Environmental Pollution 205 (2015) 350–356

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Altered immune response in mallard ducklings exposed to lead through maternal transfer in the wild

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form
9 June 2015
Accepted 11 June 2015
Available online xxx

Keywords:
Pb poisoning
Maternal transfer
Immune response
Oxidative stress
Developmental toxicity
Waterfowl

ABSTRACT

Lead (Pb) poisoning has caused significant mortality in waterfowl populations worldwide. In spite of having been banned since 2003, prevalence of Pb shot ingestion in mallards (*Anas platyrhynchos*) from the Ebro delta was still 15.5% in 2011–12. We collected mallard eggs from this area to study the effects of maternally transferred Pb on eggshell properties and on immune response and oxidative balance of ducklings. Eggshell Pb levels were positively correlated with Pb levels in the blood of ducklings. Ducklings with blood Pb levels above 180 ng mL^{-1} showed reduced body mass and died during the first week post hatching. Blood Pb levels positively correlated with humoral immune response, endogenous antioxidants and oxidative stress biomarkers, and negatively correlated with cellular immune response. Pb shot ingestion in birds can result in maternal transfer to the offspring that can affect their developing immune system and reduce their survival in early life stages.

1. Introduction

Lead (Pb) poisoning by ingestion of shot used as hunting ammunition has been identified as a frequent cause of mortality in waterbirds worldwide (Mateo, 2009). Apart from direct mortality, several sublethal effects have been described as a result of Pb shot ingestion in birds (Martinez-Haro et al., 2011a, 2011b), affecting important functions such as reproduction and immune responses (Vallverdú-Coll et al., 2015).

Pb transfer from the mother to the chicks (Burger, 1994) can be significant in species with elevated prevalence of Pb shot ingestion, as in the case of marbled teals (*Marmaronetta angustirostris*), in which maternal Pb transfer has been suggested to be a significant source of exposure for young birds (Mateo et al., 2001). The structural and functional developmental changes in the immune system of embryos and hatchlings make them especially vulnerable to Pb (Lee et al., 2001). Fair and Ricklefs (2002) found that Japanese quail (*Coturnix coturnix japonica*) chicks exposed to Pb presented elevated granulocyte numbers compared to non-exposed ones, but induced immune response was not affected. On the contrary, in ovo Pb exposure increased antibody production in chicken (*Gallus gallus domesticus*) (Bunn et al., 2000), and developing western bluebirds (*Sialia Mexicana*) showed suppressed cell-mediated responses to phytohemagglutinin (PHA) after Pb exposure (Fair and Myers, 2002). One of the main mechanisms of developing immunotoxicity of Pb is the alteration of the balance in the differentiation of T helper (Th) cells, resulting in an increased differentiation into Th2 cells (responsible of humoral-mediated immunity) at expenses of Th1 (responsible of cell-mediated immunity). This results in either immunosuppression or allergic and autoimmune reactions, depending on the type of response (Dietert et al., 2004).

Some components of the immune system, such as phagocytes, engulf microbes and produce reactive oxygen species (ROS) to kill pathogens (Hampton et al., 1998). When the production of ROS as part of the constitutive immune response overwhelms the antioxidant capacity, it results in oxidative stress, posing damage in lipids, DNA and proteins (Dowling and Simmons, 2009). The exposure to a number of chemical substances, including Pb, also results in oxidative stress. Vallverdú-Coll et al. (2015) found in

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http://dx.doi.org/10.1016/j.envpol.2015.06.014
0269-7491/

red-legged partridges (*Alectoris rufa*) that ingested Pb shot during the breeding season produced a decrease in plasma antioxidants and natural antibody levels, together with an increased PHA response. These effects of Pb exposure during the reproduction period on maternal fitness suggest that offspring could be affected not only from effects of Pb due to direct maternal transfer of this metal, but also because of the existence of a trade-off between parental health and developmental immunocompetence of nestlings, as shown by Ardia (2005). Adults exposed to Pb might allocate some energy in detoxification at expenses of reproduction.

Despite all this literature about immunotoxic effects in offspring caused by experimental Pb exposure, there are not studies addressing this issue on naturally exposed populations. Working in such ecologically realistic scenario is particularly relevant for waterfowl, given the high impact that Pb pollution exerts on these species in certain areas (Mateo, 2009). We predict that mallard ducklings (*Anas platyrhynchos*) from an area heavily contaminated with Pb shot as a consequence of an intense hunting activity could suffer altered immune response because of maternal transfer of Pb. With this purpose, we conducted a study to analyse Pb concentration in both content and shell of mallard duck eggs collected from the Ebro delta (NE Spain). We also incubated mallard eggs from this site to study the effects of maternally-transferred in ovo Pb exposure on cellular and humoral immune induced responses of ducklings, together with the study of effects on other blood biomarkers (heme group synthesis, oxidative stress and plasma biochemistry) and eggshell properties (thickness and pigmentation).

2. Material and methods

2.1. Study area

The Ebro delta is an Important Bird Area (IBA) where Pb poisoning has been found to be an important threat for waterbirds living there (Mateo, 2009). Waterfowl hunting has been carried out for more than one century resulting in high Pb shot densities in the upper 20 cm of sediment, exceeding 200 shot/m^2 in several sites (Mateo, 2009). The use of Pb ammunition is banned in the protected areas of this wetland since 2003, but Pb shot is still allowed in adjacent rice fields where waterbirds frequently forage. Consequently, the prevalence of Pb shot ingestion in mallard ducks has only dropped from 30.2% before the ban to 15.5% thereafter (Mateo et al., 2014).

2.2. Sample collection

The experimental procedure had the approval of the Universidad de Castilla-La Mancha's Committee on Ethics and Animal Experimentation. We collected 23 mallard eggs from 23 different nests during the breeding season in 2008 to study Pb concentration on the eggshell and on the content; some of those had already been incubated by mallard hens for a short time before collection. All eggs were collected from protected areas, where Pb shot is currently banned. However, because of the proximity of rice fields where Pb shot use is still allowed and where mallards usually feed, we expected variable degrees of Pb exposure depending on the time spent by each female feeding on protected wetlands or in rice fields. We weighed and measured the length and width of each egg, and stored them at 5 °C until analysis.

In addition, we collected 44 non-incubated eggs in 2009 from 29 different nests and these were artificially incubated to study developmental effects of Pb on ducklings due to maternal Pb transfer. We weighed each hatched duckling and measured tarsus length at the age of 0, 7, 14, 21 and 28 days. Ducklings were maintained in captivity at the Wildlife Rehabilitation Centre (WRC) of the Ebro delta in an indoor pen (12 m^2) with natural sunlight, lamp heating, ad libitum water and diet appropriate for developing chicks. Feed was based on corn (42%), soy flour (36%), barley (16%), pork fat (3.3%), calcium carbonate (1.25%), dicalcium phosphate (1.15%) and sodium chloride (0.3%). It had the following composition: protein (21%), fat (5.3%), cellulose (3.9%) and ashes (7%). It also contained vitamin A (10,000 UI kg^{-1}), vitamin D_3 (2000 UI kg^{-1}), tocopherol (35 UI kg^{-1}), L-lysine (0.1%) and DL-methionine (0.28%) among other additives. In order to assess exposure levels, Pb concentration was measured in blood of ducklings and eggshells. We took blood samples from tarsal vein at the age of 3 and 28 days to determine δ-aminolevulinic acid dehydratase (δ-ALAD) activity, blood Pb concentration, oxidative stress biomarkers and dietary antioxidant levels. In order to evaluate the effects of maternal Pb transfer on the immune function of the ducklings, we tested the cellular immune response (challenged at day 14 and measured at day 15) and the humoral response (challenged at day 21 and measured at day 28). We measured the Pb concentration in liver, brain and bone of ducklings that died after hatching. We also measured the thickness and the concentrations of pigments (biliverdin and protoporphyrin IX) of the eggshells. Ducklings surviving at the end of the experiment were gradually returned to the wild after a conditioning period in semicaptivity at the WRC facilities.

2.3. Blood and tissue Pb concentration and δ-ALAD analysis

We analysed Pb concentration in blood samples diluted (1:10) with triton 0.1% following Mateo et al. (1999) using graphite furnace atomic absorption spectroscopy (GF-AAS; AAnalyst800 with autosampler AS800, Perkin–Elmer). We analysed a certified blood sample (BCR-196) ($n = 3$) for Pb and the obtained percentage of recovery (mean ± SD) was 111.2 ± 0.1. The limit of detection (LOD) was <0.6 μg dL^{-1} of Pb in blood. We determined δ-ALAD activity ratio between the non-activated and the in vitro activated enzyme using a spectrophotometer (Martinez-Haro et al., 2011a). Pb analyses in lyophilized samples of duckling tissues (liver, bone and brain) and egg content were performed by GF-AAS (Rodríguez-Estival et al., 2011) (See Supporting Information for more details, SI).

2.4. Measurement of thickness and pigment concentrations in eggshells

We measured the eggshell thickness of both hatched (2009) and unhatched (2008) eggs. We cut three shell pieces (1 cm × 1 cm) from the equatorial region of each egg and separated the inner membrane. We dried shell pieces and measured three times the thickness of each piece with a micrometre to the nearest 0.001 mm. We used the average thickness of each piece, and then the average measurement of the three pieces of each egg, as a final measurement of shell thickness.

We determined eggshell porphyrins and biliverdin levels following the method described in Mateo et al. (2004) with some modifications (See SI for more details).

2.5. Immune system

We tested T-cell-mediated immune response using the PHA skin test. PHA is a mitogen lectin that produces proliferative responses of circulating T-lymphocytes that are accumulated at the injection site. We used a micrometre (Mitutoyo Absolut 547-401) to the nearest 0.001 mm to measure the thickness of one medial foot membrane at day 14. Then, we injected 50 μL of PHA in phosphate-buffered saline (PBS) (5 mg mL^{-1} dilution). We also measured the