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Altered testicular microsomal steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones

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Abstract

Androgen production in the testis is carried out by the Leydig cells, which convert cholesterol into androgens. Previously, isoflavones have been shown to affect serum androgen levels and steroidogenic enzyme activities. In this study, the effects of lifelong exposure to dietary soy isoflavones on testicular microsomal steroidogenic enzyme activities were examined in the rat. F1 male rats were obtained from a multi-generational study where the parental generation was fed diets containing alcohol-washed soy protein supplemented with increasing amounts of Novasoy, a commercially available isoflavone supplement. A control group was maintained on a soy-free casein protein-based diet (AIN93G). The diets were designed to approximate human consumption levels and ranged from 0 to 1046.6 mg isoflavones/kg pelleted feed, encompassing exposures representative of North American and Asian diets as well as infant fed soy-based formula. Activities of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD), P450c17 (CYP17), 17β-hydroxysteroid dehydrogenase (17β-HSD) were assayed on post natal day (PND) 28, 70, 120, 240 and 360 while 5α-reducatase was assayed on PND 28. At PND 28, 3β-HSD activity was elevated by approximately 50% in rats receiving 1046.6 mg total isoflavones/kg feed compared to those on the casein only diet. A similar increase in activity was observed for CYP17 in rats receiving 235.6 mg total isoflavones/kg feed, a level representative of infant exposure through formula, compared to those receiving 0 mg isoflavones from the casein diet. These results demonstrate that rats fed a mixture of dietary soy isoflavones showed significantly altered enzyme activity profiles during development at PND 28 as a result of early exposure to isoflavones at levels obtainable by humans.

Keywords: 3β-HSD; CYP17; Testicular enzyme activity; Soy isoflavones

1. Introduction

Many plants contain phytoestrogens, a class of compounds that have estrogenic properties and appear to function in anti-fungal defence [1,2]. Isoflavones are a type of phytoestrogen found in foods such as legumes, lentils and chickpeas. Soy foods such as soybeans, soy milk products, soy flour and soy-based infant formulas [3,4] have the highest recorded levels of isoflavones in food, and as such represent a large source of dietary isoflavones [3,5]. Human consumption of soy is on the rise as more soy based foods and supplements are becoming available and vegetarianism grows in popularity. Published benefits of soy isoflavones include therapeutic value for hormone replacement therapy in menopause as well as antioxidant and antipromotional effects against coronary heart disease, various cancers and osteoporosis [1,6–10].

Research examining possible deleterious effects of phytoestrogens began during the 1940s, following the discovery that consumption of plants rich in phytoestrogens caused animals to abort their fetuses [11]. Some animal studies examining the safety of isoflavone consumption have not shown impairment of male sperm production or reproductive tract development [12,13], while other studies have shown deleterious effects in terms of reproductive success and health

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[14–17]. The isoflavone genistein has been shown to inhibit the growth and proliferation of testicular cell lines [18], as well as disrupt spermiation and decrease spermatozoa number present in epididymis of rats [19]. In addition, a number of isoflavone studies have found significant alterations of serum testosterone production in rats [20–25], mice [26] and fish [27]. Previous work from this laboratory has shown that levels of serum and testicular testosterone (T) and dihydrotestosterone (DHT) levels, were significantly elevated in rats at PND 120 following consumption of isoflavones at levels comparable to those found in some human diets [28].

Testicular biosynthesis and metabolism of male steroid hormones involves a cascade of cholesterol transport proteins and steroidogenic enzymes regulated in part by the hypothalamic pituitary axis [29–31]. The mechanisms by which isoflavones may affect hormone production and circulating levels have not been investigated in detail.

However, some phytoestrogens have been shown to alter the enzyme activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) [32,33], 17β-hydroxysteroid dehydrogenase (17β-HSD) [32,34] and 5α-reductase [35,36], which could impact the production of androgens within the testis.

The following studies were designed to explore the mechanisms responsible for androgen level changes previously detected in F1 rats fed a mixture of dietary isoflavones containing genistein, daidzein, and glycitein in conjugated (glycone) and unconjugated (aglycone) states for their entire lives [28]. The rats were fed diets containing various levels of isoflavones ranging from 0 mg isoflavones (diet 1) to low amounts representative of a typical North American diet (diets 2–3) [37,38], to modest vegetarian or Asian consumption levels (diet 4) [39,40], to high dietary levels experienced by infants fed on soy-based formulae (diet 5) [41,42]. Diet 6 represents approximately 5 times the consumption of infants and 10 times the consumption of adults. The present investigation examined the effects of dietary isoflavones on the activities, expression of mRNA and protein levels of the steroidogenic enzymes 3β-HSD, 17-hydroxylase/C-17,20 lyase (CYP17), 17β-HSD and 5α-reductase.

2. Experimental

2.1. Materials

Unlabelled steroids were purchased from Steraloids Inc. (Newport, RI). Aquasol and labelled steroids: [1,2,6,7-^{3}H]DHEA (60.0 Ci/mmol), [1,2,6,7-^{3}H]progesterone (114.4 Ci/mmol), [1,2,6,7-^{3}H], [1,2,6,7-^{3}H]testosterone (95.0 Ci/mmol) and [1,2,6,7-^{3}H]androstenedione (74.0 Ci/mmol) were bought from Dupont/NEN (Boston, MA). Organic solvents were purchased from EM Science Merck KgaA (Darmstadt, Germany) with the exception of formaldehyde, which was purchased from Fisher Scientific (Nepean, ON). Plastic coated Whatman™ PE SIL G silica gel chromatography plates were bought from Chromatographic Specialties (Montreal, QC). Novasoy soy isoflavone concentrate (152–400) and alcohol washed soy protein (Pro Fam 930) were obtained from Archer Daniels Midland Company (Decatur, IL). Casein protein was purchased from ICN (Cleveland, OH). Dimethyl sulfoxide (DMSO), Tris, diethyl pyrocarbonate (DEPC), nicotinamide cofactors (NADPH and NAD^+), and β-mercaptoethanol were purchased from Sigma Chemical Co. (St Louis, MO). Sucrose was purchased from BDH Inc. (Toronto, ON). Ethidium bromide was purchased from Gibco BRL (Gaithersburg, MD). Agarose, RNAse H ribonuclease and dNTPs were purchased from Roche Diagnostics Corporation (Indianapolis, IN). Superscript II RNAse H-reverse transcriptase, oligo d(T_{15}) primers, custom PCR primers (Table 1) and TRIzol were purchased from Life Technologies Inc. (Burlington, ON). Chill wax for PCR reactions was attained from MJ Research Inc. (Boston, MA). Taq DNA polymerase and PCR buffers were purchased from Boehringer Mannheim (Laval, QC). NE-PER nuclear and cytoplasmic extraction reagents, Coomassie Plus Protein Assay reagents, bovine serum albumin standards and Supersignal ULTRA chemiluminescent Substrate were purchased from Pierce (Rockford, IL). SDS–polyacrylamide and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Richmond, CA). Immobilon-P, polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). Tris buffered saline containing 0.1% Tween-20 (TBS) were purchased from ICN Biochemicals (Cleveland, OH); and 4% skim milk powder, was purchased from Nestle (Don Mills, ON). Streptavidin was obtained from DAKO Diagnostics Canada Inc. (Missassauga, ON).

Table 1
Isoflavones were determined by HPLC in β-glucuronidase digested extracts of experimental diets

Diet	Protein	Genistein	Daidzein	Glycitein	Total isoflavones
1	Casein	ND	ND	ND	ND
2	Soy	18.6	10.5	2.6	31.7
3	Soy	21	12.3	2.8	36.1
4	Soy	39.3	27.6	7.6	74.5
5	Soy	124.4	90.9	20.5	235.6
6	Soy	544.8	412.3	89.5	1046.6

Diet 1 contained casein protein, while diets 2–6 contained an alcohol-washed soy protein concentrate, and in diets 3–6. Novasoy was added to supplement the diets with isoflavones. (N.D.: not detectable) Data depicted as isoflavones (mg)/kg pelleted feed.

2.2. Animals

The male Sprague–Dawley (SD) rats used in this study were the F1 generation produced during a multi generation study examining the effects of dietary isoflavones on growth, development and reproductive physiology. The study adhered to the protocol for the Organization for Economic Cooperation and Development (OECD) Guideline 416 (OECD TG416, 2001). Results from this larger study will be pro-

vided elsewhere (Curran, I.H.A, Cooke G.M., and Gilani, G.S., in preparation). Animal care was provided according to the guidelines of the Canadian Council for Animal Care and all procedures were reviewed and approved by the Health Canada Animal Care Committee. The parental generation (F0) were purchased from Charles River (St-Constant, PQ) at the pubescent stage of development and were pair housed with a 12 h light/dark cycle in hanging polycarbonate cages (Health Guard System, Research Equipment Company, Inc., Byran, TX) containing corncob bedding and free access to food and fresh water. Weekly food consumption was recorded at regular intervals throughout the investigation. Parental generation rats (F0) were acclimatized until postnatal day (PND) 50 at which point they were assigned randomly to experimental diets. After 70 days exposure to the experimental diets, rats were mated on PND 120 and the resulting (F1) progeny were weaned at PND 21 and provided with the same diet as their parents.

Male F1 progeny were sacrificed on PND 28, 70, 120, 240 and 360 by exsanguination, through cardiac puncture under isoflurane anaesthesia. Testes were weighed, immediately frozen in liquid nitrogen and stored at −80 °C until assay for steroidogenic enzyme activities.

2.3. Experimental diets

Six semi-purified diets were formulated according to American Institute of Nutrition (AIN) specifications [43]. One diet was casein-based (AIND93G) while the other diets were formulated by adding increasing amounts of a commercial mixture of isoflavones (Novasoy), to a base diet resembling AIN93G, but containing alcohol-washed soy protein concentrate (Pro Fam 930) in place of casein.

Once the diets were prepared, soy isoflavones content (genistein, daidzein and glycitein) was determined by HPLC [44] analysis of β-glucuronidase digested extracts of the diets as shown in Table 1 (Dr. Sarwar Gilani and Mr. Patrick Robertson of Health Canada, personal communication). All diets were provided to the animals ad libitum in pelleted form.

2.4. Specific enzyme activities

Decapsulated testes were homogenized in buffer containing 50 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 7 mM mercaptoethanol and 100 μM NADPH. For PND 28 testes, the homogenates were centrifuged ($1000 \times g_{max}$, 10 min, 4 °C) to pellet the nuclear fraction, which was then resuspended in dimethyl glutaric acid (DMGA) buffer (50 mM DMGA, 50 mM NaOH, 250 μM NADPH, 5% glycerol, pH 6.5) for immediate use in 5α-reductase enzyme assays. The $1000 \times g_{max}$ supernatant was centrifuged at $10{,}000 \times g_{max}$ for 10 min at 4°C and the supernatants recovered and recentrifuged under the same conditions to obtain a post-mitochondrial supernatant. The microsomal fractions for all assays, obtained by centrifugation of the post-mitochondrial supernatantant ($176{,}000 \times g_{max}$), were resuspended in the 50 mM Tris–HCl buffer pH 7.4 containing 250 μM NADPH for immediate use in microsomal 3β-HSD, CYP17 (17-OHase, C17,20-lyase), 17β-HSD and 5α-reductase assays. Microsomal and nuclear protein, were estimated using the method of Lowry et al. [45].

Testicular microsomal (3β-HSD, CYP17, 17β-HSD, 5α-reductase) and nuclear (5α-reductase) steroidogenic enzyme activities were assayed according to the modified methods of Cooke et al. [46,47]. At PND 28, testes from the six diet groups of rats were tested for all enzyme activities, while only 3β-HSD, CYP17 and 17β-HSD were tested at PND 70, 120, 240 and 360 as young rats aged 20–40 days of age are able to convert T to the more potent androgen DHT by the testicular enzyme 5α-reductase, but as the rat approaches adulthood, this testicular activity diminishes to unmeasurable levels [31].

Aliquots of appropriate microsomal fractions were added to: (1) 3 ml Tris–HCl buffer (pH 8.4) containing DHEA (10^{-6} M, 40,000 cpm ^{3}H/ml) for the 3β-HSD assay; (2) 3 ml Tris–HCl buffer (pH 7.4) containing progesterone (10^{-6} M, 40,000 cpm ^{3}H/ml) for the CYP17 assay; (3) 3 ml Tris–HCl buffer (pH 7.4) containing 4-androstenedione (10^{-6} M, 40,000 cpm ^{3}H/ml) for the 17β-HSD assay; and (4) 3 ml of DMGA buffer (pH 4.5 or 6.5) containing testosterone (10^{-6} M, 40,000 cpm ^{3}H/ml) for the 5α-reductase assay.

Enzyme reactions with labeled substrates were stopped after 30, 60 and 90 min incubations with hexanes and appropriate carrier steroids were added to facilitate localization of substrates and products by UV-light and exposure to iodine vapor after separation by thin layer chromatography plates using 9:1 = chloroform:methanol as a solvent. Regions corresponding to the substrate and product carrier steroids were excised and quantified by scintillation counting (Packard Liquid Scintillation Analyzer (Tri-Carb 2100TR)). Enzyme specific activities were determined by linear regression analysis ($r > 0.95$) and expressed as pmol product formed/minute/mg protein, μmol product formed/min/g testis tissue and μmol product formed/min/testis. Optimal conditions were determined for all enzymes with respect to substrate and protein concentrations and linearity of product formation with time.

2.5. RNA isolation and RT-PCR

Total RNA isolation of 20 mg of thawed testes tissue were carried out with TRIzol according to manufacturer's specifications. The concentrations and purity of total RNA isolated was determined spectrophotometrically, based on optical density at 260 nm and $A_{260/280}$ ratio, respectively. RNA quality was verified by denaturing ethidium bromide stained formaldehyde RNA electrophoresis through agarose gel [48]. All RNA samples were stored at −80 °C until reverse transcriptase reactions were carried out.

cDNA was synthesized using 5 μg of total RNA and 200 U Superscript II RNAse H-reverse transcriptase in a total reaction volume of 20 μl. The RT-reaction and subsequent steps were performed as per manufacturer's recommendations (Life Technologies Inc., Burlington, ON). The cDNA

Table 2
PCR Primer Pairs

Target gene	Forwards primer	Reverse primer	Fragment size (base pairs)	GeneBank accesion #
G3PDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452	NM_017008
SCC	ATC ACA GAG ATG CTG GCA GGA	GCA CGT TGA TGA GGA AGA TGG	481	J05156
3β-HSD	ACT GGC AAA TTC TCC ATA GCC	TTC CTC CCA GGT GAC AAG TGG	402	L17138
CYP17	GTG CTG GCA CAC GAC AAG GAG	GCC AGG ATC CAC TTG AGC ACA	478	NM_012753

was then used immediately or stored at −20 °C until needed for subsequent amplifications.

2.6. Gene amplification

Target cDNA was amplified using 50 ng of initial total RNA from the cDNA reaction in a 50 μl volume containing (1× PCR buffer + Mg) 0.25 mM each of forwards and reverse primers (Table 2). Primers were designed using SciEdCentral Clone Manager Professional Suite (Scientific and Educational Software, Durham, NC). Premature initiation of the reaction was prevented by the addition of chill wax (25 μl) before addition of 2.5 U Taq DNA polymerase. PCR reactions were optimized for each primer pair by temperature and cycle gradients (data not shown).

The following were used as PCR cycles after initial denaturing 95 °C (3 min): 95 °C (60 s), 58 °C (70 s), 72 °C (70 s). G3PDH, SCC and CYP17 were amplified with 25 cycles, while 23 cycles were used for 3β-HSD; PCR products were subjected to an extended final incubation (72 °C, 5 min) to fully extend all partial DNA fragments. Thermocycled reaction products were separated by 2% agarose gel electrophoresis followed by ethidium bromide staining and images captured (Kodak Digital Science Image Station 440 CF) for comparative densitometry analysis (Kodak ID Image Analysis Software, Eastman Kodak Company, Rochester, NY).

2.7. Western blotting analysis

Cytoplasmic and nuclear fractions from 50 mg of testis tissue were isolated with NE-PER nuclear and cytoplasmic extraction reagents according to manufacturer's instructions. Protein concentrations were determined using Pierce Coomassie Plus Protein Assay reagents using bovine serum albumin as a standard. Equal amounts of protein (35 μg) were resolved with 10% SDS–polyacrylamide gel electrophoresis as described by Laemmli [49] and transferred to Immobilon-P PVDF membranes as described by Matsudaira [50]. Proteins were visualized on 10% SDS–polyacrylamide gels and PVDF membranes by staining with Coomassie Brilliant Blue R-250 to test for equal loading of wells and equal transfer from gel to membrane (data not shown). The western blots were blocked with Tris buffered saline containing 0.1% Tween-20 (TBST) and 4% skim milk powder (1 h, room temperature.). A number of antibodies were used to probe the western blots: (1) a polyclonal rabbit antibody against rat SCC (1/10,000 dilution) (Chemicon International, Temecula, CA) (2) a polyclonal rabbit antibody against purified human placental 3β-HSD1 (1/500 dilution) (generous gift from Dr. James L. Thomas, Mercer University, School of Medicine at Macon, GA) (3) a polyclonal rabbit-anti porcine CYP17 antisera (1/10,000 dilution) (kindly supplied by Dr. Buck Hales, University of Illinois at Chicago, IL [51]). Each antibody was added with streptavidin–horseradish peroxidase conjugate (1/3000 dilution) in TBST with 4% skim-milk powder. Blots were washed 3 × 5 min with TBST before the addition of a donkey secondary anti-rabbit antibody coupled to horseradish peroxidase (1/5000 dilution) (Jackson Immuno Research Laboratories Inc., West Grove, PA) in TBST with 4% skim-milk powder. Blots were washed 6 × 5 min before the chemiluminescent detection of antigens by addition of SuperSignal ULTRA Chemiluminescent Substrate as per manufacturer's instructions in conjunction with Kodak Digital Science Image Station 440 CF with Kodak ID Image Analysis Software.

2.8. Statistical analysis

Linear and non-linear regressions were carried out using Prism V3.02 1994-2000. (Graphpad Software Inc., San Diego, CA). One-way ANOVA was used to analyze 5α-reductase enzyme activities and comparative densitometry analysis of cDNA. Two-way ANOVA was used for analysis of 3β-HSD, CYP17, 17β-HSD, 17-hydroxyprogesterone: androstenedione ratio and microsomal protein concentrations, followed up by all pairwise Tukey's tests (Sigma Stat V2.031992–1997 for windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Microsomal protein determination

The mean testis microsomal protein concentrations (mean ± S.E.) for diet 1 rats were 20.3 ± 1.7, 17.0 ± 0.5, 18.5 ± 1.5, 20.9 ± 0.7 and 17.1 ± 1.5 mg/ml for PND 28, 70, 120, 240 and 360, respectively. There were no significant differences in protein concentrations between diet groups for any time examined ($P > 0.05$).

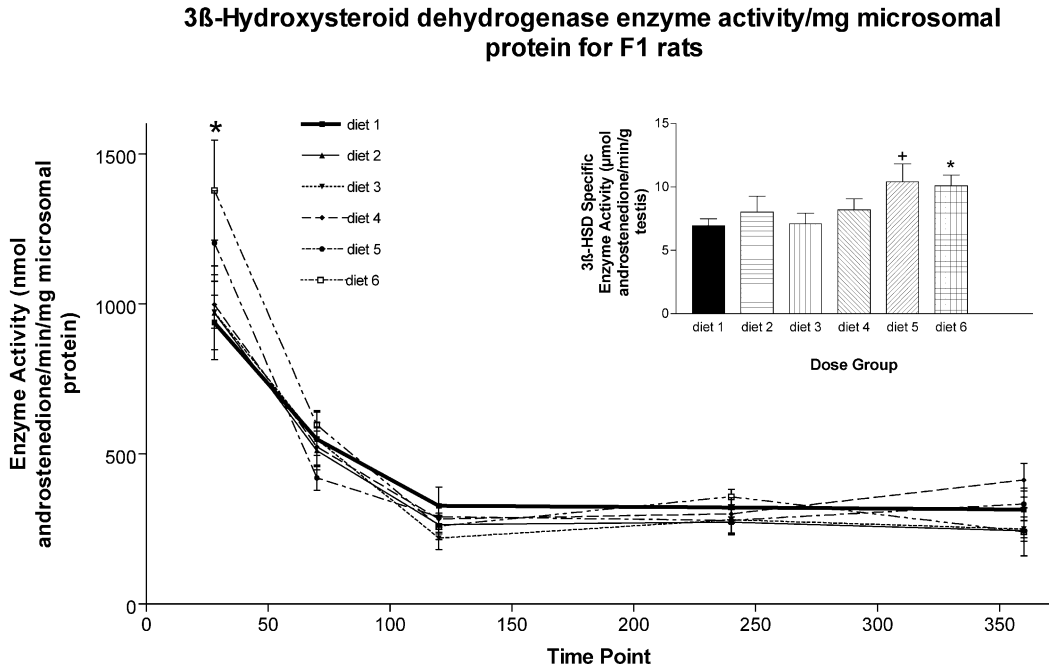

Fig. 1. 3β-Hydroxysteroid dehydrogenase (3β-HSD) enzyme activity (nmol androstenedione (A4)/min/mg protein mean ± S.E. of male rats). Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed a significant increase in enzyme activity for diet 6 (1379.3 ± 167) compared to diet 1 (938.8 ± 91) (*) ($P < 0.05$) at PND 28. The inset represents 3β-HSD activity (μmol A4/min/g testis mean ± S.E.) at PND 28 in male rats. Statistical analysis showed a significant increase in enzyme activity for diet 5 (10.4 ± 1.4) (+) and diet 6 (10.1 ± 0.9) (*) compared with diet 1 (6.9 ± 0.6) ($P < 0.05$). The number of animals sampled at PND 28 $n = 5$ for diets 1–3, $n = 6$ for diets 4–6. For PND 70 rats $n = 9$ for diets 1 and 6, $n = 8$ for 2,4 and 6, and $n = 6$ for diet 3. There were $n = 8$ animals sampled per dose for PND 120 except for diets 1 and 6 which had $n = 9$. For PND 240 there were 8 rats sampled for all groups except for diets 3 and 5 in which 7 rats were sampled. PND 360 rats had 6 rats examined per dose with the exception of diet 4, which had 5 rats examined.

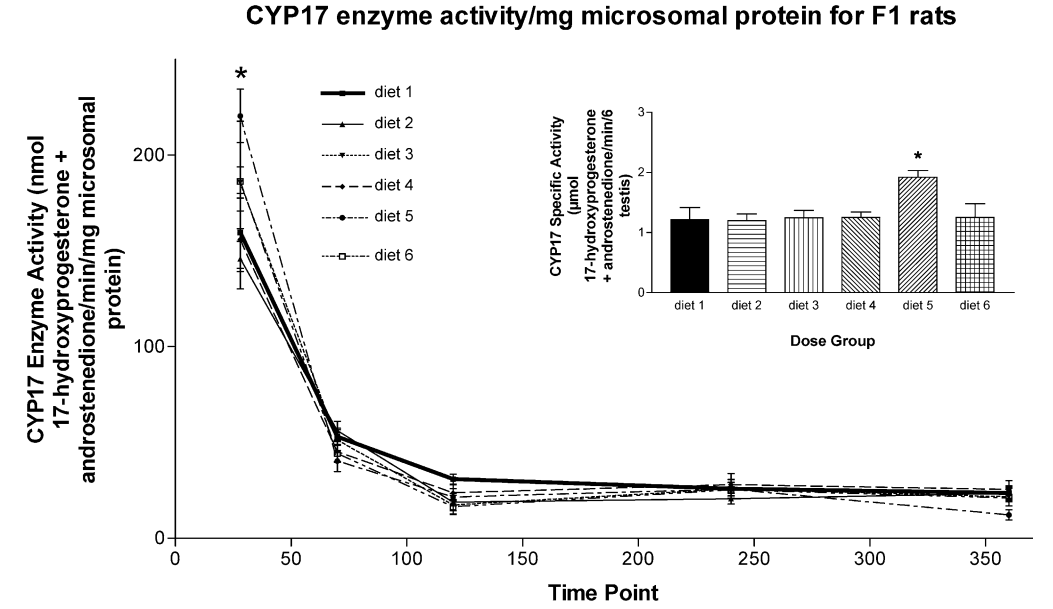

Fig. 2. CYP17 (17-OHase and C17,20-lyase) enzyme activity (nmol 17-hydroxyprogesterone (17OH-P) + androstenedione (A4)/min/mg protein mean ± S.E.) of male rats. Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed a statistically significant increase in activity seen for diet 5 (220.5 ± 14) compared with diet 1(159.7 ± 20) (*) ($P < 0.05$) at PND 28. The inset depicts CYP17 activity (μmol 17OH-P + A4/min/g testis mean ± S.E.) of PND 28 male rats. Statistical analysis showed a significant increase in activity for diet 5 (1.92 ± 0.1) compared with diet 1 (1.21 ± 0.2) (*) ($P < 0.05$). The number of animals sampled at PND 28 were $n = 5$ for diets 1–3 and 6, $n = 6$ for diets 4–5. For PND 70 rats $n = 8$ for diets 1,2,4 and 5 $n = 6$ for diet 3 and $n = 9$ for diet 6. There were $n = 6$ animals sampled from diets 5–6, $n = 7$ for diet 1, $n = 3$ for diet 3 and $n = 8$ for diet 6 for PND 120 rats. For PND 240 rats there were $n = 8$ rats sampled for all groups except for diets 3 and 6 which had 8, and diet 1 which had $n = 7$ rats. There were 6 rats examined per dose with the exception of diet 4, which had 5 rats examined at PND 360.

3.2. 3β-Hydroxysteriod dehydrogenase activity

3β-HSD activities tended to decrease with age, for example at PND 28 the lowest values were 938.8 ± 91.4 (diet 1) nmol androstenedione produced/min/mg protein, which was decreased by 50% at PND 70, and then at older time points, all values tended to range from 219.4 ± 37.3 (PND 120 diet 3) to 412.9 ± 56.2 (PND 360 diet 4) nmol androstenedione produced/min/mg protein (Fig. 1). At PND 28, rats fed 1046.6 mg isoflavones/kg feed (mg/kg) (diet 6) showed a significant increase in 3β-HSD activity compared with those consuming 0 or 31.7 mg/kg (approximately 42 and 47%), respectively ($P < 0.05$). Similarly animals, which had consumed 235.6 mg/kg (diet 5) exhibited a significant increase in 3β-HSD activity when compared with diets containing 0 or 31.7 mg /kg, respectively (approximately 28 and 24%) ($P < 0.05$). When the data were expressed as androstenedione formed/min/g testis tissue, both diets 5 and 6 were approximately 50 and 46% higher than diet 1 with 0 mg/kg ($P < 0.05$) (Fig. 1 inset). There was a statistically significant interaction between time and isoflavone levels as determined by 2 way-ANOVA ($P = 0.017$). Results obtained when expressed as androstenedione formed/min/testis showed no significant differences between different diet groups at any age examined (data not shown). Time points examined after PND 28 showed less variation between enzyme activities for the different diet groups.

3.3. CYP17 activity

Progesterone is converted in a two step process to 17-hydroxyprogesterone and then to androstenedione by one enzyme: 17-hydroxylase/C17,20-lyase (CYP17).

The CYP17 enzyme activity (nmol androstenedione/min/mg protein) detected in rats fed diet 5 at PND 28 were significantly higher (approximately 38 and 52%) compared with from those found in rats fed diets 1 and 2, respectively ($P < 0.05$). When the data were analyzed as μmol androstenedione produced/min/g testis, diet 5 activity was again found to be significantly higher than the activity of diet 1 (approximately 60%) at PND 28 (Fig. 2 inset). Furthermore, when the data were expressed as μmol androstenedione produced/min/testis, similar trends were evident at PND 28 but no statistically significant changes in activities were detected ($P > 0.05$). Statistically significant interactions occurred between time and dose of isoflavones as determined by 2 way-ANOVA for all three analyses of CYP17 activity ($P < 0.001$). Activities from ages later than PND 28 exhibited no significant differences between diet groups ($P > 0.05$). In these assays, the ratio of 17-hydroxyprogesterone: androstenedione was not significantly different indicating that there were no

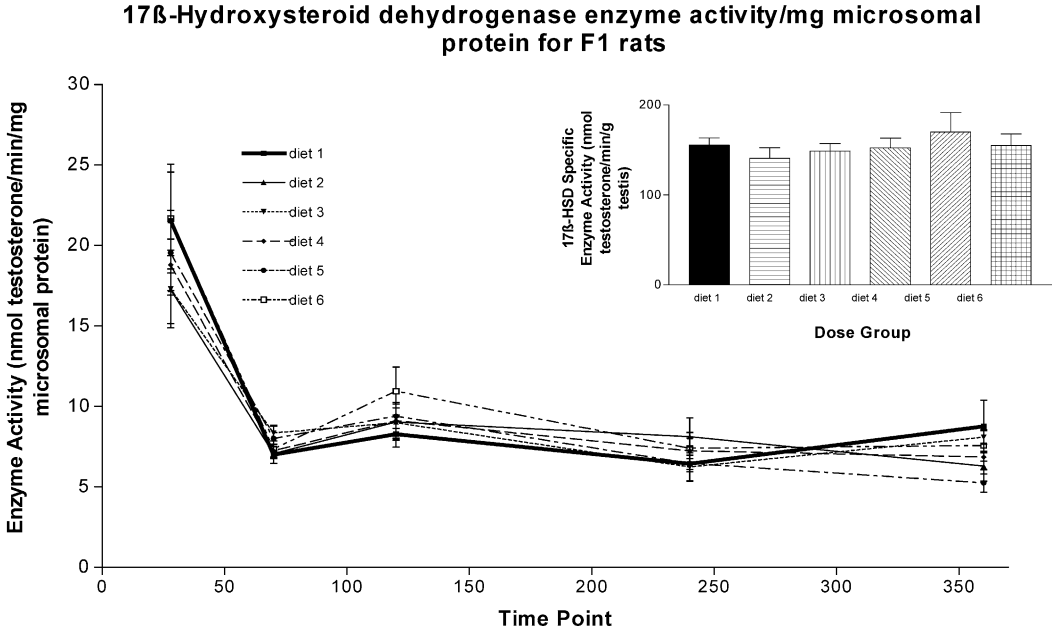

Fig. 3. 17β-Hydroxysteroid dehydrogenase (17β-HSD) enzyme activity (nmol testosterone (T)/min/mg protein mean ± S.E.) of male rats. Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed no statistically significant differences in enzyme activity seen for 17β-HSD activities ($P > 0.05$). The inset depicts 17β-HSD activity (μmol T/min/testis mean ± S.E.) of PND 28 male rats. Statistical analysis of the inset data showed no significant changes in enzyme activity ($P > 0.05$) for PND 28 rats. The number of animals sampled at PND 28 were $n = 5$ for diets 1–3, $n = 6$ for diets 4–6. For PND 70 animals $n = 9$ for diets 1 and 6, $n = 8$ for diets 2,4 and 5, and $n = 6$ for diet 3. There were $n = 8$ animals sampled per dose for PND 120 rats except for diet 1 ($n = 9$) and diet 6 ($n = 7$). For PND 240 rats there were 8 rats sampled for all groups except for diet 4 ($n = 7$). There were 6 rats examined per dose with the exception of diet 4 ($n = 5$) for PND 360.

selective effects on C17,20-lyase activity ($P > 0.05$) (data not shown).

3.4. 17β-Hydroxysteroid dehydrogenase activity

The profile of 17β-HSD activity with age resembled the profiles for 3β-HSD and CYP17. At PND 28, the 17β-HSD activities ranged from 17.3 ± 2.1 (diet 2) to 21.7 ± 3.4 (diet 6) (nmol testosterone/min/mg protein), then decreased at PND 70 and for all later time points examined, into the range of 5.2 ± 0.6 (PND 360 diet 5) to 10.9 ± 1.5 (PND 120 diet 6) nmol testosterone/min/mg protein (Fig. 3). There were no significant differences in 17β-HSD activity between dose groups at any ages when the data were examined in terms of nmol testosterone produced/min/mg protein, μmol testosterone produced/min/testis and μmol testosterone produced/min/g testis ($P > 0.05$).

3.5. 5α-Reductase activity

The testis of PND 28 animals contained measurable 5α-reductase activity within microsomal and nuclear testicular extracts at a reaction pH of 6.5 but no activity was detectable at pH 4.5. Furthermore, at pH 6.5, there were no significant differences observed for 5α-reductase activities (nmol dihydrotestosterone/min/mg protein) between the 6 diets examined for either the microsomal or nuclear 5α-reductase activities ($P > 0.05$) (Fig. 4). Similarly, when the data were examined in terms of μmol dihydrotestosterone/min/g testis and μmol dihydrotestosterone/min/testis activity, neither analysis showed any significant differences between diet groups ($P > 0.05$) (data not shown).

3.6. Steroidogenic enzyme mRNA expression levels

The initial step in androgen production is the conversion of cholesterol to pregnenolone through the action of the mitochondrial cholesterol side chain cleavage enzyme (P450 SCC). Enzyme activities were not determined for SCC, as appropriate radiolabelled substrates are not available commercially. However, isoflavone effects on SCC gene expression were examined by semi-quantitative RT-PCR in testis samples from each diet group at PND 28 (age when changes in other steroidogenic enzyme activities were observed) and at PND 120 (age when changes in serum and testicular androgen levels were evident [28]). Amplifications of SCC cDNA relative to a constitutive control (G3PDH) showed no significant changes in mean mRNA ratios of arbitrary densitometry units between the 6 diets examined at PND 28 ($P > 0.05$) (Fig. 5A) or at PND 120 (Fig. 5B). The steroidogenic enzymes that exhibited changes in activity at PND 28 (3β-HSD and CYP17) were also examined for differences in mRNA expression. RT-PCR analysis of both enzymes revealed no statistically significant differences between mean ratios of different diet groups ($P > 0.05$) (data not shown).

3.7. Western blot protein levels of steroidogenic enzymes

Western blot analysis of SCC, 3β-HSD and CYP17 was performed to test for possible differences in enzyme protein levels between rats fed different diets. Western blots showed no visible differences in intensity between immunoreactive SCC protein levels between diets 3–6 compared with intensities seen in diets 1 and 2 at both PND 28 and 120 (Fig. 6A,B). Similarly 3β-HSD and CYP17 protein lev-

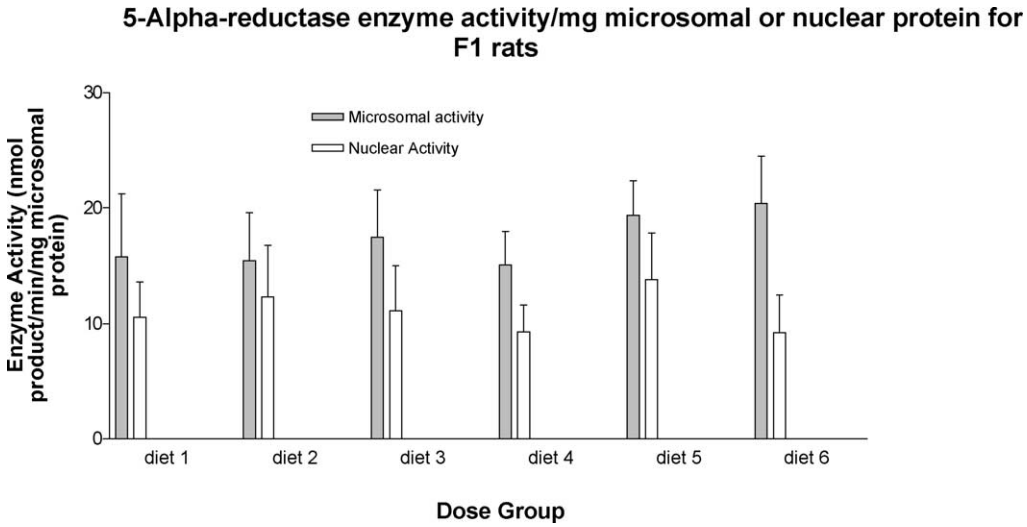

Fig. 4. 5α-Reductase enzyme activities (nmol dihydrotestosterone/min/mg protein mean ± S.E.) of PND 28 male rats. Enzyme assays were carried out both at pH 4.5 (not shown) and pH 6.5. The enzyme assays were conducted on both microsomal and nuclear testis extracts. No statistically significant differences (1 way-ANOVA) were seen between the enzymatic rates between different diet groups ($P > 0.05$). The number of animals sampled at 28 days of age were $n = 5$ for diets 1–3, $n = 6$ for diets 4–6.

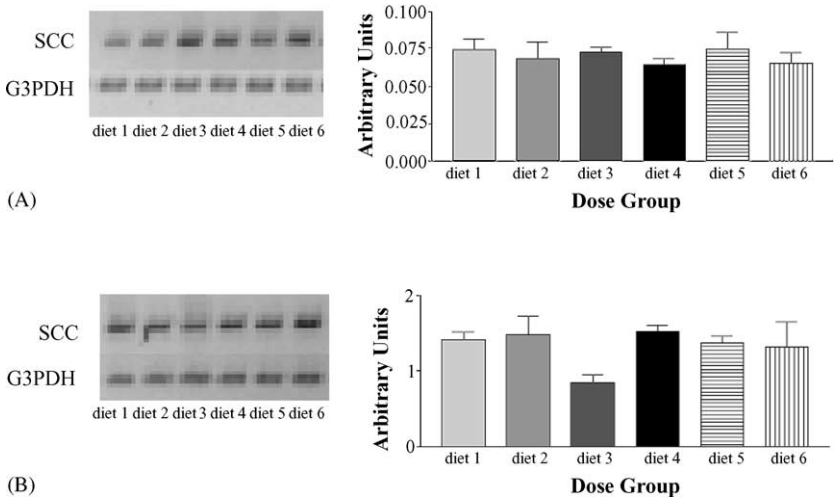

Fig. 5. Mean levels of mRNA of (A) PND 28 SCC and (B) PND 120 SCC, examined by RT-PCR. Data are depicted as an image of $n = 1$ representative (on the left) of the populations examined, statistically (on the right) is depicted (mean arbitraty units ± S.E.). The *Y*-axis represent arbitrary units developed from the ratio of densitometry of the mRNA in question divided by the densitometry of the constitutive control G3PDH. No statistically significant differences were seen between different mean dose groups expression of mRNA examined by 1 way analysis of variance ($P > 0.05$). The number of animals sampled at PND 28 were $n = 6$ for all doses examined with the exception of diet 3 ($n = 5$). The number of animals sampled at PND 120 was $n = 6$ except for diets 2 and 3 ($n = 5$).

els were not different between diets at PND 28 (data not shown).

4. Discussion

The current study was undertaken to investigate whether lifetime exposure of male rats to isoflavones that resulted in elevated serum and testicular androgen levels [28] also altered testicular enzyme activities. Earlier work from our laboratory demonstrated that exposure of rats to soy isoflavones at levels attainable by adults and infants, result in altered serum and testicular androgen levels in adulthood (PND 120) [28]. The present studies show that at PND 28 there are significantly higher enzymatic activities of 3β-HSD and CYP17 in the pathway converting cholesterol to androgens due to consumption of dietary isoflavones. CYP17 showed the greatest dose-dependent increases in enzyme activity in rats fed isoflavones, followed closely by 3β-HSD, while 5α-reductase activity and 17β-HSD activities were not affected by isoflavones. In addition, at PND 28, subtle differences were observed according to whether the 3β-HSD and CYP17 enzyme activities were expressed in terms of mg microsomal protein, g testis tissue or whole testis, possibly due to differences in testis weights between the diet groups that were seen at this age [28]. At PND 28 a dose dependent increase in testis mass was observed as the amount of soy isoflavones was increased, which did not persist at older ages [28]. At this time point the increased steroidogenic potential may have increased with the larger testis of rats fed high levels of isoflavones. These findings agree with the work of others who have found altered steroidogenic enzyme activities as a result of isoflavones [32–36], and more specifically, increased steroidogenic enzyme activity in tissues following isoflavones administration [36,52]. The results of the present studies, while not able to explain the mechanism of increased serum and testicular androgen levels seen in adult rats described previously [28], add a new layer of complexity by revealing alterations in enzyme activities during early development.

Estrogenic chemicals have been shown to alter steroid hormone enzyme activities. Majdic et al. [53], found that with fetal male rats exposed in utero to the exogenous estrogenic chemicals diethylstilbestrol (DES) or 4-octylphenol (OP), exhibited altered expression of mRNA coding for CYP17, protein levels of CYP17 and 17α-hydroxylase/C17,20-lyase activity. The effects of isoflavones on enzyme activities have shown mixed results. While some influence of genistein on

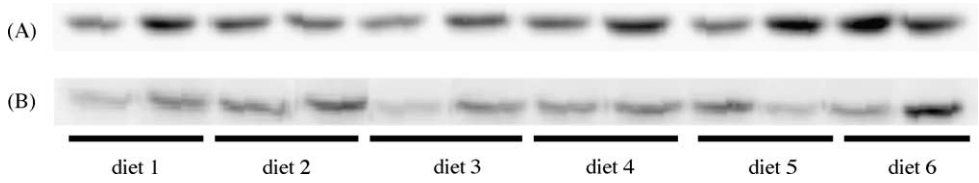

Fig. 6. Immunoreactive protein levels of (A) PND 28 SCC, (B) PND 120 SCC examined by Western blot. For all Western blots presented there was an $n = 2$ for each diet group examined at all time points.

steroid hormone signalling may be due to its potent inhibitory action on enzymes such as tyrosine kinase [54], genistein and daidzein are known inhibitors of steroidogenic enzymes in a variety of bacterial [55] and mammalian cell lines [32,33,56]. Both genistein and daidzein inhibit human placental 17β-HSD activity [56], purified human 17β-HSD type 5 [57], while genistein can inhibit human granulosa cell 17β-HSD [32]. Structural homology between genistein and daidzein compared with endogenous steroidogenic substrates is postulated as a mechanism leading to competitive inhibition at the active site of steroidogenic enzymes [58]. Recently Ohno et al. [57], have shown that daidzein is a competitive inhibitor of 3β-HSD type II enzyme activity ($K_m = 6.6\ \mu M$) within human H295R adrenocortical cells with a K_i of 2.9 μM. Krazeisen et al. [34], postulate that inhibitory potency increases with hydroxylation of the flavonoids, and inhibition occurs through interference with hydrophilic cofactor binding sites, as opposed to interference with substrate binding.

In contrast, other studies such as those by Weber et al. [36], have shown increased steroidogenic enzyme activities due to exposure to isoflavones. A short term animal study where PND 45 male SD rats were fed diets containing 200 mg isoflavones/kg (50% genistin, 40% daidzin and 10% glycitin) for 29 days, revealed increased 5α-reductase activity within the amygdala region, while medial basal hypothalamic–preoptic area 5α-reductase activity was significantly decreased [36]. Similarly, Laurenzana et al. [24], found significantly increased 5α-reductase activity in male and female rat liver microsomes, accompanied by non-significant increases in hepatic cytochrome P450 enzyme protein levels, after exposure from gestational day 7 until sacrifice at PND 50, with 250 mg/kg genistein. However, at 1250 mg/kg genistein, decreased 5α-reductase activity was found indicating a non-linear dose response in males [24]. In human fetal, postnatal and adult adrenal cell lines, genistein and daidzein lowered ACTH-stimulated cortisol production to basal levels, while postnatal and adult adrenal cells exhibited increased DHEA and DHEA-S levels [52]. The increased androgen due to genistein and daidzein may have been caused by decreasing cortisol production leading to either increased availability of substrate for androgen production or increased steroidogenic enzyme activity of P450scc or CYP17, though no changes in mRNA expression of these enzymes were found [52].

Timing and duration of exposure and the route of dosing of pure or mixed isoflavones may be important variables determining isoflavone action on steroidogenic enzymes. In vitro research involving pure isoflavones directly added to cell cultures has typically shown inhibitory actions of isoflavones on steroidogenic enzymes [32–34,55–57], while in vivo, animal studies have shown soy isoflavones increase certain steroidogenic activities [24,36], as seen in the current study.

The enzyme activities examined in the present study are down-stream of the rate-determining step of steroidogenesis, cholesterol side chain cleavage. Enzyme activities for P450 SCC were not examined due to difficulties in acquiring labelled cholesterol substrate to monitor conversion to pregnenolone. However at PND 28 and 120, no differences were found in expression levels of mRNA or protein for P450 SCC in any of the diet groups examined. Similarly mRNA and protein levels of 3β-HSD and CYP17 revealed no changes due to isoflavones at PND 28. It is possible that altered enzyme activity, independent of changes in mRNA and protein levels result from different substrate availability or alterations in the microenvironment. For example, altered P450c21 enzymatic activities due to isoflavones, could not be explained by corresponding changes in expression, and it was proposed that this was due to the upstream inhibition of 3β-HSD which lowered the amount of substrate available for P450c21 [52]. Alternatively, it has been shown that isoflavones can bind to and modify membrane environments [59,60]. Changes in microsomal membrane structure in the immediate area surrounding steroidogenic enzymes such as 3β-HSD [61] could impact enzyme activity. The membrane environment has been shown to at least partially regulate enzyme activity in vitro [62–64].

It is possible that the differences in enzyme activities seen at PND 28 are related to changes in the rate of development between rats of different diet groups. In previous work, our lab has shown increased intratesticular androgen levels in diets 1 and 2 compared with diets 3–6 at PND 28 [28]. Although these changes were not statistically significant, they could indicate a delay in androgen dependent development due to isoflavone consumption. It is possible that the increased steroidogenic enzyme activities seen with high dietary levels of isoflavones are a homeostatic overcompensation to redress delays in androgen dependent development. Isoflavones have been shown to modify endocrine regulation of androgen production by altering levels of serum LH [65–67]. Lund et al. (2004) have found increased circulating LH and statistically insignificant increased plasma DHT levels due to dosing with equol (a major metabolite of daidzein) [67]. Interestingly this group proposed that equol binds directly to DHT preventing it from providing negative feedback within the hypothalamic–pituitary–testicular (HPT) axis. Therefore, isoflavones or their metabolites (e.g. equol), could have led to changes in the normal development of the HPT axis, which in turn may have altered steroid production and circulating levels that were observed in our study. An examination of serum and tissue genistein, daidzein, glycitein and equol, is underway and will be important for deciphering mechanisms of isoflavone action and extrapolating the current findings to the human situation, where isoflavone metabolism varies between individuals who have different gut bacteria and diets leading to subpopulations who tend to be high equol excreters, similar to rodents, or who are low equol excreters [68,69].

At PND 28 testicular weights of rats fed diets 3–6 were significantly higher compared with diet 1 [28]. These isoflavone dose dependent differences could reflect altered cellular profiles. Marmoset monkeys exposed to isoflavones via infant formula exhibited increased numbers of Leydig cells, though

this was paradoxically matched with lower serum T levels [70]. In our previous findings, androgen levels at PND 28, tended to be lower for diets 3–6 compared with diets 1–2 ($P > 0.05$) [28], yet the corresponding enzyme activities are higher, possibly due to an increased number of Leydig cells in rats fed diets 5 and 6. We are presently pursuing the intriguing possibility that changes seen at PND 28 could be due to altered number of Leydig cells or altered signalling in the steroidogenic cascade.

It was of interest that steroidogenic enzyme activities were not altered at PND 120, an age where diets 5 and 6 caused elevated serum T and DHT compared with diets 1 and 2 [28]. However, other possible explanations for the increased androgens at this age may include: altered metabolism of androgens by modification of testicular and/or peripheral aromatase activity [58], changes in steroid metabolism and clearance via the liver [24,71], altered androgen binding protein/sex hormone binding globulin (ABP/SHBG) production or altered free versus bound androgen profile due to ABP/SHBG–isoflavone interactions influencing the ability of steroids to be transported [71].

In our earlier report, isoflavones significantly altered testicular T, serum T and serum DHT at PND 120 [28] and our current work reveals altered steroidogenic enzyme activities at PND 28 in rats fed isoflavones at levels obtainable by human infants [41,42,72]. Studies involving humans have found dose dependent modulation of plasma steroid levels by isoflavones, such as increased plasma DHT levels [73] or decreased serum T [74]. The impact of isoflavones on human in vivo steroidogenesis is not fully understood as present research largely focuses on cell culture [24,32,57,58]. The altered activities seen at PND 28 in the present study are at the same age as when isoflavones were seen to significantly increase testicular weights, and though not statistically significant, decreased testicular T and DHT levels [28]. From these findings it is evident that isoflavones affect early development in male rats possibly delaying the production and level of testicular androgens. We have limited our investigation of steroids to Leydig cell derived T and DHT at PND 28, as their levels were not significantly affected by isoflavones, although examination of 3α-androstanediol, a major androgen at this age, may form part of future investigations [75,76]. Additionally, the enzyme activity profiles examined in the current study do not account for the increased androgen production seen at PND 120 [28], and therefore future work will address regulatory systems upstream of the conversion of cholesterol to androgen.

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