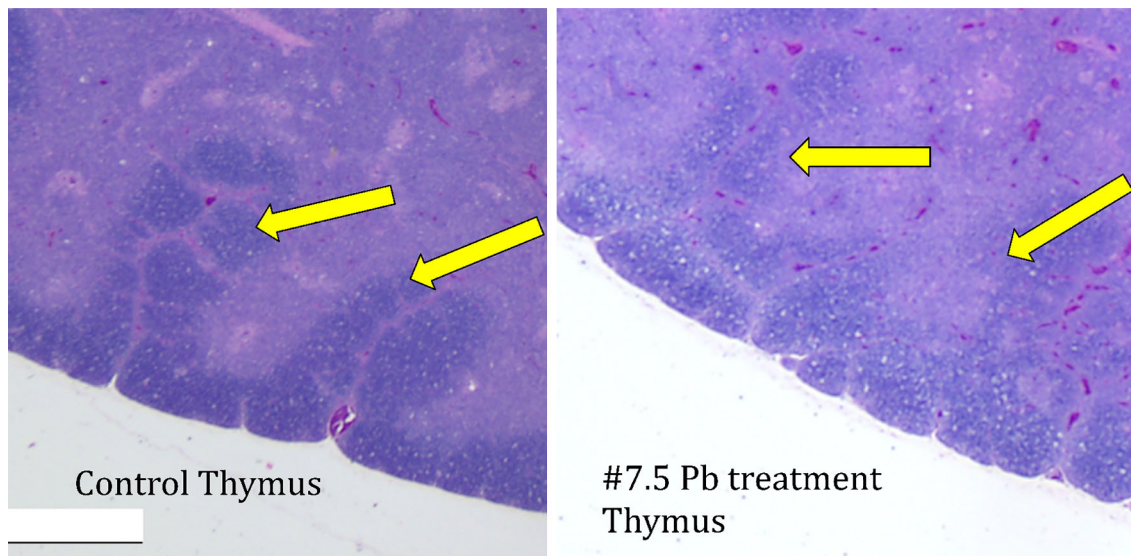


Fig. 1 Thymic cortical degeneration in #7.5 Pb treated weanling. The image on the *left* shows a healthy thymus in a control weanling. The *arrows* indicate well defined cortical regions. The *arrows* in the image

on the *right* shows cortical degeneration in a #7.5 Pb pellet treated weanling

clearly show absorption from the gut for systemic mobilization and suggest the potential for developmental toxicity to occur 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 also were modestly diminished in length and width, with both of these dimensions being 92% of control. Interestingly, Vallverdú-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 nonsignificantly 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 could not 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 6 weeks post exposure to a single Pb shoot (Holladay et al. 2012). Pb levels in these birds remained nonsignificantly elevated for an additional 2 weeks. The second clutch in the current study occurred approximately 7 weeks post exposure, with

results that again suggest blood Pb levels had dropped sufficiently not to affect egg weight, size, or hatch rate. Blood Pb levels were not followed in the present birds 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 10 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 10 days post hatch. It was observed that the hen and cock had ceased brooding the hatchlings that died within the first 10 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 (Barthalamus et al. 1977; Anders et al. 1982). Given that the exposure in the present study was a single exposure, 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.

Vallverdú-Coll et al. (2015a) recently found that mallard duck hatchlings with blood-Pb concentrations greater than 180 ng/mL died within the first 7 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 before necropsy, showed no significant differences between treatment groups. δ -ALAD activity in these birds also was not different between treatments, suggesting that 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 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, whereas 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 approximately 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 five of seven 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. Furthermore, 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 (Fig. 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 also have 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 subchronic to chronic exposures. The severity of the lesions was greater in these studies, and Pb concentrations were measurable in the tissue; however, given that Pb exposure was daily over a 1- to 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.

The present experiments were conducted, because levels of Pb that 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: 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 also are 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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References

- Anders E, Dietz DD, Bagnell CR, Gaynor J, Krigman MR, Ross DW, Leander JD, Mushak P (1982) Morphological, pharmacokinetic, and hematological studies of lead-exposed pigeons. *Environ Res* 28:344–363
- Barthalamus GT, Leander JD, McMillan DE, Mushak P, Krigman MR (1977) Chronic effects of lead on schedule-controlled pigeon behavior. *Toxicol Appl Pharmacol* 42:271–284
- Bennett JR, Kaufman CA, Koch I, Sova J, Reimer KJ (2007) Ecological risk assessment of lead contamination at rifle and pistol ranges using techniques to account for site characteristics. *Sci Total Environ* 374(1):91–101
- Berlin A, Schaller KH (1974) European standardized method for the determination of delta-aminolevulinic acid dehydratase activity in blood. *Z Klin Chem Klin Biochem* 12(8):389–390
- Beyer NW, Spann JW, Sileo L, Franson CJ (1988) Lead poisoning in six captive avian species. *Arch Environ Contam Toxicol* 17(1):121–130
- Binkowski LJ, Sawicka-Kapusta S, Szarek J, Strzyzewska E, Felsman M (2013) Histopathology of liver and kidneys of wild living Mallards *Ans platyrhynchos* and Coots *Fulica atra* with considerable concentrations of lead and cadmium. *Sci Total Environ* 450–451:326–333
- Burger TJ, Mirarchi RE, Lisano ME (1986) Effects of lead shot ingestion on captive mourning dove survivability and reproduction. *J Wildlife Manage* 50(1):1–8
- Burger J, Gochfeld M (2004) Metal levels in eggs of common terns (*Sterna hirundo*) in New Jersey: temporal trends from 1971 to 2002. *Environ Res* 94(3):336–343
- Cory-Slechta D, Garman RH, Seidman D (1979) Lead-induced crop dysfunction in the pigeon. *Toxicol Appl Pharm* 52:462–467
- Craig JR, Rimstidt JD, Bonnaffon CA, Collins TK, Scanlon PF (1999) Surface water transport of lead at shooting range. *Bull Environ Toxicol* 63:312–319
- Ferreira H, Romano M, Beldomenico P, Caselli A, Correa A, Uhart M (2014) Lead gunshot pellet ingestion and tissue lead levels in wild ducks from Argentine hunting hotspots. *Ecotox Environ Saf* 103:74–81
- Griffin TB, Couiston F, Wills H (1975) Biological and clinical effects of continuous exposure to airborne particulate lead. *Arh Hig Toksikol* 26:191–208
- Haig SM, D'Elia J, Eagles-Smith C, Fair JM, Gervais J, Herring G, River JW, Schulz JH (2014) The persistent problem of lead poisoning in birds from ammunition and fishing tackle. *Condor Ornithol Appl* 116:408–428
- Hanna-Attisha M, LaChance J, Sadler RC, Champney Schnepf A (2016) Elevated blood lead levels in children associated with the Flint drinking water crisis: a spatial analysis of risk and public health response. *Am J Public Health* 106(2):283–290
- Holladay JP, Nisnian M, Williams S, Tuckfield RC, Kerr R, Jarret T, Tannenbaum L, Holladay SD, Sharma A, Gogal RM Jr (2012) Dosing of adult pigeons with as little as one #9 lead pellet caused severe δ -ALAD depression, suggesting potential adverse effects in wild populations. *Ecotoxicology* 21(8):2331–2337
- Kendall RJ, Scanlon PF (1981) Effects of chronic lead ingestion on reproductive characteristics of ringed turtle doves *Streptopelia risoria* and on tissue lead concentrations of adults and their progeny. *Environ Pollut A Ecol Biol* 23(3):203–213
- Kerr R, Holladay J, Holladay S, Tannenbaum L, Selcer B, Meldrum B et al (2011) Oral lead bullet fragment exposure in northern bobwhite (*Colinus virginianus*). *Arch Environ Contam Toxicol* 61(4):668–676
- Lee J, Dietert RR (2003) Developmental immunotoxicity of lead: impact on thymic function. *Birth Defects Res A Clin Mol Teratol* 67(10):861–867
- Lee J, Naqi SA, Kao E, Dietert RR (2001) Embryonic exposure to lead: comparison of immune and cellular responses in unchallenged and virally stressed chickens. *Arch Toxicol* 75:717–724
- Locke LN, Bagley GE, Irby HD (1966) Acid-fast intranuclear inclusion bodies in the kidneys of mallards fed lead shot. *Bull Wildlife Dis Assoc* 2(4):127–131
- Mateo R, Beyer WN, Spann J, Hoffman D, Ramis A (2003) Relationship between oxidative stress, pathology, and behavioral signs of lead poisoning in Mallards. *J Toxicol Environ Health A* 66(17):1371–1389
- Matero R (2009) Lead poisoning in wild birds in Europe and the regulations adopted by different countries. Ingestion of lead from spent ammunition: implications for wildlife and humans. The Peregrine Fund, Boise, Idaho
- Needleman HL (2000) The removal of lead from gasoline: historical and personal reflections. *Environ Res A* 84:20–35
- Pain DJ, Fisher IJ, Thomas VG (2009) A global update of lead poisoning in terrestrial birds from ammunition sources. Ingestion of lead from spent ammunition: implications for wildlife and humans. The Peregrine Fund, Boise, Idaho
- Pant N, Upadhyay G, Pandey S, Mathur N, Saxena DK, Srivastava SP (2003) Lead and cadmium concentration in the seminal plasma of men in the general population: correlation with sperm quality. *Reprod Toxicol* 17:447–450
- Peddicord RK, LaKind JS (2000) Ecological and human health risks at outdoor firing range. *Environ Toxicol Chem* 19:2602–2613
- Rabinowitz MB, Wetherill GW, Kopple JD (1976) Kinetic analysis of lead metabolism in healthy humans. *J Clin Invest* 58:260–270
- Taggart MA, Green AJ, Mateo R, Meharg AA (2008) Metal levels in the bones and livers of globally threatened marbled teal and white-headed duck from El Hondo, Spain. *Ecotox Environ Saf* 72(1):1–9
- Tsipora N, Burger J, Newhouse M, Christian J, Gochfeld M, Mizrahi D (2011) Lead, mercury, cadmium, chromium, and arsenic levels in eggs, feathers, and tissues of Canada geese of the New Jersey Meadowlands. *Environ Res* 111:775–784
- Vallverdú-Coll N, Lopez-Antia A, Martinez-Haro M, Ortiz-Santaliestra ME, Mateo R (2015a) Altered immune response in mallard ducklings exposed to lead through maternal transfer in the wild. *Environ Pollut* 205:350–356
- Vallverdú-Coll N, Mougeot F, Ortiz-Santaliestra ME, Rodriguez-Estival J, López-Antia A, Mateo R (2015b) Lead exposure reduces carotenoid-based coloration and constitutive immunity in wild mallards. *Environ Toxicol Chem* 35(6):1516–1525
- Vallverdú-Coll N, Ortiz-Santaliestra ME, Mougeot F, Vidal D, Mateo R (2015c) Sublethal Pb exposure produces season-dependent effects on immune response, oxidative balance and investment in carotenoid-based coloration in red-legged partridges. *Environ Sci Technol* 49(6):3839–3850
- Vallverdú-Coll N, Mougeot F, Ortiz-Santaliestra ME, Castano C, Santiago-Moreno J, Mateo R (2016) Effects of lead exposure on sperm quality and reproductive success in an avian model. *Environ Sci Technol* 50(22):12484–12492
- Vigeh M, Smith DR, Hsu PC (2011) How does lead induce male infertility? *Iran J Reprod Med* 9(1):1–8
- Wang H, Li S, Teng X (2016) The antagonistic effect of selenium on lead-induced inflammatory factors and heat shock proteins mRNA expression in chicken livers. *Biol Trace Elem Res* 171:437–444