The effects on steroidogenesis and histopathology of adult male Japanese quails (*Coturnix coturnix japonica*) testis following pre-pubertal exposure to di(n-butyl) phthalate (DBP)

Umar M. Bello [a,b], Mary-Catherine Madekurozwa [a], Herman B. Groenewald [a], Tom A. Aire [a,c], Augustine Arukwe [d,]

[a] *Department of Anatomy and Physiology, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa*
[b] *Department of Veterinary Anatomy, Ahmadu Bello University, Zaria, Nigeria*
[c] *Department of Anatomy, Physiology and Pharmacology, School of Veterinary Medicine, St. George's University, True Blue, St George's, Grenada* [d] *Department of Biology, Norwegian University of Science and Technology (NTNU), Høgskoleringen 5, 7491 Trondheim, Norway*

Corresponding author : E-mail address: arukwe@bio.ntnu.no (A. Arukwe).

A B S T R A C T

In the present study, we have investigated the effects of 30-day dietary (pre-pubertal) exposure to different doses (0 (control), 1, 10, 50, 200 and 400 mg/kg bodyweight/day) of di(n-butyl) phthalate (DBP) on Leydig cells of adult male Japanese quails by quantifying the transcript levels for P450 side-chain cleavage (p450scc), P450c17 (CYP17), and 3β- a n d 1 7 β-hydroxysteroid dehydrogenase (hsd) using quantitative (real-time) poly-merase chain reaction (qRT-PCR). In addition, the plasma testosterone levels were analysed using radioimmuno-assay (RIA) and testis was examined for evidence of gross pathology and histopathology. Our data showed that pre-pubertal exposure to DBP produced alterations in testicular architecture as evident by poorly developed or mis-shaped testis, and altered spermatogenesis due to tubular degeneration and atrophy of seminiferous tubules especially in the high DBP dose (200 and 400 mg/kg) treated groups. In addition, DBP altered several key en-zymes involved in testicular steroidogenesis pathways in an apparent dose-dependent manner. For example, bi-phasic effects of DBP were observed for P450scc and 3β-hsd mRNA, that were generally increasing at low dose 10 mg/kg, and thereafter, an apparent dose-dependent decrease between 50 and 400 mg/kg. The steroidogenic acute regulatory (StAR) protein was at the lowest detectable limits and therefore not quantifiable. These effects did not parallel the non-significant changes observed for plasma testosterone levels. The present data is consis-tent with previous reports showing that DBP modulates Leydig cell steroidogenesis in several species, with a po-tential negative effect on reproduction in those avian species that are vulnerable to endocrine disrupting chemicals.

Keywords:
Di-(n-butyl) phthalate
Leydig cell
Steroidogenesis
Male Japanese quails
Endocrine disruption

1. Introduction

Dialkyl or alkyl aryl esters of 1,2-benzene dicarboxylic acid (phthalic acid), commonly referred to as phthalate esters (PEs) are synthetic chemicals ubiquitous in the environment. PEs are used extensively as plasticizers, in several consumer plastic products, including children toys, food wrappings, cosmetics, paints, some biomedical devices (e.g. dialysis tubing and intravenous bags), and enteric coatings of some pharmaceuticals (Hauser et al., 2004; Oehlmann et al., 2009). Because PEs are not covalently bound to polymer plastics, they can easily leach out over time into the environment, reaching humans and wildlife species through ingestion, inhalation, and dermal exposure (Heudorf et al., 2007). Phthalates are rapidly hydrolyzed after oral administration in the gastrointestinal tract and other tissues by non-specific esterases to produce the corresponding monoesters and alcohol (Thomas and Thomas, 1984; Mentlein and Butte, 1989). Di(n-butyl) phthalate (DBP), for instance, is metabolized into the monoester mono-butyl phthalate (MBP), which is a potent testicular toxicant (Oishi and Hiraga, 1980), and these, rather than parent compounds, are considered to be the active agents in testicular toxicity (Sjöberg et al., 1986; Mylchreest et al., 2000).

PE possesses endocrine disrupting properties (Latini, 2005), and has been shown to mimic or antagonize the action of endogenous hormones, which consequently results in adverse effects on reproduction, growth and development (Jensen et al., 1995; Crisp et al., 1998; Fisher, 2004). Humans and wildlife populations are continuously exposed to EDCs, which are constantly discharged into the atmosphere

and natural aquatic bodies (Kavlock et al., 2002). The PEs' mode of action, is not well understood, and may likely be dependent on developmental timing and dosing regimes (Akingbemi et al., 2001). Studies on rodents have identified Leydig cells as one of the main targets of phthalate-ester induced toxicity (Akingbemi et al., 2004). Phthalates interfere with the transcription of genes of several key enzymes involved in both cholesterol transport and testosterone biosynthesis. These enzymes include steroidogenic acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage (P450scc), P450c17 (CYP17), CYP19 (aromatase), and 3β- and 17β-HSD (Lehmann et al., 2004; Thompson et al., 2004; Borch et al., 2006; Ryu et al., 2007; Chauvigné et al., 2011). Interferences with steroid biosynthesis may result in impaired reproduction, alterations in sexual differentiation and growth, as well as the development of certain cancers. The combined use of gene expression analysis and histopathological examinations may provide a diagnostic tool to screen many variables required to examine gene function patterns and reproductive alterations (Adachi et al., 2002).

A number of EDCs, including DBP, are known to be anti-androgenic (Gray et al., 2001; Scott et al., 2009). Since it has been shown that phthalates do not directly bind to the androgen receptor (Parks et al., 2000; Foster et al., 2001), the anti-androgenic effects of DBP are suggested to be due to a decreased testosterone synthesis that arises from the reduction in the expression of genes involved in cholesterol transport and testosterone synthesis (Shultz et al., 2001; Barlow et al., 2003). Laboratory studies have shown that the male Japanese quail is sensitive to the effects of exogenous hormones during sexual differentiation (Ottinger et al., 2002). The testicular toxicity resulting from phthalate exposure has been shown to be age-dependent. For example, it is known that mature animals are less sensitive to phthalate toxicity than immature animals (Richburg and Boekelheide, 1996; Dalgaard et al., 2001). To date, very scant amount of literature exists on the effects of environmentally relevant concentrations of DBP, particularly in the avian wildlife species. It is now generally accepted that many species of amphibians and wild birds, that were exposed to very low doses of EDCs throughout their life, via contaminated food and water, are on the decline, and some may face extinction (Houlahan et al., 2000; Stuart et al., 2004; Lee and Veeramachaneni, 2005). Therefore, the present study was designed to test the effects of a PE (DBP) on cellular and molecular processes that underlie testicular steroidogenesis and signaling pathways, using the male Japanese quail (*Coturnix coturnix japonica*), as a sentinel avian model. Our hypothesis is that exposure of male quails to the environmental pollutant — DBP at pre-pubertal period, will produce differential expression patterns of selected genes involved in Leydig cell steroidogenesis, and may subsequently leads to alterations in testosterone levels and gonadal development.

2. Materials and methods

2.1. Chemicals and reagents

Di(n-butyl) phthalate DBP (CAS Number 84-74-2—technical grade-99% purity) was purchased from Sigma-Aldrich (Pty) Ltd (Johannesburg, South Africa). RNA stabilization reagent-RNALater® (QIAGEN) was purchased from Whitehead Scientific (Pty) Ltd (Cape Town, South Africa). TRIzol® reagent was purchased from Gibco-Invitrogen Life technologies (Carlsbad, CA, USA), and iScript™ cDNA Synthesis kit, iTaq™ Universal SYBR® Green Supermix with ROX, dNTPs and 100 bp EZ load molecular ruler were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Microtiter plates (MaxiSorp) were purchased from Nunc (Roskilde, Denmark). GelRedTM nucleic acid stain was purchased from Biotium (Hayward, CA, USA). The Radioimmunoassay kit: ^{125}I Coat-A-Count kit for total testosterone was purchased from Siemens Medical Solutions Diagnostics (Los Angeles, CA, USA). All other chemicals were of highest commercially available grade.

2.2. Animals, husbandry and exposure

A total of ninety (90) newly hatched, pre-sexed male Japanese quails (*C. coturnix japonica*) procured from the Aviary Unit, Irene Animal Improvement Research Station, Pretoria were used for the study. Prior to the experiment, the quails were acclimatized at the poultry facility (brooder cages) located in Poultry Research Unit of the Department of Production Animal Studies, (University of Pretoria) for a period of two weeks before being transferred into battery cages of dimension: 49 × 95 × 51 cm. At hatch, the temperatures were first maintained at 35–37 °C and then slowly decreased by 0.5 °C/day until a temperature of 16–23 °C was reached at 4 weeks of age. Thereafter, the birds were maintained under controlled photoperiod (16L: 8D, schedule of light–dark cycle), at 25 ± 2 °C with a relative humidity of 50 ± 5% until the age of 10 weeks. The birds were individually identified by means of wing-tags, and fed on standard commercial high protein diet (Obaro Feeds, Pretoria South Africa) and tap water was provided ad libitum. All procedures were carried out in accordance with the guidelines for the care and use of laboratory and research animals (SANS Guidelines, 2008) and the experiment was approved by the institutional Animal Ethics Committee (AEC) of the University of Pretoria (issued vide No. AEC/A065-12).

The experiment was conducted in accordance to the guidelines for avian toxicity testing studies as stipulated by the Organization for Economic Co-operation and Development (OECD guideline, 2010). DBP was dissolved in a vehicle corn oil base and used for the treatment groups. The birds were randomly divided into six dosage groups. The control group was administered a corn-oil vehicle only (a dose of 1 mL/kg), while the other five experimental groups were administered intragastrically, daily using a dosage regimen of 1, 10, 50, 200, 400 mg DBP/kg/body mass (dissolved in corn oil), for a period of 30-days. In rats, the Non-Observable-Adverse-Effect-Level (NOAEL) of DBP by intra-gastric gavage was 50 mg/kg/day (Mylchreest et al., 2000; Zhang et al., 2004), while a dose of 0, 15, and 35 μg DBP/L (Aoki et al., 2011) and 0.1, 0.5, 1.0, 5.0 or 10 ppm DBP (Lee and Veeramachaneni, 2005) has been used in fish and amphibians, respectively. Overall, the doses used in the present avian study was chosen in order to achieve a dose–response relationship to DBP exposure on reproductive effects using relatively low experimental doses (1, 10 and 50 mg/kg body mass/day) and to high doses (200 and 400 mg/kg body mass/day), in order to obtain a mechanistic overview.

2.3. Tissue collection, necropsy and histological evaluation

At the end of the 30-day dosing period, 5 individual quails each from control and experimental groups were weighed using a digital precision balance UWE Digital precision weighing balance (Algen Scale Corporation® Bohemia, NY) and immediately euthanized (asphyxiated) using carbon-dioxide (CO_2) inhalation anesthesia. At necropsy, the location of the testis was noted and the detunicated testes (left and right) from both control and DBP-exposed groups were rapidly excised (from the reminder of the adherent tissues) and visually inspected for any evidence of gross morphology and symmetry and then weighed. The gonadosomatic index (GSI = [testis weight / body weight] × 100) was calculated. The tissue samples (100–200 mg) of the left testis were immediately placed in a 1.5 mL Eppendort tube containing 1 mL RNALater® and then snap-frozen in liquid nitrogen and subsequently stored at −80 °C freezer until further processed for RNA isolation. Also, the right testis (i.e. ~1 cm^3) taken from each animal group (n = 15) were immediately fixed in 10% buffered neutral formalin solution for 24 h and processed further for routine histology using an automated tissue processor (Shandon Excelsior ES®, Thermo Scientific, Germany). The processing involved dehydration in a series of alcohol concentrations (50%, 60%, 95% to absolute alcohol), clearing was done in xylene (two changes), infiltration, as well as embedding was done using molten paraffin wax (Drury and Wallington, 1980). Tissue sections

(5 μm) were mounted on glass slides and stained with hematoxylin and eosin (H and E) using automated slide stainer (Shandon Varistain® Germini ES, Thermo Scientific, Germany). Stained sections were individually examined under a bright field Olympus BX63 light microscope (Olympus Corporation, Tokyo) at ×40 and ×100 (oil immersion) magnification for histo-pathological changes that included signs of inflammation, testicular atrophy, lymphocytic infiltration, degeneration and necrosis of Sertoli, Leydig and spermatogenic cells. All testicular evaluations were performed in accordance with the recommended approaches for the evaluation of testicular toxicity (Creasy, 2003).

2.4. Blood sampling and steroid hormone analysis

After sacrifice, blood was taken quickly from all birds (n = 5/exposure group, within 3 min of bleeds). Between 500 and 1000 μL of blood was collected from the jugular vein using a sterile 25-gauge syringe. The blood was collected into the heparinized tubes and kept on ice. The blood samples were subsequently transferred into microfuge tubes and centrifuged at 3000 rpm for 15 min at 25 °C. The plasma fraction (supernatant) was collected and stored in 1.5 mL Eppendorf vials at −20 °C. Testosterone concentrations were measured using a double antibody radioimmunoassay (RIA). The plasma was analyzed in duplicate (50 μL) using a commercially available ^{125}I Coat-A-Count kit for Total Testosterone (Siemens Medical Solutions Diagnostics, Los Angeles, CA) as directed by the manufacturer's instructions. This kit consistently provides reliable hormone concentrations and has been previously used in a number of avian species including starlings (Stevenson and Ball, 2009). The assay was highly sensitive (i.e. 100 pg/mL) and is highly specific for testosterone. The antiserum showed negligible cross-reactivity with other structurally similar steroids including 5α-dihydrotestosterone (DHT: 3.3%), 17β-estradiol (0.02%) and corticosterone (0.002%). The intra-assay coefficient of variation averaged 12%.

2.5. Quantitative (real-time) PCR

Total cDNA for quantitative real-time polymerase chain reaction (q-PCR) analysis was generated from 1 μg total RNA from all samples using a combination of poly-T and random primers from iScript™ cDNA Synthesis kit as described by the manufacturer (Bio-rad). Quantitative real-time PCR was used for evaluating gene expression profiles for Androgen receptor (AR), StAR, p450*scc*, *cyp*17, *cyp*19, and 3β- and 17β-*hsd*, using gene-specific primer pairs. The expression of individual gene targets was analyzed using the Mx3000P Real-time PCR System (Stratagene, La Jolla, CA, USA). Each 20-μL reaction contained 10 μL of iTAQ™ Universal SYBR® Green Supermix with ROX (Bio-Rad), 5 μL of 1:6 diluted cDNA template and 200 nM of each forward and reverse primer (Table 1) and the remaining volume was autoclaved MilliQ-H_2O. The three-step real-time PCR program included an enzyme activation step at 95 °C (3 min) and 40 cycles of 95 °C (30 s), 60 °C (15 s) (depending on the primers used; see Table 1), and 72 °C (15 s). Controls lacking a cDNA template were included in the analysis, to determine the specificity of target cDNA amplification as described in Arukwe (2006) and Mortensen et al. (2007). Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct-value versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency (usually >90%) with unknown samples, and this is checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using serial dilution of known amounts of plasmid containing the amplicon of interest, as described previously by Arukwe (2006). Data from each group were averaged and expressed as percentage of control samples.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 5.00 GraphPad Software Inc. Significant differences between control and exposure groups were performed using One-way ANOVA after testing for normality and variance homogeneity. Statistical differences between exposure groups were analyzed using the Tukey's Multiple Comparison Test. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Changes in body and testicular weights, and plasma testosterone levels

No significant differences in the body (not shown) and testicular weights were observed in control and DBP treated groups except in low dose (1 mg/kg) group (albeit not significant). The differences in mean testicular weight measured using the method of repeated measures one-way of analysis of variance (ANOVA) are not statistically significant ($p > 0.05$) except, in the low dose (1 mg/kg) treatment group ($p > 0.01$; data not shown). However, the biometric data showed that DBP treatment significantly affected the gonadosomatic index (GSI) values, only at the lowest DBP dose of 1 mg/kg (Fig. 1A) and plasma testosterone levels were not significantly affected (Fig. 1B).

3.2. Changes in steroidogenic enzyme transcript levels

Transcript levels for key steroidogenic proteins and enzymes were analyzed using real time q-PCR with gene-specific primers pairs, and the results showed that DBP produced dose and parameter-dependent alterations in mRNA expression levels. Specifically, DBP produced significant increase of P450scc mRNA at 10 mg/kg, and thereafter, a dose-dependent decrease was observed between 50 and 400 mg/kg bw (Fig. 2A). For *cyp*17, DBP produced an apparent dose-dependent increase of transcript levels until 200 mg/kg and then decreased below control level at 400 mg/kg (Fig. 2B). DBP produced significant increase of 3β-*hsd* mRNA at 1 and 10 mg/kg, and thereafter, showed a

Table 1
Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR.

Target gene	Primer sequence[a]		Amplicon size (nucleotides)	Annealing temperature (°C)	GenBank accession number
	Forward	Reverse			
Androgen receptor	TGAGTGATCTTCTGGAGGGA	GTGCAGAAACGATAGGCAGA	164	60	GQ369442
*cyp*19	TTGGATGAGCACATGGATT	CAGACAGAGTATCGGGAGCA	124	60	NM_001001761
*cyp*17	GGCACGTCACCCCCACCTCAG	TTATCCCACTCCTTCTCATCG	110	60	FJ267698
3β-*hsd*	AGCCACTTGATAGCAAAAGCAGTTAT	AGGTTGCACTGTCAGTACTACAGAAAG	104	60	NM_205118
17β-*hsd*	AGCAGTTGACACAACAAGCC	GCAAACAGCAGAAATCCTCA	124	60	AB710185
P450*scc*	ACGCTGGTGCAGGTTGGTCTC	GCTCAGTCCCTTGAAGTGCTT	104	60	XM_005145776
StAR	GGGCTGCTGAGTGCACAGA	GACTTACCTAACAACAAGATC	98	60	NM_204686

[a] Sequences are given in the 5–3 order.

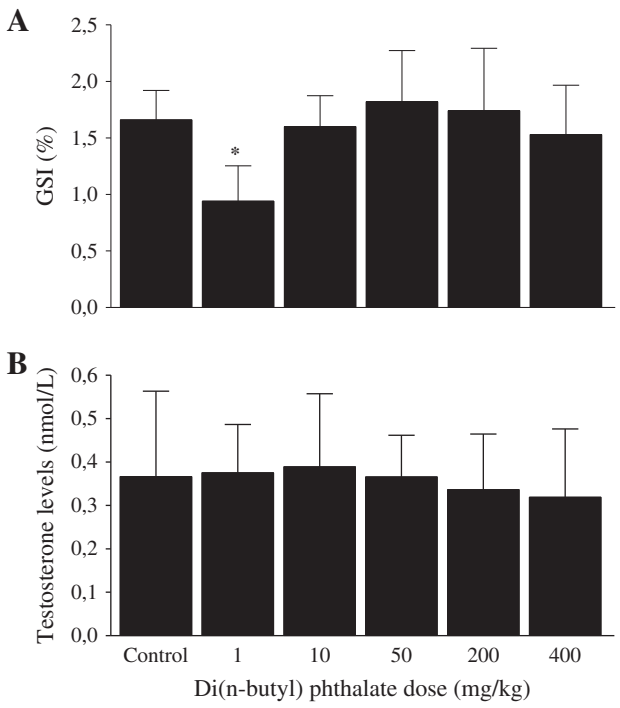

Fig. 1. Changes in gonadosomatic index (GSI, %: A) and plasma testosterone levels (B) in pre-pubertal adult male Japanese quail exposed to different dietary doses (1, 10, 50, 200 and 400 mg/kg body mass/day) of DBP. Data are given as mean ($n = 5$) ± standard deviation (SD). Asterisk (*) denotes significant difference compared with control, ($p < 0.05$), analyzed using ANOVA followed by the *t*-test.

significant decrease, that was still above control level, between 50 and 400 mg/kg (Fig. 3A). On the contrary, 17β-*hsd* mRNA showed an apparent DBP dose-dependent increase, peaking at 400 mg/kg (Fig. 3B). Apparently, we did not observe significant differences in plasma testosterone levels (Fig. 1B) between the DBP exposure and control groups. No significant differences in testicular *cyp*19 and AR mRNA expression were observed in all DBP exposure groups (Fig. 4A and B, respectively). However, there is a tendency that DBP produced reductions, compared with control, in *cyp*19 and AR mRNA levels during the exposure periods (Fig. 4).

3.3. Necropsy and histological changes

At termination of the experiment, there were relatively few testicular gross-morphological abnormalities. A poorly developed or mis-shaped testis replaced the normal, smooth and round gross-architectural outline of the testis. This gross abnormality was most often seen in the left testis of birds fed with a high DBP dose (i.e. 200 and 400 mg/kg). The gross lesion was not seen in birds fed low doses of DBP. The frequency of occurrence of a poorly developed testis was observed in two birds (15.3%) dosed with 200 mg/kg body mass of DBP. The frequency of an abnormal testis was observed in four birds (26.7%), which were dosed with 400 mg/kg body mass DBP (Not shown). Both left and right testes were affected. There was no significant difference ($p > 0.1$), data not shown, between the mean testicular weights of left and/or right testes in DBP treated groups, as compared to that of DBP control groups. Also, there was no testicular enlargement (in right or left testis) of DBP exposed groups, as compared with that of control animal, throughout the 30-day dosing period.

Testicular sections from the DBP control group had no visible histo-architectural alterations and composed of normal, well-organized and

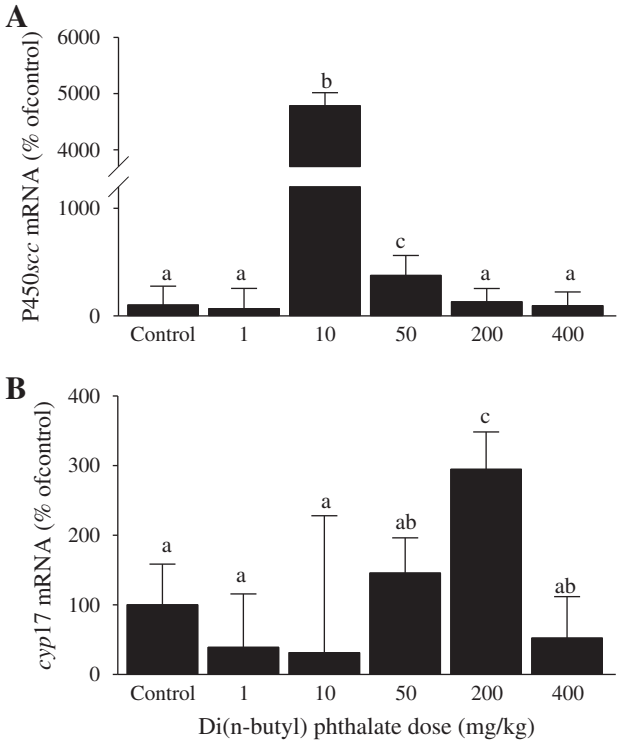

Fig. 2. Changes in testicular P450scc (A) and *cyp*17 (B), mRNA levels in pre-pubertal adult male Japanese quail exposed to different dietary doses (1, 10, 50, 200 and 400 mg/kg body mass/day) of DBP. Messenger RNA (mRNA) levels were quantified using quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) with gene-specific primer pairs. Data are given as mean ($n = 5$) ± standard deviation (SD). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA followed by the *t*-test.

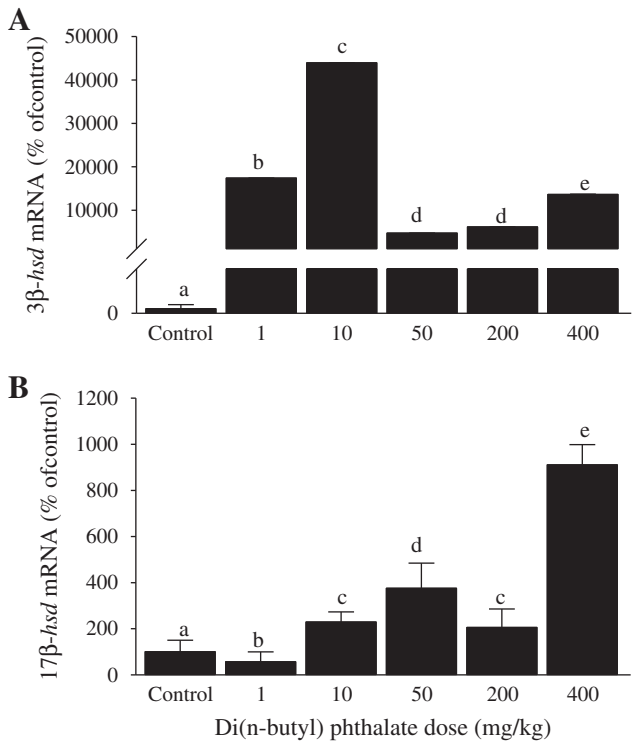

Fig. 3. Changes in testicular 3β-*hsd* (A) and 17β-*hsd* (B), mRNA levels in pre-pubertal adult male Japanese quail exposed to different dietary doses (1, 10, 50, 200 and 400 mg/kg body mass/day) of DBP. Messenger RNA (mRNA) levels were quantified using quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) with gene-specific primer pairs. Data are given as mean ($n = 5$) ± standard deviation (SD). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA followed by the *t*-test.

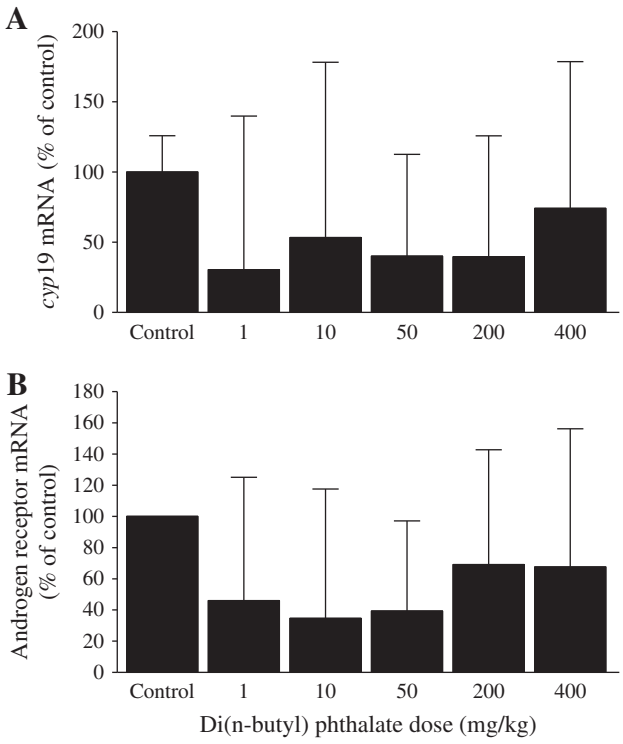

Fig. 4. Changes in testicular *cyp*19 (A) and androgen receptor (AR: B), mRNA levels in pre-pubertal adult male Japanese quail exposed to different dietary doses (1, 10, 50, 200 and 400 mg/kg body mass/day) of DBP. Messenger RNA (mRNA) levels were quantified using quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) with gene-specific primer pairs. Data are given as mean ($n = 5$) ± standard deviation (SD). No significant differences ($p < 0.05$) between exposure groups and control, analyzed using ANOVA followed by the *t*-test.

uniform seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue (Fig. 5A). The arrangement of the seminiferous tubules was regular and the tubular walls were smooth. The sertoli cells were sparsely distributed, among the spermatogenic cells (i.e. spermatogonia, spermatocytes, spermatids) at different stages of differentiation. The interstitial tissue (Fig. 5A) consisted of two main components: a narrow, compact layer of myofibroblasts and connective tissue (peritubular or boundary tissue), that closely surrounded the seminiferous tubule and the interstitial space, (i.e. area between the testicular parenchyma) which consisted of loose connective tissue septa, containing single or groups of few Leydig cells, some blood vessels, lymphatics and cells of the macrophage system such as lymphocytes, macrophages and monocytes (Fig. 5A).

There were no obvious histo-morphological differences between the control and the DBP low dose groups (i.e. 1 and 10 mg DBP/kg groups) (data not shown). However, different degrees of seminiferous tubular changes from mid to severe were observed in some DBP-treated groups. For instance, slight distortion of seminiferous tubules was recorded at the DBP — 50 mg/kg dose group, and these changes were characterized by slight sloughing (atrophy) of germ cells and vacuolar degeneration of spermatogenic, sertoli cells, as well as, the reduction in number of spermatids in the testicular parenchyma (Fig. 5B). Histopathological changes, especially in high DBP (200 and 400 mg/kg body mass) were evident by altered spermatogenesis, and this is sequel to severe atrophy of germ cells, and with spermatogenic cells being sloughed into the lumen (Fig. 5C). Also, there was vacuolar degeneration of spermatogonia and Sertoli cells, with much fluid observed in the seminiferous tubular lumen in DBP high dose groups. Although, the number of tubules exhibiting degeneration increased in the 400 mg/kg dose group, compared to that of the 200 mg/kg dose group, there was concomitant reduction of spermatogenic cells and disordered arrangement of spermatogenic cells (Fig. 5C). Furthermore, the interstitium, showed marked increase in vascularization (congestion) and hyperemia of the blood vessels (Fig. 5D) and loose connective tissue in the interstitium showed slight edema, as well as focal petechial hemorrhages (Fig. 5E and F) seen only in 400 mg/kg dose group.

4. Discussion

Because of the difficulty in establishing reliable parameters for exposure to EDCs for a variety of avian species due to a vast array of reproductive strategies, mammalian, reptiles and fish data provide insight on likely mechanisms of action for EDCs. Thus, several mammalian and lower vertebrate studies have suggested that EDCs may produce reproductive disorders at sexual differentiation, as well as disrupt the development and function of reproductive organs (ATSDR, 2001). So far, the testicular toxicity resulting from phthalate exposure has been shown to be age-dependent with mature animals being less sensitive than immature animals (Dalgaard et al., 2001). Earlier studies in the Japanese quail have demonstrated impaired male copulatory behavior and provide a reliable and sensitive indicator of embryonic gonadal hormone exposure (Adkins, 1979). In addition to the timing and progression in sexual maturation, the existence of several reports on sexual differentiation of brain circuits and behavior in Japanese quail (Panzica et al., 2001; Balthazart and Adkins-Regan, 2002) makes this species a suitable model for the evaluation of the effects of EDCs on overt reproductive impairments (Panzica et al., 2005). To our knowledge, little information is available on the underlying molecular mechanisms of interference of PEs with the transcription of key enzymes involved in both cholesterol transport and testosterone biosynthesis in the avian wildlife species.

4.1. Effects on steroidogenic enzyme genes

In order to understand the molecular basis of EDCs on developing organisms, it is necessary to understand the linkages between exposure levels, gene responsive to EDCs, and the adverse effects induced by these chemicals. The present data is consistent with previous reports showing that phthalates modulate testicular Leydig cell steroidogenesis (Akingbemi et al., 2001). For example, in recent years, phthalates have increasingly been implicated in a spectrum of reproductive disorders, due to alterations in reproductive function and development in both humans and wildlife populations (Berg et al., 2013). Effects of DBP on testosterone biosynthesis may include the inhibition of the expression of a number of key enzymes involved in cholesterol uptake or transport and steroidogenesis with downstream reproductive consequences. A number of endocrine disrupting chemicals (EDCs), including DBP, are known to be anti-androgenic (Scott et al., 2009). The androgenic effects of DBP include decrease in testosterone synthesis possibly due to reductions in the expression of genes involved in cholesterol transport and downstream synthesis (Barlow et al., 2003) or include the inhibition of the expression of a number of key enzymes involved in cholesterol uptake or transport and steroidogenesis (Stocco, 2001; Stocco et al., 2005) with downstream reproductive consequences.

Reproductive development is a continuous process throughout ontogeny and is therefore susceptible to alterations by EDCs at different life-stages. In this study, we observed that exposure of pre-pubertal adult male Japanese quail to different doses of DBP resulted to transcriptional changes in several genes involved in steroidogenesis including androgen receptor (AR), *cyp*19, p450*scc*, *cyp*17, and 3β- and 17β-*hsd* and these effects were specific for individual DBP doses (increasing at low doses and decreasing at higher doses) and did parallel the absence of effects on testosterone plasma levels. In birds, as well as other vertebrate species, the production of steroids requires the coordinated action of several steroidogenic enzymes and starts with the transport of cholesterol from the outer to the inner mitochondrial membrane by the StAR protein (Stocco, 2001 and Stocco, 2001). Backed by the

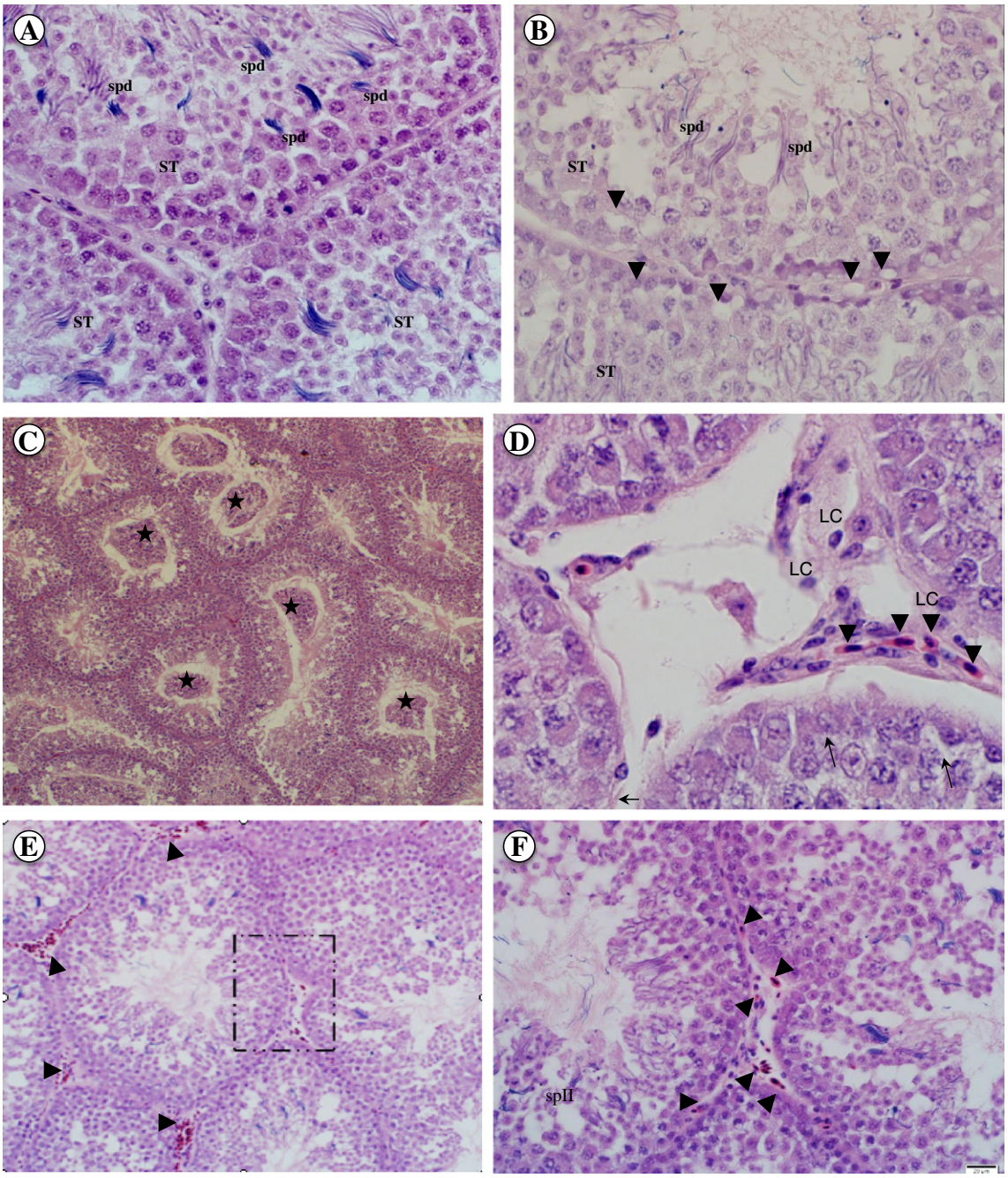

Fig. 5. Cross section of seminiferous tubule (ST) of male Japanese quail (*Coturnix coturnix japonica*) administered with corn-oil (vehicle control: A), showing normal, well organized testicular histo-architecture (Bar 100 μm) or birds administered with 50 mg DBP/kg body mass (Bar = 20 μm: B), showing altered spermatogenesis, with mild degeneration of the seminiferous tubule epithelium as evident by slight sloughing (atrophy) of germ cells and vacuolar degeneration of spermatogonia and sertoli cells (thin arrows) as well as reduction in number of spermatids (spd) in seminiferous tubules, compared to the DBP control group (image A). (C) Cross sectional view of testis from 200 or 400 mg DBP/kg body mass. Note the sloughed spermatogenic cells (asterisks) in the lumen of the seminiferous tubules. Bar 100 μm. (D) Interstitial tissue of the testis exposed to 400 mg DBP/kg body mass showing congestion of the interstitial tissue with blood vessels (see arrows on nucleated red blood cells) surrounded by fibroblasts, and cells of the macrophage system such as macrophages, lymphocytes and monocytes, also some mild interstitial oedema (represented by faintly eosinophilic material) around the Leydig cells (LC). Bar 50 μm. (E and F) Sections from 400 mg DBP treated group showing evidently interstitial tissue petechiation (arrows). The interstitial cells are also shown with petechiation (E: dashed square) showing Bars 20, 100 and 20 μm, respectively. H & E Stain.

extensive mammalian studies, Ukena et al. (1999) and Matsunaga et al. (2001) have reported that the quail brain possesses the P450 and hydroxysteroid dehydrogenase enzymes and therefore susceptible to modulations by EDCs. Previously, it has been reported that phthalates such as di(2-ethylhexyl)phthalate (DEHP) produced modulations in Leydig cell steroidogenic function and exhibit differential effects on steroidogenesis of rats (Akingbemi et al., 2001). Specifically, DEHP inhibited Leydig cell testosterone production that paralleled a decrease in pituitary luteinizing hormone (LH) secretion and reduced steroidogenic enzyme activity (Akingbemi et al., 2001). Although, the study of Akingbemi et al. (2001) did not evaluate molecular responses of enzymes, but this findings are consistent with our data showing that phthalates inhibited the transcript levels of steroidogenic enzymes.

Interestingly, the effects of DBP on transcript levels of steroidogenic enzymes (p450*scc*, *cyp*17, and 3β- and 17β-*hsd*) were biphasic, increasing at low doses or decreasing at high doses and did not parallel the

absence of changes in plasma testosterone, androgen receptor (AR) or *cyp*19 levels (see below). Our findings are consistent with the biphasic endocrine effects of phthalates that have previously been reported for the phthalates showing that exposure of juvenile rats to 10 mg/kg DEHP significantly advanced pubertal onset, whereas the 750 mg/kg DEHP dose delayed pubertal onset (Ge et al., 2007). In addition, while 10 mg/kg DEHP dose significantly increased serum testosterone levels and seminal vesicle weights, the 750 mg/kg dose decreased serum testosterone levels, as well as testes and body weights (Ge et al., 2007). Otherwise, our observation is in accordance with the of study of Brunström et al. (2009), showing that plasma testosterone concentration was not affected when Quail embryos were exposed to EDCs, suggesting that the observed impeded growth was not due to insufficient testosterone levels. Elsewhere, it was reported that fetal exposure of rats to DBP at 100 mg/kg or dexamethasone (0.1 mg/kg) alone had no effects on testis weight, ventral prostate weight, or penis length, whereas 500 mg DBP/kg significantly reduced these parameters (Drake et al., 2009). In addition, exposure to dexamethasone exacerbated 500 mg/kg DBP-mediated reduction in adult testicular weight and resulted in reduced testicular weight when given in combination with 100 mg DBP, compared with controls. Overall, these effects paralleled the reductions in plasma testosterone levels of DBP treated rats (Drake et al., 2009). Furthermore, immature male rat treated daily with di-(2 ethylhexyl) phthalate (DEHP) and flutamide (Flu) on postnatal days (PNDs) 21 to 35 showed significant decreases in the weights of the testes, prostate, and seminal vesicle and anogenital distances (AGD) in response to high DEHP dose (500 mg/kg body mass) or Flu (50 mg/kg body mass) (Vo et al., 2009). In addition, while testosterone levels were significantly decreased in all DEHP-treated groups, plasma LH levels were not altered by any of the two treatments at PND 36 (Vo et al., 2009).

In the present study, a unique effect of DBP was observed for the p450scc, *cyp*17, and 3β- and 17β-*hsd* mRNA and expression of StAR protein mRNA that was below the level of quantification. Although there are considerable diversity existing in specific steroids that are produced by different vertebrate groups or within classes of animals, some common features still apply (Stocco, 2000) and this include, the general believe that the rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the StAR protein, with subsequent conversion to pregnenolone by cytochrome P450scc (Stocco, 2000; Geslin and Auperin, 2004; Sierra, 2004). The StAR protein and P450scc are rapidly synthesized in response to acute tropic hormone stimulation, regardless of steroidogenic organ or tissue. The 3β-hsd plays integral roles in steroidogenesis by catalyzing the dehydrogenation and isomerization of pregnenolone and dehydroepiandrosterone into progesterone and androstenedione, respectively. On the other hand, 17β-*hsd* is an oxidoreductase that catalyzes the steps in the formation of all androgens and all estrogens (Lynn and Brown, 1958). These enzymes are highly expressed in classical steroidogenic organs: testes, ovary, adrenal gland, placenta and liver, and play pivotal roles in reproductive endocrinology. In accordance with the present study, it has been demonstrated that, PCB (Aroclor 1254) inhibited the activities of p450scc, cyp17, and 3β- and 17β-hsd in cultured rat Leydig cells (Murugesan et al., 2008). Elsewhere, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreased intratesticular testosterone content that paralleled a reduction in the P450scc activity and/or impairment of cholesterol mobilization by the P450scc enzyme in the testis (Moore et al., 1991; Lai et al., 2005). Similarly, in vivo injection of PCB inhibited the activity of mitochondrial P450scc in the bull testis and rat testicular Leydig cells (Machala et al., 1998). In addition, PCB directly down-regulated P450scc gene expression in neonatal mouse testis and adult rat Leydig cells (Murugesan et al., 2007). Furthermore, it has been reported that, while inorganic tin had no effect on the catalytic activity and mRNA expression of 17β-hsd I – metabolites of triphenyltin (TPT) namely- diphenyltin and monophenyltin increased 17β-hsd I activity with a concomitant increase in mRNA expression, and tributyltin (TBT) metabolites – dibutyltin and monobutyltin increased 17β-hsd I activity without a concomitant increase in mRNA expression in human choriocarcinoma JAr cells (Nakanishi et al., 2006).

Androgens, including testosterone play critical roles during growth, and differentiation of the genital tubercles into the male phenotype and sexual development through the AR. Furthermore, the cytochrome P450 aromatase (*cyp*19 or P450arom) is a crucial enzyme in the steroidogenic pathway by catalyzing the androgens to estrogen conversion. Given that P450arom catalyzes the rate-limiting step in estrogen production, it is the main regulator of local and systemic estrogen levels in the body, and as a result, plays integral roles in reproduction, sexual differentiation and male/female behavior (Cheshenko et al., 2008). Thus, the regulation of AR and *cyp*19 mRNA expression and activity is not limited to naturally occurring steroids and has been shown to be a potential target for numerous EDCs (Kishida et al., 2001; Menuet et al., 2005). In the present study, we did not observe significant testicular changes in AR, *cyp*19 or plasma testosterone levels after exposure to DBP. The *cyp*19 primer pair sequence used in the present study did not distinguish between cyp19 isoforms. But, the *cyp*19a is highly expressed in the ovary with relatively low mRNA expression and enzyme activity (Callard et al., 2001). An overlap between *cyp*19 genes has been suggested as a result of an evolutionary remnant, and it could be speculated that our primers targeted a *cyp*19 form that lacks a definite physiological function in the adult quail testis, in addition to a hormone negative feedback control mechanism. These speculations are supported by the no observable significant changes in *cyp*19 and plasma testosterone levels in the present study, suggesting a possible lack of a consensus hormone response element (HRE) in the promoter region of the quantified *cyp*19 isoform (Kazeto et al., 2001; Tchoudakova et al., 2001). For example, an auto-regulative loop for *cyp*19b expression was described in adult goldfish (Callard et al., 2001) and zebrafish embryos (Kishida and Callard, 2001; Kishida et al., 2001). HRE was also identified in the promoter region of zebrafish *cyp*19b gene (Kazeto et al., 2001; Tchoudakova et al., 2001), suggesting a direct estrogen responsiveness of the *cyp*19b gene. Nevertheless, the insignificant changes observed in the expression of testicular *cyp*19 gene and plasma testosterone levels in adult male quail further support the complexity in the prediction of biological effects of interferences with steroidogenic enzymes in intact organisms (Hilscherova et al., 2004). For example, the induction of steroidogenic enzymes is highly tissue- and cell-type specific and is controlled by different promoters and second messenger pathways and these pathways provide various targets for interaction with xenobiotics, including DBP.

Recently, Dickens et al. (2014) reported the acute changes in environmental context that rapidly paralleled region-, sex- and stimulus-specific dynamic changes in aromatase activity, providing evidence for a control of local estrogen concentrations in female quail brain, that are not dependent on ovarian secretion. The authors proposed complex dynamic conditions showing that aromatase activity is affected by experimental conditions and mismatches with local estrogen concentration that are potentially explained by several factors, including — changes in the availability of peripheral testosterone or local testosterone synthesis, time-related differences in aromatase activity and 17β-estradiol levels, localized aromatase-mediated catabolism, and differences between in vivo and in vitro aromatase activity (Dickens et al., 2014). The expression of *cyp*19a was shown previously, not to be modulated after exposure to an EDC (17α-ethynylestradiol: EE2), while *cyp*19a mRNA levels were significantly reduced by another EDC (nonylphenol: NP) in fish (Lyssimachou et al., 2006). In addition, brain *cyp*19b mRNA levels were generally 10 times higher than *cyp*19a levels (Lyssimachou et al., 2006). Thus, the lack of EDC effects on testicular *cyp*19 expression may be related to the differential tissue abundance and expression of the *cyp*19 genes after exposure to estrogenic compounds that have been reported previously (Trant et al., 2001; Kazeto et al., 2004; Lyssimachou et al., 2006; Meucci and Arukwe, 2006) or transient effect that was not captured by the timing

of sampling or sex-related effects (i.e. male vs. female). Focusing on the potential sex differences, it will be reasonable to speculate that the lack of DBP effects observed in the present study may be a result of the *cyp*19 lacking a definite physiological function in the quail testis, in addition to a hormonal negative feedback control mechanism (Meucci and Arukwe, 2006). For example, these speculations are supported by the fact that the identification of estrogen responsive elements (EREs) in the promoter region of the *cyp*19b gene points to direct estrogen responsiveness (Kazeto et al., 2001; Tchoudakova et al., 2001).

4.2. Effects on histopathology

In pre-pubertal and pubertal Japanese quail, cloacal gland hypertrophy and foam production are androgen dependent and positively correlated with testes size as well as sexual activity (Ottinger and Brinkley, 1978; Ottinger and Brinkley, 1979; San Balthazart et al., 1984). The cloacal gland is a good marker of gonadal development (Ottinger and Brinkley, 1979) and is widely used as an indicator of androgen status in males during sexual maturation. In this study, we observed that the cloacal gland that produced foam in all treated groups was reduced, compared to the control group and these signs consistently decreased, with increasing DBP dose. In addition, we observed a slight reduction in cellular testosterone levels in 200 and 400 mg DBP/kg exposed birds. Thus, the decrease in cloacal gland area in these groups may be attributed to the slight, but non-significant reduction in testosterone level. Furthermore, the intensity of crowing or vocalization decreased with increasing DBP dose in exposed birds, and this behavioral phenomenon occurs especially in the high dose (200 and 400 mg/kg) groups. In quail, males crow only during the breeding season (Guyomarch and Guyomarch, 1996) and vocalization is dependent on circulating androgens (Beach and Inman, 1965; Schumacher and Balthazart, 1983). It has been shown that the syrinx and neural sound controlling regions are sensitive to sex steroid hormones that may induce vocal changes (Burke et al., 2007), both during development and adulthood. For instance, incomplete forms of vocalization and subsequent developmental changes occur at an earlier age than normal age in testosterone-treated young Galliforms (Makler et al., 1962; Schleidt and Shalter, 1973). Moreover, testosterone can also change the vocal pattern of adults (Beani et al., 1995; Beani et al., 2000). Androgen levels rise as young quails age (Ottinger and Brinkley, 1978) and probably contribute to the development of normal adults. Consequently, intermediate stages of crowing development could be associated with reduced testosterone production stemming from social stimulation (Groothuis). Several reports (Gray et al., 1982; Barlow et al., 2003) have indicated that testosterone effects on germinal tissue are in fact, responsible for the observed decrease in seminiferous tubular height and the corresponding decrease in testis weight. In this present study, there was no difference between the mean body weight and testicular weight. Elsewhere, Gray et al. (1982) observed a decrease in testicular weight. Embryonic exposure to xenoestrogens produced a reduction in the growth of the cloacal gland at sexual maturity in male Japanese quail (Adkins, 1979; Halldin et al., 1999; Halldin et al., 2003). This effect may be induced by activation of ERα since propyl pyrazol triol (PPT) treatment during quail embryo development significantly reduced the cloacal gland area in sexually mature males (Mattsson et al., 2008b).

5. Conclusions

The present study shows that DBP produced dose and parameter-dependent alterations in mRNA expression levels of several of the key genes involved in steroidogenic pathways of pre-pubertal male adult Japanese quail. In addition, DBP exposure results in gross testicular pathology that includes mis-shaped testis and marked alteration in spermatogenesis at 200 mg/kg bw, as evident by the presence of seminiferous tubules with few germ cells and occasional sertoli cell vacuolation. Furthermore, at high dose (400 mg/kg), the testicular injuries, including slight atrophy of germ cells, tubular degeneration of seminiferous tubules, and sloughing of germ cells into the testicular lumen were also observed after DBP exposure. Overall, the alterations induced by DBP may have serious deleterious reproductive consequences.

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