Endocrine and fitness correlates of long-chain perfluorinated carboxylates exposure in Arctic breeding black-legged kittiwakes

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Abstract

Increasing levels of poly- and perfluorinated alkyl substances (PFASs) have recently been described in Arctic biota. These emerging substances are of concern given their resistance to degradation and metabolization. Some studies have reported endocrine disrupting effects for some PFASs. However, there is a gap of knowledge on the potential relationships between PFASs and hormones mediating the life-history trade-off between reproduction and survival, such as glucocorticoids. The aims of this study were to; 1) describe the concentrations of plasma perfluoroalkyl sulfonates and perfluoroalkyl carboxylates in Svalbard black-legged kittiwakes (*Rissa tridactyla*) in relation to gender and body-condition, 2) explore the relationships between PFASs and corticosterone (the major glucocorticoid in birds) and 3) assess the consequences of PFAS exposure for reproductive success. Perfluorononanoate was positively related to body-condition in male kittiwakes; perfluorotridecanoate and perfluorotetradecanoate to decreased baseline corticosterone in both sexes; and perfluorododecanoate was related to lower hatching success. These results underline the importance of considering each compound separately when investigating the hazardous effects of PFASs on wildlife.

Introduction

While most attention was directed towards the endocrine disrupting effects of legacy persistent organic pollutants (POPs)[1, 2, 3, 4], the so-called emerging POPs came into focus in the late 90's. Many emerging POPs are not regulated and comprise a wide array of everyday life products[5], but ecotoxicological data on these compounds are lacking[6]. Among them, poly- and perfluorinated alkyl substances (PFASs) are used as surface-active agents in a multitude of manufactured and consumer products (e.g., fire-fighting foam and impregnation agent for carpets, papers, and textiles). PFASs are particularly alarming, because of their special properties: they are thermally and chemically stable, have no route of degradation and cannot be metabolized by vertebrates under normal environmental conditions[7] which makes them extremely persistent in the environment. Moreover, PFASs consist of perfluorinated carbon chains that are hydrophobic and lipophobic, so they can accumulate in the blood, liver and kidney[8, 9]. Nowadays, human exposure to PFASs measured in the blood reaches the highest values observed by an exogenous chemical[9]. The occurrence of PFASs have been described in polar region such as the Arctic[10] and concentrations of some PFASs tend to increase over time in several Arctic mammal and seabird species[11, 12, 13]. In Arctic regions long-chained perfluoroalkyl carboxylic acids (PFCAs) are prevalent[11] and acute toxicity of PFCAs increases with chain length[9]. With regard to the potential endocrine disrupting properties of PFASs, laboratory studies have shown that some PFAS possess estrogenic, androgenic and thyroid-like activity[5, 9, 14, 15]. However, to date very few studies have investigated the relationships between hormones and PFAS in free living species[16]. Furthermore, the possible influence of PFAS on some major endocrine axes has only been investigated in a few studies. This is especially the case for the hypothalamo-pituitary-adrenal (HPA) axis. The HPA axis plays an important role in mediating the life-history trade-off between reproduction and survival across the release of stress hormones such as glucocorticoids[17].

However, little is known about the disruption of PFASs on glucocorticoids. The release of glucocorticoid hormones (cortisol, corticosterone: CORT) during stressful events triggers physiological and behavioral adjustments that shift energy investment away from reproduction and redirects it towards self-preservation and hence survival[17, 18]. CORT is very likely to mediate parental effort and parental investment in birds[19, 20] and any disruption of this hormone may alter the ability of an individual to adjust reproductive decisions to environmental conditions[21, 22]. Because of increasing prevalence of PFASs in the environment, especially of the most toxic long-chained PFCAs in the Arctic, and because of the pivotal role of the HPA axis, PFASs should therefore become the focus of interest as emerging endocrine disruptors for wildlife. There is also very limited information on the potential negative impact of PFASs on fitness (e.g. reproductive success, survival) of free-living organisms[23, 24, 25]. Arctic seabirds are top predators particularly at risk given the biomagnification properties of some PFASs along the trophic web[26]. The aims of this paper are to; 1) describe the concentrations of plasma PFSAs (perfluoroalkyl sulfonic acids) and PFCAs in an Arctic seabird species in relation to gender and body-condition, 2) explore their relationships with the HPA axis, and especially with plasma CORT concentrations, 3) assess the consequences of PFAS exposure on fitness traits like reproductive success. To do so, we investigated these relationships in chick-rearing adult black-legged kittiwakes (*Rissa tridactyla*) which in Svalbard are known to be exposed to PFASs[16].

Experimental

Study area and sampling collection

The sampling of birds was approved by the Governor of Svalbard, and national guidelines for ethical treatment of experimental animals were followed (NARA, FOTS id 4214, RIS 2961). The study was conducted at Kongsfjorden, Svalbard (78°54′N, 12°13′E) from July 12th to July 26th 2012 during the chick-rearing period. Twenty birds (10 males and 10 females), were caught on 20

different nests with a noose at the end of a 5 m fishing rod. A first blood sample (*ca.* 0.3 mL) was collected immediately after capture, from the alar vein with a 1 mL heparinised syringe and a 25-gauge needle to assess baseline CORT concentrations. Bleeding time (i.e. time elapsed from capture to the end of the first blood sample: 2min 27s ± 31s (SD), on average) did not affect CORT concentrations (GLM, $F_{1,18} = 0.39$, $p = 0.538$). Eighteen kittiwakes (10 males and 8 females) were then placed into a cloth bag and a second blood sample (*ca*. 2.5 mL) was collected from the alar vein at 30 minutes from capture (30min 13s ± 1min 02s) to assess stress-induced CORT and PFAS concentrations. Kittiwakes were individually marked with metal rings and PVC plastic bands engraved with a three-digit code and fixed to the bird's tarsus for identification from a distance. Birds were weighed to the nearest 2 g using a Pesola spring balance, and their skull length (head+bill) was measured to the nearest 0.5 mm with a sliding calliper. For each bird we calculated its scaled mass index[27] as a measure of body-condition. Kittiwakes were marked with spots of dye on the forehead to distinguish them from their partner during subsequent observation and then released. Prior to the beginning of the sampling period, using a mirror at the end of an 8 m fishing rod, we checked the whole plot (*ca*. 117 nests) every two days to monitor the clutch size, the number of chicks that hatched (thereafter 'hatching success') and those that reached at least 12 days of age per active nest (thereafter called ‘breeding success’). All birds studied and sampled had a clutch of two eggs.

Molecular sexing and hormone assay

Blood samples were centrifuged and stored at -20°C until used respectively in hormone assays or molecular sexing, at the Centre d'Etudes Biologiques de Chizé (CEBC). The sex was determined by polymerase chain reaction (PCR) amplification of part of two highly conserved genes (CHD) present on the sex chromosomes at the Centre d'Etudes Biologiques de Chizé (CEBC)[28]. Plasma concentrations of CORT were determined by radioimmunoassay[29] at the CEBC. The lowest detectable concentration for CORT was 1.05 ng/ml. Only one assay was performed and the intra-assay coefficient of variation was 6.7 % (N = 5 duplicates).

Chemical analyses

Analyses for perfluorinated compounds in plasma samples were performed at the Norwegian Institute of Air Research (NILU, Tromsø, Norway). We searched for 14 PFASs: perfluorobutane sulfonate (PFBS), Perfluorohexane sulphonate (PFHxS), linear perfluorooctane sulfonate (PFOSlin), perfluorobutanoate (PFBA), perfluoropentanoate (PFPA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoate (PFUnA), perfluorododecanoate (PFDoA), perfluorotridecanoate (PFTrA) and perfluorotetradecanoate (PFTeA). The method was described in detail along with instrumental settings in previous studies[30, 31]. In short, a sample (0.5 ml) spiked with internal standards was extracted in acetonitrile (1 ml) by repeated sonication and vortexing. The supernatant was cleaned up using ENVI-Carb graphitized carbon absorbent and glacial acetic acid. Extracts were analyzed by UPLC/MS/MS. Recovery of the internal standards ranged between 45% and 120%. The deviation of the target concentrations in the SRMs (NIST Human serum 1958) were within the laboratory's accepted deviation from target concentrations (< 40%) (n = 4). All blanks contained concentrations below the instrument

detection limits (three times chromatographic noise). For each compound, limit of detection (LOD) and limit of quantification (LOQ) are given in **Table 1**.

Statistical analyses

For statistics, only samples with concentrations over the analyte-specific LODs and detected in more than 70% of the samples were included. All statistical analyses were performed using R 2.13.1 and generalized linear models (GLM) with a normal/binomial error distribution and an identity/logit link function were used to test our biological assumptions. First, we tested the effects of 'sex' on 'body-condition', 'CORT' and 'PFASs'. Second we checked for relationships between 'PFASs' and 'CORT'. Third, we tested if PFAS were related to body-condition in males and females separately. Finally, we tested whether 'CORT' and 'PFASs' affected 'hatching success' and 'breeding success'. Since only one bird had a null hatching success and thus a null breeding success it was removed from statistical analyses, we thus performed analyses with a binomial response, hatching success (1 or 2 eggs have hatched) and breeding success (1 or 2 chicks have reached 12 days of age) . Model selection was performed by a step-down approach starting from the global model including all the independent variables, these latter were log-10 transformed when necessary and statistical significance was fixed to $\alpha < 0.05$. In all models we tested the effect of each compound separately.

Results and discussion

Compounds and levels of PFASs

Fourteen PFASs were analyzed of which six (PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, PFTrA) were detected in the 20 captured kittiwakes and PFTeA was detected in 19 kittiwakes. PFHxS and PFOA were detected in 9 and 4 birds, respectively (**Table 1**). They were thus excluded from statistical analyses. The dominating compound was PFTrA closely followed by PFUnA and PFOSlin then by decreasing order PFDoA > PFDcA > PFTeA > PFNA (**Table 1).** Most studies

on PFASs have concentrated on PFOS and PFOA, as they are often the most present compounds in vertebrates[32]. As a consequence there is limited information available on the toxicological effects and risk of PFCAs with longer chains than PFOA[32, 33]. Contrary to kittiwake chicks, where PFOSlin was the dominant compound[16], in adult chick-rearing kittiwakes, odd numbered longer chained PFCAs (C11 and C13) were the dominant fluorinated compounds. This difference in PFASs profile could originate from PFOS regulations taken in 2009 by the Stockholm Convention on POPs, indeed the study on chicks[16] occurred 6 years before the present one. Another explanation could be a diet difference between chicks and adults.

Hormones and PFASs in relation to sex and body-condition

CORT concentrations (baseline and stress-induced) and PFASs were not related to sex (GLM, $F<2.7$, $p>0.11$**, supporting information**) however for the PFCAs with longer chains (i.e. PFDoA, PFTrA and PFTeA), plasma concentrations tended to be higher in males than in females ($F<3.88$, $p>0.064$, **supporting information**). Body-condition was higher in males than in females (GLM, $F_{1,18}=38.7$, $p<0.001$). CORT concentrations were not related to body-condition in males or in females ($F<3.01$, $p>0.133$). PFNA was positively related to body-condition in males only (GLM, $F_{1,8}=7.19$, $p=0.028$; **Figure 1**) and no relationships were found between the other PFASs and body-condition in males or females ($F<2.43$, $p>0.158$, **supporting information**). In the results presented here, male kittiwakes with higher concentrations of PFNA were in better body- condition. Body-condition as measured by scaled mass index is a reliable predictor of body fat and proteins[27]and PFASs have high affinity for proteins[8]. The positive relationship observed between PFNA and body-condition in male kittiwakes could be related to the structural resemblance of PFASs to fatty acids and their capability to bind to nuclear receptors which play a key role in lipid metabolism and adipogenesis[34, 35]. Activation is greater as carbon backbone length increases, and carboxylates (PFOA and PFNA) have higher activation properties[35]. In

humans, PFNA can be associated with increased cholesterol and adiponectin concentrations[36, 37] and PFOA levels correlate with body weight but also insulin and leptin concentrations[38]. All these hormones are strongly associated to obesity and food intake; it is possible that similar mechanisms to those reported in humans could occur in birds. However we should be cautious in interpreting this result, as we have no evidence that PFNA disrupts lipid metabolism in birds, and the reason why no relationship was found in females remains unexplained. It may result from the ability of females to transfer elevated amounts of PFASs into their eggs[13, 39] however in the present study PFNA concentrations were not lower in females.

Relationships between PFASs and hormones

In adult chick-rearing kittiwakes baseline CORT concentrations were negatively related to PFTrA and PFTeA (**Figure 2, Table 2**). No relationships were found with the other PFASs, and no relationship was found between PFASs and stress-induced CORT concentrations (**Table 2, supporting information**). This negative relationship between PFTrA, PFTeA and CORT could be the result of several mechanisms: a negative feedback due to other hormones, hormone displacement due to high protein affinity, or a disruption of the HPA axis that has resulted in a lower ability to secrete proper baseline CORT. Some experimental studies have reported effects of PFCAs on CORT: for example in PFNA treated mice, the group receiving the higher dose had increased concentrations of both ACTH and cortisol[40]. However, in kittiwakes PFNA concentrations were not related to CORT. One possible interpretation to explain these relationships between CORT, PFTrA and PFTeA comes from the ability of PFASs to displace hormones by binding to proteins[8]. Because of their high affinity for proteins, it has been suggested that PFASs could easily bind to transport proteins and cause hormone displacement[8]. CORT is protein dependent to insure its specific role by binding to CORT-binding-globulin (CBG)[41]. This binding of CORT to CBG may serve as a tissue buffer against potentially

deleterious effects of elevated circulating CORT[41, 42]. Measuring the quantity of CBG and the number of glucocorticoid receptors (GR) could inform on the effective activity of CORT in kittiwakes contaminated by PFASs. Indeed, the observed decrease of baseline CORT concentrations with increasing PFTrA and PFTeA in kittiwakes could be a response to an increase of GR and/or a decrease of CBG. Indeed, the action of CBG is supposed to make CORT inactive[43], so if most of the CBG are bounded with PFTrA and PFTeA, more CORT will remain free and active. Very high levels of active CORT could have an important impact on health, thus a decrease in the production of CORT from the adrenals may be an adaptation to keep free CORT concentration within the normal physiological ranges. It has been suggested[8] that "*given the current environmental concentrations of PFOS, it was unlikely that PFOS would cause displacement of hormones from serum proteins in wildlife*"; indeed though no relationships between PFOS and CORT were found in the present study, the relationships with longer chain PFCAs (PFTrA and PFTeA) are still of concern. These results should be interpreted cautiously and would greatly benefit from experimental support. It would thus be interesting to measure free CORT and GR in relation to PFTrA and PFTeA. Another interesting point is that the observed pattern in the present study is the opposite of what has been found in previous studies with regard to legacy POPs and CORT in Arctic seabird species[44, 45, 46]. In these latter studies, baseline[44, 45] or stress-induced[46] CORT increased with increasing legacy POPs. The lower concentrations of CORT in relation to PFTrA and PFTeA, could have interfered with the adaptive weight loss observed in seabirds and consequently with chick feeding[47, 48]. However, in the present study PFTrA and PFTeA were not related to body-condition or reproductive success.

Relationships between PFASs and reproductive traits

Hatching success and breeding success were not related to baseline or stress-induced CORT concentrations, or with the interactions with sex ($\chi^2<1.11$, $p>0.29$). Hatching success was

significantly lower in birds with higher concentrations of PFDoA (GLM, PFDoA: χ^2 = 4.2, p=0.040; sex: χ^2 = 0.4, p=0.528 PFDoA× sex: GLM, χ^2 = 0.1, p=0.72; **Figure 3, supporting information**) and was significantly related to the interaction between PFTeA and sex (PFTeA × sex: GLM, χ^2 = 4.0, p=0.045, **supporting information**). However when analyzing males and females separately, the negative relationship between hatching success and PFTeA was only close to statistical significance in males (GLM, χ^2 = 3.4, p=0.064) and no relationship was found in females (GLM, χ^2 = 0.6, p=0.455). All the other compounds were unrelated to hatching success (p>0.35 for all tests, **supporting information**). Breeding success was not related to PFASs (p>0.07 for all tests, **supporting information**). In this study, male and female kittiwakes bearing the higher concentrations of PFDoA were more likely to hatch one egg instead of two in a two eggs clutch. In mammals, some effects of PFDoA on reproduction and development have been observed. For example, in male rats fed PFDoA lower spermatid and spermatozoa counts were observed in reproductive organs and in female rats PFDoA administration resulted in death or in the delivery of dead pups[49]. Relationships between PFASs and fitness have rarely been investigated for wildlife. In tree swallows *Tachycineta bicolor*, a similar apparent reproductive impairment was observed as in the present study, although the associated PFASs differed: PFOS concentration measured directly in eggs was negatively associated to hatching success, with PFOS concentration ≥ 150 ng/g[24, 25]. In lesser black-backed gulls *Larus fuscus* no relationships were found between PFASs and life-history traits[23]. However, in lesser black-backed gulls, whole blood concentrations for long chain PFCAs were slightly lower than in kittiwakes' plasma (1.4 ng/g versus 2.5ng/g, respectively, for PFDoA). The relationships between PFDoA and hatching success could either be the result of non-viable embryos or less efficient incubation behavior. In oviparous vertebrates, females transfer a large amount of PFASs to their eggs[39] which may result in non-viable embryos for the most contaminated females. However, in the present study high

PFDoA concentrations measured in male kittiwakes were also related to lower hatching success. Consequently the lower hatching success observed in birds with the higher concentrations of PFDoA, is more likely to result from disrupted incubating behavior. Regarding legacy POPs, an experimental study conducted on American kestrels *Falco sparverius*, reported that PCB administration resulted in altered incubation behaviors[50]: the incubation bouts realized by female and male American kestrels were not synchronized and the eggs were left unattended for longer periods[50]. In free-ranging glaucous gulls *Larus hyperboreus*, the proportion of time absent from the nest site when not incubating and the number of absences were related to blood PCB[51], and the most contaminated glaucous gulls were less able to maintain an optimal nest temperature[52]. However we have no evidence for such effects of PFDoA. Another possible explanation that could relate PFDoA to hatching success would be a disruption of the hormonal control of brood patches. Brood patches are highly vascularized featherless patches placed on the ventral surface of both male and female birds when bi-parental care is provided[53]. These patches enable the egg to be kept at an optimal temperature: if incubation patches are too small, one egg at least would probably be less exposed to parental heat. The feather loss and vascularization of those patches are under hormonal control, particularly through a synergetic association of prolactin and estrogen[53]. In rats, PFDoA administration reduces serum estradiol concentration in males and the expression of estrogen receptors in the ovaries of females[54, 55, 56]. If PFDoA reduces estradiol expression in kittiwakes, this could lead to reduced brood patches, less efficient incubation and non-viable embryos. Again, further studies are needed to test these hypotheses, such as measurement of estrogen and monitoring incubation behavior in relation to PFDoA. Another, important point is that we did not measure PFASs in the partners of our focal birds. Because kittiwakes provide bi-parental care to the brood, measuring PFASs in the partner would provide important data to explain the observed interaction between PFDoA and hatching success.

This study underlines the importance of considering each PFAS separately and their relationships with sex. To the best of our knowledge this is the first study which shows relationships between long chain PFCAs (PFNA, PFDoA, PFTrA and PFTeA), body-condition, baseline CORT concentrations and hatching success in a free-ranging seabird. Most toxicity studies of PFASs have concentrated on PFOS and PFOA, hence limited information is available on the toxicological effects and risk of other PFASs[33]. Additionally, PFCAs show dramatic increasing trends in Arctic seabird eggs[13, 57], given their hazardous effects on hormones and fitness related traits, more studies are needed. The small amount of data available makes interpreting the statistical results difficult; additionally this study is correlational; it is thus difficult to draw conclusions on the causality of these relationships, some of them could be the result of unmeasured chemicals or parameters that could confound these associations.

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Supporting Information Available

Figures depicting the non-significant relationships between PFASs, body-condition and hatching success are given in Supporting information. Additionally, we also included tables with statistics concerning the relationships between 1) sex, PFASs, CORT and body-condition, 2) PFASs and body-condition, 3) PFASs and reproductive traits. This information is available free of charge via the Internet at http://pubs.acs.org/

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Figure captions:

Figure 1: In male black-legged kittiwakes (open circles), body-condition (scaled mass index) was positively related to plasma PFNA concentrations. A relationship not found in females (closed circles). The solid line refers to a statistically significant linear regression.

Figure 2: Baseline CORT concentrations in relation to seven PFASs. Baseline CORT decreased with increasing plasma PFTrA and PFTeA concentrations, in male (open circles) and female (closed circles) chick-rearing black-legged kittiwakes. The solid line refers to a statistically significant linear regression.

Figure 3: Hatching success (1 or 2 chicks have hatched) was lower in black-legged kittiwakes with high concentrations of PFDoA (*: $p = 0.040$) open circles denote males and closed circles denote females.

482 **Table 1:** PFASs mean concentrations ± standard deviation (pg/ml ww), LOD and LOQ in the

483 plasma of female and male chick-rearing adult kittiwakes.

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				Females				**Males**			
	n° C	LOD	LOQ	N>LOD	Mean	SD	Range	N>LOD	Mean	SD	Range
Perfluoroalkyl sulfonates (PFSA)											
PFBS	4	18.5	55.5	0	n.d.	-	-	0	-	-	-
PFHxS	6	10.7	32.0	5	-	-	[<10.7; 216]	4	-	-	[<10.7; 130]
PFOSlin	8	704	2111	10	9299 ±	2611	[6804; 13581]	10	10233 ±	2685	[7002; 15183]
Perfluoroalkyl carboxylates (PFCA)											
PFBA	4	918	2754	0	n.d.	-	-	0	-	-	-
PFPA	5	36.2	109	0	n.d.	-	-	0	-	-	-
PFHxA	6	6.2	18	0	n.d.	-	-	0	-	-	-
PFHpA	7	91.2	274	0	n.d.	-	-	0	-	-	-
PFOA	8	26.5	80	2	-	-	[<26.5; 122]	2	-	-	[<26.5; 167]
PFNA	9	40.9	123	10	967 ±	704	[805; 3047]	10	1241 ±	547	[787; 2593]
PFDcA	10	61.9	186	10	1705 ±	464	[1301; 2764]	10	2162 ±	528	[1233; 3123]
PFUnA	11	83.0	249	10	10449 ±	2636	[7712; 16618]	10	11413 ±	2808	[7853; 17546]
PFDoA	12	109	327	10	2188 ±	709	[1472; 4014]	10	2658 ±	662	[1893; 3815]
PFTrA	13	360	1079	10	12960 ±	7330	[4495; 29735]	10	18156 ±	4022	[11217; 23055]
PFTeA	14	235	706	10	1167 ±	840	[289; 3258]	9	1798 ±	532	[<235; 2712]
∑7PFASs	-	-	-	-	47947 ±	11213	[29172; 66048]		41339 ±	11967	[24336; 71204]

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Table 2: Modelling the relationship between PFASs and A) baseline and B) stress-induced CORT concentrations in chick-rearing black-legged kittiwakes.

Dependent variable	Independent variable	SS	Df	F	Pr(>F)
A) Baseline CORT	linear PFOS	0.6	1,18	0.0	0.838
	PFNA	2.9	1,18	0.2	0.668
	PFDcA	18.6	1,18	1.3	0.266
	PFUnA	36.3	1,18	2.8	0.114
	PFDoA	23.8	1,18	1.7	0.207
	PFTrA	117.2	1,18	13.5	**0.002**
	PFTeA	80.1	1,17	7.7	**0.013**
B) Stress-induced CORT	linear PFOS	0.5	1,16	0.0	0.905
	PFNA	2.5	1,16	0.1	0.795
	PFDcA	0.5	1,16	0.0	0.909
	PFUnA	0.6	1,16	0.0	0.899
	PFDoA	1.9	1,16	0.1	0.819
	PFTrA	1.0	1,16	0.0	0.868
	PFTeA	46.1	1,15	1.3	0.267

492 **TOC art**

493

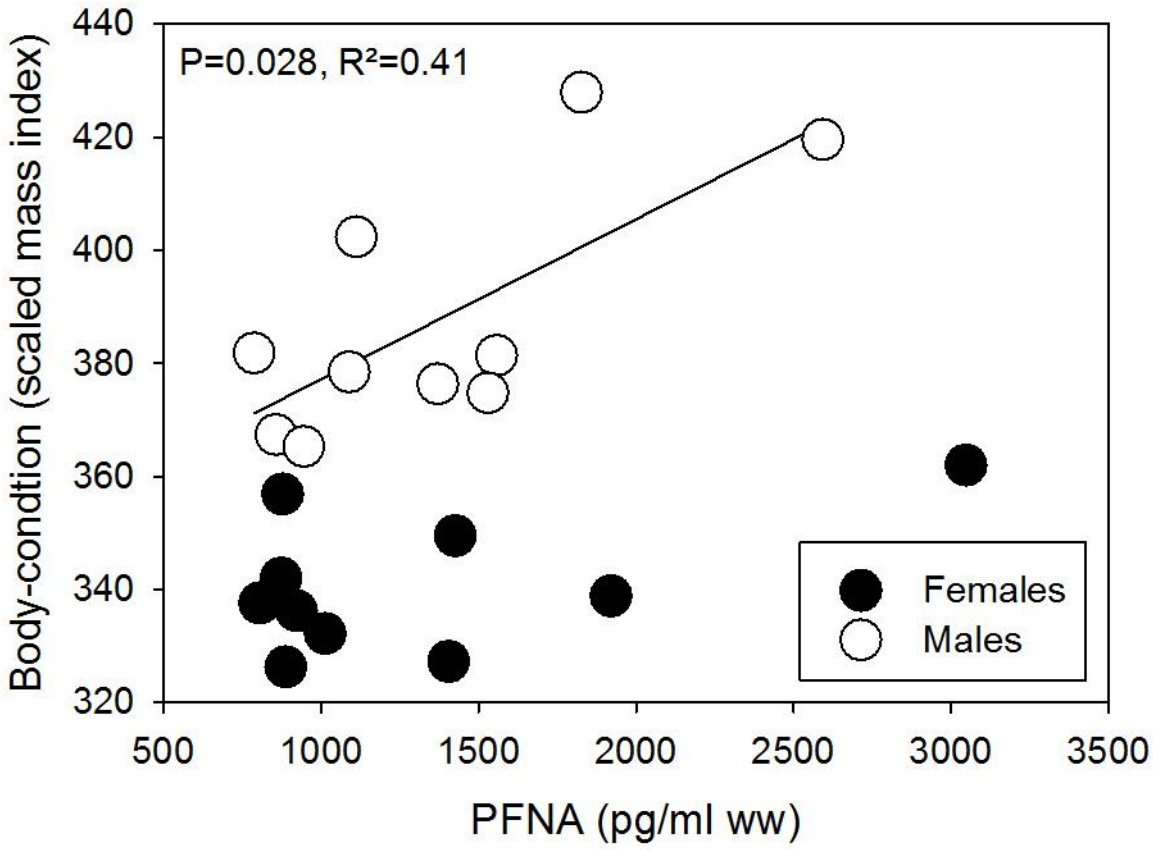

P=0.028, R²=0.41
Body-condtion (scaled mass index)
PFNA (pg/ml ww)
Females
Males
440
420
400
380
360
340
320
500
1000
1500
2000
2500
3000
3500

Figure 1

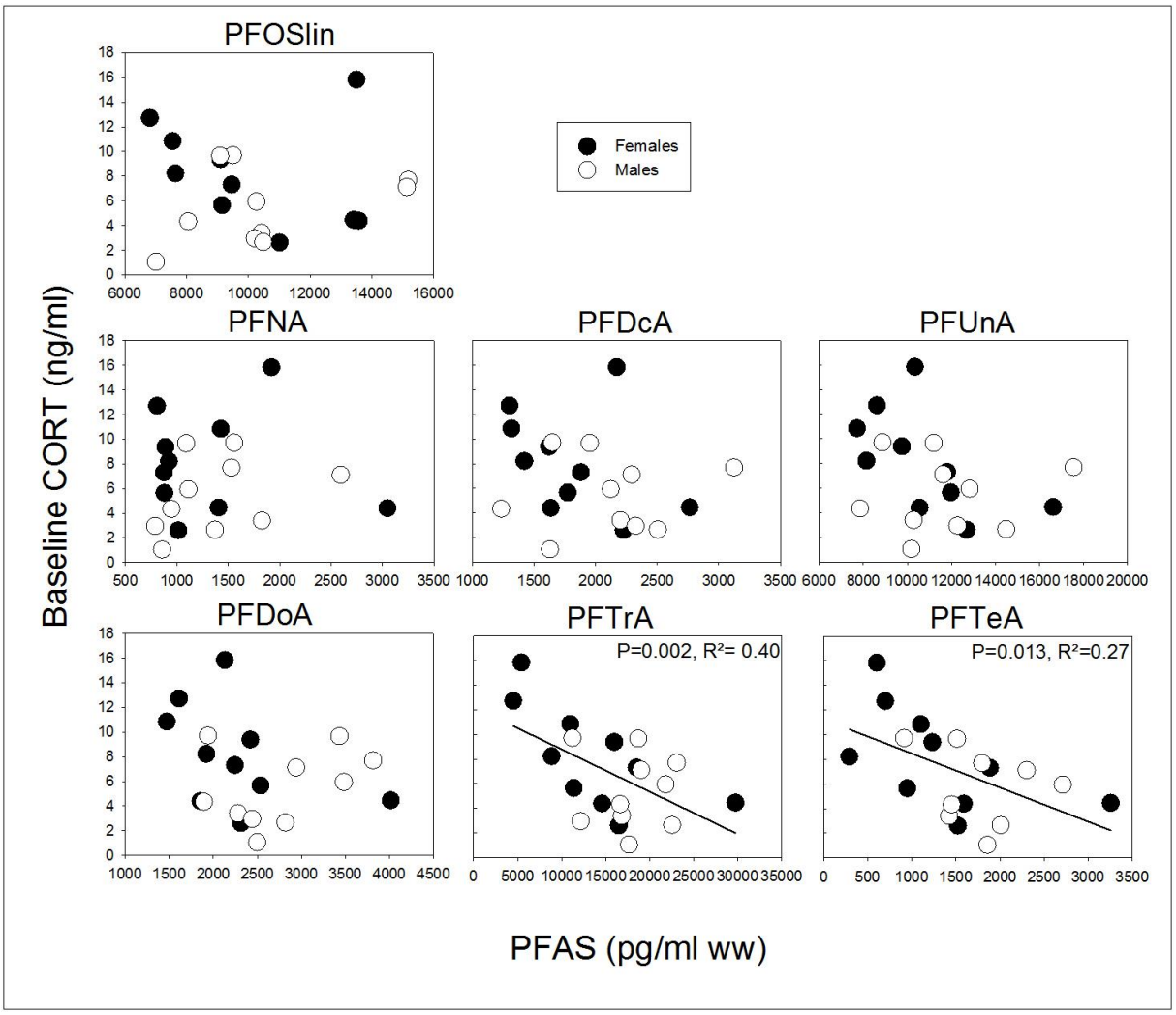
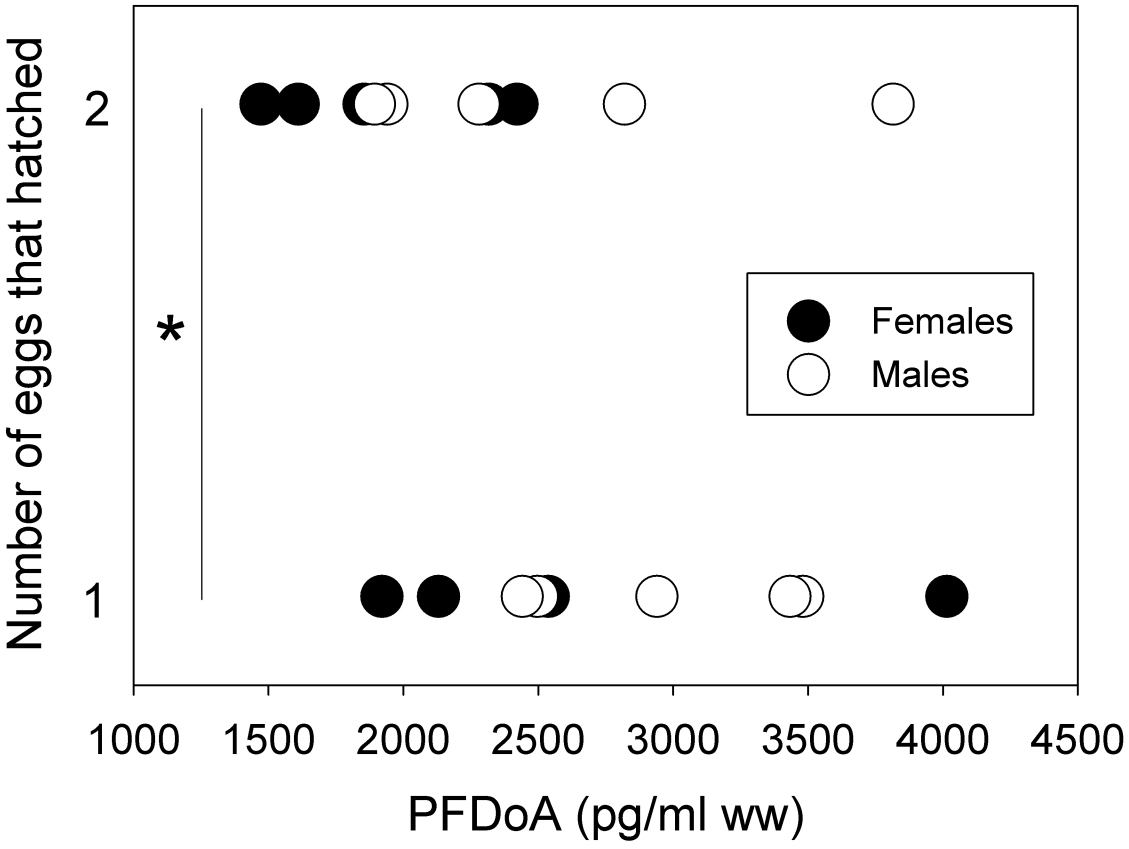

PFOSlin
Females
Males
PFNA
PFDcA
PFUnA
PFDoA
PFTrA
P=0.002, R²= 0.40
PFTeA
P=0.013, R²=0.27
Baseline CORT (ng/ml)
PFAS (pg/ml ww)

Figure 2


Number of eggs that hatched
2
1
*
Females
Males
1000
1500
2000
2500
3000
3500
4000
4500
PFDoA (pg/ml ww)