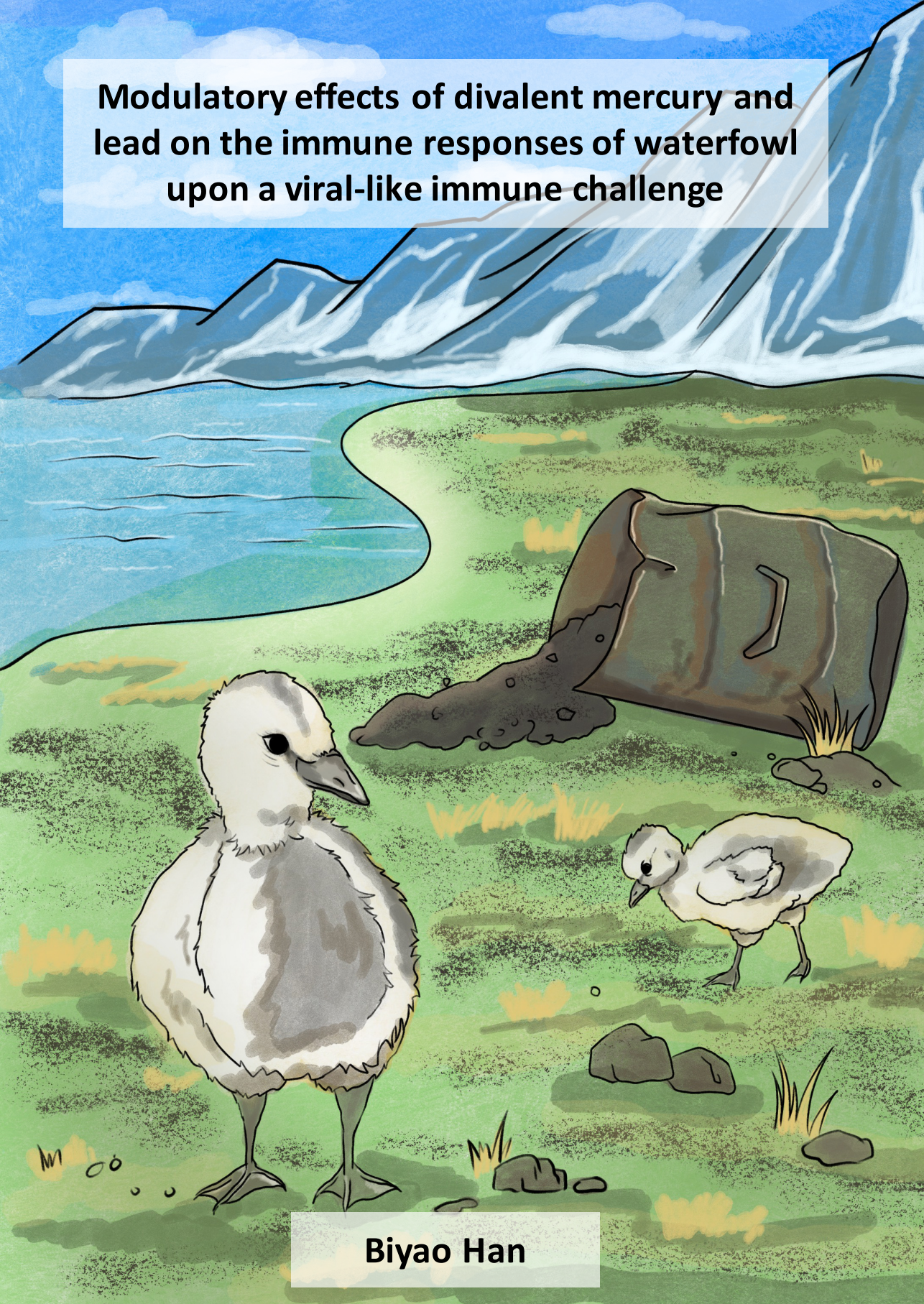
Modulatory effects of divalent mercury and lead on the immune responses of waterfowl upon a viral-like immune challenge



Biyao Han

Propositions

1. To detect immunotoxicity, effects on immune responses upon a challenge provide more meaningful endpoints than effects on baseline immunity. (this thesis)

2. To achieve better environmental risk assessments of chemicals, it is necessary to include more non-standard species and endpoints. (this thesis)

3. Artificial intelligence (AI) will reduce the number of repetitive and time-consuming analyses.

4. The adverse effects of climate change on the migratory behavior of birds are underestimated.

5. Fieldwork in remote areas contributes not only to scientific development but also to personal development.

6. Biased news causes discrimination.

Propositions belonging to the thesis, entitled

Modulatory effects of divalent mercury and lead on the immune responses of waterfowl upon a viral-like immune challenge.

Biyao Han

Wageningen, 29 March 2022

Modulatory effects of divalent mercury and lead on the immune responses of waterfowl upon a viral-like immune challenge

Biyao Han

Thesis committee

Promotors

Dr N.W. van den Brink
Associate Professor Toxicology
Wageningen University and Research

Prof. Dr I.M.C.M. Rietjens
Professor of Toxicology
Wageningen University and Research

Other members

Prof. Dr H.F.J. Savelkoul, Wageningen University and Research

Dr K.D. Matson, Wageningen University and Research

Prof. Dr M. van den Berg, Utrecht University

Prof. Dr V. Jaspers, Norwegian University of Science and Technology, Trondheim

This research was conducted under the auspices of the Graduate School of Socio-Economic and Natural Sciences of the Environment (SENSE).

Modulatory effects of divalent mercury and lead on the immune responses of waterfowl upon a viral-like immune challenge

Biyao Han

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 29 March 2022
at 1:30 p.m. in the Aula.

Biyao Han

Modulatory effects of divalent mercury and lead on the immune responses of waterfowl upon a viral-like immune challenge
184 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summary in English

ISBN: 978-94-6447-100-7
DOI: https://doi.org/10.18174/563399

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Chapter 1

General introduction

Exposure to immunotoxic contaminants, such as trace metals, could directly reduce the defence capabilities and increase the risk of infections in both animals and human beings, which may increase the prevalence of pathogens in animal reservoirs and, thereby potentially promote the transmission of zoonotic diseases (Carravieri et al., 2020; Lafferty & Kuris, 1999; Lassudrie et al., 2014). Trace metals, including mercury (Hg) and lead (Pb) have been reported to be immunotoxic to mammals (Bernier et al., 1995; Desforges et al., 2016; García-Mendoza et al., 2021; Monastero et al., 2018; Nyland et al., 2011), but relatively little is known about their effects and underlying modes of actions for avian species, especially waterfowl, who are often related to the spread of avian influenza infections (Alexander, 2007). As wild waterfowl are natural reservoirs for avian influenza viruses, impaired immune competence and pathogen resistance of wild waterfowl due to exposure to immunotoxic contaminants may increase the risk of avian influenza outbreaks, threatening public health, wildlife and ecosystems, and also the global economy (Allen et al., 2017). The "One Health" concept also highlighted the importance of interaction between environmental pollution, zoonotic diseases, and human health (Destoumieux-Garzón et al., 2018). Therefore, it is crucial to study the effects of trace metal exposure on the immune responses of wild waterfowl. In this thesis, we focus on two typical divalent trace metal ions, Hg(II) and Pb(II). To further investigate the adverse effects of Hg(II) and Pb(II) exposure on the immunity of waterfowl, a thorough understanding of the avian immune system is essential.

1.1. Avian Immune system

A well-functional immune system is crucial for recognizing foreign pathogens and for the defence and survival against these infections. The main immune organs in avian species are the bone marrow, bursa of Fabricius, and thymus (Figure 1.1), containing primary lymphoid tissues, and the spleen containing secondary lymphoid tissues (Kaiser & Balic, 2015). Bone marrow is the tissue of life-long hematopoiesis forming blood and immune cells (Fellah et al., 2013). The Bursa of Fabricius (bursa) is located dorsal to the rectum and is responsible for the amplification and differentiation of B-lymphocytes (Cooper et al., 1965; Glick et al., 1956). The bursa is active in young birds and generally disappears before sexual maturity (Fellah et al., 2013). The thymus is located in the neck, and is composed of 7-8 lobes and is the site of T-lymphocytes differentiation (Kaiser & Balic, 2015). The spleen is the largest lymphoid organ in birds, containing multiple types of immune cells, and is able to efficiently mount both innate and adaptive immune responses (Oláh et al., 2014). The avian spleen probably plays a more critical role in immune responses than the mammalian spleen, possibly related to the lack of avian lymph nodes (John, 1994).

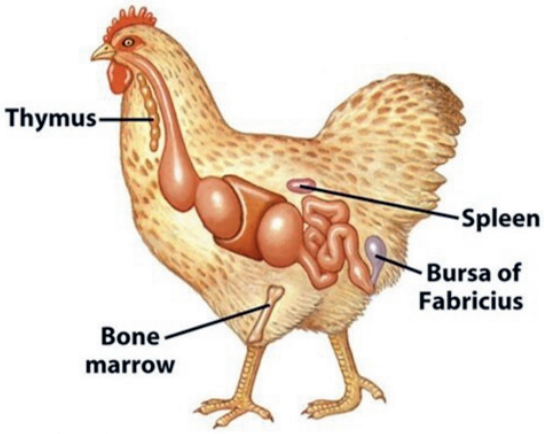


Figure 1.1. Major avian immune organs (Black, 2005).

Similar to mammals, the avian immunity consists of the non-specific immunity and the specific immunity (Figure 1.2). The non-specific immunity provides the first and second lines of immune defence and is active from birth onwards, producing a non-specific response to pathogens. The first line of defence is made up of physical barriers (e.g., intact skin and mucosal epithelia), secretion of these physical barriers (e.g., tears and sweat containing lysozyme, and stomach acid providing a low pH) and normal microbiota (e.g., in gastro-intestinal and reproductive tract) (Turvey & Broide, 2010). The physical barriers can block the pathogens from entering the body while their secretion and normal microbiota can trap, washout and kill the pathogens (Hillaire et al., 2013; Turvey & Broide, 2010). The second line of defence refers to the innate immune system containing natural antibodies, the complement system and different innate immune cells (Figure 1.2). Natural antibodies (nAbs) are immunoglobulins that are produced by B-cells in the absence of the identification of the specific pathological infections or historic immunizations (Maddur et al., 2020). As the nAbs are non-specific, they can neutralize various pathogens, and together with the lysis of microbial pathogens via the complement pathway, a broad range of pathogens can be inhibited and eliminated (Zhou et al., 2007). The cell types in the innate avian immune system include heterophils (similar to neutrophils in mammals), macrophages, monocytes, eosinophils, basophils, natural killer (NK) cells and dendritic cells (DC) (Figure 1.3). Among all the innate immune cells, heterophils and macrophages are the most abundant cell types with the functions of phagocytosis and oxidative bursts to kill pathogens (Paiva & Bozza, 2014; Sandström et al., 2014). The innate immune system can non-specifically eliminate pathogens, although it may not be extremely efficient. Therefore, antigen presenting cells (APCs) such as macrophages and dendritic cells (DC) can capture antigens and present them to naïve T cells, triggering adaptive immune responses for further, more specific and efficient responses (Gaudino & Kumar, 2019).

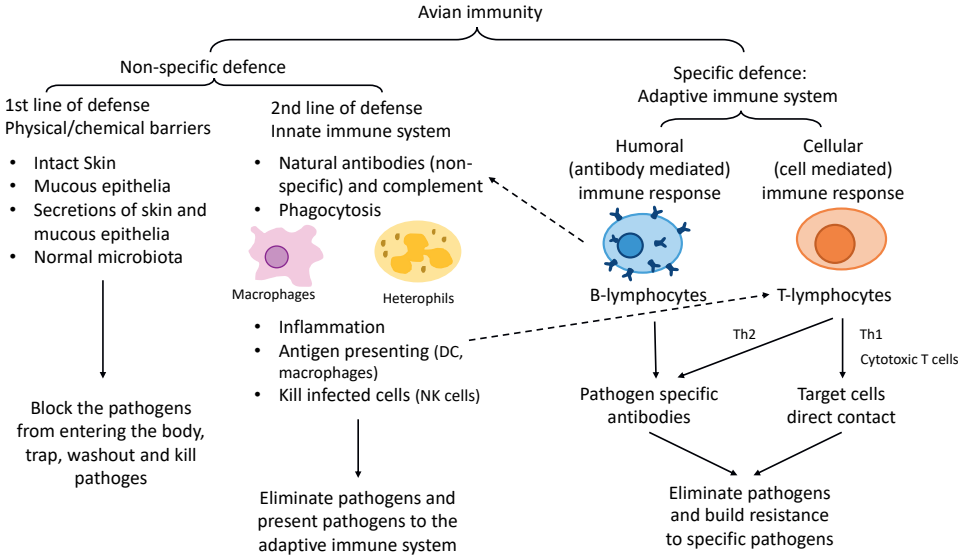


Figure 1.2. Overview of avian immune system, adapted from Vallverdú-Coll et al. (2019) and Turvey & Broide (2010). DC, dendritic cell; Th, T-helper. Dotted arrows show the interaction between the innate and adaptive immune systems.

The adaptive immune system of an organism evolves over time, depending on the infections it may experience. Major cell-types in this system are B-cells and T-cells (Figure 1.3), which develop specific immune responses (Figure 1.2, 1.3). B-cells are responsible for humoral immune responses (also called antibody mediated immune responses) while T-cells are responsible for cellular immune response (also called cell mediated immune responses). Avian B-cells produce immunoglobulins (Igs) including IgM, IgA and IgY (functionally equivalent to mammalian IgG) (Härtle et al., 2013). Among these three kind of antibodies, IgM is formed initially after exposure to antigens. Therefore, IgM is considered a primary indicator for B-cell functioning. Besides, the membrane-bound IgM (mIgM) molecules on the B-cell surface function as predominant B cell antigen receptors (BCRs) (Friess et al., 2018). Naïve T cells can differentiate into either CD8+ cytotoxic T cells or CD4+ T helper cells upon activation. Helper CD4+ T lymphocytes play a vital role in the regulation of an adequate immune response through activation and differentiation into effector helper CD4+ T lymphocytes subsets, depending on the nature of the infection and the cytokine microenvironment (Zhu et al., 2010). Effector CD4+ T helper cells can further be divided into type one (Th1) or type two (Th2) subsets, according to their cytokine profile. Th1 cells promote cell-mediated immunity by releasing pro-inflammatory cytokines such as IFN-γ and mainly response to intracellular bacterial or viral infections (Luckheeram et al., 2012). On the contrary, Th2 cells enhance antibody-mediated humoral immunity via secretion of anti-inflammatory cytokines like IL-4 mainly involved in the defence against parasites like helminthic worms (Luckheeram et al., 2012). After encountering with a given antigen for the

first time, memory B- and T-cells are differentiate and stored to mount a faster and greater secondary response upon contact with the same antigen again (Natoli & Ostuni, 2019). Overall, the adaptive immune system can remove specific pathogens and build up host resistance against these specific pathogens upon later contact.

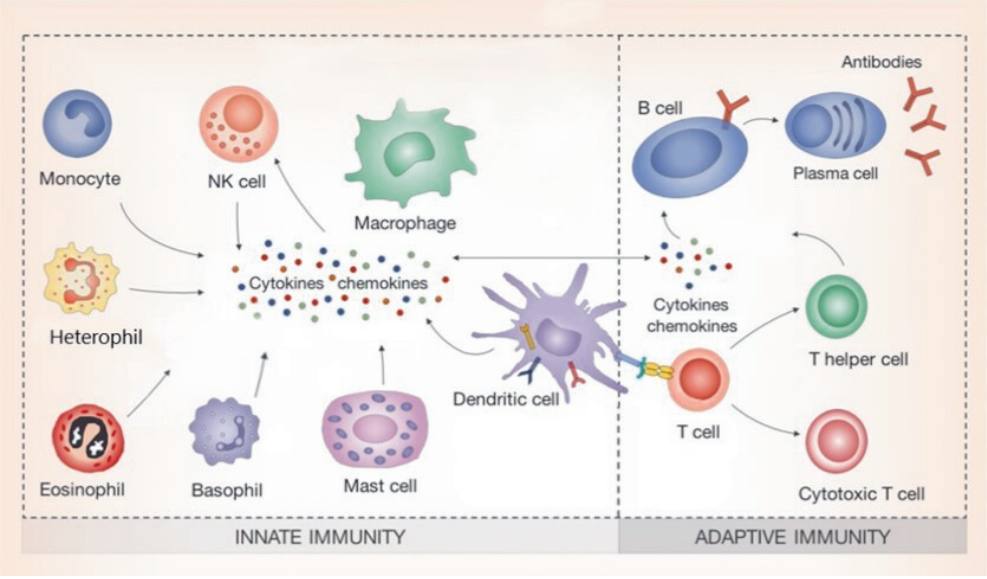


Figure 1.3. Cell types in the innate and adaptive immunity and their interaction, adapted from Silva et al. (2017).

Once infected by specific pathogens, the pattern recognition receptors (PRRs) in the host immune cells can recognise diverse pathogen-associated molecular patterns (PAMPs) and activate intracellular signalling pathways, initiating immune responses (Akira et al., 2006). Upon activation, the innate and adaptive immune systems will perform certain defence strategies within hours and days respectively (Ooi et al., 2010). In both mammals and avian species, the family of toll-like receptors (TLRs), which are expressed in multiple immune cells including macrophages, B-cells and dendritic cells, serve as key PRRs. For instance, TLR4 localized in the plasma membrane recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, while TLR3 and TLR7 localized in the endosome membrane recognize double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) from viral infections respectively (Kawai & Akira, 2007). Activation of TLRs could trigger the nuclear factor-κB (NF-κB) pathway and Interferon regulatory factors (IRFs) pathway (Figure 1.4). Activation of the NF-κB pathway promotes the expression of pro-inflammatory genes, including cytokines (e.g., interferon-γ (IFN-γ), tumour necrosis factor (TNF-α), interleukins (ILs, such as IL-18) and chemokines (recruiting immune cells to the site of infection e.g. chemokine (C-X-C motif) ligand 8, CXCL8, also called IL-8). Specifically in macrophages and heterophils, the NF-κB pathway also triggers the expression of genes related to oxidative

burst, such as inducible nitric oxide synthase (iNOS) (Davison et al., 2008; Genovese et al., 2013; Maxwell & Robertson, 1998). The activation of IRFs pathways promotes the expression of anti-viral type I interferons (IFNs), namely IFN-α and -β, leading to an anti-viral state in neighbouring uninfected cells by blocking viral replication (Abbas et al., 2015). Cytokines are crucial for communication among immune cells and regulation of immune responses (Figure 1.3). In addition to cytokines, microRNAs (miRNAs) are also important immune modulators regulating the immune responses post-transcriptionally by degrading mRNA and repressing translation (Bushati & Cohen, 2007; Fabian et al., 2010; Schickel et al., 2008). A typical immune modulating miRNA is miR-155, promoting lymphocyte proliferation and differentiation, macrophage polarization, and antibody production (Alivernini et al., 2018).

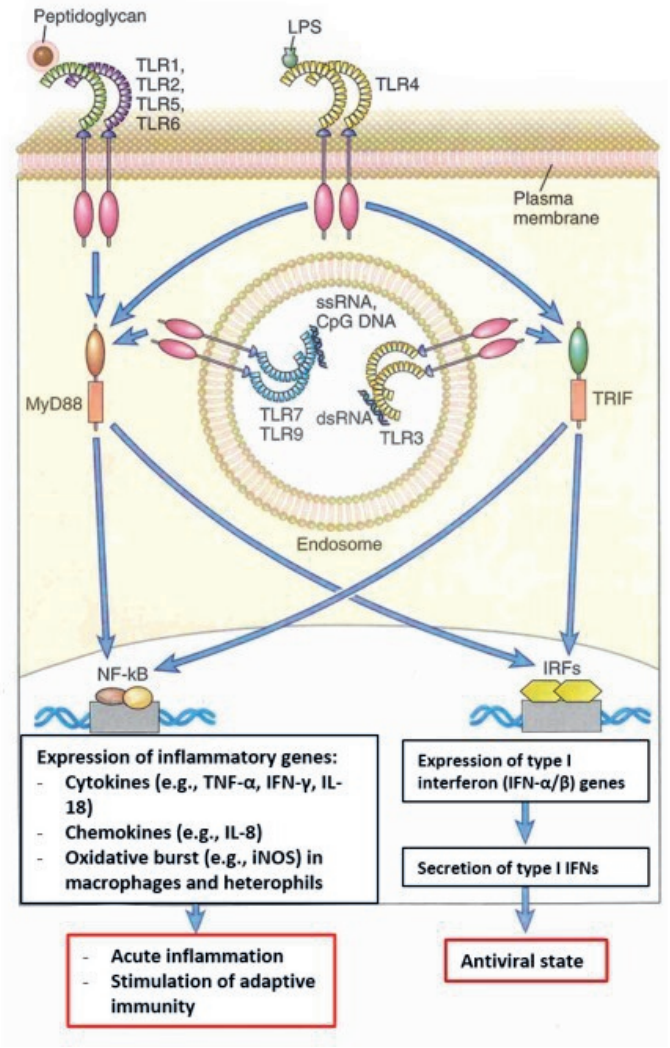


Figure 1.4. Signalling pathways and functions of TLRs, adapted from Abbas et. al. (2015).

The appropriate levels and functioning of different immune cell populations and immune mediators (e.g. cytokines, chemokines and miRNAs) are crucial for the communication and cooperation between different types of immune cells, which is the basis of an adequate immune system. Environmental stressors, such as exposure to trace metals, especially mercury (Hg) and lead (Pb), often in their divalent ionic forms, are known to affect the immune system including immune cell populations and mediators without clearly known mechanisms (Ackerman et al., 2016; Badry et al., 2020; Finkelstein et al., 2007; Vallverdú-Coll et al., 2019; Whitney & Cristol, 2018).

1.2. Immunotoxicity of trace metals

It has been reported that even low levels of trace metal exposure may affect multiple physiological functions including compromised immunity, potentially impacting immune organs, immune cell populations and signalling molecules (Ackerman et al., 2016; García-Mendoza et al., 2021; Thaxton & Parkhurst, 1973; Vallverdú-Coll et al., 2019; Whitney & Cristol, 2018; Wolfe et al., 1998). Total Hg (tHg) levels in blood have been related to decreased phagocytosis by macrophages in environmentally exposed black-footed albatrosses (*Phoebastria nigripes*) (Finkelstein et al., 2007), and higher blood tHg levels also caused weaker cellular immunity estimated with skin swelling responses after a challenge with phytohaemagglutinin (PHA) in female tree swallows (*Tachycineta bicolor*) (Hawley et al., 2009). Exposure to methylmercury (MeHg) was reported to lower lipopolysaccharide (LPS) triggered B-cell proliferation in zebra finches (*Taeniopygia guttata*) (Lewis et al., 2013) and to inhibit antibody production in common loons (*Gavia immer*) (Kenow et al., 2007). Compared to cellular immune responses, humoral immune responses in chicken showed higher susceptibility to the toxic effects of inorganic Hg(II) (Bridger & Thaxton, 1982, 1983).

Mallard ducks *(Anas platyrhynchos)* orally exposed to Pb pellets showed decreased spleen mass and decreasing numbers of heterophils and monocytes (Rocke & Samuel, 1991). Ingestion of Pb shots by red-legged partridges (*Alectoris rufa*) (Vallverdú-Coll et al., 2015) and mallard ducks *(Anas platyrhynchos)* (Trust et al., 1990; Vallverdú-Coll et al., 2016) has been associated with lower lysozyme and antibody levels. Dietary exposure to Pb(II) (as lead acetate) also activated the NF-κB pathway, and upregulated the gene expression of TNF-α and iNOS in isolated chicken heterophils (Li et al., 2017) but down-regulated the expression of IFN-γ in hearts of chicken (Huang et al., 2019), suggesting that Pb(II) exposure can potentially affect the inflammatory responses. The impacts of trace metals on the functioning of the immune system may cause higher risk of infections to pathogens including parasites (Carravieri et al., 2020; Ebers Smith et al., 2018). For example, parasite loads in female European shags (*Gulosus aristotelis*) were found to be negatively related with the Se:Hg molar ratio (Carravieri et al., 2020). Although the immunotoxic effects of different species of trace metals were reported in birds, the underlying modes of action are still unclear.

More insights in the cellular mechanisms underlying these immunotoxic effects were provided by results from *in vitro* studies, although most of these studies were performed in

mammal cell models (Das et al., 2008; Garber & Heiman, 2002; García-Mendoza et al., 2021; Heo et al., 1996; Lehmann et al., 2011; Levin et al., 2020; Loftenius et al., 1997; I. A. Silva et al., 2005; Yang et al., 2020). Both Hg(II) and Pb(II) showed high cytotoxicity to mammalian immune cells (I. A. Silva et al., 2005; Steffensen et al., 1994). Due to the high affinity of these trace metal ions with sulfhydryl (thiol; -SH) groups, one of the major mechanisms of Hg(II) and Pb(II) induced immunotoxicity was suspected to be the depletion of antioxidants such as glutathione (GSH) and consecutive induction of ROS, resulting in oxidative stress (Ajsuvakova et al., 2020; Matović et al., 2015; Robitaille et al., 2016). High levels of ROS may cause damage to cell structures including DNA, lipids and proteins. At the same time, ROS also act as defence strategies against infections and as signalling molecules (Schieber & Chandel, 2014). For example, some immune cells such as macrophages and heterophils can undergo oxidative bursts by rapid production of ROS to support the defence against microbial pathogen infections (Iles & Forman, 2002). Besides, Pb(II)-induced ROS formation was found to activate both mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) pathways, producing pro-inflammatory cytokines and triggering inflammation in rat kidney (Liu et al., 2012). The trace metal induced disturbance of redox homeostasis may also impair immune responses in avian species. Modification of cytokine profiles and immune mediators (such as miRNAs) are also reported to be related with trace metal exposure (Miguel et al., 2018). For instance, inorganic Hg(II) increased the release of pro-inflammatory cytokines (e.g. TNF-α and IL-17) from human peripheral blood mononuclear cells (PBMCs) (Gardner et al., 2010), while Pb(II) (as lead acetate) stimulated the gene expression of TNF-α, IL-8, and IL-6 in human PBMCs (Gillis et al., 2012). Given the similarity of avian and mammalian immune systems, similar mechanisms of modulatory effects upon trace metal exposure are suspected in avian species. Besides, given the high environmental levels of specific non-essential trace metals in the habitats of waterfowl (e.g. elevated levels of tHg due to historical mining activities in Svalbard, where Barnacle geese migrate for breeding; and elevated levels of total Pb (tPb) in the wetland due to the historical usage of Pb pellets), trace metal contamination may threaten the well-being of the immunity in waterfowl and may lead to a higher risk of infection and outbreak of zoonotic diseases.

1.3. Trace metal contamination

Non-essential trace metals, e.g. Pb and Hg, are ubiquitous in the environment and have no biological functions in living organisms but may cause potential adverse effects (Ali & Khan, 2018). Elevated levels of non-essential trace metals have been documented globally in aquatic systems, sediment, soil and biota (Atobatele & Olutona, 2015; Fernandes et al., 2020; Musilova et al., 2016; Pan & Wang, 2012). Human activities, including industrialization (e.g. mining and smelting) and urbanization, are the dominant sources of the non-essential trace metals in the environment (Li & Thornton, 2001; Luo et al., 2012; Nriagu & Pacyna, 1988). Based on the characteristics of non-biodegradability, persistence, potential for bioaccumulation and toxicity, trace metal exposure may threaten the wellbeing of organisms

that inhabit the contaminated areas (Ali et al., 2019; Kumar et al., 2019; Li et al., 2014; Pacyna & Pacyna, 2001). Among these non-essential trace metals, mercury (Hg, especially the organic form methylmercury (MeHg)) and lead (Pb, in its ionic form) are of great concern due to their high toxicity and elevated environmental levels in the habitat of some waterfowl species.

1.3.1. Mercury (Hg)

Industrial activities, including mining, metal smelting, fossil fuel combustion and municipal waste burning, are the major anthropogenic sources of Hg emission, which can result in total Hg (tHg) levels exceeding 1000 mg/kg in topsoil at contaminated areas (Ballabio et al., 2021; Millán et al., 2006). Long-distance transport of Hg by air or ocean currents has resulted in elevated environmental tHg levels even at remote locations that lack local anthropogenic sources, such as the Arctic and Antarctic (Douglas et al., 2012; Marquès et al., 2017; Negri et al., 2006). Hg exists in several forms, including elemental mercury (Hg(0)), inorganic mercury (mainly divalent mercury, Hg(II)), and organic mercury (e.g. methylmercury, MeHg). The elemental Hg(0) is volatile and can be oxidized to Hg(II) in the atmosphere. Hg(II) is much more water-soluble than Hg(0) and can readily enter terrestrial and aquatic systems via both wet and dry deposition. In the anoxic soil and sediment, sulphate reducing bacteria are able to methylate inorganic Hg and generate highly toxic MeHg (Erickson & Lin, 2015), which shows strong bioaccumulation and biomagnification along the food chain. Hg(0) vaper can be inhaled and taken up by the central nervous system (CNS) in the metallic form potentially causing neurotoxicity (Syversen & Kaur, 2012). Upon absorption, Hg(0) is rapidly oxidized to Hg(II) on entry to the blood and distributed over the body, potentially inducing toxicity to multiple organs and systems (Bernhoft, 2012). Although MeHg is recognised as the most toxic form, Hg(II) is more commonly and abundantly found in the environment and also in low trophic level species (Beckers & Rinklebe, 2017; Schlüter, 2000), and can also exert severe adverse effects on wild animals. Nevertheless, most of the mercury associated research has focused on MeHg, potentially ignoring the importance of Hg(II) for low-trophic level species, including herbivorous waterfowl.

1.3.2. Lead (Pb)

Pb is also released via urban and industrial discharges, similar to Hg. Historically, Pb (usually as the organic form tetraethyllead) was also used as an anti-knocking additive in gasoline, causing contamination of urban air and soils near highways (Nriagu, 1990). Inorganic Pb (usually as Pb(II)) was also added to paints to enhance colour and to improve their adherence and durability (O'Connor et al., 2018). Although the overall environmental Pb levels generally have decreased due to the ban on the use of Pb in gasoline and paints (Wade et al., 2021), in regions with historic hunting activities, Pb gunshot pellets may still occur at high concentrations in soils and sediments. Pb pellets were found in wetlands around Europe, up to 399 shots/m^2 in the upper 30 cm of sediment, in Spain (Mateo et al., 2007). Waterfowl may be exposed to Pb pellets while foraging, especially in the case of species that dabble for

their food, which have strong gizzards and use grit to help digest their food (De Francisco et al., 2003). After ingestion, Pb pellets can dissolve and be oxidized into Pb(II) in the intestinal tract of waterfowl, followed by a rapid distribution of Pb(II) over various tissues, including liver, kidneys, bones and growing feathers through the circulatory system (Ferreyra et al., 2014). Elevated total Pb levels in bones, blood, liver, feathers and even eggshells, suggesting maternal exposure of the offspring, have been detected in wild waterfowl such as mallard ducks from contaminated areas (Vallverdú-Coll et al., 2015, 2019). So far, research on Pb poisoning has mainly focused on risks on mortality in the populations affected and the potential neurotoxic risks (Williams et al., 2018). Nevertheless, Pb(II) can also cause some other sub-lethal effects in avian species, such as modulatory effects on their immune system (Vallverdú-Coll et al., 2019), for which also the underlying mechanisms are largely unknown.

1.4. Objectives of the thesis study

Despite the existing literature, there remain several knowledge gaps on the immunotoxicity of trace metal salts such as Hg(II) and Pb(II) and the modes of action underlying this immunotoxicity in avian species, especially waterfowl. As mentioned above, only a few mechanic studies have been performed in chicken so far, which however, is not an ideal model for waterfowl. Some papers have pointed out the differential immune response in chickens and ducks upon infection (Cornelissen et al., 2012; Kuchipudi et al., 2015; Wang et al., 2014), suggesting the importance of research with a specific waterfowl model. Furthermore, studies only checked the effects of exposure on the baseline immunity without challenge of the immune system (de Jong et al., 2017), which is crucial when discussing the vulnerability of birds to infections. Among the few studies concerning the challenges of the immune system, most were focused on bacterial or parasitic (Carravieri et al., 2020; Vallverdú-Coll et al., 2019) instead of viral infections. This is even more pressing in case of waterfowl, considering their role in the global environmental dynamics of avian influenza viruses.

Young birds in the developing stage are suspected to be much more sensitive to immunotoxic effects of trace metals (Vallverdú-Coll et al., 2019; Vermeulen et al., 2015). However, little is known about the immune modulatory effects of trace metal exposure in developing birds. Last but not least, there are no studies known to the author that investigate the possibilities of *in vitro* cell models to predict the immunomodulatory effects of trace metal exposure on waterfowl species.

To address the knowledge gaps, the research question for this thesis is:

> *Does exposure to realistic environmental levels of Hg(II) and Pb(II) result in modulation of the immune responses in waterfowl upon a viral-like immune challenge and what are the underlying modes of action?*

1.5. Study approach

To address the aforementioned research question, a stepwise approach including a field study, as well as controlled *in vivo* and *in vitro* studies was applied (Figure 1.5). The field study provides the relevance of potential impacts for waterfowl environmentally exposed to contaminants, however it does not allow to assess the causality of exposure-effect relationships nor the assessment of the specific modes of action. *In vivo* and *in vitro* studies however, can provide more insights in the causality and modes of action at the organism and cellular level respectively. By comparing and linking the findings from the field with *in vivo* and *in vitro* studies, the causality and modes of action underlying the adverse effects of trace metal exposure on the immune responses in waterfowl could be elucidated. The studies will also reveal whether it is possible to relate results in a domestic animal model and *in vitro* cell models to specific effects observed in environmentally exposed wild animals. If the *in vitro* cell models are proven to be applicable, they could serve as an alternative for animal tests and help reduce the use of experimental animals in the future.

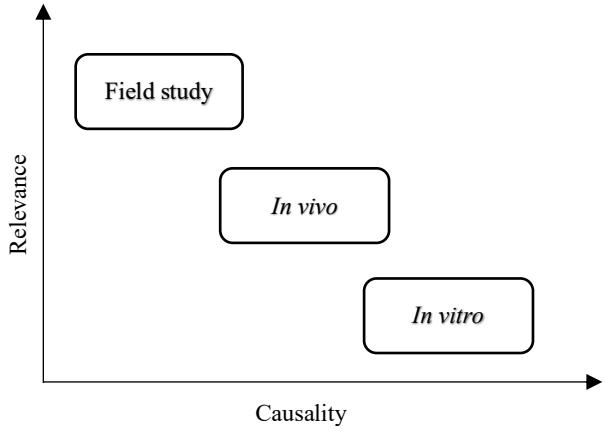


Figure 1.5. Schematic illustration of the experimental approaches of the present thesis and their potential to reflect real life environmental situations and to elucidate the modes of action underlying potential adverse effects on the immune responses.

The initial field study is based on an earlier field experiment, with modified setup. The previous study was performed during the summer of 2014 in Ny-Ålesund, Svalbard, to investigate the effects of legacy mercury from historical mining activities and social isolation on baseline immunity, neurological responses and stress behaviour in Arctic barnacle goslings (*Branta leucopsis*) (de Jong et al., 2017; Scheiber et al., 2018; van den Brink et al., 2018). Baseline immunity of gosling herded in the historical mining area was not affected by the environmental exposure to tHg (de Jong et al., 2017). However, the immune response upon challenge was not assessed. Therefore, the field study in the current thesis was designed to expose the goslings to environmental tHg (metal-species not identified but likely to be mainly Hg(II) according to previous research analysing the forms of Hg in vegetation and

soil in Ny-Ålesund (Constant et al., 2007; Douglas et al., 2012)), similarly to the earlier study (de Jong et al., 2017), and challenge their immune system with a viral-like challenge (Matsumoto & Seya, 2008). In addition, because the hepatic tHg concentrations in goslings were relatively low in the earlier study, namely 0.022 mg/kg dry weight (d.w.) for control goslings and 0.030 mg/kg d.w. for goslings herded in mining areas (van den Brink et al., 2018), an additional group of goslings was added to the experiment which received an additional exposure to Hg(II) (as $HgCl_2$) via supplementary feed (0.14 mg Hg(II) /kg feed (d.w.), similar to the tHg content in the soil samples from the historical mining area (van den Brink et al., 2018)). This additional exposure was also expected to dominate over the toxic effects due to exposure to other environmental pollutants. Similar to the previous study, new-born barnacle goslings were used as they have not yet been exposed to local Hg. The goslings were imprinted on humans and herded in the same control and mining sites as in the previous study. To minimize potential effects of genetic variations and maternal Hg exposure, three siblings were randomly distributed over the three treatment groups. We challenged their immune system 24 hours prior to the termination of the experiment through injection of double-stranded RNA (dsRNA) mimicking viral infection (Matsumoto & Seya, 2008). After 20 days of exposure, tissue and blood samples were collected to analyse the internal tHg levels, immune responses, and oxidative stress. The relationships of internal tHg levels with immune responses (including natural antibody titres, nitric oxide concentrations in plasma, leukocytes population and immune functional gene expression), and oxidative stress (assessed with mainly with GSH system) were checked with linear regression models.

To verify the findings from the field study, *in vivo* experiments were performed with Hg(II) (as $HgCl_2$) under controlled conditions. As Pb(II) is also immunotoxic, potentially threatening the well-being of waterfowl, we also included Pb(II) (as lead acetate) in the *in vivo* and *in vitro* studies. More exposure concentrations (5 exposure groups vs. 3 exposure groups in the field study) and more animals (68 ducklings for Hg(II) and 66 ducklings for Pb(II) experiment vs. 18 goslings for the field study) were included in comparison to the field study to increase the statistical power. Moreover, in the field study, most endpoints were measured only after an immune challenge with dsRNA, so it was impossible to check the effects of immune challenge and the effects of exposure on baseline immunity. To address this, non-activated groups of ducklings were included in the *in vivo* experiments. Pekin duckling (*Anas platyrhynchos domesticus*) was selected as a waterfowl model species because this species has been used earlier in immunology studies (Cornelissen et al., 2012; Fleming-Canepa et al., 2019), thus providing a basis for comparison. Furthermore, this species can be kept easily in captivity, enabling controlled exposure studies. Exposure lasted for 23 or 24 days, and the same endpoints for immune responses were measured as in the field study to enable comparison. Dose-response relationships were built using the internal liver concentrations and the immune responses.

To further understand the cellular mechanisms, *in vitro* studies with available avian immune cell lines were conducted for both Hg(II) (as $HgCl_2$) and Pb(II) (as lead acetate) exposure.

Because there are no waterfowl immune cell lines available and chicken is genetically closer related to waterfowl than mammals, two chicken immune cell lines were used. These two cell lines are the HD-11 chicken macrophage cell line as a representative for the innate immune system (Beug et al., 1979), and the DT40 chicken B-lymphocyte cell line as a representative for the adaptive immune system (Winding & Berchtold, 2001). Both cell lines express TLR3 and can therefore be stimulated with dsRNA (Peroval et al., 2013; Quan et al., 2017). General toxicity (cytotoxicity, oxidative stress) and functional immunological endpoints were quantified, including nitric oxide production by the HD-11 macrophages, antibody levels in DT40 B-cells and cell proliferation, immune gene expression (receptors, cytokines and miRNAs), and cytokine production as indicators for immunity upon Hg(II) or Pb(II) exposure and activation for both cell lines. These data reveal the effects of Hg(II) and Pb(II) exposure on specific immune cell types.

In conclusion, the experiments described in the present thesis aim to gain insights in the causality and mode(s) of action underlying the effects of trace metal exposure on the immune responses in waterfowl upon a viral-like challenge, in different model systems varying from an environmentally relevant field model, to an *in vivo* model at the individual level and *in vitro* models at the cellular level.

1.6. Outline of the thesis

Chapter 1 (the present chapter) introduces the background information, the aim of the thesis, as well as the study approach and outline of the thesis.

Chapter 2 aims to investigate the effects of low environmental tHg exposure on immune responses to a viral-like challenge in Arctic barnacle goslings (*Branta leucopsis*) in Svalbard.

New-born barnacle goslings were collected and herded in either a control or historical mining site with significantly higher tHg levels. An additional group was also herded in the mining site and exposed to extra Hg(II) via supplementary feed. The relationships between internal tHg levels in liver and the immune responses upon viral-like challenge (dsRNA injection) are assessed. Additionally, the effects of tHg exposure on oxidative stress are also discussed.

Chapter 3 describes the modulatory effects of *in vivo* Hg(II) and Pb(II) exposure on Pekin ducklings (*Anas platyrhynchos domesticus*) as a model animal for waterfowl. In the controlled conditions, dose-response relationships between trace metal exposure and immune responses upon viral-like challenge (dsRNA injection) are established. The study aims to verify the findings in the field study with barnacle goslings, and to reveal more insights in the dose-dependent effects of Hg(II) and Pb(II) exposure on the immune responses of Pekin ducklings.

Chapter 4 and **Chapter 5** address the effects of *in vitro* Hg(II) (**Chapter 4**) and Pb(II) (**Chapter 5**) exposure in two chicken immune cell lines, namely the chicken macrophage cell line HD-11 and the chicken B-lymphocyte cell line DT40. Effects of trace metal

exposure on cytotoxicity, oxidative stress, immune gene expression and cytokine production are presented. These data provide more insights in cellular mechanisms and effects on specific immune cell types.

Chapter 6 discusses the findings of the studies within the current scientific framework and presents future outlooks.

Chapter 7 provides a summary of the whole thesis.

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Chapter 2

Mercury modulated immune responses in Arctic Barnacle goslings (*Branta leucopsis*) upon a viral-like immune challenge

Biyao Han, Hans van den Berg, Maarten J.J.E. Loonen, Rafael Mateo, Nico W. van den Brink

In preparation

Abstract

Historical mining activities in Svalbard have resulted in local mercury (Hg) contamination. To address the potential immunomodulatory effects of environmental Hg on Arctic organisms, we collected new-born barnacle goslings (*Branta leucopsis*) and herded them in either a control or mining site, differing in Hg levels. An additional group was exposed to extra inorganic Hg(II) via supplementary feed, while the other groups received clean supplementary feed without Hg(II). Upon challenge of their immune system with double-stranded RNA (dsRNA) injection, endpoints for immune responses and oxidative stress were measured after 24 hours. The results indicate that Hg exposure modulated the immune responses in Arctic barnacle goslings upon a viral-like immune challenge. Hg exposure affected the immune cell populations (higher heterophil/lymphocyte ratio) and reduced the level of natural antibodies, suggesting an impaired humoral immunity. Hg exposure upregulated the expression of pro-inflammatory genes in the spleen, including inducible nitric oxide synthase (iNOS) and interleukin 18 (IL-18), suggesting Hg-induced inflammatory effects. Environmental exposure of Hg also oxidized glutathione (GSH) to glutathione disulfide (GSSG), however, goslings were capable of maintaining the redox balance by *de novo* synthesis of GSH. These adverse effects on the immune response indicated that even exposure to low, environmentally relevant levels of Hg may result in an affected immune competence at the individual level, and may even cause increased susceptibility of the population to infections.

2.1. Introduction

Mercury (Hg) is a global contaminant (Driscoll et al., 2013), potentially affecting the health of both wildlife and human beings. Elevated levels of Hg have been found in remote areas, including the Arctic, mainly due to long distance-transport via air or ocean currents, but also to local emissions (Douglas et al., 2012; Marquès et al., 2017). The major sources of local Arctic Hg emissions include coal mining, ferrous and non-ferrous metal industry, and waste incineration (Hylander & Goodsite, 2006; Outridge et al., 2008). Through the biogeochemical processes in Arctic terrestrial and aquatic environments Hg can occur in different forms, e.g. elemental (Hg(0)), inorganic forms (mainly Hg(II)) and organic forms (mainly methylmercury, MeHg) (Douglas et al., 2012; Lehnherr, 2014). Hg (II) is the predominant species in Arctic soil, tundra, snow and surface ocean (Constant et al., 2007; Douglas et al., 2012), while MeHg is of more concern for high trophic predators (e.g. beluga whales and polar bears) comprising more than 80% of their internal total Hg burden, due to its high potential for bioaccumulation and biomagnification (Bechshoft et al., 2019; Loseto et al., 2008).

Exposure to trace metals, including Hg, may modulate immune responses in wildlife, even at low environmental levels, and may, as such, potentially result in higher vulnerability to infections. For instance, in studies concerning exposure of birds, Hg was reported to weaken the T-cell mediated immunity in tree swallows, as assessed by skin swelling responses to phytohaemagglutinin (PHA) challenging (Hawley et al., 2009), to decrease macrophage phagocytosis in black-footed albatrosses (Finkelstein et al., 2007), to lower lipopolysaccharide (LPS) triggered B-cell proliferation in zebra finches (Lewis et al., 2013) and to limit antibody production in common loons (Kenow et al., 2007). In addition, parasite loads in female European shags were found to be negatively related with the Se:Hg molar ratio (Carravieri et al., 2020). Immunomodulatory effects by trace metals could be either suppression or stimulation (Krocova et al., 2000), both potentially leading to disordered immune responses. Immune suppression may result in an affected defence against pathogens, potentially posing higher risks of infection on the individuals and may lead to outbreaks of diseases in the communities (Mason, 2013; Poulsen & Escher, 2012). Similarly, undesired stimulation of the immunity can be costly, especially for migratory birds which need their energy e.g. for long distance flying (Seewagen, 2020), like geese migrating to high-arctic breeding grounds.

Svalbard (79°N/12°E) is a high-arctic archipelago, where coal mining activities were developed since 1906 (Askaer et al., 2008). However, because of several fatal accidents and declines of global coal markets (Misund, 2017), mines were gradually abandoned and closed but remains were left behind. Historical mining activity and waste piles of abandoned mines in Svalbard have resulted in continued local contamination of soil and vegetation with trace metals, including Hg (Askaer et al., 2008; Krajcarová et al., 2016; Søndergaard et al., 2007; van den Brink et al., 2018). Although there are several studies illustrating the general health

effects of mining-related heavy metal contamination on Arctic wildlife, such as Arctic hares *(Lepus arcticus)* (Amuno et al., 2016), pied flycatchers (*Ficedula hypoleuca*) (Berglund et al., 2010), voles and small birds (Brumbaugh et al., 2010), little is known about the trace metal induced immunotoxicity in Arctic migratory species.

In an earlier study, an experiment was conducted in 2014 with barnacle goslings (*Branta leucopsis*) as a model of Arctic migratory species in Ny-Ålesund, Svalbard, to investigate the effects of legacy mercury from historical mining activities and social isolation on baseline immunity, neurological responses and stress behaviours (de Jong et al., 2017; Scheiber et al., 2018; van den Brink et al., 2018). Environmental Hg exposure by herding goslings in the historical mining area did not affect their baseline immunity (de Jong et al., 2017). However, to evaluate the overall effects of Hg exposure on the immune system, not only the baseline immunity, but also the immune response upon challenge should be assessed. To assess this, a follow-up study was designed in which goslings were exposed to environmental Hg, similarly to the earlier study (de Jong et al., 2017), and their immune system was challenged with a viral-like stimulus (Matsumoto & Seya, 2008). In the earlier study, hepatic Hg levels in goslings were relatively low, namely 0.022 mg/kg dry weight (d.w.) for control goslings and 0.030 mg/kg d.w. for goslings herded in mining areas (van den Brink et al., 2018), hence in the present study an additional group of goslings was exposed to extra Hg (as $HgCl_2$) via supplementary feed, while the other groups received clean supplementary feed without Hg. Similar to the previous study, new-born barnacle goslings were used as they have not yet been exposed to local Hg. The goslings were imprinted on humans and herded in the same control and mining sites as in the previous study. To minimize potential effects of genetic variations and maternal Hg exposure, three siblings from each nest were randomly distributed over the three treatment groups. We challenged their immune system 24 hours prior to the termination of the experiment through injection of double-stranded RNA (dsRNA) mimicking viral infection (Matsumoto & Seya, 2008). After 20 days of exposure, we collected blood and tissue samples to analyse the immune responses.

2.2. Materials and methods

2.2.1. Study site and animals

The study was conducted in the area nearby Ny-Ålesund (78°55′N, 11°56′E), Svalbard (Spitsbergen). A control site (78°55′54″N, 11°50′10″E, 2.13 km to the northwest of the Ny-Ålesund village) and a mining site (78°54′55″N, 11°57′22″E, 1.36 km to the southeast of the Ny-Ålesund village) were chosen for herding the goslings (Figure 2.1). The control site is an undisturbed tundra area where wild geese and goslings were also noticed to be grazing (pers. obs.). The mining site experienced historical coal mining activities from 1916 to 1962, and has been abandoned since 1963 due to a severe incident (Dowdall et al., 2004). Although the vegetation has recovered to some extent during the past decades, remains of the mine activities such as stacks of coals, rusted installations and abandoned equipment stayed as heritage (pers. obs.). A previous study indicated that both soils and vegetation

from the mining site contained significantly higher Hg levels than the control site due to coal mining activities (van den Brink et al., 2018).



Figure 2.1. Map showing the study sites, including the control site to the northwest and the mining site to the southeast of the village of Ny-Ålesund. The map was adapted from Norwegian Polar Institute via https://toposvalbard.npolar.no/.

Eighteen 0-day-old barnacle goslings were collected from the uncontaminated island Storholmen in Kongsfjorden on 26th June 2019. Three hatchlings were collected per nest from 6 nests. Immediately upon collection, goslings were labelled with web tags on one of their feet and specific-coloured bands on their legs for easy identification during the experiment. Siblings were randomly assigned to three treatment groups, namely control, mining, and extra Hg group (6 goslings per group). Goslings were hand-reared by four humans (BH, NvdB, HvdB, AN) as foster parents, who also trained the goslings for further experimental handling. To minimize the potential effects of parenting by specific individuals, human foster parents took turns to provide care for the different groups of goslings. Body mass and total tarsus length were measured every other day to monitor the development of goslings. One gosling from the mining group was attacked by an arctic fox and dropped out from the experiment on day 13 (9th July 2019).

2.2.2. Experimental design

To investigate the effects of environmental Hg exposure on the immune response of barnacle goslings, the three groups of goslings received different treatments for 20 days. Goslings in the control group were herded in the control site and provided with clean supplementary feed. Goslings in the mining group were herded in the mining site and given clean supplementary feed while goslings in the "extra Hg" group were herded in the mining site and exposed to supplementary feed spiked with Hg (0.14 mg Hg /kg feed (d.w.), as $HgCl_2$, Because the Barnacle goslings in the experiment were fed on the vegetations in either the control or mining site, in which $HgCl_2$ was the predominant form of Hg (Constant et al., 2007), $HgCl_2$ was added to the supplementary feed for the extra Hg group. Research Diet Services BV, Wijk bij Duurstede, the Netherlands). Feed was provided from the first day. From day 5 to day 11 (1st to 7th July), goslings were herded in assigned locations for approximately 6 hours per day. From day 12 onwards, goslings from each group were left overnight in the assigned locations in a cage of approximal 2 m×2 m×0.8 m, surrounded by an electric fence to keep out predators. Shelters with electric car seat heaters were installed to keep the goslings warm, and supplemental feed as well as water were provided *ad libitum* overnight. One day prior to termination of the experiment (day 19, 15th July), one drop of blood was drawn from each gosling by puncturing the brachial vein with a 23G needle (BD Vacutainer®, Becton Dickinson, USA) to make a blood smear. The blood smear was air-dried. Then, goslings were immune challenged with 50 μg/kg bodyweight poly I:C (synthetic dsRNA analogue) via intraperitoneal (i.p.) injection. During the night of day 19, limited feed was provided to ensure their crop would be empty for euthanasia.

On day 20 (16th July), goslings were euthanized through decapitation followed by immediate dissection. The duration between immune challenge and euthanasia ranged from 22 h to 30 h, and the rank of challenge time was included in the linear regression models for nitric oxide in plasma samples. At least 3 ml of blood per gosling was collected in blood collection tubes coated with K_2EDTA (BD Vacutainer®, Becton Dickinson, USA). Another (air-dried) blood smear was made for each gosling and the rest of the blood was centrifuged at 1,000×*g* for 10 min to separate plasma and cellular factions. Red blood cells were washed with saline solution (0.9% NaCl) three times. Afterward, plasma and washed red blood cells were snap-frozen in liquid nitrogen and stored in -80 °C until further analyses. Immune organs including spleen, thymus and bursa were isolated, snap-frozen and stored at -80 °C until further analyses for gene expression. Liver tissue was collected and stored at -20 °C to determine the internal Hg and Se levels. All samples were transported to the Netherlands on dry ice.

2.2.3. Chemical analyses

Hg and Se levels were determined in both kinds of supplemental feed and in liver tissues. Briefly, liver tissues and feed were freeze-dried at -50 °C for 18 h and then digested in either 70% nitric acid (for Hg analyses) or aqua-regia (for Se analyses) assisted with microwave. The levels of total Hg were measured with cold vapor atomic fluorescence spectrometry (CV-

AFS) (da Silva et al., 2010; van den Brink et al., 2018), while the levels of Se were measured with inductively coupled plasma mass spectrometry (ICP-MS) (Forrer et al., 2001). Blue mussel (*Mytilus edulis*) tissue (ERM-CE278k, European Reference Materials, ERM, Geel, Belgium) and lichen (BCR 482, ERM, Geel, Belgium) were used as reference material for liver and feed samples respectively. Blanks were included in each batch of 10 samples. The concentrations of total Hg and Se in liver tissues are expressed as mg/kg dry weight (d.w.).

2.2.4. Immune assays

2.2.4.1. Gene expression

Organs of spleen, bursa and thymus were transferred from -80 °C freezer to RNAlater™-ICE frozen tissue transition solution (Invitrogen, Breda, the Netherlands) to avoid RNA degradation. RNA was extracted from preserved organs with RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). The purity and quantity of RNA were checked with Nanodrop (ND-1000, Themo Scientific, Delaware, US). Afterward, QuantiNova Reverse Transcription Kit (Qiagen, Venlo, the Netherlands) was used for reverse transcription reactions to synthesize cDNA with 300ng RNA input. Gene expression of immune functional genes was assessed with QuatiNova SYBR® Green PCR Kit (Qiagen, Venlo, the Netherlands) on a Rotor-Gene® 6000 cycler (Qiagen, Venlo, the Netherlands). Sequence of primer pairs, including the housekeeping gene (GAPDH), immune receptors (CD4, CD8a, MDA5, MHCIa, MHCIIa, RIGI, TLR3 and TLR7), and also immune messengers (IFN-α, IFN-ɣ, IL8, IL18, iNOS) (Biolegio, Nijmegen, the Netherlands) are listed in Table S1.1 (He et al., 2017; Xu et al., 2016). Results were normalized against the housekeeping gene GAPDH and expressed as log2 fold changes relative to the average of spleen in the control group by the -ΔΔCT method (Schmittgen & Livak, 2008). To visualize the results, a heatmap was generated with the online tool Heatmapper (Babicki et al., 2016).

2.2.4.2. Nitric oxide assay

Nitric oxide acts not only as an effector molecule defending the host against pathogens but also as a messenger regulating the immune responses (Bogdan, 2001; Coleman, 2001). Therefore, nitric oxide levels can be a good indicator to evaluate the effects of Hg exposure on the immune response upon dsRNA challenge. Nitric oxide levels in gosling plasma samples were measured as described in previous studies (de Jong et al., 2017; Sild & Hõrak, 2009). Nitric oxide is not stable in the biological tissues and could be transformed into nitrite (NO_2^-) within seconds and then to nitrate (NO_3^-) within hours (Sild & Hõrak, 2009). Thus, nitrate (NO_3^-) was reduced to nitrite (NO_2^-), and nitrite (NO_2^-) concentrations were measured to represent the nitric oxide levels in the plasma samples. In short, 20 µl plasma was deproteinized in an alkaline condition, during which the gosling plasma was diluted 20 times. Then, nitrate in the samples was reduced to nitrite by cadmium granules coated with copper. Finally, Griess reaction was used to measure nitrite (µM) with a standard curve of $NaNO_2$.

2.2.4.3. Haemolysis-haemagglutination assay

Natural antibodies (NAbs) and complement contribute to the first immune defence in animals without any infection history (Holodick et al., 2017; Reyneveld et al., 2020). A haemolysis-haemagglutination assay was used to evaluate the interaction of NAbs and complement (with lysis titres) and for NAbs activity (with agglutination titres) (de Jong et al., 2017; Matson et al., 2005). Briefly, gosling plasma was serially two-fold diluted for ten times in round (U) bottom 96-well plates with phosphate buffered saline (PBS, Gibco, Paisley, UK). Afterward, the same volume of diluted plasma and 1% rabbit blood cell suspension in PBS were mixed and incubated in a 37 °C humidified incubator for 90 min. Then, the plates were tilted to a 45° angle on the long axis at room temperature for 30 min to enhance visualization for scoring haemagglutination titres. After incubation at room temperature for another 70 min, haemolysis titres were determined. All the samples were observed and visually scored (Matson et al., 2005) by one person (BH). Half scores were given when agglutination or lysis was intermediate. Two replicates were tested for each gosling.

2.2.4.4. Haptoglobin assay

Haptoglobin is an acute-phase protein rapidly increasing in the case of inflammation, infection, or trauma (Matson et al., 2012; Quaye, 2008; Wang et al., 2001). A commercially available colorimetric haptoglobin assay kit (TP801; Tri-Delta Development Limited, Maynooth, Ireland) was used to quantify the haptoglobin-like activity (mg/ml) in gosling plasma samples with a calibration curve. As haemolysis interferes with the assay, plasma sample redness was measured as absorbance at 450nm before the addition of chromogen reagent for statistical correction (Matson et al., 2012).

2.2.4.5. Blood smear

Blood smears were stained with Hemacolor® Rapid staining (Sigma-Aldrich, Zwijndrecht, the Netherlands) and counted by one observer (BH) using a light microscope at 1000× magnification with immersion oil (Zeiss, Jena, Germany). To determine the leukocyte density, the number of leukocyte, thrombocyte and red blood cells were counted until the vision containing the 5000th red blood cell (usually 50-60 visions). For leukocyte counts, heterophils (normal or toxic), eosinophils, basophils, monocytes and lymphocytes (reactive or non-reactive) were identified according to the morphological characteristics (Jones, 2015; Mitchell & Johns, 2008), and at least 100 leucocytes were counted per slide. Changes (Δ) in leucocyte populations were calculated by subtracting the counted results of the slide before poly I:C challenge from the one after challenge.

2.2.5. Oxidative stress assays

Indicators for oxidative stress were measured in gosling red blood cells or in plasma, including superoxide dismutase (SOD), glutathione peroxidase (GPx), total glutathione (GSHt), glutathione disulfide (GSSG, oxidized from of glutathione), and malondialdehyde

(MDA) in red blood cells (RBCs); together with retinol (vitamin A), lutein (carotenoids), α-tocopherol (vitamin E) and MDA in plasma, according to the methods documented in previous studies (Lopez-Antia et al., 2015; Martinez-Haro et al., 2011). Briefly, an automated spectrophotometer A25-Autoanalyzer (BioSystems S.A., Barcelona, Spain) was used to quantify the SOD, GPx, GSHt, GSSG in RBCs. A high-performance liquid chromatography (HPLC) system was used to measure the MDA in both RBCs and plasma samples as well as retinol, lutein, and α-tocopherol in plasma samples.

2.2.6. Genetic sexing

The sex of goslings was determined genetically (Bird Genetics, Erp, the Netherlands) with DNA extracted from erythrocytes after the field experiment. The CHD-Z and CHD-W gene fragments located on the avian sex chromosome (either Z or W) were checked by polymerase chain reaction (PCR) (Griffiths et al., 1998). After separating PCR products on agarose gel, one band refers to a male (only CHD-Z gene on two Z chromosomes) and two bands refer to a female (both CHD-Z and CHD-W gene on Z and W chromosomes). Sexing results showed that we had seven males and ten females in total. In both control and extra Hg groups, there were one male and five females, while all the five goslings in the mining group were male.

2.2.7. Statistics

All statistical analyses were performed with SPSS (IBM SPSS Statistics, version 25). Firstly, we checked whether the growth rate of goslings was affected by Hg exposure during the experiment, using linear mixed models. Daily growth rates calculated as percentage change in either body mass (g) or total tarsus length (mm) were used as dependent variables. Animal ID was used as subjects while log Hg levels, sex, age and siblings were set as fixed effects.

Endpoints were analysed with either ordinal regression (for discrete data, e.g. haemolysis and haemagglutination titres) or linear regression (for continuous, e.g. gene expression, blood cell population, GSHt, etc.) models. Variables were transformed if they did not meet the model assumption of normal distribution. For instance, internal Hg and Se levels, and heterophil/lymphocyte (H/L) ratio were log-transformed, while haptoglobin and nitric oxide levels were square-root transformed (de Jong et al., 2017). Hepatic Hg levels, sex, and siblings were included in the models as independent variables. For nitric oxide, ranking of challenge time was also included in the linear regression model, while plasma redness (absorbance at 450nm) was added in the model for haptoglobin as a co-variate (Matson et al., 2012). Results were visualized with GraphPad Prism 5 (San Diego, CA, USA).

2.3. Results and discussion

The study aimed to explore the effects of environmental Hg exposure on immune responses upon a viral-like challenge in barnacle goslings. We assessed multiple immune functional endpoints, including changes in the immune cell population, plasma-based immune indicators (haemolysis, haemagglutination, haptoglobin and nitric oxide), immune gene

expression and oxidative stress. With the challenge of the immune system with dsRNA and increased Hg exposure, more specific effects were quantified than in a previous study on non-challenged barnacle goslings at the same location (de Jong et al., 2017).

2.3.1. Effects of exposure on growth

According to the output of linear mixed models, the daily growth rate of goslings calculated as either body mass (g/g/day) or tarsus length (mm/mm/day) was not influenced by Hg exposure, sex, or siblings (Table 2.1). Estimates of fixed effects are listed in Table 2.1. Age showed a negative correlation with the growth rate in both body mass and tarsus models, which indicated a slower growth rate at the late stage of the experiment (Table 2.1). No significant differences were detected in the last measurement on day 19 for body mass (g) (control group: 484.0±70.2 g (average ± stdev); mining group: 515.6±70.5 g; extra Hg group: 478.7±45.4 g) or tarsus length (mm) (control group: 70.7±2.5 mm; mining group: 73.1±3.6 mm; extra Hg group: 70.2±4.4 g). The lack of effects of exposure on growth rate is likely because the Hg exposure is low and ad libitum feed was available for the goslings.

Table 2.1. Statistical outcomes of linear models for gosling daily growth rate (%) calculated with body mass (g/g/day) and tarsus length (mm/mm/day). Underlined t and F values are significant at $p < 0.05$.

Daily growth rate	Variable	Estimate	SE	df	t
Body weight (g/g/day)	Intercept	0.1438	0.0126	80.684	**11.398**
	log tHg	5.646E-6	0.0036	52.875	0.002
	Age (day)	-0.0015	0.0007	52.018	**-2.261**
	Sex=F	-0.0055	0.0064	52.842	-0.863
	Sex=M	0[a]			
	Siblings=1	0.0039	0.0095	52.922	0.410
	Siblings=2	-0.0128	0.0108	52.920	-1.180
	Siblings=3	0.0063	0.0095	52.922	0.665
	Siblings=4	0.0093	0.0097	52.919	0.960
	Siblings=5	0.0078	0.0097	52.802	0.803
	Siblings=6	0[a]			
Tarsus Length (mm/mm/day)	Intercept	0.0615	0.0045	70.462	**13.606**
	log tHg	0.0003	0.0015	79.629	0.219
	Age (day)	-0.0019	0.0002	64.346	**-8.470**
	Sex=F	-0.0037	0.0026	79.544	-1.421
	Sex=M	0[a]			
	Siblings=1	-0.0036	0.0038	79.745	-0.947
	Siblings=2	0.0020	0.0044	79.741	0.453
	Siblings=3	0.0012	0.0038	79.745	0.300
	Siblings=4	0.0009	0.0039	79.738	0.240
	Siblings=5	0.0003	0.0039	79.444	0.078
	Siblings=6	0[a]			

a: This parameter is set to zero because it was redundant.

2.3.2. Hepatic Hg and Se levels

Total Hg (tHg) levels in gosling liver tissues ranged 3 order of magnitude among goslings (from 0.0086 up to 0.87 mg/kg d.w.) and showed a significant increase from control group (0.011±0.002 mg/kg d.w.) to mining group (0.043±0.011 mg/kg d.w.) and to extra Hg group (0.713±0.137 mg/kg d.w., Figure 2.2a). According to the linear regression, liver Hg levels were independent from Se level, sex, and siblings (Table 2.2). Compared to the previous study (van den Brink et al., 2018), the tHg levels in the control group were lower (mean current study: 0.011 mg/kg d.w. versus mean previous study: 0.022 mg/kg d.w.) while those in the mining group were higher (mean current study: 0.043 mg/kg d.w. versus mean previous study 0.030 mg/kg d.w.). The reason for these differences could be related to the different herding styles. In the previous study, goslings were walked all the way from the village of Ny-Ålesund to either the control and mining sites. Hence, the control goslings could be exposed to more Hg than in the control site while walking. In the current study, goslings were transported by the foster parents, which ensured the goslings were only exposed to local Hg in either the control or the mining sites. The higher tHg levels in the mining group of the current study were probably because of the longer grazing time as the goslings were left in the sites overnight from day 12 onwards. The extra Hg group was provided with supplementary feed with 0.14 mg Hg /kg feed d.w., which was at the same level as Hg levels found in the soil of the mining site (van den Brink et al., 2018). The average tHg levels in the extra Hg group was 0.713 mg/kg d.w., which was around 70 and 15 times higher than the control and mining groups respectively, and likely relevant for wild goslings foraging continuously in the mining area who do not receive clean additional feed like the mining group.

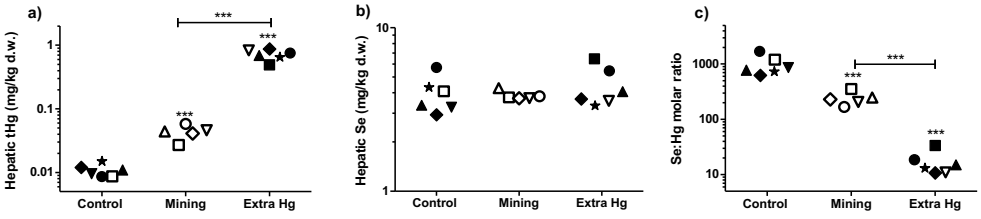


Figure 2.2. Hepatic tHg levels (a) and Se (b) levels in gosling and calculated Se:Hg molar ratio (c) of different exposure groups. Siblings from the same nest are shown as symbols with the same shape, while males are represented as closed symbols and females as black symbols. Significant differences were checked with one-way ANOVA with Tukey post hoc test (***$p<0.001$).

Selenium (Se) has been documented to be protective against Hg toxicity (Hossain et al., 2021a, 2021b; Li et al., 2014; Yang et al., 2008; Yoneda & Suzuki, 1997). Although Se levels in liver tissues did not vary among exposure groups (Figure 2.2b), Se levels differed significantly between siblings (Table 2.2), suggesting potential maternal influence (Ackerman et al., 2016). We also calculated the Se:Hg molar ratios, ranging approximately from 1000 to 10, which were significantly different among exposure groups (Figure 2.2c).

Se:Hg molar ratio can be used as an indicator to estimate Hg toxicity, as Se showed a protective effect on Hg intoxication (Carravieri et al., 2020; Li et al., 2014). In an earlier study, the number of parasites related negatively to Se:Hg molar ratios in a range from 5 to 30 in female shags (Carravieri et al., 2020). This range partly overlapped with the Se:Hg ratios we found in the goslings (10 to 1000). According to the results, the hepatic Se levels did not differ among goslings and therefore did not influence the differences of Se:Hg molar ratios among the groups (Table 2.2). Therefore, hepatic Se level was not included as one of the independent factors in the linear models for immune and oxidative stress makers.

Table 2.2. Output of ordinal (for haemagglutination) or linear regression models for hepatic element levels, immune response and oxidative stress endpoints. Listed are the $\chi 2$ (for haemolysis and haemagglutination) or F values of overall regression and Wald (for haemolysis and haemagglutination) or t values of individual parameters. Underlined t- and F-values are significant at $p < 0.05$. N (control/mining/extra Hg) = 5/6/5.

	Endpoints	log tHg (t/wald)	log Se (t)	Sex (t/wald)	Siblings (t/wald)	Challenge time order	Total df	regression (F/$\chi 2$)
Hepatic levels	Log tHg (mg/kg d.w.)	-	1.005	-0.490	0.729	-	16	0.435
	Log Se (mg/kg d.w.)	1.005	-	-0.034	**-3.539**	-	16	**4.751**
	Se:Hg molar ratio	**-6.401**	1.353	-1.652	0.172	-	16	**10.897**
Immune response	Haemolysis	1.759	-	**7.003**	3.050		93	**9.307**
	Haemagglutination	**8.071**	-	0.613	0.173	-	109	**10.536**
	Haptoglobin-like activity	-0.028	-	0.373	**-3.656**	-	16	**4.501**
	ΔHeterophil (%)	**2.257**	-	-2.015	0.458	-	16	**3.555**
	Δlymphocyte (%)	-0.495	-	**2.337**	1.298	-	16	3.152
	Δ log H/L	1.516	-	-2.054	-0.679	-	16	2.953
	log2 IL18 expression	**2.221**	-	-1.894	-0.462	-	16	**3.574**
	log2 iNOS expression	**2.555**	-	-0.150	0.879	-	16	2.496*
	Nitric oxide (μM)	-1.744	-	-0.234	0.427	**2.449**	16	**4.157**
Oxidative stress	GSHt (μmol/g RBC)	**3.002**	-	0.459	0.356	-	16	**3.062**
	GSHox (μmol/g RBC)	1.884	-	-0.773	-0.437	-	16	1.575
	GSHox%	0.197	-	-1.045	0.165	-	16	0.405
	Tocopherol (nmol/ml plasma)	-1.501	-	-0.233	**-2.255**	-	16	2.559
	Retinol (nmol/ml plasma)	0.129	-	-0.055	**-2.506**		16	2.212

*: The overall regression for iNOS expression is not significant when sex and siblings are included (F-value=2.496, p-value=0.106). When sex and siblings are excluded, the linear regression is significant (F-value=7.310, p-value=0.016) due to lower degree of freedom (df).

2.3.3. Immune responses

2.3.3.1. Gene expression

Gene expression profiles were integrated in a heatmap (Figure 2.3). Different patterns of gene expression were shown in the three organs we tested. For example, T-lymphocyte specific receptors, including the cluster of differentiation 4 (CD4) and CD8 receptors were much higher expressed in thymus than in spleen and bursa, while the toll-like receptor 7 (TLR7) was lower expressed in thymus (Figure 2.3). These variable gene expression profiles in different organs are due to the different composition of immune cell populations in the organs, namely T- and B-lymphocytes are the major cell types in thymus and bursa, respectively, while spleen contains multiple types of immune cells (Jeurissen et al., 1988).

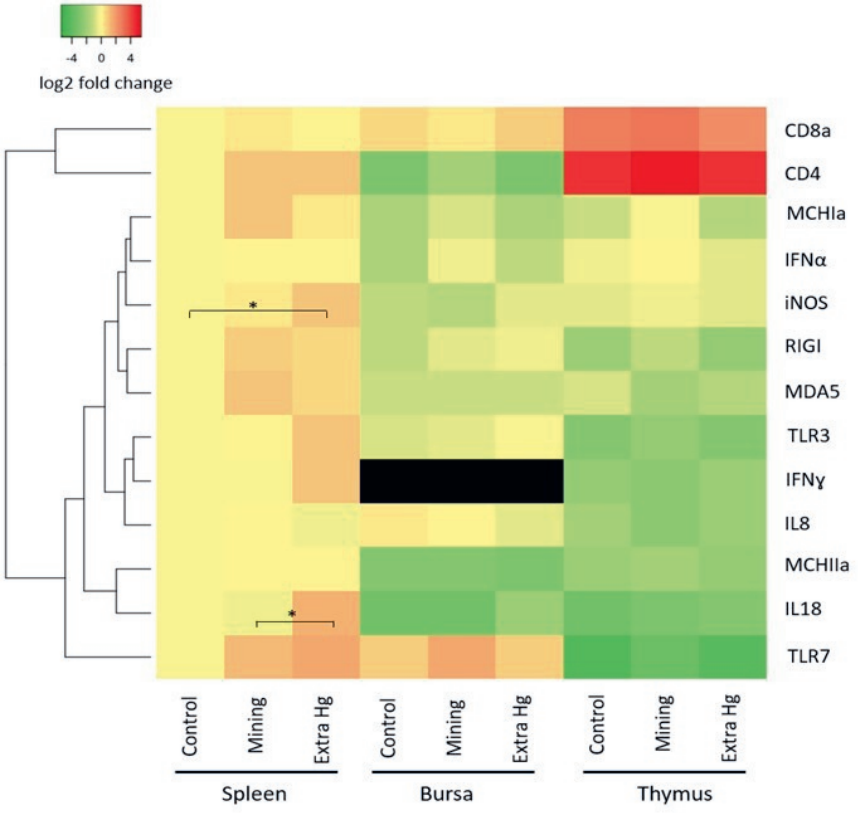


Figure 2.3. Gene expression of immune functional genes in gosling spleen, bursa and thymus tissues. Results were normalized firstly with the housekeeping gene GAPDH, and shown as log2 fold change relative to the average expression in spleen of control group. Black color indicated the non-detected genes (Ct>40, IFN-ɣ in bursa). Significant differences between groups (n=6 for control and extra Hg group, and n=5 for mining group) were tested with one-way ANOVA for each gene per organ with Tukey post hoc test (*$p<0.05$).

Among all the tested genes, only the expression of iNOS and IL18 in spleen were significantly different among exposure groups. The expression of iNOS in the extra Hg group was significantly higher than that in the control group, while the expression of IL18 was up-regulated in the extra Hg group compared to the mining group. According to the significant linear regression models, the expression of spleen iNOS and IL18 was both positively correlated with the internal tHg levels (Table 2.2). As both iNOS and IL18 are pro-inflammatory genes (Dinarello, 1999; Wood et al., 2005; Zhang et al., 2009), the upregulation suggests a potential increased inflammation due to Hg exposure upon challenge. The undesired inflammation might result in disorders in immunity such as autoimmunity (Nagy et al., 2007) and could be costly for birds, especially for the ones who need energy for migration (Eikenaar et al., 2020).

2.3.3.2. Nitric oxide

Plasma nitric oxide levels were not significantly affected by Hg exposure, but strongly related with the time interval between challenge and dissection (Table 2.2, Figure 2.4). The highest nitric oxide levels (up to 92.56 μM) were found in the goslings with the longest challenge time (around 30 h from the injection of poly I:C to dissection). Although the nitric oxide levels we examined after dsRNA challenge were already much higher (control group: 17.87±4.99 μM; mining group: 50.40±26.65 μM; extra Hg group: 17.76±6.77 μM) than the baseline nitric oxide levels measured by a previous study (de Jong et al., 2017) on barnacle goslings in the same area (mean control group: 0.69 μM; mean mining group: 0.35 μM). The nitric oxide levels were related to the timing between challenge and measurement of the levels (figured 4b), which would indicate that challenge time probably should have been longer to ensure a full induction of nitric oxide production. Barnacle goslings are waterfowl, and Pekin ducks only showed significantly higher levels of nitric oxide in serum after 72 h post-infection with H5N1 avian influenza virus, while chickens already produced significantly higher nitric oxide 24 h post-infection (Burggraaf et al., 2011). Hence, it may be that waterfowl species need a longer challenge time to build a proper nitric oxide response in comparison to the commonly studied chicken. Nevertheless, Hg exposure upregulated the gene expression of iNOS at the transcriptional level in the goslings (figure 3), which is a relatively early stage indicator of a nitric oxide response. Therefore, the non-changed plasma nitric oxide levels are not in conflict with the upregulated iNOS expression.

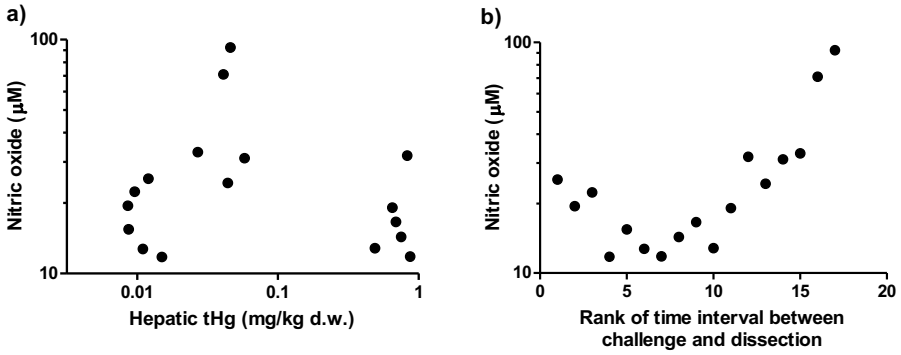


Figure 2.4. Nitric oxide levels in gosling plasma against the hepatic tHg levels (a) and the rank of time interval between challenge and dissection (b).

2.3.3.3. Haemolysis-haemagglutination

The haemolysis titre was not significantly influenced by the hepatic tHg levels, but was significantly higher in males than females (Table 2.2). Haemolysis titre reflects the activity of complement-like enzymes (Ochsenbein & Zinkernagel, 2000) and was reported to be male-biased in adult free-living wild birds during the breeding season (Valdebenito et al., 2021). Male Barnacle goslings also showed significantly higher haemolysis titres (p-value=0.024) in the previous study (de Jong et al., 2017). Due to the relatively small sample size we used, all the five goslings in the mining group were male and showing significantly higher haemolysis titres than the other two groups. Thus, although the goslings we used in the current study were at an early stage of development, and much younger than the age for sexual maturity (2 years of age) (Fjelldal et al., 2020; Forslund & Larsson, 1992), the results demonstrated that sex instead of Hg exposure was the major driver of the differences in haemolysis titre.

As for haemagglutination titre, hepatic tHg levels showed a significant negative influence according to the linear regression model (Table 2.2, Figure 2.5). Natural antibodies involved in the haemagglutination process are important for the constitutive innate immunity, providing the rapid first-line of defence to antigens (Matson et al., 2005), and are also crucial players in the humoral immunity mediated by B-lymphocytes (Baumgarth et al., 2005). In the previous study, natural antibody activity showed a decrease in goslings herded in the mining site only after social isolation, which acted as an acute stressor (de Jong et al., 2017). Higher natural antibody levels were predictive to higher survival rates in laying hens (Sun et al., 2011). Besides, natural antibodies were reported to protect mice from viral and bacterial infections by suppressing pathogen spreading and enhancing pathogen elimination in lymphoid organs (Ochsenbein et al., 1999). Therefore, in the current study, the significantly lower natural antibody activity due to Hg exposure suggests an impaired constitutive innate

humoral immunity and defence to pathogens, which might lead to a higher risk of infections for the goslings.

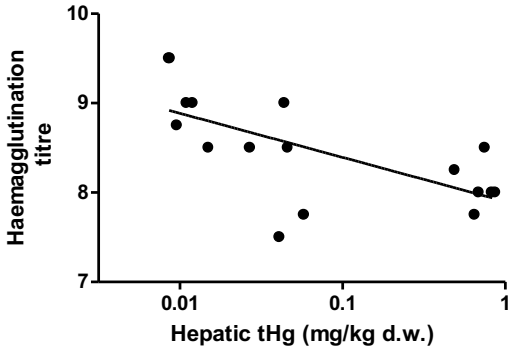


Figure 2.5. Linear regression of haemagglutination titre against tHg levels in gosling liver samples.

2.3.3.4. Haptoglobin

Haptoglobin-like activity in plasma samples showed no difference among the exposure groups (control group: 0.335±0.075 mg/mL, mining group: 0.346±0.072 mg/mL, and extra Hg group: 0.335±0.062 mg/mL). Linear regression also indicated that haptoglobin levels were not influenced by internal tHg or sex, although they differed between siblings (Table 2.2). Plasma redness (as absorbance at 450nm) was also included in the linear regression model but had no effect (t-value=-0.557, p-value=0.589).

2.3.3.5. Immune cell populations

Immune cell populations were assessed using blood smears and the percentage of each type of leukocytes within the total white blood cells (WBCs) was calculated. The change (Δ) of each cell type of leukocytes due to the immune challenge (expressed as a percentage in relation to total WBCs) was calculated as a proxy for the immune responses. The Δheterophil showed a significant positive correlation with hepatic tHg levels (Table 2.2, Figure 2.6a). ΔLymphocyte values were slightly decreased with the rise of hepatic tHg levels (not significant) and were found to be significantly influenced by sex (Table 2.2, Figure 2.6b). With the increase of Δheterophils and decrease of Δlymphocytes in the extra Hg group, the Δlog heterophil/lymphocyte ratio (Δlog H/L) raised with the higher hepatic tHg levels, although not significant (p-value=0.153), and was independent from all the factors in the model (Table 2.2, Figure 2.6c).

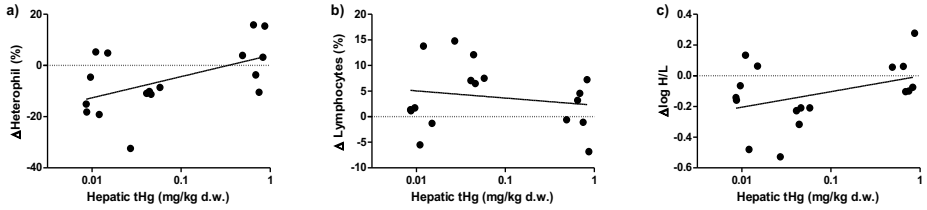


Figure 2.6. Change of heterophil population (a), lymphocyte population (b), and log heterophil/ lymphocyte ratio (c) in relation to tHg levels in liver samples according to the blood smear readings.

Heterophils in avian species have a similar function as neutrophils in mammals, being phagocytic cells, protecting the organisms against pathogens, and are also one of the major cell types producing nitric oxide (Maxwell & Robertson, 1998). Lymphocytes include B-cells that induce antibody mediating humoral immunity and T-cells, mediating cellular immunity (Sharma, 1991). However, the microscopy method used for cell type identification cannot differentiate B-cells and T-cells. Nevertheless, the H/L ratio is an indicator for humoral immune response (Gross & Siegel, 1983; Krams et al., 2012) and an increased H/L ratio, although not significant, may be related to a lower humoral immunity in exposed goslings.

2.3.4. Oxidative stress

Hg is reported to have a high affinity for the thiol group in GSH (Hultberg et al., 2001), and could induce the conversion of GSH to its oxidized form glutathione disulfide (GSSG), disturbing the redox balance and resulting in oxidative stress (Kobal et al., 2008; Ren et al., 2017; Salazar-Flores et al., 2019). Levels of total GSH (GSHt) and GSSG were qualified in red blood cells. As two GSH molecules were oxidized into one GSSG ($2GSH + H_2O_2 \rightleftharpoons GSSG + H_2O$) (Flohé, 2013), the levels of oxidized GSH molecules (GSHox) were calculated by duplicating the levels of GSSG measured (Figure 2.7b). To evaluate the redox status, the percentage of oxidized GHS molecules (GSHox%) was calculated (GSHox%=GSHox/GSHt) (Figure 2.7c). According to the linear regression models, hepatic tHg levels significantly increased GSHt levels and almost significantly increased GSHox levels (p-value=0.082) (Table 2.2, Figure 2.7a, b). Due to the increasing trend of both GSHt and GSHox with the increase of internal tHg levels, the GSHox% stayed more or less stable along with the increase of internal tHg levels (Figure 2.7b,c). Thus, the results demonstrate that goslings probably were able to compensate the Hg induced GSH depletion by triggering the *de novo* synthesis of GSH at the same time as a protective strategy. This dynamic feedback resulted in a stable GSHox% and illustrated that goslings were able to maintain redox balance upon Hg exposure at these environmental relevant levels. Similarly, increased GSHt and GSHox with stable GSHox% were found in captured mallard ducks with more than 20 µg Pb /dL blood (Martinez-Haro et al., 2011). Hg and Pb are both divalent metals, and they probably have a similar mode of action for toxicity. However, when the Hg exposure

gets higher, potentially exceeding the capacity of the buffer system, the levels of GSH could decrease, resulting in oxidative stress. For example, in surf scooter (*Melanitta perspicillata*), hepatic GSH levels showed a significant negative correlation with hepatic tHg levels ranging from 10 to 30 mg/kg d.w. (Hoffman et al., 1998), which is around ten times higher than the highest exposure in the present study.

No effect of Hg exposure was found in other indicators for oxidative stress, including SOD, GPx, MDA, retinol, lutein, and α-tocopherol.

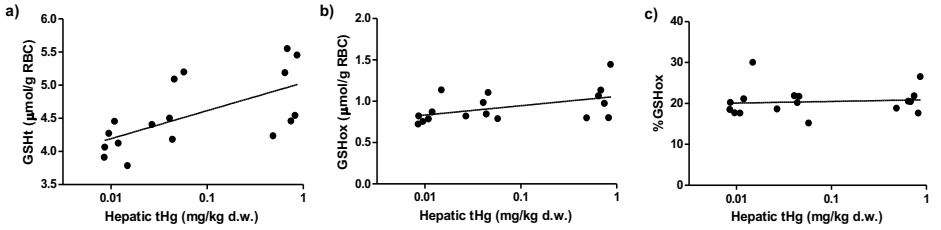


Figure 2.7. Levels of total glutathione (GSHt, a), oxidized glutathione (GSHox, b) and the percentage of oxidized glutathione (GSHox%, c) in gosling red blood cells (RBC).

2.3.5. Effects of siblings and sex

Siblings and sex were included in the statistics (Table 2.1, 2.2). Siblings influenced the internal Se levels, probably reflecting the maternal exposure (Ackerman et al., 2016). Haptoglobin-like activity, tocopherol and retinal levels were significantly correlated to siblings, but none of them were affected by internal tHg levels. Thus, the effects of siblings on these immune endpoints might be related to the maternal exposure to Se or genetic variations between nests.

Sex only showed an influence on haemolysis titres and Δlymphocytes (Table 2.2). Females were reported to usually have greater immune responses than males, such as phagocytosis and antibody responses (Klein & Flanagan, 2016). However, this sex-related difference is probably too early to be expected on most endpoints we measured at such a young age. Besides, due to the small sample size in the study, males and females were not evenly distributed in each group, especially in the mining group, where all five of the goslings turned out to be male. Larger sample size and more insights into the modes of action underlying the sex dependent immune responses are needed to confirm and explain the findings.

2.4. Conclusions

In summary, we revealed that even at low environmentally relevant levels, Hg exposure modulated the immune responses upon a viral-like immune challenge in barnacle goslings. Hg exposure led to a weaker humoral immunity with lower levels of natural antibodies, and also induced inflammation by upregulating the gene expression of iNOS and IL18. In

addition, Hg exposure in the present study oxidized GSH to GSSG, but goslings managed to compensate for this effect and maintained the redox balance by synthesizing more GSH. The observed inflammation due to Hg exposure could be costly for migratory birds like barnacle geese and influence their overall fitness. These adverse effects on the immune response, especially on humoral immunity, may result in compromised immune competence with weaker defence to infections. Nevertheless, some issues still need further research, e.g. the effects on the later stage immune response such as nitric oxide levels. Compared with the previous study on baseline immunity, more adverse effects were noticed in the challenged immune responses included in the present study, indicating that in future immunotoxicity studies attention should be focussed on the stimulated immune responses instead of on baseline immunity.

Acknowledgement

The animal experiment was performed according to the proposal approved by the Norwegian National Animal Research Authority with FOTS ID 19856. We thank Kings Bay AS and the Governor of Svalbard for their cooperation. Alice Naber (AN), Frigg Speelman, Kristle Kranenburg, Miriam van der Meulen, Marije Jousma, and Christophe Brochard are thanked for their valuable supports in the field. Pablo R. Camarero is acknowledged for the oxidative stress measurement. This study was financed by the China Scholarship Council (No. 201707720036 to BH).

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Supplementary materials

Table S1.1. List of primers used for qPCR

Gene	Forward Primer 5'-3'	Reverse primer 5'-3'	Reference
GAPDH	CATCTTCCAGGAGCGCGACC	AGACACCGGTGGACTCCACA	He et al., 2017
CD8a	AGAGACGAGCAAGGAGAA	GACCAGGGCAATGAGAAG	He et al., 2017
CD4	TTTCAACGCCACAGCAGA	GTGCCTCAACTGGATTTT	He et al., 2017
IFN-α	CAGCACCACATCCACCAC	TACTTGTTGATGCCGAGGT	He et al., 2017
IL8	CTCCTGATTTCCGTGGCTCT	AGCACACCTCTCTGTTGTCC	He et al., 2017
IL18	TGAAATCTGGCAGCGGAATGAAC	TCCCATGTTCTTCCTCACAACA	Xu et al., 2016
iNOS	GAACAGCCAGCTCATCCGATA	CCCAAGCTCAATGCACAACTT	Xu et al., 2016
MDA5	TGCTGTAGTGGAGGATTTG	CTGCTCTGTCCCAGGTTT	He et al., 2017
MHCIa	GAGCAAGCAGGGGAAGGA	CCGTTAGACACTGGGGTT	He et al., 2017
MHCIIa	CGGCCAGTTCATGTTCGAT	AAGCTGGCAAACTTCGAGA	He et al., 2017
RIGI	AGCACCTGACAGCCAAAT	AGTGCGAGTCTGTGGGTT	He et al., 2017
TLR3	CAGCAAATTTAGGATGGCAAC	ACAGATTTCCAATTGCACGTA	He et al., 2017
TLR7	CACAGAAAAATGGTACCTC	TACATCGCAGGGTAAACT	He et al., 2017

Chapter 3

Modulatory effects of inorganic mercury (Hg(II)) and lead (Pb(II)) on immune responses of Pekin ducklings (*Anas platyrhynchos domesticus*) upon a viral-like immune challenge

Biyao Han, Alan Kroeze, Hans van den Berg, Ivo Roessink, Nico W. van den Brink

In preparation

Abstract

Trace metal contamination is ubiquitous around the world and may affect the health of wildlife. Divalent trace metals, including ions of mercury (Hg) and lead (Pb), have shown to be immunotoxic to avian species. However, little is known about the immunomodulatory effects of trace metal exposure on viral infections, especially in young birds, who may be more sensitive. The current study used Pekin ducklings as an animal model to investigate effects of inorganic divalent Hg(II) and Pb(II) on avian immune responses upon a viral-like challenge with double-stranded RNA (dsRNA). The results indicate that Hg(II) altered the immune gene expression 24 h post challenge, as reflected by induction of pro-inflammatory genes IL-8, iNOS, TLR3 and TLR7, and a significant decrease of microRNA-155 (miR-155). Ducklings exposed to Pb(II) showed lower levels of natural antibodies, reduced white blood cell counts and lower heterophil proportions 24 h post challenge. Although inorganic divalent Hg(II) and Pb(II) showed specific differential effects on the immune response of Pekin ducklings, the overall adverse immunomodulatory outcomes in both cases point at inflammation, impaired B-cell function, and weaker immunocompetence.

3.1. Introduction

Non-essential trace metal contamination is widespread globally and, due to its non-biodegradability, persistence, potential for bioaccumulation and toxicity, may pose risks to organisms inhabiting contaminated environments (Ali et al., 2019; Kumar et al., 2019; Pacyna & Pacyna, 2001). Human activities such as urbanization and industrialization have major impacts on the emission and distribution of trace metals in the environment (Luo et al., 2012; Nriagu & Pacyna, 1988). Among these trace metals, mercury (Hg) and lead (Pb) are of great concern. Anthropogenic sources of Hg mainly consist of industrial activities (such as mining, fossil fuel combustion and municipal waste burning) and can lead to topsoil Hg levels exceeding 1000 mg/kg in contaminated areas (Ballabio et al., 2021; Millán et al., 2006). Long-range transport of Hg by air or ocean currents may result in elevated environmental Hg levels even at remote locations that lack local anthropogenic sources, such as the Arctic (Douglas et al., 2012; Marquès et al., 2017). In the anoxic soil and sediment, sulphate-reducing bacteria can transform inorganic Hg to organic methylmercury (MeHg) (Erickson & Lin, 2015). Although MeHg is considered as the most toxic species of Hg, inorganic divalent Hg(II) is more extensively distributed in the environment and occurs readily in low trophic level species (Beckers & Rinklebe, 2017; Schlüter, 2000), and as such Hg(II) may also be a serious risk to wildlife.

Similar to Hg, major anthropogenic sources of Pb emission are also related to urban and industrial discharges. Historically, Pb (usually as the organic form tetraethyllead) has been used as an anti-knocking additive in gasoline, which has resulted in contamination of urban areas, especially near highways (Nriagu, 1990). Inorganic Pb(II) also used to be added to paints to enhance colour and to improve their adherence and durability (O'Connor et al., 2018). In addition, the use of lead in ammunition and fishing sinkers has resulted in contamination of soils and sediments in (historic) hunting areas (Kanstrup et al., 2020; Mateo et al., 2007). Accumulated lead shots ranging from a few to more than one hundred pellets per square meter were found in sediments of European wetlands (Pain et al., 2019), potentially threatening wildlife in these habitats, such as waterfowl who can ingest the lead pellets while foraging, mistaking them with grits to aid the digestion of food in their gizzards (Ancora et al., 2008; Bianchi et al., 2011; De Francisco et al., 2003). Pb pellets can dissolve and be oxidized into Pb(II) salt in waterfowl's intestinal tract after intake, followed by fast distribution of Pb(II) over numerous tissues, including the liver, kidneys, bones, and growing feathers, via the circulatory system (Ferreyra et al., 2014). In wild waterfowl such as mallard ducks from contaminated locations, elevated total Pb (tPb) levels have been discovered in eggshells, suggesting potential maternal exposure of the nestlings (Vallverdú-Coll et al., 2015, 2019).

Even low levels of trace metal exposure may affect multiple physiological functions in organisms, including compromised immunity (Ackerman et al., 2016; García-Mendoza et al., 2021; Thaxton & Parkhurst, 1973; Vallverdú-Coll et al., 2019; Whitney & Cristol, 2018;

Wolfe et al., 1998). For instance, blood total Hg (tHg) concentrations had a negative association with phagocytosis by macrophages in black-footed albatrosses after environmental exposure (Finkelstein et al., 2007) and with weaker T-cell mediated immunity in tree swallows, as estimated with skin swelling responses after being challenged with phytohaemagglutinin (PHA) (Hawley et al., 2009). Lower lysozyme and antibody levels have been related to ingestion of Pb pellets in red-legged partridges (*Alectoris rufa*) (Vallverdú-Coll et al., 2015). In addition, the immune leukocyte populations in birds were reported to be altered by both MeHg and Pb pellet exposure (Fallacara et al., 2011; Grasman & Scanlon, 1995; Rocke & Samuel, 1991). The impacts of variable forms of trace metals on the functioning of the immune system may cause higher risks of infections to pathogens, including parasites (Carravieri et al., 2020; Ebers Smith et al., 2018), bacteria and viruses at individual levels and may result in increased prevalence of diseases (e.g., avian influenza) in the avian community. Moreover, young birds in the developing stage are suspected to be much more sensitive to immunotoxic effects of trace metals (Vallverdú-Coll et al., 2019; Vermeulen et al., 2015). However, little is known about the immunomodulatory effects of trace metal exposure upon viral challenge, especially in young birds.

As we noticed in chapter 2, even low environmentally relevant levels of total Hg (tHg) exposure did affect the immune responses in Arctic Barnacle goslings (*Branta leucopsis*) (Han et al.in prep). Effects consisted mainly of the suppression of humoral immunity and induction of inflammation. However, as only two exposure groups were included in that study and many factors could not be strictly controlled in the field study the causality of Hg(II) induced immunomodulatory effects in the goslings remains yet to be established. Therefore, the objectives of the present study are to investigate the effects of inorganic divalent trace metal exposure (both Hg(II) and Pb(II)) on an avian model animal species (Pekin ducklings, *Anas platyrhynchos domesticus*), upon a viral-like immune challenge with double-stranded RNA (dsRNA) under strictly controlled conditions. By comparing the results of the duckling experiments with the field study on goslings, we aim to provide more insights and show causality between trace metal exposure and the effects of exposure on the immune responses.

3.2. Materials and methods

3.2.1. Animals and experimental design

Two experiments were conducted at Sinderhoeve (Renkum, the Netherlands) in summer 2020 (Hg(II) experiment: 25-June to 19-July-2020; Pb(II) experiment: 20-August to 13-September-2020). One day-old ducklings (*Anas platyrhynchos domestica*) were obtained from Duck-to-Farm B.V. (Ermelo, The Netherlands), and exposed to different levels of trace metals for 23/24 days. In total 68 ducklings were used in the Hg(II) experiment and 66 in the Pb(II) experiment (Table 3.1). Upon arrival, ducklings were labelled with coloured leg bands and randomly assigned to different groups, i.e. five different exposure levels for Hg(II) or Pb(II). Within each exposure level, 50% of the ducklings was challenged 24h before

termination with dsRNA (poly I:C, Table 3.1). Besides the exposure groups, a positive control group for immune suppression (receiving non-contaminated feed and injection of cyclophosphamide 24 h before immune challenge with dsRNA) was also included in the experiment. According to former studies, the average tHg and tPb levels in the plants were 0.06 and 3.7 mg/kg respectively in Ny-Ålesund, Svalbard, where we performed the field study (Ma et al., 2020). Exposure levels ranging from low environmentally relevant levels to levels that are known to induce immunotoxicity on birds were used in the experiments, which were 0.00, 0.01, 0.10, 0.50 and 1.00 mg Hg(II)/kg feed (as $HgCl_2$) and 0.00, 0.10, 1.00, 10.00 and 100.00 mg Pb(II)/kg feed (as lead acetate, $PbAc_2$). Exposure groups are shown in Table 3.1. Six Ducklings were assigned randomly to each group, except for the highest exposure groups in the Hg(II) experiment (1.00 mg Hg(II)/kg feed) which contained 7 ducklings. In total three ducklings were excluded during the experiments. One duckling in the 0.50 mg Hg(II)/kg feed exposure groups and one duckling in the 1 mg Pb(II)/kg feed group were found dead with no signs of illness on day 18 and day 12 respectively. In addition, one duckling in the 0.10 mg Hg(II)/kg feed group was ill and was removed from the experiment at day 20, according to the humane endpoints defined in the ethical protocol. As none of the excluded ducklings were in the highest exposure groups, and no trace metal-induced impacts were noticed in the autopsy, we believe that the loss of animals was not a consequence of exposure.

Table 3.1. Exposure groups in the experiments. Concentrations of heavy metals in feed were shown in mg/kg feed. The number of animals in each group included in sample analyses is shown in the brackets.

Groups	**Concentrations of heavy metals (mg/kg feed)**					**Positive control**
Hg(II)	0 (6)	0.01 (6)	0.1 (6)	0.5 (6)	1 (7)	0 Hg(II)
Hg(II) +poly I:C	0 (6)	0.01 (6)	0.1 (5)	0.5 (5)	1 (7)	+immune suppressor + Poly I:C (6)
Pb(II)	0 (6)	0.1 (6)	1 (5)	10 (6)	100 (6)	0 Pb(II)
Pb(II) +poly I:C	0 (6)	0.1 (6)	1 (6)	10 (6)	100 (6)	+immune suppressor + Poly I:C (6)

Ducklings receiving the same exposure were housed in the same compartment. The compartments were 6m long and 1.3m wide (see Figure S3.1). A roofed area of 1 by 1 meter with an infrared light (Philips, 100 watts, continuously) provided shelter and warmth. The floors of the compartments were covered with rubber mats for two-thirds to provide grip. The remaining area (2 m by 1.3 m) was filled with water, which varied in depth from 0 to 30 cm. Compartments stood along the wall of the building and were sheltered for one-third by an overhanging roof. An electrical fence was placed around the compartments and a net was

secured over the top of the compartments to prevent predators from entering (Figure S3.1). Feed and water were provided ad libitum. Compartments were cleaned daily, and swimming water was also renewed daily. Growth of the ducklings was monitored by measuring their body weight and tarsus length every other day.

Ducklings were exposed to either Hg(II) or Pb(II) for 23 or 24 days, as the dissection was divided over two days. Exposure was achieved by premixing the feed with either $HgCl_2$ or $PbAc_2$ solutions (Research Diet Services B.V., Wijk bij Duurstede, The Netherlands), because the oral route is most relevant in environmental Hg exposure (Douglas-Stroebel et al., 2005). Every exposure level was used for both activated and non-activated groups, which were kept in the same compartment. These two groups were identical up to the penultimate day of the experiment. 24 h Before the end of the experiment, ducklings in the activated group were injected with 50 μg/kg body weight (b.w.) poly I:C (10 μg/mL in saline) via intraperitoneal (i.p.) injection, while ducklings in the non-activated group received saline solution (0.9% NaCl) via i.p. injection. The positive control group received non-contaminated feed and were injected with immune-suppressor (cyclophosphamide 50 mg/kg b.w.) at 48h before termination and immune-activator (50 μg/kg b.w. poly I:C) at 24 h before termination. Limited feed was provided in the night before termination to make sure the crop of the ducklings was empty at the time of euthanasia.

On day 23 or 24, ducklings were euthanized through decapitation and immediately dissected. The sex of the ducklings was identified during the dissection (in Hg(II) experiment: 24 males, 40 females and 2 unknowns; in Pb(II) experiment: 22 males and 43 females). At least 5ml blood per duckling was collected in tubes coated with K_2EDTA as anticoagulant (BD Vacutainer®, Becton Dickinson, USA). An air-dried blood smear was made for each duckling and the rest of the blood was centrifuged at 1,000×g for 10 min to separate plasma. Afterward, plasma was collected and stored at -80°C until analyses of nitric oxide, haemolysis-haemagglutination and haptoglobin. Spleen tissue was isolated as the major immune organ and stored in RNAlater™ stabilization solution (Invitrogen, Breda, the Netherlands) in the fridge (4°C) and transferred to -20°C until further analyses of gene expression. Liver tissue was collected and stored at -20°C to determine the internal tHg and tPb concentrations.

3.2.2. Chemical analyses

Total Hg or total Pb concentrations in liver tissues and all premixed feed were measured. Briefly, liver tissues and feed were freeze-dried at -50°C for 18 hours and then digested in a microwave with either 70% nitric acid (for tHg analyses) or aqua-regia ($HCl:HNO_3$=3:1, for tPb analyses). The concentrations of tHg were measured with cold vapor atomic fluorescence spectrometry (CVAFS) (da Silva et al., 2010; van den Brink et al., 2018) while the concentrations of tPb were measured with inductively coupled plasma mass spectrometry (ICP-MS) (Ek et al., 2004). Blue mussel (*Mytilus edulis*) tissue (ERM-CE278k, European Reference Materials, ERM, Geel, Belgium) was used as reference material for liver samples,

and lichen (BCR 482, ERM, Geel, Belgium) was used as reference material for feed samples. A quality control solution was measured every 10 samples. The concentrations of internal tHg and tPb were expressed as mg/kg dry weight (d.w.).

3.2.3. Immune assays

3.2.3.1. Gene expression

Expression of 9 immune functional genes (6 mRNA and 3 miRNA) were tested in the spleen tissues of ducklings with RT-qPCR, including anti-viral interferon-α (IFN-α), interleukins (IL-8, IL-18), inducible nitric oxide synthase (iNOS), toll-like receptors recognizing viral pathogens (TLR3 and TLR7), and microRNAs involved in immune regulation (miR-155, miR-9 and let-7). A housekeeping gene (GAPDH) and a reference gene (SNORD68) were also tested to normalize the results of mRNA and miRNA respectively. Briefly, up to 25mg spleen tissues were lysed with QIAzol Lysis Reagent (Qiagen, Venlo, the Netherlands) and the total RNA (including both mRNA and miRNA) was extracted using a miRNeasy Mini Kit (Qiagen, Venlo, the Netherlands). The quality and quantity of extracted total RNA were checked by Nanodrop (ND-1000, Themoscientific Wilmington, Delaware, US). 300 ng of total RNA was reverse translated to cDNA with the miScript II RT Kit (Qiagen, Venlo, the Netherlands) with miScript HiFlex Buffer to generate cDNA for both mRNA and miRNA. Afterward, RT-qPCR was conducted in a Rotor-Gene® 6000 cycler (Qiagen, Venlo, the Netherlands) with either a QuatiNova SYBR® Green PCR Kit (Qiagen, Venlo, the Netherlands) for mRNA or a miScript SYBR® Green PCR Kit (Qiagen, Venlo, the Netherlands) for miRNA. Commercially available miRCURY LNA miRNA PCR Assays were applied for miRNA primers (dre-miR-155, hsa-miR-9-5p, hsa-let-7a-5p and SNORD68) (Qiagen, Venlo, the Netherlands). Primers for mRNA were synthesized (produced by Biolegio, Nijmegen, the Netherlands) according to the sequence used in former studies (He et al., 2017; Wu et al., 2019; Xu et al., 2016). The sequences of primers are listed in Table S3.1. Prior to sample measurement, the efficiency of qPCR amplification was checked for each primer using a 4-fold serial dilution standard curve. Results were normalized against the housekeeping gene GAPDH for mRNA or reference gene SNORD68 for miRNA, and then expressed as log2 fold changes relative to the average expression in spleen in the non-activated and non-exposure group by -ΔΔCT method (Schmittgen & Livak, 2008).

3.2.3.2. Nitric oxide assay

Nitric oxide is an effector molecule involved in the defence against pathogens, and an immune messenger as well (Bogdan, 2001; Coleman, 2001). Thus, nitric oxide levels in plasma can be an indicator for an immune response. Nitric oxide levels in duckling plasma samples were measured as described before (de Jong et al., 2017; Sild & Hõrak, 2009). As nitic oxide is unstable and would be converted into nitrite (NO_2^-) and subsequently to nitrate (NO_3^-), nitrate (NO_3^-) was reduced to nitrite (NO_2^-), and nitrite (NO_2^-) concentrations were determined to indicate nitric oxide levels in plasma samples (Sild & Hõrak, 2009). Briefly,

20 µl plasma was deproteinized in an alkaline buffer. Then, two or three cadmium granules coated with copper were used per sample to reduce the nitrate in the samples to nitrite, which can be measured with the Griess reaction. The concentrations of nitrite oxide (µM) in plasma samples were then calculated with a standard curve of $NaNO_2$.

3.2.3.3. Haemolysis-haemagglutination assay

Natural antibodies (NAbs) and complement can non-specifically protect the animals against pathogens even without any infection history (Holodick et al., 2017; Reyneveld et al., 2020). The haemolysis-haemagglutination assay was conducted to assess the NAbs activity (with agglutination titres) and the collaboration of NAbs and complement (with lysis titres) (de Jong et al., 2017; Matson et al., 2005). In short, duckling plasma was serially diluted by 2-fold (up to 1024× dilution) in round (U) bottom 96-well plates with phosphate buffered saline (PBS, Gibco, Paisley, UK). Then, 25 µL of diluted plasma was mixed with the same volume of 1% rabbit blood cell suspension in PBS and incubated in a 37°C humidified incubator for 90 min. Afterward, the plates were tilted to a 45° angle on the long axis for 30 min at room temperature to enhance visualization for scoring haemagglutination titres. After another 70min incubation at room temperature, haemolysis titres were determined. All the samples were observed and visually scored (Matson et al., 2005). If the agglutination or lysis was partial or intermediate, half scores were given. Duplicates were measured for every duckling.

3.2.3.4. Haptoglobin assay

As an acute-phase protein, haptoglobin levels in plasma rapidly increase in case of inflammation, infection, or trauma (Matson et al., 2012; Quaye, 2008; Y. Wang et al., 2001). We used a commercially available colorimetric haptoglobin assay kit (TP801; Tri-Delta Development Limited, Maynooth, Ireland) to evaluate the haptoglobin-like activity (mg/ml) in duckling plasma samples with a calibration curve. Due to unforeseen issues during the Pb(II) experiment, some blood samples could not be centrifuged instantly on-site and were stored overnight at 4°C and centrifuged later in the lab, resulting in severe haemolysis. As haemolysis interferes with the assay (Matson et al., 2012), haptoglobin assessment was not conducted for plasma samples from the Pb(II) experiment.

3.2.3.5. Blood cell populations

Blood cell populations were quantified in blood smear on slides stained with Hemacolor® Rapid staining (Sigma-Aldrich, Zwijndrecht, the Netherlands). Slides were counted using a light microscope under an oil immersion lens (1000× magnification, Ziess, Jena, Germany). To assess the abundance of leukocytes (white blood cells, WBCs), at least 40 visions were counted per slide until reaching a total of 3000 erythrocytes. The density of leukocytes (number of white blood cells per 1000 erythrocytes) was calculated. Leukocytes, including heterophils (normal or toxic), eosinophils, basophils, monocytes and lymphocytes (reactive or non-reactive) were identified according to the morphological characteristics (Jones, 2015; Mitchell & Johns, 2008), and at least 100 leucocytes were counted per slide.

3.2.4. Statistics

Statistics were performed with SPSS (IBM SPSS Statistics, version 25) and figures were made with GraphPad Prism 5 (San Diego, CA, USA). Firstly, the relationship between the growth rate of ducklings during the experiment and trace metal exposure was checked with linear mixed models. Daily growth rates were calculated with either body mass (g/g/day) or tarsus length (mm/mm/day) and input as dependent variables. Animal ID was set as random subjects while log of internal metal (tHg or tPb) concentrations, sex and age were set as fixed effects.

To check whether the non-exposed controls from two experiments have the same responses, two-way ANOVA tests were applied with factors of Hg(II) or Pb(II) experiment and challenge. Significant differences in internal trace metal concentrations between different treatment groups were checked by one-way ANOVA with the Tukey post hoc test.

Non-continuous endpoints (haemolysis and haemagglutination titres) were analysed with ordinal regression, while continuous results were analysed with linear regression (gene expression, blood cell population and haptoglobin) models. Variables were transformed if not normally distributed to meet model assumptions. For example, internal trace metal concentrations were log-transformed, and haptoglobin together with nitric oxide levels were square root transformed (de Jong et al., 2017). Internal tHg or tPb concentrations, sex and challenge were included in the models as independent variables. If challenge did not show significant effects on the endpoints, the activated and non-activated groups were combined in the figures for visualization. Significant differences between different treatment groups were checked by one-way ANOVA with the Tukey post hoc test.

3.3. Results and discussion

3.3.1. Duckling growth

The effects of exposure, sex, and age (day) on the growth rate of ducklings calculated based on either body weight (g/g/day) or tarsus length (mm/mm/day) were examined (Table 3.2). According to the regression, exposure and sex had no effects on the growth of ducklings. The growth rates of both body weight and tarsus length were negatively related with age (day) experiments, which means the ducklings grew relatively slower in the later stage of the experiments.

Table 3.2. Statistical outcomes of mixed linear models for gosling daily growth rate calculated with body mass (g/g/day) and tarsus length (mm/mm/day). Underlined t and F values are significant at $p < 0.05$.

		Hg(II) experiment				Pb(II) experiment			
Daily growth rate	**Variable**	**Estimate**	**SE**	**df**	**t**	**Estimate**	**SE**	**df**	**t**
Body weight (g/g/day)	Intercept	69.342	0.659	688	**105.159**	64.861	0.979	711	**66.234**
	log tHg or tPb	0.157	0.249	688	0.631	0.157	0.355	711	0.443
	Age (day)	-2.922	0.040	688	**-72.515**	-2.638	0.062	711	**-42.655**
	Sex: Unknown v.s. male*	0.795	1.498	688	0.531	-	-	-	-
	Sex: Female v.s. male*	0.948	0.540	688	1.757	-0.125	0.844	711	-0.148
Tarsus Length (mm/mm/day)	Intercept	13.892	0.497	688	**27.942**	12.898	0.559	646	**23.054**
	log tHg or tPb	-0.011	0.188	688	-0.060	0.028	0.203	646	0.139
	Age (day)	-0.439	0.030	688	**-14.429**	-0.302	0.039	646	**-7.776**
	Sex: Unknown v.s. male*	-0.187	1.129	688	-0.165	-	-	-	-
	Sex: Female v.s. male*	-0.230	0.407	688	-0.564	-0.067	0.482	646	-0.140

a: This parameter is set to zero because it was redundant.

*: male was set as 0

3.3.2. Internal concentrations of trace metals

The measured concentrations of tHg or tPb in the feed were very similar to the nominal concentrations (Table S3.2). The hepatic concentrations of both trace metals showed strong correlations with the concentrations in the feed, and the internal concentrations were significantly different among the different exposure groups ($p<0.005$) (Figure 3.1). The hepatic tHg concentrations in ducklings ranged from undetectable (<0.003 mg/kg d.w.) to 3.9 mg/kg d.w., which covers the range of tHg concentrations found in gosling livers in Chapter 2 (shown as the horizontal lines in Figure 3.1a). Meanwhile, the hepatic tPb concentrations increased from 0.006 to 12.295 mg/kg d.w. with the exposure (Figure 3.1b), which are similar with hepatic concentrations found in aquatic birds from environmental exposure studies (Lucia et al., 2010; Mateo & Guitart, 2003; Vizuete et al., 2019). The hepatic tHg and tPb concentrations were independent from both sex or immune challenge (Table 3.4).

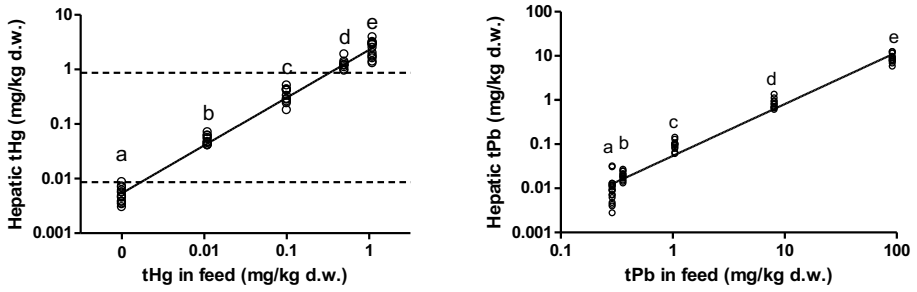


Figure 3.1. Hepatic tHg (a) and tPb (b) concentrations (mg/kg d.w.) in ducklings against the tHg or tPb concentrations in feed provided to different groups (mg/kg d.w.). Dotted horizontal lines represent the range of hepatic tHg concentrations found in Barnacle goslings in Chapter 2 (a). Significant differences were checked with one-way ANOVA with Tukey post hoc test. The same letter indicates no significant differences.

3.3.3. Comparison of non-exposed controls in two experiments

The immune endpoints of the non-exposed groups from the two experiments were compared to assess comparability of the responses between experiments. No significant differences between experiments were noticed for all endpoints, except leukocyte density, neither between the non-activated and activated ducklings (Table 3.3). Leukocyte density was significantly higher in the control ducklings of the Pb(II) exposure experiment than the ones from the Hg(II) exposure experiment (Figure S3.2), which may be due to subjective counting method or the slightly different experimental conditions (e.g. temperature). In both experiments, the dsRNA challenge significantly increased the leukocyte density by around 5 WBCs per 1000 red blood cells (RBCs), indicating induction of similar immune responses in the two experiments.

Table 3.3. Comparison of non-exposed controls in two experiments. F-values from two-way ANOVA tests are listed. Underlined F-values are significant at $p < 0.05$

		Hg(II) v.s. Pb(II) experiment	Challenged v.s. non-challenged
Gene expression	**log2 IFN-α expression**	1.20	0.61
	log2 IL-8 expression	2.07	2.83
	log2 IL-18 expression	1.21	2.04
	log2 iNOS expression	0.23	0.51
	log2 TLR3 expression	0.08	1.21
	log2 TLR7 expression	1.76	0.80
	log2 miR-155 expression	0.02	1.51
	log2 miR-9 expression	0.10	0.01
	log2 let-7 expression	0.04	0.13
plasma	**Nitric oxide (μM)**	2.26	0.58
	Haemolysis	1.21	0.01
	Haemagglutination	0.18	0.09
blood smear	**Leukocyte density**	**10.12**	**35.27**
	Heterophil (%)	4.04	0.22
	Lymphocyte (%)	1.52	0.41
	log H/L	2.79	0.27

3.3.4. Immune responses

Immune parameters in ducklings exposed to either Hg(II) and Pb(II) were assessed and their relationships with hepatic trace metal concentrations were examined with linear regression (Table 3.4). The results indicated that Hg(II) mainly affected the gene expression of ducklings while Pb(II) exposure mainly affected the natural antibody levels in plasma and immune cell populations. 24 h Immune challenge by poly I:C injection stimulated some immune responses, including the expression of some immune genes in Hg(II) exposed ducklings, the haptoglobin-like activity and leukocyte density. The effects of sex are rather limited, only influencing the heterophil population and the H/L ratios in Hg(II) exposed ducklings (Table 3.4). Although usually females have stronger overall immunity than males (Klein & Flanagan, 2016), this sex-related difference is probably not shown yet at such young age of the ducklings on the endpoints we measured.

Table 3.4. Output of ordinal (for haemolysis and haemagglutination) or linear regression models for internal levels, gene expression, plasma tests and blood smear results. Listed are the $\chi 2$ (for haemolysis and haemagglutination) or F values of overall regression and wald (for haemolysis and haemagglutination) or t-values of individual parameters. Underlined wald/t- and $\chi 2$/F- values are significant at $p < 0.05$.

		Hg					Pb				
	Endpoints	**log Hg (t/wald)**	**Gender (t/wald)**	**Activation (t/wald)**	**total df**	**regression (F/χ2)**	**log Pb (t/wald)**	**Gender (t/wald)**	**Activation (t/wald)**	**total df**	**regression (F/χ2)**
Internal levels	**log Hg (mg/kg d.w.)**	-	-1.633	0.008	59	1.349	-	-	-	-	-
	log Pb (mg/kg d.w.)	-	-	-	-	-	-	1.087	-0.002	58	0.591
Gene expression	**log2 IFN-α expression**	-1.57	1.894	**2.29**	59	**4.741**	0.51	-0.744	-0.707	58	0.41
	log2 IL8 expression	**3.974**	-0.015	**2.184**	59	**6.994**	-0.21	-0.185	-0.891	58	0.297
	log2 IL18 expression	1.761	0.056	**2.848**	59	**3.741**	1.821	0.894	-0.585	58	1.668
	log2 iNOS expression	**3.985**	-0.577	**2.381**	59	**7.661**	1.438	-0.264	-0.689	58	0.849
	log2 TLR3 expression	**4.697**	-0.997	1.411	59	**9.209**	**2.863**	-0.862	0.277	58	**2.827**
	log2 TLR7 expression	**3.907**	-1.636	1.833	59	**8.014**	-0.574	-1.959	0.421	58	1.58
	log2 miR155 expression	**-7.241**	1.469	-1.978	59	**21.521**	1.759	0.791	1.495	58	2.162
	log2 miR9 expression	**2.536**	1.339	0.088	59	2.38	-1.373	0.377	-1.519	58	1.406
	log2 let7 expression	-0.187	0.276	0.725	59	0.243	-0.992	-0.03	0.912	58	0.617
	Nitric oxide (μM)	-0.226	-0.921	1.268	59	0.742	-0.919	-1.501	0.055	58	1.192
plasma	**Haemolysis**	0.122	0.658	0.15	501	0.83	1.141	0.099	1.732	287	3.241
	Haemagglutination	0.629	0.597	0.011	333	1.004	**7.56**	0.53	0.978	403	**8.934**
	Haptoglobin	1.728	1.779	**2.554**	59	**4.192**	-1.094	0.733	**3.443**	30	**4.197**
blood smear	**W/R**	0.993	0.7	**3.297**	59	**4.214**	**-3.245**	1.074	**10.358**	58	**39.358**
	Heterophil (%)	0.987	**2.703**	-1.677	59	**3.144**	**-3.354**	0.981	-0.368	58	**3.885**
	Lymphocyte (%)	-0.493	-1.172	**3.41**	59	**4.123**	1.994	-0.659	1.018	58	0.173
	log H/L	0.798	**2.147**	**-2.566**	59	**3.419**	**-2.78**	0.858	-0.631	58	**2.783**

3.3.4.1. Gene expression

Irrespective of challenging with ds-RNA, the expression of several immune functional genes in spleen, including IL-8, iNOS, TLR3 and TLR7 was upregulated in Hg(II) exposed ducklings while Pb(II) induced the expression of just TLR3 (Table 3.4, Figure 3.2a-d, Figure 3.3a). As for the miRNAs, miR-155 was strongly suppressed by Hg(II) exposure, while let-7 was slightly but significantly induced (Table 3.4, Figure 3.2e,f). Among the tested genes, poly I:C challenge had significant positive effects on the expression of IFN-α, IL-8, IL-18, and iNOS in Hg(II) exposed ducklings (Table 3.4) without showing significant effects in the non-exposed control groups (Table 3.3). For IL-8 and iNOS, whose expression was significantly affected by both Hg(II) exposure and immune challenge, effects of the interaction between the Hg(II) exposure and challenge was also checked with the linear regression models. However, no significance of the interaction was shown for either IL-8 (t-value: -0.828; p-value: 0.411) or iNOS (t-value: 1.316; p-value: 0.193). Therefore, both Hg(II) exposure and dsRNA challenges, but not their interaction, positively affected IL-8 and iNOS expression in the ducklings.

Neither exposure nor poly I:C challenge significantly affected the expression of most genes (except for TLR3) in Pb(II) exposed ducklings (Table 3.4). However, some downstream effects were noticed to be significant, e.g. haemagglutination, leukocyte density and heterophil %. As the regulation of gene expression is a dynamic process, this lack of significance in gene expression from the Pb(II) experiment might suggest that the time period (24 h) from immune challenge to sampling is not optimal to detect the effects on gene expression. Thus, more time points may be included in the future studies.

IL-8 is a pro-inflammatory chemokine that can recruit heterophils to an infected site (Kogut, 2002). Induction of IL-8 gene expression in children has also been associated with *in utero* tHg concentrations (Stratakis et al., 2021). Although the results showed no significant effects of Pb(II) exposure on the expression of IL-8, former *in vitro* studies reported an upregulation due to Pb(II) exposure in a chicken macrophage cell line (Han et al., 2020) and human peripheral blood mononuclear cells (PBMC) (Gillis et al., 2012). The shown significant Hg(II)-induced up-regulation of another inflammatory indicator, iNOS, may lead to higher content of inducible oxide synthase at the protein level, producing more nitric oxide. Hg(II) exposure also stimulated the expression of iNOS *in vitro* in multiple cell types such as murine RAW264.7 macrophage cell line (Park & Youn, 2013), and primary mouse hepatocytes (Lee et al., 2014). Considering the induction of both IL-8 and iNOS, the results demonstrate that Hg(II) exposure has a pro-inflammatory effect on ducklings upon a viral-like challenge, while Pb(II) did not affect the expression of these pro-inflammatory genes.

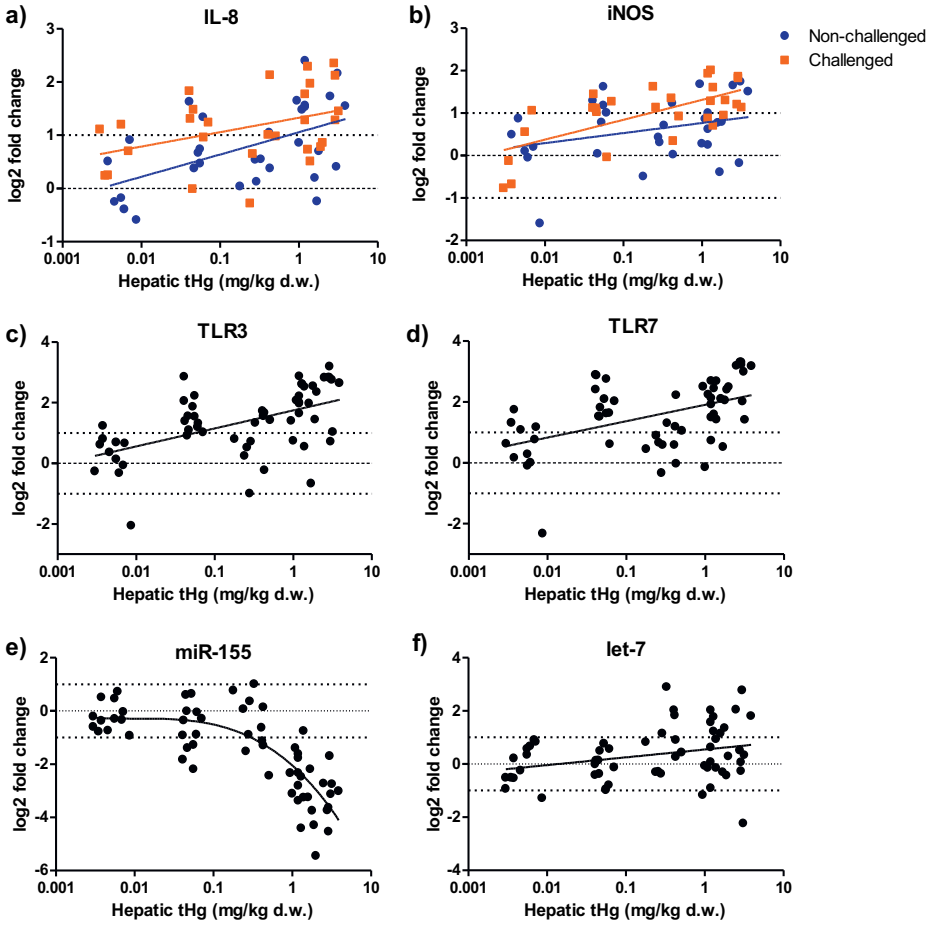


Figure 3.2. Effects of Hg(II) exposure on the gene expression of immune functional genes in duckling spleen tissues. Results were normalized firstly with the housekeeping gene GAPDH, and shown as log2 fold change relative to the average expression in spleen of control group. As the expression of IL-8 (a) and iNOS (b) was affected by activation, separate regression lines were shown for non-activated and activated results for IL-8 (a) and iNOS (b).

TLR3 and TLR7 are both endosomal toll-like receptors recognizing viral pathogen patterns, namely TLR3 recognizing dsRNA and TLR7 recognizing ssRNA (Trivedi & Greidinger, 2009). The induction of TLR3 and TLR7 expression by Hg(II) exposure showed similar patterns (Figure 3.2c, d), while the upregulation of TLR3 caused by Pb(II) exposure slightly declined in the highest exposure group (100 mg/kg Pb(II) in feed) (Figure 3.3a). The increased expression of these TLRs indicates a stimulatory effect on innate immunity, which could lead to inflammation and autoimmunity (Pollard et al., 2019). The downstream pathways of TLR3 and TLR7, including p38 mitogen-activated protein kinase (p38 MAPK)

and nuclear factor-kappa (NF-κB) signalling pathways, have been shown to be activated by Hg(II) exposure in the liver of voles, resulting in prolonged inflammation and liver damage (Assefa et al., 2012). Inflammatory genes including iNOS and NF-κB were also upregulated in peripheral blood lymphocytes of chickens, exposed to 350 mg/kg Pb(II) (as lead acetate) in feed (Sun et al., 2016). Thus, the enhanced expression of IL-8, iNOS and TLRs demonstrates that trace metal exposure, especially exposure to inorganic divalent Hg(II), could induce inflammation in ducklings.

miR-155 And let-7 are both miRNAs regulating the immune system post-transcriptionally by degrading mRNA and repressing translation (Bushati & Cohen, 2007; Fabian et al., 2010). As a multifunctional micro-RNA, miR-155 is involved in regulating multiple immune responses, including promotion of lymphocyte proliferation and differentiation, macrophage polarization, and antibody production. (Alivernini et al., 2018). The results showed a clear dose-dependent relationship of internal tHg concentrations and miR-155 expression, where miR-155 expression was strongly suppressed at hepatic tHg concentrations higher than 1 mg/kg d.w.(Figure 3.2e). Downregulation of miR-155 was also noticed in a Hg(II) exposed chicken B-cell line *in vitro* (Han et al., 2021). Decreased miR-155 levels could cause impaired B-cell function and anti-viral immunity (Babar et al., 2012; Mehta & Baltimore, 2016; Rodriguez et al., 2007; Waugh et al., 2018). Different from miR-155, let-7 suppresses B-cell activation and some cytokine production in innate immune response such as IL-6 and IL-10 (Jiang et al., 2018; Nejad et al., 2018; Schulte et al., 2011). The results showed a positive correlation of internal tHg concentrations with the expression of let-7 (Figure 3.2f), suggesting stronger immune suppressing effects at higher Hg(II) exposure. Therefore, the downregulated miR-155 and upregulated let-7 both suggest lower B-cell function and lower pathogen defence, which might result in a decreased immunocompetence.

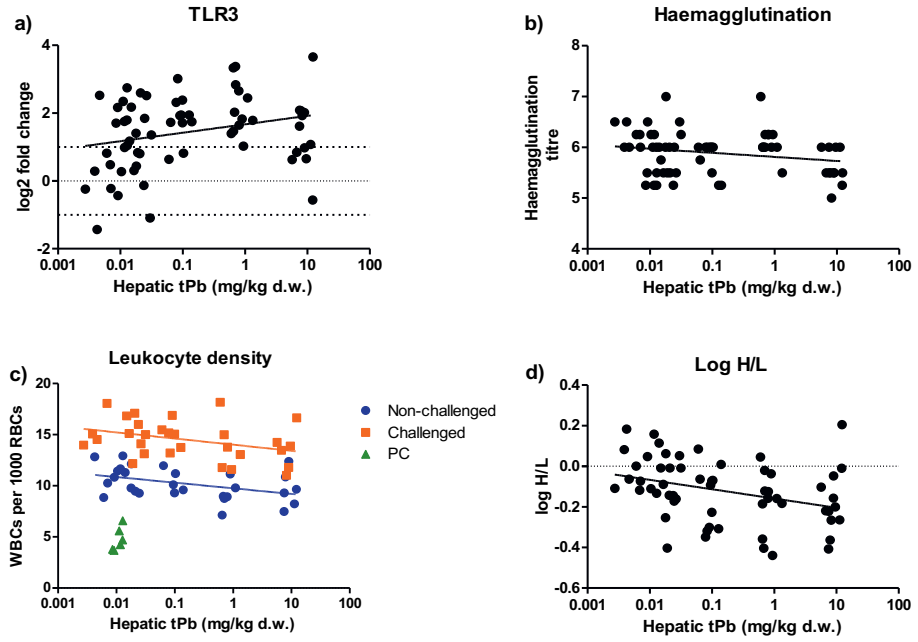


Figure 3.3. Effects of Pb(II) exposure on the immune responses in ducklings, including the gene expression of TLR3 (a), haemagglutination titres (b), leukocyte density (c) and log heterophil/ lymphocyte ratio (d). Poly I:C challenge significantly increased the leukocyte density, therefore separate regression lines were shown for non-challenged and challenged results for leukocyte density (c).

3.3.4.2. Nitric oxide

Nitric oxide levels were not influenced by any factors in the linear regression model. Nitric oxide levels in both experiments stayed near the baseline levels (Hg(II) experiment: 48.16±9.87 μM; Pb(II) experiment: 48.72±9.80 μM), which are in line with the baseline nitric oxide levels in greenfinches (Sild & Hõrak, 2009) and ducks (Wang et al., 2014). The poly I:C challenge did not show significant induction of nitric oxide in the non-exposed ducklings from both experiments, which is probably because the challenge time was not long enough for the actual nitric oxide induction. Compared with chicken, ducks have been reported to need a longer challenge time (72 h but not 24 h) until a significant increase of plasma nitric oxide could be noticed after infection of H5N1 highly pathogenic avian influenza (Burggraaf et al., 2011). Therefore, the results may merely reflect the baseline levels of nitric oxide, prior to a potential immune response. As gene expression is a relatively early-stage indicator, the upregulation of iNOS due to Hg(II) exposure might lead to an increase of plasma nitric oxide levels later, which however, needs further research with longer challenge time.

3.3.4.3. Haemolysis-haemagglutination

Natural antibody levels showed a slight but significant decrease only in Pb(II), but not in Hg(II) exposed ducklings (Table 3.4, Figure 3.3b). Impaired natural antibody levels were also noticed in red-legged partridges (*Alectoris rufa*) exposed to lead shot pellet in spring (Vallverdú-Coll et al., 2015). Besides, no influences on haemagglutination titre were found in non-activated Arctic Barnacle goslings (*Branta leucopsis*) herded in a Hg contaminated site when no other stressor (social isolation) was applied (de Jong et al., 2017). Higher natural antibody titres can increase the survival rate of laying hens (Sun et al., 2011). Although natural antibodies are not specific immunoglobulins produced by activated plasma B-cells, the decreased natural antibody levels in Pb(II) exposed ducklings may suggest impaired B-cell function.

3.3.4.4. Haptoglobin

Haptoglobin-like activity was not affected by Hg(II) exposure, but was induced by poly I:C challenge (Table 3.4). Due to the lack of data, the results of haptoglobin form Pb(II) experiment were excluded for statistical analyses. Haptoglobin could be a biomarker for acute inflammation and infection (Wang et al., 2001). Poly I:C also significantly increased serum haptoglobin levels in rats within few days after challenge (Kruger et al., 2010). The increase of haptoglobin in the results indicated a successful challenge of immune responses with poly I:C.

3.3.4.5. Blood cell populations

Appropriate immune cell populations are crucial for well-functioning immunity, and their composition can be used as primary indicators to assess immune competence (Jones, 2015; Mitchell & Johns, 2008). A significant decrease of leukocyte density and heterophil proportion, which led to lower H/L ratios, were related to Pb(II) exposure (Table 3.4, Figure 3.3c, d). The compositions of immune cell populations were not affected by Hg(II) exposure, while male ducklings had significantly more heterophils in the Hg(II) experiment (Table 3.4). Besides, in both Hg(II) and Pb(II) exposed ducklings, poly I:C challenge increased the leukocyte density, which was presented as WBCs per 1000 RBCs (Table 3.4, Figure 3.3c).

The leukocyte density reflects the relative amount of immune cells compare to RBCs in the blood. Lower leukocyte density at higher Pb(II) exposures suggests suppressed immune cell proliferation and an overall lower immune competence (Blumenreich, 1990). Decreased leukocyte densities were also documented in mallard ducks inhabiting wetlands with elevated hepatic tPb concentrations of 6.4±2.46 mg/kg d.w. (Rocke & Samuel, 1991). The stimulation effect of poly I:C challenge on leukocyte density also indicates an activated immune cell proliferation. The interaction between Pb(II) exposure and dsRNA challenge (t-value: -0.133 p-value: 0.985) is not significant when included in the linear regression model. Heterophils and lymphocytes are the two most abundant cell types observed in the blood smears. Avian heterophils have similar functions as mammalian neutrophils, playing

essential roles in innate immunity. Heterophils are pathogen phagocytic cells and also one of the major cell types producing nitric oxide (Maxwell & Robertson, 1998). Therefore, the decreased heterophil proportion in Pb(II) exposed ducklings might suggest lower innate immunity. Lymphocytes are the cells carrying out adaptive immunity, including B-cells and T-cells. B-cells produce antibodies and mediate humoral immunity, while T-cells mediate cellular immunity (Sharma, 1991). However, the microscopy method cannot separate B-cells and T-cells according to their morphology. Nevertheless, the H/L ratio is an indicator for immunity (Gross & Siegel, 1983; Krams et al., 2012). Body condition was positively correlated with H/L ratio in Burrowing parrot (*Cyanoliseus patagonus*) nestlings (Masello et al., 2009), but this parameter did not vary between individuals in the experiments. The decreased H/L ratio in Pb(II) exposed ducklings is mainly due to the decrease of heterophils and may suggest weaker innate immunity and pathogen defence.

3.4. Conclusions

Pekin ducklings were used as a model animal to assess the effects of divalent trace metal exposure on the immune responses in waterfowl with a viral-like challenge. Hg(II) exposure mainly affected the immune gene expression, including the upregulation of pro-inflammatory genes IL-8, iNOS, TLR3, and TLR7, suggesting a Hg(II)-induced inflammatory effect. Meanwhile, exposure to Hg(II) resulted in considerable downregulation of the global immune regulator miR-155, and slight upregulation of the immune suppressing miRNA let-7, suggesting an impaired B-cell function and lower pathogen defence. In the field study (Chapter 2), environmental exposure to Hg also compromised humoral immunity and increased gene expression of pro-inflammatory genes (e.g. iNOS) in Arctic barnacle goslings. Pb(II) mainly reduced the natural antibody levels, the leukocyte density and heterophils, which might also lead to compromised immunocompetence. Although the effects of Hg(II) and Pb(II) exposure on the endpoints analysed varied, the overall impacts in both cases point at induced inflammation, impaired B-cell function and weaker defence against pathogens.

Acknowledgement

The animal experiment was preformed according to the proposal approved by the Animal Experimental Committee (DEC) with the experiment number 2017.W-0065.003. We thank Menno Grouls and Aafke Saarloos for their great support in the field. This study was financed by the China Scholarship Council (No. 201707720036 to BH).

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Supplementary materials

Table S3.1. Primers used for qPCR of immune functional genes (mRNA)

Gene	Forward Primer 5'-3'	Reverse primer 5'-3'	Reference
GAPDH	ATGTTCGTGATGGGTGTGAA	CTGTCTTCGTGTGTGGCTGT	Wu et al., 2019
IFN-α	TCCTCCAACACCTCTTCGAC	GGGCTGTAGGTGTGGTTCTG	Wu et al., 2020
IL-8	CTCCTGATTTCCGTGGCTCT	AGCACACCTCTCTGTTGTCC	He et al., 2017
IL-18	TGAAATCTGGCAGCGGAATGAAC	TCCCATGTTCTTCCTCACAACA	Xu et al., 2016
iNOS	GAACAGCCAGCTCATCCGATA	CCCAAGCTCAATGCACAACTT	Xu et al., 2016
TLR3	GAGTTTCACACAGGATGTTTAC	GTGAGATTTGTTCCTTGCAG	Wu et al., 2019
TLR7	CCTTTCCCAGAGAGCATTCA	TCAAGAAATATCAAGATAATC ACATCA	Wu et al., 2020

Table S3.2. Levels of tHg or tPb in the feed

tHg		tPb	
Designed (mg/kg)	Measured (mg/kg)	Designed (mg/kg)	Measured (mg/kg)
0	<0.0076	0	0.29
0.01	0.011	0.1	0.363
0.1	0.1	1	1.053
0.5	0.5	10	8.114
1	1.1	100	92.183



Figure S3.1. Set up of the experimental compartments and the inside of the duckling enclosures.

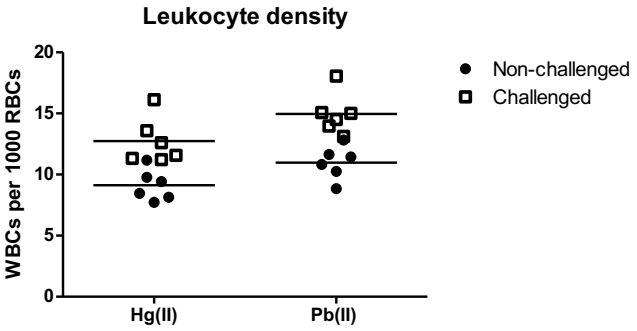


Figure S3.2. Leukocyte density of ducklings in non-exposed groups from the two experiments.

Chapter 4

Modulatory effects of mercury (II) chloride ($HgCl_2$) on chicken macrophage and B-lymphocyte cell lines with viral-like challenges *in vitro*

Biyao Han, Diego García-Mendoza, Hans van den Berg, Nico W. van den Brink.

Published in Environmental Toxicology and Chemistry. 2021; 40(10): 2813-2824. https://doi.org/10.1002/etc.5169

Abstract

Mercury (Hg) is a toxic trace metal ubiquitously distributed in the environment. Inorganic mercury (as $HgCl_2$) can cause immunotoxicity in birds, but the mechanisms of action are still not fully resolved, especially with respect to responses to viral infections. To investigate the potential immunomodulatory effects of Hg^{2+} on specific cell types of the avian immune system, chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines were applied as *in vitro* models for the innate and adaptive immune systems, respectively. The cells were stimulated with synthetic double-stranded RNA (dsRNA) which can be recognized by toll-like receptor-3 (TLR3) to mimic a viral infection. Hg^{2+} showed concentration-dependent cytotoxicity in both cell lines, with similar EC50s at 30 μM. The cytotoxicity of Hg^{2+} was closely related to glutathione (GSH) depletion and ROS induction, while the *de novo* synthesis of GSH acted as a primary protective strategy. Nitric oxide produced by activated macrophages was strongly inhibited by Hg^{2+}, and was also influenced by cellular GSH levels. Cell proliferation, gene expression of microRNA-155 (miR-155), and cellular IgM levels in B cells were decreased at non-cytotoxic Hg^{2+} concentrations. The secretion of antiviral interferon-α (IFN-α) was induced by Hg^{2+} in both cell lines. Overall, the results suggest that Hg^{2+} exposure can cause immunomodulatory effects in birds by disrupting immune cell proliferation and cytokine production, and might result in disorders of the avian immune system.

4.1. Introduction

Mercury (Hg) is one of the trace metals that ubiquitously occurs in the environment, and human activities, such as mining and industry, have considerably elevated its environmental levels and global distribution (Dorea and Donangelo 2006; Beckers and Rinklebe 2017). Similar with other non-essential trace metals, Hg is regarded as a priority environmental contaminant due to its potential for bioaccumulation and biomagnification, and also its high toxicity (Mann et al. 2011). The major species of Hg include elemental mercury (Hg(0)), inorganic mercury (predominantly divalent mercury, Hg(II)), and organic mercury (mainly methylmercury, MeHg). Although MeHg is recognised as the most toxic form, Hg(II) is more commonly and abundantly found in the environment (Schlüter 2000; Beckers and Rinklebe 2017), and can also exert severe adverse effects on wild animals. Elevated Hg levels have been detected in wild birds, affecting multiple physiological parameters including immune functions (Thaxton and Parkhurst 1973; Wolfe et al. 1998; Ackerman et al. 2016; Whitney and Cristol 2018). For example, tree swallows in a Hg contaminated area showed compromised immune competence with weaker skin swelling responses to phytohaemagglutinin (PHA) challenging (Hawley et al. 2009). Elevated levels of internal Hg were associated with decreased macrophage phagocytosis in black-footed albatrosses (Finkelstein et al. 2007). The impact of Hg on the functioning of the immune system may result in higher risk of infections at individual levels and may cause prevalence of diseases (e.g., avian influenza) in the populations.

As mentioned above, for avian species, there are some *in vivo* and wildlife studies revealing the immune modulatory effects of mercury, but very limited knowledge on the cellular mechanisms and immune functional effects of mercury toxicity was documented. More insights in the immunotoxicity of Hg were reported in studies using mammalian or human *in vitro* cell models (Loftenius et al. 1997; Silva et al. 2005; Das et al. 2008; Lehmann et al. 2011; Levin et al. 2020; Yang et al. 2020). Such *in vitro* studies can demonstrate the underlying mechanisms and can also reduce animal experiments. *In vitro* evidence has shown that the mechanisms of Hg-induced toxicity in immune cells are related to the induction of oxidative stress, the inhibition of nitric oxide and the disturbance of cytokine profiles (Kim et al. 2002; Kim and Sharma 2004; Guzzi and La Porta 2008). However, this is based on mammalian models, while little is known about the cellular mechanisms of Hg-induced immunomodulatory effects in avian species.

To evaluate the effects of Hg exposure on avian immune functions, not only the baseline immunity but also the activated immunity, which is more relevant to vulnerability to diseases, should be considered. Once infected by certain pathogens, avian immune cells recognise the pathogen-associated molecular patterns (PAMPs) with specific receptors and trigger multiple defence strategies (Mogensen 2009). For instance, toll-like receptor 3 (TLR3) can detect double stranded RNA (dsRNA) produced by most viruses during their replication (Sen and Sarkar 2005). Upon immune activation, macrophages can produce nitric oxide and undergo

oxidative burst to kill the pathogens (MacMicking et al. 1997) while B-lymphocytes can produce antibodies to neutralize the pathogens (Dörner and Radbruch 2007). To mediate the immune responses and communicate with other cells, immune cells also produce cytokines with various functions, such as anti-viral interferon-α and β (IFN-α and -β), interleukins (ILs, e.g., pro-inflammatory IL18) and chemokines (e.g., IL-8) (Kaiser and Stäheli 2013). Apart from cytokines, microRNAs (miRNAs) also play an important role in regulating the immune responses, such as miR-155 and let-7 involved in the inflammatory response, cytokine production and lymphocyte maturation (Gottwein et al. 2007; Lu and Liston 2009; Staedel and Darfeuille 2013; Kumar et al. 2015; Alivernini et al. 2018). An adequate immune response requires an appropriate population of functional immune cells, and proper levels of immune mediators (namely cytokines and miRNAs) for the communication and cooperation among different immune cells.

The aim of the present study was to investigate the modulatory effects and potential mechanisms of Hg^{2+} on the avian immune systems using chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines as *in vitro* models for the innate and adaptive immune systems, respectively with and without a mimicked viral challenge with dsRNA. Both cell lines have been documented to express TLR3 and could be stimulated with dsRNA (Peroval et al. 2013; Quan et al. 2017). General toxicity (cytotoxicity, oxidative stress) and functional endpoints were quantified, including nitric oxide production by macrophages, cell proliferation, immune gene expression (receptors, cytokines and miRNAs), and immune protein levels (cytokines and antibodies) as indicators for immunity upon Hg^{2+} exposure and activation.

4.2. Materials and methods

4.2.1. Cell culture

The HD-11 chicken macrophage cell line was a kind gift from Dr. Jurgen van Baal (Department of Animal Sciences, Wageningen University, the Netherlands) and was cultured in RPMI 1640 medium (Gibco, Paisley, UK) with 10% fetal bovine serum (FBS, Sigma-Aldrich, Zwijndrecht, the Netherlands) at 37°C in a 5% CO_2 humidified air incubator. Every two to three days, HD-11 cells were sub-cultured by detaching the cells with 5 mM ethylenediaminetetraacetic acid (EDTA, Merck, Darmstadt, Germany) and diluting cell suspension with fresh culture medium. A washing step with phosphate-buffered saline (PBS, Gibco, Paisley, UK) was performed before seeding the cells to remove EDTA.

The chicken B-lymphocyte cell line DT40 (ATCC® CRL2111™) was maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, Paisley, UK) containing 10% FBS, 10% tryptose phosphate broth (Sigma-Aldrich, Zwijndrecht, the Netherlands), 5% chicken serum (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 0.05 mM 2-mercaptoethanol (Gibco, Paisley, UK) at 37°C in a 5% CO_2 humidified air incubator. Cells were sub-cultured every two to three days by diluting the cell suspension with fresh culture medium.

4.2.2. Experimental setup

Effects of Hg^{2+} (as mercury (II) chloride, $HgCl_2$) exposure on general cytotoxicity and immune functions were investigated in HD-11 chicken macrophages and DT40 chicken B-lymphocytes. Both cell lines are able to be activated with dsRNA via the TLR3 pathway (He et al. 2007; Keestra and van Putten 2008; Zou et al. 2017; Ma et al. 2019). Therefore, we used a synthetic analogue of dsRNA, polyinosinic–polycytidylic acid sodium salt (poly I:C), (Alexopoulou et al. 2001; Matsumoto and Tsukasa 2008), to induce viral-like challenges in all assays in the current study.

Metal ions including Hg^{2+} have a high affinity for thiol groups and can deplete the major intracellular antioxidant glutathione (GSH), resulting in oxidative stress and cytotoxicity (Hultberg et al. 2001; Nuran Ercal et al. 2005; Hossain et al. 2021). To reveal the possible underlying mechanism of Hg^{2+} toxicity related with GSH depletion, co-exposure with γ-glutamylcysteine synthetase blocker L-buthionine-sulfoximine (BSO) was introduced to inhibit *de novo* GSH synthesis. The water-soluble tetrazolium-1 (WST-1) assay was used to determine the EC50 values after 24 h exposure to Hg^{2+} in cells treated under different conditions, including immune activation with 25 μg/mL poly I:C (Sigma-Aldrich, Zwijndrecht, the Netherlands) and inhibition of the *de novo* GSH synthesis by 200 μM BSO (Sigma-Aldrich, Zwijndrecht, the Netherlands). These EC50 values served as the basis for defining the concentration ranges in follow-up assays. Changes of intracellular GSH and reactive oxidant species (ROS) levels due to Hg^{2+} exposure were investigated to elucidate their potential role in the mechanism(s) of Hg^{2+} induced toxicity.

Afterwards, effects of Hg^{2+} exposure on immune functions were assessed. Production of nitric oxide by HD-11 cells was quantified as a primary functional endpoint for macrophages. Cell proliferation was examined using both the WST-1 assay for metabolic activity (cell activity), and the bromodeoxyuridine (BrdU) assay for DNA synthesis (cell division) with extended exposure time (48 hours) in both cell lines. Besides, expression of immune function genes and miRNAs after Hg^{2+} exposure was profiled with quantitative reverse transcription PCR (RT-qPCR) at the transcription level. Finally, enzyme linked immunosorbent assays (ELISAs) were employed to measure multiple immune functional protein levels, including the production of type I anti-viral IFN-α in the supernatant of both cell lines, and immunoglobulin M (IgM) levels in both supernatant (for secreted IgM) and cell lysate (for cellular IgM) of DT40 B-lymphocytes. All the assays were performed in three independent experiments and performed as described hereafter.

4.2.3. Exposure

For most of the assays, either HD-11 macrophages or DT40 B-cells were seeded at a density of 1×10^5 cells/well in a clear flat-bottom 96-well plate and incubated overnight before exposure. In proliferation assays, 1×10^4 cells/well were seeded, allowing a longer exposure time (48h). 1×10^6 cells/well were seeded in 12-well plates for RT-qPCR. A series of $HgCl_2$ (Sigma-Aldrich, Zwijndrecht, the Netherlands) stock solutions ranging from 0.2 to 2000 μM

were prepared in sterile MilliQ water and diluted 20 times in culture medium to the final exposure concentration ranging from 0.01 to 100 µM. 5 mg/mL Poly I:C stock solution was prepared in PBS and stored in -20°C. To activate the cells, poly I:C (Sigma-Aldrich, Zwijndrecht, the Netherlands) stock solution was diluted 200 times in culture medium to a final concentration of 25 µg/mL in all assays. And to block the *de novo* GSH synthesis via γ-glutamylcysteine synthetase, 200 µM (final concentration) of BSO was added to the culture medium of the cells in cytotoxicity, GSH, ROS and nitric oxide assays. Exposure to $HgCl_2$, poly I:C and BSO started at the same time. Exposure time was 24 hours for most assays, except for the proliferation assays, in which the exposure time was prolonged to 48 hours.

4.2.4. General toxicity assays

4.2.4.1 Cytotoxicity assay

Cells were exposed to Hg^{2+} in four conditions, namely with or without 200 µM BSO to block *de novo* GSH synthesis and with or without 25 µg/mL poly I:C activation. After 20 hours of exposure, 10 µL (5% v/v) WST-1 reagent was added to each well of HD-11 or DT40 cells and incubated at 37°C for 4 hours before measuring absorbance at 440 nm and a reference wavelength at 620 nm with a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale CA, USA). Cell viability was expressed as percentage of negative controls in the non-BSO, non-activated treatment group (-BSO -poly I:C group). Concentrations of Hg^{2+} used in the later assays were based on the results obtained for Hg^{2+} in cell viability assays.

4.2.4.2. GSH assay

ThiolTracker™ Violet Glutathione Detection Reagent (Life Technologies/Invitrogen, Oregon, USA) was used to measure the intracellular GSH levels after Hg^{2+} exposure. Briefly, after 20 hours of exposure, cells were washed twice with Dulbecco's phosphate-buffered saline with calcium and magnesium (D-PBS C/M, Gibco, Paisley, UK). Cells were then labelled with 20 µM ThiolTracker ™ Violet dye working solution for 30 min at 37°C. After washed for another two times with D-PBS C/M, cells were resuspended and fluorescence was measured at Ex/Em 404/526 nm with the SpectraMax M2 Microplate Reader. GSH levels were shown as percentages relative to the negative controls in the -BSO -poly I:C group.

4.2.4.3. ROS assay

ROS levels inside the cells were measured with 2`,7`-dichlorofluorescin diacetate (DCFDA, Sigma-Aldrich, Zwijndrecht, the Netherlands) reagent after 24h Hg^{2+} exposure. Once diffused into the cell and de-esterified by cellular esterase, DCFDA is oxidised to highly fluorescent 2',7'-dichlorofluorescin (DFC) by ROS (Rosenkranz et al. 1992). The exposure conditions were identical with the cytotoxicity and GSH assay (also with or without poly I:C and BSO). Cells were first exposed for 20 hours and then labelled with 25 µM DCFDA for

another 4 hours before fluorescence measurement at Ex/Em 485/535 nm. Results were presented as ratios relative to the negative controls in the -BSO -poly I:C group.

4.2.5. Immune functional assays

4.2.5.1 Nitric oxide (Griess) assay

Nitric oxide produced by HD-11 macrophages under the same exposure conditions as the ones used in the cytotoxicity, GSH and ROS assays was examined using the Griess assay (Sun et al. 2003). Briefly, after 24h exposure, 100 µL of exposed cell culture supernatant was transferred to a new 96-well plate. 50 µL 1% sulfanilamide (Sigma-Aldrich, Zwijndrecht, the Netherlands) in 5% phosphoric acid (Merck, Darmstadt, Germany) and 50 µL of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED, Sigma-Aldrich, Zwijndrecht, the Netherlands) solution were added to the supernatant successively with a 5 minutes interval. The plates were incubated for 5 minutes in the dark between the addition of the two solutions and before measuring absorbance at 540 nm with the SpectraMax M2 Microplate Reader. Nitric oxide production after treatment was calculated based on a calibration curve of $NaNO_2$ ranging from 1.56 µM to 100 µM.

4.2.5.2. Proliferation assays

Two endpoints, namely cell activity and cell division, were tested to evaluate cell proliferation levels after 48 hours Hg^{2+} exposure. The WST-1 assay was performed as described above in the cytotoxicity assay. The colorimetric BrdU assay (Roche, Manheim, Germany) was applied according to the manufacture's instruction. Briefly, during the last 4 hours of exposure, newly synthesized DNA in the cells was labelled with BrdU. Floating DT40 cells were separated by centrifugation at 300×g for 10min. Then, both HD-11 and DT40 cells were fixed and incubated with anti-BrdU-POD antibody which bound to the BrdU and reacted with the substrate. The reaction was terminated by adding stop solution after incubation with the substrate for 30 minutes at room temperature. Cell division (DNA synthesis) was quantified by absorbance at 450 nm with a reference at 690 nm and expressed as a percentage relative to the negative control in the non-activated group (-poly I:C group).

4.2.5.3. Gene expression

Expression of nine chicken genes (6 for mRNA and 3 for miRNA), including a house-keeping gene (GAPDH), the genes for type I interferons (IFN-α, IFN-β), interleukins (IL-8, IL-18) and toll-like receptors recognizing viral pathogens (TLR7), reference miRNA (SNORD68), and microRNAs involved in immune regulation (miR-155 and let7) were tested in both cell-lines by RT-qPCR. All kits used were from Qiagen (Venlo, the Netherlands). Briefly, after 24h exposure, cells cultured in 12-well plates were lysed with QIAzol Lysis Reagent and RNA was extracted using an miRNeasy Mini Kit. The quality and quantity of extracted RNA were checked by Nanodrop (ND-1000, Themoscientific Wilmington, Delaware, US). 300 ng total RNA was reverse translated to cDNA using the miScript II RT

Kit with miScript HiFlex Buffer to get cDNA for both mRNA and miRNA. Then, RT-qPCR was performed on Rotor-Gene® 6000 cycler with either QuatiNova SYBR® Green PCR Kit for mRNA or miScript SYBR® Green PCR Kit for miRNA. Commercially available primer assays were used for immune functional genes, including QuantiTect® primer assays (Gg_GAPDH_1_SG, Gg_IFNA3_1_SG, Gg_IFNB_1_SG, Gg_IL8L2_1_SG, Gg_IL18_1_SG, and Gg_TLR7_1_SG) for mRNA expression; and miRCURY LNA miRNA PCR Assay for miRNA primers (dre-miR-155, hsa-let-7a-5p and SNORD68). Efficiency of qPCR amplification was checked for each primer using a 4 fold serial dilution standard curve prior to sample measurement.

4.2.5.4. Immune protein levels

Multiple immune functional proteins produced by the two cell lines, including IFN-α by both cell lines as well as IgM (both released and cellular) by DT40 B-lymphocytes, were quantified with commercially available sandwich ELISA kits. All the ELISA kits (chicken IFN-α and chicken IgM ELISA kits) were obtained from ELISAGenie (Dublin, Ireland). Assays were conducted according to the instructions provided by manufacture for each kit.

Supernatant and cells were separated after exposure by centrifugation at 1000×g for 20min at 4°C. IFN-α levels were measured in the supernatant samples from both HD-11 and DT40 cells while IgM levels were only analysed in the supernatant of DT40 cells. Cellular IgM content was also determined in cell lysate prepared through three repeated freeze-thaw cycles after removing of supernatant. Quantities of each immune protein (pg/mL for IFN- α in and ng/mL for IgM) were calculated by comparing absorbance at 450nm with standard curves from each kit. Levels of IgM in cell lysate samples were expressed in ng/μg protein by normalizing with total protein content. Protein content was quantified with a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Landsmeer, the Netherlands) according to the manufacturer's instruction.

4.2.6. Statistics

Statistical analyses and results visualization were performed with GraphPad Prism 5 (San Diego, CA, USA). Concentration-response curves were fitted with non-linear regression (variable slope, four parameters) to calculate EC50 values. One-way ANOVA with Dunnett's post hoc test was conducted to compare treatment groups with Hg^{2+} free controls. Gene expression levels were normalized against the housekeeping gene GAPDH (for mRNA) or SNORD68 (for miRNA) and shown as log2 fold changes in comparison with the negative control of non-activated cells by -ΔΔCT method (Schmittgen and Livak 2008).

4.3. Results

4.3.1. General toxicity

4.3.1.1. Cytotoxicity

Concentration-response curves for the effect of Hg^{2+} on both HD-11 and DT40 cells were obtained and showed similar EC50 values of approximately 30 µM irrespective of the poly I:C activation when the cells were not treated with BSO (Figure 4.1). When the *de novo* synthesis of GSH was blocked by co-treatment with BSO, Hg^{2+} showed much higher cytotoxicity in both cell lines. In HD-11 macrophages, the EC50 of Hg^{2+} for cells co-treated with BSO was 2.54 µM, and the EC50 for cells treated with both BSO and poly I:C was as low as 0.75 µM. A more potent effect of Hg^{2+} exposure was noticed in BSO treated DT40 B-lymphocytes, with EC50 values that were lower than 0.5 µM. An overview of all EC50 values is shown in Table 4.1. In HD-11 macrophages, poly I:C increased the cell metabolic activity by around 50%, while no induction was noticed in DT40 B-lymphocytes (Figure 4.1). Additionally, non-cytotoxic levels of Hg^{2+} exposure resulted in a significant increase of cell metabolic activity in cells not exposed to BSO, especially in HD-11 cells.

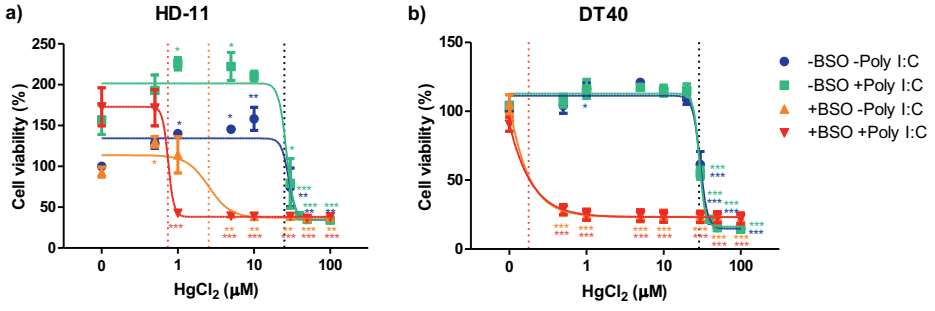


Figure 4.1. Cytotoxicity effects of Hg^{2+} on non-activated and activated (25 µg/mL poly I:C) HD-11 (a) and DT40 (b) cells with or without GSH synthesis inhibitor (200 µM BSO) after 24h exposure. Results were expressed as mean ± SEM (n=3), relative to negative control in non-activated cells for cell viability. Significant differences between treatments and Hg^{2+}-free controls were checked with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicated the EC50s for different treatment groups; red lines for the treatment groups with both BSO and poly I:C (+BSO +Poly I:C), orange lines for the treatment groups with BSO but without poly I:C (+BSO -Poly I:C), and black lines for the treatment groups without BSO as they showed similar EC50s.

Table 4.1. Overview of EC50 (95% CI) values of Hg^{2+} exposure for different endpoints

		-BSO -Poly I:C	-BSO +Poly I:C	+BSO -Poly I:C	+BSO +Poly I:C
Cytotoxicity	HD-11	29 µM (24.08-34.11)	27 µM (17.02-41.77)	2.54 µM (1.05-6.15)	0.75 µM (0.29-1.96)
	DT40	30 µM (28.28-31.35)	29 µM (27.76-30.37)	<0.5 µM n.a.	<0.5 µM n.a.
GSH	HD-11	16 µM (12.90-9.67)	15 µM (11.73-20.00)	2.28 µM (1.65-3.19)	0.82 µM (0.72 to 0.94)
	DT40	50~100 µM n.a.	50~100 µM n.a.	<0.5 µM n.a.	<0.5 µM n.a.
ROS	HD-11	6.51 µM (5.16-8.21)	6.56 µM (4.78-9.02)	0.54 µM (0.43-0.68)	0.79 µM (0.48-1.29)
Nitric Oxide	HD-11	n.a.	3.85 µM (2.70-5.49)	n.a.	0.37 µM (0.17-0.78)
Proliferation -WST-1	HD-11	5.43 µM (3.74-7.87)	5.78 µM (3.94-8.47)	n.a.	n.a.
	DT40	5.60 µM (4.68-6.69)	4.28 µM (3.55-5.16)	n.a.	n.a.
Proliferation -BrdU	HD-11	6.32 µM (5.04-7.93)	5.31 µM (5.02-5.62)	n.a.	n.a.
	DT40	2.82 µM (1.40-5.70)	2.67 µM (1.09-6.54)	n.a.	n.a.
IFN-α	DT40	0.58 µM (0.10-3.24)	0.53 µM (0.35-0.79)	n.a.	n.a.

95% CI: 95% confidential interval; n.a.: not applicable

4.3.1.2. GSH and ROS

Different patterns of intracellular GSH depletion were demonstrated in HD-11 cells under different exposure conditions (Figure 4.2a). When the cell can still synthesize GSH (without BSO), a steep decrease of GSH levels were only found close to the EC50 of Hg^{2+} cytotoxicity, regardless of the activation. However, when the *de novo* synthesis of GSH was inhibited by BSO, GSH levels declined at much lower Hg^{2+} exposure concentrations, with an EC50 of 2.3 µM (with BSO but without poly I:C), which is around 5 times lower than the EC50 obtained without BSO (approx. 15µM). Cells cultured with both BSO and poly I:C were the most vulnerable ones with an even lower EC50 of 0.82 µM Hg^{2+} and a significant decrease of their GSH level already at the lowest exposure concentration (0.5 µM). In HD-11 cells, the EC50 values for GSH depletion were in the same range as cytotoxicity for the individual treatment groups (shown as the vertical dashed lines in Figure 4.2a). As for DT40 cells, poly

I:C did not affect the GSH changes, while BSO played a major role (Figure 4.2b). Upon blocking of the synthesis of GSH, GSH levels inside the cells sharply dropped to background levels already at the lowest Hg^{2+} exposure concentration (0.5 µM). Nevertheless, GSH levels were not affected at non-cytotoxic Hg-concentrations in absence of BSO in DT40 B-cells.

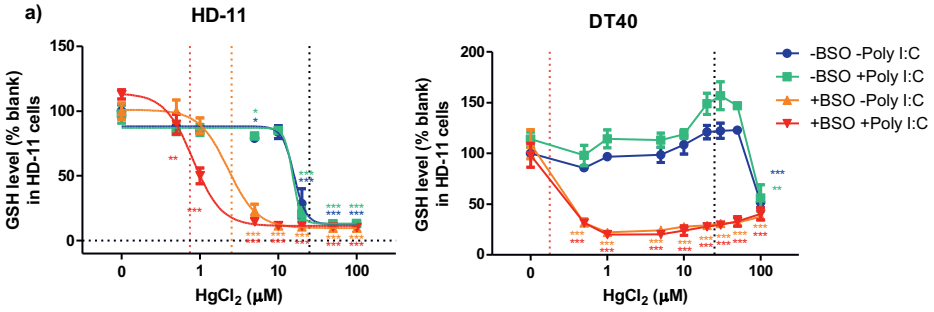


Figure 4.2. Intracellular GSH levels after 24h exposure to Hg^{2+} in non-activated and activated (25 µg/mL poly I:C) HD-11 (a) and DT40 (b) cells with or without GSH synthesis inhibitor (200 µM BSO). Results were expressed as percentage (mean ± SEM, n=3) in relative to the negative controls without GSH synthesis inhibitor and without activation (-BSO -poly I:C). Significant differences between treatments and Hg^{2+} free controls of DT40 cells were checked with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicated the EC50s of cytotoxicity after 24h exposure from WST-1 assay for different treatment groups; red lines for the treatment groups with both BSO and poly I:C (+BSO +Poly I:C), orange lines for the treatment groups with BSO but without poly I:C (+BSO -Poly I:C), and black lines for the treatment groups without BSO as they showed similar EC50s.

Similar to the results for GSH, patterns of ROS induction by Hg^{2+} exposure were influenced by the inhibition of *de novo* synthesis of GSH by BSO. In HD-11 cells, Hg^{2+} induced ROS up to around 6 fold under all four exposure conditions (Figure 4.3a). The EC50 values in cells co-exposed with BSO (without poly I:C 0.54 µM, with poly I:C 0.79 µM) were around 10 times lower than the EC50 values in cells that still could produce GSH (without poly I:C 6.51 µM, with poly I:C 6.56 µM). Poly I:C activation doubled the ROS levels in non-exposed HD-11 cells but did not affect the EC50 values of Hg^{2+} exposure. In DT40 cells, no significant induction of ROS was observed due to poly I:C activation, while ROS induction caused by Hg^{2+} exposure mainly occurred at cytotoxic concentrations above the EC50 values derived for cytotoxicity (shown as the vertical dashed lines in Figure 4.3).

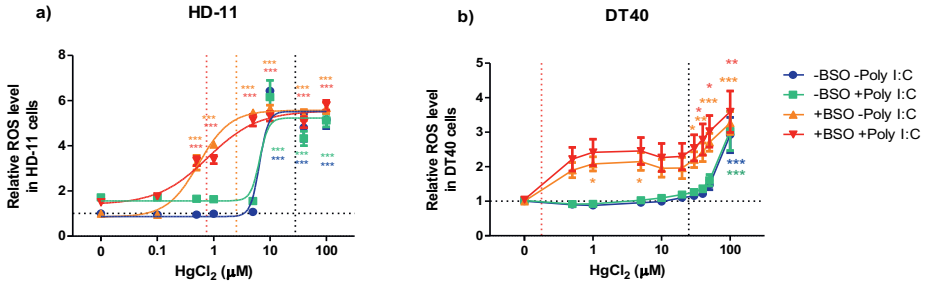


Figure 4.3. Intracellular ROS levels after 24h exposure to Hg^{2+} in HD-11 (a) and DT40 (b) cells with or without GSH synthesis inhibitor (200 μM BSO), and with or without immune stimulator (25 μg/mL poly I:C). Results were expressed as a relative ratio (mean ± SEM, n=3) to the negative controls without GSH synthesis inhibitor and without activation (-BSO -poly I:C, shown as the horizontal lines). Significant differences between treatments and Hg^{2+}-free controls were checked with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicated the EC50s of cytotoxicity after 24h exposure from WST-1 assay for different treatment groups; red lines for the treatment groups with both BSO and poly I:C (+BSO +Poly I:C), orange lines for the treatment groups with BSO but without poly I:C (+BSO -Poly I:C), and black lines for the treatment groups without BSO as they showed similar EC50s.

4

4.3.2. Immune functional endpoints

4.3.2.1. Nitric oxide

Unlike the results in GSH and ROS, the patterns of nitric oxide production by HD-11 macrophages upon Hg^{2+} exposure largely depended on poly I:C and also on BSO co-exposure (Figure 4.4). Poly I:C activation induced nitric oxide production from basal level to over 10 μM while non-activated cells hardly generated any nitric oxide. Concentration-depended inhibition of nitric oxide production was caused by Hg^{2+} exposure in activated cells at non-cytotoxic concentrations. Meanwhile, cells got more sensitive with BSO blocking the *de novo* synthesis of GSH, showing one order of magnitude lower EC50 (0.37 μM) than the one without BSO (EC50: 3.85 μM).

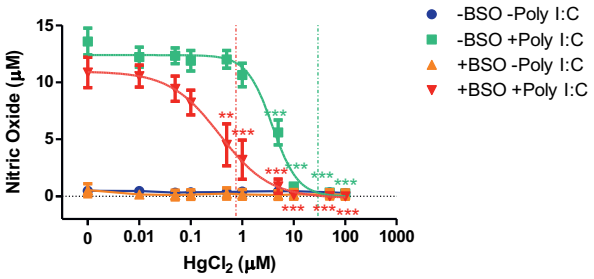


Figure 4.4. Nitric oxide (NO) production by activated (with 25 µg/mL poly I:C) and non-activated HD-11 cells with or without GSH synthesis inhibitor (200 µM BSO) were measured with Griess assay after 24h Hg^{2+} exposure. Results were expressed as mean ± SEM (n=3). Significant differences between treatments and Hg^{2+}-free controls were checked with one-way ANOVA (** $p<0.01$, *** $p<0.005$). Vertical lines indicated the EC50s of cytotoxicity after 24h exposure from WST-1 assay for different treatments; red line for the treatment group with both BSO and poly I:C (+BSO +Poly I:C), and green line for the treatment group without BSO but with poly I:C (-BSO +Poly I:C).

4.3.2.2. Proliferation

As shown in Figure 4.5, prolonged exposure (48h) to Hg^{2+} resulted in inhibition of cell proliferation at non-cytotoxic concentrations (determined by the 24h WST-1 assays, shown as vertical lines in Figure 4.5) in terms of both metabolic activity (WST-1) and DNA synthesis (BrdU) in both cell lines (Figure 4.5). No significant differences were noticed among EC50 values in both cells derived from both assays ranging from 2.7 to 6.3 µM (Table 4.1). In addition, poly I:C treatment significantly stimulated metabolic activity as well as DNA synthesis in DT40 cells (Figure 4.5c, d, $p<0.05$), while poly I:C significantly inhibited DNA synthesis in HD-11 cells (Figure 4.5b, $p<0.05$) without affecting metabolic activity (Figure 4.5a).

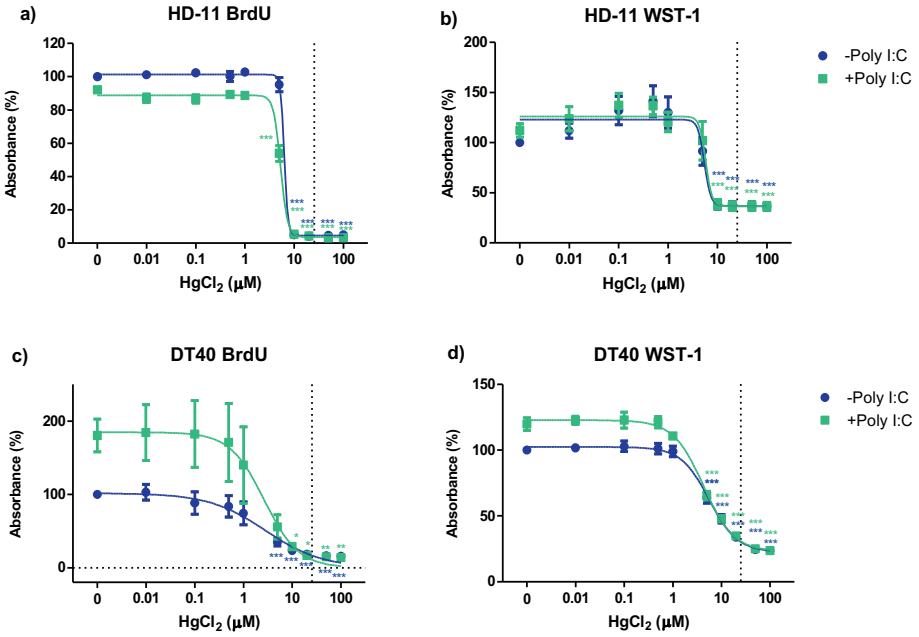


Figure 4.5. Cell proliferation of HD-11 cells (a, b) and DT40 cells (c, d) with and without 25 µg/mL poly I:C activation after 48h Hg^{2+} exposure. Results were expressed as relative absorbance to negative control in non-activated groups (mean ± SEM, n=3), and statistically compared with Hg^{2+}-free negative controls with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicated the EC50 of cytotoxicity after 24h exposure from WST-1 assay for HD-11 cells (a, b) or DT40 cells (c, d).

4.3.2.3. Gene expression

The effect of Hg^{2+} exposure on five immune functional genes and two miRNAs was analysed. Only miR155 was found to be down-regulated in activated DT40 B-lymphocytes. (Figure 4.6a, detailed results shown in Figure S4.1).

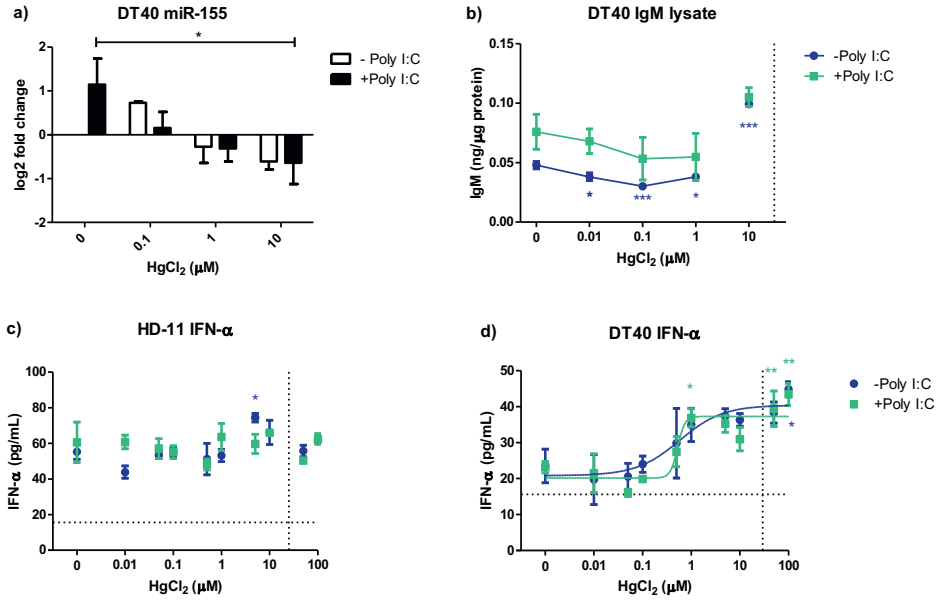


Figure 4.6. Effects of Hg^{2+} exposure with and without 25 μg/mL poly I:C activation on expression of miR-155 in DT40 B-lymphocytes (a), IgM levels in DT40 cell lysate (b), and IFN-α produced by HD-11 (c) and DT40 cells (d). Results were expressed as mean ± SEM (n=3), and significant differences between treatments and Hg^{2+}-free controls were checked with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicated the EC50 values of cytotoxicity after 24h exposure obtained from WST-1 assay. Horizontal lines showed the detection limits of the IFN-α ELISA assay (15.6 pg/mL).

4.3.2.4. Functional IFN-α and IgM levels

For DT40 B lymphocytes, IgM levels were measured in both culture supernatant and cell lysate. Secreted IgM in culture supernatant did not responds to Hg-exposure or poly I:C activation at concentrations below the EC50 for cytotoxicity (data not shown). IgM detected in cell lysate consisted of the IgM in the cytoplasm and the IgM on the cell membrane functioning as B cell receptors. Poly I: C stimulated IgM levels in the cell lysate (Figure 4.6b). Cellular IgM levels in non-activated DT40 cells significantly decreased after exposed to Hg^{2+} ranging from 0.01 μM to 1 μM followed by a remarkable increase at 10 μM. Because the results were expressed per mg protein content in the lysate samples, the increase of IgM levels at 10 μM could be due to the low protein content (Figure S4.2).

The levels of anti-viral IFN-α secreted into the culture supernatant were measure with ELISA assays for both cell lines (Figure 4.6c, d). An increasing trend if IFN-α with the increasing concentration of Hg^{2+} exposure was noted in both cell lines, especially in DT40 cells. Although significances were only found in high concentrations close to cytotoxic levels, the EC50 values for both non-activated and activated DT40 cells were as low as 0.5 μM (Table

4.1). Poly I:C activation did not affect the IFN-α production in either of the cell lines. Compared with DT40 cells, the secretion of IFN-α by HD-11 cells was less responsive to Hg^{2+} exposure but the initial levels in the non-exposed cells were higher.

4.4. Discussion

4.4.1. General toxicity

4.4.1.1. Cytotoxicity

The objective of the present study was to investigate *in vitro* cytotoxic and immune functional effects of Hg^{2+} exposure on two chicken immune cell lines (HD-11 macrophage and DT40 B-lymphocytes as models for specific avian immune cell types), upon a viral-like challenge. Similar EC50 values for cell viability (approx. 30 μM) were found for both cell lines after Hg^{2+} exposure for 24 hours (Figure 4.1, Table 4.1), which were in the same range of the EC50 values for Hg^{2+} from other *in vitro* studies using mammalian immune cells (Yamamoto et al. 1998; De Guise et al. 2000; Wataha et al. 2000; Kim and Sharma 2004; Engin et al. 2017; David et al. 2020). Poly I:C induced cell metabolic activity only in HD-11 macrophage cells but not in DT40 B-cells (Figure 4.1), however, after 48h exposure, only DT40 cells showed an increased metabolic activity upon poly I:C challenge (Figure 4.6b, d). These cell- and time-dependent responses might suggest that HD-11 macrophages build a faster response to poly I:C challenge than DT40 B-cells. Poly I:C challenge did not affect the sensitivity of either cell type to Hg^{2+} exposure as similar EC50 values were obtained. Stimulatory effects on the activity of cells by low Hg^{2+} concentration exposure were noticed, especially in HD-11 cells (Figure 4.1a). Similar hormetic effects have been observed in Hg^{2+} exposed mammalian cells *in vitro*, such as bovine leukocytes and a human mammary cell line (De Guise et al. 2000; Schmidt et al. 2004). This may point to cellular stress responses in order to detoxify the Hg^{2+} and could be important in case of chronic exposure scenarios (Damelin et al. 2000; Calabrese et al. 2007; David et al. 2020). Compared to other trace metal ions, Hg^{2+} has a relatively high toxicity. For example, the toxicity of Pb^{2+} was evaluated in the same cell types with much higher EC50 values, namely 480 μM for HD-11 macrophages and 1700 μM for DT40 lymphocytes (Han et al. 2020). Therefore, although inorganic Hg^{2+} is generally regarded to be less toxic than MeHg, it could still be a trace metal ion of concern.

4.4.1.2. GSH and ROS

Depletion of GSH and induction of ROS have been identified as major mechanisms of toxicity induced by trace metals with high affinity to thiol group (Nuran Ercal et al. 2005). In the current study, we included BSO co-exposure, blocking γ-glutamylcysteine synthetase, to investigate the function of *de novo* synthesis of GSH in the Hg^{2+} exposure experiments. As shown in Figure 4.1, 2 and 3, intracellular GSH levels dropped, ROS levels increased and cell viability decreased in both cell lines after Hg^{2+} exposure without BSO, but when BSO inhibited the *de novo* GSH synthesis, the EC50 values were even much lower. This indicates

that depletion of GSH could be one of the major mechanisms of Hg^{2+} induced toxicity in both cell lines and that the *de novo* GSH synthesis plays an import role in its detoxification. It is hypothesized that, at non-cytotoxic levels, Hg^{2+} depleted endogenous GSH, which at the same time could be compensated by the *de novo* production of GSH as a protective response. However, when the Hg^{2+} concentrations got too high, the capacity of the GSH *de novo* synthesis appeared to be not sufficient, resulting in a depletion of GSH, accumulation of ROS and loss of cell viability.

Compared to HD-11 cells, DT40 B-cells showed higher sensitivity to Hg^{2+} exposure when BSO was co-exposed in terms of cell viability, intercellular GSH and ROS levels (Figure 4.1, 2 and 3). These differences were probably due to the nature of these two type of cells. Macrophages (HD-11) are equipped with a robust and complex anti-oxidant buffer system, such as lipid mediators and metabolic reprograming, to survive from the high levels of ROS and nitric oxide produced by themselves upon activation and consecutive oxidative burst (Brüne et al. 2013; Virág et al. 2019). Meanwhile, DT40 B-cells apparently rely on *de novo* GSH synthesis more than HD-11 macrophages, showing a sharp drop of GSH and cell viability already at the lowest Hg^{2+} exposure concentration (0.5 μM) when co-exposed with BSO. However, without BSO, HD-11 macrophages were more sensitive to Hg^{2+} exposure regarding GSH levels (Figure 4.2 with a EC50 at 15 μM) than DT40 B-cells (EC50 values between 50 and 100 μM). Decreased GSH levels in DT40 cells were only noticed above cytotoxic Hg^{2+} concentrations, suggesting that there might be other mechanisms inducing cell viability besides GSH depletion. These cell-specific effects demonstrated that under normal circumstances (without BSO) HD-11 macrophages are probably more vulnerable to Hg^{2+} induced GSH depletion and ROS induction resulting in decreased cell viability with lower EC50 values than DT40 B-lymphocytes. And according to the results with BSO blocking the *de novo* GSH synthesis, macrophages are thought to be protected by more complex antioxidative protective system against ROS, which likely include other components than GSH, such as metabolic reprogramming bioactive and lipid mediators (Virág et al., 2019). These additional protective mechanisms are still effective upon BSO exposure. However, DT40 cell relies largely on GSH as antioxidant.

4.4.2. Immune functional endpoints

4.4.2.1. Nitric oxide

In addition to cytotoxic effects, modulatory effects of Hg^{2+} on some functional endpoints were also detected in both cell lines at non-cytotoxic concentrations. For instance, nitric oxide production was found to be strongly inhibited by Hg^{2+} in activated HD-11 macrophages (Figure 4.4) as also reported in murine macrophages (Tian and Lawrence 1996; Kim et al. 2002; Batista-Duharte et al. 2018). Downregulation of inducible nitric oxide synthase (iNOS) in cells of the J774A.1 murine macrophage cell line by Hg^{2+} was demonstrated in both gene expression profiles as well as protein levels (Kim et al. 2002). However, increased nitric oxide levels were found in activated macrophage cell lines after

non-cytotoxic levels of Cd^{2+} and Pb^{2+} exposure (García-Mendoza et al. 2019; Han et al. 2020), suggesting possible deviating mechanisms among divalent trace metal ions. Once the GSH synthesis was blocked by BSO, less nitric oxide was produced by activated HD-11 cells exposed to Hg^{2+} between 0.5 and 5 μM (Figure 4.4). This could be related to the decreased cell viability (Figure 4.1a). In addition, oxidized glutathione (GSSG, in this case triggered by the treatment with BSO in activated cells) may induce S-glutathionylation of nitric oxide synthase enzyme (NOS) via exchanging thiol–disulphide, leading to uncoupling of biochemically active NOS dimers and a switch from the production of nitric oxide to other types of ROS (Chen et al. 2010; Dulce et al. 2011). This might be explained by the fact that the capacity of the protective cellular antioxidant determines how much nitric oxide macrophage cells can produce, in order to prevent that the cells injure themselves. Nitric oxide is involved in more immune functions than direct antimicrobial activities, such as regulation of cytokine production and lymphocytes differentiation (MacMicking et al. 1997; Bogdan 2001; Tripathi et al. 2007). Hence, inhibited nitric oxide production upon Hg^{2+} exposure in activated macrophages can cause an impaired antimicrobial response as well as disturbed immune signalling and might lead to higher risks of infections *in vivo*.

4.4.2.2. Proliferation

Proliferation of cells was supressed by Hg^{2+} exposure in both cell lines regardless of activation, affecting both DNA replication (Figure 4.5a, c) and metabolic activity (Figure 4.5b, d). DT40 cells were slightly more sensitive to Hg^{2+} than HD-11 macrophages with around 2-fold lower EC50 in BrdU assays (Table 4.1). Decreased immune cell proliferation due to Hg^{2+} exposure was also indicated in mice *ex vivo*, dependent on age and organ (Silva et al. 2005). On the other hand, some studies suggested stimulatory effects of low level Hg^{2+} exposure on murine lymphocyte proliferation (Pelletier et al. 1985; Jiang and Möller 1996; Pollard and Landberg 2001). Although hormesis was not found in the proliferation assays upon 48 hours exposure (Figure 4.5), it was noticed in the WST-1 assays after 24 hours exposure (Figure 4.1). This might suggest that the effect on increased cell metabolic activities is a rather acute response. Interestingly, upon poly I:C activation, HD-11 cells tended to slightly decrease DNA synthesis, potentially to maintain the cell homeostasis, while DT40 cells showed enhancement in both DNA synthesis and cell activity. Besides, similar EC50 values between unstimulated and stimulated cells suggest that poly I:C challenge did not affect the sensitivity of cells to Hg^{2+} exposure (Table 4.1). The results indicate that Hg^{2+} exposure may cause a deficiency in functional immune cells and may disturb the immune cell composition in avian species *in vivo*.

4.4.2.3. Gene expression and immune protein

Gene expression and protein levels were measured as major immune parameters in the present study. Poly I:C was reported to be recognized by TLR3, triggering several pathways such as nuclear factor κB (NF-κB) and interferon receptor factors (IRFs) pathways, resulting in generation of a spectrum of cytokines including proinflammatory cytokines (e.g. IL-8 and

IL-18) via the NF-κB pathway and antiviral type I IFNs (IFN-α and -β) via IRFs pathway (Alexopoulou et al. 2001; Moynagh 2005; Sen and Sarkar 2005; Kawasaki and Kawai 2014). The results demonstrated a significant increase of IFN-α levels due to Hg^{2+} exposure in both cell lines especially DT40 cells (Figure 4.6c, d), similar to what was observed in Pb^{2+} exposed HD-11 and DT40 cells (Han et al. 2020). Although the expression of type I IFNs was documented to be lifted in HD-11 cells after 2h incubation with poly I:C (Peroval et al. 2013), different IFN-α levels upon poly I:C activation were not found in the qPCR or the ELISA assays after 24h exposure in the present study. The prolonged stimulation time could have resulted in the degradation of the induced IFNs by poly I:C. Besides, as both HD-11 and DT40 are immortal cell lines transformed with virus (MC29 virus for HD-11 cells and avian leukosis virus, ALV for DT40 cells) (Beug et al. 1979; Bezzubova and Buerstedde 1994), they might have to some extent lost their capacity for type I IFN production upon viral-like challenge with poly I:C. HD-11 cells were found not to produce more type I IFN upon infection with the highly pathogenic avian influenza virus H5N1, while both *ex vivo* (in primary chicken splenocytes) and *in vivo* (in chicken) studies showed significantly increased type I IFN production after infected with the same virus (Liniger et al. 2012). This difference could be due to the present of specialized type I IFN producing cells in primary splenocytes and *in vivo*, and might suggest a deficiency in type I IFN production in HD-11 cells upon viral challenge. Type I IFNs secreted by immune cells can pass the signal to the neighbouring cells that will develop an antiviral state to defend themselves against the infection. However, excessive type I IFNs may promote autoimmunity *in vivo* (Baccala et al. 2007). Therefore, the type I IFN induced by Hg^{2+} exposure might potentially result in a disorder of immunity.

Apart from cytokines, another group of crucial immune mediators are micro-RNAs, and they can regulate cellular activities posttranscriptional by inhibiting translation and destabilizing mRNA (Bushati and Cohen 2007). As a multifunctional micro-RNA, miR-155 is involved in regulation of lymphocyte proliferation and differentiation, macrophage polarization, and antibody production etc. (Alivernini et al. 2018). Results indicated that miR-155 was downregulated by 10 μM Hg^{2+} in activated DT40 B-lymphocytes (Figure 4.6a). Decreased miR-155 levels caused impaired B cell proliferation in mouse models (Rodriguez et al. 2007; Babar et al. 2012), which is in line with results from the current study (Figure 4.5). In addition, cellular IgM content was found to be significantly decreased by non-cytotoxic Hg^{2+} exposure in the cell lysate of non-activated DT40 lymphocytes, but not in activated cells (Figure 4.6b). IgM in cell lysate referred mainly to the IgM on the cell membrane acting as B-cell receptors (BCRs) which can recognize antigens and initiate humoral immunity (Friess et al. 2018). Therefore, Hg^{2+} affected B-cell functions probably by suppressing miR-155 expression upon activation and decreasing membrane BCRs on the cell membrane without activation. This might result in a weakened pathogen defence and higher risk of infection in avian species.

4.5. Conclusions

In summary, results in the current study revealed that Hg^{2+} had concentration-dependent toxic effects on both chicken macrophage (HD-11) and B-lymphocyte (DT40) cells and affected their immune functions (Figure 4.7). *De novo* GSH production played a vital role in protecting the cells from Hg^{2+} related GSH depletion and ROS induction, and meanwhile could influence the immune function of activated HD-11 macrophages with respect to nitric oxide production. In addition, antiviral IFN-α levels were elevated by Hg^{2+} in both cell types. In B-lymphocytes, Hg^{2+} mainly down-regulated the expression of miR155 upon activation and decreased the cellular IgM levels, which probably act as BCRs, without activation. Based on the current study, it is likely that Hg^{2+} may disturb the homeostasis of the avian immune system potentially resulting in an inadequate immune response upon viral infections which might cause a higher risk of infection for individuals and even prevalence of diseases in the group. Although we only employed two *in vitro* cell models (macrophage and B-lymphocytes) without considering the other parts of the immune system and interactions between immune cells, the results provide a starting point for future studies about molecular mechanisms, systematic immune responses and effects of long-term exposure to immune-toxic trace metals in avian species upon viral challenges.

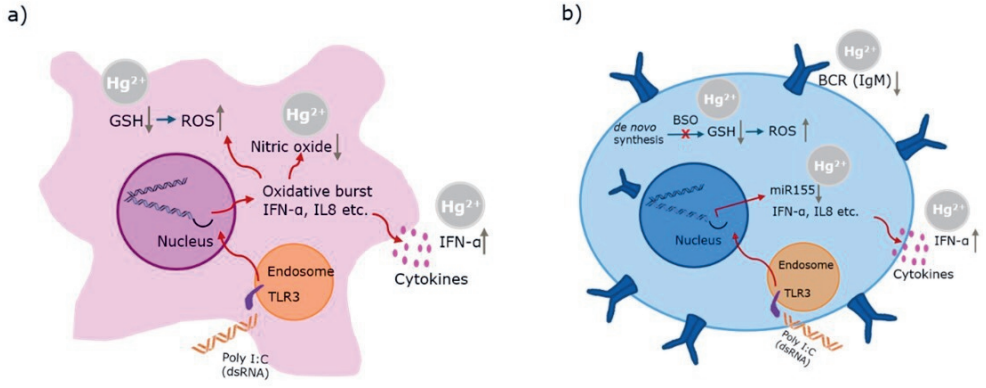


Figure 4.7. Effects of Hg^{2+} on the immune functions found in HD-11 macrophage (a) and DT40 B-lymphocyte (b). GSH; Glutathione; ROS, reactive oxygen species; IFN-α, interferon-α; IL8, interleukin 8; TLR3, toll like receptor 3; poly I:C, polyinosinic acid-polycytidylic acid; dsRNA, double-stranded RNA; BSO, buthionine sulphoximine; miR-155, microRNA 155.

Acknowledgment

Dr. Jurgen van Baal (Department of Animal sciences, Wageningen University, the Netherlands) is acknowledged for kindly providing us the HD-11 cell line. This study is funded by China Scholarship Council (Grant No. 201707720036).

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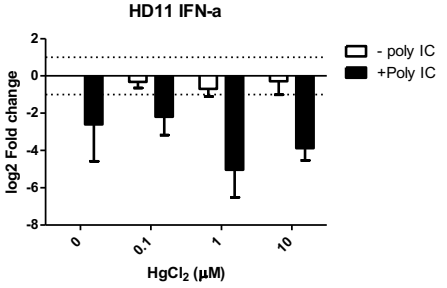
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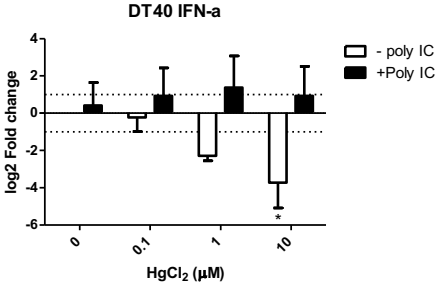
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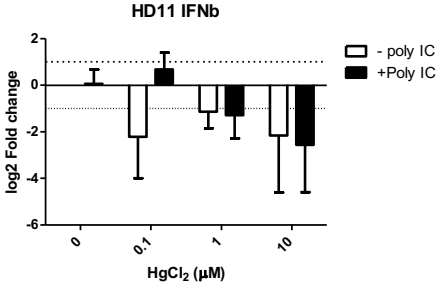
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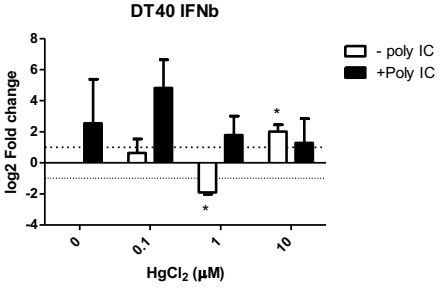
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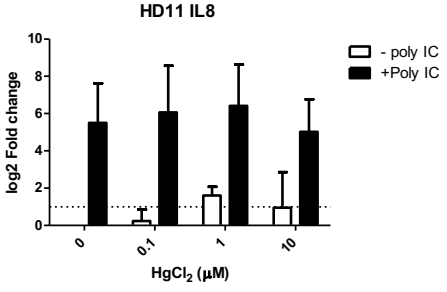
Zou H, Su R, Ruan J, Shao H, Qian K, Ye J, Yao Y, Nair V, Qin A. 2017. Double-stranded RNA induces chicken T-cell lymphoma apoptosis by TRIF and NF-κB. *Sci Rep*. 7(1). doi:10.1038/s41598-017-07919-w.

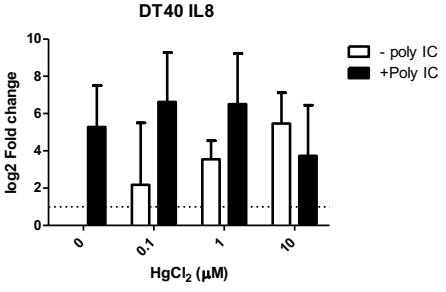

HD11 IFN-a
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)

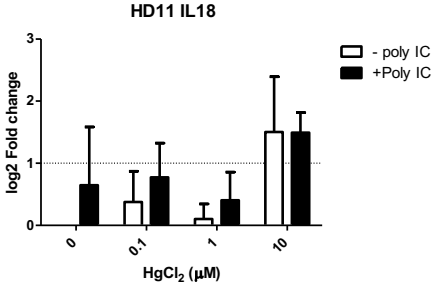

DT40 IFN-a
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)

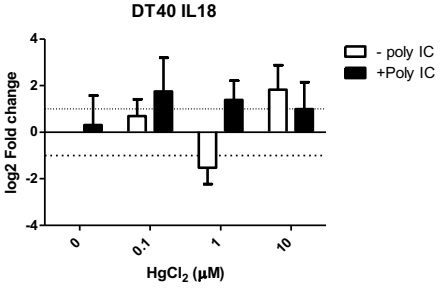

HD11 IFNb
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)


DT40 IFNb
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)


HD11 IL8
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)


DT40 IL8
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)


HD11 IL18
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)


DT40 IL18
log2 Fold change
- poly IC
+Poly IC
HgCl2 (µM)

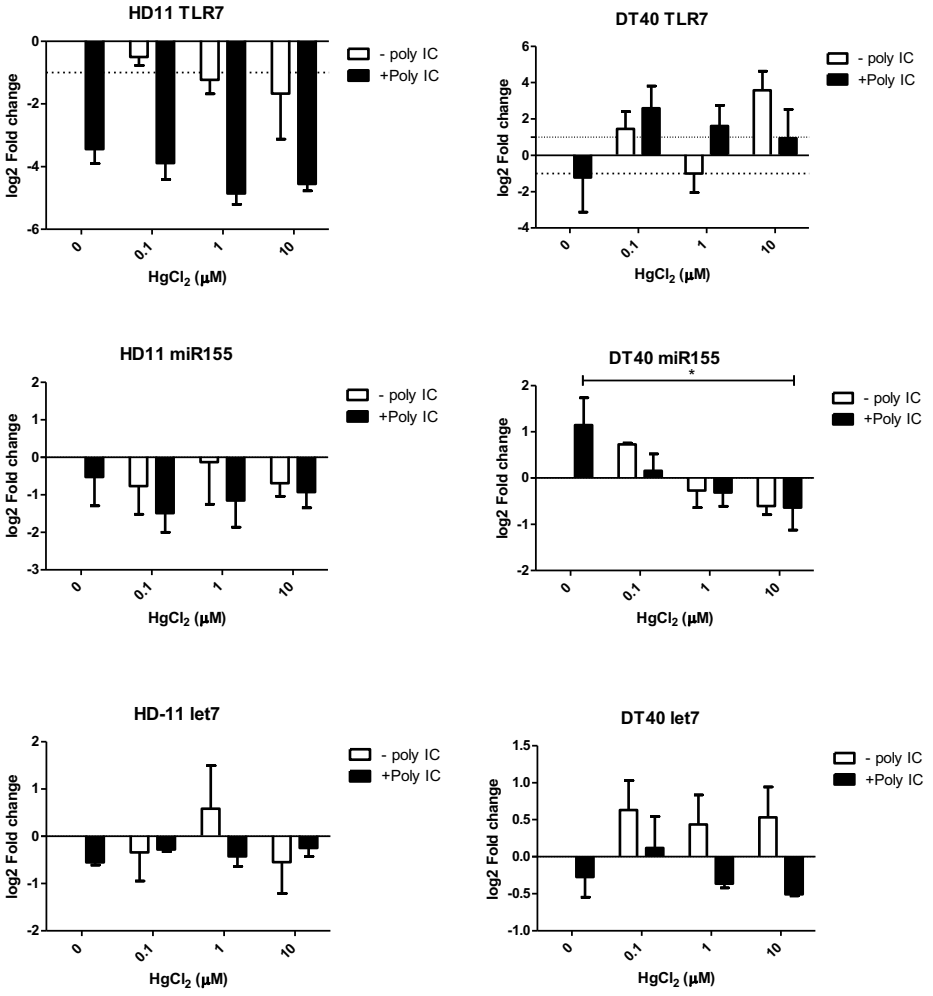


Figure S4.1. Effects of Hg^{2+} exposure on expression of immune functional genes and miRNAs in HD-11 and DT40 cells with and without poly I:C (25 µg/mL) activation for 24 hours. Results (mean ± SEM, n=3) are normalized with housekeeping gene GAPDH for mRNAs and reference gene SNORD68 for miRNA. Gene regulation is presented as log2 fold changes in comparison with the negative control of non-activated cells. Significant differences between Hg^{2+} treatment and related non-exposed controls were checked with ANOVA (* $p<0.05$).

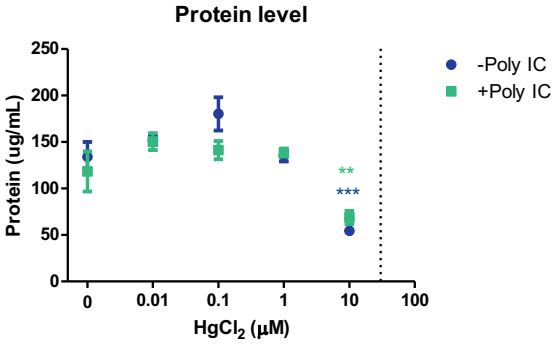


Figure S4.2. Protein levels of DT40 cell lysate samples after Hg^{2+} exposure for 24 hours. Results (mean ± SEM, n=3) are presented as protein (µg/ml) concentrations calculated with a standard curve. Significant differences between Hg^{2+} treatment and related non-exposed controls were checked with ANOVA (** $p<0.01$, *** $p<0.005$).

Chapter 5

Modulatory effects of lead acetate ($PbAc_2$) on virally challenged chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines *in vitro*

Biyao Han, Diego García-Mendoza, Hans van den Berg, Nico W. van den Brink.

Published in Environmental Toxicology and Chemistry. 2020; 39(5): 1060-1070. https://doi.org/10.1002/etc.4702

Abstract

Elevated levels of lead have been found in waterfowl, due to human activities. Lead may cause immunomodulatory effects, but the mechanisms are largely unknown, especially upon viral challenges. To characterize avian immunomodulatory hazards of Pb^{2+}, we used chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines, as *in vitro* models for the innate and adaptive immune system respectively. The cells were activated via toll-like receptor-3 (TLR3) by polyinosinic–polycytidylic acid sodium salt (poly I:C), mimicking viral infections. The results indicate that Pb^{2+} is cytotoxic to both cell lines, macrophages being more sensitive. *De novo* synthesis of glutathione (GSH) plays an important role in protecting macrophages from Pb^{2+} intoxication, which might also be closely involved in the induction of nitric oxide upon Pb^{2+} exposure. Stimulatory effects on cell proliferation were noticed at non-cytotoxic Pb^{2+} concentrations as well. Pb^{2+} exposure could also affect the inflammatory status by inhibiting the pro-inflammatory interferon (IFN)-γ while promoting the anti-inflammatory type I IFNs production in both macrophages and B-cells, and increasing intracellular IgM levels in B-cells. These results suggest that the immunomodulatory effects of Pb^{2+} in birds are probably closely associated with the disruption of immune cell proliferation and cytokine production, potentially causing disorders of the avian immune system.

5.1. Introduction

For decades, lead (Pb) has been identified as one of the most toxic environmental heavy metals (Brunekreef et al. 1983; Gadrat et al. 1966; Gavaghan 2002; Needleman 2004; Rahman and Singh 2019; Smith and Flegal 1995). Although the overall lead emission into the environment has been reduced because of, for instance the ban on the use of lead in paints, pipes and gasoline, elevated levels in the environment still occur. Especially in soils and sediments of historical hunting areas the levels are higher due to the use of lead ammunition (ECHA 2017; Williams et al. 2017). From a few, up to hundreds of lead pellets/m^2, were found in wetlands all over Europe (Pain et al. 2019), potentially threatening wildlife in these habitats. One of the most vulnerable groups of animals is waterfowl. Lead pellets are often ingested by waterfowl while foraging, mistaking them as grit used to facilitate the digestion of their food (De Francisco et al. 2003). Ingestion of lead shots by mallard ducks has been documented in Europe and the prevalence was closely related to the environmental densities of pellets (Mateo 2009). Upon ingestion by waterfowl, lead pellets dissolve in the intestinal tract often followed by rapid uptake and distribution of Pb^{2+} in various tissues including liver, kidneys and bones (Ferreyra et al. 2014). Increased lead levels were even detected in eggshells from wild waterfowl in contaminated areas, suggesting potential maternal exposure for avian embryos (Franson and Pain 2011; Pain et al. 2019; Vallverdu-Coll et al. 2015a). Lethal effects of lead poisoning are evident, causing mortality of millions of birds worldwide annually (De Francisco et al. 2003; ECHA 2017). However, the sub-lethal effects of lead exposure on the avian immune system are still largely unknown. Lead intoxication was reported to affect immunity and diminish resistance to infections in birds (Knowles and Donaldson 1997; Vallverdu-Coll et al. 2019), which might result in the prevalence of some zoonotic diseases including avian influenza.

Similar to mammals, the avian immune system consists of an innate system and an adaptive immune system. The innate immune system produces an initial non-specific response to pathogens. The adaptive immune system evolves over time, depending on the infections an organism experiences (Davison et al. 2008; Sharma 1997). Multiple types of immune cells from both the innate (e.g. macrophages, heterophils and natural killer cells) and adaptive (e.g. B- and T-lymphocytes) immune system collaborate to form a functional and effective immune response. Once infected by specific pathogens, the innate and the adaptive immune systems will be triggered and perform certain defense strategies within hours and days respectively (Abbas et al. 2014). These defense strategies include for example, production of nitric oxide by macrophages and production of antibodies (immunoglobulin, Ig) by B-lymphocytes. For communicating during immune responses, immune cells also secrete cytokines such as pro-inflammatory interferon-γ (IFN-γ), anti-viral interferon-α and β (IFN-α and -β), tumour necrosis factor (TNF-α), interleukins (ILs, e.g. pro-inflammatory IL-18) and chemokines (e.g. chemokine (C-X-C motif) ligand 8, CXCL8, also called IL-8) (Genovese et al. 2013; Maxwell and Robertson 1998; Schat et al. 2014; Speer 2016). Different cytokines are produced by different immune cells and play specific roles in the

immune response. The appropriate levels and functioning of different immune cell populations and immune mediators (e.g. cytokines and chemokines) are crucial for the communication and cooperation between different immune cells, which is the basis of an adequate immune system.

Previous studies have revealed that lead may have immune-modulating effects, potentially affecting immune organs, cellular populations and signalling molecules in birds (Vallverdu-Coll et al. 2019). For example, mallard ducks exposed to lead showed decreased spleen mass; decreased number of heterophils and monocytes (Rocke and Samuel 1991); lower antibody levels (Trust et al. 1990; Vallverdu-Coll et al. 2015a) and lower lysozyme levels (Vallverdu-Coll et al. 2016). At the cellular level, lead can alter the normal immune function indirectly by depleting antioxidants such as glutathione (GSH) and producing oxidative stress (Vallverdu-Coll et al. 2015a) which could potentially induce damage to DNA and membrane structure (Ahamed and Siddiqui 2007). Because some immune cells such as macrophages and heterophils can undergo oxidative bursts by rapid production of reactive oxygen species (ROS) to defend against microbial pathogen infections, a chemical disturbance of redox conditions may also influence the ability to produce the immune defense response related to oxidative burst (Iles and Forman 2002). Exposure to lead has also been implicated in the modification of cytokine gene expression and production in mammals both *in vivo* and *in vitro* (Cheng et al. 2002; Heo et al. 2007; Krocova et al. 2000; Yucesoy et al. 1997). More specifically, Pb^{2+} exposure was found to result in an imbalance between pro-inflammatory, cell-mediated T helper 1 (Th1) and anti-inflammatory, humoral-mediated T helper 2 (Th2) response by reducing Th1 cytokines (e.g. IFN-γ and IL-2) and enhancing Th2 cytokine (IL-4) at the same time (Iavicoli et al. 2004; Lawrence and McCabe 2002). As the major targets of Th1 responses are cellular antigens including virus and bacteria, while the major targets for Th2 responses are extracellular parasites (Degen et al. 2005), the Pb^{2+}-induced shifting to Th2 immunity could probably weaken the host defense of virus in birds. However, there is no *in vitro* model established to study the immunomodulatory effects of heavy metals in birds, which could reduce animal experiments and give more insights in the mechanisms. In addition, most studies in birds assessed the baseline immunity after lead exposure without activation of the immune system, which is crucial when discussing the vulnerability of birds to diseases. Among the few studies concerning the challenges of the immune system, most of them were focused on bacterial instead of viral infections (Vallverdu-Coll et al. 2019). Therefore, the aim of the current study is to investigate the immunomodulatory effect of Pb^{2+} exposure in two chicken *in vitro* cell lines (macrophages and B-lymphocytes) in both non-activated and virally activated conditions.

5.2. Materials and methods

5.2.1. Experimental setup

Two chicken immune cell lines, namely macrophage HD-11 cells and B-lymphocytes DT40 cells, were selected as two avian *in vitro* models for the innate and adaptive immune system

respectively. As both cell lines express toll-like receptor 3 (TLR3) which is able to recognise double strained RNA (dsRNA) related to viral infection (Peroval et al. 2013; Zou et al. 2017), polyinosinic–polycytidylic acid sodium salt (poly I:C), a synthetic analogue of dsRNA (Chen et al. 2013) was used as a viral activator via TLR3.

Both general cytotoxicity and immune functional effects of Pb^{2+} (as lead acetate, $PbAc_2$) were investigated in the two cell lines. Firstly, cell viability was measured by either water-soluble tetrazolium-1 (WST-1) assay (for free floating DT40 cells) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, for attached HD-11 cells) to quantify the EC_{50} values, used as the basis for determining the non-toxic concentration ranges in consecutive assays. Cell membrane integrity was also tested as another indicator for cytotoxicity, using the lactate dehydrogenase (LDH)-leakage assay (Adan et al. 2016). Depletion of glutathione (GSH) and induction of reactive oxidant species (ROS) in the cells were tested with or without inhibition of *de novo* GSH synthesis, as Pb^{2+} has high affinity with thiol groups (Dafre et al. 2004) and can result in oxidative stress via this mechanism (Ma et al. 2017).

Subsequently, assays were applied to assess effects of Pb^{2+} on immune functions in both non-activated cells and cells activated with 25 μg/mL poly I:C. Nitric oxide production was quantified as primary functional parameters for macrophage (HD-11 cells). Cell proliferation was tested with prolonged WST-1/MTT assays for metabolic activity (cell activity), similar as mentioned before for cytotoxicity assays and by using the bromodeoxyuridine (BrdU) assay for DNA synthesis (cell divisions). Quantitative reverse transcription PCR (RT-qPCR) was applied to profile the regulation of specific immune gene expressions by Pb^{2+} exposure at the transcription level. Finally, multiple immune functional proteins were measured with enzyme linked immunosorbent assays (ELISAs). Both released and intracellular immunoglobulin M (IgM) levels were examined for DT40 B-lymphocytes while secretion of TNF-α by HD-11 macrophages was measured. The production of IFNs, including type I anti-viral IFN- α and type II pro-inflammatory IFN-γ, and IL-8 were tested in the supernatant of both cell lines. All the assays were performed in three biological replicates.

5.2.2. Cell culture

Chicken B-lymphocyte-like cell line DT40 (ATCC® CRL2111™) derived from avian leukosis virus (ALV) (Winding and Berchtold 2001) was cultured in Dulbecco's modified Eagle media (DMEM, Gibco, Paisley, UK) supplemented with 10% tryptose phosphate broth (Sigma-Aldrich, Zwijndrecht, the Netherlands), 10% fetal bovine serum (FBS, Sigma-Aldrich, Zwijndrecht, the Netherlands), 5% chicken serum (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 0.05 mM 2-mercaptoethanol (Gibco, Paisley, UK) at 37°C in a 5% CO_2 humidified air incubator. Growth medium was renewed every two to three days.

Chicken macrophage-like cell line HD-11, transformed with MC29 virus (Beug et al. 1979) was a kind gift from Dr. Jurgen van Baal (Department of Animal sciences, Wageningen

University, the Netherlands). HD-11 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Paisley, UK) containing 10% FBS at 37°C in a 5% CO_2 humidified air incubator. Cells were sub-cultured every two to three days by detaching the cells with 5 mM EDTA (Merck, Darmstadt, Germany) followed by a washing step to remove EDTA before seeding the cells.

5.2.3. Pb^{2+} exposure

Both cell lines were exposed to similar Pb^{2+} concentrations for comparison. In a flat-bottom 96-well plate 1×10^5 cells/well were seeded and incubated overnight. In the proliferation assay 1×10^4 cells/well were used as seeding concentration to allow for a longer exposure time. For qPCR 1×10^6 cells/well were seeded in 6-well plates. A series of $PbAc_2$ (Sigma-Aldrich, Zwijndrecht, the Netherlands) stock solutions ranging from 0.02 mM to 100 mM were prepared in sterile MilliQ water and diluted 20 times to final exposure concentration in growth medium. Exposure time was 24 hours for most assays, however a 6-hour exposure was performed for the ROS assay in addition to a 24-hour exposure, while for the proliferation assay the exposure time was extended to 48 hours.

To investigate the role of newly synthesized GSH, 200 µM of L-buthionine-sulfoximine (BSO, Sigma-Aldrich, Zwijndrecht, the Netherlands) was used as GSH *de novo* synthesis inhibitor by blocking γ-glutamylcysteine synthetase in the GSH, ROS and nitric oxide assays. To trigger an anti-viral response, 25 µg/mL poly I:C (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used in all immune functional assays. The activation of the cells started at the same time with exposure. In all immune functional assays, DT40 cells were seeded and exposed in chicken-serum-free medium to eliminate the potential effects of cytokines in chicken serum, and LDH assay cells were cultured in serum-free medium to avoid the serum derived LDH.

5.2.4. General toxicity assays

5.2.4.1. Cytotoxicity assays

For HD-11 cells, 20 µL 5 mg/mL MTT solution in phosphate-buffered saline (PBS, Gibco, Paisley, UK) was added to each well after 24h exposure to Pb^{2+}, and incubated at 37°C for 2 hours. Then, the medium was aspirated and 100 µL DMSO was added to dissolve the violet formazan crystals produced by metabolically active cells. After 15-minute shaking, the plates were measured for absorbance at 562nm and 620nm using a Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale CA, USA). For DT40 cells, 20 µL WST-1 reagent was added to each well and incubated at 37°C for 2 hours before measuring absorbance at 440 nm and a reference wavelength at 620 nm. Cell viability was expressed as percentage of negative control. Concentrations used in later assays were determined by the EC_{50} obtained in cell viability assays for both cell lines.

The cell membrane integrity was evaluated after Pb^{2+} exposure with a Pierce LDH cytotoxicity assay kit (ThermoFisher Scientific, Landsmeer, the Netherlands). Briefly, after incubating the cells for 24h in serum-free medium (to avoid serum-derived LDH) with Pb^{2+} exposure, cells were centrifuged and 50 µL of supernatant was transferred into a new plate. The supernatant was incubated with 50 µL reaction mixture at room temperature for 30 minutes. Cells treated with lysis buffer served as the positive control for maximum LDH activity. Afterwards, 50 µL of stop solution was added before measurement absorbance at 490nm and 680nm. Results were expressed as percentage of positive control.

5.2.4.2. Glutathione(GSH) assay

Intracellular GSH levels were measured with the ThiolTracker™ Violet Glutathione Detection Reagent (Life Technologies/Invitrogen, Oregon, USA) in a 96-well plate setup, according to manufacturer's instructions. Briefly, after Pb^{2+} exposure for 24 hours with or without 200 µM GSH synthesis inhibitor BSO, cells were washed with Dulbecco's phosphate- buffered saline with calcium and magnesium (D-PBS C/M, Gibco, Paisley, UK) and labelled with 20 µM ThiolTracker ™ Violet dye working solution in D-PBS C/M for 30 min at 37°C. After replacing the working solution with D-PBS C/M, fluorescence was measured at Ex/Em at 404/526 nm with the Microplate Reader SpectraMax M2. GSH levels were shown as percentages in relative to the Pb^{2+}-free controls.

5.2.4.3. ROS (DCFDA) assay

2`,7`-Dichlorofluorescin diacetate (DCFDA, Sigma-Aldrich, Zwijndrecht, the Netherlands) reagent was used for measuring the intracellular level of ROS after 6h or 24h Pb^{2+} exposure. Upon diffusion into the cell, DCFDA is de-esterified by cellular esterase and oxidised to highly fluorescent 2',7'-dichlorofluorescin (DFC) by ROS (Eruslanov and Kusmartsev 2010). The exposure conditions were identical to the ones for the GSH assay (also with or without BSO). As the fluorescence of DCF is only stable for around 6 hours, the methods for measurement after 24h exposure was modified according to the recommendation from the manufacturer. For 6h exposure, cells were first washed with PBS and loaded with 20 µM DCFDA working solution in PBS, containing 2% FBS, for 60 minutes at 37°C in the dark. After loading, DCFDA working solution was replaced by exposure medium containing Pb^{2+} and incubated for 6h at 37°C in the dark until measurement. For 24h exposure, the cells were first exposed to Pb^{2+} for 20 hours and then same volume of 2× DCFDA working solution was added. The plates were then incubated for another 4 hours and measured. The ROS level was measured at 485 nm excitation 535 nm emission and presented as a relative ratio to the Pb^{2+}-free controls.

5.2.5. Immune functional assays

5.2.5.1. Nitric oxide (Griess) assay for HD-11 cells

Nitric oxide production was tested in HD-11 macrophages after Pb^{2+} exposure with the consideration of effects from *de novo* GSH synthesis (BSO) and activation (poly I:C). Briefly, after exposure, 100 µL cell culture supernatant was transferred to a new plate and incubated with 50 µL 1% sulfanilamide (Sigma-Aldrich, Zwijndrecht, the Netherlands) in 5% phosphoric acid (Merck, Darmstadt, Germany) at room temperature for 10 minutes in the dark. Then 50 µL of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED, Sigma-Aldrich, Zwijndrecht, the Netherlands) solution was added and incubated for another 10 minutes in the dark before measuring absorbance at 540 nm with a microplate reader. Nitric oxide production in µM was calculated according to a standard curve of $NaNO_2$.

5.2.5.2. Proliferation assays

MTT and WST-1 assays were applied as described before. For the colorimetric BrdU assay (Roche, Manheim, Germany), cells were labelled with BrdU during the last 4 hours of exposure. Subsequently, floating DT40 cells were spun down by centrifuge at 300×g for 10min. Then both DT40 and HD-11 cells were fixed and incubated with anti-BrdU-POD which binds to the BrdU labeled newly-synthesized DNA and reacts with the substrate. The reaction was terminated by adding stop solution after incubation for 30 minutes at room temperature. The number of proliferating cells was quantified by measuring absorbance at 450 nm and a reference wavelength at 690 nm with the microplate reader and indicated as a percentage in relative to negative controls.

5.2.5.3. Gene expression (RT-qPCR) assay

Expression of seven chicken genes, including house-keeping gene (actin-β), type I interferons (IFN-α, IFN-β), interleukins (IL-8, IL-18) and toll-like receptors recognizing viral pathogens (TLR3 and TLR7), were tested in both cell-lines by RT-qPCR with kits from Qiagen (Venlo, the Netherlands) according to the manufacturer's instruction. Briefly, after exposure for 24h, cells in 6-well plates were lysed with RLT lysis buffer and RNA was isolated using the QIAshredder and RNeasy® mini kit. The quality and quantity of obtained RNA were assessed by Nanodrop (ND-1000, Themoscientific Wilmington, Delaware, US). Subsequently 300 ng total RNA was reverse translated to cDNA with the QuantiTect® reverse transcription kit and then RT-qPCR was applied using the Rotor-Gene® SYBR® Green PCR kit and the Rotor-Gene® 6000 cycler according to the manufacture's handbook. Chicken specific primers used in this study were commercially available QuantiTect® primer assays, including Gg_IFNA3_1_SG, Gg_IFNB_1_SG, Gg_IL8L2_1_SG, Gg_IL18_1_SG, Gg_TLR3_1_SG, Gg_TLR7_1_SG and Gg_ACTB_1_SG, whose efficiency was checked prior to sample measurement.

5.2.5.4. Immune proteins

Levels of multiple immune functional macro-biomolecules produced by the two cell lines, such as TNF-α by HD-11, IgM (both released and inside the cell) by DT40, together with IFN-α, IFN-γ and IL-8 by both cell lines, were quantified with commercially available sandwich ELISA kits. The chicken IFN-γ kit was made up of a Chicken IFN-γ CytoSet™ antibody pair and a BioSource CytoSet™ Buffer Set (Invitrogen, Breda, The Netherlands), all the other ELISA kits (chicken IFN- α, TNF-α, IL-8 and IgM ELISA kits) were obtained from ELISAGenie (Dublin, Ireland).

Both non-activated and activated cells were exposed to Pb^{2+} for 24 hours followed by collecting supernatant. Specific immune protein levels were measured in the supernatant except for intracellular IgM in DT40 cells, which was tested in cell lysate prepared through repeating freeze-thaw cycles followed by centrifuging at 1000×g for 5min. ELISA assays were carried out according to the manufacture's instruction for each kit. Levels of each immune protein (in ng/mL for IgM and pg/mL for others) were determined by comparing absorbance at 450nm with standards from the kits. Quantities of IgM in cell lysate were normalized by total protein content in each sample, quantified with a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Landsmeer, the Netherlands) and expressed in ng/µg protein.

5.2.6. Data analysis and statistics

GraphPad Prism 5 (San Diego, CA, USA) was used throughout the study except for heatmaps. Concentration-response curves were fitted with non-linear regression and EC_{50} was calculated. Significant differences between experimental groups and control groups were evaluated with one-way ANOVA with Dunnett's test. Gene expression was normalized against the housekeeping gene actin-β and presented as log2 fold changes in comparison with the negative control of non-activated cells by -ΔΔCT method (Pfaffl 2001). Heatmaps and clustering analysis based on average linkage were generated by an online tool Heatmapper (http://www.heatmapper.ca/).

5.3. Results

5.3.1. General Cytotoxicity assays

5.3.1.1. Cytotoxicity assay

Both cell lines showed a concentration-dependent decrease in cell viability and an increase of cell membrane damage upon 24h Pb^{2+} exposure. Pb^{2+} inhibited the metabolic activity with an EC_{50} of 0.48 mM (95% confidence intervals 0.34 to 0.68 mM) $Pb2^{+}$ for HD-11 cells and a significantly ($p<0.05$, t-test) higher EC_{50} of 1.7 mM (95% confidence intervals 1.2 to 2.4 mM) $Pb2^{+}$ for DT40 cells (Figure 5.1a). Similarly, Pb^{2+} reduced the cell membrane integrity, especially in HD-11 cells with an EC_{50} of 1.7 mM (95% confidence intervals 1.4 to 2.2 mM)

$Pb2^+$, while no complete concentration-response curve could be obtained for DT40 cells up to 5 mM $Pb2^+$ (Figure 5.1b).

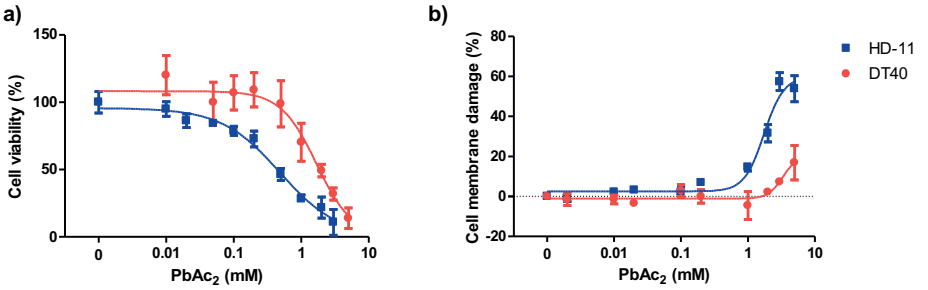


Figure 5.1. Cytotoxicity effects of Pb^{2+} on DT40 and HD-11 cells after 24h exposure, in terms of (a) Cell viability and (b) Cell membrane damage. Results are expressed as mean ± SEM (n=3), relative to negative control for cell viability and relative to the positive control for cell membrane damage.

5.3.1.2. Glutathione levels

In both cell types, GSH levels inside the cells significantly declined after 24h Pb^{2+} exposure when the *de novo* GSH synthesis was inhibited by BSO (Figure 5.2). However, no significant changes in GSH levels were observed without inhibition (Figure 5.2). When dosed with BSO, HD-11 cells showed a clear concentration-dependent response with an EC_{50} of 0.074 mM (95% confidence interval 0.061 to 0.089 mM) $Pb2^+$ which is much lower than the EC_{50} for cytotoxicity of $Pb2^+$ as quantified by MTT assay (shown as the vertical line in Figure 5.2a). In contrast, in DT40 cells, even with BSO, Pb^{2+} only decreased the GSH level at the highest concentration (5 mM $Pb2^+$) which is higher than the EC_{50} of $Pb2^+$ cytotoxicity determined by WST-1 assay (shown as the vertical line in Figure 5.2b).

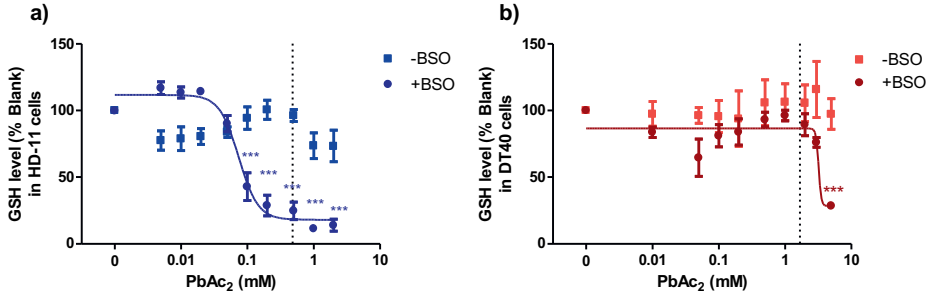


Figure 5.2. Intracellular GSH levels after 24h exposure to Pb^{2+} in HD-11 (a) and DT40 (b) cells with or without GSH synthesis inhibitor (200µM BSO). Results are expressed as percentages (mean ± SEM, n=3) in relative to Pb^{2+}-free controls. Significant differences between treatments and Pb^{2+}-free controls

were checked with one-way ANOVA (***, p<0.005). Vertical lines indicate the EC_{50} of cytotoxicity after 24h Pb^{2+} exposure from MTT assay for HD-11 cells (a) or WST-1 for DT40 cells (b).

5.3.1.3. ROS

No effects of $Pb2^{+}$ exposure on intracellular ROS levels were seen after 6h exposure in both cell lines and 24h exposure in DT40 cells (data not shown) even though GSH synthesis was blocked with BSO. When the exposure time was extended to 24 hours, ROS was only significantly induced in HD-11 macrophages after treatment with the highest $Pb2^{+}$ concentration (2 mM) and BSO (Figure 5.3) with an EC_{50} of 0.41 mM (95% confidence intervals 0.22 to 0.76 mM) $Pb2^{+}$. No effects of $Pb2^{+}$ on ROS levels was found in DT40 cells after 24h exposure even with BSO (data not shown).

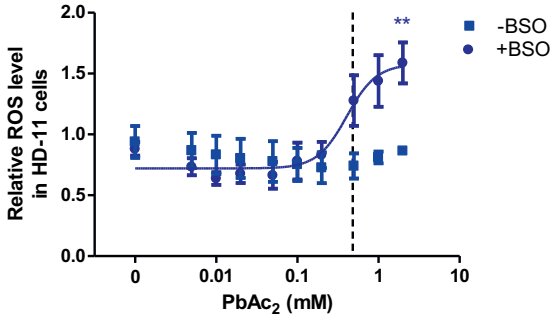


Figure 5.3. Intracellular ROS levels after 24h exposure to Pb^{2+} in HD-11 cells with or without GSH synthesis inhibitor (200µM BSO) were measured with DCFDA reagent. Results are expressed as a relative ratio (mean ± SEM, n=3) to the Pb^{2+}-free controls. Significant differences between treatments and Pb^{2+}-free controls were checked with one-way ANOVA (** p<0.01). Vertical lines indicate the EC_{50} of cytotoxicity after 24h Pb^{2+} exposure from MTT assay for HD-11 cells.

5.3.2. Immune functional assays

5.3.2.1. Nitric oxide production by macrophages

Nitric oxide produced by HD-11 macrophages after 24h $Pb2^{+}$ exposure was investigated in cell culture supernatant under different conditions including poly I:C activation and GSH synthesis inhibition (Figure 5.4). Poly I:C triggered the production of nitric oxide to approximately 9 µM in $Pb2^{+}$-free treatments. $Pb2^{+}$ significantly increased nitric oxide levels in all of the four conditions, especially in the two groups without BSO, showing induction at much lower concentrations than the EC_{50} of $Pb2^{+}$ cytotoxicity determined with MTT assay. Moreover, activated macrophages produced more $Pb2^{+}$-induced nitric oxide without BSO inhibiting *de novo* GSH synthesis.

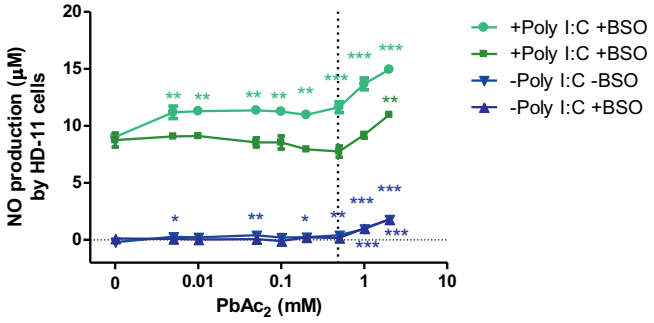


Figure 5.4. Nitric oxide (NO) production by activated (with 25µg/mL poly I:C) and non-activated HD-11 cells with or without GSH synthesis inhibitor (200µM BSO) were measured with Griess assay after 24h Pb^{2+} exposure. Results are expressed as mean ± SEM (n=3). Significant differences between treatments and Pb^{2+} -free controls were checked with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical line indicates the EC_{50} of cytotoxicity after 24h Pb^{2+} exposure from MTT assay.

5.3.2.2. Cell proliferation

Cell proliferation after 48h $Pb2^{+}$ exposure was evaluated with two indicators: metabolic activity by MTT (for HD-11 cells) or the WST-1 assay (for DT40 cells) and synthesis of DNA quantified by the BrdU assay. There was inhibition at cytotoxic concentrations in terms of both metabolic activity and DNA synthesis in both cell lines, although the inhibition effects were only significant in HD-11 cells (Figure 5.5a, b). However, $Pb2^{+}$ exposure below cytotoxic concentrations (lower than 0.02 mM for HD-11 cells and lower than 0.2 mM for DT40 cells) showed induction of proliferation in BrdU assay (Figure 5.5b, d), and this induction was only significant in non-activated HD-11 cells (Figure 5.5b). Interestingly, without an effect on the EC_{50}s, poly I:C treatment stimulated metabolic activity (Figure 5.5a) while inhibiting DNA synthesis in HD-11 cells (Figure 5.5b).

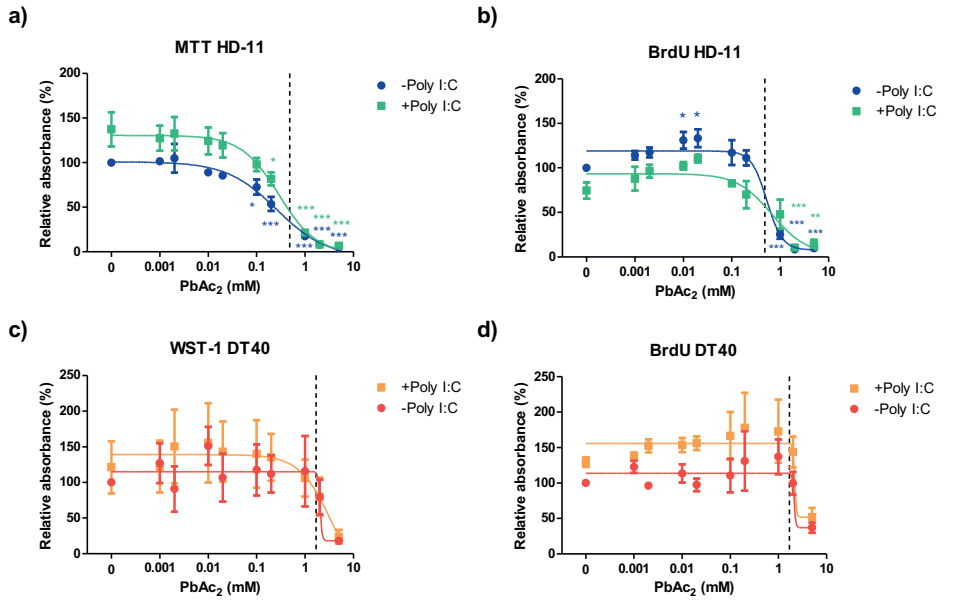


Figure 5.5. Cell proliferation of HD-11 cells (a, b) and DT40 cells (c, d) with and without 25µg/mL poly I:C activation after 48h Pb^{2+} exposure. Results were expressed as relative absorbance to negative controls in non-activated groups (mean ± SEM, n=3). The increase of proliferation in sub-toxic concentrations was statistically compared with negative controls in BrdU assay with one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicate the EC_{50} of cytotoxicity after 24h Pb^{2+} exposure from MTT assay for HD-11 cells (a, b) or WST-1 for DT40 cells (c, d).

5.3.2.3. Gene expression

Gene expression of six immune functional genes was examined and visualized with a heatmap (Figure 5.6, detailed results shown in Figure S5.1). Clustering results suggest similar effects of $Pb2^{+}$ exposure on gene expression in both DT40 B-cells and HD-11 macrophages. Pb^{2+} down-regulated the expression of TLR3 and TLR7, and meanwhile up-regulated the expression of IFN-α, IFN-β, IL-18 and especially IL-8. A significant difference was only found for IL-8 expression between the treatment of 0.5 mM $Pb2^{+}$ exposure and the $Pb2^{+}$-free control group of activated HD-11 cells.

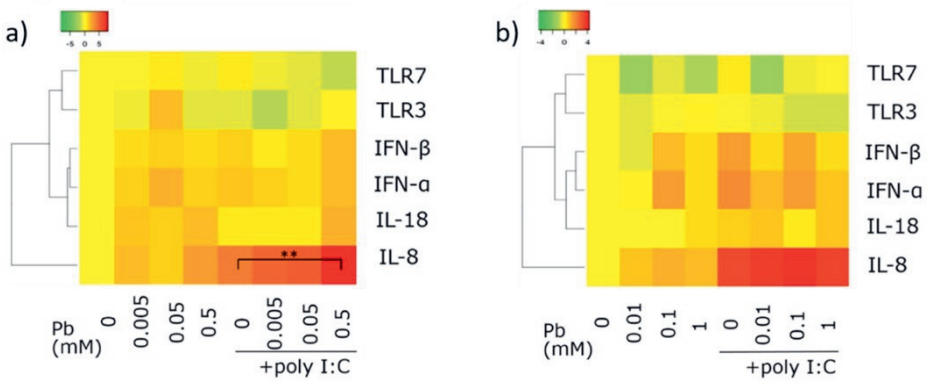


Figure 5.6. Effects of Pb^{2+} exposure on the expression of immune functional genes in HD-11 cells (a) and DT40 cells (b) with and without 25μg/mL poly I:C activation. Results are normalized with housing keeping gene actin-β and presented as log2 fold changes in comparison with the negative controls of non-activated cells.

5.3.2.4. Functional immune proteins

Several immune functional proteins were quantified with ELISA assays after 24h $Pb2^{+}$ exposure (Figure 5.7, 5.8). Concentrations of IFN-γ released by DT40 cells and IL-8 released by both cell lines were lower than the detection limits of the ELISA kits (data not shown). Pb^{2+} significantly induced IFN-α secretion from both cell lines at sub-cytotoxic concentrations irrespective of activation, and no significant effect of poly I:C activation was detected (Figure 5.7a, 5.8a). In HD-11 cells, IFN-γ production dropped after 24h Pb^{2+} treatment for both activated and non-activated cells. Activated cells were more sensitive to $Pb2^{+}$ exposure with an EC_{50} of 0.049 mM (95% confidence interval 0.039 to 0.061 mM) $Pb2^{+}$, while an EC_{50} of 0.70 mM (95% confidence interval 0.18 to 2.8 mM) $Pb2^{+}$ was obtained for non-activated ones (Figure 5.7b). HD-11 macrophages tended to release more TNF-α upon activation, yet no significance was noticed, and no statistical $Pb2^{+}$-related increase was noticed in either non-activated or activated cells compared with non-exposed cells (Figure 5.7c). As for DT40 cells, the IgM levels in non-activated DT40 cell lysate increased significantly after exposure to 0.5 mM and 1 mM $Pb2^{+}$ and exceeded the levels in activated ones, although no EC_{50} value was gained due to lack of maximum effects. However, no significant changes were found in activated cells (Figure 5.8b). IgM levels in DT40 cell culture supernatant were affected by neither poly I:C activation nor $Pb2^{+}$ exposure (Figure 5.8c).

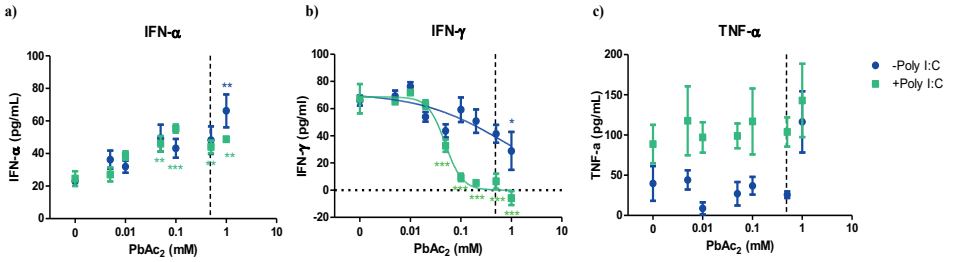


Figure 5.7. ELISA results for multiple immune functional proteins produced by HD-11 cells with and without 25µg/mL poly I:C activation after 24h Pb^{2+} exposure. All the protein levels were tested in cell culture supernatant. Results are expressed in protein concentrations (ng/mL or pg/mL) as mean ± SEM (n=3). Significant differences between treatments and Pb^{2+}-free controls were checked with one-way ANOVA and shown with different colour for different groups (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicate the EC_{50} of cytotoxicity after 24h Pb^{2+} exposure from MTT assay.

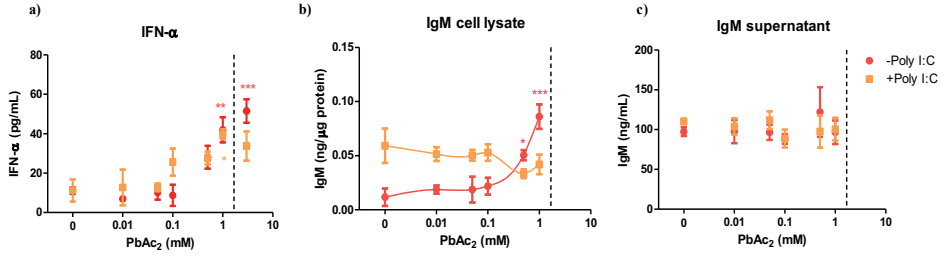


Figure 5.8. ELISA results for multiple immune functional proteins produced by DT40 cells with and without 25µg/mL poly I:C activation after 24h Pb^{2+} exposure. The protein levels were tested in cell culture supernatant, except for IgM in DT40 cell lysate (b). Results are expressed in protein concentrations (ng/mL or pg/mL) or content (ng/µg protein) as mean ± SEM (n=3). Significant differences between treatments and Pb^{2+}-free controls were checked with one-way ANOVA and shown with different colour for different groups (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). Vertical lines indicate the EC_{50} of cytotoxicity after 24h Pb^{2+} exposure from WST-1 assay.

5.4. Discussion

The present study was designed to investigate cytotoxic and immune functional effects of Pb^{2+} exposure on two avian immune cell lines as models for the avian immune system *in vitro*, toward a viral mimicking challenge. Compared to HD-11 macrophages, DT40 B-cells were significantly less sensitive to Pb^{2+} exposure with respect to cell viability (Figure 5.1a) and membrane integrity (Figure 5.1b). Similar patterns were reported in primary human immune cells isolated from blood after *ex vivo* exposure to $Pb(NO_3)_2$ for 24 hours, for which monocytes from the innate system showed an EC_{50} at approximately 3.7 mM Pb^{2+} while B-cells were not sensitive enough to get a determined EC_{50} value (Steffensen et al. 1994). In

both cell lines, the LDH leakage caused by $Pb2^{+}$ exposure occurred at higher concentrations than the inhibition of metabolic activity measured with MTT or WST-1 assay. However, the LDH leakage assays were performed in serum-free medium, which was not an optimal condition for the cells. This suggests that the damage of the cell membrane is a relatively insensitive indicator for cytotoxicity. Therefore, in the current study, results derived from MTT or WST-1 assays were used as the basis for determining the non-toxic concentration ranges in consecutive assays.

Pb^{2+} can bind to sulfhydryl (-SH) groups, which is one of the most common functional groups of many biological molecules, including the major cellular antioxidant glutathione (GSH). In this way, Pb^{2+} exposure may result in elevated ROS levels (Matovic et al. 2015). The results showed that in HD-11 macrophages non-cytotoxic concentrations of Pb^{2+} depleted intracellular GSH when the *de novo* synthesis of GSH was blocked by BSO (Figure 5.2a), but the induction of ROS was very limited (Figure 5.3). In DT40 B-cells, no effects on either GSH (Figure 5.2b) or ROS levels were noticed at non-cytotoxic concentrations of $Pb2^{+}$ even with BSO. The differences between B-cells and macrophages could be related to the nature of these two cell types. As macrophages are functionalized with inducible ROS and nitric oxide to deal with pathogens (so-called oxidative burst), they are equipped with a stronger protective anti-oxidant system for buffering oxidative stress (Brune et al. 2013). These results indicate that cells in the experiments can compensate Pb^{2+}-induced GSH depletion to prevent further damage through the induction of γ-glutamylcysteine synthetase. However, some *in vivo* studies indicated that Pb^{2+} exposure decreased GSH levels, induced oxidative stress and resulted in lipid peroxidation and DNA damages (Dai et al. 2012; Vallverdu-Coll et al. 2015b), which might be related to the prolonged *in vivo* exposure or the interaction with other elements in the organism *in vivo*.

Apart from cytotoxic effects, the results also indicated the modulatory effects of Pb^{2+} on some functional parameters in both cell lines at sub-cytotoxic concentrations. For instance, Pb^{2+} enhanced nitric oxide production in both non-activated and activated HD-11 macrophages in a concentration-dependent manner (Figure 5.4). This could be related to the up-regulation of inducible nitric oxide synthase (iNOS) by Pb^{2+} exposure (Chiang et al. 2014; Huang et al. 2019; Liu et al. 2012). However, when GSH synthesis was blocked by BSO, affecting the cells' ability to protect itself to the nitric oxide related oxidative burst, the cells produced less nitric oxide than the ones without BSO, hence it seems that the cell needs the protective antioxidants before it can mount an oxidative burst. On the other hand, the decreased synthesis of GSH may affect the uncoupling of the nitric oxide synthase enzyme, resulting in decreased nitric oxide synthase activity and reduced nitric oxide production (Kasten-Jolly and Lawrence 2014). However, contrasting effects of lead on nitric oxide production have been reported without clearly recognized mechanisms (Sharifi et al. 2005; Tian and Lawrence 1995). Induction of nitric oxide was also noticed in Cd^{2+} exposed mouse macrophage RAW264.7 cell line at non-cytotoxic concentrations, which was also associated with a depletion of GSH (Garcia-Mendoza et al. 2019). Cd^{2+} and Pb^{2+} are both non-redox

active divalent metals (Matovic et al. 2015) with high affinity to sulfhydryl (-SH) groups and are known to share similar modes of action with respect to the redox status of cells. The impact of *de novo* GSH synthesis as shown in the current experiments may indicate that the impact of Pb^{2+} on nitric oxide production could be depending on the antioxidant status of the cells. Nitric oxide has also been identified to have more functions in the immune system besides killing of microbial pathogens, such as mediating cytokines and inducing differentiation of subpopulations of T cells (Bogdan 2001; Murata et al. 2002). Hence, although the Pb^{2+}-induced nitric oxide production is rather limited compared to the induction of poly I:C, it could still have further implications on other downstream immune functions, which need to be further investigated *in vivo*.

$Pb2^{+}$ was found to alter the proliferation of both cell lines (Figure 5.5). Previous studies also highlighted similar dose-dependent proliferative effects of Pb^{2+} on primary avian (Grasman and Scanlon 1995) and murine (Razani-Boroujerdi et al. 1999) lymphocytes *in vitro* and *in vivo*. HD-11 cells tended to increase metabolic activity instead of synthesizing new DNA upon viral challenge, while DT40 cells did not follow this strategy, suggesting cell type-specific responses. These results indicate that $Pb2^{+}$-enhanced proliferation at non-cytotoxic concentrations may affect the composition of immune cell populations in avian species.

More specific functional endpoints included in the current study were the gene expression of TLRs and cytokines (Figure 5.6). Activation of TLR3 with poly I:C (mimicking viral infection) triggers multiple pathways including nuclear factor κB (NF-κB) pathway and interferon receptor factors (IRFs) pathways, which may lead to the production of series of cytokines (Abbas et al. 2014). For instance, expression of inflammatory cytokines including TNF-α and IL-8 could be induced through NF-κB pathway, and expression of type I interferons (IFN-α and -β) could be induced through IRFs pathways (Akira et al. 2006). According to the results, the only significantly upregulated gene after 24h Pb^{2+} exposure was IL-8 in activated macrophages, a pro-inflammatory chemokine recruiting heterophils (similar to neutrophils in mammals) in birds (Kogut 2002). Stimulation of IL-8 gene expression was shown to be exhibited in human peripheral blood mononuclear cells (PBMC) treated with $PbAc_2$ above 0.01mM (Gillis et al. 2012). Other studies suggested this up-regulation could be closely related to nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which also induces ROS at the same time (Dobrakowski et al. 2016; Metryka et al. 2018).

Compared to regulations of gene expression, effects on protein levels are more closely related to potential adverse outcomes at the individual level. For anti-viral cytokine IFN-α, Pb^{2+} increased its secretion in both cell lines without significant influences of poly I:C activation (Figure 5.7a, 5.8a). Although poly I:C is known to be able to trigger type I IFN production (Matsumoto and Seya 2008), the poly I:C-induced IFN-α production could be modified by many factors such as activation time as mentioned before and the characteristics of the cell line. Given that both DT40 and HD-11 cell lines are transformed to cancer cell lines by virus (Beug et al. 1979; Winding and Berchtold 2001), they may be less sensitive than primary

cells to a viral infection mimic as poly I:C, in terms of type I IFN secretion (Dauletbaev et al. 2015). Together with the slight although insignificant up-regulation of IFN-α and -β shown in qPCR results, the results indicate that Pb^{2+} could increase the production of anti-viral type I IFNs. Secreted type I IFNs can lead the neighboring cells to an "anti-viral state" as a defense response to viral infections (Kumar et al. 2011). However, prolonged production of type I IFNs could inhibit inflammation by decreasing the production of IL-1 (Guarda et al. 2011), and at the same time enhance anti-inflammatory responses by induction of Th2 cytokines like IL-10 (Ivashkiv and Donlin 2014), potentially leading to disorder of the immune system. Furthermore, Pb^{2+} inhibited IFN-γ secretion in macrophages (Figure 5.7b), similar to previous studies (Dietert and Piepenbrink 2006; Heo et al. 2007; Valentino et al. 2007). This effect has been reported to be post-transcriptional, changing the protein levels of IFN-γ without influences on gene expression, probably through selectively interfering with the translation of mRNAs or other biological processes (Guo et al. 1996; Heo et al. 2007; Metryka et al. 2018). Notably, the inhibitory effects of Pb^{2+} on IFN-γ production were more severe when stimulated with poly I:C, suggesting cells to be less capable of IFN-γ-induced inflammatory responses upon viral infections. As for DT40 B-cells, significantly increased IgM levels were found in the cell lysate of non-activated cells after exposed to non-cytotoxic concentrations of Pb^{2+}, but not in the activated ones. In addition to secreted antibody, IgM also acts as B-cell receptors (BCRs) on the cell membrane of DT40 cells recognizing antigens (Gao et al. 2002; Luo et al. 2010), which is the major component of IgM tested in the cell lysates. Hence, Pb^{2+} appeared to stimulate B-cells by increasing intracellular IgM levels and thereby potentially facilitating more surface BCRs, which might boost B-cell functions and humoral mediated Th2 response.

In summary, results from the present study indicated that Pb^{2+} showed concentration-dependent toxic effects on both chicken B-lymphocyte and macrophage cell lines, with macrophages being more sensitive. Pb^{2+} exposure could also affect the inflammatory status by inhibiting the pro-inflammatory IFN-γ and promoting anti-inflammatory type I IFNs, and stimulating B-cells. The immunomodulatory effects of Pb^{2+} could likely result in disorder of immune systems and inappropriate immune responses to viral pathogens, which also have been proved for bacterial and parasitic pathogens by *in vivo* studies (Knowles and Donaldson 1997; Vallverdu-Coll et al. 2015b). Although only two cell types from the immune system were studied *in vitro* without interaction between cell lines, these results provide a starting point for further mechanistic, long-term and systematic studies about the immunomodulatory effects of heavy metals in avian species upon viral challenges.

Acknowledgment

We thank Dr. Jurgen van Baal (Department of Animal sciences, Wageningen University, the Netherlands) for kindly providing us the HD-11 cells. China Scholarship Council is acknowledged for funding this study (Grant No. 201707720036).

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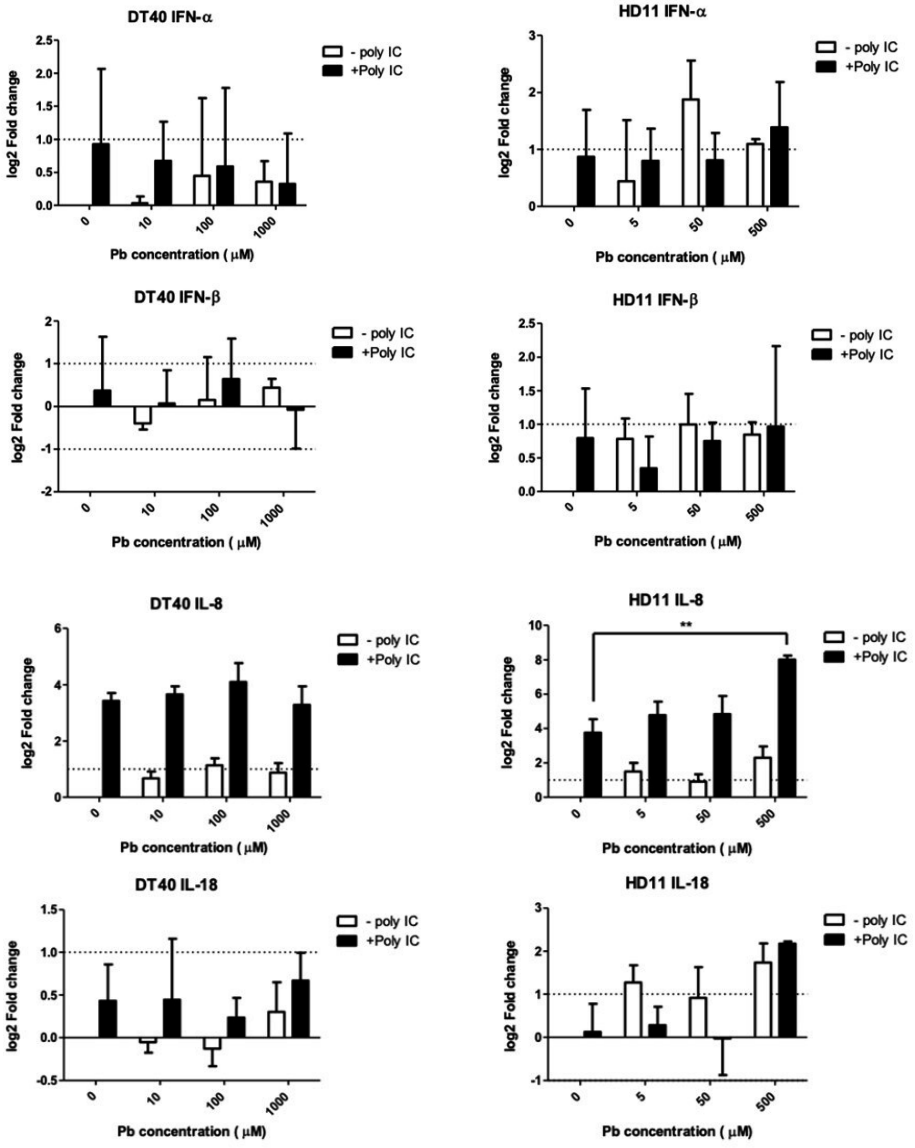
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Supplementary materials



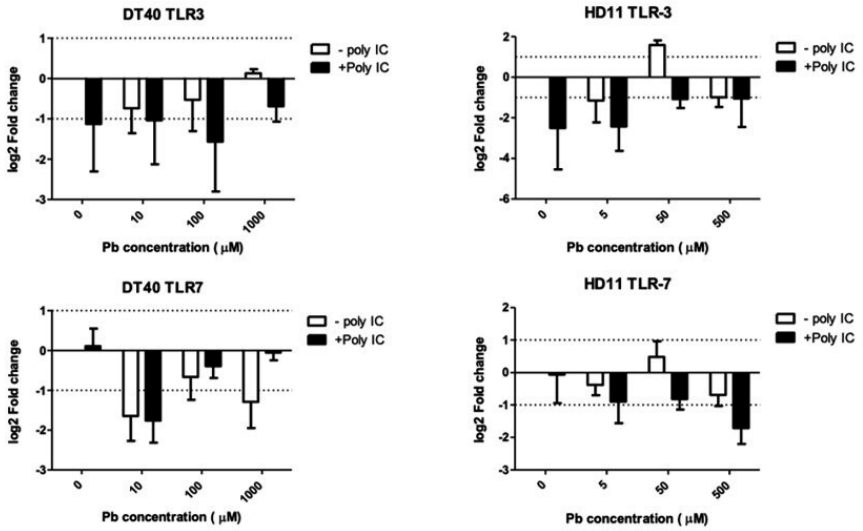


Figure S5.1. Effects of Pb exposure on expression of immune functional genes in DT40 and HD-11 cells with and without poly I:C activation for 24 hours. Results (mean ± SEM, n=3) are normalized with the housekeeping gene actin-β and presented as log2 fold changes in comparison with the negative control of non-activated cells.

Chapter 6

General discussion

6.1. Overview of results

In the current thesis, a stepwise approach was used to investigate the effects and modes of action of Hg(II) and Pb(II) exposure on avian immune responses upon a viral-like immune challenge. Firstly, a case study of environmental Hg exposure at a historical mining area with Arctic Barnacle goslings (*Branta leucopsis*) (Chapter 2) pointed out that even at low environmentally relevant levels, environmental Hg exposure can impair immune competence by affecting B-cell functions and inducing inflammation upon poly I:C challenge. The field study provided highly relevant information for risk assessment. Then, we used both controlled *in vivo* experiments with Pekin ducklings (Chapter 3) and *in vitro* experiments with chicken immune cells lines (Chapter 4) to verify the findings from the field study and to investigate the cellular modes of action underlying the adverse effects. Chicken instead of waterfowl immune cells were used because they are the only available ones. Pb is also an immunotoxic trace metal potentially affecting the immune system of dabbling waterfowl, especially in historical hunting areas due to the use of Pb ammunition (Mateo et al., 2007). Therefore, we also performed *in vivo* (Chapter 3) and *in vitro* studies (Chapter 5) to investigate whether Pb(II) has similar modulatory effects on avian immune responses as Hg(II). Following this top-down approach, we were able to integrate the results from the field study, studies under controlled *in vivo* conditions and *in vitro* studies and build causality at individual and cellular levels.

In this chapter, the effects of Hg(II) and Pb(II) on oxidative stress are discussed, then the effects of Hg(II) exposure on the immune responses observed in the different experimental approaches are compared and discussed, and finally, the effects of Pb(II) exposure are compared with the effects of Hg(II). In the end, further considerations for future work and future perspectives are presented.

6.1.1. Oxidative stress

Divalent trace metals, including Hg(II) and Pb(II), have been reported to have a high affinity for the thiol group in GSH (Hultberg et al., 2001), oxidizing GSH to its oxidised form GSSG (Fig 1a), potentially disturbing the redox balance and inducing oxidative stress (Kobal et al., 2008; Ren et al., 2017; Salazar-Flores et al., 2019). Hepatic total Hg (tHg) levels in goslings significantly increased total GSH (GSHt, GSHt=GSH+GSHox) levels and almost significantly increased GSHox levels (p-value=0.082), resulting in a more or less stable GSHox% with the increase of internal tHg levels (Chapter 2). This demonstrated that goslings probably were able to compensate the Hg-induced GSH oxidation with the *de novo* synthesis of GSH as a protective strategy (Figure 6.1a). However, at higher tHg exposures, this may potentially exceed the capacity of the buffer system, depleting the levels of reduced GSH, resulting in oxidative stress. For instance, hepatic GSH levels showed a significant

decrease in surf scooter (*Melanitta perspicillata*) with hepatic tHg levels ranging from 10 to 30 mg/kg d.w. (Hoffman et al., 1998), a level much higher than the ones in the present study.

In vitro studies with chicken immune cell lines also highlighted the crucial protective role of *de novo* synthesis of GSH against trace metal exposure (Chapter 4, 5, Figure 6.1b). L-buthionine-sulfoximine (BSO) co-exposure was used to block the *de novo* synthesis of GSH via inhibition of γ-glutamylcysteine synthetase (Drew & Miners, 1984). In both chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines, this block of *de novo* GSH synthesis upon Hg(II) (Chapter 4) or Pb(II) (Chapter 5) exposure resulted in depletion of cellular GSH, induction of ROS, and loss of cell viability at much lower concentrations than exposure to trace metal ions alone.

Therefore, *de novo* synthesis of GSH appears to protect organisms and cells from trace metal-induced oxidative stress both *in vivo* and *in vitro*. However, in case trace metal exposure exceeds the capacity of this buffer system, oxidative stress may occur and may cause adverse effects such as DNA damage and lipid peroxidation (Bandyopadhyay et al., 1999). The environmental Hg exposure in the gosling study (Chapter 2) was relatively low, so the goslings were able to cope with the Hg-induced GSH depletion and maintain redox balance with *de novo* GSH synthesis. Specific ROS may act as an essential messenger in both innate and adaptive immune systems, and are involved in multiple immune processes such as inflammation and lymphocyte activation (Schieber & Chandel, 2014). Thus, the potential disturbance of redox balance by high exposures to trace metals may not only cause tissue damage, but may also impair the immune responses.

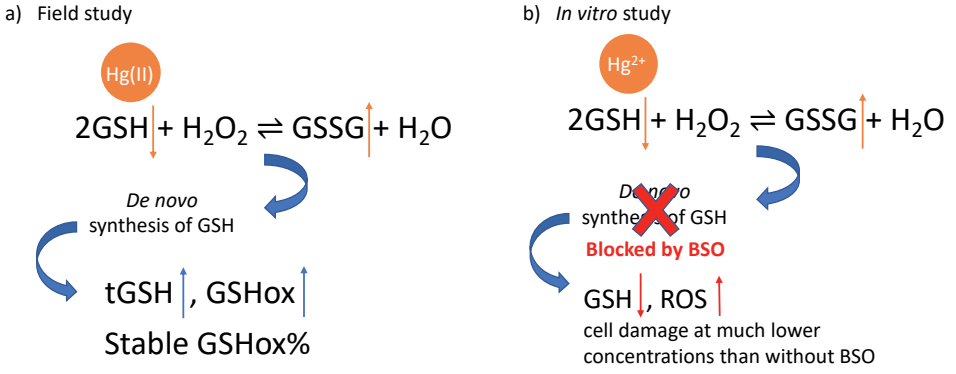


Figure 6.1. Illustration of Hg-induced GSH depletion and protective effects of *de novo* GSH thesis in the field study (Chapter 2) and *in vitro* (Chapter 4).

6.1.2. Effects of Hg(II)

The results on the effects of Hg(II) exposure in the field study, and the controlled *in vivo* and *in vitro* experiments are listed in Table 6.1. The major effects appeared to include promoting inflammation and impairing B-cell functions. The results from the controlled *in vivo* study with Pekin ducklings showed high similarity to the result obtained in the field study with Barnacle goslings, while the *in vitro* experiments indicated no effects on inflammation but did reveal increased levels of IFN-α secretion. This difference may probably be ascribed to the fact that the *in vitro* studies only involved a single cell type while the field and controlled *in vivo* studies involved the whole immune system and organism.

Table 6.1. Overview of effects of Hg(II) exposure on immune endpoints related to inflammation and B-cell function in a field study with goslings, a controlled *in vivo* study with ducklings and *in vitro* studies with both chicken macrophage and B-lymphocyte cell lines (please note: Hg-species in the field study were characterised as total Hg (tHg)).

Endpoints		Hg(II)			
		Field study goslings	*in vivo* ducklings	*in vitro* (macrophages)	*in vitro* (B-lymphocytes)
Inflammation	Nitric oxide	too short challenge		-	n.a.
	iNOS expression	+	+	n.a.	n.a.
	IL-8 expression	no effect	+	no effect	no effect
	IL-18 expression	+	no effect	no effect	no effect
	TLR3 expression	no effect	+	no effect	no effect
	TLR7 expression	no effect	+	no effect	no effect
	Heterophils%	+	no effect	n.a.	n.a.
B-cell functions	Natural antibody	-	no effect	n.a.	n.a.
	miR-155 expression	n.a.	-	no effect	-
	Lymphocytes%	no effect	no effect	n.a.	n.a.
	B-cell proliferation	n.a.	n.a.	n.a.	-
	Cellular IgM	n.a.	n.a.	n.a.	-
Anti-viral cytokine	IFN-α expression	no effect	no effect	no effect	no effect
	IFN-α secretion	n.a.	n.a.	+	+

n.a.: not applicable (endpoints not measured or not relevant to the functions). "+" with orange colour indicates positive effects/increase of endpoints, while "-" with green colour indicates negative effects/decrease of endpoints.

6.1.2.1. Inflammation

In both the field study and the controlled *in vivo* study, Hg(II) exposure induced the expression of pro-inflammatory genes such as iNOS, IL-8, IL-18 and TLRs. However, such pro-inflammatory effects were not noticed in the *in vitro* studies (Table 6.1). This indicates the *in vitro* cell lines used may not be suitable to study this endpoint.

Environmental tHg exposure upregulated the gene expression of iNOS and IL18 in spleen which may result in inflammation (Chapter 2). As both iNOS and IL18 are pro-inflammatory genes (Dinarello, 1999; Wood et al., 2005; X. Zhang et al., 2009), the upregulation suggests a potential Hg-induced inflammation upon immune challenge. In addition, the increased ΔHeterophils (%) with higher internal Hg concentrations also indicated a possible pro-inflammatory effect of Hg exposure (Maxwell & Robertson, 1998). The induced inflammation might result in disorders in immunity such as autoimmunity, in which the immune system might attack own healthy cells (Nagy et al., 2007). In addition, the Hg(II)-increased inflammation could be costly for birds, especially for migratory birds such as Barnacle goose who need energy for migration (Eikenaar et al., 2020). Barnacle geese in the high Arctic are known to invest less energy in their immune systems than Barnacle geese breeding in the Netherlands (Sandström, 2017). This lower immune activity is possible because of the lower pathogen pressure in the high Arctic, which is beneficial for the birds migrating to Svalbard (Gilg & Yoccoz, 2010). Thus, migrating Barnacle geese in Svalbard may also invest less in their immune system, thereby saving energy for the migration. However, the Hg-increased inflammation might impair this strategy, potentially resulting in lower energy supply during migration which would influence their overall fitness and survival rate during migration.

Similar to the results from the field study, Hg (II) showed modulatory effects on the immune responses in the controlled *in vivo* studies with Pekin ducklings upon a viral-like challenge mainly by promoting inflammation and impairing B-cell function (Chapter 3, Table 6.1). For instance, Hg (II) exposure upregulated the expression of pro-inflammatory genes such as iNOS, IL8, TLR3 and TLR7 in the spleen of Pekin ducklings (Chapter 3). IL-8 is a pro-inflammatory chemokine that can recruit heterophils to infected sites in avian species (Kogut, 2002). TLR3 and TLR7 are both endosomal toll-like receptors (TLRs) recognizing viral pathogen patterns, namely dsRNA and ssRNA, respectively (Trivedi & Greidinger, 2009). The downstream pathways of TLR3 and TLR7, including p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor-kappa (NF-κB) signalling pathways, have been shown to be activated in the liver of voles exposed to inorganic Hg(II) exposure, resulting in prolonged inflammation and liver damage (Assefa et al., 2012). Therefore, the upregulated expression of iNOS, IL8 and TLRs demonstrates that exposure to inorganic Hg(II), could also increase inflammation in Pekin ducklings.

Nitric oxide production is an endpoint that was tested throughout this thesis, from the field study with goslings (Chapter 2) to the controlled *in vivo* study with Pekin ducklings (Chapter

3) and the *in vitro* studies with chicken immune cell lines (Chapter 4, 5). Nitric oxide can be produced by macrophages and heterophils upon immune challenges to kill or inhibit replication of pathogens (Bogdan, 2001). In the field study, plasma nitric oxide levels in goslings were not affected by tHg exposure, but were strongly related to the duration between dsRNA challenge and termination of the experiment (ranging from 22 to 30 hours). This time-dependent effect indicated that challenge time probably should have been longer to ensure a full induction of nitric oxide production. This could also explain the reason why only baseline levels of nitric oxide were detected in the plasma of ducklings 24 hours post dsRNA immune challenge (Chapter 3). In an earlier study, Pekin ducks only showed significantly higher levels of nitric oxide in serum after 72h post-infection with H5N1 avian influenza virus, while chickens already produced significantly higher nitric oxide 24h post-infection (Burggraaf et al., 2011). Hence, waterfowl species, such as Barnacle geese and Pekin ducks, probably need a longer challenge time than chicken to build a proper nitric oxide response. Furthermore, 24 hours challenge appeared enough to strongly induce nitric oxide production in a chicken macrophage cell line (HD-11) *in vitro* (Chapter 4, 5). This successful induction of nitric oxide in chicken macrophages may be ascribed to the species, but may also be related to the direct and faster immune stimulation *in vitro* than *in vivo*.

Inducible nitric oxide synthase (iNOS) is the major enzyme producing nitric oxide upon immune activation (Burggraaf et al., 2011). Hg exposure upregulated the gene expression of iNOS in both goslings (tHg, Chapter 2) and ducklings (Hg(II), Chapter 3) (Table 6.1). The gene expression of iNOS is at the transcriptional level, which is a relatively early stage indicator, while the nitric oxide levels measured either in plasma (Chapter 2, 3) or cell culture supernatant (Chapter 4, 5) is a later stage indicator, downstream of iNOS. As both immune activation and gene expression are dynamic processes, more time points would probably provide more information on the time-dependent effects of trace metal exposure on immune responses, including nitric oxide production. Nevertheless, it can be concluded that exposure to Hg results in an increased iNOS response.

No indicators of pro-inflammatory effects were found after exposure to Hg(II) *in vitro* in chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines (Chapter 4, Table 6.1). On the contrary to the upregulated iNOS gene expression in goslings and ducklings *in vivo*, nitric oxide production was found to be strongly inhibited by Hg(II) in activated HD-11 macrophages (Chapter 4), as also reported in murine macrophages (Batista-Duharte et al., 2018; Kim et al., 2002; Tian & Lawrence, 1996). Downregulation of iNOS in cells of the J774A.1 murine macrophage cell line by Hg(II) was demonstrated in both gene expression profiles as well as at the protein level (Kim et al., 2002). In the field and controlled *in vivo* studies, the up-regulation of iNOS expression was found in spleen tissue, and the nitric oxide levels in plasma stayed at the baseline levels, probably due to too short challenge time (Chapter 2, 3). The spleen tissue contains multiple types of immune cells, so as in the blood. Apart from macrophages, heterophils can also produce nitric oxide upon immune challenges and are the most abundant immune cell type in blood circulation (Jones, 2015). Therefore,

the differences in nitric oxide production between the *in vivo* and *in vitro* studies in this thesis may be related to the different cell types involved. Future studies on the effects of Hg(II) exposure on heterophils may provide more information about cell-specific effects.

6.1.2.2. B-cell functions

Natural antibody responses upon challenge in gosling plasma (field study) significantly decreased due to the tHg exposure, suggesting impaired B-cell function and weaker defence against infections (Chapter 2). Natural antibodies produced by B-cells are important for the constitutive innate immunity, providing the rapid non-specific defence to antigens (Matson et al., 2005). In a previous study, natural antibody activity showed a decrease in goslings herded in the mining site only after social isolation, which acted as an acute stressor (de Jong et al., 2017). The different effects on the natural antibody levels between the previous study (de Jong et al., 2017; van den Brink et al., 2018) and the field study of the present thesis are probably related to the immune challenge, and the wider range of internal hepatic tHg concentrations due to the additional extra Hg group and longer overnight stays in the contaminated area. Higher natural antibody levels were related with higher survival rates in laying hens (Sun et al., 2011), and were reported to protect mice from viral and bacterial infections by suppressing pathogen replication and enhancing pathogen elimination in lymphoid organs (Ochsenbein et al., 1999). Therefore, the significantly lower natural antibody levels due to tHg exposure in the present study suggested an impaired constitutive innate humoral immunity and defence to pathogens, which might lead to a higher risk of infections.

Strong inhibition of miR-155 expression in the spleen of ducklings was observed when hepatic tHg concentrations exceeded 1 mg/kg d.w. (Chapter 3). Downregulation of miR-155 was also noticed in Hg(II) exposed cells of the chicken B-cell line *in vitro* upon challenge (Chapter 4). MiR-155 is a multifunctional micro-RNA regulating multiple immune responses, including promoting lymphocyte proliferation and differentiation, regulating macrophage polarization, and boosting antibody production (Alivernini et al., 2018; Bushati & Cohen, 2007; Fabian et al., 2010). Decreased miR-155 levels could inhibit B-cell proliferation, which was also shown in the *in vitro* experiment (Chapter 4) as well as in mouse models *in vivo* (Babar et al., 2012; Rodriguez et al., 2007). Thus, the downregulated miR-155 levels by Hg(II) exposure suggest potentially impaired B-cell function (Babar et al., 2012; Mehta & Baltimore, 2016; Rodriguez et al., 2007; Waugh et al., 2018). Cellular IgM, mainly IgM on the cell membrane as B-cell receptors (BCRs) recognizing antigens and initiating humoral immunity (Friess et al., 2018), showed a significant decrease upon Hg(II) exposure in non-activated DT40 B-lymphocytes, however, not in activated cells (Chapter 4).

Taking together the decreased natural antibody levels, miR-155 expression, and cellular IgM levels, Hg(II) exposure appeared to impair B-cell functions, which could result in a weaker humoral immunity.

6.1.2.3. Anti-viral cytokine IFN-α

IFN-α is the major anti-viral interferon. The effects of Hg(II) exposure on IFN-α gene expression were measured in all experiments, however, no significant effects were found (Table 6.1). The production of anti-viral cytokine IFN-α was only measured with species-specific chicken ELISA kits *in vitro,* due to the lack of available ELISA kits for ducks and geese. Secretion of IFN-α by both chicken immune cell lines was significantly increased upon Hg(II) exposure irrespective of immune challenge (Chapter 4). The lack of effects on gene expression may be related to the timing of the measurement, and/or may be due to the different biological levels of gene expression and protein production. Type I IFNs secreted by immune cells can pass the signal to the neighbouring cells that will develop an antiviral state to limit the replication of virus and defend the host cells against the infection (Abbas et al., 2015). However, excessive type I IFNs may induce autoimmunity *in vivo* (Baccala et al., 2007). Hence, the Hg(II) induced type I IFNs production in the *in vitro* cell models might point at potential immunomodulatory effects.

Although the overall effects of Hg(II) exposure turned out to be promoting inflammation and impairing B-cell functions, effects on specific endpoints differed. Lower natural antibody levels were only detected in environmentally exposed goslings (Chapter 2) but not in Hg(II)-exposed ducklings under controlled conditions (Chapter 3), and upregulation of pro-inflammatory genes was only detected *in vivo* in both goslings and ducklings (Chapter 2, 3) but not *in vitro* (Chapter 4). These differences may result from species differences, differences in biological systems (cell versus organism), or the timing of measurements. As mentioned above, barnacle goslings may suppress their immune system in the high Arctic to save energy for the migration (Sandström, 2017). Nevertheless, the duck strain we used has probably been selected for generations in order to get optimal meat production by growing fast (House et al., 2021) and maybe also for lower sensitivity to infection. The potentially different sensitivity to infections or immune challenges between wild goslings and Pekin ducklings might be relevant to the different effects found in the field and the controlled *in vivo* experiment. In addition, the immune response is a highly dynamic process, and some processes are probably more direct and faster in the *in vitro* model systems.

6.1.3. Pb(II) vs. Hg(II)

To investigate whether other divalent trace metals have similar immunotoxic effects as Hg(II), Pb(II) was included in the controlled *in vivo* study with ducklings (Chapter 3) and with chicken immune cell lines *in vitro* (Chapter 5). Pb is an environmental pollutant, potentially threatening the wellbeing of wild birds, especially dabbling waterfowl, due to the historical use of Pb ammunition for hunting in wetlands (De Francisco et al., 2003). The controlled *in vivo* and *in vitro* experiments allowed comparison between the effects of Hg(II) and Pb(II). Table 6.2 compares the effects of Pb(II) exposure with the effects we found in Hg(II) exposure experiments.

Similar to Hg(II), Pb(II) also showed general pro-inflammatory effects and impairment of B-cell functions both *in vivo* and *in vitro*. Pb upregulated the expression of some pro-inflammatory genes, such as the expression of TLR3 in spleen of ducklings *in vivo* and IL-8 in challenged cells of the HD-11 chicken macrophage cell line *in vitro* (Table 6.2). Although the upregulated genes were not exactly the same in each experiment, both of them promote inflammation, and significant upregulation of these two genes was also found in Hg(II) exposed ducklings (Chapter 3). As for the effects on B-lymphocyte functions, Pb(II) exposure decreased the natural antibody levels in the plasma of ducklings and inhibited the proliferation of cells of the chicken B-lymphocyte (DT40) cell line *in vitro*. Besides, Pb(II) exposure also increased the secretion of IFN-α in both cell lines without affecting the corresponding gene expression, which was in line with the effects found in Hg(II) exposed cells. Furthermore, *de novo* synthesis of GSH played important protective roles in both Pb(II) and Hg(II) exposed cells *in vitro*, suggesting GSH depletion is one of the major biological processes related to trace metal exposure. Pb(II) also showed some effects different from Hg(II). For instance, in the controlled *in vivo* experiments, Hg(II) exposure mainly affected the immune gene expression, including the upregulation of pro-inflammatory genes IL-8, iNOS, TLR3, and TLR7, suggesting a Hg(II)-induced inflammatory effect, while Pb(II) mainly reduced the natural antibody levels, the leukocyte density and heterophils, which might also lead to weaker immunocompetence, albeit via different pathways (Chapter 3). As the gene expression is a rather early stage effect while the natural antibodies and immune cell population changes are more downstream, the different effects between Pb(II) and Hg(II) may alternatively be ascribed to ion specific differences in the timing of the relevant pathways, which calls for measurements at more time points.

Besides, according to the results from the *in vitro* experiments, Hg(II) had remarkably higher ($p<0.05$) cytotoxicity than Pb(II) in both cell lines, with significantly lower EC50s (Table 6.2). In addition, the EC50 values for these two cell lines were comparable after Hg(II) exposure. However, HD-11 cells were more sensitive to Pb(II) exposure with an EC50 value three times lower than DT40 cells (Table 6.3), pointing at potentially different modes of action underlying the cytotoxicity of Hg(II) and Pb(II).

As mentioned before, Pb(II) enhanced the nitric oxide production by cells from the chicken macrophage (HD-11) cell line, which was different from Hg(II). Increased nitric oxide levels were also found in Cd(II) exposed cells from the RAW264.7 murine macrophage cell line (García-Mendoza et al., 2019) upon activation, suggesting possible deviating mechanisms among divalent trace metal ions.

Table 6.2. Comparison of the Pb(II)-induced effects with the Hg(II)-induced effects on immune endpoints related to inflammation and B-cell function in the field study with goslings, a controlled *in vivo* study with ducklings and *in vitro* studies with both chicken macrophage and B-lymphocyte cell lines (please note: Hg-species in the field study were characterised as total Hg (tHg)). The left part of the form for Hg(II)-induced effects is the same with Table 6.1.

Endpoints		Hg				Pb		
		Field study goalings	*in vivo* ducklings	*in vitro* (macrophages)	*in vitro* (B-lymphocytes)	*in vivo* ducklings	*in vitro* (macrophages)	*in vitro* (B-lymphocytes)
inflammtion	Nitric oxide	too short challenge		-	NA	too short challenge	+	NA
	iNOS expression	+	+	NA	NA	no effect	NA	NA
	IL8 expression	no effect	+	no effect	no effect	no effect	+	no effect
	IL18 expression	+	no effect	no effect	no effect	no effect	no effect	no effect
	TLR3 expression	no effect	+	no effect	no effect	+	no effect	no effect
	TLR7 expression	no effect	+	no effect	no effect	no effect	no effect	no effect
B-cell function	Natural antibody	-	no effect	NA	NA	-	NA	NA
	miR15 5expression	no effect	--	no effect	-	no effect	NA	NA
	Lymphocytes%	no effect	no effect	NA	NA	no effect	NA	NA
	Proliferation	NA	NA	-	-	NA	-	-
	Cellular IgM	NA	NA	NA	+	NA	NA	+

n.a.: not applicable (endpoints not measured or not relevant to the functions). "+" with orange colour indicates positive effects/increase of endpoints, while "-" with green colour indicates negative effects/decrease of endpoints.

Moreover, significantly increased cellular IgM levels were found in the cell lysate of non-activated DT40 B-cells after exposure to non-cytotoxic concentrations of Pb(II), unlike the inhibiting effects of Hg(II) on the cellular IgM production by non-activated cells (Chapter 5). The increased intracellular IgM levels upon Pb(II) exposure may facilitate more surface BCRs, which might boost the pathogen recognition by B-lymphocytes. These differences suggested that apart from the common pathways, Hg(II) and Pb(II) may also have some different modes of action underlying their immunotoxicity.

To sum up, Pb(II) also showed modulatory effects on both ducklings *in vivo* and on chicken immune cell lines *in vitro* mainly by inducing inflammation and disturbing B-cell functions. In the controlled *in vivo* experiments, different from Hg(II), Pb(II) showed modulatory effects on more later stage endpoints, causing a decrease of natural antibodies and a change in immune cell composition. In the *in vitro* study, Pb(II) showed lower toxicity than Hg(II), and some opposite effects were also noticed, such as increased nitric production and cellular IgM levels upon Pb(II) exposure but not upon Hg(II) exposure, suggesting that there might be, at least to some extent, different modes of action underlying Pb(II) and Hg(II) induced immunotoxicity.

Table 6.3. Comparison of EC50s (95% confidence interval, 95% CI) for the cytotoxicity of Hg(II) and Pb(II) to chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines.

		Hg(II)	Pb(II)
Cytotoxicity	HD-11	29 µM (24.08-34.11)	480 µM (340-680)
	DT40	30 µM (28.28-31.35)	1700 µM (1200-2400)

6.2. Future perspectives

The results obtained in this thesis show that the exposure to divalent trace metals including Hg(II) and Pb(II) could result in modulatory effects on the immune responses upon a viral-like challenge, even at low environmentally relevant levels of exposure. Currently, the risk assessment for birds in the European regulatory system mostly focuses on lethal and reproductive effects (EFSA, 2009). The results of the present thesis indicate that birds could be vulnerable to immunotoxicity induced by environmental exposure to heavy metal pollutants and that immunotoxicity may be a relevant endpoint to be taken into account in the risk assessment for birds. As illustrated in the present thesis, a stepwise approach provides the possibility to link findings from field studies with results from controlled *in vivo* and *in vitro* studies in avian species, thus evaluating the use of *in vitro* models as an alternative to animal experiments. Based on the results from the present thesis, some further considerations and future perspectives can be presented, especially on the following topics: use of pathogen-specific immune responses as an endpoint in immunotoxicity studies; development of *in vitro* models for avian species; development of additional advanced assays; application of PBK

modelling to facilitate quantitative *in vitro* to *in vivo* extrapolation (QIVIVE), and environmental risk assessment. More details on these topics are presented in the following sections.

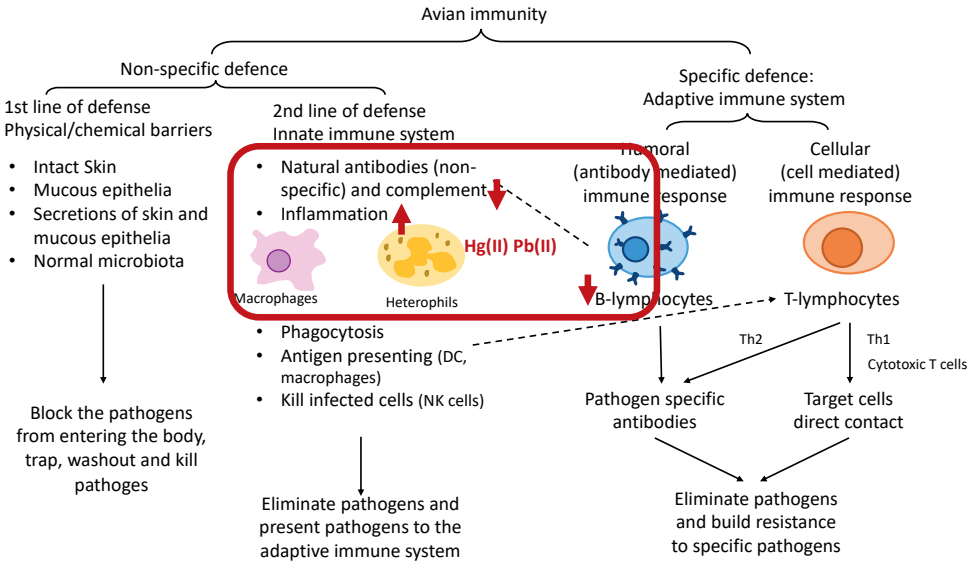


Figure 6.2. Overview of the avian immune system and the effects of Hg(II) and Pb(II) on the immune functions. The red square shows the immune functions included in the current thesis. The red arrows show the decreased or increased functions by Hg(II) and Pb(II) exposure. Adapted from Vallverdú-Coll et al. (2019) and Turvey & Broide (2010). DC, dendritic cell; Th, T-helper. Dotted arrows show the interaction between the innate and adaptive immune systems.

6.2.1. Use of pathogen-specific immune responses as endpoints

Given the complexity of the immune system and the method of immune challenge, in this thesis, the focus was on the non-specific immune responses upon a viral-like immune challenge with double-stranded (dsRNA) via TLR3 (Figure 6.2). In the current thesis, synthetic dsRNA (poly I:C) was used to achieve the viral-like challenge, and rabbit red blood cells as a non-specific foreign antigen to assess the natural antibodies. The specific immunity, such as pathogen-specific antibody production and T-cell mediated immune responses (Figure 6.2), are not included in the studies in this thesis and provide an interesting endpoint for future studies.

As an intermediate product during virus replication, dsRNA can be recognized by TLR3 and trigger both innate and adaptive immune responses via the same pathways used by some viral infections (Jin et al., 2010). However, in addition to TLR3 also other receptors are involved in the recognition of viruses. For example, TLR1/2/4 can recognize some viral proteins while retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) and TLR7/9 can recognize viral nucleic acids (Bowie & Unterholzner, 2008). Therefore, infection with an actual virus instead

of dsRNA, as in the current study, probably triggers more pathways than just the TLR3 pathway, and the application of specific pathogens likely triggers a more comprehensive immune response.

In addition, immune challenges with real pathogens such as specific viruses, bacteria or foreign red blood cells can provide more insights into the effects of trace metal exposure on the specific humoral immunity to the relevant pathogens. For example, environmental exposure to total cadmium (Cd) impaired the primary humoral response against sheep red blood cells (SRBC) in wood mice after an immune challenge with SRBC once for five days, showing less total IgG in plasma and a lower number of SRBC specific antigen producing cells in the spleen. (García-Mendoza et al., 2021). Due to the variable features of pathogens (e.g. highly pathogenic avian influenza vs. low pathogenic avian influenza), the consequences of the trace metal exposure might be different, and the overall effects might also be different, suggesting that exposure to contaminants might result in differential effects on the sensitivity to specific pathogens. Effects of trace metal exposure on immunological memory could also be assessed by measuring the indicators for secondary immune responses such as antibody production upon a second challenge with the same pathogen.

In the case of avian influenza, infection with avian influenza virus as the challenge to study the immunotoxic effects of environmental contaminants in the controlled *in vivo* study would provide information about the effects of the environmental contaminants on the avian immune competence against avian influenza. For the field study with wild birds, collection of information on the actual exposure to also other relevant environmental contaminants, as well as on immune indicators and infections from certain populations, will provide further insight into the relationships between exposure, immunotoxic effects and the prevalence of the infections, and thus provide important topics for future work. For instance, one could compare the immune status and infections of the bird population living in a trace metal contaminated area with a control population living in a clean area. Site-specific effects on immune responses were noticed in wild wood mice environmentally exposed to Cd, and differences between effects at different sites were linked to site-specific antigen profiles (García-Mendoza et al., 2021). Thus, the local pathogenic infections should also be considered in field studies, and this may require site-specific assessments. Such studies would be more complicated for migratory birds, because of the multiple stopovers along the long distance, where exposure and infections can happen, but highly relevant given the potentially reduced immune competence of these birds because of use of energy for other physiological functions during migration (Eikenaar et al., 2020). In such studies on migrating bird populations, there are much more factors potentially influencing their immunity that need to be considered than in the controlled *in vivo* studies, such as the migration route (S. Yin et al., 2021), season, site of exposure and exposure to other contaminants, so that the field study could provide important insights relevant for real-life situations.

6.2.2. Development of *in vitro* models for avian species

Compared to mammalian systems, there are only very few cell models for birds. In this thesis, two available chicken immune cell lines were used to investigate the effects of trace metal exposure, namely the chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines. However, the results of the present thesis suggested that these cell models may not be suitable to evaluate some endpoints relevant for immunotoxicity, such as inflammation. Based on the available literature, Hg(II) and Pb(II) were anticipated to modulate the development of mammalian CD4+ T-cell subsets (Y. Zhang & Lawrence, 2016). However, due to the lack of available avian T-cell models, the effects of Hg(II) and Pb(II) on T-cells were not included in the studies of the present thesis. Nevertheless, the results obtained in the two avian immune cell types already point out that the effects of the trace metals may be cell-specific, with different functional endpoints affected and different sensitivity in the different cell lines. A much wider and better-studied spectrum of mammalian immune cell lines is available, such as the HL-60 human neutrophil cell line (Blanter et al., 2021), the Jurkat human T-cell line (Abraham & Weiss, 2004), the D10.G1.4 mouse helper T-cell line (W. Yin et al., 2019) and the CW13.20-3B3 mouse B-cell line (Lin et al., 2000). The development of more avian cell lines will greatly boost the research in understanding the cellular mechanisms underlying the adverse effects of pollutants on specific avian immune cell types. The application of more *in vitro* models can also reduce the use of experimental animals.

Another option is to develop isolation protocols for primary immune cells from birds, which may be more representative and species-specific than the immortal cell lines. Most of the immortalized cell lines are cancer cell lines that might lose some of the functional characteristics of the original cells (Masters, 2000). For instance, HD-11 chicken macrophages were found not to produce more type I IFN upon infection with the highly pathogenic avian influenza virus H5N1, while both primary chicken splenocytes and *in vivo* (in chicken) studies showed significantly increased type I IFN production after infection with the same virus (Liniger et al., 2012). This is in line with the results that the dsRNA challenge did not trigger the IFN-α secretion from either of the chicken cell lines (Chapter 4, 5). The biggest challenges for using primary cells are the isolation of the single cell type, culture and exposure conditions and the relatively short lifespan of the cells.

6.2.3. Development of additional advanced assays

The application of specific assays, commonly used in mammalian immunology, may also boost the immunology tests for wild birds, such as ELISA, flow cytometry, and omics tools. These assays have been widely used in immune studies on mammals but not yet in avian species. An enzyme-linked immunosorbent assay (ELISA) can measure the protein content such as immunoglobins, cytokines and receptors in various biological samples such as cell culture supernatant, cell lysate and plasma. However, most ELISA assays are usually species-specific depending on the capture antibody (Garnier et al., 2017). In the current thesis, ELISA assays were only available for use in the *in vitro* experiments with chicken cell lines due to

the lack of available ELISA kits for ducks and geese. The development of ELISA assays for non-model species (e.g., avian species other than chicken) and more general ELISA assays interacting with specific protein across multiple species would be of great help for the studies in non-model animals, including wild birds. Apart from ELISA, there are also some other approaches quantitatively analyzing immune functional proteins, such as western blot, liquid chromatography-mass spectrometric (LC-MS) assays (Yuan et al., 2015), and antibody arrays (can simultaneously measure multiple proteins) (Kupcova Skalnikova et al., 2017), which may also be developed for use in future studies on avian species.

Flow cytometry has been used for immunophenotyping in mammals of cells according to their size, granularity, and cell surface receptors (e.g. CD3 for T-cells, CD4 for T-helper cells, and CD8 for cytotoxic T-cells) labelled with fluorescent probes (Adan et al., 2017). With specific fluorescent probes, flow cytometry can also measure intracellular cytokines and biological processes such as apoptosis and DNA damage (Adan et al., 2017). However, comparable to the antibodies used in ELISA assays, most fluorescent probes in flow cytometry are also highly species-specific antibodies (Huang et al., 2012). For the field study and the controlled *in vivo* study of the present thesis, no suitable fluorescent probes or flow cytometry methods were available for phenotyping immune cells in goslings and ducklings, and therefore blood smears were manually evaluated with microscopy to characterize the immune cell populations (Chapter 2, 3), which was time-consuming, potentially subjective and not able to distinguish B- and T-cells. Hence, if a flow cytometry method could be established to identify the immune cell types in avian species, more detailed information could be gained, especially for T-cell subsets.

Furthermore, the omics tools, including transcriptomics (with e.g., RNA-seq) and proteomics (with e.g., liquid chromatography (LC)/ gas chromatography (GC) coupled with quantitative mass spectrometric (MS) methods) can provide insights into the effects and related modes of action at the transcriptional and protein levels. However, the use of omics tools needs libraries for identifying certain genes or proteins. Integrated omics tools with both transcriptomics and proteomics have been applied in studying indicators of immune responses such as receptors and cytokines in fish (Natnan et al., 2021) and chicken (Xu et al., 2019), but not yet in non-model birds.

6.2.4. Application of PBK modelling to facilitate QIVIVE

Although the stepwise approach of the present thesis allowed the comparison of the effects from the field study, to the effects observed in the controlled *in vivo* and *in vitro* studies, this comparison could only be made in a qualitative way. This is because it is complicated to translate the effect concentrations in the *in vitro* studies to the *in vivo* dose levels. For example, the Hg(II) concentration inducing the significant miR-155 reduction in cells of the chicken B-cell line *in vitro*, which was 10 μM, can be roughly translated to an *in vivo* level by multiplying with the molecular weight of Hg (200.6 μg/μmol) and dividing by the tissue dry/wet ratio (0.27 kg d.w./L), resulting in an *in vivo* hepatic tHg concentration of 7.4 mg/kg

d.w. (=10 µmol/L × 200.6 µgram/µmol × 1/0.27 L/kg d.w.), which is around 7 times higher than the hepatic tHg level at which miR-155 expression was significantly inhibited in the ducklings from the controlled *in vivo* experiment. However, this back-of-the-envelope translation relates the hepatic tHg concentrations with the *in vitro* exposure for B-cells, while B-cells are mainly localized in the bursa instead of the liver and not all the Hg ions in the liver are in the free fraction and thus available for reaction with molecular targets.

More precise translation between *in vitro* concentrations and *in vivo* dose levels is possible on the basis of the use of physiologically based kinetic (PBK) modelling. A PBK model describes the body as composed of multiple physiological compartments, describing for each compartment with a differential equation the fate of the chemical of interest in that compartment. Once validated, the PBK model can either predict the concentrations of the chemical of interest in the compartment of interest upon exposure (forward dosimetry), or can be used to calculate the dose level that will result in a certain concentration in a certain compartment (reverse dosimetry) (Bartels et al., 2012). By relating the free fraction of the compound of interest in the culture medium from the *in vitro* assay with the free fraction of the compound of interest in the blood, this model may be used to convert *in vitro* concentration-response curves into *in vivo* dose-response curves, thereby providing an *in vitro* - *in silico* alternative to animal experiments in risk assessment (Louisse et al., 2017). To simulate the absorption, distribution, metabolism and excretion (ADME) of the compound of interest, three types of parameters are necessary as input for the PBK model, including physiological parameters (e.g., tissue volumes and tissue blood flows, which vary depending on the species), physicochemical parameters (e.g., blood/tissue partition coefficients, depending on the chemical) and kinetic parameters (e.g., kinetic constants for the metabolic reactions, depending on both the species and the chemical) (Rietjens et al., 2011). PBK models have been developed and used frequently for mammals, including mice, rats and human, but their application in studies on birds is still very limited (Cortright et al., 2009).

For most trace metals, metabolism is usually not considered due to the lack of biotransformation in animals (Dogruer et al., 2021; O'Flaherty, 1995). However, there are some exceptions. For instance, the demethylation of MeHg in liver and brain was included in a PBK model for human (Ruiz et al., 2010). Some PBK models also included the gut microbiota or parasites as compartments (Le et al., 2018; Mendez-Catala et al., 2020), which would probably be helpful for a correct prediction with PBK model based QIVIVE for immunotoxicity as the microbiota may be involved in the conversion of trace metals, and parasites may accumulate trace metals.

6.2.5. Environmental risk assessment

Besides the trace metal ions studied in the current thesis (Hg(II) and Pb(II)), many other compounds have also been reported to be immunotoxic, such as MeHg, poly- and perfluoroalkyl substances (PFAS), polycyclic aromatic hydrocarbons (PAH), and

formaldehyde (Neagu et al., 2021; Suzuki et al., 2020). So far, there are only murine models to evaluate the immunotoxicity of chemicals in the frameworks of regulations. For instance, a local lymph node assay (LLNA) to assess the skin sensitization effects is required in Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations, and a T-cell dependent antibody reaction (TDAR) in mice or rats is required in the United States Environmental Protection Agency (US EPA) guidelines for pesticide regulations (Boverhof et al., 2014; Rossi & Basketter, 2014). Non-standard species, including birds, are not included in the guidelines for toxicity testing within the chemical regulations for immunotoxic compounds. At the same time, the risk assessment for birds in the European regulatory system mostly focuses on lethal and reproductive effects, while immunotoxicity is not included as a standard endpoint (EFSA, 2009). However, the findings in this thesis pointed out that even low realistic environmental exposure to contaminants could impair the immune competence of avian species. Therefore, more non-standard species (e.g., avian species) and endpoints (e.g., immunotoxicity) need to be also included for environmental risk assessment and chemical regulations.

6.3. Conclusions

The results from the present thesis revealed that the major effects of both Hg(II) and Pb(II) exposure on the immune responses in waterfowl are promoting inflammation and impairing B-lymphocyte functions, which may result in lower immune competence against pathogens and higher risks of infections. The controlled *in vivo* study with Pekin ducklings showed similar results as the field study with Barnacle goslings. However, the *in vitro* experiments with chicken immune cell lines did not show the pro-inflammatory effects due to Hg(II) or Pb(II) exposure, suggesting that the *in vitro* cell models used in the present thesis may not be suitable for evaluating the effects on inflammation. Nevertheless, this thesis mainly focused on the innate immune responses and the non-specific humoral immune responses, and additional studies on other compartments of the immune system (e.g., T-cell functioning) are still needed for a comprehensive understanding of immunotoxicity of the divalent trace metals on waterfowl. Besides, the inclusion of actual pathogens in future studies could probably verify the hypothesis that the impaired immune competence by exposure to environmental pollutants can increase the risk of infection.

Altogether, the results of the current thesis emphasise the potential need to include immunomodulatory effects on birds in the environmental risk assessment of chemicals. This would contribute not only to the better protection of wildlife populations, but also towards sustaining environmental and human health.

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Chapter 7

Summary

Summary

Trace metals, such as mercury (Hg) and lead (Pb), have been reported to be immunotoxic to animals, however, relatively little is known about their effects and modes of action for avian species, especially waterfowl, which are frequently linked to the transmission of avian influenza infections. Given the fact that wild waterfowl are natural reservoirs for avian influenza viruses, impaired immune competence and pathogen resistance due to trace metal exposure in wild waterfowl may raise the risks of infections and might result in avian influenza outbreaks. Therefore, it is crucial to investigate the modulatory effects of trace metals, namely Hg(II) and Pb(II) in this thesis, on the immune responses of waterfowl. The objective of this thesis was to investigate whether realistic environmental levels of Hg(II) and Pb(II) exposure could modulate the immune responses in waterfowl upon a viral-like immune challenge and to explore the underlying modes of action.

Chapter 1 provided background information on the avian immune system, as well as an overview of the immunotoxicity of different species of trace metals that has been reported by previous studies. Divalent mercury (Hg(II)) and lead (Pb(II)) salts were selected as the most relevant model trace metals due to their widespread contamination and potential immunotoxicity to waterfowl. The knowledge gaps in the field of immunotoxicity on waterfowl induced by these divalent trace metal ions were highlighted. A stepwise approach from environmental to *in vivo* and to *in vitro* studies was introduced to reveal the effects and potential modes of action of Hg(II) and Pb(II) exposure on immune responses in waterfowl upon a viral-like immune challenge.

Chapter 2 presented a case study of low environmental total Hg (tHg) exposure on immune responses in Arctic barnacle goslings (*Branta leucopsis*) upon a viral-like challenge in Svalbard. Historical mining activities in Svalbard have resulted in local tHg contamination. New-born barnacle goslings were collected and herded in either a control or mining site, differing in tHg levels. An additional group was exposed to extra Hg(II) (as $HgCl_2$) via supplementary feed. Endpoints for immune responses and oxidative stress were measured 24 h post a viral-like immune challenge with a double-stranded RNA (dsRNA) injection. The results indicated that even low, environmentally relevant tHg exposure modulated the immune responses in Arctic barnacle goslings upon a viral-like immune challenge. Higher hepatic tHg concentrations were related to upregulated expression of pro-inflammatory genes in the spleen, including inducible nitric oxide synthase (iNOS) and interleukin-18 (IL-18), suggesting increased inflammation in higher exposed goslings. Hg exposure also led to a weaker humoral immunity by affecting the immune cell populations (higher heterophil/lymphocyte ratio) and reducing the level of natural antibodies. Goslings were capable of maintaining the redox balance by increased synthesis of glutathione (GSH) upon exposure to tHg. Therefore, these results demonstrated that environmental exposure to low levels of tHg could result in an affected immune competence at the individual level in Arctic

goslings, pointing at a potential increase of the susceptibility of avian populations to infections.

To verify the findings from the field study and to reveal the underlying modes of action, *in vivo* studies were performed in **Chapter 3,** in which Pekin ducklings (*Anas platyrhynchos domesticus*) were used as a model animal for waterfowl to investigate the effects of inorganic divalent Hg(II) (as $HgCl_2$) and Pb(II) (as lead acetate, $PbAc_2$) on avian immune responses upon a viral-like challenge with double-stranded RNA (dsRNA). The results indicated that Hg(II) altered the immune gene expression 24 h post-challenge, as reflected by induction of pro-inflammatory genes IL-8, iNOS, TLR3 and TLR7, and a significant decrease of microRNA-155 (miR-155). Ducklings exposed to Pb(II) showed lower levels of natural antibodies, reduced leukocyte density and lower heterophil proportions 24 h post-challenge. Although inorganic divalent Hg(II) and Pb(II) showed specific differential effects on the immune response of Pekin ducklings, the overall adverse immunomodulatory outcomes in both cases were mainly inflammation, impaired B-cell function, and weaker immunocompetence.

Chapter 4 and **Chapter 5** aimed to address immunomodulatory effects of Hg(II) (as $HgCl_2$) (**Chapter 4**) and Pb(II) (as $PbAc_2$) (**Chapter 5**) at the cellular level upon a viral-like immune challenge with dsRNA. Two chicken immune cell lines were used, namely the chicken macrophage cell line HD-11 representing the innate immune system and the chicken B-lymphocyte cell line DT40 representing the adaptative immune system. Effects of Hg(II) and Pb(II) exposure on cytotoxicity, oxidative stress, immune gene expression and cytokine production were assessed. These data provided more insights into cellular mechanisms and effects on specific immune cell types.

The results showed similar effects of Hg(II) and Pb(II) exposure on these chicken immune cell lines. However, Hg(II) showed higher cytotoxicity with lower EC50 values when compared to Pb(II). *De novo* synthesis of glutathione (GSH) plays an essential role in protecting immune cells from both Hg(II) and Pb(II) intoxication. Furthermore, immune cell proliferation was strongly inhibited while the secretion of antiviral interferon-α (IFN-α) was induced by both trace metal ions in both cell lines. Some different effects between Hg(II) and Pb(II) exposure were also noticed. For instance, Hg(II) strongly inhibited the production of nitric oxide by activated HD-11 macrophages, while Pb(II) slightly enhanced it. Hg(II) downregulated the gene expression of miR-155 in activated DT40 B-lymphocytes, while Pb(II) upregulated the expression of pro-inflammatory chemokine IL-8 in activated HD-11 macrophages. Overall, results from this thesis suggested that Hg(II) and Pb(II) both caused immunomodulatory effects in chicken immune cell lines mainly by impairing cell proliferation and cytokine production.

Chapter 6 presented a general discussion of the results gained in the thesis and provided perspectives for future studies. With the stepwise approach, it is possible to compare the effects of Hg(II) and Pb(II) in the field study with the controlled *in vivo* and *in vitro* studies.

The major effects of Hg(II) and Pb(II) were promoting inflammation and impairing B-lymphocyte functions upon a viral-like immune challenge. This disturbance of the avian immune system potentially hampers the immune response upon viral infections, which might cause a higher risk of infection for individuals and even the prevalence of diseases in the population as a whole. Possibilities of potential future studies, such as studies focusing on T-cell functions and inclusion of actual pathogens, were also discussed.

Altogether, this thesis indicates that even at low environmentally relevant levels, Hg(II) and Pb(II) exposure affected the immune responses in waterfowl upon a viral-like immune challenge, mainly by promoting inflammation and impairing B-cell functions. The current study emphasises the potential need to include immunomodulatory effects on birds in the environmental risk assessment of chemicals. This is relevant not only for the protection of wildlife populations, but also towards sustaining environmental and human health.

Appendix

Acknowledgements

About the author

List of publications

SENSE Diploma

Acknowledgements

The journey of doing a PhD is never easy, requiring a lot of dedication form oneself and also help from other people. I am so lucky that I have so many supportive people around me all the time, who made my journey fun and enjoyable. Therefore, I want to express my gratitude to everyone who has helped me during this journey.

Firstly, I would like to thank my promotors, Nico and Ivonne, for giving me the opportunity to pursue my PhD in Toxicology at Wageningen University. I have learnt so much from you on how to be an outstanding researcher. Thank you Ivonne for always being so efficient, sharp and responsive. Special thanks to Nico for being such a great supervisor, who always encourages me and believes in me. You always cheered me up every time I started doubting myself. I really enjoyed our discussions and working together in the field.

I would also like to acknowledge everyone who helped me with the field work, both in Svalbard and also in the Netherlands. Nico, many thanks for initiating the project in Svalbard and giving me this precious memory! Thank you Maarten for your guidance and tours. I learned a lot about Arctic birds from you! Alice and Hans, thanks for taking care of the goslings together, and chasing the foxes away together! Also thanks to Frigg, Kristle, Marije, Miriam, and Christophe for supporting during the field work in Svalbard. Ivo, Alan, Hans, Nico, Menno and Aafke, I really appreciated your help with the duckling experiments in Sinderhoeve. I could not have conducted the experiments without you. I would also like to acknowledge my master students, Xinyao, Viky and Alan. Thank you for your contributions in the project. I enjoyed our time working together and you helped me figure out how to become a better coworker and supervisor.

I would also like to thank the PhD thesis committee, Prof. Savelkoul, Dr. Matson, Prof. van den Berg and Prof. Jaspers for your critical review on this PhD thesis.

To everyone from the TOX family, thank you so much for giving me such great time! Hans B., thank you for the nice talks and I enjoyed when we organized the lab trip together. Lidy, thank you for all your help and kindness during my Master thesis until your last day at TOX. Carla and Gerda, thanks a lot for your help with all the administrative matters. I would like to thank all our brilliant technicians. Hans, I cannot thank you enough. You are always so calm and organized, and you are someone I can always talk to and rely on. I hope now you are enjoying your retirement with your wife a lot, maybe camping and watching birds around Europe? Laura, big thanks for your support in the cell culture lab, with my PCR tests and also with the IT stuff. I had an amazing time chatting with you! Bert, many thanks for your help and heartwarming emails even after your retirement! Sebas, thanks for all your hugs and jokes! And Wouter, thank you for your nice cakes, I am so happy that we share the same birthday!

To all the *intoxicated PhDs*, big hugs for everyone! It is my great pleasure to share the beautiful memories with you, including the PhD trip to Japan, dinners, drinks, birthdays, movie/pizza nights, etc. Nacho and Marta, thank you so much for being my big brother and sister, always there when I want to talk or need help! Diego, my kind daily supervisor for my master thesis, my officemate, and the pioneer of eco-immunotox, you helped me improve so much in both knowledge and personal aspects. Mengying and Diana, my dearest ladies, I really enjoyed our lunches, and the super relaxing and enjoyable nights. Big thanks for the EcoToxer, Marta, Nacho, Diego, Yiming, Merel, Aafke, Orsi, Edith, Bohan, Shivani and Mateo. I think we made a great team, helping each other and assisting the practical courses. Also our "dinner dinner" group, Akanksha, Menno, Chen, Aafke, Merel, I am so grateful to have had you during the hard times of COVID, hanging out with you guys did save me from the loneliness and stress. Miaoying and Shuo, thanks for always being so kind to help me both in work and personal life. Qianrui, thanks for the milk tea and all the tasty food you shared with me, good luck with the last stretch of your PhD! Shensheng and Danlei, it was so nice to have you as my peers and thanks for all the support and friendship. Hope all of us can enjoy our life as postdocs! My officemates in 4032, Nacho, Aziza, Menno, Shensheng, Jing Jin, Diego, Liang, Jiaqi, Shivani and Ghalia, it was my great pleasure to share the office with you and thanks for the heartwarming chats and drinks! Ixchel, Georgia, Annelies, Katja, and Suparmi, thank you for caring for me and always brining me positive energies. Hugo, Yasser, Isaac, Felicia, Alexandra, Frances, Jingxuan, Qiuhui, Maartje, Nina, Tessa, Veronique, Wisse, Xiyu, Thijs, Weijia, Xukun, Katharina, thank you for being such wonderful colleagues. Although we might not have talked a lot due to the pandemic, I am really happy that we get to know each other and let's keep in touch!

Special thanks to my best paranymphs, Menno and Merel. Menno, I remember telling you that I was afraid of you the first time I met you. However, shortly after that, I found out you are actually a very kind, helpful and independent guy with a special sense of humor :P. I am so lucky to have had you as my officemate and friend. I will also remember the time that we were the PhD representatives and organized Sinterklaas party. Wish you can always have time for the things you like, such as cycling, drinking and partying! Merel, you are the most kind-hearted girl I have ever met. Your sweet smile always melts my heart! I also admire that you can balance your work and sports so well! Thank you so much to both of you for being my paranymphs on one of the most remarkable days in my life.

I would also like to thank my friends in the Netherlands. My housemates, Qi, Yuyun, Fengjiao, Wei, Jun, Zhuoshi, Xinya, Siwei, Xinping, Du, and Yang, thank you for sharing the house with me in Lombardi, and giving me so many wonderful memories, eating together and celebrating traditional Chinese festivals and Dutch ones too. I didn't realize I had so many super lovely housemates until I wrote the long list. Wish you all the best either with finishing PhD or with work! My friends in Wageningen, Jun Qiu, Yuxi Deng, Anna, Min, Yidong, Yu Lei, Zhihong, Yanjun, Shanshan, Zhijun, and anyone I might have forgotten, I wish you all the best in your life!

My close friends, Pei, Bingnan, Yue, Jiayu, I am so lucky to have you with me since six and a half years ago, when we came to Wageningen for our master studies in Environmental Sciences. There are so many lovely trips, dinners, drinks and talks, that I will remember forever. I am so happy that I can pretty much discuss everything within the group. Bingnan, you were always my biggest supporter and you are always there if I need any help! Pei and Yue, although you live a bit far away from Wageningen, you always gave me advice and strength in both academic aspects and my personal life. Jiayu, you are the one in the group who has a lot of experience outside of academia. I got to know a lot about working in an environmental consultancy company from you. By the way, I am really jealous of your delicious food, dogs and cats, and all the fun stuff you do in Shanghai :P. I hope we can keep in touch no matter where we are around the world!

亲爱的“论坛群”小伙伴们，裴裴，冰囡，玥玥，佳雨，能在六年半前来瓦村读硕士时结识你们真的太幸运了！作为志同道合的环科小伙伴，我真的很开心我能跟你们分享不管是学习工作还是生活上的所有事。我也很享受咱们一起的旅行（虽然好像并没有大家一起，但是好像跟每个人都有旅行过呢），聚餐，和聊天。冰囡，你是我在瓦村一直可以依靠的朋友，不管什么事情，你总是第一个愿意伸出援手。裴裴和玥玥，虽然你们离瓦村有点远，你们总是能在学术和生活上都给我很好的建议。佳雨，你是咱们群里唯一一个打工人，通过你我也了解了很多国内环境咨询行业的信息。我也很嫉妒你在上海有美食，丰富的娱乐和猫狗双全的生活呀哈哈。希望我们不管在世界的哪个角落都能一直保持联系，支持彼此！真老铁都很稳！

My long-lasting best friends in China, Yimeng, Haoliang, Yuexin, Yubing, Jing, Lin and Yifan, thank you for caring about me even with the long distance and time differences. Yimeng, Haoliang and Jing, it was a pity that I could not manage to attend your weddings due to corona. I wish all of you good luck with your academic careers as well as your personal lives! Haoliang, you always make me laugh with your funny jokes. Yuexin, thanks for always cheering me up! Yubing, thank you for your endless friendship, wish you a lot of luck with your work! Lin and Yifan, wish you enjoy your work and life, looking forward to visiting you in Shanghai or Nanjing!

我也想感谢我在国内的长久的朋友们，梦梦（依梦），大嘴（昊亮），孙儿（悦昕），冰冰（煜冰），婧婧（郭婧），霖霖（顾霖）和凡凡（一凡）。谢谢你们跨越距离和时差地关心我！梦梦, 大嘴和婧婧，因为疫情没能参加你们的婚礼真的是太可惜了，祝你们的学术顺利，多发文章，生活美满！大嘴，你总是能用你的玩笑逗笑我哈哈。孙儿，谢谢你总是能鼓舞我，要活出自己的色彩呀～冰冰，谢谢你给我的无尽友谊，祝你工作顺利，升职加薪呀～霖霖和凡凡，希望我们能在上海或者南京相聚哦！

My beloved boyfriend, now I should call you my beloved husband, Jeffrey, thank you for being in my life. I feel secure when we are together, and you always relieve my stress and calm me down. You encouraged me to chase my dreams. I cannot tell you with words how happy and moved I was when you told me you were willing to go to Canada and then to China with me. I am extremely excited about our upcoming adventures! I would also like to extend my gratefulness to your family, Anna, Paul, Michiel, Bert, Ilse and the children Vince and Marie, for their supports and blessings.

Finally, I want to give my deepest gratitude to my family in China. It is a pity that you could not come to the Netherlands for my defense, but I know you are always with me. I could never achieve all the things that I achieved without your support! Many thanks to my parents, who always respect me and support the decisions I make. Although we are not too good at expressing our love for each other, I deeply love you, mom and dad, and I know it is the same for you! My lovely grandma and grandpa, time is always too short to be with you, please take care and be healthy! My dearest cousin Jingting, thank you so much for being so sweet and I really appreciate that you came and lived in Germany, that way I am not all alone in Europe. Our Christmas trips to Spain and Portugal were perfect. We should definitely travel together again in the future! Although it is a bit sad that I will leave Europe for a while, I believe you will manage very well and I will always be on your side! My dear cousin Yanying, thank you for supporting me all the time and taking care of my mom's birthday celebrations. I am so excited and cannot wait to see your baby! I would also like to thank all the family members (uncle Wei, aunt Cailing, aunt Xiangnyu, aunt Caiqin, uncle Bin, cousin Zhenyu, aunt Caifeng, uncle Jingsen, cousin Yanbo, uncle Guojun, aunt Hua and cousin Shaowei). Many thanks to everyone!

最后，我想要向我在中国的家人们表达我最深的感谢。因为疫情，你们没能来荷兰参加我的答辩和毕业典礼有点可惜，但是我知道你们一直与我同在。没有你们的支持，我不可能取得现在的成就。我想要感谢我的父母，他们总是尊重我并且支持我的每一个人生的重大决定。尽管我们都不太擅长表达对彼此的爱意，我还是想说，爸爸妈妈，我爱你们！我知道你们也深深地，无条件地爱我。亲爱的爷爷奶奶，跟你们在一起的时间总是太短，希望你们一定要健康开心！我最亲爱的静婷妹妹，谢谢你总是这么可爱这么甜，笔芯！你能来德国我真的很开心，我就不是一个人在欧洲了。咱们一去西班牙葡萄牙的旅行简直完美！希望以后也能再跟你一起旅行。虽然我要离开欧洲一段时间，有点小小的伤心，但是我相信你能很好地照顾自己，我也会一直支持你的！彦颖姐姐，谢谢你一直以来的关心和支持，还有替我帮我妈妈庆祝生日！我已经迫不及待的想见你的宝宝了哈哈。我也要感谢其他的家人（二爹，二妈， 姑姑，大姨，大姨夫，小宇哥哥，二姨，二姨夫，彦波哥哥，老舅，舅妈和薇薇妹妹）。谢谢每一个人！

About the author



Biyao Han was born on July 20, 1993 in Nei Mongol, China. She studied Environmental Sciences at Nanjing University, China for four years. After obtaining her bachelor's degree in 2015, she continued to pursue a master's degree in Environmental Sciences at Wageningen University & Research in the Netherlands. In her second year of her master, she did her MSc thesis at the Division of Toxicology at Wageningen University & Research and her internship at Helmholtz Centre for Environmental Research (UFZ) in Leipzig, Germany. In November 2017, Biyao started her PhD at the Division of Toxicology at Wageningen University & Research under the supervision of Dr. Nico W. van den Brink and Prof. Dr. Ivonne M.C.M. Rietjens with the financial support of the Chinese Scholarship Council (CSC). During her PhD, she followed a postgraduate education in Toxicology, which enables her to register as a European Toxicologist. Biyao will soon continue her interests in avian toxicology research at University of Saskatchewan, Canada, as a postdoc fellow.

List of publications

Han, B., García-Mendoza, D., van den Berg, H., & van den Brink, N.W. (2021), Modulatory effects of mercury (II) chloride ($HgCl_2$) on chicken macrophage and B-Lymphocyte cell lines with viral-like challenges *in vitro*. Environmental toxicology and chemistry, 40(10): 2813-2824. https://doi.org/10.1002/etc.5169

Han, B., García-Mendoza, D., van den Berg, H., & van den Brink, N. W. (2020). Modulatory effects of Pb^{2+} on virally challenged chicken macrophage (HD-11) and B-lymphocyte (DT40) cell lines *in vitro*. Environmental toxicology and chemistry, 39(5): 1060-1070. https://doi.org/10.1002/etc.4702

Han, B., van den Berg, H., Loonen, M. J. J. E., Mateo, R., van den Brink, N. W. Mercury modulated immune responses in Arctic Barnacle goslings (*Branta leucopsis*) upon a viral-like immune challenge (In preparation)

Han, B., Kroeze, A., van den Berg, H., Roessink, I., van den Brink, N. W. Modulatory effects of inorganic mercury (Hg(II)) and lead (Pb(II)) on immune responses of Pekin ducklings (*Anas platyrhynchos domesticus*) upon a viral-like immune challenge (In preparation)

García-Mendoza, D., Han, B., van den Berg, H. J., & van den Brink, N. W. (2019). Cell-specific immune-modulation of cadmium on murine macrophages and mast cell lines in vitro. Journal of Applied Toxicology, 39(7), 992-1001. https://doi.org/10.1002/jat.3788

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SENSE PhD Courses

- Environmental research in context (2018)
- Research in context activity: 'Making a video to introduce my PhD project to the general audience' (2021)

Other PhD and Advanced MSc Courses

- Immunotoxicology , Utrecht University (2018)
- Epidemiology, Utrecht University (2018)
- General Toxicology, Wageningen University (2018)
- Mutagenesis and Carcinogenesis, Leiden University (2019)
- Toxicogenomic, Maastricht University (2019)
- Species-specific birds course, KNAW-NIOO (2019)
- Pathobiology, Utrecht University (2019)
- Presenting with Impact, Wageningen Graduate Schools (2019)
- Introduction to R for Statistical Analysis, PE&RC and WIMEK (2019)
- Organ Toxicology, Radboud University (2020)
- Cell Toxicology, Leiden University (2020)
- Molecular Toxicology, Amsterdam University (2021)
- Career perspectives, Wageningen Graduate Schools (2021)

Management and Didactic Skills Training

- Supervising three MSc students with thesis (2019-2020)
- Assisting practicals of the MSc course 'Environmental Toxicology' (2018-2021)

Oral Presentations

- *Mercury modulated immune responses in Arctic Barnacle goslings (Branta leucopsis) with viral challenge*. Society of Environmental Toxicology and Chemistry (SETAC) Europe's 30th Annual meeting (Online), 3-7 May 2020

SENSE coordinator PhD education

Dr. ir. Peter Vermeulen

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The research described in this thesis was financially supported by China Scholarship Council (No. 201707720036 to Biyao Han).

Financial support from Wageningen University for printing this thesis is gratefully appreciated.

Cover design by Biyao Han, 二柱酱.

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