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The domestic fowl (*Gallus gallus domesticus*) embryo as an alternative for mammalian experiments – Validation of a test method for the detection of endocrine disrupting chemicals

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HIGHLIGHTS

- The first study to systematically examine the variability of individual parameters in control groups of chicken embryos.
- The low natural variability of the test system results in a good reproducibility in control and substance-treated groups.
- Reference values for developmental and gonadal endpoints, suggested as validity criteria, are provided.
- The chicken embryo is a suitable system for the detection of EDCs and a promising alternative to mammalian experiments.

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ABSTRACT

In recent decades the embryo of *Gallus g. domesticus* has been widely used as a model for the study of early sexual development and the potential impact of substances affecting development, including endocrine disrupting chemicals (EDCs). Since there is no standardized procedure available for experiments with the chicken embryo, the objective of our project is to expedite the protocol to assess the potential effects of EDCs on early sexual differentiation. The main aim of the present study was to systematically investigate the natural variability of individual developmental and histological key parameters in untreated and solvent-treated control groups, since this has been insufficiently addressed so far. A further aim was to provide robust values for all parameters investigated in control and substance experiments, using two known estrogenic compounds, bisphenol A (75/150/300 μg/g egg) and 17α-ethinylestradiol (20 ng/g egg). On embryonic day 1 eggs were injected with the estrogenic compounds. On embryonic day 19 histological gonadal data as well as morphological parameters were noted. In baseline experiments with control groups the selected endpoints showed reproducible results with low variabilities. Furthermore, gonadal endpoints responded sensitively to the treatment with the two model EDCs. Thus, these endpoints are recommended for the assessment of suspected EDCs in which the values provided for all parameters can serve as validity criteria in future experiments. The embryo of *G. domesticus* has shown to be a suitable alternative to currently accepted mammalian bioassays for the impact assessment of EDCs on reproductive tissues.

1. Introduction

Reproductive disorders in animals and humans caused by chemical substances that are suspected as endocrine disrupters have gained major interest for science and society. Especially the interference of these chemicals with sexual development and reproduction plays a major role. Endocrine disrupting chemicals (EDCs) are naturally or synthetically occurring compounds and may affect the natural balance of hormones or alter the endocrine control in animals. Various studies confirm the suspicion that EDCs may adversely affect wildlife and human health (Colborn et al., 1993; Giesy et al., 2003; Vandenberg et al., 2012; Mallozzi et al., 2016). In view of the large number of constantly used chemicals, e.g. in agriculture, industry or medicine, it is expected that EDCs

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end up in the environment and may be incorporated by animals and humans and, thus, may impact their hormonal systems. Only a small proportion of these substances has been tested for potential effects on the hormonal system. The pending investigation of further substances will therefore produce a high demand for animal experiments in the coming years, since most of these substances have to be tested *in vivo*.

The Hershberger assay and uterotrophic assay are two internationally standardized tests for EDCs with androgenic or estrogenic activity, which are based on rodents (OECD, 2007, 2009). These tests use juvenile or adult rats or mice and, thus, not the most sensitive life stage for EDCs, the developing embryo (Lan and Katzenellenbogen, 1976; Cook et al., 1997; Grote et al., 2004). Moreover, the critical effect on sexual differentiation in higher vertebrates is currently studied by a standardized multigenerational test, using quail and other avian species (OECD, 1984). However, this test is time intensive and costly and a very high number of individuals is needed. As a result, nearly 2.7 million vertebrates are annually consumed for animal testing throughout Germany, almost 256,600 of them for toxicological studies (BMELV, 2015). This high number is hardly acceptable for animal welfare reasons, and this is why the search for a suitable animal replacement system is of great importance.

As early as 1959, Russel and Burch dealt with the subject of treating experimental animals in a more human way (Russel and Burch, 1959, reprinted 1992). Their principle of the 3Rs represents the widely accepted ethical standard for the use of animal experiments, which is already being implemented and applied in many laws and technical guidelines. The 3Rs stand for Replacement, Reduction and Refinement.

An interesting and promising approach to avoid animal testing is the development of a standardized procedure for the testing of potential EDCs in avian embryos. In recent years, the avian embryo has emerged as a model for the study of environmental pollutants, including EDCs (Fry and Toone, 1981; Fry, 1995; Berg et al., 1998, 1999, 2001a, 2001b, 2004; Berge et al., 2004; Biau et al., 2007; Brunstrom and Halldin, 2000). The present study is part of a project aiming to advance a replacement method for testing hormonally active compounds in birds, where fertilized eggs of the domestic fowl (*Gallus gallus domesticus*) are used (Berg et al., 1998, 1999). Chicken eggs provide significant advantages in the testing of chemicals, as they are available throughout the year and the injection of substances directly into the yolk allows specific and standardized dosages (Berg et al., 1999). As the hen affects the development of its offspring by transferred genetic materials as well as hormones (Carere and Balthazart, 2007), substances incorporated by the mother may consequently also influence the development of the offspring even originally or as metabolites in the allantoic fluid (Kamata et al., 2006). However, in contrast to developing mammals or aquatic species, the chicken egg is a closed system lacking any exchange with its environment except for the interchange of gases. Thus, one injection of a test compound results in chronic chemical exposure, because no exchange or loss of the substance is possible. A single injection may therefore be sufficient to influence the developing embryo (Davies et al., 1997; Gooding et al., 2003; McAllister and Kime, 2003; Zhang et al., 2007). The embryonic development is fully described (Keibel and Abraham, 1900; Hamburger and Hamilton, 1992; Starck and Ricklefs, 1997) and the individual developmental stages are clearly visible and easily accessible. Also the endocrine system of adult birds is largely similar to that of mammals (Lange et al., 2002) which allows a limited transfer of the resulting data to humans, as little differences still exist, e.g. the genetic and endocrine control of gonadal development is not identical to humans. It is known that the influence of xenohormones in birds during embryonic development can lead to irreversible malformations of the gonads or later to a disturbed gender-related behavior, whereas EDCs may exert less severe and often reversible effects in the less sensitive adult stage (Adkins-Regan, 1990; Ottinger and Abdelnabi, 1997). Therefore, the chicken embryo is a suitable model for the study of early sexual development and the potential impact of EDCs regarding the replacement for other vertebrate or even mammalian models.

However, the characterization of the normal development of the test organism without exposure to EDCs should be a first and fundamental step for the successful development of a test design based on chicken embryos. Based on previously published studies we decided for different common and gonad-based endpoints which have already been shown to be affected by EDCs (Scheib and Reyssbrion, 1979; Scheib and Baulieu, 1981; Scheib, 1983; Berg et al., 1998, 1999; Halldin et al., 2003). These endpoints are the surface area of left and right ovary or testis, the cortex thickness of left ovary or testis and the percentage of seminiferous tubules of left testis. In an extensive series of experiments, we investigated these endpoints and worked out a detailed morphological and histological description of embryonic gonads. Special focus was on the systematic examination of the variability of individual parameters in untreated and solvent-treated control to demonstrate the natural variability of the test system. Furthermore, this may allow the determination of reliable reference values for each endpoint which may serve as validity criteria in future experiments. Also, these values could be used for the comparison between controls and substance-treated groups. Moreover, the present study draws a comparison between untreated controls and solvent controls, which received dimethyl sulfoxide (DMSO) as a solvent. At last this should allow a statement about the conformity and validity of the data of both test groups and whether DMSO is a suitable solvent for the chicken egg test. With the knowledge about the normal development of the chicken embryos it will be possible to obtain a reliable comparison between control groups and test groups treated with different EDCs.

The second step in our investigations was to evaluate the effects of the chosen EDCs on gonadal differentiation as assessed by the previously chosen endpoints. After profound analysis of existing literature we determined two promising compounds and analyzed their effects on embryonic development with special focus on potential gross morphological and histological changes of the gonads. The selected estrogenic substances 17α-ethinylestradiol (EE_2), a synthetic hormone primarily used for contraception, and bisphenol A (BPA), a monomer used as basic material for polycarbonate plastics, have already been widely used in the study of EDC-related effects on different groups of organisms (Watts et al., 2001; Oehlmann et al., 2006; Pettersson et al., 2006; Birceanu et al., 2015) including the bird embryo (Berg et al., 1998, 1999, 2001a, 2001b, 2004; Biau et al., 2007; Oshima et al., 2012).

In the end we want to demonstrate that the test method based on chicken embryos is a suitable system for the detection of EDCs. If the chicken embryo proves to be similarly sensitive or even more sensitive to EDCs than already established tests, the successful development of a standardized test system based on chicken embryos is very promising. As an alternative to current assays, this test system could then contribute to a reduction in the number of consumed experimental animals.

2. Materials and methods

2.1. Dosing

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. Fertilized eggs of white Leghorn chicken (*Gallus gallus domesticus*) were obtained from a local breeder (LSL Rhein-Main, Dieburg, Germany). The eggs were incubated at 37.5 °C ± 0.5 °C and 60% ± 10% relative humidity and turned over eight times a day in a fully automated incubator (J. Hemel Brutgeräte, Verl, Germany). For the baseline experiments all trials involve the formation of two control groups, the negative control (NC), which includes unmanipulated eggs and the solvent control (SC) which received DMSO (CAS: 67-68-5; purity = 99.5%; Applichem, Darmstadt, Germany) as a carrier. In these experiments, either 15 μL or 60 μL of the solvent were used. In ovo-exposure experiments investigating the effects of EDCs additionally include an EE_2-treated group (20 ng/g egg weight; CAS: 57-63-6; purity: ≥ 98%; Sigma Aldrich Chemie GmbH, München, Germany) and three BPA-treated groups (75, 150, 300 μg/g egg weight; CAS: 80-05-7; purity: ≥ 99%; Sigma Aldrich Chemie GmbH, München, Germany). In previous studies, these substance concentrations have proven to strongly affect the reproductive organs of chicken embryos (Berg et al., 2001b, 2004). The corresponding doses were administered to the eggs dissolved in 15 μL of the solvent.

Solvent and substances were injected into the yolk via a small hole on the circle of the widest diameter of the egg using Hamilton microliter syringes and needles (ga22s/51mm/pst2). The injection was performed on day one of incubation. After injection the hole in the shell was sealed with agarose gel (3%, in phosphate buffered saline). During incubation, eggs were periodically checked by candling to identify and discard dead embryos or unfertilized eggs.

2.2. Dissection, tissue preparation and evaluation

The embryos were dissected on day 19 of incubation, two days before anticipated hatching. Unfertilized eggs were excluded from statistics. All embryos were examined for deformations of body or inner organs. Ovaries and testes were examined for deformations under a stereo microscope. Photos of all gonads (Diskus, Carl H. Hilgers, Königswinter, Germany) were taken for further analyses of the gonad surface area, in which the entire visible surface of each single gonad was determined with an image editing program (Fiji is just ImageJ, Open Source). Gonads were dissected and fixed in Bouin's solution for 24 h. The fixative was rinsed repeatedly with 80% ethanol. Ethanol was removed by saccharose solution (10, 20 and 30% in phosphate buffered saline) before gonads were embedded in Tissue-Tek® (Sakura Finetek Europe B·V., Alphen aan den Rijn, Netherlands). Gonads were sectioned (6 μm) by a freeze microtome (Microm HM 500 O, Thermo Fisher Scientific Germany, Bonn, Germany) at −23 °C. Tissue sections were stained with hematoxylin and eosin. Histological examination was performed using a light microscope (Olympus BX50, Olympus, Tokyo, Japan) and a camera (JVC Digital Camera, KY-F75U, Yokohama, Japan). In both sexes the thickness of the cortex and in male embryos additionally the percentage of the area of seminiferous tubules were measured with an image analysis system (Diskus, Carl H. Hilgers, Königswinter, Germany).

2.3. Determination of sexual genotype

DNA isolation for each individual was carried out with a tissue sample from the heart taken during dissection. Dead embryos, identified and removed before dissection, were also sampled. All embryos were typed for their sexual ZZ or ZW genotype according to Fridolfsson and Ellegren (1999), based on PCR with a single set of primers. Amplification was performed using qPCR and the primers 2550F "5′-GTT ACT GAT TCG TCT ACG AGA-3′" and 2718R "5′-ATT GAA ATG ATC CAG TGC TTG-3′". Following amplification, all qPCR products underwent a melting curve, which resulted in characteristic bands for each sex. Male embryos had a single 600-bp CHD1-Z specific fragment with a melting temperature of nearly 84 °C, while females had a 600bp-CHD1-Z specific fragment and an additional 450-bp CHD1-W female-specific fragment, with a melting temperature of nearly 82 °C.

2.4. Measurements and statistics

For the examination of the surface area of left and right gonads the data from 15 (baseline experiments with controls) or 4 (experiments with BPA and EE_2) individual experiments were analyzed. For the histological endpoints cortex thickness of left gonads as well as the percentage of seminiferous tubules in left testis, the data from 11 (baseline experiments) or 4 (experiments with BPA and EE_2) experiments were analyzed. For the baseline experiments in particular, the range of variation within the control groups, as well as any deviations among the control groups was in the focus of investigation. For experiments with BPA and EE_2 it was examined whether and how the endpoints specified in the baseline experiments respond to the treatment with EDCs.

The cortex thicknesses of male and female left gonads as well as the percentage of the area of seminiferous tubules in male left testes were measured. 10 (baseline experiments) or 5 (experiments with BPA and EE_2) sections for each embryo and endpoint were evaluated. The selected sections were exclusively taken from the gonads middle sectional plane. To determine the cortex thickness different representative areas around the gonad were chosen. From each section five measurements were performed to determine the cortex thickness. For males the area of all seminiferous tubules in a defined image section were measured to determine a representative percentage in the male left testis. Therefore random representative image sections were selected which showed only the medullary tissue but not the cortex region.

For each endpoint of the baseline experiments the mean value of each of the 11 (cortex thickness and seminiferous tubules) or 15 (gonad surface areas) individual experiments was calculated. From the mean values of the individual experiments, the arithmetic means of NC and SC were calculated. Statistical evaluation was carried out with GraphPadPrism® (version 5.01, GraphPad Software Inc., San Diego, USA). Data of all endpoints following normal distribution were verified by *t*-test. If data did not follow normal distribution, Man-Whitney-U-test was used for analysis. Quantal data were evaluated using Fisher's exact test. In the baseline experiments we first determined within the respective control groups whether the 11 (cortex thickness and seminiferous tubules) or 15 (gonad surface areas) individual experiments statistically differ from each other (one-way ANOVA with Newman-Keuls post test). It was also determined within the respective control groups whether the individual experiments statistically differ from their arithmetic mean (one-way ANOVA with Dunnett's post test). Finally, a direct comparison of the individual experiments of NC and SC was made to analyze whether they differ from each other statistically (one-way ANOVA with Newman-Keuls post test).

For the test series with BPA and EE_2, the results of 4 test runs were merged and analyzed. Statistical evaluation was carried out according to Green and Wheeler (2013). When the untreated negative control did not differ statistically from the solvent-treated control (unpaired *t*-test; $p > .05$), these two groups were merged as a common control. When both controls differed statistically from each other (unpaired *t*-test; $p < .05$) the solvent control was used as the reference-control. For the endpoints gonadal cortex thickness and area of seminiferous tubules as well as for the endpoint gonad surface area data were normalized to the control. Data were analyzed using Fisher's exact test, one-way ANOVA with Dunnett's

Table 1
Embryonic mortality in untreated (NC) and solvent-treated (SC) controls.

		NC	SC
Σ experiments		15	15
Mortality (%) [a] [b]	0	53.3 (8)	20.0 (3)
	>0-10	6.67 (1)	0.00 (0)
	>10-20	40.0 (6)	33.3 (5)
	>20-30	0.00 (0)	33.3 (5)
	>30-40	0.00 (0)	0.00 (0)
	>40-50	0.00 (0)	6.67 (1)
	>50-60	0.00 (0)	6.67 (1)
Σ fertilized eggs		256	258
Sex-specific mortality (%) [c]	Σ males	46.9 (120)	43.4 (112)
	males vital	44.9 (115)	36.8 (95)
	males dead	1.95 (5)	6.59 (17)
	Σ females	50.0 (128)	47.6 (123)
	females vital	48.0 (123)	42.2 (109)
	females dead	1.95 (5)	5.43 (14)
	sex not verified [d]	3.13 (8)	8.91 (23)

[a] Experiments were classified according to the percentage of mortality in the individual experiments (e.g. “>0–10” means a mortality rate between zero and ten percent).
[b] The number in parentheses represents the number of experiments with a defined mortality rate.
[c] The number in parentheses represents the number of affected embryos.
[d] The phenotypic and genetic sex of these embryos could not be determined, because they died in an early developmental stage in which no reproductive organs were visible and no sufficient tissue sample were available for genetic sexing.

multiple comparison test (normal distribution of data) or Kruskal-Wallis test with Dunn's multiple comparison test (no normal distribution of data) with GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, USA).

3. Results

3.1. Baseline experiments in control groups

3.1.1. Embryonic mortality

The fertility rate in the individual experiments was at least 89% in both control groups. In the untreated control a maximum of 16% of embryos per experiment died. SC showed a higher variability of mortality up to 54%, whilst the majority of the experiments were in a range up to 25% mortality (Table 1). Mortality was significantly different between the two control groups ($p < .001$).

In both controls, the genetic sex matched 100% with the phenotypic sex, in males as well as in females. The sex ratio was balanced within both test groups, showing values around 1:1 for the total number of male and female embryos (Table 1). There was no indication for a sex-related effect of the solvent on mortality. In both control groups an equivalent proportion of embryos of both sexes died: 2% in the untreated control and around 6% in the solvent-treated control.

There was no correlation between the injection volume used for the SC and the mortality. Experiments performed with an injection volume of 60 μL per egg showed no statistically significant increase in mortality compared with experiments performed with an injection volume of 15 μL per egg.

3.1.2. Malformations

Observations of the different kinds of malformations were made on the treated embryos dying during the incubation period or stopped on embryonic day 19. In general, different types of malformations were noted in the same embryo. In the untreated control 3 of 256 embryos (1.17%) were malformed with a total number of 4 malformations. These malformations were exclusively found to be celosomia (75.0%) or affected the limbs (left wing; 25.0%). In the solvent-treated control 15 of 258 embryos (5.81%) were malformed with a total number of 22 malformations. These malformations mainly affected the eyes (left/right anophthalmia; 27.3%) often in context with malformations of the beak (torsion of beak or atrophy of upper beak; 18.2%), celosomia (22.7%) and less often malformations of the limbs (legs/feet; 13.6%), edema and exencephalia (9.10%, respectively). Statistical analysis showed that the number of malformed embryos and the total number of individual malformations in the DMSO-treated control increased significantly compared to untreated embryos ($p < .01$ and $p < .001$, respectively). There was no difference in the rate of malformations between the different volumes of DMSO.

3.1.3. Morphological observation of the gonads – gonad surface area

The left and right gonads of male and female chicken embryos are positioned on the ventromedial surface of the respective mesonephros with the dorsal aorta between the gonads. In female embryos, the difference in size between left and right ovary was clearly evident on embryonic day 19. The left ovary was significantly larger and more differentiated than the right one. On average, the right ovary attained around 20% of the surface area of the left ovary in both control groups (Table 2).

In male embryos, the difference in size between left and right gonad was less prominent compared to females. Both testes were fully differentiated and had about the same size, although the left testis was slightly larger. On average, the right testis attained about 90% of the surface area of the left one for both control groups (Table 2).

Regarding the parameters surface area of left and right testis and ovary, little statistical deviations were detected when comparing the mean values in the control groups of the 15 individual experiments against each other or against the arithmetic means of the respective control group. This shows a high statistical contingency, i.e. a high portion of untreated or solvent-treated controls in individual experiments with no statistical difference compared to the other controls of the respective group or compared with the arithmetic mean value of the respective control group. Within the untreated and solvent-treated control group, at least 93% of the experiments showed no statistically significant differences from each other or from the arithmetic mean of the respective control group ($p > .05$) (Table 3). Between untreated and solvent-treated control group, at least 94% of the experiments showed no statistically significant differences from each other ($p > .05$) (Fig. 1 A, B and

Table 2
Gonad surface area, cortex thickness and percentage of seminiferous tubules of untreated (NC) and solvent-treated (SC) chicken embryos on embryonic day 19.

Sex	Group	Gonad surface area			Cortex thickness [μm]	Seminiferous tubules [%]
		left [mm^2]	right [mm^2]	right/left [%]		
Male	NC	4.32 ± 0.58	4.00 ± 0.56 [b]	92.5 ± 7.81 [a]	9.32 ± 1.43	30.6 ± 2.75
	SC	4.17 ± 0.70	3.74 ± 0.71 [b]	89.8 ± 9.50 [a]	9.81 ± 1.59	30.1 ± 3.02
Female	NC	10.8 ± 1.37 [c]	2.24 ± 0.50 [b]	20.8 ± 4.60	158 ± 24.2	–
	SC	9.70 ± 1.53 [c]	2.04 ± 0.50 [b]	21.1 ± 4.14	163 ± 24.5	–

Statistical analysis by unpaired *t*-test. Identical superscripted letters indicate a significant difference (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$) between NC and SC.

Table 3
Statistical consistency as the percentage of experiments with no statistically significant difference of the mean values of individual experiments within untreated (NC) and solvent-treated (SC) control groups compared with each other (all vs. all) or against the arithmetic mean of the respective control group (individual vs. arithmetic mean) or between NC and SC.

Sex	Endpoint	Consistency of individual experiments (%)				
		within NC		within SC		between NC and SC
		all vs. all [a]	individual vs. arithmetic mean [b]	all vs all [a]	individual vs. arithmetic mean [b]	all vs. all [c]
Female	Surface area of left ovary	100%	100%	94%	93%	94%
	Surface area of right ovary	100%	100%	100%	100%	100%
	Cortex thickness	98%	100%	93%	91%	99%
Male	Surface area of left testis	100%	100%	99%	93%	100%
	Surface area of right testis	97%	93%	100%	100%	99%
	Cortex thickness	78%	82%	100%	100%	93%
	Seminiferous tubules	98%	91%	100%	100%	99%

[a] Statistical analysis by one-way ANOVA with Newman-Keuls post test. Comparison of all mean values of individual experiments within the respective control group.
[b] Statistical analysis by one-way ANOVA with Dunnett's post test. Comparison of all mean values of individual experiments with the arithmetic mean of the pooled control group.
[c] Statistical analysis by one-way ANOVA with Newman-Keuls post test. Comparison of all mean values of individual experiments between untreated and solvent-treated control group.

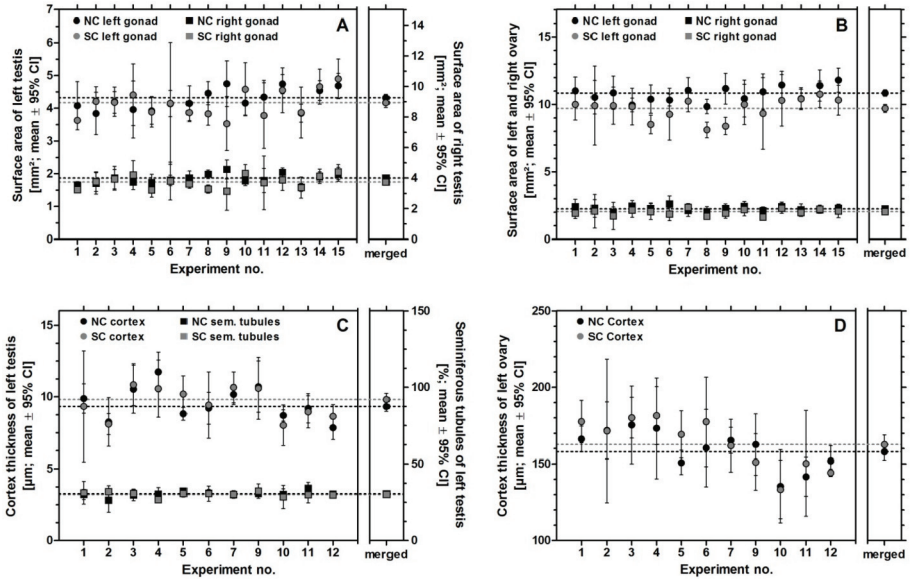

Fig. 1. Surface area of left and right testis (A) and ovary (B), cortex thickness and percentage of seminiferous tubules of left testis (C) and cortex thickness of left ovary (D) of embryos of the domestic fowl (*Gallus g. domesticus*) on embryonic day 19. Data points represent the mean ± 95% confidence interval (95% CI) of untreated negative control (NC) and solvent-treated control (SC) in each single experiment; dashed lines and data points "merged" represent the arithmetic mean of all NC and SC data from the 15 (A, B) or 11 (C, D) experiments. Experiment no. 8 (C, D) was not considered, since the gonadal tissues were used for other examinations.

Table 3).

Overall, the left and right gonads were slightly smaller in the solvent-treated control compared to the untreated control, for males as well as for females. In a predominant proportion of the experiments (left and right ovary: 100% and 87%, respectively; left and right testis: 53% and 67%, respectively) the means of the individual experiments of the untreated control were higher than those of the solvent-treated control. This effect was found to be more pronounced in female gonads.

Comparing the gonad surface area of male and female gonads in the solvent-treated group, experiments that were performed with an injection volume of 60 μL per egg showed a statistically significant difference compared with experiments that were performed with an injection volume of 15 μL per egg for the area of the left

ovary ($p < .001$) and the area of the right testicle ($p < .05$). The surface area of these gonads decreased with increasing volume of the solvent.

3.1.4. Histological observation of the gonads – left testis and ovary

On day 19 of incubation the female left ovary showed a well-differentiated medulla and cortex, while the right ovary had only medulla tissue with an outer thin layer of flattened cells, comparable to the cortex of male testicles. The medulla of the left ovary was loosely arranged and crossed by lacunar channels. The left and right male testes were nearly identically sized, mirror-inverted formed and characterized by a thin cortex layer of 2–3 cells and interstitial space and seminiferous tubules in the medulla, representing the location of spermatogenesis in postnatal developmental stages.

As already shown for the endpoint gonad surface area in section 3.1.3, also the parameters cortex thickness of left testis and ovary and percentage of seminiferous tubules in the left testis exhibited a high statistical consistency when comparing the mean values of the 11 individual experiments against each other or against the arithmetic means of the respective control groups. Within untreated and solvent-treated control group, at least 78% of the experiments showed no statistically significant differences from each other or from the arithmetic means of the respective control group ($p > .05$) (Table 3). Between untreated and solvent-treated control groups, 93–99% of the experiments showed no statistically significant differences from each other ($p > .05$) (Fig. 1C, D and Table 3).

Comparing the histological endpoints in gonads of male and female embryos, experiments that were performed with a solvent volume of 60 μL showed no statistically significant increase compared with experiments that were performed with 15 μL of the solvent.

3.2. Effects of in ovo exposure to BPA and EE_2

3.2.1. Embryonic mortality

In the experiments investigating the effects of BPA and EE_2 the fertility rate of the individual treatment groups was ≥87%. The untreated control showed the lowest mortality of nearly 6%, the solvent control a mortality rate of nearly 20% (Table 4). With a decreasing concentration of BPA a rising mortality was observed, which was between 17% and 30%. Only in the test group receiving 75 μg BPA/g egg weight, which showed the highest mortality rate with 30%, the deviation from the solvent control was statistically significant ($p < .01$). The mortality in the test group receiving EE_2 was 23.4%. There were no statistical differences between the individual experiments of the respective treatment groups. There was also no indication that one of the sexes was more affected by substances treatment than the other.

The genetic sex ratio was balanced within all test groups, showing a proportion of males between 37% and 59%. In both controls, as well as in treatment groups receiving different concentrations of BPA, the genetic sex matched 100% with the phenotypic sex. The group receiving 20 ng EE_2/g egg showed a significantly increased number of embryos, which were determined as phenotypic females but identified as genetic males. Almost 90% of the genetic males (17 of 19 vital males) were identified as intersex-males with ovotestes. The right testes of the affected males were noticeably smaller than those of control males while left testes visibly changed in shape and structure.

3.2.2. Malformations

Different kinds of malformations were observed for all treated embryos dying during the incubation period or stopped on embryonic day 19. While no malformations could be found in the control groups, in the EE_2-treated group 2 of 47 embryos (4.26%) showed malformations which were found to be celosomia or exencephalus (50.0%, respectively). Taking all groups receiving different concentrations of BPA together, 8 of 143 embryos (5.59%; 75 μg BPA/g egg: 4 of 50 embryos (8.00%); 150 μg BPA/g egg: 2 of 46 embryos (4.35%); 300 μg BPA/g egg: 2 of 47 embryos (4.26%)) showed malformations which were found to be celosomia (50.0%), exencephalus (12.5%) or in general deformations of body or head (37.5%). Compared to the control, only the test group receiving 75 μg BPA/g egg weight showed an increase in the incidence of malformations ($p < .01$).

3.2.3. Morphological observations of the gonads - gonad surface area

The gonad surface area (Fig. 2 A Table 5) was especially influenced by EE_2, which caused a significant reduction of female right ovaries by about 26% and of male right testes by about 55%. Female left gonad surface areas significantly increased when treated with 75 μg BPA/g egg. Left and right gonad surface areas of males and right gonad surface areas of females of all groups receiving BPA did not differ statistically from the control. In females, the right ovary attained around 20% of the surface area of the left ovary with marginal differences between controls and treatment groups receiving EE_2 or BPA as well as between the different treatment groups. In males, the right testis of controls and treatment groups receiving different concentrations of BPA attained around 90% of the surface area of the left. Since the gonad surface area of the right testis was significantly decreased when treated with EE_2, the ratio of right to left testis was significantly reduced to 42% ($p < .001$). The gonad surface area of right testes of EE_2-treated males was similar to right ovaries in control groups. Although the surface area of left testes was only marginally changed by EE_2 affected testes showed a female-like shape and a well visible female-typical thickened

Table 4
Embryonic mortality after in ovo exposure to EE_2 (20 ng/g egg) and bisphenol A (75, 150 or 300 μg/g egg).

	NC	SC	EE_2	BPA 75	BPA 150	BPA 300
Σ fertilized eggs	66	41	47	50	46	47
Σ males	45.5% (30)	48.8% (20)	48.9% (23)	54.0% (27)	37.0% (17)	48.9% (23)
males vital	42.4% (28)	43.9% (18)	40.4% (19)	44.0% (22)	34.8% (16)	38.3% (18)
males dead	3.03% (2)	4.88% (2)	8.51% (4)	10.0% (5)	2.17% (1)	10.6% (5)
Σ females	54.5% (36)	51.2% (21)	46.8% (22)	42.0% (21)	58.7% (27)	51.1% (24)
females vital	51.5% (34)	36.6% (15)	36.2% (17)	26.0% (13)	43.5% (20)	44.7% (21)
females dead	1.52% (1)	14.6% (6)	10.6% (5)	16.0% (8)	15.2% (7)	6.38% (3)
sex not verified [a]	1.52% (1)	0.00% (0)	4.26% (2)	4.00% (2)	4.35% (2)	0.00% (0)

[a] The phenotypic and genetic sex of these embryos could not be determined, because they died in an early developmental stage in which no reproductive organs were visible and no sufficient tissue sample were available for genetic sexing.

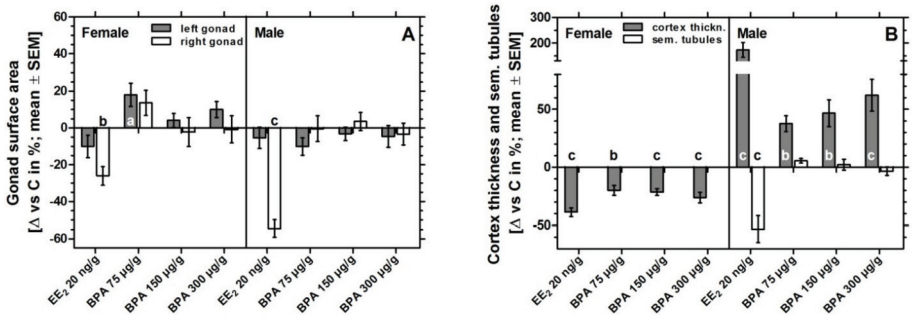

Fig. 2. Effects of in ovo exposure to bisphenol A (BPA) and 17α-ethinylestradiol (EE_2) on left and right gonad surface area (A) and cortex thickness and percentage of seminiferous tubules (B) of embryos of the domestic fowl (*Gallus g. domesticus*) on embryonic day 19. Statistical analysis by one-way ANOVA with Dunnett's multiple comparison test (B) or Kruskal-Wallis test with Dunn's post test (A, B). Lowercase indicate significant differences compared to control. Level of significance: a: $p < .05$; b: $p < .01$; c: $p < .001$.

Table 5
Gonad surface area, cortex thickness and percentage of seminiferous tubules of chicken embryos after in ovo exposure to EE_2 (20 ng/g egg) and bisphenol A (75, 150 or 300 µg/g egg).

Sex	Group	Gonad surface area			Cortex thickness [µm]	Seminiferous tubules [%]
		left [mm^2]	right [mm^2]	right/left [%]		
Male	NC	4.61 ± 0.85	4.03 ± 0.68	87.9 ± 6.62	11.9 ± 2.18	27.9 ± 3.42
	SC	4.48 ± 0.86	4.17 ± 0.81	91.7 ± 8.74	11.0 ± 2.40	26.8 ± 4.62
	C #	4.56 ± 0.85	4.09 ± 0.73	89.4 ± 7.62	11.6 ± 2.26	27.4 ± 3.92
	EE_2	4.31 ± 1.11	1.86 ± 0.83 [c]	42.0 ± 19.3 [c]	31.6 ± 11.1 [c]	12.8 ± 10.0 [c]
	BPA 75	4.10 ± 0.95	4.07 ± 1.32	94.0 ± 8.68	15.9 ± 3.07 [b]	28.9 ± 2.13
	BPA 150	4.41 ± 0.66	4.23 ± 0.80	95.9 ± 10.7	17.0 ± 4.44 [b]	28.0 ± 3.83
	BPA 300	4.35 ± 1.19	3.95 ± 1.09	89.5 ± 6.83	18.8 ± 5.03 [c]	26.5 ± 2.69
Female	NC	10.6 ± 1.38 [b]	2.36 ± 0.65	22.6 ± 6.16	158 ± 40.1	–
	SC	8.83 ± 1.80	2.02 ± 0.62	21.7 ± 3.71	154 ± 20.6	–
	C #	–	2.25 ± 0.65	22.3 ± 5.49	157 ± 35.7	–
	EE_2	7.94 ± 2.29	1.67 ± 0.47 [b]	22.3 ± 8.90	95.8 ± 26.1 [c]	–
	BPA 75	10.4 ± 2.14 [a]	2.56 ± 0.60	24.9 ± 4.36	125 ± 22.2 [b]	–
	BPA 150	9.19 ± 1.50	2.20 ± 0.79	23.6 ± 6.73	123 ± 18.1 [c]	–
	BPA 300	9.71 ± 1.73	2.23 ± 0.74	22.2 ± 5.59	116 ± 27.8 [c]	–

If NC and SC were not statistically different (unpaired *t*-test, $p > .05$), they were pooled to a merged control C and tested against the treatment-groups. If NC and SC were statistically different (unpaired *t*-test, $p < .05$), C was not calculated and treatment-groups were tested against SC.
Statistical evaluation with One-way ANOVA and Dunnett's post test (female cortex thickness, male seminiferous tubules) or Kruskal-Wallis test with Dunn's post test (male cortex thickness, male and female gonad surface areas). Identical superscripted letters indicate asignificant difference (a: $p < 0.05$; b: $p < 0.01$; c: $p < 0.001$) compared to the pooled control C or the solvent control SC.

translucent cortex region when viewed under a stereomicroscope.

3.2.4. Histological observations of the gonads – left testis and ovary

All concentrations of BPA as well as the single concentration of EE_2 resulted in a significant reduction of the cortex thickness of female left ovaries ($p < .01$ and $p < .001$; Fig. 2 B, Table 5). EE_2 differed by nearly 39% and BPA concentrations by up to about 26% from the control. Estrogen-mediated effects in females were found to be concentration-independent as differences between EE_2 and BPA or between the different concentrations of BPA were marginal. In contrast to females, the cortex thickness of male left gonads was significantly increased by the administration of BPA and EE_2. EE_2-related effects were markedly stronger than those of BPA. Compared to the control the male cortex thickness was increased by up to 62% in BPA-treated groups and by 173% in the EE_2-treated group. The BPA-related increase of male cortex thickness was found to be concentration dependent. In the affected cortices of both treatment groups, BPA and EE_2, dividing cells were found which resembled the female oogonia. Since the study of these cells was very complex due to the relatively narrow cortex thickness of male embryos this observation was not quantified. The percentage of seminiferous tubules in male left gonads, however, was affected by EE_2 alone. Compared to the control, EE_2-treatment resulted in a significant drop by about 53%. BPA concentrations differed marginally ($p > .05$) by up to 5.5%.

4. Discussion

The aim of our project is the further development of a standardized test protocol for the assessment of the effects of EDCs in chicken embryos. Therefore, the focus of the present study was on the systematic investigation of the variability of individual parameters in untreated and solvent-treated control groups to provide therefore an important basis for a further validation of the test. In a large number of experiments ($n = 15$), carried out over a period of 3 years, the natural variability of the test system was determined. By merging the data of the 15 experiments we also compared for differences inside and between untreated and solvent-treated control groups. We determined normal mortality rates and gonadal parameters of 19-days-old embryos to establish validity criteria for future standardized test series. In a second step, we investigated the effects of two model estrogens, BPA and EE_2, on developmental and gonadal endpoints to provide robust values for all parameters investigated and to show good reproducibility of the method. This is to demonstrate that the test method based on the chicken embryo is a suitable system for the detection of EDCs.

4.1. Embryonic mortality

It has been shown that the mortality of embryos from unmanipulated control eggs in the 15 baseline experiments was up to a

maximum of 16%. Based on the studies of Romanoff and Romanoff (1972), expected mortality in untreated embryos is about 20%. Comparable low mortality rates were also confirmed in other studies (Wyatt and Howarth, 1976; DeWitt et al., 2005a, 2005b). In comparison, mortality of solvent-treated embryos was below 25% in a predominant proportion of the 15 experiments while in two experiments more than 30% of embryos died, suggesting that DMSO induces an increased mortality compared to the untreated control. Our data indicate that a mortality of 30% in the solvent group as well as in the untreated group should be considered as validity criterion for a future test design.

In studies on avian development different carriers have been used, such as organic solvents, vegetable oil, just water or an emulsion. Each carrier with its special characteristics has its advantages and disadvantages. DMSO is used in a variety of experiments because of its solubility properties and good miscibility with other solvents. In toxicological studies and pharmacological screenings it is used to enhance the solubility of hydrophobic chemicals (Castro et al., 1995) but it may increase membrane permeability and sustance uptake (Notman et al., 2006). Although a solvent such as DMSO is often inevitable care should be taken as an intrinsic toxicity of DMSO has been reported in chicken (Caujolle et al., 1967; Carew and Foss, 1972; Landauer and Salam, 1972; Morgan, 1974; Wyatt and Howarth, 1976) and various other organisms (Anderson et al., 2004; Chen et al., 2011; Galvao et al., 2014; Stevens et al., 2015). It has been shown that an increasing dose of the solvent is associated with an increase in mortality. Different studies indicate that higher injection volumes in general have harmful effects on developing chicken embryos, irrespective of the solvent used (Landauer and Salam, 1972; Morgan, 1974; Wyatt and Howarth, 1976; DeWitt et al., 2005b). However, it must be mentioned that also the day of application can affect embryonic mortality. Regarding the two volumes of DMSO (15 μL, 60 μL) used in the present study, there was no difference between the mortality in the individual developmental stages of the embryos. This suggests that even a higher volume of up to 60 μL DMSO administered into the egg yolk of one day old embryos does not cause an increase in mortality compared to the lower volume of 15 μL. However, based on our own data and the results of other studies mentioned above, the volume of the solvent used should not exceed 60 μL. It is rather useful to keep the volume as low as possible to minimize toxic effects of the solvent. Nevertheless, the volume of the solvent is generally dependent on the chemicals to be dissolved therein and the concentration of the chemical.

In ovo-treatment of chicken embryos with BPA and EE_2 led to a slight increase in mortality as expected. Nevertheless, mortality rates of controls and treatment groups were below 30%, which is in a reasonable range since low mortality rates or, conversely, high survival rates result in a sufficient number of vital embryos for follow-up examinations of gonadal tissues. In the 4 individual experiments the mortality rates of the untreated control were below 14% and the mortality rates of the solvent control below 27% which is in the same range as baseline experiments. According to the proposed validity criteria, as derived from the baseline experiments, all 4 experiments investigating the effects of EE_2 and BPA can be considered as valid.

The natural sex ratio of *Gallus g. domesticus* varies around 50%. The genetic sex ratio in the baseline experiments was balanced within both control groups, in vital embryos as well as in dead embryos. The genetic sex agreed 100% with the phenotypic sex for both control groups. There was no indication that one of the sexes was more affected by the solvent than the other.

In ovo-treatment to 20 ng EE_2/g egg, however, resulted in a large number of embryos that were phenotypic females but found to be genetic males. The size of the right testis of the affected males was noticeably decreased with the structural appearance of a right ovary. The left testis, although not changed in size, showed shape and structure of a left ovary. This indicates that already the visual evaluation of the gonads, together with the information about the genetic sex, can indicate possible influences of EDCs on sex differentiation in chicken embryos. From this it can be concluded that a more detailed examination of the gonads is potentially suitable to detect possible effects of EDCs.

4.2. Malformations

Baseline experiments with control groups show a malformation rate of 1.2% in unmanipulated embryos and 5.8% in solvent-treated embryos. Compared to the unmanipulated group the significant increase in the solvent-treated group indicates a slight teratogenic activity of DMSO. This finding is in line with the results of Dresser et al. (1992) who assessed the teratogenic activity of four solvents in the Frog Embryo Teratogenesis Assay with *Xenopus laevis* (FETAX) and reported DMSO to be the least toxic and teratogenic solvent examined. Caujolle et al. (1967) noted that spontaneous malformations may exist in the chicken embryo with an incidence of about 2% what coincides with the data shown here for the untreated control. However, Alsop (1919) and Byerly (1930) showed malformation rates around 6%. Although the number of malformed embryos in the solvent-treated control increased compared to the untreated control, the SC is in the same range as in the studies mentioned above.

Malformations in untreated embryos were exclusively found to be celosomia or malformations of the limbs. DMSO-treated embryos showed malformations which mainly affected the eyes (left/right anophthalmia) often in context with malformations of the beak (torsion of the beak or atrophy of the upper beak), celosomia and less often malformations of the limbs (legs/feet), edema or exencephalia. Byerly (1930) found different types of malformations in unmanipulated eggs of the white leghorn breed, e.g. terata of the eyes (mono-/microphthalmia) or the brain (exencephalia, hyperencephalia). The study of Caujolle et al. (1967) showed that the nature of spontaneous malformations in chicken embryos exhibits a common pattern: anophthalmia, crossed beak with or without anencephalia and celosomia but never malformations of the limbs. Furthermore, they found that a 50% solution of DMSO in 0.9% physiological saline caused malformations at doses approaching the LD_{50} (10.3 mg/embryo at E3 or 12.2 mg/embryo at E4). The most typical DMSO-induced malformations of embryos treated at E3 were left anophthalmia and left torsion of the beak with reduction of the upper beak and only to very small extent malformations of the limbs. DMSO-treatment at E4 produced almost 26% malformed embryos and generally caused lesions of the limbs and in lower percentages malformations of the beak and the eyes, anurous embryos and celosomia. In the baseline experiments of the present study the malformations found in the solvent-treated group support the findings of Caujolle et al. (1967) although our percentage of malformed embryos is significantly lower. There was no difference in the rate of malformation between the DMSO-treated groups of different volumes suggesting that also a dosage of 60 μL can be used without increasing the number of malformed embryos. A malformation rate of almost 6% appears to be acceptable, as there is a sufficient number of embryos remaining for subsequent histological examinations.

Analyzing the frequency of malformations in the 4 experiments investigating the effects of EDCs, malformations are found neither in the untreated control nor in the solvent-treated control while malformation rates in the BPA- and EE_2-treated groups are around 5%. The significant increase in the malformation rate of the substance-treated group receiving 75 μg BPA/g egg compared to the

control has to be assumed as concentration-independent as higher concentrations of the estrogen show marginal differences. However, all values are still in the range of the reported spontaneous malformation rate in chicken embryos of about 2% (Caujolle et al., 1967) to around 6% (Alsop, 1919; Byerly, 1930). Also, the malformation rates of around 5% in the estrogen-treated groups are in the same range as the malformation rates of the solvent control in our baseline experiments. In this context, the incidence of malformations in the substance-treated groups can be considered as inconspicuous. However, almost all of the BPA- or EE_2-treated groups show a marginally increased incidence of celosomia, compared to the control. In addition, exencephalia are exclusively found in substance-treated groups, while the formation of edema is completely absent there. Various malformations have already been described, among them, for example, terata of the eyes, the beak, the brain or the formation of celosomia (Byerly, 1930; Caujolle et al., 1967). These terata largely coincide with the malformations found in the solvent- and substance-treated groups. Although the pattern of malformations from solvent-treated control to substance-treated groups is slightly shifted, there is no statistical evidence that treatment with BPA or EE_2 specifically favors particular terata or generally results in increased malformation rates. It can be concluded that in ovo-exposure to both estrogenic substances does not increase the rate of malformations.

4.3. Morphological observations of the gonads - gonad surface area

In the baseline experiments the treatment with DMSO results in reduced gonad surface areas in both sexes. This effect is more distinct in female than in male gonads, as untreated control and solvent control differ significantly from each other. The size ratio of right to left gonad, however, remains unaffected for female embryos. The solvent seems to affect the growth of the female gonads in some way. For male embryos treated with DMSO there is only a slight tendency of smaller left and right gonad surface areas and the ratio of left to right gonad differs marginally between untreated control and solvent control. The reason may be a growth-inhibiting effect caused by the low basic toxicity of the solvent. An alternative explanation is a possible endocrine-mediated effect of the solvent. This is supported by the fact that the effect on the surface area of the gonads is sex-specific with female embryos being more affected while the change in male embryos is marginal. The hypothesis of a solvent-induced influence on the endocrine system of organisms is supported by various studies. In their review about the effects of different OECD-recommended carrier solvents, Hutchinson et al. (2006) describe an influence of DMSO on the reproduction of different fish species and an impact on biomarkers of endocrine disruption. The results of Pawlowski et al. (2004a, 2004b) show that DMSO did not affect different reproduction-related endpoints such as spawning and biomarker response of the fathead minnow, but a distinct reduction in the mean egg production. Further studies demonstrate an inhibition of various cytochrome P450 enzymes by DMSO (Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999; Easterbrook et al., 2001). It is concluded that since these enzymes are involved in the metabolism of endogenous substances such as steroid hormones interactions with solvents can result in a change in circulating hormone concentrations with subsequent effects on reproductive functions. This probable DMSO effect cannot be excluded for the chicken embryo used in our experiments.

Furthermore, data show that the larger the volume of the solvent used, the smaller the gonad surface areas, which again leads to the point that the solvent volume should be kept as low as possible. Similar studies measuring reproductive endpoints or endocrine disrupter biomarker responses also propose a maximum solvent concentration for the testing of aquatic organisms (Hutchinson et al., 2006).

As a consequence of this, in the testing of chemical substances, a comparison should always be drawn between untreated embryos and solvent-treated embryos in order not to over- or underestimate any possible influence of the solvent. The arithmetic means of the gonad surface area of male and female embryos found in the baseline experiments can serve as key values for future tests.

The phenotypic sex of birds can be determined by screening the sex organs. Along with the information about the genetic sex the endpoint gonad surface area indicates whether a test substance influences the differentiation of the sexes. In ovo-exposure of male and female chicken embryos to EE_2 causes a significant decrease in the surface area of right testis and ovary. This effect is more pronounced in males and although the left testis does not change significantly in size, it shows a completely different appearance as it develops into an ovotestis with a translucent cortex as is typical for the left ovary. On the contrary, BPA had marginal effects on the gonad surface area of male and female chicken embryos. Only the concentration of 75 μg BPA/g egg resulted in an increase of the female left gonad surface area, which was found to be concentration-independent. For chicken and quail it is known that substances with endocrine potential can induce morphological changes in the sex organs (Scheib, 1983; Berg et al., 1998, 1999, 2001a, 2001b; Matsushita et al., 2006; Razia et al., 2006). The knowledge about the change in the gonads in shape, size and structure leads to the conclusion that gonad-related endpoints are useful for the testing of potential endocrine active substances. Although BPA and EE_2 are both estrogens, their activity differs considerably (Metcalfe et al., 2001; Oehlmann et al., 2006). However, it was in our expectation that in the chicken egg test the observed effect profile is substance-specific as shown here. Nevertheless, we have successfully shown the suitability of the endpoint gonad surface area for the detection of endocrine potentials as already the low dosage of 20 ng EE_2/g egg causes a significant change in shape and size of male testes. As a next step it should be investigated whether further classes of EDCs such as androgens, anti-androgens and anti-estrogens are able to cause comparable effects in gonads of the chicken embryo regarding the focused endpoints. All in all the endpoint gonad surface area could help facilitate the screening of potential EDCs as possible effects can be detected easily. However, subsequent histopathological analysis of the gonads will then give a more detailed description of the effects of possible EDCs on sex organ development of chicken embryos.

4.4. Histological observation of the gonads – left testis and ovary

Gonad tissue-related endpoints appear to be useful in the investigation of endocrine compounds, because they specifically influence the differentiation of the sexes. Since this represents a system in which the chemical is in direct contact with the embryo throughout development, it is likely that any toxic or teratogenic effect will be readily observed. However, there is always the possibility that the chicken will not be a species susceptible to a particular compound, just as it has been shown that other commonly used species of animals do not respond to all chemicals in a similar manner. It is also possible for the chicken to be more sensitive to a chemical than other species. Finally, this technique may be applied also to the study of (over-)additive effects of chemicals. Through the detailed investigation of the untreated and solvent-treated controls, a reliable statement about substance-related deviations from these can be made.

In the baseline experiments there was a marginal tendency of an enhanced cortex thickness in the SC compared to the NC, for males as well as for females. Analogous to the endpoint gonad surface area, females tended to be more affected than males. The

percentage of seminiferous tubules was not affected by the solvent. For both sexes only a small percentage of the means of the individual experiments within a test group as well as between the individual experiments between NC and SC differed significantly from each other. It can be assumed that this deviation is within natural fluctuation and indicates a good reproducibility for the method used. Therefore, the arithmetic means of the endpoints cortex thickness and percentage of seminiferous tubules are proposed as key values for follow-up tests.

The role of natural hormones in gonadal differentiation of birds is still partly unknown. Their possible function may become clearer when the hormone level is artificially influenced, for example by in ovo application of EDCs that potentially interact with steroidogenic enzymes. It is known that synthetic hormones may induce irreversible malformations of the gonads in birds during embryonic development or can alter gender-related behavior later in life, whereas other, less potent EDCs may exert less severe and often reversible effects in the less sensitive adult stage (Adkins-Regan, 1990; Ottinger and Abdelnabi, 1997). Therefore, the chicken embryo appears as a suitable model for the study of early sexual development and the potential impact of EDCs.

Depending on their hormonal system, the response of male and female chicken embryos to certain substances or substance classes can be very different. In birds, the genetic male is homozygous (ZZ), while the genetic female is heterozygous (ZW). Initially, all embryos have the same basic sex, regardless of their genetic gender: they are designed as males. The differentiation in one of the sexes during embryonic development depends on the level of circulating steroid hormones. Without external influence the undifferentiated gonads of genetic males develop into testes. In genetic females the synthesis of P450 aromatase finally results in the production of estrogens (Kagami and Hanada, 1997) which is substantial for the formation of female sex organs.

The results of the experiments assessing the effects of in ovo exposure to BPA and EE_2 show that both estrogens cause a significant reduction of the female cortex thickness. In males both substances cause a significantly thickened cortex with oocyte-like cells and a female-like structure, which coincides with previously published studies (Berg et al., 1998, 1999, 2001a, 2001b). While BPA does not affect the percentage of seminiferous tubules in male gonads, testicular tissue of EE_2-treated embryos appears significantly altered with a visibly lower number and degree of differentiation of seminiferous tubules and female-typical structures as lacunae. Since there are marginal differences between untreated and solvent-treated control in the baseline experiments as well as in substance experiments, it can be concluded that the differentiation of cortex and seminiferous tubules is unaffected by the solvent in both sexes. Therefore, it can be assumed that the shown effects are substance-specific and do not originate from the solvent.

Comparing both estrogenic substances, EE_2-caused effects are much more pronounced than those of BPA indicating the higher estrogenic potency of EE_2. This coincides with the studies of Metcalfe et al. (2001) which rates EE_2 to be much more potent than BPA in the *yeast estrogen screen* and in experiments with Japanese medaka (*Oryzias latipes*). Various studies on domestic fowl (*Gallus g. domesticus*) and Japanese quail (*Coturnix japonica*) demonstrate the effective feminization of male embryos when treated with estrogens or estrogen-active EDCs (Romanoff, 1960; Scheib and Reyssbrion, 1979; Wolff, 1979; Samsel et al., 1982; Sotonyi and Csaba, 1986; Etches and Kagami, 1997; Berg et al., 1998, 1999, 2001a, 2001b; Shibuya et al., 2004). While the left testis is formed into an ovotestis or ovary, the differentiation of the right testis is largely inhibited, resembling a right ovary. In contrast, the treatment of female embryos with estrogens showed fewer effects on gonad differentiation. Since the key enzyme P450arom is not synthesized in male gonads (Ayers et al., 2013; Scheider et al., 2014), constitutional estrogen concentrations in testes are very low (Woods and Erton, 1978; Tanabe et al., 1979, 1983) and not sufficient to cause an effect. Though, for a short time during embryonic development the estrogen receptor is detectable in male gonads, which makes them basically vulnerable to estrogens (Gasc, 1980; Smith et al., 1997; Nakabayashi et al., 1998). The artificial presence of estrogen at this critical time point therefore causes the differentiation towards the phenotypically female sex. Therefore, male gonads are basically able to develop ovarian tissue, whereby the absence of estrogen is essential for the testes formation. Since the estrogen level in females is continuously high, the administration of additional estrogen such as EDCs does not affect gonadal differentiation as strongly as in males.

Our as well as other investigations (Berg et al., 1998, 1999, 2001a, 2001b) have shown that the gonad-related endpoints cortex thickness and percentage of seminiferous tubules reliably respond to the treatment with the model estrogens BPA and EE_2. Also we provide reference values for future experiments. As mentioned above the next step of our investigations will focus on whether these endpoints can display the effects of other classes of EDCs as androgens, anti-androgens and anti-estrogens.

5. Overall conclusions

The present study is part of a project aiming to improve a replacement method for testing hormonally active compounds in birds, where fertilized eggs of the domestic fowl (*Gallus gallus domesticus*) are used. In baseline experiments we focused on the investigation of untreated and solvent-treated control groups to study normal development of the embryos without exposure to EDCs. We examined developmental and gonadal endpoints and determined reliable reference values for each endpoint which can serve as validity criteria in future experiments. Both controls were easily reproducible with low variability within as well as between both control groups. Since solvent-related effects were low we recommend DMSO as solvent for subsequent experiments. In further experiments investigating the effects of two estrogenic EDCs, BPA and EE_2, we provided robust reference values for all endpoints which are suggested to serve as positive control values in future experiments. Overall, the chicken embryo has proven to be a suitable and reliable test system for the investigation of the effects on toxicology and reproductive tissues of various chemical substances. Based on these results the study of the effects of further substance classes as androgens, anti-androgens and anti-estrogens on sexual differentiation of the chicken embryo is very promising.

6. Declarations

Conflict of interest

None.

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