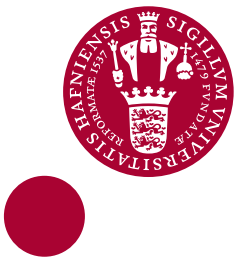

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The influence of natural variation and organohalogenated contaminants on physiological parameters in white-tailed eagle (*Haliaeetus albicilla*) nestlings from Norway

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ABSTRACT:

Environmental exposure to organohalogenated contaminants (OHCs), even at low concentrations, may cause detrimental effects on the development and health of wild birds. The present study investigated if environmental exposure to OHCs may influence the variation of multiple physiological parameters in Norwegian white-tailed eagle (*Haliaeetus albicilla*) nestlings. Plasma and feather samples were obtained from 70 nestlings at two archipelagos in Norway in 2015 and 2016. The selected physiological parameters were plasma concentrations of thyroid hormones (thyroxine, T4 and triiodothyronine, T3), plasma proteins (prealbumin, albumin, α_1-, α_2-, β- and γ-globulins) and selected blood clinical chemical parameters (BCCPs) associated with liver and kidney functioning. Feather concentrations of corticosterone ($CORT_f$) were also included to investigate the overall stress level of the nestlings. Concentrations of all studied physiological parameters were within the ranges of those found in other species of free-living birds of prey nestlings and indicated that the white-tailed eagle nestlings were in good health. Our statistical models indicated that perfluoroalkyl substances (PFASs) and legacy OHCs, such as polychlorinated biphenyls, organochlorinated pesticides and polybrominated diphenyl ethers, influenced only a minor fraction of the variation of plasma thyroid hormones, prealbumin and $CORT_f$ (5–15%), and partly explained the selected BCCPs (< 26%). Most of the variation in each studied physiological parameter was explained by variation between nests, which is most likely due to natural physiological variation of nestlings in these nests. This indicates the importance of accounting for between nest variation in future studies. In the present nestlings, OHC concentrations were relatively low and seem to have played a secondary role compared to natural variation concerning the variation of physiological parameters. However, our study also indicates a potential for OHC-induced effects on thyroid hormones, $CORT_f$, prealbumin and BCCPs, which could be of concern in birds exposed to higher OHC concentrations than the present white-tailed eagle nestlings.

1. Introduction

Organohalogenated contaminants (OHCs), such as perfluoroalkyl substances (PFASs) and legacy persistent organic pollutants (POPs) like polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), have been shown to interfere with physiological processes involved in development and growth of birds (Cassone et al., 2012; Champoux et al., 2017; Nøst et al., 2012). Even low concentrations of OHCs have been associated with decreased plasma concentrations of thyroid hormones (Cesh et al., 2010; Champoux et al., 2017), increased feather concentrations of corticosterone (stress hormone) (Monclús et al., 2019) and immunomodulation (Fernie et al., 2005; Grasman et al., 1996) in nestling birds.

In all vertebrates, the liver and kidneys are responsible for the majority of detoxification and excretion of metabolites and xenobiotics such as OHCs. However, these organs may also accumulate OHCs

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(Lehman-McKeeman, 2008). In turn, the OHC burdens may interfere with organ function and thus influence blood clinical-chemical parameters (BCCPs) associated with liver and kidney damage (Sonne et al., 2012, 2010). Plasma proteins are primarily produced in the liver to maintain osmotic pressure and transport nutrients and hormones in the blood circulation. However, certain plasma proteins may increase in response to physiologic changes, such as disease, injury or stress (Lee et al., 2015; Murata et al., 2004). Certain plasma proteins are classified as negative or positive acute-phase proteins (APPs), as their concentrations either decrease (albumin and prealbumin) or increase (globulins) during an immune or inflammatory response (Cray et al., 2009). Thus, these plasma proteins are used as inflammatory biomarkers both in domesticated and wild birds (Lee et al., 2015; Tatum et al., 2000), and may also indicate potential immunomodulation in OHCs exposed nestlings (Grasman et al., 2000).

White-tailed eagles (*Haliaeetus albicilla*) have an apex trophic position and can accumulate high concentrations of OHCs, even at an early age (Bustnes et al., 2013; Løseth et al., 2019a,b). Their diet depends on the prey species available within their territories and consists mainly of marine and terrestrial carrion, fish, seabirds and small mammals (Nadjafzadeh et al., 2016). Adult white-tailed eagles are territorial and mostly resident within their breeding areas. Thus, the contaminant burden of their nestlings reflects contaminant levels in local prey, making white-tailed eagle nestlings useful sentinels of local environmental pollution (Helander et al., 2008; Olsson et al., 2000). The internal OHC concentrations previously found in Norwegian white-tailed eagle nestlings are relatively low (Løseth et al., 2019b; Sletten et al., 2016; Sonne et al., 2012) compared to nestlings of its close North American relative, the bald eagle (*Haliaeetus leucocephalus*, Cesh et al., 2010; Pittman et al., 2015; Route et al., 2014). Despite these low concentrations of OHCs, studies have found indications of induced oxidative stress (Sletten et al., 2016) and impaired liver enzyme activity (Sonne et al., 2012) in Norwegian white-tailed eagle nestlings. This may indicate that even low OHC concentrations can have the potential to induce physiological effects in nestlings.

The aim of the present study was to investigate if environmental exposure to OHCs influences multiple physiological parameters in white-tailed eagle nestlings. The selected physiological parameters were thyroid hormones, corticosterone, plasma proteins, and selected BCCPs; of which the latter may indicate impaired liver or kidney function. The concentrations of thyroid hormones, plasma proteins and BCCPs were analysed in plasma, while the corticosterone concentrations were analysed in feathers ($CORT_f$) to avoid the potential stress-induced peak in plasma corticosterone during sampling and handling (Bortolotti et al., 2008). The plasma OHC concentrations, age estimation and stable isotope ratios were gathered from two previous studies related to levels and variation of OHCs in the same Norwegian white-tailed eagle nestlings (Løseth et al., 2019a,b). The plasma protein concentrations from the same nestlings were obtained from Flo et al. (2019).

We hypothesised negative relationships between OHCs and thyroid hormones, indicating possible thyroid disruption (McNabb, 2007), and positive relationships between OHCs and $CORT_f$, as individuals with high OHC burdens may experience more stress (Monclús et al., 2019, 2018). Positive relationships were also hypothesised between OHCs and selected BCCPs, implying potential OHC-induced perturbations in hepatic and renal function (Sonne et al., 2012, 2010). We also hypothesised positive relationships between OHCs and globulins, and negative relationships between OHCs and prealbumin and albumin, indicating increased susceptibility to diseases with higher OHC burdens (Fairbrother et al., 2004). Biological factors such as nest identity, sex and age, and dietary tracers indicating dietary carbon sources ($\delta^{13}C$) and trophic positions ($\delta^{15}N$) of the nestlings were accounted for as these factors may influence all physiological parameters (Jerzak et al., 2010; McNabb, 2007; Whalan, 2015).

2. Materials and methods

2.1. Field sampling

A total of 70 white-tailed eagle nestlings were sampled at two archipelagos in Norway, Smøla (63.35°N; 8.03°E) and Steigen (67.93°N; 14.98°E) in June–July in 2015 (Smøla: $n = 13$ and Steigen: $n = 14$) and 2016 (Smøla: $n = 22$ and Steigen: $n = 21$). See Table S1 in the supplementary information (SI) for detailed info. The nestlings were on average 69 days old (range: 44–87 days old), and the age and sex of the nestlings were estimated based on morphometric measurements as described in detail in Løseth et al. (2019a). All nestlings were sampled for body feathers (10–12) and blood (8 mL) as described in Løseth et al. (2019b). The body feathers were still growing and connected to the blood circulation at the time of sampling. Blood samples were collected in heparinised vacutainers and centrifuged at 860 *g*. The plasma was then transferred to cryotubes and stored at −20 °C. The sampling was approved by the Norwegian Food Safety Authority (Mattilsynet, 2015/6432 and 2016/8709) and the handling of the birds was in accordance with the regulations of the Norwegian Animal Welfare Act and EU legislation (3R).

2.2. Thyroid hormone analyses

In plasma, the thyroid hormones (THs) thyroxine (T4) and triiodothyronine (T3) are mainly bound to transport proteins, but a small fraction of the THs are also freely circulating (McNabb, 2007). The plasma samples were therefore analysed for free and total (bound) T4 (FT4 and TT4, respectively) and free and total T3 (FT3 and TT3, respectively). The THs were analysed at the Department of Biology at the Norwegian University of Science and Technology (NTNU), Norway, using a commercially available solid-phase radioimmunoassay (MP Biomedicals, LLC, USA) according to the assay description. The sensitivity of the assays was 7.6 ng/mL for TT4, 0.45 pg/dL for FT4, 0.067 ng/mL for TT3 and 0.06 pg/mL for FT3. Samples ($n = 70$) were analysed in triplicate and the mean (± standard error, SE) coefficient of variation (CV) between parallels was 11.1 ± 1.1% for TT3, 7.9 ± 0.9% for FT3, 5.4 ± 0.5% for TT4 and 5.5 ± 0.5% for FT4. Commercially available standard reference materials (SRMs) (Lyphochek Immunoassay Plus Control, Lot: 40330, Biorad Laboratories, CA, USA) and the laboratory's own reference material (chicken serum) were analysed for quality assurance. The reference materials were also analysed in triplicate and used to control for the performance of the kits for every fourth sample. The recovery of the commercial SRM ranged from 66–91% for TT4, 76–121% for FT4, 84–106% for TT3 and 88–112% for FT3. The molar concentrations of THs in plasma are available in the SI Table S4.

2.3. Feather corticosterone analyses

Concentrations of the nestlings' feather corticosterone ($CORT_f$, pg/mm) were assessed as an integration of their stress responses in the nest, as corticosterone hormones are deposited into the nestling feathers during growth (Bortolotti et al., 2008). The analyses were conducted at the Department of Biology at NTNU, Norway. One feather from the back of each nestling ($n = 69$) was washed twice with MilliQ-water, its calamus was removed, and the remaining feather was weighed (feather mass: 30–85 mg) and measured (feather length: 66–108 mm) before extraction. The feather was cut into small fragments ($< 3\ mm^2$) and extracted with 10 mL methanol (LiChrosolv®, HPLC grade, Sigma-Aldrich, USA) in polypropylene tubes according to Bortolotti et al. (2008). Samples were sonicated for 30 min at room temperature in a water bath, incubated overnight at 50 °C on a shaker at 45 rpm. The supernatant was collected and filtered using synthetic polyester fibre in a filtration cartridge (6 mL, Supelco, USA). Extracts were evaporated to dryness at 50 °C under a N_2 stream, reconstituted in

0.5 mL phosphate buffered saline (Sigma-Aldrich, USA) and frozen at −20 °C until analysis. A commercial enzyme immunoassay kit (Enzo Life Sciences, ADI-900-097, USA) was used to quantify corticosterone in the feathers according to the product manual and in accordance with previous studies (Bourgeon et al., 2014; Cruz-Martinez et al., 2015). The performance of the kit was validated for accuracy, precision and reproducibility using extracts from homogenised pools of feathers from white-tailed eagle nestlings, and also using extracts from pools of adult Western capercaillie (*Tetrao urogallus*) feathers because of low sample amount available for white-tailed eagle feathers. The sensitivity of the assay was 27 pg/mL, and samples were analysed in two assays with a mean (± SE) intra-assay CV of 8.8 ± 1.6% and an inter-assay CV of 12.7 ± 2.2%. Each sample was analysed in duplicate, and the mean CV ± SE was 5.5 ± 0.5%. The molar concentrations of corticosterone in the feathers are available in the SI Table S4.

2.4. Immune proteins

The method for the analyses of plasma proteins has been described in detail in a previous paper (Flo et al., 2019) and were performed at the Veterinary Central Laboratory at the Norwegian University of Life Sciences (NMBU), Norway. In brief, concentrations of total protein (n = 70) was determined by a biuret test using Siemens ADIVA 1800 automated chemistry analyser (Siemens Healthineers, Germany) and the plasma protein fractions were determined by a serum protein separation kit and Capillarys 2 instrument (Sebia®, Lisses, France). Concentrations of prealbumin, albumin, α_1-, α_2-, β- and γ-globulin fractions are expressed as mg/mL.

2.5. Blood clinical-chemical parameter analyses

The analyses of BCCPs were conducted using an automated spectrophotometrical analyser containing ion-selective electrodes (ADVIA, 1800; Siemens Healthineers, Germany) at the Central Laboratory at the Department of Small Animal Clinical Sciences, University of Copenhagen, Denmark. All plasma samples (n = 70) were analysed within six months after sampling according to Sonne et al. (2010). Daily internal quality controls and quarterly external quality controls were performed on the selected assays. Only results from accepted analytical runs are reported here.

The analyses included alkaline phosphatase (ALP; U/L), alanine aminotransferase (ALT; U/L), gamma-glutamyltransferase (GGT; U/L), total bilirubin (TB; mmol/L), creatinine (CRE; mmol/L) and bile acids (BA; mmol/L). For interpretation of the results, liver status was assessed by a panel of three enzymes (ALP, ALT and GGT) and two waste products metabolized by the liver (TB and BA), which may increase during liver impairment or disease. Kidney status and glomerular filtration was assessed by a panel of one muscle break-down product (CRE), an enzyme (GGT) and a waste product (BA), which may all increase during kidney impairment or disease. For biological relevance, all components of each panel should be interpreted together (Whalan, 2015). Further details on BCCP analysis can be found in Sonne et al. (2010, 2012).

2.6. Stable isotope analyses

The stable isotope analyses of nitrogen and carbon, as dietary tracers, have been described in detail in Løseth et al. (2019a) and were analysed at the MARE Centre of the University of Liège, Belgium. In brief, bulk stable carbon (^{12}C and ^{13}C) and nitrogen isotopes (^{14}N and ^{15}N) were analysed in a homogenate of cleaned feather material by an element analyser coupled to a mass spectrometer. The reported stable carbon and nitrogen isotope values are expressed as δ (‰) relative to the international reference standards Vienna PeeDee Belemnite and atmospheric nitrogen, respectively.

2.7. OHC analyses

The procedures used for the contaminant analyses of plasma samples has been described in detail in Løseth et al. (2019b). A total of 60 OHCs were targeted for the analyses, which were 23 PCBs, nine chlorinated pesticides (OCPs), seven PBDEs and 19 PFASs (see SI Tables S2–S3 for complete list). The samples were analysed for PCBs, OCPs and PBDEs at the Toxicological Centre at the University of Antwerpen, Belgium and for PFASs at the Norwegian Institute for Air Research in Tromsø, Norway. In brief, PCBs, OCPs and PBDEs were extracted from plasma using *n*-hexane:dichloromethane (DCM, 1:1, v:v). Fractionation of compounds was performed on Supelclean™ ENVI Florisil cartridges (500 mg, 3 mL, Supelco® Analytical), the compounds were then eluted with *n*-hexane:DCM and quantified according to Eulaers et al. (2011). PFASs were extracted from plasma using the Powley method (Powley et al., 2005) and quantified according to Herzke et al. (2009). Internal standards were added to all samples and concentrations were corrected for eventual losses during extraction. The recoveries ranged from 30–118% for PCBs, 41–90% for OCPs, 74–97% for PBDEs, and 59–101% for PFASs (Løseth et al., 2019b). For every tenth sample, a procedural blank was analysed to control for background contamination. To control the performance of the analytical method for the PCB, OCP and PBDE extraction, a human plasma sample from the Arctic Monitoring and Assessment Programme interlaboratory exercise was analysed for every 20th sample. To control the performance of the PFAs extraction, a commercially available human plasma sample (NIST SRM, 1957; USA) was analysed for every tenth sample. For the PCBs, OCPs and PBDEs, the limits of quantification (LOQs) were set to ten times the signal-to-noise ratio of sample runs or calculated as three times the standard deviation of the procedural blanks for each compound. For the PFASs, the LOQs were calculated as three times the signal-to-noise ratio for each compound. No background contamination of PFASs were encountered in the blanks. The concentrations of the compounds included in this study were previously reported in Løseth et al. (2019a) and were detected in over 50% of the plasma samples from each year and location.

2.8. Statistical analyses

The statistical analyses were performed using R (v. 3.4.2; R Development Core Team, 2008). All variables were investigated for influential outliers, normality and homoscedasticity (Zuur et al., 2010). Statistical significance was set to $\alpha = 0.05$ for all analyses. First, a principal component analysis (PCA) using the *vegan* package (Oksanen et al., 2018) was used to explore the relationships between all OHCs (Fig. S1). Since there was a clear separation between these groups in the PCA, separate PCAs were performed for the legacy OHCs (PCBs, OCPs and PBDEs) and PFASs. Then, we extracted the first principal component (PC1) from each PCA, $PC1_{OHCs}$ and $PC1_{PFAS,}$ which explained 71.4% and 63.7% of the variation of compounds within each group, respectively. The two PCs are referred to as legacy OHCs and PFASs throughout the results and discussion.

Due to the structure of the data, with multiple nestlings in some nests (Table S1), statistical tests from the *nlme* package (Pinheiro et al., 2018) were applied with nest identity as a random variable to control for pseudoreplication introduced by sampling multiple nestlings in the nests (Løseth et al., 2019a). Linear mixed effect models (LMM) were performed for each response variable separately with the explanatory variables and nest identity as a random factor, to investigate the direction and significance of the effects. The response variables were the THs, $CORT_f$, each fraction of plasma proteins and BCCPs. Outliers were removed from TB (nestling #70 = 47.1 nmol/mL and #60 = 6.1 nmol/mL), GGT (#29 = 28 U/L and #1 = 24 U/L) and BA (#13 = 0 nmol/mL), as these were several folds lower or higher than the mean concentrations of TB, GGT and BA, which were 19.0 nmol/mL, 6.6 U/L and 17.1 nmol/mL, respectively. The removed outliers were regarded as

Table 1
Median, minimum and maximum concentrations of thyroid hormones, plasma proteins, blood clinical-chemical parameters and feather corticosterone from white-tailed eagle nestlings sampled at Smøla and Steigen (Norway) in 2015 and 2016. The plasma protein concentrations are obtained from Flo et al. (submitted). The summed concentrations of 26 legacy OHCs congeners (Σ_{26}L.OHCs) and of 8 PFASs (Σ_8PFASs) which contributed to PC_{OHC} and PC_{PFASs}, respectively, were calculated from Løseth et al. (2019b). Mean and standard deviation of all concentrations are available in the Supplementary Information (SI, Table S5).

		Smøla						Steigen					
		2015 (n = 13)			2016 (n = 22)			2015 (n = 14)			2016 (n = 21)		
	unit	median	min	max	median	min	max	median	min	max	median	min	max
Total triiodothyronine (TT3)	ng/mL	2.37	0.95	4.52	2.17	1.43	3.69	2.08	0.96	2.61	1.67	0.96	3.32
Free triiodothyronine (FT3)	pg/mL	10.31	5.91	21.64	10.34	5.92	14.45	9.48	4.46	16.23	8.34	5.15	15.80
Total thyroxine (TT4)	ng/mL	16.42	2.44	14.07	18.84	2.25	14.78	15.82	11.25	20.84	19.95	31.41	27.53
Free thyroxine (FT4)	pg/mL	7.23	2.41	11.66	7.87	3.23	11.07	7.90	4.18	9.50	8.97	5.48	10.83
Feather corticosterone ($CORT_f$)	pg/mm	3.26	2.12	5.45	3.70	2.04	7.52	2.62[a]	1.48	3.81	2.88	1.52	5.88
Total protein	mg/mL	31.4	28.1	34.8	31.5	26.0	35.9	30.8	25.1	33.8	33.0	20.3	39.6
Prealbumin	mg/mL	4.95	2.72	7.89	4.21	2.83	6.62	4.22	2.89	4.88	3.37	1.85	6.63
Albumin	mg/mL	14.11	11.87	17.23	14.93	11.52	19.07	13.96	10.64	15.18	15.67	9.71	19.35
α_1-globulin	mg/mL	0.59	0.43	1.01	0.69	0.36	1.45	0.76	0.45	1.38	0.70	0.42	1.34
α_2-globulin	mg/mL	3.56	2.77	4.03	3.55	2.88	4.41	3.58	2.96	4.21	4.15	2.80	5.25
β-globulin	mg/mL	4.88	4.12	7.68	4.78	4.03	6.06	4.80	4.07	5.68	5.24	3.51	6.35
γ-globulin	mg/mL	2.72	2.22	3.50	2.48	2.00	4.45	3.14	2.21	5.23	3.60	2.01	4.52
Alanine aminotransferase (ALT)	U/L	15.00	8.00	21.00	19.00	14.00	40.00	15.50	9.00	29.00	19.00	12.00	32.00
Alkaline phosphatase (ALP)	U/L	1241	1024	1717	1551	1094	2442	1166	912	1801	1536	1026	2248
Total Bilirubin (TB)	nmol/mL	14.00	7.70	28.70	18.30	6.10	47.1	18.30	12.30	28.70	19.00	9.00	29.30
Gamma-glutamyl transpeptidase (GGT)	U/L	7.00	4.00	24.00	5.00	0.00	12.00	6.50	2.00	12.00	4.00	2.00	28.00
Bile Acid (BA)	nmol/mL	16.00	3.00	64.00	16.50	2.00	82.00	14.00	0.00	39.00	9.00	1.00	39.00
Creatinine (CRE)	mmol/mL	13.00	9.00	19.00	17.00	12.00	27.00	12.50	10.00	20.00	15.00	13.00	19.00
Σ_{26}Legacy OHCs[b]	ng/mL	4.02	1.77	15.21	7.40	2.92	51.14	9.91	6.08	113.88	9.20	2.89	49.28
Σ_8PFASs[c]	ng/mL	24.28	10.82	45.00	8.84	4.38	12.70	31.21	17.63	52.07	12.61	6.96	32.61

[a] Sample size, n = 13.
[b] Σ_{26}Legacy OHCs: CB 99, CB 101, CB 105, CB 118, CB 138, CB 153, CB 156, CB 170, CB 171, CB 177, CB 180, CB 183, CB 187, CB 194, OxC, TN, CN, *p,p'*-DDE, *p,p'*-DDT, HCB and *β*-HCH, BDE 47, BDE 99, BDE 100, BDE 153 and BDE 154 (Løseth et al., 2019b).
[c] Σ_8PFASs: L-PFOS, B-PFOS, PFOA, PFNA, PFDcA, PFUnA, PFDoA and PFTriA (Løseth et al., 2019b).

aberrations and not of biological relevance, as these individuals did not have elevated levels of the other BCCPs within the liver or kidney panel.

The response variables TT3, FT3, $CORT_f$, prealbumin, α_1-globulin, BA, ALT and CRE were $\log_e$ transformed prior to LMM to ensure normality of the residuals as required by the model assumptions. The initial full models included location, year, sex, PC_{OHCs}, PC_{PFAS}, $\delta^{13}C$, $\delta^{15}N$ and age at sampling as explanatory variables. The most parsimonious model for each response variable was selected based on AIC for small sample sizes (AICc) using *dredge* (*MuMIn* package; Barton, 2018) on models fitted with maximum likelihood (ML). Models were validated by normally distributed residuals, homogeneity of variances, and no collinearity between variables.

Because of the spatial and temporal differences found for OHCs, dietary tracers and age at sampling (Løseth et al., 2019a), model selection was also performed on models without location and year as we were interested in the variation of responses explained by OHCs, dietary tracers, sex and age. Parameter estimates were extracted from the final models fitted with restricted maximum likelihood (REML). The models from the final selection with ΔAICc of 0 are presented in Table 2, while the other most parsimonious candidate models with ΔAICc ≤2 from both the initial and final model selections are presented in the Supporting Information (Tables S6–S41). To complement AICc in Table 2, the marginal pseudo-R^2 (R^2_m; explaining the variation by the fixed factors) and conditional pseudo-R^2 (R^2_c; explaining the variation by both fixed and random factors) were extracted according to Nakagawa and Schielzeth (2013). Linear mixed effect ANOVAs from the *nlme* package (Pinheiro et al., 2018) were used to investigate potential differences in physiological variables between nestlings within the same nest and between nests of different brood sizes.

3. Results and discussion

3.1. Explanatory variables affecting the endocrine and physiological parameters

The concentrations of plasma THs, $CORT_f$, plasma proteins and selected BCCPs of the nestlings are listed in Table 1. The model selections with and without year and location as explanatory variables are further referred to as the initial and final model selection, respectively. The most parsimonious models from each selection are discussed and were highly comparable with each other.

3.1.1. Thyroid hormones

The concentrations of TT4 and TT3 (Table 1) in the present study were similar to those previously found in free-living bald eagle nestlings (aged 44 ± 15 days old, Cesh et al., 2010), and may indicate normal circulating thyroid concentrations of nestling eagles.

As hypothesised, significant negative relationships were found between legacy OHCs and FT3 and TT3, in both the initial and final model selection (Table 2 and S5-S9). The negative relationships detected between legacy OHCs and T3s are in accordance with studies on bald eagle (Cesh et al., 2010), Eurasian dipper (*Cinclus cinclus*; Morrissey et al., 2014), peregrine falcon (*Falco peregrinus;* Fernie et al., 2017; Smits and Fernie, 2013) and great blue heron (*Ardea herodias*; Champoux et al., 2017) nestlings.

The initial model selection of T4s indicated that the variation of plasma TT4 (t = 3.4, $p < 0.01$, Table S10) and FT4 (t = 1.8, $p = 0.08$, Table S12) was affected by sampling year. Thus, it is possible that the observed plasma variation of TT4 is linked to interannual variation of

external factors, such as prey availability and hence food consumption or weather conditions, as THs are involved in metabolism and thermoregulation (McNabb, 2007).

The final model selection indicated a significant negative relationship between PFASs and TT4 concentrations (Table 2) and a non-significant relationship between PFASs and FT4. As both legacy OHC and PFAS concentrations were significantly higher in nestlings sampled in 2015 than in 2016 at both locations (Løseth et al., 2019a, Table 1) it is likely that PFASs may contribute to some of the interannual variation of the T4s. The proteinophilic properties of PFASs cause these compounds to have high binding affinity to plasma proteins, which may in turn cause endocrine disruption by displacement of their natural ligands such as T4 and T3 (Jones et al., 2003; Ren et al., 2016). PCBs and PBDEs have also been shown to affect the binding of T3 to its main transport protein, prealbumin (Hill et al., 2018; Ucan-Marin et al., 2010). This may result in displacement of T3 from prealbumin and hence increased metabolism and excretion of FT3, resulting in lower T3 concentrations with higher OHC burdens (Scanes and McNabb, 2003).

Numerous studies have found negative relationships between OHCs and circulating TH concentrations in nestlings (Cesh et al., 2010; Champoux et al., 2017; Fernie et al., 2017; Morrissey et al., 2014; Smits and Fernie, 2013). However, Nøst et al. (2012) found positive relationships between PFASs and TT4 in black-legged kittiwake (*Rissa tridactyla*) and northern fulmar (*Fulmarus glacialis*) nestlings, and between T3s and legacy OHCs in black-legged kittiwake nestlings. Birds have different strategies during early development and are characterized along a gradient describing the degree of development at hatching, from altricial (incomplete) to precocial (advanced) (Starck and Ricklefs, 1998). Precocial birds hatch with open eyes, down, and leave the nest within a few days as they rapidly develop good thermoregulatory control following hatching. Altricial birds hatch with closed eyes, with little or no down, and require complete parental care as they develop thermoregulatory control at a later stage (Starck and Ricklefs, 1998). As thyroid hormones regulate parts of the metabolism associated with heat generation, the developmental patterns of thyroid function are markedly different in precocial versus altricial nestlings (McNabb, 2007). Of the species mentioned in the discussion above, the black-legged kittiwakes are semi-precocial, while white-tailed and bald eagles are semi-altricial, and northern fulmars, Eurasian dippers and Great blue herons are altricial (Starck and Ricklefs, 1998). Such differences in developmental strategies may influence the direction of relationships between THs and OHCs in nestlings of different species (McNabb et al., 1984; Starck and Ricklefs, 1998; Vjboh et al., 1996). However, the sampling of nestlings in field studies usually occurs when the nestlings are older, fully capable of thermoregulation, and the developmental differences related to TH may therefore be less pronounced. Differences in relationships between plasma THs and OHCs may also originate from the cyclic patterns of circulating TH induced by thyroid stimulating hormone (TSH). Production and release of TSH from the pituitary increase TH release from the thyroid gland and may thereby introduce fluctuations of plasma TH concentrations (McNabb, 2007). Regardless of the direction of the correlations (negative/positive), these previously reported significant correlations between THs and OHCs in nestlings mentioned above collectively suggest a potential for OHCs to affect the TH system at some level.

3.1.2. Feather corticosterone

The corticosterone concentrations in the feathers (Table 1) were in the same range as those found in feathers of common buzzard (*Buteo buteo*, Martínez-Padilla et al., 2013) and red kite (*Milvus milvus*) nestlings (Monclús et al., 2018). These concentrations may indicate that the nestlings did not experience chronic stress during their time in the nests, as they are not particularly high compared to concentrations previously reported in feathers of birds of prey (López-Jiménez et al., 2017).

The initial model selection of $CORT_f$ identified significant differences in $CORT_f$ between locations (Table S14) and indicated that the Smøla nestlings may have experienced more stress during their time in the nests than the Steigen nestlings ($t = -3.3, p < 0.01$). Yet, the final model selection of $CORT_f$ indicated a significant negative relationship between $CORT_f$ and PFASs (Table 2).

Exposure to OHCs has previously been proposed to interact with stress responses in birds (Boonstra, 2004; Tartu et al., 2014a). However, no papers were available for specific comparisons on the effects of PFASs on $CORT_f$ concentrations in birds, and as far as we know this is the first study investigating this potential relationship. Tartu et al., 2014 found significant negative effects of perfluorotridecanoic acid (PFTriA) and perfluorotetradecanoic acid (PFTeA) on baseline plasma

Table 2
Model estimates from the most parsimonious models (ΔAICc = 0.00) of the final model selection explaining the variation of endocrine and physiological parameters in plasma of white-tailed eagle nestlings ($n = 70$) from Smøla and Steigen. The table includes the model intercept (β_0), model estimates (β_x), significance values (p), marginal pseudo-R^2 (R^2_m), which presents variation explained by the explanatory variables, and conditional pseudo-R^2 (R^2_c), which presents the total variation explained by explanatory variables, and the random factor (nest). The sex variable represents males. Beta estimates follow the order of the factors in the models. Statistical significances ($\alpha = 0.05$) are marked with *. Additional candidate models (ΔAICc < 2) are listed in the supplementary information (Table S5 – S40).

Endocrine and physiological parameters	Explanatory variables	β_0	β_1	β_2	β_3	*p-values*	R^2_m	R^2_c
Total triiodothyronine (TT3)	Legacy OHCs	0.69	−0.90			0.04*	0.05	0.76
Free triiodothyronine (FT3)	Legacy OHCs + age	−4.27	−0.16	−0.01		< 0.01*; 0.11	0.15	0.77
Total thyroxine (TT4)	PFASs	18.85	−2.06			0.01*	0.12	0.61
Free thyroxine (FT4)	PFASs	0.01	−0.001			0.12	0.04	0.56
Feather corticosterone ($CORT_f$)[a]	PFASs	1.10	−0.15			0.02*	0.08	0.14
Total protein	1	31.70					0.00	0.30
Prealbumin	Legacy OHCs + age	1.03	0.10	0.01		0.04*; 0.10	0.08	0.45
Albumin	1	14.77					0.00	0.60
α_1-globulin	$\delta^{15}N$ + sex	−1.55	0.09	−0.15		0.05*; 0.04*	0.11	0.36
α_2-globulin	1	3.70					0.00	0.65
β-globulin	$\delta^{13}C$	2.23	0.03			0.11	0.04	0.38
γ-globulin	1	3.05					0.00	0.44
Alanine aminotransferase (ALT)	PFASs + Legacy OHCs + sex	2.81	−0.18	0.08	0.13	< 0.01*; 0.04*; 0.03*	0.25	0.55
Alkaline phosphatase (ALP)	PFASs + Legacy OHCs	1503.2	−267.66	−89.44		< 0.01*; 0.06	0.23	0.71
Total Bilirubin (TB)[b]	$\delta^{13}C$ + sex	−35.67	−2.83	2.78		0.01*; < 0.01*	0.15	0.90
Gamma-glutamyl transpeptidase (GGT)[b]	PFASs + $\delta^{15}N$	26.70	2.37	−1.49		< 0.01*; < 0.01*	0.26	0.37
Bile Acid (BA)[c]	Legacy OHCs + sex	14.68	5.57	5.09		0.02*; 0.10	0.11	0.66
Creatinine (CRE)	PFASs	2.69	−0.16			< 0.01*	0.17	0.65

[a] Sample size, $n = 69$.
[b] Two outliers were removed from these models, $n = 68$.
[c] One outlier was removed from this model, $n = 69$.

corticosterone in breeding black-legged kittiwakes, which indicate a potential interference by PFASs on stress responses in birds. However, corticosterone measured in feathers and plasma should not be directly compared as they present different time points (Bortolotti, 2008), even though studies have found significant correlations between the two matrices (Fairhurst et al., 2013). Previous studies have reported positive correlations between legacy OHCs and corticosterone concentrations measured in feathers (Monclús et al., 2019, 2018) and plasma of birds (Bowerman et al., 2002; Nordstad et al., 2012; Verboven et al., 2010). However, a lack of correlations has also been reported between legacy OHCs and corticosterone in both feathers and plasma (Bourgeon et al., 2012; Tartu et al., 2014a). Even though the nestlings sampled at Steigen had significantly higher concentrations of both legacy OHCs and PFASs than those from Smøla (Løseth et al., 2019a), no relationships were found between legacy OHCs and $CORT_f$.

Both external (i.e. predators, parental investment, weather, disturbance) and internal factors (i.e. infections, starvation, competition) have been shown to induce stress in birds (Boonstra, 2004; Fairhust et al., 2012a,b; Legagneux et al., 2013; Romero et al., 2000). The different topography of the two sampling locations generates more prevalent and stronger winds at Smøla than Steigen. Smøla is also further south than Steigen, and the temperatures at Smøla were roughly 1 °C higher than at Steigen during the nestling periods in 2015 and 2016 (data obtained from The Norwegian Meteorological Institute, 2018). Weather and other environmental conditions have been shown to induce variation in corticosterone concentrations in birds (Fairhurst et al., 2012b; Legagneux et al., 2013; Romero et al., 2000), and may offer an explanation of the differences in $CORT_f$ between the locations.

3.1.3. Plasma proteins

The concentrations of plasma proteins in the nestlings have been discussed in a previous paper (Table 1; Flo et al., 2019) and were within the ranges of those previously reported in apparently healthy free-living bald eagle (Bowerman et al., 2000) and peregrine falcon nestlings (Lanzarot et al., 2001). As previously suggested by Flo et al., 2019, these values do not indicate suppression or activation of the immune system in the nestlings.

Significant relationships were detected between OHCs and prealbumin in both the initial and final model selection (Table 2 and S18-S19). In the initial selection, significant positive relationships were detected between prealbumin and both PFASs ($t = 2.47$, $p = 0.02$) and legacy OHCs ($t = 2.73$, $p = 0.01$) when location was included in the model ($t = -3.94$, $p < 0.01$). However, the final model selection only included a significant positive relationship between prealbumin and legacy OHCs (Table 2), a relationship which was contrary to our hypothesis. This relationship may be by chance as there are no indications of immunomodulation in nestlings with higher OHC burdens, as none of the other plasma proteins displayed significant relationships with OHCs (Table 2, Table S16-S17 and S20-S29).

The final model selection of α_1-globulins indicated significantly higher concentrations in nestlings feeding at higher trophic positions ($\delta^{15}N$) and in males than females (Table 2). As a positive APP, α_1-globulins may increase in response to inflammation (Cray et al., 2009; Reilly and Eckersall, 2014). The white-tailed eagles feed at higher trophic positions in marine environments, and prey items such as migrating birds may increase their exposure to inflammatory pathogens and parasites (Krone et al., 2004; Leung and Koprivnikar, 2016).

The final models of the remaining plasma proteins indicated that the variation of total protein, albumin, α_2-and γ-globulins were best explained by the random factor (nest identity), which indicates that the variation of plasma proteins was related to differences between the nests, rather than any of the explanatory variables (Table 2). These findings correspond with a previous study on white-tailed eagle nestlings, which did not find significant effects of OHCs on immunoglobin Y concentrations (Sletten et al., 2016), one of the major components of the γ-globulins in birds. In contrast, a study on peregrine falcon nestlings from the Canadian Great Lakes area found significant relationships between plasma proteins and OHCs in nestlings with OHC burdens several folds higher than the present nestlings (Smits and Fernie, 2013). This may suggest that the absence of significant relationships between plasma proteins and OHCs in the present nestlings may be due to their relatively low OHC burdens.

3.1.4. Blood clinical-chemistry parameters

The plasma concentrations of ALT, TB, BA and CRE (Table 1) were similar to those previously found in free-living white-tailed eagle nestlings (Sonne et al., 2010, 2012). The ALP and GGT concentrations (Table 1) were also similar to the previous studies, however some of the ALP and GGT concentrations measured in nestlings from 2015 to 2016, respectively, exceeded previously recorded ranges (Sonne et al., 2010, 2012). Nevertheless, the concentrations of the selected BCCPs indicated normal liver and kidney function as the values were within the reference ranges for nestlings and birds of prey (Campbell, 2012; Whalan, 2015). Year to year variations are likely explained by differences in e.g., diet, or driven by external conditions such as temperature, precipitation and wind (Sonne et al., 2012).

The initial model selection of the BCCPs indicated significant differences between years for ALT (Table S30) and GGT (Table S34) concentrations in the liver panel, as well as for CRE (Table S40) in the kidney panel. The initial model selection of GGT and BA concentrations (Table S38), which are present in both the liver and kidney panel, indicated significant differences between nestlings at the two locations.

Contrary to our hypothesis of increased concentrations of BCCPs in the liver panel with increasing OHCs, the final model selections indicated significant negative effects of PFASs on ALT and ALP concentrations (Table 2). Similarly, a previous study on white-tailed eagle nestlings also found a significant negative correlation between ALT and PFASs (Sonne et al., 2012). However, when considering all components of each panel collectively to determine possible biological significance (Whalan, 2015), the present values indicate no signs of PFASs-induced organ effects or lesions in the studied nestlings.

Interestingly, the final models of the liver panel also indicated significant positive relationships between legacy OHCs and ALT and BA according to our hypothesis (Table 2). The significant relationship between ALT and legacy OHCs correspond with previous studies on white-tailed eagle nestlings (Sonne et al., 2010, 2012) and adult great skuas (*Stercorarius skua*, Sonne et al., 2013). No other significant positive correlations were found between OHCs and the remaining four components of the liver panel, so it is unclear exactly how and if hepatoxicity occurs in the present nestlings with higher OHC burdens (Whalan, 2015).

For the last parameter in the liver panel, TB, the models indicated a significant negative relationship with $\delta^{13}C$, and increased concentrations in plasma TB of males compared to females. The final ALT and BA models also indicated differences between female and male nestlings (Table 2). Additional models were run for each sex separately to further investigate if these differences could influence the relationship between explanatory variables and TB, ALT and BA. However, the three most parsimonious models for TB, ALT and BA for each sex included similar relationships (negative/positive) with the same factors as shown in Table 2. Previous studies regarding BCCPs in nestling birds of prey have not found differences between sexes (Bowerman et al., 2000; Hernández and Margalida, 2010; Limiñana et al., 2009), which is suggested to be a result of sexual immaturity at the time of sampling. As there are no other significant differences between sexes regarding physiological parameters in the present study, the reason for the sex differences of TB, ALT and BA in the present nestlings is unknown. Similar to TB and $\delta^{13}C$, a significant negative relationship was also found for GGT and $\delta^{15}N$. No previous studies have reported similar relationships, and again it is unclear if it could be related to trophic position or the amount of lipid-rich or marine versus terrestrial prey species in their diets (Sonne et al., 2012, 2010).

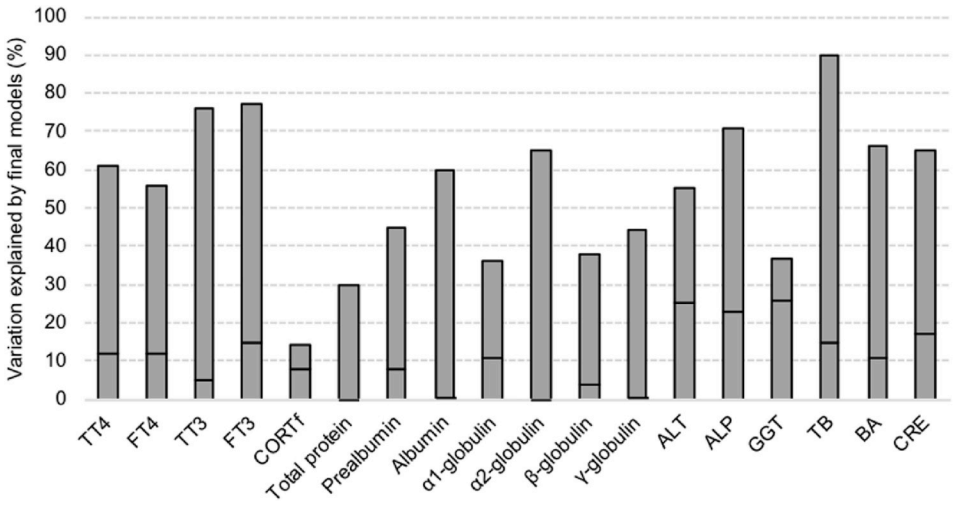

Fig. 1. Bar chart indicating the total variation of the physiological parameters explained by the final models presented in Table 2. The bars present the variation explained by both explanatory variables and the random factor (nest variation) in the models (the conditional pseudo-R^2, R^2_c). The black line in the bars indicate the variation explained by only the explanatory variables (the marginal pseudo-R^2, R^2_m).

Both BA and GGT contribute to the liver and the kidney panel. The final BA model mentioned above, with a positive relationship between BA and legacy OHCs, corresponds to our hypothesis and may indicate potential liver and/or kidney effects induced by legacy OHCs. The final GGT model also corresponds with our hypothesis and indicates a significant positive relationship between PFASs and GGT. However, the final CRE model indicated a significant negative relationship between PFASs and CRE, the last component of the kidney panel. No significant relationships have previously been found for BA, GGT and CRE with legacy OHCs or PFASs in white-tailed eagle nestlings (Sonne et al., 2012, 2010). As all BCCP components of each panel should normally be interpreted together to indicate biological relevance (Whalan, 2015), the contrasting relationships with components in both panels suggest that there are likely no clear contaminant induced effects to the liver or kidneys of the present nestlings, although differential effects of contaminants on different parameters cannot be ruled out. Thus, the current study identifies to some extent effects on certain BCCP components of each panel, but these cannot be characterized as clear OHC-induced effects.

3.2. Biological relevance

Despite significant relationships between OHCs and the analysed parameters in the present study, legacy OHCs and PFASs only explained between 5 and 15% of the variation in TH, $CORT_f$ and prealbumin, and less than 26% of the variation in ALT, ALP, GGT, BA and CRE (Fig. 1 and Table 2). Most of the variation of the physiological parameters in the models were introduced by variation between the nests (R^2_c, Table 2), which was included as a random factor. This variation between nests could be influenced by parental experience and investment (Fairhust et al., 2012a) and thus food availability (Sonne et al., 2012). Sibling rivalry may also influence physiological parameters in nestlings from broods with multiple nestlings (López-Jiménez et al., 2016). However, in the present study there were no statistically significant differences regarding brood size or between siblings for the investigated physiological variables ($F = 0.01–3.5$, $p > 0.06$). This random nest factor may therefore represent natural variation of the physiological parameters between nestlings, which can be expected as all parameters were within the ranges previously found for seemingly healthy and free-living nestlings (Cesh et al., 2010; Flo et al., 2019 ; Martínez-Padilla et al., 2013; Sonne et al., 2012). Overall, the initial models including year and location offered more parsimony with lower AICc than the final models (excluding year and location). This indicates that spatial and temporal variation may have significantly contributed to the variation of the analysed physiological parameters. Some of these variations are expected as the nestlings may have different physiological responses to changes in the environment. As the models indicate only a minor influence of OHCs on the physiological parameters, it is unknown if these relationships present biologically relevant information of potential OHC-induced effects in the present white-tailed eagle nestlings. However, these relationships may indicate potential detrimental effects on the health of nestlings when exposed to higher OHC concentrations than the present study, and/or to other environmental challenges such as low food availability or infections. As birds accumulate more pollutants over time (Jaspers et al., 2013; Løseth et al., 2019a), the biological relevance of these potential OHC-induced effects cannot be ruled out later in life.

4. Conclusion

Our study indicated that PFASs and legacy OHCs have only a minor influence on the variance of thyroid and corticosterone hormones, prealbumin and parameters reflecting liver and kidney status (ALT, ALP, GGT, BA and CRE) in the white-tailed eagle nestlings. However, the plasma concentrations of PFASs and legacy OHCs in these nestlings were generally low, as previously reported (Løseth et al., 2019a). Most of the variation of the physiological parameters was explained by variation between nest sites (the random factor), which may be due to natural variation of physiological parameters between nestlings. The concentrations of the investigated physiological parameters were in the range of those previously reported for other free-living nestlings and indicated that the present nestlings were in generally good health. Nevertheless, we cannot rule out a greater potential of OHC-induced effects on the investigated parameters in nestlings or adults exposed to higher OHC concentrations than the present nestlings.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108586.

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