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Steroid hormones and persistent organic pollutants in plasma from North-eastern Atlantic pilot whales

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ABSTRACT

Persistent organic pollutants (POPs) are known to have endocrine disruptive effects, interfering with endogenous steroid hormones. The present study examined nine steroid hormones and their relationships with the concentrations of selected POPs in pilot whales (*Globicephala melas*) from the Faroe Islands, NE Atlantic. The different steroids were detected in 15 to all of the 26 individuals. High concentrations of progesterone (83.3–211.7 pmol/g) and pregnenolone (PRE; 4.68–5.69 pmol/g) were found in three adult females indicating that they were pregnant or ovulating. High androgen concentrations in two of the males reflected that one was adult and that one (possibly) had reached puberty. In males a significant positive and strong correlation between body length and testosterone (TS) levels was identified. Furthermore, positive and significant correlations were found between 4-OH-CB107/4'-OH-CB108 and 17β-estradiol in males. In adult females significant positive correlations were identified between PRE and CB149 and t-nonachlor, between estrone and CB138, -149, -187 and p,p'-DDE, between androstenedione and CB187, and between TS and CB-99 and -153. Although relationships between the POPs and the steroid hormones reported herein are not evidence of cause-effect relationships, the positive correlations between steroids and POPs, particularly in females, suggest that POPs may have some endocrine disrupting effects on the steroid homeostasis in this species.

1. Introduction

Long-finned pilot whales (*Globicephala melas*) from the Faroe Islands (NE Atlantic) are highly contaminated with persistent organic pollutants (POPs) such as PCBs, organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs), and persistent metabolites such as hydroxyl (OH)-containing PCBs (Hoydal et al., 2015). Some of these POPs and/or their metabolites have structural similarities with endogenous hormones, and can interact with hormone transport proteins or disrupt hormone metabolism. They can thus mimic or in some cases interfere with the activity of endogenous hormones (Jenssen, 2006; O'Connor and Chapin, 2003). Previously, we have shown that in pilot whales some POPs may have minor effects on circulating levels of thyroid hormones and vitamin concentrations (Hoydal et al., 2016). However, POPs can also perturb the reproductive hormone system, and thus the reproductive function of wildlife mammals (Colborn et al., 1993). Possible mechanisms for endocrine disruption are multiple and complex, and involve alterations in receptor-mediated signalling and post-receptor activation and alterations in hormone synthesis, transport, storage, release and metabolism (O'Connor and Chapin, 2003).

Endocrine disruptive effects of POPs related to reproductive hormones have frequently been reported in fish, amphibians and reptiles (Guillette, 2000). Contaminants can have estrogenic or anti-estrogenic effects (Yordy et al., 2010). For example p,p'-DDE and t-nonachlor have estrogenic properties whereas CB-138 and CB-180 have anti-estrogenic properties (Yordy et al., 2010). Thus the effects of exposure to a mixture of contaminants may be difficult to interpret. In wild mammals and surrogate wildlife mammalian model species, it has been suggested that POPs, and in particular PCBs and their OH-PCB metabolites) can influence circulating levels of steroid hormones (Gustavson et al., 2015; Hallanger et al., 2012; Haave et al., 2003; Ropstad et al., 2006; Sonne et al., 2014). Disturbance of steroid levels elicited by POPs has also been indicated in studies of cetaceans (Subramanian et al., 1987; Yordy et al., 2010). The developing foetus is uniquely sensitive to endocrine

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disruption and studies using experimental animals have indicated that exposure to PCB at environmentally relevant concentrations in utero, and in the suckling period, can influence reproductive functions (O'Connor and Chapin, 2003; Ropstad et al., 2006). In a recent study, high and stable PCB burdens have been associated with small populations and low fecundity rates in killer whales (*Orcinus orca*) from European waters (Jepson et al., 2016). However, it is not known if this low fecundity rate is linked to endocrine disruption caused by pollutants.

Although high body concentrations of POPs were reported to disrupt levels of testosterone in Dall's porpoise males in 1984 (Subramanian et al., 1987), only one other study appears to have followed up this work. Applying an in vitro approach (E-Screen) to investigate estrogenic or anti-estrogenic activity in contaminant mixtures extracted from the blubber of bottlenose dolphins, significant estrogenic activity were reported for some contaminant groups, such as the DDTs (Yordy et al., 2010). In spite of these indications that POPs may act as steroid disrupting chemicals in whales, and the implications of such endocrine disruption on fitness and population dynamics of cetaceans, to our knowledge there are no other reports of possible effects of POPs on levels of steroid hormones in neither male nor female cetaceans.

Taking into consideration the relative high concentrations of POPs reported in pilot whales from the Faroe Islands (Hoydal et al., 2015), the aim of the present study was to examine possible relationships between POP concentrations and steroid hormones in these whales. Thus, we determined the concentrations of steroid hormones constituting the circulatory steroid profile in female and male pilot whales from the Faroe Islands taking into account age and size differences, and analysed the relationships between the hormone concentrations and the POP concentrations previously analysed in the same individuals (Hoydal et al., 2015).

2. Materials and methods

Blood plasma from 26 pilot whales (12 females and 14 males) was sampled in connection with traditional hunt in the Faroe Islands on two different occasions; on 23/7-2010 and 02/09-2011 (Table S1). Immediately post-mortem, blood samples were collected into clean heat treated (at 450 °C for four hours) glass jars containing heparin and kept on ice until further sample preparation. The blood was centrifuged at 1500*g* for five minutes and plasma was transferred into cryovials and frozen in liquid N_2. The samples were stored at − 80 °C until analysis. The length of the animals was measured and the sex was registered. Age was determined by counting growth layer groups formed annually in dentine and cement of teeth as described in Lockyer (1993). The individuals were grouped into sex and age groups according to their length and/or age, based on the mean length and age of sexual maturity (Desportes et al., 1993; Martin and Rothery, 1993). Thus males smaller than 494 cm and younger than 14 years and females smaller than 375 cm and younger than eight years were categorized as juveniles. The sampling in 2010 consisted of 5 adult females, 1 juvenile female, 2 adult males and 3 juvenile males, and the sampling in 2011 consisted of 5 adult females, 1 juvenile female and 9 juvenile males (Table S1). For more information on the sampling procedures, see Hoydal et al. (2015).

2.1. Steroid hormone analysis

Plasma samples were analysed for steroid hormones using solid phase extraction (SPE) followed by GC-MS/MS according to the fully validated method, including quality criteria, described by Hansen et al. (2011). The steroids analysed were the progestagens pregnenolone (PRE) and progesterone (PRO), the androgens androstenedione (AN), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT) and testosterone (TS), and the estrogens estrone (E1), 17α-estradiol (αE2) and 17β-estradiol (βE2).

Briefly, SPE columns (Agilent Technologies, Bond Elut – C18, 500 mg, 3 ml) were conditioned with 2 × 2.5 ml heptane, 2 ml acetone, 2 × 2.5 ml methanol and 2 × 3 ml of pH 3 adjusted water. Approximately 2–2.5 ml of sample was added to 6 ml of pH 3 adjusted water and 50 µl internal standard (mixture of six deuterated steroid analogues 0.4 ng/µl) and the pH in the solution was adjusted to approximately 3 with 1 M H_2SO_4 and the sample solutions were applied on the columns. The samples were eluted with 2 × 2.5 ml acetone and the eluate was evaporated to dryness under a gentle stream of N_2 and at 60 °C. The evaporated samples were reconstituted in 200 µl $CHCl_3$ and applied on new SPE columns (aminopropyl cartridges (Waters Sep-Pak, Vac 6cc (500 mg) NH_2 cartridges)), which had been prepared by adding 2 × 2 ml heptane. The samples were eluted by adding 5 ml of $CHCl_3$:isopropanol (2:1), and the fatty acids and phospholipids were retained in the column. The samples were evaporated to dryness under a gentle N_2 flow and at 60 °C. The evaporated samples were reconstituted in 50 µl $CHCl_3$ + 450 µl heptane and applied to the silica column (1 g of silica dissolved in heptane in glass columns (Merck LiChrolut, 3 ml) with filters (Macherey-Nagel Chromabond filters) on the bottom). Then, 5 ml of heptane were added into the column to remove sterol esters, followed by addition of 10 ml of heptane:acetone (90:10) to remove sterols and stanols. Then the steroids were eluted from the columns using 5 ml of heptane: acetone (65:35).

Derivatisation of the samples was performed by evaporating the samples to appr. 1 ml (N_2, 60 °C). A volume of 100 µl of the derivatisation standard (0.2 ng/µl AE2 (MeOH)) was added and the samples were evaporated to dryness. A derivatisation reagent was made by 1 ampule of N-methyl-N-(trimethyl-silyl)-trifluoro acetamide, 2 µl of N-trimethylimidazole and 50 µl of 20 mg 1,4 dithioerythritol in 500 µl pyridine (DTE) and 50 µl of the reagent was added to the evaporated samples. The vials were placed in the oven at 60 °C for one hour for derivatisation and the samples were evaporated to dryness under N_2 and at 60 °C. After that they were dissolved in 200 µl heptane with 0.1 ng/µl MeE1 (injection standard) and transferred to GC vials and analysed by GC-MS/MS (Bruker Scion TQ). Limits of quantification for individual steroids and procedures for quality control are described in details in Nossen et al. (2016). Recoveries are reported in Hansen et al. (2011).

2.2. Analysis of POPs in plasma

The plasma samples were previously analysed for 175 different POPs (i.e. PCBs, PBDEs and OCPs) and their relevant metabolites (i.e. OH-metabolites) at the Organic Contaminants Research Laboratory/Letcher Labs at the National Wildlife Research Centre, Carleton University in Ottawa, Canada. The samples were cleaned up in multiple steps and three fractions were extracted; phenolic, neutral and sulfonic fractions. The extractions were based on methods described elsewhere (Gabrielsen et al., 2015; Gebbink et al., 2008a, 2008b; McKinney et al., 2006) with modifications and analysed using gas chromatography (GC) and quadrupole mass spectrometry (MS). The results are fully reported elsewhere (Hoydal et al., 2015).

Quality control (QC) samples consisted of bovine (*Bos taurus*) serum spiked with known concentrations of many of the targeted neutral, phenolic and sulfonate analytes. Based on the spiked internal standards, the mean percent recoveries of all analytes from plasma were generally > 80%. Method limits of quantification (MLOQs) were based on 10 times the signal to noise ratio, and method limits of detection were based on 3 times the signal to noise ratio. MLODs for plasma of PCBs, OCs and FRs generally ranged from 0.02 to 0.5 ng/g wet weight (ww) for halogenated phenolic contaminants. MLOQs for plasma of PCBs, OCs and FRs generally ranged from 0.1 to 3 ng/g ww, and 0.1–1.7 ng/g ww for halogenated phenolic contaminants.

The lipid content in blood was determined by a sulfo-phospho-vanillin reaction using an olive oil-derived calibration curve ranging from 2 to 12 mg/ml. The absorbance was measured at 540 nm, the maximum absorbance. Of the 175 different POPs analysed for, 27 were detected as