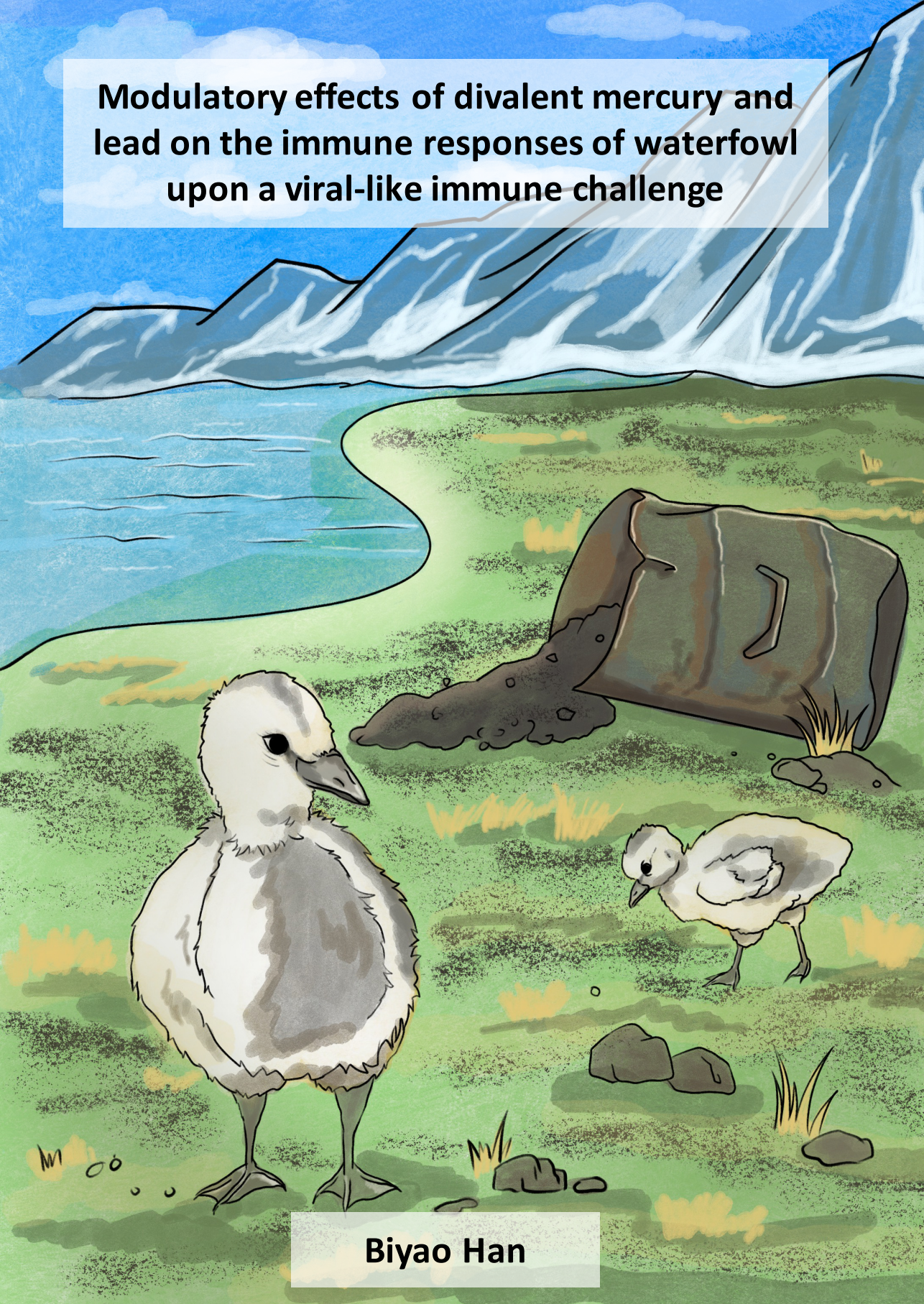
Modulatory effects of divalent mercury and lead on the immune responses of waterfowl upon a viral-like immune challenge



Biyao Han

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

Biyao Han

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.

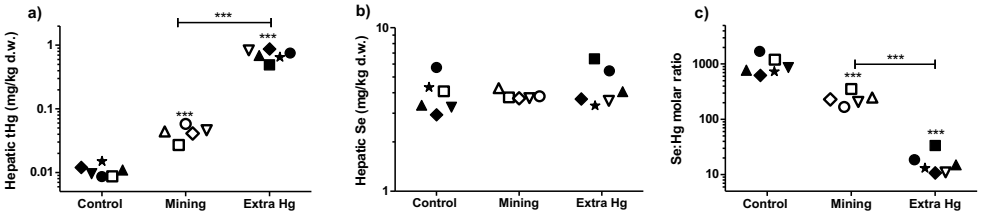

Figure 2.2. Hepatic tHg levels (a) and Se (b) levels in gosling and calculated Se:Hg molar ratio (c) of different exposure groups. Siblings from the same nest are shown as symbols with the same shape, while males are represented as closed symbols and females as black symbols. Significant differences 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).

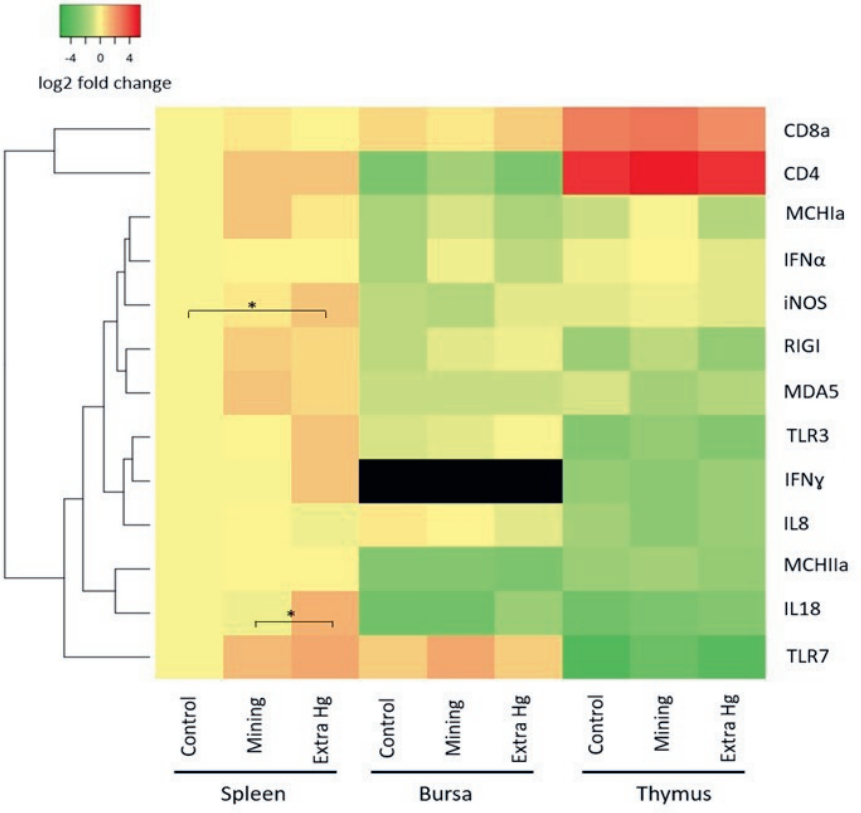

Figure 2.3. Gene expression of immune functional genes in gosling spleen, bursa and thymus tissues. Results were normalized firstly with the housekeeping gene GAPDH, and shown as log2 fold change relative to the average expression in spleen of control group. Black color indicated the non-detected genes (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.

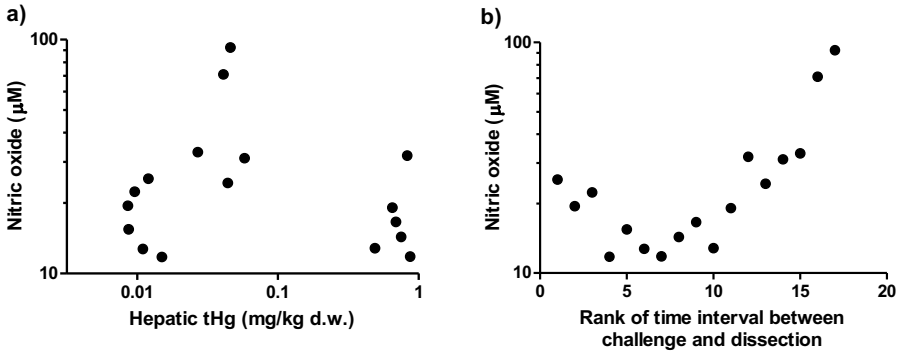

Figure 2.4. Nitric oxide levels in gosling plasma against the hepatic tHg levels (a) and the rank of time interval between challenge and dissection (b).

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.

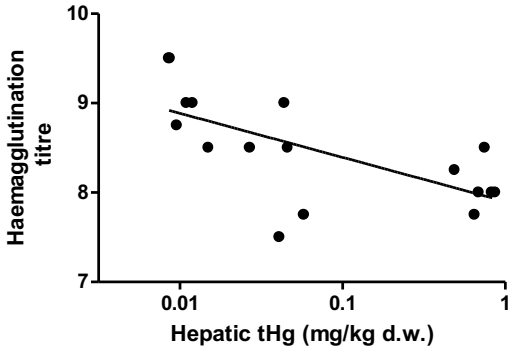

Figure 2.5. Linear regression of haemagglutination titre against tHg levels in gosling liver samples.

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).

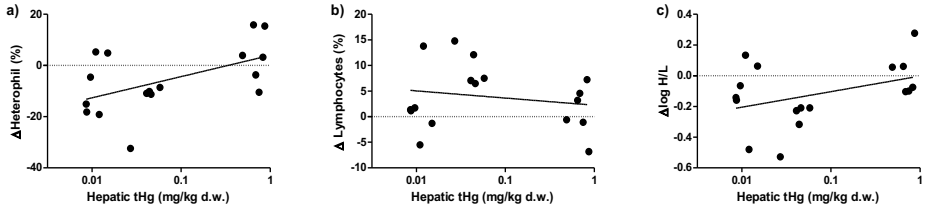

Figure 2.6. Change of heterophil population (a), lymphocyte population (b), and log heterophil/ lymphocyte ratio (c) in relation to tHg levels in liver samples according to the blood smear readings.

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.

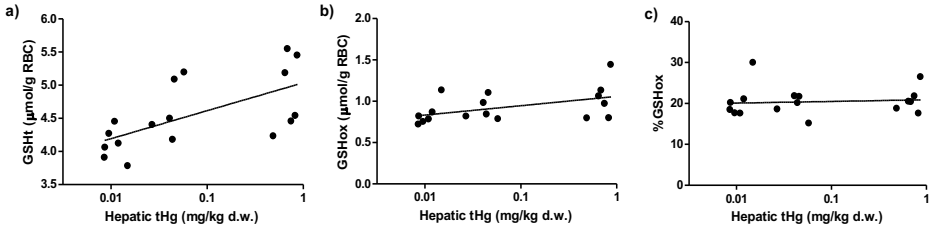

Figure 2.7. Levels of total glutathione (GSHt, a), oxidized glutathione (GSHox, b) and the percentage of oxidized glutathione (GSHox%, c) in gosling red blood cells (RBC).

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.

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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