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In vitro and in vivo endocrine disrupting effects of the azole fungicides triticonazole and flusilazole

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Running Title: Triticonazole and flusilazole elicit endocrine disrupting effects

Key words: conazoles, fungicides, endocrine disruptors, anogenital distance, anti-androgen, steroidogenesis

30 **ABSTRACT**

31 Azoles are effective antifungal agents used in both medicine and agriculture. They typically work
32 by inhibiting cytochrome P450 enzymes, primarily CYP51 of the ergosterol biosynthesis pathway,
33 thus damaging the fungal cell membrane. However, apart from their desired antifungal properties,
34 several azoles also exhibit endocrine disrupting properties in mammals, both *in vitro* and *in vivo*.
35 Here, we have tested two currently used agricultural azole fungicides, triticonazole and flusilazole,
36 for their *in vitro* anti-androgenic activity and potential effects on reproductive parameters. Both
37 fungicides showed strong androgen receptor (AR) antagonism and disruption of steroid biosynthesis
38 *in vitro*. Following gestational exposure to flusilazole (15 or 45 mg/kg bw/day) or triticonazole (150
39 or 450 mg/kg bw/day) in time-mated Sprague Dawley rats, triticonazole induced shorter male
40 anogenital distance (AGD). Flusilazole exposure did not affect the AGD, but altered fetal male
41 blood hormone profile, with increased androstenedione and decreased estrone levels. Flusilazole
42 and triticonazole have dissimilar effects on reproductive parameters *in vivo*, but both show
43 endocrine disrupting activities.

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51 INTRODUCTION

52 Prenatal and early postnatal development is considered particularly vulnerable periods for endocrine
53 disruption, because this is when reproductive organs and neural systems are developing largely
54 under the control of hormone signaling. Exposure to endocrine disrupting chemicals (EDCs) during
55 fetal life is thus of great concern as it can perturb normal development and cause disease later in
56 life. Potential EDCs comprise a plethora of compounds from diverse chemical classes, including
57 phthalates, parabens, bisphenols, and azole fungicides.

58 Azoles are widely used as anti-fungal agents for crop protection. Their residues are frequently
59 found in agricultural food products (EFSA, 2017; Petersen, 2017) and wastewater (Kahle et al.,
60 2008). Azoles are also used in medicine for treating a range of human fungal infections including
61 vaginal mycoses in pregnant women, which can result in high exposure concentrations. The broad
62 use of azole compounds stems from their ability to inhibit the fungal enzyme Sterol 14 α -
63 demethylase (CYP51), causing a disruption of fungal membrane integrity (Zarn et al., 2003).
64 However, several azoles also target mammalian CYP51 (Lamb et al., 1999) and other members of
65 the CYP450 family (Zhang et al., 2002), including enzymes catalyzing crucial steps in
66 steroidogenesis such as CYP19 (aromatase) (Ian Mason et al., 1985; Vinggaard et al., 2000), which
67 converts androgens to estrogens. Furthermore, several azoles can affect hormone receptors such as
68 the androgen receptor (AR) (Kjærstad et al., 2010; Roelofs et al., 2014). Azole fungicides can thus
69 have off-target effects in mammals and perturb, for instance, reproductive development, a process
70 that is dependent on sex hormone synthesis and signaling.

71 In humans, maternal use of oral azole anti-mycotic drugs during pregnancy has been associated
72 with anti-androgenic effects such as shorter anogenital distance (AGD) in male offspring
73 (Mogensen et al., 2017). This is supported by several animal studies where, for instance in rodents,

prochloraz, tebuconazole and ketoconazole can induce feminization of male offspring, with effects such as increased nipple retention and shorter AGD (Hass et al., 2012; Laier et al., 2006; Taxvig et al., 2008, 2007; Vinggaard et al., 2005). Two other azoles, epoxiconazole and propiconazole, have been shown to hyper-masculinize male rat offspring (Goetz et al., 2007; Hass et al., 2012), which displayed longer AGD at some doses, which contradicts an anti-androgenic mode of action only. In fact, prochloraz, tebuconazole and epoxiconazole are azoles that also disrupt female reproductive development and cause masculinization effects (Hass et al., 2012; Laier et al., 2006; Melching-Kollmuss et al., 2017; Taxvig et al., 2007), suggesting an androgenic mode of action. Together, these studies paint a more complex picture of adverse outcomes following exposure to commonly used azole fungicides, raising concerns about human exposure to these compounds.

We have tested two widely used azole pesticides, triticonazole and flusilazole, in an *in vivo* rat developmental toxicity study, as well as by endocrine relevant *in vitro* assays; the overall aim being to test whether these two azoles harbor endocrine disrupting properties. Triticonazole and flusilazole were chosen because they are current use fungicides, but also because they have shown differing activities in a zebrafish embryo model. Triticonazole was seen to primarily affect steroid biosynthesis, whereas flusilazole predominantly affected retinoic acid signaling as assessed by altered gene expression profiles (Hermsen et al., 2012). We report the adverse effects of triticonazole and flusilazole on male reproductive development in rats and present, to our knowledge, the first rodent *in vivo* toxicity study on triticonazole specifically targeting hormone-sensitive endpoints, which can be used for future risk assessment or classification of this azole.

MATERIALS AND METHODS

Chemicals

97 In this study we used flusilazole ($\geq 95\%$ pure, CAS No. 85509-19-9) from BOC Sciences (batch no.
98 B18LN07171, New York, USA) and triticonazole ($>95\%$ pure, CAS No. 131983-72-7) from
99 Abcam (#ab143728, lot no. GR3232419-3(N/A), Cambridge, UK). LC-MS grade acetonitrile,
100 methanol, formic acid, ethyl acetate and 25% ammonium hydroxide were from Sigma Aldrich
101 (Schneldorf, Germany). Isolute bulk C-18 sorbent was obtained from Biotage, Sweden, the sorbent
102 was washed with acetonitrile and ethyl acetate and dried prior to use. Water was purified on a Milli
103 Q system (Millipore Corporation, US).

104

105 *AR-EcoScreenTM assay*

106 The stably transfected AR-EcoScreenTM cell line (#JCRB1328, Japanese Collection of Research
107 Bioresources Cell Bank) was used to test human AR (hAR) agonistic and antagonistic effects of
108 flusilazole and triticonazole largely as described by the OECD test guideline no. 458 (OECD,
109 2016). Briefly, cells were cultured in DMEM/F-12 medium supplemented with 5 % fetal bovine
110 serum, 1% Penicillin-Streptomycin, 200 $\mu\text{g/mL}$ Zeocin, and 100 $\mu\text{g/mL}$ Hygromycin (all purchased
111 from Thermo Fisher Scientific). We used cells from passage 3-6, with cultures maintained at 37°C
112 with a humidified atmosphere of 5 % CO₂ and 95 % air.

113 The day before exposure, cells were seeded in DMEM/F-12 supplemented with 1% Penicillin-
114 Streptomycin and 5% dextran-coated charcoal-treated fetal bovine serum (Sigma-Aldrich®) at a
115 density of 90,000 cells/mL using 100 μL cell solution/well in white 96-well plates (Perkin Elmer).
116 Cells were exposed for 24 h in 2-fold serial dilutions with concentrations between 0.2-50 μM . For
117 controls, the AR agonist R1881 (Perkin Elmer) was used in 2-fold serial dilutions from 0.008-1 nM
118 and the AR antagonist hydroxyflutamide (OHF, Toronto Research Chemicals) was used in 3-fold
119 serial dilutions from 4-9000 nM. When testing for AR antagonism, 0.1 nM R1881 was added to all

wells, and for assessment of non-AR-mediated luminescence signals from triticonazole exposure, 1 μ M OHF was added to all wells, and tested in 2-fold serial dilutions in concentrations from 1.6-50 μ M. DMSO was used as vehicle for test compounds and control compounds, with DMSO concentration kept constant (0.1%) in all wells. Luciferase activity was measured on a Lumistar Galaxy luminometer (BMG LABTECH, Offenburg, Germany) using the Dual-GloLuciferase Reagent. Cytotoxicity was assessed by measuring *Renilla* luminescence using the Dual-Glo stop and Glo reagent (Promega, WI, USA). Experiments were performed in triplicate reactions and repeated thrice, except for non-AR-mediated luminescence signal testing which was repeated twice.

H295R steroidogenesis assay

The human adrenocortical carcinoma cell line H295R (CRL-2128TM, ATCC®, Virginia, USA) was used for testing effects on steroidogenesis as previously described (Rosenmai et al., 2013). We used cells from passage 4-6, cultured in DMEM/F-12-medium with L-glutamine and HEPES without phenol red (InvitrogenTM, Life TechnologiesTM, CA, USA), supplemented with 1% Corning® ITSTM+ Premix Universal Culture Supplement (Corning, MA, USA, #354352) and 2.5% Corning® NuSerumTM Culture Supplement (Corning, MA, USA, #355100) at 37°C in a 5 % CO₂ atmosphere.

The day before exposure, cells were seeded 300,000 per well in clear 24-well plates (VWR-Corning®, NY, USA). Cells were exposed for 48 h to controls (Forskolin: 1 μ M, 10 μ M (Sigma-Aldrich) and Prochloraz: 0.3 μ M, 3 μ M (VWR-Bie & Berntsen, Denmark) or test compounds (triticonazole and flusilazole in 2-fold serial dilutions with concentrations between 0.8-50 μ M). Vehicle (DMSO) concentration was kept constant in all wells (0.1%). After the incubation period, cytotoxicity was assessed by visual inspection and the medium transferred to new 24-well plates and stored at -80°C. Assay sensitivity was confirmed by measuring testosterone and estradiol in

controls with Cayman ELISA kits (#582701 and #582251, Cayman Chemical, MI, USA) according to manufacturer's instructions and OECD test guideline no. 456 (OECD, 2011). All experiments were performed in triplicate reactions and repeated thrice.

Hormone analysis in cell medium by HPLC-MS/MS

Medium from the H295R steroidogenesis assay was collected and analyzed for steroid hormone levels. Before LC-MS/MS analysis, deuterated internal standards were added to the cell extracts and samples centrifuged at 15,000 g for 10 min. Steroid hormones were separated, detected, and quantified using on-line-SPE LC-MS/MS, using an Oasis HLB column (2.1 × 20 mm, 15 µm). For 17β-estradiol and estrone analysis, a Kinetex C₁₈ column (2.1×100 mm, 2.6µm) was used with an injection volume of 100µl, measuring in ESI- mode using methanol, and 1mM ammonia in water as the mobile phases (gradient flow rate was 0.4 ml/min). For the other hormones, an Ascentis Express C₈ column (2.1×100 mm, 2.7µm) was used with an injection volume of 100µl, measuring in ESI-/ESI+ mode with acetonitrile and 0.1% formic acid in water as the mobile phases (gradient flow rate was 0.25 ml/min).

We included 15 hormones in the assay, of which 11 were detected (underlined): aldosterone, androstenedione, corticosterone, cortisol, dehydroepiandrosterone (DHEA), 11-deoxycortisol, dihydrotestosterone (DHT), epitestosterone, 18-hydroxycortisol, 17α-hydroxyprogesterone, progesterone, pregnenolone, testosterone, estrone, and 17β-estradiol.

The limit of quantification (LOQ) was 1.0 ng/ml for pregnenolone; 0.25 for deoxycortisol: 0.1 ng/ml for corticosterone, DHEA, and DHT; 0.05 ng/ml for epi-testosterone and hydroxyprogesterone; 0.03 ng/ml for aldosterone; 0.02 ng/ml for corticosterone, estradiol, testosterone and androstenedione; 0.01 ng/ml for hydroxycortisol, progesterone, and estrone. For

quantification, external calibration standards were run before and after the samples at levels of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 10.0 and 20 ng/ml, with 4.0 ng/ml internal standards: (testosterone-d2, methyltestosterone-d3, progesterone-c2 and estradiol-d3 from EURL Wageningen and cortisol-d4 and deoxycortisol-d8 from LGC Standards). For testosterone, cortisol, deoxycortisol, estradiol and progesterone dedicated internal standard was used. Furthermore, cortisol-d4 was used for aldosterone, and hydroxyprogesterone; deoxycortisol-d8 was used for cortocosterone; progesterone-c2 was used for pregnenolone and estradiol-d3 was used for estrone. However, some measured values in the groups with high azole-exposures were below the LOQ or the limit of detection. Values below the detection limit were set as zero. The limit of detection and LOQ were estimated as the concentrations corresponding to three and ten times signal-to-noise, respectively. The mass spectrometer was an EVOQ Elite Triple Quadrupole Instrument from Bruker (Bremen, Germany) and the UPLC system was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump. Data handling was done using the software MS Workstation v. 8.2.1.

Animal Study

Animal experiments had ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553). All methods were performed in accordance with relevant guidelines and regulations. Forty 10 weeks old, time-mated Sprague-Dawley specific pathogen free rats (Charles River, Germany, via SCANBUR, Karlslunde) were delivered to the Technical University of Denmark animal facilities at gestational day (GD) 3 (with the day following overnight mating denoted GD1). On GD4, dams were weighed and assigned to 5 groups of 4 animals each with similar body weight (bw) distributions. Animals were housed in pairs until GD17, thereafter individually under standard conditions in High Temperature polysulfone (H-TempTM) cages

(Tecniplast, Italy) with wood chip bedding, nesting material and a wooden shelter (Tapvei, Denmark). Animals were kept in a controlled environment with 12h light/dark cycles, $55 \pm 5\%$ humidity, $22 \pm 1^\circ\text{C}$ temperature, and 10 air changes per hour. Animals were fed a standard soy and alfalfa-free diet based on Altromin 1314 (Altromin GmbH, Germany). Tap water in Bisphenol A-free bottles (Polysulfone 700 ml, 84-ACBT0702SU Tecniplast, Italy) was provided ad libitum.

An acclimatization period of 4 days was allowed before starting exposure on GD7. The rats were weighed and gavaged each morning with vehicle (corn oil, Sigma-Aldrich, #C867-2.5L, Denmark), flusilazole (15 or 45 mg/kg bw/day) or triticonazole (150 or 450 mg/kg bw/day) until GD21. The dams were given a dosing volume of 2 ml/kg bw.

Necropsy

Dams were exposed 1 hr $\pm$ 15 min before decapitation under CO_2/O_2 -anesthesia. Uteri were collected, weighed, and the number of live fetuses, location in uterus, resorptions, and implantations were registered. Body weight and any anomalies of the fetuses were recorded. Maternal and fetal trunk blood was collected and transferred to heparin-coated vials. Fetal blood was pooled for each sex within each litter. Blood samples were kept on ice before centrifugation at 4000 rpm at 4°C for 10 min after which the plasma was transferred to new tubes and stored at -80°C .

Amniotic fluid was collected from all fetuses using disposable glass Pasteur pipettes, pooled within each litter, snap frozen in liquid nitrogen and subsequently stored at -80°C . Maternal livers were weighed, and three standard pieces were cut and fixed in 10% formalin. Anogenital distance (AGD), the distance between the genital papilla and the anus, was measured using a stereomicroscope with a micrometer eyepiece by an experienced technician blinded with respect to

211 treatment groups. Fetal testes were dissected under a stereomicroscope and immediately placed in
212 Bouin's fixative.

213

214 *Triazole concentrations in amniotic fluid and fetal plasma measured by LC-QTOF*

215 Flusilazole and triticonazole concentrations in fetal plasma and amniotic fluid were measured by
216 Liquid Chromatography-Mass Spectrometry (LC-MS). 50 µl sample was transferred to an
217 Eppendorf vial containing 150 µl acetonitrile. Approximately 50 mg Isolute C18 sorbent was added
218 and the vial vortexed briefly, incubated at 4°C for 30 min, and centrifuged at 10,000 $\times$ g for 10 min
219 at 4°C (Ole Dich Instrument makers, Denmark). Prior to analysis, 20 µl of the supernatant was
220 transferred to an HPLC vial and mixed with 80 µl 75% acetonitrile.

221 LC was performed on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) with a Poroshell SB C-8
222 (100 $\times$ 2.1 mm, 2.7 µm particle size) column held at 40°C (Agilent technologies, Walbron,
223 Germany). The solvent system consisted of A: 2.5 mM ammonium hydroxide + 0.1 % formic acid
224 in water and B: acetonitrile. Solvent programming were: 0% B from 0 to 1 min followed by a linear
225 gradient to 5% B to 3 min and a linear gradient to 98% B to 10 min, isocratic 98% B from 10 to 12
226 min followed by reversal to initial conditions to 12.1 min and re-equilibration of the column to 15
227 min. The flow rate was 0.3 ml/min from 0 to 1 min followed by a linear gradient to 0.4 ml/min to
228 10 min, which was held to 12 min followed by reversal to initial conditions.

229 The LC system was connected to a Bruker Daltonics, maXis qTOF MS equipped with an
230 electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). The
231 ion source settings were: nebulizer pressure 2 bars, drying gas flow 8 l/min, dry gas temperature
232 200 °C, capillary voltage 2500 V. The scan range was from 80 to 700 m/z with an acquisition rate
233 of 2 Hz. Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2-0.4

min time segment and used for internal calibration of the data files. Hexakis(perfluorooctoxy)phosphazene was used as lock mass calibrant to compensate for drift in the mass axis during analysis.

Matrix matched standard samples were prepared in blank plasma at 4-5 different levels in the concentration range of: flusilazole 0.6 to 74.5 ng/ml, triticonazole 0.73 to 910 ng/ml. Standards and blanks were analyzed in the beginning of a sequence and after each set of 20 samples. Data files were processed using QuantAnalysis (Bruker Daltonics, Bremen, Germany). Extracted ion chromatograms of m/z 316.1076 ± 0.003 Da (flusilazole) and m/z 318.1368 ± 0.003 Da (triticonazole) were constructed and integrated. Plasma concentrations were calculated based on linear calibration curves constructed using 1/x weighing.

Plasma steroid hormone analysis by HPLC-MS/MS

Plasma samples were analyzed for steroid hormone levels by LC-MS/MS as previously described (Hadrup et al., 2016). The extraction procedure and LC-MS/MS setup were slightly changed: Plasma samples were thawed on ice and 100 μ l added to an Eppendorf vial containing 300 μ l ice-cold, 2% formic acid in acetonitrile with 3.33 ng/ml internal standard (deoxycortisol-d8 and cortisol-d4 from Cerilant, methyltestosterone-d3, beta-testosterone-d2, beta-estradiol-d3 and progesterone-c2 from Rikilt), then vortexed for 5 s. The vial was then placed at -20°C for 20 min and centrifuged at 10,000 g for 7 min at 4°C. The supernatant was subsequently transferred to a tube containing approximately 50 mg SupelTM QuE Z-Sep powder (Supelco, Bellefonte, PA, USA, #55418-U) and briefly vortexed, then shaken for 60 s, before centrifugation for 3 min at 3500 g. The supernatant was transferred to a centrifuge glass and dried using a nitrogen evaporator at 50°C

256 after which it was reconstituted in 50µl acetonitrile, vortexed briefly, and mixed with 450 µl milliQ
257 water.

258 Steroid hormones were separated, detected, and quantified using on-line-SPE LC-MS/MS exactly
259 as described above for analysis of the H295R steroidogenesis assay. Out of 14 hormones assayed
260 for, we detected 6 (underlined): testosterone, epitestosterone, androstenedione,
261 dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), corticosterone, cortisol,
262 hydroxycortisol, deoxycortisol, pregnenolone, progesterone, 17α-OH-progesterone, estradiol, and
263 estrone.

264 Limit of quantification (LOQ) was estimated as the concentration corresponding to 10 times the
265 signal to noise ratio of plasma samples spiked with analyte. LOQs were estimated to be 0.1 ng/ml
266 for androstenedione, progesterone, testosterone and estrone, 0.3 ng/ml for corticosterone, and 2.0
267 ng/ml for pregnenolone. For quantification, external calibration standards were run before and after
268 the samples at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ng/ml, with 2.0 ng/ml
269 internal standard. Blank plasma samples were spiked with analyte at three concentration levels: 0.0
270 (blank sample), 0.5, and 2.0 ng/ml and run for quality control. The mass spectrometer was an
271 EVOQ Elite Triple Quadrupole Instrument from Bruker (Bremen, Germany) and the UPLC system
272 was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump. Data handling was
273 performed in MS Workstation v. 8.2.1 software.

274

275 *Histopathology*

276 One Bouin's fixed testis from each litter in all dose groups (7-8 testes per dose group, 39 testes in
277 total), was randomly selected for histopathological examination. Two sections per testis were
278 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. Maternal livers (one section per liver) were stained with H&E and evaluated (dose group blinded to observer) by light microscopy for the presence of vacuolization, infiltrations and hypertrophy.

Statistical analyses

Data from the *in vitro* assays, analysis of *in vivo* internal triazole concentrations, analysis of hormone levels in fetal plasma, maternal parameters and litter data were assessed for normal distribution by residual statistics. Data not normally distributed were log-transformed and assessed again to confirm normality. Normally distributed data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Homogeneity of variance was confirmed by residual statistics and applying Bartlett's test. Non-normally distributed data was analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

In vivo data on fetal body weight, AGD and AGD index (AGDi, AGD/cube root of bw) at GD21, were analyzed using one-way ANOVA followed by Dunnett's post hoc test using SAS[®] (SAS Enterprise Guide 6.1, SAS Institute, USA). Statistical analyses of AGD and AGDi were adjusted using litter as an independent, random and nested factor and analyzed using body weight as a covariate. Fetal body weights were analyzed using the number of offspring per litter as covariate. For all analyses, the litter was the statistical unit.

RESULTS

301 *Flusilazole and triticonazole antagonizes AR in vitro*

302 In the AR-EcoScreenTM assay, both flusilazole and triticonazole were strong hAR antagonists.
303 Flusilazole had a lowest observed effect concentration (LOEC) of 0.8 μ M and an IC₅₀ of 2.8 ($\pm$ 0.1)
304 μ M, whereas triticonazole had a LOEC of 0.2 μ M and an IC₅₀ of 0.3 ($\pm$ 0.01) μ M (Fig. 1A). In
305 addition, triticonazole showed hAR agonistic activity at the highest doses; 25 and 50 μ M, whereas
306 flusilazole did not. However, addition of 1 μ M of the AR antagonist OHF did not diminish the
307 triticonazole-induced luciferase response at 25 and 50 μ M (Fig. 1B). Flusilazole induced
308 cytotoxicity at the two highest doses of 25 and 50 μ M (Fig. 1C), thus these data points were
309 excluded from statistical analyses. No cytotoxicity was observed for triticonazole at any of the
310 tested doses (Fig. 1D).

311

312 *Triticonazole and flusilazole disrupts steroidogenesis in vitro*

313 Flusilazole affected all steroid hormones in the H295R Steroidogenesis assay (Fig. 2A).
314 Pregnenolone, 17 α -hydroxyprogesterone, corticosterone, cortisol, 11-deoxycortisol, DHEA,
315 androstenedione, testosterone, estrone, and 17 β -estradiol levels were decreased with LOECs of
316 down to 0.78 μ M. Progesterone levels were strongly increased with a LOEC of 3.13 μ M.
317 Triticonazole affected most steroid hormones, except DHEA, but showed a different pattern of
318 effects than flusilazole (Fig. 2B): pregnenolone, corticosterone, cortisol, deoxycortisol, and
319 androstenedione were all decreased, whereas estrone, 17 β -estradiol, and testosterone were
320 increased at low doses, but decreased at 50 μ M. Progesterone was slightly decreased between 0.78
321 and 6.25 μ M, whereas 17 α -hydroxyprogesterone was increased with a LOEC of 12.5 μ M.

322

323 *Flusilazole and triticonazole were present in both amniotic fluid and fetal plasma*

Flusilazole and triticonazole were measured in amniotic fluid and plasma from GD21 fetuses by LC-MS. As seen in Table 1 and Fig. 3, concentration of flusilazole and triticonazole increased in a dose-dependent manner relative to exposure levels, both in plasma and amniotic fluid. For flusilazole, the concentration in plasma was around 3.5-fold higher than in amniotic fluid, and there was a 3-fold increase in internal dose from the 15 to the 45 mg/kg dose groups. For triticonazole, the concentration in plasma was 3- to 4-fold higher than in amniotic fluid, and there was around a 2-fold increase in internal doses from 150 to the 450 mg/kg bw dose groups.

Administered doses of triticonazole and flusilazole induced only mild toxicity at highest doses

We observed no external signs of maternal toxicity, no significant differences in maternal bw gain (from GD7-21) or adjusted maternal bw (bw after subtracting uterus weight), nor irregular litter sizes between groups at GD21 (Table 2). However, both flusilazole and triticonazole induced an increase in number of late resorptions in the highest dose groups, albeit not statistically significant (Table 2). One occurrence of a very late resorption (around GD18) was also observed in the highest flusilazole exposure group. In addition, we observed mild hepatocellular centrilobular hypertrophy consistent with an increased induction of hepatic enzymes in the dams from the high dose groups for both fungicides, along with incidences of focal necrosis in the flusilazole high dose group (data not shown).

Male fetuses exposed to triticonazole in utero display shorter anogenital distance

AGD was shorter in male fetuses at GD21 after *in utero* exposure to triticonazole, also when accounting for bodyweight (AGDi, AGD/cube root of bw): 10% shorter AGD and 8% shorter AGDi in the 450 mg/kg bw group, and 4% smaller AGDi in the 150 mg/kg bw group. Female AGD was not significantly affected by triticonazole exposure. For flusilazole, neither male nor female

offspring displayed significant changes to AGD in any of the exposure groups, 15 or 45 mg/kg bw (Table 2, Fig. 4).

Flusilazole affects androstenedione and estrone levels in fetal male plasma

Androstenedione levels were significantly elevated in male fetuses exposed to either 15 or 45 mg/kg bw flusilazole. Estrone levels were lower in the 45 mg flusilazole/kg bw group. We observed no changes for pregnenolone, progesterone, corticosterone or testosterone levels in fetal male plasma. Triticonazole had no significant effect on the measured hormone levels in male fetuses (Fig. 6).

Flusilazole or triticonazole exposure did not affect testis histology

Histopathological examination of GD21 testes did not reveal any histological changes in response to azole exposure.

DISCUSSION

We have examined potential anti-androgenic and prenatal reproductive effects in rats caused by fetal exposure to the azole fungicides triticonazole and flusilazole. Many azoles can disrupt reproductive development in rodents, likely through endocrine disrupting mechanisms of action. Furthermore, rodent studies have revealed a complex pattern of effects, for instance with shorter AGD and increased nipple retention in males (Hass et al., 2012; Laier et al., 2006; Taxvig et al., 2008, 2007; Vinggaard et al., 2005), but also longer AGD in both males and females (Goetz et al., 2007; Hass et al., 2012; Laier et al., 2006; Melching-Kollmuss et al., 2017; Taxvig et al., 2007). It

therefore seems difficult to predict *in vivo* effects of the various azole compounds, which also proved to be the case for triticonazole and flusilazole.

Since many azoles have shown AR antagonistic effects *in vitro*, we tested both triticonazole and flusilazole using the human AR-EcoScreenTM assay (Kjærstad et al., 2010). Both compounds proved to be strong hAR antagonists, with triticonazole being the most potent with a 10-fold lower IC₅₀ value of 0.3 µM, compared to 2.8 µM for flusilazole. These values are smaller than reported in a human breast cancer cell line, where IC₅₀ were 11.9 µM for flusilazole and 10.7 µM for triticonazole (Roelofs et al., 2014), suggesting less hAR antagonistic potency than was seen in our assay. This discrepancy, however, could be caused by simple assay and cell type variations. Regardless, flusilazole and triticonazole can both interfere with the hAR, which can have detrimental effects for reproductive development and function.

Apart from showing anti-androgenic potential, triticonazole also showed agonistic activity at high concentrations (from 25-50µM) without signs of increased cell proliferation. This could indicate that triticonazole is a selective hAR modulator, but could also be due to a variety of other reasons. Firstly, triticonazole could mediate a stabilization of the luciferase reporter, as it has previously been reported for other chemicals tested in this assay (OECD, 2016). Secondly, the AR-EcoScreenTM cell line expresses the glucocorticoid receptor, which has affinity for the androgen-responsive element, and could thus increase luminescence upon activation and lead to false positive results (OECD, 2016; Satoh et al., 2005). However, addition of the AR antagonist OHF indicated that the observed rise in luciferase reporter activation at high triticonazole concentrations was not AR-mediated.

Azole fungicides are frequently reported to interfere with steroidogenesis. This includes *in vitro* down-regulation of testosterone and estradiol (Kjærstad et al., 2010; Taxvig et al., 2013), and up- or

391 down-regulation of progesterone (Kjærstad et al., 2010; Taxvig et al., 2013). We thus analyzed the
392 effects of flusilazole and triticonazole on steroid biosynthesis using the H295R steroidogenesis
393 assay. Both triazoles strongly disrupted steroid hormone synthesis. Flusilazole downregulated
394 pregnenolone, corticosteroid, androgen and estrogen synthesis. Estrone and estradiol levels were
395 remarkably low already at the lowest flusilazole concentration tested, indicating a potent inhibitory
396 effect of flusilazole on aromatase (CYP19), which converts androstenedione and testosterone to
397 estrone and estradiol. This fits well with previous studies where inhibition of aromatase,
398 demonstrated in *in vitro* assays, has been suggested to underlie steroidogenic disturbances in
399 response to azole exposure (Vinggaard et al., 2000). However, these effects on the steroidogenic
400 pathway could also indicate that flusilazole inhibits 17 α -hydroxylase (CYP17A1), 17,20-lyase
401 (CYP17A1), 21-hydroxylase (CYP21A2), 11 β -hydroxylase (CYP11B1), cholesterol side-chain
402 cleavage enzyme (CYP11A1), and possibly 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and 3 β -
403 HSD. Notably, with regard to the more than 5-fold increase in progesterone levels, we speculate a
404 strong inhibitory effect on 17 α -hydroxylase activity. Overall, flusilazole seems to affect sex-
405 hormone synthesis, but also corticosteroid and progestagen biosynthesis.

406 Triticonazole seemed to have strongest effect on corticosteroid and progestagen synthesis,
407 inhibiting pregnenolone synthesis and all corticosteroids measured, potentially via 21-hydroxylase,
408 11 β -hydroxylase, and cholesterol side-chain cleavage enzyme. 17-hydroxyprogesterone levels were
409 elevated, indicating stimulatory effects on 17 α -hydroxylase or inhibitory effects on 21-hydroxylase
410 or 17,20-lyase. Effects on androgens and estrogens, on the other hand, were differing between lower
411 and higher triticonazole concentrations, with an increase of testosterone, estrone and estradiol levels
412 but a decrease in these hormones at the highest concentration tested, 50 μ M; a decrease that was
413 also seen in androstenedione levels at the highest concentration. It cannot be excluded that these
414 lower hormone levels at high azole exposure concentrations is a consequence of cellular

415 cytotoxicity; however, it is worth noting that in the AR-EcoScreenTM assay, triticonazole had strong
416 antagonistic effects on the hAR, but showed agonistic effects on the hAR at these same high
417 concentrations.

418 Having shown clear anti-androgenic activity of both triticonazole and flusilazole *in vitro*, we tested
419 their endocrine disrupting effects on pregnant rats. *In utero* exposure to triticonazole induced short
420 fetal male AGD, whereas flusilazole exposure did not affect fetal AGD in either sex. A short male
421 AGD is a sign of androgen insufficiency during the masculinization programming window (Welsh
422 et al., 2008), and is used as a broad biomarker in reproductive toxicity studies screening for anti-
423 androgenicity as it is associated with several male reproductive disorders (Schwartz et al., 2019a).
424 Having displayed anti-androgenic effects both *in vitro* and *in vivo*, triticonazole thus emerges a
425 clear anti-androgenic compound that is predicted to have detrimental effects on male reproductive
426 development, depending on exposure context. Although it is highly unlikely that human fetuses will
427 be exposed to such high doses, triticonazole adds to the overall burden of environmental chemicals
428 with anti-androgenic potential. It is important to note that these endocrine disruptive effects were
429 not shown in the toxicity studies used for the European approval of the fungicide, where it was
430 concluded, that any signs of effects on hormonal balance were likely the consequence of general
431 toxicity. This shows the importance of conducting *in vitro* screenings followed by targeted studies
432 of hormone-sensitive endpoints (EFSA, 2005).

433 In contrast to triticonazole, flusilazole did not induce a shorter male AGD following *in utero*
434 exposure at the applied doses. Based on available toxicity data for flusilazole, however, a dose
435 higher than 45 mg/kg bw/day would likely cause maternal and fetal toxicity (WHO, 1996).

436 Triticonazole and flusilazole are examples of azole compounds that probably have different
437 mechanisms of action. In a zebrafish embryo model, triticonazole exposure was shown to primarily

affect the expression of genes related to steroid biosynthesis, whereas flusilazole exposure seemed to have bigger effects on genes involved in retinoic acid signaling (Hermsen et al., 2012), an effect of flusilazole, which has also been observed in rat embryos (Dimopoulou et al., 2016). In support of dissimilar mechanistic profiles, we observed no changes to plasma steroid hormone levels in the triticonazole-exposed male fetuses at GD21, yet shorter male AGD. Flusilazole, on the other hand, caused significantly higher levels of androstenedione and lower levels of estrone in exposed fetuses, which could indicate inhibition of aromatase (CYP19) activity. This fits with results from the *in vitro* steroidogenesis assay, where flusilazole completely repressed estrone and estradiol levels. In contrast to the *in vivo* flusilazole-induced higher levels of androstenedione and no effects on the other hormones, flusilazole exposure resulted in lower levels of androstenedione as well as lower levels of the other androgens and corticosteroids analyzed *in vitro*. It is possible that feedback mechanisms are able to restore hormone levels to normal, despite inhibitory effects of flusilazole on several enzymes in the steroid biosynthesis, whereas the very strong inhibitory effect on aromatase results in the low estrone levels and consequently increased androstenedione levels.

Intriguingly, fetal flusilazole exposure did not result in altered AGD despite predicted strong anti-androgenic effects from the *in vitro* assays. It could be speculated that flusilazole induces opposing endocrine disruptive mechanisms of action, where an inhibitory effect on the androgen receptor is counteracted by an inhibitory effect on aromatase causing high fetal androgen levels. Notably, recent data suggests that estrogen signaling also plays a role in male reproductive development (Sathyanarayana et al., 2012; Schwartz et al., 2019b; Stewart et al., 2018) beyond its role in mouse penile development (Govers et al., 2019; Zheng et al., 2015). This warrants further studies on the possible consequences of flusilazole-induced lower estrogen levels in male fetuses.

We could detect both flusilazole and triticonazole in amniotic fluid and fetal plasma at GD21, confirming that both compounds can cross the placental barrier. Concentrations were generally

lower in amniotic fluid than in plasma, indicating extensive metabolism of both flusilazole and triticonazole before excretion. The large standard errors in the high dose groups indicate that the azoles have been absorbed or metabolized to different degrees in each litter, likely caused by biological differences in pharmacokinetics.

The fetal plasma concentration of triticonazole was more than 17-fold higher than the IC_{50} of 0.3 μ M for hAR antagonism observed *in vitro*, whereas fetal plasma flusilazole levels were below the IC_{50} for hAR antagonism, but the high-dosed animals did show values more than 2 times above the LOEC of 0.8 μ M. In addition, plasma concentrations of triticonazole were above LOEC for *in vitro* effects on corticosteroid, progestagen, testosterone, and estrogen biosynthesis in both dose groups, whereas flusilazole plasma concentrations only reached levels with *in vitro* effects for corticosteroid, testosterone and estrogen biosynthesis, but contrary to triticonazole did show effects on *in vivo* androstenedione and estrone levels.

The endocrine-disrupting effects, in rats, for the two azoles are seen at doses above no observed adverse effect levels (NOAELs) used to set acceptable daily intake (ADI) values, whereas human exposure from foods is far lower than these doses (EFSA, 2017; Jensen et al., 2019). However, several other EDCs, including, but not limited to, azole compounds, are known to affect the male reproductive system (Ribeiro et al., 2017; Rodprasert et al., 2019), and each of these compounds may contribute to a combined effect. The exact contribution of triticonazole and flusilazole to the human load of male reproductive toxicants would require further studies on toxicokinetics, species differences, and lowest effective concentrations *in vivo*. However, triticonazole, in particular, fits the pattern of an anti-androgenic compound that can affect male reproductive development. Flusilazole, on the other hand, seems to affect fetal development in a different manner than triticonazole at the applied doses. It would be of interest to further characterize how flusilazole affects reproductive tissues during development to improve chemical risk assessment.

486

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FIGURE LEGENDS

Fig. 1: Flusilazole and triticonazole are androgen receptor (AR) antagonists *in vitro*: Dose-response curves for flusilazole and triticonazole in the AR-EcoScreen™ assay. A) Cells were incubated with increasing concentrations of flusilazole or triticonazole (μM) in the presence of 0.1 nM R1881. The values are mean ± SEM (n=3) and are presented as fold change in firefly luminescence relative to R1881 response. B) Cells were incubated with increasing concentrations of flusilazole or triticonazole alone or triticonazole in the presence of 1 μM hydroxyflutamide (OHF) (μM). The values are mean ± SEM (n=3 for flusilazole and n=2 for triticonazole) and are presented as fold change in firefly luminescence. C) Induction of cytotoxicity following flusilazole exposure, and D) Induction of cytotoxicity following triticonazole exposure. C = cytotoxicity. The values are mean ± SEM (n=3) and are presented as fold change in Renilla luminescence. *p< 0.05.

Fig. 2: Triticonazole and flusilazole disrupt steroidogenesis *in vitro*: Relative steroid hormone levels synthesized by H295R (CRL-2128™) cells exposed to increasing concentrations (μM) of A) flusilazole or B) triticonazole for 48 hours. HSD = hydroxysteroid dehydrogenase. Data are represented as mean ± SEM (n=3). *p< 0.05.

Fig. 3: Triticonazole and flusilazole levels in amniotic fluid and fetal plasma: Concentration (μM) of A) triticonazole and B) flusilazole in amniotic fluid or combined plasma from male and female fetuses at GD21. Fetuses were exposed via mothers from GD7-21 to 15 or 45 mg/kg bw/day of flusilazole or to 150 or 450 mg/kg bw/day of triticonazole. Controls were zero (data not shown). Results shown are mean ± SEM of pooled samples from 4 litters.

655 **Fig. 4: Fetal exposure to triticonazole induces shorter male anogenital distance:** The anogenital
656 distance index (AGDi, AGD/cube root of bw) of control males (Control) and flusilazole (15 and 45
657 mg/kg bw/day) or triticonazole (150 and 450 mg/kg bw/day) exposed males. Results are shown as a
658 box and whiskers plot with the minimum, the 25th percentile, the median, the 75th percentile, and
659 the maximum of the data, n=7-8 litters/group. *p < 0.05, ***p < 0.0001 (with bw as covariate).

660

661 **Fig. 5: Flusilazole affects androstenedione and estrone levels in fetal male plasma:**

662 Fetal male plasma hormone concentrations at GD21 (ng/mL) following exposure to 15 or 45 mg/kg
663 bw/day of flusilazole or to 150 or 450 mg/kg bw/day of triticonazole from GD7-21. Results shown
664 are mean $\pm$ SEM of pooled samples from 7-8 litters (n=7-8).

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TABLES

Table 1 – Internal concentrations of flusilazole and triticonazole

Data represent group means $\pm$ SEM 1 hour $\pm$ 15 min. after exposure

Fluz-15 and Fluz-45 = 15 and 45 mg/kg bw/day flusilazole. Triz-150 and Triz-450 = 150 and 450 mg/kg bw/day triticonazole

	Control (μ M)	Fluz-15 (μ M)	Fluz-45 (μ M)	Triz-150 (μ M)	Triz-450 (μ M)
GD21 Caesarean section					
Amniotic Fluid (N=4)	0	0.20 $\pm$ 0.05	0.59 $\pm$ 0.13	1.34 $\pm$ 0.17	2.98 $\pm$ 1.01
Male and female plasma (N=8)	0	0.68 $\pm$ 0.11	2.11 $\pm$ 0.35	5.26 $\pm$ 0.74	9.01 $\pm$ 1.76

Table 2 - Pregnancy and litter data

Data represent group means, based on litter means $\pm$ SD.

Fluz-15 and Fluz-45 = 15 and 45 mg/kg bw/day flusilazole. Triz-150 and Triz-450 = 150 and 450 mg/kg bw/day triticonazole

	Control	Fluz-15	Fluz-45	Triz-150	Triz-450
GD21 Caesarean section					
No. of litters	N=8	N=8	N=8	N=7	N=8
Maternal bw (g), GD7	245.3 $\pm$ 31.5	243.5 $\pm$ 15.9	236.8 $\pm$ 29.1	245.1 $\pm$ 23.2	246.6 $\pm$ 25.3
Maternal bw gain (g), GD7-21	115.6 $\pm$ 12.3	131.7 $\pm$ 6.6	113.6 $\pm$ 13.7	135.3 $\pm$ 24.6	118.2 $\pm$ 26.2
Adjusted maternal bw (g), GD21 ^a	281.5 $\pm$ 25.6 ^b	275.7 $\pm$ 20.5	257.8 $\pm$ 21.7	285.2 $\pm$ 27.9	275.9 $\pm$ 36.1
No. of implantations	14.5 $\pm$ 1.5	15.1 $\pm$ 1.0	14.4 $\pm$ 2.4	16.1 $\pm$ 2.0	15.0 $\pm$ 2.4
No. of fetuses	13.4 $\pm$ 1.6	14.1 $\pm$ 1.6	13.1 $\pm$ 2.5	16.0 $\pm$ 2.2	14.3 $\pm$ 2.7
% postimplantation loss	7.8 $\pm$ 5.9	6.7 $\pm$ 7.1	8.6 $\pm$ 8.7	2.7 $\pm$ 5.0	5.2 $\pm$ 5.8
% late resorptions	0.8 $\pm$ 2.2	0.8 $\pm$ 2.36	4.3 $\pm$ 8.1	0.9 $\pm$ 2.4	5.2 $\pm$ 5.8
Fetal bw male (g)	4.2 $\pm$ 0.3	4.5 $\pm$ 0.4	4.4 $\pm$ 0.3	4.2 $\pm$ 0.4	4.2 $\pm$ 0.3
Fetal bw female (g)	4.0 $\pm$ 0.2	4.3 $\pm$ 0.3	4.0 $\pm$ 0.4	3.9 $\pm$ 0.3	4.0 $\pm$ 0.2
Male AGD (mm)	4.0 $\pm$ 0.1	4.1 $\pm$ 0.2	4.0 $\pm$ 0.3	3.9 $\pm$ 0.2	3.6 $\pm$ 0.1**
Male AGD index	2.5 $\pm$ 0.1	2.5 $\pm$ 0.1	2.5 $\pm$ 0.1	2.4 $\pm$ 0.1*	2.3 $\pm$ 0.1***
Female AGD (mm)	2.2 $\pm$ 0.1	2.3 $\pm$ 0.2	2.3 $\pm$ 0.1	2.2 $\pm$ 0.1	2.3 $\pm$ 0.1
Female AGD index	1.4 $\pm$ 0.1	1.4 $\pm$ 0.1	1.4 $\pm$ 0.1	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1

Values in **bold** are statistically significantly different from Control

* Significantly different from Control, $p < 0.05$

** Significantly different from Control, $p < 0.001$

*** Significantly different from Control, $p < 0.0001$

^a Adjusted maternal bw (g) is bw at GD 21 after subtracting the uterus weight

^b N=7 dams, as one uterus was not weighed.

AGD (mm) was analysed with fetal weight as covariate.

AGD index = AGD divided by cube root of the bodyweight

HIGHLIGHTS

- Flusilazole and triticonazole have endocrine disrupting activities
- *In vitro* effects don't fully predict flusilazole and triticonazole *in vivo* effects
- Triticonazole displays anti-androgenic properties
- Flusilazole exposure reduces estrogen synthesis, likely by aromatase inhibition

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

	Flusilazole	Triticonazole
<i>In vitro</i>		
AR Antagonism		
AR Agonism		
Steroidogenesis Disruption		
<i>In vivo</i>		
Anogenital Distance		
Androstenedione		
Estrone		

No effect

Decrease

Increase/effect

A

Cholesterol

cholesterol side-chain
cleavage enzyme

Pregnenolone

Progesterone

17 α -hydroxylase17 α -Hydroxypregnenolone

17, 20 lyase

DHEA

Androstenedione

17 β -HSD17 β -HSD

Androstenediol

5 α -reductase

Dihydrotestosterone

21-hydroxylase

11-Deoxycorticosterone

Aromatase

Estrone

17 β -HSD17 β -Estradiol

Aldosterone

Aldosterone synthase

Corticosterone

11 β -hydroxylase

Cortisol

Fetal male rat plasma hormone levels