Dissimilar effects of organohalogenated compounds on thyroid hormones in glaucous gulls

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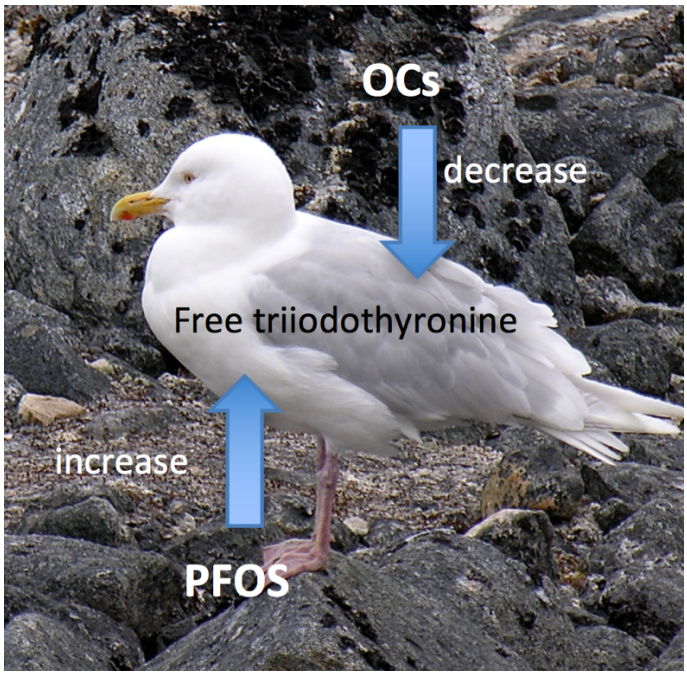
Highlights

- The influence of POPs on thyroid hormones (THs) in glaucous gulls were studied.
- There were inverse associations between several chlorinated compounds and FT3.
- There was a contrasting positive association between PFOS and FT3.
- POPs could affect THs in a complex manner involving both additive and antagonistic effects.

Graphical Abstract



Abstract

The glaucous gull (*Larus hyperboreus*) is an arctic top predator and scavenger exposed to high levels of mixtures of organohalogenated contaminants (OHCs) of which many interfere with the thyroid hormone (TH) system. In the present study, we applied statistical modelling to investigate the potential combined influence of the mixture of chlorinated, brominated and perfluorinated organic compounds in plasma of glaucous gulls on their plasma TH concentrations. In females, there were significant negative associations between several organochlorinated compounds (OCs) and free thyroxin (FT4) and triiodothyronine (FT3), indicating additive negative effects on FT4 and FT3. However, in these females there was also a significant positive association between perfluorooctane sulfonate (PFOS) and FT3. The inverse associations between several OCs and FT3 and the contrasting positive association between PFOS and FT3, indicate that these two groups of OHCs may have dissimilar and antagonistic effects on FT3 in female glaucous gulls. In males, there were no associations between any of the OHCs and the THs. That OHCs affect THs in a complex manner involving both additive and antagonistic effects add to the challenge of interpreting the overall functional effect of thyroid disruptive chemicals in wildlife. However, experimental studies are needed to confirm or disprove such effects.

Keywords: Arctic, Svalbard, pollution, additive effects, antagonistic effects, TDC

INTRODUCTION

Arctic wildlife is exposed to complex mixtures of organohalogenated contaminants (OHCs) such as organochlorinated compounds (OCs) which includes polychlorinated biphenyls (PCBs) and organochlorinated pesticides, organobrominated compounds such as brominated flame retardants (BFRs), and poly- and perfluorinated alkylated substances (PFASs) (Letcher et al., 2010). Chlorinated and brominated organic compounds have lipophilic properties and are associated with lipids in organisms, whereas PFASs have amphipathic properties and are associated with proteins in organisms (Jones et al., 2003). The physicochemical properties of many OHCs cause them to be present in biota in pristine arctic areas even decades after their production and use was banned (Letcher et al., 2010). High concentrations of OHCs may cause reproductive, behavioral and developmental stress by having disruptive effects on endocrine systems in arctic animals (Letcher et al., 2010).

The glaucous gull (*Larus hyperboreus*) is a predator and scavenger occupying a top position in the arctic marine food web (Anker-Nilssen et al., 2000). Since the 1970s, high levels of long-transported OHCs have been reported in this species (Bourne and Bogan, 1972; Letcher et al., 2010). Exposure to OHCs through the diet combined with the somewhat restricted capacity for biotransformation of these compounds in glaucous gulls makes the species susceptible for bioaccumulation of high levels of such compounds (Verreault et al., 2005). Previous studies have demonstrated thyroid disruptive potential of OHCs in glaucous gulls (Ucan-Marin et al., 2010; Ucán-Marín et al., 2009; Verreault et al., 2004; Verreault et al., 2007) and a growing body of evidence proposes that arctic wildlife, including glaucous gulls, are being adversely affected by high body burden of various OHC compounds at the population level (Erikstad et al., 2013; Nuijten et al., 2016).

The thyroid hormone (TH) system controls pre- and postnatal development, thermoregulation, lipid metabolism, moulting and several other physiological processes

(McNabb, 2000). Thyroxine (T4) is secreted by the thyroid gland and transported by TH-transporting proteins, such as transthyretin (TTR) and albumin, to peripheral tissues where it undergoes enzymatic deiodination to the more biologically active triiodothyronine (T3) (McNabb, 2000). Maintenance of normal functioning circulatory concentrations of THs is essential in sustaining good health.

OHCs have been reported to cause lowered plasma concentrations of TH, and concentrations of circulating THs are proposed to be useful biomarkers of effect of these compounds in wildlife studies (Skaare et al., 2002). Thyroid disruption by OHCs may occur by direct toxic effects in the thyroid gland, interference with the TH synthesis, enzymatic deiodination or metabolism, binding to TH receptors in tissues, or transport mechanisms in the plasma. Previous studies have shown that several OHCs may displace T4 and T3 in glaucous gull TTR and albumin (Ucan-Marin et al., 2010; Ucán-Marín et al., 2009). Competitive displacement of THs from TTR have also been demonstrated for PCBs, polybrominated diphenyl ethers (PBDEs), and their hydroxylated metabolites (OH-PCBs and OH-PBDEs), PFASs, hexachlorobenzene (HCB) and metabolites of dichlorodiphenyltrichloroethane (DDT) in other species (Meerts et al., 2000; Van den Berg et al., 1991; Weiss et al., 2009). Such displacement of THs from transport proteins is thought to increase the biliary excretion rates of particularly T4, and has been suggested as a central mechanism of TH disruption by OHCs.

Since thyroid disruptive compounds (TDCs) may act via several different modes of actions, the complex mixtures of OHCs that wildlife are exposed to may cause combined or interactive effects on the plasma concentrations of THs, which are considered as physiological important endpoints for TDC effects. The presence of different compounds with similar and dissimilar modes of actions (Bliss, 1939; Loewe and Muischneck, 1926; Plackett and Hewlett, 1952) with respect to thyroid effects may cause complex combined effects, such

as additive, antagonistic, synergistic and potentiation effects. In addition, there may be complex non-monotonous dose-dependent variations in TH-effects caused by exposure to multiple chemicals (Crofton et al., 2005).

Mixture effects on THs of multiple compounds and groups of chlorinated and brominated organic compounds have been studied in arctic animals, indicating that both additive and antagonistic effects are present (Villanger et al., 2011). A study on northern fulmar (*Fulmar glacialis*) and black-legged kittiwake (*Rissa tridactyla*) chicks showed positive associations between levels of PFASs and plasma concentrations of free and total T4 (Nøst et al., 2012). This is in contrast to the previously reported negative associations between OCs and plasma T4 in adult male glaucous gulls (Verreault et al., 2004). Since levels of OCs, BFRs and PFASs are high in glaucous gulls from the Norwegian Arctic (Verreault et al., 2005; Verreault et al., 2007) there may be mixture effects of these contaminant groups on circulating TH concentrations in this species.

The aim of the present study was to apply statistical modeling to investigate the potential combined effects on thyroid hormones in glaucous gulls caused by the mixture of chlorinated, brominated and fluorinated anthropogenic compounds in their plasma. Thus, plasma concentrations of 28 chlorinated, 10 brominated, 16 per-and polyfluorinated compounds, and total (T) and free (F) T4 and T3 were analyzed in glaucous gulls breeding in Kongsfjorden, Svalbard. Associations between these contaminants and the THs were examined using principle component analysis (PCA) and correlation analyses. The effects of the contaminants on the THs were also modeled using orthogonal partial least-squares (OPLS) regression analysis. The effects of possible confounding factors, such as sex and body condition were also investigated and taken into consideration.

MATERIALS AND METHODS

Sampling

A total of 39 breeding glaucous gulls were collected during the second half of the incubation period at the Kongsfjorden area, Spitsbergen, Norway (78°55′N 11°56′E) in June 2011, 2012 and 2013. All glaucous gulls sampled were breeding, thus the age of the birds were expected to be four years or older (Gaston et al., 2009). Blood was drawn from the branchial veins and kept cool and dark until centrifugation within 8 hours, and the plasma was frozen at -20 °C. Biometric measures (body mass, bill length, gonys height, head length and wing length) were recorded. Sex was determined biometrically or by molecular determination. Further details on sampling procedures and sex determination are given in the Supporting Information. The project was approved by the Governor of Svalbard (2010/00053-8, 2011/01095-42 and 2013/00050-28) and the sampling and handling of the birds were in accordance with the regulations of the Norwegian Animal Welfare Act.

Contaminant and lipid content analysis

Analysis of OHCs in plasma samples were performed at the Norwegian Institute of Air Research (NILU) in Tromsø, Norway. The compounds analyzed included PCBs (12 congeners), chlorinated pesticides (chlordanes [CHLs], DDT and its metabolites, hexachlorocyclohexane [HCH] and its isomers, hexachlorobenzene [HCB]), PBDEs (10 congeners) and PFASs (16 compounds). Quantification was conducted by the internal standard method. Because of the differing properties of the compounds (lipophilic vs amphipilic) and because OHC concentrations in wildlife studies and in ecological wildlife risk assessments of OHCs usually are provided on a mass/mass basis (Letcher et al. 2010, Dietz et al. 2015, Nuijten et al. 2016) contaminant concentrations are given on a wet weight (ng/g ww) basis assuming that the density of the plasma was 1.025g/mL. In addition, information on

plasma lipid content (%) is given in the results, allowing for estimation of the lipid weight concentrations.

The method for analysis of chlorinated and brominated organics is described previously (Herzke et al., 2009). Briefly, plasma samples (500 µL) were extracted with *n*-hexane after denaturation with ethanol and a saturated ammonium sulfate solution in water. Clean-up on Florisil columns, separation on an Agilent Technology 7890 gas chromatograph and detection on an Agilent Technology 5975C mass spectrometer were performed (Herzke et al., 2009). Limits of detection (LOD) were defined as three times the noise or blank signal if these exceeded the instrumental detection limits. Blanks and standard reference materials (SRM) (1958 human serum, NIST, USA) were analyzed every fifteenth sample for quality assurance of the results. Blank contamination was observed for γ-HCH, HCB, *trans*-chlordane, *cis*-chlordane, *oxy*-chlordane, *trans*-nonachlor, *cis*-nonachlor, *p,p*'-DDE and PCB-28. The SRM analyses were within the given limits of accuracy.

The methods for analysis of PFAS are described in detail previously (Powley et al., 2005). Briefly, extraction of plasma samples (200 µL) was conducted using methanol, before matrix removal by ENVI-Carb graphitized carbon absorbent and glacial acetic acid. Quantification was conducted by an ultra-high pressure liquid chromatography triple-quadrupole mass spectrometry (UPLC-MS/MS). LOD was defined as three times the noise or blank signal. Blanks and SRM (1958 human serum, NIST, USA) were analyzed every fifteenth sample for quality assurance of the results. No contamination of the blanks was observed. The SRM analyses were within the given limits of accuracy.

Enzymatic quantification of lipid content of the blood was performed by Unilab Analysis AS, Tromsø, Norway based on levels of triglycerides, free cholesterol, total cholesterol and phospholipids (Akins et al., 1989). The total lipid concentration was converted to plasma lipid percentage of the wet weight samples.

Thyroid hormone analysis

TH analysis by commercially available radioimmunoassay (RIA) Coat-A-Count kits (Siemens medical solution, Diagnostics, LA, USA) was performed at NTNU, Trondheim, Norway. The method is a sensitive technique also for analysis in birds and the kits have been verified for glaucous gulls (Verreault et al., 2004; Verreault et al., 2007). The procedure was followed as described in the kit descriptions. Plasma samples were analyzed for free and total T3 (FT3 and TT3) and free and total T4 (FT4 and TT4). Average coefficient of variation between parallels was 3 % for TT3 (two parallels), 5 % for FT3 (two parallels), 5 % for TT4 (three parallels) and 6 % for FT4 (three parallels). Standard reference materials (SRMs) (human serum, Biorad Laboratories, CA, USA) and the laboratory's own SRM (bovine serum) were analyzed for quality assurance.

Statistical analysis

Compounds detected in minimum 60 % of the individuals were included in the statistical analysis. Samples in which compounds were under LOD were assigned a random concentration between 0 and the compound specific LOD. Univariate and descriptive statistics were conducted using software SPSS (version 21.0, IBM SPSS Inc., IL, USA). Multivariate data analyses were performed using SIMCA P+ (version 12.0.0.0, Umetrics, Sweden).

Shapiro-Wilk's W-test was used to test for normality. Variables that were not normally distributed were log 10-transformed for use in parametric statistics. Pearson correlations were used for bivariate correlation analyses. Statistical significance was set to $p \leq 0.05$, and p values were two-tailed. The Levene's test was used to assess equality of variance. One-way ANOVA was applied to examine if there were differences in OHC or TH

concentrations among the sampling years. Independent samples T-tests were conducted to test differences in variables between males and females. Bonferroni correction was not applied when comparing associations between multiple variables because of the increased probability of producing false negatives (Moran, 2003).

A body condition index (BCI) was calculated for each individual using principal component analysis (PCA). A single measure of size was calculated from total head length, wing length and gonys height separately for males and females, since the glaucous gulls are sexually dimorphic (Sagerup et al., 2009b). The first principal component (PC1) from the size measure was regressed against the body mass in a linear regression model and the individual BCI was defined as the standardized residual of the regression.

Orthogonal partial least-squares (OPLS) regression was used to model the effects of OHC concentrations and biological variables (X, predictor variables) on TH levels (Y, response variables), and the effect of the OHCs, plasma lipid content and THs on BCI. OPLS regression is useful when the dataset is characterized by a limited number of observations and high degree of multicolinearity among the variables. For each OPLS model, a calculated R^2Y describes the dispersion of the data from the model, and Q^2 shows the cross-validation of the model. An $R^2Y>0.7$ and $Q^2>0.4$ characterize an acceptable, or good model for biological data (Lundstedt et al., 1998). A coefficient plot describes the relationships between the X variables and the Y variable, and a variable importance in projection (VIP) plot reflects the relative importance of each *X* in explaining *Y*. Predictors with VIP value>1 have the most explanatory power of *Y* (Sørmo et al., 2011). An ANOVA of the cross-validated residuals (CV-ANOVA) was applied for each OPLS model to test significance (Eriksson et al., 2008). The OPLS models were optimized by step-wise removing X-variables with lowest VIP values until achieving a significant model.

RESULTS

The body mass (mean, standard deviation, range) of the male and female gulls were 1760 g (standard deviation [SD] =109, range 1535 – 20130) and 1442 g (SD = 86, range 1315 – 1700), respectively. The BCI of the males and females were 0.00 (SD = 0.96, range -1.77 – 2.26) and 0.0 (SD = 0.96, range -1.34 – 2.24), respectively. The plasma lipid content of the male and female gulls were 1.44% (SD = 0.16, range 1.17 – 1.69) and 1.47% (SD = 0.22, range 0.97 – 1.98), respectively.

Plasma concentrations of OHCs

There were no significant differences in OHC or TH levels between individuals captured in 2011, 2012 and 2013, respectively (one-way ANOVA), and data from the three years were thus combined to increase statistical power. A PCA showed that differences between male and female glaucous gulls were evident for both OHC levels and patterns, and results are thus presented separately for the two sexes (Table 1). Median plasma sum (∑) PCB and ∑pesticides concentrations were 61% and 41% higher in males than in females, respectively. Although concentrations of PFOA were significantly higher in females than in males, and concentrations of PFDoA, PFTrA and PFTeA were significantly higher in males than in females, there were no difference in ∑PFASs between the sexes (Table 1). Furthermore, ∑PBDE concentrations did not differ between the sexes (Table 1). The order of the concentrations (ng/g ww) of the main OHC groups relative to ∑OHC in male glaucous gulls was: ∑PCBs (69%) > ∑pesticides (18 %) > ∑PFASs (12 %) > ∑PBDE (1%). For female glaucous gulls, the order was: ∑PCBs (46%) > ∑PFASs (37%) > ∑pesticides (16%) > ∑PBDE (1%). Regarding the PFASs, perfluorooctane sulfonate (PFOS) was the dominating compound in both sexes (Table 1). It should be noted that presenting the results on a molar

basis may have resulted in some changes in the relative (molar) concentrations among the compounds.

Table 1. Mean, standard deviation (SD), median and range plasma concentrations (ng/g wet weight) of organohalogenated contaminants quantified in more than 60% of the samples of male and female glaucous gulls (*Larus hyperboreus*) breeding in Kongsfjorden, Svalbard during the summer of 2011, 2012 and 2013. Asterisks devote statistical differences between males and females, * $p<0.05$, ** $p<0.01$.

	Males				Females			
Compound	N	Mean ± SD	Median	Range	N	Mean ± SD	Median	Range
β-HCH**	15	0.56 ± 0.30	0.45	0.17 – 0.99	24	0.28 ± 0.17	0.24	0.08 – 0.69
HCB*	15	10.04 ± 3.78	9.93	4.36 – 15.90	24	7.49 ± 2.40	7.45	3.97 – 15.10
c-chlordane	15	0.02 ± 0.01	0.02	0.01 – 0.33	22	0.02 ± 0.01	0.02	0.01 – 0.08
Oxy-chlordane*	15	17.26 ± 12.56	11.40	4.74 – 41.90	24	8.68 ± 6.19	5.32	1.95 – 25.20
t-nonachlor	15	0.99 ± 0.72	0.87	0.07 – 2.67	24	1.15 ± 1.08	0.68	0.26 – 4.82
c-nonachlor*	15	0.66 ± 0.38	0.54	0.14 – 1.41	24	1.16 ± 0.91	0.99	0.34 – 4.74
mirex**	15	8.01 ± 5.44	5.41	2.79 – 19.40	24	3.59 ± 2.84	2.29	0.85 – 10.20
p,p'-DDE**	15	6.00 ± 3.52	4.77	2.13 – 11.90	24	2.81 ± 2.08	1.82	0.74 – 9.00
∑OCPs*	15	43.53 ± 25.24	33.14	17.91 – 89.96	24	25.12 ± 13.71	19.68	8.72 – 60.92
PCB-28**	15	0.24 ± 0.10	0.24	0.09 – 0.41	24	0.15 ± 0.07	0.14	0.05 – 0.33
PCB-52	12	0.16 ± 0.14	0.16	0.04 – 0.50	18	0.29 ± 0.23	0.24	0.03 – 0.75
PCB-99**	15	7.52 ± 4.29	5.80	2.40 – 13.90	24	3.49 ± 2.62	2.33	0.51 – 11.10
PCB-105*	15	2.98 ± 1.86	2.36	0.31 – 6.15	24	1.68 ± 1.11	1.19	0.35 – 4.56
PCB-118**	15	13.97 ± 8.35	10.60	4.14 – 30.2	24	6.51 ± 4.53	4.55	1.39 – 17.20
PCB-138**	15	31.71 ± 19.36	23.90	9.68 – 66.40	24	14.07 ± 9.41	9.34	2.87 – 37.10
PCB-153**	15	68.12 ± 47.92	45.80	20.10 – 162.00	24	28.51 ± 21.91	19.10	6.53 – 87.00
PCB-180*	15	32.21 ± 24.41	21.80	9.78 – 82,40	24	14.29 ± 13.81	8.31	2.63 – 52.80
PCB-183**	15	4.60 ± 3.03	3.49	1.41 – 9.95	24	2.05 ± 1.68	1.28	0.44 – 6.59
PCB-187**	15	5.41 ± 2.59	4.62	2.24 – 9.35	24	2.62 ± 1.99	1.93	0.82 – 9.72
PCB-194*	15	4.40 ± 3.42	2.90	1.17 – 12.20	24	1.99 ± 2.04	1.13	0.32 – 7.57
∑PCBs**	15	171.30 ± 113.36	122.35	52.48 – 378.27	24	75.57 ± 56.11	47.79	16.61 – 219.55
PBDE-47	15	2.50 ± 1.29	1.97	1.26 – 4.47	24	1.90 ± 1.56	1.33	0.47 – 6.68
∑PBDEs	15	2.50 ± 1.29	1.97	1.26 – 4.47	24	1.90 ± 1.56	1.33	0.47 – 6.68
PFHxS	14	0.80 ± 0.52	0.58	0.20 – 1.89	20	2.34 ± 4.40	0.63	0.15 – 18.72
PFOS	15	14.26 ± 8.01	12.23	5.99 – 33.34	24	47.22 ± 110.60	10.09	2.56 – 507.66
PFOSA	13	1.03 ± 0.89	0.78	0.35 – 3.66	19	0.74 ± 0.33	0.72	0.33 – 1.81
∑PFSAs	15	15.87 ± 8.90	12.71	6.62 – 35.77	24	49.69 ± 114.64	11.16	2.78 – 526.95
PFOA**	9	0.13 ± 0.09	0.12	0.03 – 0.34	19	0.30 ± 0.17	0.27	0.09 – 0.75
PFNA	15	2.67 ± 1.68	2.23	0.95 – 6,25	24	2.27 ± 1.04	2.20	0.30 – 4.42
PFDcA	15	1.06 ± 0.59	0.83	0.44 – 2.56	24	0.86 ± 0.43	0.72	0.31 – 2.09
PFUnA	15	4.42 ± 1.59	3.81	2.47 – 7.76	24	3.76 ± 2.09	3.27	1.28 – 10.60
PFDoA*	15	1.16 ± 0.37	1.05	0.65 – 1.89	24	0.80 ± 0.48	0.74	0.21 – 2.49
PFTrA**	15	3.96 ± 1.31	3.68	2.52 – 7.73	24	2.69 ± 1.23	2.48	0.98 – 5.88
PFTeA**	15	0.57 ± 0.26	0.52	0.35 – 1.36	18	0.35 ± 0.12	0.29	0.16 – 0.62
∑PFASAs	15	13.91 ± 5.00	11.50	8.50 – 23.20	24	10.90 ± 5.09	9.93	3.28 – 26.04
∑PFASs	15	29.79 ±12.68	22.86	17.58 – 58.97	24	60.58 ±113.86	25.49	6.87 – 533.22

Associations between OHCs and hormones

Table 2. Mean concentrations of total and free T3 (TT3 and FT3) and T4 (TT4 and FT4), standard deviation (SD), median and range measured in plasma of male and female glaucous gulls (*Larus hyperboreus*) breeding in Kongsfjorden, Svalbard during the summer of 2011, 2012 and 2013.

	Males (N=15)			Females (N=24)		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range
TT3 (nmol/L)	2.21 ± 1.02	1.95	1.72 – 4.38	2.38 ± 0.97	2.33	0.82 – 4.59
TT4 (nmol/L)	24.52 ± 11.53	22.18	13.16 – 43.71	30.69 ± 9.59	29.36	10.62 – 50.90
FT3 (pmol/L)	3.23 ± 1.83	3.45	0.80 – 6.12	2.97 ± 1.61	2.98	0.55 – 6.57
FT4 (pmol/L)	30.32 ± 41.35	14.83	4.94 – 133.15	22.64 ± 9.37	20.62	6.26 – 41.47

Plasma concentrations of TT3, FT3, TT4 and FT4 are presented in Table 2. There were no significant differences in TH levels between male and female glaucous gulls.

In males, TT3 correlated significantly negatively with β-HCH, mirex, *p,p'*-DDE, PCB-28, 99, 118, 138, 153, 180, 183 and 187 ($p<0.05$, $r>-0.503$). However, there was also a strong positive correlation between BCI and TT3 in males ($p=0.002$, $r=0.727$). Thus, an OPLS model with BCI as *Y* was created to assess how THs and OHCs affected the condition of the male birds (Figure 1). In this model BCI correlated negatively with TT4, PBDE-47, PCB-153, 183, 187, PFDcA, PCB-138, β-HCH, PCB-180, mirex and *p,p'*-DDE, and positively with PFOA, PFHxS, TT3, PFOSA, PCB-28 and PCB-118 (Figure 1, $p=0.049$, $R^2X=0.707$, $R^2Y=0.945$, $Q^2=0.729$). A partial correlation analysis was therefore performed to control for the influence of BCI on the relationship between OHCs and TT3 in males. Controlling for BCI resulted in no significant relationships between any of the OHCs and TT3 in males. No significant OPLS model with TT3 as *Y* in males could be obtained without BCI as covariable. Thus, it can be concluded that there were no statistical associations between any of the OHCs and TT4, FT4, TT3 or FT3 in males. However, the results indicate that in general male gulls with high BCIs had high concentrations of several PFASs, but low concentrations of several OCs (Figure 1).

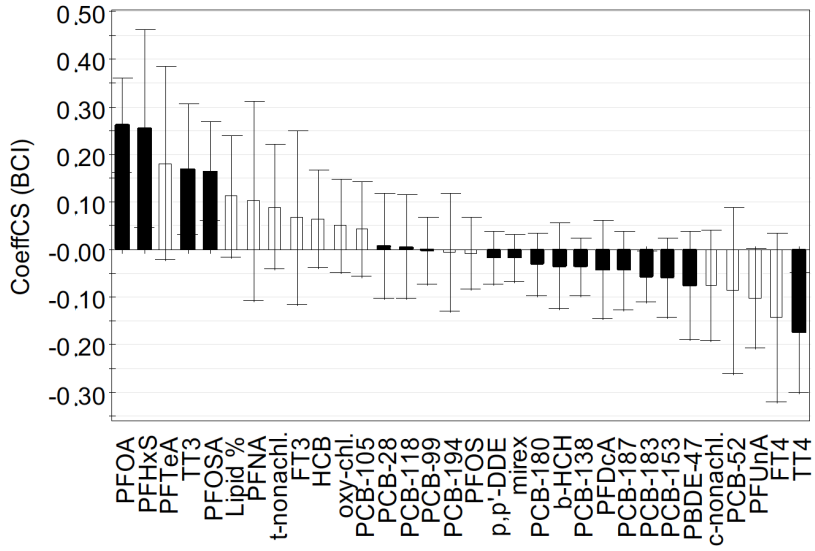


Figure 1. Orthogonal partial least-squares (OPLS) regression coefficient plot showing regression coefficient (CoeffCS) values of each X-variable, describing the direction of the relationship between the X-variables and body condition index (BCI) in male glaucous gulls (*Larus hyperboreus*) (n=15) breeding in Kongsfjorden, Svalbard. The filled bars present the coefficient values of variables with variable importance in projection (VIP) values ≥1, which indicates high importance of that X-variable in predicting BCI. The error bars represent the 95% confidence interval.

In females there were no associations between BCI and THs, but a significant negative relationship was found between lipid% and FT4 (p=0.015, r=-0.492). No correlations were found between BCI and OHCs in females. Partial correlation analysis revealed that lipid% did not confound the relationship between most OHCs and THs in females.

Applying FT4 as *Y* in females resulted in a significant OPLS model which included 13 chlorinated compounds, 2 PFASs and lipid% (Figure 2, CV-ANOVA; p=0.022, R^2X=0.781, R^2Y=0.621, Q^2=0.437). The following variables had VIP values >1.0 and were thus of greatest importance for predicting FT4 levels in females in descending order: HCB, mirex, lipid%, *p,p'*-DDE, PCB-118, PCB-138 and *oxy*-chlordane. All these six OCs correlated inversely with FT4 (Table 3). Although the two PFASs, perfluorononanoic acid (PFNA) and perfluorooctaonic acid (PFOA), were included as predictors for FT4 in the OPLS model, their influence was low due to low VIP values, and there were no individual correlations between these two compounds and FT4 in females (Table 3). Furthermore, although there was a

significant relationship between PCB-105 and FT4 (Table 3), this compound was not identified as an important explanatory variable in the OPLS (Figure 2). This is because PCB-105 had a very low VIP in the OPLS and was removed during the optimalization of the OPLS model.

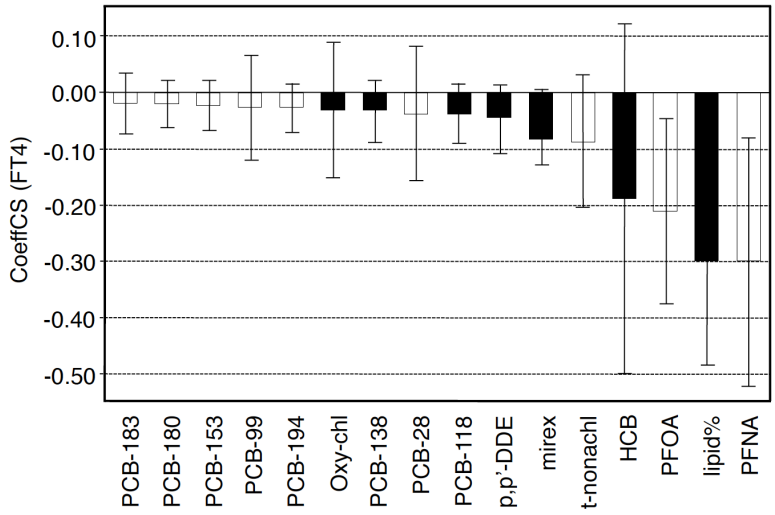


Figure 2. Orthogonal partial least-squares (OPLS) regression coefficient plot showing regression coefficient (CoeffCS) values of each X-variable, describing the direction of the relationship between the X-variables and FT4 levels in female glaucous gulls (*Larus hyperboreus*) (n=24) breeding in Kongsfjorden, Svalbard. The filled bars present the coefficient values of variables with variable importance in projection (VIP) values ≥1, which indicates high importance of that X variable in predicting FT4 levels. The error bars represent the 95% confidence interval.

The relationship between the summed concentrations of all the 15 OHCs ($\sum OHC_{15}$) included in the *Y*=FT4 (females) OPLS model and FT4 (Figure 2) is depicted in Figure 3. The negative relationship between ($\sum OHC_{15}$) and FT4 (p=0.023, R=-0.463) thus represent the modelled additive effects of these OHCs on FT4 levels in females (Figure 3), where each of the compounds contributes differently (Figure 2).

Significant OPLS models could not be obtained with FT3, TT3 or TT4 as *Y* in females. Thus, the relationships between the OHCs and these variables were examined more in detail using correlation analysis. There were no significant correlation between any of the OHCs and TT4. However, there were significant positive correlations between PFOS and

both FT3 and TT3 in the females (Table 3), and there were significant inverse correlations between 13 of the OCs and FT3 ($p<0.05$, Table 3).

Table 3: Bivariate relationships (Pearson correlations) between THs and OHCs in female (N=24) glaucous gulls (*Larus hyperboreus*) breeding in Kongsfjorden, Svalbard during the summer of 2011, 2012 and 2013. Significance levels (*p*) and correlation coefficient (*r)* are included. Borderline significance is marked in italic when $0.05 < p \leq 0.07$; ns = non-significant ($p > 0.07$).

	FT4		FT3		TT3	
Compound	*p*	*r*	*p*	*r*	*p*	*r*
β-HCH	*0.067*	*-0.388*	0.021	-0.468	ns	ns
HCB	0.004	-0.574	*0.070*	*-0.377*	ns	ns
oxy-chlordane	0.020	-0.480	0.016	-0.487	ns	ns
t-nonachlor	0.032	-0.440	ns	ns	ns	ns
mirex	0.009	-0.531	0.013	-0.500	ns	ns
p,p'-DDE	0.023	-0.471	0.014	-0.495	ns	ns
PCB-28	*0.052*	*-0.409*	0.046	-0.411	ns	ns
PCB-99	ns	ns	0.022	-0.360	ns	ns
PCB-105	0.038	-0.435	0.034	-0.435	ns	ns
PCB-118	0.027	-0.461	0.018	-0.479	ns	ns
PCB-138	0.032	-0.447	0.022	-0.464	ns	ns
PCB-153	0.025	-0.465	0.023	-0.461	ns	ns
PCB-180	0.044	-0.414	0.028	-0.450	ns	ns
PCB-183	0.029	-0.454	0.020	-0.472	ns	ns
PCB-187	*0.064*	*-0.384*	0.012	-0.504	ns	ns
PCB-194	0.037	-0.438	0.045	-0.413	ns	ns
PFOS	ns	ns	0.019	0.476	0.008	0.525
PFNA	ns	ns	ns	ns	ns	ns
PFOA	ns	ns	ns	ns	ns	ns

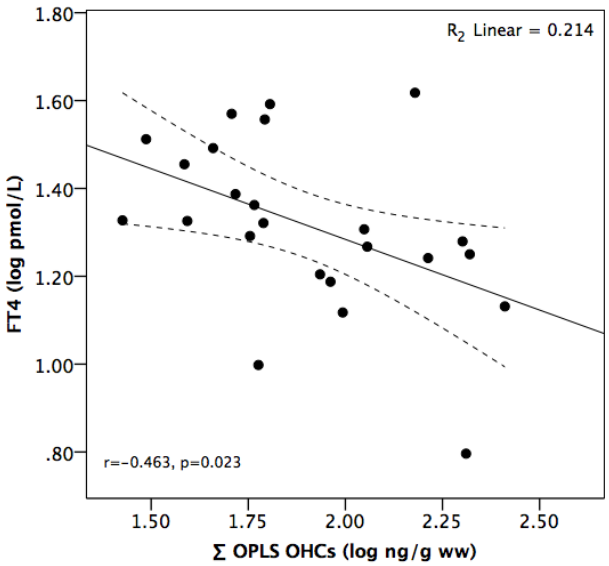


Figure 3. The linear relationships (± 95% confidence interval) between FT4 levels and the summed concentration of plasma OHCs included in the Y=FT4 orthogonal partial least-square (OPLS) regression model in female glaucous gulls (*Larus hyperboreus*) (n=24) breeding in Kongsfjorden, Svalbard. The R^2, pearson correlation coefficient (*r*) and significance level (*p*) are shown in the plot. ∑ OPLS OHCs includes HCB, *oxy*-chlordane, *t*-nonachlor, mirex, *p,p'*-DDE, PFOA, PFNA and PCB-28, -99, -118, -138, - 153, -180, -183 and -194.

The association between the sum of these 13 OCs ($\sum OC_{13}$) and FT3 is shown in Figure 4A (*p*=0.017, R=-0.481), whereas the positive correlation between PFOS and FT3 is shown in Figure 4B (*p*=0.019, R=0.476). Thus, there were dissimilar associations between $\sum OC_{13}$ and FT3 (negative) and PFOS and FT3 (positive) in female glaucous gulls. Since the birds were exposed to all these chemicals, we also tested the correlation between the sum of all these OHCs ($\sum OHC_{14}$, i.e. $\sum OC_{13}$ + PFOS) and FT3 in the females. The results showed that there were no correlation between $\sum OHC_{14}$ and FT3 (Figure 4C). This may indicate antagonistic mixture effects of these OCs and PFOS.

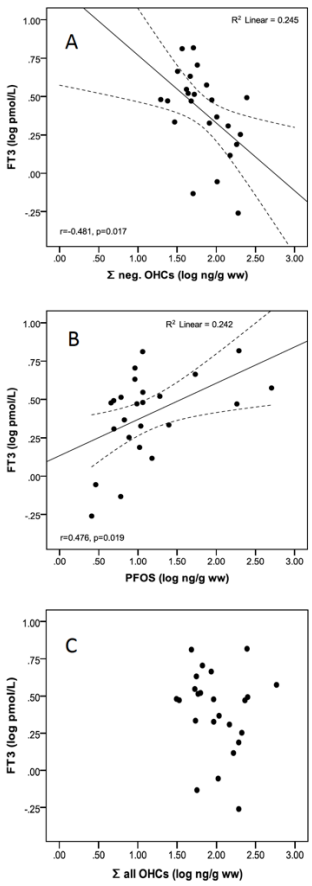


Figure 4. The linear relationships (± 95% confidence interval) between FT3 levels and (*A*) the concentration sum of plasma OHCs negatively correlated with FT3, (*B*) PFOS and (*C*) the concentration sum of all OHCs correlating with FT3 (both positively and negatively) in female glaucous gulls (*Larus hyperboreus*) (n=24) breeding in Kongsfjorden, Svalbard during the summer of 2011, 2012 and 2013. The R^2, Pearson correlation coefficient (*r*) and significance level (*p*) are shown in the plots. ∑ neg. correlated OHCs includes β-HCH, HCB, *oxy*-chlordane, mirex, *p,p'*-DDE and PCB-28, -105, -118, -138, -153, -180, -183, -187 and -194. ∑ all OHCs denotes includes β-HCH, HCB, *oxy*-chlordane, mirex, *p,p'*-DDE and PCB-28, -105, -118, -138, -153, -180, -183, -187, -194 and PFOS.

DISCUSSION

In female glaucous gulls, there were significant negative associations between several OCs and plasma concentrations of FT4 and FT3, indicating additive negative effects of several OCs on FT4 and FT3 in female glaucous gulls. However, in these females there was also a significant positive association between PFOS and plasma concentrations of FT3, indicating that these two groups of OHCs may have dissimilar and antagonistic effects on FT3 in female glaucous gulls. Thus, the magnitude of the effects on FT4 and FT3 is most likely influenced by the relative concentrations of various OHCs in plasma.

The mechanisms explaining the effects of OHCs on FT4 and FT3 include multiple modes of action. One such suggested mechanism is competitive binding affinities of OHCs to the TH-plasma transport protein TTR, and to albumin which is the major plasma protein in birds, and a resultant excretion of T4 (Davison et al., 1978). Competitive binding of OHCs to both TTR and albumin have been reported in glaucous gulls (Ucan-Marin et al., 2010; Ucán-Marín et al., 2009). This has been presumed to facilitate the excretion of FT4 from the plasma via urine or bile, thereby decreasing the plasma concentrations (van den Berg et al., 1991). Thus, in the female glaucous gulls, exposure to OCs may cause reduced plasma levels due to competitive binding on plasma transport proteins. Such a displacement could alter the ratio between TT4 and FT4. However, there were no correlations between the TT4-FT4 ratio and the OCs in the females. It should also be noted that in the gulls, TT4 concentrations did not correlate with the OHCs. Thus, other mechanisms may be involved. Another such mechanism may be that OHCs affect deiodinase activities in tissues and organs. In polar bears (*Ursus maritimus*) PCB concentrations were reported to be positively correlated with activities of deiodinase type I (D1) and type 2 (D2) in muscle, liver and kidney tissues (Gabrielsen et al., 2015). Furthermore, in an *in vitro* study on ringed billed gulls (*Larus delawarensis*), gulls with high concentrations of high-brominated BDEs had high hepatic D1 activity (Franscois et al., 2016). Since D1 and D2 converts T4 to T3 and/or reverse T3, and D1 also converts T3 to T2 (Bianco et al., 2002), it may be possible that increased tissue activities of these deiodinases due to OHC exposure may result in lowered plasma concentrations of FT4 and FT3. It is also possible that the OHC affected other steps of the hypothalamic-pituitary-thyroid (HPT) axis regulation, thus no conclusions on the mechanisms of action of the OHCs on FT4 and FT3 can be deduced from the present study.

No associations between any of the OHCs and the THs were identified in the male glaucous gulls. We therefore speculate that in glaucous gulls breeding in Kongsfjorden,

Svalbard, females are more susceptible to thyroid disruptive effects from OHCs than males. This is in contrast to the previously reported correlations between OCs and THs in glaucous gull males, but not in females breeding at Bjørnøya (Verreault et al., 2004). It is possible that the sex related differences in TH susceptibility to OHCs in the two studies is due to ecological factors that influence the endocrine status of the birds, such as nutritional status, breeding status and environmental conditions. Possible sex-differences in effects of OHCs on THs have previously been indicated in American kestrels (*Falco sparverius*) (Fernie and Marteinson, 2016), and the significance of such sex-differences should be investigated further.

The identifications of the generally negative associations between individual OCs and FT4 and FT3 and the contrasting positive associations between PFOS and FT3 and TT3 in the female glaucous gulls emphasizes the complex actions of thyroid disruption. Reduced levels of THs associated with OCs have been reported in other wildlife bird species, including glaucous gulls (Fernie and Marteinson, 2016; Verreault et al., 2004). Also, positive correlations between PFASs and plasma TH concentrations have been reported in wildlife studies of Arctic birds (Nøst et al., 2012). The negative relationships between the many OCs and FT3 in the present female glaucous gulls might indicate that OCs have additive effects on FT3 (Figure 4A), as previously reported for TT4 in rats (Crofton et al., 2005). Individual chemicals in a mixture may cause additive effects by simple similar action (dose addition) and simple dissimilar action (response addition) (Bliss, 1939; Loewe and Muischneck, 1926; Plackett and Hewlett, 1952).

Individual chemicals in a mixture can also cause antagonistic effects through complex similar action or complex dissimilar action (Bliss, 1939; Loewe and Muischneck, 1926; Plackett and Hewlett, 1952). The differing slopes of the associations between the OCs and FT3 (negative), and between PFOS and FT3 (positive) (Figure 4A, B), indicate that these two groups of OHCs may have antagonistic effects on FT3 in female glaucous gulls. The very

different chemical nature of OCs and PFOS, nonpolar and neutral compounds versus very polar and ionic compounds, suggests no mechanistic interlinkage or competition via similar modes of action. It is possible that these two groups of OHCs have dissimilar modes of actions that results in antagonistic effects of mixtures of these compounds on FT3 (Figure 4C). It should, however, be noted that the present study is correlative, and thus not evidence of any cause-effect relationships. Thus, experimental studies are needed to confirm or disprove the hypothesized antagonistic effects between the OCs and PFOS on FT3, and the possible modes of actions involved.

One important unknown factor in assessing the effects of PFASs in wildlife, is that the animals may be exposed to unstable PFASs that are not detectable in the organisms. These precursors may be metabolised to PFASs analysed in the present study. It is therefore possible that these precursors may be the active endocrine disrupting compounds, although not being detected in the plasma. To unveil if this is the case, experimental exposure studies are needed.

Population differences in OHC and hormone concentrations

A previous study on glaucous gulls from Bjørnøya in Svalbard (540 km south of Kongsfjorden) conducted in 2001 (Verreault et al., 2004) reported median whole blood concentrations of PCBs (sum of 14 congeners) in males and females that were 2.9 and 4.3 times higher, respectively, than those reported in the plasma of the present glaucous gulls (sum of 11 congeners). Furthermore, median whole blood concentrations of *p,p'*-DDE were 21.6 and 34.8 times higher in Bjørnøya males and females as compared to in the plasma samples herein. Although it is difficult to compare levels of OHCs in whole blood and plasma directly, the circulating concentrations of most of these compounds appear to have been much higher in Bjørnøya glaucous gulls in 2001 than in Kongsfjorden gulls in 2011-2013. This may be due to a spatial difference in OHC concentrations in their prey, dissimilar diets between the

populations in Bjørnøya and Kongsfjorden, or since the birds in Bjørnøya were sampled in 2001, it may reflect the suggested slow decline in legacy pollutant contamination in the Arctic (Bytingsvik et al., 2012). The clear pattern of male glaucous gulls having higher levels of OCs than females is consistent with other studies, and is explained by the transfer of lipid associated contaminants to the egg yolk during egg formation (Verreault et al., 2006).

The high levels of PFOS compared to the other PFASs in the present glaucous gulls is in accordance previous reports in glaucous gulls from Bjørnøya (Verreault et al., 2005). The significantly lower concentrations of PFDoA, PFTrA and PFTeA in the female gulls as compared to in the male gulls, is likely due to that these compounds are readily offloaded to the egg, as previously shown in herring gulls (*Larus argentatus*) (Gebbink and Letcher, 2012) In contrast, the significantly higher concentrations of PFOA in the females as compared to in the males, is most likely due to that PFOA does not appear to be easily transferred from female gulls to their eggs (Gebbink and Letcher, 2012). This results that the PFOA in females are not offloaded to their eggs, and the concentrations of this compound in females and males should therefore be expected to be similar. Nevertheless, the PFOA concentrations were significantly higher in females than in males. This may be due to sex-differences in elimination, although female rats (*Rattus norvegicus*) have been reported to have significantly higher rates of urinary elimination of PFOA than male rats (van den Heuvel et al., 1991). It is, however, possible that the urinary elimination of PFOA in mammals and birds differ due that mammals excrete nitrogen as urea, whereas nitrogen is excreted as uric acid in birds. Sex-differences in exposure due to dietary differences, or differences in intestinal uptake mechanisms between the sexes may also be causes for the higher concentrations of PFOA in the female gulls than in the male gulls. The causes for the significantly higher concentrations of PFOA in female glaucous gulls than in the males should be investigated further to assess if this is generic for birds or just a species-specific phenomenon.

If high concentrations of OCs are associated with a depression of THs, lower plasma concentrations of affected THs in the gulls from Bjørnøya (Verreault et al., 2004) than in our gulls should be expected. The TH analyses in these two studies were conducted in the same laboratory using the same analytical kits, and a comparison of plasma TH concentrations is therefore possible. In spite of the apparent higher OC concentrations in the Bjørnøya study, there appeared to be no differences in any of the THs between these two studies (Verreault et al., 2004). This is intriguing, and in contrast to reports in northern fulmars, where negative associations between OHCs and THs were reported to correlate inversely across several breeding populations (Verreault et al., 2013). It is possible that the similar plasma TH concentrations in the gulls in the two studies despite their presumed large differences in OC burdens were caused by high concentrations of compounds with antagonistic TH effects in the population at Bjørnøya in 2001. Indeed, in glaucous gulls from Bjørnøya that were sampled in 2004, plasma concentrations of PFOS, were 9.4 times and 2.8 times higher, respectively, than in the present male and female glaucous gulls from Kongsfjorden (Verreault et al., 2005). This may indicate that the high plasma concentrations of PFOS in glaucous gulls at Bjørnøya in 2001 may have "compensated" the negative effects caused by the OCs, causing plasma TH concentrations to be similar in the Bjørnøya study (Verreault et al., 2004) and in the present study. Dissimilar and antagonistic effects of various OHCs on THs may thus indicate that THs are less suitable as biomarkers of effect, as previously suggested (Skaare et al., 2002).

Associations between OHCs and BCI

In males, the OPLS model showed that in addition to TT3, TT4 and several of the pollutants were predictors for the BCI. Several OCs and PBDE-47 were modelled to contribute to a low BCI, whereas three of the PFASs (PFOA, PFHxS and PFOSA) contributed to a high BCI of males. No significant OPLS model for BCI was identified in females. Inverse correlations between BCI and OCs have previously been reported in glaucous gulls (Sagerup et al., 2009a). Although it is possible that high levels of OCs may directly influence the BCI negatively, it is more likely that the inverse relationships between the plasma concentrations of the OCs and the BCI is due to that high energy costs during breeding causes emaciation and a resultant release of the lipophilic OCs from the adipose tissue into the bloodstream.

With respect to the modelled positive effects of the PFASs on the BCI of male glaucous gulls, positive relationship between BCI and PFNA has previously been reported in male black-legged kittiwakes, but not in female kittiwakes (Tartu et al., 2014). It has been suggested that PFASs can promote adipogenesis and thus act as obsesogens (de Cock and van de Bor, 2014), which could theoretically explain the positive association between some PFASs and BCI in our male gulls. However, there are conflicting results on the role of PFASs as obesogens, and their possible obesogenic properties are therefore unclear (de Cock and van de Bor, 2014). An alternative, and perhaps more likely explanation for the positive associations between some PFASs and the BCI in the present male gulls, could be that birds with high BCIs also have high levels of plasma proteins, possibly due to a more protein and/or calorie rich diet.

Since PFASs are strongly bound to plasma proteins such as albumin (Jones et al., 2003), which is present in high concentrations in bird plasma, this could indicate that there are no causal effects of PFASs on the BCI, but that the positive correlations are caused by the

high affinity of the PFASs for plasma proteins. Thus, individuals with high BCI and high plasma protein concentrations would have high PFASs concentrations. It should, however, be noted that in female hooded seals (*Cystophora cristata*), blood plasma protein content had no effect on the variations in PFASs levels (Grønnestad et al., 2017). Unfortunately, plasma protein concentrations were not analyzed in the present study. Nevertheless, the reported positive correlations between some PFASs and the BCI in male gulls herein and in male kittiwakes (Tartu et al., 2014) warrant further studies on possible cause-effect relationships.

In the present males, TT3 and BCI were positively correlated. This is in accordance with the general assumption that body condition influences the concentration of circulating THs in birds (McNabb, 2000), although BCI was not found to correlate with THs in the present females. In the males, the plasma TT3 concentrations also correlated significantly with several OCs prior to controlling for BCI, but the inclusion of BCI in the model revealed that there were no significant relationships between OCs and TT3 in the glaucous gull males. Thus, the influence of BCI on plasma TT3 levels illustrates the importance of accounting for confounding factors when discussing effects of contaminants on circulating THs.

In summary, female glaucous gulls seemed to be more subjected to thyroid disruption than males, as plasma concentrations of both FT3 and FT4 were altered by a variety of OHCs individually and in mixtures. For both FT3 and FT4 there appeared to be additive negative effects of several OCs. However, the results indicate that for FT3, antagonistic effects between PFOS and OCs, resulted in no overall effect of the mixture of OHCs that affected FT3. Although it is important to note that the present study is correlative, and thus not evidence of any cause-effect relationships. Thus, experimental studies are needed to confirm or disprove the hypothesized antagonistic effects. Nevertheless, the present study indicates that OHCs could affect THs in a complex manner involving both additive and antagonistic

effects. Such interactive effects may add to the challenge of interpreting the overall functional effect of TDCs in wildlife.

Supporting Information - The Supporting Information are available on xxxx

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SUPPORTNG INFORMATION

Dissimilar effects of organohalogenated compounds on thyroid hormones in glaucous gulls

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Description of sampling and sex determination

The glaucous gulls were captured using an automatic triggered nest trap or a net canon. Biometrical measures were recorded for all individuals, including bill length, gonys height, total head length (±0.1 mm), wing length (±0.1 mm) and body mass ±10 g). Blood was drawn from the branchial veins on the inside of the wings, and kept cool and dark until centrifugation at the laboratory (10 000 rpm, 10 minutes) within 8 hours after sampling. Plasma samples were kept frozen at -20°C until time of analysis. Sex determination was conducted biometrically using total head and bill length, as recommended for *Laridae* species (males having bill length > 61.5 mm and total head length > 142 mm) (Bustnes et al., 2000; Coulson et al., 1983). Molecular sex determination by polymerase chain reaction (PCR) was performed at NTNU, Trondheim, for individuals having indistinguishable sizes. The principle of sexing by PCR is detection of the female W-chromosome, visualized as two bands in contrast to the single band of males. This method is previously described elsewhere (Griffiths et al., 1998).

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