Environment International 142 (2020) 105861

Contents lists available at ScienceDirect

Environment International

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

# A novel use of the leukocyte coping capacity assay to assess the immunomodulatory effects of organohalogenated contaminants in avian wildlife

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ARTICLE INFO

Handling Editor: Olga Kalantzi

*Keywords:*
Bird of prey
Innate immune system
Organochlorines
Poly- and perfluoroalkyl substances
Sub-lethal effects
White-tailed eagle

ABSTRACT

Apex predators are characterized by high levels of biomagnifying organohalogenated contaminants (OHCs) which have been found to induce detrimental health effects in wildlife, such as immune system impairment. The leukocyte coping capacity (LCC) assay is a functional real-time measure of an innate immune response essential in pathogen resistance, known as the respiratory burst. The current study suggests the novel use of this tool to test whether OHCs impair the innate immune system of a sentinel top predator, the white-tailed eagle (*Haliaeetus albicilla*; WTE). The LCC analysis was performed in the field on WTE nestlings ($n$ = 84) from northern Norway over two breeding seasons. Poly- and perfluoroalkyl substances (PFAS) dominated the total OHC load, surpassing the levels of legacy organochlorines. In addition, we detected significant negative correlations between concentrations of all polychlorinated biphenyls, *p,p′*-dichlorodiphenyldichloroethylene, perfluorohexane sulfonic acid and long-chain perfluorocarboxylic acids and the LCC of WTE nestlings. Based on our current findings reflecting a potential negative effect of both emerging and legacy OHCs on innate immune capacity, we suggest LCC to be a relevant and accessible test expanding the ecotoxicological toolbox to assess sub-lethal effects of OHCs in apex avian wildlife.

## 1. Introduction

Organohalogenated contaminants (OHCs), such as organochlorines (OCs) and poly- and perfluoroalkyl substances (PFAS), are anthropogenic chemicals utilized for a wide array of industrial and commercial purposes (Buck et al., 2011; Walker, 2001). OHCs are of concern because of their persistence, ubiquitous distribution through long-range transport, capacity to bioaccumulate in organisms and biomagnify through food chains, and reported toxic effects in both humans and wildlife (Jones and de Voogt, 1999). Consequently, apex predatory species are characterized by high exposure and are therefore more prone to suffer from severe health effects (Letcher et al., 2010). High exposure to OHCs has been associated with detrimental effects in wildlife through reproductive impairment in females, lower survival rates or increased mortality of offspring (Letcher et al., 2010; Rattner, 2009) as seen in the white-tailed eagle (*Haliaeetus albicilla*; hereafter WTE) on the Swedish coast of the Baltic Sea (Helander et al., 2002). Due to its apex position, the WTE functions as a sentinel species to monitor environmental pollution (Helander et al., 2008).

Immune system impairment is one potential sub-lethal effect induced by OHC exposure (Desforges et al., 2016). Immune impairment is manifested either directly (Bustnes et al., 2004; Jara-Carrasco et al., 2015; Sagerup et al., 2009) or indirectly through endocrine disruption (DeWitt and Patisaul, 2018; Kuo et al., 2012), and may potentially lead to increased susceptibility to diseases and pathogens (Badry et al., 2020; Jepson et al., 1999). In addition to the physiological impact from OHC exposure, stress (manifested by elevated corticosterone levels, i.e. the major stress hormone in birds; Romero and Romero, 2002) can have pervasive effects on both immunity (Bourgeon and Raclot, 2006; McEwen and Wingfield, 2003) and oxidative stress (i.e. the imbalance

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https://doi.org/10.1016/j.envint.2020.105861
Received 24 January 2020; Received in revised form 24 April 2020; Accepted 20 May 2020

between free radicals and antioxidants; Costantini et al., 2008; Stier et al., 2009). Simultaneously, the induction of an immune response can lead to higher oxidative stress (Costantini and Moller, 2009) which can induce damage to biomolecules such as lipids, proteins and DNA, and increase the rate of telomere erosion (Houben et al., 2008; Watson et al., 2015; Young et al., 2016). The modes of action of biomarkers of physiological stress, oxidative stress and immunocompetence are therefore highly inter-connected and should ideally be assessed simultaneously and/or through an integrated measure.

The leukocyte coping capacity (LCC) assay represents an integrated measure of the patho-physiological state of the organism (Huber et al., 2019; Lilius and Marnila, 1992) making it a potential biomarker for ecotoxicological studies. Polymorphonuclear leukocytes (PMNLs), i.e. heterophil granulocytes in birds (Harmon, 1998), constitute one of the first lines of innate immune defense against invading pathogens and represent the most abundant cellular component of the immune system (Abbas et al., 2014; Rungelrath et al., 2020). Activated PMNLs release an array of factors and mediators, including reactive oxygen species (ROS; Quinn and Gauss, 2004), which are produced in an anti-pathogenic defense cascade known as the respiratory burst (Nathan, 2006; Shelton-Rayner et al., 2010). In the LCC analysis, the respiratory burst is stimulated *in vitro* by a potent PMNL activator, phorbol myristate acetate (PMA; McLaren et al., 2003). Several studies show that PMNLs of individuals previously exposed to environmental (external) or organismal (internal) stressors have a reduced capacity to produce ROS in response to a PMA challenge (Huber et al., 2017a, b; McLaren et al., 2003; Moorhouse et al., 2007; Shelton-Rayner et al., 2010, 2012). Lower LCC scores indicate a lower potential to produce a respiratory burst and individuals with a decreased LCC response are therefore physiologically less responsive towards invading pathogens (McLaren et al., 2003; Shelton-Rayner et al., 2012). Simultaneously, studies on the immunomodulatory properties of OHCs in birds have found impairments of the innate and the adaptive arm of the immune system such as altered leukocyte composition (Bustnes et al., 2004; Jara-Carrasco et al., 2015), lowered ability of lymphocytes to proliferate *in vitro* in response to antigens (Sagerup et al., 2009), and abnormal antibody function (Sagerup et al., 2009).

The present study aimed to test the novel use of the LCC as a measure of early innate immunocompetence in relation to OHC exposure in WTE nestlings. We predicted that nestlings with higher OHC exposure have a reduced LCC, i.e. a lower early innate immune defense. Prior to evaluating whether OHC exposure in WTE nestlings can affect the leukocytes' ability to produce a respiratory burst (LCC-method), we identified potential ecological and environmental drivers of plasma LCC and OHC levels in nestlings.

## 2. Material and methods

### 2.1. Field sampling

Fieldwork was conducted between the 17th of June and 6th of July 2017 and the 24th of June and 6th of July 2018 in different geographical areas of northern Norway: Steigen (67°44′ N 14°48′ E), Harstad (68°48′ N 16°32′ E) and Tromsø (69°39′ N 18°57′ E). Morphological measurements (body mass [kg], wing and tail length [both measured in mm]), body feathers and a blood sample were collected from 84 WTE nestlings at approximately 5–9 weeks since hatching, before fledging. All nestlings in a brood were captured on their nest, accessed from the land or the sea. Brood size ranged between 1 and 3 chicks per nest. The field protocol was approved by the Norwegian Food Safety Authority.

A lithium-heparinized syringe with needle was used to puncture the brachial vein and approximately 5–10 mL of blood was drawn. After aliquoting 20 μL of whole blood for immediate LCC measurements, the remaining blood was stored under dark and cold conditions until centrifugation (8000 rpm for 10 min) within 12 h. Plasma and red blood cells (RBCs) were separated after centrifugation and kept frozen at −20 °C until chemical analysis.

### 2.2. LCC-analysis

Out of 84 sampled WTE nestlings, 78 were successfully assayed for their LCC. Measurement of LCC in the nestlings was initiated within 30 min of blood sampling. Based on the description by Huber et al. (2017b), 10 μL of heparinized whole blood and 90 μL of $10^{-4}$ mol $L^{-1}$ lucigenin (bis-N-methylacridinium nitrate; Sigma Aldrich, Vienna, Austria) dissolved in dimethyl sulfoxide (VWR International, Stockholm, Sweden) and diluted with phosphate-buffered saline (PBS, pH 7.4), were transferred into two silicon anti-reflective tubes, respectively (Lumivial, EG & G Berthold, Germany). Into the first tube, we added 10 μL of PBS to measure unstimulated blood chemiluminescence levels, which provides information on individual baseline levels of superoxide anion and acts as a control. In the second tube, we added 10 μL of $10^{-5}$ mol $l^{-1}$ phorbol-myristate-acetate (PMA; Sigma Aldrich, Vienna, Austria) to assess whole blood chemiluminescence in response to this secondary chemical challenge (the first constant challenge occurring in the circulation in vivo). The tubes were swiveled gently to mix the solutes. Blood chemiluminescence for each tube was assessed for 30 s every 5 min for a period of 60 min using a portable high sensitivity chemiluminometer (Junior LB 9509, E G & G Berthold, Germany) and expressed in relative light units (RLUs) whose values equal to total photon count divided by 10 (Huber et al., 2017a). Between single measurements, the tubes were kept under a constant temperature of 39 °C in a light-proof metal bead bath (Grant Instruments, JB Nova5, UK).

### 2.3. Leukocyte differentiation

Blood smears were prepared from whole blood in the field using the standard two-side wedge procedure (Houwen, 2000). Total and differential leukocyte counts were performed at the INVITRO Labor für Veterinärmedizin, Diagnostik und Hygiene (Vienna, Austria; Appendix A, Table A.1). Heterophil numbers from the individual total leukocyte counts were used to correct for a potential mass effect of leukocytes on the measured LCC response in the WTE nestlings.

### 2.4. Molecular sexing

The sex of nestlings was genetically determined using RBCs at the Institut Pluridisciplinaire Hubert Curien – Département Ecologie, Physiologie et Ethologie (IPHC-DEPE) in Strasbourg, France. Molecular sexing analysis followed the protocol described by Helander et al. (2007). In summary, DNA was extracted from RBCs using a commercial kit (NucleoSpin® Blood QuickPure, Macherey Nagel, Germany) and the sections of the sex-linked chromo-helicase-DNA-binding gene (CHD-Z and CHD-W) were amplified by polymerase chain reaction using primers 2550F and 2718R (Sletten et al., 2016).

### 2.5. Contaminant analyses

Chemical analyses of organochlorines (OCs) and poly- and perfluoroalkyl substances (PFAS) in blood plasma were carried out at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. All solvents used were of Suprasolv® grade and purchased from Merck-Schuchardt (Hohenbrunn, Germany). The concentration of compounds are expressed in ng $g^{-1}$ wet weight (*ww*) and a full list of the targeted compounds are given in Table A.2.

#### 2.5.1. OC analysis

The method used has previously been described in detail by Herzke et al. (2009). In brief, 0.98–1.02 g of plasma was spiked with internal standard solutions containing all compounds of interest in their isotopic labelled version, before being denatured with ethanol and further

treatment with saturated ammonium sulphate solution. Next, the samples were extracted twice with *n*-hexane before volume reduction, and finally re-dissolved in hexane and cleaned up by a Florisil column to remove biological matrix. A recovery standard ($^{13}$C-PCB-159) was added to all samples prior to analysis. The extracts were quantitatively analyzed by gas chromatography coupled to mass spectrometry by using the internal standard dilution method. All samples were analyzed for 12 polychlorinated biphenyl congeners (PCB-28/31, 52, 99, 101, 105, 118, 138, 153, 180, 183, 187 and 194), dichlorodiphenyltrichloroethanes (*p,p′*-DDT and *o,p′*-DDT) and their metabolites (*p,p′*-DDE, *o,p′*-DDE, *p,p′*-DDD and *o,p′*-DDD), hexachlorobenzene (HCB), hexachlorocyclohexanes (*α*-, *β*- and γ-HCH), chlordanes (*cis*-chlordane, *trans*-chlordane, *trans*-nonachlor, oxychlordane), and Mirex.

### 2.5.2. PFAS analysis

The method used is reported in more detail by Sletten et al. (2016). First, 200 μL of plasma sample was spiked with an isotopic labelled internal standard solution ($^{13}C_4$-PFBA, $^{13}C_5$-PFPA, $^{13}C_5$-PFHxA, $^{13}C_4$-PFHpA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_6$-PFDA, $^{13}C_7$-PFUnDA, $^{13}C_2$-PFDoDA, $^{13}C_2$-PFTeDA, $^{13}C_3$-PFHxS, $^{13}C_4$-PFOS, $^{13}C_8$-FOSA, and $^{13}C_2$-6:2 FTS; Wellington Laboratories, Ontario, Canada), then extracted with methanol by repeated sonication in an ultrasonic bath and vortexing. Next, the samples were centrifuged until sedimentation, and the supernatant was purified using ENVI-Carb 120/400 (Supelco 57210-U) and glacial acetic acid as described by Powley et al. (2005). Finally, 0.5 mL of supernatant solution was spiked with 3,7-diMeo-PFDcA recovery standard, and the sample was vortexed. The analysis of compounds was performed by ultrahigh-pressure liquid chromatography triple-quadrupole mass spectrometry and quantification was done by internal standard method using the isotopically labelled PFAS.

### 2.5.3. Quality assurance and quality control

Reference materials (OCs: human serum, NIST SRM 1958; PFAS: AM-S-Y 1804) and blanks were prepared and treated in the same way as the plasma samples to assure the quality of the method.

The limit of detection (LOD) was defined as the average signal to noise ratio for the analyzed matrix, or average concentration of the procedural blanks (if a signal was detected), plus 3 times the standard deviation. The limit of quantification (LOQ) was set to 3 times the average signal to noise ratio in plasma concentrations, or average concentration of the procedural blanks plus 10 times the standard deviation.

## 2.6. Data analysis

We performed statistical analyses and plotting of results in R (R Development Core Team, 2019). All tests were two-tailed and the null-hypotheses were rejected at an α-level of 0.05. Contaminant concentrations and LCC scores were $\log_e$-transformed prior to analyses in order to meet the assumption of normality. All the selected models were visually examined through standard diagnostics plots (e.g. visual examination of residual plots in order to assess the normality assumption).

Since nestlings were sampled at different developmental stages, an index for body condition (hereafter BC) was calculated based on the residuals from the regression between body mass and wing length (Helander et al., 2002; Stevenson and Woods, 2006). Sex was included as an additive predictor in the regression since body mass significantly differed between sexes (Welch's two-sample *t*-test, $P < 0.01$; Table A.3).

To express the unadulterated LCC response, the control sample score was subtracted from the PMA-challenged sample in order to correct for baseline leukocyte activity and potential background noise. Subsequently, the cleansed LCC response curve was fitted with a piecewise linear regression (PLR) model using the *seg.mod* function in the Segmented package (Fig. A.1; Muggeo, 2008). A positive slope of the first segment and a negative slope of the second segment were set as our *a priori* selection criteria for the LCC response (supplementary material including all curves that show both the raw data and fitted lines; Fig. A.1. for curve explanation). These selection criteria were fulfilled for 62 out of 78 fitted LCC curves, highlighting that the majority of the nestlings responded to the PMA challenge following a similar pattern. Only the latter 62 selected LCC curves were used in further statistical analyses.

Using Pearson's Product-Moment correlation (*r*), we documented that the slope of segments and the peak of LCC (i.e. the amplitude of the maximum LCC, hereafter LCC-peak) were linearly related to each other (Table A.4). Therefore, the extracted LCC-peak was used as a variable for all subsequent analyses as it is highly representative for the entire LCC response curve. Finally, the LCC-peak was corrected for the total number of heterophils (LCC-peak divided on the number of heterophils for each individual nestling) to exclude a potential mass-effect and resulted in a final sample size of 56.

Only OHCs detected in more than 75% of the samples were statistically analyzed, after substitution of non-detects by half the compound-specific LOQ-value. Based on their degree of correlation (Table A.5a–c), their physio-chemical properties, relative contribution to the total contaminant load, including visual examination of potential clusters revealing correlated compounds through Principal Component Analysis biplots (*prcomp* function; Fig. A.2a–c; Zuur et al., 2007), we selected the final compounds in the present study to compose: $\Sigma_{12}$PCBs, HCB, *cis*-chlordane, *trans*-nonachlor, *p,p′*-DDE, PFHxS, PFOS (branched and linear isomers), PFOA and long-chained perfluorocarboxylic acids (LC-PFCAs; PFDoDA, PFUnDA and PFTrDA).

The majority of the nests, i.e. 25 out of 39 nests (65%), contained only one nestling, and this prevented us from estimating inter- and intra-nest variability in our analyses using Linear Mixed-Effect Models (LMEs: Pinheiro and Bates, 2000; Zuur et al., 2009). Bustnes et al. (2013) outline the rationale for this where they chose to apply LME in statistical analyses of goshawks (*Accipiter gentilis*), a species that commonly produce more chicks per nest, but not for WTEs (since 60% of the nests in their study contained single chicks). Consequently, we follow Bustnes et al.'s (2013) assessment and decided to apply standard linear regression models (LMs) in our analyses. Nonetheless, we fitted and compared LMEs (using the *nlme*-library: Pinheiro et al., 2017) and LMs, and documented that our biological conclusions were not affected by the choice of modelling approach. Even though the estimated parameters slightly differed, both the estimates ($r = 0.95$, $P < 0.01$) and the predictions ($r = 1$, $P < 0.01$) from the models were highly correlated.

A set of *a priori* candidate linear regression models using *lm* function were created to explain variation in 1) OHC levels (each compound tested separately) and 2) LCC-peaks (Table A.6). Predictor variables for the models were chosen based on their biological relevance, i.e. year, BC, brood size and sex while avoiding collinearity (Zuur et al., 2009), by not including correlated variables in the same models (tested by using the *ancova* function in the HH package; Heiberger and Holland, 2004). A model selection of the set of candidate models was performed using the *aictab* function in the AICcmodavg package (Mazzerolle, 2019), and the most parsimonious model was defined as the one with the lowest second-order Akaike Information Criterion ($AIC_c$; Burnham et al., 2002; Table A.7a-b). Finally, a linear model was tested to investigate the relationship between the LCC-peak and the different OHC concentrations.

## 3. Results and discussion

### 3.1. Variation in OHC levels

ΣOCs and ΣPFAS represented 48.3% and 51.7%, respectively, of the total targeted plasma contaminant load of the WTE nestlings in northern Norway in 2017 and 2018 (Table 1). PFOS was detected in

**Table 1**
Mean ± standard error (*SE*), median and range (min–max) of the plasma concentrations (in ng $g^{-1}$ wet weight) of the targeted organohalogenated compounds detected in at least 75% of white-tailed eagle nestlings sampled in northern Norway during 2017 and 2018. Compounds are categorized as organochlorines (OCs) and poly- and perfluoroalkyl substances (PFAS).

| | Mean ± *SE* | Median | Min-max |
|---|---|---|---|
| OCs | | | |
| HCB | 1.46 ± 0.10 | 1.11 | 0.37–5.40 |
| *cis*-chlordane | 0.26 ± 0.02 | 0.22 | 0.04–1.06 |
| *trans*-chlordane | 0.19 ± 0.01 | 0.16 | 0.04–0.55 |
| *cis*-nonachlor | 0.33 ± 0.02 | 0.28 | 0.08–1.17 |
| *trans*-nonachlor | 0.90 ± 0.07 | 0.71 | 0.19–4.10 |
| Mirex | 0.44 ± 0.08 | 0.23 | 0.04–4.56 |
| *p,p′*-DDD | 0.15 ± 0.01 | 0.13 | 0.00–0.60 |
| *p,p′*-DDE | 7.68 ± 0.84 | 5.46 | 0.94–46.47 |
| PCB-28/31 | 0.19 ± 0.01 | 0.18 | 0.06–0.47 |
| PCB-52 | 0.13 ± 0.01 | 0.12 | 0.02–0.37 |
| PCB-99 | 1.04 ± 0.12 | 0.67 | 0.12–4.80 |
| PCB-101 | 0.24 ± 0.02 | 0.19 | 0.07–0.82 |
| PCB-105 | 0.5 ± 0.05 | 0.38 | 0.08–2.60 |
| PCB-118 | 1.97 ± 0.21 | 1.31 | 0.31–9.96 |
| PCB-138 | 4.85 ± 0.54 | 3.04 | 0.57–22.53 |
| PCB-153 | 7.90 ± 0.98 | 4.97 | 0.54–51.05 |
| PCB-180 | 3.36 ± 0.54 | 1.97 | 0.39–33.06 |
| PCB-183 | 0.55 ± 0.08 | 0.33 | 0.03–4.65 |
| PCB-187 | 1.33 ± 0.15 | 0.86 | 0.15–7.97 |
| PCB-194 | 0.42 ± 0.09 | 0.22 | 0.04–5.73 |
| $\Sigma_{12}$PCBs | 22.50 ± 2.70 | 14.12 | 3.00–141.76 |
| ΣOCs | 33.77 ± 3.70 | 22.77 | 4.77–199.58 |
| PFAS | | | |
| PFHxS | 0.69 ± 0.07 | 0.59 | 0.09–3.52 |
| PFOS | 24.26 ± 1.82 | 19.86 | 1.22–99.60 |
| ΣPFSAs | *24.94 ± 1.87* | *20.44* | *1.53–103.12* |
| PFNA | 3.63 ± 0.30 | 2.76 | 0.99–15.82 |
| PFOA | 0.76 ± 0.05 | 0.65 | 0.05–1.98 |
| PFDA | 1.67 ± 0.11 | 1.40 | 0.45–6.81 |
| PFUnDA | 3.51 ± 0.18 | 3.21 | 1.09–8.49 |
| PFDoDA | 0.50 ± 0.04 | 0.39 | 0.03–1.67 |
| PFTrDA | 1.12 ± 0.09 | 0.99 | 0.06–3.71 |
| ΣPFCAs | *11.19 ± 0.62* | *10.05* | *3.35–33.60* |
| LC-PFCAs[1] | 5.13 ± 0.30 | 4.56 | 1.18–13.42 |
| ΣPFAS | 36.13 ± 2.38 | 30.08 | 5.53–119.86 |

Complete list of contaminants and their abbreviations are given in Table A.2.
[1] The combined concentrations of the long-chain perfluorinated carboxylic acids PFUnDA, PFDoDA and PFTrDA.

overall the highest concentration of all targeted compounds (Table 1), which is consistent with previous observations on WTE nestlings in northern Norway (Loseth et al., 2019; Sletten et al., 2016) and bald eagle (*Haliaeetus leucocephalus*) nestlings in North America (Elliott et al., 2019). Fluorinated sulfonates (PFSAs), such as PFOS, have been found to be the dominating fluorinated compounds in wildlife species although fluorinated carboxylates (PFCAs) are found to have comparable concentrations (Butt et al., 2010). In the present study, PFSAs represented approximately 70% of the total PFAS load in the WTE nestlings and PFCAs the remaining 30% (Table 1). Concentrations of PFOS in avian and mammalian wildlife have shown decreasing trends over the last decades (Ahrens et al., 2011; Riget et al., 2019; Sun et al., 2019), most likely as a result of large reductions in its production and utilization in the early 2000s (Buck et al., 2011). On the contrary, PFCAs have shown increasing trends (AMAP, 2016; Sun et al., 2019; Vorkamp et al., 2019). Decreasing and increasing trends of fluorinated contaminants reflect a change in use of such compounds, with certain compounds being phased out following restrictions while being substituted with other compounds sharing similar properties.

Further, PCBs still constitute a major group of compounds detected in the WTE nestlings (Bustnes et al., 2013; Sletten et al., 2016), with PCB-153 displaying the highest concentrations among the $\Sigma_{12}$PCBs (Table 1). PCB-153 is found in relatively high levels in many avian apex predators due to its high persistence and bioaccumulation potential (Bustnes et al., 2004; Bustnes et al., 2005; Leat et al., 2019). Concurrently, *p,p′*-DDE displayed similar high concentrations as PCB-153 in the nestlings (Table 1). Plasma concentrations of *p,p′*-DDE in northern Norwegian WTE nestlings in 2017 and 2018 were found to be higher compared to the same population in 2011–2012 and 2015–2016 (Loseth et al., 2019; Sletten et al., 2016), but lower compared to 2008–2010 (Bustnes et al., 2013). Plasma concentrations are considered a short-term measure of contaminant levels in the nestlings that mainly reflect recent dietary exposure and are therefore highly dependent on the time of sampling (e.g. right before/just after feeding; Espín et al., 2016). Nevertheless, temporal trends of legacy organochlorines such as PCBs and *p,p′*-DDE show significant decreasing trends in biota, reflecting the ban of these compounds 20–30 years ago (AMAP, 2016).

Elevated plasma levels of $\Sigma_{12}$PCBs, HCB, *cis*-chlordane, *trans*-nonachlor, *p,p′*-DDE and PFOA were significantly associated with a lower BC in the WTE nestlings ($P < 0.05$; Table 2). Maternal transfer is an important source of contaminants in nestlings, but as the nestlings grow, such contaminants become diluted in their body tissues as long as the dietary intake is not compensating for this effect (Bustnes et al., 2013). As a result, nestlings in poor body condition often have higher contaminant levels than nestlings in good condition due to less dilution.

In 2018, plasma levels of LC-PFCAs and PFHxS were significantly elevated compared to plasma collected in 2017 ($P < 0.01$; Table 2), implying across-year variation in exposure of some contaminants. Variation in OHC levels between years is likely to be caused by a multitude of factors, e.g. location, diet and/or environmental fluctuations (Bustnes et al., 2015).

Although brood size was included in the most parsimonious model explaining variation in both PFOS and PFHxS concentrations, it was never found to have a significant effect ($P = 0.33$ and $P = 0.06$, $P = 0.22$ and $P = 0.09$, respectively; Table 2). Sex was the only predictor variable not selected in any of the retained candidate models explaining variation in OHC levels in the WTE nestlings.

### 3.2. Sex and inter-year differences in LCC-peak amplitudes

LCC-peak amplitudes ranged from 19.59 to 4368.74 RLUs, with a mean of 677.99 ± 93.99 RLUs (Fig. 1). Predominantly, there was a significant difference in the amplitude of the LCC-peak of WTE nestlings in northern Norway between the two sampling years, showing LCC-peaks to be approximately 5 times higher in 2017 compared to 2018 ($P < 0.01$; Fig. 1). Stressful conditions, such as lowered food availability, inclement weather or increased exposure to pathogens, are known to affect the immune system either by immunoenhancement or immunosuppression (McEwen and Wingfield, 2003). However, we observed no difference in BC between years (One-way ANOVA, $F_{1, 73} = 0.60$, $P = 0.44$) that could support between year differences in food availability, nor do we have information regarding pathogen exposure. Nevertheless, weather conditions appeared more challenging in 2018 compared to 2017 (data visually examined; Norsk Klimaservicesenter, n.d.) and we might speculate that less favorable weather conditions may have contributed to the large difference observed in LCC responses between years, regardless of pollution exposure.

Furthermore, the LCC-peak amplitude was generally found to be higher in females than in males, though only significantly so in 2018 ($P < 0.05$; Fig. 1), likely due to relatively low sample size and high inter-individual variation in LCC scores. Sex differences in LCC have previously been reported for captive house sparrows (*Passer domesticus*), with males having higher LCC-peak amplitudes than females (Huber et al., 2017b). Sex differences could be a result of sexual hormones, which have been found to contribute to the differential regulation of immune response between sexes (Klein and Flanagan, 2016).

**Table 2**
Parameter estimates from the most parsimonious linear models explaining variation in plasma contaminant levels ($log_e$-transformed) in white-tailed eagle nestlings sampled in northern Norway across two years (2017 [baseline] and 2018 acting as levels). The significant relationships are **bolded**.

| Parameter | Estimate | Confidence Interval (95%) | *P* |
|---|---|---|---|
| $\Sigma_{12}$PCBs | | | |
| (F = 18.57, $R^2$ = 0.20, $P < 0.01$) | | | |
| Intercept | 2.77 | 2.59–2.94 | < 0.01 |
| **Body condition** | **$-8.94e^{-04}$** | **$-1.30e^{-03}$ to ($-4.80e^{-04}$)** | **< 0.01** |
| HCB | | | |
| (F = 16.75, $R^2$ = 0.18, $P < 0.001$) | | | |
| Intercept | 0.23 | −5.35 to 0.96 | 0.17 |
| **Body condition** | **$-5.85e^{-04}$** | **$-8.73e^{-04}$ to ($-2.96e^{-04}$)** | **< 0.01** |
| *cis*-chlordane | | | |
| (F = 7.46, $R^2$ = 0.09, $P < 0.01$) | | | |
| Intercept | −1.49 | −1.63 to (−1.36) | < 0.01 |
| **Body condition** | **$-4.30e^{-04}$** | **$-7.45e^{-04}$ to ($-1.16\ e^{-04}$)** | **< 0.01** |
| *trans*-nonachlor | | | |
| (F = 16.44, $R^2$ = 0.18, $P < 0.01$) | | | |
| Intercept | −0.25 | −0.37 to (−0.13) | < 0.01 |
| **Body condition** | **$-5.81e^{-04}$** | **$-8.67e^{-04}$ to ($-2.95e^{-04}$)** | **< 0.01** |
| *p,p′*-DDE | | | |
| (F = 14.92, $R^2$ = 0.16, $P < 0.01$) | | | |
| Intercept | 1.76 | 1.59–1.92 | < 0.01 |
| **Body condition** | **$-7.51e^{-04}$** | **$-1.13e^{-03}$ to ($-3.63e^{-04}$)** | **< 0.01** |
| LC-PFCAs | | | |
| (F = 11.85, $R^2$ = 0.24, $P < 0.01$) | | | |
| Intercept | 1.32 | 1.18–1.47 | < 0.01 |
| **Year 2018** | **0.44** | **0.24–0.65** | **< 0.01** |
| Body condition | $-2.30e^{-04}$ | $-4.71e^{-04}$ to $63e^{-05}$ | 0.06 |
| PFOA | | | |
| (F = 4.66, $R^2$ = 0.06, $P = 0.03$) | | | |
| Intercept | −0.47 | −0.62 to (−3.16) | < 0.01 |
| **Body condition** | **$-3.85e^{-04}$** | **$-7.41e^{-04}$ to ($-2.97e^{-05}$)** | **0.03** |
| PFOS | | | |
| (F = 2.90, $R^2$ = 0.07, $P = 0.06$) | | | |
| Intercept | 3.09 | 2.89–3.28 | < 0.01 |
| Brood size (2) | −0.13 | −0.39 to 0.14 | 0.34 |
| Brood size (3) | 0.45 | −0.04 to 0.94 | 0.07 |
| PFHxS | | | |
| (F = 5.60, $R^2$ = 0.19, $P < 0.01$) | | | |
| Intercept | −0.93 | −1.30 to (−0.56) | < 0.01 |
| **Year 2018** | **0.68** | **0.26–1.10** | **< 0.01** |
| Brood size (2) | −0.27 | −0.70 to 0.17 | 0.22 |
| Brood size (3) | 0.69 | −0.12 to 1.49 | 0.09 |

### 3.3. Amplitude of LCC-peaks in relation to OHC exposure

Negative relationships between OHC concentrations and the amplitudes of the LCC-peaks were found for all nine OHCs tested except *cis*-chlordane (Estimate < 0, Table 3). Yet, the latter negative relationships were only significant for $\Sigma_{12}$PCBs, *p,p′*-DDE, LC-PFCAs and PFHxS ($P < 0.05$; Table 3; Fig. 2), indicating elevated plasma concentrations to be associated with a reduced amplitude of the LCC-peak. These findings support our prediction that individuals exposed to higher OHC levels have a reduced LCC compared to less contaminated nestlings. Interestingly, contaminants detected in both relatively low and high concentrations in the WTE nestlings (Table 1) displayed the same lowering effect on the amplitude of the LCC-peak with increasing concentrations, indicating that some OHCs might be potent also at low levels. A reduced LCC, represented by a reduction in the amplitude of the LCC-peak, reflects a lowered innate immunity through a lowered potential of circulating leukocytes to release ROS in response to immune challenges. Since the heterophil respiratory burst represents an early innate immune defense to pathogen exposure, our results therefore suggest a possible immunomodulatory effect of $\Sigma_{12}$PCBs, *p,p′*-DDE, LC-PFCAs and PFHxS, which ultimately might be critical to the survival and fitness of WTE nestlings.

Exposure to PCBs and *p,p′*-DDE has been previously linked to immune impairment in several avian species (Grasman and Fox, 2001; Grasman et al., 1996; Jara et al., 2018; Mayne et al., 2004; Smits et al., 2002; Smits and Bortolotti, 2001), but studies on innate immune endpoints are scarce. Bustnes et al. (2004) and Jara-Carrasco et al. (2015) reported a positive relationship between PCB and *p,p′*-DDE concentrations and leukocyte indices in glaucous gulls (*Larus hyperboreus*) and chinstrap penguins (*Pygoscelis antarcticus*), respectively. The latter studies hypothesized these positive relationships to be a result of either a contaminant-mediated alteration of the leukocyte functions, or a contaminant-mediated immunosuppression contributing to infections, both resulting in a compensatory increased production of leukocyte numbers (Bustnes et al., 2004; Jara-Carrasco et al., 2015). Although our study reported negative relationships, it is noteworthy that the present and latter studies are based on different immunological endpoints, i.e. leukocyte responsiveness as opposed to absolute cell counts further explaining the discrepancies between studies. Nevertheless, either positive or negative, a significant association between contaminant exposure and immunological endpoints can lead to a suboptimal function of the immune system, potentially increasing the susceptibility to pathogens and diseases. In conclusion, legacy organochlorines such as PCBs and *p,p′*-DDE which still dominate the body burden of apex predators, keep acting as possible stressors on the immune system.

While immune system impairment is known to be associated with PFAS exposure in mammals, few studies have focused on immune endpoints and PFAS in birds (Castano-Ortiz et al., 2019; DeWitt et al., 2012). Experiments on chicken cells detected immunomodulation of PFOS on innate immunity signaling pathways (Castano-Ortiz et al., 2019). Additionally, negative effects of long-chained PFCAs have been

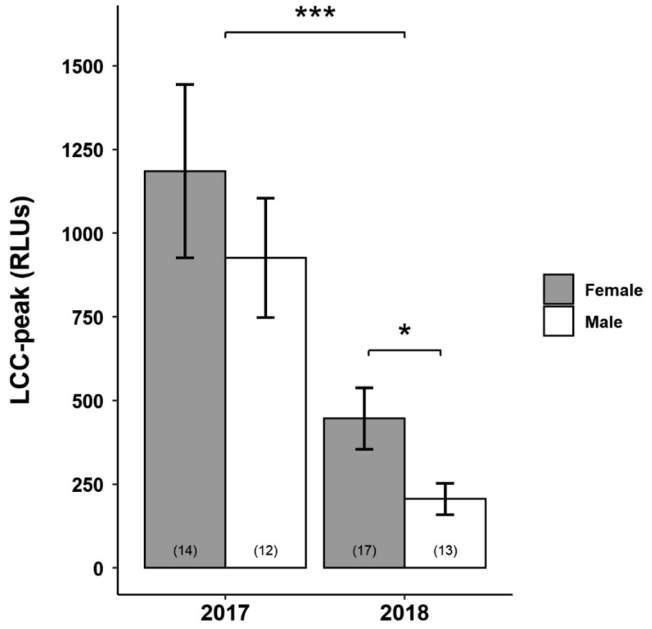


**Fig. 1.** Mean ± *SE* intensity of the leukocyte coping capacity (LCC) peak amplitudes between female and male white-tailed eagle nestlings sampled in northern Norway during 2017 and 2018. Asterisks (*) indicate statistical significant differences between the variables (*$P < 0.05$, ***$P < 0.01$) and the bracketed numbers represent sample size for each group.

**Table 3**
Parameter estimates for the linear models of the relationship between LCC-peak amplitude (response variable) and contaminant concentrations (predictor variables) in northern Norwegian white-tailed eagle nestling plasma sampled during 2017 and 2018. The LCC-peak and contaminant concentrations were $\log_e$-transformed prior to analysis, and the contaminants were tested separately. The significant relationships are **bolded**.

| Predictor variable | Estimate | Confidence Interval (95%) | *P* |
|---|---|---|---|
| **$\Sigma_{12}$PCBs** | **−0.37** | **−0.69 to (−0.05)** | **0.02** |
| HCB | −0.31 | −0.83 to 0.20 | 0.23 |
| *cis*-chlordane | 0.23 | −0.35 to 0.82 | 0.43 |
| *trans*-nonachlor | −0.19 | −0.71 to 0.33 | 0.47 |
| ***p,p′*-DDE** | **−0.35** | **−0.69 to (−0.00)** | **< 0.05** |
| **LC-PFCAs** | **−0.91** | **−1.50 to (−0.32)** | **< 0.01** |
| PFOA | −0.17 | −0.59 to 0.24 | 0.41 |
| PFOS | −0.07 | −0.64 to 0.49 | 0.80 |
| **PFHxS** | **−0.43** | **−0.71 to (−0.15)** | **< 0.01** |

reported on reproduction and physiological stress in black-legged kittiwakes (*Rissa tridactyla;* Tartu et al., 2014). Current and previous findings suggest that increasing trends of PFHxS and LC-PFCAs could be of concern to wildlife health, despite their relatively low concentrations.

Although our correlational study cannot reveal causal relationships, our results contribute to the weight of evidence suggesting immunomodulation of some OHCs in avian wildlife. Yet, our results do not help understand the underlying mechanisms of OHC exposure on the LCC of WTE nestlings. Further experimental studies are therefore needed to elucidate the possible proximate mechanisms causing contaminants to lower the immunological responses. Longitudinal studies should also examine the long-term effects of lowered innate immune response on survival and reproductive success, and ideally consider variables such as BC and annual variations in contaminant concentrations, as they might ultimately influence the relationships between the innate immune response (LCC) and contaminant exposure in WTE nestlings.

### 3.4. Relevance of the LCC in wildlife ecotoxicology

Based on our results, the LCC-method has clear methodological advantages in wildlife ecotoxicology as it is conducted almost instantly after blood sampling, allowing a real-time perspective of the leukocytes' ability to produce a respiratory burst. This *in vitro* method avoids both the potential impact of centrifugation and freezing on cell reactivity (Bortolin et al., 2017) as well as the stress related to any prior injection with an immune activator (e.g. antigens) or recapture. Finally, the method is cheap and requires only a droplet of blood for the analyses.

## 4. Conclusion

The present study reported significant negative associations between $\Sigma_{12}$PCBs, *p,p′*-DDE, LC-PFCAs and PFHxS and the LCC in nestlings of an established apex predatory bird species, the WTE. Our results contribute to the weight of evidence for immunomodulatory properties of OHCs in avian wildlife and suggest that LCC represents a relevant and accessible test to expand the toolbox of wildlife ecotoxicology. In the context of multiple stressors, future research should also consider environmental variables such as annual variations in OHC concentrations and/or food availability, as they might ultimately influence the relationships between the innate immune response (LCC) and contaminant exposure in WTE nestlings.

## Funding

The present study was part of the Norwegian Research Council (NFR) funded project ECOSTRESS - Ecological consequences of environmentally persistent pollutants in a marine sentinel species: A multi-stressor approach (project number 255681).

## CRediT authorship contribution statement

**Elisabeth Hansen:** Formal analysis, Investigation, Writing - original draft, Visualization. **Nikolaus Huber:** Methodology, Investigation, Validation, Writing - review & editing. **Jan O. Bustnes:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Dorte Herzke:** Formal analysis, Validation, Writing - review & editing. **Bård-Jørgen Bårdsen:** Formal analysis, Writing - review & editing. **Igor Eulaers:** Writing - review & editing. **Trond V. Johnsen:** Investigation, Resources. **Sophie Bourgeon:** Conceptualization, Investigation, Resources, Writing - review & editing, Supervision, Project administration.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors thank Aasmund Gylseth for his expertise and help during fieldwork in Steigen, and Einar Veimoen for assistance during fieldwork in Harstad. We acknowledge Linda Hanssen and Arntraut Götsch at NILU Tromsø for their help on the contaminant analyses, as well as Sandrine Zahn and Francois Criscuolo for performing molecular sexing.

## Appendix A. Supplementary material

The following files are available free of charge: Leukocyte data, targeted organohalogenated contaminants (OHCs), morphological data, correlation matrix of leukocyte coping capacity (LCC) curve estimates,

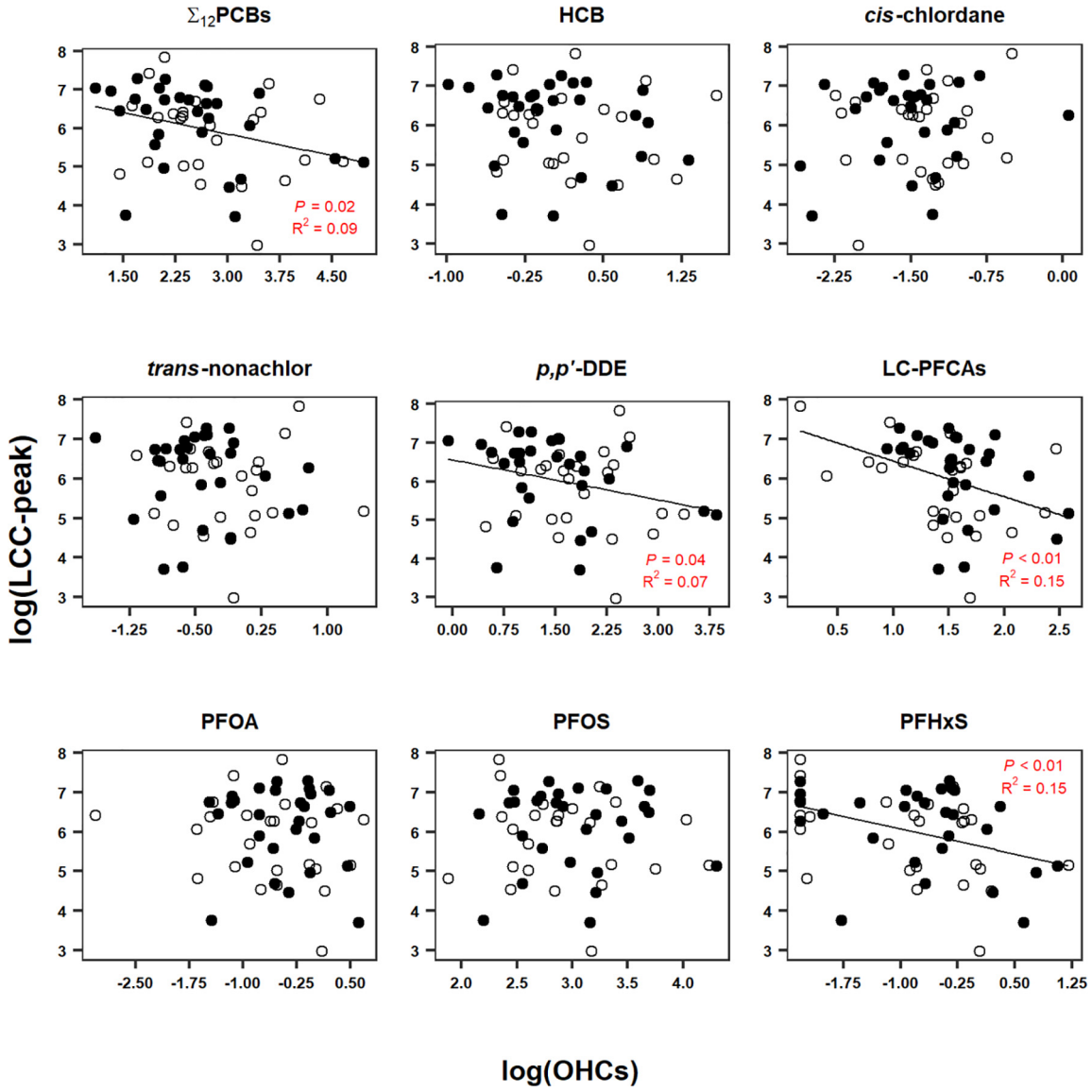


**Fig. 2.** Relationship between leukocyte coping capacity (LCC) peak amplitudes and organohalogenated contaminant (OHC) concentrations in female (filled circles) and male (open circles) white-tailed eagle nestlings in northern Norway in 2017 and 2018. Regression lines indicate a significant association. *P*-values and adjusted $R^2$ of the significant linear models are reported for each compound.

correlation matrices and principal component analysis (PCA) biplots of OHCs, candidate linear models, model selection output, and LCC response curve (with additional zip-file "LCC_curves" including all the individual LCC responses). Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105861.

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