UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

**PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA**

# EFEITOS DA CONTAMINAÇÃO POR CHUMBO NO SISTEMA IMUNE INATO DE *Coturnix coturnix japonica*

**GABRIEL MELHADO**

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Zoologia.

**Rio Claro-SP**

**2020**

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Rio Claro – SP
2020

M521e

Melhado, Gabriel

Efeitos da contaminação por chumbo no sistema imune inato de Coturnix coturnix japonica / Gabriel Melhado. -- Rio Claro, 2020

37 p. : tabs.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências, Rio Claro

Orientador: Ariovaldo Pereira da Cruz Neto

1. Fisiologia. 2. Sistema imunológico. 3. Codornas. 4. Poluição. 5. Chumbo. I. Título.

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# CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: EFEITOS DA CONTAMINAÇÃO POR CHUMBO NO SISTEMA IMUNE INATO DE Coturnix coturnix japonica

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Rio Claro, 15 de dezembro de 2020

aos meus pais e melhores amigos Helena Evangelista Melhado e Valter Melhado. Foram eles que me conduziram e me trouxeram paz em todo o meu percurso até aqui

ao meu irmão de sangue, Lucas Melhado, aquele que carrega o meu maior amor e admiração

à minha amada companheira, Luiza Freitas Silva, que escolheu compartilhar seu brilho comigo e caminhar sempre ao meu lado

DEDICO.

**“Se não podes entender, crê para que entendas. A fé precede, o intelecto segue.”**

**Santo Agostinho**

## AGRADECIMENTOS

Ao Deus, que criou todos os seres e nos presenteou com a vida para que pudéssemos ser ferramentas em suas mãos para estudar, compreender e proteger a natureza de sua obra.

Sem dúvidas foram anos de teste da minha fé, pois meu caminho teve nuances que não estavam em meu planejamento. Porém, se cheguei até aqui foi graças a Ele que projetou todas as experiências que eu deveria viver e o conhecimento que eu deveria aprender e compartilhar.

Ao meu orientador, Ariovaldo Pereira da Cruz Neto, pela figura de professor e mestre.

À Professora Drª. Renata Guimarães Moreira Whitton pelas correções, ideias e sugestões dadas na qualificação que enriqueceram esse trabalho.

Ao querido Professor Dr. Amauri Antonio Menegário, pela paciência, acolhida, alegria, discussões de futebol e apoio total a esta presente contribuição científica. Sinto-me em casa quando estou no CEA.

Ao meu padrinho da ciência, Tiago Gabriel Correia (TG), por todas as oportunidades oferecidas, confiança, carinho, companheirismo, ensinamentos, aventuras pelo Brasil, ideias e correções que foram fundamentais para a realização dessa pesquisa.

Ao meu grande amigo Ayrton Nascimento (um dos maiores presentes que ganhei com essa pesquisa), pela ajuda nos experimentos, pelos projetos visionários, pelas aulas de finanças, pelas tardes de futebol (e domínio do Marcelo), pela companhia, pela amizade, pelas ideias e conhecimento.

Aos meus amigos do CEA (Centro de Estudos Ambientais) que me instruíram, me ensinaram um novo mundo na ciência e me acolheram. Em especial ao meu amigo Jorge Henrique Pedrobom, que foi um grande professor e amigo durante esse mestrado.

A todos os meus colegas do LaFA (Laboratório de Fisiologia Animal – UNESP Rio Claro) que me ajudaram e preencheram meus dias com boas conversas.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001, e da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Processo 2014/16320-7.

Ao Departamento de Zoologia da Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), em Rio Claro, pelas facilidades logísticas.

À minha família maravilhosa que sempre me deu suporte em minha busca por novos sonhos.

À minha amada esposa, Luiza Freitas Silva (Preta), que me ajudou em todas as etapas desse projeto e me amou mesmo nos momentos mais desafiadores que passei. Meu porto seguro.

Às minhas rezadeiras, que elevaram preces em meu bem maior.

Muito obrigado!

**RESUMO GERAL**

As aves tem apresentado um declínio populacional nas últimas décadas ligado as atividades antrópicas, como industrialização, atividades agrícolas e processo de urbanização. Associada a essas atividades, a poluição por metais tem aumentado, evidenciada principalmente pela presença do chumbo (Pb) nos ecossistemas em elevadas concentrações. A preocupação perante à poluição por Pb é decorrente da capacidade de bioacumulação e toxicidade desse metal, e seu potencial efeito deletério sobre a saúde e sobrevivência das aves. Por exemplo, exposição ao Pb pode acarretar em um declínio na condição corpórea e alterações em processos fisiológicos que mantém a homeostase desses animais, como disfunção nas respostas imunológicas. No presente estudo, analisamos os efeitos do Pb sobre parâmetros indicativos da condição geral de saúde e elementos constitutivos e resposta imunológica induzida em codornas (*Coturnix coturnix japonica*). Para tanto, avaliamos os efeitos do Pb sobre a massa corpórea, consumo alimentar, número de células brancas, razão heterofilo/linfócito e produção da proteína haptoglobina. O Pb mostrou afetar a reposta imune constitutiva e a homeostase das aves à medida que codornas contaminadas por Pb tiveram aumento do número total de leucócitos. Além disso, os efeitos da contaminação por Pb em respostas induzidas do sistema imunológico podem estar associados a efeitos horméticos, já que houve aumento dos leucócitos, da razão H/L e da produção de haptoglobina, em aves contaminadas pelo metal e desafiadas imunologicamente com injeção de lipopolissacarídeo de *Escherichia coli*.

**Palavras-chave:** haptoglobina, chumbo, metal, resposta imune, hormese.

## ABSTRACT

Birds have shown a population decline in recent decades related to human activities, such as industrialization, agricultural activities and the urbanization process. Associated with these activities, chemical pollution by metals has increased, evidenced mainly by the presence of lead (Pb) in natural ecosystems in high concentrations. The concern regarding Pb pollution is due to its bioaccumulation capacity and toxicity, and its potential deleterious effect on the birds' health and survival. For example, exposure to Pb can lead to a decline in body condition and changes in physiological processes that maintain homeostasis in these animals, such as dysfunction of immune responses. In the present study, we analyzed the effects of Pb on parameters indicative of general health condition and constitutive elements and induced immune response in quails (*Coturnix coturnix japonica*). For this purpose, we evaluated the effects of Pb on body mass, food intake, total number of white cells, heterophil/lymphocyte ratio and production of the protein haptoglobin. Pb has been shown to affect the constitutive immune response and avian homeostasis as quails contaminated by Pb have increased the total number of leukocytes. In addition, the effects of Pb contamination on induced responses of the immune system may be associated with hormertic effects, since there was an increase in leukocytes, the H/L ratio and the production of haptoglobin, in birds contaminated by the metal and immunologically challenged with injection of lipopolysaccharide from *Escherichia coli*.



**Key-words:** haptoglobin, lead, metal, immune response, hormesis.

**SUMÁRIO**

## INTRODUÇÃO GERAL

As atividades antrópicas tem desempenhado pressões no meio ambiente que culminaram no atual padrão global de perda de biodiversidade. Como principais causas dessa tendência temos a fragmentação, deterioração e sobre-exploração do habitat, introdução de espécies invasoras, poluição e mudanças climáticas (Gaston et al., 2003; MEA, 2005). Tais atividades não são distribuídas uniformemente e podem ter efeitos sinérgicos sobre diferentes táxons, uma vez que são desempenhadas concomitantemente nos habitats (Brook et al., 2008).

As aves correspondem ao táxon com maior diversidade de funções ecológicas dentre os vertebrados e são responsáveis por muitos serviços ecossistêmicos, que beneficiam o bem-estar humano (Sekercioglu, 2006). No entanto, elas também são um dos grupos que foram maior e negativamente impactados por alterações no meio ambiente causadas pelo homem (Gaston et al., 2003; Newbold et al., 2015; Northrup et al., 2019). A população global de aves tem apresentado um declínio de 20% nas últimas décadas (Gaston et al., 2003), associado principalmente as mudanças climáticas e uso do solo (Gaston et al., 2003). No entanto, a análise em larga escala sugere fortemente que a alteração do habitat causada pelo homem é, de fato, o principal fator subjacente ao padrão observado de declínio da população de aves (Eglington and Pearce-Higgins, 2012; Gaston et al., 2003). Dada a importância ecológica das aves, entender os efeitos das atividades antrópicas na redução populacional e da perda da diversidade de espécies, é fundamental para prever futuros cenários afim de planejar ações de proteção e conservação dos animais (Chown and Gaston, 2008).

Não obstante as causas específicas por trás da alteração do habitat, a poluição química por metais é um subproduto conspícuo dessas atividades, ao participar no desenvolvimento de novas tecnologias, intenso processo de urbanização e atividades agropecuárias (Wang and Chen, 2009). Uma vez nos ecossistemas, os metais são capazes de contaminar corpos d’água, solo e o ar. A toxicidade desses químicos nos animais expostos é potencializada por seus efeitos cumulativos ao longo da cadeia alimentar (Dedourge-Geffard et al., 2009; Nolet et al., 1994; Rajeshkumar and Li, 2018; Roggeman et al., 2013).

Dentre os metais encontrados em elevadas concentrações na natureza, o chumbo (Pb) está entre os mais tóxicos e é de especial preocupação (Plaza et al., 2018). O Pb provém principalmente da fabricação de baterias, minas extrativistas, queima e processamento de combustíveis fósseis, despejos de lixo e projéteis de armas de fogo (Hoffman et al., 1985; Singh et al., 1994). A contaminação por Pb tem sido relacionada a milhões de mortes anuais na população mundial de aves aquáticas e terrestres (De Francisco et al., 2003). Qualquer que seja a atividade antropogênica subjacente a um aumento de Pb no ambiente, os efeitos da contaminação por Pb têm sido amplamente associados ao declínio da população global de aves.

Como outros vertebrados terrestres, a principal fonte de exposição ao Pb para as aves é a ingestão (Fisher et al., 2006). Quando ingerido, o Pb é absorvido pelos intestinos, acumula-se no sangue e, subsequentemente, em diferentes órgãos e tecidos, comprometendo as funções de diversos sistemas (nervoso, digestivo e reprodutivo - Burger, 1995; Fisher et al., 2006; Mehrota et al., 2008). Em aves, a meia-vida do Pb no sangue é de 13 a 15 dias (Fry and Maurer, 2003; Pain, 2009), enquanto órgãos como o fígado e os rins geralmente retêm o Pb por vários meses após a ingestão (Rainio et al., 2015). Portanto, os efeitos tóxicos da bioacumulação de Pb na saúde das aves dependem, de fato, da interação entre a concentração de Pb e o grau de exposição (agudo ou crônico). Por exemplo, a exposição aguda a baixas concentrações de Pb causa mudanças hematológicas sutis, mas se essa concentração aumentar e/ou persistir por longos períodos, o Pb se acumula nos tecidos e pode interromper gravemente os processos básicos associados à capacidade das aves de manter a homeostase (Vallverdú-Coll et al., 2019).

Em meio aos processos básicos de manutenção da homeostase, o sistema imunológico desempenha funções fundamentais na proteção dos animais contra traumas, infecções e agentes estressores externos. Entretanto, o Pb é associado, dentre outros efeitos tóxicos, a capacidade imunossupressora (Fair and Ricklefs, 2002; Fisher et al., 2006; Ma, 1989; Nain and Smits, 2011; Scheuhammer, 1987). A contaminação por Pb acarreta em alterações em componentes que atuam na manutenção e ativação do sistema imunológico das aves, ao passo que modifica a atuação das respostas humoral e celular (Fair and Ricklefs, 2002; Kenow et al., 2010; Lewis et al., 2013).

Muitas dessas disfunções no sistema imunológico correspondem às alterações dos componentes que atuam durante a resposta imune inata, a qual funciona como a primeira linha de defesa contra processos infecciosos (Abbas, 2015). Nas primeiras horas de infecção, o sistema imune inato combate a proliferação de patógenos por meio da ativação do seu principal componente: a Resposta de Fase Aguda (RFA).

A RFA envolve a produção e mobilização de elementos celulares e proteínas de fase aguda, além de acionar uma cascata de respostas fisiológicas e comportamentais (i.e. diminuição na massa corpórea, frequência alimentar e aumento na produção de leucócitos), que simultaneamente otimizam o processo de contenção e eliminação de patógenos (Baumann and Gauldie, 1994; Cray et al., 2009; Owen-Ashley and Wingfield, 2007). Grasman e Scanlon (1995) observaram aumento da razão heterofilo/linfócito e redução na produção de anticorpos em codornas (*Coturnix coturnix japonica*) expostas de 100 a 400 µg/ml de Pb na água durante 7 dias. Os efeitos de supressão do sistema imunológico também foram observados em aves aquáticas (*Anas platyrhynchos* - Rocke and Samuel, 1991) e terrestres (*Gallus gallus* - Bunn et al., 2000) após serem expostos a pequenos grãos de Pb (5 a 10 µg), à medida que essas aves reduziram a produção de células brancas no sangue.

Esses estudos avaliaram os efeitos do Pb *per si* nos elementos constitutivos, responsáveis pela manutenção do sistema imune e homeostase das aves. Entretanto, mesmo sabendo dos efeitos do Pb sobre os elementos constitutivos, pouco se sabe sobre os efeitos da contaminação por esse metal na imunocompetência das aves diante de um processo infeccioso. De fato, estudos que avaliaram a ativação do sistema imunológico de aves silvestres em áreas contaminadas por Pb sugerem interações entre Pb e doenças infecciosas (Locke and Bagley, 1967; Rocke and Samuel, 1991).

Portanto, com o intuito de entender a influência do Pb não só nos elementos constitutivos do sistema imunológico, mas também na ativação e atuação do mesmo no combate de doenças infecciosas, nós avaliamos codornas (*Coturnix coturnix japonica*) expostas ao Pb e posteriormente induzidas à ativação de respostas imunes inatas por meio da injeção de lipopolissacarídeo (LPS). Nossos resultados sugerem que a contaminação por Pb tem efeitos na resposta imunológica constitutiva, à medida que aumenta a produção de células brancas do sangue. Ademais, o Pb também alterou respostas imunológicas induzidas pelo aumento dos leucócitos, da razão H/L e da produção de haptoglobina. Tais aumentos desses componentes atuantes na resposta imune inata podem estar associadas a um efeito hormético do Pb no processo de ativação do sistema imunológico das aves.

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Paper title: The effects of sub-lethal Pb exposure on constitutive and induced immune response in birds

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**ABSTRACT**

Lead (Pb), one of the most common metal found in natural ecosystem in elevated concentrations due to anthropogenic activities, is known by bioaccumulating in the liver, bones and kidneys causing deleterious effects to animal physiology. Pb toxicity can affect potentially animal welfare and immunocompetence. To understand the effects of Pb exposure in birds – sentinels of environmental health – in this current work, we exposed Japanese quails (*Coturnix coturnix japonica*) to Pb in drinking water (250mg/mL) during 7 days and injected them with lipopolysaccharide (LPS) from *Escherichia coli*. The results showed that Pb contamination affects constitutive immune response and birds's homeostasis by increasing the number of circulating white blood cells (WBC). Besides, birds that were previously exposed to Pb, and then had innate immune response induced with LPS, reduced food intake, increased WBC count, the heterophil/lymphocyte ratio (H/L) and concentrations of plasma haptoglobin, an acute phase protein that assists the innate immune response. This study suggests that Pb has harmful effects on birds' welfare and on induced responses of immune system as it affects leukocyte profile and food intake. Moreover, the effects of Pb exposure on induced immune responses seemed to be dose-dependent and a hormetic effect might be associated during the innate response activation process, whereas quails responded to an imminent infection increasing the main components of innate immune system.

**Key-words:** lead contamination, metal, innate immune system, acute phase protein, hormesis.

## INTRODUCTION

Among the metals found in high concentrations in nature due to anthropogenic activities, lead (Pb) is amongst the most toxic and it is of special concern (Plaza et al., 2018). The main source of Pb exposure for avian species is through inhalation and ingestion (Fisher et al., 2006) and its toxic effects is potentialized by its cumulative effects along the food chain (Dedourge-Geffard et al., 2009; Nolet et al., 1994; Rajeshkumar and Li, 2018; Roggeman et al., 2013). After ingestion, Pb is readily absorbed by the guts, accumulates in the blood, and subsequently on different organs and tissues, disrupting the functions of several systems (nervous, digestive and reproductive - Burger, 1995; Fisher et al., 2006; Mehrota et al., 2008). Such disruption can be severely enough to affect fitness capacity and even lead contaminated individuals to death (De Francisco et al., 2003). Whatever the underlying anthropogenic activity associated with an increase in Pb in the environment, the effects of Pb contamination have been extensively associated with avian population declines (Eglington and Pearce-Higgins, 2012; Gaston et al., 2003).

The effects of Pb exposure on the capacity of birds to maintain viable populations depends, ultimately, on the individual resilience to maintain their health when confronted with this pollutant. Thus, a first step to understand the consequences of Pb exposure requires understanding how Pb affects underlying processes associated with the maintenance of homeostasis. Amid the basic processes of maintaining homeostasis, the immune system plays a fundamental role in protecting animals against trauma, infections and external stressors. However, Pb is associated, among other toxic effects, with an immunosuppressive capacity (Fair and Ricklefs, 2002; Fisher et al., 2006; Ma, 1989; Nain and Smits, 2011; Scheuhammer, 1987).

The immune system has a fundamental role in birds' welfare and survival as it protects them against pathogens by the action of humoral and cellular responses (Fair and Ricklefs, 2002). Pb affects constitutive components of both responses by reducing the production of antibodies (Fair and Ricklefs, 2002) and the total number of circulating leukocytes in the bloodstream (Bunn et al., 2000; Rocke and Samuel, 1991). Changes in humoral and cellular responses decrease the competence of animals in dealing with imminent infectious diseases, since these responses are part of innate immune system, which works as the first line of defense (Vallverdú-Coll et al., 2019).

During the first hours of an infection, the cellular components combat the proliferation of pathogens and release cytokines as proinflammatory signals that are responsible to induce a core part of the innate immune system: the Acute Phase Response (APR – Janeway, Travers & Shlomchik, 2004). Once induced, the APR triggers a cascade of physiological and behavioral responses (i.e. body mass loss, decreasing in food intake, and increasing production of leukocytes) (Baumann and Gauldie, 1994; Cray et al., 2009; Owen-Ashley and Wingfield, 2007). The APR also relies on the secretion of Acute Phase Proteins (APPs) produced potentially in the liver (Owen-Ashley and Wingfield, 2007). These proteins have antimicrobial properties, performing the opsonization of bacteria, enhancement of phagocytosis and, activation of complement system (Baumann and Gauldie, 1994; Owen-Ashley and Wingfield, 2007). In response to an infectious process, APPs may have their production increased (positive proteins), such as Haptoglobin (Hp). The Hp acts reducing oxidative damages (Jelena et al., 2013; Quaye, 2008) by bounding to free hemoglobin prevenient from hemolysis caused by infections (Armour et al., 2020) and enhances the innate immune response with its bacteriostatic and immunomodulation capacities (Cray et al., 2009).

Many avian immunotoxicology studies have helped to understand how Pb contamination affects birds' wealth and homeostasis by evaluating constitutive elements of immune system. However, little is known regarding the effects of Pb contamination on the induced immune responses. In fact, studies that evaluated the activation of the immune system of wild birds in areas contaminated by Pb have suggested interactions between Pb and infectious diseases (Locke and Bagley, 1967; Rocke and Samuel, 1991).

To provide more information to fill this gap, our objectives included to *(1) examine the effects of Pb exposure on the constitutive immune system and their relation with bird welfare and (2) to evaluate the effects of Pb contamination on the induced immune response*. To conduct our tests, we chose Japanese quails (*Coturnix coturnix japonica*) as model because their life history and genetic are well-known and they have not been genetically selected like other poultry birds (i.e. *Gallus gallus*). Therefore, quails can provide similar physiological responses to infectious diseases and Pb exposure in comparison with wild birds (Huss, 2008; Nain and Smits, 2011; Romijn et al., 1995).

## MATERIAL AND METHODS

*Animal housing*

Adult females (n = 48; $M_b$ = 140.72g ± 9.71) of Japanese quails (*Coturnix coturnix japonica*) were obtained from commercial producer of poultry birds and kept in individual bird cages (60 x 56 x 81 cm) placed inside a climatic chamber (Eletrolab® – São Paulo, Brazil) at controlled temperature (25ºC) and photoperiod (12h day:12h night). Feed (Supra®, Brazil) and water were provided *ad libitum*.

*Experimental protocols*

After one week that quails were obtained and placed inside the climatic chamber, the experiments started by randomly assigned each individual to one of one of the six treatment groups: (a) control (n = 9), (b) lead exposed group (Pb; n = 9), (c) immune challenged group (LPS; n = 9), (d) lead exposed + immune challenged group (Pb-LPS; n = 9), (e) phosphate-buffered saline solution group (PBS; n = 6), and (f) lead exposed + phosphate-buffered saline solution group (Pb-PBS; n = 6). After this assignment, experimental trails lasted for 9 days, being day 0 the day before treatments and day 8 the last day of experiment. Quails assigned to the Pb groups (Pb, Pb-PBS and Pb-LPS) received 250 mg/mL of lead acetate dissolved in drinking water during 7 days (day 1 to day 7), a subchronic and sub-lethal exposure (Grasman and Scanlon, 1995). The volume of water available to these birds each day were the same (30 ml), and we observed that, regardless the group, all birds drank all the water that was offered every day. Quails assigned to the LPS groups (LPS and Pb-LPS) received, on day 7, an intraperitoneal injection of 50 µL of 2 mg/kg solution of lipopolysaccharide from *Escherichia coli* (LPS –O127:B8, Sigma-Aldrich, USA; Koutos and Klasing, 2001) diluted in phosphate-buffered saline solution (PBS, Sigma-Aldrich). As control groups for LPS and Pb-LPS treatments, individuals from groups PBS (PBS and Pb-PBS) were injected with 50 μl of phosphate-buffered saline solution on day 7 as well. We chose day 7 to inject birds with LPS and PBS to assess body mass, food intake and hematological parameters 24 hours post-injections to guarantee the activation process of the Acute Phase Response (Matson et al., 2005).

*Body mass and food intake*

Quails were weighed with an Ohaus Precision Balance twice a day (at 06h00 and 18h00) on day 0 (pre-treatments) and day 8 (post-treatments). We calculated the relative daily body mass change as $\Delta M_b = (Mb_{18h00} - Mb_{6h00})/Mb_{6h00}$. A known amount of food was placed at 6h00 and kept all day long in a plastic bird container every day. At 18h00 we retired the food container to measure food waste and calculate daily food intake as the difference between initial (06h00) and final weights (18h00). No food was available overnight and the plastic containers were properly covered and only a small opening was provided to birds access the food avoiding spillovers.

*White blood cell (WBC) and Haptoglobin (Hp)*

We collected 1 mL of blood from brachial vein of all quails with a heparinized 22G needle (0.07mm) at 18h00 on day 8. A volume of ~15-20uL of this blood were used to prepare two blood smears to count the total number of white blood cells (WBC). We counted WBC in 20 different field views of each blood smear at 400X magnification in a microscope (Olympus CX41 - Tokyo, Japan). In addition, one hundred leukocytes were counted and identified in two replicates, and then we calculated and analyzed the heterophil/lymphocyte ratio (H/L).

The remaining blood was centrifuged (Sigma®) to obtain plasma that was used to assess the concentration of haptoglobin (Hp – µg/mL). We assessed plasma Hp concentrations by measuring the peroxidase activity of hemoglobin as this reaction is directly proportional to the concentrations of Hp (Nazifi et al., 2011). Thus, the circulating Hp was measured following the instructions provided by a commercial colorimetric assay kit (TP801; Tri-Delta

Diagnostics, NJ – USA; Bailly et al., 2016). Samples of plasma (7.5µL) were deposited on 96-well plates and then, hemoglobin (100µL) and a chromogen solution (140µL) were added in each well. We agitated circularly the entire plate and let to incubate for 5 minutes. Afterwards, we read the plate in a colorimeter (Chameleon, HIDEX® - Finland) at 600-630 nm.

*Lead concentration*

On the day 8, quails were euthanized through cervical dislocation (Protocol 6283 – "Ethics Committee on the Use of Animals" – Sao Paulo State University - Rio Claro) and the liver was collected to assess the concentration of Pb accumulated in this tissue to guarantee that our protocols of pollutant exposure truly worked. First, we lyophilized all samples to obtain dry weight and sequentially, we digested 0.175 g of each sample in double distillated nitric acid ($HNO_3$) in a microwave (Berghof, SW4 model, Germany) under high temperature (~200ºC) and pressure conditions (≤30 bar). These conditions allowed us to liberate the Pb present in the dried tissue. The extracts obtained from acid digestion were diluted in ultra-pure water and analyzed by the mass spectrometry (ICP–MS – Thermo Scientific, Germany). Results and statistical analysis for lead concentration in the liver can be found in the *supplementary materials* section (Table 1).

*Data Analysis*

First, to analyze the effects of Pb on birds' welfare, we evaluated body mass changes ($\Delta M_b$) and food intake. Besides, we analyzed WBC count as a constitutive component of immune system of quails from control and Pb groups. For $\Delta M_b$ and food intake, we compared values that occurred during pre-treatment (day 0) and post-treatment (day 8) periods with a two-way analysis of variance (2-way ANOVA). We tested the effects of the treatments (control and Pb exposure) and the period (pre-treatment and post-treatment), and also the interaction treatment-period. For WBC count, we compared both treatments with a t-test.

Furthermore, in order to evaluate the effects of Pb exposure on the induced immune response, we compared $\Delta M_b$, food intake, WBC count, H/L ratio and Hp of individuals from PBS, LPS, Pb-PBS and Pb-LPS groups. For $\Delta M_b$ and food intake, we run 2-way ANOVA as described above. For WBC count, H/L ratio and Hp, we performed one-way analysis of variance (1-way ANOVA). The H/L ratio data did not have normal distribution. Therefore, we arcsine transformed (arcsine– square root [H/L ratio]) all data before statistical analysis. When factors or their interactions tested in each ANOVA were significant, we conducted post-hoc pairwise comparisons using the Holm-Sidak method. All values were reported mean ± SE and a fiducial level of 0.05 was used to test for significance of the results.

## RESULTS

*Effects of Pb exposure on birds' welfare and constitutive component of immune system*

Birds did not show any change in body mass between treatments ($F_{1,35}$ = 1.316; $P$ = 0.260; Fig. 1A), periods ($F_{1,35}$ = 0.0913; $P$ = 0.764) and their interaction treatments-periods ($F_{1,35}$ = 0.602; $P$ = 0.443). On the other hand, contaminated quails increased food intake after subchronic exposure to metal (Treatments: $F_{1,35}$ = 17.910; $P$ < 0.001; Periods: $F_{1,35}$ = 6.006; $P$ = 0.020; Fig. 2A). Moreover, t-test showed that birds exposed to Pb increased WBC count in comparison with control group ($P$ < 0.001; Fig. 3A).

*Effects of Pb exposure on induced immune response*

Body mass did not change among all treatments ($F_{3,59}$ = 1.572; $P$ = 0.207; Fig. 1B), periods ($F_{1,59}$ = 0.775; $P$ = 0.383) and their interaction treatments-periods ($F_{3,59}$ = 0.496; $P$ = 0.687). On the other hand, birds decreased food intake after being injected with LPS; or exposed to Pb and then injected with LPS ($F_{3,59}$ = 5.112; $P$ = 0.004; Fig. 2B). Quails treated with LPS, Pb-LPS and Pb-PBS had an increase on their WBC production ($F_{3,29}$ = 12.374; $P$ < 0.001; Fig. 3B), whereas LPS and Pb-LPS treatments induced higher H/L ratios ($F_{3,29}$ = 7.234; $P$ = 0.002; Fig. 4). In addition, birds that received LPS and Pb-LPS as treatments increased their production of haptoglobin ($F_{3,29}$ = 23.218; $P$ < 0.001; Fig. 5). There was no mortality due to Pb dose offered to the birds.

## DISCUSSION

We measured body mass and food intake as assessments of birds' welfare and WBC count as assessment of constitutive immune response under contamination by lead (Pb). Animals can mobilize their energy budget as an attempt to deal with toxicity caused by metals (Dedourge-Geffard et al., 2009), that would decrease body mass and increase the need of higher food consumption. In fact, we observed that quails contaminated with Pb had an increment in their food intake, but no evidence of changes in body mass was found. Changes in body mass can reduce immune responsiveness (Snoeijs et al., 2004) which may be injurious to animal welfare. Snoeijs et al (2004) did not find any variation in body mass of wild great tits (*Parus major*) living in a Pb-contaminated area. This might happen because birds may balance their resource budget and no changes in body mass occur even in animals contaminated by metals (Daan et al., 1996).

Moreover, quails responded to Pb increasing the total number of circulating white blood cells (WBC). This increase on WBC count may suggest that Pb somehow elicited the activation of constitutive response in birds. Furthermore, the WBC count can also be used as assessment of physiological stress, once an increase in glucocorticoid hormones affects the leukocyte profile (Davis et al., 2008). Thus, the increase found in WBC count after Pb exposure may characterize a response to deal with stressful conditions due to Pb toxicity. This immunologic response, under effects of Pb contamination, seems to be affected by dose and via of exposure to Pb. Japanese quails (*Coturnix coturnix japonica*) that were exposed to single shots of 0.05g or 0.2g of Pb did not show any increase on WBC count (Fair and Ricklefs, 2002), in contrast to this current study which conducted a subchronic exposure to higher dose of Pb in drinking water.

Indeed, Pb contamination is closely related to immunocompetence as its toxicity can elevate the risk of infections (Lindström et al., 2005). Consequently, to evaluate the effects of Pb contamination on the induced innate immune responses, we also injected lipopolysaccharide (LPS) from *E. coli* in birds that were previously exposed to Pb. As elements of the Acute Phase Response (APR) - main component of innate immune system - we measured body mass, food intake, WBC count, H/L ratio and plasma Hp. Mounting the APR is a high-energy demand physiological process (Martin et al., 2003). Besides, it has been suggested that there are also energetic costs to repair tissue damaged due to Pb bioaccumulation (Fair and Ricklefs, 2002). Then, we expected to find reduction in body mass and increase in food intake. However, we found no changes in body mass in contaminated quails inoculated with LPS. Individuals that received only LPS reduced food intake (28%) as well as birds exposed to Pb and posteriorly injected with LPS (18%), as expected. It seems to be contradictory to reduce food intake even facing an imminent infection and its energetic costs added to metal exposure, but this might be assumed to be part of an adaptive strategy that includes reduction in foraging activity to save energy and lower the availability of important macronutrients for pathogens proliferation (Johnson, 2002).

Moreno-Rueda (2014) injected house sparrows (*Passer domesticus*) with 0.1 mg of LPS from *E. coli* and did not found any change in body mass after 4 hours of injection. Birds are likely mobilizing energetic reserves (Armour et al., 2020) and suppressing other metabolic energy-consuming functions (Martin, 2005; Moreno-Rueda, 2014), once dealing with costs of mounting an induced immune response. This might be underlying strategy related to the absence of changes in body mass in our results. Moreover, changes in body mass as response to LPS immune-challenge have been dose-dependent as lower and higher doses (100µg to 1mg/kg) of LPS injection used in other studies have affected differently birds' body mass (Armour et al., 2020; Sköld-Chiriac et al., 2015).

The activation of the innate immune response was evidenced after LPS injection once we found leukocytosis in *C. coturnix japonica*. Leukocytosis is considered to be widespread physiological responses following APR activation whereas animals face an imminent infection. The same was observed in birds exposed to Pb and then injected with LPS or PBS. Thus, both LPS and Pb induced an increase in WBC counts at the same magnitude. Previous studies have described immunotoxic effects caused by Pb (Grasman and Scanlon, 1995; Rocke and Samuel, 1991). However, we found that Pb may be immunostimulatory rather than immunosuppressive.

Studies that have observed suppressive effects of Pb contamination in birds tested higher doses during longer period of exposure (chronic assays), in contrast to our protocols. Thus, the effects of Pb contamination on induced immune response can be dose-dependent. Our results show that Pb has seemed to elicit the activation of innate responses (= hormetic effects). Hormesis can be characterized by an U-shaped dose-response which represents either an immunostimulation at low doses of contaminant, but an immunosuppression at higher doses; or an inverted U-shaped dose-response including immunosuppression at low doses, but an immunostimulation at higher doses (Calabrese and Baldwin, 2003; Davis and Svendsgaard, 1990; Rainio et al., 2015; Vallverdú-Coll et al., 2019). In a study conducted with a wild species (*Sialia mexicana*) exposed to lead shots (0.05 – 0.15g) early in the development of nestlings have observed possibilities that Pb contamination might increase immunologic activity enhancing the capacity of immune response against pathogens (Fair and Myers, 2002). However, this early immune activation has energetic costs that can compromise other activities. Furthermore, Nain and Smits (2011), after subchronic exposed Japanese quails to different concentrations of Pb (5pmm – 50ppm) in drinking water, have found evidences that Pb may enhance immune functions, suggesting hormetic effect as well.

The H/L ratio increased in quails that were injected with LPS as well as quails exposed to Pb firstly, and then inoculated with LPS. In birds, heterophils are one type of granulocytes and the most abundant phagocytic cells (Vallverdú-Coll et al., 2019) that are part of the first line of defense against *E. coli* infections (Roitt et al., 1998; Olkowski et al., 2005). These cells control the proliferation of bacteria during the APR. Kankova et al (2019) also found significant increase in H/L ratio after injection of 1.5mg/kg of LPS in Japanese quails. On the other hand, our results showed that Pb did not affect the H/L ratio. The same was observed in *Sialia mexicana* that received single shots of 0.05g, 0,1g or 0.15g (Fair and Myers, 2002). Few studies have counted and identified specific types of granulocytes to assess H/L ratio as an immunocompetence measurement, in avian immunotoxicological studies with Pb as contaminant. Fair and Ricklefs (2002) found an increase in production of granulocytes in Japanese quails that ingested ~0.05 or ~0.2 g of Pb single shot pellets and then were immunologic challenged by New-castle disease virus (NDV). Thus, our current work add information about this cellular component of innate immune response facing a bacterial induced infection after Pb contamination.

Quails treated with only injection of LPS increased circulating Hp as well as birds exposed to Pb and then injected with LPS. Haptoglobin is an anti-inflammatory protein that eliminates pro-oxidant hemes (Quaye, 2008) generated by infectious process and presents bacteriostatic capacity that assists the innate immune system to control the proliferation of bacteria during the APR. Northern bobwhite quails (*Colinus virginianus*) injected with 1mg/kg of LPS also increased plasma haptoglobin concentration (Armour et al., 2020). Previous studies and our results have showed that LPS elicits the production of acute phase proteins (Armour et al., 2020; Lee et al., 2012), and suggested that Hp plays an important role during the induced innate responses by bacterial infection. In addition, we expected that bioaccumulation of Pb in the liver would affect the production of Hp by hepatocytes. However, our results showed that Pb did not interfere in the synthesis of this acute phase protein in the liver. Same result was found in wild great tits (*Parus major*) living in a Pb-contaminated area (Vermeulen et al., 2015). They found no positive relationship between Hp concentrations and Pb bioaccumulated in that species.

In conclusion, our study sums to understand that Pb contamination affects birds' welfare and constitutive immune response, indeed, once quails had their leukocytes profile and food intake changed after exposed to this metal. Our results made clearer that effects of Pb toxicity on immune system are dependent of dose, degree (acute, subchronic or chronic) and via of exposure. We also bring here evidence that effects of Pb on the induced innate immune responses can be dose-dependent and Pb may be imunnostimulatory, suggesting hormetic effect. Although, further studies must be conducted considering a gradient of Pb exposure (lower and higher concentrations than we tested here) to validate whether Pb has hormetic effects on the innate immune system activation.

**ACKNOWLEDGMENTS**

We thank Amauri A. Menegário and all colleagues from Centro de Estudos Ambientais (CEA) for helping us with Pb analysis; and Ayrton Nascimento for helping us with lab facilities. This study was supported by grants to A.P.C.N. from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant # 2014/16320-7). G.M. was supported by a grant from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Grant # 88888.434131/2019-01).

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## FIGURES CAPTIONS

Figure 1. Body mass change (mean ± SE) of Japanese quails (*Coturnix coturnix japonica*) after treatments **(A)** control and lead exposure (Pb); **(B)** phosphate-buffered saline injection (PBS), lipopolysaccharide injection (LPS), lead exposure + PBS injection (Pb-PBS), lead exposure + LPS injection (Pb-LPS).

Figure 2. Food intake (mean ± SE) of Japanese quails (*Coturnix coturnix japonica*) after treatments **(A)** control and lead exposure (Pb); **(B)** phosphate-buffered saline injection (PBS), lipopolysaccharide injection (LPS), lead exposure + PBS injection (Pb-PBS) and lead exposure + LPS injection (Pb-LPS).

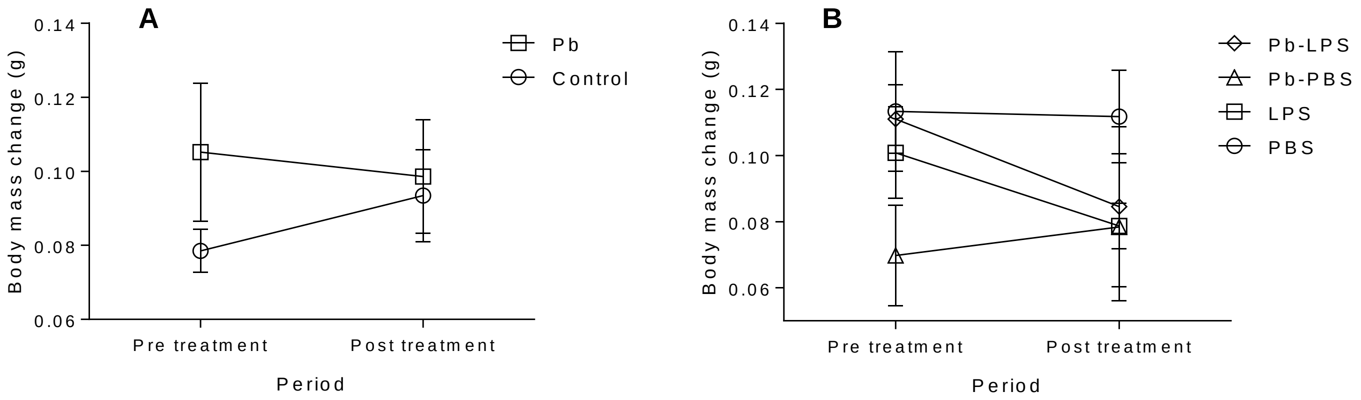
Figure 3. White blood cell count (WBC - mean ± SE) of Japanese quails (*Coturnix coturnix japonica*) after treatments **(A)** control and lead exposure (Pb); **(B)** phosphate-buffered saline injection (PBS), lipopolysaccharide injection (LPS), lead exposure + PBS injection (Pb-PBS) and lead exposure + LPS injection (Pb-LPS).

Figure 4. Heterophil/lymphocyte ratio (H/L - mean ± SE) of Japanese quails (*Coturnix coturnix japonica*) after treatments **(A)** control and lead exposure (Pb); **(B)** phosphate-buffered saline injection (PBS), lipopolysaccharide injection (LPS), lead exposure + PBS injection (Pb-PBS), lead exposure and LPS injection (Pb-LPS).

Figure 5. Plasma haptoglobin (Hp) concentration  (mean ± SE) of Japanese quails (*Coturnix coturnix japonica*) after treatments phosphate-buffered saline injection (PBS), lipopolysaccharide injection (LPS), lead exposure + PBS injection (Pb-PBS) and lead exposure + LPS injection (Pb-LPS).

## FIGURES

Figure 1.

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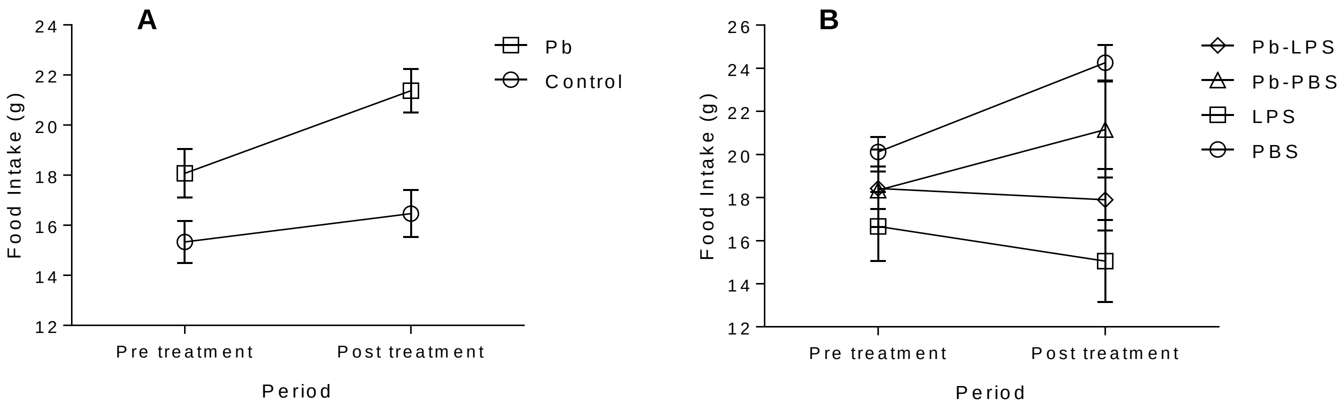

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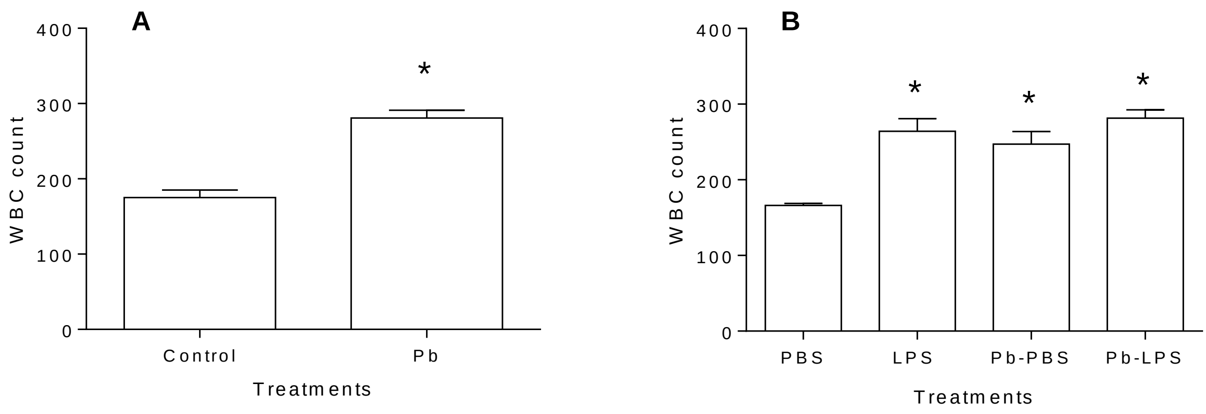
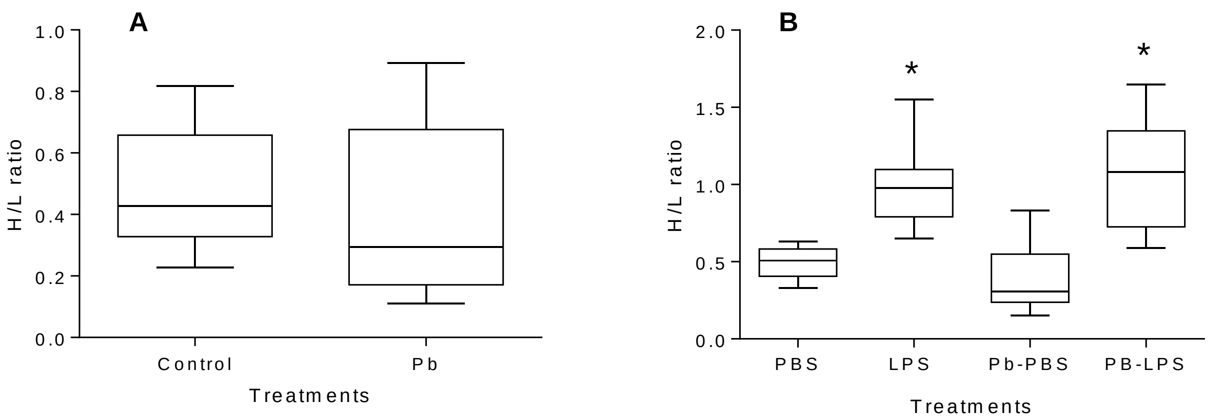



Figure 4.

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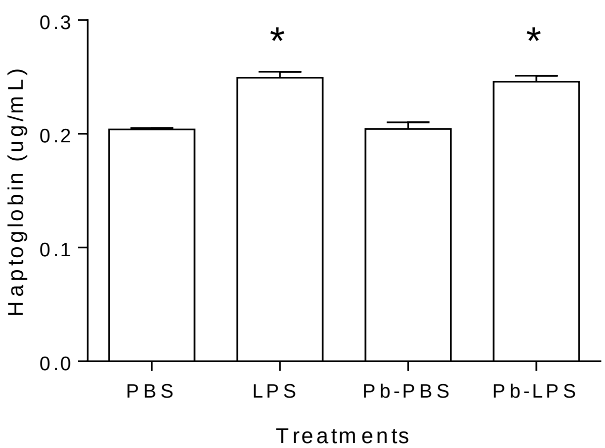

**SUPLEMENTARY MATERIALS**

Table 1. Concentrations of lead (Pb) bioaccumulated in the liver (ug/mg ± SE) of Japanese quails (*Coturnix coturnix japonica*) after treatments: phosphate-buffered saline injection (PBS), lipopolysaccharide injection (LPS), lead exposure + PBS injection (Pb-PBS) and lead exposure + LPS injection (Pb-LPS). Quails were exposed to Pb by drinkable water containing 250mg/L of lead acetate during 7 days.

Data for bioaccumulation of Pb in the liver of *Coturnix coturnix japonica* were compared among treatments with a non-parametric test Kruskal-Wallis, since values did not follow normal distribution. There was a significant difference among treatments ($p < 0.001$). Individuals contaminated with Pb (Pb, Pb-PBS and Pb-LPS) had great concentrations of Pb in comparison with other groups. Among all groups that were exposed to Pb, no difference were found.

| Treatment | Mean ± SE | Max. | Min. |
|---|---|---|---|
| Control | 0.0448 ± 0.008 | 0.0827 | 0.0140 |
| PBS | 0.0378 ± 0.005 | 0.0478 | 0.0300 |
| LPS | 0.0396 ± 0.006 | 0.0703 | 0.0163 |
| Pb | 7.423 ± 0.349 | 9.429 | 6.388 |
| Pb-PBS | 2.013 ± 0.203 | 2.481 | 1.197 |
| Pb-LPS | 4.737 ± 1.413 | 11.363 | 1.192 |