No effects of the antiandrogens cyproterone acetate (CPA), flutamide and *p,p´*-DDE on early sexual differentiation but CPA-induced retardation of embryonic development in the domestic fowl (*Gallus gallus domesticus*)

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Because a wide range of environmental contaminants are known to cause endocrine disorders in humans and animals, *in vivo* tests are needed to identify such endocrine disrupting chemicals (EDCs) and to assess their biological effects. Despite the lack of a standardized guideline, the avian embryo has been shown to be a promising model system which responds sensitively to EDCs. After previous studies on the effects of estrogenic, antiestrogenic and androgenic substances, the present work focuses on the effects of *in ovo* exposure to *p,p´*-DDE, flutamide and cyproterone acetate (CPA) as antiandrogenic model compounds regarding gonadal sex differentiation and embryonic development of the domestic fowl (*Gallus gallus domesticus*). The substances were injected into the yolk of fertilized eggs on embryonic day one. On embryonic day 19 sex genotype and phenotype were determined, followed by gross morphological and histological examination of the gonads. Treatment with flutamide (0.5, 5, 50 µg/g egg), *p,p´*-DDE (0.5, 5, 50 µg/g egg) or CPA (0.2, 2, 20 µg/g egg) did not affect male or female gonad development, assessed by gonad surface area and cortex thickness in both sexes and by the percentage of seminiferous tubules in males as endpoints. This leads to the conclusion that antiandrogens do not affect sexual differentiation during embryonic development of *G. gallus domesticus*, reflecting that gonads are not target organs for androgens in birds. *In ovo* exposure to 2 and 20 µg CPA/g egg, however, resulted in significantly smaller embryos as displayed by shortened lengths of skull, ulna and tarsometatarsus. Although gonadal endpoints were not affected by antiandrogens, the embryo of *G. gallus domesticus* is shown to be a suitable test system for the identification of substance-related mortality and developmental delays.

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ABSTRACT

Because a wide range of environmental contaminants are known to cause endocrine disorders in humans and animals, *in vivo* tests are needed to identify such endocrine disrupting chemicals (EDCs) and to assess their biological effects. Despite the lack of a standardized guideline, the avian embryo has been shown to be a promising model system which responds sensitively to EDCs. After previous studies on the effects of estrogenic, antiestrogenic and androgenic substances, the present work focuses on the effects of *in ovo* exposure to *p,p´*-DDE, flutamide and cyproterone acetate (CPA) as antiandrogenic model compounds regarding gonadal sex differentiation and embryonic development of the domestic fowl (*Gallus gallus domesticus*). The substances were injected into the yolk of fertilized eggs on embryonic day one. On embryonic day 19 sex genotype and phenotype were determined, followed by gross morphological and histological examination of the gonads. Treatment with flutamide (0.5, 5, 50 µg/g egg), *p,p´*-DDE (0.5, 5, 50 µg/g egg) or CPA (0.2, 2, 20 µg/g egg) did not affect male or female gonad development, assessed by gonad surface area and cortex thickness in both sexes and by the percentage of seminiferous tubules in males as endpoints. This leads to the conclusion that antiandrogens do not affect sexual differentiation during embryonic development of *G. gallus domesticus*, reflecting that gonads are not target organs for androgens in birds. *In ovo* exposure to 2 and 20 µg CPA/g egg, however, resulted in significantly smaller embryos as displayed by shortened lengths of skull, ulna and tarsometatarsus. Although gonadal endpoints were not affected by antiandrogens, the embryo of *G. gallus domesticus* is shown to be a suitable test system for the identification of substance-related mortality and developmental delays.

Key words: Chicken embryo, endocrine disruption, gonad, developmental toxicant, histology, dwarfism

1. INTRODUCTION

Among the substances in constant use, there is a group of chemicals with structural similarity to natural sex hormones. Contaminants with hormonal action, so called endocrine disrupting chemicals (EDCs), are suspected to affect the development and health status of humans and animals with special focus on sex differentiation and reproduction. As agonists and antagonists of androgen (AR) and estrogen (ER) receptors, EDCs can activate or block corresponding receptors, potentially affecting all systems controlled by the endocrine system. A growing number of reports underlines the assumption, that EDCs pose a threat to the ecosystem and to animal and human health *(Delbes et al., 2022; Ho et al., 2022; Marlatt et al., 2022; Metcalfe et al., 2022)*. In order to assess possible effects and to weigh risks, the testing of chemicals for their endocrine potential is of great importance.

Currently, there are several internationally standardized biotests for the testing of androgenic and estrogenic EDCs in mammals, among them two frequently used rodent-based tests, namely Hershberger assay *(OECD, 2009)* and uterotrophic assay *(OECD, 2007)*. Since mainly juvenile and adult animals with full pain perception are used in these tests, the search for a suitable animal replacement system is of great significance. Moreover, these tests do not adequately reflect the impact of EDCs on the most sensitive stage of life, the developing embryo.

There is a long tradition of using avian embryos to study sexual development and potential effects of environmental pollutants including EDCs *(Berg et al., 1998; Berge et al., 2004; Biau et al., 2007; Eising et al., 2001; Fry & Toone, 1981)*. It is well known that the exposure of xenobiotics during avian embryonic development can induce irreversible deformities or malformations of the sex organs and disrupt gender-specific behavior *(Farhat et al., 2020; Ottinger et al., 2008; Quinn et al., 2008)*. One advantage is that the avian egg can be considered as a closed system lacking any

exchange with its environment except for the interchange of gases. The single administration of a specific and standardized dose, often injected directly into the egg *(Berg et al., 1999)*, may be sufficient to affect the developing embryo *(Davies et al., 1997; Gooding et al., 2003; McAllister & Kime, 2003; Zhang et al., 2007)*. Since no exchange or loss of the substance is possible, this injection results in chronic chemical exposure.

The embryo of domestic fowl (*Gallus gallus domesticus*) is particularly suitable for our experiments as its developmental stages are fully described *(Hamburger & Hamilton, 1992; Keibel & Abraham, 1900; Starck & Ricklefs, 1997)*.

Since there is no standardized procedure available, the present study is part of a project aiming to expedite a protocol to assess the potential effect of EDCs on early sexual differentiation in the chicken embryo. In earlier publications we presented the effect of estrogens, antiestrogens, and androgens on embryonic gonad sex development. In the present study we finally analyzed the effects of antiandrogenic compounds on embryonic development with special focus on potential gross morphological and histological changes of the gonads. Cyproterone acetate (CPA), flutamide and *p,p´*-dichlorodiphenyldichloroethylene (*p,p´*-DDE) were chosen as antiandrogenic model compounds.

An important class of antiandrogens are synthetic drugs such as CPA and flutamide that were specifically designed to competitively bind to androgen receptors *in vivo (Bhatia et al., 2014b)*. These AR antagonists have been used to treat androgen-dependent prostate cancer in men and menstrual cycle irregularities in women with polycystic ovarian syndrome *(Heinlein & Chang, 2004; Paradisi et al., 2013)*. Furthermore, both compounds have been used for the testing of potential effects on different non-target organisms including the bird embryo *(Adkinsregan & Garcia, 1986; de Gregorio et al., 2021; Fitzgerald et al., 2020; Gismondi et al., 2019; Jin et al.,*

2019; Mentessidou et al., 2021; Rangel et al., 2006; Rolon et al., 2019; Utsumi & Yoshimura, 2009, 2011; Yu et al., 2020; Yu et al., 2021).

P,p′-DDE is the primary metabolite of the insecticide DDT, which was used widely from the 1940s until its ban in most industrial countries in the 1970s *(Quinn et al., 2008)*. DDT and its metabolites are known to induce eggshell thinning and developmental disorders in fish-eating and raptorial seabirds *(Bouwman et al., 2019; Buck et al., 2020; Fry & Toone, 1981; Hickey & Anderson, 1968; Holm et al., 2006; Peakall & Lincer, 1996; Ratcliffe, 1970)* and affect sexual development in quail and chicken *(Blomqvist et al., 2006; Halldin et al., 2003; Kamata et al., 2020; Quinn et al., 2008)*.

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.

Cyproterone acetate (CPA; CAS: 427-51-0), flutamide (CAS: 13311-84-7) and *p,p′*-dichlordiphenyldichlorethen (*p,p′*-DDE; CAS: 72-55-9) were purchased from Sigma Aldrich Chemie GmbH (München, Germany). Fertilized eggs of white Leghorn (*G. gallus domesticus*) were obtained from a local breeder (LSL Rhein-Main Geflügelvermehrungsbetrieb, Dieburg, Germany). Eggs were incubated at 37.5 ± 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). CPA (0.2, 2, 20 µg/g egg), flutamide (0.5, 5, 50 µg/g egg) and *p,p′*-DDE (0.5, 5, 50 µg/g egg) were dissolved in 15 µL (CPA) or 60 µL (*p,p′*-DDE and flutamide) of the solvent dimethyl sulfoxide (DMSO; CAS: 67-68-5; purity: 99.5%; Applichem, Darmstadt, Germany) and injected into the yolk on day one of incubation via a small hole at the widest diameter of the egg using Hamilton microliter syringes and needles (ga22s/51mm/pst2). Following injection, the shell was sealed with agarose gel (3%, in phosphate buffered saline). During incubation, eggs were periodically checked by candling to identify unfertilized eggs or dead embryos.

2.2 Dissection, tissue preparation and evaluation

Dissection was performed on day 19 of incubation. All embryos were examined for external deformations and malformations of inner organs with special focus on ovaries and testes. Gonad

surface areas were analyzed by determining the entire visible surface of each single gonad with an image editing program (Fiji is just ImageJ, Open Source). Gonads were dissected and fixed in Bouin´s solution for 24 hours. The fixative was removed by repeated rinsing with 80% ethanol. Ethanol was removed by saccharose solution (10, 20 and 30% in phosphate buffered saline). Gonads were embedded in Tissue-Tek® (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) and sectioned (6 µm) by a cryomicrotome (Microm HM 500 O, Thermo Fisher Scientific Germany, Bonn, Germany) at -23°C. Tissue sections were stained with hematoxylin and eosin.

2.3 Determination of sexual genotype

A tissue sample from the heart was used for DNA isolation. Dead embryos, identified and removed before dissection, were also sampled. All embryos were typed for their sexual ZZ or ZW genotype, using the PCR-based method of *Fridolfsson and Ellegren (1999)*. DNA-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. Both sexes had a single 600-bp CHD1-Z specific fragment with a melting temperature of ~84°C. Females had an additional 450-bp CHD1-W females-specific fragment with a melting temperature of ~82°C.

2.4 Determination of embryonic energy metabolism

In order to study the impact of CPA-treatment on embryonic energy metabolism, protein, glycogen, and lipid contents were determined using a tissue sample from the liver taken during dissection. Six embryos of untreated and solvent control group as well as CPA-treated groups were

used. The liver was weighted and homogenized with a 2% sodium sulfate solution. Part of the homogenate was used to determine the protein content according to *Bradford (1976)*. Another part of the homogenate was used to determine glycogen and lipid contents according to *(Van Handel, 1965; Van Handel, 1985a*, *1985b)*. For each fraction calibration curves using standards in five ascending concentrations were created using 0.1% BSA solution (protein), 0.1% glucose solution (glycogen) or 0.1% rape solution (lipid). Standards were treated analogously to samples. The absorbance of standards and samples was determined using a photometer (BioSpecrometer®, Eppendorf, Hamburg, Germany; protein: 595 nm; glycogen/lipid: 625 nm). The absorbance of the respective standards was plotted against protein, glucose, or lipid content, respectively, calculating linear calibration curves. Based on the respective calibration curve, protein, glycogen, or lipid contents of the samples (µg/g liver) were determined and extrapolated to the total volume of the homogenate. Using the specific calorific values (protein: 17 kJ/g; glucose: 17 kJ/g; lipid: 37 kJ/g) the energy content of protein, glycogen and lipid reserves in J/mg embryo was calculated.

2.5 Measurements and statistics

For histological examination of embryonic gonads, a light microscope (Olympus BX50, Olympus, Tokyo, Japan) and a camera (JVC Digital Camera, KY-F75U, Yokohama, Japan) were used. Cortex thickness (both sexes) and the percentage of seminiferous tubules (males) in left gonads were measured (Fiji is just ImageJ, Open Source). Ten sections per embryo were evaluated, exclusively taken from the gonad's middle sectional plane. Five measurements per section were performed to determine the cortex thickness. Since cortex thickness is not constant over the whole organ, different representative areas around the gonad were chosen. The area of all seminiferous tubules in a defined image section was measured to determine a representative percentage of

156 seminiferous tubules in the male left testis. For this a random representative image section was
157 selected which showed only the medullary tissue but not the cortex region.
158 One experiment was performed with different concentrations of CPA (0.2, 2, 20 µg/g egg), another
159 experiment was performed with different concentrations of flutamide and *p,p´*-DDE (0.5, 5, 50
160 µg/g egg). Solvent control was used as the reference-control. Data were analyzed using Fisher´s
161 exact test and one-way ANOVA with Dunnett´s multiple comparison test with GraphPad Prism
162 5.03 (GraphPad Software Inc., San Diego, USA).

3. RESULTS

3.1 Embryonic mortality and malformations

In ovo exposure to all concentrations of *p,p´*-DDE and flutamide caused a concentration-dependent increase in embryonic mortality which was found to be significantly different from the solvent control for 50 µg *p,p´*-DDE/g egg ($p<0.05$) and 5 and 50 µg flutamide/g egg ($p<0.05$ and $p<0.01$, respectively). Mortality rates of *p,p´*-DDE-treated groups were between 45% and 55%, mortality rates of flutamide-treated groups were between 53% and 70% (see figure 3A).

Different types of single or multiple malformations were found in the control-, *p,p´*-DDE or flutamide-treated groups. In the untreated control group one embryo (6.67%) showed celosomia. In the solvent control two embryos (13.3%) showed either malformations of the extremities or celosomia. *In ovo* exposure to 0.5 µg *p,p´*-DDE/g egg led to two malformed embryos (10.5%), one of them with both-sided anophthalmia, the other one a "twin embryo" conjoined at the head, showing various malformations. *In ovo* exposure to 5 µg *p,p´*-DDE/g egg led to one malformed embryo (5.3%) with crossed beak and a cyclops-like eye at the front of the head. *In ovo* exposure to 0.5 µg flutamide/g egg led to two malformed embryos (10.0%) with celosomia, crossed beak and left-sided or both-sided anophthalmia while *in ovo* exposure to 5 µg flutamide/g egg led to one embryo (5.0%) with celosomia. In the highest concentration (50 µg/g egg) of both *p,p´*-DDE and flutamide, no malformations were detected. None of the *p,p´*-DDE- or flutamide-treated groups showed a statistically significant difference from the solvent or the untreated control group.

In ovo exposure to all concentrations of CPA resulted in a concentration-dependent increase in mortality which was found to be significantly different from the solvent control group for 20 µg CPA/g egg ($p<0.01$). In the group treated with the highest concentration of CPA (20 µg/g egg),

this resulted in nearly 78% mortality leaving only a few embryos for follow-up analyses (see figure 1A).

Different types of single or multiple malformations were found in the solvent control and CPA-treated groups. In the untreated control group, none of the embryos showed malformations. In the solvent control one embryo (8.3%) showed celosomia. *In ovo* exposure to 0.2 µg CPA/g egg led to one embryo (4.3%) with left-sided anophthalmia and *in ovo* exposure to 2 µg CPA/g egg led to two malformed embryos (8.3%) with exencephalia, right-sided anophthalmia and celosomia. None of the CPA-treated groups showed a statistically significant difference from the solvent or the untreated control group.

Remarkably, an increased incidence of significantly delayed development was found, which especially occurred at concentrations of 2 and 20 µg CPA/g egg. In order to analyze this statistically, the parameters length of skull (from the tip of the beak to the back of the head), length of ulna (right side) and length of tarsometatarsus (right side) were measured. Embryos exposed to the lowest concentration of 0.2 µg CPA/g egg showed no effects on body lengths, while higher concentrations of 2 and 20 µg CPA/g egg resulted in a statistically significant reduction of all three parameters ($p<0.001$, respectively) compared to the solvent control (see figure 2A).

To investigate the potential impact of CPA-treatment on embryonic energy reserves, we determined the content of protein, glycogen, and lipid in liver samples of control and CPA-treated groups. Compared to the solvent control, all CPA treatments were characterized by a significantly decreased content of glycogen (0.2 µg CPA/g egg: $p<0.01$; 2 and 20 µg CPA/g egg: $p>0.001$, respectively). In addition, embryos exposed to the highest concentration of 20 µg CPA/g egg showed a significantly decreased content of protein ($p<0.001$). However, the content

of lipids in this group was marginally but not statistically significantly increased ($p>0.05$) (see figure 2B).

3.2 Morphological observation of the gonads – gonad surface area

Exposure to higher concentrations of *p,p′*-DDE or all concentrations of flutamide had no statistically significant effect on the surface areas of male or female gonads. Only *in ovo* exposure to 0.5 µg *p,p′*-DDE/g egg resulted in a statistically significant decrease of the surface area of the right ovary and a statistically significant increase of the surface area of the left testis ($p<0.05$, respectively) compared to the solvent control. In control groups there was a statistically significant difference between the untreated and the solvent control for the female left gonad surface area ($p<0.001$) and left and right male gonad surface areas ($p<0.05$, respectively) (see figure 3B). Compared to the solvent control, *in ovo* exposure to CPA did not affect male or female gonad surface areas (see figure 1B).

3.3 Histological observation of the gonads – left testis and ovary

None of the examined antiandrogenic substances induced any effect on male or female gonadal sex differentiation. Neither *p,p′*-DDE, flutamide or CPA had a statistically significant effect on the percentage of seminiferous tubules in left testes or the cortex thickness in left testes or ovaries at any of the tested concentrations (see figures 1C and 3C). The mean values for these endpoints in the antiandrogen-treated groups varied around the mean value of the respective solvent control group. The high mortality rate in the highest concentration of CPA (20 µg/g egg) resulted in a lack of usable tissue samples for the investigation of the histological parameters mentioned.

4. DISCUSSION

4.1 Embryonic mortality and malformations

In ovo exposure to higher concentrations of *p,p´*-DDE, flutamide and CPA resulted in significantly increased mortality rates. Steroid hormone-like drugs including CPA and flutamide are known to potentially induce hepatotoxicity when administered at high doses *(Rojas et al., 2020)*. Following administration, reactive metabolites of these drugs are formed, which may lead to hepatitis *(Giorgetti et al., 2017; Kassid et al., 2022)*. In humans, various case studies report about hepatotoxicity following treatment with flutamide or CPA (e.g., reviewed by *Giorgetti et al. (2017)* and *Kumar et al. (2021)*). The hepatotoxic effect of antiandrogenic substances is also proven by *in vivo* and *in vitro* experiments *(de Gregorio et al., 2021; de Gregorio et al., 2016; Ding et al., 2021; Legendre et al., 2014; Leone et al., 2014; Snouber et al., 2013)*. Therefore, it is traceable that the tested substances adversely affect embryonic development and result in increased mortality rates when administered in higher doses.

Furthermore, *in ovo* exposure to 2 and 20 µg CPA/g egg resulted in significantly smaller embryos as displayed by shortened lengths of skull, ulna and tarsometatarsus. A number of experiments with mammals suggest that antiandrogenic substances can affect bone maturation and elongation resulting in an extension of the growth phase. *Neumann (1982)* and *Neumann and Topert (1986)* state that antiandrogens act in all target organs for androgens and principally affect all functions which are influenced by androgens. Some of these effects, such as the delay of puberty, inhibition of spermatogenesis, the loss of libido or the atrophy of accessory glands, are more sex-specific, while other effects such as delayed bone maturation or the inhibition of body weight development are less sex-specific. Especially under the influence of CPA, a retardation of bone maturation and

249 longitudinal growth is shown in experiments with rodents *(Hertel et al., 1969; Schenck &*
250 *Neumann, 1973)*. This coincides with the findings of the present study. Delayed embryonic growth
251 of *G. gallus domesticus* can thus be directly attributed to the treatment with CPA. This suggests
252 that the chick embryo is a suitable test system for the identification of substance-related mortality
253 and developmental delays.
254 Embryonic energy reserves were determined to investigate the impact of CPA treatment on
255 embryonic development. With increasing concentrations of CPA, a significant decrease in the
256 levels of glycogen and protein was observed. However, 20 µg CPA/g egg resulted in a significantly
257 increased content of lipids. As CPA is known to potentially induce hepatotoxicity *(Kumar et al.,*
258 *2021; Leone et al., 2014)* it can be suspected that higher contents of lipids in the liver of embryos
259 of *G. gallus domesticus* are signs of an incipient liver damage. In reverse, lower contents of
260 glycogen could be a result of increased metabolic activity for detoxification.

261 **4.2 Morphological observation of the gonads – gonad surface area and left testis and ovary**

262 *In ovo* exposure to flutamide or CPA did not affect male and female gonad surface areas. *In ovo*
263 exposure to 0.5 µg *p,p´*-DDE/g egg resulted in significantly smaller left male and right female
264 gonad surface areas. However, we assume that these results are due to the small number of embryos
265 analyzed per experimental group.
266 The significant difference in gonad surface area between the untreated and the solvent control as
267 found in the experiment with flutamide and *p,p´*-DDE confirms the previous findings of our project
268 group. In *Jessl et al. (2018a)* we intensively analyzed untreated and solvent control groups and
269 found that treatment with DMSO resulted in reduced gonad surface areas in both sexes. Gonad
270 surface areas of these gonads decreased with increasing volume of the solvent. Although we cannot

clarify the cause of this effect, we suspect a growth-inhibiting effect caused by the low basic toxicity of the solvent or a possible endocrine-mediated effect of the solvent *(Jessl et al., 2018a)*.

Summarized, the present study shows that the antiandrogens flutamide, *p,p´*-DDE and CPA have no effects on the tested endpoint gonad surface area. This raises the question of whether the gonads of *G. gallus domesticus* are target organs for antiandrogenic substances. It is known that in mammals, antiandrogens principally affect all androgen-dependent functions and organ systems. In rats, AR antagonists such as flutamide, *p,p´*-DDE and CPA are known to be potent inhibitors of androgen dependent reproductive organs *(Neri & Peets, 1975)* resulting in reduced anogenital distance *(Fussell et al., 2015; Pallares et al., 2014)*, hypospadias *(Sinclair et al., 2017)*, atrophy of seminal vesicles *(Pallares et al., 2014)*, nipple retention *(Fussell et al., 2015)*, delayed onset of puberty and reduced ventral prostate weight in male rats *(Kelce et al., 1995)*. In fish, flutamide adversely affects male and female sex differentiation. In females it causes hastened ovarian development with distorted morphology *(Chakrabarty et al., 2012)*, a reduction of relative gonads size *(Milsk et al., 2016)* and disturbs female reproduction *(Bhatia et al., 2014b)*. In males, flutamide affects secondary sex characteristics *(Milsk et al., 2016)* and testicular growth *(Bhatia & Kumar, 2016; Bhatia et al., 2014a; Yin et al., 2017)*.

Also, in birds antiandrogens are known for their hormonal disruptive potential with DDT and its derivates as the most popular representatives. Enriched through the food chain, this compound leads to egg shell thinning in seabirds *(Fry & Toone, 1981; Hickey & Anderson, 1968)* and intersex testes and oviducts in gull embryos *(Fry & Toone, 1981)*. While many species of raptorial and fish-eating birds are shown to be highly sensitive to DDT-related eggshell thinning, other species such as chicken and quail are almost completely insensitive to this end point *(Peakall & Lincer, 1996)*.

Considering gonadal endpoints, antiandrogenic effects may be quite different, depending on the test substance and the species used. *O,p´*-DDT for example adversely affects the gonads of domestic roosters resulting in cloacal defects, deformations of one or both testes and smaller diameters of seminiferous tubules *(Blomqvist et al., 2006)*. In quail *o,p´*-DDT leads to a significant reduction of plasma testosterone levels and the area of the cloacal gland while testis weight and diameter of seminiferous tubules were not affected. Ovaries appeared unaffected although the right oviduct was regressed and the left oviduct was shortened *(Halldin et al., 2003)*. *Quinn et al. (2008)* report that *p,p´*-DDE has no significant effect on gonadal physiology and morphology in both sexes of quail. Studying the effects of *p,p´*-DDE and *p,p´*-DDT on avian reproduction no change in the morphology of reproductive organs was found *(Kamata et al., 2013)*, which coincides with the results of the present study.

(Wollman & Hamilton, 1968; Wollman & Hamilton, 1967) for example demonstrate an inhibitory effect of CPA on comb size of chicks explained by the antagonization of androgenic effects. Furthermore, *Utsumi and Yoshimura (2009)* described CPA to have inhibitory effects on the development of cloacal gland structures in quail. Since AR and mRNA are produced in this tissue cloacal glands, these are target organs for androgens. On the contrary, CPA did not cause significant structural differences in quail ovaries and testes. In quail, flutamide-treatment affects male copulatory behavior as demonstrated by reduced TP-activated strutting representing a central nervous system effect of the drug *(Adkinsregan & Garcia, 1986)*.

The lack of antiandrogenic effects on gonad-based endpoint seems to be related to the avian hormonal system. In birds, sexual differentiation is dependent on estrogen *(Brunstrom et al., 2009; Vaillant et al., 2001b)*. The presence of estrogen causes the differentiation toward the female sex, whereas the absence of estrogen causes differentiation towards the male sex. In contrast, androgens

appear to play a minor role in avian sex differentiation *(Estermann et al., 2021; Groenendijk-Huijbers & Van Schaik, 1976)*. Furthermore, the selected gonad-based endpoints in embryos of *G. gallus domesticus* seem to be insensitive to antiandrogens as they are not target organs/tissues for androgens. However, this does not mean that the endpoints studied are generally useless for the investigation of the effects of potential EDCs on embryonic sexual differentiation. In further investigations we found that especially estrogens but also antiestrogens and androgens can adversely affect embryonic sexual differentiation of *G. gallus domesticus*. In *(Jessl et al., 2018b; Jessl et al., 2018a)* we have shown that *in ovo* exposure of chick embryos to EE_2, a synthetic estrogen, resulted in a distinct feminization of genetic males which formed female-like cortex tissue in their left gonads. In addition, EE_2 treatment resulted in a reduction of the percentage of seminiferous tubules. In *Jessl et al. (2018b)* we demonstrated that the antiestrogen tamoxifen affected female embryonic sex differentiation and caused a size reduction of the left ovary and malformations of the ovarian cortex. In *Scheider et al. (2018)* we investigated the effects of the functional androgen tributyltin (TBT) and found it to affect sex differentiation as it led to virilization effects of female embryos which were mainly characterized by a significant reduction of the left cortex.

5. OVERALL CONCLUSIONS

The focus of the present work was to study the effects of the antiandrogenic compounds CPA, *p,p´*-DDE and flutamide on embryonic gonadal sex differentiation of chicken (*Gallus gallus domesticus*). *In ovo* exposure to all three substances had no effects on gonadial endpoints. In contrast to test results with estrogenic, antiestrogenic and androgenic compounds, these endpoints were not affected by antiandrogenic EDCs and are therefore shown no suitable parameters for the detection of chemicals with antiandrogenic properties in chick embryos.

However, *in ovo* exposure to CPA resulted in significantly smaller embryos than in control groups as displayed by shortened lengths of skull, ulna and tarsometatarsus. This suggests that the chick embryo is a suitable test system for the identification of substance-related mortality and developmental delays.

6. DECLARATIONS

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Figure 1

Effects of *in ovo* exposure to cyproterone acetate (CPA; 0.2, 2, 20 µg/g egg) on embryos of the domestic fowl (*Gallus gallus domesticus*) on embryonic day 19.

Endpoints shown: mortality (A), left and right gonad surface area (B) and cortex thickness and percentage of seminiferous tubules of left gonad (C). Statistical analysis by Fisher´s exact test (A) and one-way ANOVA with Dunnett´s multiple comparisons test (B, C). NC: untreated control group. Lowercase indicates significant differences compared to the solvent control (SC). Level of significance: b, $p<0.01$. Skull symbol: high mortality in the group resulted in an absence of usable gonad tissue for measurements.

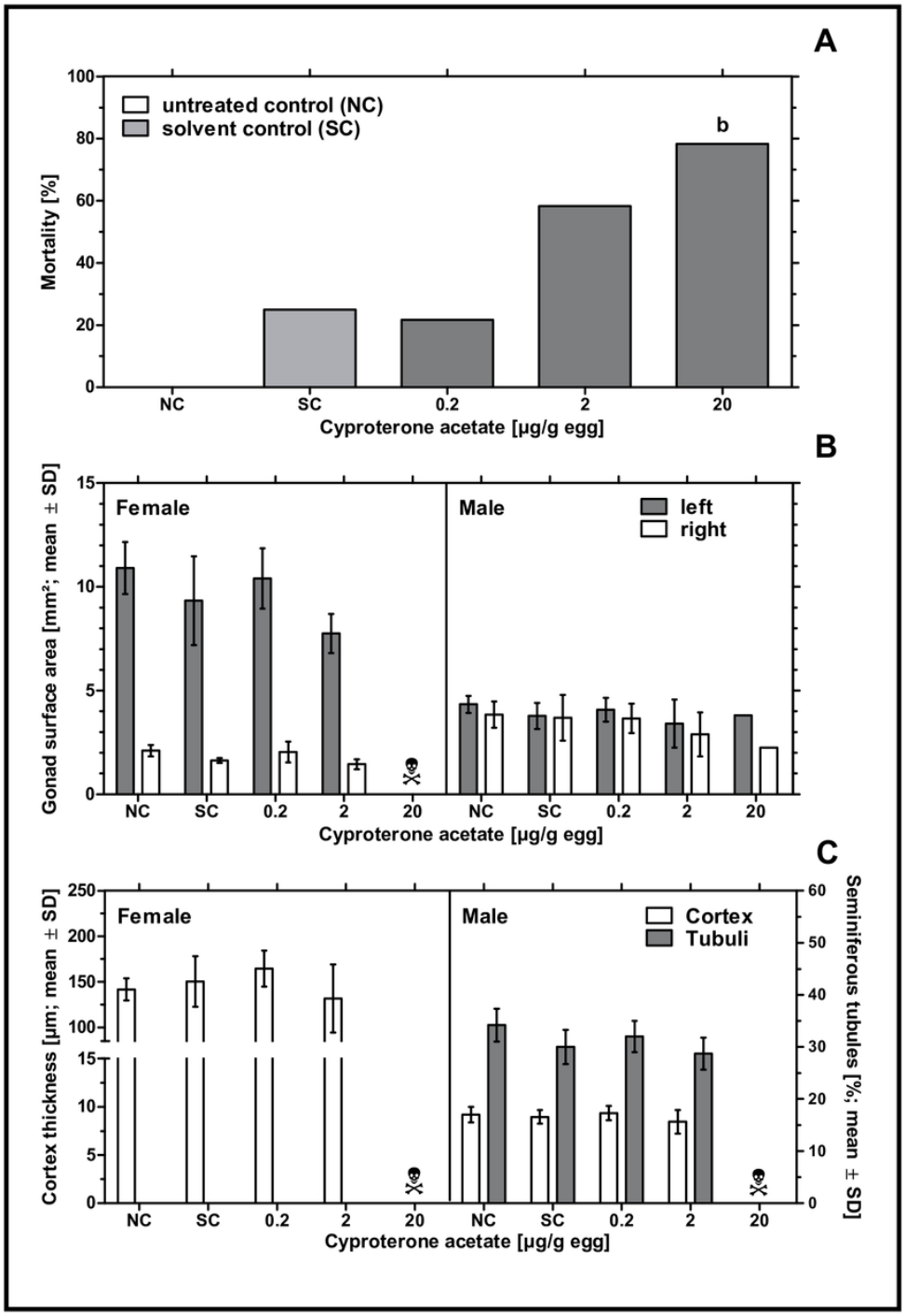

A
untreated control (NC)
solvent control (SC)
b
Mortality [%]
NC
SC
0.2
2
20
Cyproterone acetate [µg/g egg]
B
Female
Male
left
right
Gonad surface area [mm²; mean ± SD]
Cyproterone acetate [µg/g egg]
C
Female
Male
Cortex
Tubuli
Cortex thickness [µm; mean ± SD]
Seminiferous tubules [%; mean ± SD]
Cyproterone acetate [µg/g egg]

Figure 2

Effects of *in ovo* exposure to cyproterone acetate (CPA; 0.2, 2, 20 µg/g egg) on body lengths and energy levels of embryos of the domestic fowl (*Gallus gallus domesticus*) on embryonic day 19.

Endpoints shown: length of skull, tarsometatarsus and ulna (A) and energy levels (lipid, protein, and glycogen) of liver (B). Statistical analysis by one-way ANOVA with Dunnett´s multiple comparisons test. NC: untreated control group. Lowercase indicates significant differences compared to the solvent control (SC). Level of significance: b, $p<0.01$; c, $p<0.001$.

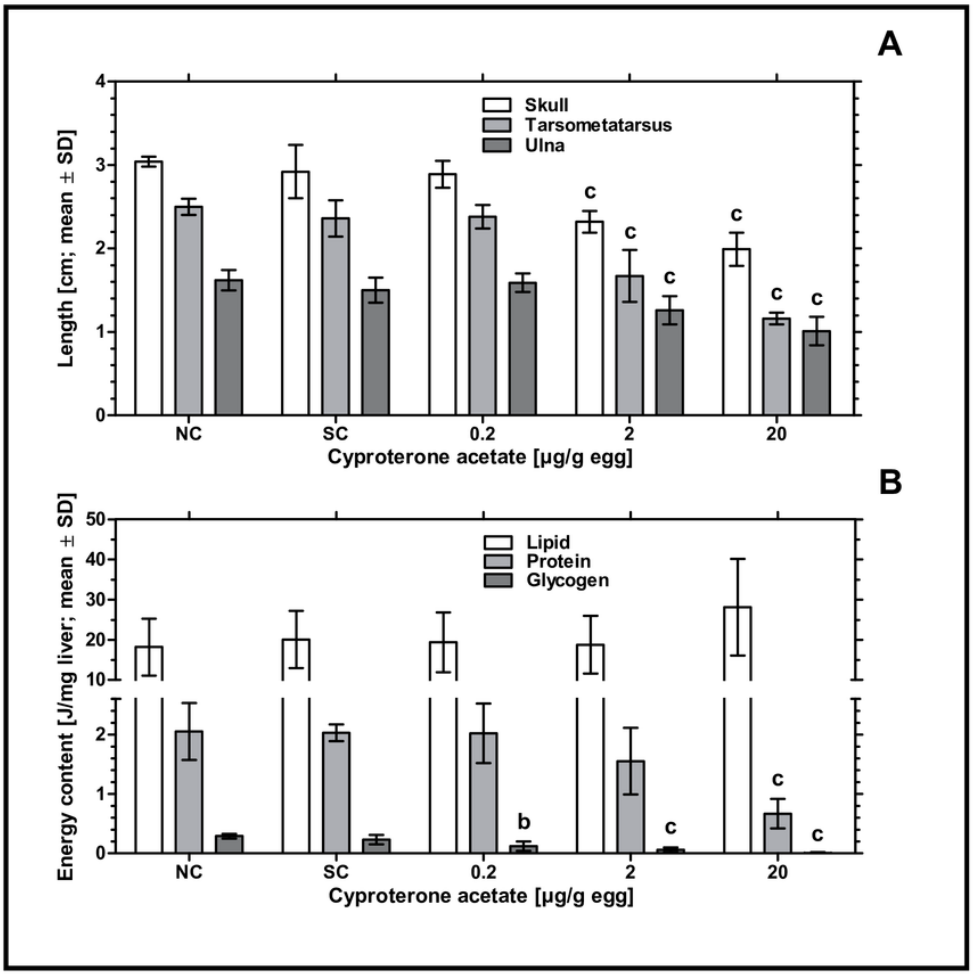
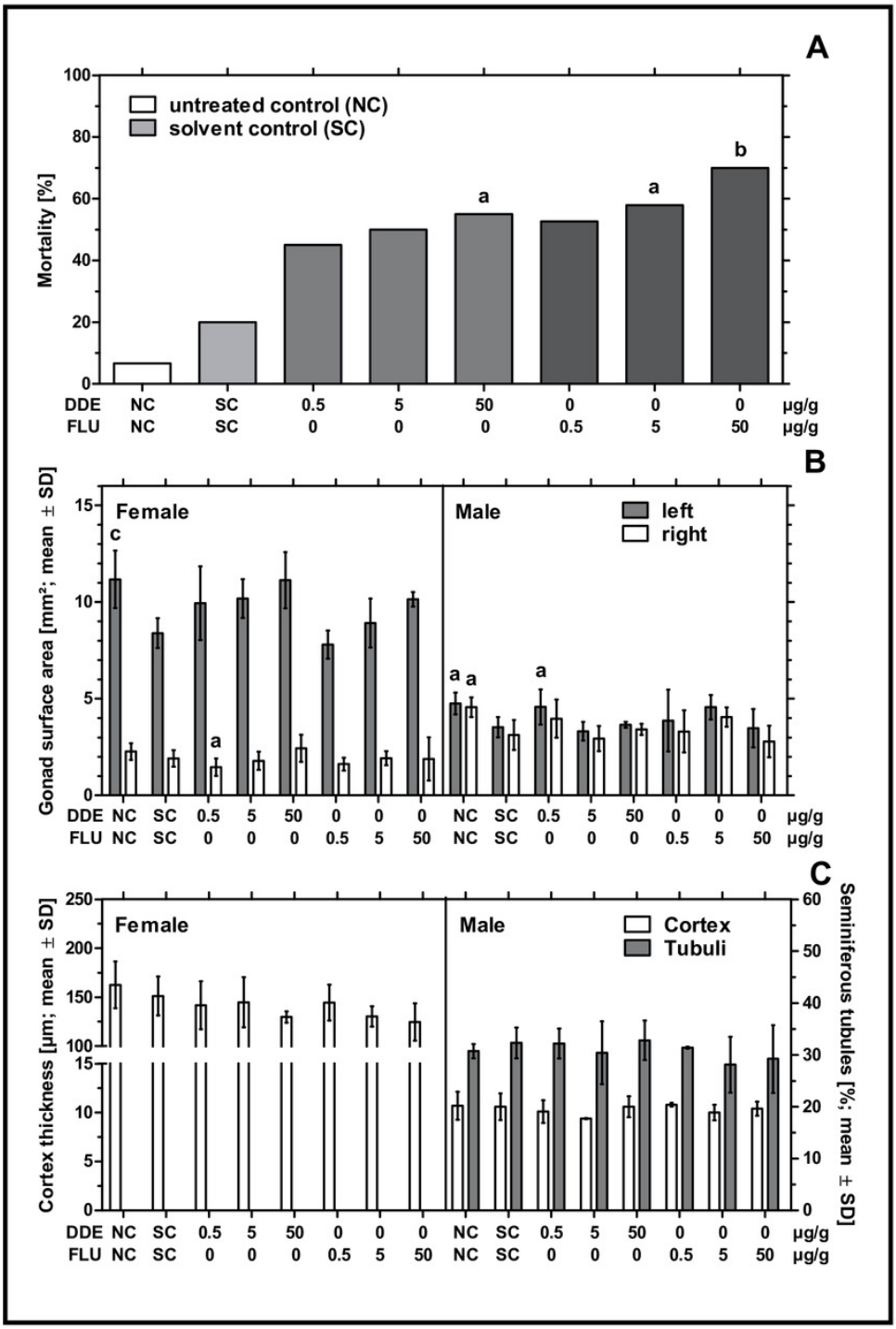

A
Skull
Tarsometatarsus
Ulna
Length [cm; mean ± SD]
0
1
2
3
4
c
c
c
c
c
c
NC
SC
0.2
2
20
Cyproterone acetate [µg/g egg]
B
Lipid
Protein
Glycogen
Energy content [J/mg liver; mean ± SD]
10
20
30
40
50
0
1
2
b
c
c
c
NC
SC
0.2
2
20
Cyproterone acetate [µg/g egg]

Figure 3

Effects of *in ovo* exposure to *p,p´*-DDE (DDE; 0.5, 5, 50 µg/g egg) and flutamide (FLU; 0.5, 5, 50 µg/g egg) on embryos of the domestic fowl (*Gallus gallus domesticus*) on embryonic day 19.

Endpoints shown: mortality (A), left and right gonad surface area (B) and cortex thickness and percentage of seminiferous tubules of left gonad (C). Statistical analysis by Fisher´s exact test (A) and one-way ANOVA with Dunnett´s multiple comparisons test (B, C). NC: untreated control group. Lowercase indicates significant differences compared to the solvent control (SC). Level of significance: a, $p<0.05$; b, $p<0.01$.


A
untreated control (NC)
solvent control (SC)
Mortality [%]
DDE NC SC 0.5 5 50 0 0 0 µg/g
FLU NC SC 0 0 0 0.5 5 50 µg/g
B
Female
Male
left
right
Gonad surface area [mm²; mean ± SD]
C
Female
Male
Cortex
Tubuli
Cortex thickness [µm; mean ± SD]
Seminiferous tubules [%; mean ± SD]