



Developmental exposure to a real-life environmental chemical mixture alters testicular transcription factor expression in neonatal and pre-pubertal rams, with morphological changes persisting into adulthood

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ABSTRACT

Environmental chemical (EC) exposure may be impacting male reproductive health. The translationally relevant biosolids treated pasture (BTP) sheep model was used to investigate gestational low-level EC mixture exposure on the testes of F1 male offspring. Adult rams from ewes exposed to BTP 1 month before and throughout pregnancy had more seminiferous tubules with degeneration and depletion of elongating spermatids, indicating possible “recovery” from previously reported testicular dysgenesis syndrome-like phenotype in neonatal and pre-pubertal BTP lambs. Expression of transcription factors *CREB1* (neonatal) and *BCL11A* and *FOXP2* (pre-pubertal) were significantly higher in the BTP exposed testes, with no changes seen in adults. Increased *CREB1*, which is crucial for testes development and regulation of steroidogenic enzymes, could be an adaptive response to gestational EC exposure to facilitate the phenotypic recovery. Overall, this demonstrates that testicular effects from gestational exposure to low-level mixtures of ECs can last into adulthood, potentially impacting fertility and fecundity.

1. Introduction

The past eight decades have seen a consistent decline in the reproductive health of humans and wildlife (Harrison et al., 1997). In men, this presents as reducing sperm counts and semen quality (Levine et al., 2022; Nelson and Bunge, 1974; Swan et al., 2000) concurrent with

increasing rates of male reproductive disorders, including testicular cancer (Adami et al., 1994; Chia et al., 2010; Møller, 1998) and anomalies of the male external genitalia, most noticeably cryptorchidism and hypospadias (Campbell et al., 1987; Chilvers et al., 1984; Matlai and Beral, 1985; Paulozzi et al., 1999; Toppari et al., 2010). While endeavours to elucidate the underlying causes of these adverse trends in male

Abbreviations: B, Biosolids; BTP, Biosolids treated pasture; CREB, cAMP response element-binding protein; FFPE, Formalin-fixed paraffin embedded; C, Control; DEGs, Differentially expressed genes; ECs, Environmental chemicals; TF, Transcription factor; GO, Gene ontology; GD, Gestation Day; HIF1 α , Hypoxia Inducible Factor 1 Alpha; SCO, Sertoli-cell-only; TDS, Testicular dysgenesis syndrome; TDI, Tolerable daily intake.

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reproductive health, now termed testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001), have identified many contributory factors, such as malnutrition, sedentary lifestyle, and stress (Crean and Senior, 2019; Ilacqua et al., 2018), much research has focussed on the role of exposure to environmental chemicals (ECs) (Skakkebaek, 2002; Skakkebaek et al., 2022). Epidemiological evidence has shown links between gestational exposure to ECs and negative reproductive health outcomes for male offspring (Rodprasert et al., 2021), which is mirrored in animal models of gestational exposure to individual ECs, for example, phthalates (Hu et al., 2009; Repouskou et al., 2021).

While many investigative studies have concentrated on potential effects of specific chemicals or families of chemicals, there is increasing attention to the vast numbers of chemicals in the environment to which the population is constantly co-exposed. This is of concern as EC mixture effects can be seen even when the individual mixture components are administered at doses lower than their tolerable daily intake values (TDI) (Buñay et al., 2018; Kortenkamp, 2014). While efforts are being made to model more realistic EC exposure scenarios (Tsatsakis et al., 2017), it is logistically impossible in terms of both the numbers and doses of chemicals to simulate actual EC exposure by traditional component-based methodologies. The biosolids treated pasture (BTP) sheep model, however, utilises a more realistic EC exposure paradigm relative to the human populations. Biosolids are a byproduct of wastewater treatment, biosolids commonly utilised as agricultural fertiliser; approximately 8500 tonnes are produced daily in England, 87% of which are utilised by agriculture (Water UK, 2022). Biosolids are applied to around 150,000 ha annually in the UK, which equates to approximately 1.3% of UK farmland (BAS, 2019). As biosolids are derived from domestic and industrial human waste water treatment, they contain a complex mixture of chemicals which reflects human EC exposure (Rhind et al., 2010, 2002, 2013; Venkatesan and Halden, 2014a, 2014b). These include alkylated phenols, dioxin-like compounds, pharmaceuticals, personal care products, plasticising agents such as bisphenol A and phthalates, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polybrominated diphenyl ethers, and metabolites thereof. Grazing of sheep on BTP during pregnancy results in measurable concentrations of many ECs in maternal and offspring organs (Bellingham et al., 2012; Filis et al., 2019; Rhind et al., 2010, 2009, 2005). A TDS-like phenotype (reduced germ cell numbers and greater rates of Sertoli-cell only (SCO) seminiferous tubules) has been reported previously in neonatal (1-day-old) Aberdale, and pre-pubertal (8-weeks-old) EasyCare lambs whose mothers were grazed on BTP prior to and during pregnancy, with lower plasma testosterone concentrations also reported for the neonatal male offspring (Elcombe et al., 2022b, 2021). In the Texel foetus (GD110 and GD140), across various exposure periods, fewer germ cells, Leydig cells, and Sertoli cells, and lower plasma testosterone concentrations have been reported (Lea et al., 2022; Paul et al., 2005). Results from adult (19-months-old) Texel rams suggest that BTP exposure (gestationally, and for 7 months during post-natal life, i.e., lactational / direct oral exposure) results in a TDS-like phenotype in a subset of rams (Bellingham et al., 2012). However, the progression of the maternal BTP exposure induced testicular phenotype seen in male offspring has not been characterised into adulthood. Testicular transcriptomic profiles have been produced from foetal, neonatal, and pre-pubertal testes, which have indicated perturbations in multiple pathways and led to evidence of alterations in transcription factor (TF) activation, specifically cAMP response element-binding protein (CREB) and hypoxia inducible factor 1 alpha (HIF1 α), however, the extent of TF perturbation and the permanence of such alterations is not yet known.

The current study aimed to investigate the morphological and transcriptomic changes in adult (11-months-old) ram testes after pre-conceptual and gestational BTP exposure of their mothers. This is compared with already published data from pre-pubertal rams of the same exposure cohort of animals (i.e., siblings and half-siblings), and already published data from neonatal rams of a separate exposure cohort, also born following maternal gestational BTP grazing. HIF1 α

activity is investigated in the neonatal and adult animals and compared with already published data from pre-pubertal rams. TF analysis is then performed on samples from animals of all ages and both exposure cohorts, and evidence of increased TF activity investigated.

2. Methods

2.1. Ethics statement

All procedures were carried out in line with the UK Home Office Animals (Scientific Procedures) Act (A(SP)A) 1986 regulations, under project licence PF10145DF. The project was also approved by the University of Glasgow School of Biodiversity, One Health, and Veterinary Medicine Research Ethics Committee. Animals were maintained under normal husbandry conditions at the Cochno Farm and Research Centre, University of Glasgow.

2.2. Experimental animals

All adult study animals were EasyCare sheep, and were siblings or half siblings of the pre-pubertal rams described by Elcombe et al. (2022b), which were also used. All neonatal animals were Aberdale sheep, and from a separate exposure cohort described in Elcombe et al. (2021). For one month prior to mating by artificial insemination with semen from four rams (four sire groupings within the same genotype), and for the entirety of pregnancy, ewes were maintained on either biosolids treated pasture (BTP) (biosolids exposed (B)) or pastures fertilised with inorganic fertiliser (control (C)). Pastures were fertilised twice yearly (April and September). BTPs used conventional rates of biosolids (4 tonnes / hectare) as a fertiliser, and C pastures used conventional fertiliser with equivalent amounts of nitrogen (225 kg N/ha per annum). Pregnant ewes were brought indoors two weeks prior to parturition. While maintained indoors, ewes were fed forage harvested from their respective pasture types (i.e., Control vs BTP), supplemented with concentrates as per normal husbandry practice. After birth, pre-pubertal and adult male offspring were maintained on control pastures, whereas neonatal male offspring did not leave birthing pens. At 1 day (neonatal, n = 7 control, n = 17 biosolids), 8 weeks (pre-pubertal, n = 11 control, n = 11 biosolids) or 11 months (adult, n = 11 control, n = 10 biosolids) of age, male offspring were weighed and euthanised by IV barbiturate overdose (140 mg/kg Dolethal, Vetroquinol, UK).

2.3. Tissue collection

For all three age groups, at necropsy, testes were dissected. From the left testes, two slices were taken transversely from the centre, quartered, and fixed overnight in 10% neutral buffered formalin (Thermo Scientific – 16499713) before being transferred to 70% ethanol (VWR – 20821.330). Fixed sections of testes were trimmed and processed for embedding in paraffin wax for histology (Excelsior AS, Thermo Scientific). Formalin-fixed, paraffin embedded (FFPE), testicular tissues were stored at room temperature until analysis. From the right testes, transverse slices 5 mm thick were taken, quartered, and frozen in liquid nitrogen prior to storage at – 70 °C for later RNA extraction.

2.4. Immuno-histochemistry

Two Section (5 μ m) of FFPE testicular tissues were taken for each adult animal and mounted on Polysine® coated glass slides. One section per animal underwent immuno-histochemistry (DAB staining), as previously described (Elcombe et al., 2022b), but using a rabbit anti-Sox9 antibody (Sigma-Aldrich AB5535) diluted at 1:1000 to identify Sertoli cells. The other section, and equivalent sections taken from FFPE neonatal testicular tissues, underwent fluorescent immuno-histochemistry for HIF1 α as previously described (Elcombe et al., 2022b).

2.5. Image capture and analysis

2.5.1. SOX9 immuno-histochemistry

Six images from separate areas of the lobuli testis were captured at 100x magnification (Leica DM4000B microscope, Leica DC480 digital camera) for each 11-month-old animal. Individual tubule sections entirely captured within image boundaries ($n = 1483$ Control, 1423 Biosolids) were manually counted. SOX9 positive Sertoli cells were identified by DAB staining and counted. Spermatogonia, spermatocytes and round spermatids were identified by circular nuclei, and elongating spermatids and spermatozoa were identified by condensed and elongated nuclei with darker haematoxylin staining. Normal seminiferous tubule sections at any stage should have a generation of elongating spermatids or spermatozoa. As per OECD guidelines (OECD, 2009), tubule sections with no or few (<5) elongating spermatids and spermatozoa were classified as showing degeneration and depletion of elongating spermatids, whereas those with many (>5) elongated spermatids and spermatozoa were classified as showing typical spermatogenesis.

2.5.2. HIF1 α fluorescent immuno-histochemistry

Image capture and analysis of HIF1 α fluorescent immuno-histochemistry was performed as previously described (Elcombe et al., 2022b). Briefly, for each section, four images from separate areas of the lobuli testis were captured at 400x magnification (Leica DM4000B microscope, Leica DC480 digital camera). Nuclear HIF1 α staining was quantified on areas outside the seminiferous tubules using the JACoP plugin (Bolte and Cordelières, 2006) for ImageJ.

2.6. RNA extraction, cDNA library preparation, sequencing, and data analysis

Nanopore transcriptome sequencing and analysis was performed on adult testicular tissues as previously described for the neonatal (Elcombe et al., 2021) and pre-pubertal (Elcombe et al., 2022b) testes. Briefly, cDNA was synthesised from approximately 30 mg of frozen tissue (from frozen transverse central testis sections, using areas away from the tunica albuginea and rete testis) and barcoded for multiplexed sequencing. In two batches, each batch containing half the samples and comprised of an even mix of C and B samples, pooled barcoded sample cDNA was sequenced using a MinION and an R9.4.1 flow cell (Oxford Nanopore – FLO-MIN106D). Reads were basecalled, demultiplexed, barcodes and adapters trimmed, aligned to a reference transcriptome generated using NCBI's Oar_v4.0 reference genome and annotation files, and counted. Differential gene expression analysis was performed on counts by edgeR (Robinson et al., 2010) with differential expression thresholds of \log_2 fold change < -1 or > 1, p-value < 0.05, and false discovery rate (FDR) < 0.1. Differentially expressed gene (DEG) lists were subjected to gene ontology (GO) and KEGG pathway analyses in DAVID (version 6.8). The DEG list from the adult ram testes, and those previously generated from neonatal (Elcombe et al., 2021) and pre-pubertal (Elcombe et al., 2022b) ram testes, were submitted to ChEA3 for transcription factor (TF) enrichment analysis (Keenan et al., 2019), which produces a list of TFs whose gene products may be over-represented within DEG lists. Sequencing data for gene products of identified transcription factors were extracted from each dataset, geometric means of fold change values calculated, and results filtered for TFs where \log_2 of the geometric mean fold change was ≥ 1 or ≤ -1 in any age group.

2.7. Quantitative qPCR

RNeasy Mini Kits (Qiagen – 74104) were used to extract RNA from approximately 30 mg of frozen neonatal, pre-pubertal, and adult testicular tissues. Tissue was taken from frozen transverse central testis sections, from areas away from the tunica albuginea and rete testis. A

QuantiTect Reverse Transcription Kit (Qiagen – 205311) was used to degrade genomic DNA and synthesise cDNA. qPCR was performed on a Stratagene 3000 qPCR system using Brilliant II SYBR Master Mix (Agilent – 600828). Primer sequences are in Supplementary Data 1. Primer efficiencies and Ct values for each sample were calculated by regression of raw fluorescent data by PCR Miner (Zhao and Fernald, 2005). These were used in $\Delta\Delta C_t$ analysis to calculate \log_2 fold change values.

2.8. Statistical analysis

R (version 4.1.1) base functionality was used for all calculations and statistical analyses. For each cohort (i.e., neonatal separate to pre-pubertal and adult), data were fitted to generalised linear models using a gamma distribution with the `glm()` function, and groups compared by *t*-tests on coefficient estimates against their respective, same age controls using the `summary()` function. For the adult and prepubertal cohorts, sire was incorporated into models to account for the genetic structure of data. The R package `ggplot2` (version 3.3.5) was used to produce plots. In text, data are reported as mean \pm SD.

3. Results and discussion

3.1. Greater proportions of seminiferous tubules with degeneration and depletion of spermatids in testes of adult rams exposed gestationally to BTP

In adult (11-months-old) ram testes, examination of histological images (Fig. 1 A) revealed a higher ($p = 0.0225$) proportion of seminiferous tubule sections displayed degeneration and depletion of spermatids in B ($31.62 \pm 9.92\%$) relative to C ($21.96 \pm 6.48\%$) rams (Fig. 1B). However, there were no differences between B and C ram testes in terms of germ cell numbers or germ cell: Sertoli cell ratios, and no incidences of SCO tubules observed in any animal.

Offspring of sheep grazed on BTP before and during pregnancy exhibit a TDS-like phenotype (i.e., reduced germ cell numbers and greater rates of Sertoli-cell only seminiferous tubules) in neonatal Aberdale rams (Elcombe et al., 2021), and in the pre-pubescent siblings and half-siblings of the adult EasyCare rams presented here (Elcombe et al., 2022b). Following similar exposure, reductions in germ cell, Sertoli cell, and Leydig cell numbers have been seen in the mid-gestation (GD110; unknown breed) foetus (Paul et al., 2005), and in the late-gestation Texel foetus (GD140) various exposure timings led to reductions in testes weight, total Sertoli cell numbers, and plasma testosterone concentrations (Lea et al., 2022), which demonstrate gestational BTP exposure induces teratogenic effects on the male reproductive system. These studies also suggest that genetic differences between breeds of sheep does not greatly impact the pathogenesis of the TDS-like phenotype, which implies selective breeding practices would not be able to confer resistance to TDS development. Adult cell count data was therefore compared to similarly analysed, previously published data, shown in Supplementary Data 2A and B. Comparing adult data to that from pre-pubertal siblings and half-siblings of the adult animals (from Elcombe et al., 2022b), it is clear in terms of seminiferous tubule cellular proportions, and frequency of SCO seminiferous tubules, that the TDS-like phenotype described in the pre-pubertal B rams was not present in the same manner in the adult B rams. This could suggest (partial) recovery from a TDS-like phenotype with age, and therefore greater time without exposure. Although from a different breed stock and exposure cohort, comparing these data to previously published data from similarly exposed neonatal ram testes (from Elcombe et al., 2021), the greatest histological differences were seen in the 1-day-old B rams, which could also suggest (partial) recovery with time. Despite this apparent phenotypic recovery, adult B rams exhibited an increased proportion of seminiferous tubules showing degeneration and depletion of elongating spermatids. This phenotype is regarded as the end stage lesion of low intratesticular testosterone (OECD, 2009), which agrees with previously published observations of lower plasma testosterone

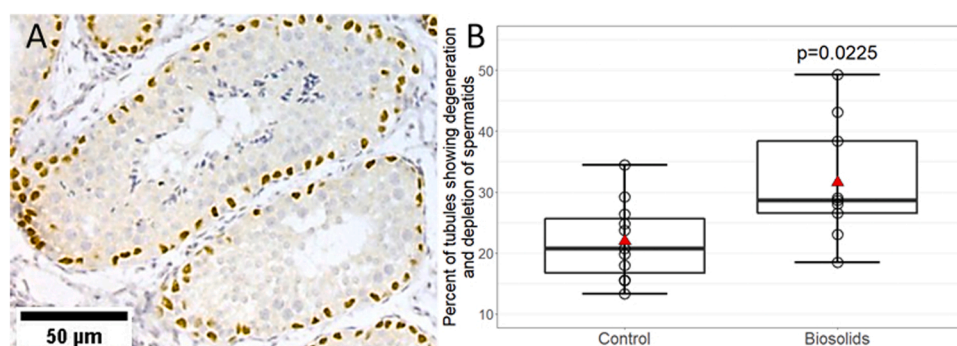


Fig. 1. (A) Examples of seminiferous tubule sections stained with anti-SOX9 DAB and haematoxylin, showing typical morphology (top) and degeneration and depletion of elongating spermatids (bottom). (B) Biosolids rams have a higher proportion of seminiferous tubules showing degeneration and depletion of elongating spermatids. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range, circles show individual data points, and red triangles show means.

concentrations in the late gestation foetus and neonatal male BTP offspring (Elcombe et al., 2021; Lea et al., 2022), and may have an impact on sperm production, semen quality, fertility, and fecundity.

The persistence of a TDS-like phenotype in a subset of adult (19-months-old) Texel rams born to ewes grazed on BTP during pregnancy, that were also grazed on BTP for 7 post-natal months (Bellingham et al., 2012), contrasts with the results seen in the adults in this study. While this phenotypic difference in adult BTP sheep testes could be a factor of susceptibility differences between breeds, as this phenotype has been observed in multiple breeds of sheep, it also suggests that while the TDS-like phenotype may be teratogenic in nature (i.e., originating during foetal development), continued EC exposure post-parturition may be required to maintain the TDS-like phenotype into adulthood. Indeed, this latter scenario is more reflective of consistent real-life exposure in humans.

3.2. Gestational BTP exposure alters testicular transcriptome in adult rams

Analysis of Nanopore sequenced testicular transcriptomes in adult rams identified 1183 differentially expressed genes (DEGs) between B and C rams (562 with greater levels of expression and 621 with lower levels of expression in B relative to C). Thirty-three DEGs had a false discovery rate ≤ 0.1 (13 with greater levels of expression and 20 with lower levels of expression in B relative to C). The results of transcriptomic analyses are another indication of the enduring effects of foetal exposure. Once again, when adult data are compared to similarly analysed, previously published data (Supplementary Data 2 C), the reduction in morphological differences observed with increasing time without B exposure was mirrored by the decreased number of DEGs. The greatest number of DEGs observed was in the neonatal testes (from Elcombe et al., 2021; 296 DEGs: expression in B rams were higher for 21 genes, and lower for 275 genes, compared to C). The number of DEGs in pre-pubertal ram testes was about one third of the neonatal (from Elcombe et al., 2022b; 99 DEGs: expression in B rams were higher for 60 genes, and lower for 39 genes, compared to C). Furthermore, the number of DEGs identified in the adult ram testes was one third of that seen in the pre-pubertal rams, which were from the same exposure cohort and therefore only differed in age and time since exposure. There was very little overlap between DEGs identified between age groups: seven in common between the neonatal and pre-pubertal rams, three in common between neonatal and adult rams, with only one in common between the pre-pubertal and adult rams, which were derived from the same exposure cohort of animals. There were no genes in common between all three ages (Supplementary Data 3).

Gene ontology (GO) analysis of the 33 DEGs identified in the adult testicular transcriptome indicated five GO terms and one KEGG pathway as enriched ($p < 0.05$) (Table 1). Of specific note is the identification of the mTOR signalling pathway as a site of disruption. mTOR has previously been identified as affected by EC exposure in transcriptomic analyses of foetal, neonatal, and pre-pubertal biosolids ram testes

Table 1

GO terms and KEGG pathways identified as enriched by DAVID, from the list of 33 DEGs in the adult biosolids ram testicular transcriptome.

| Category | Term | Fold Enrichment | p Value |
|------------------------|--|-----------------|---------|
| GO: Cellular Component | Cytosol | 3.2 | 0.0016 |
| KEGG | mTOR signalling pathway | 14.7 | 0.0140 |
| GO: Cellular Component | STAGA complex | 104.6 | 0.0182 |
| GO: Cellular Component | Transcription factor TF1C complex | 97.6 | 0.0195 |
| GO: Biological Process | Histone H3 acetylation | 59.4 | 0.0317 |
| GO: Biological Process | Cellular response to hydrogen peroxide | 39.6 | 0.0472 |

(Elcombe et al., 2022b, 2021; Lea et al., 2022). Alterations with mTOR signalling pathways due to low-level exposure should be of concern as mTOR is a crucial component of proper testicular development and spermatogenesis (Correia et al., 2020) and its disruption by chemical exposure (e.g., 4NP, DEHP, or BPA) has been shown to induce testicular autophagy in pubescent rodents (Duan et al., 2017; Fu et al., 2020; Quan et al., 2017). Additionally, mTOR is also crucial in spermatogonial stem cell maintenance, and induction or inhibition of mTOR activity can deplete the pool of spermatogonial stem cells (Hobbs et al., 2015; Xiong et al., 2015). Based on the results of our previous work in which investigations into disrupted mTOR signalling led to evidence of Hypoxia Inducible Factor 1 Alpha (HIF1 α) activation and nuclear localisation in Leydig cells of biosolids pre-pubertal lambs (Elcombe et al., 2022b), this was examined in the current study in both neonatal lambs from a separate exposure cohort, and adult animals from the same exposure cohort as the pre-pubertal offspring.

3.3. Deactivation of HIF1 α , and the normalisation of HIF1 α gene product expression by adulthood in sheep gestationally exposed to BTP

Evidence of exposure induced changes to the nuclear localisation of HIF1 α within Leydig cells, as previously reported in the pre-pubertal biosolids EasyCare rams (Elcombe et al., 2022b), was investigated in the neonatal Aberale rams, and the adult EasyCare rams from the same exposure cohort as the pre-pubertal rams. There was significantly ($p = 0.0048$) greater proportion of HIF1 α nuclear localisation in the Leydig cells of the testes from the neonatal B rams (0.452 ± 0.156) relative to C (0.241 ± 0.087), whereas there was no statistically significant difference in the proportion of HIF1 α nuclear localisation in the Leydig cells of adult B rams (0.277 ± 0.113), compared to control (0.249 ± 0.093) (Fig. 2). In Supplementary Data 2D, this data is compared to the previously reported data from pre-pubertal ram testes (same exposure cohort as the adults) where activation was previously observed (Elcombe et al., 2022b). The proportion of HIF1 α nuclear

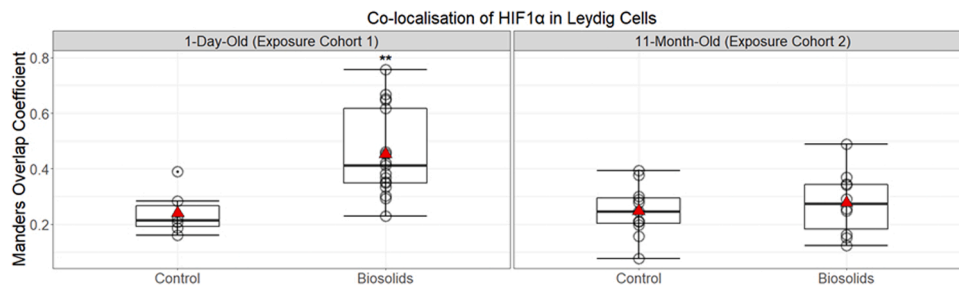


Fig. 2. Co-localisation analysis of IHC shows significantly ($p = 0.0048$) more HIF1 α nuclear localisation in Leydig cells of neonatal biosolids ram testes compared with control, but no difference in Leydig cells of adult biosolids ram testes. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

localisation in the C ram testes was consistent across the neonatal, pre-pubertal, and adult rams (0.241 ± 0.087 , 0.251 ± 0.097 , and 0.249 ± 0.093 , respectively). Within the B exposed rams, the greatest proportion of HIF1 α nuclear localisation was in the Leydig cells of the testes from the neonatal rams, which was slightly higher than that previously published for the pre-pubertal rams from the same exposure cohort as the adults (Elcombe et al., 2022b). A greater proportion of HIF1 α activation and nuclear localisation in Leydig cells of the neonatal lambs indicates this disruption by ECs is likely to be of foetal origin. Leydig cells contain the highest amounts of HIF1 α in the testes (Palladino et al., 2011) and, through binding site blocking, HIF1 α activation in Leydig cells can reduce the transcription of STAR, the rate limiting step in steroidogenesis, and lower testosterone production (Manna et al., 2016; Wang et al., 2019). In this respect, changes in HIF1 α activation may have a role in the pathogenesis of the TDS-like phenotype seen in younger biosolids animals, where lower testosterone levels were observed in the GD140 foetus and neonatal lamb (Elcombe et al., 2021; Lea et al., 2022). However, the primary function of HIF1 α is that of angiogenic and metabolic reprogramming in response to hypoxia (Child et al., 2021), and HIF1 α activation can occur via changes in biochemical pathways (e.g. mTOR) (Dodd et al., 2015) or in direct response to xenobiotics (Bonello et al., 2007; Xia et al., 2009).

To assess the impact greater or equal HIF1 α nuclear localisation had on the expression of HIF1 α gene products, as before in the pre-pubertal rams, qPCR was performed for *HK1*, *PDPK*, and *VEGFA* on cDNA synthesised from neonatal and adult ram testes (Fig. 3). Significantly

greater expression of *HK1* ($p = 8.4e-07$) and *VEGFA* ($p = 0.001$) was seen in the neonatal B ram testes compared to same age C, and no statistically significant differences in gene expression were seen in the adult testes. However, curiously, *PDPK* expression was not different between B and C rams in the neonatal ram testes. When these results are compared to those for the pre-pubertal B ram testes (previously published in Elcombe et al., 2022b) from the same exposure cohort as the adult rams, fold change in expression levels of *HK1* and *VEGFA* for neonatal B rams compared to same age C were greater than in the pre-pubertal B ram testes, but expression levels in the neonatal B ram testes were of similar magnitude to the pre-pubertal B ram testes (Supplementary Data 4).

3.4. Gestational BTP exposure alters transcription factor expression in testes of neonatal and pre-pubertal rams, but not adult rams

The persistent changes in activation of the transcription factor (TF) HIF1 α in biosolids animals at different ages led to the investigation of alterations in other TFs. ChEA3 TF analysis of DEG lists from neonatal, pre-pubertal, and adult ram offspring, and subsequent interrogation of sequencing data, identified fifty TFs potentially affected by exposure (Supplementary Data 5). Of these, 43 showed lower expression of gene products in the neonatal B lamb compared to same age C, with no differences seen in the pre-pubertal or adult offspring that were derived from a different exposure cohort to the neonatal. 1 TF (CREB1) showed higher gene product expression in the neonatal B testes compared to same age C, and 6 TFs (BCL11A, FOSL1, FOXA1, FOXP2, GATA3, and

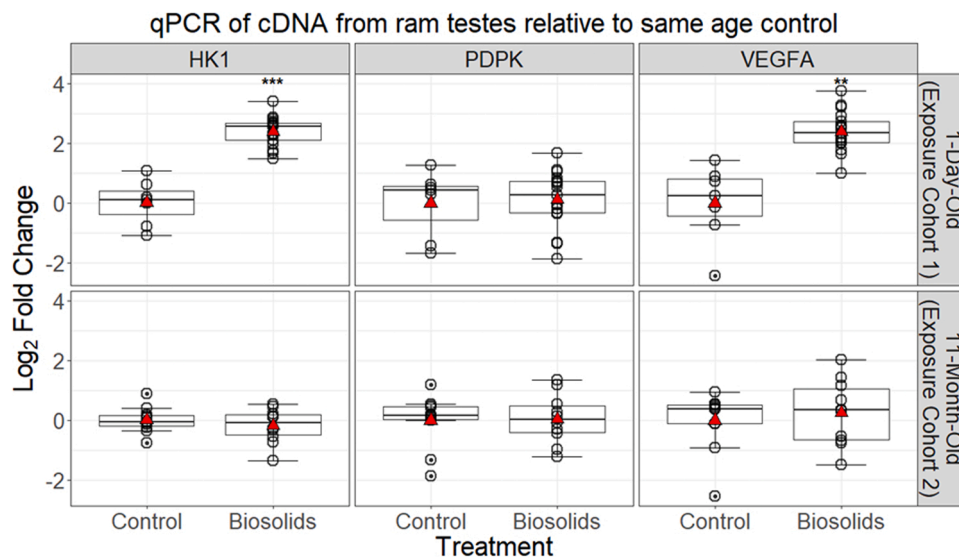


Fig. 3. qPCR for the genes *HK1*, *PDPK*, and *VEGFA* in neonatal and adult ram testes expressed as Log₂ Fold Change in gene expression relative to same age control. Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

JUND) showed altered gene product expression in either B pre-pubertal or B adult ram testes, which were of the same exposure cohort, compared to same age C (Fig. 4). Of these, BCL11A, FOXA1 and FOXP2 showed lower gene product expression in the neonatal B rams and higher in the pre-pubertal B rams (different exposure cohorts) compared to same age C, and GATA3 showed higher gene product expression in the pre-pubertal and adult rams (same exposure cohort) compared to same age C.

To assess if differences in gene product expression were due to changes in TF expression, qPCR was performed on these TFs (Fig. 5). There were no statistically significant differences in expression levels of *FOSL1*, *FOXA1*, *GATA3*, or *JUND* between B and same age C in any age group. In the neonatal testes, *CREB1* expression was significantly ($p = 1.3e-04$) greater in B lambs than in C. In the pre-pubertal testes, significantly greater expression of *BCL11A* ($p = 0.018$) and *FOXP2* ($p = 0.0069$) was seen in the testes of B lambs than same age C. Higher transcription levels of *CREB1* in the neonatal, and *BCL11A* and *FOXP2* in the pre-pubertal testes matched the increased transcription of gene products of those TFs identified within the sequencing data for those age groups. While there is no literature on the roles of *BCL11A* or *FOXP2* within the testes, Cyclic AMP (cAMP) signalling and CREB (cAMP-response element binding protein) have previously been identified as affected by EC exposure in the neonatal biosolids lamb (Elcombe et al., 2021), and are known to play important roles within the testes. Within Sertoli cells, cAMP signalling and CREB are crucial in testicular development and spermatogenesis (Don and Stelzer, 2002), and within Leydig cells CREB regulates the expression of important steroidogenic genes (Kumar et al., 2018). It is therefore possible that the activation and increased transcription of *CREB1* in the neonatal biosolid ram testes is an adaptive response to EC exposure.

3.5. Conclusions

The long-term consequences of exposure to mixtures of ECs on male reproductive health is of continuing concern. Increasing attention towards the effects of low-dose chemical mixtures has shown that effects can often be seen following exposure to a mixture of chemicals at doses which, individually, would not be expected to elicit an effect (Elcombe et al., 2022a). Low dose gestational exposure to even simple mixtures (containing less than 10 chemicals) have been shown to cause genital malformations, alterations in testicular morphology, inhibited steroidogenesis, and impaired spermatogenesis, in male rodents (Buñay et al., 2018; Christiansen et al., 2009; Jacobsen et al., 2012). However, such component-based studies fall short of simulating the exposure scenario to which humans are constantly exposed, i.e., low concentrations of many hundreds of chemicals. The ability for gestational

exposure to a complex, low-level, real-life chemical mixture to affect testicular development, with effects lasting into adulthood, is demonstrated in the present study.

A challenge and limitation in the interpretation of the present results are that two age groups (pre-pubertal and adult) are from the same exposure cohort, are of the same breed of sheep, and are full or half-siblings, whereas the other age group (neonatal) were different in all these respects. As the chemical contents of biosolids has batch variability, and chemical uptake varies based on factors of soil content (Clarke and Smith, 2011; Rhind et al., 2013; Zhang et al., 2015), there were undoubtedly differences in exposure between that received by the neonatal rams, and by the pre-pubertal and adult rams. The genetic differences between Aberdale (neonatal rams) and EasyCare (pre-pubertal and adult rams) sheep is another variable of unknown impact on the results, which could affect susceptibility or resistance to exposure-induced effects and must be considered while interpreting. However, as similar BTP phenotypes have been previously reported in multiple breeds of sheep from separate exposure cohorts, the pre-pubertal rams morphologically resembled the neonatal rams more than their older exposure cohort counterparts, and the pre-pubertal and neonatal rams showed very similar HIF1 α activation patterns, these concerns were not considered to be major factors with regards to the present study. Therefore, this allowed us to compare the effects of exposure over multiple ages, which allows considerations of directionality and longevity, which is a strength of the present study. An additional strength comes from using a more translationally relevant animal system, rather than traditional laboratory rodents. Sheep are precocial, with organ development more similar at birth to humans than altricial rodents. Sheep are also more physiologically like humans in terms of reproductive cycle, gestational periods, lifespan, steroidal biosynthesis, and start and duration of puberty. Crucially, similarities in testicular development between sheep and humans, in terms of hypothalamic-pituitary-gonadal axis function onset, plasma androgen and anti-Mullerian hormone levels, genital tubercle formation, and external genitalia differentiation, are much greater than for other species, including rats and mice (O'Shaughnessy and Fowler, 2011).

It is well recognised that foetal development is a period of increased risk to xenobiotic induced toxicity, especially that mediated by endocrine disruption. The present study exemplifies this, evidencing that exposure to an environmental chemical mixture, at realistically low doses, during gestation alone is sufficient to produce observable morphological and molecular effects in the testes, which persist into adulthood. Differences between the adult rams in this study and adult rams from a similar study, with a period of post-natal exposure, indicates a crucial period of life whereby, with continued exposure, TDS-like traits persist into adulthood. This also suggests adverse effects evident in early

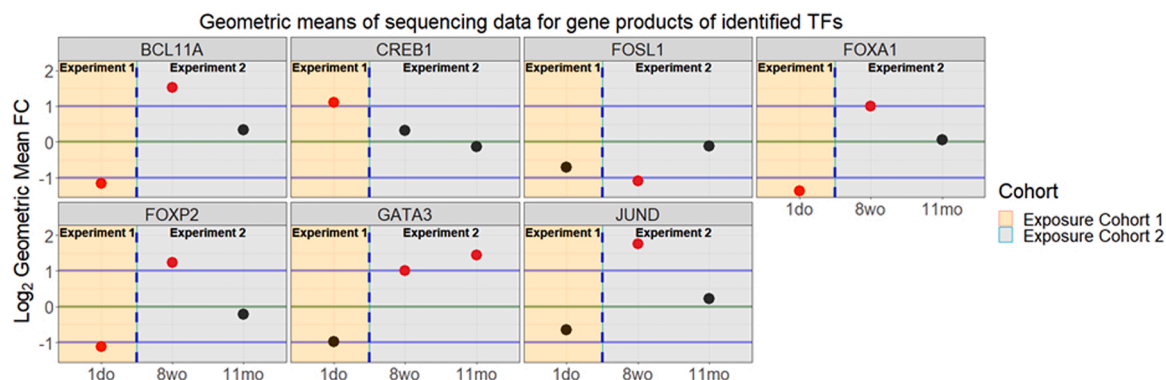


Fig. 4. Changes in expression of gene products for transcription factors identified by ChEA3. Transcription factor analysis was performed on gene lists from transcriptomic sequencing analysis of 1-day-old (1do), 8-week-old (8wo), and 11-month-old (11mo) ram testes, by ChEA3. Transcriptomic sequencing data of the gene products of identified transcription factors were interrogated, and log₂ of geometric means of fold change relative to same age control plotted. A threshold of ± 1 log₂ fold change was applied for inclusion. Red dots indicate data points which passed the threshold.

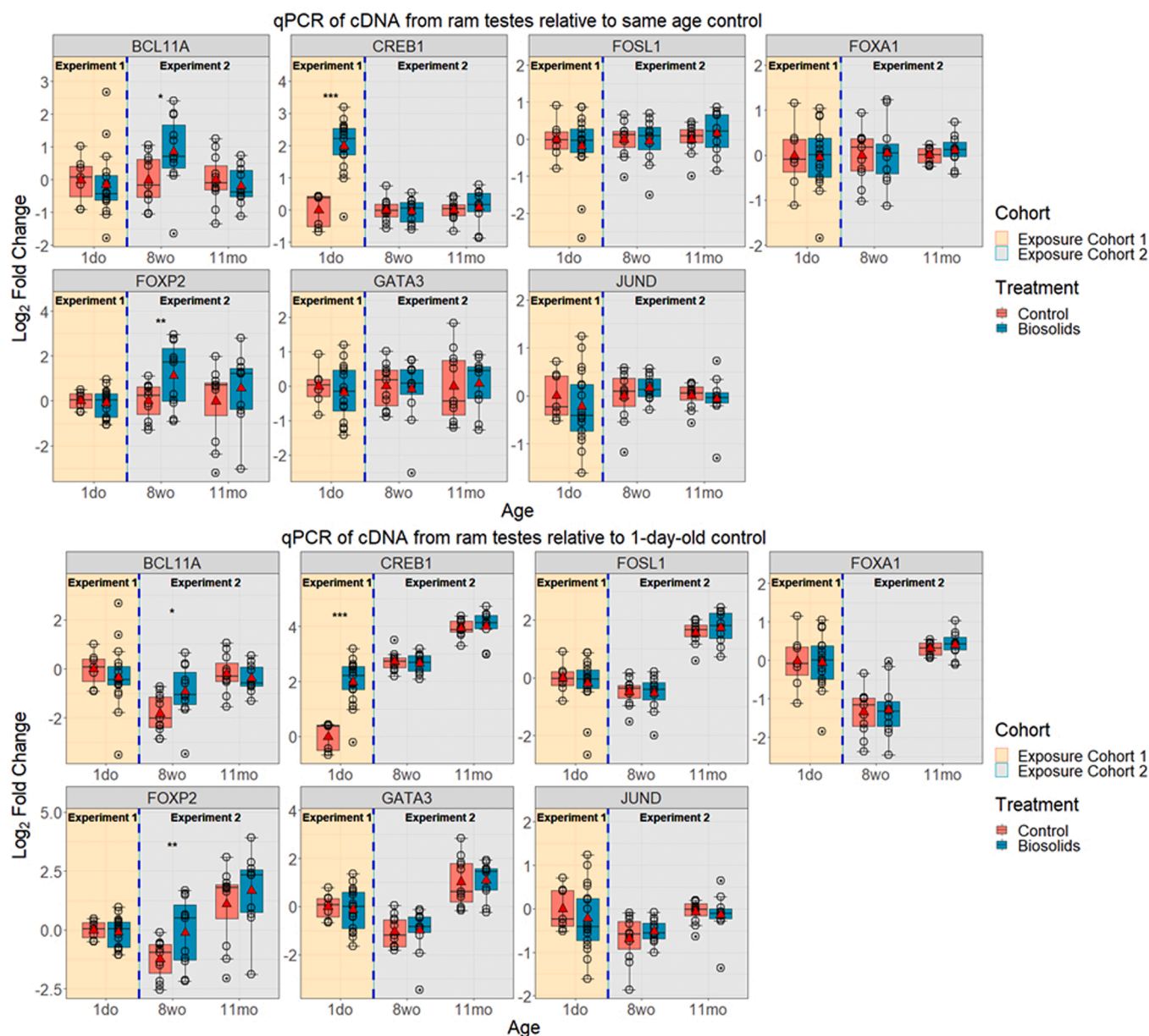


Fig. 5. qPCR for the genes BCL11A, CREB1, FOSL1, FOXA1, FOXP2, GATA3, and JUND in neonatal (1do), pre-pubertal (8wo), and adult (11mo) ram testes expressed as Log_2 Fold Change in gene expression relative to same age control (top) or neonatal control (bottom). Boxes represent 25th to 75th percentile, horizontal bar indicates 50th percentile, whiskers indicate range excluding outliers, solid filled circles show outliers, open circles show individual data points, and red triangles show means.

life are not necessarily permanent, and may be at least partially recoverable, dependant on removal from the source of exposure. Increased HIF1 α activation in Leydig cells is shown to be present from birth in exposed offspring, which may be linked to lowered steroidogenesis during foetal development and early life. Increased transcription of CREB1 could be a compensatory mechanism against this action, and therefore may be important in the partial recovery observed. The current findings add to the increasing body of evidence suggesting that exposure to real-world levels of environmental chemical mixtures during pregnancy may be having a negative impact on the reproductive health of male offspring, contributing to declining male reproductive health, including sperm counts, semen quality, fertility, and fecundity.

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CRedit authorship contribution statement

Chris S. Elcombe: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Ana Monteiro:** Methodology, Investigation. **Mohammad Ghasemzadeh-Hasankolaei:** Writing – review & editing. **Vasanth Padmanabhan:** Writing – review & editing. **Richard Lea:** Writing – review & editing. **Kevin D. Sinclair:** Writing – review & editing. **Neil P. Evans:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Michelle Bellingham:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.etap.2023.104152](https://doi.org/10.1016/j.etap.2023.104152).

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