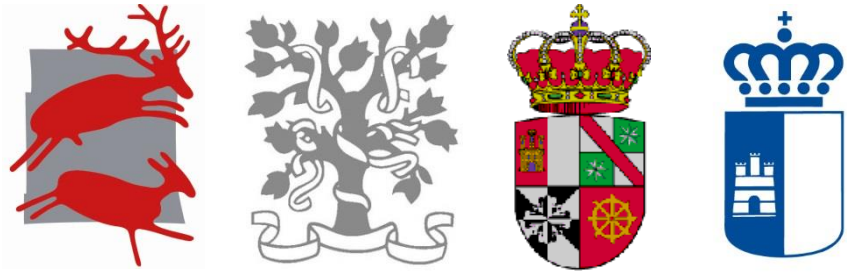

INSTITUTO DE INVESTIGACIÓN EN RECURSOS CINEGÉTICOS

(IREC–CSIC–UCLM–JCCM)

Immunotoxic and reproductive effects of lead on avifauna affected by shot ingestion

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Memoria presentada por

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para optar al grado de Doctor

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GENERAL INDEX

ABSTRACT

The ingestion of spent lead (Pb) shot by waterbirds is a common cause of poisoning in heavily hunted wetlands. Although Pb-based ammunition was banned in protected Spanish wetlands in 2001, spent Pb shot remains unaltered for decades in sediments, and consequently the risk of poisoning remains present. Thus Pb poisoning is an important issue in the conservation of waterfowl even in protected areas, which produces not only physiological and behavioural alterations, but also a wide range of sublethal effects or even death.

Incidence of Pb shot ingestion and mortality caused by Pb poisoning have been widely described. Instead, there is a great lack of knowledge concerning the sublethal effects of Pb poisoning on birds, which will be the focus of the present work. This thesis aims to estimate sublethal effects of Pb on birds concerning relevant functions for the individual as well as for population dynamics such as immunity and reproduction. The involvement of oxidative stress in the pathogeny of Pb adverse effects on these two functions was used as the meeting point among all the analysed responses.

To achieve these goals, we firstly assessed the degree of compliance with the ban on the use of Pb ammunition in a Spanish protected wetland, the Ebro delta, and its effects on the prevalence of Pb shot ingestion in waterbirds and on game meat consumers from a public health perspective. Secondly, we studied possible Pb-induced immunological and reproductive abnormalities in environmentally Pb exposed wild waterbirds from the same area considering the possible involvement of oxidative stress as a mechanism involved in Pb toxicity, in parallel with the study of effects of a sublethal Pb exposure on more specific immune system components and reproductive outcomes under experimental conditions by using an avian model to better understand these mechanisms.

Nine years after the ban on the use of Pb ammunition in the Ebro delta, reduced Pb poisoning in waterbirds and exposure for game meat consumers were found. However, the enforcement of the ban did not fully eliminate the risk of Pb

exposure. Although Pb levels in trapped mallards (*Anas platyrhynchos*) from the Ebro delta were reduced 9 years after the Pb restriction, a significant proportion of individuals from this area were still affected by Pb poisoning. Moreover, blood Pb levels were still associated with a range of gender-specific effects on wild mallards such as changes in levels of oxidative stress biomarkers and antioxidants, impairment of the constitutive immune response, and reduced carotenoid-based coloration in males. Furthermore, we demonstrated that Pb shot ingestion in mallards can result in maternal transmission of Pb to the offspring through eggs. A greater exposure to Pb was associated with a decreased survival of ducklings, induction of oxidative stress and the impaired developing immune function.

Under experimental conditions, we found season dependent effects of Pb exposure on immune responses in red-legged partridges (*Alectoris rufa*). Exposure to Pb slightly affected constitutive immunity during the breeding season, whereas it was deeply altered during the non-breeding season which was associated with changes in gut microbiota. Cellular induced response similarly increased after Pb exposure in both seasons which was closely related to the levels of antioxidants and oxidative stress. Females exposed to Pb seemed to use antioxidants to cope with oxidative stress during the breeding season, whereas Pb-exposed males increased carotenoid-based coloration which could reflect an increased breeding investment. During the non-breeding season, both genders prioritized oxidative balance maintenance at the expense of coloration.

Further experiments showed that prelaying Pb exposure induced the production of heavier and larger eggs, heavier chicks and reduced hatching success when females were exposed. In males, Pb exposure decreased acrosomal integrity and sperm motility, and increased sperm vigour, but did not affect sperm viability, concentration, overall progress or fertility. Several sperm parameters showed positive relationships with carotenoid-based coloration and levels of antioxidants that were influenced by Pb exposure, suggesting that redder males may be more capable to preserve sperm from oxidative stress.

This thesis is a step forward for the understanding of effects and mechanisms of sublethal Pb exposure on interrelated functions and allocation trade-offs among them in birds.

Introduction

Lead (Pb) poisoning has been identified as a health problem since ancient times. Nikander, a Greek physician, reported on the colic and anaemia resulting from Pb poisoning in 250 BC (Needleman, 1999), and this disease was very common in Roman times because of the use of Pb in water pipes, earthenware containers and for wine storage (500 BC-300AC) (Nriagu, 1983). Mateu Orfila, considered as one of the founding fathers of modern toxicology, also described the risks of using Pb compounds and the associated clinical signs in 1817, and identified Pb poisoning as the most important medical subject to be known at that present time (Goyer and Clarkson, 2001). Several sources of Pb contribute in the environmental contamination of natural and anthropic environments (e.g.: mines, smelters, leaded gasoline). In some cases, these sources can represent an exposure high enough to produce clinical signs (Green and Pain, 2012) and even the death (Kaufmann et al., 2003) in humans. In the case of wild birds, three sources of exposure are the most important in terms of potential toxicity and impact on populations: Pb-based ammunition, Pb fishing weights and Pb contaminated soils from mining activities. This thesis will be focused on some epidemiological aspects of the first one, although the observed adverse effects on biomarkers or animal functions could be extrapolated to the other sources of exposure.

This thesis has been organized to answer several unresolved questions about Pb poisoning in wild birds at a national and world scale. Firstly, the ban on the use of Pb ammunition in protected wetlands in Spain since 2001 needs an evaluation in terms of the compliance by hunters and the effects on the reduction of Pb poisoning incidence and contamination of game meat. This is a local issue that can give us valuable information for other regions or countries. This thesis further explores the links between the mechanisms of toxicity of heavy metals, in particular the induction of oxidative stress, and the sublethal effects observed at a functional level, focussing on two functions of high importance for the demography of the species: reproduction and immunity.

The following sections describe the state-of-the-art of these four main points of the study: (i) epidemiology of Pb poisoning in wild birds, Pb effects on (ii) immune and (iii) reproductive functions and (iv) the involvement of oxidative stress in the pathogeny of the Pb adverse effects on these two functions.

1. An overview of lead poisoning in wild birds: the situation in Spain

This review of the literature is focused on Spanish studies on Pb poisoning in wild birds, because other works covered this information at continental or even world levels (Mateo, 2009; Pain et al., 2009). Moreover, as our species under study were mallards (*Anas platyrhynchos*) and red-legged partridge (*Alectoris rufa*), this review is also focused on waterbirds and galliforms.

A common route of exposure leading to clinical Pb poisoning in wild birds is the ingestion of Pb shot used for hunting (Guitart et al., 1999). Wild birds ingest Pb shot for two main reasons. Firstly, species with a developed muscular stomach (gizzard) (e.g.: waterfowl, galliforms) usually feed on hard vegetal matter (e.g.: seeds) or animals with exoskeleton, which require a regular intake of grit (gastroliths) to break and grind food (Mateo et al., 2000b). Pb shot accumulated in hunting areas are ingested by these species when they confuse them with particles of grit (Mateo et al., 2000b; Mateo and Guitart 2000). The second cause of ingestion of Pb pellets concerns birds of prey, especially scavengers, which feed on prey or carcasses with Pb embedded ammunition (shot or bullet fragments) in their flesh (Cerradelo et al., 1992; Mateo et al., 1997b). Once absorbed by birds, Pb adversely affects the circulatory and nervous systems, kidneys and digestive tract, and can also alter other functions, such as immunity or reproduction (Mateo et al., 1998b, 2003a, Rodriguez et al., 2010).

1.1. Lead poisoning in waterbirds

How waterfowl hunting is practiced varies widely among species and locations in Spain, and determines the risk of Pb poisoning in a specific wetland. For instance, the method for coot (*Fulica atra*) hunting in the Ebro delta consists of corralling birds with boats to force them to fly above the circle of hunters located in the same boat or on the shore of the lagoon, whereas ducks are mainly hunted from hides located on the edge of ponds or rice fields. Hunting activity in the lagoons in the Ebro delta is restricted to the early morning hours, while hunting in the rice fields takes place during the four nights around each full moon throughout the hunting season. Hunters usually

bait with cereal grain to attract birds around the areas where they hunt, such as on the fringes of the rice fields. In other wetlands like Doñana, flocks of ducks were followed through swamps by horse and hunted with guns capable of firing large loads of pellets. In Doñana, in areas such as the dune Cerro de los Ánsares, greylag geese (*Anser anser*) were also hunted when they went to the dune to eat sand of a specific thickness to grind bulrush (*Scirpus* sp.) in the gizzard (Mateo et al., 2000a).

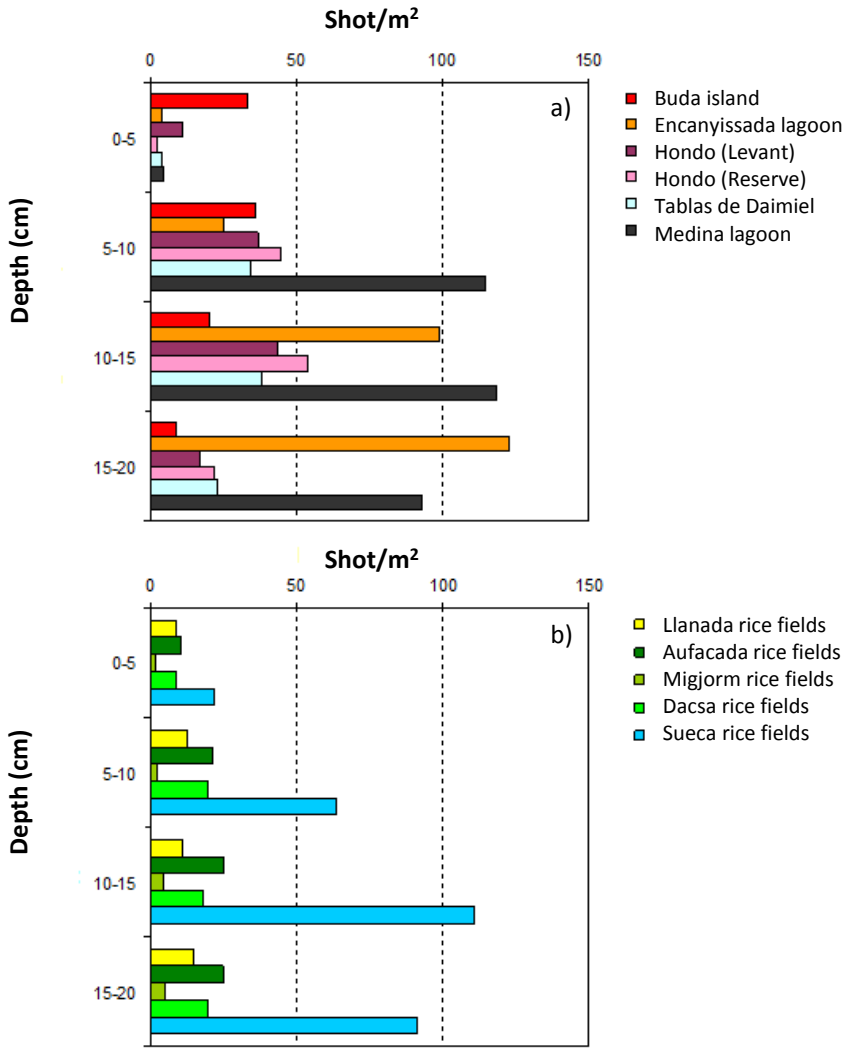

Figure 1. *Densities of Pb shot in the top 20 cm of sediments of lagoons (a) and rice fields (b) in Spain (Guitart et al., 1994, Bonet et al., 1995; Mateo et al., 1997a, 1998a). Adapted from Mateo et al., 2013.*

The highest densities of Pb shot in these wetlands are located in sites where most intense hunting activity has been practiced, from fixed positions and for a long period of time. Due to the high persistence of Pb shot on the environment, presence of Pb shot in sediments has been detected in almost all studied Spanish wetlands, at different depths depending on areas (Figure 1). Maximum densities of pellets in sediment produced by hunting activity have been detected in the "Medina lagoon" (Cádiz) (399 pellets/m^2) (Rodriguez et al., 2010). Even greater densities can be found in wetlands with clay pigeon shooting areas, such as "El Hondo Natural Park" (1,432 pellets/m^2) (Bonet et al., 2004). In "Tablas de Daimiel National Park" about 100 shot/m^2 were still detected in sediments nearly 30 years after the ban on hunting (Mateo et al., 1998a), reflecting the high shot persistence (between 30 and 300 years, depending on soil characteristics and use; Jørgensen and Willems 1987).

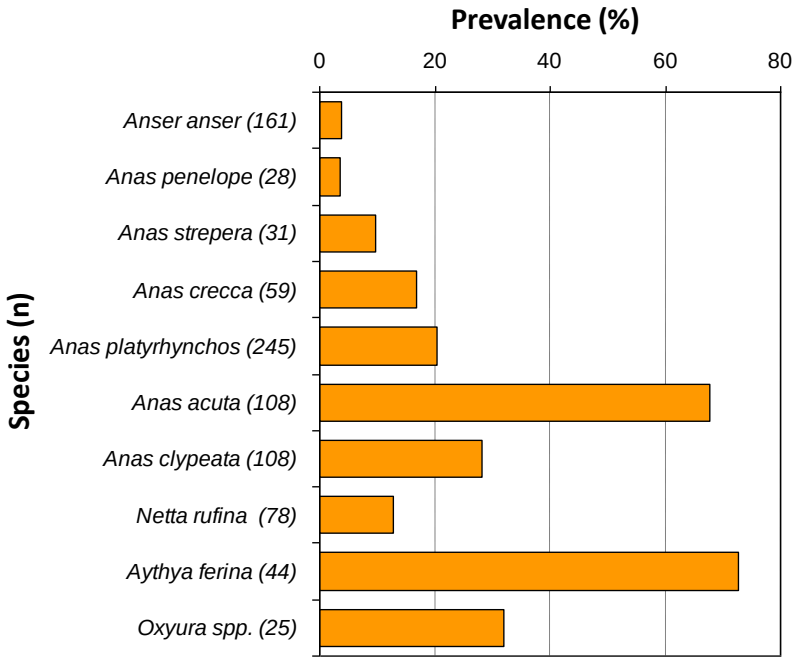

***Figure 2**. Prevalence of Pb shot ingestion in waterfowl from Spanish wetlands between 1977 and 2004 (Lofts 1984, Guitart et al., 1994, Mateo et al., 1997a, 1998a, 2000b, 2007a). Adapted from Mateo et al., 2013.*

The prevalence of ingested Pb pellets has been studied since the late 70's by examining gizzards of birds shot by hunters (Llorente 1984, Guitart et al., 1994, Mateo

et al., 1997a, 1998a, 2000b, 2007a). Some species, such as northern pintail (*Anas acuta*) and pochard (*Aythya ferina*) presented prevalence of Pb shot ingestion around 70%. Other granivorous ducks, such as mallard, shoveler (*Anas clypeata*) and teal (*Anas crecca*), presented a prevalence close to 25% (Figure 2). Species with diets based on grain and selecting a grit size below 3 mm in diameter presented higher ingestion rate of pellets (Mateo et al., 2000b). The amount of grit in the gizzard depends on species, while the grit sizes depends on locality, and may be related to factors such as the availability of different sizes of grit into the environment or the diet of species in a given area (Figuerola et al., 2005).

Other methods more recently used to assess the degree of Pb exposure in wetlands include trapping of live birds (Martinez-Haro et al., 2011b) and non-invasive sampling of faces, which allows to identify the frequency of Pb shot ingestion and sources of Pb poisoning (Martinez-Haro et al., 2010).

Marbled teal (*Marmaronetta angustirostris*), a waterfowl species categorized as vulnerable by the International Union for Conservation of Nature (IUCN), has been shown to be affected by Pb poisoning (Mateo et al., 2001b, Svanberg et al., 2006). Moreover, Pb poisoning has been identified as a main cause of death in white-headed duck (*Oxyura leucocephala*), a globally endangered species according to the IUCN (BirdLife International 2012). Mortality due to Pb poisoning in European waterfowl is estimated around one million of waterbirds, belonging to 17 different species, of a total population of more than eleven million individuals (Mateo, 2009). In the Ebro delta, mortality from Pb poisoning within a largely sedentary population of mallards was estimated in the early 1990s at about 9,600 birds throughout the winter season (Mateo et al., 1997a). In addition, population trends of wintering waterfowl in Europe in recent decades (BirdLife 2004) have been negative for northern pintail and pochard, which presented the highest prevalence of Pb shot ingestion in South Europe in the 90's (Mateo, 2009). Considering all species of waterfowl hunted in Europe, a significant relationship between the trend of the wintering population size and the prevalence of Pb shot ingestion has been observed (Figure 3; Mateo, 2009).

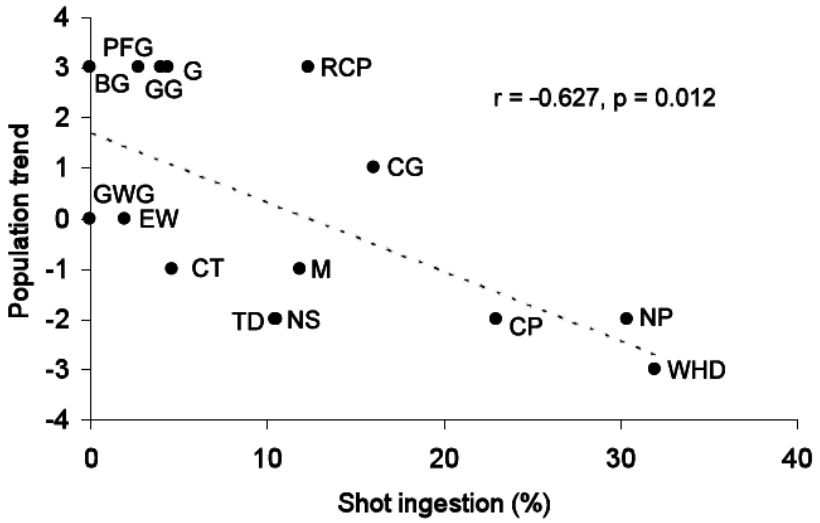

Figure 3. *Correlation between the prevalence of lead shot ingestion and the trend of the wintering population in Europe of 15 species of waterfowl. Pink-footed Goose: PFG, Greater White-fronted Goose: GWG, Greylag Goose: GG, Barnacle Goose: BG, Eurasian Wigeon: EW, Gadwall: G, Common Teal: CT, Mallard: M, Northern Pintail: NP, Northern Shoveler: NS, Red-crested Pochard: RCP, Common Pochard: CP, Tufted Duck: TD, Common Goldeneye: CG, White-headed Duck: WHD. Adapted from Mateo, 2009.*

1.2. Lead poisoning in upland granivorous birds

In terrestrial habitats, unlike in wetlands, density of Pb shot has generally been little studied. Only Ferrandis et al. (2008) studied density of pellets on a private upland hunting estate in central Spain dedicated to red-legged partridge hunting by driven shooting. A density of 7.4 pellets/m^2 to 1 cm soil depth was reported. Because of the low frequency of driven shooting per season in this specific estate, such density is probably low compared to other intensive partridge hunting estates where farm raised partridges are released and driven shooting can be carried out more frequently throughout the hunting season. Regarding Pb shot ingestion, Soler-Rodriguez et al. (2004) examined gizzards from seven hunted red-legged partridges and one of them had ingested 14 pellets and presented 35.6 µg/g wet weight of Pb in liver. More recently, Ferrandis et al. (2008) examined 76 hunted red-legged partridges from the same estate mentioned above, and found a 3.9%, prevalence of ingestion of Pb shot,

with significant variations between years (20% in 2004, 1.5% in 2006) that could be due to differences in the type of grain used to bait animals in the field.

1.3. Lead contamination of game meat

The use of Pb ammunition poses a risk not only to birds but also to public health because birds that have eaten Pb shot present high Pb levels in their tissues (Guitart et al., 2002; Mateo, 2011). In most cases, these levels exceed the maximum residue limits established by the European Union for Pb in viscera (0.5 µg/g fresh weight) and meat (0.1 µg/g fresh weight) of farm animals for human consumption. In addition, Pb ammunition in game meat can release significant amounts of Pb during cooking, especially if they have been cooked with recipes that include vinegar (Mateo et al., 2007b), which increases Pb bioavailability and thus the risk to the consumer (Mateo et al., 2011).

1.4. Adopted measures to reduce lead poisoning from lead-based ammunition

The most important way to reduce the incidence of Pb poisoning in birds, and in turn protect game meat consumers, involves using alternative Pb-free ammunition. This measure allows (i) stopping Pb shot deposition on wetlands and intensive hunting preserves, (ii) eliminating contamination risk from the presence of Pb ammunition in game meat as well as (iii) reducing the likelihood of Pb poisoning in raptors. However, residual contamination may still remain from already accumulated pellets in the environment. Physical elimination of already accumulated Pb pellets in the soil is practically unfeasible in aquatic environments, and only has been carried out successfully in substrates in which it is easier to work, such as in Cerro de los Ánsares (Doñana) (Mateo et al., 2007a). Another possible measure to reduce Pb shot ingestion in waterfowl is the supplementation of grit in areas where this material is limited, although experimental results indicate that it would be effective only in feeding areas (Mateo and Guitart 2000, Martinez-Haro et al., 2009, 2011a).

The use of Pb ammunition for hunting is restricted in 15 countries of the European Union, at least for waterfowl hunting or hunting in wetlands (Thomas and Guitart 2010), to which Norway was added (Mateo, 2009). Among these European

countries, only five (Belgium, Denmark, Netherlands and Sweden) have fully or partially extended the ban on Pb shot for upland hunting (Mateo, 2009). In Spain, the ban on the use and possession of Pb ammunition in Ramsar sites and other protected wetlands was established in 2001 (Royal Decree 581/2001) and extended in 2007 to all wetlands included in the Natura 2000 network (Law 42/2007). This measure has not affected hunting bags in areas such as the Ebro delta, where compliance of such Pb prohibition on hunting areas is almost complete and is managed by regional authorities (Mateo, 2011) (Figure 4).

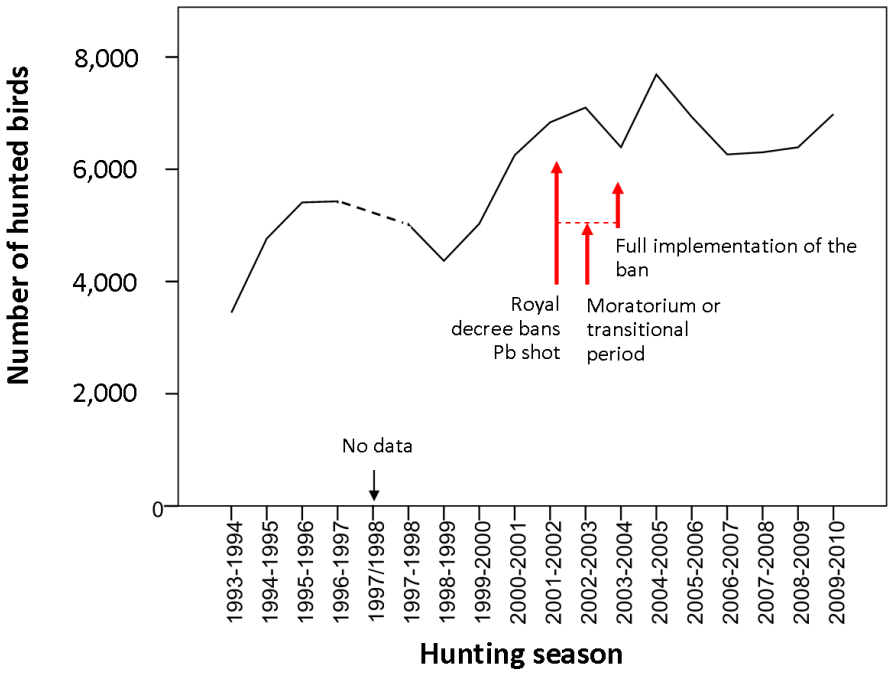

Figure 4. *Evolution of number of hunted waterbirds in three hunting areas with public management (Encanyissada, Buda Island and Garxal) in the Ebro Delta in relation to the ban on Pb ammunition. Adapted from Mateo et al., 2013.*

Regarding hunting in the uplands, the use of copper bullets has been promoted for large game by stalking only in Catalonia (J. Ruiz-Olmo, pers. Comm.). Similarly, in order to protect the bearded vulture (*Gypaetus barbatus*), some trials for the use of copper bullets in large game hunting have been organized in Sierra de Cazorla (Fundación Gypaetus 2010).

Despite the EU being responsible for design, implementation and management of the policy and legislation in the regulation of chemicals, in which Pb would be

included (Thomas and Guitart 2005), the European Commission (EC) has not taken any action on Pb ammunition. The Agreement on the Conservation of African-Eurasian Migratory Waterbirds (AEWA), dependent of the Convention on the Conservation of Migratory Species of Wild Animals (CMS, Bonn Convention), has been the most explicit regarding the prohibition of Pb pellets by saying that "the signatory parties shall endeavour to eliminate the use of Pb shot for hunting in wetlands by the year 2000". At a meeting of the AEWA in 2012 it was included in its resolutions that by 2017 the use of Pb shot for hunting in wetlands should have been banned by signatory countries and effectiveness of national measures to ban Pb shot and implement non-toxic alternatives in wetlands should be assessed, and there must exist a collaboration between all stakeholders, including hunters and bullet manufacturing industries among others, to understand and address the obstacles of the ban implementation, and to establish and implement joint communication strategies. The Secretariat and the Technical Committee of the AEWA will support the implementation and evaluation of such collaboration (AEWA 2012). More recently (November 2014), the CMS celebrated the 11th Conference of the Parties (COP) in Quito, and it was recommended in the guidelines of the conference resolution a rapid worldwide phase-out of the use of Pb ammunition in all habitats and its replacement with non-toxic alternatives, in order to protect migratory birds from being poisoned (UNEP/CMS 2014).

Once introduced the epidemiological scale, it is clear that incidence and mortality caused by Pb poisoning have been widely described, but there exist important knowledge gaps regarding sublethal effects. This thesis aims to fulfil these gaps, and estimate sublethal effects of Pb on birds. The following sections will focus on the sublethal effects on functions that can compromise the population dynamics of exposed birds, either by increasing the risk of mortality by diseases (i.e. immune function) or reducing the recruitment (i.e. reproductive function). In addition, oxidative stress, which is known to be induced by Pb, has been used as the meeting point among all the analysed responses.

2. Effects of lead on the immune function

2.1. Introduction to the immune system

Because environmental pollution can act on the immune system (Eeva et al., 2005), immuno-competence may be used as an indicator of the effects of contaminants on the organism. One of the main principles of immunotoxicological studies lies in the trade-off involving energetic resource allocation, consisting of an additional spending of energy in metabolizing toxicants that would otherwise be used in the maintenance of the immune system, in addition to damage directly caused by the pollutant (Sheldon and Verhulst, 1996).

Immune defences are divided into constitutive and induced components. Constitutive immune response provides the first line of defence against infection and includes disease-resistance mechanisms that are present in the organism without the need of previous stimulation with an antigenic agent (reviewed in Lee, 2006). They are not specific to a particular pathogen, but include cellular and molecular components that recognize classes of molecules frequently encountered in pathogens. Constitutive immunity acts through processes of phagocytosis by fixed or circulating macrophages and the inflammatory response (reviewed in Fairbrother et al., 2004). Induced immune responses are non-specific and specific mechanisms with a high degree of specificity, which are activated by an antigenic challenge, and are responsible for memorizing the antigen for future occasions. Lymphocytes are highly specialized cells that interact with several cells to initiate immune responses (Blakley and kouassi, 2005).

Specific induced immunity is classified into cell-mediated and humoral mediated responses. Cell-mediated immunity acts through the development and proliferation of T-lymphocytes (T-cells), which regulate the function of the humoral-mediated and non-specific immune responses. T-cell subpopulations regulate immune processes by enhancing (T-helper cells (T_{h2}) with CD4+ surface antigens) or supressing (T-suppressor cells (T_{h1}) with CD8+ surface antigens) immune responses (reviewed in Fairbrother et al., 2004). Communication between macrophages and T-lymphocytes is mediated by cytokines. T_{h1} and Th_2 cells produce different types of cytokines and there exists a cross-regulation between the two subsets in which cytokines produced by one

population of cells inhibits the proliferation of the other one (Hemdan et al., 2007). The imbalance in the T_{h1}/T_{h2} influences host responses affecting immune balance, causing immunosuppression or hyperactivity (Dietert et al., 2004). On the other hand, B-lymphocytes are precursors of antibody-secreting cells and can be activated directly with cross-linkage with the antigen (T-independent humoral responses) or indirectly by interaction of T-lymphocytes (T-dependent responses). The humoral response is characterized by the production of antibodies, which are glycoproteins with specific receptors for binding to particular pathogens and neutralize them to facilitate its removal (Ochsenbein and Zinkernagel, 2000). Primary humoral response is generated by naive B-lymphocytes (natural antibodies; NAbs) the first time they encounter an antigen. NAbs activate antibody secreting cells that produce membrane-bound immunoglobulins (IgM) specific to that antigen, while an expanded clone of memory B-lymphocytes is generated. Memory cells quickly proliferate and differentiate the next time the same antibody is detected, producing antigen-specific antibodies (IgG) (secondary humoral response) (Ochsenbein and Zinkernagel, 2000).

Cells such as macrophages, dendritic cells and B-lymphocytes are called antigen-presenting cells (APCs) and display major histocompatibility complex (MHC) genes, a set of genetic loci encoding many of the proteins involved in antigen presentation to T cells, which aid in the ability of the immune system to determine self from non-self (e.g.: to avoid autoimmunity) (Gleichmann et al., 1989).

A balance between different types of immune response may exist and the exposure to an immunomodulatory agent may then induce compensatory responses with the potential to produce diseases (Blakley and Kouassi, 2005). In the past, the immune system was considered as an isolated system that rarely interacts with other functions, but nowadays it is considered one of the most complex systems which is influenced by stress, nervous and endocrine systems, and several physiological factors (Blakley and Kouassi, 2005). As a result, effects caused by some pollutants may vary according to sex, season, or the physical condition of the animal (Møller et al., 2003).

2.2. Effects of lead on the immune system

Because this thesis focuses on avian species, adverse effects of Pb on different aspects of the immune system in birds are summarized in this section (Figure 5). In addition, due to the studies with birds are much fewer in number than those conducted with mammalian models, this information has also been added summarized to give an overview of state-of-the-art in this area (Figure 5).

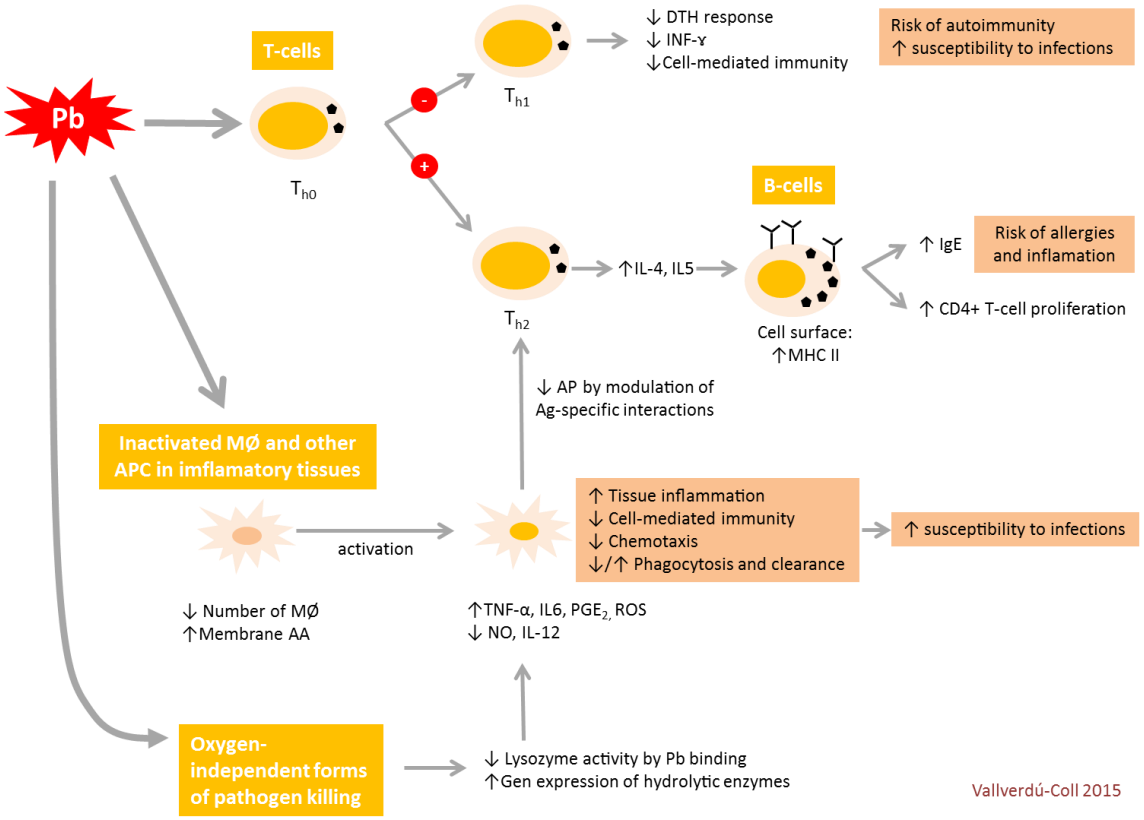

Figure 5. *Effects of Pb on different components of the immune system and the main consequences of these effects (adapted from Dietert and Piepenbrink, 2006). T_h: T-helper cells. DTH: delayed-type hypersensitivity. INF: interferon. IL: interleukin. MHC: major histocompatibility complex. Ig: immunoglobulin. APC: antigen presenting cell. Ag: antigen. TNF: tumour necrosis factor. PG: prostaglandin. NO: nitric oxide. MØ: macrophage. AA: arachidonic acid. Red circles indicate the effects of lead in a given response and the sign indicates whether this is positive or negative. Black arrows indicate whether the Pb effect is an increase or decrease on a specific parameter.*

2.2.1. Effects of lead on B-lymphocytes and humoral immune response

Concerning the production of antibodies against specific inoculated antigens in birds, some authors did not find any effect of Pb exposure on T-dependent (Redig et al., 1991) and T-independent (Nain and Smits, 2011) antibody production. However, experimental Pb shot ingestion has been shown to decrease T-dependent humoral response in mallard ducks (Trust et al., 1990), and in Japanese quails (*Coturnix coturnix*) with inadequate or deficient diets (Grasman and Scanlon, 1995). Similarly, great tits (*Parus major*) from a heavy metal polluted area showed decreased T-dependent humoral responses compared with birds from the control area (Snoeijs et al., 2004), while pied flycatcher (*Ficedula hypoleuca*) males showed greater T-dependent responses in a polluted area than birds from the control site (Eeva et al., 2005).

These effects have been studied more in depth in experiments with mammal species, in which reduced (Blakley et al., 1980; Koller and Kovacic, 1974; Koller, 1980) as well as increased (Lawrence, 1981; McCabe et al., 1991) T-dependent responses have been reported in laboratorial rodents exposed to Pb. The enhanced B-cell proliferation accompanied by an increased B-cell MHC class II expression suggests a Pb modulation of T_{h1} versus T_{h2} activity (Mccabe and Lawrence, 1991) (Figure 5). Lead is known to modulate APCs functions, inhibiting T_{h1} cells (responsible of cell mediated responses) and promoting T_{h2} cell (responsible for humoral responses) development (Gao et al., 2007) (Figure 5). In agreement, several studies have demonstrated a Pb-induced increase in IgE production in mammals (Heo et al., 1996; Miller et al., 1998; Snyder et al., 2000), which is frequently accompanied by increased levels of T_{h2} cytokines potentially related to allergies and inflammation (Tepper et al., 1990; Wood et al., 2004). In avian species, the Pb-induced T_{h1}/T_{h2} imbalance has only been studied in embryonic or developing Pb exposure, which suppressed T_{h1} cytokine production (Hussain et al., 2005; Lee and Dietert, 2003; Lee et al., 2001, 2002) (e.g.: *in ovo*, after hatching), for which there is a special section below (Figure 5).

2.2.2. Effects of lead on T-lymphocytes and cell-mediated immune response

Effects of lead on delayed-type hypersensitive response

A normal delayed-type hypersensitivity (DTH) requires antigen specific T-lymphocytes that will be primed and expanded (T_{h1} dependent), and then recruited to a local site of antigen deposition (dependent of locally produced chemotactic signs) (Dietert and Piepenbrink, 2006). The response develops in a sensitized individual after re-exposure to the sensitizing antigen and is characterized by induration at the site of antigen deposition. Pb-induced suppression of DTH was reported in mice since the last 70s (Laschi-Loquerie et al., 1984; Müller et al., 1977), but its relationship with the skewed T_{h1}/T_{h2} was not found until the last 90s (McCabe et al., 1999). In birds, DTH response has only been studied in developmental Pb exposure. In *in ovo* Pb exposure, DTH has been shown to decrease in chicken (*Cornell K strain white leghorn*) (Lee and Dietert, 2003) but only when Pb was administered between days 9 and 12 of embryonic development (Lee et al., 2001, 2002), whereas others did not find Pb effects on such response (Bunn et al., 2000).

Effects of lead on T lymphocyte proliferation

Mitogens are substances that stimulate proliferation of T-cells, which is an early step in immunological reactions. Mitogens are able to activate T-lymphocytes (e.g.: Concanavalin A (Con A), phytohemagglutinin (PHA)), B-lymphocytes (e.g.: lipopolysaccharide; LPS) or both (Pokeweed mitogen; PWM) (Koller, 1980).

Results obtained with this type of test have not been consistent when experimental avian models have been used. For instance, some experimental studies reported no effects of Pb exposure on PHA and LPS induced responses in mallard ducks and Japanese quails (Nain and Smits, 2011; Trust et al., 1990). However, Redig et al. (1991) found suppressed responses induced by PHA and Con A in Pb-exposed red-tailed hawks (*Buteo jamaicensis*). Similarly, Grasman and Scanlon (1995) found a reduced PHA response in Pb-exposed Japanese quails.

The same inconsistency was found regarding studies with laboratory rodents in the 70s and early 80s (Gaworski and Sharma, 1978; Koller et al., 1979; Lawrence, 1981;

Shenker et al., 1977). Further studies demonstrated that Pb indirectly enhances CD4+ T-cell proliferation (McCabe et al., 2001) by targeting APCs and modulating antigen-specific interactions (Farrer et al., 2005). The specific mechanism by which Pb enhances CD4+ proliferation involves the inhibition of an inducible isoform of nitric oxid (NO) synthase (Figure 5).

2.2.3. Effects of lead on macrophages

Knowles and Donaldson (1997) conducted an interesting study focused on effects of Pb on one of the major component of macrophages surface, arachidonic acid, which is a precursor of eicosanoids. These authors reported that the concentration of arachidonate in phospholipids of macrophages from Pb exposed turkey poults (*Meleagris gallaparvo*) increased comparing to controls. *In vitro*, these macrophages increased the production of eicosanoids ($PGF_{2\alpha}$, PGE_2, and $tromboxane_2$), which was associated with subsequent decreases of activated macrophages and phagocytosis (Figure 5). Eicosanoids are immunomodulatory metabolites. A main action of PGE_2 is the downregulation of cellular activities, such as MHC expression, cytokine production, and cytotoxicity reactions (Goodwin and Ceuppens, 1983). As reviewed by Dietert and Piepenbrink (2006), an increased sensitivity to endotoxin has been linked to the increased production of TNF-α (Guo et al., 1996) and other pro-inflammatory cytokines (e.g.: IL1β, IL6) (Kim and Lawrence, 2000; Kishikawa et al., 1997) by macrophages.

There are an extensive number of functions and mechanisms involved in the homeostasis of macrophages that are targets of Pb exposure, which have been studied only in mammals and are summarized below (Figure 5). One of the most important functions of NO produced by macrophages is the destruction of intracellular pathogenic organisms, and the impairment of its production (Tian and Lawrence, 1995) by inhibiting the function of the inducible form of NO-synthase (iNOS) (Farrer et al., 2008) is considered one of the most sensitive endpoints for Pb immunotoxicity (reviewed in Dietert and Piepenbrink 2006). Exposure to Pb has additionally been shown to suppress the antigen presentation capability (Kowolenko et al., 1988), chemotaxis (Bishayi and Sengupta, 2003) and phagocytic activity (Bussolaro et al., 2008) in murine macrophages. After intravenous Pb exposure, carbon clearance has

been shown to decrease in rats (Filkins and Buchanan, 1973; Trejo et al., 1972), whereas Indian ink clearance increased in intraperitoneal Pb exposed mice (Schlick and Friedberg, 1981).

2.2.4. Effects of lead on granulocyte cells

In avian species, heterophils provide an important first line of defence against bacteria and were reduced in mallards experimentally exposed to Pb shot (Rocke and Samuel, 1991). In contrast, Villagra et al. (1997) found that Pb exposure increased neutrophil population in rats, and increased eosinophil degranulation in Pb exposed females.

2.2.5. Effects of lead on oxygen-independent forms of pathogen killing

Although this topic has not been studied in adult birds (see section 2.4 for developmental immunotoxicity of Pb), a review in the existing literature considering mammals has been included because it is one of the aspects to be seen in this thesis. Lysozyme is an enzyme of leukocytic origin with antibacterial and antiviral activity that has a protecting role involved in intracellular pathogen killing in birds (Callewaert and Michiels, 2010). Pesek and Schneider (1988) firstly demonstrated Pb binding to lysozyme, which produces changes in its conformation. It results in a decreased lysozyme activity in Pb-exposed rats (Olmo et al., 2012; Teijón et al., 2003).

Another way to kill pathogens is by the secretion of hydrolytic enzymes (e.g.: proteases) by activated macrophages, which can promote an inflammatory response (Steinhoff et al., 2005). Developmental Pb exposure of mice was shown to increase the gene expression of a number of hydrolytic enzymes in the spleen due to the continual destruction of erythrocytes induced by Pb (Kasten-Jolly et al., 2010). The high expression and secretion of proteases by macrophages in the spleen due to Pb exposure would result in peripheral inflammatory responses (Kasten-Jolly et al., 2010) (Figure 5).

2.3. Effects of lead on diseases resistance

In general, Pb exposure has been associated with an increased susceptibility to viral infections, which may be related to a reduced T_{h1} capacity, and with the increased production of TNF-α, PGE_2 and ROS by macrophages that result in reduced viral clearance (Dietert and Piepenbrink, 2006).

Knowles and Donaldson (1997) conducted a pathogen challenge study inoculating chicken with *Salmonella gallinarum* and observed that macrophage function was reduced by Pb due to an induced fragility and loss of integrity of the phospholipid cell membrane. On the contrary, Nain and Smits (2011) reported a reduced morbidity and mortality against *Escherichia coli* in experimentally Pb exposed Japanese quail.

Several works have reported reduced resistance to virus in mice (Gupta et al., 2002; Thind and Khan, 1978). Regarding bacteria, host-resistance to *Listeria monocytogenes* is generally suppressed by Pb (Dyatlov and Lawrence, 2002; Kim and Lawrence, 2000; Lawrence, 1981). A proper response against these bacteria requires an effective antigen presentation, a robust macrophage response and subsequent appropriate production of IL2, IFN (interferon)-ɣ and NO, and a robust T_{h1} driven protection (reviewed in Dietert and Piepenbrink 2006), all them potentially impaired by Pb exposure (Farrer et al., 2008; Gao et al., 2007; Heo et al., 1996; Kim and Lawrence, 2000). Others have reported increased susceptibility to other bacteria (Bishayi and Sengupta, 2003; Cook et al., 1975; Fernandez-Cabezudo et al., 2007; Hemphill et al., 1971) in experimentally exposed rodents.

2.4. Developmental immunotoxicity of lead

Dietert et al. (2004) suggested that Pb exposure during some specific stages of development can induce immunological changes in juveniles and adults, which can be apparently delayed or latent, appearing only after the co-occurrence of other environmental stressors. In addition, developing individuals seem to be sensitive to much lower Pb exposure levels than those that are immunotoxic to the adult. Immunotoxic responses to Pb appear to differ across life stages, and the latter stages of embryo development are a period of considerable sensitivity for Pb-induced

immunotoxicity (reviewed in Dietert and Piepenbrink, 2006), which corresponds to the seeding of the thymus with bone marrow-derived precursors followed by thymocyte maturation (Dietert et al., 2000; Landreth, 2002). Because of the anatomical and physiological differences between birds and mammals, developmental Pb effects have been reviewed regarding birds only, as there is a large literature in this topic.

The most recent study examining the effects of Pb on avian developmental immune system was performed by Vermeulen et al. (2015) under field conditions, who studied the relationships between constitutive immunity (i.e.: agglutination, lysis, haptoglobin levels and NO concentration) and metal exposure along a gradient of pollution in great tit nestlings. Despite these authors did not find any pattern corresponding to pollution gradient in studied immune components, they found that Pb levels were negatively correlated to lytic response in a specific area when they tested these relationship at an individual level. Other studies conducted under field conditions reported neither relationship between blood Pb levels and PHA response in white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) nestlings, nor Pb effects on phagocytic activity after dietary exposure in early-life great tits.

Lee et al. (2001) conducted an *in ovo* Pb administration in chicken eggs at different stages of embryonic development, which resulted in different immunotoxic outcomes in the juvenile birds. For instance, the negative effects of Pb on DTH function appeared to emerge between days 9 and 12 of *in ovo* development. Other observed effects were supressed production of IFN-ɣ and macrophage production of NO in Pb treated groups (Lee et al., 2001). Another experiment was performed to study effects of Pb on the host immune response to an infectious agent (inoculation with infectious bronchitis virus (IBV)) after developmental *in ovo* Pb exposure in chickens. Antibody response to IBV in juvenile chicks was unaffected by Pb treatment whereas DTH response was decreased after the late exposure (day 12 of embryonic development), but not after an early exposure (day 5 of embryonic development) (Lee et al., 2002).

Regarding specific induced immune responses, *in ovo* Pb exposure increased antibody production in chickens (Bunn et al., 2000). Fair and Ricklefs (2002) found that Japanese quail chicks exposed to Pb presented elevated granulocyte numbers compared to non-exposed ones, whereas induced immune response was not affected (T-dependent and T-independent humoral responses, cellular response against PHA).

In contrast, Lohman chickens (*Gallus gallus domesticus*) experimentally exposed to Pb showed suppressed T-independent and PHA responses (Youssef et al., 1996). Similarly, developing western bluebirds (*Sialia Mexicana*) showed suppressed cell-mediated responses to PHA after Pb exposure (Fair and Myers, 2002).

Bunn et al., (2000) showed decreased total leukocyte counts in *in ovo* Pb exposed chicken males, although Pb exposure did not affect the DTH response or the INF-ɣ activity. In a similar study, Fair and Myers (2002) curiously found that *in ovo* Pb-exposed western bluebirds immunized with SRBC and BVD survived better than controls and suggested that this result may be due to an adjuvant activation of the immune system.

In summary, the literature review for mammals and birds highlights that Pb exposure can affect many different components of the immune system (Figure 5). Some Pb-induced effects may only appear in the presence of stressors or may be delayed with variable latency time. Part of the difficulty of the study of Pb-induced immunotoxicity lies in the fact that it depends on species, doses, administration route, gender, season, physiological status, individual variability and a wide range of other external stressors (e.g.: weather, migration, parasite load), known as confounder factors. In addition, this heavy metal has been associated with hormesis in some occasions (Nain and Smits, 2011), which is a dose-dependent response phenomenon characterized by a U-shaped dose–response relationship, with low dose stimulation and high dose inhibition (Davis and Svendsgaard, 1990).

Despite the increasing amount of literature based on experimental studies testing sublethal effects of Pb shot ingestion in birds, little is known about chronic Pb exposure under natural conditions and its subclinical effects on waterfowl populations. Taking into account all these considerations, one of the aims of this thesis was to investigate possible Pb-induced immunological abnormalities in environmentally exposed wild birds, in parallel with the study of Pb effects on different immune system components under experimental conditions, in order to better understand these mechanisms.

3. Effects of lead on reproductive system

This section reviews the effects of Pb exposure on male and female reproductive parameters (Figure 6). Male reproduction may be affected by Pb, leading in the most extreme cases to infertility. In the case of females, Pb effects have been reported on fertility and embryonic development. Here, I focus on the literature concerning avian species. Secondly, because there is hardly any literature on the effects of Pb on reproductive function in male birds, I will briefly expend this review to findings of studies conducted on rats, mice and other mammal species. These animal models, mainly rodents, are those that have provided most information and have allowed a better understanding of the effects of Pb on the reproductive system. Finally, as the literature regarding female birds is mainly based on reproductive outcomes without considering involved mechanisms, a brief mention of the effect of Pb on folliculogenesis and hormone regulation in mammals is added at the end.

3.1. Hormonal regulation of the reproductive system

A brief description of the hormonal regulation of the reproductive system is presented here (Figure 6). Gonads, which greatly vary in size in seasonally breeding species, are responsible for the proliferation of gametes and the secretion of steroid sex hormones that control the development and functional activity of the accessory sexual structures and secondary sexual characteristics. These functions are regulated by hypothalamic control on the secretion of two gonadotropic hormones from the pituitary gland (adenohypophysis), the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). FSH is responsible of gametogenetic activity of germinal epithelium, whereas LH regulates secretory activity of Leydig cells in males, and induces ovulation and formation of ovarian corpus luteum in females.

Testes are male gonads that consist of a mass of seminiferous tubules and they are lined by the germinal epithelium consisting on developing germ cells (FSH regulation) and Sertoli cells (LH regulation). Once the spermatozoa have been released from Sertoli cells, they migrate from the tubule lumina into the efferent ducts, and are discharged in the seminal sacs from which they are finally ejaculated.

In the case of females, germinal epithelium in ovary starts gametogenesis during embryonic development and no further oocyte formation occurs in the adult life. Thecal cells of the developing follicle are involved in secretion of oestrogens. Granulosa cells are involved in progesterone secretion after its transformation into the granulosa lutein tissue of the corpus luteum. In birds, vitellogenesis and the consequent deposition of yolk within the developing oocyte is an oestrogen-dependent phenomenon, as well as ovulation. The ovum is then received from the ovarium in the infundibulum, where it is fertilized, and then albumin is deposited to the yolk shell is secreted.

3.2. Effects of lead on avian reproduction

Up to now, to the best of my knowledge, no study has tested the effects of Pb exposure on sperm quality in any avian species. Concerning Pb effect on sperm, the only existing work is the one performed by Dauwe et al. (2004), who used a non-destructive method to indirectly determine sperm quality by quantifying the number of spermatozoa trapped on the perivitelline layers of the egg, which may be an indicator of the ejaculate size of males and positively correlates with the number of spermatozoa transferred to the female (Bramwell and Howarth, 1992). These authors (Dauwe et al., 2004) found that male blue tits (*Parus caeruleus)* from heavily polluted areas presented lower concentrations of sperm cells in perivitelline layers than the ones from least polluted areas.

In avian species, females are especially vulnerable to Pb toxicity during the pre-breeding period, due to the increased absorption of calcium (Ca) within the perspectives of eggshell formation and the similarity between the two cations, Ca and Pb (Tejedor and Gonzalez, 1992). While there is no literature on the direct effects of Pb on sperm quality in exposed males, there is a large number of works that studied the effects on reproductive parameters on females and pair reproductive outcomes in environmentally exposed populations compared with control populations, mainly in songbirds. Moreover, females can transfer Pb through eggs to the offspring (Dauwe et al., 1999; Mora, 2003), and this may affect embryonic development during a highly sensitive period for Pb toxicity (Lee et al., 2001). Transfer of Pb 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 (Mateo et al., 2001). For instance, great tit nestlings from areas polluted with high levels of heavy metals (including Pb) showed lower body condition, more leg abnormalities and fledged later than nestlings from non-polluted areas (Eeva et al., 2009; Janssens et al., 2003a). An earlier laying date, an increased number of females interrupting their laying period and a reduced hatching success and clutch size than in less polluted sites were also reported (Eeva et al., 2009; Janssens et al., 2003b).

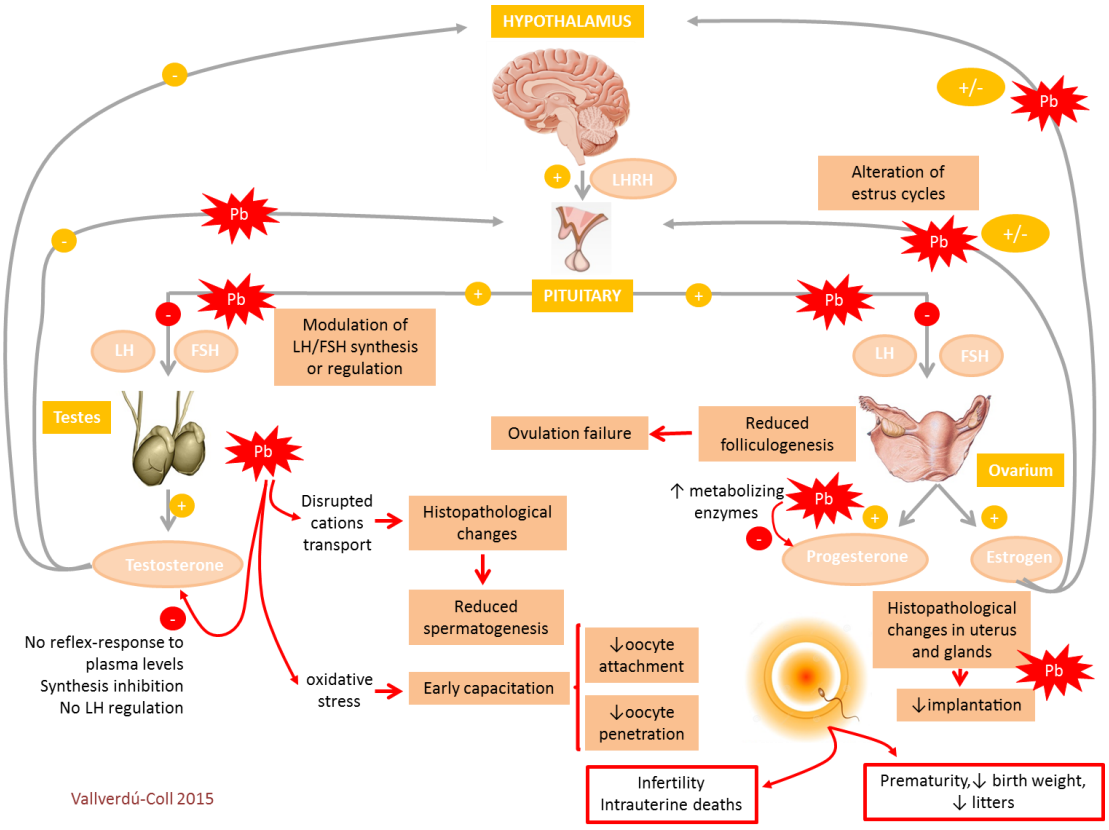

***Figure 6**. Effects of Pb on male and female reproduction and main consequences of these effects. Yellow circles indicate physiological stimuli (positive or negative depending on the sign) by which different components of hypothalamic-pituitary-hormonal axis is regulated. Red circles indicate the effects of lead on a given response.*

Moreover, several monitoring studies have described that impaired reproductive parameters that are crucial in explaining observed population trends

(e.g.: reduced eggshell thickness, egg size and volume, clutch size and hatching success) in heavily polluted areas were reversed when the emission of heavy metals was restricted (Eeva and Lehikoinen, 2000, 2015). Although clutch sizes and fledgling numbers remained below the levels of the reference area, Eeva and Lehikoinen (2015) found a recovery of various reproductive parameters (egg shell quality, clutch size, hatchability, and fledgling number) in a metal polluted area especially after metal-rich dust emissions from the smelter were markedly reduced. In another study, nestlings from a wild population breeding in the vicinity of a copper smelter showed decreased growth rates, lower haematocrit, higher corticosterone levels, less colourful plumage and lower survival probabilities (Eeva et al., 2014). In a similar experiment no major long-term effects of Pb on the oxidative status or phagocytic activity were found in great tits chicks from a population breeding in the vicinity of a metal smelter on oxidative status and phagocytic activity (Rainio et al., 2015).

Developmental exposure to Pb has been shown to induce differences in righting response, locomotion, thermoregulation, begging and feeding behaviour in the chicks of herring gulls (*Larus smithsonianus*) (Burger and Gochfeld, 1996). These authors found that Pb exposed herring gulls nestlings were less able to compete for food with their siblings, with a consequent reduction of weight, which was compensated by an extra parental care (Burger and Gochfeld, 1996). Effects of Pb exposure during the reproductive period on maternal fitness suggest that offspring could be affected not only by effects of Pb due to direct maternal Pb transfer, but also because of the existence of a trade-off between parental health and developmental immunocompetence of nestlings, as shown by Ardia (2005).

The structural and functional developmental changes in the immune system of embryos and hatchlings make them especially vulnerable to Pb (Lee et al., 2001). Elevated granulocyte numbers (Fair and Ricklefs, 2002), increased antibody production (Bunn et al., 2000) and suppressed cell-mediated responses (Fair and Myers, 2002) were observed after experimental developmental Pb exposure in birds (See section 2 for more detail), which may compromise the ability to fight against adversity and survival of individuals.

3.3. Effects of lead on the reproductive system of mammals

3.3.1. Males

Effects of lead on spermatogenesis

Macroscopic changes in accessory sex organs (e.g.: reduced weight of testes, seminal vesicles, epididymis, and prostate) have been demonstrated in various studies in Pb-exposed rats (Ronis et al., 1996; Sokol, 1990). Exposure to Pb have been shown to produce histopathological changes in testes (Ghelberg and Bordas, 1981) accompanied by a disruption of spermatogenesis (Batra et al., 2001; Eyden et al., 1978; Graça et al., 2004) in laboratorial rodents. The disruption of cellular energetics and cation transport in the testicular tissue at initial stage of Pb exposure may be responsible for altering the germinal function of the testis (Saxena et al., 1984), while others have demonstrated that Pb-induced germ cells to undergo apoptosis in the seminiferous tubules of rats (Adhikari et al., 2001).

Other effects of Pb on sperm associated with male infertility have been frequently reported in mammals, such as reduced sperm concentration, increased dead of sperm cells (Akinola et al., 2015) and increased sperm chromatin condensation (Hernández-Ochoa et al., 2006) and fragmentation (Castellanos et al., 2015).

Effects of lead on sperm functional parameters

Successful fertilization of an ovum by spermatozoa depends not only on sperm count and morphology but also on functional parameters. Exposure to Pb have been seen to induce an increased frequency of acrosome-reacted spermatozoa and an early onset of capacitation by modifying the membrane fatty acid profile trough oxidative stress (Hsu et al., 1998), which might consequently result in premature acrosome reaction (Castellanos et al., 2013, 2008) and reduced oocyte penetration capability (Hsu et al., 1998; Isaksson et al., 2008). Other mechanisms through which Pb may affect the membrane permeability have been proposed such as by alteration of membrane potential (Kurtyka et al., 2011) or by Pb competition with Ca^{2+} in physiological processes involved in ion transport in the membrane (Chen et al., 2009; Evans et al.,

2003) and which would also be related by Pb-induced decrease on sperm motility (Akinola et al., 2015; Oliveira et al., 2009).

In addition, several authors have reported Pb-induced functional disorders, related to the interaction between sperm and oocytes and implantation (reviewed by Vigeh et al., 2011). For instance, decreased number of sperm cells attaching to the ovum that resulted in a low frequency of sperm attachment to the ovum were reported in Pb exposed mice (Chowdhuri et al., 2001).

Lead-induced hormonal disruption

Reproductive hormones play a key role in the regulation of spermatogenesis and sperm development. The most important Pb-induced disorders may occur in the hypothalamic-pituitary-testosterone (HPT) axis. For instance, Pb has been shown to modulate levels of testosterone, LH and FSH by affecting the hypothalamic-pituitary unit and gonadal steroid biosynthesis in rats (Ronis et al., 1996; Sokol et al., 1985) (Figure 6).

Suppression of testicular testosterone has been described in Pb exposed male rats (Nathan et al., 1992; Ronis et al., 1996) (Figure 6). Other pathways apart from Pb disruption of the HPT axis have been suggested to explain Pb effects on testosterone, such as a lack of reflex in response to plasma testosterone, direct inhibitory androgen biosynthesis in Leydig cells (Wiebe et al., 1983) or defects in LH regulation at the pituitary level (Sokol et al., 1985).

3.3.2. Females

Exposure to Pb is a cause of folliculogenesis dysfunction in mice (Junaid et al., 1997; Taupeau et al., 2001) (Figure 6). Chronic Pb exposure blocks ovarian and luteal functions by reducing progesterone, LH and FSH levels in primates (Franks et al., 1989), and reduces the frequency of implanted ovum and of pregnancies in mice (Odenbro and Kihlström, 1977) (Figure 6). Reduced levels of progesterone after Pb administration (Nakade et al., 2014) have been attributed to an increased activity of a progesterone-metabolizing enzyme in rats (Abdou and Newairy, 2006).

4. Oxidative stress in lead toxicity

4.1. Introduction to oxidative stress

Oxidative stress is a condition whereby the production of reactive oxygen and nitrogen species (ROS/RNS) overwhelms the capacity of antioxidant defences. Reactive oxygen species (ROS) are free radicals (molecules containing unpaired electrons) produced as by-products of oxidation-reduction (REDOX) reactions during essential cellular processes (Freeman and Crapo, 1982). In healthy individuals and at low levels of ROS, they play roles of signalling molecules. Antioxidants serve to balance ROS intracellular production, inhibiting the destructive oxidation of molecular components within the cellular milieu (Brigham, 1986). At high levels, ROS induce oxidative stress on the cell.

As reviewed by Dowling and Simmons (2009), mitochondria are a primary site of ROS production, where most of energy production is generated via oxidative phosphorylation. Electrons are transported through proteins via REDOX reactions to end up incorporating into an oxygen molecule. Under normal conditions, oxygen is then converted to water and the energy stored is used to ATP production. During this process, a small percentage of the oxygen consumed by the mitochondria is converted to ROS, instead of water. Other REDOX reactions have been shown to generate ROS, for instance, in constitutive immune responses (Hampton et al., 1998). When ROS production overwhelms the capacity of antioxidant defences, it results in oxidative stress that damages lipids, DNA and proteins (Dowling and Simmons, 2009) (Figure 7).

The undesirable accumulation of ROS is prevented by a wide range of enzymatic and non-enzymatic antioxidant mechanisms (Apel and Hirt, 2004). Among antioxidant enzymes, the metalloenzyme superoxide dismutase (SOD) is the main responsible to scavenge ROS, by dismutating superoxide to hydrogen peroxide (H_2O_2). The H_2O_2 is eliminated by catalase (CAT), which results in two water molecules (Figure 7).

Another enzymatic way to eliminate such H_2O_2 involves the presence of the antioxidant metalloenzyme glutathione peroxidase (GPx), a cytoplasmic Se-dependent enzyme, and the presence of reduced glutathione (GSH), an endogenous antioxidant. GSH is a tripeptide which contains a sulfhydryl group (SH) that provides a great redox

capacity. It is responsible for maintaining an optimal intracellular redox environment and then plays a key role in protecting cells against oxidative damage. GSH can act directly interacting with ROS, or act as a cofactor in enzymatic reactions (Schafer and Buettner, 2001) (Figure 7). Then, in presence of 2 GSH molecules and GPx, H_2O_2 is eliminated and a molecule of glutathione disulphide (GSSG; oxidized form of GSH) is generated. GSSG can be reconverted to GSH in the presence of the enzyme glutathione reductase (GR) and NADPH (Figure 7).

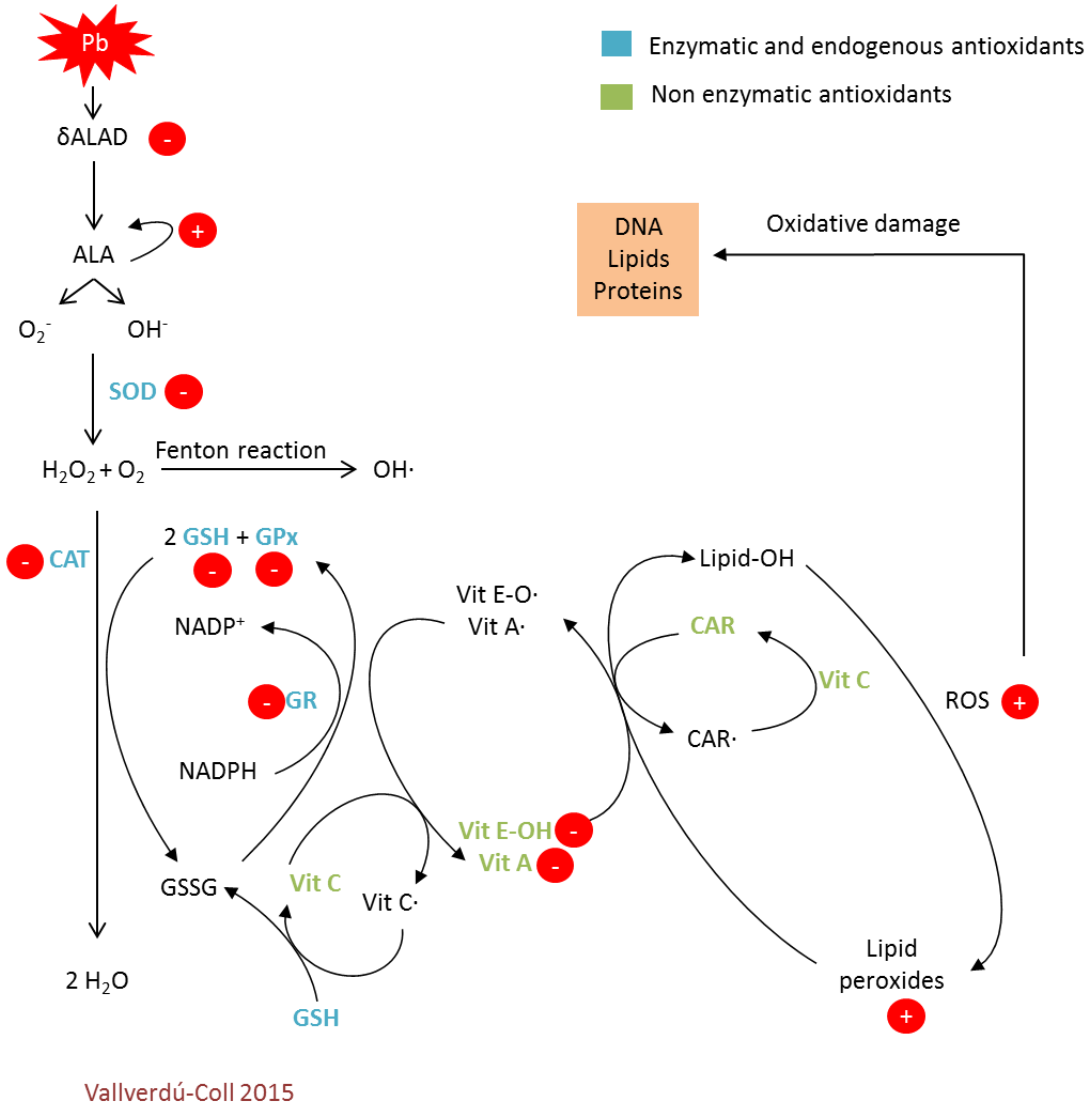

***Figure 7**. Effects of Pb on different components of the antioxidant system and mechanisms of Pb-induced oxidative stress. Red circles indicate positive and negative effects of Pb (according to the sign) on a given parameter. δ ALAD: δ-aminolevulinic acid dehydratase. ALA: aminolevulinic acid. SOD: superoxide dismutase. CAT: catalase.*

GSH: reduced glutathione. GPx: glutathione peroxidase. GR: glutathione reductase. GSSG: oxidized glutathione. Vit: vitamin. ROS: reactive oxygen species.

Non-enzymatic mechanisms to scavenge ROS include exogenous antioxidants (Apel and Hirt, 2004) like carotenoids and vitamins acquired from diet. These different types of antioxidants interact with each other. For instance, when reacting with ROS vitamin E (α-tocopherol) becomes a radical itself, which is then reduced by carotenoids and other antioxidants (Figure 7), highlighting the necessity of a balance between levels of vitamins and carotenoids to keep the power of antioxidant defences (Koivula and Eeva, 2010). In addition, a great number of carotenoids can be used as a source of vitamin A (Surai, 2002), as this vitamin can be synthesized *de novo* from the symmetrical cleavage of β-carotene by an oxygenase enzyme in the liver (reviewed in Hill and Johnson, 2012).

Carotenoids play important biochemical roles in animals and cannot be synthesized *de novo* by Vertebrates, which must acquire them through their diet (Goodwin, 1984). Carotenoids actively interact with radicals within the membranes (i.e.: the lipid bilayer) in which they reside undergoing both electron abstraction and electron transfer reactions (i.e.: free radical scavengers) (reviewed in Hill and Johnson, 2012). In addition, dietary carotenoids can be used by some animal species to provide colour to their integuments (e.g.: skin, feathers) and have been shown to present immune-stimulatory properties (Rühl, 2007).

Vitamins can also have antioxidant and immune-stimulant properties, as it is the case of vitamin A (retinol) (Hill and Johnson, 2012; Rühl, 2007) and vitamin E. For instance, vitamin E has an anti-inflammatory function and has been shown to induce a decrease in monocyte pro-inflammatory activity, release cytokines and decrease plasma C-reactive proteins (Singh and Jialal, 2004). On the other hand, retinoic acid (RA: vitamin A metabolite) plays a main role in mucosal immune responses, as it is required for generating gut-tropic lymphocytes and antibody-secreting cells (Cassani et al., 2012). Retinol deficiency has been associated with increases in oxidative damage to mitochondria in rats (Barber et al., 2000), because this vitamin has been suggested to act as a cofactor in the homeostatic control of redox state of mitochondria (Hoyos et al., 2012). Furthermore, RA regulates DNA transcriptional activity of several genes,

many of which participate in the maintenance of cellular redox balance (reviewed in Hill and Johnson, 2012).

Cell membranes are prone to lipid peroxidation (i.e. oxidative degradation of the lipids in cell membranes due to free radical attack). Lipid peroxidation causes decomposition of polyunsaturated fatty acids (PUFA), basic constituents of cell membranes, causing cell damage and even affecting the integrity of membranes. Vitamins E and C (ascorbic acid or ascorbate) directly interact with free radicals (Burton and Ingold, 1986; Jones et al., 1995) and work together to protect membrane lipids from oxidative damage (Packer et al., 1979). On the one hand, vitamin E terminates the chain reaction of lipid peroxidation in membranes and lipoproteins (Dieber-Rotheneder et al., 1991), whereas ascorbic acid scavenges aqueous-phase ROS by electron transfer and inhibiting lipid peroxidation, and also reduces the oxidized α-tocopherol (Halliwell et al., 1987)(Figure 7).

4.2. Lead induction of oxidative stress

4.2.1. Overload of pro-oxidants

In the case of Pb poisoning, blood is not only a transporter but also a critical target for Pb toxicity (Matović et al., 2015). Delta-aminolevulinic acid dehydratase (δ-ALAD) is a citosolic Zn-metalloenzyme; it is the second enzyme in the heme biosynthesis pathway and it catalyses an asymmetric addition of two molecules of aminolevulinic acid (ALA) to form the monopyrrole porphobilinogen, which is the precursor of heme (Kelada et al., 2001). δ-ALAD is expressed in all tissues, but the highest levels of expression are found in erythrocytes and the liver (Wetmur, 1994). Once absorbed into the bloodstream, Pb accumulates in erythrocytes due to the high affinity for δ-ALAD by displacing Zn^{2+} at the metal binding site, changing the enzyme's structure by binding to its SH groups, which results in an inhibition of its activity (Kelada et al., 2001). Consequently, there is an accumulation of ALA and a depressed heme formation, which stimulates production of more ALA that accumulates in blood (Gurer and Ercal, 2000). Accumulation of ALA is linked to the generation of H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$, as well as lipid peroxides (Bechara, 1996) (Figure 7). Erythrocyte δ-ALAD inhibition has been used as a routine Pb-poisoning test in many studies and in clinical laboratories, being

considered a specific biomarker of Pb exposure due to its high sensitivity to this pollutant (Goyer and Clarkson, 2001).

4.2.2. Interaction with thiol groups and enzyme cofactors

Other enzymes related to the antioxidant system such as GR have their activity inhibited by Pb binding to SH groups, which results in decreased levels of GSH (Gurer-Orhan et al., 2004). This inhibition leads to decreased GSH:GSSG ratios that will render cells more susceptible to oxidative damage. GSH also possesses a SH group where Pb binds (Christie and Costa, 1984), which can deplete the available GSH in its reduced form (Korsrud and Meldrum, 1988) (Figure 7).

Toxicity of Pb is partially due to its ability to antagonize other trace elements metabolism (Figure 7). Such antagonisms consist of the inhibition of numerous metalloenzymes that depend on the free availability of certain trace elements for optimal activity (Gelman et al., 1978), as in the case of δ-ALAD. Metalloproteins like GPx, CAT and SOD accomplish their antioxidant functions by enzymatically detoxifying peroxides, H_2O_2 and $O_2^{\cdot-}$, respectively. These antioxidant enzymes depend on various essential trace elements for proper molecular structure and enzymatic activity, which makes them potential targets for Pb toxicity (Gelman et al., 1978). For instance, Pb-Se antagonism affects GPx activity, which requires Se as a cofactor (Schrauzer, 1987).

Field studies have reported changes in levels of exogenous antioxidants associated with Pb exposure. These relationships are in some cases positive, whereas in other cases negative correlations between levels of Pb and dietary antioxidants have been found in wild waterfowl (Martinez-Haro et al., 2011b) and mammals (Rodríguez-Estival et al., 2011a). These effects may be due to disturbances of antioxidant enzymes (Rodríguez-Estival et al., 2011b) associated with Pb ability to replace trace elements acting as cofactors (Flora et al., 2012), or to a compensation response from the organism to cope with the oxidative stress generated by Pb poisoning (Martinez-Haro et al., 2011b; Matović et al., 2015).

4.2.3. Lipid peroxidation and changes in lipid composition

Some adverse effects produced by Pb are attributed to membrane damage by lipid peroxidation. More specifically, Pb effects on red blood cell (RBC) membranes have been deeply studied because of its important presence in the blood stream (Leggett, 1991). Lipids are main constituents of membranes composed by fatty acid side-chains. Fatty acids with zero, one, or two double bonds are more resistant to oxidative attack than PUFA that have more than two double bonds (Halliwell, 1989). Several studies have described Pb-induced changes in the fatty acid composition of membranes. Knowles and Donaldson (1997) found increased concentration of arachidonic acid in phospholipids of membranes from avian tissues, which may be responsible for the enhanced lipid peroxidation in those membranes (Lawton and Donaldson, 1991). Mateo et al. (2003b) also found an increased proportion of n-6 PUFA in liver and brain from Pb exposed mallards. Altered lipid composition of membranes may result in altered membrane integrity, permeability, and function, which may increase the susceptibility to lipid peroxidation (Gurer and Ercal, 2000).

Koivula and Eeva (2010) reviewed literature regarding metal-related oxidative stress in birds, which suggests that avian species have some unique molecular mechanisms which allow them to tolerate and eliminate noxious compounds and to defence themselves against free radicals on oxidative stress, based on their low rate of mitochondrial oxygen radical production that may decrease oxidative damage (Cohen et al., 2008), and due to their ability to modulate their enzyme activities and detoxification systems in relation to pollution levels (Fossi et al., 1991). According to these authors (Koivula and Eeva, 2010), these features make birds especially interesting as a model when considering the induction of oxidative stress by metals.

4.3. Oxidative stress involved in lead toxicity

4.3.1. Immune response

<u>Oxidative stress induction of DNA damage</u>

This topic has been poorly studied in birds regarding heavy metals (Koivula and Eeva, 2010), and to my knowledge no study exists regarding Pb induction of DNA damage in

birds. Increased levels of DNA damage have been observed after Pb exposure in experimental studies with rodents (Devi et al., 2000; Valverde et al., 2002). Genotoxicity through indirect mechanisms involving DNA synthesis and repair (Restrepo et al., 2000), as well as increased the likelihood of fixed damage to DNA (reviewed in Silbergeld, 2003; Xu et al., 2008). It has been suggested that Pb damages DNA indirectly by participating in a Fenton reaction to generate $OH^{\cdot}$ in the presence of H_2O_2, and a singlet oxygen might be the principal oxygen species involved to induce the damage (reviewed in Silbergeld, 2003). Kasten-Jolly et al. (2010) performed a gene expression microarray analysis on RNA from the spleens of developmentally Pb-exposed mice, and found that Pb exposure affected the expression of genes associated with innate immunity and increased apoptosis, B-cell differentiation, and T_{h2} development. Pb was shown to up-regulate the expression of genes encoding the heme-regulated inhibitor, which could generate immunogenic self-peptides that could enhance the expression of caspases, cytokines, and other immunomodulators.

Many of the proposed mechanisms through which Pb affect the immune response by oxidative stress or vice versa involve different macrophage components and functions, which are explained below.

Effects on phagocytic activity of macrophages

In a recent review, Kasten-Jolly and Lawrence (2014) proposed a possible mechanism by which Pb-induced oxidative stress may alter the phagocytic activity of macrophages. According to Kasten-Jolly and Lawrence (2014), damaged erythrocyte membranes makes RBCs more likely to be phagocytised by macrophages present in the spleen. Increased destruction of the Pb-exposed erythrocytes would result in anaemia with a concomitant increase in free iron (Fe) that will perturb cellular Fe homeostasis, increasing oxidative stress within the macrophages. By lowering the cellular GSH content, Pb could increase the concentration of $O_2^{\cdot -}$ in the cell, which will be converted to H_2O_2 by SOD. This H_2O_2 will be converted to highly reactive $HO^{\cdot}$ and OH^{-} in the presence of Fe excess produced by the increased erythrocyte destruction (reviewed in Kasten-Jolly and Lawrence, 2014).

Effects on NO production by macrophages

The production of NO by macrophages is important for the destruction of intracellular pathogenic organisms, and has been shown to be inhibited by Pb exposure (Tian and Lawrence, 1995). Pb can affect NO synthase activity at multiple levels, such as through the loss of GSH or heme, among others (reviewed in Kasten-Jolly and Lawrence, 2014). In addition, macrophages with more GSH produce NO and promote T_{h1} responses, whereas macrophages with less GSH preferentially promote T_{h2} responses (Murata et al., 2002). Pb is known to favour T_{h2} responses, related to auto-immunity and anti-inflammatory diseases (Dietert et al., 2004).

Effects on membrane composition of macrophages

Exposure to Pb has been shown to increase the concentration of arachidonate and the secretion of PGE_2 in the phospholipid membrane of macrophages (Knowles and Donaldson, 1997; Lee and Battles, 1994). PGE_2 is synthesized from arachidonic acid with the concomitant oxidation of GSH to GSSG (Kasten-Jolly and Lawrence, 2014). One hand PGE_2 synthesis represents an expense of GSH, and on the other hand the lack of this compound (GSH) may interfere with the actions modulated by PGE_2.

4.3.2. Sperm quality

It has been demonstrated that Pb is able to induce seminal oxidative stress and a subsequent decrease of male fertility. Negative correlations have been found between Pb concentration in sperm and levels of CAT, GSH, SOD and total antioxidant status, but this relationship was positive between Pb and MDA levels in bovine seminal plasma (Tvrdá et al., 2013, 2012). This may be in part because spermatozoa are particularly susceptible to oxidative stress due to their high levels of PUFA in their membranes (Castellanos et al., 2008; Vernet et al., 2004). The low antioxidant capacity of spermatozoa make them more prone to peroxidative damage (Sanocka and Kurpisz, 2004). Thus, oxidative stress also affects male fertility by damaging sperm membranes, decreasing both sperm motility and its ability to fuse with the oocyte (Tremellen 2008). Furthermore, the generation of ROS in sperm of Pb-exposed mice has been linked to

an increase in acrosome reacted spermatozoa (Hsu et al., 1998), suggesting that oxidative stress induced by Pb is implicated in premature capacitation (Figure 7).

4.3.3. Secondary sexual traits

Because of its disruptive effects on oxidative balance, Pb also has the potential to affect the expression of the coloured ornaments displayed by birds, in particular, those produced by carotenoid pigments. These secondary sexual traits play key roles in social signalling, mate choice and bird reproduction by reliably advertising individual quality (Andersson, 1994). The expression of these brightly coloured ornaments can be mediated by sexual hormones, such as testosterone (Martinez-Padilla et al., 2010; Mougeot et al., 2009).

Many of the yellow, orange or red ornaments displayed by birds are pigmented by carotenoids and function as social signals (Hill and McGraw, 2006).

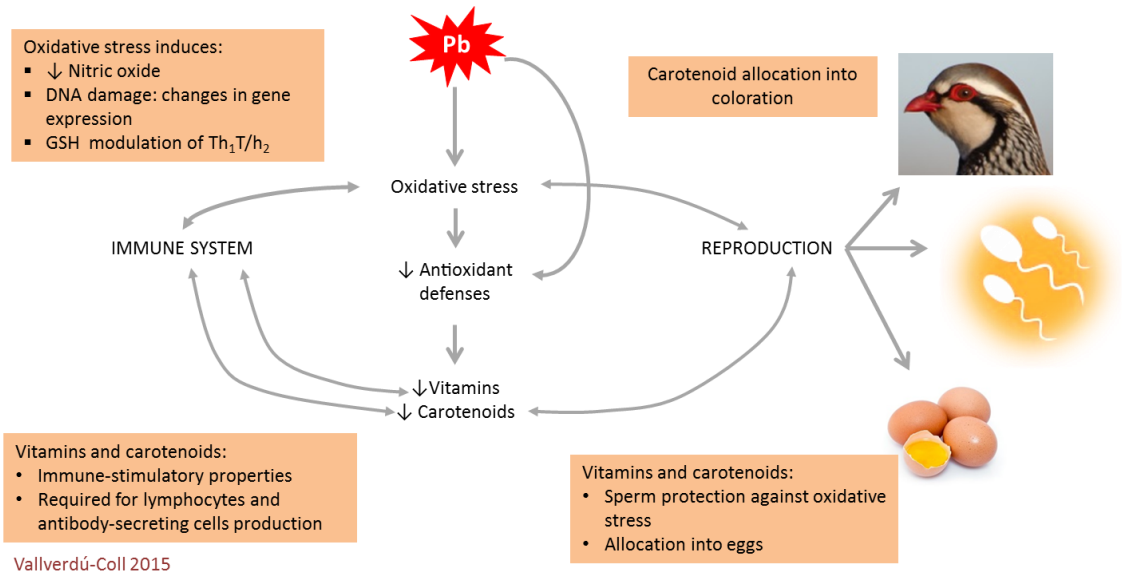

***Figure 8**. Effects of Pb on immune and reproductive functions considering their bidirectional relationship with oxidative stress induced by Pb poisoning, and the trade-offs between oxidative balance maintenance, immunity and reproduction*

The production costs of carotenoid-based colouration in integuments such as beak or eye-rings have received particular attention because, unlike plumage, their

colour can change rapidly. Hence, these traits could be useful indicators of current health (Blount et al., 2003), parasite infection levels (Mougeot et al. 2007; Martinez-Padilla et al. 2007), immunocompetence (Mougeot 2008) or physiological stress (Mougeot et al. 2010b). Carotenoid pigments have been suggested to mediate the honesty of signals through allocation trade-offs with other traits such as the immune system and reproduction (Blount, 2004) (Figure 8). Carotenoids have immuno-stimulant properties (Rühl, 2007), so individuals may trade allocation of available carotenoids to their ornaments or towards self-maintenance and immune function (Peters et al., 2004a, 2004b). The carotenoid-based ornaments of birds are particularly sensitive to changes derived from parasite infestations (Lozano, 1994; Brawner et al., 2000; Hõrak et al., 2004; Mougeot et al. 2007; Martínez-Padilla et al., 2007) and are also very sensitive to oxidative stress (Alonso-Alvarez et al., 2008; Perez-Rodriguez et al. 2010). Oxidative stress is also intimately linked with immune function and disease or parasite resistance (Dowling and Simmons, 2009) as we have seen above. Carotenoids can scavenge free radicals and cytotoxic molecules produced during immune response (Chew and Park, 2004) and can thereby reduce auto-reactivity (Von Schantz et al., 1999). Immune system activation consumes carotenoids (Perez-rodriguez et al., 2008), while carotenoid supplementation can increase immune responsiveness (Alonso-Alvarez et al., 2004; Blount et al., 2003) and thereby alleviate the costs of mounting an immune response (Hõrak et al., 2007). In birds, carotenoids appear as relatively weak antioxidants compared to many other antioxidant compounds, but recent work showed that carotenoid-based traits are sensitive to and respond to oxidative stress (Pérez-Rodríguez, 2008; Mougeot et al. 2010a) and therefore can reflect the levels of oxidative stress caused by parasite infection or immune challenges (Mougeot et al., 2009; Perez-Rodriguez et al. 2010). To date, the effect of Pb exposure on carotenoid-based ornamentation is virtually unknown, but given that the expression of these coloured traits is tightly related to immune function and oxidative stress, carotenoid-based ornaments should be sensitive to Pb exposure (Figure 8). One study reported that nestlings from a wild population of great tit breeding in the vicinity of a copper smelter had less colourful plumage than nestlings from a control area (Eeva et al., 2014), although authors attributed such effect to decreased food quality/quantity at the smelter site.

The expression of carotenoid-based ornaments displayed by birds could thus be affected by both oxidative stress and Pb exposure. Furthermore, due to the great energy cost of reproduction, adults exposed to Pb during the breeding season might allocate some energy in detoxification at expenses of reproduction. During the reproductive period, Pb may therefore affect not only maternal fitness, but also offspring fitness, through maternal Pb transfer (Dauwe et al., 1999; Mora, 2003) or trade-offs between parental health and developmental immunocompetence of nestlings (Ardia, 2005).

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Objectives and structure of this thesis

The first part of the thesis focussed on the assessment of the degree of compliance with the ban on the use of Pb ammunition in a Spanish wetland, the Ebro delta, and its effects on the prevalence of Pb shot ingestion in waterbirds, as well as the evaluation of the potential impact of this measure on game meat consumers from a public health perspective (Chapter 1). Trapped Mallards (*Anas platyrhynchos*) from the same area, the Ebro delta, were used to monitor Pb levels from a wild waterfowl population 6 and 9 years after the ban, and to study effects of such subchronic Pb exposure under natural conditions and its subclinical effects. The working hypothesis was that Pb exposure would be associated with increased oxidative stress, which would in turn affect other potential endpoints of Pb-induced toxicity, like carotenoid-based ornamental coloration and constitutive immunity (Chapter 2). Finally, the effects of parental Pb exposure and maternal Pb transfer on the development of immune function and immunocompentence in the offspring, and its possible link to oxidative stress, were studied by collecting mallard eggs from this area (Chapter 3).

In the second part, experimental studies were used to assess the possible immunotoxic effect of Pb shot ingestion on birds, using captive red-legged partridges (*Alectoris rufa*). This was based on the analysis of the constitutive and induced components of the immune system and the evaluation of effects on body condition and parasite loads, as well as their relationship with immune status, carotenoid-based coloration and oxidative status (Chapter 4). Experiments were also conducted on the same species to study the effects of Pb exposure on reproduction (sperm quality, maternal effects, laying performance, reproductive success, levels of dietary antioxidants and carotenoid-based coloration) and the relationships between carotenoid-based coloration and sperm quality considering Pb toxicity (Chapter 5).

CHAPTER 1

REDUCING Pb POISONING IN BIRDS AND Pb EXPOSURE IN GAME MEAT CONSUMERS: THE DUAL BENEFIT OF EFFECTIVE Pb SHOT REGULATION

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ABSTRACT

The use of lead (Pb) ammunition in the form of shot pellets has been identified as a Pb exposure risk in wildlife and their human consumers. We explore the hypothesis that Pb shot ban enforcement reduces the risk of avian Pb poisoning as well as Pb exposure in game meat consumers. We assessed compliance with a partial ban on Pb shot commencing in 2003 by examination of 937 waterbirds harvested by hunters between 2007 and 2012 in the Ebro delta (Spain). Prevalence of Pb shot ingestion was determined, as were Pb concentrations in liver and muscle tissue to evaluate the potential for Pb exposure in game meat consumers. Hunted birds with only embedded Pb shot (no steel) declined from 26.9% in 2007–08 to <2% over the following three hunting seasons after ban reinforcement. Pb shot ingestion in mallards decreased from a pre-ban value of 30.2% to 15.5% in the post-ban period. Liver Pb levels were predominantly defined by the presence of ingested shot, whereas muscle levels were defined by the presence of both ingested and embedded shot. Only 2.5% of mallard muscle tissue had Pb levels above European Union regulations for meat (0.1 μg/g wet weight) in the 2008–09 season, when Pb shot ingestion prevalence was also at a minimum (5.1%). Effective restrictions in Pb ammunition use have a dual benefit since this reduces Pb exposure for game meat consumers due to embedded ammunition as well as reducing Pb poisoning in waterbirds.

Keywords: Dietary exposure, environmental policy, regulatory compliance, Pb poisoning, non-toxic ammunition, waterfowl

1. INTRODUCTION

The use of lead (Pb) ammunition has been clearly identified as a Pb exposure risk in wildlife and their human consumers (Green and Pain, 2012; Gustavsson and Gerhardsson, 2005; Lévesque et al., 2003). While the first articles regarding Pb poisoning in wild birds date back to the 19th century (Friend et al., 2009), it was not until the end of the 20th century that the use of Pb shot for waterfowl hunting was banned in several North American and European countries (Avery and Watson, 2009; Thomas and Guitart, 2010). In Spain, the use of Pb shot for hunting in wetlands was banned in protected areas in October 2001, but, in some wetlands (such as the Ebro delta) this ban was not fully implemented until the 2003–04 hunting season (Mateo et al., 2013).

Long use of Pb shot in wetlands has left a significant legacy because Pb shot can remain virtually unaltered in the environment for decades (Jorgensen and Willems, 1987; Takamatsu et al., 2010), as is the case in the Ebro delta. Here, waterfowl hunting has taken place for over a century, resulting in Pb shot densities (in the upper 20 cm of sediment) of 97–266 shot/m^2 in wetland lagoons and 6–83 shot/m^2 in surrounding rice fields (Guitart et al., 1994; Mateo et al., 1997, 2013). As a consequence, prevalence of Pb shot ingestion in this area has been very high. For example, between 1991 and 1996 it was 74.2% in Northern pintail (*Anas acuta*), 69.2% in common pochard (*Aythya ferina*) and 30.2% in mallard (*Anas platyrhynchos*) (Mateo et al., 2000). These rates of Pb shot ingestion in the Ebro delta and other Spanish wetlands meant that up to 40.4% of hunted waterbirds have been shown to hold liver Pb levels above the maximum residue level (MRL, 0.5 µg/g wet weight, w.w.) established for offal for human consumption in the European Union (EU) (Guitart et al., 2002).

It is now recognized that consumption of waterbirds with embedded Pb shot in their muscle tissue, or, that have ingested Pb shot, may pose a significant risk to human health (Green and Pain, 2012; Mateo et al., 2011). Indeed, regular consumption of meat from game animals hunted with Pb ammunition has been associated with increased human blood Pb levels (Bjerregaard et al., 2004; Iqbal et al., 2009); and recently, the Spanish Agency for Food Safety and Nutrition recommended that children

under six years old and pregnant women do not eat meat from animals killed with Pb ammunition because of the potential for negative effects on the developing central nervous system (AESAN, 2012).

The most widely used alternative to Pb shot is steel, but, as noted by Jules Verne (1874) in his book "The Mysterious Island", its ballistic properties do differ to that of Pb: "As Smith had not discovered any lead in the island he substituted iron shot, which were easily made. As they were not so heavy as leaden ones they had to be made larger, and the charges contained a less number, but the skill of the hunters counterbalanced this defect". Such ballistic differences between Pb and steel can also reduce the tendency for hunters to comply with regulations regarding Pb shot use (Friend et al., 2009). With this in mind, comparisons between existing data regarding Pb shot prevalence in waterfowl with new data obtained after a regulatory ban could be informative (as recently recommended by the African–Eurasian Waterbird Agreement; AEWA, 2012). Hence, between 2007 and 2012 we monitored the presence of embedded shot in hunted birds in the Ebro Delta. We recorded the prevalence of Pb shot and non-toxic shot ingestion, and Pb concentrations in liver and muscle tissue of waterfowl. In considering this information, the aim of this study was to (i) assess the degree of compliance with a ban on Pb ammunition, (ii) study the effect of the partial ban restricted to the protected areas on the prevalence of Pb shot ingestion in waterbirds, and (iii) evaluate the impact of this measure on Pb levels in game meat from a food safety perspective. The annual results of this study were also passed to the Regional Government (Department of Environment, Generalitat de Catalunya) which allowed them to assess on-going compliance with the Pb shot ban, and where needed, improve associated enforcement measures.

2. MATERIAL AND METHODS

2.1 Study area

The Ebro delta is an alluvial plain situated in the NE of Spain with a surface area of 320 km^2 (see Supplemental material, Figure S1). It is designated as an Important Bird Area

(IBA) and included in the Ramsar Convention List of Wetlands of International Importance. The use of Pb ammunition is banned in the protected areas (lagoons and marshes) of the Ebro delta (24%), but it is still allowed in adjacent unprotected crop fields (76%, mostly rice fields).

2.2 Sampling and Pb analysis

A total of 523 waterfowl carcasses from 11 waterbird species (Table 1) were collected from hunting bags between 2007 and 2011. The hunting season in the Ebro delta begins in mid-October and ends on the first week of March. Carcasses were all X-rayed to detect shot pellets, which were then removed during necropsy (Figure S2). Steel shot was easily distinguished from Pb shot because the former is usually larger, rounder and is attracted to a magnet. Steel shot is the only non-toxic alternative used in the study area at the moment. The embedded pellet data were used as an indicator of compliance with the ban on Pb use. Some animals had embedded steel and Pb shot, which is a likely consequence of repeated encounters with different hunters (Guillemain et al., 2007). In addition, 414 gizzards were collected by hunters during the 2011–12 season, which increased our sample size regarding shot pellet ingestion. Gizzard examinations (n = 885 (some gizzards were lost)) were performed as described by Mateo et al. (1997) in order to obtain comparable data with that reported for Pb shot ingestion between 1991 and 1996 (Figure S3). Liver and pectoral muscle samples were also obtained and analysed for Pb concentrations. Muscle samples were only collected in three of the hunting seasons (2007–08, 08–09, 10–11). Livers and muscles were stored at − 20 °C until analysis. A total of 465 liver (58 were too damaged by shot to be analysed) and 327 muscle samples were dried and analysed for Pb following the methodology described by Mateo et al. (2007); using a graphite furnace-atomic absorption spectroscopy system (AAnalyst 800, Perkin Elmer). Blanks (n = 31) were analysed in each batch of digestions. Limit of detection (LOD, back-calculated in tissue concentrations using 3 × SD of the blanks) was 0.030 μg/g dry weight (d.w.). Values <LOD were assigned as LOD/2 (0.015 μg/g d.w.) within statistical analysis. Bovine liver (BCR 185R, Community Bureau of Reference) with a certified Pb level (mean ± S.D.) of

0.172 ± 0.012 µg/g d.w. was analysed with samples and the Pb recovery was 94.5% (n = 18, mean ± S.E. = 0.162 ± 0.007 µg/g d.w.). All Pb concentrations are presented as µg/g d.w.

2.3 Statistical analyses

All the X-rayed waterbirds were hunted in the protected lagoons. Hence, the presence of embedded Pb shot alone, without steel shot, was considered as a failure to comply with the ban. The presence of steel alone was considered compliance. We conservatively assumed that birds with steel and Pb were most probably killed with the latter, and were only carrying Pb shot as a result of previous hunter encounters in non-protected areas. Therefore, the data used to evaluate the trend in ban compliance was the percentage of birds with embedded Pb shot only. Percentages for non-compliance were compared among seasons (2007–08 to 2010–11) for the whole sample and for mallards only (as a bioindicator species) with chi-square (χ^2) tests. The number of embedded pellets was compared between mallards shot with steel only, or, with Pb only, with a Mann–Whitney test. This non-parametric test was used because the data did not fit a normal distribution (even after a logarithmic transformation). As the number of shot that impacts a bird may depend on the size of the animal, we also studied this relationship with a Spearman correlation coefficient (r_s). Differences in the prevalence of Pb shot ingestion and in liver Pb levels >5 µg/g d.w. were compared with χ^2 tests among species, between pre and post-ban periods for all species, and among the post-ban hunting seasons for mallards. The percentage of waterbirds with liver and muscle Pb concentrations above the MRL set for offal (0.5 µg/g w.w. ≈ 1.5 µg/g d.w.) and meat (0.1 µg/g w.w. ≈ 0.32 µg/g d.w.) for human consumption in the EU (European Commission, 2006) was calculated and compared among species and among seasons for mallards with χ^2 tests. One-way analysis of variance (ANOVA) was used to determine differences among species and hunting seasons in ln-transformed (natural logarithm) liver and muscle Pb concentrations. This transformation was used to adjust the data to a normal distribution to permit the use of parametric tests. Tukey tests were used to establish post-hoc differences among groups. The influence of the

presence of ingested or embedded Pb pellets on Pb concentrations in liver and muscle was analysed with general linear models (GLMs) using Pb concentration as the dependent variable, and the presence/absence of embedded Pb shot, the presence/absence of ingested Pb shot, and species as factors. The level of significance was set at $p \leq 0.05$. Statistical procedures were carried out with IBM SPSS Statistics 19.

3. RESULTS

Compliance with the ban on Pb shot increased during our study period. In 2007–08, the percentage of waterbirds shot with Pb pellets only was 26.9%, and this declined significantly in the following three seasons to ≤2% (χ^2=86.8, p<0.001; Figure 1).

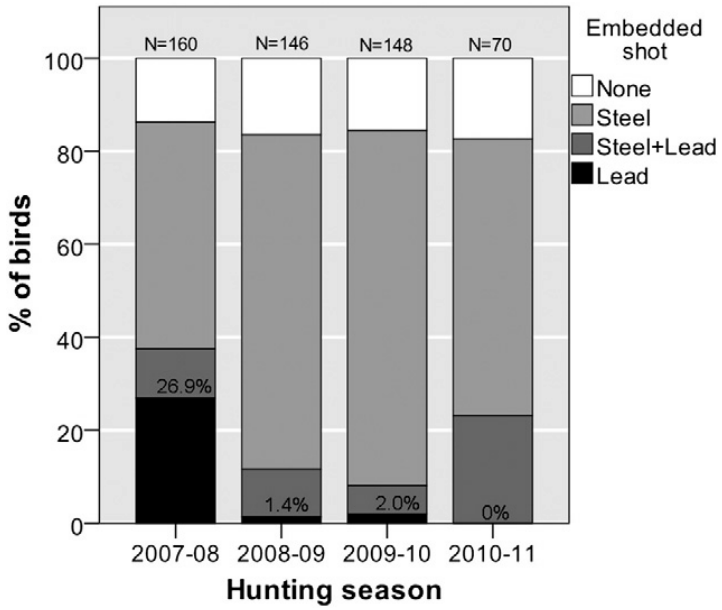

Figure 1. *Percentage of waterbirds with embedded steel and/or lead shot after the partial ban on Pb shot in the Ebro delta. Sample size (N) and % with only embedded Pb shot are shown for each season.*

In mallards specifically, these values dropped from 35.7% in 2007–08 to ≤3.1% (χ^2_3=55.8, p<0.001; Figure S4). The number of embedded shot also depended on species body mass (n=9, r_s=0.683, p=0.042; Figure S5). The number of embedded

pellets was similar in mallards shot with Pb only (n=24, median=4, range=1–24) and in those shot with steel only (n=164, median=5, range=1–30) (Mann–Whitney test, Z=1.186, p=0.236).

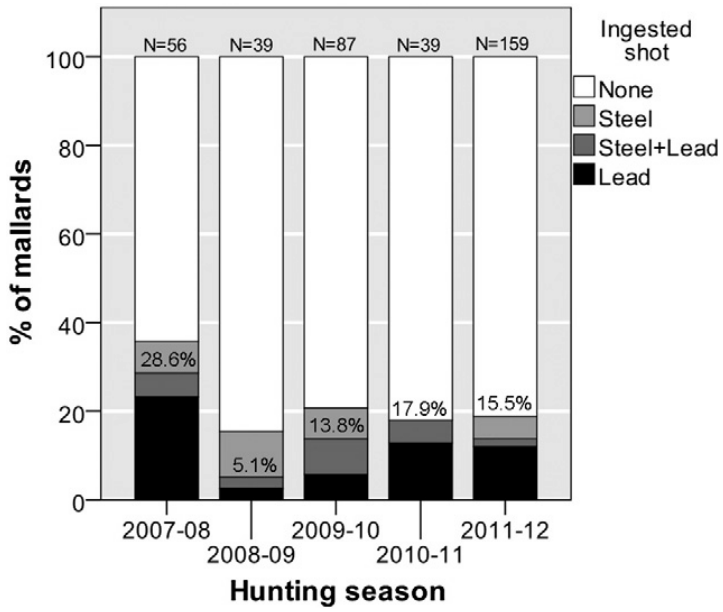

Figure 2. *Prevalence of steel and/or lead shot ingestion in mallards after the partial ban on Pb shot in the Ebro delta. Sample size (N) and % with ingested Pb shot (Pb and Pb + steel) are shown for each season.*

The prevalence of Pb shot ingestion decreased significantly after the ban in Northern shoveler (*Anas clypeata*), common teal (*Anas crecca*), common pochard and mallard (χ^2 tests, all $p \leq 0.047$) and a similar trend was observed for the percentage of birds with liver Pb levels >5 µg/g d.w. (Table 1). Shot ingestion differed among species ($\chi^2_{13}=116$, $p<0.001$, Table 1). For mallard, during the 2007–08 hunting season shot ingestion was 28.6%, not significantly different to the pre-ban value of 30.2% (Table 1 and Figure 2). However, a significant decrease was found in the 2008–09 season (5.1%) ($\chi^2_1=8.227$, $p=0.004$) after an increase in ban compliance (Figure 2). During the post-ban period, steel shot ingestion was found in all species except 3 (of 11; Table 1). The mean number of pellets (± SD, range) detected in the gizzards examined was 4.74 (±10.54, 1–86) for Pb, and 2.57 (±4.83, 1–33) for steel.

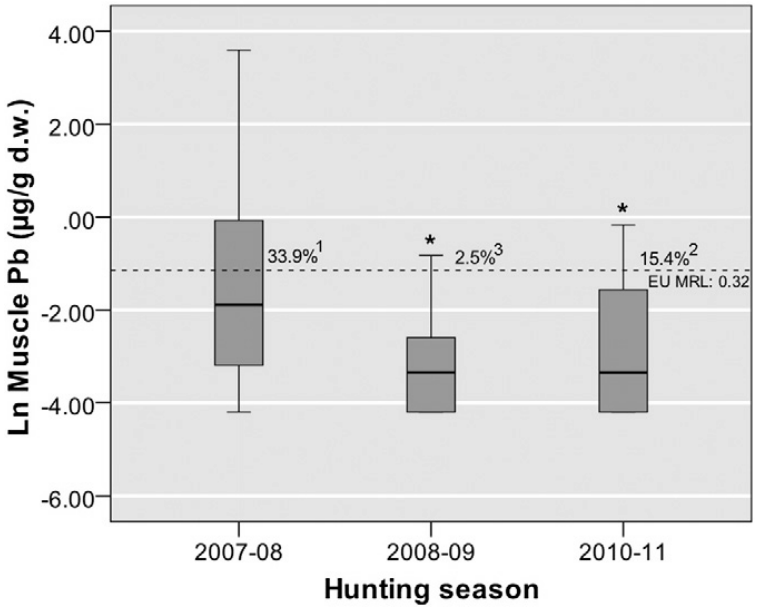

Figure 3. *Box-plots (median, 25–75%, range) of muscle Pb levels (ln-transformed) in mallards hunted during three different hunting seasons. The dashed line represents the maximum residue level set for meat for human consumption in the European Union (European Commission, 2006). The percentage of samples above this MRL is shown for each season. *Significant differences with 2007–08 season ($F_{2,132}$=12.7, p<0.001).* [1,2,3] *Differences in superscripts show differences among seasons in percentages above MRL (χ^2_2=15.35, p<0.001).*

Muscle Pb concentrations decreased significantly between 2007–08 and the subsequent seasons in mallard ($F_{2,132}$=12.7, p<0.001) (Figure 3) and in the entire pool of hunted birds ($F_{2,324}$=21.3, p<0.001). Liver Pb was significantly lower in 2008–09 compared to other seasons in mallards ($F_{3,212}$=3.12, p=0.027). In terms of EU MRL values, the mean dry mass of the muscle analysed was (mean ± SD) 30.9 ± 2.6%. Pb concentrations differed among waterbird species in muscle ($F_{9,317}$=6.1, p<0.001) and in liver ($F_{9,455}$=18.6, p<0.001; Table 2). Muscle Pb was largely explained by the presence of ingested Pb shot ($F_{1,311}$=95.5, p<0.001; Figure 4), but there was also a significant effect due to the presence of embedded Pb shot ($F_{1,311}$=15.7, p<0.001). Liver Pb was explained only by the presence of ingested Pb shot in the gizzard ($F_{1,449}$=175.6, p<0.001; Figure S6). The number of ingested shot positively correlated with Pb levels in muscle (rs=0.521, p<0.001) and in liver (r_s=0.505, p<0.001).

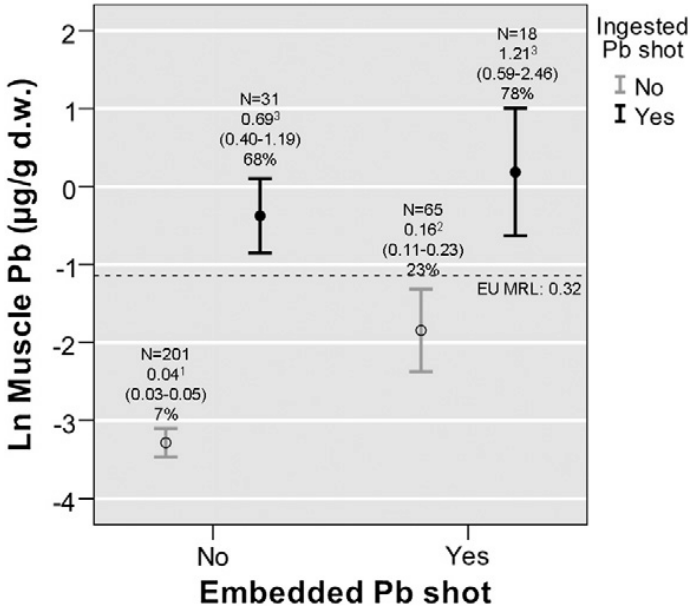

Figure 4. *Muscle Pb levels (ln-transformed, mean with 95% CI) in waterbirds with ingested and/or embedded Pb shot. The dashed line represents the maximum residue level (MRL) set for meat for human consumption in the European Union (European Commission, 2006). Values adjacent to bars are N, back-transformed geometric mean with 95% CI and % of samples above the EU MRL (0.32 µg/g d.w. ≈ 0.1 µg/g w.w.). Means sharing a superscript do not differ significantly.*

Table 1. Comparing the prevalence of Pb and steel shot ingestion and the proportion of animals with Pb levels in liver >5 µg/g between the pre-ban period (1991-1996) and the post-ban period (2007-2012).

Species	1991-1996[a]				2007-2012[b]					
	Shot ingestion		Liver Pb		Shot ingestion				Liver Pb	
	n	Pb (%)	n	>5 ppm (%)	n	Pb (%)	Steel (%)	Either (%)	n	>5 ppm (%)
A. acuta	97	74.2	24	75.0	25	76	20	84	15	100.0
A. clypeata	36	27.8	36	22.2	102	7.8*	2.9	7.9	37	5.4*
A. crecca	35	22.9	31	19.3	170	10.6*	0.6	11.1	77	10.4
A. ferina	26	69.2	26	53.9	20	35*	30	45	6	33.3
A. fuligula	4				1					
A. penelope	25	4	20	10.0	16	12.5	0	12.5	11	9.1
A. platyrhynchos	86	30.2	43	27.9	380	15.5*	10	21	216	20.4
A. strepera	25	8	24	8.3	40	0	2.5	2.5	9	0.0

F. atra	28	3.6	28	3.6	93	2.2	23.7	25.8	91	0.0
G. gallinago	-	-	2	0.0	18	5.6	0	5.6	-	-
N. rufina	21	19	21	9.5	16	12.5	25	31.3	2	0.0

[a] Guitart et al. (1994), Mateo et al. (1997, 2000). [b] Present study. * Significantly different between 1991-1996 and 2007-2012 periods (χ^2 tests).

Table 2. Concentrations of Pb (µg/g d. w.) in liver and muscle by species.

	Liver					Muscle				
Species	n	G Mean	95% C.I.	Min-Max	>1.5 µg/g (%)	n	G Mean	95% C.I.	Min-Max	>0.3 µg/g (%)
A. acuta	15	41.57[a]	25.62-68.81	6.95-166.25	100.0	15	1.43[a]	0.79-2.59	0.12-5.60	86.7
A. clypeata	37	0.3[b]	0.16-0.61	0.02-175.22	16.2	25	0.05[b]	0.02-0.10	<LOD-2.89	12.0
A. crecca	77	0.19[b]	0.10-0.28	0.02-52.89	16.9	43	0.09[b]	0.05-0.19	<LOD-20.82	30.2
A. ferina	6	1.44[b]	0.17-12.06	0.08-23.70	50.0	6	0.37[ab]	0.10-1.35	0.07-1.69	50.0
A. fuligula	1	0.63			0	1	0.10			
A. penelope	11	0.30[b]	0.09-0.88	0.02-11.95	9.1	12	0.07[b]	0.02-0.19	<LOD-1.14	16.7
A. platyrhynchos	216	1.10[b]	0.84-1.39	0.02-180.17	42.1	135	0.08[b]	0.06-0.11	<LOD -36.31	19.3
A. strepera	9	0.37[b]	0.22-0.60	0.18-1.49	0	10	0.09[b]	0.04-0.20	<LOD -1.38	10.0
F. atra	91	0.40[b]	0.31-0.46	0.02-2.25	1.1	78	0.04[b]	0.03-0.06	<LOD -444.00	6.4
N. rufina	2	0.37[b]		0.26-0.52	0	2	0.10[b]		0.06-0.15	0.0

LOD=Limit of detection. [a.b] Means sharing a letter do not differ significantly.

4. DISCUSSION

4.1 Pb shot ban compliance

During the 2007 to 2011 period there was a significant increase in compliance with a ban on the use of Pb shot in the Ebro delta. Non-compliance values declined from 26.9% in 2007–08 to ≤ 2% in the three subsequent seasons (Figure 1). The Pb shot ban has thus translated into a significant reduction in the prevalence of Pb shot ingestion in four waterfowl species, and a significant decrease in Pb levels in game meat. This latter decrease can be attributed to both (a) a reduction in the prevalence of Pb shot ingestion, and (b) the reduced risk of Pb ammunition contamination of meat around wounds because steel shot is being used instead.

The contribution of non-toxic shot regulations to waterfowl conservation has been evaluated in the USA, where the use of Pb shot for waterfowl hunting was completely banned in 1991. There, compliance values based on counts of Pb and steel shot shell wads found in the field ranged from 54.8 to 92.2% in different USA locations, and five years after the Pb ban in Illinois, hunter compliance based on embedded shot was 98.9% in mallard and 96.5% in Canada goose (*Branta canadensis*) (Havera et al., 1994). Minimum hunter non-compliance was just 1.1% (for mallard) and 1.8% (for goose), which is similar to the compliance values observed here for the Ebro delta. In Canada, where ban compliance based on anonymous hunter surveys was >80%, bone Pb levels in hunted waterfowl declined significantly from 1989–90 to 2000 (Stevenson et al., 2005). In the USA and Canada, legislative compliance appears to be high, which has been attributed to the general support of waterfowl hunters for the non-toxic shot program and to active enforcement led by conservation police officers (Anderson et al., 2000; Stevenson et al., 2005). Compliance values from North America contrast quite starkly against the low level of compliance recently documented in England, where 68% (in 2001–2002) and 70% (in 2008–2010) of mallards had been shot with Pb despite the fact that this ammunition was banned for hunting over wetlands in 1999 (Cromie et al., 2010). During our first season of monitoring, we also detected relatively high non-compliance values in the Ebro delta (26.9%, Figure 1). However, this was corrected in subsequent seasons as enforcement and vigilance from park rangers

increased, and, as local authorities threatened to ban hunting in the protected areas if non-compliance persisted. Improved enforcement was undertaken without any new laws or changes to regulations regarding Pb shot use. Instead, stricter controls on ammunition carried by hunters at entry points to hunting areas were put in place, random carcass sampling was undertaken at the end of shoots, and, national ID numbers were recorded for the hunters who harvested each bird. Such measures acted as simple but effective deterrents against non-compliance. Carcasses were also X-rayed for the present study, and results obtained were regularly passed to the regional government so that the effectiveness of enforcement actions could be assessed and, if needed, adjusted during the subsequent hunting season. As a consequence, a marked decrease in the percentage of embedded Pb shot (≤ 2%) was observed in 2008–2009, and in subsequent hunting seasons (Figure 1).

One argument often used by hunters against replacing Pb ammunition with non-toxic alternatives is that Pb shot tends to remain embedded in the target, while steel may pass through and thus leave badly injured birds but not kill them. However, this was not supported by our data as we found that the number of embedded pellets in mallards was similar in individuals shot with Pb only and in those shot with steel only. Moreover, the performance of steel shot appears to be entirely acceptable for waterfowl hunting, in as far as the number of harvested birds was similar before and after the Pb shot ban in the Ebro delta (Mateo et al., 2013). Likewise, the crippling rate found in the USA was only found to increase temporarily, during ban implementation (Schulz et al., 2009); and presumably, after this point, hunters had made slight adaptations to their style and technique which facilitated the effective use of the steel shot alternative.

4.2 Risk for waterbirds

Inter-specific differences observed here in the prevalence of Pb shot ingestion are most likely a function of variability among species in terms of diet, and the type of grit ingested (Figuerola et al., 2005; Mateo et al., 2000; Pain, 1990). In this regard, mallard is a very useful biomonitoring species for Pb poisoning, since it holds a near worldwide

and abundant distribution (Guitart et al., 1994), and the prevalence of Pb shot ingestion in this species is commonly moderate to high among waterfowl (Mateo, 2009). Here, the prevalence of Pb shot ingestion in the 2007–08 hunting season (28.6%, Figure 2) did not differ when compared to the pre-ban value (30.2%; Mateo et al., 2000). However, a significant decrease in Pb shot ingestion was found in the following seasons (mean 2008–12: 15.5%, Figure 2), after ban reinforcement. Similar reductions in Pb shot ingestion in waterbirds after a Pb shot ban have also been observed in the Mississippi flyway (USA), where rates of Pb shot ingestion declined from 7.8–8.4% for the pre-ban period (1938–79) to 2.8% (1996–97) after the 1991 nationwide ban (Anderson et al., 2000). Samuel and Bowers (2000) also found a reduction in the prevalence in waterfowl with elevated blood Pb levels (>20 μg/dL) from 11.7% before the ban to 6.5% six years after the ban in Tennessee (USA). In contrast, the proportion of waterbirds birds dying due to Pb poisoning has yet to decline in England despite a Pb shot ban (Newth et al., 2013).

An on-going risk for waterbirds exists due to the high density of Pb shot pellets already accumulated in sediments, even in the protected areas within the Ebro delta. In addition to this, Pb shot pellets can still be used legally in areas regularly used by waterfowl for feeding such as in adjacent rice fields, which are not considered wetlands by the Catalan authorities. This essentially means that a partial Pb shot ban is in force which is only commonly observed in protected wetlands. With this in mind, it is unsurprising that overall Pb shot ingestion rates in mallards only decreased by ~ 50% during the period from 1991–96 (30.2%) to 2011–12 (13.8%) (Figure 2).

4.3 Risk for human consumers

Pb exposed animals represent a significant hazard for human consumers due to the elevated Pb levels held in their tissues (Guitart et al., 2002; Taggart et al., 2011). Here, 68–78% of birds (Figure 4) with ingested Pb shot also had muscle Pb levels above EU MRLs established for livestock meat (0.1 μg/g w.w., European Commission, 2006). In addition, birds that had been killed with Pb shot tended to have higher muscle Pb than birds shot with steel, or, without pellets embedded in the flesh (Figure 4). It is well

recognized that Pb shot and bullets can suffer from fragmentation on impact with game animals. This can result in quite widespread contamination around wounds and increase the risk of exposure to Pb in human consumers of game meat (Hunt et al., 2009; Johansen et al., 2004; Pain et al., 2010; Scheuhammer et al., 1998). Moreover, common recipes that use acidic ingredients, like vinegar or wine, to cook game meat can further increase the transfer of Pb from ammunition to the meat and enhance the subsequent bioavailability of that Pb (Mateo et al., 2007, 2011). Logically, biologically incorporated Pb held within poisoned bird tissues might also be expected to have a higher bioavailability for consumers when compared to embedded Pb in a metallic form. In addition to dietary intake, regular physical contact with Pb ammunition during hunting is also an additive and important route of Pb exposure for hunters (Tsuji et al., 2008).

In broader terms, restrictions in Pb ammunition use can have an immediate effect on the reduction of Pb exposure in game consumers from embedded ammunition, and, a mid-term effect due to the decrease in the prevalence of Pb poisoning in waterbirds harvested by hunters. Here, we have found that the percentage of muscle samples of mallards above the EU MRL for Pb declined from 33.9% in 2007–08 to 2.5% in 2008–09 (Figure 3); and this reduction directly coincided with increased Pb shot ban compliance and the lowest observed prevalence of Pb shot ingestion (5.1%, Figure 2). However, there was an increase in the 2010–11 season in the prevalence of lead shot ingestion (17.9%, Figure 2) and the muscle Pb levels recorded above EU MRL (15.4%, Figure 3). This finding is difficult to explain, because the compliance with the Pb shot ban in the protected areas was high in all seasons after 2008–09. Several factors may have acted to increase the prevalence of Pb shot ingestion in 2010–11. One issue may relate to the increasing number of permits given to scare birds away from the rice fields during spring and summer. This may have had a negative effect on efforts to reduce the impact of Pb shot in surrounding protected areas, especially since farmers and hunters predominantly used Pb shot gun fire for this purpose.

Although consumption of game meat in the general population within Spain (and elsewhere in Europe) is generally low, it can be significant among hunters, their

families and their social group (Sevillano Morales et al., 2011). Unlike in the USA, wild game meat in Spain (and other EU countries) can be/is widely sold and is available on the menus of restaurants. Likewise, even sporadic/occasionally elevated intake of Pb ammunition fragments in particularly susceptible groups within the general population (pregnant women, children) may well be toxicologically relevant. In cases where gamebird consumption is greater than once per week, an effective ban on Pb shot may reduce significantly the risk of dietary exposure to elevated Pb. For example, Green and Pain (2012) found that consumption of <1 meal per week may be associated with a reduction in intelligence quotient (IQ) in children, and that 1.2–6.5 meals per week may be associated with increased systolic blood pressure, occurrence of chronic kidney disease, and rates of spontaneous abortion.

Although the results of this study demonstrate the clear beneficial effects that can occur when a ban on Pb ammunition in a protected wetland is effectively enforced, the partial implementation of this ban (which excludes adjacent rice fields/feeding grounds) will not fully and effectively eliminate the risk of Pb exposure in humans due to consumption of contaminated meat from poisoned birds or those physically containing Pb ammunition residues. It is critical that hunters themselves are engaged in efforts to ameliorate the impacts of Pb on wildlife in general, and on game species in particular. Collaboration with, and educational programs for, the hunting community should therefore be promoted (Friend et al., 2009); and these programs must highlight the dual benefits of using non-toxic shot, as well as the potential risk that Pb contaminated food poses to human health, especially to foetuses and to young children (Carlisle et al., 2009; Lanphear et al., 2005). We suggest that in order to effectively reduce the risk of Pb exposure in humans and poisoning in waterbirds, “protected wetland only” bans on Pb shot should be extended to adjacent feeding grounds. The fact that reasonably good ban compliance was obtained in protected areas within a short period, and, that hunting bag counts were apparently unaffected by a change to steel ammunition (Mateo et al., 2013), also supports the goal to extend the Pb ban to other ecologically important habitats.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL MATERIAL

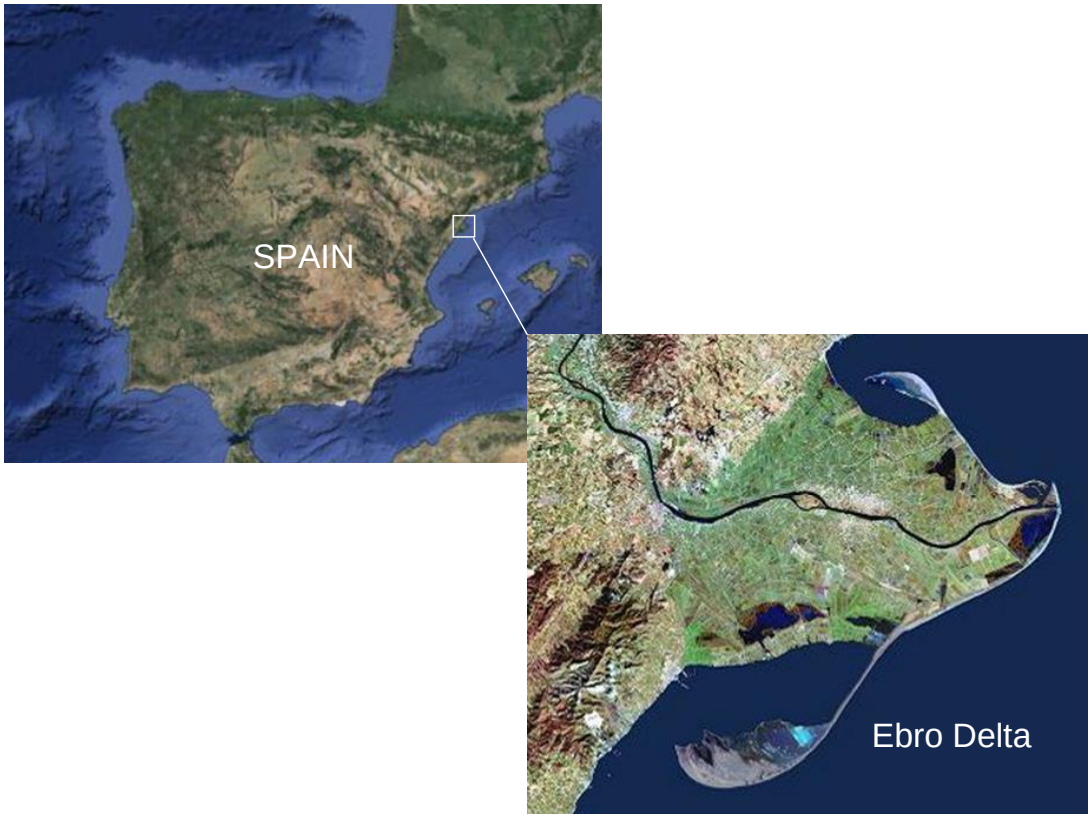

Figure S1. *Map of the study area in the Ebro delta (NE Spain). Source: Goggle Maps and the Catalan Institute of Cartography.*

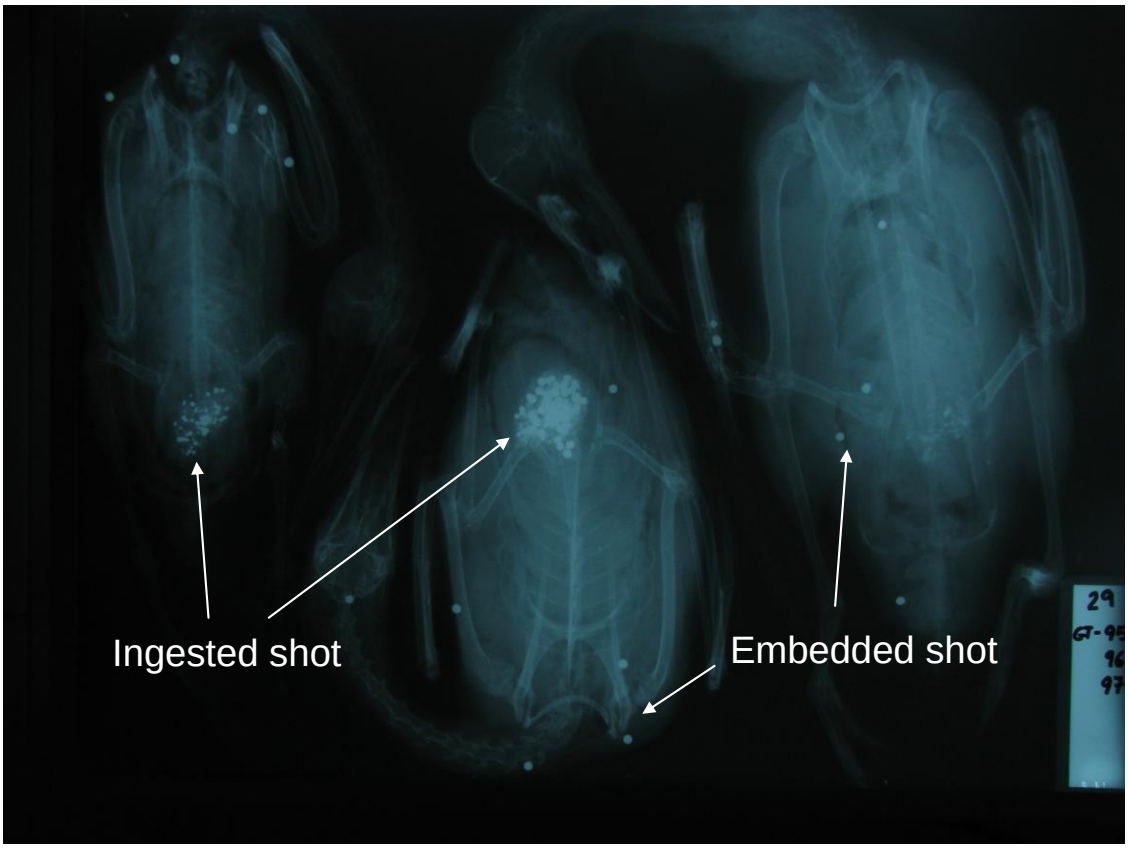

Figure S2. *X-ray of birds with ingested and embedded shot. Author: R. Mateo.*

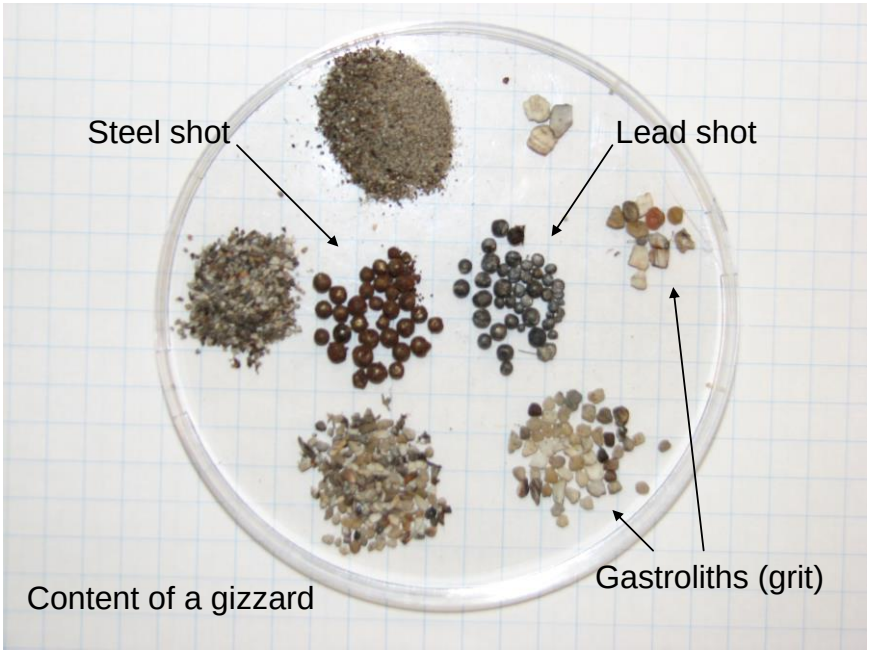

Figure S3*. Content of a gizzard of common pochard (Aythya ferina) with ingested Pb and steel shot pellets. This species selects gatroliths (grit) with similar size to shot pellets. Author: R. Mateo.*

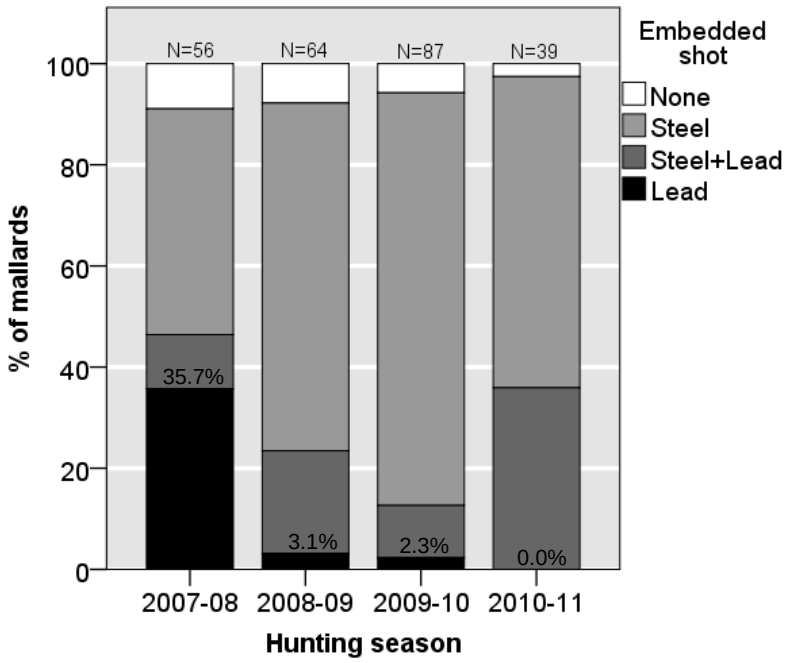

Figure S4. *Percentage of mallards with embedded steel and/or lead shot after the partial Pb shot ban in the Ebro delta.*

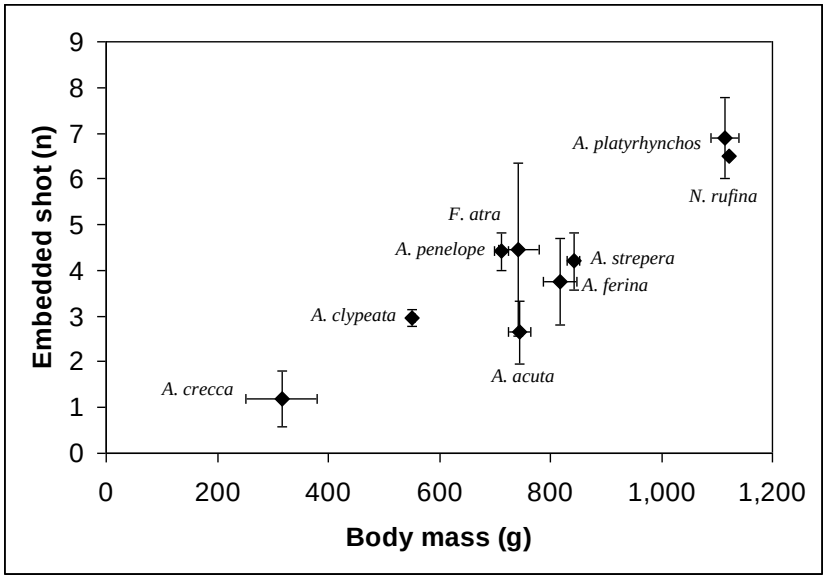

Figure S5. *Relationship between the body mass and the number of embedded shot in the studied waterbird species.*

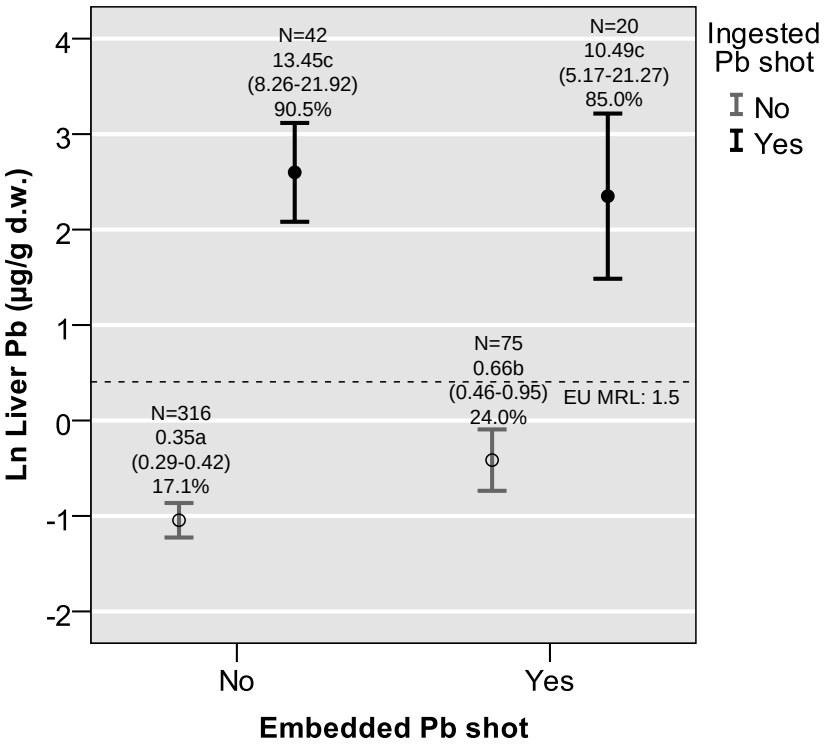

Figure S6. *Liver Pb levels (ln-transformed) in waterbirds with ingested and/or embedded Pb shot. Values adjacent to bars are N, geometric mean, 95% CI and % of samples >EU MRL (1.5 µg/g d.w. ≈ 0.5 µg/g w.w.).*

CHAPTER 2

LEAD EXPOSURE REDUCES CAROTENOID-BASED COLORATION AND CONSTITUTIVE IMMUNITY IN WILD MALLARDS

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ABSTRACT

The ingestion of spent lead (Pb) from ammunition is a known cause of mortality in waterfowl, but little is known about sublethal effects produced by Pb poisoning on birds, especially in wild populations. We studied potential sublethal effects associated with Pb exposure in mallards *Anas platyrhynchos* from the Ebro delta (NE Spain) after a ban on Pb ammunition. We analysed the relationships between blood Pb levels and oxidative stress, immune response and carotenoid-based coloration, which are known to be influenced by oxidative stress. Lead levels were reduced by half from 6 to 9 years after the ban. Lipid peroxidation was positively related to Pb levels in females. δ-aminolevulinic acid dehydratase activity was suppressed by Pb exposure, and negatively associated with the activity of antioxidant enzymes. Carotenoid levels were positively associated with blood Pb concentration in both sexes, and males with higher Pb levels presented a less intense coloration in legs and beak. Lead levels were positively related to hemolytic activity of circulating immune system components, and negatively related to lysozyme levels. In summary, Pb exposure was associated in a gender-specific way with increased oxidative stress, with consequences on colour expression and impaired constitutive immunity. In females, antioxidants seemed to be allocated mostly in reproduction rather than in self-maintenance, while males seemed to better maintain oxidative balance to the detriment of coloration.

Keywords: Pb poisoning, immune response, carotenoid-based coloration, waterfowl, shot

1. INTRODUCTION

The ingestion of spent lead (Pb) shot by waterbirds is a common cause of poisoning in heavily hunted wetlands (Mateo, 2009), which produces not only physiological and behavioural alterations, but also a wide range of sublethal effects (Martinez-Haro et al., 2011) or even death (Mateo, 2009). Among the most commonly described effects, waterbirds usually exhibit Pb toxicosis including neurological signs (e.g.: drooping posture, behavioural changes), distension of the proventriculus, green watery faces, weight loss and anemia (Mateo et al., 2003b). In recent years, there has been an increasing interest regarding the sublethal effects caused by Pb on immune function (Fair and Myers, 2002; Fair and Ricklefs, 2002) and reproduction (Dauwe et al., 2004; Vallverdú-Coll et al., 2015a). The impairment of these functions may inconspicuously affect population dynamics by increasing the susceptibility to pathogens and decreasing reproductive success (Pain, 2009a).

One of the mechanisms involved in Pb toxicity is the induction of oxidative stress through pro-oxidant mechanisms leading to the generation of reactive oxygen species (ROS) and/or the impairment of antioxidant defences (Matović et al., 2015). Lead exposure can promote the overload of tissues with the pro-oxidant aminolevulinic acid (ALA) and induce lipid peroxidation by several pathways (Gurer and Ercal, 2000). Regarding antioxidant defences, Pb can inhibit several antioxidant enzymes because of competitive substitution of essential elements in these enzymes or through the binding to their functional sulfhydryl (SH) groups (Matović et al., 2015).

Lead-induced oxidative stress has been described in experimental (Mateo and Hoffman, 2001; Mateo et al., 2003b) and field studies conducted with waterbirds (Martinez-Haro et al., 2011). Oxidative stress can cause haemolytic anaemia by lipid peroxidation and loss of membrane integrity of red blood cells (RBCs) (Flora et al., 2012). Damaged RBCs are more vulnerable to phagocytosis by macrophages, which can in turn undergo oxidative stress as a consequence of the overload of iron from phagocytized RBCs (Jang et al., 2011; Jomova and Valko, 2011). In red-legged partridges (*Alectoris rufa*), phagocytic activity of blood was shown to increase after experimental exposure to Pb via shot ingestion (Vallverdú-Coll et al., 2015b), and Pb exposure was also related to other alterations of the immune system such as

decreased lysozyme activity, reduced levels of natural antibodies (NAbs), or increased cellular responses, all of which were linked to changes in levels of antioxidants and oxidative stress biomarkers.

Lead exposure may also affect carotenoid-based ornamental coloration (Vallverdú-Coll et al., 2015b), which is known to play key roles in social and sexual signalling in birds (Hill and McGraw, 2006). Carotenoid-coloured traits can advertise the quality of the individual, such as immune function (Peters et al., 2004) and even influence mate investment on reproduction (K. E. Omland, 1996). This signal reliably indicates the quality of the individual because carotenoids have both antioxidant (Burton, 1989) and immune-stimulant properties (Rühl, 2007), and cannot be synthetized by birds, which must acquire them from diet (Goodwin, 1984). Birds therefore face an allocation trade-off between using available carotenoids for self-maintenance needs (immune function, oxidative balance maintenance) or for increasing carotenoid-based signalling (yellow-red coloration of integuments or feathers). The expression of carotenoid-based ornaments displayed by birds could thus be affected by both oxidative stress (Pérez-Rodríguez et al., 2013) and Pb exposure (Vallverdú-Coll et al., 2015b).

Despite an increasing number of experimental studies on sublethal effects of Pb shot ingestion in birds, little is still known about chronic Pb exposure under natural conditions and its subclinical effects on waterfowl populations. We studied wild mallards *Anas platyrhynchos* from the Ebro delta (NE Spain) and hypothesised that Pb exposure would be associated with increased oxidative stress, which would in turn affect other potential endpoints of Pb-induced toxicity, including carotenoid-based ornamental coloration and constitutive immunity. We studied the possible role of oxidative stress as a mechanism involved in Pb toxicity, immune response and carotenoid allocation trade-offs. In addition, we studied how other parameters indicative of health status (body condition, plasma biochemistry profile, sexual hormones levels) varied with Pb levels, in order to better understand the overall effects of Pb exposure on wild mallards.

2. MATERIAL AND METHODS

2.1 Study area and species

The Ebro delta (NE Spain) is an Important Bird Area (IBA) and has been included in the Ramsar Convention List of Wetlands of International Importance. In this alluvial plain, high Pb shot densities (>200 shot/m^2) have been reported in the upper 20 cm of sediment due to waterfowl hunting, and Pb poisoning has been found to be an important threat for waterbirds (Mateo, 2009). The use of Pb ammunition was banned in the protected areas (lagoons and marshes) of the Ebro delta in 2003, but it is still allowed in adjacent, unprotected rice fields where waterbirds frequently forage (Mateo et al., 2014). The sampling site in the present study was the Canal Vell lagoon, which is a protected area currently subjected to Pb shot ban.

Mallard ducks have been considered as a good bio-indicator for Pb poisoning because they are a common widely distributed species and are characterised by a moderately high prevalence of Pb shot ingestion (Mateo, 2009). Before the ban, the prevalence of Pb shot ingestion by mallards averaged 30.2% in the Ebro delta, and it subsequently dropped to 15.5% five years after the ban (Mateo et al., 2014). Lead exposure through shot ingestion is therefore still a threat to waterbirds. This is likely due to the high density of spent Pb shot accumulated in sediment, even in the protected areas (Mateo et al., 2014).

Male and female mallards are sexually dimorphic. Birds acquire their nuptial plumage after the molt in September, before pair formation and breeding that takes place in spring (Cramp and Simmons, 1980). Males display a bright green head, white collar, dark brown breast, black tail curl, and a bright yellow bill, which is pigmented by carotenoids (Cramp and Simmons, 1980; K. E. Omland, 1996). Females have a brown plumage over most of their body. Both sexes display bright orange legs (Cramp and Simmons, 1980). Lutein is the main carotenoid deposited in the bill integument, with a variable quantity of zeaxanthin and 3′-dehydro-lutein (Peters et al., 2004). Trade-offs between the immune system and sexual signalling have been reported in male mallards in which an increased immune investment has been associated with reduced testosterone and carotenoid levels, as well as carotenoid-based coloration (bill yellowness) (Peters et al., 2004).

2.2 Field procedures and sample collection

Wild mallards were captured using baited funnel traps in the 2009 and 2012 breeding seasons (March-May). Traps were set on shorelines frequently used by waterbirds, baited with sorghum and checked three times per day to retrieve and process captured birds. Captured birds were held in a cloth bag prior to processing and were individually marked (banding with metal rings). A total of 108 mallards (65 males and 43 females individuals) were sampled (136 captures in total, from which 28 were recaptures).

Mallards were sexed from plumage and weighed. Tarsus, wing, and beak length were measured, and a 2 mL of blood sample from the brachial vein was collected. Blood was stored in heparinised tubes to avoid coagulation and kept refrigerated until processed within 1-2 h. Haematocrit was recorded using a capillary tube reader after centrifugation at 4,000 *g* for 5 min. Blood samples were then separated into three aliquots. One aliquot was kept for blood Pb analysis, another for analysis of δ-aminolevulinic acid dehydratase (δ-ALAD) activity, and a third one was centrifuged to separate plasma from the cellular fraction (RBCs) for further immunological, biochemical, and antioxidant analysis. These aliquots were stored in liquid nitrogen. In 2012, carotenoid-based coloration (see below) was also measured before releasing the birds at the capture site.

2.3 Blood Pb levels and δ-ALAD activity analysis

For blood Pb assays, whole blood samples were diluted (1:10) with 0.1% triton and analysed using graphite furnace atomic absorption spectroscopy (GF-AAS; AAnalyst 800 with autosampler AS800, Perkin Elmer) (Mateo et al., 1999). Calibration standards were prepared from commercial solutions containing 1 g/L of Pb (Panreac) and Mili-Q grade water. A certified reference material for Pb (blood BCR-196) was analysed to ensure the quality of the methodology with a recovery (mean ± SD) of 98 ± 12% (n=46). The detection limit was <0.6 µg Pb/dL in blood. δ-ALAD activity was determined using a spectrophotometric assay and the ratio of non-activated/in vitro-activated enzyme (δ-ALAD activity ratio) was then calculated (Martinez-Haro et al., 2011).

2.4 Oxidative stress biomarkers and antioxidant levels

Oxidative stress biomarkers were analysed using RBCs homogenates. We measured glutathione (GSH) levels in the oxidized (oxGSH) and total (tGSH; reduced + oxidized) forms by spectrophotometric assays (Reglero et al., 2009b). Lipid peroxidation was determined measuring thiobarbituric acid-reactive substances (TBARS) using spectrophotometric assays (Aust, 1985) in 2009, and by measuring malondialdehyde (MDA) levels in 2012 using high performance liquid chromatography (HPLC) coupled to a fluorescence detector (Romero-Haro and Alonso-Alvarez, 2014). GSH peroxidase (GPx, EC, 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1) activities were measured using spectrophotometric assays with Ransel and Ransod kits (Randox Laboratories), respectively. Antioxidant enzyme activities were expressed relative to mg of protein (Bradford, 1976). Plasma was used to measure levels of dietary antioxidants, specifically vitamins (retinol, α-tocopherol) and carotenoids (lutein, zeaxanthin), using an HPLC coupled to a photodiode detector and a fluorescence detector (Rodríguez-Estival et al., 2010). Total carotenoid levels (tCAR) were calculated as the total sum of circulating carotenoid pigments.

2.5 Carotenoid-based coloration

In 2012, we measured the coloration of legs (males and females) and of the beak (males only) of all sexually mature mallards that were captured (but not recaptures) using a portable spectrophotometer (Minolta CM-2600 d). In order to assess repeatability and obtain more accurate measurements, the color measurements of each body part (beak and leg for males, leg only for females) was measured three times. Reflectance data were extracted and processed using SpectraMagic™ NX software (Konica Minolta). For each color measurement and body part, "*L*", "*a*", "*b*" ("Lab" color space) values were obtained and used to calculate the hue and chroma of each trait. Hue and chroma measurements were highly repeatable (all *R*-values>0.95; all $p<0.001$) and the average values were used to characterise the color of study traits. Chroma values are indicative of color saturation (the greater the chroma, the greater the color purity and perceived color intensity). Color hue is indicative of the perceived color (e.g. green, yellow, orange, red). Hue values are inversely related to the red shift,

so to ease the interpretation of the results, we calculated beak yellowness (the inverse of beak hue; with greater values characterising a more orange-yellow beak and lower values a more greenish-yellow beak). Female mallards have been shown to prefer males with orange-yellow bills rather than green-yellow ones, which are displayed by better quality males (K. E. Omland, 1996). Green-yellow beaks may reflect a reduced allocation of lutein (the carotenoid responsible for the yellow bill coloration of mallards) into ornaments. Similarly, we calculated leg orangeness (the inverse of leg hue), with greater values describing red-orange legs and lower values describing more yellow-orange legs. It is not known whether leg coloration relates to individual quality, but individuals of better quality are presumably those displaying red-orange legs. Yellow-orange legs may reflect a reduced allocation of zeaxanthin (the carotenoid responsible for the orange coloration) into leg integuments.

2.6 Constitutive immune responses

Constitutive immune responses are disease-resistance mechanisms present in the organism without prior stimulation with an antigenic or immunogenic agent. Constitutive responses were characterised in samples from 2012 by quantifying NAbs in plasma and their interaction with complement proteins to lyse foreign cells by using hemolysis-hemagglutination tests against sheep red blood cells (SRBCs) (Vallverdú-Coll et al., 2015b). Lysis reflects the interaction of complement and NAbs, whereas agglutination results from the action of NAbs only.

Lysozyme is an enzyme of leukocytic origin with antibacterial and antiviral activity. Lysozyme levels were measured in plasma by determining bacterial lysis using agar-bacteria suspension (Vallverdú-Coll et al., 2015b).

2.7 Plasma biochemistry and sexual hormones

Plasma biochemistry was measured spectrophotometrically using commercial available reagent kits (BioSystems). For the 2009 and 2012 samples, levels of albumin, total proteins, creatinine, uric acid, triglycerides, cholesterol, glucose, calcium (Ca), phosphorus (P), magnesium (Mg), alkaline phosphatase (ALP), creatine kinase (CK),

lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. For the 2009 samples, levels of ɣ-glutamyl transferase (ɣG-T) and urea were also measured. Testosterone and estradiol were measured in 2009 plasma samples using enzyme-linked immunosorbent assay (ELISA) commercial kits (DRG Instruments GmbH).

2.8 Statistical analysis

Continuously distributed variables were checked for normality and log-transformed to fit a normal distribution when necessary. Mallards were classified into two groups according to their blood Pb concentration (<20 μg/dL or ≥20 μg/dL) that correspond to background and elevated levels (Pain, 1996), respectively. Differences in the prevalence of elevated levels were compared between sampling years using a Fisher one-tailed test. Differences in the average blood Pb concentration between sampling years were compared using T tests. Factors influencing variation in blood Pb-levels (log-transformed) were analysed using generalized linear mixed models (GLMMs) that included sex, year and month (March vs. April-May) as predictors. The "individual identity" was included as a random factor to consider those individuals that were sampled more than once (recaptures).

Lipid peroxidation measures taken in 2009 (TBARS) and 2012 (MDA) were combined into a single variable (lipid peroxidation, LPO) by normalizing their respective values for each year (mean=0; SD=1). We thus obtained a variable indicative of the relative lipid peroxidation index for each sampling year. In order to summarize the overall oxidative balance, a reduction of variables was performed by means of a principal component analysis (PCA) including all the oxidative stress and antioxidant variables (i.e. LPO, GPx, SOD, tGSH, oxGSH, retinol, lutein, zeaxanthin and tocopherol). The resulting PCs were subsequently used as indicators of oxidative stress in order to study whether they could explain the potential effects of Pb exposure on carotenoid-based coloration, immune response and other variables (δ-ALAD activity ratio, plasma biochemistry, hormones levels).

The possible associations between carotenoid-based coloration and blood Pb levels were investigated using generalized lineal models (GLM) that included year, sex,

body condition, blood Pb concentration and the interaction between sex and blood Pb as explanatory variables. The interaction between sex and blood Pb level was considered because of marked differences between sexes in some studied variables during our sampling period in spring. Body condition (mass corrected for size) was calculated using the scaled mass index (Peig and Green, 2009), using tarsus length as the most reliable measure of body size.

The co-variation among other study variables (δ-ALAD activity ratio, oxidative stress biomarkers, immune function, plasma biochemistry and sexual hormones) and Pb concentration was studied using GLMMs that included the same explanatory variables as above, and "individual identity" as random factor in order to take into account the non-independence of repeated measurements on recaptured individuals.

In order to study a possible mediating effect of oxidative stress on the associations between Pb levels and carotenoid-based coloration, immune function, δ-ALAD activity ratio or plasma biochemistry (dependent variables), we used GLMMs that included the Principal Components derived from oxidative stress variables (see above) as covariates. Immune function parameters were added as covariates in GLMs to test their relationship with carotenoid-based coloration. Final models were selected using a backward deletion procedure in which interactions and main effects were sequentially removed when non-significant. Linear correlation coefficients between blood Pb concentration and δ-ALAD activity ratio, oxidative stress biomarkers, components of immune response or carotenoid-based coloration were calculated considering only data from the first capture per individual.

Statistical significance was set at $p \leq 0.05$. All statistical analyses were performed using the IBM SPSS Statistics 20.0 software.

3. RESULTS

Table 1 summarizes the means of each parameter related to oxidative balance, carotenoid-based coloration and constitutive immunity in mallards with low (<20 µg/dL) or high (≥20 µg/dL) Pb blood levels. The linear relationship between these variables and blood Pb levels are shown in this table based on GLMMs results and further details are given below.

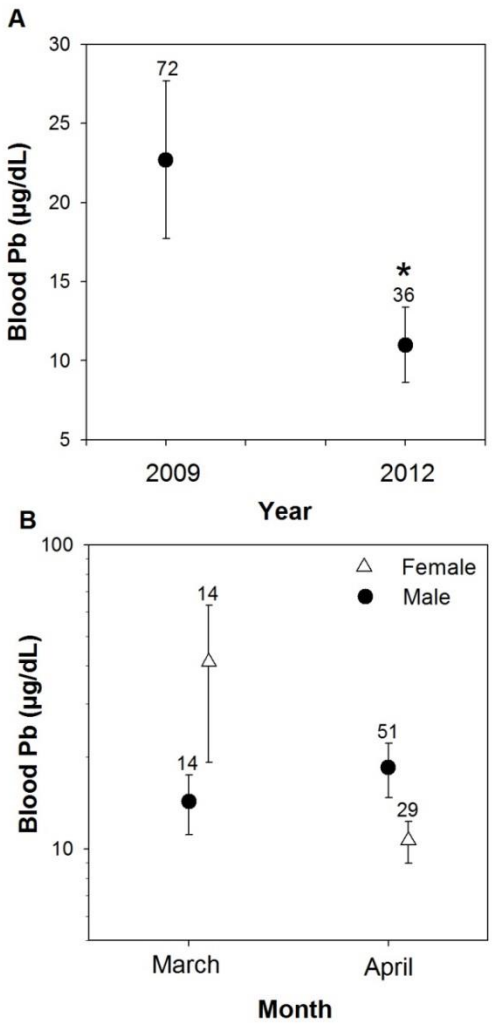

***Figure 1**. Temporal variations in blood Pb levels in wild mallards from the Ebro delta showing the decrease of Pb concentrations over time. (**A**) Mean (± S.E.) blood Pb levels in 2009 (6 years after the ban on the use of Pb ammunition) and in 2012 (9 years after the ban); (**B**) Mean (± S.E.) blood Pb levels according to gender and month. Note the log scale in the "y" axis (blood Pb). Numbers above error bars refer to sample size.*

3.1 Blood Pb levels, δ-ALAD activity ratio and hematocrit

The prevalence of elevated Pb exposure associated with subclinical or clinical poisoning (≥20 µg/dL in blood) was significantly lower in 2012 (13.9%, n=36) than in 2009 (30.6%, n=72) (Fisher one-tailed test, p=0.046). The average blood Pb concentration (mean ± SE) was also lower in 2012 (11.0 ± 2.40 µg/dL) than in 2009 (22.7 ± 4.99 µg/dL) (t_{106}=2.61, p=0.010; Figure 1A). The model that best explained blood Pb levels included sampling year ($F_{1,131}$=5.90, p=0.017), month ($F_{1,131}$=4.50,

p=0.036), sex and the interaction between sex and month ($F_{1,131}$=4.38, p=0.038). Marginal means showed that blood Pb levels were greater in females than in males in March, but not in April, when males and females had similar Pb levels (Figure 1B). δ-ALAD activity ratio was negatively correlated with blood Pb levels ($F_{1,39}$=117.6, p<0.001) (Table 1). The relationship between Pb concentration and δ-ALAD activity ratio was steeper in males than in females (Pb×sex interaction: $F_{1,39}$=7.33, p=0.010; Figure 2). We did not detect significant associations between Pb levels and hematocrit.

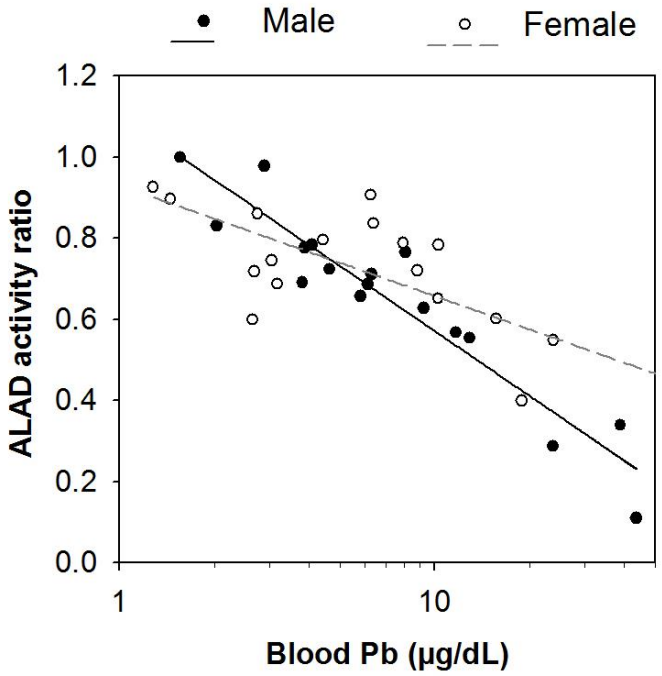

Figure 2. *Relationship between blood Pb levels and δ-aminolevulinic acid dehydratase (δ-ALAD) activity ratio (n=35, r=-0.836, p<0.001) in male and female mallards. The inhibition of this enzyme is commonly used as an indicator of Pb exposure. Note the log scale in the "x" axis (blood Pb).*

3.2 Oxidative stress biomarkers and antioxidant levels

Three Principal Components (PCs) summarized oxidative stress and antioxidant parameters (Table 2), accounting for 58.2% of total variance. PC1 was associated with increasing levels of dietary antioxidants, PC2 with increasing levels of total and oxidized GSH, and PC3 with increased activities of antioxidant enzymes. δ-ALAD

activity ratio was negatively associated with PC3 ($F_{1,33}$=6.01, p=0.020), indicating that greater antioxidant enzyme activities were associated with increased Pb exposure.

A positive relationship between LPO and blood Pb levels was found in females but not in males (blood Pb: $F_{1,132}$=4.35, p=0.039; Pb×sex: $F_{1,132}$=4.667, p=0.032). Overall levels of carotenoids were greater in males than in females ($F_{1,125}$=13.2, p<0.001; Supplemental material, Table S1). Zeaxanthin levels were positively associated with blood Pb levels ($F_{1,123}$=4.04, p=0.047) and were greater in males than in females ($F_{1,123}$=10.1, p=0.002; Supplemental material, Table S1). Lutein, retinol and tocopherol levels were unrelated to Pb levels (Table 1). Similarly, we did not detect significant associations between Pb levels and SOD, GPX, tGSH or oxGSH.

3.3 Carotenoid-based coloration

We found significant relationships between beak and leg hue (i.e. perceived coloration) and blood Pb levels in males. Legs were redder in males than in females (χ^2_1=11.6, *p*=0.001). Males with lower blood Pb levels displayed redder legs (χ^2_1=9.74, *p*=0.002), while leg hue was unrelated to Pb in females (Pb×sex: χ^2_1=6.58, *p*=0.010) (Figure 3A). In males, beak yellowness was negatively correlated with blood Pb levels (χ^2_1=13.6, *p*<0.001) (Figure 3B) and positively associated with body condition (χ^2_1=5.41, *p*=0.020). When oxidative stress PCs were included as covariates, PC1 (dietary antioxidants) was positively correlated with beak yellowness (χ^2_1=6.56, *p*=0.010), and the negative association between beak coloration and Pb levels remained significant (*p*<0.001). We found no associations between blood Pb levels and beak or leg chroma.

3.4 Constitutive immune function

Lytic response against SRBCs was positively correlated with blood Pb concentration ($F_{1,42}$=4.40, p=0.042), while lysozyme levels were negatively correlated with blood Pb ($F_{1,40}$=6.91, p=0.012) (Figure 4). Agglutination response to SRBCs was negatively associated with leg orangeness (χ^2_1=9.36, p=0.002) and chroma (χ^2_1=5.82, p=0.016), while beak coloration was not associated with any of the immune response variables studied here. No differences in constitutive immune function were observed between sexes.

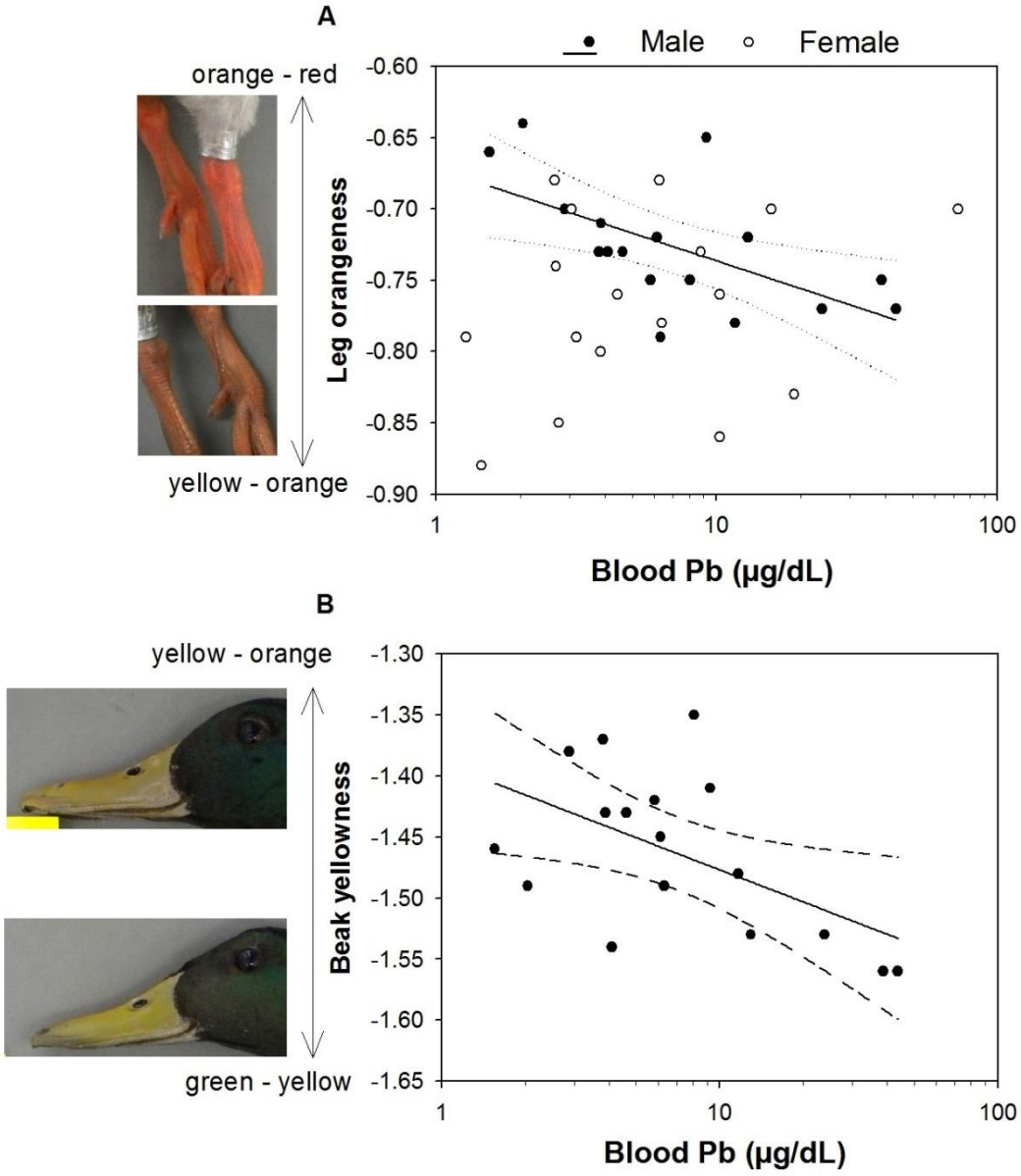

***Figure 3**. Relationship between carotenoid-based coloration and blood Pb levels in wild mallards from the Ebro delta. Males exposed to Pb had reduced carotenoid-based coloration. (**A**) Leg orangeness in males (n=17, r=-0.604, p=0.010) and females, (**B**) beak yellowness (n=17, r=-0.541, p=0.025) in males. Note the log scale in the "x" axis (blood Pb).*

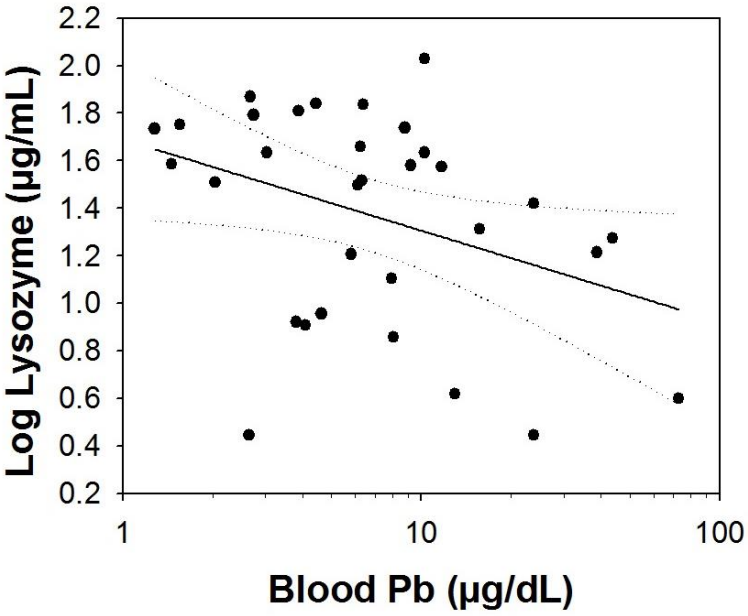

***Figure 4**. Relationship between lysozyme concentration and blood Pb levels (n=32, r=-0.371, p=0.037) in wild mallards from the Ebro delta. Note the log scale in the "x" axis (blood Pb). The Pb-mediated reduction of lysozyme has been associated with changes in the gut microbiota.*

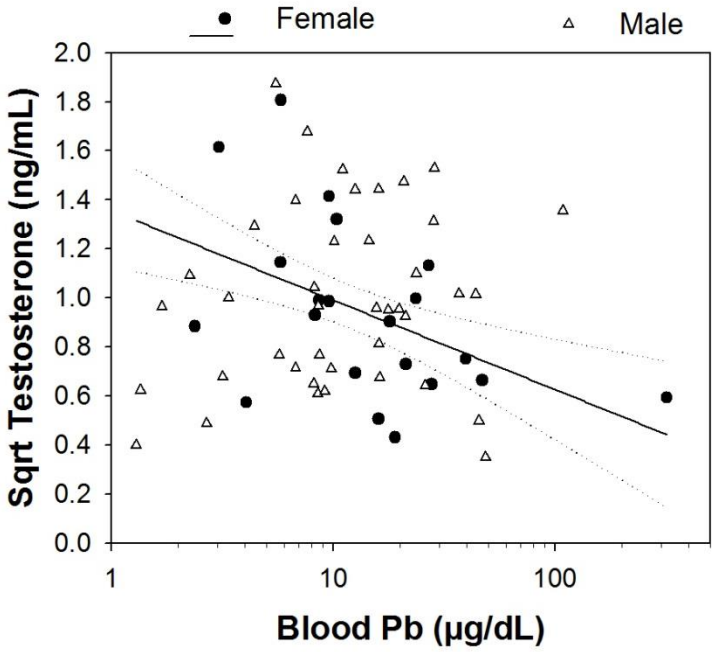

***Figure 5**. Relationship between testosterone concentration and blood Pb levels in male and female (n=21, r=-0.468, p=0.033) mallard. Note the log scale in the "x" axis (blood Pb).*

Table 1. Summary of variables associated with Pb exposure, oxidative status, carotenoid-based coloration and constitutive immunity measured in blood and plasma of wild mallards having blood Pb levels above or below the abnormal exposure threshold (≥20 µg/dL in blood). The right part of the table shows the results of GLMMs with individual identity as random factor (for Pb exposure variables, oxidative stress biomarkers, dietary antioxidants and constitutive immune response) or GLMs (for carotenoid-based coloration) using each parameter as dependent variable and blood Pb levels, sex, and the interaction sex*blood Pb levels as factors. Only models showing significant Pb effects are included (ns: not significant, p*>0.05*).

Studied Function	Parameter	Blood Pb				Model significance			
		<20 µg/dL		≥20 µg/dL		Pb	Pb Effect[a]	Sex	Pb*sex
		N	Mean ± S.E.	N	Mean ± S.E.				
Pb Exposure	Blood Pb (µg/dL)	82	7.87 ± 0.559	27	51.5 ± 11.8	-	-	-	-
	δ-ALAD[b] activity ratio	30	0.742 ± 0.024	5	0.328 ± 0.0702	p<0.001	(-) all	ns	p=0.010
	Hematocrit (%)	65	45.0 ± 0.813	18	48.79 ±1.011	ns			
Oxidative stress biomarkers	Lipid peroxidation index	78	0.013 ± 0.107	23	0.371 ± 0.221	p=0.039	(+) females	ns	p=0.032
	GPx[c] (IU/mg)	72	0.889 ± 0.038	22	0.826 ± 0.066	ns			
	SOD[d] (IU/mg)	75	1.27 ± 0.046	23	1.21 ± 0.102	ns			
	tGSH[e] (µmol/g)	77	5.62 ± 0.170	23	5.61 ± 0.381	ns			
	oxGSH[f] (µmol/g)	65	0.713 ± 0.068	19	0.951 ± 0.164	ns			
	oxGSH[g] %	65	12.6 ± 1.011	19	16.5 ± 2.54	ns			

Dietary antioxidants	Retinol (nmol/mL)	79	8.06 ± 0.344	23	7.99 ± 0.514	ns			
	Tocopherol (nmol/mL)	79	50.8 ± 2.53	23	53.5 ± 3.84	ns			
	CAR[h] tot	79	22.8 ± 1.53	23	26.4 ± 2.59	ns			
	Lutein (nmol/mL)	79	17.6 ± 1.34	23	20.3 ± 2.38	ns			
	Zeaxanthin (nmol/mL)	78	4.44 ± 0.367	23	5.61 ± 0.586	p=0.047	(+) all	p=0.002	ns
Carotenoid-based coloration	Beak hue	14	1.45 ± 0.015	3	1.55 ± 0.010	p<0.001	(+) males	-	-
	Beak chroma	14	30.5 ± 1.04	3	25.2 ± 2.36	ns			
	Leg hue	30	0.746 ± 0.011	4	0.748 ± 0.017	p=0.002	(+) males	p=0.001	p=0.010
	Leg chroma	30	37.1 ± 1.19	4	38.6 ± 3.69	ns			
Constitutive immune response	Lysozyme (µg/mL)	30	34.8 ± 4.99	5	13.6 ± 4.50	p=0.012	(-) all	ns	ns
	SRBCs[i] agglutination titer	29	3.07 ± 0.228	5	2.80 ± 0.200	ns			
	SRBCs[i] lysis titer	29	2.345 ± 0.145	5	2.60 ± 0.245	p=0.042	(+) all	ns	ns

[a]Relationship between the parameter and blood Pb levels: (+) positively related to blood Pb levels, (-) negatively related to blood Pb levels. [b]δ-ALAD: δ-aminolevulinic acid dehydratase. [c]GPx: Glutathione peroxidase. [d]SOD: Superoxide dismutase. [e]tGSH: Total glutathione. [f]oxGSH: Oxidized glutathione. [g]% oxGSH: Percentage of oxidized glutathione. [h]CAR tot: total carotenoids. [i]SRBCs: Sheep red blood cells.

Table 2. Results of the Principal Component Analysis of oxidative stress and antioxidant biomarkers. The variance explained by each PC, as well as the correlation coefficients of the PCs with the original variables are shown.

	Principal Components		
Study variables	PC1	PC2	PC3
Lipid peroxidation	0.12	0.069	0.241
GPx[a] (IU/mg protein)	-0.031	-0.187	0.784
SOD[b] (IU/mg protein)	-0.123	0.168	0.75
tGSH[c] (µmol/g RBCs)	0.115	0.754	0.007
oxGSH[d] (µmol/g RBCs)	0.104	0.827	0.039
Retinol (nmol/mL)	0.284	0.262	0.39
Lutein (nmol/mL)	0.775	-0.238	0.207
Zeaxanthin (nmol/mL)	0.807	0.298	-0.285
Tocopherol (nmol/mL)	0.829	0.303	-0.151
Variance explained			
Proportion (%)	23.1	18.1	17.1
Cumulative (%)	23.1	41.1	58.2

[a]GPx: Glutathione peroxidase. [b]SOD: Superoxide dismutase. [c]tGSH: Total glutathione. [d]oxGSH: Oxidized glutathione

Table 3. Summary of biochemical parameters and hormones in plasma of wild mallards having blood Pb levels above or below the abnormal exposure threshold (≥20 µg/dL in blood). Statistical results of GLMMs using each parameter as dependent variable and blood Pb levels, sex, and the interaction sex*blood Pb levels as factors, and individual identity as random factor are shown. Only models showing significant Pb effects are included (ns: not significant, *p>0.05*).

Parameter	Blood Pb				GLMMs			
	<20 µg/dL		≥20 µg/dL		Pb	Pb Effect[a]	Sex	Pb*sex
	N	Mean ± S.E.	N	Mean ± S.E.				
Albumin (g/L)	75	18.0 ± 0.65	26	14.3 ± 1.33	ns			
ALP[b] (U/L)	77	152 ± 14.4	26	81.0 ± 11.5	p=0.010	(-) all	ns	ns
ALT[c] (U/L)	77	108 ± 5.76	26	80.7 ± 8.46	ns			
AST[d] (U/L)	77	163 ± 13.3	26	142 ± 23.75	ns			
CK[e] (U/L)	45	1557 ± 219	13	991 ± 341	p=0.006	(-) males	ns	p=0.041
Creatinine (mg/dL)	70	0.333 ± 0.015	20	0.304 ± 0.028	ns			
g-GT[f] (U/L)	23	28.8 ± 7.95	10	16.9 ± 9.26	ns			
LDH[g] (U/L)	50	2463 ± 327	10	2038 ± 432	ns			
Urea (mg/dL)	42	10.4 ± 0.985	17	10.6 ± 1.99	ns			
Calcium (mg/dL)	68	11.7 ± 0.675	20	10.8 ± 0.73	p=0.008	(-) all	p=0.008	ns
Cholesterol (mg/dL)	70	312 ± 14.3	20	299 ± 25.6	ns			

Glucose (mg/dL)	70	386 ± 14.2	19	370 ± 18.9	p=0.033	(-) males	p=0.003	p=0.027
Magnesium (mg/dL)	68	2.76 ± 0.226	19	2.11 ± 0.187	ns			
Phosphorus (mg/dL)	52	3.70 ± 0.368	17	3.19 ± 0.461	ns			
Total proteins (U/L)	66	51.7 ± 1.26	19	49.1 ± 1.56	ns			
Triglycerides (mg/dL)	70	319 ± 19.9	20	266 ± 40.5	ns			
Uric acid (mg/dL)	69	13.5 ± 0.717	20	12.3 ± 1.49	p=0.013	(-) all	ns	ns
Testosterone (ng/mL)	45	1.16 ± 0.127	18	0.976 ± 0.158	p=0.047	(-) females	ns	p=0.037
Estradiol (pg/mL)	45	51.3 ± 7.61	17	43.5 ± 8.82	ns			

[a]Relationship between the parameter and blood Pb levels: (+) positively related to blood Pb levels, (-) negatively related to blood Pb levels. [b]ALP: Alkaline phosphatase. [c]ALT: Alanine aminotransferase. [d]AST: Aspartate aminotransferase. [e]CK: Creatinine phosphokinase. [f]ɣ-GT: γ-Glutamyl transferase. [g]LDH: Lactate dehydrogenase.

4. DISCUSSION

Following a partial ban enforcement on the use of Pb ammunition in 2003 in the lagoons of the Ebro delta, NE Spain, the percentage of mallards with elevated blood Pb levels (≥20 μg/dL) decreased over time (30.6% in 2009 and 13.2% in 2012). However, despite the ban, a significant proportion of mallards from this area were still affected by Pb shot ingestion and presented moderately high blood Pb levels. Moreover, we found correlative evidence that blood Pb levels were still associated with a range of gender-specific effects on wild mallards such as changes in levels of oxidative stress biomarkers and antioxidants, alteration of the constitutive immune response, and reduced carotenoid-based coloration in males.

Female mallards tended to have greater blood Pb levels than males at the beginning of the breeding season (March), but not later in spring (April-May) (Figure 1B). Egg laying may represent a way of Pb elimination in females (Kerr et al., 2011; Vallverdú-Coll et al., 2015a), as we have previously observed in this population (Vallverdú-Coll et al., 2015a). This could explain the greater Pb concentration limited to the onset of the breeding season in March. Plasma calcium levels were greater in female than in male mallards, and were negatively correlated with blood Pb concentration. During the breeding season, females can increase Ca^{2+} absorption and mobilization for eggshell formation and, at the same time, they can also increase Pb^{2+} absorption and mobilization because of the similarity between the two cations (Tejedor and Gonzalez, 1992).

Among the several pathways of oxidative stress induction by heavy metals, the inhibition of δ-ALAD enzyme activity is one highly specific to Pb exposure (Gurer and Ercal, 2000). Such inhibition increases the levels of its substrate ALA, which has a pro-oxidant activity leading to production of ROS (Gurer and Ercal, 2000). As expected, a reduced δ-ALAD activity was associated with increased blood Pb levels in mallards, and was related to greater PC3 values, indicative of greater levels of antioxidant enzymes. Similarly, Espín et al. (Espín et al., 2015) reported inverse relationships between δ-ALAD activity and levels of TBARS and tGSH in Griffon vultures (*Gyps fulvus*), as well as between activities of δ-ALAD and catalase (antioxidant enzyme) in eagle owls (*Bubo bubo*) naturally exposed to Pb. Moreover, the negative relationship between δ-ALAD

activity and blood Pb levels reported here was more pronounced in male than in female mallards, which could be explained by a reduced Pb assimilation in females that can eliminate it through egg laying (Vallverdú-Coll et al., 2015a). Similar sexual differences were observed in northern bobwhites (*Colinus virginianus*) orally exposed with Pb shot (Kerr et al., 2011).

Lipid peroxidation in RBCs membranes due to Pb exposure can result in hemolytic anaemia (Flora et al., 2012). However, we did not find an association between Pb levels and hematocrit. Lead-induced anaemia is characterized by a high polychromatophylic index, as occurs in regenerative anaemias (Mateo et al., 2003b). The positive trend between RBCs lipid peroxidation index and Pb levels in females could be indicative of a greater vulnerability of females to Pb-induced oxidative stress, which may be due to their lower plasma carotenoid levels in comparison to males. Females must allocate carotenoids to eggs during the laying period (Isaksson et al., 2008) to increase offspring survival at the expense of their own oxidative balance (Velando et al., 2014), so the carotenoid allocation trade-off between oxidative stress and reproductive investment could be more pronounced than in males. Our results showed a positive relationship between blood Pb and plasma zeaxanthin levels in both genders. This could be explained by the up-regulation of protein-facilitated transport of carotenoids in the intestine under oxidative stress conditions (Hill and Johnson, 2012). A similar Pb-induction can exist in the case of antioxidant enzymes (Mateo and Hoffman, 2001), as suggested by the negative association between PC3 and δ-ALAD activity (indirect indicator of Pb exposure) found in wild mallards. Among the other biochemical biomarkers analysed, uric acid was the only molecule with remarkable antioxidant capacity in birds that presented a negative relationship with blood Pb levels, as previously found in coot (Martinez-Haro et al., 2011).

Male mallards display several ornamental carotenoid-based traits (e.g.: yellow bill) (Goodwin, 1984), whose coloration is testosterone-dependent (F. Mougeot et al., 2009), and plays a key role during pairing and mating (K. E. Omland, 1996). In the current study, both bill yellowness and leg orangeness were negatively related to blood Pb levels in males. This suggests that males compensated Pb-induced oxidative stress at the expense of ornamental coloration, as expected for carotenoid-based traits that advertise an individual´s health or quality (Pérez-Rodríguez, 2009). In addition to

modulating carotenoid-based coloration (Peters et al., 2004), testosterone can increase ROS production (F. Mougeot et al., 2009). Hence, only birds in good condition and health should be able to simultaneously maintain a high reproductive effort and brightly colored integuments. In drakes, Pb exposure reduced carotenoid-based coloration, in particular beak yellowness, making these less attractive for mating (K. E. Omland, 1996) and thereby potentially reducing their reproductive success. Interestingly, a negative association between levels of Pb and testosterone was found in females, but not in males. Chaube et al. (Chaube et al., 2010) reported that the in vitro administration of Pb inhibited testosterone production in the ovaries of catfish (*Heteropneustes fossilis*), and suggested that it may result from an alteration of enzymes involved in its production. Although others (Dumitrescu et al., 2014) have also suggested a Pb-inhibition of enzymes (e.g.: aromatase cytochrome P-450) to produce changes in testosterone levels in female rats, these authors found that Pb exposure was associated with an increase rather than a decrease in plasma levels of this hormone.

Regarding constitutive immunity, other studies have shown that lysozyme activity decreases in the presence of Pb (Olmo et al., 2012). In a recent study, our research group found a dose-dependent reduction in lysozyme activity in red-legged partridges after the oral administration of 1 and 3 Pb shot pellets (Vallverdú-Coll et al., 2015b). In addition, these partridges showed an increased blood phagocytic activity and changes in gut microbiota that were associated with the observed changes in these two parameters of the constitutive immunity. Lysozyme has a protective role against pathogens in the gut of birds (Callewaert and Michiels, 2010), so the reduction of its activity by Pb exposure may increase susceptibility to infections.

The level of NAbs, measured as the agglutination against SRBCs, was not related to blood Pb levels in the present study. In contrast, our research group recently found a reduced agglutination response of NAbs against SRBCs in red-legged partridges experimentally exposed to 1 Pb shot (Vallverdú-Coll et al., 2015b). There is little published information on the effects of Pb on the response of NAbs, as most studies are focused on the primary and secondary responses after a prior exposure to an antigen (Fair and Myers, 2002; Fair and Ricklefs, 2002). Interestingly, leg orangeness and chroma were negatively associated with NAbs levels. By contrast, a positive

association between antibody production and beak chroma has been previously reported in mallards (Peters et al., 2004). Similarly, Velando et al. (2014) found that blue-footed booby males (*Sula nebouxii*) maintained feet coloration after an immune challenge at expenses of body condition (i.e. investing in mating and reproduction at expenses of self-maintenance). Here, no direct relationship was found between Pb levels and body condition in mallards. Furthermore, Pb levels reported here were positively related to specific parameters such as lipid peroxidation and levels of circulating antioxidants. This suggests that mallards may be capable of compensating for Pb-related oxidative stress by allocating fewer carotenoids to integuments or egg yolk, prioritizing maintenance (Vallverdú-Coll et al., 2015b) at expenses of coloration in the case of males.

The binding of antibodies to antigen activates complement proteins, resulting in the lysis of the SRBCs (Matson et al., 2005). In the current study, lytic response was positively associated with blood Pb levels. Kasten-Jolly et al. (Kasten-Jolly et al., 2010) found an up-regulation by Pb exposure of the protein C4bp, which is responsible for the regulation of the classical complement pathway. According to Johnson et al. (Johnson et al., 1986), the lytic process appears to have an oxidative basis that depends on the concentration of antibodies and density of macrophages. Lead-induced oxidative stress alters RBCs membrane integrity and makes these cells more vulnerable to macrophage phagocytosis (Jang et al., 2011). Moreover, the continuous destruction of erythrocytes by Pb can induce the up-regulation of hydrolytic enzymes genes expression and increase apoptosis (Kasten-Jolly et al., 2010). Thus, the observed increased lytic response of mallards with greater Pb levels could also be the result of the immunomodulation of genes associated with innate immunity (Kasten-Jolly et al., 2010) that could increase lytic capacity or alter the complement pathway (Kasten-Jolly and Lawrence, 2014).

In the Ebro delta, a maternal transfer of Pb through the egg to the offspring has been reported in mallards (Vallverdú-Coll et al., 2015a). In that study, Pb-induced alterations on offspring affected not only the developing immune system (increased humoral and decreased cellular responses), but also oxidative balance and survival. Thus, despite the observed reduction in Pb levels, mallards from the Ebro delta still

suffer sublethal effects that could potentially affect reproductive success and offspring survival prospects.

Regarding plasma biochemistry variables, glucose levels were negatively associated with blood Pb level. Karamala et al. (2011) suggested that hypoglycaemia observed in experimentally Pb-exposed rats may result from a dysfunction of liver, thyroid and pancreatic damage. A negative relationship between levels of blood Pb and ALP activity has been previously described in wild waterbirds (Martinez-Haro et al., 2011) and in experimentally Pb-exposed red-legged partridges (Vallverdú-Coll et al., 2015b). This finding is relevant because ALP is also an indicator of osteoblastic activity, and Pb exposure has been associated with altered bone mineralization in birds (Álvarez-Lloret et al., 2014).

In summary, we have found evidence for Pb exposure to be associated with increased oxidative stress in wild mallards from the Ebro delta, and with reduced constitutive immunity and carotenoid-based coloration in males. The impairment of these two functions may have population level effects, as the latter two endpoints imply compromised responses against pathogens and reduced mating opportunities, respectively. Other multifunctional alterations observed (e.g. hepatic enzymes and other biochemical parameters) could also compromise the survival of individuals. In terms of subclinical effects, Pb exposed females seemed to prioritize the investment of antioxidants in reproduction over self-maintenance and oxidative balance. In contrast, Pb exposed males seemed to better maintain oxidative balance, at the detriment of carotenoid-based colored traits that play an important role in mate attraction and mating success.

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SUPPLEMENTAL MATERIAL

Table S1. Summary of variables measured in blood and plasma of wild mallards having blood Pb levels above or below the subclinical toxicity threshold (≥20 µg/dL in blood) as a function of gender.

	Blood Pb level							
	<20 µg/dL				≥20 µg/dL			
	Females		Males		Females		Males	
Parameter	N	Mean ± S.E.	N	Mean ± S.E.	N	Mean ± S.E.	N	Mean ± S.E.
Blood Pb (µg/dL Pb)	33	7.99 ± 0.902	48	7.95 ± 0.715	10	62.2 ± 29.0	17	45.2 ± 8.49
Hematocrit (%)	27	46.04 ± 1.07	39	44.5 ± 1.16	8	46.5 ± 0.872	10	50.6 ± 1.48
δ-ALAD[a] activity ratio	16	0.744 ± 0.034	14	0.739 ± 0.035	2	0.452 ± 0.095	3	0.245 ± 0.070
TBARS[b] (µmol/g pellet)	15	0.048 ± 0.004	31	0.049 ± 0.003	7	0.066 ± 0.006	11	0.044 ± 0.005
MDA[c] (nmol/g)	16	44.3 ± 3.62	14	40.0 ± 3.14	2	53.5 ± 10.5	3	52.1 ± 6.37
GPX[d] (UI/mg protein)	30	1.04 ± 0.050	42	0.78 ± 0.048	9	0.881 ± 0.101	13	0.789 ± 0.896
SOD[e] (UI/mg protein)	31	1.33 ± 0.066	44	1.22 ± 0.063	9	1.39 ± 0.161	14	1.09 ± 0.127
tGSH[f] (µmol/g pellet)	32	5.60 ± 0.253	45	5.63 ± 0.231	9	6.30 ± 0.757	14	5.16 ± 0.373
oxGSH[g] (µmol/g pellet)	29	0.665 ± 0.084	36	0.751 ± 0.103	9	1.11 ± 0.264	10	0.803 ± 0.202
oxGSH[h] (%)	29	12.0 ±1.34	36	13.0 ± 1.48	9	18.4 ± 3.45	10	14.8 ± 3.77
Retinol (nmol/mL)	32	8.87 ± 0.525	46	7.38 ± 0.431	9	8.23 ± 0.714	14	7.86 ± 0.727
Tocopherol (nmol/mL)	32	41.2 ± 3.72	46	57.3 ± 3.17	9	52.1 ± 6.41	14	54.4 ± 4.95
Lutein (nmol/mL)	32	14.3 ± 1.48	46	21.2 ± 2.03	9	15.9 ± 2.93	14	23.9 ± 3.34
Zeaxanthin (nmol/mL)	32	3.12 ± 0.395	45	5.42 ± 0.528	9	4.65 ± 0.697	14	6.23 ± 0.829
CAR[i] tot (nmol/mL)	32	17.5 ± 1.65	46	26.6 ± 2.22	9	20.6 ± 3.36	14	30.1 ± 3.39
Albumin (g/L)	29	19.8 ± 1.07	45	16.9 ± 0.795	10	14.7 ± 2.31	16	14.1 ± 1.66
ALP[j] (U/L)	31	177 ± 27.6	45	137 ± 15.5	10	92.3 ± 23.9	16	73.9 ± 11.6
ALT[k] (U/L)	29	112 ± 8.96	47	107 ± 7.504	10	93.4 ± 12.8	16	72.8 ± 11
AST[l] (U/L)	30	126 ± 17.5	46	190 ± 18.2	10	152 ± 30.7	16	136 ± 34.19
CK[m] (U/L)	20	1784 ± 341	24	1409 ± 295	4	1528 ± 1052	9	752 ± 212
Creatinine (mg/dL)	28	0.325 ± 0.023	41	0.340 ± 0.020	8	0.339 ± 0.042	12	0.280 ± 0.038
ɣ-GT[n] (U/L)	7	50.0 ± 17.0	16	20 ± 8.0	4	36.0 ± 21.0	6	4.0 ± 2.0
LDH[o] (U/L)	25	2897 ± 532	24	2069 ± 385	4	2398 ± 897	6	1799 ± 457
Urea (mg/dL)	12	10.3 ± 1.92	29	10.7 ± 1.17	7	8.36 ± 1.78	10	12.2 ± 3.14
Calcium (mg/dL)	27	12.3 ± 1.37	40	11.3 ± 0.690	8	11.04 ± 1.56	12	10.7 ± 0.722
Cholesterol (mg/dL)	28	281 ± 24.0	41	331 ± 17.0	8	288 ± 38.0	12	307 ± 35.0
Glucose (mg/dL)	28	355 ± 24.0	41	407 ± 17.0	8	376 ± 25.0	11	366 ± 28.0
Magnesium (mg/dL)	27	3.09 ± 0.380	40	2.57 ± 0.284	7	2.41 ± 0.436	12	1.93 ± 0.150

Phosphorus (mg/dL)	18	4.65 ± 0.758	33	3.29 ± 0.376	7	2.57 ± 0.582	10	3.63 ± 0.659
Total proteins (U/L)	27	54.3 ± 1.52	38	50 ± 1.85	8	52.0 ± 2.29	11	47.0 ± 1.96
Triglycerides (mg/dL)	28	369 ± 36.6	41	290 ± 21.3	8	297 ± 74.9	12	246 ± 47.3
Uric acid (mg/dL)	27	13.9 ± 1.06	41	13.4 ± 0.965	8	14.4 ± 2.39	12	10.9 ± 1.88
Testosterone (ng/mL)	14	1.19 ± 0.245	31	1.14 ± 0.151	7	0.655 ± 0.131	11	1.18 ± 0.229
Estradiol (pg/mL)	14	96.8 ± 14.97	30	25.9 ± 3.25	7	81.02 ± 8.28	10	17.2 ± 4.09
Lysozyme (µg/mL)	16	43.5 ± 7.55	14	24.7 ± 5.40	2	15.1 ± 11.1	3	12.6 ± 4.97
SRBCs[p] agglutination titer	16	3.50 ± 0.50	13	2.50 ± 0.20	2	2.50 ± 0.050	3	3.0 ± 0.0
SRBCs[p] lysis titer	16	2.30 ± 0.20	13	2.30 ± 0.20	2	2.50 ± 0.50	3	2.70 ± 0.030
Beak hue		-	14	1.45 ± 0.154		-	3	1.55 ± 0.01
Beak chroma		-	14	30.5 ± 1.04		-	3	25.2 ± 2.36
Leg hue	16	0.771 ± 0.016	14	0.719 ± 0.012	1	0.70	3	0.76 ± 0.007
Leg chroma	16	34.4 ± 1.73	14	40.4 ± 1.13	1	37.70	3	39.0 ± 5.20

[a]δ-ALAD: δ-aminolevulinic acid dehydratase. [b]TBARS: Thiobarbituric acid reactive substances. [c]MDA: malondialdehyde. [d]GPx: Glutathione peroxidase. [e]SOD: Superoxide dismutase. [f]tGSH: Total glutathione. [g]oxGSH: Oxidized glutathione. [h]% oxGSH: Percentage of oxidized glutathione. [i]CAR tot: total carotenoids. [j]ALP: Alkaline phosphatase. [k]ALT: Alanine aminotransferase. [l]AST: Aspartate aminotransferase. [m]CK: Creatinine phosphokinase. [n]ɣ-GT: γ-Glutamyl transferase. [o]LDH: Lactate dehydrogenase. [p]SRBCs: Sheep red blood cells.

CHAPTER 3

ALTERED IMMUNE RESPONSE IN MALLARD DUCKLINGS EXPOSED TO LEAD THROUGH MATERNAL TRANSFER IN THE WILD

Núria Vallverdú-Coll, Ana López-Antia, Monica Martínez-Haro, Manuel E. Ortiz-Santaliestra. Rafael Mateo. Environmental Pollution 205 (2015) 350-356

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 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.

Keywords: Pb poisoning, maternal transfer, immune response, oxidative stress, developmental toxicity, waterfowl

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 (T_h) cells, resulting in an increased differentiation into T_{h2} cells (responsible of humoral-mediated immunity) at expenses of T_{h1} (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 oxigen 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 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 ºC 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), vitamin D_3 (2,000 UI/kg), tocopherol (35 UI/kg), 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 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 antioxidants 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 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 Supplemental Material for more details, SM).

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 x 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 SM for more details).

2.5 Immune system

We tested T-cell-mediated immunity 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 dilution). We also measured the medial foot membrane of the opposite leg and injected 50 µL of PBS. After 24 h, we measured again foot membrane thickness in both paws, estimating the intensity of cell-mediated response subtracting the thickness produced by PBS to the thickness produced by PHA injection.

Humoral immune response was estimated using a haemagglutination test after antigen injection to stimulate the synthesis of specific antibodies. We tested the primary response against sheep red blood cell (SRBC) antigens to assess the T-lymphocyte dependent antibody response. At the age of 21 days, we injected 100 µL of SRBC dilution (1% in PBS; R3378, Sigma) in the tarsal vein of each duckling. We took blood samples 7 days after SRBC administration to determine the haemagglutination response. We centrifuged the blood samples and prepared serial dilutions of plasma with PBS in wells of microtiter plates for a final volume of 50 µL of plasma dilution; then, we added 50 µL of SRBC (0.5% in PBS) to each well obtaining a final volume of 100 µL. As a negative control, we added 50 µL of PBS instead of plasma in a well in each row. Plates were tilted 45º to improve visualization of haemagglutination and incubated for 1 h at 37 ºC. The antibody concentration was calculated as the logarithm of the lowest plasma dilution causing agglutination.

2.6 Oxidative stress biomarkers and biochemistry analysis

We measured oxidative stress biomarkers in red blood cell (RBC) homogenates by spectrophotometry following the methods described in Reglero et al. (2009). We quantified thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation. We determined the levels of total glutathione (tGSH) and oxidized glutathione (oxGSH), calculated as moles of GSH in form of disulfide (GSSG). The ratio between the oxGSH and tGSH indicates the redox balance of this important

intracellular antioxidant. We measured GSH peroxidase (GPx, EC, 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1) activities, both antioxidant enzymes, using Ransel and Ransod kits (Randox Laboratories), respectively. Antioxidant enzyme activities were calculated relative to mg of protein of the homogenates, which were quantified following the Bradford (1976) method. We also determined the plasmatic levels of dietary antioxidants such as free retinol, α-tocopherol, lutein and zeaxanthin by HPLC coupled to photodiode detector and fluorescence detector (Rodríguez-Estival et al., 2010). In order to obtain a plasma biochemistry profile, albumin, total protein, creatinine, uric acid, triglycerides, cholesterol, glucose, calcium (Ca), phosphorous (P), magnesium (Mg), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), ɤ-glutamyl transferase (g-GT) and urea, were measured with an automatic spectrophotometer analyser A25, using the reaction kits available for each determination (BioSystems, Barcelona, Spain).

2.7 Statistical analysis

Regarding Pb concentration on different tissues, data below the detection limit were assigned values of half of the respective LOD. Normality of all variables was tested by Kolmogorov-Smirnov tests, and non-normal variables (eggshell and egg content Pb concentrations in 2008, and blood, liver and bone Pb concentrations in 2009) were log-transformed (natural logarithm) to fit a normal distribution. Even after log-transformation, we were not able to normalize egg content, blood and liver Pb concentrations. As in some cases two eggs were collected from the same nest in 2009, the relationship between Pb concentration in eggshell and blood, and between these Pb levels and δ-ALAD activity ratio and immune responses, were tested with generalised linear mixed models (GLMMs) including the nest as a random factor. The relationships between Pb levels in blood and tissues of ducklings were also tested with GLMMs. Spearman linear correlation coefficients (r_S) were calculated among these variables and between Pb levels in eggshell and egg content. In order to study the effects of Pb on oxidative stress biomarkers and plasma biochemistry, a reduction of variables was performed by means principal component analysis (PCA). These PCA

were performed separately for samplings performed at day 3 and 28. Oxidative stress PCA included TBARS, GPx, SOD, tGSH, oxGSH, retinol, lutein, zeaxanthin and tocopherol. Plasma biochemistry PCA included the profile cited above. The obtained principal components (PCs) were used as dependent variables in GLMMs with Pb as the independent variable. In order study the interaction of oxidative stress on immune function, we used GLMMs with immune responses as dependent variables and oxidative stress principal components (only data of day 3 for PHA and data of days 3 and 28 for SRBC test) as independent variables. Finally, we tested if oxidative stress PCs used as covariates in GLMMs were able to remove the effects of Pb on the immune function, which may indicate an oxidative stress involvement on the mechanism of toxicity of Pb. Normality of Pearson residuals of each GLMM was tested by Kolmogorov-Smirnov tests.

We classified ducklings according to their blood Pb concentration in two groups based on levels <100 ng/mL and ≥100 ng/mL. This threshold (100 ng/mL) is defined by the Centers for Diseases Control as harmful for children (Prevention and Centers for Disease Control, 1997). We analysed the survival of ducklings in these two groups differing in blood Pb levels using a Kaplan–Meier analysis. Statistical significance was set at $p \leq 0.05$. A marginally significant effect was defined as a p value within the range $0.10 > p > 0.05$. The statistical analyses were performed using SPSS Statistics 19.0 Software.

3. RESULTS

3.1 Maternal Pb transfer and effects on heme metabolism

In 2008, levels of Pb in the eggshell and in the egg content were positively correlated ($n=23$, $r_S=0.439$, $p=0.036$) (Figure 1a). In 2009, hatching rate of the artificially incubated eggs was 70.5%. Blood Pb levels were significantly correlated with eggshell levels of Pb in ducklings at day 3 ($F_{1,27}=17.02$, $p<0.001$) (Figure 1b). Early Pb effect on ducklings was shown by the negative association between δ-ALAD activity ratio in blood at day 3 with eggshell Pb concentration ($F_{1,27}=3.27$, $p=0.064$; Figure 1c; data shown in Tables S1 and S2, SM) and with blood Pb concentration at day 3 ($F_{1,28}=6.35$, $p=0.018$; Figure S1a, SM). This relationship between δ-ALAD activity ratio and Pb in blood was also significant at

day 28 ($F_{1,17}$=28.85, p<0.001; Figure S1b, SM). Ducklings with blood Pb levels above 100 ng/mL (the minimum detected Pb concentration above this threshold was 180 ng/mL) died during the first 7 days post hatching (Log rank test, χ^2=7.896, p=0.005) (Figure 2a). The ratio between the body mass at day 0 and the egg mass was lower in ducklings with blood Pb concentration >100 ng/mL than in those with lower levels ($F_{1,27}$=13.7, p=0.001) (Figure 2b). The model that better explained the body mass at day 0 included the egg mass ($F_{1,26}$=9.5, p=0.005) and the blood Pb concentration (not log-transformed) ($F_{1,26}$=7.35, p=0.012) at day 3 as covariates. In dead ducklings, blood Pb levels at day 3 were positively associated with Pb concentration in liver ($F_{1,7}$=6.9; p=0.034), bone ($F_{1,7}$=18.0, p=0.004) and brain ($F_{1,7}$=9.7, p=0.017) (Table S1, SM). Eggshell thickness was not related to eggshell Pb levels (unhatched and hatched eggs from 2008 and 2009) or content Pb levels in unhatched eggs (not incubated, 2008) (Figure S2a). Eggshell thickness in hatched eggs (incubated, 2009) was positively associated with Pb concentration in blood of ducklings at day 3 ($F_{1,27}$=10.05, p=0.004; Figure S2b) and negatively with their δ-ALAD activity ratio ($F_{1,27}$=6.65, p=0.016; Figure S2c). No relationship was observed in mallard eggshells between Pb concentration and pigmentation (protoporphyrin and biliverdin levels; Table S1).

3.2 Relationship between maternal Pb transfer and immune response of ducklings

Cellular immune function at day 15 in ducklings was negatively related to Pb levels in eggshell ($F_{1,16}$=5.48, p=0.033 Figure 3a) and blood at day 3 ($F_{1,17}$=11.39, p=0.004; Figure 3b; data shown in Table S1, SM).

On the contrary, humoral immune response at day 28 was positively associated with blood Pb levels at day 3 ($F_{1,17}$=5.75, p=0.028; Figure 3c) and negatively correlated with cellular immune response at day 15 (n=18, r_S=-0.513, p=0.030; Figure 3d).

3.3 Interactions between oxidative stress parameters and Pb effects on immune response.

Three oxidative stress PCs were obtained at day 3 and 28, accounting for 72.8 and 74.8% of total variance, respectively (matrix of rotated components show in Table S3, SM). A significant positive relationship between blood Pb level and PC2 was found at day 3 ($F_{1,15}$=7.103, p=0.018), which mainly indicates an increase of endogenous

antioxidants (tGSH) at these levels of Pb exposure, and at lower extent higher levels of oxidative stress biomarkers (TBARS and oxGH) and tocopherol. No effect of Pb was found on other PCs at day 3 or PCs at day 28. At day 3, PHA response was related negatively with PC2 ($F_{1,14}$=6.1, p=0.027), which indicates a higher response with lower levels of antioxidants and oxidative stress biomarkers. The inclusion of PC2 as covariate in GLMM eliminated the significant effect of Pb on PHA response ($F_{1,13}$=3.95, p=0.068), although the effect of PC2 was neither significant. No effect of oxidative stress PCs was found on humoral response. No effect of Pb was found on PCs obtained with the profile of plasma biochemistry (Table S4, SM).

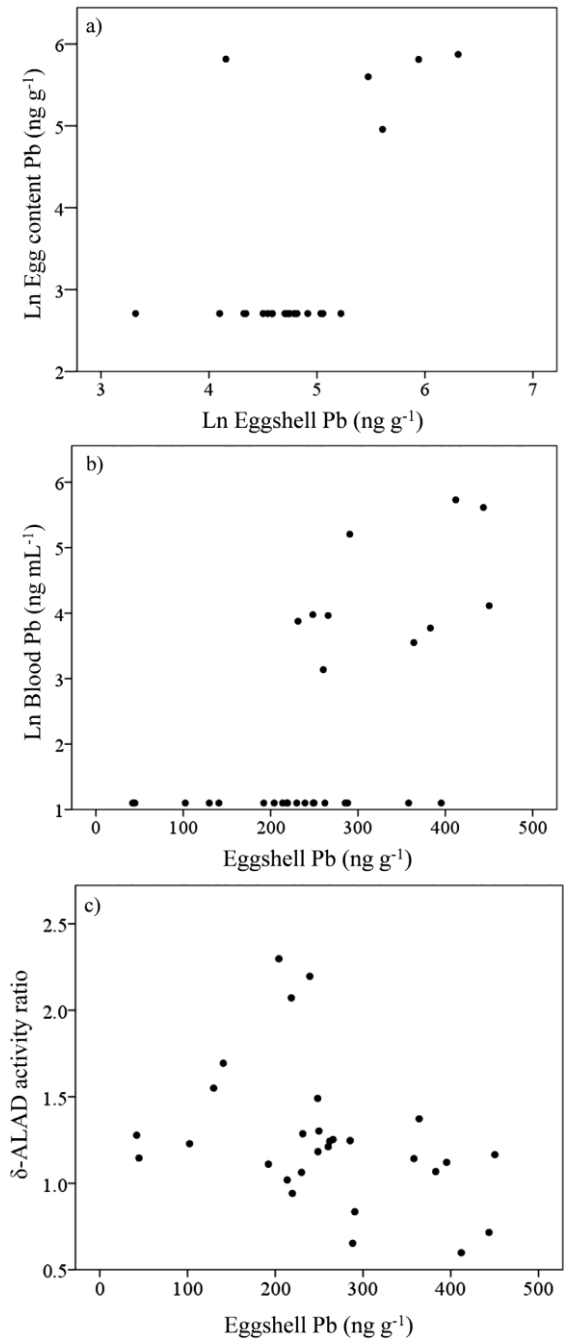

Figure 1. *Correlation between eggshell lead (Pb) concentration and egg content Pb concentration (n=23, r_S=0.439, p=0.036) in wild mallard eggs collected in the Ebro delta in 2008 (a). Correlation between eggshell Pb concentration and either (b) blood Pb concentration (n=29, r_S=0.608, p <0.001) or (c) d-ALAD (d-aminolevulinic acid dehydratase) activity ratio (n=29, r_S=-0.418, p=0.024) measured at day 3 in samples (eggshells and ducklings) of 2009.*

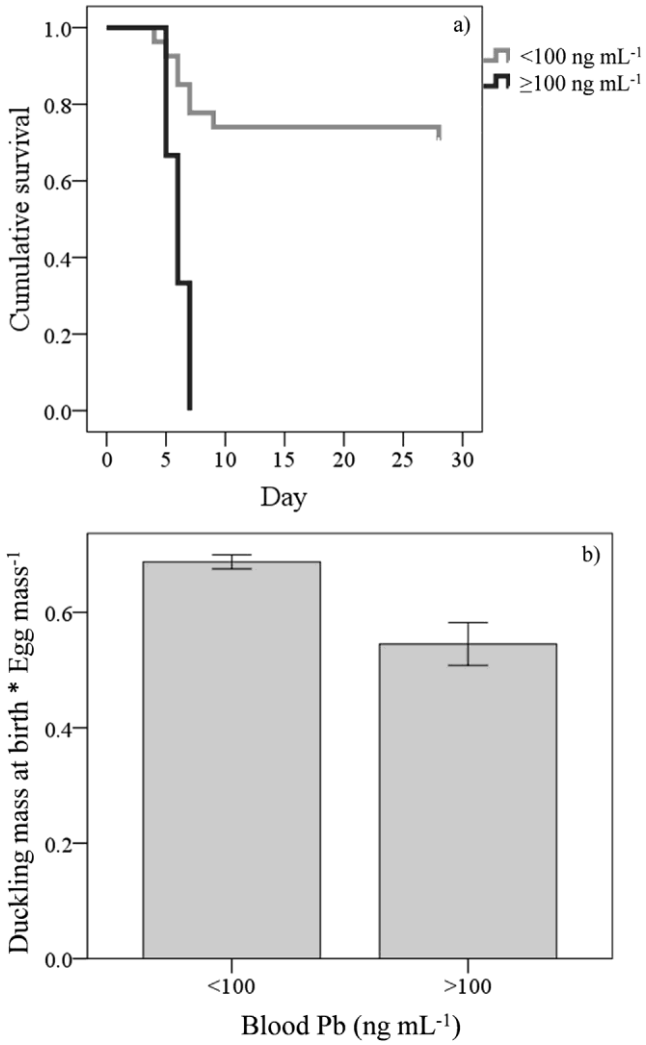

Figure 2. *Duckling survival (n=30, χ^2=7.896, p=0.005, log rank test) in 2009 as a function of blood Pb levels at day 3 (a), and ratio between body mass at birth and egg mass as a function of blood Pb levels at day 3 (n=29, $F_{1,27}$= 13.67, p=0.001, GLMM) (b). Error bars indicate standard error type 1.*

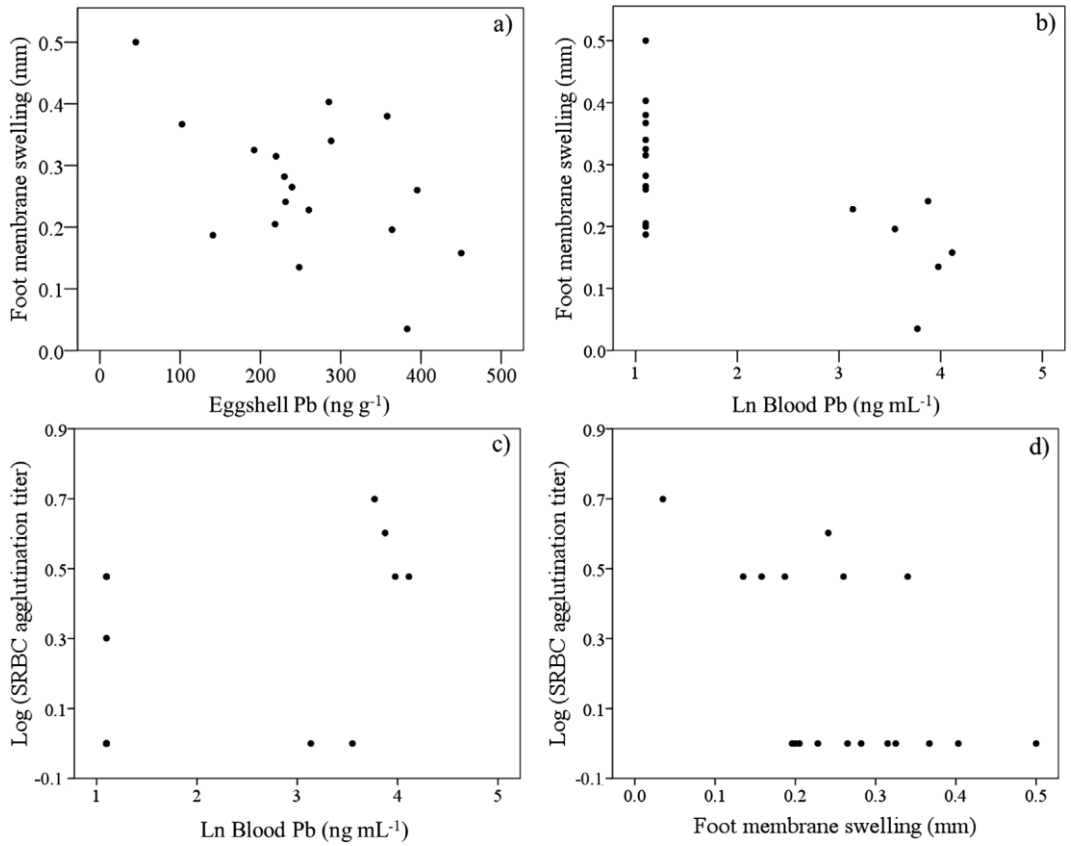

Figure 3. *Correlation between cellular immune response (foot membrane swelling) and eggshell (a) ($F_{1,16}$=5.48, p=0.033) or blood (b) (n=19, r_S=-0.674, p=0.002) Pb concentration, correlation between humoral immune response (agglutination titer of antibodies against Sheep Red Blood Cells) and blood Pb concentration (n=19, r_S=0.517, p=0.023) (c), and correlation between humoral and cellular immune responses (n=18, r_S=-0.513, p=0.030) (d) in ducklings from hatched eggs collected in the Ebro delta in 2009.*

4. DISCUSSION

Levels of Pb detected in eggs and tissues from ducklings reported here confirm the maternal transfer of Pb in bird species with high prevalence of Pb shot ingestion, such as the case of mallards in the Ebro delta. Higher exposure to Pb was associated with a decreased body mass at birth and survival of ducklings, and with changes in the immune function.

4.1 Maternal Pb transfer and effects on heme metabolism

Maternal transfer of Pb resulted in measurable levels of this element in eggs and duckling tissues. Although some authors have reported transference of Pb from polluted water through the eggshell (Kertész et al., 2006), and females could bring Pb polluted water on the eggs when arriving to the nest, we may expect that Pb present in eggs and offspring was maternally transferred. The origin of Pb contamination in the Ebro delta is the use of Pb ammunition, and the main cause of Pb poisoning in waterfowl from this area is shot ingestion due to high Pb shot densities in sediments (Mateo, 2009). Thus, contamination of eggs by contact with polluted water or sediments is unlikely, and surely irrelevant in comparison with exposure because of maternally derived Pb.

Pb exposed females may increase Pb^{2+} absorption for eggshell formation during the breeding season, because of the similarity with Ca^{2+} (Tejedor and Gonzalez, 1992). Bone is efficient to store Pb in chronic exposure by substitution of Ca (Suzuki et al., 1981). Furthermore, female bones act as a reservoir for the supply of eggshell Ca and they may be able to mobilize it during the breeding season (Hertelendy, 1980). Therefore, massive Ca mobilization from female bone for formation of both the eggshell and egg content may also induce Pb mobilization, as suggested by Guirlet et al. (2008) in turtles.

Pb levels shown here in both the eggshell and egg content were lower than those reported in other contaminated areas (Mora, 2003; Dawe et al., 1999) or experimental studies (Jeng et al., 1997). To our knowledge, this is the first study positively relating the eggshell thickness and Pb levels from ducklings. Circulating prostaglandin E_2 (PGE_2) levels increase after ovulation and have a role in eggshell calcification (Hertelendy, 1980). Several authors have reported an increased production of PGE_2 due to Pb exposure (Dietert and Piepenbrink, 2006) and the arachidonic acid (their precursor) was found at higher levels in the liver of Pb-exposed mallards than in controls (Mateo et al., 2003). However, it is important to emphasize that eggshell thickness of unhatched eggs (non-incubated eggs from 2008) was unrelated to Pb levels in eggshell or egg content. It was in the hatched eggs (incubated eggs from 2009) when we found a positive association between eggshell thickness and

blood Pb levels in ducklings. Therefore, we may suspect that differences in shell thickness related to Pb exposure might occur during incubation in the eggshell resorption process. Eggshell thickness tends to decrease throughout the incubation period because it is the primary source of Ca for the developing embryo and this also weakens the eggshell to allow hatching (Johnston and Comar, 1955; Chien et al., 2009). Ducklings with higher blood Pb levels may have suffered some impairment on development, as evidenced by their lower body condition at day 3 after hatching, and this could be related with a lower eggshell resorption for embryo Ca uptake.

We confirmed the eggshell Pb levels as a good non-invasive indicator of local exposure of wild bird populations (Dauwe et al., 1999), due to the positive correlation detected with blood Pb concentration from ducklings. Moreover, the offspring with elevated Pb eggshell levels showed depressed δ-ALAD activity. Individuals with higher Pb levels showed signs of intoxication such as lower body condition, which has already been reported in the offspring of rats exposed to Pb during pregnancy (Teijón et al., 2006). In agreement with our results, Kertész et al. (2006) reported an increased mortality rate of mallard embryos after the immersion of eggs into Pb-polluted water. On the contrary, other authors did not find the same effects of maternal Pb exposure that we observed here. For instance, no effect of Pb on body mass or survival was reported in western bluebird and Japanese quail chicks orally exposed to Pb shot at the age of 3 and 8 days, respectively (Fair and Myers, 2002; Fair and Ricklefs, 2002). Furthermore, Eeva et al. (2014) showed no effects of Pb exposure on δ-ALAD activity, survival rate or body mass of great tits (*Parus major*) orally exposed to Pb acetate from 3 to 14 days of age. It is remarkable that in cited literature nestlings were Pb-exposed after hatching, at ages at which we have already reported Pb alterations in the present study. In the scenario considered in the current work, exposure to Pb happened during embryonic development, and adverse effects were found shortly after hatching.

4.2 Effect of maternal Pb transfer on duckling immune response

The two components of induced immunity studied here were affected by embryonic Pb exposure. Regarding the two distinct populations of Th cells, T_{h1} mediates cell-mediated immunity, whereas T_{h2} mediates humoral-mediated immunity and there

exists a cross-regulation between the two subsets (Hemdan et al., 2007). The imbalance in the T_{h1}/T_{h2} differentiation after developmental Pb exposure, resulting in increased T_{h2} differentiation at expenses of T_{h1}, influences host responses causing immunosuppression or hyperactivity (Dietert et al., 2004). We found that blood Pb concentration negatively related to cellular immune response and positively related to humoral response, which supports this skewed response towards T_{h2}.

Ducklings showed a negative association between cell-mediated response to PHA and concentration of Pb in blood from ducklings, posing a decreased capacity to react after an antigenic challenge to the organism. Similarly, Fair and Myers (2002) found a suppressed cellular response against PHA in western bluebird nestlings exposed to Pb shot ingestion at 3 days of age, and other authors reported a depressed DTH response (indicator of T_{h1} immunity) in embryonic Pb exposed chicken (Lee et al., 2001).

On the contrary, we have observed a positive association between Pb levels and the humoral immune response in mallard ducklings. Bunn et al. (2000) also found such positive association between Pb embryonic exposure and the humoral response in chicken, and an increased T_{h2} development in spleen has been reported in embryonic Pb exposed mice (Kasten-Jolly et al., 2010), supported by the reduction of T_{h1}-associated cytokines and the increase of T_{h2}-associated interleukins .

4.3 Relationship between Pb effects on immune response, oxidative stress parameters and antioxidants levels

Pb produces δ-ALA accumulation (Fossi, 1994) and this has a pro-oxidant effect that can alter the redox status (Oteiza and Bechara, 1993). In fact, we found a negative correlation between δ-ALAD activity ratio and oxGSH (Figure S3a, SM). We found that blood Pb concentration was positively related to the levels of oxidative stress PC2 at day 3, which reflects higher levels of tGSH, and at lower extent higher levels of TBARS, oxGSH and tocopherol. This increase of tGSH level has been also observed in other experimental studies with Pb-exposed birds (Mateo and Hoffman, 2001; Vallverdú et al., 2015), probably because of the induction of γ-glutamylcysteine synthetase by Pb (Griffith 1999). The relationships between the cellular immune response and oxidative

stress biomarkers in blood at day 3 after PCA reduction indicate a higher PHA skin reaction in ducklings with lower antioxidant levels. This can be explained by the regulatory role of antioxidants on PHA response (Hasselquist and Nilsson, 2012). Other authors have also studied the effects of Pb on oxidative balance and immune function in birds and their interactions. Rainio et al. (2015) studied the effects of experimental Pb dietary exposure on antioxidant defence and phagocytosis activity in early-life great tits (Pb acetate administered from 3 to 14 days of age) and found no major effects of Pb in any of these functions. Vallverdú-Coll et al. (2015) found an increase of PHA response and blood GSH levels in Pb-exposed adult red-legged partridges, though both parameters were inversely related when Pb treatment was considered in the analysis as a factor. These authors also found that oxGSH levels were positively related to blood Pb levels as well as to PHA response. The negative association between GSH levels and the PHA response observed here may highlight the importance of GSH levels to understand the effects of Pb on the immune function. In this regard, Townsend et al. (2003) reviewed several studies reporting the importance of GSH in human disease, and they suggested that levels of this endogenous antioxidant can determine whether T_{h1} or T_{h2} response predominates. Furthermore, some authors have suggested that the generation of ROS as a consequence of heavy metal exposure inactivates transcription factors responsible of T_{h1} and T_{h2}-driven immune responses (Hemdan et al., 2007). Then, the Pb-induced immunotoxicity observed here may be in part modulated by antioxidant levels (i.e. tGSH) that can be induced or depleted by this heavy metal.

In summary, our results show that the sublethal exposure to ingested Pb shot in birds can result in a significant maternal transfer through the eggs to the offspring that can affect their developing immune system and reduce their survival in early life stages. Further research should be focused on the implications of these sublethal effects on the populations of waterfowl or other bird species under the risk of Pb exposure, because adults surviving to acute or chronic poisoning could transfer Pb to their eggs, resulting in adverse effects on the progeny.

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SUPPLEMENTAL MATERIAL

1. Tissue Pb concentration

Limit of detection (LOD) (back-calculated in tissue concentrations) was 0.05 µg/g of d.w. for duckling tissues and 0.03 µg/g of d.w. in the content of the eggs. Blanks were processed in each batch (n=3) of digestions. Pb standards were used to perform calibrations and to determine Pb concentrations in samples by linear regression. Certified samples of bone ash (SRM 1400, National Institute of Standards and Technology) (n=1), lobster hepatopancreas (TORT-2, National Research Council Canada) (n=1) and bovine liver (BSR 185R, Community Bureau of Reference) (n=2) were also analysed in each batch of tissue digestions and the obtained percentages of recovery were 82.8%, 88.2% and 97.5 ± 2.1%, respectively. For analysis of eggshells, 0.6 g of lyophilized samples were digested during 12 h at room temperature with 1 mL of HNO_3 (69% Analytical grade, Panreac, Spain) and 2 mL of H_2O (Milli-Q grade) in Pyrex tubes in a heating block with an electronic controller. The next day, 1 mL of H_2O_2 was added, the tubes where heated 45 min at 90 °C, and then 2 h at 120 °C. Digests were diluted to 50 mL with H_2O (Milli-Q grade). LOD was 0.04 µg/g of d.w. Blanks were processed in each batch (n=3) of digestions. Pb standards were used to determine Pb concentrations in samples by linear regression. Bone ash (SRM 1400, National Institute of Standards and Technology) samples were analysed in each batch of digestions to assure the quality of the methodology. The percentages of recovery (mean ± SD) were

78.3 ± 1.3% in 2008 (n=3) and 81.4 ± 10.4% in 2009 (n=5). Sample concentrations were not corrected with the recovery value.

2. Measurement of pigment concentrations in eggshells

Aliquots of eggshell from each individual were lyophilized and we froze them at -20 ºC until analysis. For the extraction, we mixed 0.1 g of lyophilized eggshell with 500 µL of acetonitrile (ACN) and 250 µL of hydrochloric acid (HCl) 3N in 1.5 mL plastic tubes, and we let them resting uncovered for 15 min. Then the tubes were closed and vortexed for 15 min, and sonicated for 10 min. Samples were centrifuged for 10 min at 12,000 *g* and 400 µL of supernatant were transferred to glass vials for high performance liquid chromatography (HPLC) analysis. We repeated a second and a third extraction by using 400 µL of ACN instead of 500 µL. We quantified samples obtaining calibration curves constructed with standard solutions using non-pigmented eggshell as matrix. We prepared working solutions dissolving free acid porphyrins and protoporphyrin IX in 3N HCl, coproporphyrin III in methanol, and biliverdin in ACN. Concentrations in the five calibration points were 0.38, 0.75, 1.50, 3.00 and 6.00 pmol of biliverdin and 0.62, 1.24, 2.49, 4.98 and 9.95 pmol for protoporphyrin IX, that were added to 0.1 g of non-pigmented eggshell. Coproporphyrin III was diluted in methanol (1:1) as an internal standard and 50 µL of this dilution were added to each standard calibration and processed sample. Standard calibrators were processed in the same way as the samples. The recovery of the extraction procedure, calculated by comparing standard solutions (n=5) with samples spiked with biliverdin and protoporphyrin IX, was 118 ± 15.91% (mean ± SD) and 85.21 ± 17.07%, respectively. The analytical system used to quantify porphyrins was formed by a liquid chromatograph Agilent 1100 series equipped with a photodiode detector and an Agilent 6110 Quadrupole LC/MS with a multimode (MM-ES) source. High purity nitrogen generator (N_2-Whisper 0-40) supplied the nitrogen for mass detector. The injection volume was 40 µL. The flow rate was 0.7 mL/minute and a solvent gradient was used. Initially, the mobile phase composition was ammonium acetate (0.1M, pH 5.16) 75% and methanol 25%, reaching to methanol 100% at 24 min. It was maintained until 31 min, returning to the initial conditions until 36 min. The total time of each run was 36 min. We used a Waters (Milford MA)

Spherisorb c18 ODS 2 (5 µL particle size, 100 mm x 4.5 mm) chromatographic column maintained at 68.0 ºC. Detection was done at 400 nm wavelength. Porphyrins were detected using positive ion monitoring with the following MM-ES source settings: Nebulizer pressure was set at 50 psi, drying gas flow was 5 L/min, drying gas temperature was 350 ºC, vaporizer temperature was 200 ºC, capillary voltage was 2,000 V, and charging voltage was 1,000 V. The ions used for identification and quantification were 583.2 for biliverdin, 655.2 for coproporphyrin III and 563.2 for protoporphyrin IX. The fragmentation voltages were 240 V, 210 V and 300 V, respectively. In addition, we monitored samples in parallel with UV-vis spectrometry detection as additional peak identification evidence. We used ChemStation software (B.03.02) to control chromatographic conditions.

Table S1. Mean (± S.E.) lead (Pb) concentration in eggshell and egg content, and eggshell thickness in unhatched eggs collected in the Ebro delta in 2008. Mean (± S.E.) eggshell thickness and concentration of Pb, biliverdin and protoporphyrin IX in hatched eggs from the Ebro delta (2009). Mean (± S.E.) Pb concentration on tissues of dead ducklings from hatched eggs and increased foot membrane thickness after PHA injection.

Parameter	Year	N	Mean ± S.E.
Eggshell Pb concentration ng/g	2008	23	150 ± 24.2
Egg content Pb concentration ng/g		23	74.3 ± 25.2
Eggshell thickness (mm)		23	0.264 ± 4.51
Eggshell Pb concentration ng/g	2009	42	248 ± 15.6
Eggshell thickness (mm)		33	0.244 ± 0.0032
Eggshell biliverdin pmol/g		33	5.87 ± 0.462
Eggshell protoporfirin IX pmol/g		33	8.99 ± 0.516
Bone Pb concentration µg/g		12	0.778 ± 0.461
Liver Pb concentration µg/g		12	0.0653 ± 0.0219
Brain Pb concentration µg/g		12	0.094 ± 0.0279
Δ Foot membrane thickness (mm)		19	0.264 ± 0.0249

Table S2. Mean (± S.E.) blood lead (Pb) and δ-ALAD ratio, oxidative stress biomarkers and biochemical parameters of ducklings from hatched eggs collected in the Ebro delta at days 3 and 28 of age.

Parameter	Sampling day			
	Day 3		Day 28	
	N	Mean ± S.E.	N	Mean ± S.E.
Blood Pb concentration (ng/mL)	32	35.7 ± 13.4	20	21.7 ± 13.6
δ-ALAD ratio[a]	30	1.29 ± 0.08	19	1.20 ± 0.05
TBARS (µmol/g)[b]	20	0.041 ± 0.003	18	0.037 ± 0.003
GPx (UI/mg)[c]	20	0.407 ± 0.021	18	0.409 ± 0.027
SOD (UI/mg)[d]	20	1.85 ± 0.109	18	1.93 ± 0.139
tGSH (µmol/g pellet)[e]	20	4.73 ± 0.129	18	4.73 ± 0.203
oxGSH (µmol/g pellet)[f]	17	0.569 ± 0.073	17	0.634 ± 0.060
% oxGSH[g]	17	11.8 ± 1.45	17	14.3 ± 1.85
Retinol (nmol/mL)	20	7.89 ± 0.715	18	8.23 ± 0.831
Lutein (nmol/mL)	20	7.79 ± 1.005	18	6.31 ± 0.972
Zeaxanthin (nmol/mL)	20	5.404 ± 0.827	18	4.12 ± 0.728
Tocopherol (nmol/mL)	20	22.8 ± 1.86	18	20.9 ± 2.13
Albumin (g/L)	15	9.60 ± 1.65	7	3.86 ± 1.42
Alkaline phosphatase (U/L)	15	673 ± 65	12	630 ± 70
Alanine aminotransferase (U/L)	18	81.8 ± 7.8	13	71 ± 8.0
Aspartate aminotransferase (U/L)	18	77.3 ± 12.9	12	68.4 ± 16.0
Creatinine phosphokinase (U/L)	3	1550 ± 170	6	1559 ± 16.0
Creatinine (mg/dL)	13	0.167± 0.046	11	0.091 ± 0.039
ɤ-Glutamyl transferase (U/L)	17	9.77 ± 2.053	12	10.5 ± 5.78
Lactate dehydrogenase (U/L)	15	1069 ± 102	10	408 ± 141
Urea (mg/dL)	15	6.25 ± 1.23	9	5.13 ± 1.56
Calcium (mg/dL)	9	14.5 ± 0.5	2	12.3 ± 2.1
Cholesterol (mg/dL)	12	236 ± 14	2	100 ± 96
Glucose (mg/dL)	11	174 ± 26	2	87 ± 97

Magnesium (mg/dL)	12	2.10 ± 0.22	2	1.72 ± 0.86
Phosphorous (mg/dL)	11	9.42 ± 1.15	2	7.27 ± 7.27
Total protein (g/L)	10	35.1 ± 5.0	2	0.7 ± 0.7
Triglycerides (mg/dL)	10	209 ± 39	2	13 ± 3
Uric acid (mg/dL)	9	8.27 ± 2.12	2	ND

[a]δ-ALAD: δ-aminolevulinic acid dehydratase.

[b]TBARS: Thiobarbituric acid reactive substances.

[c]GPx: Glutathione peroxidase.

[d]SOD: Superoxide dismutase.

[e]tGSH: Total glutathione.

[f]oxGSH: Oxidized glutathione.

[g]% oxGSH: Percentage of oxidized glutathione.

ND: Not detected.

Table S3. Rotated component matrix of oxidative stress parameters from ducklings from the Ebro delta at days 3 and 28 of age. Rotation method used was Varimax with Kaiser Normalization. Five and four iterations converged in rotations at days 3 and 28 of age, respectively.

	Day 3			Day 28		
	Principal Component			Principal Component		
Parameter	1	2	3	1	2	3
TBARS	0.242	0.630	0.284	-0.043	-0.086	0.835
GPx	-0.702	-0.273	0.288	0.223	0.823	-0.228
SOD	-0.016	-0.011	-0.891	0.137	0.833	0.061
tGSH	-0.123	0.877	-0.078	0.162	0.131	0.710
oxGSH	0.254	0.454	0.524	-0.162	0.571	0.317
Retinol	0.889	0.205	0.197	0.851	0.337	0.268
Tocopherol	0.370	0.585	0.042	0.262	0.574	0.546
Lutein	0.945	0.050	0.201	0.973	0.066	0.049
Zeaxanthin	0.964	0.063	0.104	0.982	-0.005	-0.027

Table S4. Rotated component matrix of plasma biochemistry parameters from ducklings from the Ebro delta at days 3 and 28 of age. Rotation method used was Varimax with Kaiser Normalization. 9 and 6 rotation converged in iterations at days 3 and 28 of age, respectively. Only parameters tested in more than 5 individuals were included in the analysis.

	Day 3			Day 28		
	Principal Component			Principal Component		
Parameter	1	2	3	1	2	3
Albumin	-0.174	0.859	0.390	0.937	0.081	0.308
Alkaline phosphatase	-0.159	0.557	0.252	-0.040	0.920	0.099
Alanine aminotransferase	-0.476	0.618	0.038	0.402	-0.789	-0.388
Aspartate aminotransferase	-0.115	0.114	-0.030	-0.175	-0.083	-0.953
Creatinine phosphokinase		NI		0.083	0.948	-0.308
Creatinine	-0.174	0.508	0.062	0.868	-0.065	0.493
γ-Glutamyl transferase	-0.027	0.275	0.701	0.961	-0.164	-0.003
Lactate dehydrogenase	0.330	-0.177	-0.018	0.643	-0.431	0.552
Urea	0.168	0.953	-0.216	0.576	-0.008	0.792
Calcium	0.582	-0.153	0.614		NI	
Cholesterol	-0.199	0.799	-0.316		NI	
Glucose	0.902	-0.127	0.357		NI	
Magnesium	0.933	-0.024	-0.123		NI	
Phosphorous	0.957	0.009	-0.118		NI	
Total protein	0.858	-0.131	0.443		NI	
Triglycerides	0.873	-0.131	0.297		NI	
Uric acid	0.250	-0.090	0.935		NI	

NI: Parameter not included in the analysis because N≤5

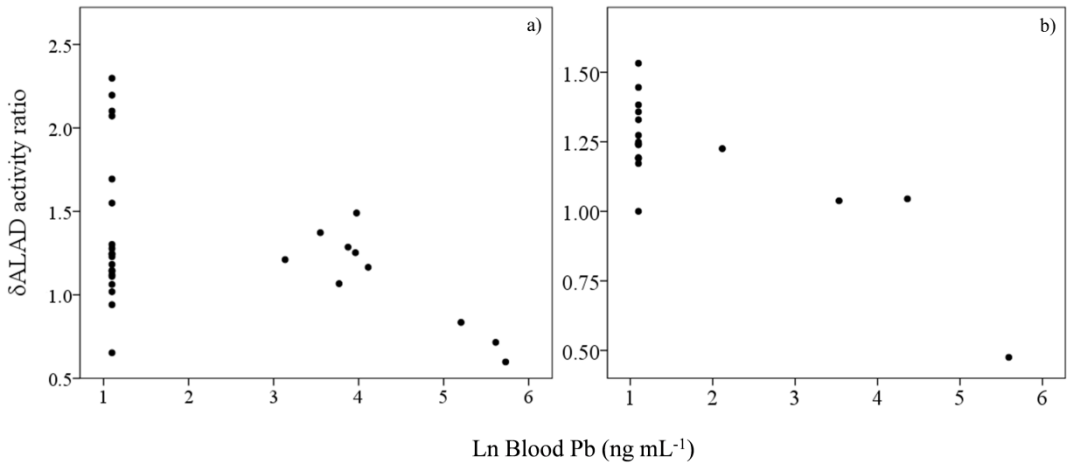

***Figure S1**. Correlation between δ-ALAD (δ-aminolevulinic acid dehydratase) activity ratio and blood lead (Pb) concentration of ducklings, hatched from eggs collected in the Ebro delta, at day 3 (n=30, $F_{1,28}$=6.35, p=0.018) (a) and 28 (b) (n=19, r_S=-0.566, p=0.012) of age.*

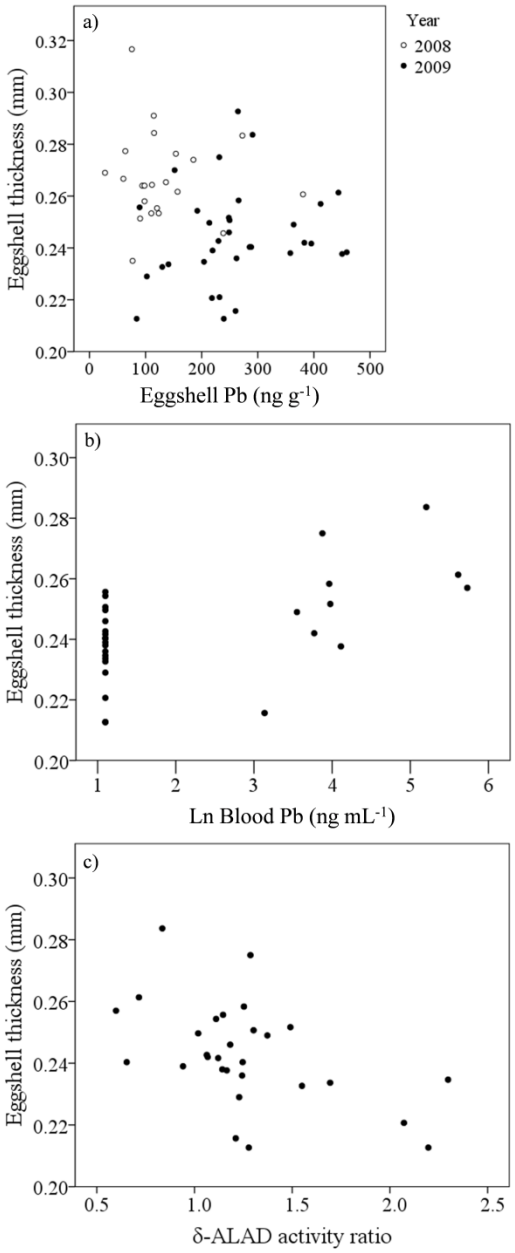

Figure S2. *Association between eggshell thickness and eggshell Pb concentration in 2008 and 2009 (a). Correlation between eggshell thickness and (b) blood Pb concentration (n=29, r_S=0.539, p=0.003), and (c) δ-ALAD (δ-aminolevulinic acid dehydratase) activity ratio of ducklings (n=29, r_S=-0.457, p=0.013) at day 3 of age, hatched from eggs collected in the Ebro delta.*

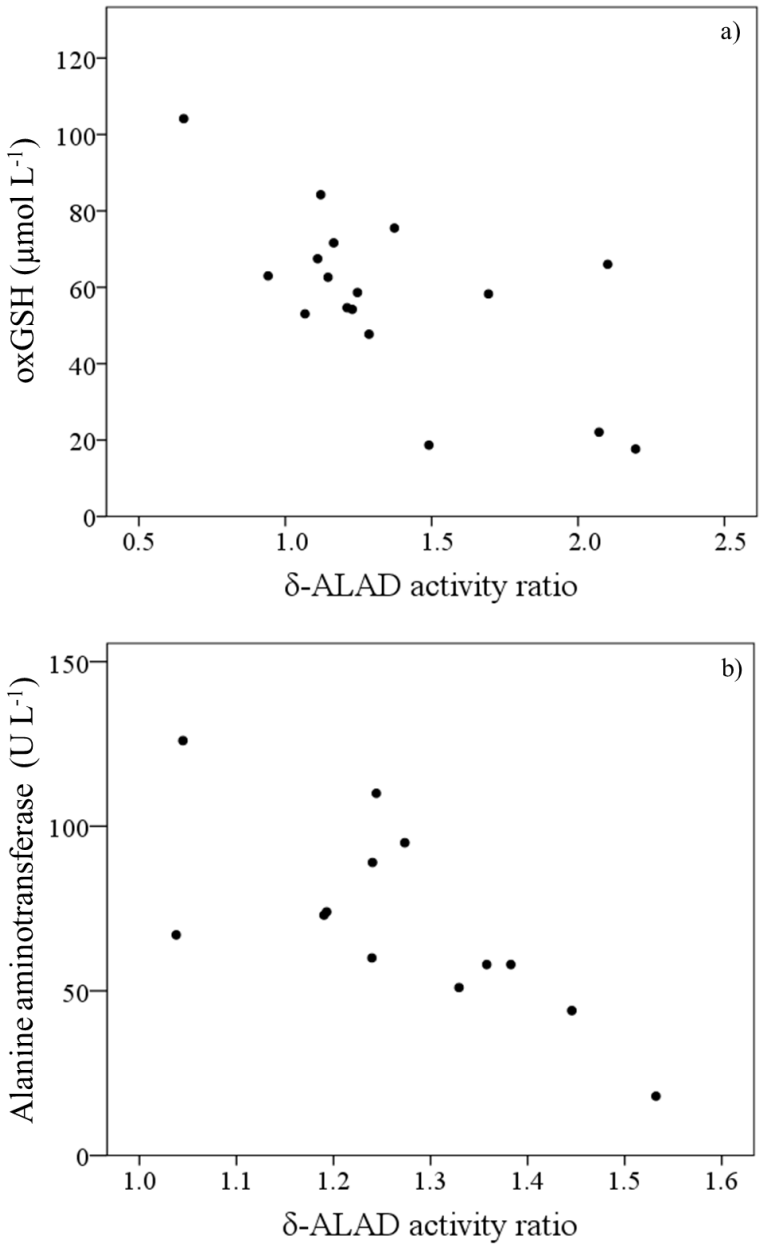

***Figure S3**. Correlations found in ducklings, hatched from eggs collected in the Ebro delta, between δ-ALAD (δ-aminolevulinic acid dehydratase) activity ratio at day 3 and oxGSH (oxidized glutathione) levels at day 28 (n=17, r_S=-0.532, p=0.028) (a), and between δ-ALAD at day 28 and levels of alanine aminotransferase (n=13, r_S=-0.680, p=0.011) at day 28 (b).*

CHAPTER 4

SUBLETHAL Pb EXPOSURE PRODUCES SEASON-DEPENDENT EFFECTS ON IMMUNE RESPONSE, OXIDATIVE BALANCE AND INVESTMENT IN CAROTENOID-BASED COLORATION IN RED-LEGGED PARTRIDGES

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Rafael Mateo. Environmental Science and Technology 49 (2015) 3839-50

ABSTRACT

Ingestion of lead (Pb) shot pellets constitutes the main cause of Pb poisoning in avifauna. We studied the effects of sublethal Pb exposure on immunity, carotenoid-based coloration, oxidative stress and trade-offs among these types of responses during spring and autumn in red-legged partridges (*Alectoris rufa*). We evaluated constitutive immunity testing lysozyme and natural antibody levels, and blood bactericidal and phagocytic activities. We studied induced immunity by testing PHA and humoral responses. We analyzed fecal parasite and bacterial abundance and oxidative stress biomarkers. Pb exposure in spring reduced natural antibody levels, whereas in autumn, it reduced lysozyme levels and increased phagocytic activity. Pb exposure increased PHA response in both seasons, and decreased T-independent humoral response in autumn. Pb exposure also increased noncoliform and decreased coliform Gram-negative gut bacteria. In spring, Pb exposure decreased antioxidant levels and increased coloration in males, whereas in autumn, it increased retinol levels but reduced coloration in both genders. Our results suggest that in spring, Pb-exposed females used antioxidants to cope with oxidative stress at the expense of coloration, whereas Pb-exposed males increased coloration, which may reflect an increased breeding investment. In autumn, both genders prioritized oxidative balance maintenance at the expense of coloration.

1. INTRODUCTION

Lead (Pb) poisoning by the ingestion of ammunition used for hunting is frequent in waterbirds and raptors (Espín et al., 2014; Mateo et al., 2014) and a lethal threat for many endangered species (e.g., white-headed duck *Oxyura leucocephala*; California Condor *Gymnogyps californianus*), compromising conservation efforts (Stroud and Hunt, 2009; Taggart et al., 2009). Apart from direct mortality, a wide range of sublethal effects has also been associated with Pb exposure in birds (Mateo et al., 1998).

One of the most relevant targets of Pb exposure in birds is the immune system, with potential immunosuppressive effects on constitutive or induced components that can reduce resistance to pathogens (Dietert and Piepenbrink, 2006; Fairbrother et al., 2004). Pb exposure may affect constitutive immunity by reducing macrophage function (Knowles and Donaldson, 1997) or circulating white blood cell levels (both heterophils and lymphocytes)(Rocke and Samuel, 1991). In birds, most studies found little or no effect of Pb on constitutive immune response (Fair and Myers, 2002; Hussain et al., 2005; Lee and Dietert, 2003; Lee, 2006; Vodela et al., 1997), but have highlighted an immunosuppressive potential on induced components of the immune humoral (Trust et al., 1990) and cellular (Fair and Myers, 2002; Grasman and Scanlon, 1995) responses. However, both immunosuppression and immunostimulation are mechanisms of Pb-induced immunotoxicty (Gleichmann et al., 1989) and dose-dependent hermetic effects have also been reported (Nain and Smits, 2011). Because of the complexity of the immune system, interdependencies in responses and inconsistencies in the direction of pollutant effects on each response, resistance to infection has been proposed as a useful endpoint to evaluate overall immunotoxic effects (Friend and Trainer, 1970). Some authors (Dietert and Piepenbrink, 2006) have suggested that some Pb effects on immune components, such as the increase of tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2) and reactive oxygen species (ROS) produced by macrophages, and the decrease in T-helper (Th) type 1 lymphocyte capacity would contribute to increase sensitivity to infectious diseases. A better understanding of immunotoxic effects produced by chemicals at doses below those associated with overt toxicity therefore requires the use of various assays, integrating

cell-mediated, humoral and constitutive immunity tests, together with disease resistance evaluation (Karol, 1998).

Immune system activation in response to infections generates ROS to fight invading pathogens. When ROS production overwhelms the capacity of antioxidant defenses, it results in oxidative stress that damages lipids, DNA and proteins (Dowling and Simmons, 2009). Pb exposure has been shown to cause oxidative stress in captive (Mateo et al., 2003; Hoffman et al., 2000; Mateo and Hoffman, 2001) and wild birds (Martinez-Haro et al., 2011). Plasmatic levels of dietary carotenoids differ during the breeding and nonbreeding seasons (Pérez-Rodríguez, 2008a) because of their strong regulation by sexual hormones (Pérez-Rodríguez, 2009). Oxidative stress also affects the expression of carotenoid-based ornaments displayed by birds, which play key roles in social and sexual signalling (Pérez-Rodríguez et al., 2013). Carotenoids cannot be synthesized by vertebrates and must be acquired from diet (Goodwin, 1984). Carotenoids have antioxidant and immune-stimulant properties, so birds have to trade-off their use for ornamental coloration, immune function or oxidative balance maintenance (Alonso-Alvarez et al., 2008; Pérez-Rodríguez, 2009; Peters et al., 2004). Immunity, oxidative stress and carotenoid-based ornaments are therefore tightly interconnected, and are all potential targets of Pb toxicity. We hypothesized that sublethal Pb exposure will affect how birds solve the trade-offs among these three types of functions.

Here we report on the effects of experimental Pb shot ingestion at sublethal doses in a bird displaying carotenoid-based ornaments, the red-legged partridge *(Alectoris rufa*). This medium size game bird is of high socio-economic value and at risk of Pb shot ingestion (e.g., 7.8% prevalence in wild partridges in Central Spain), given the high densities of shot pellets usually found in its habitats (73 600 pellets/ha) (Ferrandis et al., 2008). Because allocation priorities in resource investment (immunity, carotenoids) may vary seasonally (Møller et al., 2003), we replicated the experiment during the breeding and nonbreeding seasons. We evaluated Pb effects on constitutive and induced immunity, parasite infestation (as an endpoint of Pb-induced immunotoxicity) and carotenoid-based coloration. We also studied the role of

oxidative stress as a mechanism involved in Pb toxicity, immune response and carotenoid allocation trade-offs.

2. MATERIAL AND METHODS

2.1 Experimental design and sample collection

We conducted the experiments in the Dehesa de Galiana experimental facilities (Ciudad Real, Spain), with the approval of the Universidad de Castilla-La Mancha's Committee on Ethics and Animal Experimentation. For each experiment (spring and autumn), we used three randomly assigned treatment groups: low dose (oral ingestion of 1 Pb shot via gastric gavage), high dose (ingestion of 3 Pb shot via gastric gavage) or control (gastric gavage, without shot ingestion). These numbers of ingested Pb shot pellets (1−3) are frequently found in gizzards of upland gamebirds and waterbirds in Spain (Ferrandis et al., 2008; Mateo et al., 1997). The total masses of administrated Pb were of 0, 109 and 327 mg in the control, low dose and high dose group, respectively. For the spring experiment (breeding season), we used 60 captive-born adult partridges housed in outdoor cages in pairs (10 pairs per treatment group). Prior to experiment, we weighted each partridge and measured tarsus length, to calculate body condition (scaled mass index (Peig and Green, 2009)). Birds were fed ad libitum with a mixture of wheat and commercial partridge feed containing corn, wheat, barley, soy, sunflower and composed by protein (15.5%), fat (2.1%), fiber (6.5%), ashes (6.0%), calcium (0.75%), phosphorus (0.68%), sodium (0.15%), methionine (0.45%) and lysine (0.80%) (Partridge maintenance fodder, Nanta-Nutreco, Tres Cantos, Spain). Tap water was also provided ad libitum. After 1 week of acclimation, birds in the low and high dose groups were exposed to Pb (exposure day: January 24, 2012). We subsequently measured body mass and collected blood and feces samples weekly, until 31 days postexposure. We measured the following endpoints at different sampling times (for a chronogram of the experiment, see Table S1 of the Supplemental material): blood and feces Pb concentrations (weekly), constitutive immunity (i.e., day 7, natural-antibody levels; days 7 and 21, whole blood bactericidal activity and plasma lysozyme levels), induced immunity (T-dependent antibody response at day 21; short and long-term

PHA response measured at days 14 and 28, respectively), oxidative stress biomarkers, activity of antioxidant enzymes, dietary and endogenous antioxidant levels, δ-aminolevulinic acid dehydratase (δ-ALAD) activity, plasma biochemistry (all these on days 7 and 21) and carotenoid-based coloration (days 0 and 21). Because of sample volume limitations, not all variables could be measured for each individual at each sampling time. For the autumn experiment (nonbreeding season), we used a different set of 90 partridges (15 males and 15 females per treatment group) individually housed in outdoor cages. After 1 week of acclimation, we measured body condition (as above) and treated birds (exposure day: October 23, 2012). We collected blood 0, 7, 21, and 55 days after exposure and measured the following endpoints at different sampling times (see Table S1 for a chronogram of the experiment, Supplemental Material): blood Pb concentration (all sampling times), constitutive immunity (i.e., whole blood phagocytic activity at days 0, 7 and 21; plasma lysozyme levels at days 0 and 21), induced immunity (day 21; i.e., PHA response, T-dependent and T-independent specific humoral responses), dietary antioxidant plasma levels, carotenoid-based coloration and fecal parasite and Gram-negative bacterial abundance (all these on days 0 and 21).

2.2 Blood and feces Pb Concentrations, δ-ALAD and plasma biochemistry analyses

For blood Pb measurement, whole blood samples were diluted (1:10) with 0.1% triton and analyzed using graphite furnace atomic absorption spectroscopy (GF-AAS; AAnalyst800 with autosampler AS800, Perkin Elmer) (Mateo et al., 1999).36 We prepared calibration standards from commercial solutions containing 1 g/L of Pb (Panreac) and Mili-Q grade water. A certified reference material for Pb (blood BCR-196) was analyzed to ensure the quality of the methodology within a recovery (mean ± SD) of 98 ± 12% (n=46). The detection limit was <0.6 µg/dL of Pb in blood. Fecal Pb measurement was performed using a graphite furnace atomic absorption spectroscopy system (Martínez-Haro et al., 2010) (see the Supplemental Material for further details).

δ-ALAD activity and the ratio of nonactivated/in vitro activated enzymes (δ-ALAD activity ratio) were determined using a spectrophotometric assay (Martinez-Haro et al., 2011; Pain, 1989).

Plasma biochemistry was measured with an automatic spectrophotometer analyzer A25, using the reaction kits available for each enzyme or analyte (BioSystems, Barcelona, Spain) (see the Supplemental Material for further details).

2.3 Immune responses

2.3.1 Constitutive immune responses

Constitutive immune responses are disease-resistance mechanisms present in the organism without the necessity of prior stimulation with an antigenic or immunogenic agent. They include cellular (NK, phagocytic cells) and molecular components that recognize classes of molecules frequently encountered in pathogens. We characterized constitutive responses using functional in vitro tests measuring the phagocytic and bactericidal activity in fresh blood samples.

We quantified natural-antibodies (NAbs), their interaction with complement proteins to lyse foreign cells and lysozyme concentrations in plasma. When phagocytes are activated, they produce ROS for killing pathogens generating electronically excited states, which, on relaxation to the ground state, emit photons. This emission is referred to as phagocyte chemiluminescence (CL). We measured phagocytic activity in fresh blood performing a whole-blood chemiluminescence assay (Papp and Smits, 2007), to assess the production of ROS by circulating phagocytes (see the Supplemental Material for more details). We performed a bactericidal assay to measure bactericidal activity of fresh blood in vitro against *Escherichia coli* following a previous procedure (Millet et al., 2007) with slight modifications (see the Supplemental Material for more details). Lysozyme is an enzyme of leukocytic origin with antibacterial and antiviral activity. This protein is widely distributed in the organism, especially in lysosomes of monocytes, macrophages and granulocytes (Gill, 1995), and it is also present in lymphocytes. We measured plasmatic lysozyme levels

using lysis tests against *Micrococcus lysodeikticus* (Millet et al., 2007) (see the Supplemental Material for more details). NAb levels in plasma and their joint activity with the complement proteins were estimated using hemolysis–hemagglutination tests before injection of any antigen. This assay is useful for characterizing NAb-mediated complement activation (Matson et al., 2005). Lysis reflects the interaction of complement and NAbs, whereas agglutination results from the actions of Nabs only, which are the only immunoglobulins (Ig) that do not need previous exposure to a particular antigen.

2.3.2 Induced immune responses.

Induced immune responses are unspecific and specific mechanisms activated with an antigenic challenge. We characterized unspecific mechanisms including the response to phytohemagglutinin (PHA), indicative of cell-mediated immune responsiveness. We also characterized the specific mechanisms, including the synthesis of antibodies in primary response to the inoculation with immunogenic agents, specifically sheep red blood cells (SRBC) and *Brucella abortus* vaccine (BA) for T-dependent and T-independent responses, respectively. T-cell-mediated immune responsiveness was measured using the PHA-skin test. PHA is a lectin that produces a proliferative response of circulating T-lymphocytes that are accumulated at the injection site (Goto et al., 1978). We used a micrometer (Mitutoyo Absolut 547-401) to measure wing web thickness (nearest 0.01 mm) prior to and 24 h after subcutaneous injection of 100 µL of PHA in PBS (1 mg/mL dilution). We estimated PHA response intensity as the change in wing web thickness 24 h post injection. In the spring experiment, we measured PHA response twice: 14 days (short-term response) and 28 days (long-term response, when blood Pb levels were expected to have dropped (Jordan and Bellrose, 1951) after Pb administration. In the autumn experiment, PHA response was measured once (21 days after exposure).

We measured humoral response using different tests, depending on the type of response. B-Lymphocytes are precursors of antibody secreting cells and can be activated directly by cross-linkage between Ig and antigen, or indirectly by interaction

with T-lymphocytes (Blakley and kouassi, 2005). We tested responses against two different antigens, SRBC and BA, to assess Tlymphocyte dependent and T-lymphocyte independent antibody responses, respectively (see the Supplemental Material for more details).

2.4 Intestinal parasite and bacterial abundances

As an endpoint assessment of Pb imunotoxicity, we estimated parasite and bacterial abundance in fresh fecal samples. We collected samples from individual partridges by placing a clean filter paper under each cage 24 h before collection. We carefully picked up 3 pellets of fresh feces in a sterile 1.5 mL tube and one cecum sample placed in a zip-lock plastic bag. Samples were kept refrigerated and analyzed within 24 h from collection.

We estimated the abundance of coccidian oocsysts (*Eimeria sp.*) and nematode parasite eggs (*Heterakis gallinarum*, *Capillaria sp*.) in cecum using a McMaster counting chamber and saline flotation medium (Mougeot and Redpath, 2004). For intestinal microbiota, fresh feces were analyzed by microbiological culture, comparing number and presence of Gram-negative bacilli (GNB) upon day 0 and 21. Counting of GNB was done by serial dilution method, by duplicate, plating 100 µL of each dilution in McConkey agar and incubating for 18−24 h at 37 °C. Then, colony-forming units (CFU) were calculated by the average of those plates that contained between 30 and 300 CFU. GNB were classified as coliform or noncoliform according to biochemical properties. All coliform bacilli were further confirmed biochemically by the API 20E system (BioMérieux, Marcy L'Étoile, France).

2.5 Oxidative stress biomarkers

Oxidative stress biomarkers were assayed using red blood cell homogenates (RBC) or plasma samples. Using RBC, we measured total (tGSH) and oxidized (oxGSH) glutathione levels (an endogenous antioxidant) by spectrophotometric assays (Reglero et al., 2009b), and malondialdehyde (MDA, an indicator of lipid peroxidation) by high

performance liquid chromatography (HPLC) coupled to a fluorescence detector (Romero-Haro and Alonso-Alvarez, 2014). GSH peroxidase (GPx, EC, 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1) activities were measured using spectrophotometric assays with Ransel and Ransod kits (Randox Laboratories, Cornellà de Llobregat, Spain), respectively (Reglero et al., 2009b). We expressed antioxidant enzyme activities, relative to mg of protein (Bradford, 1976). Using plasma, we measured free retinol, α-tocopherol and lutein levels (dietary antioxidants; see Table S2 in the Supplemental Material) by HPLC coupled to a photodiode detector and a fluorescence detector (Rodríguez-Estival et al., 2010).

2.6 Carotenoid-based coloration

Partridges display red beak and eye-rings, which are pigmented by carotenoids, function as indicators of health status and individual quality and play key roles in sexual selection and reproduction (Alonso-Alvarez et al., 2012; Pérez-Rodríguez et al., 2013). We used high resolution digital photography to measure the redness of the beak and eye-rings (Mougeot et al., 2009) (see the Supplemental Material for more details). Images were taken under standardized conditions, using the same gray reference (Kodak Gray Scale, Kodak, New York) to adjust color measurements. Using Adobe Photoshop v7.0, we measured the relative amount of the eye-ring area pigmented by carotenoids and the red, green and blue components of the beak, eye-ring and gray reference, and calculated standardized beak and eye-ring redness values for individual partridges (Mougeot et al., 2009).

2.7 Statistical analysis

Continuous variables were checked for normality and log-transformed to fit a normal distribution when necessary. We used general lineal models (GLM) with the experimental dose, gender and their interaction as fixed factors to test for Pb effects on oxidative stress biomarkers, immune responses and carotenoid-based coloration. When significant differences were found, we checked for the dose causing effects on

marginal means through least significant difference (LSD) tests. In autumn, when most study variables were also measured before Pb exposure, initial values were used as covariates in GLM analyses, and marginal means were calculated for each variable. Linear correlation coefficients for blood Pb concentration and δ-ALAD activity and ratio, oxidative stress biomarkers, components of immune response and carotenoid based coloration were calculated to explore the relationships among them and study the inter-relationships of Pb effects on immune function, oxidative stress and coloration. We also used GLMs to explore these relationships, where immunological variables significantly affected by Pb were included as dependent variables, and oxidative stress and coloration measurements as covariates. Binary logistic regression was used to study Pb effects on parasite prevalence, using the immune responses as covariates to understand Pb mechanisms.

Statistical significance was set at $p \leq 0.05$. A marginally significant effect was defined as a p value within the range $0.05<p<0.10$. All statistical analyses were performed using the IBM SPSS Statistics 19.0 software.

3. RESULTS

3.1 Blood and feces Pb, δ-ALAD and plasma biochemistry

Differences in blood Pb concentration were observed between treatment groups since the first exposure week in both experiments, and these Pb levels remained elevated after 28 days (all $p<0.001$; Table 1). Blood Pb levels were significantly higher in females than in males in spring ($p=0.014$), and a similar trend was observed in autumn, but only at day 55 ($p=0.053$) (Figure S1, Supplemental Material). The δ-ALAD activity ratio, measured during the spring experiment, was negatively correlated with blood Pb levels at day 7 ($r=-0.682$, $p<0.001$) and at day 21 ($r=-0.674$, $p<0.001$; Figure S2, Supplemental Material). Differences in feces Pb concentrations were observed between treatment groups since the first exposure week in spring, and these differences remained after 31 days (all $p<0.025$; Table S3, Supplemental Material). Body condition was significantly lower in partridges exposed with the high Pb dose in

spring at day 7 (p=0.020) but not thereafter (Table 1), and was unaffected by Pb in autumn. During spring, Pb exposure also reduced triglyceride and total protein levels at day 7, and reduced phosphorus and ALP levels at day 21 (see the Supplemental Material, Table S4).

3.2 Constitutive immunity.

The levels of NAbs in plasma decreased in exposed partridges after 7 days of exposure in spring (p=0.046; Figure 1a, Table 2). By contrast, Pb exposure had no effect on the interaction of NAbs with proteins of the complement (Table 2). In spring, Pb treatment had no effect on bactericidal activity in fresh blood or on plasmatic lysozyme levels (Table 2). In autumn, lysozyme levels differed among treatment groups (p<0.001), with lower lysozyme levels in exposed individuals than in controls (Table 3, Figure1b). In autumn, phagocytic activity of the whole blood differed between sexes (p=0.022; higher activity in females) and increased after Pb exposure (p=0.013; Figure 1c, Table 3).

3.3 Induced immunity and pathogen resistance

Pb exposure increased the short-term response to PHA (measured at day 14) in the low dose group in spring (p=0.018; Figure 2, Table 2). The long-term PHA response (measured at day 28) tended to be higher in Pb exposed birds than in controls (marginally significant effect; p=0.089; Figure 2). In autumn, Pb exposure also increased PHA response measures at day 21 (p=0.044, Table 3), although this effect was significant only in males (p=0.013; Figure 2). Pb exposure had no effect on T-dependent antibody response, quantified as the agglutination of SRBC (Tables 2 and 3). However, Pb exposure tended to impair the T-independent humoral response against BA in autumn (p = 0.067) (Table 3, Figure 3). After Pb exposure, noncoliform GNB abundance in feces was higher in the low (UFC × 103/g, mean ± SE, 32.3 ± 14.2) and high (23.6 ± 13.2) dose groups than in controls (1.7 ± 1.7; p = 0.007; estimated

marginal means of log-transformed variables and LSD test, with values before Pb exposure included as covariates) (Table 4).

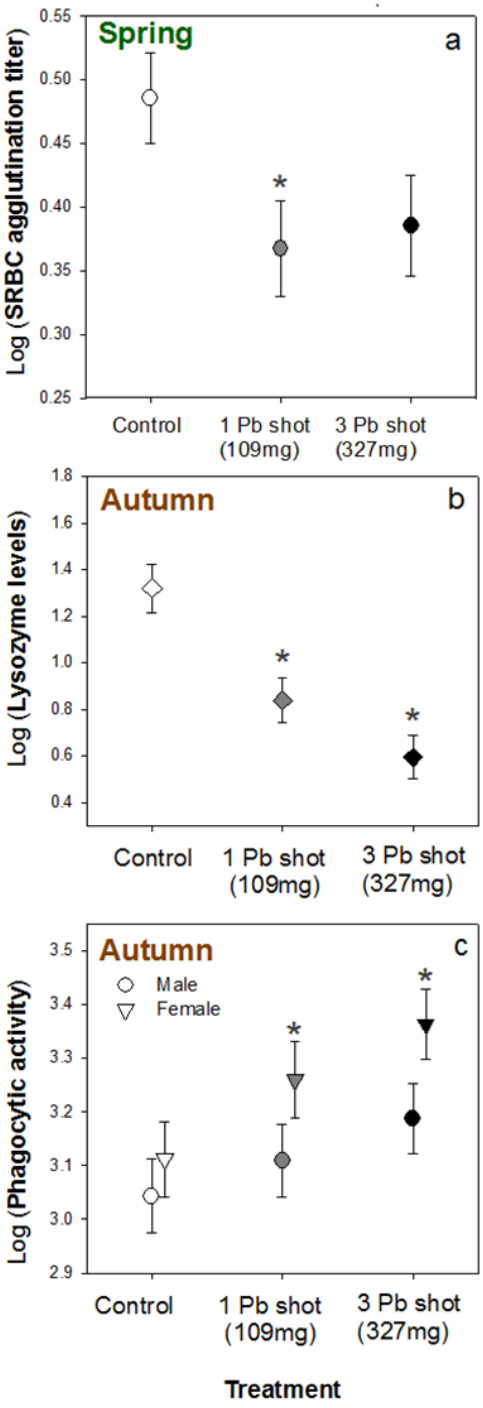

Figure 1. *Mean (±SE) agglutination of sheep red blood cells generated by natural antibodies (NAbs) as a function of Pb treatment after 7 days of exposure in spring (a), mean (±SE) plasmatic lysozyme levels as a function of Pb treatment (b), and mean (±SE) phagocytic activity as a function of Pb treatment and gender (c) in autumn after 21*

days of exposure. Control=0, no Pb shot; low dose=1 Pb shot (109 mg); high dose=3 Pb shot (327 mg). Asterisk indicates significant differences with the control group (LSD, p<0.05).

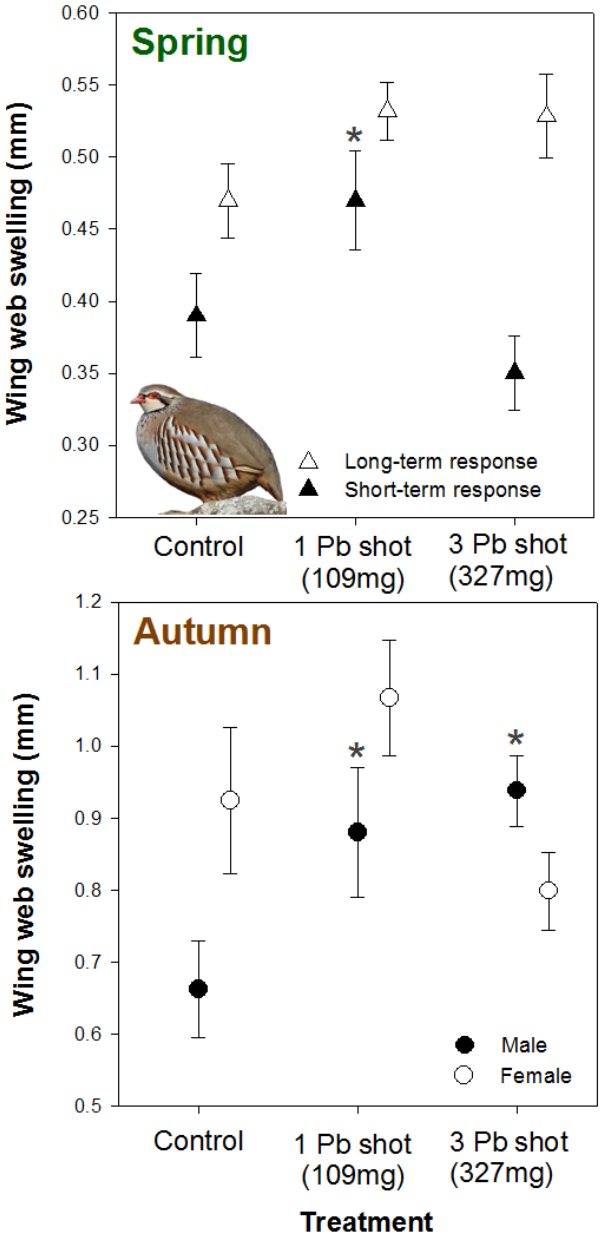

Figure 2. *Cellular immune response (mean wing web thickness increase ± SE) to phytohemagglutinin after 14 (short-term response) and 28 (long-term response) days of Pb exposure as a function of Pb treatment in spring (top) and after 21 days of exposure as a function of Pb treatment and gender in autumn (bottom). Control=0, no*

Pb shot; low dose=1 Pb shot (109 mg); high dose=3 Pb shot (327 mg). Asterisk indicates significant differences with the control group (LSD, p<0.05).

Interestingly, lysozyme levels negatively correlated with noncoliform GNB abundance (p=0.001), and the effect of Pb exposure on noncoliform GNB was no longer significant when we added this covariate in the model. By contrast, Pb treatment tended to reduce coliform GNB abundance in the low dose group as compared with controls (p=0.065). In this case, coliform GNB abundance correlated positively with lysozyme levels (p=0.038) and negatively with phagocytic activity (p=0.047), and including these two covariates improved the significance of Pb effects on coliform GNB (p=0.054). Although Pb exposure had no significant effect on Coccidia or nematode parasite abundances (Table 4), the prevalence of *Heterakis sp*. was marginally higher in the high-dose (20%) than in the low-dose (3%) and control (2%) groups (p=0.097). *Heterakis* prevalence was also negatively related to lysozyme levels (p = 0.054).

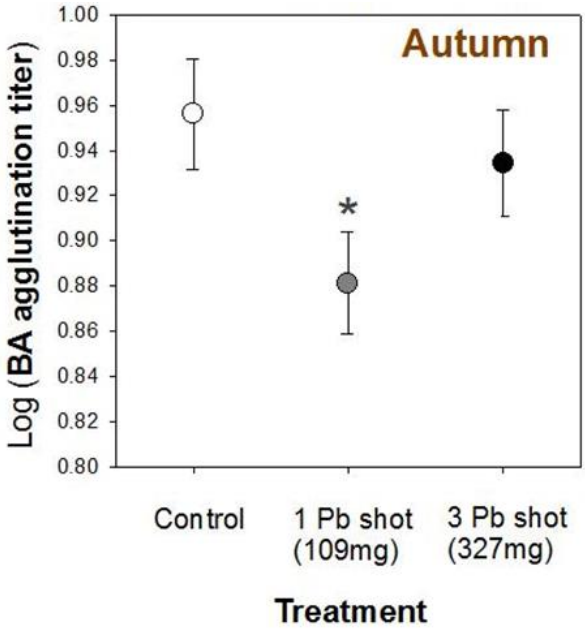

Figure 3. *Mean (±SE) antibody response of specifically synthesized antibodies against Brucella abortus vaccine after 21 days of exposure in autumn as a function of Pb treatment. Control=0, no Pb shot; low dose=1 Pb shot (109 mg); high dose=3 Pb shot (327 mg). Asterisk indicates significant differences with the control group (LSD, p<0.05).*

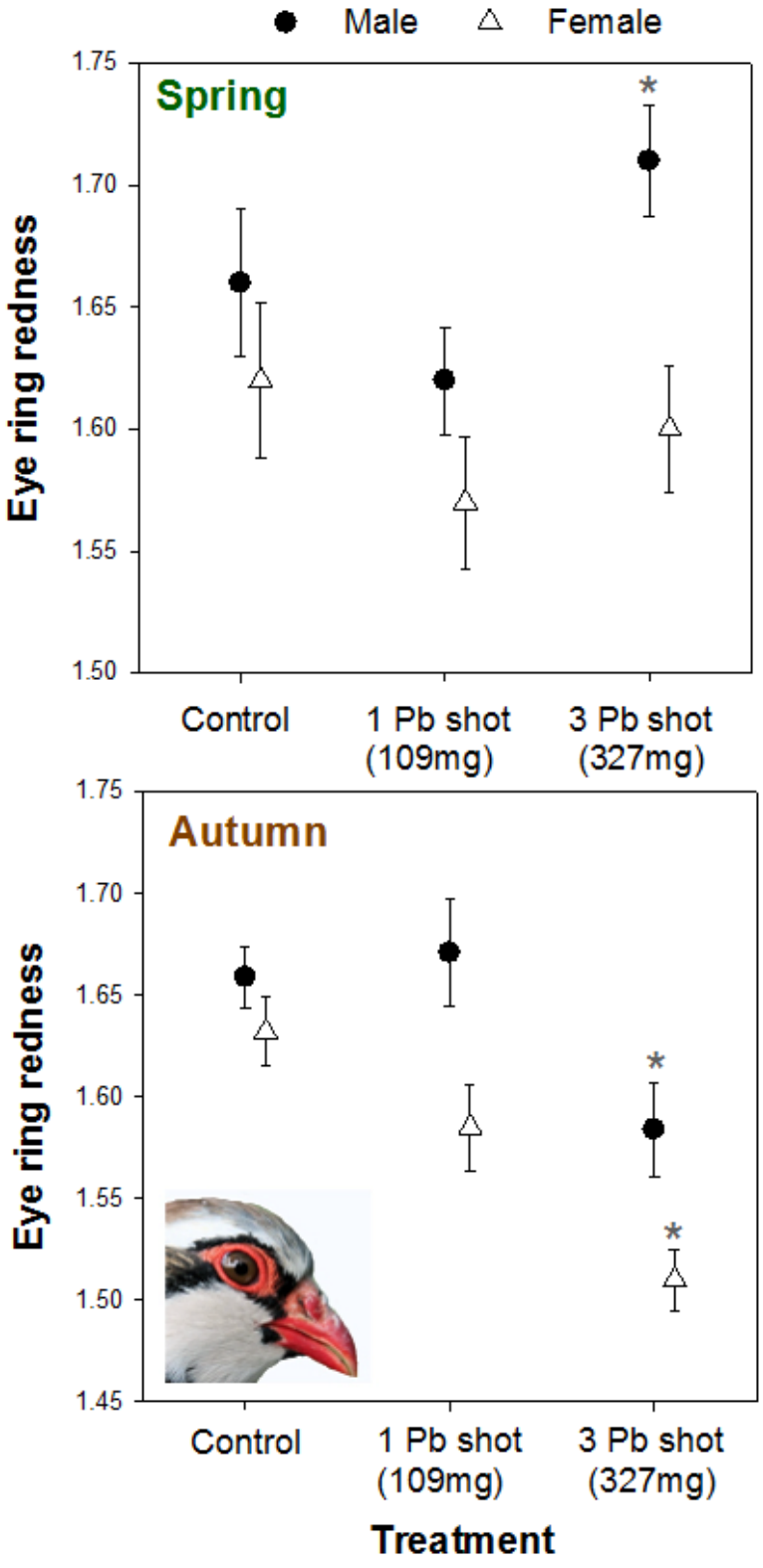

Figure 4. *Mean (±SE) eye-ring redness of partridges according to gender and season (top, spring experiment; bottom, autumn experiment) after 21 days of exposure. Control=0, no Pb shot; low dose=1 Pb shot (109 mg); high dose=3 Pb shot (327 mg). Asterisk indicates significant differences with the control group (LSD, p<0.05).*

3.4 Oxidative stress biomarkers

In spring, Pb treatment had significant effects on oxidative stress biomarkers at day 7 (Table 1). Exposed individuals had higher tGSH levels than controls ($F_{1,59}$=4.484, p=0.038). Blood Pb concentration positively correlated with tGSH (r=0.312, p=0.014) and oxGSH (r=0.338, p=0.010) levels. No effects of Pb were found on MDA levels. Retinol plasma levels in males (p=0.011) and lutein levels in both genders decreased in Pb exposed groups (p=0.027 and p<0.001, respectively), and a similar trend was found for tocopherol (p=0.053). During the autumn experiment, Pb exposure at both doses increased retinol plasma levels at day 21 (p<0.001), and had no significant effect on lutein or tocopherol levels.

3.5 Carotenoid-based coloration

In spring, beak redness was positively correlated with blood Pb concentration at day 21 (r=0.324, p=0.020). Pb exposure increased eye-ring redness in males exposed at the high dose (p=0.008; Figure 4), but had no effect on beak or eye-ring redness in females. However, in females, the percentage of pigmented eye-ring area was negatively correlated with blood Pb concentration at day 7 (r=−0.429, p=0.023). In autumn, eye-ring and beak redness were greater in males than in females (p=0.004 and p=0.027, respectively), and were reduced 21 days after Pb exposure at the high dose (all p<0.001) in both genders (Figure 4). Pb exposure also reduced the percentage of pigmented eye-ring area, but only in females of the high dose group (p<0.001).

3.6 Relationship between Immunotoxic effects of Pb, oxidative stress and carotenoid-based coloration

In autumn, lysozyme levels positively correlated with plasma lutein at day 21 (p=0.044), and the effect of Pb exposure on this immune function remained significant when including lutein as a covariate (p<0.001). Phagocytic activity measured at day 21 correlated negatively with both eye-ring and beak redness (p<0.001 and p=0.009, respectively). The effects of Pb exposure on phagocytic activity were no longer

significant when color variables were included as covariates. In spring, lysozyme levels measured at day 7 and NAb levels were not related to any oxidative stress or color endpoint. Plasma lysozyme concentration at day 21 negatively correlated with tocopherol plasma levels ($r=-0.325$, $p=0.033$) at day 21 in spring. The short-term PHA response, measured at day 14, negatively correlated with tGSH levels measured 7 days before ($p=0.088$), and the effect of Pb on this immune function remained significant when including tGSH as a covariate ($p=0.018$). Likewise, the longterm PHA response, measured at day 28, positively correlated with oxGSH ($p=0.078$) and lutein ($p=0.098$) levels. The effect of Pb on long-term PHA response became significant when these variables were included as covariates ($p=0.038$; $p=0.030$, respectively). Hemagglutination of SRBC (T-dependent antibody response) was not related to oxidative stress, carotenoid or coloration variables.

Table 1. Pb blood levels (µg/dL), body condition, δ-ALAD ratio, oxidative stress biomarkers and antioxidant levels of control and Pb exposed partridges in spring (7 and 21 days after exposure) and autumn (21 and 55 days after exposure).

SPRING	Day 7						Day 21					
Variable	Control		Low dose		High dose		Control		Low dose		High dose	
	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
Blood Pb (µg/dL)	24	0.9 ± 0.8	20	232.2 ± 32.4**	19	396.7 ± 56.4**	22	<LOD	15	58.7 ± 13.1**	16	135.7 ± 28.7**
Body condition	23	419 ± 4.76	19	420 ± 5.23	20	402 ± 5.11**	21	433 ± 18.4	17	441 ± 20.7	18	435 ± 21.6
δ-ALAD Ratio	21	0.998 ± 0.022	18	0.029 ± 0.010**	16	0.080 ± 0.059**	20	0.938 ± 0.05	13	0.317 ± 0.08**	15	0.207 ± 0.071**
RBC tGSH mM	23	6.95 ± 0.222	20	7.76 ± 0.249*	18	7.55 ± 0.393	21	6.17 ± 0.331	15	6.37 ± 0.284	12	6.61 ± 0.342
RBC oxGSH mM	20	0.546 ± 0.014	20	0.58 ± 0.011	17	0.567 ± 0.023	19	0.539 ± 0.016	14	0.563 ± 0.021	12	0.544 ± 0.028
RBC oxGSH (%)	20	7.97 ± 0.256	20	7.58 ± 0.229	17	7.75 ± 0.398	21	8.25 ± 0.558	14	9.07 ± 0.457	15	7.25 ± 0.587
Blood SOD IU/mg	24	1.59 ± 0.058	20	1.56 ± 0.087	19	1.69 ± 0.148	21	1.78 ± 0.099	16	1.85 ± 0.073	13	2.0 ± 0.087
Blood GPx IU/mg	20	0.382 ± 0.035	17	0.345 ± 0.033	16	0.366 ± 0.042	20	0.379 ± 0.032	15	0.353 ± 0.027	13	0.429 ± 0.042
Total protein mg/g	24	353 ± 9.0	20	343 ± 14.9	19	337 ± 14.5	21	319 ± 12.9	16	301 ± 12.6	13	309 ± 13.0

MDA (nmol/g)	20	38.9 ± 2.51	17	36.3 ± 3.03	16	39.3 ± 2.69	20	45.5 ± 2.71	15	41.3 ± 2.88	13	44.0 ± 4.39
Retinol (nmol/ml)	20	22.7 ± 0.899	17	20.3 ± 1.11	16	18.5 ± 1.54**	21	16.9 ± 0.701	14	18.1 ± 0.889	13	16.4 ± 0.829
Lutein (nmol/ml)	20	27.3 ± 2.25	17	19.3 ± 1.66**	16	17.9 ± 1.99**	21	21.4 ± 1.45	14	22.8 ± 1.63	13	19.7 ± 2.07
Tocoferol (nmol/ml)	20	26.7 ± 2.35	17	20.9 ± 1.39*	16	22.2 ± 1.98	21	24.5 ± 1.65	14	23.1 ± 1.55	13	21.4 ± 2.42
AUTUMN				Day 21						Day 55		
Blood Pb (µg/dL)	29	<LOD	30	103.9 ± 16.9	30	224.7 ± 34.5	26	<LOD	24	1.6 ± 0.7	26	8.5 ± 1.0
Body condition	30	445 ± 3.43	30	448 ± 3.43	30	443 ± 3.47						
Retinol (nmol/ml)	30	1.35 ± 0.112	29	1.89 ± 0.072**	30	2.06 ± 0.057**						
Lutein (nmol/ml)	30	0.047 ± 0.004	29	0.060 ± 0.003	30	0.058 ± 0.003						
Tocoferol (nmol/ml)	30	2.60 ± 0.236	29	3.16 ± 0.206	30	2.88 ± 0.181						

Significantly different from controls at *p≤0.05 or **p≤0.01.

Table 2. Immunological responses of control and Pb exposed partridges in spring.

Variable	Day	Treatment					
		Control		Low dose		High dose	
		N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
Lysozyme (µg/mL)	7	19	44.1 ± 24.9	17	23.3 ± 8.8	16	13.7 ± 4.4
Lysozyme (µg/mL)	21	19	23.1 ± 5.4	13	13.6 ± 4.5	11	52.2 ± 22.3
Blood CFU (bactericidal assay)	7	14	95.3 ± 4.9	17	100.0 ± 8.1	16	90.3 ± 4.9
Blood CFU (bactericidal assay)	21	22	73.6 ± 2.6	14	74.8 ± 5.6	15	72.8 ± 3.2
Short-term PHA response	14	22	0.39 ± 0.03	20	0.47 ± 0.03*	18	0.35 ± 0.03
Long-term PHA response	28	21	0.47 ± 0.03	18	0.53 ± 0.02	17	0.53 ± 0.03
Natural antibodies agglutination titer	7	19	2.45 ± 0.43	17	1.41 ± 0.15*	15	1.50 ± 0.15
Natural antibodies-complement lysis	7	19	0.45 ± 0.13	17	0.82 ± 0.19	15	0.77 ± 0.18
SRBC primary agglutination titer	21	21	3.24 ± 0.46	14	3.18 ± 0.36	13	2.62 ± 0.23

Significantly different from controls at *p≤0.05 or **p≤0.01.

Table 3. Immunological responses of control and Pb exposed partridges during autumn experiment.

Variable	Day	Treatment					
		Control		Low dose		High dose	
		N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
Lysozyme (µg/mL)	21	30	27.08 ± 4.27	29	23.50 ± 9.95	30	5.01 ± 1.13*
Phagocytic activity	7	30	4099 ± 565	30	4165 ± 525	30	3718 ± 285
Phagocytic activity	21	29	1390 ± 173	30	1981 ± 295	30	2235 ± 242**
PHA response	21	29	0.79 ± 0.06	30	0.97 ± 0.05*	30	0.87 ± 0.05
Brucella abortus agglutination titer	21	30	8.37 ± 0.29	30	7.07 ± 0.34**	30	7.63 ± 0.03
SRBC agglutination titer	21	29	3.14 ± 0.31	30	3.08 ± 0.27	30	2.77 ± 0.24

Significantly different from controls at *p≤0.05 or **p≤0.01.

Table 4. Parasite and bacterial abundances (means ± SE) in feces of control and Pb exposed partridges in autumn before (day 0) and 21 days after Pb exposure. All N=30 for each period and treatment group.

Variable	Period	Treatment		
		Control	Low dose	High dose
		Mean ± SE	Mean ± SE	Mean ± SE
Coccidia (oocysts×10^3/g)	Day 0	73 ± 19	164 ± 59	76 ± 23
Heterakis sp. (eggs/g)		308 ± 216	1168 ± 1043	550 ± 498
Capillaria sp. (eggs/g)		1527 ± 1427	0	0
Coliform GNB (CFU×10^3/g)		13585 ± 10410	571 ± 308	824 ± 406
Non-coliform GNB (CFU×10^3/g)		0	0	434 ± 430
Coccidia (oocysts/g)	Day 21	244 ± 113	103 ± 60	85 ± 66
Heterakis sp. (eggs/g)		737 ± 623	1542 ± 1098	1595 ± 1433
Capillaria sp. (eggs/g)		107 ± 107	28 ± 28	0
Coliform GNB (CFU×10^3/g)		440 ± 423	1.2 ± 1.2	9.3± 5.6
Non-coliform GNB (CFU×10^3/g)		1.7 ± 1.7	32.3 ± 14.2	23.6 ± 13.2

Significantly different from controls at *p≤0.05or **p≤0.01.

4. DISCUSSION

Experimental Pb shot ingestion in red-legged partridges produced significant changes in some of the immune response variables studied here, involving both constitutive and induced components. Interestingly, Pb effects on immune function, antioxidant status and carotenoid-based coloration differed between seasons, probably reflecting differences in allocation trade-offs involving antioxidants and carotenoids during the breeding and nonbreeding seasons.

Blood Pb levels observed after experimental exposure were similar to those found in the field (Martinez-Haro et al., 2011), through natural Pb shot ingestion. When ingested, the shot remains in the gizzard of the bird and becomes eroded during 3 weeks (Jordan and Bellrose, 1951). During this period, Pb is dissolved by the action of gastric fluids and the formed Pb salts can be absorbed through the intestine wall into the bloodstream, or remain unabsorbed and excreted in feces (Pain, 2009). Blood Pb has a half-life of around 2 weeks in birds, and the concentration of Pb is generally elevated in blood immediately after exposure; afterward, Pb is progressively deposited in soft tissues and bone (Pain, 2009). In agreement, we observed high blood Pb concentrations 7 days after exposure, which markedly decreased after 21 days. Experimental studies in birds have reported that >88% of Pb dissolved in the gizzard from ingested pellets is excreted in feces (Irwin, 1977), which explains the high fecal Pb levels detected here. We found maximum blood Pb levels immediately after shot administration, when the elevated quantity of dissolved Pb may result in a high absorption rate through the intestinal wall. In feces, we detected a similar temporal pattern, with Pb levels remaining elevated several weeks after Pb shot dosing.

Sublethal Pb exposure also significantly affected δ-ALAD activity, particularly in females, which showed higher blood Pb concentration than males during spring until 21 days after exposure. Such differences between sexes have been frequently observed in Pb exposed birds (Scheuhammer, 1996) and may be explained by the increased Ca^{2+} and Pb^{2+} absorption or mobilization for eggshell formation during the breeding season, because of the similarity between these two divalent cations (Minnema et al., 1988; Tejedor and Gonzalez, 1992). Regarding other physiological variables, we observed a

decrease in body condition in Pb exposed birds 7 days after exposure, and a recovery after 21 days (Mateo et al., 2003).

Several components of the constitutive and induced immune responses were affected by Pb exposure, with some differences between genders and seasons, as observed elsewhere (Apanius, 1998; Kendall et al., 2001; Møller et al., 2003; Rocke and Samuel, 1991). Regarding constitutive responses, the lower NAb-related agglutination observed in spring could result in increased infection sensitivity (Ochsenbein et al., 1999), because NAbs enhance antigen presentation and initiate specific responses of B and T cells (Ochsenbein and Zinkernagel, 2000). Blood bactericidal activity in spring, which gives an overall measure of the constitutive response (involving phagocyticactivity of leuckocytes and microbicidal activity of antibodies and lysozyme ; Millet et al., 2007) was unaffected by Pb exposure here, suggesting that birds were able to compensate Pb effects on constitutive immunity in the breeding season (Blakley and kouassi, 2005). In autumn, lysozyme plasmatic levels were reduced in exposed partridges at both doses. We are not aware of other studies relating Pb exposure with lysozyme levels in birds, but a decrease in lysozyme levels was observed in the spleen of Pb-exposed rats (Teijón et al., 2003). The increased phagocytic activity could be explained by a Pb-induced synthesis of arachidonic acid, which is precursor of eicosanoids involved in phagocytosis (Knowles and Donaldson, 1997). Lysozyme is mostly synthesized in monocytes and macrophages (Gordon et al., 1974). The Pb mediated decrease in lysozyme could have been compensated by an increase in the number of these circulating, lysozymeproducing leukocytes, which would explain the higher phagocytic activity of Pb exposed animals. Regarding induced immunity, PHA response increased in both experiments as a consequence of Pb exposure. Similarly, Pb has been found to increase the macrophage stimulation of T-lymphocytes in mice, and to interfere with antigen-specific interactions between these two types of cells, and decreasing the ability of antigen presentation (Kowolenko et al., 1988). Other studies in birds have shown no Pb effects on the PHA response (Fair and Ricklefs, 2002; Nain and Smits, 2011; Trust et al., 1990) or a suppressed PHA response (Fair and Myers, 2002; Grasman and Scanlon, 1995). It has been suggested such T cell dependent responses stimulated by mitogens may depend on the dose, method and duration of Pb administration (Redig et al., 1991). The increased

cell-mediated response, similarly to the increased phagocytic activity, could also be related to Pb-induced increase of eicosanoids that mediate macrophage acute inflammatory responses(Knowles and Donaldson, 1997). Antibody T-dependent response was unaffected by Pb exposure, as found in other studies in birds (Fair and Ricklefs, 2002; Redig et al., 1991). By contrast, other authors (Trust et al., 1990) found a decreased agglutination of SRBC. Here, we observed that the induced response mediated by T-independent antibodies was slightly suppressed, whereas other studies reported no effect (Fair and Ricklefs, 2002; Nain and Smits, 2011). The primary humoral response studied here is usually slightly above background levels, whereas the secondary response (not tested here) is a more biologically meaningful reaction, indicative of the memory component of the B cell response (Smits and Baos, 2005) and should be considered in future experiments. This T-independent response depends on the activation of the complement system, which links constitutive and induced immunity (Ochsenbein and Zinkernagel, 2000). In a similar experiment (Nain and Smits, 2011) studying the effects of Pb exposure in drinking water in 4 week old Japanese quails (*Corturnix coturnix japonica*) a hormetic and immunostimulatory effect was found, although in this case, lower doses of Pb were used (daily Pb intake of 1.1 and 10.7 mg/kg during 7 weeks) than the ones administered in the current work (109 mg and 327 mg of Pb in the low and in the high dose groups at the beginning of the experiment, respectively).

Unlike other host resistance studies (Knowles and Donaldson, 1997; Nain and Smits, 2011), in the current experiment, partridges were naturally exposed to environmental microorganisms present in their housing pen. Here, we measured abundance of fecal bacteria and parasites prior to and after Pb exposure, to consider individual background levels and variability, offering a better approximation to a real scenario. We observed a change in gut microbiota in Pb exposed partridges, with an increase in noncoliform and a decrease trend in coliform GNB. Pb is known to affect lipopolysaccharides (LPSs) of the cell membrane of *E. coli* (Peng et al., 2007). Our results are consistent with a previous study (Nain and Smits, 2011) showing a reduced morbidity after *E. coli* inoculation in Pb exposed Japanese quail. Changes in gut microbiota produced by Pb can be due to in situ effects on bacteria development or to immunomodulation. Here, changes in bacterial richness appeared mediated by lysozyme

and phagocytic activity. The Pb-induced increase of noncoliform abundance seems to be directly related to altered lysozyme levels. Lysozyme, which was strongly suppressed in Pb exposed partridges, has a protecting role against pathogens in the gut of birds (Callewaert and Michiels, 2010), but this protection may disappear when lysozyme levels decrease (Rzucidlo, 1978). The increased prevalence of *Heterakis sp*. in partridges exposed at the high dose may be related to lower lysozyme levels, a protein known to inhibit viruses, parasites and fungi (Benkerroum, 2008). Furthermore, although NAb response was not determined in the same experiment that fecal bacterial counts in the current work, decreased levels of NAb after Pb exposure may be involved in observed changes in gut microbiota. IgM (NAbs) are among the most abundant immunoglobulins in intestinal secretions of chickens,6 and some IgG appears to leak into the gut through the lymphatic vessels during some infections (Pastoret et al., 1998). Other studies, using higher doses than ours, reported oral Pb exposure effects on the gut microbiota composition mediated by nonabsorbed heavy metals or before their absorption (Breton et al., 2013). In Pb exposed partridges, the high concentration of Pb in the intestine several weeks after exposure and Pb effects on constitutive immunity could explain observed changes in the gut bacteria.

Pb may induce oxidative stress through δ-ALA accumulation generating ROS (Kendall et al., 2001) but also through alterations of antioxidant system mechanisms (Martinez-Haro et al., 2011; Rodríguez-Estival et al., 2011). Partridges exposed to Pb in spring had altered antioxidant levels, specifically increased tGSH and reduced dietary antioxidant levels (lutein, retinol and tocopherol), with an apparent recovery after 21 days. Heavy metals and oxidative stress can induce γ-glutamyl cysteine synthetase, an enzyme involved in GSH synthesis (Griffith, 1999). A similar increase of GSH levels has been reported with the experimental Pb exposure in mallard ducklings (*Anas platyrhynchos*) and Canada goslings (*Branta canadensis*), but also a negative relationship between the resultant blood Pb levels and GSH (Mateo and Hoffman, 2001). This can indicate that the organism increases the synthesis of GSH and uses it for Pb excretion by binding the element to the GSH's thiol group (Mateo and Hoffman, 2001). Moreover, oxGSH levels were positively correlated with blood Pb concentration, which may be indicative of Pb-induced stress, as reported in other birds (Martinez-Haro et al., 2011).

However, % of oxGSH was unaffected by Pb levels, so the increased tGSH probably prevented further redox unbalance in this endogenous antioxidant. The decrease in dietary antioxidant plasma levels in Pb-exposed partridges during spring is consistent with field data on Pb-exposed waterbirds (Martinez-Haro et al., 2011). The strong seasonal variation in plasmatic levels of dietary antioxidants observed here has been previously observed (Pérez-Rodríguez, 2008a), with higher levels during the mating-breeding season than during the rest of the year. Oxidative stress and coloration are linked to antioxidants (Pérez-Rodríguez et al., 2013), and in our experiment appeared linked to the reduction of dietary lipophilic antioxidants (vitamins A and E and carotenoids) and a compensatory increase of the endogenous antioxidant (tGSH) at the beginning of Pb exposure in spring, when plasma levels of these antioxidants were higher.

Interestingly, the high Pb dose increased eye-ring carotenoidbased coloration in males at the end of spring experiment (breeding season). Male redness is known to positively influence breeding investment by female red-legged partridges (Alonso-Alvarez et al., 2012). Since retinol levels decreased only in males, Pb exposed male partridges may have increased ornamental coloration at the expense of plasma antioxidants, possibly as a "terminal investment" strategy, which occurs in birds when survival or future breeding opportunities are compromised (Velando et al., 2014). The increase in male coloration may have been caused by oxidative stress at low intensity. The latter was shown to favour the transformation of dietary carotenoids (zeaxantin and lutein) into ketocarotenoids (astaxanthin and papilioerythrinone, the pigments ultimately responsible for the red coloration of partridges), a metabolic transformation that requires oxidation steps (García-de Blas et al., 2014). By contrast, Pb exposed female partridges may have prioritized oxidative stress compensation to the detriment of coloration. In spring, the redness and percentage of pigmentation of female eye-rings did not differ between treatment groups, but negatively correlated with blood Pb levels. During the nonbreeding season, Pb exposure reduced carotenoid-based coloration in both genders at the end of the autumn experiment. Moreover, we found a negative association between the constitutive (phagocytosis) immune response and carotenoid-based coloration levels. Neither Pb exposure nor the stimulation of any immune

response decreased plasma carotenoid levels. This suggests that, in the nonbreeding season, both genders prioritize keeping circulating carotenoid levels to fight against oxidative stress at the expense of carotenoid-based ornamentation (Alonso-Alvarez et al., 2008).

We also found some interactions between oxidative stress and immune function for induced components. Antioxidant levels in spring were negatively associated with the short-term PHA response and lysozyme levels at day 21, whereas oxGSH levels were positively associated with long-term PHA response. As the energetic costs of constitutive responses are lower than those of induced immunity (Lee, 2006), the induced response should be more affected under oxidative stress. Constitutive and induced cellular immune responses involve an oxidative burst, and ROS production to fight pathogens (Blakley and kouassi, 2005). In agreement, we found that lower antioxidant levels were associated with greater PHA response. Furthermore, GSH plays a role in cellular immunity, by affecting Th receptors and breaking the balance between T_{h1} and T_{h2} responses (Townsend et al., 2003) (e.g., depletion of intracellular GSH reduces its negative regulatory mechanism of protein kinase C isozymes, which may induce cell proliferation and differentiation) (Ward et al., 1998).

Interestingly, some variables affected by Pb, such as spring antioxidant levels, body condition and components of constitutive response (NAbs levels in spring, and lysozyme levels in autumn) were only altered immediately after Pb exposure, i.e., when blood Pb levels were highest. By contrast, we observed a lagged effect on other parameters like carotenoid-based coloration and noncoliform GNB abundance, components of both constitutive and induced immune responses (phagocytic activity, PHA response, T-independent humoral response) or antioxidant levels in autumn. This suggests that immune function and carotenoid-based coloration can be appropriate endpoints to study Pb adverse effects on birds, with important implications on their resistance to diseases and intraspecific social signaling.

In conclusion, exposure to Pb similarly affected the induced immune response in both seasons. By contrast, the effects of Pb on constitutive response appeared compensated in spring, and strongly affected in autumn. During the breeding season

(spring), Pb-exposed females may use antioxidants to cope with oxidative stress at the expenses of carotenoid-based coloration, and may be less sensitive to immunologic challenges. Pb exposed males increased ornamental coloration in spring, possibly as a short-term strategy to increase breeding investment. In autumn, Pb strongly impaired the constitutive response of males and females. Then, Pb-exposed partridges mobilized free retinol and reduced carotenoid allocation to ornaments, without altering other plasma antioxidant levels, and prioritized oxidative balance maintenance.

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SUPPLEMENTAL MATERIAL

1. MATERIAL AND METHODS

1.1. Housing condition of captive birds used for experiments

For the spring and autumn experiments, captive partridges were housed in outdoor cages (95×40×42 cm). Birds were fed ad libitum with a mixture of wheat and commercial partridge feed (Partridge maintenance fodder, Nanta-Nutreco, Tres Cantos, Spain). Tap water was also provided ad libitum.

The partridges used for the autumn experiment were birds other than those used in the previous spring experiment. The partridges used in autumn were previously housed together (since July of 2012) in a large outdoor pen (40 m x 9 m), where they were naturally exposed to environmental pathogens.

1.2. Blood sampling

For each blood sampling, we extracted 1 mL of blood from the jugular vein. Blood samples were separated into four aliquots collected with a heparinized tube to avoid coagulation and used as follows: one was kept fresh, the second one was stored at -80ºC for Pb analysis, the third one was stored in liquid nitrogen for analysis of δ-aminolevulinic acid dehydratase (δ-ALAD) activity, and the fourth one was centrifuged to separate plasma from the cellular (red blood cells, RBC) fraction for further immunological, biochemical, and antioxidant analysis.

Chronograms of the spring and autumn experiments

Table S1. Temporal scheme of sampling times (Day 0 = initial exposure, January 24, 2012 and October 23, 2012 for the spring and autumn experiments, respectively) and of the variables measured at each sampling time.

Variable		Experimen								
		Sampling day in					Sampling day in			
		0	7	14	21	28	0	7	21	55
Biometry and	Tarsus length	x					x			
	Body mass	x	x	x	x	x	x			x
	Plasma biochemistry		x		x					
Pb concentration	Blood Pb		x	x	x	x	x	x	x	x
	δ-ALAD		x		x					
Constitutive immunity	Blood bactericidal activity		x		x					
	Blood phagocytic activity						x	x	x	
	Plasma lysozyme		x		x		x		x	
	Complement-mediated hemolysis		x							
	Natural antibodies (SRBC agglutination)		x				x			
Induced immunity	Cellular response (PHA)			x		x			x	
	T-dependent humoral response (SRBC				x				x	
	T-independent humoral response (BA								x	
	Fecal parasites and bacteria						x		x	
Oxidative stress	Endogenous antioxidants in RBC		x		x					
	Dietary antioxidants in plasma		x		x		x		x	
	Antioxidant enzymes in RBCs		x		x					
	Lipid peroxidation (MDA)		x		x					
Color	Carotenoid-based coloration (beak and eye ring)	x			x		x		x	

1.3. Feces collection and Pb analysis

During the spring experiment, feces from each pair of partridges were collected at days 7, 19, 25 and 31. A plastic bag was placed under each cage 24 h before collection. We carefully picked up 4 pellets of fresh feces from each cage, trying to get samples from both the male and the female. The pool of feces of each cage was homogenized. Samples were kept in a zip-lock plastic bag and stored at -80 ºC until analysis.

Feces Pb assays were performed following Martínez-Haro et al. (2010). Feces were freeze-dried, and 0.2–0.3 g of dry weight was digested with 2.5 ml of HNO_3 (69% Analytical grade, Panreac, Spain) in Pyrex tubes in a heating block (Micro, for 40 tubes, Selecta) with an electronic controller (RAT-2, Selecta). Tubes were heated for 5 h at 50 °C, and for 5 h at 100 °C. After being left overnight at room temperature, 2.5 ml of H_2O_2 (30% v/v Suprapur, Merck, Germany) were added. Tubes were heated for 45 min at 90 °C, 2 h at 120 °C and another 1 h at 150 °C. Digests were diluted to 50 ml with H_2O (Milli-Q grade).

Lead concentration was determined using a graphite furnace atomic absorption spectroscopy system (AAnalyst800 equipped with an autosampler AS800, Perkin–Elmer), using 50 µg of $NH_4H_2PO_4$ and 3 µg of $Mg(NO_3)_2$ as matrix modifiers for each atomization. A certified reference material for Pb (DC73349 Bush Branches and Leaves) was analyzed to ensure the quality of the methodology, with a recovery (mean ± SD) of 106 ± 4.15% (n=8). The detection limit was <0.1 µg/g of Pb in feces.

1.4. Immune responses

Details of the specific assays performed to evaluate the immune response are given below.

When phagocytes are activated, they produce reactive oxygen species (ROS) for killing pathogens generating electronically excited states, which, on relaxation to the ground state, emit photons. This emission is referred to as phagocyte chemiluminescence (CL). We measured phagocytic activity in fresh blood performing a whole-blood chemiluminescence assay (Papp and Smits, 2007) with

slight modifications, to assess the production of ROS by circulating phagocytes. The light enhancer luminol (3-aminophthalhydrazide, 98%, Alfa Aesar, Germany) as a 100 mM stock solution was prepared in dimethyl sulfoxide; we added 50 µL of stock solution to 4.95 mL of Hank's Balanced Salt Solution (HBSS) and quickly mixed it to obtain a 1 mM working solution, which was stored at -80 ºC. We prepared a working solution (20 mg/mL) of zymosan A from *Saccharomyces cerevisiae* (Sigma, Germany). Zymosan A contains some substances that are recognized by the complement receptor III complex (Unkeless and Wright, 1988), which plays an important role in regulating phagocyte migration and activation. Blood aliquots of 17 µL were diluted in 230.5 µL of HBSS and added to plastic lumivials (Berthold Technologies). We added 2.5 µL of the zymosan A working solution to the diluted blood samples, and 250 µL of luminol 1 mM. We run a blank duplicate of each sample replacing the volume corresponding to zymosan A by HBSS. The final reaction volume in each vial was 500 µL. We incubated lumivials at 37 ºC for 3 h and determined the CL of each sample and its blank with a luminometer (Lumat^3 LB 9508, Berthold Technologies). The incubation time was selected during a preliminary test after checking that samples reached their CL peak at that specific time. The phagocytic activity of each sample was calculated as the difference in CL between the sample and its blank.

We performed a bactericidal assay to measure bactericidal activity of fresh blood in vitro (Millet et al., 2007) with slight modifications. We prepared CO_2-independent media enriched with 5% complement-inactivated serum and 4 mM glutamine, and we diluted 60 µL of each blood sample in 180 µL of medium, preheated at 37 ºC, in a sterile 1.5 mL Eppendorf tube. We prepared a working culture with 10^4 colony-forming units (CFU)/mL of living *Escherichia coli,* and we added 24 µL of this suspension in each blood mixture. We incubated tubes at 37 ºC in constant shaking for 30 minutes. We spread 100 µL of each tube onto petri dishes by duplicate, and then covered plates with PCA (plate count agar) preheated at 46 ºC and moved gently to spread the sample. Plates were incubated upside down at 37 ºC for 24 h, at the end of which we counted the number of CFU. For calculations of bactericidal activity, CFU averages of the two plates from each

sample were compared to CFU measured in control plates, where blood was replaced by medium.

Lysozyme is an enzyme of leukocytic origin with antibacterial and antiviral activity. This protein is highly distributed in the organism, especially in lysosomes of monocytes, macrophages and granulocytes (Gill, 1995), and it is also present in lymphocytes. We measured plasmatic lysozyme levels (Millet et al., 2007) to determine the lysis of bacteria by lysozyme as indicated by a decrease in opacity of an agar-bacteria suspension. We added 5 mg of dried *Micrococcus lysodeikticus* (Sigma, USA) to 125 mL of sterilized 1% agarose and kept at a temperature of 50-60 ºC. In a 96-well plate, we added 150 µL of this suspension to a 10 µL plasma sample. At the same time, we obtained a standard curve by adding the bacterial suspension of *M. lysodeikticus* to serial dilutions of a standard chicken lysozyme (Roche, Germany) solution. We measured absorbance at 850 nm in a microplate reader after 1 h, 24 h and 48 h, and estimated lysozyme concentration in samples by comparison with the standard curve. We used as lysozyme activity indicator the decrease in absorbance from 1 h to 48 h measures. We used the 48 h lecture instead of the 24 h because it provided a better adjustment of the calibration curve, as at 24 h no response pattern to calculate the calibration curve was observed.

The levels of natural antibodies (NAbs) and the activity of the complement proteins were estimated using hemolysis–hemagglutination tests before injection of any antigen. This assay is useful for characterizing NAbs-mediated complement activation (Matson et al., 2005). Lysis reflects the interaction of complement and NAbs, whereas agglutination results from NAbs only, which are the only immunoglobulins (Ig) that do not need previous exposure to a particular antigen.

T-cell-mediated immunity response was tested using the phytohemagglutinin (PHA)-skin test. PHA is a lectin that produces a proliferative response of circulating T lymphocytes that are accumulated at the injection site. We used a micrometer (Mitutoyo Absolut 547-401) to the nearest 0.01 mm to measure wing web thickness and injected 100 µL of PHA in PBS (1 mg/mL dilution). After 24 h, we measured again the wing web thickness, estimating the intensity of cell-mediated response as the difference between initial and final thickness.

We also tested the response at the end of the spring experiment by performing a second PHA-skin test.

With regards to the humoral response, we used different tests depending on the type of response. B-lymphocytes are precursors of antibody secreting cells and can be activated directly by cross-linkage between Ig and antigen, or indirectly by interaction with T- lymphocytes (Blakley and kouassi, 2005). We tested responses against two different antigens, sheep red blood cells (SRBC) and *Brucella abortus* (BA), to assess T-lymphocyte dependent and T-lymphocyte independent antibody responses, respectively. For immunization, we injected intraperitoneally 100 µL of a SRBC dilution (20% in PBS) prepared with blood extracted from a sheep housed in the same farm as we conducted the experiments, or intramuscularly in the thigh 50 µL of a commercial preparation of BA vaccine (Plasmatec Stained Febrile Antigens, United Kingdom). To analyze the T-cell dependent response we used a hemagglutination test similar to that used for the quantification of NAbs, although in this case the animals were previously immunized by intraperitoneal injection of SRBC. At the beginning of the spring experiment, we used plasma from a first blood sample collected 7 days before immunization with SRBC to determine NAb concentration, and a second plasma sample collected 7 days after SRBC administration to determine specific, T-cell dependent antibody production. In autumn, only specifically induced antibodies were measured, using a blood sample taken 14 days after injecting SRBC. We prepared serial dilutions of plasma with PBS (total volume 50 µL) in wells of microtiter plate, and added 50 µL of SRBC (0.1%) to each dilution obtaining a final volume of 100 µL. The SRBC used in the humoral response assays were obtained from the sheep used for immunization. A negative control was prepared for each sample adding 50 µL of PBS-only instead of the plasma dilution. Plates were incubated for 1 h at 37 ºC followed by another 2 h at room temperature, at the end of which we checked them to estimate the hemolysis (in the case of pre-immunization samples) and hemagglutination. To improve visibility of the agglutination, plates were tilted 45º during incubation. The lower plasma concentration causing hemolysis (sample transparent) and hemagglutination (difuminated RBCs) were used as titers for complement-mediated lysis and

concentration of antibodies, respectively. The titers were calculated as the $\log_2$ of the corresponding plasma concentration. In the case of T-cell independent response, we used an agglutination test for titration of BA similar to that described for SRBC with slight modifications. The plasma serial dilutions (25 µL) were mixed with 25 µL of a 40% dilution of BA vaccine in PBS, for a final volume of 50 µL. Plates were incubated at 37 ºC for 24 h and titers determined and calculated as with the SRBC hemagglutination tests.

1.5. Carotenoid-based ornamentation

We used digital photographs to measure the red coloration of the beak and eye ring of partridges before and after Pb exposure, in both experiments (spring and autumn). For each bird, we took a high resolution digital picture of the left side of the head using the same digital camera (Canon EOS 7D) and under standardized conditions (same light illumination and gray background, same distance (40 cm) between the body part to analyze and the camera). For each picture, we placed the same gray reference (Kodak Gray Scale, Kodak, New York) near the body parts that were measured.

We analyzed the digital pictures using Adobe Photoshop v7.0. From each picture, we measured the RGB components (the average intensity of red (R), green (G) and blue (B) components of pixels) of the eye ring and beak (upper mandible), and of the gray reference. We considered the eye ring and beak colors separately because the eye rings are soft tissues, whereas the beak is a more keratinized structure. The intensity of carotenoid-based red coloration, or RGB value (referred as to ‘‘redness’’) was calculated as R divided by the average of R, G and B (Villafuerte and Negro, 1998). RGB values of the gray reference were used to standardize all color measurements and correct for possible differences in coloration between pictures. In addition, we calculated the relative amount (% pixels) of the eye ring area pigmented by carotenoids (Mougeot et al., 2009). From the digital pictures, we thus obtained the following color measurements for each male: (1) beak redness, (2) eye ring redness, and (3) eye ring pigmentation (% of eye ring area pigmented).

1.6. Precision of vitamins and carotenoids analysis

In order to study the coefficient of variation of results (precision), we prepared a pool mixing plasma samples obtained from 4 different untreated partridges. We analyzed 14 replicates of pool in one day to calculate the intra-day repeatability. We also obtained the inter-day repeatability values analyzing the pool in 6 different days.

Table S2. Intra and inter-day repeatability of vitamins and carotenoids analyses from a pool of plasma from different untreated partridges. Mean concentration in plasma, Standard Deviation (SD) and Relative Standard Deviation (RSD).

		Variable			
Repeatability		Retinol	Lutein	Zeaxanthin	Tocopherol
Intra-day	Mean (nmol)	1.851	0.519	0.181	2.235
	SD	0.118	0.045	0.013	0.110
	RSD (%)	6.388	8.590	7.388	4.914
Inter-day	Mean	1.720	0.533	0.182	2.124
	SD	0.212	0.057	0.017	0.127
	RSD (%)	12.339	10.739	9.238	5.982

7. Plasma biochemistry analyses

Albumin, total proteins, creatinine, uric acid, triglycerides, cholesterol, glucose, calcium (Ca), phosphorous (P), magnesium (Mg), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in plasma with an automatic spectrophotometer analyzer A25, using the reaction kits available for each enzyme or analyte (BioSystems, Barcelona, Spain).

2. RESULTS

2.1 Feces Pb concentration

Significant differences in feces Pb concentration were observed between treatment groups at day 7, 19, 25 and 31 post exposure ($p<0.001$, $p=0.004$, $p=0.024$, $p=0.006$, respectively; Table S3).

2.2 Plasma biochemistry analyses

During spring, Pb exposure reduced triglyceride levels ($p=0.005$; Table S3) and total proteins ($p=0.058$) at day 7, and reduced phosphorus ($p=0.020$) and ALP levels ($p=0.001$) at day 21.

Table S3. Feces Pb concentration (µg/g) of control and Pb exposed partridges 7, 19, 25 and 31 days after Pb exposure.

Timing (Days after exposure)	Feces Pb (µg/g)					
	Control		Low dose		High dose	
	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
7 Days	13	3.07 ± 1.73	10	1,134 ± 229**	10	2,426 ± 385**
19 Days	12	1.11 ± 0.717	9	248 ± 144	9	613 ± 227**
25 Days	12	5.57 ± 1.22	9	12.3 ± 6.34	9	83.3 ± 44.1**
31 Days	13	1.70 ± 0.952	9	4.0 ± 1.27	9	55.5 ± 29.3**

Significantly different from controls at *p≤0.05 or **p≤0.01

Table S4. Plasma biochemical variables and hematocrit of control and Pb exposed partridges in spring (7 and 21 days after exposure).

	7 Days after exposure						21 Days after exposure					
	Control		Low dose		High dose		Control		Low dose		High dose	
Variable	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE
Albumin (g/L)	14	16.86 ± 1.58	12	17.58 ± 0.61	13	17.08 ± 1.89	14	17.36 ± 1.32	9	14.22 ± 2.28	9	15.56 ± 0.78
Creatinin (mg/dL)	13	0.32 ± 0.04	14	0.28 ± 0.02	13	0.41 ± 0.11	15	0.34 ± 0.03	7	0.35 ± 0.08	7	0.38 ± 0.03
Calcium (mg/dL)	17	11.33 ± 0.44	16	10.68 ± 0.43	15	10.84 ± 0.31	17	10.53 ± 0.31	12	10.38 ± 0.68	9	10.76 ± 0.26
Phosphorous (mg/dL)	17	6.28 ± 0.45	15	7.31 ± 0.75	14	6.52 ± 0.92	15	6.42 ± 0.45	11	4.77 ± 0.50**	8	5.43 ± 0.42
Cholesterol (mg/dL)	18	202.94 ± 18.45	16	214.81 ± 13.04	14	190.79 ± 10.56	17	207.12 ± 10.98	11	206.45 ± 17.21	9	199.56 ± 11.24
Alkaline phosphatase (U/L)	11	3020.72 ± 10.47	9	2431.56 ± 907.93	5	2276.8 ± 1081.57	6	2697.33 ± 658.71	3	940 ± 449.76**	6	881.33 ± 165.43**
Creatine kinase (U/L)	14	495.21 ± 62.72	13	410.08 ± 84.80	12	449.75 ± 60.62	12	491.58 ± 55.79	9	439.33 ± 68.15	9	645.11 ± 129.25
Total protein (U/L)	18	48.28 ± 2.01	16	45.56 ± 2.10	14	41.29 ± 2.01*	17	45.12 ± 1.88	11	44 ± 3.54	9	40 ± 1.94
Lactate dehydrogenase (U/L)	8	1732.75 ± 263.77	8	1367.75 ± 218.86	5	1266.8 ± 188.97	2	781 ± 723.00	6	1207.17 ± 391.40	3	1622 ± 241.85
Magnesium (mg/dL)	18	2.76 ± 0.20	15	2.70 ± 0.89	15	2.66 ± 0.16	2	2.78 ± 0.09	7	2.85 ± 0.13	3	3.09 ± 0.22
Alanine Aminotransferase (U/L)	15	22.6 ± 3.62	15	21.27 ± 2.02	14	20.07 ± 2.77	17	18.82 ± 2.45	10	17.9 ± 2.53	9	20.22 ± 4.88

Triglycerides (mg/dL)	16	245.5 ± 25.25	16	260.25 ± 24.38	14	166.36 ± 18.94*	17	254.35 ± 19.09	11	231.27 ± 17.71	9	210.33 ± 22.78
Aspartate aminotransferase (U/L)	12	186.75 ± 8.98	15	183.67 ± 13.60	12	205.17 ± 7.99	15	154.4 ± 15.55	11	143.18 ± 20.19	9	177.67 ± 11.60
Uric Acid (mg/dL)	16	5.99 ± 0.56	16	4.63 ± 0.50	14	6.75 ± 1.21	17	5.66 ± 0.23	11	5.32 ± 0.65	9	5.22 ± 0.67
Glucose (mg/dL)	14	522.57 ± 27.64	13	529.85 ± 45.54	10	453.4 ± 23.38	13	471.23 ± 60.99	8	493.5 ± 30.00	9	496.89 ± 29.92
Haematocrit (%)	23	41.43 ± 2.06	19	39.23 ± 1.77	15	37.19 ± 1.91	22	39.65 ± 1.04	15	41.23 ± 1.85	15	38.28 ± 1.68

Significantly different from controls at *p≤0.05 or **p≤0.01

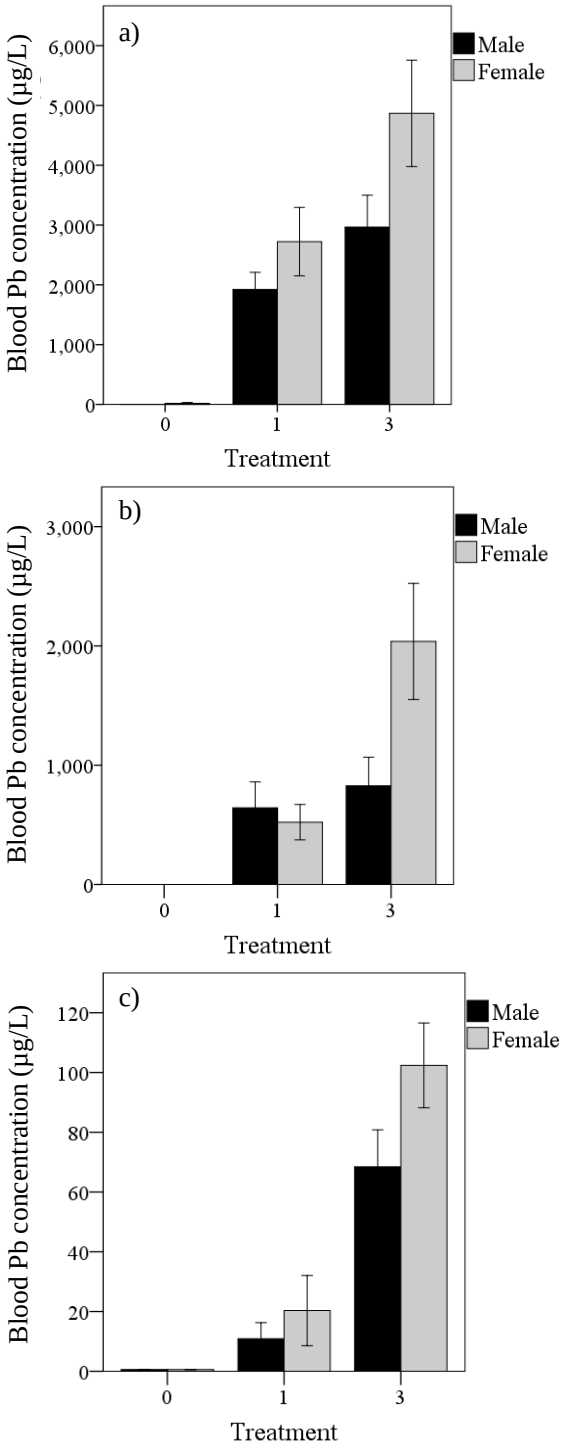

Figure S1. *Mean (± SE) Pb concentration in blood (a) 7 days and (b) 21 days after exposure during spring, and (c) 55 days after exposure during autumn, according to treatment group (Control=0; low Pb dose=1 shot; high Pb dose=3 shots) and gender.*

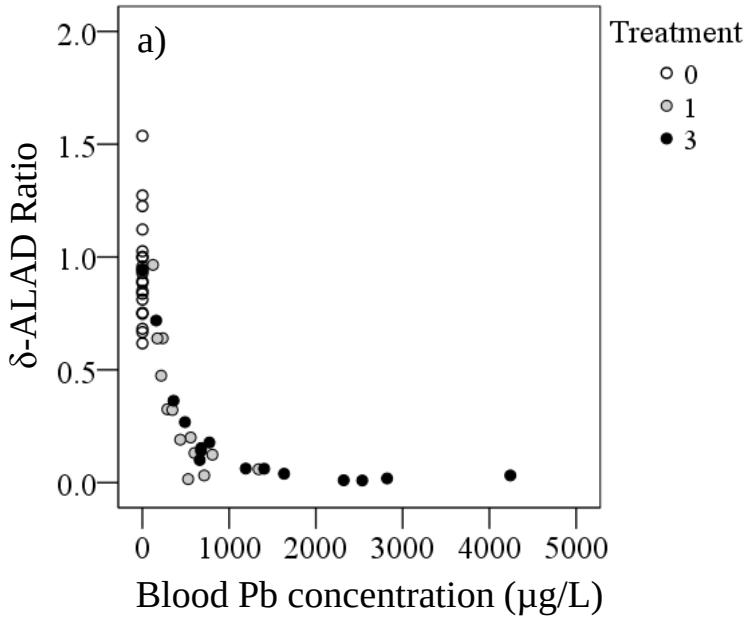

Figure S2. *Relationship between blood Pb level and δ-ALAD ratio in control (0: white) and Pb exposed (1=low dose, grey; 3=high dose, black) partridges in spring 21 days after exposure.*

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CHAPTER 5

SEX-SPECIFIC EFFECTS OF A PRELAYING Pb EXPOSURE ON THE REPRODUCTION OF THE RED-LEGGED PARTRIDGE

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ABSTRACT

Lead (Pb) poisoning by the ingestion of shot pellets, bullet fragments or fishing weights is a frequent cause of death in wild birds, but also have a wide range of sublethal effects. Here we report on the effects that a sublethal Pb exposure during the prelaying period have on the breeding performance of the red-legged partridges (*Alectoris rufa*). We studied separately the effects of exposing males or females on egg properties, laying performance, reproductive success, levels of dietary antioxidants and carotenoid-based coloration. We also studied the effects of male exposure on sperm quality and the relationship between sperm quality, carotenoid-based ornaments and antioxidant levels. We show that the prelaying Pb exposure induced the production of heavier and larger eggs, heavier chicks and reduced hatching success when females, but not males, were exposed. Fecundation rate and other laying performance parameters were unaffected. In males, Pb exposure decreased acrosomal integrity and sperm motility, and increased sperm vigour, but did not affect sperm viability, concentration or overall progress. Moreover, clutch size was increased in pairs in which the male had been exposed to Pb in comparison to unexposed pairs. Pb exposure increased levels of circulating antioxidants in males, whereas the percentage of eye-ring area pigmented by carotenoids decreased in exposed females. Overall, the sub-lethal Pb doses used here did not induce spermatozoon death or infertility in males, but rather caused an increase in reproductive investment. Pb exposed females also exhibited increased investment in reproduction, laying larger and heavier eggs and chicks, but had reduced carotenoid-based coloration and hatching rate. Several sperm parameters showed positive relationships with carotenoid-based coloration and levels of antioxidants that were influenced by Pb exposure, suggesting that redder males may be more capable to preserve sperm from oxidative stress.

Key words: Pb poisoning, reproduction, sperm quality, eggs, carotenoids, coloration.

1. INTRODUCTION

The ingestion of spent lead (Pb) ammunition used for hunting has been identified as a cause of mortality in wild birds around the world (Mateo, 2009). Although the use of Pb shot has been limited, or even banned, in several countries (Mateo, 2009), large amounts of shot pellets have accumulated in sediments, where they remain unaltered for decades and are available for foraging waterbirds (Mateo et al., 2014). Recent studies have highlighted that sublethal Pb exposure still happens in protected areas and negatively affects important avian fitness components such as immunity or reproduction (Vallverdú-Coll et al., 2015a).

Several studies have reported male infertility associated with Pb exposure. Dauwe et al. (2004) reported a reduced number of spermatozoa on the periviteline layer of eggs from a heavy metal polluted area, which is a sensitive effect that may occur before the impairment of other more noticeable breeding parameters (e.g., fecundation and hatching rates). Moreover, experiments conducted on rodents have reported effects of Pb exposure on the histology and cell kinetic in testis (Batra et al., 2004, 2001), increased chromatin condensation (Hernández-Ochoa et al., 2006), reduced sperm density and motility and an increase in the percentage of deformed and dead sperm cells (Akinola et al., 2015; Hsu et al., 1998a). All these effects may result in infertility, and a proper assessment of Pb effect on reproduction should contemplate both toxic effects on sperm quality (concentration, motility, morphology) and their consequences in terms of reproductive success (Friedler, 1996).

In birds, females appear to be especially vulnerable to Pb toxicity during the pre-breeding period, because of the increased absorption of calcium (Ca) for eggshell formation and the similarity between Ca^{2+} and Pb^{2+} (Tejedor and Gonzalez, 1992). The effects of Pb and other heavy metals on reproductive success have been reported on birds from polluted areas, with effects on several parameters such as clutch size, hatchability, laying date and eggshell quality (Dauwe et al., 2004; Eeva and Lehikoinen, 2015; Eeva et al., 2009). These reproductive parameters have direct effects on population dynamics, and the reduction of heavy metal emission has been associated with an improvement of reproductive outcome in some bird species (Eeva and Lehikoinen, 2015; Eeva et al., 2009). Females have an extra via of Pb elimination,

through eggshell formation, which results in maternal Pb transmission to the offspring during embryonic development (Lee et al., 2001; Vallverdú-Coll et al., 2015a), with negative consequences for chick health (Bunn et al., 2000; Fair and Ricklefs, 2002; Berglund et al., 2010; Vallverdú-Coll et al., 2015a).

Exposure to Pb can also affect the expression of secondary sexual traits displayed by birds, such as carotenoid-based ornaments (Vallverdú-Coll et al., 2015b). These yellow-red coloured traits function in social and sexual signalling (Goyer and Clarkson, 2001) by advertising the quality of their bearers and influencing mate choice and investment in reproduction (Alonso-Alvarez et al., 2012; K. Omland, 1996). Our previous results showed season and gender-dependent Pb effects on carotenoid-based coloration in red-legged partridges (*Alectoris rufa*) orally-exposed to Pb shot (Vallverdú-Coll et al., 2015b). We have also found negative relationships between blood Pb levels and carotenoid-based coloration in mallards (*Anas platyrhynchos*) under field conditions (Vallverdú-Coll et al., under review).

It is well known that Pb induction of oxidative stress is one of the main mechanisms of its toxicity. This consists on pro-oxidant mechanisms that promote the generation of reactive oxygen species (ROS) (e.g.: by promoting accumulation of pro-oxidant substances such as aminolevulinic acid) (Gurer and Ercal, 2000; Bechara, 1996) and/or the impairment of antioxidant defences (e.g.: inactivating enzymes by binding to sulfhydryl groups or inhibitiing metalloenzymes by antagonizing trace element metabolism) (Gurer-Orhan et al., 2004; Schrauzer, 1987), and may damage lipids, DNA and proteins (Dowling and Simmons, 2009). Carotenoids have antioxidant properties and oxidative stress can affect the expression of carotenoid-based ornaments (Pérez-Rodríguez et al., 2013). A recent experiment has shown that Pb effects on carotenoid allocation trade-offs could be mediated by Pb-induced oxidative stress (Vallverdú-Coll et al., 2015b). Furthermore, it has been shown that oxidative stress generated by Pb can affect not only the secondary sexual traits, but also sperm quality, reducing male fertility (Castellanos et al., 2015; Tvrdá et al., 2013, 2012). Moreover, sperm quality and ornaments have been suggested to be linked through oxidative stress. Helfenstein et al. (2010) proposed that the sperm of colourful males is better protected against oxidative damage, as they found that less colourful males under oxidative stress

conditions suffered greater reduction in sperm motility and increased levels of sperm lipid peroxidation compared to colourful individuals.

In Spain, shot densities above 200 shot/m^2 have been reported in sediments from some wetlands (Mateo et al., 1997) and wild birds may accidentally ingest Pb shot more than once in heavily contaminated areas. For this reason, we have evaluated here the effects of an experimental Pb shot exposure at sublethal doses on several key reproductive traits. We conducted the experiment on the red-legged partridge, a medium size game bird of high socio-economic value and at moderate risk of Pb shot ingestion in Spain (Ferrandis et al., 2008). Despite the considerable amount of literature reporting Pb effects on reproductive success in birds, very few studies have been published regarding Pb effects on avian male fertility and, to our knowledge, no literature exists on the effect of Pb on avian sperm parameters. We investigated dose-dependent effects of Pb exposure (ingestion of Pb shot) on sperm quality, egg laying process, egg quality, hatching and reproduction success, levels of dietary antioxidants and carotenoid-based coloration in both males and females, as well as Pb effects on the body condition of the offspring at birth. We also studied the relationships between carotenoid-based coloration and sperm quality considering Pb toxicity, to better understand carotenoid allocation trade-offs.

2. MATERIAL AND METHODS

2.1 Experimental design and Pb exposure

The experiment was conducted in the Dehesa de Galiana experimental facilities (Ciudad Real, Spain), with the approval of the Universidad de Castilla-La-Mancha's Committee on Ethics and Animal Experimentation. Partridges used in the current experiment were exposed to Pb for the first time during the non-breeding season in autumn, as part of a study to test Pb effects on immune responses, oxidative stress and carotenoid-based coloration (see Vallverdú-Coll et al., 2015b for more details). Partridges were individually housed in outdoor cages (95×40×42 cm) and dosed via gastric gavage with either 0 (control group, not exposed to Pb), 1 (low dose group) or 3 (high dose group) Pb shot (N = 15 males + 15 females per treatment group; exposure

day: October 23, 2012). After the autumn Pb exposure, these partridges were released together to a large outdoor pen (40 m×9 m), where they remained until the end of February 2013, when they were moved back to the cages and each one was paired with a mate of the opposite sex that had not been previously exposed to Pb. Some unexposed pairs were added to balance the experimental groups and in order to have replacement individuals (because with this type of pairing, males sometimes attack and hurt females). We divided individuals in two main groups in order to test sex-specific effects of Pb exposure on reproduction. A first group (exposed males) consisted of males used in the autumn experiment (either controls, low dosed or high dosed) paired with new, unexposed females. This group was used to study the effects of Pb on sperm quality, egg properties, pair reproduction success parameters and carotenoid-based coloration. A second group (exposed females) consisted of females used in the autumn experiment paired with new, unexposed males. This group was used to study egg properties, pair reproductive success parameters and carotenoid-based coloration. Therefore, we had two series (one per group) of control pairs (non-exposed male + non-exposed female), which were necessary to control for disturbance effects during sample collection (see below). On April 21, 2013, partridges were dosed again with the same number of Pb shot as they had received in autumn (repeated exposure). These doses were based on previous knowledge about the numbers of ingested Pb shot pellets frequently found in gizzards of upland gamebirds and waterbirds in Spain (Ferrandis et al., 2008; Mateo et al., 1997). The average mass of shot pellets used both in autumn and spring was 109 mg per shot. The group of exposed females consisted on 17 control pairs, 13 pairs exposed at the low dose and 13 pairs exposed at the high dose. The group of exposed males consisted on 18 control pairs, 11 pairs exposed at the low dose and 11 pairs exposed at the high dose. From now on, we will refer to this experiment as the spring Pb exposure. Birds were fed *ad libitum* with a mixture of wheat and commercial partridge feed (Partridge maintenance fodder, Nanta-Nutreco, Tres Cantos, Spain), with a mixture of wheat and commercial partridge feed containing corn, wheat, barley, soy, sunflower and composed by protein (15.5%), fat (2.1%), fiber (6.5%), ashes (6.0%), calcium (0.75%), phosphorus (0.68%), sodium (0.15%), methionine (0.45%) and lysine (0.80%). Tap water was also provided *ad libitum*.

Because the autumn exposure had caused treatment-related effects on carotenoid-based coloration (Vallverdú-Coll et al., 2015b), we recorded this parameter right before the spring exposure. One month after Pb exposure (May 21, 2013), we measured carotenoid-based coloration again and collected blood from the jugular vein to determine levels of Pb, vitamins and carotenoids (see below). In the group of exposed males, semen was collected once per week (see below), starting from early March in order to train males for obtaining semen samples during the experiment.

2.2 Blood Pb concentration

Whole blood samples were diluted (1:10) with 0.1% triton and analysed using graphite furnace atomic absorption spectroscopy (GF-AAS; AAnalyst800 with autosampler AS800, Perkin Elmer) (Mateo et al., 1999). We prepared calibration standards from commercial solutions containing 1 g/l of Pb (Panreac) and Mili-Q grade water. A certified reference material for Pb (blood BCR-196) was analysed to ensure the quality of the methodology within a recovery (mean ± SD) of 98 ± 12% (n=46). The detection limit was <0.51 µg/dl of Pb in blood.

2.3 Sperm collection and quality

Starting in March, we collect sperm from individuals from the exposed males group on a weekly basis. These males were separated from their females (placed in another individual cage next to their female´s cage) for two days before a sperm collection, and placed back with their female after the sampling. We collected sperm 4, 11, 16, 25, 32, 38, 44, 49, 60, 66, 72 days after Pb exposure. We used a massage technique (Burrows and Quinn, 1937; Santiago-Moreno et al., 2015) to collect semen. We did not always obtain sperm every time from every sampled male, and we recorded the % of males that ejaculated in each sampling following the standard procedure (hereafter sperm donation rate).

Following ejaculation, a semen sample was collected by capillarity using non-heparinized micro haematocrit capillary tubes (Deltalab S.L., Soda Lime Glass). The collected sperm was immediately diluted 1:1 at ambient temperature in a medium

containing glutamate (1.92 g), glucose (0.8 g), magnesium acetate ($4{\cdot}H_2O$; 0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone (Mr 10,000; 0.3 g) and 100 ml H_2O (Lake and Ravie, 1984) in 200 µl micro-eppendorf tubes. Diluted semen samples were kept refrigerated until transported to the laboratory, within a couple of hours.

To measure sperm concentration, we diluted samples in Mili-Q grade water (1:50) and used a Bürker chamber after two minutes of resting for spermatozoa counting under a microscope at 400×. We estimated the proportion of sperm with integral acrosome (acrosomal integrity, in %) using an acidic aniline blue staining method, following Santiago-Moreno et al. (2009). In a drop of 5µl of diluted semen sample, we counted between 100 and 200 cells under oil immersion at 1000× with a phase-contrast microscope. Spermatozoa classified as not showing acrosome integrity were those with a hooked, swollen, thinned or no acrosome (Wakely and Kosin, 1951) (Image 1).

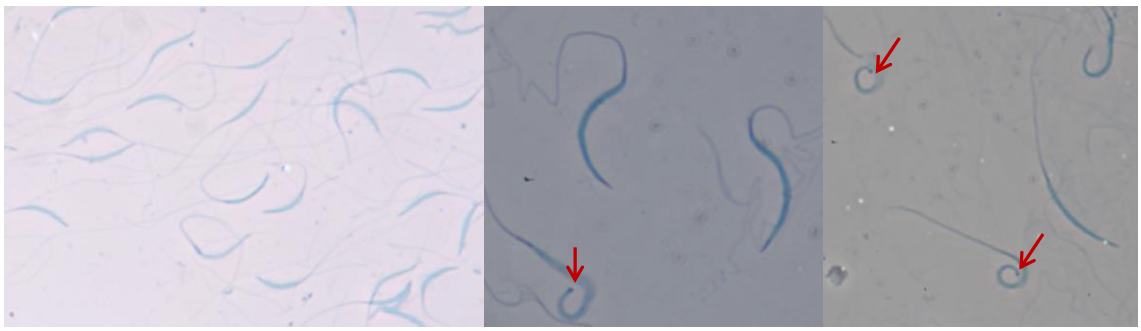

Image 1. *Red-legged partridge spermatozoa stained with acidic aniline blue staining to reveal acrosome integrity. Red arrows indicate abnormal acrosomes.*

Sperm viability was assessed using a nigrosine-eosine staining (NE) method (Tamuli and Watson, 1994). Diluted sperm (5 µl) was mixed with the NE stain at 37ºC, smeared and dried on a warm plate at 37ºC. For each sample, we counted between 100 and 200 cells under oil immersion using bright field microscopy at 1000×. Live spermatozoa remained unstained, while dead cells were dull pink. Viability (%) was expressed as the percentage of live spermatozoa (Image 2).

Sperm samples collected 5, 17, 45, 67 and 73 days after Pb exposure were transported under refrigeration after collection to the Instituto Nacional de

Investigación y Tecnología Agraria y Alimentaria (INIA) in order to measure sperm concentration and motility. Following the method described by Santiago-Moreno et al. (2012), we used a computer-aided sperm analysis (CASA) system coupled to a phase contrast microscope (Nikon Eclipse model 50i, Nikon Instruments Europe B.V., IzasaS.A.; negative contrast) and the Sperm Class Analyzer (SCA, Barcelona, Spain) v.4.0. software (Microptic S.L., Barcelona, Spain). The percentage of motile spermatozoa and the percentage showing progressive motility (spermatozoa swimming forward quickly in a straight line) were recorded. The following sperm movement characteristics were also recorded: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF). The following progression ratios (%) were calculated from the velocity measurements described above: linearity of forward progression (LIN=VSL/VCL×100), straightness (STR=VSL/VAP×100), and wobble (WOB=VAP/VCL×100). Mean values of VSL, VCL, VAP, ALH, and BCF parameters describe sperm vigour, while LIN, STR and WOB describe progressiveness.

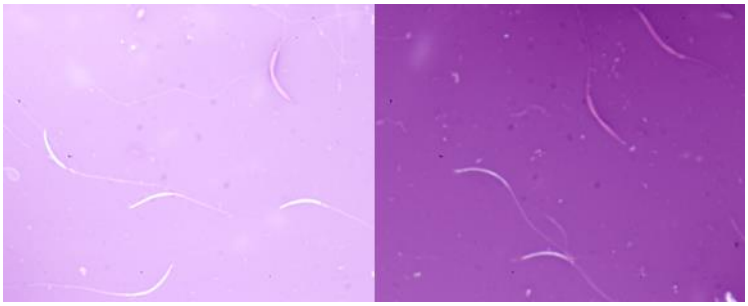

Image 2. *Red-legged partridge spermatozoa stained with nigrosine-eosine staining to reveal viability. Live spermatozoa are white, while dead cells are pink.*

2.4 Egg and hatching parameters

The group with exposed females was located in a pen where visits and disturbances were kept at minimal levels (as opposed to the group with exposed males, which was visited weekly for sperm collections). By using in each case the set of control pairs corresponding to each group, we were able to compare only individuals housed in similar conditions (i.e. visited and disturbed at similar levels).

After Pb administration, all eggs laid by each breeding pair were collected daily, individually marked, weighed, measured (maximum length and width) and stored at 15 ºC. Every 15 days, batches of eggs were transferred to an automatic incubator (Masalles Valltrade), where they were incubated at 37.7 ºC and 45% humidity with constant movement. After 21 days of incubation, eggs with a developed embryo were then transferred to a hatching chamber at 37.7 ºC and constant humidity without movement, and placed in individual boxes until hatching. Unhatched eggs were opened and we determine whether they were fecundated or not (based on the presence of an embryo or germinal disk). We calculated the volume of each egg following (Hoyt, 1979) ($V=0.00051\times length\times width^2$), egg density (d=weight/volume), and elongation index (E.I.=length/width) and determined the developmental stage of unhatched embryos in a categorical scale from 1 (germinal disk) to 5 (fully developed embryo). For each pair, we calculated laying duration (number of days from the start to the end of the laying sequence), the relative laying date end (number of days from the Pb exposure to the end of laying), the clutch size (total number of laid eggs), the frequency of laying (total number of laid eggs/laying duration) and the percentage of fecundated and hatched eggs.

At the time of Pb exposure, 11 couples had already initiated laying, and a total of 13 eggs were previously laid before treatment. These 13 eggs were taken into account when calculating clutch size, duration and frequency of laying, but were not considered when assessing individual egg parameters such as weight, width and length nor when calculating fecundation and hatching rates.

2.5 Levels of dietary antioxidants and measurement of carotenoid-based coloration

We used plasma samples to measure levels of free forms of vitamins (retinol and α-tocopherol) and carotenoids (lutein and zeaxanthin) by high performance liquid chromatography (HPLC) coupled to a photodiode detector and a fluorescence detector, following Rodríguez-Estival et al. (2010). Lutein and zeaxanthin are positional isomers. This difficult their chromatographic separation due to the similarity in their retention times because they frequently co-elute. In the current study, we found overlapped chromatographic peaks of these two carotenoids, thus we created a single

variable (lutein-zeaxanthin) representing the sum of the amounts of the two compounds.

We used high resolution digital photography to measure the redness of the beak and eye-rings of partridges (Mougeot et al., 2009). Images were taken under standardized conditions, using the same grey reference (Kodak Gray Scale, Kodak, New York) to adjust colour measurements. Using Adobe Photoshop v7.0, we measured the relative amount of the eye-ring area pigmented by carotenoids (hereafter eye-ring pigmentation, in %) and the red, green and blue (RGB) components of the beak, eye-ring and grey reference. From these, we calculated standardized beak and eye-ring redness values for each individual partridge, following Mougeot et al. (2009).

2.6 Statistical analysis

Continuous variables were checked for normality (Kolmogorov-Smirnov tests) and log-transformed (levels of Pb and lutein-zeaxanthin) or arcsine-transformed (percentages and variables related to sperm properties) to fit a normal distribution when necessary. Blood Pb concentration data below the limit of detection (LOD) were assigned values of LOD/2.

The two main groups (exposed males and exposed females) were analysed separately. We used generalized lineal models (GLM) with blood Pb levels as a dependent variable and the experimental dose as a factor to check the effects of Pb exposure. We also used GLM with blood Pb levels from the whole pool of individuals as dependent variable and sex as a factor to study possible gender differences.

The effects of Pb on sperm quality (dependent variables: viability, acrosomal integrity, concentration and parameters related to sperm motility) were analysed using generalized linear mixed models (GLMMs) including the treatment dose, a categorical "time" variable and the interaction between the treatment dose and time as fixed factors. The "time" variable was set up to reflect consecutive spermatogenesis cycles, based on the available information of spermatogenic cycle in rooster (*Gallus gallus domesticus*) (14 days of spermatogenesis, Reviers, 1968) and Japanese quail (*Coturnix coturnix japonica*) (25 days of spermatogenesis and sperm transit though the deferent ductus, (Jones and Jackson, 1972)). It was established as follows: T1: 0-20 days post Pb

exposure; T2: 21-40 days post exposure; T3: 41-60 days post exposure: T4:61-72 days post exposure. The “individual identity” was included in the GLMMs as a random factor to account for repeated measurements on the same males. Binary logistic regression was used to test for Pb effects on sperm donation rate, using the treatment dose, time and the interaction between the treatment dose and time.

We tested for effects of Pb exposure on individual egg parameters (dependent variables: mass, length, width, volume, density, elongation index, eggshell thickness, stadium of embryo development) and chick condition at birth (dependent variables: chick mass and ratio chick mass/egg mass at birth) using GLMMs including the treatment dose, time (established by categories the same way as for the sperm analysis, considering the moment when the egg was laid) and the treatment by time interaction as fixed factors. When significant effects of Pb were found on egg or chick parameters, we explored how the time elapsed since female exposure affected these parameters by running partial GLMMs for each time period after female exposure (T1: 0-20 days post Pb exposure; T2: 21-40 days post exposure; T3: 41-53). Binary logistic regression was used to study Pb effects on fecundation and hatching success, using the treatment dose, time and the interaction between the treatment dose and time. The “pair identity” was included as a random factor in all these models to account for the non-independence of eggs laid by the same female.

The treatment effects on clutch/laying process parameters (laying duration, date of end of laying, laying frequency or clutch size) were analysed using GLMs that included the treatment dose, the time and their interaction.

Spearman linear correlation coefficients (subsequently referred to as r_s) for blood Pb concentration and levels of antioxidants were calculated. We used GLMs with the experimental dose as fixed factor to test for Pb effects on carotenoid-based coloration.

In order to study relationships between sperm quality and both the carotenoid-based coloration and antioxidant levels, we run GLMs with sperm parameters (mean value of all sampling for each parameter of each individual) as dependent variables and antioxidant levels and coloration parameters as covariates. Treatment and the interaction between treatment and each of the covariates were also included to test whether Pb toxicity influenced these relationships. As the carotenoid-based coloration

was a single measurement after spring Pb exposure, the mean values sperm quality parameters were calculated to obtain a single value for each individual.

Possible effects of handling stress on individual egg parameters (dependent variables) were tested by comparing the “frequently manipulated” and “non-manipulated” control groups. We used GLMMs that included the factor “manipulation” (categorical variable), time, and the “pair identity” as a random factor. The effects of manipulation on clutch/laying process parameters (dependent variables) were tested by comparing manipulated and non-manipulated control groups using GLMs including the factor “manipulation” and time.

Normality of Pearson residuals of each model was tested by Kolmogorov-Smirnov tests. We selected final models using a backward deletion procedure in which interactions and main effects were sequentially removed when non-significant. When significant differences were found, we checked for the dose causing effects on marginal means through least significant difference (LSD) tests.

Statistical significance was set at $p \leq 0.05$. A marginally significant effect was defined as a p value within the range $0.05 < p < 0.10$. This marginal significance threshold was considered because of being studied sublethal effects of Pb, some effects may not be as obvious or marked as might occur in an acute or more continuous exposure to this pollutant as sometimes occurs under field conditions. When we found these "marginal effects" that may indicate trends or subtle variations, we studied them by marginal mean tests to detect significant differences between treatments. All statistical analyses were performed using the IBM SPSS Statistics 22.0 software.

3.RESULTS

3.1 Levels of Pb 1 month after Pb exposure

Differences among treatments in blood Pb concentration were observed in both exposed males (χ^2_2=173, p<0.001) and exposed females (χ^2_2=721, p<0.001) groups, and no differences were detected by gender. Blood Pb levels one month after exposure averaged 34.5 ± 5.31, 557 ± 29.5 and 758 ± 75.2 in the control, low dose and high dose

groups of exposed females, respectively (mean ± SE). In the exposed males group, blood Pb levels averaged 38.0 ± 4.98, 821 ± 149 and 920 ± 202 (mean ± SE) in the control, low dose and high dose groups of exposed males, respectively.

3.2 Effects of Pb on sperm quality

Table 1 summarizes the average sperm quality parameters according to male treatment group. We found a positive effect of time on ALH ($F_{2,32}$=4.21, p=0.024) and BCF ($F_{2,32}$=4.64, p=0.017). Exposure to Pb significantly affected ALH ($F_{2,32}$=4.45, p=0.020), which was higher in males exposed to 1 Pb shot than in controls (p=0.014) and in males exposed to 3 Pb shot (p=0.011). Similarly, we observed a significant effect of treatment on BCF ($F_{2,32}$=3.93, p=0.030), which was higher in males exposed to 1 Pb shot than in controls (p=0.017) and in males exposed to 3 Pb shot (p=0.019). We also found a marginal negative effect of Pb treatment on acrosomal integrity ($F_{2,86}$=2.45, p=0.096), although marginal means showed that significant differences occurred only between the high and the low dose treatment groups (p=0.030). Between 41 and 60 days post spring Pb exposure, the sperm donation rate was marginally positively affected by Pb shot ingestion (χ^2_2=4.66, p=0.097), being higher in males exposed to 1 Pb shot than in controls (p=0.026) (Figure 1). This effect coincided with the end of the period of maximum sperm production of the breeding season, as sperm donation rate was much reduced in the following period in all groups (Figure 1).

3.3 Effects of Pb exposure on egg parameters

In the case of egg mass, length, width and volume, we found significant effects of time ($F_{2,330}$=50.1, p<0.001; $F_{2,330}$=10.5, p<0.001; $F_{2,330}$=3.2, p=0.042; $F_{2,330}$=4.55, p=0.011, respectively) and significant time×treatment interactions ($F_{4,330}$=6.49, p<0.001; $F_{4,330}$=6.25, p<0.001; $F_{4,330}$=3.65, p=0.006;$F_{4,330}$=5.96, p<0.001, respectively). We further explored these effects by considering three time-periods post Pb exposure. Marginal means showed that at T1 (0-20 days post exposure), females exposed to 1 Pb shot laid eggs with increased mass (p=0.002), length (p=0.005), width (p=0.032) and volume (p=0.007) with respect to controls. At T2 (21-40 days post exposure), females exposed to 3 Pb shot laid eggs with increased mass (p=0.026), length (p=0.015) and

volume (p=0.046) when compared to controls. Eggshell thickness was unaffected by Pb exposure.

Individual egg parameters were unaffected by Pb treatment in the group of exposed males, and no effect of manipulation was observed for these study parameters when comparing the two sets of controls ("frequently manipulated" vs. "un-manipulated"; table 2).

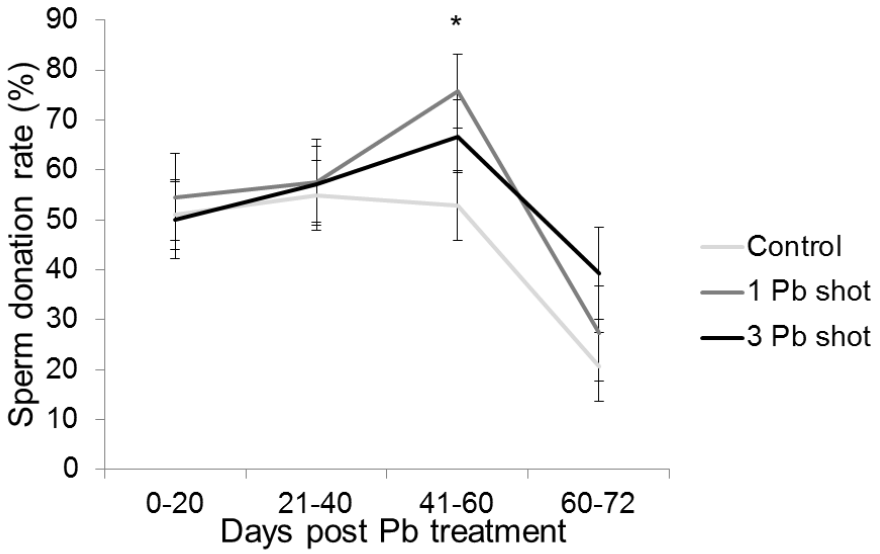

Figure 1. *Sperm donation rate (± S.E.) as a function of the time elapsed since the spring Pb exposure and treatment (exposed males group). Asterisk indicates significant differences with the control group (LSD, p<0.05).*

3.4 Effects of Pb on clutch/laying process

Hatching rate (percentage of eggs hatched out of the total laid) was negatively affected by Pb treatment in exposed females ($F_{2,341}$=3.20, p=0.042). This percentage varied from 80.5% (controls) to 62% in females exposed to 3 Pb shot (Table 2). Pb exposure did not affect the fecundation rate, clutch size or any parameter related to laying (frequency, duration, date of end) in the group of exposed females.

In the group of exposed males, we found a marginally significant Pb treatment effect on clutch size (χ^2_2=5.84, p=0.054), which was larger in pairs with males exposed

to 3 Pb shot than in controls (marginal effect, p=0.053) or pairs with males exposed to 1 Pb shot (p=0.023) (Table 2).

No effect of the manipulation was observed on the parameters studied when comparing the two sets of controls ("frequently manipulated" vs. "un-manipulated").

3.5 Effects of Pb on the offspring

In the group of exposed females, we found a significant effect of time ($F_{2, 238}$=13.78, p<0.001) and a significant time×treatment interaction ($F_{4, 238}$=2.97, p=0.020) on chick mass at birth (Figure 2a). Marginal means showed that at T1, weight of chicks from mothers exposed to 1 Pb shot was greater than weight of chicks from controls (p=0.017) and mothers exposed to 3 Pb shot (p=0.035). The chick mass/egg mass ration was unaffected by Pb treatment.

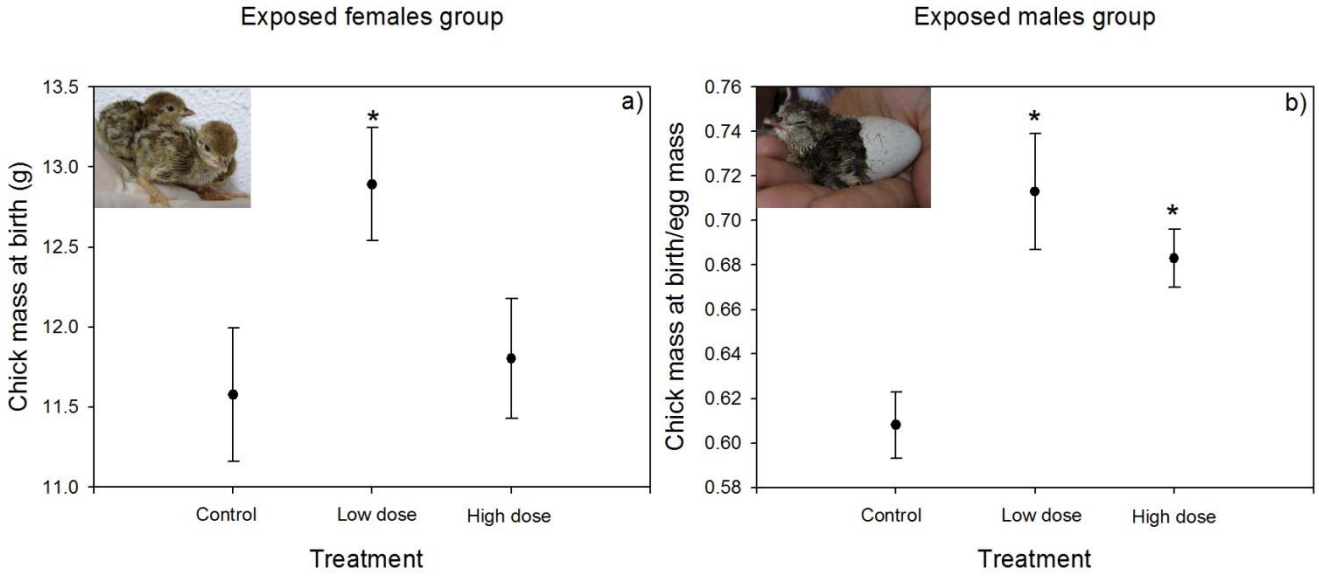

Figure 2. *Marginal means (± S.E.) for chick mass at birth (exposed females group) (a) and ratio between chick mass at birth and egg mass (exposed males group) (b) from eggs laid between 0 and 20 days after Pb exposure as a function of treatment group. Asterisk indicates significant differences with the control group (LSD, p<0.05).*

In the exposed males group, we found a significant effect of time ($F_{2, 222}$=60.4, p<0.001), a positive effect of treatment ($F_{2,222}$=6.51, p=0.002) and a significant time×treatment interaction ($F_{4,222}$=5.34, p<0.001) on chick mass/egg mass ratio (Figure

2b). Marginal means showed that the ratio was greater in chicks exposed to 1 (p=0.001) and 3 (p<0.001) Pb shot than in controls at T1. Chick mass at birth was unaffected by Pb treatment.

3.6 Plasma antioxidants and carotenoid-based coloration

In the group of exposed males, we found positive correlations between blood Pb concentration and levels of retinol (n=37, r_s=0.401, p=0.014; Figure 3a), tocopherol (n=37, r_s=0.480, p=0.003; Figure 3b) and lutein-zeaxanthin (n=37, r_s=0.548, p<0.001; Figure 3c). By contrast, in the exposed females, blood Pb levels were unrelated to levels of these dietary antioxidants.

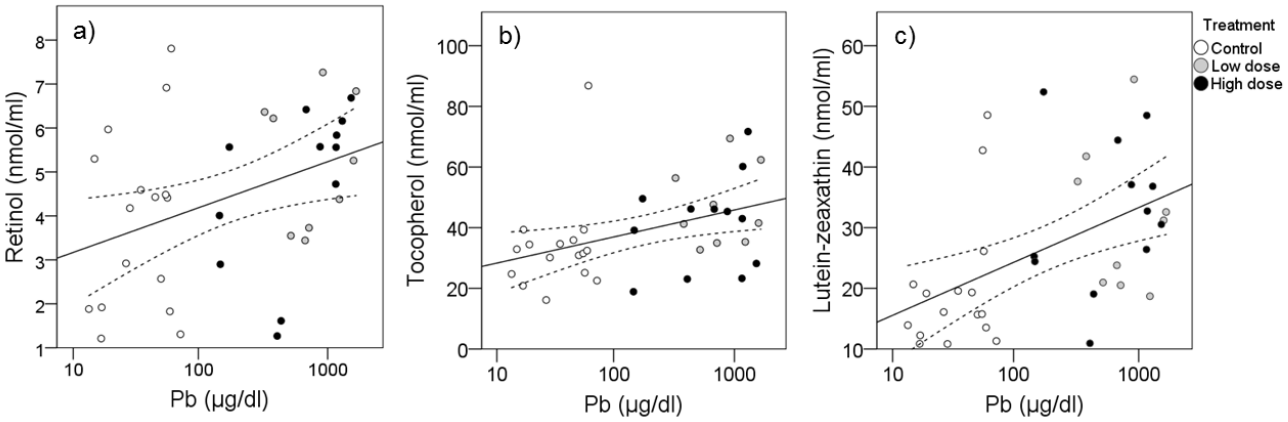

Figure 3. *Relationship between blood Pb concentration and levels of retinol (a) (n=37, r_s=0.401, p=0.014), tocopherol (b) (n=37, r_s=0.480, p=0.003) and lutein-zeaxanthin (c) (n=37, r_s=0.548, p<0.001) in plasma according to treatment (exposed males group). Note the log scale in the "x" axis (blood Pb).*

In the group of exposed females, we found differences in eye-ring redness among treatments before the spring Pb exposure (i.e. 6 months after autumn Pb exposure) (χ^2_2=9.93, p=0.007), with redder eye-rings in females corresponding to the treatments with 1 (p=0.014) and 3 (p=0.04) Pb shot than in control females (Figure 4a).

After the spring Pb exposure, the percentage of pigmented eye-ring area positively correlated with lutein-zeaxanthin levels (χ^2_1=6.37, p=0.012) and differed between treatment groups (χ^2_2=7.99, p=0.018): females exposed to 1 (p=0.015) and 3

(p=0.011) Pb shot had reduced pigmented eye-ring areas compared with controls (Figure 4b).

In the group of exposed males, we found differences in eye-ring redness between treatment groups before the spring Pb exposure (6 months after autumn Pb exposure) (χ^2_2=6.22, p=0.045). The eye-rings displayed by males of the treatment with 3 Pb shots were less red (p=0.015) than those of control males (Figure 4c). Other male coloration traits were unaffected by Pb exposure neither before nor after the spring Pb exposure.

No effect of the manipulation was observed in the parameters studied when comparing the two sets of controls ("frequently manipulated" vs. "un-manipulated").

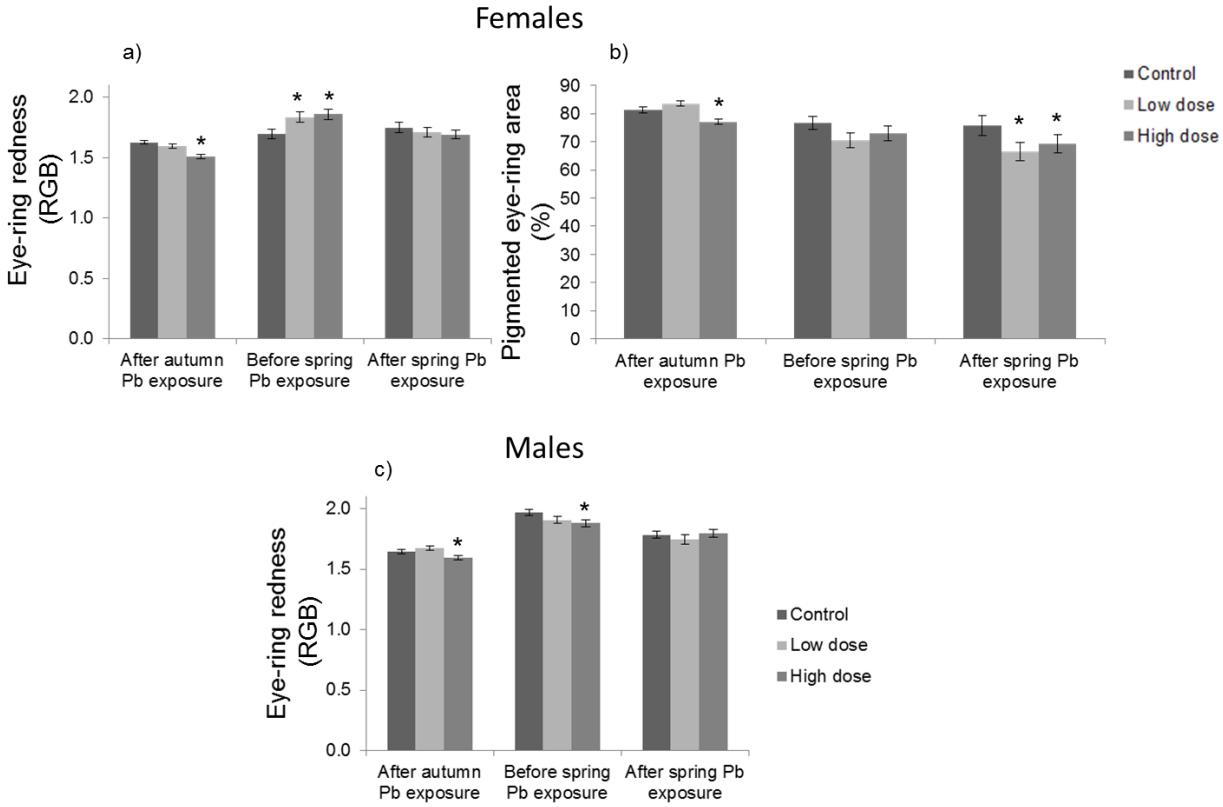

Figure 4. *Marginal means (± S.E.) for the eye-ring redness of females (a), the percentage of pigmented eye-ring area of females (b) and for the eye-ring redness of males (c) according to treatment and sampling time. See more details of "After autumn exposure" in Vallverdú-Coll et al. (2015b).*

3.7 Relationship between sperm quality, carotenoid-based coloration and Pb treatment

Acrosomal integrity (%) was positively associated with retinol levels (χ^2_1=3.84, p=0.05) and negatively affected by Pb treatment (χ^2_2=6.22, p=0.045). Marginal means showed that acrosomal integrity was lower in males exposed to the high dose than in males exposed to the low dose (p=0.049) and controls (p=0.024) (Figure 5a). Sperm concentration was positively associated with retinol levels (χ^2_1=8.52, p=0.004) and negatively associated with lutein-zeaxanthin levels (χ^2_1=10.5, p=0.001).

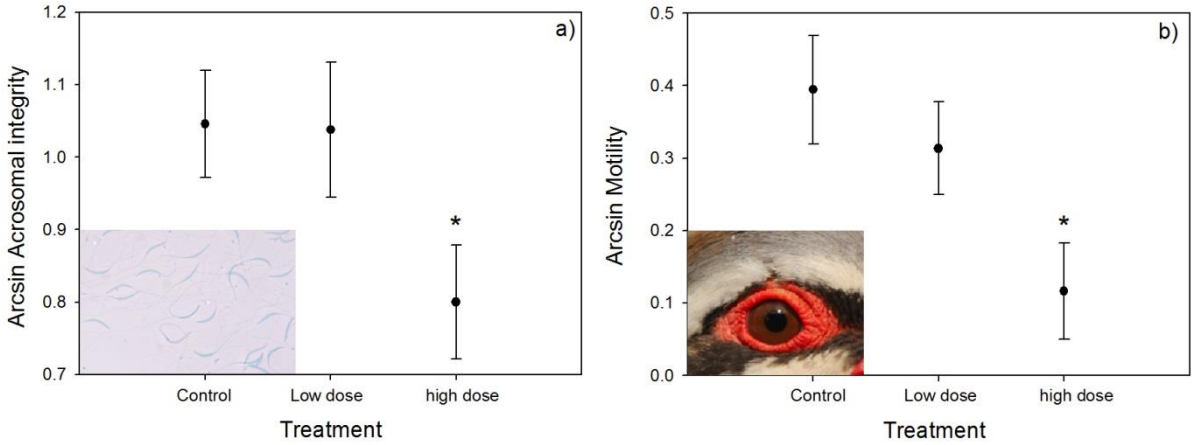

Figure 5. *Marginal means (± S.E.) of the percentage of sperm acrosomal integrity (arcsin transformation) (a) and the percentage of sperm motility (arcsin transformation) (exposed males group) as a function of treatment group. Asterisk indicates significant differences with the control group (LSD, p<0.05).*

Sperm motility (%) was positively associated with levels of tocopherol (χ^2_1=7.56, p=0.006) and eye-ring redness (χ^2_1=4.05, p=0.044). In addition, we found a negative effect of Pb treatment, which became significant only when these two covariates were considered into the GLM. Males with redder eye-ring had higher sperm motility, and marginal means showed that sperm motility was lower in individuals exposed to 3 Pb shot than in males exposed to 1 Pb shot (p=0.027) and controls (p=0.008) (Figure 5b). Percentage of non-progressive motility positively related to tocopherol (χ^2_1=3.96, p=0.047) and eye-ring redness (χ^2_1=8.23, p=0.004).

Regarding VSL and VAP, we found a significant effect of Pb treatment (χ^2_2=6.53, p=0.038; χ^2_2=6.31, p=0.043, respectively) and the interaction between treatment and

eye-ring redness (χ^2_2=6.45, p=0.040; χ^2_2=6.28, p=0.043, respectively). Marginal means showed that Pb treatment tended to increase VSL and VAP at the low dose of Pb, but non-significant differences among treatments were found. Furthermore, we detected an outlier in both parameters corresponding to the same individual that could be interfering with the results (see Figure 6). So we re-analysed data without considering this individual. In the new analyses, we found a positive association with eye-ring redness (χ^2_1=5.21, p=0.023) and a significant effect of Pb treatment (χ^2_2=7.81, p=0.020) on VSL. Marginal means showed that sperm from males exposed to 1 Pb shot presented larger VSL than controls (p=0.005). Similarly, we found a positive association between VAP and eye-ring redness (χ^2_1=6.28, p=0.043) and a significant effect of treatment (χ^2_2=6.31, p=0.043). Marginal means showed that sperm from males exposed to 1 Pb shot presented larger VAP than controls (p=0.004).

All the other coloration parameters or measures of antioxidant levels were unrelated to sperm quality parameters or between them.

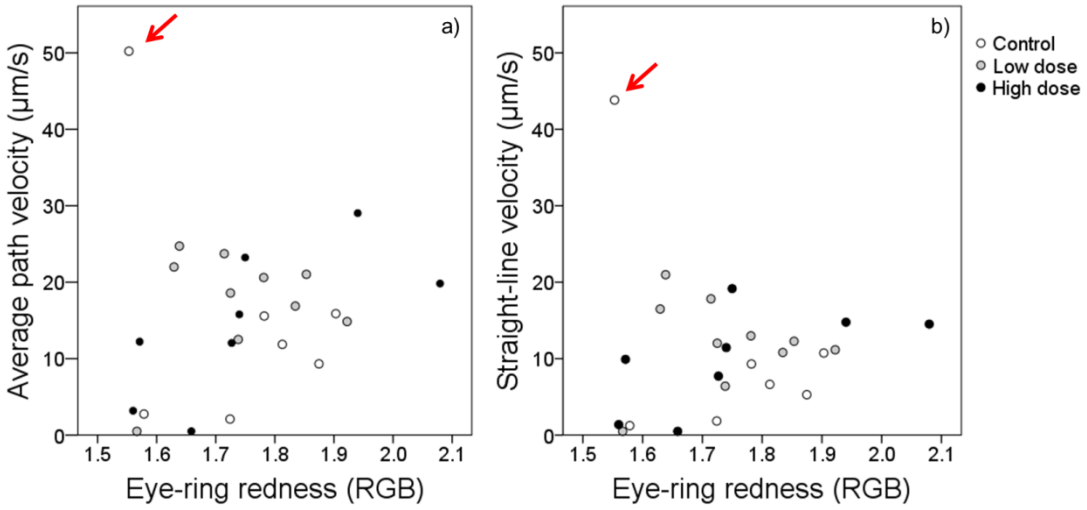

Figure 6. *Relationship between eye-ring redness and average path velocity (AVP) (a) and straight-line velocity (VSL) (b) of sperm according to treatment (exposed males group). Outliers are marked by red arrows.*

Table 1. Sperm quantitative, qualitative, morphological and motility variables for the control and Pb exposed red-legged partridges.

	Treatment					
	Control		Low Pb dose		High Pb dose	
Parameter	N	Mean ± S.E.	N	Mean ± S.E.	N	Mean ± S.E.
Number of pairs		18		11		11
Sperm donation rate (%)	18	47.1 ± 3.7	11	56.2 ± 4.5	11	54.5 ± 4.0
Concentration (spermatozoa/ml)	13	703 ± 287	10	653 ± 214	14	457 ± 221
Viability (%)	29	72.4 ± 3.21	23	78.5 ± 3.45	26	71.8 ± 3.86
Acrosomal integrity (%)	36	65.7 ± 4.58^{ab}	25	75.6 ± 3.61^{a}	28	56.3 ± 6.70^{b}
Motility (%)	12	17.7 ± 5.7	10	20.1 ± 6.7	14	14.6 ± 6.4
Progressive motility (%)	13	0.87 ± 0.41	10	1.89 ± 0.56	14	2.69 ± 2.0
Non progressive motility (%)	13	12.7 ± 2.86	10	15.32 ± 3.89	14	12.1 ± 2.98
Curvilinear velocity (VCL) (μm/s)	13	21.6 ± 4.5	10	27.3 ± 3.7	14	22.1 ± 4.7
Straight-line velocity (VSL) (μm/s)	13	8.8 ± 3.1	10	11.6 ± 1.8	14	10.4 ± 3.1
Average path velocity (VAP) (μm/s)	13	12.9 ± 3.6	10	17 ± 2.3	14	14.9 ± 3.6
Amplitude of lateral head displacement (ALH) (μm)	13	0.8 ± 0.3^{a}	10	2.1 ± 0.4^{b}	14	0.8 ± 0.3^{a}
Beat-cross frequency (BCF) (Hz)	13	2.7 ± 1^{a}	10	6.5 ± 1^{b}	14	3 ± 0.9^{a}
Linearity of forward progression (LIN) (%)	13	33 ± 5.2	10	38.2 ± 5.2	14	35.3 ± 6.2
Straightness (STR) (%)	13	56.6 ± 6.3	10	60.3 ± 7.4	14	53.5 ± 7.8
Wobble (WOB) (%)	13	51.6 ± 5.4	10	56.4 ± 6.5	14	53.8 ± 6.8

[a,b]Different letters indicate differences in marginal means at $p \leq 0.05$. The N size for percentages of viability and acrosome integrity are higher than the number of pairs because they correspond to repeated, weekly samplings, while the other parameters were only studied on specific dates (see methods).

Table 2. Mean (± S.E.) reproductive parameters and dietary antioxidant levels one month after the spring Pb exposure for control and red-legged partridges exposed at the low and high dose. Effects of manipulation were also checked by comparing controls between the two groups, and no significant differences were detected for any variable.

	Exposed females group			Exposed males group		
Parameters	Control	Low dose	High dose	Control	Low dose	High dose
Number of pairs	17	13	13	18	11	11
Number of laying females	16	13	12	18	11	11
Total number of eggs	133	111	100	121	64	107
Egg length (mm)	38.6 ± 0.11[a]	39.0 ± 0.13[b]	39.2 ± 0.16[b]	38.7 ± 0.134	38.5 ± 0.167	38.9 ± 0.14
Egg width (mm)	29.6 ± 0.08[a]	29.7 ± 0.07[b]	29.53 ± 0.1[a]	29.7 ± 0.072	29.3 ± 0.136	29.4 ± 0.095
Egg mass	17.4 ± 0.15[a]	17.8 ± 0.12[b]	17.6 ± 0.18[b]	17.5 ± 0.138	17.1 ± 0.223	17.5 ± 0.149
Elongation Index	1.31 ± 0.003	1.31 ± 0.004	1.33 ± 0.004	1.31 ± 0.004	1.31 ± 0.006	1.32 ± 0.005
Egg volume	17.3 ± 0.12[a]	17.6 ± 0.11[b]	17.5 ± 0.18[b]	17.4 ± 0.123	16.9 ± 0.203	17.2 ± 0.144
Egg density	1.006 ± 0.005	1.012 ± 0.004	1.007 ± 0.005	1.005 ± 0.004	1.010 ± 0.006	0.011 ± 0.005
Duration of laying (days)	33.4 ± 3.80	38.0 ± 3.47	33.7 ± 4.37	27.2 ± 4.35	26.09 ± 5.28	35.3 ± 4.7
End of laying (days since Pb exposure)	50.3 ± 0.597	49.8 ± 0.833	49.3 ± 0.585	48.7 ± 1.097	45.6 ± 3.84	51.5 ± 0.50

Clutch size per laying female	8.18± 0.9	9.15 ± 0.697	7.54± 1.07	6.89 ± 0.89[ab]	6.0 ± 1.28[a]	9.73 ± 1.22[b]
Frequency of laying	0.278 ± 0.020	0.257 ± 0.023	0.234 ± 0.024	0.278 ± 0.026	0.248 ± 0.030	0.284 ± 0.019
Fertility (%)	91.7 ± 0.23	93.8 ± 0.023	78.0 ± 0.041	96.7 ± 1.6	90.6 ± 3.6	93.8 ± 2.2
Hatching rate (%)	80.5 ± 0.034[a]	83.9 ± 0.035[a]	62.0 ± 0.049[b]	86.0 ± 3.2	73.4 ± 5.5	86.0 ± 3.4
Development stage of un-hatched embryos (1-5)	4.16 ± 0.236	3.57 ± 0.582	3.07 ± 0.511	2.91 ± 0.364[a]	3.45 ± 0.427	4.13 ± 0.493
Chick mass at birth (g)	12.3 ± 0.102[a]	12.5 ± 0.110[b]	12.3 ± 0.19[a]	12.3 ± 0.138	12.3 ± 0.231	12.3 ± 0.131
Chick mass at birth/egg mass	0.705 ± 0.007	0.701 ± 0.006	0.702 ± 0.008	0.697 ± 0.006[a]	0.712 ± 0.009[b]	0.708 ± 0.006[b]
Retinol (nmol/ml)	4.73 ± 0.50	5.44 ± 0.324	4.8 ± 0.32	3.86 ± 0.050	5.23 ± 0.50	4.69 ± 0.533
Lutein-Zeaxanthin (nmol/ml)	6.03 ± 0.54	6.83 ± 0.59	7.39 ± 0.55	19.8 ± 2.74[a]	31.3 ± 3.95[b]	32.4 ± 3.52[b]
Tocopherol (nmol/ml)	28.7 ± 3.04	37.7 ± 2.36	34.02 ± 3.94	33.6 ± 3.91[a]	46.8 ± 4.37[b]	41.2 ± 4.56[a]

[a,b] Indicate differences in marginal means in GLM at $p \leq 0.05$.

4. DISCUSSION

Red-legged partridges experimentally treated with a second Pb exposure showed significant changes in some of the reproductive parameters studied here, such as sperm quality in exposed males, egg properties in exposed females, and final reproductive outcome in both males and females. Moreover, we found sex-specific effects of Pb exposure on carotenoid allocation and carotenoid-based coloration.

In Pb exposed males, we detected several effects on sperm characteristics that could be hormetic responses, especially at the low exposure dose. We found that males exposed to Pb at the high dose had sperm with lower percentages of acrosomal integrity as observed before in mammals (Castellanos et al., 2015; Oliveira et al., 2009; Reglero et al., 2009), but this effect was not associated with lower sperm viability or reduced fertilization rate. Exposure to Pb was also associated with a reduction of the percentage of sperm motility (Akinola et al., 2015; Oliveira et al., 2009) when coloration and levels of antioxidants were considered. On the contrary, exposure to Pb at the low dose was related to the increase of four parameters (VSL, VAP, ALH and BCF) related to the velocity and the type of motility of spermatozoa. Overall, we found a lower percentage of moving sperm in Pb exposed males, but those who move did it more intensely. These alterations did not result in changes in the overall progressiveness of spermatozoa, as none of the three studied progression ratios were affected, which reflects an increase in the vigour of spermatozoa but not in progressiveness. Similar effects of Pb on ALH have been reported in rabbits (Moorman et al., 1998) and humans (Wildt and Berlin, 1983; Osorio et al., 1993). Several studies have confirmed Pb-induced oxidative stress in semen and its relationship with impaired sperm quality (Tvrda et al., 2012; Tvrdá et al., 2013; Castellanos et al., 2015). Antioxidant supplementation has also been shown to improve motility parameters such as ALH (Xia et al., 2012). In fact, we have found that acrosomal integrity, sperm concentration and motility were positively related to antioxidant levels. The observed increases in ALH, BCF, VAP and VSL could be therefore in part associated with increases in the levels of plasmatic antioxidants that we observed in Pb exposed males. This may also be an attempt to protect the organism against oxidative stress, which has been shown to occur in Pb exposed red-legged partridges during the breeding

season (Vallverdú-Coll et al., 2015b). In species that are not strictly monogamous, like red-legged partridges (Casas et al., 2006), factors related to sperm motility are crucial in determining which male will fertilize an egg (Denk et al., 2005). Sperm competition promotes qualities such as faster spermatozoon swimming velocity (Santiago-Moreno et al., 2014). A sperm donation rate of Pb exposed males at the end of the maximum ejaculatory period of the breeding season, which is supposed to last until May (Santiago-Moreno et al., 2015), may possibly reflect a strategy to ensure egg fertilization.

A Pb-induced reduction of sperm concentration has been described by others (Akinola et al., 2015; Dauwe et al., 2004), but has not been observed here. Contrary to our findings, Akinola et al. (2015) reported increased percentages of dead sperm cells in juvenile rats. In addition, Pb is known to increase sperm chromatin condensation, which is related to male infertility (Hernández-Ochoa et al., 2006). Moreover, the use of fertility as an indicator of toxic effect to spermatozoa should be accompanied with its relationship with reproductive outcome (Friedler, 1996). In our experiment, we observed some slight sperm alterations, possibly because the Pb doses were not enough to induce death of spermatozoa. This is also consistent with a lack of effects on fecundation or hatching rates in the group of exposed males. It should be considered that in most samplings we were able to collect sperm from approximately 50% of individuals (see Figure 1). Near the end of the experiment, we collected sperm from most males in some samplings, but several males did not ejaculate regularly (we were able to get weekly samples from only a few individuals (see Figure 1). This could be explained by a lack of a long-term training of these individuals to ejaculation massage in order to reduce the stress. The use of trained individuals to ensure an appropriate sample size should be considered in future experiments.

Furthermore, the Pb exposure of males affected the laying performance of their mate at the high dose, which increased clutch size. The hypothesis of reproductive compensation predicts that females should increase reproductive effort when they mate with males of lower quality, in order to compensate potential negative effect on offspring fitness (Gowaty et al., 2007). Although only one of the mates was exposed to Pb in our experiment, field exposure to heavy metal pollution

usually affects both genders of wild birds and this finally results in a decrease in clutch size (Eeva et al., 2009; Janssens et al., 2003a, 2003b).

The hypothesis of reproductive compensation has been also used to explain the increased sperm quality (i.e. sperm number per ejaculate) in males mated with females that did not prefer these males (Gowaty et al., 2007). In our case, we detected increased sperm motility parameters in Pb exposed males, but we did not found an increase in the sperm concentration. An explanation of the increased vigour and velocity of sperm motility in Pb exposed males may come from the interaction of Pb^{2+} on the physiological mechanisms mediated by Ca^{2+} able to produce the capacitation process of spermatozoa, which includes increased motility at early stages as well as increased membrane permeability (Suarez, 2008) followed by the acrosome reaction (Castellanos et al., 2013, 2008). Effects of Pb on capacitation process may be produced by competition with Ca^{2+} in the uptake on Ca^{2+} and voltage-gated potassium channels. These effects may be involved in cytoplasm alkalinisation and membrane hyperpolarization that are necessaire to activate flagellar movement (Demarco et al., 2003; Visconti et al., 2011). Another way to modify capacitation is by directly affecting antioxidant defence which derives in membrane peroxidation (Hsu et al., 1998b).

The Pb exposure of females affected egg properties, with similar effects on eggs from females exposed at the low and high Pb doses, but at different moments of the laying period. Females exposed to 1 Pb shot laid larger and heavier eggs during the first 20 days post exposure, and the same effect was observed in females exposed to 3 Pb shot but at 20-40 days post exposure. This difference in time when Pb effects occur could be explained because such effects on eggs could be associated with a specific blood Pb level. Blood Pb levels in females exposed to the high Pb dose are very high during the first 20 days, but may progressively diminish as the body assimilates or eliminates Pb, thus blood Pb levels in females exposed to the high dose of Pb might be similar at T2 to Pb levels of females exposed at the low dose at T1. Mass differences between eggs of the same size have been attributed to increases in water content in the albumen fraction (Pearson et al., 2002), but in the current study the increased mass was associated with an increased volume. The laying of larger eggs has been associated with disproportionately larger yolks and lipid stores in precocial species, which results in higher hatchling energy densities and yolk reserves (Østnes et al.,

1997) and may imply a greater investment of carotenoids into eggs from the mother. In fact, chicks from females exposed to the low Pb dose presented greater mass at birth. These chicks proceeded from eggs laid between 0 and 20 days post exposure that were also heavier than eggs from the other groups. On the contrary, larger and heavier eggs from females exposed at the high Pb dose did not result in heavier chicks, and this hypothetical higher input of energy was not enough to keep the hatching rate at the same level than in control females. Exposed males did not induce the production of larger or heavier eggs in their mates. Curiously, these females mated with exposed males produced chicks with greater ratio between chick mass at birth and egg mass, which may reflect a higher chick development, again in agreement with the hypothesis of reproductive compensation. Contrary to the results reported here, Vallverdú-Coll et al. (2015a) observed a lower ratio between body mass at birth and egg mass in mallard ducklings affected by maternal Pb transfer under field conditions. They additionally found a positive relationship between blood Pb levels of ducklings and both eggshell Pb levels and thickness (post-hatching). Here we did not find differences in eggshell thickness regarding Pb treatment. The reduced hatching rate could be explained by an altered eggshell composition due to Pb deposition which may interfere with water vapour transport through the eggshell, as its permeability is determined by pore geometry that is fixed during eggshell formation (Wangensteen and Rahn, 1970). Any significant change in the gas permeability of the shell can have a deleterious effect upon the embryo and the survival. A too low permeability would produce hypoxia and hypercarbia, whereas a too high permeability would result in water loss (Wangensteen and Rahn, 1970). Some authors have similarly reported reduced hatching success in great tits (*Parus major*) breeding in areas polluted with heavy metals, including Pb (Eeva et al., 2009; Janssens et al., 2003a, 2003b), but others found no effect (Dauwe et al., 2005). Smaller clutches and decreased health status and survival of the offspring have also been described in great tits affected by heavy metal pollution (Eeva et al., 2009; Janssens et al., 2003a, 2003b). Furthermore, Eeva & Lehikoinen (2015) found an improvement of several breeding parameters (i.e.: eggshell quality, clutch size, hatchability, fledgling number) in pied flycatcher (*Ficedula hypoleuca*) after the reduction of smelter emissions.

The secondary sexual traits or ornaments displayed by birds, including carotenoid-based traits, have been shown to indicate individual quality and are often used to optimize mate choice and reproductive decisions (Horvathova et al., 2012). Due to their role as immune-stimulants and antioxidants (Pérez-Rodríguez, 2009), carotenoids may be key molecules in the physiological trade-off between reproduction (i.e. coloration) and self-maintenance (Alonso-Alvarez et al., 2008). When the same individuals studied here were exposed to Pb for the first time during the non-breading season (autumn Pb exposure), eye-ring and beak redness were reduced in both genders, and the percentage of pigmented eye-ring area was reduced in females (Vallverdú-Coll et al., 2015b). During the non-breeding season, Pb-exposed partridges therefore prioritized self-maintenance (i.e.: oxidative balance) at the expense of investment in ornamental coloration (Vallverdú-Coll et al., 2015b). In the current experiment, we have shown that among the above-mentioned effects of the autumn exposure on coloration, only a reduced eye-ring redness in high-exposed males remained until the next breeding season (six months later). We have also found that eye-ring redness was greater in Pb exposed females than in controls before the spring Pb treatment, which may be a strategy to increase mate investment in reproduction. Alonso-Alvarez et al. (2012) showed that female red-legged partridges increased their breeding investment when male red ornamentation was artificially increased. Our observations suggest that such an effect could be reciprocal (i.e. female coloration may also modulate male investment in reproduction), although this would need to be experimentally tested. In addition, resources allocated to the current reproduction provide an immediate biological gain, but at the expense of survival and future reproduction (Bell, 1980). Therefore, our observation could also reflect a “terminal investment” strategy, which occurs in birds when survival or future breeding opportunities are compromised (Velando et al., 2014). After the spring exposure, Pb exposed females maintained levels of circulating carotenoids and eye-ring redness similar to those of controls, but had a reduced percentage of pigmented eye-ring area. This reduced allocation of carotenoid to colour the eye rings may be explained by an increased carotenoid allocation to the egg yolk in order to protect the embryo from oxidative stress (Pérez-Rodríguez, 2008), which would be consistent with the

observation that these females also laid larger and heavier eggs, and produced heavier chicks.

In another study, we have seen that in partridges exposed to Pb for the first time during the breeding season, Pb exposure increased eye-ring redness in exposed males and reduced the percentage of pigmented eye-ring area in females, and reduced levels of dietary antioxidants in both genders (Vallverdú-Coll et al., 2015b). On the contrary, males exposed at the high Pb dose had reduced eye-ring redness before the spring exposure coinciding with the period of highest red colour expression in this species, prior to the laying period (Pérez-Rodríguez, 2008b), but this effect did not last as the breeding season progressed and after the spring Pb exposure. Throughout the laying period, eye-ring redness tended to decrease in all groups (Figure 4) and differences between treatments disappeared, so we have not found any Pb effect on coloration of males in the spring exposure studied here. However, unlike the autumn exposure, the spring Pb exposure increased levels of circulating antioxidants, which may be a mechanism to keep these available to fight against oxidative stress (Alonso-Alvarez et al., 2008). The reduction on the percentage of pigmented eye-ring area in females seems to be a consistent effect of Pb in this gender, observed during both the autumn and the spring exposure.

We confirmed our hypothesis regarding positive relationships between sperm quality and carotenoid-based coloration as we found positive relationships between sperm parameters and coloration that were also interrelated with antioxidant levels. Our results suggest that colourful males may have greater sperm motility and velocity associated with higher levels of tocopherol. In addition, proper antioxidant levels (e.g.: higher levels of retinol) seem to be related with higher sperm concentration and acrosomal integrity which might be related to a greater capacity to keep oxidative balance. Furthermore, Pb toxicity seems to modulate these relationships, as it is responsible for the increase of circulating levels of such antioxidants but at the same time induces negative effects on these functions, as in the case of reduced motility and acrosomal integrity that are related to higher levels of retinol and tocopherol, respectively.

In summary, a second Pb exposure during the prelaying period reduced the acrosomal integrity and the percentage of sperm motility at the high dose, and

increased the vigour of spermatozoa at the low dose, but did not affect the viability, concentration or overall progressiveness of spermatozoa. Females mated with Pb exposed males increased clutch size and ratio between chick mass at birth and egg mass, reflecting an enhanced reproductive effort. Exposure to Pb increased levels of circulating antioxidants in males possibly as a way to cope with Pb-induced oxidative stress. Pb exposed females laid heavier and larger eggs that resulted in heavier chicks at the low dose, but had a lower hatching success than non-exposed females at the high dose, while keeping a similar fecundation ratio. At the beginning of the breeding season, females previously exposed to Pb during autumn display redder eye-rings, which may reflect an increased investment in reproduction. However, after the second Pb exposure, the percentage of eye-ring pigmented area decreased, and available carotenoids were allocated to needs other than coloration, such as antioxidant defences or allocation into the egg-yolk. Several sperm parameters showed positive relationships with carotenoid-based coloration and levels of antioxidants that were influenced by Pb, suggesting that redder males may be more capable to preserve sperm from oxidative stress.

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General discussion

1. Use and accumulation of lead ammunition: regulation and compliance

Despite international efforts to limit the use of lead (Pb) ammunition and several attempts to reduce the involved risks, this issue is not solved and remains a worldwide concern for human and wildlife health. The Agreement on the Conservation of African-Eurasian Migratory Waterbirds (AEWA) dependent of the Convention on the Conservation of Migratory Species of Wild Animals (CMS) has celebrated several conferences promoting actions in this direction. For instance, the Parties of this agreement should have eliminated the use of Pb shot for hunting in wetlands by the year 2000, and it has to be banned by 2017 through collaboration between all stakeholders to establish and implement joint communication strategies (AEWA, 2012). More recently, the Parties of the AEWA were asked to proceed to a rapid full phase-out of the use of Pb ammunition in all habitats (wetlands and terrestrial) and its replacement with non-toxic alternatives to protect migratory birds from poisoning (UNEP/CMS, 2014). The problem with these guidelines is that, according to the European Commission, each Party may determine whether or not and how to implement them. For instance, although some Parties implemented a full ban on the use of Pb pellets in the past (e.g.: Norway, Denmark, Netherlands, Sweden) (Mateo, 2009), the Norwegian parliament recently resolved to reintroduce Pb shot for hunting outside wetlands (February 2015) after a lengthy campaign by the Norwegian Association of Hunters and Anglers, and the same happened in Finland. In the case of Spain, where the use of Pb shot for hunting in wetlands was banned in protected areas in October 2001, it is still unclear how the full Pb restriction will be implemented, and such decision might depend on regional governments.

Risks to humans in addition to environmental and wildlife health have also been considered. Two groups of scientific experts in Pb and environmental health from the US (2013) (Bellinger et al., 2013) and Europe (2014) (Bernhoft et al., 2014) signed a consensus statement highlighting the scientific evidence on the toxic effects of Pb on human and wildlife health and supported the reduction and elimination of Pb ammunition, based upon (1) evidence for the toxic effects of Pb in humans and wildlife, even at very low exposure levels, (2) convincing data that the discharge of Pb-based ammunition into the environment poses significant risks of exposure to humans

and wildlife, and (3) the availability and suitability of several non-Pb alternative products for hunting (Bernhoft et al., 2014), and empathized that other sources of Pb exposure (e.g.: plumbing, paints, petrol) have been reduced by regulation. This information has been recently updated with further independent research carried out in countries across the European Union (EU) in 2015, which further strengthens the arguments mentioned before and shows that the availability and suitability of non-Pb alternative products for hunting can be ensured (Gremse et al., 2015).

The effectiveness of a properly enforced ban on Pb ammunition in protected wetlands with compliance levels that exceeded 80% has been demonstrated in the US and Canada (Havera et al., 1994; Stevenson et al., 2005). In the Ebro delta, the high degree of compliance (98%) was accompanied by beneficial effects such as reduced Pb exposure for game meat consumers as well as reduced Pb poisoning in waterbirds (Chapter 1). By contrast, a lower degree of compliance (about 30%) (Cromie et al., 2010) and a high percentage (34%) of trapped waterbirds showing elevated blood Pb levels (i.e. >20.0 µg/dL) (Newth et al., 2013) were found 10 years after the ban on Pb shot in England. Similarly, 30.6% of mallards (*Anas platyrhynchos*) trapped in the Ebro delta six years after the prohibition showed elevated blood Pb levels that dropped to 13.9% thereafter (Chapter 2). In the USA and Canada (Anderson et al., 2000; Stevenson et al., 2005), as well as in the Ebro delta (Chapter 1), compliance with the Pb ban appears to be high because of the cooperative attitude of waterfowl hunters for the non-toxic shot program and of active enforcement led by conservation police officers or local authorities.

It has been reported (Chapter 1) that the percentage of hunted mallards showing Pb levels above the EU maximum residue level (MRL) declined coinciding with increased compliance and the lowest observed prevalence of Pb shot ingestion, but increased again at the end of the study period. It poses a risk for hunters and their families (Sevillano Morales et al., 2011), as Pb-contaminated game meat intake has been associated with a reduction in intelligence quotient (IQ) in children, increased systolic blood pressure, occurrence of chronic kidney disease, and rates of spontaneous abortion (Green and Pain, 2012). In order to effectively reduce the risk of Pb exposure to humans and poisoning of waterbirds, prohibitions applied in protected

wetlands should be extended to adjacent feeding grounds such as in adjacent rice fields (Newth et al., 2013). Hence, the overall Pb shot ingestion rates by mallards only decreased by ~50% during the period from 1991–96 to 2011–12 (Chapter 1). The fact that a reasonably good ban compliance was obtained in protected areas within a short period, and that hunting bag counts were apparently unaffected by the change to steel ammunition (Mateo et al., 2013), also supports the goal to extend the Pb ban to other ecologically important habitats.

2. Level of lead exposure and induction of oxidative stress

An assessment of blood Pb levels in wild mallards trapped in the Ebro delta allowed us to report real values of Pb exposure in this partially protected wetland, and to conduct subsequent correlative studies between these levels and the studied parameters. Blood Pb concentration tends to reflect recent exposure, within 35–40 days of testing (Halloran et al., 1988), and the values observed in wild mallards (Chapter 2) were comparable to Pb levels detected in blood of red-legged partridges (*Alectoris rufa*) experimentally dosed with 1 and 3 Pb shot after 21 days of exposure (Chapter 4).

Females tended to present greater blood Pb levels than males at the beginning of the breeding season (Chapters 2 and 4), probably because of an increased Pb absorption associated with its similarity to calcium (Ca) (Tejedor and Gonzalez, 1992). Mobilization of Ca from female bone for formation of both the eggshell and egg content may also induce Pb mobilization (Guirlet et al., 2008), as it was confirmed with the presence of maternally transferred Pb into the eggshell of mallard eggs collected from the Ebro delta (Chapter 3), which may represent a way of Pb elimination in females.

One of the main objectives of this thesis was to study the induction of oxidative stress as a mechanism of Pb toxicity that produces adverse, sublethal effects on birds. The suppression of delta-aminolevulinic acid dehydratase (δ-ALAD) is a very Pb-specific pathway to induce oxidative stress and has been a consistent effect reported throughout this work (Espín et al., 2015; Martinez-Haro et al., 2011b; Chapters 2, 3 and

4). δ-ALAD activity has been negatively associated here with activity of antioxidant enzymes and levels of oxidative stress biomarkers in wild mallards (Chapters 2 and 3), which supports the idea that the suppression of its activity is related to oxidative imbalance (Figure 1). In addition, greater levels of blood Pb were positively associated with levels of oxidative stress biomarkers and endogenous antioxidant (total glutathione, tGSH) (Chapters 2, 3 and 4) (Figure 1). Increased levels of tGSH (Mateo and Hoffman, 2001) may result from the induction of g-glutamylcysteine synthetase, an enzyme involved in the hepatic synthesis of GSH, by Pb (Griffith, 1999). Similarly, levels of dietary antioxidants have been positively associated with levels of Pb during the breeding (Chapters 2 and 5) and non-breeding seasons (Chapter 4) (Figure 1). This may be due to a compensation response of the organism to cope with the oxidative stress generated by Pb poisoning (Alonso-Alvarez et al., 2008; Martinez-Haro et al., 2011b; Matović et al., 2015) and it appears to be associated with repeated or chronic exposure to Pb. As reviewed by Koivula and Eeva (2010), birds may be more resistent to axidative stress than other species due to their ability to modulate their enzyme activities and detoxification systems in relation to pollution levels (Fossi et al., 1991).

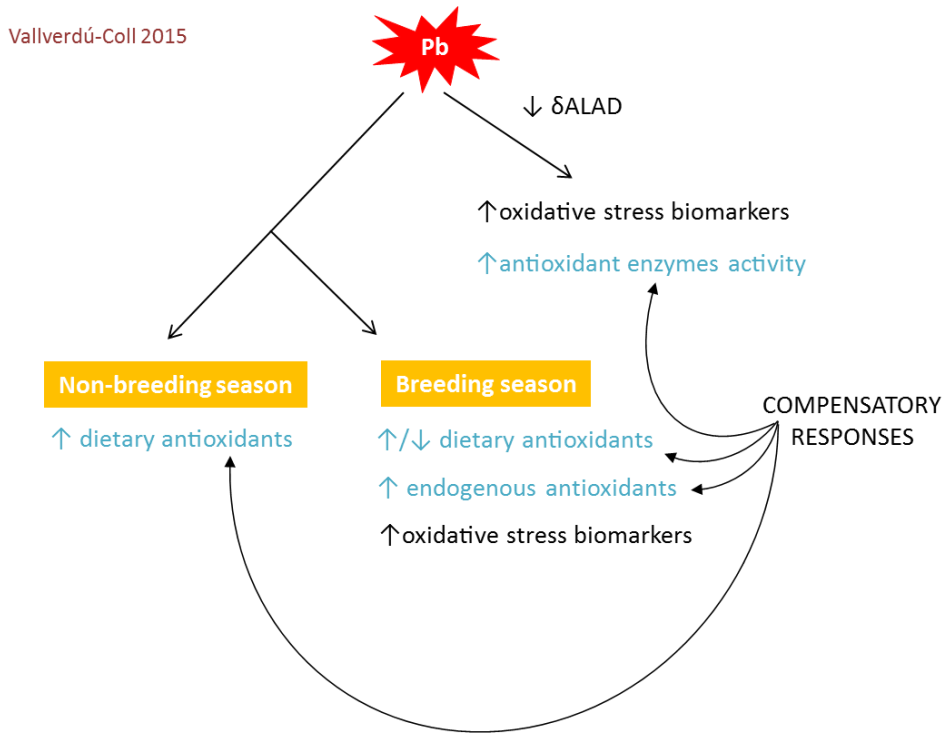

***Figure 1**: Alterations of the antioxidant system observed after Pb exposure. δ-ALAD: delta-aminolevulinic acid dehydratase.*

3. Effects of Pb shot ingestion on immune response and its relationship with oxidative stress

In wild adult mallards, we were not able to directly relate the observed associations between constitutive immune responses and blood Pb levels to oxidative stress biomarkers (Chapter 2). In environmentally Pb exposed ducklings and Pb treated partridges, some interrelationships between blood Pb levels, induced immune response and biomarkers of oxidative stress were found. Cellular immune response was shown to relate negatively to levels of antioxidants and oxidative stress biomarkers for short-term responses (Chapters 3 and 4), while these same relationships were positive in long-term responses (Chapter 4) (Figure 2). Our results suggested a Pb-induced imbalance between T helper cell types 1 (T_{h1}) and 2 (T_{h2}) in maternally exposed ducklings skewed towards T_{h2}. By contrast, experimentally Pb exposed partridges showed Pb-induced altered responses compatible with T_{h1}/T_{h2} imbalance skewed towards T_{h1} (Chapter 4) (Figure 2). We observed a negative association between tGSH levels and cellular response. Levels of this endogenous antioxidant can determine whether T_{h1} or T_{h2} response predominates (Townsend et al., 2003), as macrophages with more GSH produce nitric oxide and promote T_{h1} responses, whereas macrophages with less GSH preferentially promote T_{h2} responses (Murata et al., 2002). In addition, the generation of reactive oxygen species (ROS) as a consequence of heavy metal exposure inactivates transcription factors responsible for T_{h1} and T_{h2}-driven immune responses (Hemdan et al., 2007). Another explanation for the different Pb effects on the T_{h1}/T_{h2} balance is that such effect may depend on the Pb dose and the absorption pathway (i.e.: shot ingestion or maternal transfer), as well as physiological and developmental status (Dietert and Piepenbrink, 2006) (Figure 2).

In fact, gender and physiological status seem to have important roles in terms of shaping immunity (Apanius, 1998; Møller et al., 2003), which may account for variations in susceptibility to Pb (Rocke and Samuel, 1991). Several components of the constitutive and induced immune responses were affected by experimental Pb exposure in partridges with some differences between seasons. The observed effects of Pb on the induced immune response remained constant throughout the year and consisted of increased cellular responses (Figure 3). Regarding constitutive immunity,

birds seemed to be able to compensate the effects of Pb during the breeding seeson. As reviewed by Nam and Lee (2006), the energetic costs of constitutive immunity are low when compared with those of induced responses, and induced responses could thus be more sensitive to oxidative stress. Partridges were not able to maintain an intact "low cost" constitutive response after Pb exposure in autumn, even when they did not have to invest energy into reproduction (a function that has a high energetic cost), suggesting a greater immune susceptibility during the non-breeding season (Figure 3).

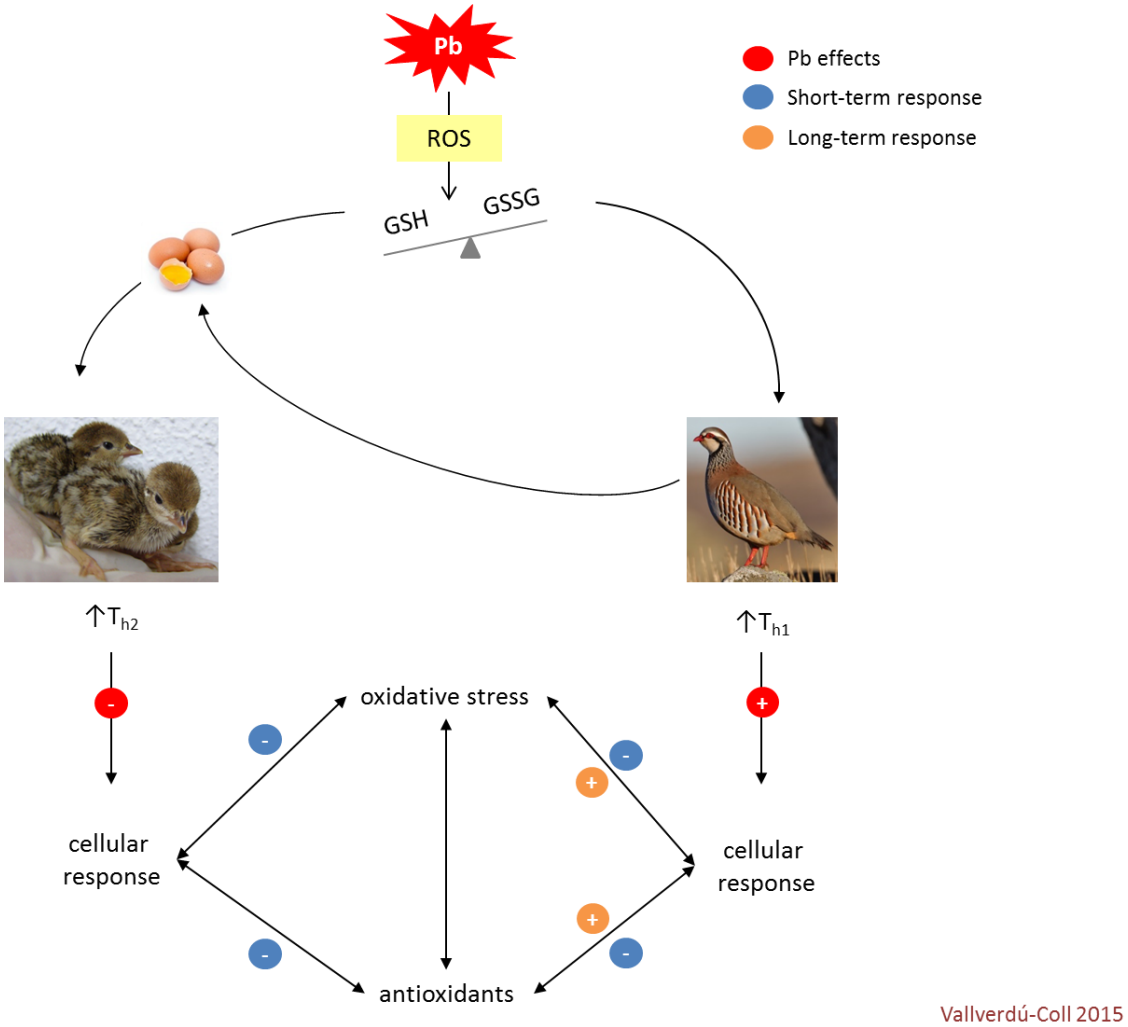

Figure 2: *Effects of Pb on short- and long-term cellular immune response to phytohemagglutinin depending on the age of exposure (i.e.: developmental exposure through maternal transfer vs Pb shot ingestion in adulthood) and its relationships with antioxidants and oxidative stress. Signs indicate whether the relationships are positive*

(+) or negative (-). ROS: Reactive oxygen species. GSH: total glutathione. GSSG: oxidized glutathione. T_h: T helper cells.

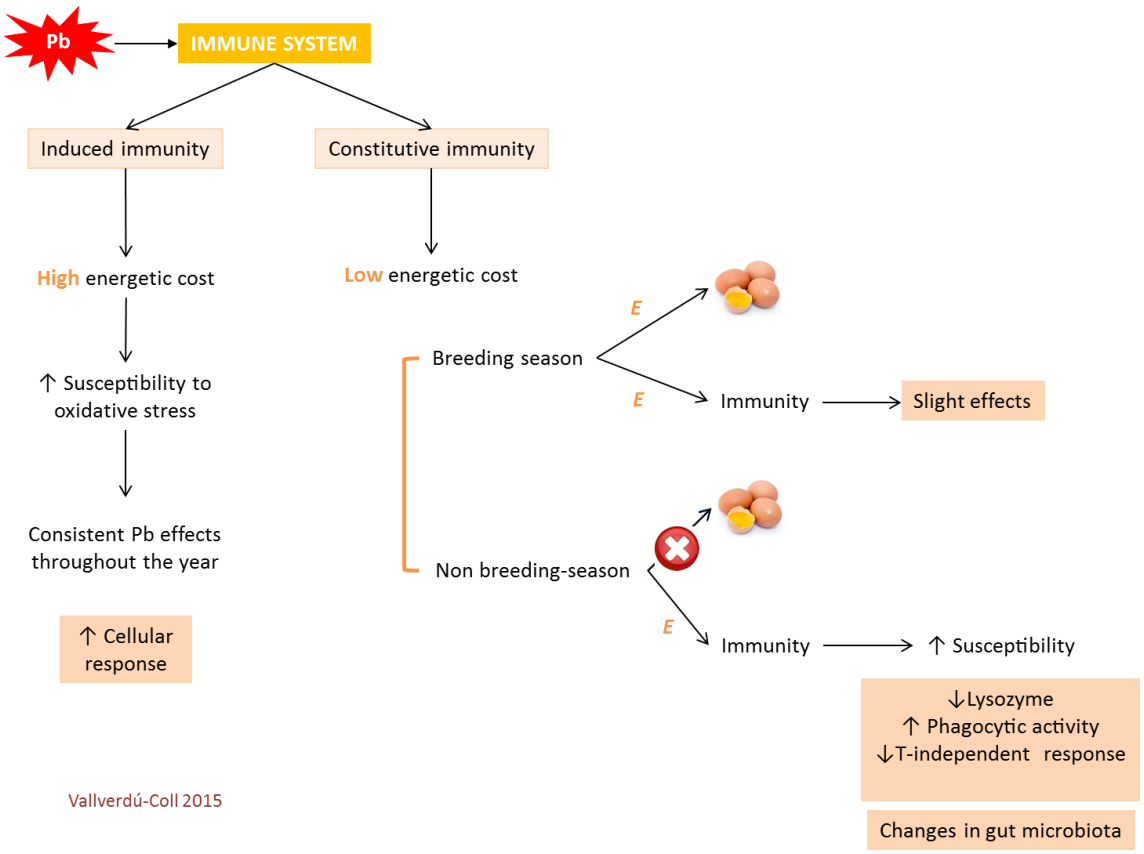

Figure 3: *Differences between Pb effect of on constitutive and induced immune responses considering the energetic cost of each function. E: Energy.*

In the non-breeding season, Pb-induced effects on constitutive immunity were associated with changes in gut microbiota that may be related to pathogen resistance (Chapter 4). Phagocytic activity increased in exposed individuals (Jang et al., 2011), which may be associated with an increased oxidative stress within the macrophages (Kasten-Jolly and Lawrence, 2014). Furthermore, the inhibition of lysozyme (Pesek and Schneider, 1988) seems to be a consistent effect (Chapters 2 and 4) and could be used as an immunological biomarker of Pb exposure.

The induced response mediated by T-independent antibodies was suppressed in partridges exposed to Pb in the non-breeding season, which depends on the

activation of the complement system (Ochsenbein and Zinkernagel, 2000). This contrasts with the increased lytic response observed in Pb exposed wild mallards (Chapter 2) that is also a complement activation dependent function (Ochsenbein and Zinkernagel, 2000). Because the immune system consists of a large number of interrelated components, unexpected positive or negative correlations may be observed when studying Pb effects on different types of immune responses, considering trade-offs between cross-regulated mechanisms, each one with different inherent costs (Lee, 2006). Hence, the general view is that Pb generates an immune disruption by up-regulating some components and suppressing others under the influence of a wide range of factors, which can be translated into a final decreased capacity to combat pathogen attack or a lower disease resistance. Considering the overall results of the different studies presented here, we have found evidence for an association between Pb immunotoxicity and the oxidative stress induced by this heavy metal. While it is true that we have not managed to justify all the alterations caused by Pb on immune responses, there are certain mechanisms that can form the basis for these changes. The well-known (and here confirmed) oxidative stress induction by Pb appears to promote the use of antioxidants (especially exogenous) to combat ROS generation. Such antioxidants invested in maintaining the oxidative balance are then not available for other functions and this deficit may result in impaired immune responses (Hasselquist and Nilsson, 2012). This was the case for the cellular response of wild ducklings that presented a negative relationship with antioxidant levels and was lower in individuals with higher levels of Pb. In other occasions, it seems that the organism may try to compensate for this pro-oxidant Pb activity by increasing levels of the endogenous antioxidant GSH, which also seems to be related to altered cellular response in adult Pb exposed partridges, although in this case such response is increased (Figure 2).

4. Effects of Pb shot ingestion on reproduction and its relationships with oxidative stress and immune response: the carotenoid allocation trade-offs

The ornaments displayed by birds, such as carotenoid-based traits, have been shown to reliably indicate individual quality and are often used to optimize mate choice and

reproductive decisions (Horvathova et al., 2012). Due to their role as immune-stimulants and antioxidants (Pérez-Rodríguez, 2009), carotenoids may be key molecules in the physiological trade-off between reproduction (i.e. coloration; egg production, sperm quality) and self-maintenance (oxidative balance, immunity) (Alonso-Alvarez et al., 2008). Furthermore, transformation of dietary carotenoids (zeaxanthin and lutein) into ketocarotenoids (astaxanthin and papilioerythrinone, the pigments ultimately responsible for the red coloration of partridges), a metabolic transformation that requires oxidation steps, has been shown to be favoured by oxidative stress at low intensity (García-de Blas et al., 2014; Chapter 4). One of the main focuses and novel aspects of the research conducted in this thesis lies in the study of the effects of Pb on immune and reproductive functions considering their bidirectional relationship with Pb induced oxidative stress, and of the trade-offs in the use of available carotenoids for oxidative balance maintenance, immunity and reproduction.

In mallards from the Ebro delta, levels of dietary antioxidants were greater in Pb exposed individuals in the breeding season (Chapter 2). In Pb exposed males, available antioxidants may be used for maintaining oxidative balance, since males with higher levels of Pb displayed less coloured carotenoid-based traits and did not show increased levels of oxidative stress (Figure 4a). The observed negative association between leg orangeness and humoral immune response may reflect a trade-off in the allocation of carotenoids between the two functions (immunity and reproduction). In Pb exposed females, coloration was not affected but showed greater levels of oxidative stress despite the increased levels of plasma antioxidants, which may then be allocated to eggs (Figure 4b).

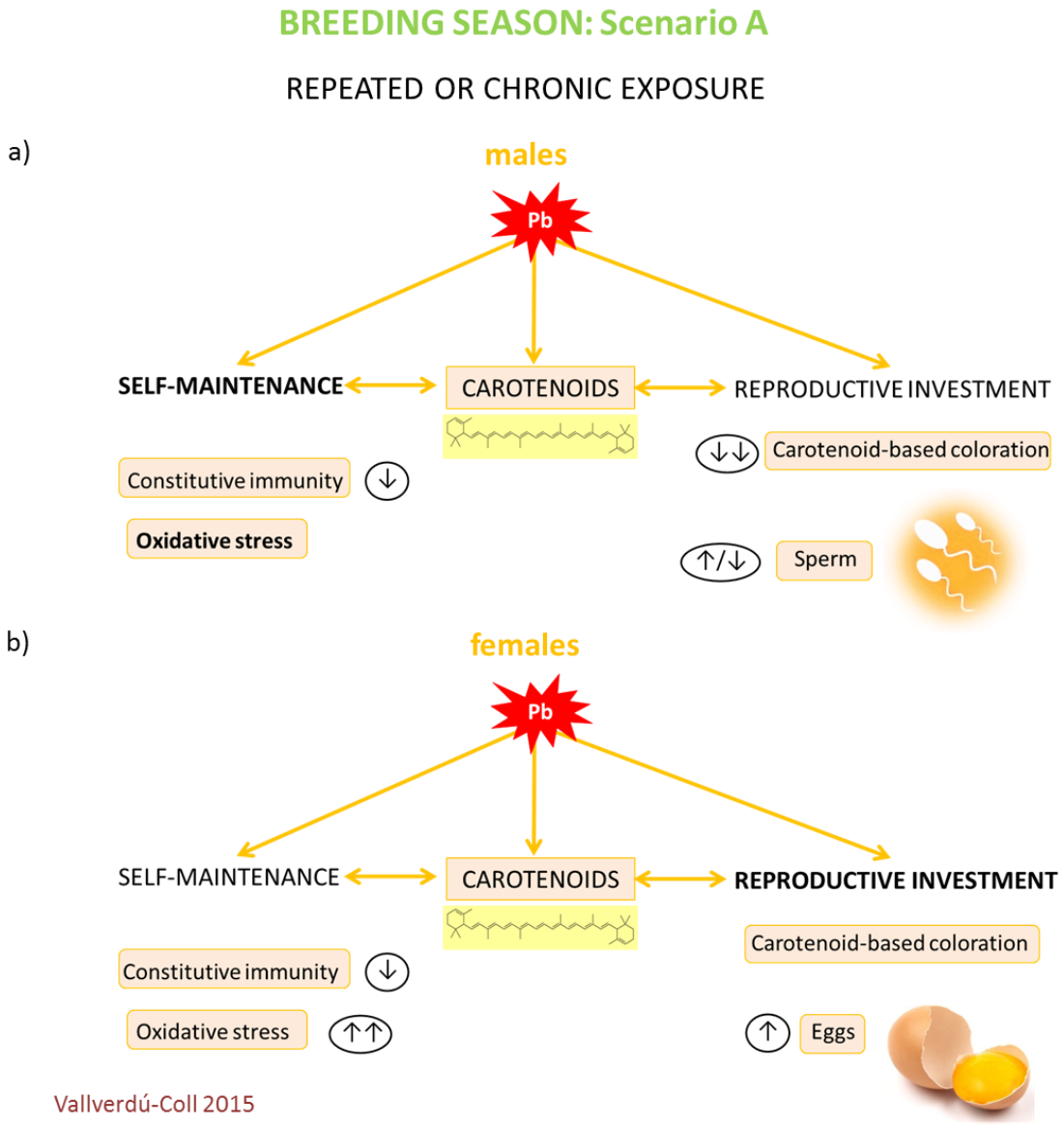

Figure 4: *Carotenoids allocation trade-off between self-maintenance and reproduction in repeatedly or chronically Pb exposed males (a) and females (b) summarizing results from field (Chapter 2) and experimental (Chapter 5) studies during the breeding season. Functions in bold indicate the investment priorities. Arrows indicate the direction of Pb effects and the number of arrows reflects the intensity of the effect.*

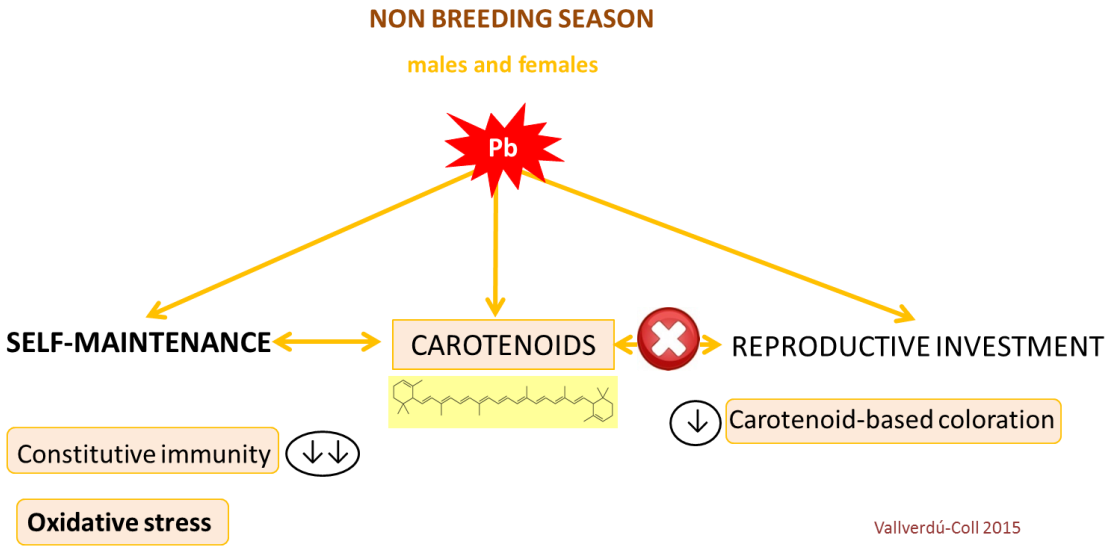

Figure 5: *Carotenoids allocation trade-off between self-maintenance and reproduction in Pb exposed birds during the non-breeding season. Functions in bold indicate the investment priorities. Arrows indicate the direction of Pb effects and the number of arrows reflects the intensity of the effect.*

By contrast, Pb exposure reduced eye-ring redness in both genders during the non-breeding season (Figure 5). Moreover, we found a negative association between the constitutive (phagocytosis) immune response and carotenoid-based coloration levels, suggesting a trade-off between them. Neither Pb exposure nor the stimulation of any immune response decreased plasma carotenoid levels in the autumn experiment, suggesting that during the non-breeding season both genders prioritized keeping circulating carotenoid levels to fight against oxidative stress at the expense of carotenoid-based ornamentation (Alonso-Alvarez et al., 2008) and constitutive immunity (Figure 5). This experiment allowed us to better understand allocation priorities that depended on the gender and physiological status of birds. In addition, it allowed us to detect immediate Pb effects after shot ingestion (i.e.: when blood Pb levels were highest) that can be quickly reversed and could be difficult to detect under field conditions (e.g.: antioxidant levels in spring, body condition, levels of natural antibodies). We also observed a lagged (delayed) effect on other parameters (e.g.: coloration, bacterial abundance, cellular response, phagocytic activity and levels of T-

independent antibodies), which may be considered as more appropriate endpoints in future field studies.

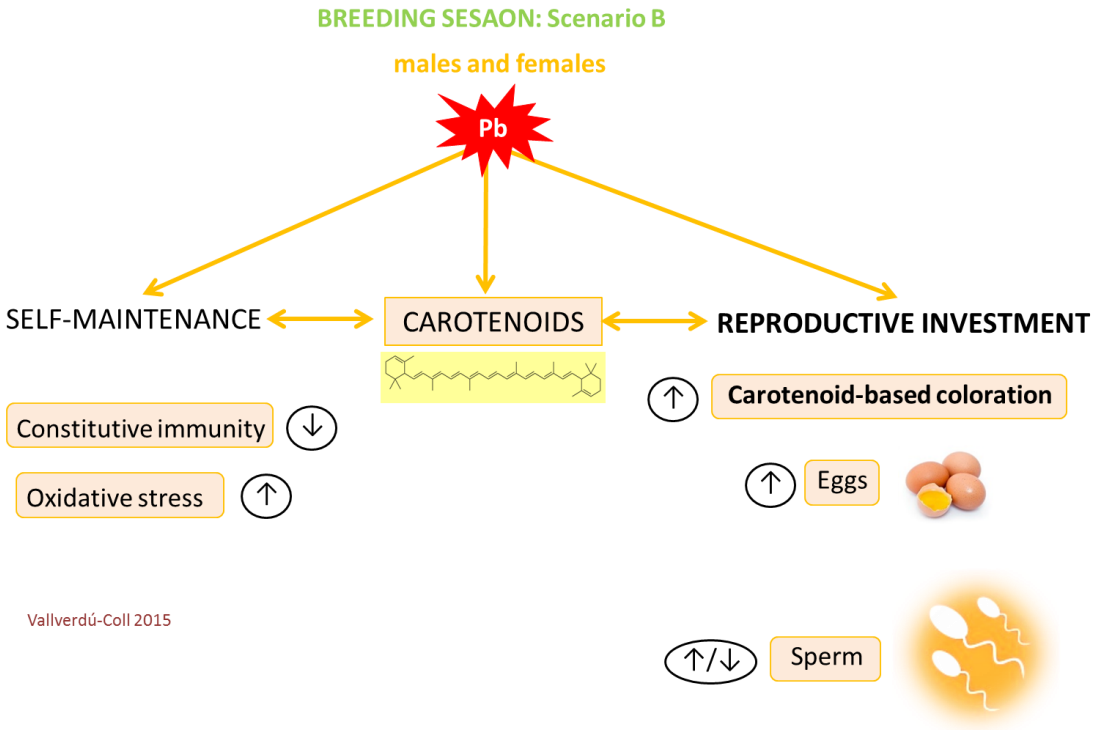

Figure 6: *Carotenoids allocation trade-off between self-maintenance and reproduction in Pb exposed birds during the breeding season. Functions in bold indicate the investment priorities. Arrows indicate the direction of Pb effects and the number of arrows reflects the intensity of the effect.*

The reduced eye-ring redness in Pb exposed males observed in non-breeding season exposure (Chapter 4) remained until the next breeding season (6 months after first Pb exposure) (Chapter 5) (Figure 4a). In addition, eye-ring redness was greater in Pb exposed females than in controls (6 months after first Pb exposure) at the beginning of laying period. This may be a strategy to increase mate investment in reproduction, possibly at the expense of survival and future reproduction (Bell, 1980) (Figure 6). After a second Pb exposure in spring (Chapter 5), Pb exposed females had a reduced percentage of pigmented eye-ring area. This reduced allocation of carotenoid to colour the eye rings in females may be explained by greater vulnerability to Pb-induced oxidative stress than males (Chapter 2) due to an increased carotenoid

allocation to the egg yolk in order to protect the embryo from oxidative stress (Pérez-Rodríguez, 2008). This would increase offspring survival prospects, but at the expense of the female´s own oxidative balance (Velando et al., 2014) (Figure 6). In agreement, partridge females exposed to Pb for the second time not only showed reduced allocation of carotenoids to eye rings, but also laid larger and heavier eggs and produced heavier chicks (Chapter 5) (Figure 6). The laying of larger eggs has been associated with disproportionately larger yolks and lipid stores in precocial species, which results in higher hatchling energy and yolk reserves (Østnes et al., 1997) and may imply a greater investment of carotenoids into eggs from the mother. In fact, eggs from females exposed to 1 Pb shot were larger and heavier and produced chicks that presented greater mass at birth than chicks from other groups. Despite the higher mass of eggs laid by females exposed to 3 Pb shot between 20 and 40 days after Pb exposure, no differences in hatchling mass were found with respect to controls. However, despite of the higher input of energy, the hatching rate was lower in females exposed to 3 Pb shot than in controls. Interestingly, females mated with exposed males produced relatively heavier chicks considering mass at birth respect to egg mass (Figure 6). This may reflect a better chick development, supporting the hypothesis of reproductive compensation that predicts that females should increase reproductive effort when mated with males of lower quality, in order to compensate potential negative effect on offspring fitness (Gowaty et al., 2007). The reduced hatching rate in exposed females could be explained by an altered eggshell composition due to Pb deposition, which may interfere with water vapour transport through the eggshell affecting the embryo and its survival (Wangensteen and Rahn, 1970), or due to Pb toxicity on the embryo. In mallard ducklings from the Ebro delta (Chapter 3), the body mass at birth relative to egg mass was reduced by maternal Pb transfer under field conditions, which could be related to a lower embryo resorption of Ca from the eggshell, due to the Pb deposition into the shell instead of Ca. A greater exposure to Pb was also associated with a decreased survival of ducklings, induction of oxidative stress and the impaired immune functions. In this field study (Chapter 3), we confirmed the eggshell Pb levels as a good non-invasive indicator of local exposure of wild bird populations (Dauwe et al., 1999) and, to our knowledge, this is the first study positively relating the eggshell thickness and Pb levels from ducklings. These

differences between results obtained from field and experimental studies may be due to the fact that under environmental conditions both parents may be exposed to Pb through shot ingestion. The species, but also the level, frequency and time elapsed since Pb exposure may all influence how Pb affects reproductive outcome.

Regarding male reproduction, the Pb-induced reduction of carotenoid-based coloration observed at the beginning of the breeding period (Chapter 2 and 5) may decrease their opportunities to mate (Omland, 1996; Pérez-Rodríguez et al., 2013). We confirmed our hypothesis regarding positive relationships between sperm quality and carotenoid-based coloration (Chapter 5) as we found positive relationships between sperm parameters and coloration that also appeared to be mediated by antioxidant levels. Our results suggest that colourful males may have greater sperm motility and velocity, and that this is also associated with higher levels of tocopherol. Furthermore, Pb toxicity seems to modulate these relationships, as it is responsible for the increase of circulating levels of such antioxidants but at the same time induces negative effects on these functions. Regarding the direct effects of Pb on semen, we found that males exposed to 3 Pb shot had sperm with lower motility (Akinola et al., 2015; Oliveira et al., 2009) and percentages of acrosomal integrity (Castellanos et al., 2015; Oliveira et al., 2009; Reglero et al., 2009). However, these effects were not associated with a reduced sperm viability or fertilization rate. On the contrary, exposure to 1 Pb shot was related to an increased vigour of spermatozoa but not in progressiveness. Sperm alterations observed in our experiment did not induce death of spermatozoa. This is also consistent with a lack of effects on fecundation or hatching rates in the group of exposed males.

5. Summary and proposals for the future

This work has demonstrated that the ingestion of Pb pellets produces various sublethal effects on birds, affecting their oxidative balance and their immune and reproductive functions. The latter two effects were frequently mediated or at least related to oxidative stress mechanisms and alterations of the antioxidant system. Both immune and reproductive outcomes were affected under experiment at very low doses of 1 and 3 shot, which is a number of pellets frequently found in gizzards of upland

gamebirds and waterbirds in Spain (Ferrandis et al., 2008; Mateo et al., 1997). These effects of Pb also appeared to be season-dependent and gender-specific, especially those related to carotenoid allocation trade-offs, which differ during the breeding and non-breeding seasons. Our results suggest that females seem to present a greater vulnerability to Pb-induced oxidative stress than males, which may be related to the allocation of carotenoids to eggs during the laying period. By contrast, males invested more carotenoids for oxidative balance and reproduction. The observed effects of Pb on components of induced immunity were constant throughout the year, while constitutive immunity tended to be more affected during the non-breeding season, when birds of both genders seemed to prioritize the maintenance of oxidative balance to the detriment of coloration and immunity. Regarding carotenoid-based coloration, we hypothesized that only birds in good condition and health should be able to simultaneously maintain a high reproductive effort and brightly coloured integuments. This hypothesis was confirmed as we found that coloration was a good indicator of sperm quality and reproductive investment in males, but not of immune status during the breeding season. During the breeding season, both males and females can increase the coloration of their integuments (increasing their investment in reproduction) at the expense of other functions.

The levels of Pb exposure observed in protected wetlands in the Ebro delta have been associated with changes in various components of the immune system that may be related to increased infection sensitivity by compromised responses against pathogens. In addition, these Pb levels have been associated with effects involved in mate choice, sperm quality, hatchability or chick immune-competence and survival. These results highlight that despite the good degree of compliance of the ban on Pb ammunition, legal hunting with Pb in unprotected feeding areas and the large accessibility of pellets that still remain in the environment still induce sublethal effects on birds. These may have negative consequences at the population level and pose a risk for game meat consumers. Some measures to reduce the incidence of shot ingestion have been proposed, such as the physical elimination of already accumulated Pb pellets in the soil, which is practically unfeasible in aquatic environments, or the supplementation of grit in feeding zones from humid areas where this material is limited (Mateo and Guitart 2000, Martinez-Haro et al., 2009, 2011a). One of the best

ways to reduce the incidence of Pb poisoning in birds, and to protect game meat consumers, would be to extend the ban on Pb ammunition not only to adjacent feeding grounds but also to terrestrial habitats, in order to definitely stop spreading Pb into the environment, as suggested by the UNEP/CMS (2014).

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Conclusions

1. A ban on the use of lead (Pb) ammunition in the Ebro delta has resulted in reduced Pb poisoning in waterbirds and exposure for game meat consumers. A good degree of compliance with the ban has been demonstrated, due to the cooperative attitude of waterfowl hunters and an active enforcement by local authorities. However, the partial implementation of the Pb ban did not fully eliminate the risk of Pb exposure.

2. Negative effects of Pb on immunity and reproduction in birds have been demonstrated under field and experimental conditions and appear related to oxidative stress mechanisms and alterations of the antioxidant system.

3. In captive red-legged partridges (*Alectoris rufa*), Pb effects on induced immunity components consisted of an increased cellular response modulated by levels of antioxidants. Pb effects on constitutive immunity were season-dependent, with greater effects during the non-breeding season when birds prioritized the maintenance of oxidative balance to the detriment of coloration and immunity.

4. A gender specific carotenoid allocation occurred during the breeding season in wild mallards (*Anas platyrhynchos*). Breeding females, who must allocate carotenoids into eggs, seem more vulnerable to oxidative stress, whereas males invest greater amounts of carotenoids into oxidative balance maintenance at expenses of carotenoid-based coloration.

5. Pb exposure in wild mallards can result in maternal Pb transfer through the eggs to the offspring that can induce oxidative stress, affect immune system development, and reduce survival during early life stages of ducklings.

6. A first exposure to Pb in captive male partridges increased their carotenoid-based coloration during the breeding season, whereas it was decreased in the non-breeding season, and this last effect remained until the following breeding period.

7. In captive partridges, Pb exposed females can increase their investment in reproduction at the expense of other functions. At the short term of Pb exposure, females exposed at the low dose resulted in the production of heavier eggs and chicks when compared to non-exposed or high-dosed individuals. Exposure at the high Pb dose resulted in a decreased hatching rate.

8. Pb exposure under experimental conditions in red-legged partridges decreased acrosomal integrity and sperm motility, and increase sperm vigour, but do not affect sperm viability, concentration, overall progress or fertility.

9. Carotenoid-based coloration was positively correlated with some aspects of sperm quality and reproductive investment in males or red-legged partridge, but not of immune status during the breeding season.

10. Despite the good degree of compliance of the ban on the use of Pb ammunition, legal hunting with Pb in unprotected feeding areas and the large accessibility of pellets that still remain in the environment continues to induce sublethal effects on wild birds that may have negative consequences at the population level and still poses a risk for game meat consumers.