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Morphological and transcriptomic effects of endocrine modulators on the gonadal differentiation of chicken embryos: The case of tributyltin (TBT)

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ABSTRACT

Morphological malformations induced by tributyltin (TBT) exposure during embryonic development have already been characterized in various taxonomic groups, but, nonetheless, the molecular processes underlying these changes remain obscure. The present study provides the first genome-wide screening for differentially expressed genes that are linked to morphological alterations of gonadal tissue from chicken embryos after exposure to TBT. We applied a single injection of TBT (between 0.5 and 30 pg as Sn/g egg) into incubated fertile eggs to simulate maternal transfer of the endocrine disruptive compound. Methyltestosterone (MT) served as a positive control (30 pg/g egg). After 19 days of incubation, structural features of the gonads as well as genome-wide gene expression profiles were assessed simultaneously. TBT induced significant morphological and histological malformations of gonadal tissue from female embryos that show a virilization of the ovaries. This phenotypical virilization was mirrored by altered expression profiles of sex-dependent genes. Among these are several transcription and growth factors (e.g. FGF12, CTCF, NFIB), whose altered expression might serve as a set of markers for early identification of endocrine active chemicals that affect embryonic development by transcriptome profiling without the need of elaborate histological analyses.

1. Introduction

Endocrine disrupting chemicals (EDCs) represent a potential risk for animals because of their ability to interfere with endogenous endocrine pathways through various molecular processes. Tributyltin (TBT) is a known endocrine disrupter and a highly toxic compound with a complex toxicity profile. This organotin compound affects *inter alia* calcium homeostasis, inhibits oxidative phosphorylation as well as ion transport processes, interferes with the cytochrome P450 dependent monooxygenase system by inhibition of aromatase as the key enzyme for the conversion of androgens to estrogens and is a potent agonist of the retinoid X receptor (RXR) (Alzieu, 2000; Dmetrichuk et al., 2008; Gooding and LeBlanc, 2001; Nishikawa et al., 2004; OECD, 2010; Oberdörster and McClellan-Green, 2000; Sekizawa et al., 2017). TBT has widely been used as an antifouling. Environmental levels of TBT have been found up to 197 ng TBT-Sn/L in sea water and up to 1,198 ng TBT-Sn/g dry weight in blue mussels (*Mytilus edulis*) (Chau et al., 1997; Rodríguez-González et al., 2006). Accelerated by UV radiation, increasing temperature, and biological activity by microorganisms, TBT may degrade through sequential dealkylation to dibutyltin (DBT), monobutyltin (MBT), and inorganic tin, becoming progressively less toxic in the process (Antizar-Ladislao, 2008; Gadd, 2000). Indeed, information on the mechanisms of TBT detoxification is still limited (Dubey and Roy, 2003). Since 2008, the use of TBT in antifouling paints is banned internationally (Antizar-Ladislao, 2008).

TBT affects a wide range of non-target organisms from various invertebrate phyla to different classes of vertebrates. It causes adverse effects by disruption of their endocrine system such as abnormal shell growth, imposex and intersex development (Alzieu, 2000; Oehlmann et al., 1998; Stroben et al., 1992). TBT may bioaccumulate in higher trophic levels (EFSA, 2004), and thus may pose a threat for predators, although data on TBT tissue concentration in birds is scarce and information on TBT concentration in bird eggs is not available. Mollusk and fish-eating coastal seabirds seem to be particularly concerned by

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accumulating TBT from their prey from a theoretical standpoint (Kannan and Falandysz, 1997; Kannan et al., 1998). In livers of the Japanese common cormorant (*Phalacrocorax carbo*) up to 9 ng Sn/g wet weight were found (Guruge et al., 1997) and in birds (grebes, tufted ducks, cormorants) in the Netherlands measured TBT concentrations ranged from 9 to 28 ng Sn/g dry weight (Stäb et al., 1996). Measured levels were considerably lower than TBT levels in fish and invertebrates from the same area serving as food for the birds. These results indicate a lower biomagnification potential for TBT in birds than expected which reflects their good capability to metabolize TBT via phase I pathways (cytochrome P450-dependent debutylation) (Veltman et al., 2006) and the seasonal moulting as an efficient elimination route of organotins. Feathers of cormorants have shown to contain up to 30% of the total body burden of butyltins probably due to feather proteins that have a high affinity for organotins (Guruge et al., 1997).

The adverse effects of TBT on the gonads have already been shown for both, sexually mature animals and developing embryos in a broad range of species. Virilization and even sex-reversal of female gastropods, as well as effects on sex differentiation in other invertebrate and vertebrate groups are clear examples of endocrine disruption by TBT in early and later life stages starting at 1–20 ng TBT-Sn/L in gastropods (Davies et al., 1997; Gooding et al., 2003; His and Robert, 1985; Matthiessen and Gibbs, 1998; OECD, 2010; Wang et al., 2010). At similar exposure levels, effects on sex ratio were also observed in a sexual development test with the zebrafish *Danio rerio* (McAllister and Kime, 2003). In mice, prenatal TBT-exposure resulted in lower body weight, prolonged diestrus or extended periods of estrus during adulthood (Si et al., 2012). In human placenta, Heidrich and co-workers (Heidrich et al., 2001) demonstrated an inhibition of aromatase activity in response to TBT with an IC_{50} of 0.7 µgTBT-Sn/L.

In general, ovarian follicles from lower vertebrates such as fish and amphibians exhibited a comparable sensitivity to butyltins like mammalian ovary tissue cultured *in vitro* (Ahn et al., 2007; Yamazaki et al., 2005). For many of the observed TBT effects on sexual differentiation in the various wildlife groups aromatase seems to be the most prominent target. The modulation of aromatase activity is likely to result in a hormonal imbalance with an altered estrogen/androgen level and finally in the formation of ovotestes during development (Ahn et al., 2007; Heidrich et al., 2001; Nakanishi et al., 2006). However, sensitivity of steroidogenic enzymes to exogenous chemicals differs between ovaries and testes. In mammals, male sexual differentiation is dependent on testosterone (Cunha et al., 2004; Marker et al., 2003; Ricke et al., 2004). In contrast, differentiation of reproduction organs and sexual behaviour in birds is regulated by estrogens. If estrogen synthesis is inhibited, genetically female birds may develop into phenotypic males (Bannister et al., 2011). Higher levels of estrogen, on the other hand, feminise male embryos: In Japanese quails (*Coturnix japonica*), Berg and colleagues showed that administration of synthetic estrogens results in development of an ovary-like tissue in the left testis, while malformations occur in the Müllerian duct of female quails (Berg et al., 1999). In line with this, the treatment of genetic females with male hormones like e.g. 17α-methyltestosterone (MT) results in a characteristic virilization that affects the gonads, accessory sex organs as well as behaviour in a wide range of organisms, such as molluscs, fish, turkeys, and rats (Alzieu, 2000; Feist et al., 1995; Memon et al., 2008; Singh, 2013; Yamasaki et al., 2003).

Like in mammals, the avian mother affects the development of its offspring not only by transferred genetic material, but also by transferring hormones (Carere and Balthazart, 2007). Substances incorporated by the mother may consequently also influence offspring development even originally or as metabolites in the allantoic fluid (Kamata et al., 2006). Contrasting to other test systems that employ mammals or aquatic species, however, the chicken egg represents a closed system that lacks any exchange with its environment except for the interchange of gases. A unique injection of a testing compound results in chronic chemical exposure, because no exchange or loss of the test compound is possible. A single test compound injection at a very early developmental phase may therefore be sufficient to significantly influence the developing embryo. Consequently, chicken eggs provide a different system for assessment of the effects of chronic chemical exposure compared to aquatic systems, where the test compound is introduced into the environment (Ahn et al., 2007; Bannister et al., 2011; Berg et al., 1999; Carere and Balthazart, 2007; Cunha et al., 2004; Davies et al., 1997; Feist et al., 1995; Gooding et al., 2003; Heidrich et al., 2001; His and Robert, 1985; Kamata et al., 2006; Marker et al., 2003; McAllister and Kime, 2003; Memon et al., 2008; Nakanishi et al., 2006; Ricke et al., 2004; Si et al., 2012; Singh, 2013; Yamasaki et al., 2003; Yamazaki et al., 2005; Zhang et al., 2007; Zhang et al., 2011; Zhang et al., 2013).

Here we present a comprehensive morphological and histological study of chicken embryonic gonads after exposure to the endocrine modulator TBT along with a genome-wide transcriptome analysis of sex-specific gene expression. We investigated whether effects of TBT on embryonic survival and development before hatching, with special emphasis on the structure of the gonads in the common domestic hen (*Gallus gallus domesticus*), are accompanied by characteristic shifts in gene expression of the gonads.

2. Materials and Methods

2.1. Substances

The tested substances comprised tributyltin chloride (TBT-Cl, CAS-no. 1461-22-9, 96% p.a., Sigma-Aldrich, Munich) and 17α-methyltestosterone (MT, CAS-no. 58-18-4, ≥97% HPLC, Sigma-Aldrich, Munich), which served as positive control. For histological analysis chicken embryos were exposed to four doses of TBT (0.5, 3, 10 and 30 pg TBT-Sn/g egg), while gene expression profiling was performed after exposure to 10 pg TBT-Sn/g egg. All TBT doses are provided as tributyltin as tin (TBT-Sn). These doses can be converted to tributyltin chlorid (TBT-Cl) by multiplying with factor 2.44 (1 pg TBT-Sn/g = 2.44 pg TBT-Cl/g). MT was injected at a dose of 30 pg MT/g egg. For both test substances sesame oil (Oleum Sesame Raffinatum, CAS-no. 8008-47-0, Sigma-Aldrich, Munich) was used as solvent with an injected volume of 8 µL per egg, irrespective of the dose. To simulate the maternal transfer, the solvent and tested substances were injected directly into the yolk, right under the embryonic tissue, with a Hamilton syringe. Injection holes were subsequently sealed with 3% agarose to allow the passage of air and humidity.

2.2. Ethics statement

All experiments were carried out with respect for the principles of laboratory animal care, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the German Animal Welfare Act. All guidelines have been followed, complying with the ARRIVE guidelines. These experiments are pre-tests for the development of a test system using younger chicken embryos within the scope of a new animal replacement (3R) system (Jessl et al., 2017; Russel and Burch, 1959; Scholz et al., 2013).

2.3. Exposure of embryos

Six exposure experiments were performed with a total of 301 newly laid fertile chicken eggs (*Gallus gallus domesticus*, White Leghorn), which were obtained from a commercial local supplier (LSL Rhein-Main, Schaafheim). The eggs were incubated in batches of up to 20 eggs per day, while remaining eggs were stored up to 5 days at 16 +- 1 °C to prevent the start of development. Eggs were bred in a ThermoStar 100 egg incubator (J. Hemel Brutgeräte, Verl, Germany) and spaced so that they were not in contact with one another. Incubation was performed at 37.6 +- 0.1 °C and 60 +- 5% humidity, and eggs were turned once

every 2 hours. After 24 hours of incubation (embryonic day (ED) 1), the eggs were candeled and randomized into 7 treatment groups: control (untreated/not injected), solvent control (only sesame oil injected), positive control (MT), and four TBT exposure groups (doses: 0.5, 3, 10 and 30 pg TBT-Sn/g egg), not fertilized eggs were excluded. Eggs were candled on ED 7, 14, and 17 and dead embryos observed during candling were removed from egg shells and examined for possible causes of death. Embryos were decapitated 2 days before anticipated hatching on embryonic day 19 (ED19), and dissected under a microscope. Phenotypical sexing and morphologic judgment was blindly performed according to the respective gonad morphology before further processing for histological and genetic analyses. If a female showed a smaller left and/or a bigger right gonad than normal, it was used as a first marker for virilization.

2.4. Gonad tissue isolation and processing for histological analysis

Gonads were isolated together with the underlying mesonephros to provide a landmark for stereoscopic adjustment after histological processing. Tissues were fixed using Bouin's solution (15 mL saturated aquatic solution of picric acid with 5 mL formalin 40% and 1 mL glacial acetic acid) followed by repeated EtOH 70% rinsing until picric acid was removed (picric acid colors EtOH yellowish, thus, all picric acid was removed if EtOH stayed colorless). 6 µm cryo-slices from the middle of the gonads were stained by Hematoxylin-Eosin followed by measuring of cortex thickness and counting of *Tubuli seminiferi* in female gonads under a microscope (Olympus BX 50) using image processing (Diskus 4.5, Hilgers, Königswinter, Germany). 10 slices per individual were used for anlaysis. If a female gonad showed a noticeable thinner cortex and/or more than 10 seminiferous cords (cut off ration to exclude false positives), it was considered as virilized in a blind treatment examination.

2.5. Genetic sexing

For genetic sex determination, blood samples from each embryo were collected in absolute EtOH. and stored at −20 °C until isolation of the DNA with the DNEasy isolation kit (Qiagen, Hilden, Germany). Genetic sexing was carried out by a PCR using the primers 2550F (5'-GTT ACT GAT TCG TCT ACG AGA-3') and 2718R (5'-ATT GAA ATG ATC CAG TGC TTG-3') (Fridolfsson and Ellegren, 1999). These primers target at CHD1 introns, located on the Z (CHD1Z, 600 bp) and W chromosome (CHD1W, 450 bp). Thermal cycling comprised DNA polymerase activation at 95 °C for 1 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 1 min and a final extension step at 72 °C for 3 min. All amplifications were performed on an advanced primus 96 thermocycler (Peqlab). Amplicons derived from this modified protocol were separated on a 1.4% agarose gel, where they resolved into one band (Z) in the case of male or two bands (Z + W) in the case of female embryos.

2.6. Gonad tissue isolation for transcriptome analysis

As gonads could be used either for histology or genentic analyzing, 2-3 individuals per test series, sex and group were taken for genetic analysis. Because of the six test series, we used ten individuals altogether for transcriptome analysis. Their individual right and left gonads were excised, cleaned from adhesive tissue and immediately frozen in 200 µl RNA lysis buffer (Promega) for later RNA isolation. Total RNA of whole right and left gonads (ten individuals of each group: control, solvent control, TBT 10 pg TBT-Sn/g egg and MT 30 pg/g egg) was extracted using the SV Total RNA Isolation System Kit according to manual 048 (Promega). Deviating from the protocol, on-column DNaseI digestion of genomic DNA was elongated from original 15 min to 30 min. A second DNaseI digestion was carried out with Baseline-Zero™ DNase (Epicentre; provided by Biozym Scientific GmbH, Hessisch Oldendorf, Germany) in solution to ensure that the samples were completely free of DNA. Total RNA concentration was estimated in a dilution series with the LabelGuard NanoPhotometer (Implen, München, Germany). RNA quality and quantity was further determined using a Caliper lab-on-a-chip system (Agilent – CALIPER, Hopkinton MA, USA). All isolated total RNA samples had an RNA Integrity Number (RIN) ranging from 8.5 to 10.0 (highest quality).

2.7. Library preparation and bioinformatics

DeepSuperSAGE libraries were constructed and validated by qPCR as previously described (Scheider et al., 2014a). Sequencing was performed on Illumina's Genome Analyzer IIx, and subsequent base calling was carried out by Illumina's GAPipeline. The obtained raw data were further analyzed with GenXPro's SuperSAGE data processing pipeline. First, distinct libraries were sorted out from the bulked sequencing data according to their respective indices, followed by elimination of PCR-derived tags identified by TrueQuant technology (GenXPro). Next, 26 bp SuperTAGs were extracted from the remaining sequences, and subsequently mapped on the *Gallus gallus* genome Galgal4 (Version 72.4, published in November 2011 by the International Chicken Genome Consortium) using the short read mapper Novoalign v2.07.13 (Novocraft Technologies). Feature annotation for the mapped loci was performed on the basis of the standard Ensembl genebuild pipeline (please consult http://www.ensembl.org/info/docs/genebuild/2013_04_chicken_genebuild.pdf for a detailed description of the annotation), and tags were counted via the Python package HTSeq v0.5.4p2 (EMBL Heidelberg, https://pypi.python.org/pypi/HTSeq). The unambiguously mapped reads for each library were normalized to a million sequenced reads in total (tags per million; TPM). Read numbers for the pooled datasets from left and right gonads were obtained by *in silico* pooling of the raw sequencing data from the respective left and right gonad prior to the analysis via the GenXPro SuperSAGE data processing pipeline. Fold changes were determined by pair-wise comparison of the normalized tag numbers in two particular libraries, and the statistical significance was assessed by *chi* square tests according to Man and colleagues (Man et al., 2000). TPM counts of zero were adjusted to 0.05 to allow for calculation of fold changes, even if a given tag was only present in one of the libraries (see supplementary table T1).

2.8. Data accessibility

The pre-processed raw data are submitted to the Genome Expression Omnibus (Accession number: GSE108141).

3. Results and Discussion

3.1. Effect of sesame oil on gonadal differentiation and gene expression

In (eco-) toxicological studies the selection of the best suited solvent for a given substance is a critical decision. Ideally, the solvent should resolve the substance completely but should not elicit an effect on its own. Since TBT is lipophilic, we had to use an oily carrier. Because we did not want to use an emulsion of several compounds as previously described for chicken studies (Berg et al., 1998; Berg et al., 2001a; Berg et al., 2001b), we used sesame oil as solvent, which was previously applied in several vertebrate endocrine disruption studies (Chataigneau et al., 2004; Demissie et al., 2008; Fail et al., 1998; Liang et al., 2013). In other bird studies, sesame oil did not show any significant morphological effect (Eising et al., 2001; Muller et al., 2005; von Engelhardt et al., 2006). Our histological analysis revealed a slight but notable reduction in the cortex thickness of gonadal tissue from female chicken embryos treated with sesame oil (Fig. 1C), resulting in the occurrence of 21% virilized females in the solvent control compared to 0% in the untreated control (Table 1). This difference is not statistically significant (Fisher's exact test, $p = 0.061$). The treatment did not induce

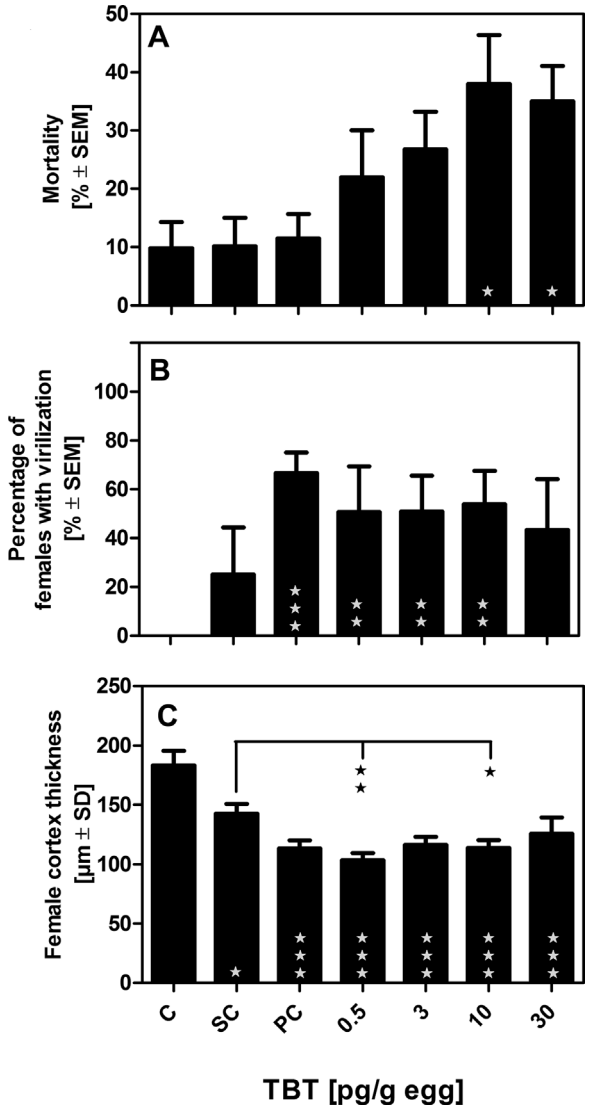

Fig. 1. Effect of TBT on chicken embryos. A: Mortality in response to TBT until embryonic day 19. B: Numbers of morphologically virilized genetic females based on structural anomalies in gonads. C: the corresponding cortex thickness of female animals. C = Control, SC = Solvent control; PC = Positive Control (MT). Statistics: 1-way ANOVA with Dunnett's Post test for A and C, Fisher's exact test for B * = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$.

any other changes in gonadal structure and was apparently non-toxic (Fig. 1A), which confirms the previous findings.

In line with the observed histological alterations, gene expression profiling of sesame oil-treated embryos revealed a strong impact of the solvent on the identified transcription profiles. In total, 417 and 606 genes were significantly differentially expressed in males and females, respectively (Fig. 4). Serum response factor (SRF) is a transcription factor that participates in regulation of embryonic development, and is found to be down-regulated by 50% in male embryos, while its abundance in female embryos corresponds to approximately a third of its abundance in completely untreated embryos. Homeobox protein HoxA4 (HOXA4), another transcription factor that is involved in embryonic development, is significantly down-regulated in sesame oil-treated embryos (log_2 fold change of 2 in both sexes). The deregulated expression of both *SRF* and *HOXA4* may contribute to the observed reduction of cortex thickness in female embryos, and underlines the importance of a thorough characterization of the employed solvent to account for off-target effects in toxicological studies.

3.2. Effects of TBT on gonadal morphology and differentiation based on histological alterations

In total, gonadal morphology and differentiation on E19 was analyzed in 241 embryos in six test series. At the two highest TBT doses (10 and 30 pg TBT-Sn/g egg) mortality increased significantly by more than 30% relative to the untreated and the solvent control (approx. 10%, Fig. 1A). Embryos often died before embryonic day 7 at first candling.

In all TBT and the MT (positive control) exposed groups anatomical virilization of female gonads was observed. Virilization comprised the development of a longer right gonad and/or a rounder (less folded and less flat) left gonad (Figs. Fig. 1B, Figure 2B), resembling anatomical characteristics of a developing ovotestis (Koba et al., 2008). On the histological level, virilization effects were mainly characterized by a significant reduction of the cortex (Fig. 1C, Fig. 2D + E). Overall, around 50% of the female embryos were affected by virilization of the gonads in the three lower TBT exposure groups (0.5, 3 and 10 pg/g), while 30% were affected by the highest TBT dose (30 pg/g) and 68% in the positive control (30 pg MT/g) (Table 1). Both, the lacking significance of cortex reduction (regarding 3 and 30 pg/g) and the decrease in the percentage of virilization in the ovaries of females from the higher exposure groups does not necessarily reflect a reduced effect of higher concentrations of TBT, but might be linked either with the increasing dose-dependent mortality rate of exposed embryos (Fig. 1a), or related to the typical non-monotonic dose-response relationship of endocrine disrupters, where the effect is not increasing in a linear way but characterized for example by an u-shaped (Fig. 1C) or inverted u-shaped (Fig. 1B) response (Lagarde et al., 2015; Vandenberg et al., 2012) Contrary to ovaries, testes did not exhibit any anatomical or histological alterations in the TBT-treated groups and the positive control when compared to the untreated and solvent control (Fig. 2C + F).

3.3. Gene expression profiling

Of the sequenced 32.2 million reads 85% could be mapped to the chicken genome. In total 14,074 genes are represented by unambiguously mapped reads that did not return more than one hit (supplementary tab T1). As previously reported, gene expression in the gonads of untreated embryos strongly differs between sexes and between the left and right testes and ovaries (Scheider et al., 2014a).

Table 1
Total number of injected fertile eggs, surviving embryos until E 19, number of genetic males and females and number and proportion of virilized females in the different treatment groups. Virilized females exhibited a thinner cortex and/or at least 10 Tubuli seminiferi in histological sections of the gonad. Embryos that died before E19 are not listed.

	Σ fertile eggs	Σ surviving embryos	Σ genetic male	Σ genetic female	Σ virilized females (%)
Untreated control	51	47	27	20	0 (0)
Solvent Control	31	27	13	14	3 (21)
MT 30 ng/g egg	54	48	23	25	17 (68)
TBT-Sn 0.5 pg/g egg	40	33	14	19	10 (53)
TBT-Sn 3 pg/g egg	45	34	18	16	10 (63)
TBT-Sn 10 pg/g egg	44	28	10	18	8 (44)
TBT-Sn 30 pg/g egg	36	24	14	10	3 (30)

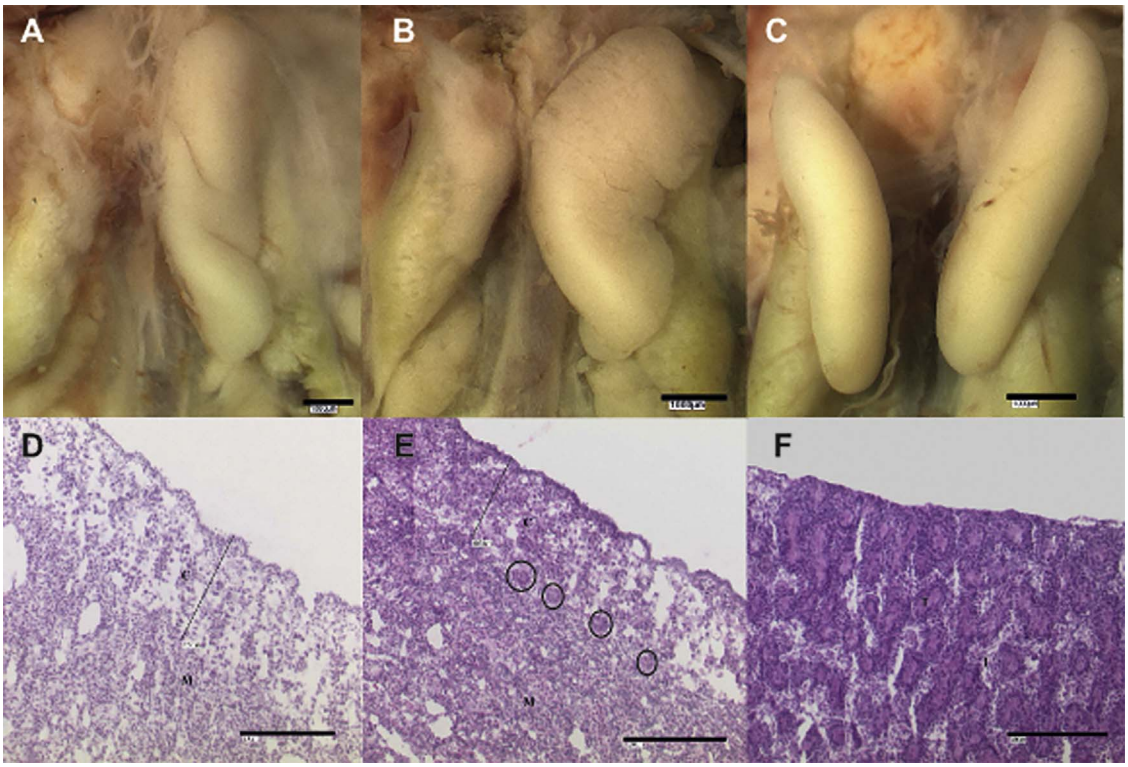

Fig. 2. Morphological (A-C; bar = 1 mm) and histological (D-F; bar = 200 µm) photographs of gonads in chicken embryos (E19) A + D: Control female with large and folded left and regressing right gonad, section through left gonad with cortex and medulla. B + E: Genetic female (10 pg TBT-Sn/g egg) with masculinized gonads: the left gonad gets rounder, more firm, and the right gonad gets taller. The section indicates Tubuli seminiferi in the outer medulla region (encircled) and a reduced cortex. C + F: Control male with two uniform testes and seminiferous cords and interstitial space.

Fig. 3 illustrates the most differentially expressed genes between the sexes in control as well as solvent, TBT or MT-treated chicken embryos. Apparently, the natural sex-specific gene expression is only partially continued in the response pattern caused by the exposure to the test chemicals. Most of the affected genes are activated or inactivated regardless of the sex, but some genes exhibit a sex-dependent differential expression under substance influence, partially differing between MT and TBT.

3.4. MT-induced changes in gonadal differentiation and gene expression

MT is the equivalent of endogenous testosterone, which is transformed to estrogen by the aromatase enzyme. In female embryos, the natural level of estrogen is higher than the testosterone level due to an increased activity of aromatase. The balance of both, estrogen and testosterone, is crucial for normal embryonic development of gonadal tissues in males and females. In our study, the cortex of female chicken gonads was still present, but thinner in MT-treated embryos (Fig. 1C). The change in cortex thickness in these embryos implies that the testosterone-estrogen relationship is shifted in favor of androgens such that the volume of the whole gonad is changed together with cortex thickness, while the gonad is not completely sex-reversed. Apparently, only complete left-side ovariectomy (Wallenburg, 1982) or *in ovo* treatment with the high-potential, non-steroidal aromatase inhibitor fadrozole (Abinawanto et al., 1998; Vaillant et al., 2001; Wartenberg et al., 1992) results in embryonic phenotypic female-to-male sex-reversal. The latter, however, has to be maintained by further treatment within juvenile development, otherwise this transformation is reversible in mature birds (Burke and Henry, 1999). Additionally, sex-reversed female chicken cannot be transformed into completely functional neo-males (Abinawanto et al., 1998; Vaillant et al., 2003; Yang et al., 2011). We therefore conclude that the *in ovo* application of 30 pg MT/g egg does generally not influence the development of the female gonad up to a neo-male, but nevertheless induces histomorphological changes of the tissue.

Even though MT affected development in several studies with

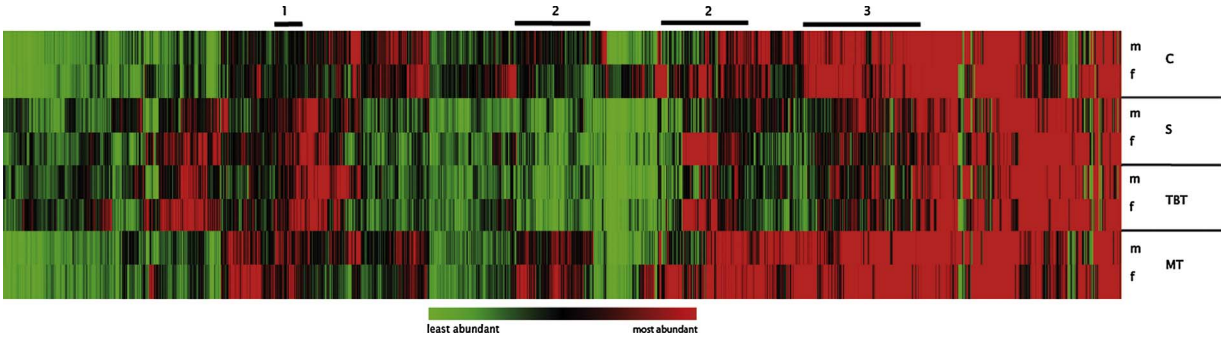

Fig. 3. Heat map of the most differential gene expression in the gonads of genetic males and females. $p_{max} < 9E^{-3}$, hierarchical clustering. 1 = S, TBT and MT differ from C, 2 = S, TBT and MT differ from C sex-dependently, 3 = S and TBT differ from MT and C.

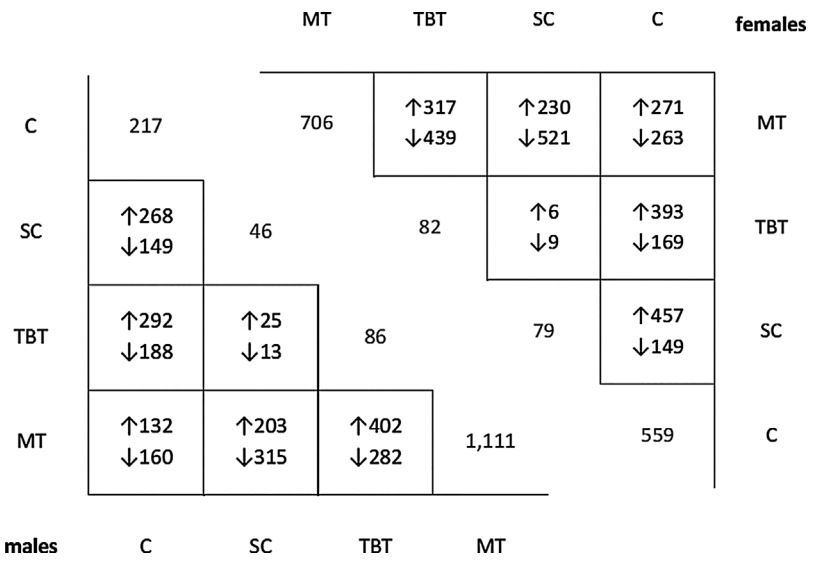

Fig. 4. Pair-wise comparison of differentially expressed transcripts in male (left) and female (right) gonads along with the number of exclusively expressed transcripts (gray numbers) for each group. Listed are unambiguously annotated reads with $\log_2$ fold changes stronger than |1.5| and p-values below 0.000001. Up- and downregulated transcripts are indicated by corresponding arrows for the library on the ordinate, respectively. Please consult Fig. 1 for further information.

different taxa (Cevasco et al., 2008; Faucounau et al., 1995; Feist et al., 1995; Giusti et al., 2013), MT and testosterone propionate did not have any morphological effects on the gonads of treated male and female quails (Shibuya et al., 2004; Utsumi and Yoshimura, 2009). In addition to the observed reduction of cortex thickness in our study, several MT- and TBT-treated females showed *Tubuli seminiferi* in the medulla, especially close to the cortex (Fig. 2E), and this did not occur in control and solvent-treated embryos. Our results therefore deviate from the studies in quails and confirm the findings of the androgen studies that reported an impact of MT during development. We assume that the technique of injection of the substance, the day of application of the compound as well as the microscopic analysis might be possible causes for this. Firstly, we injected the endocrine disrupter directly into the yolk, just below the embryo, while the injection mode in the quails studies (Shibuya et al., 2004; Utsumi and Yoshimura, 2009) was either into the egg white or into the air cell. Secondly, we injected on embryonic day 1 to imitate the maternal transfer and to fully assess the impact of our tested compounds during the whole embryonic development. Contrasting to this, Utsumi and colleagues (Utsumi and Yoshimura, 2009) applied the substance on embryonic day 13, followed by histological examination only three days later. The substances were therefore available for a shorter period compared to our study. Moreover, we administered the substance at a much earlier stage of development where changes in a few genes in the few cells that represent the gonads at E1 may have effects that have consequences in all subsequent cell divisions. Additionally, we processed an intensive histological investigation which was not considered in the other studies.

Gene expression profiling of gonadal tissue from male and female embryos subsequent to MT treatment indicates that the exposure to MT evokes transcriptional alterations that are distinct from those induced by the solvent. In comparison to the sesame oil-treated group more than 200 genes were significantly upregulated in both sexes, while 315 and 521 genes are downregulated in male and female chicken embryos, respectively (Fig. 4). Among the transcripts that are upregulated in both sexes upon MT treatment are the mRNAs that encode the two transcription factors V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog F (MAFF) and transcription factor CP2 (TFCP2). Both are involved in embryonic development, and especially TFCP2 is known to regulate expression in chicken embryonic stem cells via activation of the cENS-1 promoter (Acloque et al., 2004; Lecoin et al., 2004). We additionally found a highly significant upregulation of the gene encoding (Sex Determining Region Y)-Box 9 (SOX9) in MT-treated males compared to the solvent control. SOX9, a transcription factor related to the mammalian testis-determining factor SRY, is expressed shortly after the Anti-Mullerian Hormone, which represents a key player for sexual differentiation (Oréal et al., 1998), and was identified as androgen-responsive gene in the trout testis (Rolland et al., 2013). Transforming Growth Factor Beta 3 (TGFB3) is involved in embryonic gonadal development (Memon et al., 2008), and the encoding mRNA is strongly downregulated in both sexes after MT treatment.

In our precedent study the mRNA encoding Gallinacin 10 (GAL10) was highly differential expressed between the sexes with an increased expression in the ovaries (Scheider et al., 2014b). Here, we report a dramatic, about 10-fold upregulation of *GAL10* transcription in male MT-treated embryos compared to the solvent control group while in female embryos it is not even doubled. The sex-dependent changes in *GAL10* expression therefore reflect the hormonal imbalance that leads to the observed histomorphological changes of gonad tissue in MT-treated chicken embryos. Taken together, the MT-induced differential expression seems to affect embryonic development by altered expression of important transcription factors such as MAFF, TFCP2, and SOX9, which in turn affect expression of secreted proteins such as GAL10.

3.5. Altered gonadal differentiation and gene expression in TBT-treated chicken embryos

TBT affects embryonic tissue in two different ways: the toxic and the hormonal component. Egg mortality increased significantly after treatment with the two highest TBT doses, which is clearly linked to increased toxicity. The second component of TBT is the hormonal reaction. As with MT, the cortex of female chicken gonads is generally thinner in TBT-treated embryos and likewise several females developed seminiferous cords in the medulla.

On the transcriptional level we find a significantly increased expression of *GAL10* in both sexes subsequent to TBT treatment compared to the solvent control whereof the male gonads reacted most prominently (Table 2). The upregulation in gonadal tissue from male embryos that were exposed to TBT corresponds to the 10-fold upregulation that is present subsequent to MT treatment. Interestingly, MT-treated female embryos do not show a strong differential expression of *GAL10*, while TBT treatment evokes a 3.3-fold upregulation. The fact that MT and TBT-treated males show an almost identical expression pattern of *GAL10*, which was previously identified as sex-specifically expressed in female chicken embryos, is an indicator of the hormonal reaction that is evoked by exposure to TBT. Two other members of the antimicrobial gallinacin family react to TBT treatment in a sex-dependent manner. The mRNAs encoding GAL1 and GAL6 are significantly downregulated after TBT treatment in females, while expression in male embryos is not

Table 2
Fold change of selected pluripotency and differentiation markers in response to MT and TBT in male and female gonads relative to the solvent control (S) in SuperSAGE data. Bonferroni correction * $p < 0.1$ ** $p < 0.5$ *** $p < 0.01$, ns = not significant, na = not available, red = upregulated, green = downregulated.

gene symbol	male				female			
	S-MT		S-TBT		S-MT		S-TBT	
CTCF	1.12	ns	1.37	ns	-1.14	ns	2.20	***
CTGF	1.05	ns	1.66	ns	-1.49	ns	2.54	***
CYP19A1	221.92	ns	na	na	-1.21	ns	-1.34	ns
FGF12	-3.13	***	-1.15	ns	-1.53	ns	2.57	***
GAL1	1.53	**	2.00	ns	1.16	ns	-2.29	***
GAL10	9.75	***	11.57	*	1.36	***	3.33	***
GAL6	1.03	ns	-1.55	ns	-2.01	***	-3.31	***
HSP70	-7.40	***	-1.57	ns	-6.54	***	-1.95	ns
MAFF	34.20	***	1.94	ns	2339.40	***	102.88	ns
NFIB	1.04	ns	9.87	***	-7.27	***	1.58	***
SOX9	3.97	***	-1.72	ns	272.62	ns	102.88	ns
TFCP2	14.29	***	-81.20	ns	6.49	ns	-63.54	ns
TGFB3	-12.03	***	1.33	ns	-10.02	***	4.20	ns

affected. Since *GAL6* is also significantly downregulated in MT-treated female embryos, downregulation of this gene is likely linked with the endocrine potential of TBT by provoking female-specific genes to be expressed in males. Downregulation of *GAL1*, on the other hand, might contribute to the stress response that is evoked by the toxic component of TBT.

The genes encoding connective tissue growth factor precursor (CTGF) and fibroblast growth factor 12 (FGF12) also display a sex-dependent differential expression subsequent to TBT treatment. The abundance of the mRNAs encoding both growth factors is significantly changed by the treatment and found to be more than doubled in female embryos. In contrast to the increased expression of *FGF12* in TBT-treated females, *FGF12* is also downregulated in gonadal tissue from MT-treated male embryos. Both growth factors are involved in embryogenesis and the increased expression might therefore contribute or result from the altered cortex structure that is observed in gonadal tissue from TBT-treated females at E19 (Pizette and Niswander, 1999). CCCTC-Binding Factor (CTCF) is an important transcriptional regulator that binds to tens of thousands of genomic sites in mammalian cells (Holwerda and de Laat, 2013), and the encoding mRNA is significantly upregulated in female gonad tissue compared to the solvent control. TGGCA-Binding Protein (NFIB) represents another important transcriptional regulator that is differentially expressed between the sexes subsequent to TBT treatment. The encoding mRNA is approximately 10-fold more abundant in male embryos in comparison to the sesame oil-treated group, but there is no differential expression between the corresponding female groups. However, we find an almost 7-fold downregulation of *NFIB* in gonadal tissue from MT-treated female embryos compared to the solvent control. TBT thus seems to evoke a contrary differential expression of *NFIB* in exposed female embryos as in MT-treated male embryos.

Interestingly, aromatase (CYP19A1) expression seems to be affected only by the solvent control. While its expression is increased about 4-fold subsequent to injection of the solvent, no significant differences are evoked by additional injection of TBT or MT. Since TBT is known to inhibit aromatase activity this implies that the induced expression of *CYP19A1* by the applied solvent forestalls a further differential expression or that this response has already passed by at embryonic day 19 (Bruggeman et al., 2002).

Other surveys that investigated the influence of organotin compounds on RNA expression generally focused on a small set of predefined genes. One of the few studies with a bigger set of genes was a 24 hour exposure of adult tunicates to 100 nM TBT where the expression of 13,400 protein coding genes was tested on a cDNA microarray (Azumi et al., 2004). Subsequent analysis revealed the differential expression of genes that are mainly involved in stress responses and detoxification. Due to the lack of a positive control the hormonal component of the reaction could not be determined in the data, and apparently the applied concentration of 100 nM of TBT already caused strong toxic effects. Among the stress-responsive genes in tunicates were members of the cytochrome P450 family as well as the 70 kd heat shock protein (HSP70). Although there are some indications for differentially expressed mRNAs that encode cytochrome P450 none of these genes exhibit any significant differential expression in comparison to the solvent control in our survey. *HSP70*, on the other hand, exhibits a strong differential expression in gonadal tissue from MT-treated embryos of both sexes, but also in tissue from the solvent control group. However, we did not detect a significant deregulation of *HSP70* expression in the TBT- groups.

4. Conclusions

Individuals are often most sensitive to endocrine disrupters in their early life stages (embryos, fetuses and juveniles). During these stages, endocrine disrupters interfere with developmental and organizational processes such as sexual differentiation (see e.g. (Ankley and Johnson, 2004; Holwerda and de Laat, 2013; Rolland et al., 2013)). In contrast to mammals, where steroid synthesis is initiated in the gonads after the termination of their differentiation, estrogen synthesis in birds begins in undifferentiated embryo gonads, which are thus more sensitive to hormonal manipulation (Trukhina et al., 2015). Exposure during this critical window of development may induce effects on sexual differentiation which are irreversible and thus persist in later life, whereas identical exposure levels during adulthood often result in temporary and reversible alterations of reproductive performance or of somatic parameters (Nichols et al., 2011). However, effects of early life stage exposures may only become apparent in the sexually mature organisms during the reproductive phase (Matthiessen and Johnson, 2007; Nichols et al., 2011). In view of their potentially serious consequences such delayed irreversible effects cause greatest concern (Si et al., 2012).

The present study provides the first survey linking the morphological alterations of gonadal tissue from chicken embryos that are induced by the known endocrine disrupter TBT with the underlying gene expression. We applied a single test compound injection at a very early developmental phase to simulate maternal transfer of the organotin compound. This treatment results in virilization of female gonads in a significant number of individuals and allowed us to identify differentially expressed transcription and growth factors that are associated with this phenotype in E19 embryos. Although the effect of the solvent cannot be neglected, MT that served as positive control allowed for discrimination of the toxic and hormonal effects induced by TBT. Surprisingly, we identified an aberrant expression of sex-dependent genes such as GAL10, but not of the mRNA encoding the TBT-inhibited aromatase. Since gonad development is well progressed at E19 aromatase inhibition might have already been compensated for. Our following studies will therefore aim to elucidate the transcriptional landscape after exposure to endocrine disruptive compounds in earlier embryonic stages of *Gallus gallus*. The final goal is to find parallel gene expression shifts in younger embryos to find a technique for an animal replacement study within the scope of a potential new 3R animal replacement method (Russel and Burch, 1959).

Conflict of interest statement

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxlet.2017.11.019.

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