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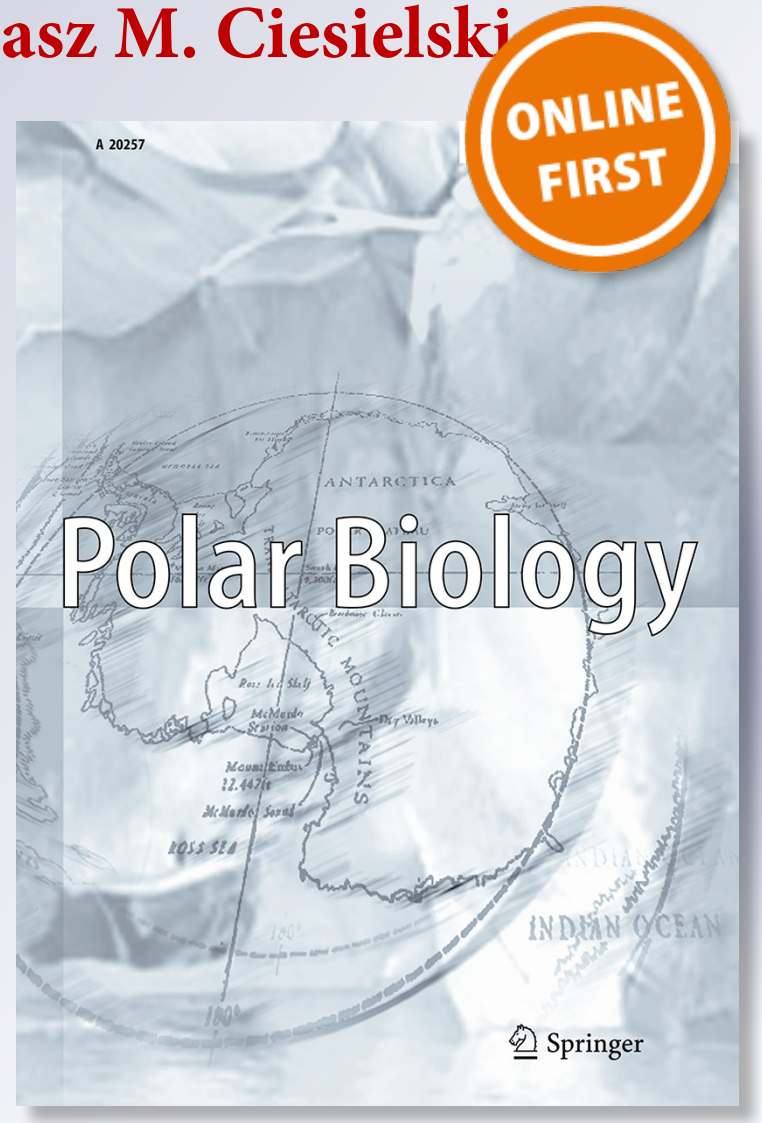

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ORIGINAL PAPER

Steroid hormone profile in female polar bears (*Ursus maritimus*)

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Abstract The polar bear is an iconic Arctic species, threatened by anthropogenic impacts such as pollution and climate change. Successful reproduction of polar bears depends on a functioning steroid hormone system, which is susceptible to effects of persistent organic pollutants. The present study is the first study to report circulating concentrations of nine steroid hormones (i.e., estrogens, androgens and progestagens) in female polar bears (*Ursus maritimus*). The aim of the study was to investigate the effects of age, condition, location and reproductive status on steroid profile in female polar bears. Levels of pregnenolone (PRE), progesterone, androstenedione (AN), dehydroepiandrosterone (DHEA), testosterone, dihydrotestosterone, estrone (E1), 17α-estradiol (αE2) and 17β-estradiol (βE2) were quantified in blood (serum) of free-living female polar bears ($n = 15$) from Svalbard, Norway, by gas chromatography–tandem mass spectrometry (GC–MS/MS). Concentrations of androgens, estrogens and progestagens were in the range of 0.02–166, 0.01–1.49 and 0.16–17.1 nmol/L, respectively. A statistically significant negative correlation was found between E1 and DHEA, and a positive correlation between E1 and βE2. Additionally, negative relationships were found between body mass and cholesterol, contour body length and cholesterol, and head length and PRE, while a positive relationship was found between PRE and cholesterol. The steroid profile suggests that AN and the sex steroids are primarily synthesized through the Δ-4 pathway in polar bears, similar to rodents. The large individual variability in steroid levels reported here most likely reflects the differences in reproductive status of the female polar bears during mating season. The steroid data establish reference values of steroid hormones and may be applied in further studies on polar bears endocrine system and anthropogenic threats to polar bear reproduction.

Keywords Steroid hormones · Steroidogenesis · Androgens · Estrogens · Progestagens · Polar bears · GC–MS/MS · Svalbard

Introduction

Polar bears are threatened by current climate changes in the Arctic because of their strongly ice-associated life history, depending on the ice for feeding, mating and locomotion (Ramsay and Stirling 1986; Molnár et al. 2010; Regehr et al. 2010). In addition, it has been shown that polar bears have high body burdens of persistent organic pollutants (POPs) that may act as endocrine disrupters and thus adversely affect their fertility and fecundity (Jenssen 2006; Letcher et al. 2010). Climate change and pollution are considered as two major anthropogenic threats to polar bear populations (Jenssen et al. 2015). The International Union for Conservation of Nature (IUCN) has red-listed

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the polar bear as vulnerable due to suspected population reduction (>30 % within three generations) and a decline in the area of suitable habitats (Schliebe et al. 2008).

Polar bears (*Ursus maritimus*) are long-lived apex predators in the Arctic marine food chain with a seasonal reproductive pattern. Polar bears usually mate in April or May, followed by a delayed implantation taking place during autumn, at the time when the females enter the den (ca. October) and start their fasting period, which lasts for approximately 6 months (Ramsay and Stirling 1988). Steroid hormones are crucial in vertebrates for numerous physiological processes, including regulation of growth, development, homeostasis and reproduction (Evans-Storms and Cidlowski 1995; Hadley 1996). These lipophilic, endogenous hormones are mainly produced and secreted from steroidogenic glands such as the adrenals, gonads (ovary and testis) and the placenta (Hadley 1996). Adipose tissue is an active endocrine organ, capable of steroid conversion (Siiteri 1987; Quinkler et al. 2004). It is an important organ of prereceptor androgen metabolism capable of both active androgen synthesis and inactivation regulating its exposure to androgens in an autocrine manner (O'Reilly et al. 2014). In addition, several steroids such as pregnenolone are synthesized in high concentrations in the brain, and these neurosteroids are involved in regulation of sexual behavior, feeding, aggressiveness, locomotion and stress (Baulieu 1997; Do Rego et al. 2009).

In animals, including polar bears, steroids in blood (i.e., plasma and serum) are traditionally investigated for monitoring endocrine function (Palmer et al. 1988; Haave et al. 2003; Oskam et al. 2003; Heistermann 2010). Circulatory steroid concentrations represent the biologically active steroid fraction (Kuijper et al. 2007). However, different biological and environmental factors such as age, sex, body mass, stress, dietary status and thus body condition, day-length and contaminants may influence circulating steroid levels and affect fertility of animals (Meikle et al. 1986; Lintelmann et al. 2003; Oskam et al. 2003; Schneider 2004; Parikh et al. 2006; Ceglarek et al. 2010).

All five steroid classes, i.e., glucocorticoids (e.g., cortisol), mineralocorticoids (e.g., aldosterone), androgens (e.g., testosterone, dihydrotestosterone and its precursors dehydroepiandrosterone and androstenedione), estrogens (e.g., estradiol, estrone) and progestagens (e.g., progesterone, pregnenolone), are derived from cholesterol (Miller 1988). Steroidogenesis (Fig. 1) is a dynamic sequential pathway, and alteration in flux in one part of the pathway may thus affect steroid production in other parts of the pathway (Nielsen et al. 2012). The conversion of cholesterol to pregnenolone by CYP11A1 is usually the first enzymatic step in the sequential steroid biosynthesis pathway (Stocco and Clark 1996). Following synthesis of pregnenolone, the sex steroids (androgens and estrogens) may be produced through two pathways (Fig. 1): the Δ-4 pathway (e.g., progesterone, 17-hydroxyprogesterone, androstenedione to estradiol) or the Δ-5 pathway (e.g., 17-hydroxypregnenolone, dehydroepiandrosterone to estradiol) (Conley and Bird 1997; Lintelmann et al. 2003). The preferred pathway is reported to be species dependent (Conley et al. 2012). For instance, the Δ-5 pathway for the formation of sex steroids appears to be the dominating pathway in human follicular cells, while the Δ-4 pathway dominates in rats (Conley and Bird 1997). Which of the two pathways is the preferential one for steroid synthesis in polar bears is not known.

Selective mass spectrometry (MS)-based methods are very suitable for steroid measurements (Xu et al. 2007; Soldin and Soldin 2009), because they can simultaneously determine minute titer levels of multiple key steroid hormones in a single sample. MS technology overcomes some of the limitations of immunoassays, especially the lack of specificity (cross-reactivity), and frees the laboratory from dependence on suppliers of assay-specific reagents. The methodology also plays an important role in the standardization of measurements among laboratories and serves as a comparison between different analytical techniques. This approach may reveal disorders related to hypothalamic–pituitary–adrenal (HPA) or hypothalamic–pituitary–gonadal (HPG) axes, which may cause homeostatic or reproductive problems of individuals (Kao et al. 2001; Rauh 2010; Weltring et al. 2012). In addition, steroid profiling may provide information about gonadal activity, sexual maturity and reproductive physiology (Lasley and Savage 2007).

Because a successful reproduction of polar bears in the future might be threatened by climate change, POPs exposure or a combination of both (Jenssen et al. 2015), the aim of the present study was to examine the steroid hormone profile in female polar bears and investigate the relationships between hormone levels and biological and environmental factors [i.e., body mass (BM), body condition index (BCI), straight and contour body lengths, head length, axial girth, zygomatic width, capture date, latitude, longitude and blood lipid content]. Thus, serum concentrations of cholesterol, pregnenolone (PRE), progesterone (PRO), androstenedione (AN), dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT), estrone (E1), 17α-estradiol (αE2) and 17β-estradiol (βE2) were simultaneously analyzed for the first time in live-caught female polar bears from Svalbard (Norway).

Materials and methods

Field sampling

Blood samples were collected from 15 female polar bears in Svalbard between 11th and 27th of April 2008. Details

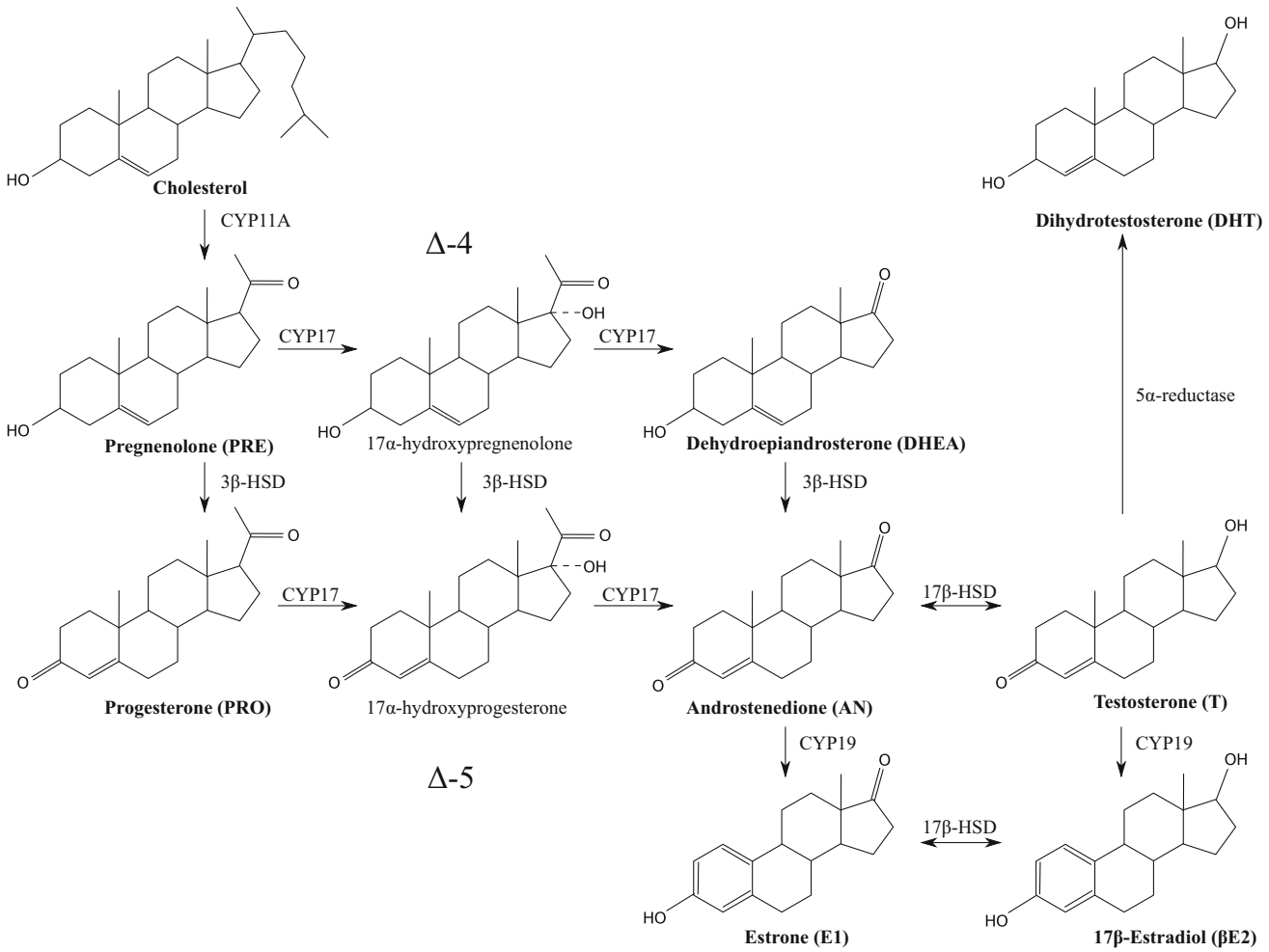

Fig. 1 Steroidogenesis. In vertebrates, the biosynthesis of reproductive hormones (androgens and estrogens) is a complex pathway starting with conversion of the precursor cholesterol to PRE by CYP11A enzyme. Enzyme abbreviations: CYP11A, cholesterol side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; CYP17, cytochrome P45017 (17α-hydroxylase/17,20 lyase); 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, cytochrome P45019 (aromatase) (Sanderson and van den Berg, 2003). Steroids analyzed in the present study are given in *bold letters*

on the sampling are described elsewhere (Bytingsvik et al. 2012). In brief, sedation of the bears was performed from a helicopter by remote intramuscular injection by a dart containing Zoletil® (200 mg/mL; Virbac Laboratories, Carros, France). Blood was collected from the femoral vein and kept cold until centrifugation. After centrifugation (1164×*g*, 10 min), serum was stored at −20 °C in the field, and later at −70 °C until analysis. A vestigial premolar tooth was extracted from captured bears upon first capture. Age was estimated by counting annual growth layers in cementum of an extracted vestigial premolar tooth (Calvert and Ramsay 1998). The biometric variables body mass (BM), straight body length (SBL), contour body length (the distance from the tip of the nose to the tip of the tail along the contour of the spine while the bear is aligned laterally), head length, axial girth and zygomatic width were also measured (Table 1). Body condition index (BCI) was estimated based on Cattet et al. (2002) according to the following equation: $BCI = (\ln BM - 3.07 \times \ln SBL + 10.76) \div (0.17 + 0.009 \times \ln SBL)$, where BM is the bear's body mass and SBL is the straight body length. The capture location (latitude and longitude) and capture date were recorded (Table 1). Furthermore, it was recorded whether the females were observed together with their offspring or an adult male, and whether they were lactating or not. All capture and handling methods were performed in accordance with the National Animal Research Authority (Oslo, Norway).

Analysis of fat content and cholesterol level

The method used for extraction, cleanup and determination of lipid content is based on the procedure previously described by Brevik (1978) with modifications described by

Table 1 Mean ± standard deviation (SD), median and range (min–max) of biometric variables, capture date (1–366), and capture location of the female polar bears ($n = 15$) sampled at Svalbard (Norway) in 2008

Variables	Mean ± SD	Median	Range Min–max
Age (years)	8.8 ± 3.5	8	4 to 16.0
Body mass (kg)	169 ± 29	169	133 to 236
Body condition index[a]	−1.30 ± 0.53	−1.28	−2.07 to −0.54
Straight body length (cm)	193 ± 7	193	180 to 205
Contour body length (cm)	205 ± 7	205	192 to 215
Axial girth (cm)	112 ± 7	110	101 to 127
Head length (mm)	343 ± 11	341	331 to 366
Axial girth (cm)	112 ± 7	110	101 to 127
Zygomatic width (mm)	197 ± 10	196	183 to 216
Capture date (1–366)	102 ± 11	104	77 to 114
Latitude (°N)	78.4 ± 1.1	78.5	76.7 to 79.7
Longitude (°E)	16.8 ± 3.5	16.3	12.2 to 22.3

[a] The body condition index was estimated from the following formula: BCI = (ln(BM) − 3.07 × ln(SBL) + 10.76) − (0.17 + 0.009 × ln(SBL)) (Cattet et al. 2002) in which *BM* body mass and *SBL* straight body length

Bernhoft et al. (1997) and Andersen et al. (2001). Briefly, the samples were extracted twice with cyclohexane and acetone (3:2 proportion) using an ultrasonic homogenizer (4710 Series, Cole Parmer Instruments Co., Chicago, IL) followed by cleanup with sulfuric acid (H_2SO_4, 96 %). The supernatant was evaporated to approximately 1 mL using a Zymark evaporation system (Hopkington, MA). The extractable lipid content in each plasma sample was determined based on an aliquot of the extract that was evaporated to dryness on a 40 °C sand-bath under a gentle flow of nitrogen, and the lipid content was determined gravimetrically and expressed as percentage (%) of total sample weight. Cholesterol levels were determined using a clinical chemistry analyzer with test strip device (Reflotron® Cholesterol strips, Roche Diagnostics, Mannheim, Germany).

Analyses of steroid hormones

Chemical analyses of steroid hormones in the serum samples were conducted at the Department of Pharmacy at University of Copenhagen (Copenhagen, Denmark).

Extraction

The procedure used for extraction, cleanup, derivatization and quantification of the steroid hormones is based on a methodology originally described by Hansen et al. (2011). Briefly, approximately 3 g of the serum samples were weighed and pH-adjusted to 3.0 ± 0.1 using diluted sulfuric acid in order to stabilize the steroid hormones. The samples were further spiked with an internal standard (IS) mix (50 µL of a 0.4 ng/µL solution in methanol) of the following ultra-high-pure deuterated analogues: d7-androstenedione, d4-estrone, d5-17β-estradiol, d9-progesterone, d3-testosterone and d3-dihydrotestosterone, followed by extraction. Solid-phase extraction (SPE) was performed using C18 cartridges (500 mg, 10 mL reservoir, Varian Inc, California). The cartridges had been preconditioned with heptane (2 × 3 mL), acetone (3 mL), methanol (2 × 3 mL) and tap water (2 × 3 mL) adjusted to pH 3.0 ± 0.1. After enrichment, the cartridges were air-dried by suction (60 min) using a vacuum manifold (IST Vacmaster from Biotage, Uppsala, Sweden). Subsequently, the analytes were eluted from the SPE cartridges with 5 mL acetone. Finally, the eluate was evaporated to dryness under a stream of nitrogen, followed by reconstitution in 100 µL chloroform.

Cleanup and derivatization

To remove interfering compounds including fatty acids, phospholipids and sterol esters, the SPE extracts (reconstituted in 100 µL chloroform) were cleaned by a two-step procedure. Aminopropyl cartridges (500 mg, Water Sep-pak, Ireland) were used as the first cleanup step. The cartridges were preconditioned with heptane (2 × 2 mL) prior to sample application, and analytes were then eluted from the cartridges by a 2:1 v/v mixture of chloroform/isopropanol (5 mL). The eluates were evaporated to dryness and reconstituted in chloroform (50 µL), followed by addition of heptane (450 µL). For the second cleanup step, a freshly prepared silica gel (1 g in heptane, Kieselgel 60, Merck, Darmstadt, Germany) was packed in a glass cartridge (3 mL, LiChrolut, Merck, Darmstadt, Germany) with a Chromabond filter in the bottom (Macherey–Nagel, Dren, Germany).

The reconstituted samples from first cleanup step were added to the silica gel, followed by addition of heptane (5 mL) and a 90:10 v/v mixture of heptane/acetone (10 mL). Finally, the steroids were eluted from the cartridge by a 65:35 v/v mixture of heptane/acetone (5 mL).

The derivatization quality control standard, estradiol-17-acetate (>99 % purity), was added (100 μL of a 0.2 ng/μL solution in methanol) to the cleaned sample extracts, followed by evaporation to dryness under a stream of nitrogen at 60 °C. The analytes were derivatized using a silylating reagent mixture (50 μL) of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), *N*-trimethylsilylimidazole (TMSI) and 1,4-dithioerythriol (DTE) (1000:2:50). Subsequently, the samples were left to react in an oven (60 °C, 1 h) and thereafter evaporated to dryness by nitrogen (60 °C). An instrument control standard, estrone-3-methyl ether (>98 % purity), was dissolved in heptane (0.10 ng/μL) and thereafter added as a reconstitution solvent (200 μL). Finally, the samples were transferred to GC vials for quantification.

Quantification

The processed samples were analyzed for concentrations of PRE, PRO, DHEA, AN, T, DHT, E1, βE2 and αE2 using a Varian CP-3800 gas chromatograph (Varian Inc, California, USA) with a large-volume programmable temperature vaporizer (PVT) injector, operated in split–splitless mode. This system was connected to a Varian 1200 triple-quadrupole mass spectrometer (Varian Inc, California, USA) operated in a selective reaction monitoring (SRM) mode with electron ionization (70 eV). The column was a Zebron-5HT Inferno (30 m × 0.25 mm, 0.25 mm, Phenomenex Inc., CA), operated at a constant carrier gas flow of 1.0 mL/min. Total chromatographic time was 30 min using the column oven temperature program: 150 °C (2-min hold), 150–230 °C (25 °C/min), 230–248 °C (1 °C/min) and finally 248–325 °C (25 °C/min) with a 3.7-min hold. Further technical details can be found in Hansen et al. (2011). Quantification was based on a 6-point calibration curve obtained from analysis of nine standard solutions. The calibration standards were diluted (0.100–20.0 ng), spiked with IS and derivatized using the same procedure as described for the serum samples. The steroid concentrations are given in nmol/L.

Validation of steroid hormone analysis

To ensure reliable results from analytical measurements, a quality assurance (QA) system with identification criteria based on retention time, peak area of qualifier and transition ions of analytes from the GC–MS/MS was applied as described by Hansen et al. (2011). Limits of detection (LOD) was defined as 3.3 times the residual standard deviation of the regression divided by the slope of the calibration curve and ranged from 0.69 to 1.25 nmol/L, 0.29–0.59 nmol/L and 1.14–1.36 nmol/L for the androgens, estrogens and progestagens, respectively (Hansen et al. 2011). In addition, if chromatographic signal-to-noise ratio for a given steroid hormone in sample was higher than three, the concentration was estimated using the calibration curve. The used tranquilizer may result in plasma concentrations reaching up to 10,000 ng/mL of the two active pharmaceuticals zolazepam and tiletamine (Semple et al. 2000). As AN and zolazepam share the same mass (286 Da), we investigated the potential interference on the chemical analysis by comparing neat low-level steroid hormone calibration standards without and with the used tranquilizer and observed no apparent effect on the analytical result for AN.

Statistical analysis

Statistical analyses were conducted using SPSS Statistical Software (version 19 for Mac, SPSS Inc., Chicago, IL) and STATISTICA (version 12, StatSoft. Inc., 2013, Tulsa, OK 74104, USA). Steroids with concentrations below limit of detection (LOD) when quantifiable in ≥60 % of the samples were given a random value between zero and LOD. The data were assessed for normality using Shapiro–Wilk test ($n < 50$). Because $\log_{10}$ transformations did not result in normal distribution of the variables, nonparametric analysis was applied. Bivariate Spearman's rank correlation test (two-tailed) was used to assess the relationship among steroid hormone levels, biological and environmental variables. Since correlations between steroid levels and age and between steroid levels and capture date were expected, these relationships were tested using one-tailed test. We applied two-tailed tests for examining the relationships between the individual hormones. Unless otherwise specified, the p values are two-tailed. For the analysis of the reproductive status of female polar bears in relation to steroid concentrations, principal component analysis (PCA), Mann–Whitney U test and Kruskal–Wallis ANOVA by ranks were applied. The reproductive status evaluation was based on whether females lactated or not and whether males were present in close proximity to the females. The significance level in all statistical tests was set to $\alpha = 0.05$. All numerical variables are presented as mean ± standard deviation (SD) with medians and ranges.

Results

The sampled female polar bears were from 4 to 16 years old. Polar bears are assumed to be adults at the age of 5 years, the initial age of reproduction in the Barents Sea population (Derocher et al. 2005).

Concentrations of steroid hormones and cholesterol

Steroid hormone and cholesterol levels, as well as the lipid content, all in serum, are given in Table 2. All steroids were detected in all females except for T, αE2 and PRO that were detected above LOD in one, 8 and 14 of the females, respectively (Table 2). The least potent androgen, AN, was the hormone with the highest concentrations (mean 33.19 nmol/L). One single female (i.e., not observed together with a male) had an AN concentration of 165.6 nmol/L. It should be noted that there was a large variation in the concentrations of AN between the individuals (Table 2). Together with AN, also PRO and PRE were the hormones that were detected in highest concentrations.

Relationship between steroid hormones and biological variables

A statistically significant positive relationship was found between the steroid precursor cholesterol and PRE (Fig. 2a, $r_s = 0.669$, $p = 0.006$), the first steroid synthesized in steroidogenesis (Fig. 1). Additionally, a statistically significant negative relationship was found between body mass and cholesterol (Fig. 2b, $r_s = -0.565$, $p = 0.028$), contour body length and cholesterol ($r_s = -0.520$, $p = 0.047$) and head length and PRE ($r_s = -0.542$, $p = 0.037$). There were no statistically significant correlations between any of the steroids and age (Online resource 1), body mass, body condition index, straight body length, axial girth, head length, zygomatic width, capture date, latitude, longitude or lipid content ($p > 0.05$). The PCA model (total explained variance for two significant PCs was 51 %) did not reveal any clusters related to lactation or male presence in proximity of female besides age groups of the bears. The loading plot indicates that this effect could be mostly attributable to the body size parameters but not steroid hormone concentrations (Online resource 2).

Table 2 Lipid content (%), and concentrations of cholesterol (mmol/L) and of steroid hormones (nmol/L)

Variables	Mean ± SD	Median	Range Min–max	*n*
Lipid	1.35 ± 0.21	1.40	0.82–1.56	15
Cholesterol	9.07 ± 1.64	9.10	5.70–13.1	15
Androgens				
AN	33.2 ± 45.0	10.2	2.10–166	15
DHEA	0.57 ± 0.31	0.43	0.26–1.28	15
T[b]	0.02			1
DHT	0.07 ± 0.02	0.07	0.05–0.12	15
Estrogens				
E1	0.29 ± 0.38	0.06	0.01–1.49	15
αE2[b]	0.09 ± 0.04	0.11	0.03–0.14	8
βE2	0.25 ± 0.30	0.13	0.02–1.03	15
Progestagens				
PRE	1.63 ± 0.47	1.61	0.94–2.56	15
PRO[a]	4.83 ± 4.70	3.50	0.16–17.1	14

Androgens, estrogens and progestagens, in serum samples of 15 female polar bears (*Ursus maritimus*). The results are presented as mean, standard deviation (SD), median, range (min–max) and number of individuals with levels above LOD (*N*)

[a] Steroid hormones detected in ≥60 % of the samples. Missing values were given a random number between zero and the limit of detection (LOD) and included in the calculations. Thus, in the statistics, $n = 15$

[b] For steroid hormones detected in <60 % of the samples, the presented statistics are only based on individuals with detectable levels. *T* was only detected in one individual

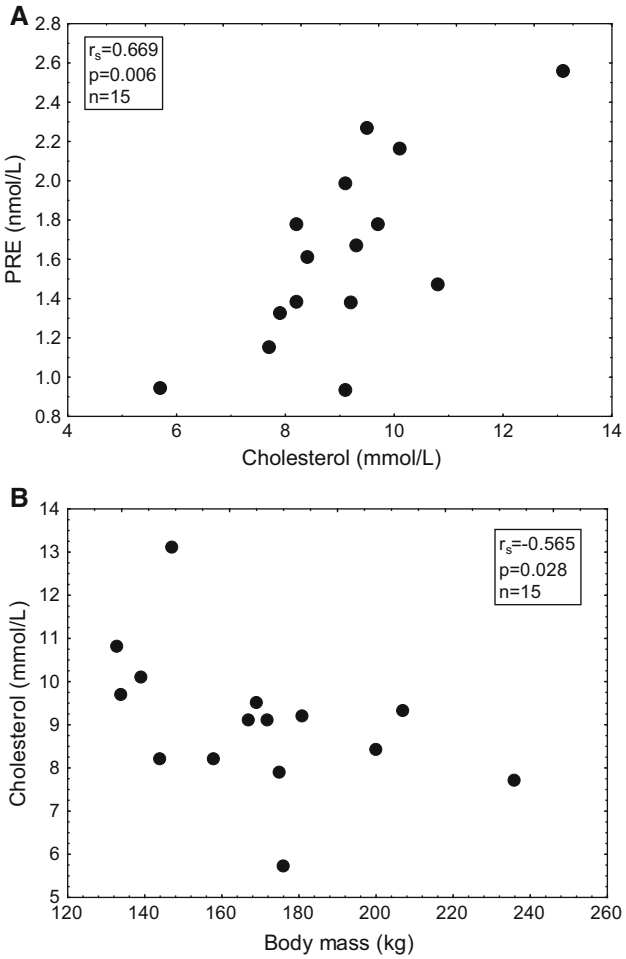

Fig. 2 Scatter plots illustrating the correlation between serum steroid levels and biological factors in female polar bears (*Ursus maritimus*) from Svalbard (Norway), collected in 2008. Correlation between PRE and cholesterol (**a**), and correlation between PRE and head length (**b**). r_s = Spearman's rank correlation coefficient

With respect to relationships between the individual steroid hormones, E1 was negatively correlated with DHEA (Fig. 3a, $r_s = -0.540$, $p = 0.038$) and E1 was positively correlated with βE2 (Fig. 3b, $r_s = 0.809$, $p = 0.0003$). In the present study, five females were observed to lactate or to have some fluid present in their nipples. Out of these five females, two females had cubs: One female had one 2-year-old cub and the other female had two 2-year-old cubs. The other three lactating females had presumably lost their cubs before they were sampled. Among the five lactating females, four were accompanied by males. Ten of the females were non-lactating, and of these, only one was accompanied by male. E1 was found in statistically significantly higher concentrations ($p = 0.01$) in the blood of lactating females in comparison with non-lactating individuals. In contrast, levels of DHEA were lower ($p = 0.04$) in lactating females when compared to non-lactating ones (Table 3).

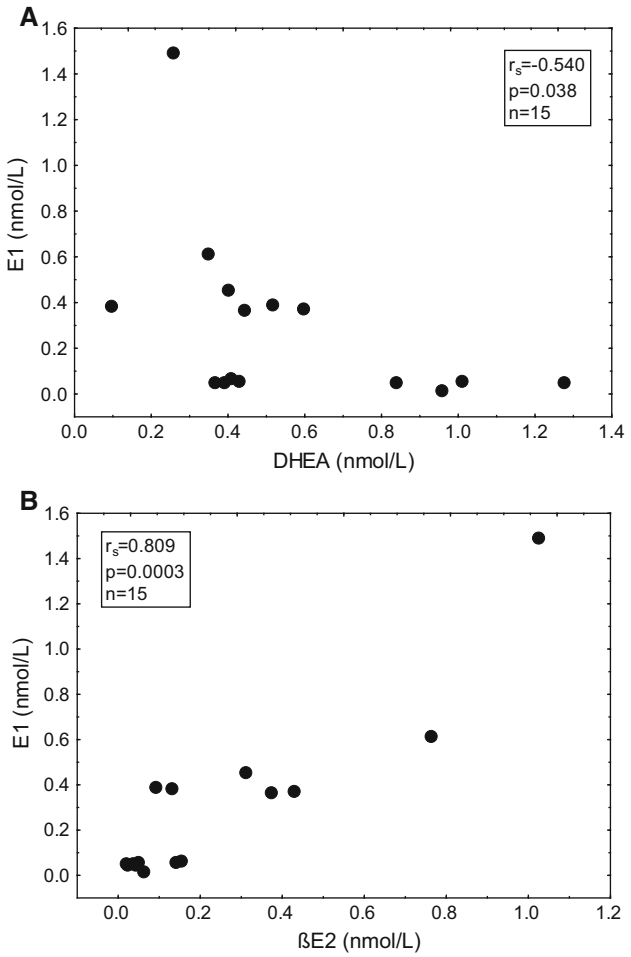

Fig. 3 Scatter plots illustrating significant correlation between steroid hormones in female polar bears (*Ursus maritimus*) from Svalbard (Norway), collected in 2008. Correlation between E1 and DHEA (**a**), and correlation between E1 and βE2 (**b**); r_s = Spearman's rank correlation coefficient. The p values are two-tailed

Ten of the females were single, i.e., observed without being accompanied by a male. Five of the females were observed together with an adult male. As previously mentioned, two of these females were also accompanied by their offspring, which were 2-year-old cubs. No differences in sex steroid concentration were found between females observed with males and those observed without males (Table 3).

Discussion

Steroid hormone concentrations

Previous studies on female polar bears have mainly included the circulating levels of only two steroids, namely PRO and βE2 (Palmer et al. 1988; Ramsay and Stirling 1988; Tsubota et al. 1998; Haave et al. 2003). Here, circulating concentrations of nine steroid hormones (i.e., estrogens, androgens and progestagens) in female polar bears are reported.

Estrogens

The concentrations of βE2 (mean 0.25 nmol/L, range 0.02–1.03 nmol/L) are in accordance with those previously reported in female black bears (*Ursus americanus)* from the North America (mean 0.26 nmol/L: Tsubota et al. 1998). Somewhat higher levels were reported in Canadian female polar bears (approximately 0.07–0.09 nmol/L: Palmer et al. 1988) and in female polar bears from Svalbard (mean 0.03 nmol/L, range 0.01–0.07 nmol/L: Haave et al. 2003). However, it should be noted that differences in the concentrations between studies may be due to different analytical techniques.

As a part of follicle maturation, the secretion of βE2 increases before ovulation (Hadley 1996). In female black bears, the level of βE2 is considered to peak during estrus, when the female is receptive to mating (Tsubota et al. 1998). In addition, circulating βE2 concentration decreases after ovulation, possibly due to the secretion of PRO in the developing *corpus luteum* (Tsubota et al. 1998). During the mating season, βE2 concentrations would be expected to be higher in single females than those with cubs. However, in the study by Haave et al. (2003) where plasma samples from 86 adult females (≥4 years of age) live-captured in the spring (between 27 of March and 8 of May) were collected, no statistically significant difference in βE2 between single females and females with cubs and females with yearlings was observed. In the present study, there were no statistically significant differences in βE2 between lactating and non-lactating females, and between females that were observed with and without accompanying males.

Table 3 Reproductive status of female polar bears in relation to steroid serum concentrations (nmol/L)

Status	*N*	AN	DHEA	DHT	E1	βE2	PRE	PRO
Lactating	5	32.3 ± 34.7	**0.37 ± 0.11**	0.09 ± 0.03	**0.59 ± 0.54**	0.44 ± 0.43	1.55 ± 0.53	4.92 ± 7.13
Non-lactating	10	33.6 ± 51.1	**0.67 ± 0.33**	0.06 ± 0.01	**0.15 ± 0.17**	0.15 ± 0.16	1.67 ± 0.47	4.84 ± 3.44
With male	5	34.4 ± 37.1	0.49 ± 0.29	0.07 ± 0.02	0.47 ± 0.60	0.30 ± 0.41	1.59 ± 0.50	3.98 ± 7.33
Without male	10	32.6 ± 50.3	0.61 ± 0.32	0.07 ± 0.02	0.21 ± 0.22	0.22 ± 0.25	1.65 ± 0.49	5.30 ± 3.14

Steroid concentrations are shown for lactating and non-lactating females and whether or not they were accompanied by a male polar bear. Values in bold indicate statistically significant differences between lactating and non-lactating bears ($p < 0.05$)

However, to clarify whether there is a difference in βE2 levels, we would need larger sample size from both groups. All the females, including the two females with 2-year-old cubs and the three other females that were lactating (but had no cubs), should have been available for mating during the sampling period. The reported E1 concentrations in the present polar bear females (mean 0.29 nmol/L, range 0.01–1.49 nmol/L) were similar to the values reported in pre- and postmenopausal women (0.026–0.74 nmol/L: Nelson et al. 2004) and in other marine mammals such as northern fur seals (*Callorhinus ursinus*) from Alaska (0.01–0.92 nmol/L: Browne et al. 2006). In the human female, E1 secretion is higher during the preovulatory phase than in the luteal phase (Hadley 1996). In the present study, either the lactating females had lost their offspring ($n = 3$), or their offspring were 2 years of age ($n = 2$) and, thus, all these females would therefore be available for mating. The statistically significantly higher E1 concentrations in lactating females compared to the non-lactating females may indicate that lactating females were in the preovulatory phase, whereas the non-lactating females to a larger extent had mated and, thus, were in the luteal phase, but the role of E1 in polar bear reproduction is not well understood. Moreover, small sample size does not allow us to unequivocally confirm this finding.

The reported levels of αE2 in the polar bears were low. Low or non-detectable concentrations of αE2 have been reported in women (Moos et al. 2009). It is assumed that αE2 is synthesized from epitestosterone (Moos et al. 2009). Therefore, a low enzymatic activity of 17α-hydroxy-steroid oxidoreductase converting androstenedione to epitestosterone or a low aromatization of epitestosterone in female polar bears could explain the low levels of αE2 in the present study. Our findings may therefore indicate that αE2 is less important than βE2 in this species, possibly due to its lower estrogenic potency (Moos et al. 2009).

Androgens

In female vertebrates, androgens are precursors for the synthesis of estrogens and are thus needed for maintaining female fertility (Staub and DeBeer 1997; Wylie et al. 2010). In the present study, T was detected in only one of the females, while DHT was detected in low concentrations in all individuals. In women, most DHT originates from peripheral transformation of T, with levels in pre-menopausal women (0.317 nmol/L) constituting ca. 50 % of T (0.635 nmol) in blood (Rothman et al. 2011). Thus, it may be suggested that a low conversion rate of AN to T and of T to DHT by 17β-HSD and 5-reductase, respectively, results in low concentrations of these androgens (Stanczyk 2006) in female polar bears.

Androstenedione and DHEA were the major androgens in female polar bears. These compounds have also been reported to be the major androgens in human females (Abraham 1974). However, these two steroids have low androgen potency and are usually regarded as precursor hormones due to weak binding to androgen receptors (Conley et al. 2012). It should be noted, however, that the levels of DHEA reported in the present polar bear females (mean 0.57 nmol/L, range 0.26–1.28 nmol/L) were only within the lower range of levels reported for human females (range 0.55–26.70 nmol/L: Kushnir et al. 2010) and slightly lower than those in other marine mammals exhibiting seasonal breeding such as northern fur seals (range 0.59–4.85 nmol/L, Browne et al. 2006). In humans, DHEA is mainly synthesized by the adrenal gland, but in other mammals, DHEA secretion takes place exclusively in gonads and coincides with low levels of DHEA in blood (Cutler et al. 1978). Hence, the low DHEA levels in female polar bear blood compared to human females may result from DHEA mainly being synthesized in the gonads and may also be attributed to the lack of a dominant *zona reticularis* in the adrenal glands (Cutler et al. 1978; Conley and Bird 1997). Moreover, in a study on dairy cattle, it was suggested that the lactating mammary gland can decrease circulating DHEA levels (Marinelli et al. 2007) which could explain the observed difference in DHEA levels between lactating and non-lactating females in polar bears.

With respect to circulating AN concentrations, the levels in female polar bears (mean 33.2 nmol/L, range 2.10–166 nmol/L) differed substantially from ranges

reported in northern fur seals (0.05–1.14 nmol/L: Browne et al. 2006). Additionally, the levels were 12–22 times higher than the reported reference range for AN in human females (0.17–7.47 nmol/L: Kushnir et al., 2010). The higher levels of Δ-4 steroids (PRO, AN) compared to Δ-5 steroids (PRE, DHEA) in female polar bears could indicate that AN is primarily synthesized through the Δ-4 pathway in polar bears, similar to rodents (Payne and Hales 2004). This is in contrast to humans or bovine animals, where the Δ-5 pathway is reported as the dominating route for synthesis of AN (Conley and Bird 1997; Conley et al. 2012). Steroid synthesis differences between the species can affect their sensitivity to environmental chemicals or drugs. Scott et al. (2009) suggest that the difference between Δ-4 or Δ-5 pathway in rats and humans is a logical explanation for the higher vulnerability of the testicular steroidogenesis of humans to inhibition by the antifungal agent, ketoconazole, as compared to that of the rats. In contrary, the same difference has been proposed as a cause for humans being less susceptible than rat to steroidogenesis inhibition by the imidazole fungicide prochloraz (Scott et al. 2009). This finding might have an implication for selection of mammalian models (e.g., rodents) for studying steroidogenesis in polar bears or predicting impact of pollutants on steroid hormones.

The relative steroid contribution from gonads, adipose tissue and adrenal glands is reported to depend on reproductive status (Hillier and Tetsuka 1997). The ovarian contribution of AN is reported to increase from 30 % during the follicular phase to 60 % during mid-cycle of the female menstrual cycle (Hillier and Tetsuka 1997). This indicates that AN is involved in the female polar bear estrus cycle, perhaps functioning as a non-masculinizing reservoir during ovulation when positive feedback controlled by LH stimulates a rapid increase in βE2. However, the role of AN in female polar bears is not known.

Progestagens

The reported levels of PRO (mean 4.83 nmol/L, range 0.16–17.1 nmol/L) did not differ substantially from previously reported levels in samples of female polar bears from Svalbard and Canada collected during the spring (Palmer et al. 1988; Ramsay and Stirling 1988; Derocher et al. 1992; Haave et al. 2003). Moreover, the levels of PRE reported here (mean 1.63 nmol/L) were close to previously reported concentrations of PRE in human female serum (means of 2.89–3.09 nmol/L: Keski-Rahkonen et al. 2011). Pregnenolone is considered a neurosteroid, with anti-neurodegenerative effects (Vallee et al. 2001; Goncharova and Lapin 2002). Pregnenolone is also an important precursor for the synthesis of all steroid hormones (Stocco et al. 2005). A low variation in PRE levels (2.7-fold) in female polar bears was found in the present study. This could reflect a stable steroidogenic flow of PRE for further synthesis of glucocorticoids, mineralocorticoids and sex steroids.

Associations between steroid hormones and biological variables

There was a statistically significant inverse association between DHEA and E1 in the female polar bears. This is in contrast to humans, in which a positive correlation between DHEA and E1 has been reported (Audet-Walsh et al. 2011). This inter-species difference between polar bears and humans may be due to differences in synthesizing routes of E1. In humans, there is a concentration-dependent conversion of DHEA to E1 in the Δ-5 pathway associated with the positive correlation between these steroids (Audet-Walsh et al. 2011). Since the Δ-4 pathway may be the dominating route in polar bears, the inverse correlation between DHEA and E1 in polar bears could suggest that AN is the ultimate precursor for estrogens. However, a strong correlation was found between E1 and βE2 (Fig. 3b). The positive correlation between these estrogens here may reflect the concentration-dependent conversion of E1 to βE2 by the 17β-HSD enzyme (Missmer et al. 2006). The estrogenic potency of E1 is much less than that of βE2, and E1 may therefore be regarded as a reservoir for βE2.

The positive relationship between cholesterol and PRE is most likely due to the fact that cholesterol is the precursor of PRE, and conversion of cholesterol to PRE is a rate-limiting step in the steroidogenesis (Stocco et al. 2005). However, the role of cholesterol and PRE in polar bear growth is not well understood. The negative relationships between cholesterol and body mass and between cholesterol and contour body length may be explained by the diet of polar bears. In a study of Kaduce and Folk (2002), plasma lipid cholesterol concentrations were lower in fed compared to fasting polar bears. This was explained by the protective role of n-3 fatty acids (which were not available to fasted bears) against high serum lipids; thus, whether an animal is fasting or feeding is reflected in blood cholesterol levels. Body mass (and thus contour body length) dramatically changes in polar bears during the year (Atkinson and Ramsay 1995) and is low during fasting, in comparison with feeding period. Thus, cholesterol concentrations might be directly related the changes in body mass.

In the present study, there were no correlations between age and PRO, and between age and βE2. These results are consistent with a previous study in Svalbard female polar bears (Haave et al. 2003). Interestingly, studies of female polar bears from East Greenland have indicated declining bone mineral density (BMD) with increasing age of female

polar bears (Sonne et al. 2004). Especially, females between 14 and 23 years of age had indications of declining BMD with age (Sonne et al. 2004). Because sex steroids, and in particular βE2, are involved in regulation of bone mass, it was suggested (Riggs et al. 2002; Sonne et al. 2004) that the age-related decline in BMD could indicate an age-specific change in reproductive performance possibly due to reproductive senescence. Since the oldest polar bear in the present study was only 16 years of age, the present study does therefore not provide any information on possible reproductive senescence in polar bears.

Several POPs have been documented to affect circulating concentrations of reproductive hormones in polar bears (Haave et al. 2003; Oskam et al. 2003), and this may adversely affect their fecundity and fertility (Jenssen et al. 2015). Normal reproductive development and fertility depend on a proper balance between androgen and estrogen synthesis in primary steroidogenic and peripheral tissues in all species. Therefore, basic knowledge on the reproductive physiology of polar bears, including information on the steroidogenesis and how it is affected by intrinsic factors such as sex, age, diet and fasting, is important to unravel the possible reproductive disrupting effect of POPs in polar bears.

Conclusions

This study suggests that there are large variations in steroid hormones between individuals in female polar bears, and we suggest that this likely reflects differences in reproductive status. Furthermore, our study provides evidence that supports the hypothesis that AN and the sex steroids are primarily synthesized through the Δ-4 pathway in polar bears. Moreover, an inverse relationship was identified between head length and PRE. The steroid data establish reference values of steroid hormones and may be applied in further studies of polar bears, related to effects of contaminants on steroid levels.

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