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Transcriptomic changes upon epoxiconazole exposure in a human stem cell-based model of developmental toxicity

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A B S T R A C T

Conazole fungicides such as epoxiconazole are mostly used on cereals of crops to inhibit fungal growth through direct inhibition of sterol 14α-demethylase (CYP51A1). However, this enzyme is highly conserved and in humans it is part of the steroid hormone biosynthesis pathway. Endocrine disrupting effects of epoxiconazole have been shown in rodents and have been substantiated by invitro data, however, the underlying molecular mechanisms are not clear. We took advantage of a human stem cell based invitro model for developmental toxicity to study the molecular effects of epoxiconazole. This model is based on 3D cultures of embryoid bodies and differentiation into cardiomyocytes, which mimics the early stages of embryonic development. We have previously shown that epoxiconazole impairs differentiation of these embryoid bodies and therefore has the potential to affect human embryonic development. We employed global transcriptome analysis using RNA sequencing and found that the steroid biosynthesis pathway including CYP51A1, the human sterol 14α-demethylase, was highly deregulated by epoxiconazole in our model. We confirmed that most genes of the steroid biosynthesis pathway were upregulated, including CYP51A1, suggesting a compensatory mechanism at the gene expression level. Our data suggest that epoxiconazole acts mainly by decreasing cholesterol biosynthesis in the cells. We conclude that epoxiconazole bears the potential to harm human embryonic development through inhibition of the steroid biosynthesis pathway. As this may be a common feature of compounds that target sterol 14α-demethylase, we add evidence to the assumption that conazole fungicides may be human developmental toxins.

1. Introduction

Studying molecular mechanisms underlying the adverse effects of chemicals can be challenging, especially in the case of developmental toxicity. In vitro models are a good option not only for studying human developmental toxicity but also the causative molecular mechanisms (Liu et al., 2017; Zink et al., 2020). Human induced pluripotent stem cells (hiPSC) can be used to model early embryonic development by formation of embryoid bodies (EBs), in which cells can differentiate into early cell types, such as cardiomyocytes (Liu et al., 2017; Takahashi et al., 2007). The heart is the first functional organ of the body and starts beating after three weeks of pregnancy (Carlson, 2014). Accordingly, cardiomyocyte differentiation in EBs is one of the most widely used developmental toxicity test systems (Seiler and Spielmann, 2011; Worye et al., 2018). We have previously shown that hiPSC differentiating into cardiomyocytes using an eight-day protocol can detect developmental toxicity of thalidomide. Moreover, we detected the pesticide epoxiconazole as a positive in our assay, suggesting its known adverse effects in rodents might also happen in the human (Lauschke et al., 2020).

Epoxiconazole belongs to a group of fungicides called conazoles. Its mode of action in fungi is through direct inhibition of sterol 14α-demethylase (in humans encoded by the CYP51A1 gene) and aromatase. Blocking of sterol 14α-demethylase leads to a lack of ergosterol, which is essential for yeast and fungal cell membranes (Zarn et al., 2003). However, sterol 14α-demethylase is highly conserved among organisms and epoxiconazole also inhibits the human CYP51A1 enzyme (Trisken et al., 2006). In humans, CYP51A1 is involved in the production of cholesterol that is the precursor of steroid hormones, and the endocrine disrupting activity of epoxiconazole and other conazoles has been...
investigated during the last years (Chambers et al., 2014; Draskau et al., 2021; Hass et al., 2012; Kjærstad et al., 2010; Munkboel et al., 2019; Taxvig et al, 2007, 2008). Epoxiconazole has been shown to have weak estrogenic activity, to be an androgen receptor antagonist and to affect sex steroid hormone synthesis in vitro (Kjærstad et al., 2010; Lucia Pinto et al., 2018). Furthermore, epoxiconazole was shown to be feto-toxic at high doses and to increase birth weight at lower doses upon prenatal exposure in rats (Taxvig et al, 2007, 2008). Epoxiconazole is used on crops such as wheat and barley, which are used for human food production, as well as on beetroot. Residues in grains have been shown to be very low and unlikely to lead to exposure of human consumers above the acceptable daily intake (Chambers et al., 2014; DAR, 2006). However, farmers are closer to exposure levels that could impose a health risk (Chambers et al., 2014). Due to the classification of epoxiconazole as an endocrine disruptor and increasing health concerns (ANSES, 2018), epoxiconazole was abandoned for use in the EU with effect after October 2021 (National Farmers Union of England and Wales, NFU, 2020).

We set out to investigate the molecular mechanism of epoxiconazole in a human stem cell based model of development. To this end, we compared the global gene expression levels in differentiating stem cells upon exposure to epoxiconazole and the solvent control using RNA-Seq. We exposed differentiating hiPSC to epoxiconazole at the highest efficacious, non-cytotoxic exposure level we had previously published i.e. 20 μM (Lauschke et al., 2020) and collected samples on day two (D2) and day seven (D7) of cardiomyocyte differentiation as well as from the pluripotent cells. We further analyzed the differentially expressed genes in the epoxiconazole groups compared to the control and found the steroid biosynthesis pathway to be enriched. We confirmed these gene expression changes using qRT-PCR, showing that most genes of the steroid biosynthesis pathway were affected as well as genes involved in steroid hormone biosynthesis. Our results indicate that epoxiconazole disrupts cholesterol synthesis in human cells, which supports the human relevance of the well-known endocrine disrupting effects shown in rodents.

2. Methods

2.1. Cell culture

The hiPSC line BIONi010-C was used for all experiments (Bioneer A/S, Horsholm, Denmark). hiPSC were cultured in mTeSR™1 medium (STEMCELL Technologies, Vancouver, Canada) on hESC-Qualified Matrigel (Corning, Corning, USA) coated cell culture dishes (Thermo Fisher Scientific, Waltham, USA). Medium was exchanged daily except for weekends (weekend-free culture) and cultured in 5% CO₂ at 37 °C. Cultures were split approximately once a week using 0.02% EDTA in DPBS.

2.2. Cardiomyocyte differentiation

HiPSC were differentiated into cardiomyocytes as described in Lauschke et al., (2020). Briefly, cells were harvested as single cells by incubation in Gibco™ TrypLE™ Select (Thermo Fisher Scientific) for 1–2 min. Cells were resuspended as single cells and a suspension of 5×10⁴ cells/mL seeded at 100 μL per well into a 96-well Polystyrene Conical Bottom MicroWell™ Plate (249,952, Thermo Fisher Scientific) in mTeSR-ROCK. The plates were centrifuged at 500 g for 5 min at room temperature and incubated overnight. After 20 h, 80 μL/well old medium were removed and 80 μL/well D0 medium added. After this, medium was exchanged daily (24 h ± 2 h) with respective medium on the following days: TS-medium on day 1, Wnt-medium on day 2, TS-medium on day 3 and TS-medium on day 4. 80 μL/well old medium were removed and 80 μL/well new medium added, except for day 6, where only 60 μL/well were removed. Media are listed in Supplementary Table 1.

2.3. Chemicals exposure and sampling

Epoxiconazole was purchased from Sigma Aldrich (St. Louis, USA) and prepared as 600 mM stock solution in dimethyl sulfoxide (DMSO, cell culture grade) (Sigma Aldrich). The stock solution was diluted in DMSO to create a 20 mM solution. As described in Lauschke et al. (2020), exposure of differentiating cells was started on D2, as earlier exposure lead to disintegration of the EBs, and repeated on D3 and D6 by diluting the 20 mM solution or DMSO (as a control) 1:1000 in medium of the respective day (Wnt- or TS-medium).

Cells were harvested for RNA extraction by the following procedure: Pluripotent samples were harvested from the single cell suspension created for seeding into 96-well conical bottom plates. Approximately 1×10⁵ cells from the excess single cell suspension were centrifuged at 200 g for 5 min, supernatant removed and the cell pellet snap frozen in liquid nitrogen. For D2 and D7 samples, EBs were collected from the 96-well plates on day 2 and day 7, respectively, using a P1000 pipette with wide orifice tips and collected in 15 ml centrifugation tubes. Cells were spun down at 200 g for 5 min and the supernatant discarded except for approximately 500 μL. In this, the cells were resuspended, transferred to 1.5 ml centrifugation tubes and centrifuged at 1000 g for 5 min. The supernatant was removed carefully and the pellet made as dry as possible before snap freezing in liquid nitrogen. All cell samples were stored at −80 °C for few weeks until RNA extraction.

All experiments were conducted three times i.e. using three independent cardiomyocyte differentiation (biological triplicates), unless stated differently. Experiments for RNA Seq and qRT-PCR rely on different biological replicates. Each experiment was based on a pool of 32–96 EBs per treatment.

2.4. RNA extraction

RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s specifications. Briefly, the cell pellets were thawed slightly and 400 μL RLT buffer containing 1% beta-mercaptoethanol added. The cells were lysed and homogenized by passing the sample through a 20-gauge needle attached to a sterile 1 ml plastic syringe at least 10 times. One volume of 70% ethanol was added, mixed with the lysate and the sample transferred to an RNeasy spin column. All steps of the subsequent RNA extraction protocol were followed as suggested by the manufacturer, including DNase digestion. RNA was eluted in 50 μL RNaše free water and concentration measured on a Nanodrop (Thermo Fisher Scientific). For qRT-PCR analysis, 200 ng of RNA with an A260/A280 ratio > 1.8 were used.

For RNA-Seq, RNA integrity was measured on a Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer’s instructions. Samples with a RIN value < 9.0 were further cleaned up using the Qiagen RNeasy Micro Kit (Qiagen). Briefly, samples were thawed on ice and the volume adjusted to 100 μL using RNaše-free water. Then, 350 μL RLT buffer containing 1% beta-mercaptoethanol were added followed by 250 μL 96–100% ethanol. The samples were mixed by pipetting, transferred to a MinElute spin column and centrifuged. All following steps were conducted according to the manual except for DNase digestion, which was not included in the cleanup. RNA was eluted in 16 μL RNaše-free water and RNA integrity measured again on a Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies). Final concentrations of all samples with RIN > 9.0 were measured using the Quibit™ RNA Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Samples with a total of >300 ng RNA in at least 24 μL volume and a RIN value > 9.0 were used for RNA sequencing.

2.5. Library preparation

Sequencing libraries were prepared at Novogene Co. Ltd. (Beijing, China) according to their standard procedures. Libraries were generated
Remaining overhangs were converted into blunt ends and 3'-random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-) mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in NEB Next Strand Synthesis Reaction Buffer (5X). For cDNA synthesis, the first strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-) and the second strand cDNA using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends and 3'-ends of the retrieved cDNA adenylated, to enable ligation with NEB Next Adaptors with hairpin loops. To select cDNA fragments of 150–200 bp, the fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB) was incubated with the size-selected, adapter-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Lastly, PCR products were purified using an AMPure XP system and library quality assessed on the Agilent Bioanalyzer 2100 system.

2.6. Clustering and sequencing

These steps were performed after library preparation at Novogene according to their standard procedures. Briefly, clustering was performed on a Biot Cluster Generation System using PE Cluster Kit eBot-HS (Illumina) according to the manufacturer’s instructions. Sequencing was performed on an Illumina platform and paired-end reads generated.

2.7. Bioinformatics

After sequencing, bioinformatics analysis was performed at Novogene according to their standard procedures. Briefly, clustering was performed on a Biot Cluster Generation System using PE Cluster Kit eBot-HS (Illumina) according to the manufacturer’s instructions. Sequencing was performed on an Illumina platform and paired-end reads generated.

2.8. qRT-PCR and gene expression analysis

For confirmatory qRT-PCR, 200 ng RNA were reverse transcribed into cDNA using the Omniscript® Reverse Transcription Kit (Qiagen). Then, 3.75 ng cDNA were used per well in a 384-well microtiter plate for qRT-PCR. This was performed with the TaqMan Assay Kits (Thermo Fisher Scientific) listed in Supplementary Table 2 on a QuantStudio 7 Flex (Applied Biosystems, Foster City, USA). Each sample was measured in technical duplicates. Samples with cycle threshold (CT) values < 30 had to reach a CT difference between technical duplicates < 1. Samples with CT values > 35 were regarded as non-detectable. Relative gene expression was calculated with the ∆∆CT method, normalized to the average of the two house-keeping genes, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and ACTB (β-actin). Experiments were performed in biological triplicates for D2 samples and biological duplicates for D7 samples, each with a pool of 32–96 EBs per group.

2.9. Statistics

Statistical analysis was performed in GraphPad prism version 8. Relative gene expression of epoxiconazole and DMSO control of each day was compared using an unpaired two-tailed t-test. If SDs were significantly different in the two data sets, Welch’s correction was performed. The data were checked for normal distribution and if the data were not normally distributed they were log-transformed for statistical analysis. Stars indicate the following significance: * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

3. Results

We investigated an early and a late time point of cardiomyocyte differentiation, namely day 2 (D2) and day 7 (D7). D2 samples had been exposed for 24 h to epoxiconazole or DMSO solvent control and D7 samples for six days (144 h). The cells on D2 represented mostly cardiac mesoderm and progenitor cells, while they had committed to cardiomyocyte identity on D7 (Lauschke et al., 2020) (Fig. 1A). We hypothesized that pathways disturbing cardiac mesoderm development into cardiac progenitor cells would be visible in D2 samples, while effects on cardiomyocyte development would be revealed in D7 samples. Our principle component analysis confirmed that the samples clustered according to experimental day, with the pluripotent samples showing the largest distance along PC2, and the D2 and D7 samples along PC1 (Fig. 1B). There was no apparent grouping according to control or epoxiconazole exposure on either day, suggesting that the differentiation stage had a larger impact on the transcriptome than exposure to the test chemical.

When comparing the differentially expressed genes between epoxiconazole and solvent group on D2 and D7, respectively, we found 440 significantly differentially regulated genes on D2 and 2,410 on D7. On D2, 296 genes were upregulated and 144 genes downregulated (Fig. 2A, left panel). On D7, as many as 911 genes were upregulated and 1,499 downregulated (Fig. 2A, right panel). We also assessed the genes that were commonly deregulated in both groups and found that these were 103 genes (Fig. 2B).

In order to identify genetic pathways targeted by epoxiconazole we conducted a KEGG pathway analysis of the differentially regulated genes and found that on both days, the steroid biosynthesis pathway was among the most significantly enriched pathways (Fig. 3).

This pathway caught our attention because epoxiconazole inhibits one of the enzymes in this pathway, namely CYP51A1 (Trosken et al., 2006). Therefore, we analyzed this pathway more closely and found ten genes to be deregulated on either one or both days upon epoxiconazole treatment (Fig. 4A). These were almost all genes included in the steroid biosynthesis pathway, as indicated in red in Fig. 4B. The steroid biosynthesis pathway produces cholesterol, which in turn is the precursor for all steroid hormones (Fig. 4B). Therefore, we also investigated this pathway and found four genes to be deregulated upon epoxiconazole treatment (Fig. 4C).

This suggested that epoxiconazole treatment affects the expression of genes involved in steroid and steroid hormone biosynthesis and we set...
out to confirm this using qRT-PCR. We were able to detect all candidate genes from our RNA-Seq analysis using qRT-PCR with the exception of \textit{HSD17B6} and \textit{SULT2B1} (both part of the steroid hormone biosynthesis pathway). The expression levels of these genes were too low to be detected with our conditions in qRT-PCR (data not shown). As shown in Fig. 5 A, all investigated genes of the steroid biosynthesis pathway were upregulated in the epoxiconazole treated samples compared to controls on both days. This confirmed our RNA-Seq data except for \textit{MSMO1} and \textit{SC5D}, for which the RNA-Seq analysis had showed downregulation on D2 or D7, respectively (Fig. 4 A). In the steroid hormone biosynthesis pathway, \textit{CYP1B1} did not appear to be differentially expressed in epoxiconazole and control samples, and for \textit{HSD17B12} we only showed a non-significant trend in upregulation on both days (Fig. 5 B). All together, we confirmed that most genes involved in steroid biosynthesis were upregulated upon epoxiconazole treatment, whereas the steroid hormone biosynthesis genes were either too low expressed or not significantly changed.

Lastly, there are also other proposed modes-of-action for epoxiconazole as for example through inhibition of CYP26 or CYP19, as well as effects on glucocorticoid production (Chambers et al., 2014; Marotta and Tiboni, 2010; Menegola et al., 2005), and we therefore analyzed our RNA-seq data for DEGs involved in these pathways. However, the only CYP26 enzyme expressed in our system was CYP26A1 and this was not deregulated by epoxiconazole. CYP19 was not expressed in our system. Lastly, we did not find any enzymes involved in the production of glucocorticoids to be deregulated. With this, we did not find evidence for other CYP450-related modes-of-action of epoxiconazole than cholesterol biosynthesis. In support of this, the KEGG-pathway “cholesterol metabolism” was among the most significantly affected pathways in the D2 samples (Fig. 3 A). When investigating this pathway closer, we identified genes encoding for proteins that lead to higher intracellular cholesterol levels upregulated (LDLR, NPC1, NPC2, LIPA), while those encoding for proteins for cholesterol efflux (CETP, ABCA1) were downregulated (data not shown). This indicates a compensatory mechanism in the cells to balance potentially lower cholesterol levels resulting from the inhibition of CYP51A1.

4. Discussion

In this study, we found genes of the steroid biosynthesis pathway to be deregulated upon exposure to epoxiconazole. Endocrine disrupting effects of epoxiconazole are evident in rodents and effects on the human endocrine system have been suggested using in vitro assays, but our study is the first to investigate the molecular mechanism of epoxiconazole in human stem cells. We used a concentration of 20 μM epoxiconazole in this study that we previously identified as the highest non-cytotoxic efficacious concentration (Lauschke et al., 2020). This concentration is above relevant human exposure levels, but was used to ensure detection of potentially subtle effects at the gene expression level, which might not be detectable at lower exposure levels.
Epoxiconazole inhibits the human CYP51A1 enzyme (Tröskén et al., 2006) and our data suggests that this manifests at the gene expression level. We showed that not only CYP51A1 but most genes upstream (FDFT1, SQLE and LSS) and downstream (TM7SF2, NSDHL, HSD17B7 and DHCR7) of it were upregulated. This indicates a compensatory mechanism in the cells at the transcriptional level to repair loss of enzyme activity.

We hypothesize that epoxiconazole exposure disturbs steroid hormone biosynthesis mainly at the level of cholesterol production. Moreover, we also found the KEGG-pathway “cholesterol metabolism” significantly enriched in the D2 samples, emphasizing the role of cholesterol in the effects of epoxiconazole. Epoxiconazole decreases
serum levels of cholesterol in adult rats and mice (Hester et al., 2012; US Environmental Protection Agency, 2003) and inhibitory effects on cholesterol biosynthesis have been shown for other conazole fungicides as well (Kan et al., 1985).

Cholesterol, besides being a precursor for steroid hormones and bile acids, is an important component of cell membranes and crucial in cell signaling pathways (Sheng et al., 2012; Subczynski et al., 2017; Yeagle, 1985). Moreover, it is involved in key developmental pathways such as Hedgehog signaling and the fetoprotein transcription factor (Incardona and Eaton, 2000; Ingham, 2000; Paré et al., 2004). Low levels of cholesterol in infants are connected with Smith-Lemli-Opitz syndrome, a severe developmental disease characterized by multiple birth defects and mental retardation (Irons et al., 1993). It is caused by mutations in DHCR7 (Waterham et al., 1998), the enzyme that catalyzes the conversion of 7-dehydrocholesterol to cholesterol (see Fig. 4B), which we also identified to be deregulated upon epoxiconazole exposure. Also loss of other enzymes of the cholesterol biosynthesis pathway are fatal to embryonic development. For example, SQUE knockout in mice is lethal at the embryonic stage (Tozawa et al., 1999) and SC5D mutations are lethal in fetuses which furthermore have craniofacial defects (Krakowiak et al., 2003). Moreover, mutations in DHCR24 lead to desmosterolosis, a syndrome characterized by congenital malformations (Waterham et al., 2001), and mutations in EBP underlie Conradi-Hünermann syndrome (skeletal malformations) and the CHILD syndrome (limb malformations and skin anomalies) (Braverman et al., 1999; Derry et al., 1999; König et al., 2000). With this, cholesterol biosynthesis appears necessary for normal embryonic development and chemicals disrupting cholesterol biosynthesis are potentially hazardous to the human embryo.

In this study, we used an in vitro model for developmental toxicity that is based on the differentiation of hiPSC into cardiomyocytes. The cells undergo stages similar to those in the developing embryo such as cardiac mesoderm and cardiac progenitor formation, before they become cardiomyocytes (Lauschke et al., 2020). Our model can predict developmental toxicity (Lauschke et al., 2020), however, it is not clear yet to which extent endocrine disrupting chemicals can be detected. Nevertheless, the majority of chemicals with endocrine disrupting activities also impose developmental toxicity (Choi et al., 2004) and the fact that we detect a positive response with the endocrine disrupting chemical epoxiconazole in our assay, may indicate that our assay has a potential to detect other endocrine disrupting chemicals as well. Additionally, cholesterol is important for the contraction of cardiomyocytes.

Fig. 4. Deregulated genes in the steroid and steroid hormone biosynthesis pathways. A) Deregulated genes of the steroid biosynthesis pathway in a heat map depicting Log2FoldChange of epoxiconazole vs. control samples of day 2 (D2) and day 7 (D7). B) Main part of the steroid biosynthesis pathway, the end product cholesterol is the precursor for steroid hormone biosynthesis. Significantly deregulated genes are indicated in red, the molecular target of epoxiconazole, CYP51A1, is highlighted in orange. C) Deregulated genes of the steroid hormone biosynthesis pathway in a heat map depicting Log2FoldChange of epoxiconazole vs. control samples from D2 and D7. For B) and C), gene expression was analyzed by RNA-Seq, only significantly regulated genes with p-values < 0.05 are shown. If no significant change on one of the days, the value 0 (no change) was given. Red = upregulation, green = downregulation, black = no significant change. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
and potentially also functions in cardiomyocyte differentiation (Hissa et al., 2017; Shi et al., 2017). Therefore, we hypothesize that our assay detects effects on endocrine hormones through effects on the precursor, cholesterol.

To put our results into perspective, we can compare with the effects of statins that are drugs with cholesterol lowering as their major mechanism of action. Statins have deleterious effects on fetal development and show developmental toxicity in animal studies, although the mechanisms of action are not well understood (Jyoti and Tandon, 2015). Stains have been investigated in an in vitro differentiation model of murine embryonic stem cells and were found to affect differentiation of especially the mesodermal lineage (with weaker effects on differentiation into endo- and ectodermal lineages), leading to changes in the transcript levels of mesoderm-specific markers (Jyoti and Tandon, 2015). However, effects of statins on genes involved in cholesterol biosynthesis were not investigated in this paper, but this finding stresses the importance of further investigating the link between mesoderm differentiation and developmental toxicity and the mechanisms of action involved, including cholesterol reductions.

5. Conclusions

In conclusion, we provide evidence that the molecular mechanism of epoxiconazole’s developmental toxicity is through deregulation of the steroid biosynthesis pathway. Our data connect the previously shown inhibition of human CYP51A1 enzyme with endocrine disrupting effects in rodents. This further supports the notion, that epoxiconazole is not only a developmental toxicant to rodents but also to humans, which might also be applicable to other conazole fungicides. We therefore suggest that other conazole fungicides that target sterol 14α-demethylase are studied at the molecular level and are relevant candidate compounds for future investigations with our stem cell based in vitro model.

Author contributions

Karin Lauschke: Conceptualization; Formal analysis; Data curation; Investigation; Methodology; Visualization; Writing – original draft, Marlene Danner Dalgaard: Formal analysis; Data curation; Methodology, Jenny Emnēus: Funding acquisition; Supervision; Writing – review & editing, Anne Marie Vinggaard: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing – review &

Fig. 5. Confirmatory qRT-PCR of differentially expressed genes identified by RNA-Seq. A) Genes of the steroid biosynthesis pathway. B) Genes of the steroid hormone biosynthesis pathway. Gene expression relative to the house-keeping genes GAPDH and β-actin, in the control (Ctrl) and epoxiconazole (Epoxi) treated samples from day 2 (D2) and day 7 (D7). Statistically significant differences between the epoxiconazole and control samples on each day are indicated with stars. Error bars indicate SEM (standard error of means).
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131225.

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