Steroids in house sparrows (*Passer domesticus*): effects of POPs and male quality signaling

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ABSTRACT

At high trophic levels, several environmental contaminants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated flame retardants (PBDEs) have been found to affect endocrinological processes governing growth and reproduction. Less attention has been paid to species at lower trophic levels, and it is presently unclear whether or not the lower levels of contaminants observed at lower trophic levels are able to exert negative effects on important endocrinological process governing growth and reproduction.

The aim of the present study was to use the house sparrow (*Passer domesticus*) as a low trophic level model species to study the effects of environmental contaminants on endocrinology. We analysed the levels of some selected PCBs (PBC-28, -52, -101, -118, -138, -153, -180), PBDEs (BDE-47, -99, -100, -153, -154, -209, hexabromocyclododecane [HCBD]) and OCPs (hexachlorobenzene [HCB], dichloro-diphenyl-dichloroethylene [*p,p'*-DDE]) and investigated the possible effects of these contaminants on circulating levels of steroid hormones (estrone [E1], 17α-estradiol [αE2], 17β-estradiol [βE2], progesterone [PRO], pregnenolone [PRE], 17-hydroxyprogesterone [OH-PRO], 17-hydroxypregnenolone [OH-PRE], androstenedione [AN], dehydroepiandrosterone [DHEA], dihydrotestosterone [DHT], testosterone [TS]) in adult house sparrows from a population on the island Leka, mid Norway. Plasma samples were analysed for steroid hormones by gas chromatography tandem mass spectrometry (GC-MS/MS) and liver samples were analysed for environmental contaminants by gas chromatography-electron capture detection (GC-ECD) and gas chromatography mass spectrometry (GC-MS). It was hypothesized that POPs may have endocrine disrupting effects on the local house sparrow population and can thus interfere with the steroid hormone homeostasis.

Bivariate correlations revealed negative relationships between PCB-118 and βE2 and *p,p'*-DDE, ∑OCPs and E1 among female house sparrows. Among male sparrows, bivariate correlations indicted positive relationships between DHT levels and PCB-118, BDE-100 and ∑PCBs. Furthermore, positive relationships were found between AN and TS levels and beak length, as well as ornamental traits such as badge size category and area of the total badge size in males. Although sparrows are in the mid-range trophic levels, the present study indicates that POPs may affect steroid homeostasis in house sparrows, in particular for females. For males, circulating steroid levels appears to be more associated with biometric parameters related to elaborate traits.

1. Introduction

High levels of contaminants are well documented in species in the top part of the food chain. Among the most important classes of persistent organic pollutants (POPs) accumulating in wildlife are chlorinated and brominated aromatic compounds such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs) and different organochlorine pesticides (OCPs: dichloro-diphenyl-trichloroethane [DDT] and its metabolites). In birds, occurrence of POPs has been documented in particular for birds of prey (Luzardo et al., 2014) but a few studies on POPs in passerines have also been conducted (Dauwe et al., 2003; Eens et al., 2013). Even though passerines appear to be less sensitive to POP exposure than other birds, (Van den Steen et al., 2009) the fact that these contaminants are present in low trophic level bird species is a cause for concern. Thus, effects of pollutants and POPs have been reported in passerines, including house sparrows, in both urban and agricultural areas (Bishop et al., 2000; DeLeon et al., 2013; Herrera-Duenas et al., 2014).

The house sparrow (*Passer domesticus*) is a small semi-colonial passerine and one of the most abundant bird species globally. A fraction of juvenile House sparrow disperse during their first year of living but later in life the site fidelity is very high (Altwegg et al., 2000; Tufto et al., 2005; Anderson, 2006; Pärn et al., 2009; Pärn et al., 2012). The adult male and female house sparrows are easily distinguished from each other; females are pale brown and grey whereas the males have brighter black, white and brown markings (Møller et al., 1996; Anderson, 2006). Males also develop a black badge on their chest during their first autumn moult in late September or early October, which have been shown to be heritable but also influenced by environmental effects such as cohort effects and climatic effects prior to moulting (Jensen et al., 2006; Jensen et al., 2008). After moulting the badge is partially covered with grey feather tips, but these are worn off due to preening and dust bathing making it bigger and darker.

Females are known to choose mates on the basis of male badges among other qualities (Møller et al., 1996). Female preference for mates with elaborated traits has often been explained on the basis that exaggerated secondary sexual traits may reflect individual male quality and females might gather direct and indirect benefits in mating with such males (Gonzalez et al., 2001, Ringsby et al., 2009). The badge size also seems to be related to social status and is hence a signal of dominance (Møller, 1987; Solberg and Ringsby, 1997). Studies have shown that badge size is positively selected because male mating success increases with badge size (Jensen et al. 2008), and that males with a large badge size have higher lifetime reproductive success (Jensen et al., 2004). It is believed that androgens, especially testosterone (TS), directs the secondary sexual traits like the chest badge in house sparrows (Buchanan et al., 2001), however there have been contradicting reports on whether this is a direct relationship. Some previous studies found a positive correlation between TS levels and size of the chest badge of house sparrows (Evans et al., 2000; Gonzalez et al., 2001) indicating that testoserone is important not only for dominance, but also for attracting female sparrows. In a study by Laucht et al (2010), however, TS levels were positivily related to bill colour and not badge size.

The biosynthesis of steroid hormones is termed the steroidogenesis and includes synthesis of mineralocorticoids, glucocorticoids and sex steroid hormones. In vertebrates, the steroidogenesis is a complex sequential pathway starting with the degradation of cholesterol to progestagens (pregnenolone [PRE], progesterone [PRO]) by CYP11A and further conversions to androgens (TS, androstenedione [AN], dihydrotestosterone [DHT], dehydroepiandrosterone [DHEA]) and finally estrogens (17β-estradiol [βE2], estrone [E1]). Vertebrate steroid hormones are mainly synthesized in gonads, adrenal glands, placenta, brain and adipose tissue and regulate a variety of different biological functions such as carbohydrate metabolism, salt and water balance, blood pressure, stress, reproduction, fertility, development of secondary sex characteristics, growth, pregnancy, behaviour, breeding etc. (Stocco, 2001). Such interactions may have detrimental effects and a potential cause for decreasing bird populations worldwide. Furthermore, due to its sequential dependence, effects in one part of the pathway may cause changes in other parts of the pathway, thereby increasing or decreasing steroid synthesis downstream (Harvey and Everett, 2003; Nielsen et al., 2012).

The aim of the present study was to determine levels of PCBs and OCPs, and to investigate the possible effects of these contaminants on circulating levels of steroid hormones in house sparrows from the island Leka in mid- Norway. It may be hypothesised that POPs may interfere with the steroid hormone homeostasis and cause negative effects on population dynamics in local house sparrow populations. Since little information is available on the endocrine disruptive aspects of contaminants on hormone homeostasis in passerines, the present study may therefore provide important information on the subject.

2. Materials and methods

2.1 Field work

2.1.1 Study site and sampling

The sparrows used in the present study were collected on the island Leka (65°5'N, 11°37'E) located in Nord-Trøndelag county in mid Norway during February 2013. The house sparrows in this population live in close contact with human settlements and are often found associated with dairy farms where they live in and around cowsheds all year roundand food is available. The estimated number of adult house sparrows present on Leka in February 2013 was 137 individuals, distributed among 12 farms with varying degree of distance from each other (maximum approx. 9 km). Typically, about 10-20 % of the recruits disperse from their natal farm and settle in other farm populations before their first breeding season (Pärn et al., 2012). Most of the dispersing individuals move short distances, and the number of dispersers decline rapidly with distance (Tufto et al., 2005). As part of an artificial selection experiment on basal metabolic rate (BMR) practically all house sparrows (>90%) were captured in field by mist nets during February 2013,

measured for morphological traits and ringed with individually numbered metal rings. Unique colour combinations of plastic colour rings were attached to the tarsi, which allowed for recognition of the birds in the field (Ringsby et al., 2009). Subsequently, the captured sparrows were placed indoors in an isolated barn with access to fresh water and food (1 week) until >90% of all birds on the island were captured.

Sparrows with high basal metabolic rate (BMR) were selected for toxicological and steroid analysis on Leka (BMR selection threshold (equal to the mean before selection): males > 78.53 mlO_2/h, females > 76.48 ml O_2/h) whereas birds with low BMR were used in another experiment not related to the toxicological study. Birds with high BMR have higher food intake and it is therefore also likely that this group of the population would have a higher intake of POPs than the group of birds with low BMR. Thus, we expect that the high BMR group represent the high contaminated part of the population.

2.1.2 Measuring morphological traits

Body mass was measured using a 50 g Pesola spring balance (to the nearest 0.1 g). Tarsus length, bill length and bill depth where all measured to the nearest 0.01 mm using a digital Vernier slide calliper. Wing length was measured using a ruler with an accuracy of 0.5 mm. Digital slide callipers were also used to measure length and width (to the nearest mm) of the black chest badge of adult males and two types of badge measurements were taken. The first was total badge size, defined as the area covered with black feather and feathers with light bases and light grey feather tips (Solberg and Ringsby, 1997; Ringsby et al., 2009). The second was visible badge size, defined as the area covered with black feathers without light tips. Badge sizes were then calculated according to Møller (1987) and Jensen et al. (2008).

2.1.3. Sampling for toxicological analysis

Both females (n = 22) and males (n = 25) were selected (total n = 47) for toxicological analysis. The sparrows were killed by decapitation and blood was collected in heparinized tubes and subsequently centrifuged (3000 rpm, 10 minutes) to separate the plasma from the blood cells. The plasma (200 µl) was transferred to blood vials with a Pasteur pipette for steroid analysis and 50 µl internal standard was added (IS: 50 µl of a 0.1 ng/µl stock solution). Plasma samples were then stored at –20 °C for later analysis. Immediately after collection of blood tissue dissection was carried out to obtain the liver, which was weighed and wrapped in aluminum foil before storage in liquid nitrogen until further analysis.

2.2 Chemical analysis

2.2.1 Steroid hormone analysis

Plasma samples from all birds were analysed for steroid hormones. The steroid hormone analysis was conducted at the Department of Pharmacy at the University of Copenhagen, Denmark. The plasma samples were analysed for eleven steroid hormones; pregnenolone (PRE), progesterone (PRO), 17-hydroxyprogesterone (OH-PRO), 17-hydroxypregnenolone (OH-PRE) androstenedione (AN), dehydroepiandrosterone (DHEA), dihydrotestosterione (DHT), testosterone (TS), estrone (E1), 17α-estradiol (αE2), 17β-estradiol (βE2). The procedure is based on the method originally described by Hansen et al. (2011) with some modifications.

2.2.1.1 Solid phase extraction (SPE)

Plasma samples were filtrated using glass microfiber filters (Watman™ GF/C, 25 mm Circles, Cat No; 1822-025) to ensure that the samples did not clot in the cartridges during solid phase extraction (SPE). Approximately 2 ml plasma was diluted with tap water (7 ml) and pH-adjusted (pH = 3) by dilution of sulphuric acid (1M H_2SO_4) in order to stabilize the steroid hormones.

The steroid hormones were isolated using C18 cartridges (500 mg, 10mL reservoir, Varian Inc., California). Prior to isolation, the SPE columns were conditioned with heptane (5 ml), acetone (2 ml), methanol (5 ml) and pH adjusted tap water (pH=3, 7 ml). Subsequently, the filtrated samples were added and after isolation, the columns were air dried by suction (1h) using a vacuum manifold (IST VacMaster, Uppsala Sweden). Finally the analytes were eluted from the SPE cartridges with acetone (5 ml) and evaporated to dryness using a heat-block (60 °C) and nitrogen gas.

2.2.1.2 Derivatization

After evaporation to dryness, a derivatization control standard (DCS: 50 µl of a silylating reagent mixture of N-methyl-N-trietylsilyl-trifluoroacetamide (MSTFA), N-trimethylsilylmidazole (TMSI) and 1,4-dithioerythriol (DTE)) was added to the extracts containing the analytes followed by evaporation to dryness on heat-block (60 °C) and nitrogen gas. The vials were left in an oven (60 °C, 1h) and thereafter the mixture was evaporated to complete dryness using the heat-block (60 °C) and nitrogen gas. Finally, an instrumental control standard (ICS; esterone-3-methyl-ether MeE1 0.1 ng/µl in heptane) was added (200 µl) and samples were transferred to GC-vials for quantification by gas chromatography tandem mass-spectrometry (GC-MS/MS).

2.2.1.3 Quality control

Linear calibration curves were established for each steroid hormone following the derivatization procedure on neat standard dilutions (0.1-100 ng/ml). Absolute and relative recoveries for the analysed hormones and the quality assurance system using ICS, DCS, compound retention times and SRM ion ratios are previously described in Hansen et al (2011). Series of blanks and spiked procedural controls were analysed following each sample-batch. A derivatization quality control

standard (DCS) and an instrumental control standard (ICS) were added to each sample to insure the quality during the derivatization step and during instrumental quantification. The present method has previously been shown to determine steroid hormones in the low part per billion ranges with high selectively (Hansen et al., 2011).

2.2.1.4 Quantification

The details of the quantification are as described in Hansen et al. (2011) with some modifications. The GC was a Bruker Scion™ GC with a Zebron-5HT Inferno (30m × 0.25mm, 0.25 µm, Phenomenex Inc., Torrance, CA, USA) column. The GC was connected to SCION TQ GC triple quadrupole (Bruker Daltonics, Bremen, Germany) equipped with an EI interface. For the hydroxy-steroids (OH-PRE and OH-PRO) not included in in the original method, the following ion transitions were used; OH-PRE: m/z 433.00 to m/z 253.10 (T) and m/z 343.20 (Q); OH-PRO: m/z 359.00 to m/z 145.10 (T) and m/z 269.20 (Q). The limits of detection (LOQs) were as follows: PRE: 0.009 ng/ml; PRO: 0.05 ng/ml; OH-PRE: 0.1 ng/ml; OH-PRO: 0.01 ng/ml; DHEA: 0.05 ng/ml; AN: 0.008 ng/ml; TS: 0.01 ng/ml; DHT: 0.009 ng/ml; E1: 0.05 ng/ml; αE2: 0.05 ng/ml; βE2: 0.05 ng/ml.

2.2.2 Persistent organic pollutants analysis

Liver samples from the sampled birds where analysed for environmental contaminants ~~at the Norwegian School of Veterinary Science (NVH), laboratory of Environmental Toxicology, Oslo Norway~~. The laboratory is accredited by the Norwegian Accreditation for testing analysed chemicals in biological material according to the requirements of the NS-EN ISO/IEC 17025 (Test 137). The method used is based on the procedure originally described by ~~Brevik and Bjerk,~~ (1978) and optimized by Polder et al. (2008).

Liver samples were analysed for seven PCBs (PCB-28, -52, -101- 118, -138, -153, -180), six BFRs (BDE -47, -99, -100, -153, -154, -209) and OCPs (HCB and pp`-DDE).

2.2.2.1 Quality control:

For each series of 17 samples; 3 blank samples, one blind and two recovery samples were analysed. The blank samples consisted of solvents and a mixture of internal standards (I.S.); PCB I.S. (PCB-29, -112 and -207, Ultra Scientific, RI USA) and BFR I.S. (BDE-77, -119, -181 and -13C12-209, Cambridge isotope laboratories, Andover, MA, USA. The blind and recovery samples consisted of spiked samples of cattle liver. Also, in-house reference of seal blubber (LRS) was used in the quality control.

2.2.2.2 Homogenization and extraction:

Sparrow livers (0.3-0.6 g) were cut into fine pieces using a scalpel and transferred to centrifuges tubes (80 ml) and weighed. Internal standards (IS) were added to the samples according to which analytes being analysed (PCB I.S [PCB-29, -112 and -207, Ultra Scientific, RI, USA] and BFR

I.S. [BDE-77, -119, -181 and -13C12-209], Cambridge isotope laboratories, Andover, MA, USA]). After homogenization and addition of IS, the lipids and the lipid soluble contaminants were extracted using liquid-liquid extraction. NaCl (6 %, 2 ml), grade 1 water (10 ml), acetone (15 ml) and cyclohexane (20 ml) were added to the centrifuge tubes followed by sonication by ultrasound probe (2 min), (Cole Parmer CPX 750, Vernon Hills IL, USA). After sonication the samples were centrifuged (10 min, 3000 rpm), (Allegra X-12R Beckman Coulter, Fullerton, CA, USA) and the supernatant was transferred to Zymark glass for evaporation by nitrogen gas (TurboVap II, Zymark Corporation, Hopkinton, MA, USA) to approximately 1 ml. The procedure was repeated for the remaining phase in the centrifuge tubes. The concentrated lipid extracts where then quantitatively transferred to volumetric flasks and adjusted to the respected volume (5 ml) with cyclohexane.

2.2.2.3 Gravimetric lipid determination

Aliquots of the ~~concentrated~~ lipid extract (1 ml) were used for gravimetric lipid determination and added to pre-weighed 8-grams glasses. The glasses were left on sand-bath (40 °C) overnight, weighed and evaporated to dryness on sand-bath with nitrogen gas before the glasses was weighed once more.

The lipid concentration was calculated according to the formula:

$$\frac{(\text{Weight of glass containing lipids - weight of empty glass}) \times \text{flask volume} * 100}{\text{weighted amount} \times \text{excised volume}}$$

2.2.2.4 Lipid clean-up

Prior to quantification on GC, it is necessary to remove interfering substances like lipids from the extract. This was achieved by clean-up with sulphuric acid (H_2SO_4) of the remaining lipid extract; the extract was transferred to 10 mL test tubes and H_2SO_4 (96 %, 2 mL), (Fluka analytical, Sigma-Aldrich, St. Louis, USA) was added and stored at darkness for 1h. Finally, the test tubes were centrifuged (3000 rpm, 10 min) and the supernatant ~~was transferred~~ to GC vials for quantification by gas chromatography mass spectrometry (GC-MS) and gas chromatography electron capture detection (GC-ECD).

2.2.2.5 Quantification

The details of the GC- quantification are as described in Polder et al. (2008) with some technical modifications of GC conditions. The constant flow of hydrogen carrier gas was increased to 1.2 mL/min and final holding time at 257 °C was increased to 21 min, changing the total run time to 72.6 min. The lowest level of detection (LOD) for individual compounds was set at three times the noise level, except for *p,p'*-DDT, PCB -28, - 52 and -101. Due to co-eluting compounds, the

LOD of these compounds was set to ten times the noise level. Compounds not detected in the analysis, thereby being under LOD, were excluded from further statistical evaluations. The LODs were as follows: PCB-28: 0.097 ng/g ww, PCB-52: 0.057 ng/g ww, PCB-101: 0.052 ng/g ww, PCB-118: 0.098 ng/g ww, PCB-153: 0.099 ng/g ww, PCB-180: 0.064 ng/g ww, BDE-28: 0.125 ng/g ww, BDE-47: 0.105 ng/g ww, BDE-99: 0.095 ng/g ww, BDE-100: 0.075 ng/g ww, BDE-153 and BDE-154: 0.120 ng/g ww, BDE-209: 0.965 ng/g ww, HBCD: 1.130 ng/g ww.

2.3 Statistical analysis

Statistical analysis was conducted using SPSS Statistical Software (Version 21 for Windows, IBM, SPSS Inc., Chicago, IL) and SIMCA P+ (Version 12.0, Umetrics, Umeå, Sweden). POPs and steroid hormones detected in less than 60 % of the individuals were excluded from the statistical analysis. These where: BDE-28, BDE-153, BDE-154, BDE-209, HBCD, OH-PRO, OH-PRE, αE2. For those variables detected in more than 60 % of the samples, potential values being under LOD were replaced by random numbers between 0 and LOD prior to analysis.

The data was also assessed for normality using Shapiro-Wilk test and homogeneity of variance in SPSS. Variables that were not normally distributed were transformed using natural log (ln) transformation and if normality was not obtained, non-parametric analysis was performed. Independent T-test and Mann-Whitney U test was applied to assess differences between sexes with a significance level $p<0.05$. Bivariate Spearman's rank correlation test was applied to assess possible correlations between steroid hormones, contaminants and biometrical variables with a significance level of $p<0.05$. Negative or positive correlations from the Spearman's rank correlation test were further illustrated by scatter plots.

3. Results

3.1 Morphological traits

Morphological traits measured in both female and male house sparrows from Leka (n = 47) are listed in Table 1. Significant differences between females and males were observed for wing length and body mass (Mann-Whitney U test, $p<0.005$, n = 47) where males had significantly longer wings and higher body mass (Table 1). No other biometrical data differed significantly between the sexes.

3.2 Environmental contaminants

For both males and females the contaminant groups found in the highest concentrations (ng/g ww) in the liver samples were, in decreasing order; $\sum_7$PCBs > $\sum_2$Pesticides > $\sum_3$PBDEs. The most prevalent congeners for $\sum_7$PCB were PCB-153 > PCB-180 > PCB-138 > PCB 118 > PCB-

28 > PCB-101 > PCB-52. As for $\sum_2$Pesticides the most prevalent compound was *p,p'*-DDE > HCB and for $\sum_3$PBDEs; BDE-99 > BDE-47 > BDE-100 (Table 2). The overall concentrations of the environmental contaminants were low with *p,p'*-DDE (range: 0.15 – 5.74 ng/g, ww) and PCB-153 (range: 0.04 – 32.2 ng/g, ww) found in the highest concentrations. PCB-52, PCB101 and BDE-100 were found in the lowest concentrations. BDE-28 and HBCD were not detected in any of the birds, whereas BDE-153 (range: 0.120-2.288 ng/g ww) BDE-154 (0.121-0.274 ng/g ww) and BDE-209 (1.053-2.478 ng/g ww) were detected in 18, 4 and 11 birds, respectively. Since these compounds were detected in <60% of the individuals they were not included in the statistical analyses.

For both females and males statistically significant correlations were observed between all the PCB congeners (Spearman, $p<0.005$), except for PCB-52, that did not correlate with any other PCBs. The PBDE congeners also correlated significantly with each other (Spearman, $p<0.05$), in both sexes. Statistically significant differences between females and males were observed for PCB-28, *p,p'*-DDE and HCB (independent t-test, $p<0.05$, Table 2).

3.3 Steroid hormones

The highest steroid hormone concentration detected in both females and females was TS, which also had high variability among individuals, and the variations among individuals were very high. Statistical significant differences between females and males were only observed for PRO (Mann-Whitney U test, $p<0.033$). Among females, statistically significant correlations between hormone levels were observed for AN and βE2 (Spearman, $r = 0.469$, $p = 0.028$). Among males, significant correlations between AN and E1 (Spearman, $r = 0.485$, $p = 0.014$) and TS and PRE (Spearman, $r = 0.483$, $p = 0.014$) were identified. No other significant correlations between steroid hormones were detected.

3.4 Spearman's rank correlation coefficient test and Correlation plots

All observations and variables were investigated with Spearman's rank coefficient test among males and females, and significant correlations ($p<0.005$) are listed in (Table 4).

3.4.1 Females

Significant negative correlations between single contaminants and steroid hormone levels, were observed between PCB-118 and βE2 levels ($r = -0.426$, $p = 0.048$) and *p,p`*-DDE and E1 ($r = -0.607$, $p = 0.003$) (Table 4). The correlations were maintained even after removal of the potential outliers. Moreover, a negative relationship was also observed between E1 and $\sum_2$OCPs ($r = -0.533$ $p = 0.011$) (Table 4) and the negative relationship was maintained even after removing the potential outlier. In addition, some of the biometrical data also correlated significantly with steroid hormone levels (Table 4); TS was negatively correlated with beak length ($r = -0.600$, $p = 0.003$) and PRE correlated positively with body mass ($r = 0.470$, $p = 0.027$). Significant correlation was almost obtained for DHEA and body mass, thus this association was only

considered as a trend (r = 0.413, p = 0.056). In addition, PCB-52 was the only contaminant which correlated with age, and this was a negative correlation (r = -0.704, p = 0.001), indicating no age-related accumulation of the other contaminants.

3.4.2 Males

Significant positive correlations were observed between PCB-118 and DHT (r = 0.408, p = 0.043) and BDE-100 and DHT (r = 0.400, p = 0.048) (Table 4). Moreover, a positive relationship was also observed between DHT and ∑PCBs (r = 0.402, p = 0.046) and the positive relationship was maintained even after removal of the potential outlier. In addition, some of the biometrical data also correlated with steroid hormones level (Table 4): AN was positively correlated with beak length (r = 0.548, p = 0.005), TS was positively correlated with chest category (r = 0.402, p =0.046) and total badge size (r = 0.434, p = 0.030). In addition, PCB-52 was the only contaminant which correlated with age (r = -0.633, p = 0.001) indicating no age-related accumulation of the other contaminants.

4. Discussion

In the present study, the reported male TS levels (mean = 1.461 ng/ml) did not differ substantially from levels previously reported for house sparrows in New York during the winter period (Hegner and Wingfield, 1986) and levels reported in house sparrows from France in spring prior to and during early breeding (mean = 1.02 ng/ml), (Chastel et al., 2003). Seasonal differences in the TS levels have been reported in several bird species (Moore et al., 2002) and plasma TS levels is reported to peak during the onset of the breeding season which may vary among species. Moreover, large individual variations in TS levels as reported in the present study are expected due to normal individual differences. In addition to differences in reproductive status, the variation might also reflect differences in dominance status where some individuals may have higher TS levels due to more aggressive and competitive behaviour than less dominant males (Solberg and Ringsby, 1997; Buchanan et al., 2001).

In female house sparrows, TS levels (mean = 0.930 ng/ml) were lower than in males, but this was not statistically significant. The female TS levels in the present study were higher than levels found in female house sparrows sampled during February in New York) (Hegner and Wingfield, 1986). However, the annual reproductive cycle reported in that particular study refers strictly to the study population in New York, and may explain why females in the present study have higher TS levels. In addition, when comparing the female TS levels with levels presented by Ketterson et al. (2005) who compared data from the literature on different avian species, the levels reported herein do not differ substantially from the levels reported for pre-laying females. High levels of TS in females have, in some bird species, also been associated with aggressive behaviour in periods of territorial establishment and defense of nest sites (Sandell, 2007) and such female

aggression might explain the relative high TS levels in the present study. For instance, Mazuc et al. (2003) demonstrated that TS levels in female house sparrows were positively related to breeding population density. Also, female sparrows are known to defend their nests against intruding females particularly during egg-laying period. This was experimentally demonstrated by Veiga (1992) who suggested that the female-female aggression could be related to the avoidance of nest parasitism, or expelling a potential female in order to maintain its monogamous status. In addition, the fact that all birds were maintained in a barn for one week could have resulted in some variations of TS levels due to stress (Deviche et al., 2012).

AN levels were low in both males and females (male mean = 0.092 ng/ml, female mean = 0.088 ng/ml) compared to levels reported in Japanese quail (male mean = 1.9 ng/ml, female mean = 2.4 ng/ml) (Ramenofsky, 1984), but did not differ substantially from AN levels reported for pre-laying female blue-throats (*Luscinia svecica)*, (Pärn et al., 2008). Circulating levels of DHT were similar to levels reported in house sparrows from New York during winter (Hegner and Wingfield, 1986). DHEA was the steroid with the lowest concentrations in both male and female sparrows and was lower than that reported in white-throated sparrows (*Zonotrichia albicollis*), (Spinney et al., 2006). Low DHEA levels may indicate that DHEA is not the primary pathway of conversions to more potent androgens or estrogens in the sparrows herein. However, the role of DHEA and other androgens and the natural variation in relation to the breeding cycle is not known in house sparrows and awaits further investigations.

Not many publications are available on estrogen levels in male house sparrows so comparisons within the species are difficult. Concentrations in plasma of males are typically low, ranging 0.002-0.180 ng/ml depending on the species (Hess, 2003). In the present study, βE2 levels (mean = 0.139 ng/ml) did not differ substantially from levels reported in male white-crowned sparrows (*Zonotrichia leucophrys*) from Canada, late in the breeding season (Charlier et al., 2011). Female levels of βE2 (mean = 0.252 ng/ml) corresponded with levels found in female house sparrows from New York (Hegner and Wingfield, 1986) and pre-breeding house sparrows from France (mean = 0.41 ng/ml), (Chastel et al., 2003). There was no large individual variation in βE2 levels, except for one female having very high βE2 concentrations compared to the others (2.65 ng/ml). Since βE2 secretion is dependent on reproductive state, all females in the present study seemed to be in the same stage of their reproductive cycle (except for one), which is expected since breeding usually is synchronized in birds (Verhulst and Nilsson, 2008). To our knowledge, no studies have investigated the level of E1 in female house sparrows, clearly illustrating a gap in our knowledge regarding estrogens in house sparrows.

Limited information is available on progestagens in passerines, in particular for PRE for which no previous data has been identified. PRE is the product in the first and rate-limiting step of the steroidogenesis and furthermore the dominating brain neurosteroid. Understanding the role of PRE is therefore pivotal in obtaining a better understanding as to how steroids control growth and reproduction in passerines. PRO was the only steroid hormone which differed significantly between the sexes (Mann-Whitney U test, $p = 0.033$) showing that males had higher PRO levels

than females. In comparison to other species, PRO levels in male sparrows (mean = 0.91 ng/ml) were lower than those reported in breeding male black kites (*Milvus migrans*), (mean pre-laying = 2.0 ng/ml, mean peak = 7.73 ng/ml) (Blas et al., 2010). Blas et al. (2010) reported PRO levels in breeding individuals while PRO levels in the present study were from pre-breeding males. Furthermore, PRO levels did not differ substantially from PRO levels reported for male white crowned sparrows (mean = 0.8 ng/ml), (Charlier et al., 2009). In female sparrows, PRO levels (mean = 0.412 ng/ml) were lower than reported in the plasma of control female song sparrows investigated by Elekonich and Wingfield (2000) as well as for breeding female black kites (mean pre-laying = 4.0 ng/ml, mean peak = 7.52 ng/ml), (Blas et al., 2010), though differences in breeding-stages may again explain lower PRO levels reported herein.

To our knowledge, this is the first study to investigate levels of POPs in liver tissue of the house sparrow, except for a study that reported levels of BDE-209 (Ciesielski et al., 2008). In general, the contamination burden in both male and female house sparrows in the present study was low compared to studies of birds of prey (Naso et al., 2003). Nevertheless, negative relationships between POPs and estrogens were observed in female house sparrows. This indicates that POPs have the potential to affect steroid homeostasis at relatively low POP concentrations in species in the low to mid trophic levels. Further investigations are therefore needed to clarify whether the present findings could indicate a general trend. It should be noted that these birds were those in the population that presumably had the highest POP intake based on the BMR.

The distribution of congeners in house sparrows were similar to congener distribution found in great tit eggs and nestlings (Dauwe et al., 2003) as well as Caspian terns (*Hydroprogne caspia*) and great blue herons (*Ardea herodias*), (Mora, 1996). Furthermore, the predominance of higher chlorinated congeners found in the present study is also typical for species high in the food chain like polar bears and glaucous gulls (Gabrielsen et al., 1995; Kannan et al., 2005). This predominance is most likely due to the lipophilic and persistent nature of hepta-, hexa- and penta PCBs to facilitate absorption and accumulation. In addition, it is well known that higher chlorinated PCBs exhibit higher bioaccumulation in avian tissue than lower chlorinated congeners (Naso et al., 2003) due to their molecular structure and chemical properties.

Only two OCPs were analysed in the present study and *p,p'*-DDE was the most abundant which is consistent with a previous study of OCPs in great tit eggs (Van den Steen et al., 2009) and a study of grassland passerines (Bartuszevige et al., 2002). Males also had statistically significantly higher levels of OCPs than females which could be due to males having slightly higher body mass compared to females. Bigger males usually have a higher food intake than smaller individuals and might increase body burdens of some contaminants. In addition, females may transfer some of their contamination load on to their eggs during breeding (Robinson et al., 2012).

In the present study, the HCB levels were similar to levels reported in the lower range in birds from different trophic levels in Italy (Naso et al., 2003). The fact that HCB is still detected in

animal tissue despite EUs ban of HCB use indicates its persistence in the environment, as a by-product from production of various chlorine-containing chemicals and as an impurity in several pesticides (Bailey, 2001). In addition, OCP levels in the present study were well below levels associated with toxic effects on reproduction in the most sensitive avian species (Custer et al., 2003) and are expected due to little application of pesticides at Leka.

Few data exist on PBDE levels in passerine birds and in the present study, PBDEs were detected in low concentrations compared to other contaminant groups. PBDE levels have been reported to be higher in urban than rural areas (Hale et al., 2006). Leka is located far from industrial sites and PBDE levels would thus be expected to be lower than other contaminant groups that are more prevalent in rural areas, such as OCPs. Highly brominated BDEs were detected in <60% of the sparrows in the present study (BDE-209, -154, -153). In a previous study, BDE-209 was reported in a higher proportion of the sampled sparrows from the same area (Ciesielski et al. 2008), and levels appeared to be somewhat higher. It is possible that the high number of individuals herein with BDE-209 levels below the detection limit reflects decreasing environmental concentrations of this compound. Lower brominated congeners were more prevalent (BDE-47, -99, -100) in the sparrows and comparable with other studies on eggs of low trophic birds (Dauwe et al., 2009). However, this is in contrast to what was found in eggs of peregrine falcons (*Falco peregrinus*), in Sweden (Lindberg et al., 2004) where higher brominated BDEs were detected. Thus, the detection of the lower brominated BDEs (penta-BDE) in the present study might reflect the fact that the house sparrow is a bird low on the food chain compared to the peregrine falcon and differences in congener profiles could be expected.

The negative relationship between βE2 levels and PCB-118 reported in the present study have also been reported in female polar bears from Svalbard (Haave et al., 2003), though only as a borderline significance. However, polar bears are known to accumulate much higher levels of contaminants. In addition, polar bears are able to metabolize PCBs to OH-PCBs and might therefore be more susceptible to endocrine disruption (Bytingsvik et al., 2012; Gustavson et al., 2015). Also, positive correlations between androgens (DHT) and contaminants (PCB-118, BDE-100 and ∑PCBs) were observed among males. However, the mechanism behind this is unknown. One possibility could be an induction of the 5α-reductase activity, responsible for converting TS to DHT but this awaits further investigations.

Positive correlation between TS and total badge size and TS and chest category were observed among males and are in agreement with previous studies on house sparrows (Gonzalez et al., 2001). Such a positive correlation is expected as androgens are known to play a part in development of sexual secondary characteristics in birds (McGraw et al., 2006). Furthermore, it is also known that more dominant male house sparrows, having higher levels of TS, often molt into larger badges and thus be more successful in attracting female sparrows (Møller, 1990). Female preference for elaborated traits illustrates the importance of maintaining a large badge. However, sexual signals are known to be associated with some costs and to be reliable for individual quality. Thus, sparrows may not be able to display high levels of TS without

experiencing negative costs. High TS levels have been linked with immunosuppressive aspects and males with big badges illustrate their individual male quality by coping with the internal stress of maintaining a big badge (Evans et al., 2000; Gonzalez et al., 2001; Buchanan et al., 2003). Females might thus, gather direct and indirect benefits by mating with such males. Males with higher TS and thus probably a bigger badge may be more successful in reproduction than less ornamented males. If a direct coupling exists between TS levels and Darwinian Fitness, in male house sparrows, then this species qualifies as an excellent model species for investigating the effects of steroid modulations from e.g. POPs on Darwinian Fitness.

It should be mentioned, however, that the coupling between male quality signaling and TS levels may be more complex than first anticipated. For example, Laucht et al. (2010) found bill colour and not badge size to correlate with plasma TS levels. Although the study by Laucht et al. (2010) was conducted on captive house sparrows which may affect male behaviour and interactions, it still indicates that male quality signaling is complicated and involves several morphological and endocrine processes not yet known in details. This aspect needs further investigation.

Due to the limited knowledge on endocrine disruption on steroid hormone homeostasis in house sparrows, future studies should be conducted on different house sparrow populations from various locations to assess whether the results reported herein refers only to the particular population on Leka or whether they indicate general trends. Both wildlife and experimental studies may help to better understand the endocrinology of house sparrows. Furthermore, studies could also include investigations on urban populations versus rural populations to consider whether there are differences in contamination and possible endocrine disruption from a more industrialized area compared to rural area. Since birds are seasonal breeders further studies could also include investigations on whether the house sparrow is more susceptible for endocrine disruption during the breeding season compared to the non-breeding season.

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Figure 1: Chest category determination of the chest badge in male house sparrows (Møller, 1987).

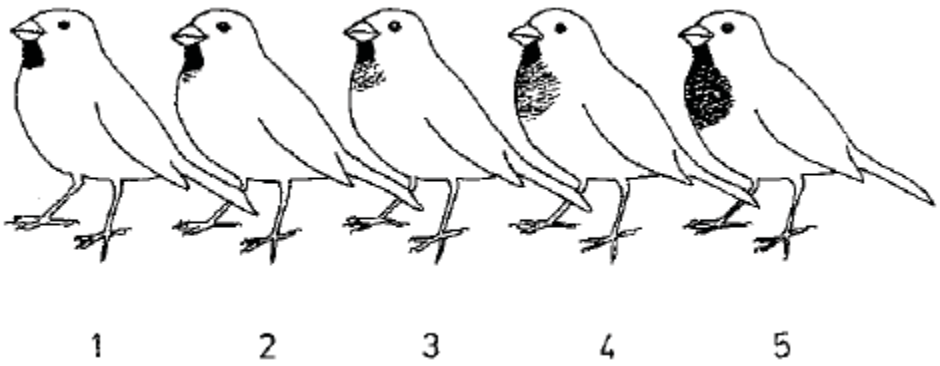

Table 1: Mean ± standard deviation (SD), median and minimum and maximum values of morphological variables measured in 22 female (n = 22) and 25 male (n = 25) house sparrows (*Passer domesticus*) collected at Leka, Helgeland Norway 2013. The average value of both right and left tarsus bone length, as well as average value of left and right wing length was used. BMR: Basal metabolic rate. *Indicates significant differences between sexes ($p<0.05$). [a]: Phenotypic traits existing only for male house sparrows. B: Badge and beak category were visually ranged from 1-5 (Figure 1).

Variables	*Females*			*Males*		
	Mean ±SD	**Median**	**Min-max**	**Mean ±SD**	**Median**	**Min-max**
Age (years)	1.50±1.30	1	1.0-7.0	1.60±1.16	1	1.0-5.0
Body mass (g)*	31.1±1.423	31.2	28.7-34.1	32.18±1.680	32.1	29.2-35.9
Gonads (g)	0.02±0.017	0.02	0.001-0.090	0.09±0.136	0.042	0.005-0.559
Tarsal bone	19.77±0.613	19.82	18.50-20.78	19.66±0.707	19.77	17.27-20.43
Wing length*	78.40±1.610	78.11	75.80-82.93	81.05±1.251	81.21	70.76-82.71
Beak height	7.85-0.225	7.810	7.48-8.31	7.84±0.204	7.85	7.45-8.15
Beak length	13.27±0.507	13.35	11.79-14.06	13.29±0.465	13.23	12.58-14.27
Liver mass (g)	0.41±0.087	0.396	0.291-0.672	0.38±0.065	0.389	0.278-0.581
Lipids (% in liver)	2.0±0.942	2.03	0.46-3.85	2.57±1.176	2.4	1.13-5.68
BMR (mlO_2/h)	86.55±6.954	87.24	76.53-106.2	89.07±6.483	87.77	79.15-101.76
Eye-mask[a] (mm)				14.11±1.145	14.3	11.40-16.70
Total badge[a] (mm^2)				19.79±1.351	19.83	16.84-22.83
Visible badge[a] (mm^2)				14.36±0.693	14.67	13.09-15.30
Badge category[ab]				2.60±0.645	3	1.0-4.0
Beak category[ab]				3.44±1.158	4	1.0-5.0

Table 2: Mean, standard deviation (SD), median and minimum and maximum values of concentrations (ng/g ww) of polychlorinated biphenyls (PCBs), chlorinated pesticides (OCs) and polybrominated diphenyl ethers (PBDEs) found in liver samples from 22 female and 25 male house sparrows collected at Leka, Helgeland Norway 2013. Only contaminants detected in ≥ 60 % of the samples were included and n denotes the number of individuals with detectable concentrations > LOD. *Indicates significant differences between sexes ($p<0.05$). [a]: Contaminants containing concentration values under detection limit of the analysis.

POPs	*Females*				*Males*			
	Mean ±SD	**Median**	**Min-max**	**n**	**Mean ±SD**	**Median**	**Min-max**	**n**
PCB-28*[a]	0.142±0.043	0.140	0.044-0.230	21	0.210±0.200	0.161	0.102-1.141	25
PCB-52[a]	0.128±0.081	0.130	0.025-0.246	16	0.166±0.172	0.105	0.032-0.812	16
PCB-101[a]	0.129±0.076	0.119	0.026-0.268	19	0.165±0.109	0.14	0.026-0.488	20
PCB-118[a]	0.295±0.283	0.234	0.029-0.900	15	0.505±0.645	0.34	0.039-2.737	22
PCB-138	0.893±0.726	0.704	0.135-3.211	22	1.796±2.841	1.018	0.165-12.262	25
PCB-153[a]	1.760±1.925	1.217	0.040-9.141	21	3.881±7.230	1.821	0.216-32.259	25
PCB-180[a]	1.007±1.286	0.562	0.028-5.944	21	2.302±5.131	0.773	0.065-19.665	25
$\sum_7$PCBs	4.354±4.420	3.106	0.327-19.672		9.025±16.328	4.358	0.645-69.364	
HCB*	0.794±0.277	0.682	0.507-1.640	22	1.081±0.517	0.968	0.465-2.924	25
p-p'-DDE*	1.380±0.972	0.988	0.154-3.194	22	2.415±1.450	2.252	0.598-5.738	25
$\sum_2$OCPs	2.174±1.249	1.67	0.661-4.834		3.496±1.967	3.22	1.063-8.662	
BDE-47	0.298±0.215	0.253	0.036-0.796	17	0.827±2.051	0.204	0.048-10.333	19
BDE-99	0.661±0.424	0.70	0.035-1.654	20	1.478±2.643	0.518	0.063-12.938	23
BDE-100	0.116±0.068	0.116	0.031±0.299	16	0.179±0.176	0.090	0.035±0.586	17
$\sum_3$PBDEs	1.066±0.707	1.071	0.102-2.749		2.484±4.870	0.812	0.146-23.157	

Table 3: Mean, standard deviation (SD), median and minimum and maximum values of concentrations (ng/ml plasma) of steroid hormones (progestagens, androgens, estrogens and mineralocorticoid) detected in plasma samples from 22 female and 25 male house sparrows collected at Leka, Helgeland Norway 2013. Only hormones detected in ≥ 60 % of the samples were included and n denotes the number of individuals with detectable concentrations > LOD. *Indicates significant differences between sexes ($p<0.05$). [a]: Individuals with steroid hormone concentrations under detection limit of the analysis.

Steroid hormone	*Females*				*Males*			
	Mean ±SD	**Median**	**Min-max**	**n**	**Mean ±SD**	**Median**	**Min-max**	**n**
Progestagens								
PRE	0.220±0.312	0.121	0.015-1.370	22	0.228±0.466	0.097	0.009-2.317	25
PRO*	0.412±0.517	0.279	0.049-2.456	22	0.913±0.863	0.620	0.055-3.325	25
Androgens								
DHEA[a]	0.024±0.033	0.017	0.004-0.157	22	0.030±0.048	0.014	0.002-0.188	24
AN[a]	0.087±0.164	0.040	0.005-0.767	21	0.092±0.165	0.040	0.012-0.797	25
TS[a]	0.930±0.841	0.713	0.004-3.446	19	1.461±1.524	0.881	0.004-7.160	24
DHT[a]	0.161±0.091	0.188	0.028-0.307	22	0.118±0.097	0.065	0.002-0.346	24
Estrogens								
E1	0.525±0.320	0.217	0.093-6.391	22	0.193±0.104	0.155	0.091-0.480	25
βE2[a]	0.252±0.536	0.140	0.111-2.649	22	0.139±0.039	0.141	0.026-0.229	24

Table 4: Statistically significant correlations (Spearman's rank correlation test) between steroid hormones and contaminants, steroid hormones and biometry and contaminants and biometry for male (n=25) and female (n=22) house sparrows sampled at Leka, Helgeland Norway 2011. [a]: Border line significance between DHEA and body mass.

Correlated variables	Sex	Correlation coefficient (r)	Significance level (p)
βE2 - PCB-118	Female	-0.426	0.048
E1 - *p,p'*-DDE	Female	-0.607	0.003
E1 - ∑OCPs	Female	-0.533	0.011
DHT - PCB-118	Male	0.408	0.043
DHT - ∑PCBs	Male	0.402	0.042
TS - beak length	Female	-0.600	0.003
PRE - body mass	Female	0.470	0.027
DHEA - body mass[a]	Female	0.413	0.056
AN - beak length	Male	0.548	0.005
TS - chest category	Male	0.402	0.046
TS - total badge	Male	0.434	0.030
PCB-52 - age	Female	-0.704	0.001
PCB-52 - age	Male	-0.633	0.001

Supplement material:

Supplemental Table 1:

Morphological variables:

Abbreviations – morphological measurements

ID	Number of individual metal ring
Location:	Name of farm the bird was caught
Colour (L):	Colour combination, left tharsal bone
Colour (H):	Colour combination, right tharal bone
BeakCat:	Beak category (blackness of beak) – males only
ChestCat:	Chest category (blackness of chest badge) – males only
Tars (L):	Length of left tharsal bone
Tars (R):	Length of right tharsal bone
Wing (R):	Length of right wing
Wing (L):	Length of left wing
Mask:	Length of the black eye-mask – males only
Total badge:	Size of the potential chest badge, with light feather tips
Visual badge:	Size of the visual chest badge, without light feather tips