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Transcriptome analysis of fetal rat testis following intrauterine exposure to the azole fungicides triticonazole and flusilazole reveals subtle changes despite adverse endocrine effects

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HIGHLIGHTS

- Triticonazole and flusilazole have endocrine disrupting effects *in vivo*.
- Triticonazole induces few transcriptional changes to fetal rat testis.
- Flusilazole induces few transcriptional changes to fetal rat testis.
- *Calb2*, a putative biomarker, is upregulated in fetal testis by flusilazole.

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ABSTRACT

Azoles are used in agriculture and medicine to combat fungal infections. We have previously examined the endocrine disrupting properties of the agricultural azole fungicides triticonazole and flusilazole. Triticonazole displayed strong androgen receptor (AR) antagonism *in vitro*, whereas *in utero* exposure resulted in anti-androgenic effects *in vivo* evidenced by shorter anogenital distance (AGD) in fetal male rats. Flusilazole displayed strong AR antagonism, but less potent than triticonazole, and disrupted steroidogenesis *in vitro*, whereas *in utero* exposure disrupted fetal male plasma hormone levels. To elaborate on how these azole fungicides can disrupt male reproductive development by different mechanisms, and to investigate whether feminization effects such as short AGD in males can also be detected at the transcript level in fetal testes, we profiled fetal testis transcriptomes after *in utero* exposure to triticonazole and flusilazole by 3'Digital Gene Expression (3'DGE). The analysis revealed few transcriptional changes after exposure to either compound at gestation day 17 and 21. This suggests that the observed influence of flusilazole on hormone production may be by directly targeting steroidogenic enzyme activity in the testis at the protein level, whereas observations of shorter AGD by triticonazole may primarily be due to disturbed androgen signaling in androgen-sensitive tissues. Expression of *Calb2* and *Gsta2* was altered by flusilazole but not triticonazole and may pinpoint novel pathways of disrupted testicular steroid synthesis. Our findings have wider implication for how we integrate omics data in chemical testing frameworks, including selection of non-animal test methods and building of Adverse Outcome Pathways for regulatory purposes.

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Author contribution

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1. Introduction

Male sexual development is dependent on androgen signaling during fetal life. After gonadal sex determination, the XY genital ridges differentiate into testes and rapidly start to synthesize androgens (Svingen and Koopman, 2013). In turn, androgen production, which mainly takes place in the fetal Leydig cells, influences development of the secondary male sex organs and general sex characteristics (Schwartz et al., 2019a). Male reproductive development is thus dependent on sex hormones produced by the developing testes. Consequently, exposure to endocrine disrupting chemicals (EDCs) during fetal life, particularly anti-androgens, can have serious consequences for normal development and cause reproductive disorders later in life (Skakkebaek et al., 2016).

Many chemicals have anti-androgenic potential and can thus disrupt male reproductive development. Fetal and perinatal exposure to such compounds, including phthalates, parabens and azole fungicides, can cause feminization effects in male offspring, for instance short anogenital distance (AGD). The feminization effects in the male offspring is often attributed to suppressed testosterone levels (Borch et al., 2006; Carruthers and Foster, 2005; Foster, 2006; Gray et al., 2000; Swan et al., 2005; Vinggaard et al., 2005) or blocked androgen receptor (AR) action (Gray et al., 2001; Kelce et al., 1995, 1994; Ostby et al., 1999). Suppressed testosterone levels may suggest that the compounds act directly on the testes by disrupting steroidogenesis. Effects mediated through the AR, on the other hand, suggest that the compounds exert their effects in other androgen-sensitive tissues where AR action is required for cell differentiation. Notably, several studies report on disrupted fetal testis histology and gene expression profiles after exposure to anti-androgenic chemicals, not least phthalates (Barlow et al., 2004; Boberg et al., 2011; Borch et al., 2006; Di Lorenzo et al., 2020; Foster et al., 1980; Liu et al., 2005). This suggests a more complex picture of how different chemicals affect male reproductive development than simply disrupting testosterone synthesis or AR action. In fact, predicting *in vivo* anti-androgenic effects from *in vitro* data derived from testing steroid synthesis and AR action is not always straight forward and can lead to 'surprises' in terms of *in vivo* outcomes. This can be because of unpredicted toxicokinetics, but also because of likely involvement of molecular pathways driving reproductive development other than the testosterone-AR axis.

Azole fungicides represent a group of compounds where *in vivo* endocrine disrupting effects can be difficult to predict from *in vitro* data. Azoles are widely used in medicine and agriculture because of their ability to suppress fungal growth, mainly by inhibiting the fungal cytochrome P450 sterol 14 α -demethylase (CYP51) enzyme (Zarn et al., 2003). Unfortunately, azoles also cause various adverse effects in mammals, including anti-androgenic effects such as shorter AGD and nipple retention in male offspring (Hass et al., 2012; Laier et al., 2006; Taxvig et al., 2008, 2007; Vinggaard et al., 2005). Enigmatically, some azole fungicides can also induce longer AGD in both female and male rodents (Goetz et al., 2007; Hass et al., 2012; Laier et al., 2006; Melching-Kollmuss et al., 2017; Rockett et al., 2006; Taxvig et al., 2007). So, although it may be speculated that the anti-androgenic effects would arise from interference with mammalian CYP enzymes, not least in the steroidogenic pathways, current knowledge points to a more complex picture of effect modalities. For instance, we recently reported that the two azole fungicides triticonazole and flusilazole have endocrine disrupting effects in rats, but through different modes of

action; and with *in vivo* effects not entirely predictable based on *in vitro* test data (Draskau et al., 2019). Triticonazole displayed strong AR antagonism *in vitro*, and *in utero* exposure resulted in anti-androgenic effects *in vivo*, evidenced by shorter male AGD. Flusilazole displayed strong AR antagonism, but less potent than triticonazole, and disrupted steroidogenesis *in vitro*, but *in utero* exposure only disrupted fetal male plasma hormone levels in rats.

To better understand the mechanisms of action underpinning the reproductive effects seen after *in utero* exposure to azoles – and thereby enable the design of more robust alternative test strategies for chemicals – we follow up on these previous results and investigate putative molecular effects in the fetal male testis at the transcriptional level. Using the 3' Digital Gene Expression (3'DGE) technology (Cacchiarelli et al., 2015), fetal testis transcriptomes from flusilazole- and triticonazole-exposed rats were profiled to i) gain new knowledge about how these azole fungicides may affect testis development by different mechanisms and ii) to scrutinize the general question: does changes in AGD always predict testicular dysgenesis in rodents. This can lead to valuable information on toxicity mechanisms for azoles in particular, but also anti-androgenic chemicals more broadly. Omics approaches have been proposed to be valuable tools in the search for new molecular insights that could help us identify molecular events leading to apical effect endpoints (Darde et al., 2018a). In turn, this new mechanistic insight offers means of integrating molecular events with regulatory risk assessment, including aiding in chemical grouping and developing novel adverse outcome pathways (AOPs), which are pragmatic descriptions of the mechanistic events that lead from molecular initiating trigger to adverse outcome (Ankley et al., 2010). In this way the AOP concept represents a platform to integrate fundamental biological knowledge on molecular events with health risk assessment (Draskau et al., 2020); biological knowledge that may be accessible by using high-throughput omics approaches.

2. Materials and methods

2.1. Chemicals

Flusilazole ($\geq 95\%$ pure, CAS No. 85509-19-9) was purchased from BOC Sciences (batch no. B18LN07171, New York, USA). Triticonazole ($>95\%$ pure, CAS No. 131983-72-7) was purchased from Abcam (#ab143728, lot no. GR3232419-3(N/A), Cambridge, UK).

2.2. Animal study

The *in vivo* rat experiment was previously described in (Draskau et al., 2019). Briefly, time-mated Sprague-Dawley rats (CrI:CD(SD)) (Charles River Laboratories, Sulzfeld, Germany) were delivered at gestational day (GD) 3, with the day following overnight mating denoted GD1. On GD4, dams were weighed and assigned to treatment groups with similar body weight (bw) distributions. Animals were kept under standard conditions with 12 h light/dark cycles and fed a standard soy- and alfalfa-free diet based on Altromin 1314 (Altromin GmbH, Germany) along with tap water in Bisphenol A-free bottles (Polysulfone 700 ml, 84-ACBT0702SU Tecniplast, Italy) provided *ad libitum*. Animals were kept in pairs until GD17, thereafter individually.

Rat dams were exposed to compounds from GD7. The rats were weighed and gavaged each morning with vehicle (corn oil, Sigma-Aldrich, #C867-2.5L, Denmark), flusilazole (45 mg/kg bw/day) or triticonazole (450 mg/kg bw/day) until necropsy at GD17 or GD21. The dams were given a dosing volume of 2 ml/kg bw. Compounds were administered 1 h $\pm$ 15 min before decapitation under CO₂/O₂-

anesthesia. Fetal testes were harvested under a stereomicroscope and immediately placed in either formalin or Bouin's fixative for histological analyses or RNAlater for gene expression analyses. Tissue in RNAlater (Thermo Fisher Scientific, Lithuania) was stored at -80°C until further processing.

Animal experiments had ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553) and were overseen by the in-house Animal Welfare committee. All methods were performed in accordance with relevant guidelines and regulations.

2.3. RNA extraction

Total RNA was extracted from fetal testes at GD17 and GD21 ($n = 9-13$ testes/group) with an RNeasy Micro Kit (Qiagen, Germany) following manufacturer's instructions, including on-column DNase I digestion. For GD17 samples, both testes from each animal were pooled for RNA extraction, whereas for GD21 samples, only one testis from each animal was used for RNA extraction. RNA quantity and quality was assessed using a 2100 Bioanalyzer Instrument (Agilent Technologies, CA, USA) according to manufacturer's instructions. Only samples with an RNA integrity number (RIN)-score >7 were included for further analyses.

2.4. 3'Digital Gene Expression sequencing (3'DGE)

Sample preparation for the 3'DGE experiment was performed at the Research Institute for Environmental and Occupational Health (Irset, Rennes, France) as previously published (Pham et al., 2020; Saury et al., 2018). Briefly, poly(A) tailed mRNAs were tagged with universal adapters, barcodes and unique molecular identifiers (UMIs) during reverse transcription. Barcoded cDNAs from multiple samples were then pooled, amplified, and tagged using a transposon-fragmentation approach that enriches for 3' ends of cDNA. The resulting library was sequenced on an Illumina HiSeq 2500 using a TruSeq Rapid SBS kit (Illumina, San Diego, CA, USA) by the GenoBiRD Platform (IRS-UN, Nantes Cedex, France). Quality control and preprocessing of data were performed by GenoBiRD. The first 16 bases of the first reads must fully correspond to a specific barcode. The second reads were aligned to the rat reference transcriptome from the UCSC website (release rn6, downloaded in December 2019) using the pipeline described in (Saury et al., 2018). A gene expression matrix was generated by counting the number of unique UMIs associated with each gene (lines) for each sample (columns). The resulting UMI dataset was further normalized by using the rlog transformation implemented in the DESeq2 package (Love et al., 2014). Sequencing-derived data and processed data were deposited at the GEO repository under the accession number GSE154012. The resulting transcriptomic signatures of each compound were also deposited at the TOXsign repository (<https://toxsign.genouest.org>) (Darde et al., 2018b).

2.5. Differential gene expression analysis

The following statistical comparisons were made in the AMEN suite of tools (Chalmel and Primig, 2008): GD17 controls vs GD21 controls, GD17 controls vs GD17 flusilazole-exposed samples, GD17 controls vs GD17 triticonazole-exposed samples, GD21 controls vs GD21 flusilazole-exposed samples, and GD21 controls vs GD21 triticonazole-exposed samples. Briefly, genes showing an expression signal higher than a given background cutoff (corresponding to the overall median of the rlog-transformed UMI dataset, 1.29) and at least a 1.5-fold change between the control and exposed expression values were selected. To define a set of 596 genes

significant statistical changes across comparisons, the linear models for microarray data (LIMMA) package was used (F-value adjusted with the false discovery rate method, $p \leq 0.05$) (Smyth, 2004).

2.6. Functional analysis

A Gene Ontology enrichment analysis was performed with AMEN. A specific annotation term was considered enriched in a gene cluster when the FDR-adjusted p-value was <0.01 (Fisher's exact probability).

2.7. cDNA synthesis and RT-qPCR analysis

Using an Omniscript RT Kit (Qiagen, Germany) and random primer mix (New England Biolabs, MA, USA) cDNA synthesis was performed in accordance with manufacturer's instructions from 500 ng total RNA. The same testes samples used in the 3'DGE analysis were analyzed by RT-qPCR in technical duplicates and repeated with 10–12 biological replicates. 11 μl reactions containing diluted cDNA(1:20), TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG and TaqMan™ Gene Expressions Assay (Life Technologies, CA, USA) were run on an Applied Biosystems QuantStudio 7 Flex Real-Time PCR System (Thermo Fischer Scientific, MA, USA). TaqMan assays used were *Calb2* #Rn00588816_m1, *Gsta2* #Rn00566636_m1, *Rps18* #Rn01428913_gH, and *Sdha* #Rn00590475_m1 (Life Technologies, CA, USA). Cycling conditions were: 95°C for 20 s followed by 45 cycles of 95°C for 1 s and 60°C for 20 s. The relative gene expression was calculated using the ΔCT method selecting *Rps18* and *Sdha* as normalizing genes based on previously shown stable expression in rat testis (Svingen et al., 2015). The RT-qPCR data were assessed for homogeneity of variance and normal distribution by residual statistics. Non-normally distributed data were log-transformed and assessed again to confirm normality. The data were then analyzed using a two-tailed, unpaired *t*-test comparing control vs flusilazole or triticonazole at both GD17 and GD21 using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

2.8. Immunofluorescence (IF)

IF was performed on one formalin-fixed, paraffin-embedded testis from each of three litters/dose group at both GD17 and GD21. Two 5 μm sections per testis were blocked using 5% Bovine Serum Albumin (BSA, Sigma-Aldrich®, MO, USA) in phosphate-buffered saline (PBS) and stained with primary and secondary antibodies diluted in PBS with 1% BSA. Primary antibodies were CALB2 (used at 1:2000 dilution; Thermo Fisher Scientific, cat.no. PA5-16681) and CYP11A1 (used at 1:100 dilution; Santa Cruz Biotechnology, cat.no. Sc-18043). Secondary antibodies were donkey anti-rabbit Alexa Fluor 568 and donkey anti-goat Alexa Fluor 488 (used at 1:500 dilution; product no. A10042 and A11055, Molecular Probes). Nuclei were counterstained with DAPI (1:1000, Thermo Fisher Scientific, product no. 62248) in PBS and positive controls (adult testis tissue and brain tissue) as well as negative controls (without antibody and only with secondary antibody). Protein expression was evaluated (dose group blinded to observer) by fluorescence microscopy using an Olympus BX-53 microscope fitted with a QImage Retiga-6000 camera. Images were captured using Cell Sense Dimensions software (Olympus Ltd, UK).

2.9. Histopathology

Histopathological examinations were performed on one Bouin's

fixed, paraffin-embedded testis from each litter in all dose groups at GD17 (4 testes per dose group, 12 testes in total). Two 5 μ m sections per testis were stained with Meyer's hematoxylin and eosin (H&E) and evaluated (dose group blinded to observer) by light microscopy for the presence of multinucleated gonocytes, small or large Leydig cell clusters, edemas, and testis cord dysmorphology. Slides were analyzed using a Leica DMR microscope fitted with a Leica DFC295 digital camera for image capture in Image Pro Plus 7.0 software (Media Cybernetics, MD, USA).

3. Results and discussion

3.1. Temporal changes to the rat testis transcriptome between GD17 and GD21

When comparing testes from control animals only, we identified 590 differentially expressed genes between GD17 ($n = 10$) and GD21 ($n = 13$) testes (Appendix A). Of these, 122 genes displayed a significantly decreased expression pattern from GD17 to GD21 testes (Fig. 1). Conversely, the remaining 468 genes showed an increased expression from GD17 to GD21 (Fig. 1). Although not the focus of this study, these developmentally regulated genes attest to the robustness of our 3'DGE dataset. It is also the first 3'DGE dataset available from rat testes at these developmental stages and the raw data is a valuable resource for mining the transcriptional landscape in rat fetal testes at late gestation, and comprise 17,349 genes (GEO repository accession number GSE154012).

3.2. Transcriptional changes in fetal rat testis transcriptome after exposure to flusilazole or triticonazole

With the robustness of the 3'DGE data established, we next analyzed the transcriptome of fetal testes collected from flusilazole- and triticonazole-exposed fetuses. At GD17, one gene (*Lamp1*) was dysregulated in flusilazole-exposed testes, and only one gene (*Mmp14*) was differentially expressed in triticonazole-exposed testes (Fig. 1). At GD21, four genes (*Crygb*, *Gsta2*, *Calb2*, *Serp1b13*) were differentially expressed in flusilazole-exposed testes, and six genes (*Ass1*, *Serp1b13*, *Tmem255b*, *Fabp12*, *Cyp11b1* and *MGC115197*) were differentially expressed in triticonazole-exposed testes (Fig. 1). Hence, only one gene (*Serp1b13*) was dysregulated in exposed testes from both compounds at GD21.

Considering that we analyzed more than 17,000 genes, and the fact that we previously have shown adverse male reproductive effects in these exposed rat fetuses, this very low number of affected genes were somewhat surprising, yet illuminating. Clearly, both flusilazole and triticonazole have anti-androgenic potentials *in vitro* with potential to affect both steroidogenesis and AR action and *in vivo* with triticonazole causing short male AGD and flusilazole affecting male steroidogenesis (Draskau et al., 2019). Therefore, the limited effects at the transcriptional level in the testes strongly suggests that the two azoles affect testis function at the protein level, or exert their disruptive action in tissues other than the testes themselves.

It is not surprising that chemicals that mainly target CYP enzymes or AR activity does not disrupt the testis transcriptome significantly. Adverse effects at the organismal level such as short AGD culminates from too little androgen being synthesized or AR action being blocked. More marked changes to the transcriptome would thus likely take place in the target tissue, as for instance the perineum as recently reported with the AR antagonist finasteride (Schwartz et al., 2019b). Notably, this is in contrast to other anti-androgenic phthalates that are known to cause feminization effects by reducing testosterone levels and concomitantly induce

more significant changes to testis gene expression levels (Beverly et al., 2014; Gray et al., 2016; Hannas et al., 2012; Liu et al., 2005). Thus, we still need to invest more into delineating the modalities by which EDCs cause reproductive effects *in vivo*. Our results suggests that flusilazole targets CYP enzyme activity in the testis without disrupting various other gene pathways or general testis integrity, which may not be the case for other anti-androgenic EDCs. Likewise, disruption of the AR by triticonazole, and to some degree flusilazole, will occur in peripheral target tissues and not leave a transcriptional footprint in the testes proper.

3.3. Histological assessment of rat fetal testes after exposure to flusilazole or triticonazole

Before looking more closely at some of the gene transcripts that were dysregulated, we examined the testes from exposed animals to determine if there were any obvious histological changes despite lack of marked transcriptional changes. At GD17 we observed no obvious histopathologies, with no signs of multinucleated gonocytes, small or large Leydig cell clusters, edemas, or testis cord dysmorphology in response to either flusilazole or triticonazole exposure (Fig. 2). We have previously reported that testes from GD21 fetuses exposed *in utero* to either flusilazole or triticonazole displayed no histopathological changes (Draskau et al., 2019).

3.4. Verification of dysregulated *Calb2* and *Gsta2* expression in exposed fetal rat testes by RT-qPCR

Although only a very small number of gene transcripts were affected in exposed testes, they could potentially be of interest to pinpoint other pathways disrupted following exposure to azoles. We thus verified two of the dysregulated genes, Calbindin 2 (*Calb2*) and Glutathione s-transferase a2 (*Gsta2*), by RT-qPCR analysis. The two genes were selected as interesting targets of flusilazole due to their potential involvement in regulation of steroidogenesis. *Calb2* showed an increased expression in the flusilazole-exposed group at GD17 and GD21 (Fig. 3A), whereas *Gsta2* was undetected at GD17, but showed decreased expression by flusilazole at GD21 (Fig. 3B). The results verified the expression pattern observed by DGE-Seq analysis (Fig. 3C and D). Both *Calb2* and *Gsta2* are expressed in Leydig cells and appear to play roles in steroidogenesis. Thus, the effects of flusilazole on these genes are likely related to the ability of flusilazole (but not triticonazole) to alter steroid synthesis as seen both *in vitro* and *in vivo* (Draskau et al., 2019).

Gsta2 encodes an enzyme that mediates detoxification by conjugation with glutathione (Hayes and Pulford, 1995), so a downregulation in flusilazole-exposed testes could indicate a decrease in the detoxification response. However, GSTA is also regulated by Steroidogenic factor 1 (SF-1), and human GSTA isoforms regulate progesterone and androstenedione production in coordination with 3 β -hydroxysteroid dehydrogenase (3 β -HSD) as shown *in vitro* (Matsumura et al., 2013). Interestingly, flusilazole regulates male plasma steroid hormones *in vivo*, including upregulation of androstenedione levels (Draskau et al., 2019). In pigs, GSTA2-2 have also shown noticeable steroid isomerase activity (Fedulova et al., 2010) and is expressed in both Leydig and Sertoli cells, where it is under the control of androgens in Sertoli cells (Benbrahim-Tallaa et al., 2002). Finally, decreased GSTA correlate with a lack of Sertoli cell maturation in rat testis after *in utero* exposure to the anti-androgen flutamide (Benbrahim-Tallaa et al., 2008). Overall, this indicates that *Gsta* plays a prominent role in steroidogenesis and may be part of a general feedback mechanism in response to flusilazole-induced effects on CYP enzymes.

Calb2 (Rogers, 1987), which encodes the calcium-binding protein Calretinin, plays a significant role in the nervous system

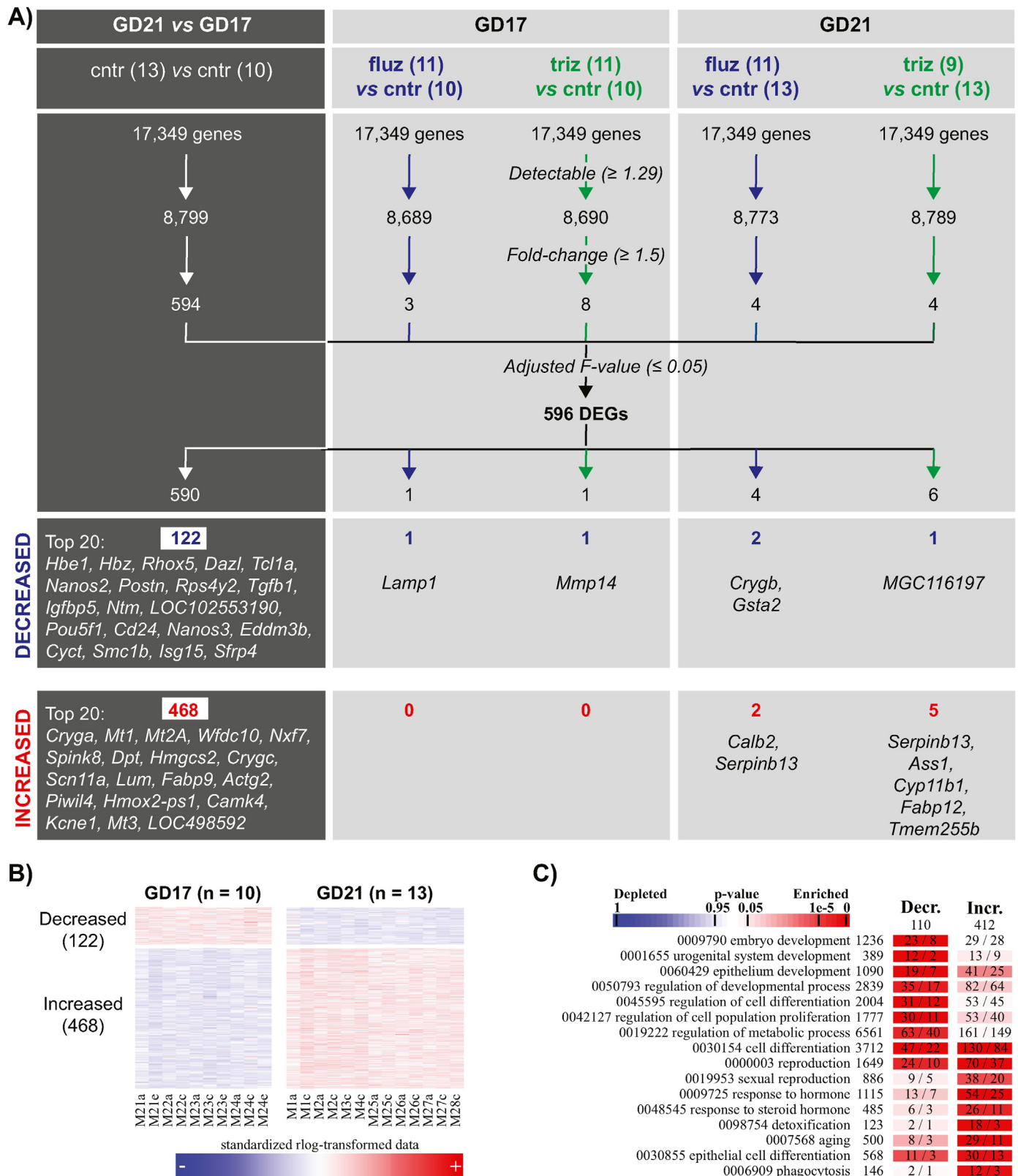


Fig. 1. Differentially expressed genes in controls, flusilazole (fluz)- and triticonazole (triz)-exposed fetal testes. **A)** Differentially expressed genes revealed by 3'DGE in controls at GD21 compared to controls at GD17 and in exposure groups compared to control at GD17 and at GD21. 3'DGE profiling detected 590 differentially expressed genes between GD17 and GD21 controls. At GD17, one gene was dysregulated in testes in the flusilazole exposure group and one in the triticonazole exposure group compared to controls. At GD21, four genes were dysregulated in testes in the flusilazole exposure group and six genes were dysregulated in the triticonazole exposure group, with one gene (*Serpinb13*) dysregulated in both exposure groups, relative to control testes. N = 9–13 testes per age group. **B)** Heatmap representation of transcriptional changes in rat testes from GD17 to GD21 of development. 3'DGE profiling detected 590 differentially expressed genes between GD17 and GD21, showing that the fetal testes continue to differentiate throughout fetal life. Of the differentially expressed genes, 122 were decreased at GD21 compared to GD17. The remaining 468 transcripts were increased at GD21 compared to GD17. N = 9–13 testes per age group. **C)** Significantly enriched biological process terms (Appendix B) and their identification numbers are given followed by the total number of genes associated with the term and the numbers of genes observed vs. expected by chance for the decreased and increased classes. The total number of genes associated with a biological process term and the genes in the classes are shown at the top. A color scale of p-values for enriched (red) and depleted (blue) terms is shown at the bottom. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

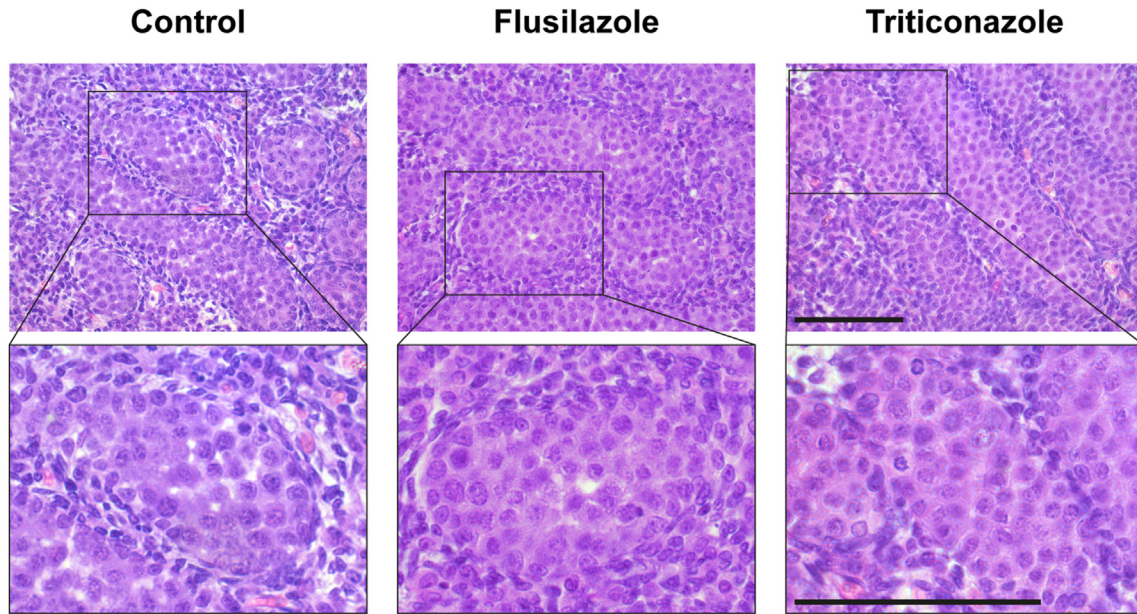


Fig. 2. Histopathological evaluation of testes from GD17 fetuses. Testes were stained with H&E and analyzed by brightfield. Neither flusilazole- nor triticonazole-exposed testes showed any sign of histological aberrations compared to control testes. N = 3 testes per treatment group; scale bars = 50 μ m.

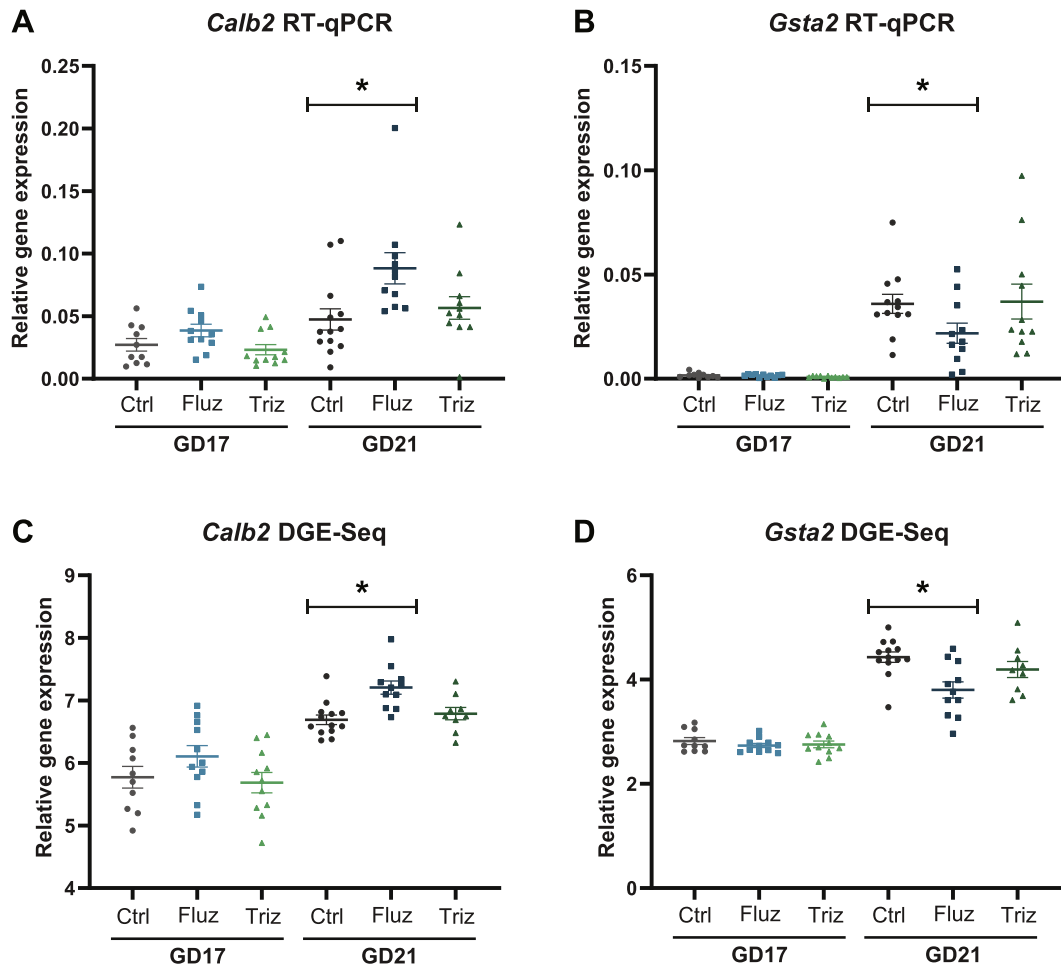


Fig. 3. RT-qPCR validation of selected differentially expressed genes. RT-qPCR validation of *Calb2* (A + C) and *Gsta2* (B + D). Top panels (A,B) shows RT-qPCR data for the relative expression levels of *Calb2* and *Gsta2*, * $p < 0.05$ (two-tailed, unpaired *t*-test) whereas bottom panels (C,D) show DGE-Sequencing data from fetal rat testes at GD17 and GD21, * $p < 0.05$ (F-value adjusted with false discovery rate method). Animals were exposed *in utero* to either vehicle (Ctrl), 45 mg/kg bw/day flusilazole (Fluz) or 450 mg/kg bw/day triticonazole (Triz). Data depict mean $\pm$ SEM (n = 9–13 testes per group).

(Schwaller, 2014) but has also been implicated as a diagnostic marker in ovarian and testicular tumors (Lugli et al., 2003; Portugal and Oliva, 2009; Radi and Miller, 2005). Already in 1994, *Calb2* was suggested to play a role in testosterone synthesis after being found expressed in rat Leydig cells (Strauss et al., 1994). In the fetal testis, *Calb2* expression was shown to follow the number of Leydig cells, with some additional expression detected in Sertoli cells and the pattern of *Calb2* expression during fetal development suggested a role in gonad differentiation alongside promoting steroidogenesis (Altobelli et al., 2017; Xu et al., 2018). Interestingly, correlations between *Calb2* and Sertoli cell differentiation in patients with spermatogenic failure has been reported (Bar-Shira Maymon et al., 2005), as well as an important role in protecting Leydig cells against apoptosis (Xu et al., 2018, 2017). This highlights the likely importance of *Calb2* for androgen production and testicular function, and supports effects of flusilazole on fetal Sertoli cell maturation and steroidogenesis through effects on *Gsta2* and *Calb2*. Finally, *Calb2* was recently suggested a novel biomarker for adverse reproductive effects in rat ovaries (Johansson et al., 2020). Hence, looking more closely at how *Calb2* may serve as a general biomarker for adverse reproductive effects could be fruitful, not least as it appears intricately linked with androgenic cell integrity and function.

3.5. Spatial expression pattern of CALB2 in rat fetal testis

As an early effort towards elucidating the role for *Calb2* in testis androgen function, we analyzed the expression pattern of CALB2 in the testes at GD17 and GD21, both in control animals and those exposed to flusilazole. This analysis confirmed the localization of CALB2 in fetal Leydig cells (Fig. 4). Apart from the obvious localization of CALB2 signal in the testis interstitium, we further confirmed specific cell localization by also staining the tissue for the Leydig cell marker CYP11A1, which showed clear colocalization at both developmental stages. Notably, although all CALB2-positive cells were also expressing CYP11A1, not all CYP11A1-positive cells were expressing CALB2. This suggests that CALB2 is not activated in all fetal Leydig cells. Whether this is related to differentiation stage or different functional status of the mature Leydig cell population requires additional studies to illuminate.

4. Conclusions

Taken together, our analysis suggests that flusilazole and triticonazole do not act through transcriptional changes in testes. We suggest that flusilazole influences hormone production in fetal male rats by directly targeting steroidogenic enzyme activity in the testis at the protein level, whereas triticonazole shortens fetal male rat AGD primarily due to disturbed androgen signaling in androgen-sensitive tissues. The altered expression of *Calb2* and *Gsta2* after exposure to flusilazole, but not triticonazole, may pinpoint novel pathways of disrupted testicular steroid synthesis.

Our results highlight four important points that are relevant when testing the potential adverse effects on the male reproductive system; both with regard to the azole fungicides flusilazole and triticonazole, but also to EDCs more broadly.

- Adverse male reproductive effects associated with testicular dysgenesis or functional impairment, as observed *in vivo*, is not necessarily reflected by changes to the testis transcriptome.
- Classical male reproductive adverse effects such as short AGD may not be related to any changes to the testes themselves. This, of course, is well established with the knowledge that AR action is exerted in peripheral tissues. Yet, it is important to reiterate this point, so that it is fully accounted for in alternative test strategies for anti-androgenicity.
- Chemicals that disrupt steroidogenesis, particularly when measured *in vitro* such as in the H295 assay, do not necessarily alter gene expression in the testis. Rather they may specifically target enzyme activity at the protein levels resulting in altered hormone synthesis.
- Chemicals that induce marked transcriptional changes in the fetal testes, such as phthalates, and simultaneously alter steroid hormone synthesis may reflect broader disruption to testis differentiation rather than direct interference with steroidogenesis. In other words, changes to expression of a substantial number of genes may instead reflect that the Leydig cells are compromised and thus cannot maintain adequate steroid production.

In a time where much effort is being put towards elaborating alternative test strategies for EDCs, and chemicals more broadly -

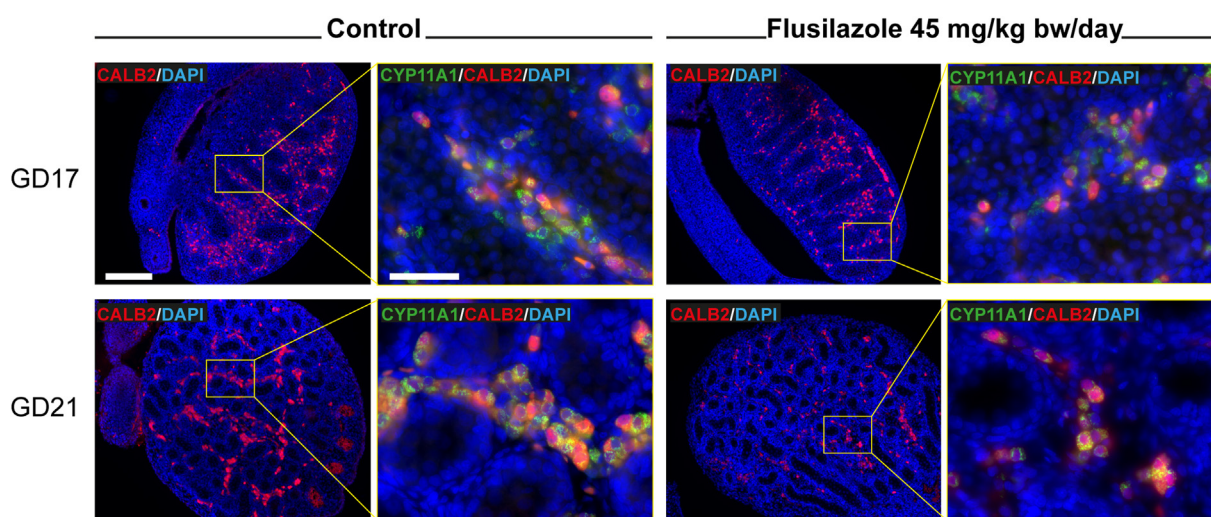


Fig. 4. Protein expression profiling of CALB2 in rat fetal testis at GD17 and GD21. Flusilazole-exposed testes were stained with CALB2 and CYP11A1 antibodies and analyzed by fluorescence microscopy. Staining confirmed the localization of CALB2 in fetal Leydig cells with obvious localization of CALB2 signal in the testis interstitium, and confirmed by colocalization with Leydig cell marker CYP11A1. N = 3 testes per treatment group; scale bars: low magnification = 200 μ m, high magnification = 50 μ m.

for instance through the development of complex AOP frameworks for chemical risk assessment – the full appreciation of how chemicals induce adverse effects at the molecular and cellular level is of paramount importance. This study has shown that, although transcriptomics approaches can be powerful tools in toxicology (Darde et al., 2019), they must be applied to the correct tissue for *in vivo* characterization of effect mechanisms.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2020.128468>.

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