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Endocrine disrupting chemicals and their possible effects on the bovine and human ovarian follicle

Endocriene verstoorders en hun mogelijke effecten op de boviene en humane ovariële follikel

Thesis submitted in fulfilment of the requirements for the degree of Doctor in Veterinary Sciences (PhD)
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by

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LIST OF ABBREVIATIONS

A-1254	Aroclor-1254
AhR	Arylhydrocarbon receptor
AMAP	Arctic Monitoring and Assessment Program
APEOs	Alkylphenol polyethoxylates
ART	Assisted reproductive technology
BDE	Brominated diphenyl ether
BMI	Body mass index
BOF-UA	Bijzonder onderzoeksfonds – Universiteit Antwerpen
BPA	Bisphenol-A
cAMP	Cyclic adenosine monophosphate
CB	Chlorinated biphenyl
CGs	Cortical granules
COC	Cumulus oocyte complex
CPA	Cyclophosphamide
DCM	Dichloromethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di-(2-ethylhexyl) phthalate
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
E2	Estradiol
ECM	Extracellular matrix
ECNI	Electron-capture negative ionization
EDC(s)	Endocrine disrupting chemical(s)
ER	Estrogen receptor
ESM-MS/MS	Tandem mass spectrometer with an electrospray interface in negative ion mode
EU	European Union
FASFC	Federal Agency for the Safety of the Food Chain
FAVV	Federaal Agentschap voor de Veiligheid van de Voedelketen
FSH	Follicle stimulating hormone
FWO	Fonds voor Wetenschappelijk Onderzoek
GC/MS	Gas chromatography / mass spectrometry
GnRH	Gonadotropin releasing hormone
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
hCG	Human chorionic gonadotropin
HCH	Hexachlorocyclohexane
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LC/MS	Liquid chromatography / mass spectrometry
LH	Luteinizing hormone
LOAEL	Lowest observed adverse effect level
LOQ	Limit of quantification

lw	Lipid weight
MEHP	Mono(2-ethylhexyl)phthalate
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
Na_2SO_4	Sodium sulphate
NEB	Negative energy balance
NIST	National Institute of Standards and Technology
NOAEL	No observed adverse effect level
OCPs	Organochlorine pesticides
OPU	Ovum pick-up
P4	Progesterone
PBDEs	Polybrominated diphenyl ethers
PC	Principal component
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PET	Polyethylene terephthalate
PFAAs	Perfluoroalkyl acids
PFBA	Perfluoro-butanoic acid
PFBS	Perfluoro-butane sulfonic acid
PFDoA	Perfluoro-dodecanoic acid
PFDA	Perfluoro-decanoic acid
PFDS	Perfluoro-decanoic sulfonic acid
PFHpA	Perfluoro-heptanoic acid
PFHxA	Perfluoro-hexanoic acid
PFHxS	Perfluoro-hexane sulfonic acid
PFNA	Perfluoro-nonanoic acid
PFOA	Perfluoro-octanoic acid
PFOS	Perfluoro-octane sulfonate/sulfonic acid
PFTeA	Perfluoro-tetradecanoic acid
PFTrA	Perfluoro-tridecanoic acid
PFUnDA	Perfluoro-undecanoic acid
PN	Pronuclei
POPs	Persistent organic pollutants
PP	Polypropylene
PPAR(s)	Peroxisome proliferator-activated receptor(s)
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
PVC	Polyvinylchloride
SD	Standard deviation
SPDE	Solid-phase disk extraction
StAR	Steroidogenic acute regulatory-protein
TBBP-A	Tetrabromobisphenol A
TCDD	2,3,7,8-tetrachlorodibenzo-ρ-dioxin
TEF	Toxic equivalency factor
TL	Total lipids
UPLC	Ultra performance liquid chromatography
VCD	4-vinylcyclohexene diepoxide
VCH	4-vinylcyclohexene
WHO	World Health Organization
ZNA	Ziekenhuis Netwerk Antwerpen

“Perseverance is not a long race; it is many short races one after another.”

Walter Elliott

GENERAL INTRODUCTION

ENDOCRINE DISRUPTION AND LIVESTOCK REPRODUCTION: MYTH OR MENACE?

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SUMMARY

The global reproductive performance of high producing dairy cows has decreased significantly the last years. In the same period, the presence of chemicals in the environment has strongly increased. Some of these substances exhibit endocrine disrupting properties, whereby they are able to disturb the reproductive system. As a result, these endocrine disrupting chemicals (EDCs) were recently suggested as one of the possible causes of the multifactorial fertility problem of high producing dairy cows.

This review describes the properties and action mechanisms of EDCs and outlines the evolution of EDC-research. A possible link between the presence of EDCs in the environment and their possible influence on the fertility of livestock is also discussed.

INTRODUCTION

Although environmental pollution has been around for several ages, the 'Industrial Revolution' has led to a substantial environmental contamination with synthetic chemicals, as it is known today. Consequently, living organisms are exposed to much larger amounts and variety of chemicals than in the past. Chemical compounds can be detected in air, water and soil samples and in animal and human tissues (Covaci *et al.*, 2002; Ying *et al.*, 2002; Jaspers *et al.*, 2006; Covaci *et al.*, 2008a; Petro *et al.*, 2010; Josefsson *et al.*, 2011; Onofrio *et al.*, 2011). Besides common cytotoxic actions, some of these substances can act as 'endocrine disrupting chemicals' (EDCs) by interfering with the synthesis, function, storage and/or metabolism of hormones (Sweeney, 2002). EDCs have the ability to alter steroidogenesis (Sanderson, 2006) and can mimic or antagonize the effects of natural hormones by binding to their receptors (estrogen, androgen and thyroid receptors) (Janosek *et al.*, 2006). EDCs can also disrupt the physiological endocrine balance in the body through binding to other regulatory nuclear receptors such as the arylhydrocarbon receptor (AhR) (Pocar *et al.*, 2005; Janosek *et al.*, 2006) and the Peroxisome Proliferator-Activated Receptor family (PPARs) (Lovekamp-Swan and Davis, 2003; Latini *et al.*, 2008; Kwintkiewicz *et al.*, 2010). Humans and animals are exposed to these chemicals via contaminated food and water, inhalation of polluted air or through (dermal) contact with contaminated soil (Brevini *et al.*, 2005). In literature, it has already been hypothesized that EDCs can have a negative influence on the thyroid gland, the immune system and reproductive functions (Gandolfi *et al.*, 2002).

WHICH COMPOUNDS ARE CURRENTLY CONSIDERED AS ENDOCRINE DISRUPTING CHEMICALS?

Currently, about 60 chemicals have been identified as EDCs (Brevini *et al.*, 2005). This group can be divided in synthetic (industrial chemicals, pesticides, detergents, plastic additives, synthetic hormones and preservatives) and natural EDCs (phyto- and mycoestrogens) (Figure 1a-b).

Synthetic endocrine disrupting chemicals

Industrial chemicals

Polychlorinated biphenyls (PCBs) and dioxins are by far the best-known endocrine disruptors in this group of chemical compounds. In Belgium, these compounds gained attention because of the "dioxin

crisis" in 1999, whereby recycled lipids, used for the production of animal feed, were contaminated with a PCB mixture containing dioxins (Covaci *et al.*, 2008b). Less known, but certainly no less abundant, are the so-called "flame retardants" and the increasingly more important perfluoroalkyl acids.

Polychlorinated biphenyls (PCBs) are a group of aromatic compounds consisting of 209 'congeners', whereby the number and location of the chlorine atom(s) determine the congener type and properties. PCBs were produced during approximately 50 years until the late seventies of the previous century (Meeker, 2010). Because of their inflammability and stability, PCBs were used for many purposes, including in transformers, for the production of adhesives, textiles and plastics, in printing and for producing coolants (Boerjan *et al.*, 2002). Although the production of PCBs is banned in many countries, they continue to leak into our environment because of leakage from installations or the use of an incorrect disposal procedure (La Rocca and Mantovani, 2006). Polychlorinated biphenyls can be divided in two groups on the basis of their mechanism of action: dioxin-like (coplanar) and non-dioxin-like (non-coplanar) PCBs. As is suggested by the name, dioxin-like PCBs are also able to bind the AhR, whereby their effects are largely similar to those caused by dioxins. Studies also indicate that only coplanar PCBs induce embryotoxic effects in different species (Kuchenhoff *et al.*, 1999; Pocar *et al.*, 2005). The possible endocrine disrupting properties of non-coplanar PCBs can be explained by the action of their metabolites: hydroxylated PCB metabolites are able to inhibit sulphotransferases, resulting in an increase of the amount of free estradiol in the organism (Kester *et al.*, 2000). In addition, PCBs may also interfere with thyroid hormone metabolism (Miller *et al.*, 2009). Although it took until January 2012 to define maximal levels in food for 6 non-coplanar PCBs (EC, 2006), it is especially this type of PCBs that are found in our food chain (La Rocca and Mantovani, 2006).

Polychlorinated dibenzo-p-dioxins (PCDDs) are a group of environmental contaminants including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a prototype and most toxic substance. Dioxins are formed in incinerators, during forest fires and during industrial processes such as the production of herbicides and bleaching of paper pulp (Boerjan *et al.*, 2002). Exposure to dioxins can cause chloracne in humans, cleft palate in mice, an impaired function of the immune system and increases cancer risk (Bock and Kohle, 2006). Dioxins have also teratogenic, embryotoxic and neurotoxic properties (Mandal, 2005). They exert these negative effects mainly through binding to the AhR. This receptor is a ligand activated transcription factor, which, following ligand binding, moves from the

cytoplasm to the nucleus where it dimerizes with the AhR nuclear translocator. This heterodimer in turn binds to specific DNA enhancer sequences, known as xenobiotic response elements (Pocar *et al.*, 2006). The toxicity of dioxins can probably be attributed to an impaired activation of the AhR due to the binding of an exogenous ligand, thereby disrupting the physiological functions of the receptor (Bock and Kohle, 2006). To 'score' the toxicity of compounds that act through the AhR, a toxic equivalency factor (TEF) is used which expresses the toxicity of a compound as a function of TCDD-toxicity (TEF for TCDD = 1) (Larsen, 2006).

Flame-retardants are chemical compounds used in polymers, textile, building materials, furniture and electrical installations to increase their resistance against fire (Vos *et al.*, 2003). Their global production in 1992 was estimated to be more than 600 000 tons per year (IPCS, 1997). Widely used flame-retardants are polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBP-A) and hexabromocyclododecane (HBCD). In contrast to PCB and dichlorodiphenyltrichloroethane (DDT) levels, the environmental concentration of some brominated flame retardants, such as PBDEs, has increased strongly during recent decades (Vos *et al.*, 2003). Human exposure to PBDEs probably mainly occurs through food. In Belgium, the daily amount of PBDE intake through food is estimated at 23-48 ng (Voorspoels *et al.*, 2007). Studies indicate that brominated flame retardants are able to influence the endocrine system by interacting with thyroid hormone homeostasis, AhR and estrogen receptors (ERα and ERβ) (Darnerud *et al.*, 2001; Meerts *et al.*, 2001). As most endocrine toxicity studies were performed with PBDEs, knowledge about possible endocrine disorders caused by HBCD and TBBP-A is currently almost non-existent. In January 2003, the European Parliament published a directive that stated that from the 1st of July 2006 onwards, no more electrical and electronic equipment containing PBDEs should enter the market (EC, 2003).

A final group of industrial chemicals with possible endocrine disrupting effects are the **perfluorinated alkylated substances (PFASs)**. As suggested by their name, these chemicals consist of a carbon backbone on which multiple fluorine atoms are covalently bound. The carbon-fluorine bond is the strongest covalent bond that is known, rendering PFASs very stable and practically non-degradable. PFASs are used as surfactants, polymers and as flame-retardant foams in industrial and commercial products (Dauwe *et al.*, 2007). Perfluoro-octane sulfonate (PFOS), the end-metabolite of PFASs, has been detected in mammals, fish and birds all over the planet, with the highest concentrations found in organisms living in densely populated and industrialized regions (Giesy and Kannan, 2001). In human, PFASs, being both hydrophilic and hydrophobic, are readily absorbed, able to bind to serum

proteins like albumin (Han *et al.*, 2003), poorly eliminated and distributed primarily in serum and liver (Lau *et al.*, 2004), with long half-lives of around 4 and 5 years for perfluoro-octanoic acid (PFOA) and PFOS, respectively (Olsen *et al.*, 2007). The liver is generally considered as being the target organ for these compounds (OECD, 2002). Research has shown that large doses of PFOS and PFOA induce tumors, and also lead to an impaired development, endocrine disruption, and neonatal mortality in experimental animals (Betts, 2007). The mechanisms underlying these defects are largely unknown. A possible explanation can be the binding of PFASs on the already mentioned PPARs (Abbott *et al.*, 2007). However, it is highly likely that these compounds can also disrupt the normal physiology through other biochemical processes.

Pesticides

Pesticides are able to induce a whole set of harmful effects in man, such as headache, respiratory problems, neurologic or physic complaints and skin irritation (Bretveld *et al.*, 2006). Exposure to pesticides has also been associated with decreased fetal growth, shortened pregnancies and spontaneous abortions (Stillerman *et al.*, 2008). Several pesticides also exhibit endocrine disrupting properties, e.g. various imidazole fungicides inhibit or induce *in vitro* the actions of the aromatase enzyme responsible for the conversion of androgens to estrogens (Vinggaard *et al.*, 2000). Other pesticides, like methoxychlor, chlordecone (Kepone), dieldrin and endosulfan possess estrogen-like properties (Bretveld *et al.*, 2006). Because of this wide range of adverse effects, many pesticides have been taken from the market and are replaced by less harmful products. Some pesticides, however, are so efficient, making a ban on these products in third world countries less obvious. DDT is still one of the most effective and inexpensive products in the battle against Anopheles mosquitoes, responsible for the transmission of malaria which makes more than 1 million victims every year (IEH, 1999; WHO, 2006). Therefore, the World Health Organization (WHO) decided in 2006 that treatment of walls and roofs with DDT in risk areas should again be recommended in the fight against malaria.

Detergents and plastic additives

In this large group of chemicals, several widely used compounds possess an endocrine activity; the most important and/or most studied substances are phthalates, bisphenol-A and alkylphenol polyethoxylates.

Phthalates are primarily used as plasticizers to keep plastic or related materials flexible for food and construction industries, in the manufacture of toys, medical equipment and pharmaceuticals

(Lovekamp-Swan and Davis, 2003; Latini *et al.*, 2006). They are also present in personal care products and paints. More than 8 million tons phthalates are being consumed each year (Lovekamp-Swan and Davis, 2003). Because phthalates are not bound to plastics in a covalent way, they are easily released in the environment (Latini *et al.*, 2006). Of all phthalates, di-(2-ethylhexyl) phthalate (DEHP) is the most widespread and best known (Harris and Sumpter, 2001). Prenatal exposure to phthalates leads to a reduced foetal testosterone synthesis in laboratory animals (Parks *et al.*, 2000), which is described as 'the phthalate syndrome' and characterized by malformations of the epididymis, vas deferens, seminal vesicles, prostate and external genitals often in conjunction with cryptorchidism and feminization (Latini *et al.*, 2006). Phthalates are acting through the PPARs and the inhibition of the Follicle Stimulating Hormone (FSH) stimulated cyclic adenosine mono phosphate (cAMP) production and therefore also capable to disrupt folliculogenesis in the ovary (Lovekamp-Swan and Davis, 2003).

Bisphenol-A (BPA) is used in the production of polycarbonate plastics, epoxy resins and as an additive to other plastics like polyvinylchloride (PVC) and polyethylene terephthalate (PET), meaning that BPA can be found in many plastic items such as storage boxes, bottles and toys. BPA is also present in the inner coating of food cans and dental resins (Maffini *et al.*, 2006; Welshons *et al.*, 2006). Nearly 3 million tons of BPA were produced worldwide in 2003 (Welshons *et al.*, 2006). Due to frequent washing, heating and/or contact with acidic or alkaline substances, BPA monomers are able to enter the food chain (Brotons *et al.*, 1995; Lim *et al.*, 2009). BPA is able to bind to both ER-subtypes and can provoke rapid cellular responses through binding on membrane-bound ERs (Kuiper *et al.*, 1998; Wozniak *et al.*, 2005). It can decrease sperm production and motility, increase the expression of the estrogen and progesterone receptor in the endometrium and cause aneuploidy in oocytes (Maffini *et al.*, 2006). In addition, BPA interferes with thyroid function as an antagonist of the thyroid hormone trijodothyronin (Moriyama *et al.*, 2002). BPA is metabolised in mammals through hepatic glucuronidation or sulfation. Degradation of BPA is species and oxygen tension specific: it is degraded under aerobic, but not under anaerobic conditions (Kang *et al.*, 2006). In murine *in vivo* experiments, the observation that BPA induces physiologic effects in concentrations that are lower than the tolerable daily intake (50 µg/kg/day) is very alarming (Nagel *et al.*, 1999). Moreover, BPA-levels in human blood samples are higher than the concentrations observed to be harmful in *in vitro* experiments (Welshons *et al.*, 2006), which is why researchers propose a new, more complete BPA risk assessment (vom Saal and Hughes, 2005).

The final class of chemicals in this group that deserve more attention are the **alkylphenol polyethoxylates (APEOs)**. They are used for the production of plastics and paper, in the textile industry and as emulsifiers in the fabrication of liquid pesticides. APEOs are also present in detergents and health care products (Markey *et al.*, 2001). The worldwide production of these compounds can be estimated to be more than 500 000 tons a year (Ying *et al.*, 2002). In general, it is accepted that APEOs disappear through bio-degradation by forming short chain APEO-metabolites that in turn can fully de-ethoxylate in alkylphenols like nonylphenol and octylphenol (Montgomery-Brown and Reinhard, 2003). While the estrogenic action of APEOs can be explained by their binding capacity to the ER (White *et al.*, 1994), octylphenol showed to possess the strongest estrogen activity. Because they mainly contaminate the environment via wastewater from water treatment plants, APEOs are usually found in surface water and aquatic organisms (Ying *et al.*, 2002). Fish living downstream of these plants show many reproductive disorders from which intersexuality is an important one (Jobling and Tyler, 2003). Male fish produce for example vitellogenin, a protein that is normally produced only by female fish (Purdom *et al.*, 1994). Analyses conducted in Great Britain showed that APEO and its metabolites, combined with natural and synthetic estrogens are the most important estrogen active chemicals in this 'purified water' (Jobling and Tyler, 2003). The concentrations of APEOs and metabolites were even higher (ng/ml) than the natural and synthetic estrogens (pg/ml) (Ternes *et al.*, 1999; Ying *et al.*, 2002). Next to reproductive disorders, these fish display disturbances in kidney function and the immune system, and illustrate evidence of genotoxicity (Liney *et al.*, 2006). In wastewater treatment plants, APEO-metabolites attach to the present sludge (Ahel *et al.*, 1994; La Guardia *et al.*, 2001) and subsequently enter the food chain because the sludge can be applied under certain conditions on pastures as a fertilizer. For that reason, the Scientific Committee of the 'Federal Agency for the Safety of the Food Chain' (FASFC) advised to expand the list of chemicals for which maximum values in sludge have to be determined, with chemicals being listed in an European work document, including DEHP, PCBs, dioxins and nonylphenol(ethoxylates) (EC, 2000; FAVV, 2002).

Synthetic hormones

Unlike the European ban of 1988, the USA and Canada still allow the use of certain **synthetic hormones**, like trenbolone-acetate, zeranol (=α-zearalanol) and melengestrol-acetate (USA only), for the use in livestock production industry (Shier *et al.*, 2001; Wilson *et al.*, 2002; Chung and Johnson, 2009). Most of the time, growth hormones are gradually released in the blood from subcutaneous implants, metabolized to their active forms and finally discharged in the environment through the

feces (Schiffer *et al.*, 2001; Wilson *et al.*, 2002). In (veterinary) medicine, synthetic estrogens are used for different purposes, sometimes with unexpected adverse effects. Since 1948, diethylstilbestrol (DES) was prescribed to prevent spontaneous abortions. However, when daughters of DES-treated mothers developed various reproductive disorders, DES was taken off the market (Colborn *et al.*, 1993). One of the most famous and widespread applications of synthetic estrogens is their use as a contraceptive. While these synthetic compounds are excreted through the feces, as are natural estrogens, they persist in the effluents of wastewater treatment plants (Ternes *et al.*, 1999) and therefore add to natural sex hormones entering our environment. Whether this extra amount of oestrogen and androgen active compounds is harmful to the eco-system remains a major point of discussion for the scientific community (Caliman and Gavrilescu, 2009).

Preservatives

Parabens are a group of preservatives consisting of 7 alkylesters of p-hydroxybenzoic acid, their common metabolite. They are mainly used in cosmetics, health care products and also as food additives, because of their antimicrobial activity (Byford *et al.*, 2002; Darbre and Harvey, 2008). Different maximum levels have been determined for parabens used in cosmetics and health care products on the one hand and parabens used as food additives on the other hand (VITO, 2009). While parabens enter the body orally and through the skin (Darbre and Harvey, 2008), their concentrations are usually higher in urine than in blood, which indicates a rapid metabolization and excretion of these substances in the body (Boberg *et al.*, 2010). Epidemiological research has shown that more breast cancers are present in the quadrant closest to the armpit, which is in closest contact with deodorants. Moreover, parabens are found in breast cancer tissue (Darbre and Harvey, 2008). In utero exposure of rats to butylparaben causes a decreased expression of ERβ in the ovaria and of the 'Steroidogenic Acute Regulatory'-protein (StAR) in the adrenal glands of female foeti. This disturbed StAR-expression can influence steroidogenesis because StAR is responsible for cholesterol transport to the inner mitochondrial membrane, an essential step in steroidogenesis (Taxvig *et al.*, 2008).

Both (anti-)estrogen and anti-androgen effects are being attributed to parabens (Chen *et al.*, 2007). The estrogen action of parabens increase with chain length and branching (Okubo *et al.*, 2001; Byford *et al.*, 2002). Parabens also possess an indirect estrogen action, through inhibition of sulphotransferases, resulting in higher free estradiol concentrations (Prusakiewicz *et al.*, 2007). On the contrary, parabens apparently inhibit aromatase, the enzyme responsible for the conversion of testosterone to estradiol (Boberg *et al.*, 2010).

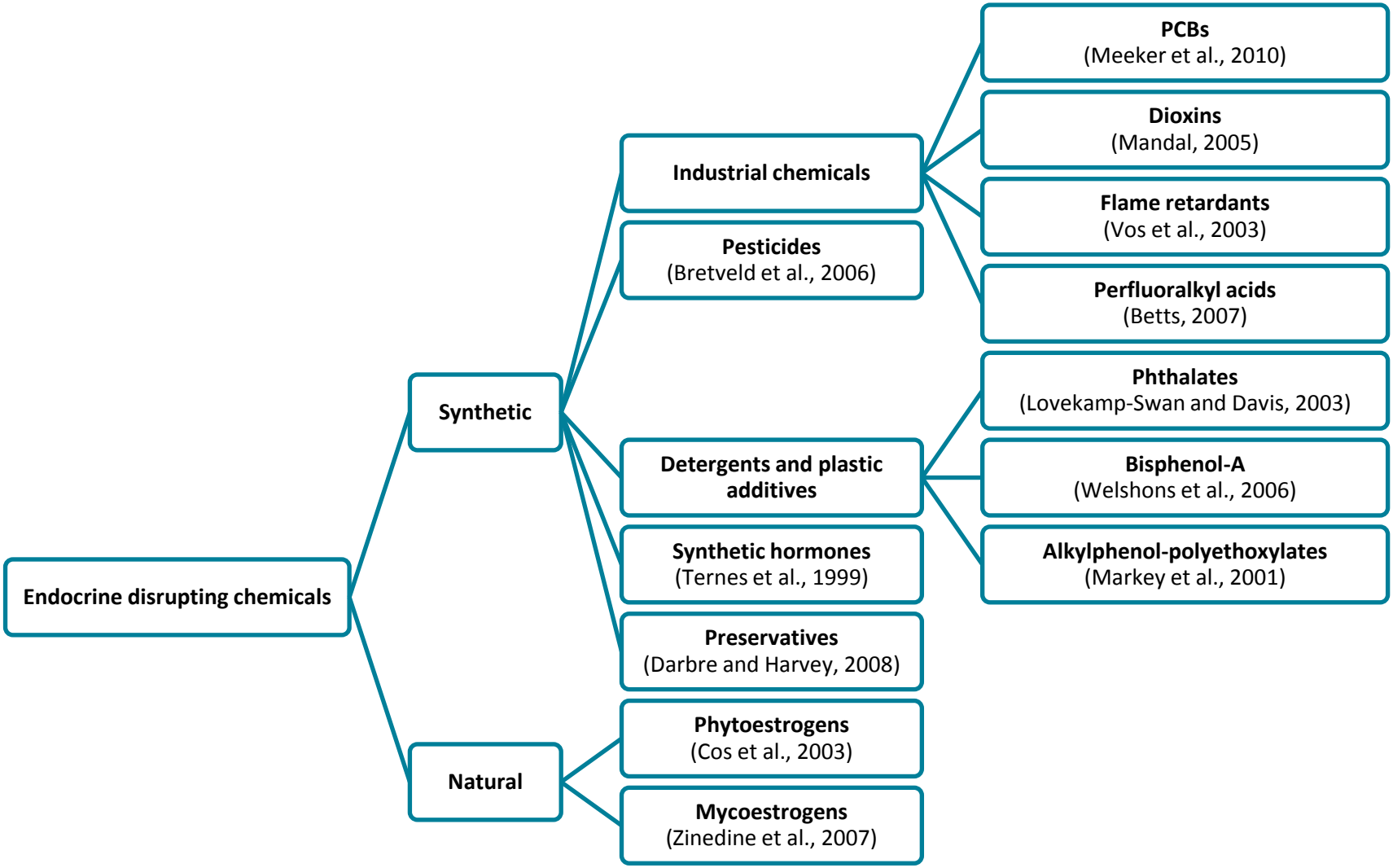

Figure 1a Overview of endocrine disrupting chemicals

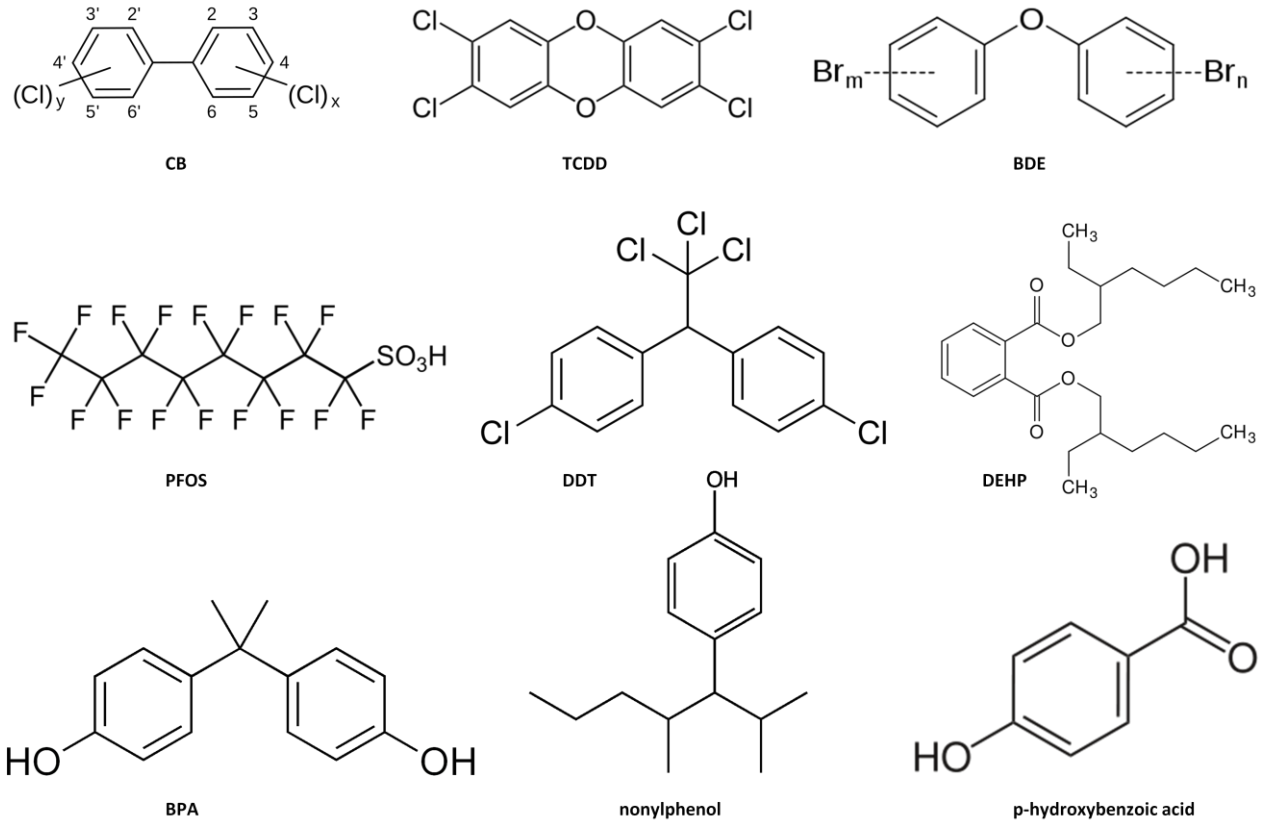

Figure 1b Chemical structures of the most important EDC representatives
(CB = chlorinated biphenyl, TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, BDE = brominated diphenylether, PFOS = perfluoro-octane sulfonate, DDT = dichlorodiphenyltrichloroethane, DEHP = di(2-ethylhexyl)phthalate, BPA = bisphenol-A)

Natural endocrine disrupting chemicals

Phytoestrogens

Phytoestrogens are endocrine active substances naturally contained in plants. The endocrine effects of these compounds are primarily based on their ability to bind to the estrogen receptor (Cos *et al.*, 2003). Next to the influence of the age of the exposed animal and the exposure dose, consequences of exposure to phytoestrogens can also differ among species, which can probably be attributed to the concentration of natural estrogens in the blood. Phytoestrogens act agonistically in species with low circulating natural estrogen concentrations (sheep, cattle), while species with relative high circulating estrogen levels, like humans, have to deal with anti-estrogen effects of these compounds (Adams, 1995). Phytoestrogens are also suggested to have protective effects on estrogen-related conditions, such as menopausal symptoms, and estrogen-related diseases (e.g. prostate and breast cancers, osteoporosis, and cardiovascular disease) (Cos *et al.*, 2003). Despite these positive effects, an excessive intake of phytoestrogens is questionable. Meat replacers are often made of soybean (*Glycine max*), an important source of phytoestrogens like the isoflavones daidzein and genistein (Baber, 2010) while a substantial amount of babies drink soybean-based milk. At the moment, the intake of soybean-derived products is considered safe for children (Klein, 1998), but it is still unclear if consuming high amounts of phytoestrogens at a very young age can be harmful later in life. Red clover (*Trifolium pratense*) and hop (*Humulus lupulus L.*) are other sources of phytoestrogens, in which daidzein, formononetin, biochanin A and genistein (isoflavones) and the flavonoids 8- and 6-prenylnaringenin can be found (Chadwick *et al.*, 2006; Overk *et al.*, 2008). 8-Prenylnaringenin is known as one of the most potent phytoestrogens (Zanoli and Zavatti, 2008). *In vitro* experiments revealed that phytoestrogens, next to their hormonal activity, also have pro-apoptotic effects and genotoxic characteristics by inducing the formation of micronuclei, small nuclei that are formed during cell division when (parts of) chromosomes are not incorporated in the daughter cells (Stopper *et al.*, 2005).

Mycoestrogens: Zearalenone

Zearalenone is a non-steroidal mycotoxin with estrogenic activity, synthesized by fungi from the genus Fusarium (Bennett and Klich, 2003) that can infect different crops during growth and/or storage in a humid atmosphere. As a consequence, this mycotoxin can be present in diverse grain-derived products, like flour, malt and beer (Zinedine *et al.*, 2007). Although zearalenone has a low acute toxicity, long-term toxicity studies in mice indicated that it might display chronic toxicity

including a possible role in the development of liver cancer. Rats, however, did not show an increased number of tumors (NTP, 1982). Moreover, it appears that zearalenone is able to bind both ERα and ERβ (Kuiper *et al.*, 1998), being one of the most potent naturally occurring estrogens (Stopper *et al.*, 2005). After oral ingestion, zearalenone is readily absorbed and reduced to α- and β-zearalenol, which are further metabolized to α- and β-zearalanol. The observation that α-zearalenol has a stronger estrogen activity than zearalenone or β-zearalenol (Shier *et al.*, 2001), combined to the fact that the α-/β-zearalenol ratio is species specific can explain the different species sensitivity following zearalenone exposure (Fink-Gremmels and Malekinejad, 2007). Given the worldwide high concentrations in food and the knowledge that zearalenone is capable of inducing *in vitro* MCF-7 breast cancer cell proliferation in the absence of estrogens, the question arises whether this compound also contributes to the increasing incidence of breast cancer observed over the last decades (Yu *et al.*, 2005).

ENDOCRINE DISRUPTING CHEMICALS, AN UNDERESTIMATED PROBLEM?

In recent years, endocrine disruptors were studied through both *in vivo* and *in vitro* experiments. The first reports were epidemiological studies which linked reproductive disorders in wildlife to exposure to specific toxic substances, only later described as endocrine disruptors (Colborn *et al.*, 1993). These studies were mostly conducted in animals which already showed reproductive abnormalities and were located at the top of the food chain or living in an environment with exceptional high concentrations of EDCs (Rhind, 2005; Guillette and Moore, 2006). Well-known examples are the alligators from Lake Apopka, Florida. After years of large-scale pesticide use on adjacent agricultural land and through leakage of pesticides, mainly dicofol and DDT-derivatives, the gonads of these animals showed several morphological abnormalities (Milnes and Guillette, 2008). While multi-oocyte follicles and polynuclear oocytes were found in females, smaller phallus sizes were found in male alligators compared to animals from less polluted lakes. In addition, the eggs of these animals showed a decreased viability with a subsequent decline in the number of young alligators.

Following these findings in wildlife, experiments were started, exposing laboratory animals to known EDC-levels to study possible abnormalities and determine different toxicity levels of chemicals, such as the 'No Observed Adverse Effect Level' (NOAEL) and 'Lowest Observed Adverse Effect Level' (LOAEL) (Chaffin *et al.*, 1997; Fisher *et al.*, 1999). In addition, *in vitro* exposure experiments using different cell types were executed to unravel the molecular mechanisms responsible for the harmful effects of EDCs and to detect and identify new EDCs (Todd *et al.*, 1995; Maras *et al.*, 2006; Vanparys

et al., 2010). According to the increasing number of publications, the attention for the potentially harmful effects of EDCs on welfare and health of humans and animals increased substantially over the last years (Figure 2). However, EDC-research has been neglected for a long time for several reasons. Initially, it was assumed that EDCs were not toxic in the rather low environmental concentrations detected, which were far below those causing adverse physiological effects in acute toxicity studies (IEH, 1999). Moreover, the production of several actually known EDCs, such as DDT and PCBs, was already banned since the 60s and 70s of the previous century (Walker, 2001; Rhind, 2005). On top of that, abnormalities in wildlife were only observed in animals living in heavily contaminated areas (Colborn *et al.*, 1993; Guillette *et al.*, 1994). Finally, EDCs have a much lower affinity for the estrogen receptor compared to natural estrogens, which makes EDCs less biologically potent than estradiol and other estrogens (Rhind, 2005).

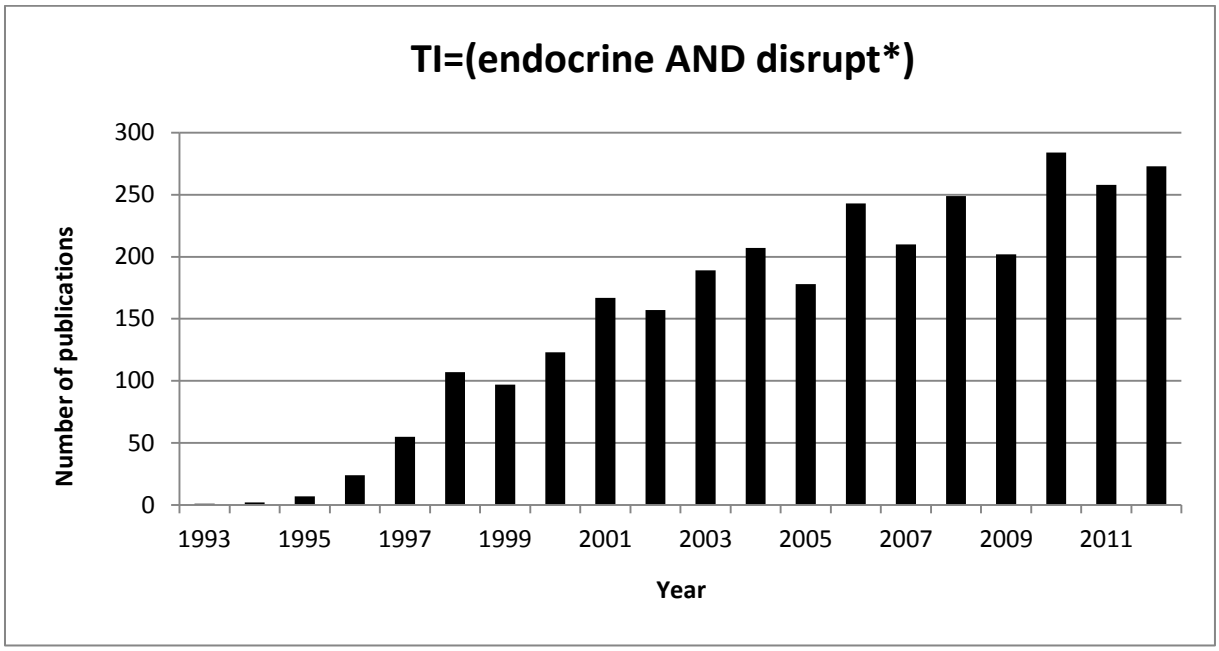

Figure 2 Number of publications per year (1993 – 2012) whereby 'Endocrine' AND 'disrupt*' are mentioned in the title of the publication according to the search engine "Web of Science". Our search started in 1993, the year in which the first review about endocrine disruption was published (Colborn *et al.*, 1993). It should be noted that the publications in which the exact name of the endocrine disrupting chemical is mentioned, are not included, which means that the actual amount of published articles regarding endocrine disruption is much higher.

Taking into account the very complex nature and behavior of EDCs, it becomes clear that more in depth research on the possible harmful effects of these compounds is highly recommended. First of all, many EDCs have **long half-lives** so they remain in the environment several years after they were produced, albeit in relatively low concentrations (Colborn *et al.*, 1993; Pocar *et al.*, 2003). While the

production of DDT and PCBs is already banned for decades in many countries, these compounds are still found in the environment because of these long half-lives (Chu *et al.*, 2003; Voorspoels *et al.*, 2004). In addition, the majority of EDCs are extremely **lipophilic**, which is why they tend to accumulate in adipose tissue (Brevini *et al.*, 2005). Mammals mobilise large quantities of fat during lactation, meaning that EDCs, stored in the adipose tissue of the dam, are possibly released in circulation. In this way, both the dam and the neonate (through suckling of contaminated milk) are exposed to higher EDC-concentrations (Sweeney, 2002). By the combination of their long half-lives and being lipophilic, EDCs undergo a **bioaccumulation** whereby the highest EDC levels are found in animals at the top of the food chain. Moreover, EDCs have a **higher bioavailability** in the body as compared to endogenous hormones, because the latter are bound to a higher extent to binding proteins present in the blood (Dechaud *et al.*, 1999; Nagel *et al.*, 1999). Humans and animals are exposed to continuously varying complex **EDC-mixtures**, so it can be assumed that these chemicals can influence each other's action in an additive, adverse or synergistic way. The harmful dose of environmental relevant EDC mixtures turned out to be even significantly lower as compared to individual administration of the different chemicals (Rajapakse *et al.*, 2002). It has already been shown that **metabolites** of EDCs can have a higher endocrine activity than the parent compounds (Kester *et al.*, 2000; Meerts *et al.*, 2001). These metabolites however are hardly taken into account when the parent compounds are administered as in the majority of *in vitro* experiments. Moreover, the *in vivo* metabolism of many compounds is often unknown and/or species specific (Boerjan *et al.*, 2002). For the worse, EDCs are able to induce **transgenerational effects** through their action on the epigenome. This way, the consequences of exposure are being transferred to the next generations without modifying the DNA-sequence, but through alterations of other molecular mechanisms controlling gene expression, such as DNA methylation and histone protein modifications (Anway *et al.*, 2005; Skinner, 2007). For example, the administration of the pesticides vinclozoline and methoxychlor to pregnant rats induced a higher apoptotic rate in spermatogonia and a decrease in the number and motility of sperm cells until the F4-generation (Anway *et al.*, 2005). Finally, studies suggested that a **chronic exposure** to environmental relevant EDC concentrations could induce the same or even more harmful effects than an acute exposure to high EDC levels (Borman *et al.*, 2000). Because mainly acute toxicity studies were performed, it is possible that long term effects of EDCs have been underestimated in the past, whereby the maximum allowable EDC concentrations in current regulations may be based on incomplete or even incorrect knowledge. This is one of the reasons why the European Union approved the 'Registration, Evaluation, Authorisation and

Restriction of Chemicals (REACH)' program, through which they are committed to submit a whole group existing and new chemicals to a new, more complete toxicological screening (EC, 2007).

ENDOCRINE DISRUPTING CHEMICALS AND THEIR EFFECTS ON FARM ANIMALS

During the last decades, a spectacular decrease of dairy cow fertility has been observed, more specifically in high producing herds, which is mainly manifested through a prolonged calving interval (Opsomer *et al.*, 1998; Shrestha *et al.*, 2004) and an increase of the number of inseminations per gestation. In Belgium, these parameters have increased over the past years from 409 to 420 days and from 1.53 to 1.71 inseminations, respectively (CRV, 2010). High energetic and protein rich diets (Butler, 1998), larger herd sizes (Fahey *et al.*, 2002), genetic selection in favor of milk yield (Snijders *et al.*, 2000) and the negative influence of metabolic disorders occurring shortly after parturition on oocyte and subsequent embryo development (Leroy *et al.*, 2005) are the most proposed causes for this declining trend in fertility.

However, over the last years, it has already been suggested that the effects of EDCs can be an additional factor contributing to the fertility problems observed in domestic animals (Sweeney, 2002; Rhind, 2005). Literature data on potential adverse effects of EDCs on farm animals are very scarce (Boerjan *et al.*, 2002). However, it is more than likely that these animals are exposed to EDCs through food and/or drinking water uptake. An excessive intake of natural phytoestrogens by ruminants, already described in 1946 in sheep grazing on clover rich pastures, is a clear example of this. These sheep showed various reproductive disorders, including a decreased birth rate and uterus prolapse (Bennetts *et al.*, 1946). Later, it was determined that cattle were also susceptible to the effects of phytoestrogens (Adler and Trainin, 1960). Cows exhibited a swollen vulva, cervical mucus secretion, an enlarged uterus and irregular estrous cycles with periods of anoestrus and reduced fertilization rates. Both in sheep and cattle, the effects disappeared after the animals had been relocated to pastures without phytoestrogen containing plants. In sheep, however, the effects turned out to be permanent if the animals were exposed for more than 3 years to these phytoestrogen-containing plants (Adams, 1995). Farm animals also appear to be sensitive to zearalenone (Minervini and Dell'Aquila, 2008). Sows fed with zearalenone-contaminated feed showed a prolonged inter-estrus interval (Edwards *et al.*, 1987). Pigs and sheep showed to be more susceptible to the effects of zearalenone as compared to rodents, because the NOAEL in this latter group was higher than in pigs and sheep during exposure experiments (Zinedine *et al.*, 2007). In cows, infertility, reduced milk production and hyperestrogenism have already been linked to exposure to zearalenone (D'Mello *et*

al., 1999). However, neither zearalenone nor its metabolites could be detected in muscle, kidney, liver, bladder or dorsal fat of bulls fed a zearalenone-contaminated diet (0.1 mg/day/kg) (Dänicke *et al.*, 2002). The use of sewage sludge on pastures as a fertilizer caused a clear disruption of the development of both the fetal ovaries and testes in sheep (Paul *et al.*, 2005; Fowler *et al.*, 2008). In addition, cows drinking sewage sludge contaminated surface water had a lower milk production and a higher age at first calving (Meijer *et al.*, 1999).

In recent years, *in vitro* experiments were performed to investigate the (possible) direct adverse effect of EDCs on farm animal gametes. The exposure of bovine and porcine cumulus oocyte complexes (COCs) during *in vitro* maturation to respectively Aroclor 1254 (A-1254, PCB-mixture) and a mixture of chlorinated organic chemicals disrupted both oocyte maturation and further embryonic development (Campagna *et al.*, 2001; Pocar *et al.*, 2001). Moreover, the same chemical mixture also led to a decline in motility and viability, an increased level of capacitation, the occurrence of spontaneous acrosome reactions and increased cytosolic calcium levels in porcine spermatozoa (Campagna *et al.*, 2009). Porcine granulosa cells also appear to be susceptible to the effects of EDCs since their steroid production was disrupted after exposure to BPA (Mlynarcikova *et al.*, 2005). Because primordial germ cells are already present in the fetus, these cells can be exposed to EDCs for a very long time. Moreover, in several species of farm animals (e.g. dairy cow) the lactation peak coincides with the best moment of establishing a new gestation (Leroy *et al.*, 2008). Therefore, EDCs, possibly released during lactation, can have an adverse impact on the quality of the (ovulated) oocyte and embryo with a hampered gestation as a result. In addition, research has also shown that embryos and fetuses are more sensitive to the actions of EDCs than adult specimens (Boerjan *et al.*, 2002; Sweeney, 2002; Brevini *et al.*, 2005).

When performing *in vitro* studies, it is very important to mimic the *in vivo* situation as much as possible. Reliable and recent data on the presence and concentration of EDCs in different tissue types and body fluids of farm animals is therefore truly essential, but currently only scarcely available (Boerjan *et al.*, 2002; Hirako *et al.*, 2005; Rhind, 2005). The need for such descriptive studies is therefore imperative in order to formulate a structured opinion on the possible impact of EDCs on the fertility of livestock. The few data available (Table 1) show that detectable EDCs levels are of similar magnitude in several European countries (Kamarianos *et al.*, 2003; Covaci *et al.*, 2004; Rhind, 2005; Voorspoels *et al.*, 2007; Blanco-Penedo *et al.*, 2008; Glynn *et al.*, 2009). Finally, transparent research regarding the possible harmful effects of EDCs on farm animals is extremely important for the public opinion, not in the least from a consumer point of view.

Table 1 Concentrations (median or range of endocrine disrupting chemicals in tissues and body fluids of domestic animals.
(CB = chlorinated biphenyl, HCB = hexachlorobenzene, DDE = dichlorodiphenyldichloroethylene, PCB = polychlorinated biphenyls, PBDE = polybrominated diphenylether)

Species	Tissue	Chemical	Concentration	Sampling year	Country	Reference
Bovine (calf)	Liver	CB 153	10.5 ng/g ww	2006	Asturias, Spain	Blanco-Penedo *et al.*, 2009
			15.3 ng/g ww	2006	Galicia, Spain	
Bovine	Follicular fluid	HCB *p,p'*-DDE Sum PCBs	1.77 ng/ml 1.50 ng/ml 3.05 ng/ml	?	Greece	Kamarianos *et al.*, 2003
Bovine	Muscle	Sum PBDEs	< 20 pg/g ww	2005	Belgium	Voorspoels *et al.*, 2007
Bovine	Adipose tissue	CB 153 HCB *p,p'*-DDE	1.8 ng/g lw 2.7 ng/g lw 2.4 ng/g lw	2004	Sweden	Glynn *et al.*, 2009
Sheep	Follicular fluid	Sum PCBs HCB *p,p'*-DDE	1.25 ng/ml 0.73 ng/ml 2.97 ng/ml	?	Greece	Kamarianos *et al.*, 2003
Sheep	Liver Muscle Kidney fat	Alkylphenols Phthalates	< 10- 400 µg/kg > 20 000 µg/kg	?	Great-Britain	Rhind, 2005
Porcine	Follicular fluid	Sum PCBs HCB *p,p'*-DDE	0.78 ng/ml 0.80 ng/ml 0.45 ng/ml	?	Greece	Kamarianos *et al.*, 2003
Porcine	Liver	Sum PCBs *p,p'*-DDE	27.8-145.9 ng/g lw 12.7-27.6 ng/g lw	?	Romania	Covaci *et al.*, 2004
Porcine	Adipose tissue	Sum PCBs *p,p'*-DDE	38.4-176.3 ng/g lw 2.6-6.3 ng/g lw	?	Romania	Covaci *et al.*, 2004
Porcine	Adipose tissue	CB 153 DDE	0.5 ng/g lw 0.5 ng/g lw	2004	Sweden	Glynn *et al.*, 2009

ww = wet weight, concentration relative to the total tissue weight
lw = lipid weight, concentration relative to the lipid content of the tissue

CONCLUSION

It has been sufficiently illustrated that EDCs are capable of influencing the endocrine system of humans and animals. Because of their relatively low environmental concentrations, the mainly subtle

effects of these compounds stayed unnoticed for years. However, new action mechanisms of these substances are frequently brought to light. Research on the effects of EDCs is complex, because of the nature of these compounds, mostly occurring in the environment as mixtures consisting of the original substance and its metabolites that can influence each other in an additive, adverse or synergistic way. Therefore, the availability of reliable experimental *in vitro* methods and models is crucial.

EDCs were already even linked to the decreasing fertility of high producing dairy cows observed nowadays. However, before definite conclusions can be drawn regarding this possible involvement, more information is strongly needed about the *in vivo* EDC-contamination status of our livestock.

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ENDOCRINE DISRUPTING CHEMICALS AND FEMALE FERTILITY: FOCUS ON (BOVINE) OVARIAN PHYSIOLOGY

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SUMMARY

Throughout the previous century, the production, use and, as a result, presence of chemicals into the environment increased enormously. Consequently, humans and animals are exposed to a wide variety of chemical substances of which some possess the ability to disrupt the endocrine system in the body, thereby denominated as 'endocrine disrupting chemicals' (EDCs).

Because the reproductive system is a target organ for endocrine disruption, EDCs are postulated as one of the possible causes of human subfertility. Within the reproductive system, the ovarian follicle can be considered as an extremely fragile micro-environment where interactions between the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte.

In this review, we explore how EDCs can interfere with the well-balanced conditions in the ovarian follicle. In addition, we highlight the bovine ovarian follicle as an alternative *in vitro* model for EDC- and broader toxicology research.

INTRODUCTION

Although environmental contamination has been around for several ages, the Industrial Revolution led to chemical environmental pollution as we know it today. Consequently, living organisms are exposed to a much larger amount and variety of chemicals than in the past. Chemical compounds can be detected in air and soil samples, in water from rivers and oceans and in animal and human tissues (Covaci *et al.*, 2002; Ying *et al.*, 2002; Jaspers *et al.*, 2006; Covaci *et al.*, 2008; Petro *et al.*, 2010; Josefsson *et al.*, 2011; Onofrio *et al.*, 2011). Besides common cytotoxic actions like apoptosis, some of these substances can act as endocrine disrupting chemicals (EDCs) by interfering with the synthesis, function, storage and/or metabolism of hormones (Sweeney, 2002). EDCs show the ability to alter steroidogenesis (Sanderson, 2006) and mimic or antagonize the effects of natural hormones by binding to their receptors (estrogen, androgen and thyroid receptors) (Janosek *et al.*, 2006). They also disrupt the endocrine balance in the body through binding to other regulatory nuclear receptors like the arylhydrocarbon receptor (AhR) (Pocar *et al.*, 2005a; Janosek *et al.*, 2006) and the Peroxisome Proliferator-Activated Receptor family (PPARs) (Lovekamp-Swan and Davis, 2003; Latini *et al.*, 2008; Kwintkiewicz *et al.*, 2010).

In addition, EDCs possess some crucial characteristics which increase their harmful potential. First of all, many EDCs have long half-lives so they stay present in the environment years after they were produced (Colborn *et al.*, 1993; Pocar *et al.*, 2003b). Because many of the EDCs are lipohilic, they accumulate in adipose tissue (Brevini *et al.*, 2005). Mammals mobilise large quantities of fat during lactation which means that the EDCs, stored in the adipose tissue of the dam are released. In this way, both the dam and the neonate (through suckling contaminated milk) are exposed to higher EDC-concentrations. Moreover, different EDCs cross the placental barrier, directly exposing the developing foetuses (Barr *et al.*, 2007). By the combination of their long half-lives and lipophilicity, EDCs undergo a bioaccumulation process whereby the highest EDC-levels are found in animals at the top of the food chain. Also, EDCs have a higher bioavailability in the body than endogenous hormones, because the latter possess a higher affinity for binding proteins in the blood (Dechaud *et al.*, 1999; Nagel *et al.*, 1999). Human and animals are exposed to continuously varying complex mixtures of EDCs which can influence each other's actions in an additive, adverse or synergistic way. The harmful dose of environmental relevant EDC mixtures turned out to be even significantly lower than when individual chemicals were administered (Rajapakse *et al.*, 2002). It has already been shown that metabolites of EDCs can have a higher endocrine activity than the parent compound (Kester *et al.*, 2000; Meerts *et al.*, 2001). These metabolites are however not taken into account

when the parent compounds are administered as is done in the majority of *in vitro*-experiments. Moreover, the *in vivo* metabolisation of many compounds is often unknown and/or species specific (Boerjan *et al.*, 2002). As EDCs are able to cross the placenta, EDCs are able to induce transgenerational effects through action on the epigenome, whereby the consequences of exposure are being transferred to the next generations without modifying the DNA-sequence (Anway *et al.*, 2005; Skinner, 2007). Finally, exposure studies suggest that a chronic exposure to environmental relevant EDC-concentrations could induce the same or even more harmful effects than an acute exposure to high EDC-levels (Borman *et al.*, 2000).

Chemicals which are suspected or specifically known for their endocrine disrupting properties are dioxins (PCDDs) (Mandal, 2005), polychlorinated biphenyls (PCBs) (Fonnum and Mariussen, 2009), pesticides (Bretveld *et al.*, 2006), flame retardants (Vos *et al.*, 2003), parabens (Darbre and Harvey, 2008), perfluorinated compounds (Jensen and Leffers, 2008), plastic additives like phthalates (Lovekamp-Swan and Davis, 2003), bisphenol-A (BPA) (Welshons *et al.*, 2006) and alkylphenols (Markey *et al.*, 2001). In addition, besides synthetic hormones used as pharmaceutical drugs (Caliman and Gavrilescu, 2009), several natural endocrine active substances (e.g. isoflavones) (Cos *et al.*, 2003) are also released in the environment.

The presence of EDCs has already frequently been associated with reproductive malfunction in wildlife species (Vos *et al.*, 2000; Bernanke and Kohler, 2009; Hamlin and Guillette, 2010). Egg-shell thinning in predatory birds due to *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), the most persistent metabolite of dichlorodiphenyltrichloroethane (DDT), was the first indication for EDCs to be an important cause of reproductive abnormalities in wildlife (Vos *et al.*, 2000). Furthermore, following a large pesticide spill, alligators exhibited abnormal ovarian physiology with increased numbers of multi-oocyte follicles and polynuclear oocytes (Guillette and Moore, 2006). Similarly, male fish residing in the effluents of sewage water treatment plants, were shown to produce vitellogenin, a protein which is normally detected only in female fish (Jobling and Tyler, 2003). Despite these examples of unambiguous influences of EDCs on wildlife, it remains difficult to prove causal relationships between the presence of EDCs and specific reproductive problems *in vivo*. Nonetheless, experiments with laboratory animals and *in vitro* research indicate the ability of different chemicals to influence the endocrine system (Diamanti-Kandarakis *et al.*, 2009).

In the reproductive system, the tightly endocrine regulated ovary can be considered as a target organ for the actions of EDCs. Moreover, EDCs have already been detected in human follicular fluid (Table

1) (Trapp *et al.*, 1984; Baukloh *et al.*, 1985; Schlebusch *et al.*, 1989; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Ikezuki *et al.*, 2002; Younglai *et al.*, 2002; De Felip *et al.*, 2004; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010). This presence in the ovarian follicular micro-environment clearly implicates direct contact between EDCs, the oocyte and its surrounding somatic cells at a crucial state of their growth and development. Therefore, although direct evidence for cause and effect is currently lacking, EDCs are possibly playing a role in the substantial rise in the incidence of human subfertility (Massaad *et al.*, 2002; Toft *et al.*, 2004; Diamanti-Kandarakis *et al.*, 2009). While a considerable amount of scientific research reports are available regarding the possible effects of EDCs on spermatogenesis and semen quality (Delbes *et al.*, 2006; Varghese *et al.*, 2008; Meeker, 2010), much less is known about their influence on the oocyte and its follicular micro-environment. Such information is particularly important given the fact that female mammals are born with a definite number of oocytes in the ovaries, although a few recent publications suggest the idea of post-natal oogenesis in mice (Johnson *et al.*, 2004; Lee *et al.*, 2007; Zou *et al.*, 2009).

This review paper focuses on both endocrine related and other harmful mechanisms of action of EDCs on ovarian follicular physiology, from the primordial follicle stage until ovulation. The influences of EDCs on whole follicles as well as on individual components of the follicle like granulosa cells and cumulus oocyte complexes (COCs) will be discussed (Table 2). In addition, we will comment on the bovine ovarian follicle and its components as a potential new model for *in vitro* reproductive toxicology research.

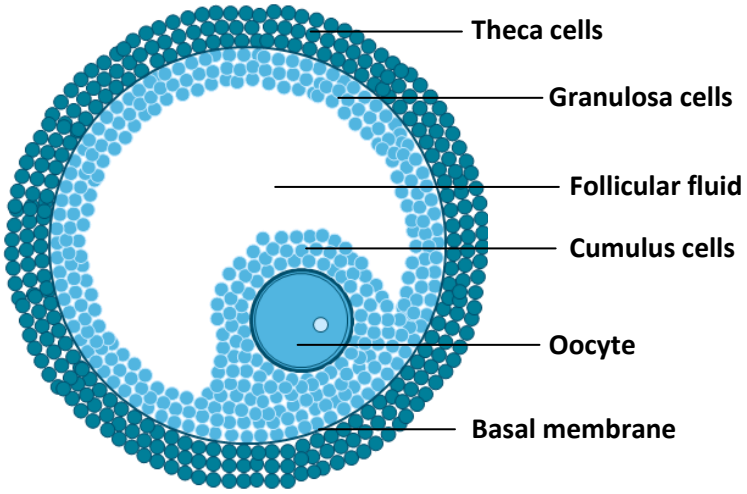

Figure 1 Antral follicle

Table 1 Literature data of human follicular fluid contamination with different EDCs
(DDT = dichlorodiphenyltrichloroethane, PCB = polychlorinated biphenyls, HCB = hexachlorobenzene, DDE = dichlorodiphenyldichloroethylene, BPA = bisphenol A, LOQ = limit of quantification)

Country	Sampling period	N patients	Compounds	Mean [a]	SD or Range [a]	Reference
Germany	?	15	Total DDTs	3.37	-	Trapp *et al.*, 1984
			PCB	8.03	-	
			HCB	2.59	-	
Germany	?	27	Total DDTs	± 6.5	-	Baukloh *et al.*, 1985
Austria	?	20		± 3.4	-	
Germany	?	37	Σ PCBs [b]	5.8	0.5 – 24.2	Schlebusch *et al.*, 1989
Canada (Halifax)	?	25	DDE	0.61	0.47	Jarrell *et al.*, 1993
			PCB	< LOQ	-	
			HCB	0.11	0.11	
Canada (Hamilton)	?	29	DDE	0.73	0.40	
			PCB	4.08	5.68	
			HCB	0.14	0.08	
Canada (Vancouver)	?	20	DDE	1.07	0.84	
			PCB	2.72	4.91	
			HCB	0.20	0.13	
Belgium	1998	8	Σ PCBs [c]	0.553	0.303 – 1.257	Pauwels *et al.*, 1999
Japan	?	32	BPA	2.4	0.8	Ikezuki et al, 2002
Canada	?	21	Σ PCBs [d]	0.198	-	Younglai *et al.*, 2002
			p,p'-DDE	2.677	1.584	
Italy	2000	12 [e]	Σ PCBs [c]	0.391	-	De Felip *et al.*, 2004
			p,p'-DDE	0.22	-	
			HCB	0.022	-	
Germany	?	21	Σ PCBs [f]	0.26	0.02 [g]	Weiss *et al.*, 2006
			p,p'-DDE	0.78	0.75	
Tanzania	?	16	Σ PCBs [f]	0.22	0.06 [g]	
			p,p'-DDE	4.89	4.8	
USA	1994–2003	72	Σ PCBs [h]	0.350 [i]	-	Meeker *et al.*, 2009
			p,p'-DDE	0.384 [i]	-	
			HCB	0.032	-	
Czech Republic	2003–2004	99	Σ PCBs [j]	33.2 [k]	0.6 – 375 [k]	Jirsová *et al.*, 2010
			DDE	3303.3 [k]	122.7 – 35228.8 [k]	

[a] ng/ml or ng/g wet weight, otherwise mentioned
[b] CB 138 + CB 153 + CB 180
[c] CB 118 + CB 138 + CB 153 + CB 180
[d] CB 49 + CB 153 + CB 180
[e] 2 pools of 6 samples
[f] CB 138 + CB 153
[g] = SEM
[h] Sum of 57 PCB congeners
[i] Geometric mean
[j] CB 44 + CB 47 + CB 101 + CB 158
[k] ng/g lipid weight
[l] CB 118 + CB 138 + CB 153 + CB 170 + CB 180 + CB 183 + CB 187

EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON OVARIAN FOLLICULAR PHYSIOLOGY

In female mammals, the ovaries of the newborn contain a large follicular reserve of non-growing primordial follicles, which consist of an immature, quiescent oocyte, surrounded by a single layer of flattened (pre-) granulosa cells. The transition from non-growing to growing follicles is a gradual process, which begins shortly after the formation of the primordial follicles and continues through reproductive life (Fortune, 2003). 'Initiation' of growth or 'activation' of primordial follicles is the transition from the primordial to the primary follicle and is, like the early stages of follicular growth, a gonadotropin independent process (Hunter *et al.*, 2004). This is accompanied by the proliferation and differentiation of granulosa cells that transform from a flattened to a cuboidal shape. In the subsequent growth stages, the oocyte undergoes volume expansion, a zona pellucida develops between the oocyte and proliferating granulosa cells (van Wezel and Rodgers, 1996) and follicle growth becomes dependent on the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Hunter *et al.*, 2004). The majority of activated follicles evolve to the antral stage, characterized by the formation of a cavity or 'antrum', filled with follicular fluid (Figure 1). FSH is the main hormone controlling follicle growth and its production is in turn regulated through the main secretory products of large antral follicles, estradiol (E2) and inhibin A. Successive dominant follicle selection depends on the expression of LH-receptors on granulosa cells and thus the follicle's ability to become LH-dependent for its growth, because FSH-concentrations decline due to the inhibitory feedback loop by E2 and inhibin A. Also, the degree of follicular vascular development is believed to play a critical role in the selection of the dominant follicle (Hunter *et al.*, 2004). Follicles which do not develop into a dominant one, undergo atresia.

EDCs can act directly on the follicle or indirectly by action on the hypothalamus-pituitary axis (for review: Gore, 2010). Given the fact that primordial follicles are present in the female ovary throughout the whole reproductive lifespan, the damaging effects of EDCs on the ovarian follicle can act during a very long exposure time.

Indirect endocrine disrupting effect of chemicals through pre-antral follicles

Direct endocrine disrupting effects on primordial or primary follicles are highly unlikely because these first stages of folliculogenesis are gonadotropin independent. However, chemicals with no direct endocrine disrupting properties, but which are able to compromise the normal growth of primordial and primary follicles through a general cytotoxic action, can have an irreversible and

indirect endocrine disrupting effect on the reproductive system (Table 2). One of the most studied ovotoxic chemicals is 4-vinylcyclohexene (VCH). Daily intraperitoneal treatments with its bioactive form, 4-vinylcyclohexene diepoxide (VCD), for 30 days leads to primordial and primary follicle loss in both mice and rats, resulting in a smaller amount of recruitable follicles for activation and follicle growth and thus, a decline in the number of E2-producing antral follicles (Mayer *et al.*, 2002). Subsequently, the negative feedback system to the pituitary can be hampered, with increased FSH-levels as a result. In the end, an endocrine distortion could be generated which may lead to irregular reproductive cycles, possibly resulting in subfertility and an early onset of the menopause (Mayer *et al.*, 2002).

The underlying toxic mechanism involves the AhR and the apoptotic caspase cascade in rats (Hu *et al.*, 2001; Thompson *et al.*, 2005), while BCL2-associated X protein (Bax) and caspase-2 and 3 pathways are involved in mice (Takai *et al.*, 2003; Thompson *et al.*, 2005). Apparently, only mice are capable of metabolizing VCH to its bioactive form, VCD (Hoyer and Sipes, 2007), which underlines the species specific behavior related to EDCs. Only recently, it was suggested that VCD acts as an ovotoxin directly by interfering with the KIT-mediated signaling pathway of the oocyte, essential for its survival before functional FSH-receptors are expressed (Keating *et al.*, 2011). However, because human exposure to VCH and its metabolites is unlikely, VCH is basically considered as a model toxicant for the study of primordial follicle loss and ovotoxicity (Hoyer and Sipes, 2007), rather than being harmful per se.

On the other hand, there are several groups of chemicals to which ovotoxic effects have been attributed and that are real threats to human/mammal reproductive health. For example, 2-bromopropane, which has been described as the causal agent for ovarian dysfunction in female factory workers (Lim *et al.*, 1997), has the ability to destroy primordial follicles due to the induction of apoptosis in oocytes and granulosa cells (Yu *et al.*, 1999). The bioactive cyclosphosphamide (CPA) metabolites, 4-hydroperoxy-CPA and phosphoramide mustard, used as chemotherapeutic agents, possess ovotoxic effects by inducing cell death through a caspase-independent pathway (Desmeules and Devine, 2006). Finally, mice exposed to cigarette smoke showed a reduction in the number of primordial follicles (Tuttle *et al.*, 2009), while components of cigarette smoke, such as 9,10-demethylbenzanthracene and benzo[a]pyrene, showed to be even more potent than VCD in damaging primordial follicles (Borman *et al.*, 2000).

Endocrine disrupting chemicals and somatic supporter cells in the ovarian antral follicle

The function of granulosa and theca cells is crucial in the process of normal folliculogenesis and oocyte growth and development. Not only are they responsible for the delivery of nutrients to the oocyte, they also attribute to the ovarian steroidogenesis (Scaramuzzi *et al.*, 2011) [Figure 2 (Jones, 2005)] and establish a link between the oocyte and the surrounding ovarian tissue in which the follicle is embedded. Theca cells surround the follicle in close contact with the basal lamina and therefore belong to the vascularized portion of the follicular wall (Figure 1). They can easily take up cholesterol, the substrate for steroidogenesis (Young and McNeilly, 2010), which they turn into androgens in an LH-dependent way. These androgens diffuse through the basal lamina and enter the avascular granulosa cells, which turn them into estrogens (Edson *et al.*, 2009), a process which is stimulated by FSH (Figure 2). Given that *in vivo* several EDCs are in direct contact with the somatic cells of the antral follicle, due to their presence in human follicular fluid (Table 1), and their ability to impair steroidogenesis (for review: Sanderson, 2006; Whitehead and Rice, 2006), it is reasonable to assume that EDCs can interfere with the function of theca and granulosa cells, thereby compromising the oocyte's survival and normal follicle growth. Unfortunately, studies on the influence of EDCs on the theca cells' androgen production are very scarce (Gregoraszczuk *et al.*, 2008; Young and McNeilly, 2010), which is why we will focus on the effects of EDCs on granulosa cell function (Table2).

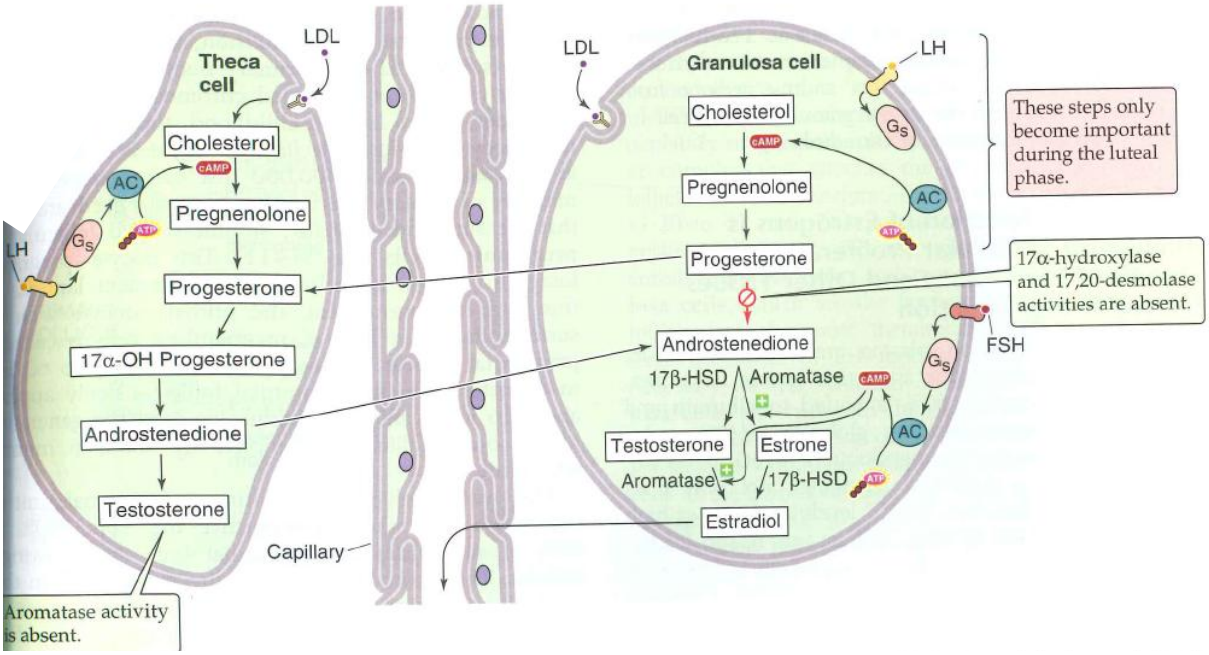

Figure 2 Steroidogenesis in the human and bovine ovarian follicle by theca and granulosa cells (Jones, 2005)

Different *in vitro* models have already been used to investigate the effect of EDCs on granulosa cells. The use of human luteinized granulosa cells, collected during Assisted Reproductive Technology (ART) procedures, showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) could decrease E2-concentration without an effect on the aromatase activity, thereby suggesting a potential role for TCDD toxicity upstream in the steroidogenesis pathway (Moran *et al.*, 2000). Furthermore, the pesticides *p,p'*-DDE, Kepone and methoxychlor can mimic the rapid non-genomic (i.e. increase of intracellular Ca^{2+}-concentration) response to ovarian hormones like E2 and progesterone (P4) in the same cells (Younglai *et al.*, 2004b; Wu *et al.*, 2006). Recently, exposing human luteinized granulosa and KGN cells (an ovarian granulosa-like tumor cell line) to BPA provoked a decrease in FSH-induced aromatase and E2-expression, through the induction of PPARγ (Kwintkiewicz *et al.*, 2010), while *p,p'*-DDE enhances basal and FSH-stimulated aromatase activity in these cells (Younglai *et al.*, 2004a).

In addition, a whole set of ovarian granulosa cell lines has been developed in the past, each with its own advantages and shortcomings (for review: Havelock *et al.*, 2004). Although not frequently reported, these cell lines can also be used to investigate the underlying molecular mechanisms through which EDCs exert their damaging effects on granulosa cells (Kwintkiewicz *et al.*, 2010). Primary granulosa cell cultures are yet another way to study the impact of EDCs on granulosa cell function. In contrast with the immortalized cell lines, primary cells derive directly from tissues without subculture or transformation/transfection. In this way, primary cells mimic more closely the *in vivo* situation and generate more physiologically relevant data than cell lines, although they are more difficult to sustain under *in vitro* culture conditions compared to these immortalized cells. In rat granulosa cells, TCDD was responsible for the downregulation of the FSH-receptor (Hirakawa *et al.*, 2000), probably through binding to the AhR. Exposure of the same cells to mono(2-ethylhexyl)phthalate (MEHP), the active metabolite of di(2-ethylhexyl)phthalate (DEHP), revealed a role for PPARs in the observed decreased aromatase transcription and concomitant lower E2-production (Lovekamp-Swan and Davis, 2003). Due to an increased transcription of genes involved in oxytocin synthesis following exposure to PCBs, DDT and its metabolites, the oxytocin production in bovine granulosa and lutein cells increased (Mlynarczuk *et al.*, 2009), which can result in delayed luteal regression *in vivo* (Tallam *et al.*, 2000). In porcine granulosa cell cultures, different EDCs were shown to induce alterations of steroid production (Mlynarcikova *et al.*, 2005; Ptak *et al.*, 2005; Gregoraszczuk *et al.*, 2008; Ranzenigo *et al.*, 2008; Grasselli *et al.*, 2010). These *in vitro* experiments show that EDCs can influence the steroidogenic capacity of the granulosa cells, thereby demonstrating their ability to disturb the critical well-balanced endocrine regulation of the developing follicle, which is essential to reach the preovulatory stage.

To (partially) overcome the problem of the limited *in vitro* lifespan of primary cultures, serum is added most of the time (before and/or during exposure) to facilitate cell attachment and to assist the *in vitro* growth of primary cells. However, granulosa as well as theca cells luteinize spontaneously in serum-supplemented cultures (Murphy, 2000), a process which is accompanied by ultrastructural and morphological cell changes (Gutierrez *et al.*, 1997b). Additionally, E2-production could only be maintained in bovine granulosa cells cultured in serum-free conditions (Gutierrez *et al.*, 1997a). Therefore, primary granulosa cells cultured in the presence of serum, may only have limited physiological resemblance to the granulosa cells of an *in vivo* growing follicle. These cells, together with the human luteinized granulosa cells, probably serve better as a model for the study of the effects of EDCs on the corpus luteum. Another critical point to keep in mind is that cell plating density has been found to influence E2- and P4-production in primary granulosa cell cultures (Portela *et al.*, 2010), which implies that variable cell numbers under the same experimental conditions have to be avoided.

Endocrine disrupting chemicals and the cumulus oocyte complex (COC)

During the oocyte's final maturation, which starts shortly before ovulation, both the nucleus and cytoplasma undergo changes to prepare the oocyte for fertilization. Oocytes, which are quiescent in the prophase of the first meiotic division since birth, resume meiosis after the LH-surge. This process is characterized by the dissolution of the nuclear envelope, also known as the process of germinal vesicle breakdown. The first meiotic cleavage ends with the development of a secondary oocyte and the extrusion of the first polar body. Complete maturation is established when oocytes reach the metaphase of the second meiotic cleavage (Edson *et al.*, 2009).

Through *in vitro* maturation (IVM) of COCs, it is possible to mimic these final maturation steps which take place *in vivo* in the preovulatory follicle after the LH-surge. Following IVM, the matured oocytes can be fertilized *in vitro*, cultured until blastocyst stage and even implanted in receptor animals to generate offspring. A substantial amount of studies reported on the inability of oocytes to fulfill complete maturation following exposure to generally high EDC-concentrations during IVM (Alm *et al.*, 1998; Krogenaes *et al.*, 1998; Campagna *et al.*, 2001; Pocar *et al.*, 2001b) (Table 2). Using a mouse follicle bioassay, BPA (30 µM) showed its ability to affect every step of the final nuclear maturation process of the oocyte without affecting follicular steroidogenesis (Lenie *et al.*, 2008). In light of this, BPA was shown to interfere with the normal pattern of Ca^{2+}-oscillations, suggested to be of importance for normal final murine oocyte maturation and subsequent fertilization (Mohri and

Yoshida, 2005). Moreover, *in vivo* exposure in mouse models to environmental relevant BPA concentrations caused oocyte meiotic abnormalities (Hunt *et al.*, 2003). Also, the AhR signaling pathway has been suggested to be directly involved in the resumption of meiosis in mammalian oocytes, through the induction of CYP1A1 which was shown to be indispensable to complete IVM until metaphase II (Pocar *et al.*, 2004).

Next to changes in the nucleus, cytoplasmic maturation is equally necessary to complete a successful maturation process of the oocyte. Pocar and co-workers (Pocar *et al.*, 2001a) found that exposing bovine cumulus oocyte complexes to 0.1 µg/ml Aroclor 1254 (A-1254, a commercially available PCB-mixture) during IVM altered the length of the 3'-poly(A) tail, important for the control of mRNA-translation, of gene transcripts involved in early differentiation. In the same study, the dispersion of cortical granules (CGs) underneath the plasma membrane was delayed in exposed oocytes, which resulted in a failed CGs-release after fertilization and in the end, because their products normally prevent the penetration of multiple sperm cells, a higher degree of polyspermy (Pocar *et al.*, 2001b). Porcine oocytes did not succeed in producing a functional cytoplasmic microtubule network to relocate mitochondria properly after PCB exposure during IVM (Brevini *et al.*, 2004). Additionally, exposure to A-1254 disturbed gap-junction communication between the oocyte and its surrounding cumulus cells. While these observations did not seem to affect maturation rate, they did significantly decrease blastocyst formation. Recently, it was shown that in utero exposure to DEHP and PCBs caused a reduced maturation rate and negatively affected the oocyte's developmental capacity (Pocar *et al.*, 2012a; Pocar *et al.*, 2012b).

Next to the oocyte, it seems that cumulus cells themselves are potential targets for indirect EDC-action (Table 2). For example, a simultaneous increase of Bax and decrease of Bcl-2 transcription were observed in bovine cumulus cells after A-1254 exposure during IVM, which resulted in a higher percentage of apoptotic cumulus cells in the exposed group (Pocar *et al.*, 2005b). In the same study, a lower maturation rate was reported for COCs compared to naked oocytes, both exposed during maturation, which suggests a key role for cumulus cells in mediating PCB-induced toxicity during maturation. Several studies also mentioned reduced cumulus expansion following EDC-exposure (Alm *et al.*, 1998; Campagna *et al.*, 2001; Mlynarcikova *et al.*, 2005). During cumulus expansion, cumulus cells produce an extracellular matrix (ECM), which consists of an hyaluronan oligosaccharide backbone, cross-linked by ECM-proteins, and proteoglycans and which is indispensable for normal ovulation and fertilization (Russell and Salustri, 2006). Mlynarcikova and co-workers (Mlynarcikova *et*

al., 2005) suggest that an impaired incorporation of hyaluronic acid in the ECM is the main reason for the diminished cumulus expansion following exposure to EDCs.

As mentioned earlier, relatively high concentrations of EDCs are needed to induce an effect on oocyte maturation. However, research has shown that *in vitro* exposure to lower EDC-concentrations, which in some cases almost approached the *in vivo* levels, was already sufficient to affect fertilization rate and subsequent blastocyst developmental capacity (Krogenaes *et al.*, 1998; Pocar *et al.*, 2001b; Pocar *et al.*, 2003a). These findings strongly suggest that even moderate EDC-exposure within the follicular environment can decrease oocyte quality and jeopardize further embryo development.

Taken into account the potential influences of EDCs on granulosa cells and on COCs and with the understanding that ovulation is a finely tuned process of endocrine interactions in the hypothalamus-pituitary-ovary axis, major endocrine disruptions during the follicular growth pattern due to the action of EDCs will predominantly lead to anovulation, cystic deformation or atresia of the dominant follicle, rather than resulting in the ovulation of an inferior oocyte.

Table 2 Overview of functional pathways leading to altered ovarian oocyte growth and maturation effects due to exposure to different EDCs.
(VCH = vinylcyclohexene, 2-BP = 2-bromopropane, CPA = cyclosphosphamide, DMBA = dimethylbenzanthracene, TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, BPA = bisphenol-A, DDE = dichlorodiphenyldichloroethylene, MEHP = mono(2-ethylhexyl)phthalate, PCB = polychlorinated biphenyl, DDT = dichlorodiphenyltrichloroethane, DEHP = di(2-ethylhexyl)phthalate)

Cell type	Compound	Physiological process	Physiological effects
Primordial and primary follicles [a]	• VCH • 2-BP • CPA-metabolites • DMBA • Benzo[a]pyrene	• Activation of apoptotic pathways (caspase, Bax, caspase independent) • Activation of AhR pathway	Follicle destruction, irregular reproductive cycles, ovarian dysfunction
Human luteinized granulosa cells [b]	• TCDD • Pesticides • BPA • *p,p'*-DDE	• Aromatase independent decrease of [E2] • Mimic rapid non-genomic responses to E2 and P4 • Decrease FSH-induced aromatase expression through induction of PPARγ • Increase FSH-induced aromatase activity	Imbalance of the physiological steroidogenic system
Primary granulosa cells [c]	• TCDD • MEHP • PCBs, DDT and metabolites • α-zearalenol, BPA, PCBs	• Downregulation FSH-receptor activity through AhR-binding • Decrease aromatase transcription through PPARs • Increase of oxytocin production • Impair steroid production	Disturbance of the endocrine regulation of the developing follicle
Cumulus oocyte complexes (oocytes) [d]	• BPA • DEHP • PCBs	• Affect final nuclear maturation • Interfere with Ca^{2+}-oscillations • Meiotic abnormalities • Alter 3'-poly(A) tail • Delay in cortical granules dispersion	Impaired maturation, fertilization, blastocyst formation and lower developmental capacity

		• Failure to produce a functional cytoplasmic microtubule network • Disturb gap-junction communication between oocyte and cumulus cells	
Cumulus oocyte complexes (cumulus cells) [e]	• PCBs	• Bax increase + Bcl-2 decrease	• Higher amount of apoptotic cumulus cells
	• BPA	• Impaired incorporation hyaluronic acid in the ECM	• Diminished cumulus expansion during maturation

[a] (Lim *et al.*, 1997; Yu *et al.*, 1999; Borman *et al.*, 2000; Hu *et al.*, 2001; Mayer *et al.*, 2002; Takai *et al.*, 2003; Thompson *et al.*, 2005; Desmeules and Devine, 2006; Hoyer and Sipes, 2007; Tuttle *et al.*, 2009; Keating *et al.*, 2011)
[b] (Moran *et al.*, 2000; Younglai *et al.*, 2004a; Younglai *et al.*, 2004b; Wu *et al.*, 2006; Kwintkiewicz *et al.*, 2010)
[c] (Hirakawa *et al.*, 2000; Lovekamp-Swan and Davis, 2003; Mlynarcikova *et al.*, 2005; Ptak *et al.*, 2005; Gregoraszczuk *et al.*, 2008; Ranzenigo *et al.*, 2008; Mlynarczuk *et al.*, 2009; Grasselli *et al.*, 2010)
[d] (Pocar *et al.*, 2001; Hunt *et al.*, 2003; Brevini *et al.*, 2004; Mohri and Yoshida, 2005; Lenie *et al.*, 2008; Pocar *et al.*, 2012)
[e] (Mlynarcikova *et al.*, 2005; Pocar *et al.*, 2005)

FUTURE 'ENDOCRINE DISRUPTING CHEMICAL RESEARCH': THE BOVINE OVARIAN FOLLICLE AS AN ALTERNATIVE MODEL?

This review specifically concentrates on the possible influences of EDCs acting within the ovarian follicular micro-environment. In addition, it aims to raise some concerns on endocrine disruption research, which exponentially gained more attention since the first review on this topic was published nearly two decades ago (Colborn *et al.*, 1993). Undoubtedly, the abnormalities observed in wildlife species, most of them located at the top of the food chain, created relevant concerns related to human (reproductive) health and necessitated continuous monitoring and research (Hotchkiss *et al.*, 2008; Marty *et al.*, 2011). However, almost simultaneously, criticism about the real impact of EDCs in humans was raised (Ross, 2004; Caliman and Gavrilescu, 2009; Vandenberg *et al.*, 2009), based on the scarcity of information on causal exposure-effect relationships in the field. Furthermore, the endocrine related abnormalities in wildlife are almost exclusively present in animals living in highly contaminated geographical locations (Rhind, 2005). On top of that, *in vitro* exposure experiments provide often ambiguous results, which only nourishes the controversy about EDCs and their possible deleterious effects. Clearly, this controversy should not imply that EDC-research is unnecessary as long as EDCs demonstrate harm *in vitro*. Even if the reported damage is subtle, it has to be documented, mainly because problems such as subfertility are extremely complex and without any doubt multifactorial. The effects caused by EDCs could contribute to the overall fertility decrease and may also interact with other causes such as dietary factors. It has already been suggested that EDCs play a role in obesity (Newbold *et al.*, 2007; Chen *et al.*, 2009), which in turn is also linked to subfertility (Robker, 2008; Brewer and Balen, 2010). This link becomes even more interesting considering PPARs which are involved in normal fatty acid metabolism, but also described as the gateway through which several relatively new EDCs (BPA, phthalates, perfluorinated compounds) can execute their effects (Froment *et al.*, 2006).

The main concern for the immediate future, related to research on possible endocrine disrupting effects of the numerous chemicals still to be (re)tested (e.g. within the REACH program), is the development of relevant and robust *in vitro* experimental systems which are in line with the 3R-principle (Russell and Burch, 1959; Cortvrindt and Smitz, 2002; Combes *et al.*, 2003; Luciano *et al.*, 2010). At this moment, only three embryotoxicity assays have been validated within REACH, i.e. the micromass test, the whole rat embryo culture test and the embryonic stem cell test (Genschow *et al.*, 2002). One of the first challenges is to include environmentally relevant EDC-concentrations in

exposure experiments, both through individual exposure to unravel the working mechanisms of a chemical and through exposure to environmental relevant mixtures. This means that in case of the (human) follicular micro-environment, extensive monitoring of the follicular fluid is without a doubt indispensable to obtain knowledge about the different EDC-concentrations to which the follicular cells and the oocyte are exposed (Table 1). It however cannot be completely ruled out that next to the detected compounds in the follicular fluid, other currently undetectable chemicals are present which can possibly also exert toxic effects. Despite the use of model toxicants to provoke typical pathological responses (e.g. VCD for primordial follicle loss), the *in vivo* relevance of experiments where follicular cells are exposed to chemicals which are not or unlikely to be detected in follicular fluid and/or added in concentrations which will never be reached *in vivo* is questionable.

In our opinion, the use of primary cells to investigate endocrine disruption and even broader toxicology research needs to be encouraged. Cell lines are valuable in revealing new mechanisms of action for different chemicals, but these results will gain even more significance when they can be repeated in primary cultures. From this point of view, the use of follicular cells from domestic animals, besides laboratory rodents, has to be taken into consideration as an innovative approach in reproductive toxicology research. Indeed, farm animals have already been suggested several times as alternative species to investigate the effects of endocrine disruption (Krogenaes *et al.*, 1998; Gandolfi *et al.*, 2002; Magnusson, 2004; Petro *et al.*, 2010). An important advantage of using domestic animal follicular cells compared to the micromass and whole embryo culture test, is the fact that no (extra) laboratory animals are needed as ovaries are easily accessible in the slaughterhouse. Therefore, in view of the '3R-principle' (Russell and Burch, 1959), a significant reduction of the number of laboratory animals can be accomplished (Petro *et al.*, 2010). Also, it is not unthinkable that before follicle retrieval, laboratory animals were already exposed to higher concentrations of EDCs or other reproductive toxicants compared to domestic animals due to their indoor housing in plastic cages and highly processed feed intake, which may contain multiple EDCs (Hunt *et al.*, 2003).

Within the domestic animal group, the bovine species is of particular interest because of its striking similarities in early reproductive physiology as compared to human. Not only do humans and cows have the same length of pregnancy/gestation and are they both single ovulators, their ovarian function and oocyte characteristics are remarkably comparable, in contrast to major differences in ovarian physiology and reproductive function between rodents and humans (for review: Neuber and Powers, 2000; Campbell *et al.*, 2003). Furthermore, bovine and human embryos are strongly related with respect to microtubule formation during fertilization, the timing of embryonal genome

activation, metabolic requirements, interactions with the culture medium and duration of pre-implantation development (Navara *et al.*, 1995; Anderiesz *et al.*, 2000; Menezo *et al.*, 2000). The bovine model already contributed substantially to research on the effects of hyperlipaedemia and elevated non-esterified fatty acid concentrations on embryo quality and physiology (Leroy *et al.*, 2010; Van Hoeck *et al.*, 2011), uterine function and immunity (Herath *et al.*, 2006) and reproductive aging (Malhi *et al.*, 2005). As mentioned above, bovine follicles have already been used to examine the effects of different toxic substances, e.g. PCBs, on oocyte maturation and subsequent embryo development (Pocar *et al.*, 2001b), while exposure of bovine oocytes to chemicals during IVM and fertilization is already used as a test within the EU-funded 'ReProTect' project (Lazzari *et al.*, 2008). Regarding the follicular micro-environment, the contamination status of the follicular fluid is a useful parameter to check if the oocyte and the somatic follicular cells, usually freshly aspirated from follicles of slaughterhouse derived ovaries, already came in contact with significant higher EDC-concentrations than the unavoidable background levels *in vivo*, which could possibly influence the outcome of subsequent *in vitro* experiments. Therefore, we recommend to examine the presence of EDCs in the follicular fluid of the slaughtered animals prior to the start of *in vitro* experiments (Petro *et al.*, 2010).

In order to be fully accepted as a reliable model in reproductive toxicology research, experiments with bovine follicular cells have to be executed in completely defined culture media which automatically rules out the routine use of serum. Bovine granulosa cells can already be cultured in a completely defined serum free medium in which E2-production can be maintained (Gutierrez *et al.*, 1997a). Also, maturation, fertilization and culture of bovine COCs in group can be established under serum-free conditions (George *et al.*, 2008). Recently, progress has been made in individually maturing, fertilizing and culturing bovine embryos, which gives us the capacity to track each oocyte individually until development to the blastocyst stage (Goovaerts *et al.*, 2011). In this way, neighboring oocytes/embryos cannot influence each other through paracrine signaling, which brings us again closer to the *in vivo* situation. However, to our knowledge, serum is still necessary to obtain acceptable numbers of singly matured, fertilized and cultured bovine embryos.

CONCLUSIONS

In conclusion, it is clear that *in vitro* exposure to EDCs can induce permanent follicle loss, provoke modifications in the steroidogenesis of granulosa cells, influence the interaction of the oocyte with its surrounding cumulus cells and disturb final maturation, fertilization and even subsequent embryo

development. Therefore, although real cause-effect relationships remain difficult to prove, the presence of EDCs in our environment and food may not be neglected in subfertility research. We also pointed out the importance of reliable and (environmental) relevant experimental *in vitro* models. From this point of view, we recommend the bovine ovarian follicle and its individual components as a promising *in vitro* reproductive toxicology model, with broader applications than only EDC-research.

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AIMS OF THE STUDY

Endocrine disrupting chemicals (EDCs), substances which are able to interfere with the endocrine system, have been put forward as one of the causes for the reduced fertility of high producing dairy cows observed nowadays. This decrease in reproductive performance of dairy cows is a huge economic problem for the dairy cattle industry. Studies on the possible involvement of EDCs as a determining factor in the decline of reproductive success of dairy cows are however only scarcely available. In addition, data on *in vivo* concentrations of these compounds in dairy cows are even more difficult to find. As a consequence, the collection of information about the contamination status of these animals with EDCs is urgently needed to estimate the possible causal relationship between the presence of EDCs and the reproductive problems of which dairy cows are suffering from.

Also human reproduction is possibly hampered by the presence of EDCs. Within the female reproductive system, the ovarian follicular micro-environment is a relevant target for EDCs, due to its strict endocrine control. Although the presence of well-known EDCs in human follicular fluid is already documented, almost no data are currently available regarding the *in vivo* consequences of this direct interaction of EDCs with important follicular components such as the maturing oocyte, granulosa and theca cells. In addition, reports regarding the possible presence of newly emerging EDCs are also lacking.

Furthermore, to investigate how EDCs can interact with the human ovarian follicular micro-environment *in vivo*, reliable *in vitro* models which closely mimic the *in vivo* situation are indispensable, yet very difficult to establish. In other research fields, the bovine ovary and its individual components (e.g. cumulus oocyte complexes, pre-antral follicles) have already been successfully used as a model for the human ovarian follicular micro-environment due to the striking similarities between bovine and human early pre-implantation embryo and reproductive physiology. Within the ovarian follicle, granulosa cells are crucial for *in situ* steroidogenesis and thus as a result, can be considered as important target cells for potential direct EDC interference.

The main objective of this thesis is to gain a better understanding of the impact of EDC-contamination on the physiology of both the bovine and human ovarian follicle.

Therefore, the specific aims of this thesis are defined as follows:

1. Study the EDC-contamination status of different tissues and body fluids, including follicular fluid, of high producing dairy cows, in order to
    - assess the possible role of EDCs as one of the reasons for the declining high producing dairy cow fertility **(Chapter 3A)**
    - evaluate the use of the bovine ovarian follicle and its contents as a relevant model for EDC-research **(Chapter 3A)**

2. Investigate the current contamination of the human follicular micro-environment with well-known and newly emerging EDCs **(Chapters 3B + 3C)**

3. Assess the possible impact of this contamination on the oocyte's developmental competence **(Chapters 3B + 3C)**

4. Investigate if and how environmentally-relevant EDC-concentrations can influence granulosa cell function, one of the key components of the ovarian follicle for maintaining the correct physiological endocrine balance **(Chapter 3D)**

RESULTS

OCCURRENCE OF ENDOCRINE DISRUPTING CHEMICALS IN TISSUES AND BODY FLUIDS OF BELGIAN DAIRY COWS AND ITS IMPLICATIONS FOR THE USE OF THE COW AS A MODEL TO STUDY ENDOCRINE DISRUPTION

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SUMMARY

BACKGROUND: The reproductive performance of high producing dairy cows has dropped severely throughout the last decades. It has already been suggested that the presence of endocrine disrupting chemicals (EDCs) in the environment could be one of the reasons for this declining fertility. Reliable data concerning tissue and body fluid concentrations of these chemicals are thus crucial, but currently only scarcely available.

METHODS: Therefore, we selected dairy cows (≥ 6 year) from diverse locations in Belgium and analyzed tissues (liver, adipose tissue, muscle, kidney, ovaria) and body fluids (serum, follicular fluid, milk) for their content of potential EDCs, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs). Furthermore, we collected milk and serum samples from high producing dairy cows 2 - 3 weeks post-partum to verify if the massive lipolysis required to sustain milk production is accompanied with an increase in EDC concentrations in milk and serum.

RESULTS: Overall, contamination was very low (median sum PCBs liver: 11.7 ng/g lipid weight), with follicular fluid samples showing no detectable contamination. CB 153 was present in each tissue sample. Strong correlations could be found between EDCs in the same tissue. The increased PCB-concentrations observed in milk samples from high producing dairy cows could indicate that massive lipolysis can play a role in liberating and thereby increasing EDC-concentrations in milk.

CONCLUSION: Because concentrations of the most prevalent EDCs in dairy cow tissues and body fluids are very low, exposure to EDCs can hardly be considered as a major cause of declining fertility in high producing dairy cows in Belgium. As a result of this low contamination and the similarities between the female bovine and human reproductive physiology, *in vitro* studies based on Belgian dairy cow ovarian follicles can be considered as a valuable model to study the effects of EDCs on human reproduction.

INTRODUCTION

During the last decades, the number of high producing dairy cows suffering from reduced fertility has risen dramatically, indicated by a prolonged calving interval, by delayed first ovulation after calving and a rise in the number of artificial inseminations per conception (Leroy and de Kruif, 2006). Different causes have already been suggested, such as the use of high energy and protein-rich rations (Butler, 1998), increased herd sizes (Fahey *et al.*, 2002), genetic selection towards milk yield (Snijders *et al.*, 2000) and metabolic disorders during the negative energy balance (NEB) period post-partum with a potential negative effect on oocyte and subsequent embryo quality (Leroy *et al.*, 2005).

Throughout the previous century, the production, use and, as a result, the presence of chemicals into the environment has increased enormously. Consequently, animals are exposed to a much larger amount and variety of chemicals than in the past. Research has shown that some of these substances can act as endocrine disrupting chemicals (EDCs) by interfering with the synthesis, function, storage or metabolism of hormones (Sweeney, 2002). Hormones play a crucial role in the physiologic mechanisms controlling fertility; therefore, it is reasonable to assume that these EDCs can be an additional factor in the disappointing fertility outcome in modern dairy cow industry (Rhind, 2005). Much information about the possible deleterious effects of EDCs on the reproductive capacity of dairy cows arises from *in vitro* research. Pocar and co-workers (2001) demonstrated that the addition of Aroclor-1254 (A-1254), a commercial polychlorinated biphenyl (PCB) mixture, to the maturation medium of bovine oocytes disrupted oocyte maturation and embryo development. Furthermore, organohalogenated pesticides (OCPs) altered the function of bovine granulosa cells maintained in primary culture (Tiemann *et al.*, 1996).

The use of the bovine ovarian follicle as an *in vitro* model has already been recommended as a valuable instrument to unravel reproductive events in women due to the similarities in ovarian follicular dynamics and endocrine control (Campbell *et al.*, 2003). However, the intention of using the bovine follicle as a toxicological screening tool implies knowledge about the degree of contamination of this model. Moreover, EDCs have to be present *in vivo* to exert their actions. Data about EDC-concentrations in multiple dairy cow tissues and body fluids are thus truly indispensable to generate an overview of the contamination status of Belgian dairy cows. Specific data about the presence of EDCs in the follicular micro-environment are also required. However, at the moment, these are only scarcely available (Kamarianos *et al.*, 2003; Rhind, 2005; Glynn *et al.*, 2009).

Hence, the objectives of this present study were 1) to assess the content of EDCs, well-known in Belgium for their high concentrations in animals at the top of the food chain and their environmental

abundance (Jaspers *et al.*, 2006; Covaci *et al.*, 2008a), in tissues and body fluids from Belgian dairy cows and 2) to investigate the amount of contamination in the follicular micro-environment by analysis of the follicular fluid from Belgian dairy cows.

MATERIALS AND METHODS

Sample collection

In the slaughterhouse, dairy cows (Holstein Friesian, n = 20) originating from different parts of Belgium were selected upon their age (≥ 6 year) (December 2007 and June 2008). Blood samples (n = 20) were captured in polypropylene (PP) tubes (without heparine) during exsanguination. Milk samples (n = 7) were collected in PP-tubes before udder removal and tissue samples [liver (±200 g), adipose tissue (±150 g), muscle (±200 g), kidney (±200 g) and ovaries (±10 g)] were obtained from each cow as well. After slaughterhouse sampling, blood (n = 19) samples were taken from lactating high producing dairy cows 2 - 3 weeks post-partum from two dairy herds chosen randomly in Flanders. From one herd, milk samples (n = 8) were taken as well. Blood was taken from the tail vein and milk samples were obtained after milking.

Each sample was identified using the unique and official ear tag number of the cow. In the lab, coagulated blood samples were centrifuged (15 min, 2000 g) and serum was collected in PP-tubes. Ovaries were washed with 0.9 % NaCl and blotted dry. Follicular fluid was aspirated from the ovarian follicles with an 18 G needle and a 10 ml syringe and pooled per cow. All tissue samples were homogenised using a kitchen blender and the homogenised samples were stored in PP-recipients. All samples were kept frozen at –20 °C until analysis.

Chemical analysis of tissues

In all samples, 25 PCB-congeners (28/31, 52, 74, 95, 99, 101, 105, 110, 118, 128, 132, 138/163, 149, 153, 156, 167, 170, 180, 183, 187, 194, 196/203, 199), 7 PBDE-congeners (28, 47, 99, 100, 153, 154, 183), hexachlorobenzene (HCB), chlordanes (cis-chlordane, trans-chlordane, trans-nonachlor and oxychlordane), hexachlorocyclohexanes (α-, β-, γ-HCHs), *p,p'*-dichlorodiphenyl-trichloroethane (*p,p'*-DDT) and its metabolites (*p,p'*-DDD and *p,p'*-DDE) were analyzed. All standards were obtained from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). All solvents were of pesticide-grade purity and

were, together with concentrated sulphuric acid (analytical grade), silica gel 60 (63-230 mesh) and anhydrous Na_2SO_4, available from Merck (Darmstadt, Germany).

Before analysis, tissue samples were weighed (adipose tissue ±0.3 g, liver ±5 g, kidney ±7 g, muscle ±8 g, ovary ±9.5 g), mixed with anhydrous Na_2SO_4 and spiked with internal standards (CB 46, CB 143, BDE 77, BDE 128 and ε-HCH). Further sample treatment was carried out based on a previously described method (Covaci *et al.*, 2002). In brief, extraction was performed with 100 ml hexane/acetone (3:1, v/v) in an automated Soxhlet extractor (Büchi, Flawil, Switzerland) operated in hot extraction mode for 2 h. After gravimetric determination of the lipid content (using an aliquot of the extract, dried for 1 h at 105 °C), the extract was cleaned on 8g acidified silica (44 %, w/w) and analytes were eluted with 15 ml n-hexane and 10 ml dichloromethane (DCM). The eluate was concentrated under a gentle nitrogen stream to near dryness and redissolved in 100 µl iso-octane. The analysis of extracts was carried out on an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass spectrometer (GC/MS) operated in electron ionization (EI) mode, in combination with a HT-8 capillary column (25 m x 0.22 mm x 0.25 µm) (SGE, Zulte, Belgium).

Chemical analysis of body fluids

Body fluids (serum, milk and follicular fluid) were treated following a slightly adjusted different method (Covaci and Schepens, 2001). In short, samples (3 - 4 ml) were spiked with internal standards (CB 143 and ε-HCH), mixed with 2 ml deionized water and 750 µl formic acid (used for protein denaturation) and then equilibrated by ultrasonic treatment for 20 min. The C18 Empore™ solid-phase disk extraction (SPDE) cartridges (3M Company, St. Paul, MN, USA) were washed with DCM (2 x 500 µl) and activated with 1 ml methanol and two portions of 500 µl deionized water. Next, fluid samples were loaded on the cartridges and each cartridge was rinsed with 2 x 500 µl deionized water. The sorbent bed was dried under vacuum pressure for 10 min. Clean-up of the samples was performed on PP-cartridges filled with 500 mg acid silica and 100 mg anhydrous Na_2SO_4 on top (pre-washed with 2 ml hexane). The SPDE-cartridges were eluted with 2 x 500 µl hexane and 2 x 500 µl DCM followed by the elution of the acid silica cartridges with 3 ml hexane and 3 ml DCM. The eluate was than concentrated to near dryness and redissolved in 80 µl iso-octane. The analysis of extracts was carried out by GC/MS operated in electron-capture negative ionization (ECNI) mode, in combination with a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm) (J&W Scientific, Folsom, CA, USA).

Quality assurance

Multi-level calibration curves in the linear response interval of the detector were created for the quantification, and good correlation (r^2 > 0.999) was achieved. The identification of target analytes was based on the relative retention times to the internal standard used for quantification, on ion chromatograms and intensity ratios of the monitored ions. A deviation of the ion intensity ratios within 20 % of the mean values obtained for calibration standards was considered acceptable. Procedural blanks were consistent (RSD < 15 %) and therefore the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 x SD of the blank (which ensures >99 % certainty that the reported value is originating from the sample) taking into consideration the amount of sample analyzed. The quality control was performed by regular analyzes of procedural blanks, by random injection of standards, spiked samples, solvent blanks and certified reference materials [SRM 1945 – Persistent Organic Pollutants (POPs) in whale blubber]. The quality control scheme was also assessed through regular participation to inter-laboratory comparison exercises organized by AMAP (POPs in serum) (AMAP, 2008) and NIST (POPs in tissues) (Schantz *et al.*, 2008). Obtained values were deviating with less than 10 % from the consensus values.

Data treatment

Statistical analysis was performed using SPSS 15.0 for Windows (Chicago, IL, USA). Samples with levels below the LOQ were assigned a value of f x LOQ with f being the detection frequency or the proportion of measurements above the LOQ (Voorspoels *et al.*, 2002). By doing so, data below LOQ can still be used in the statistical data treatment. However, compounds with < 50 % of the measurements above their LOQs were excluded from statistical analysis. Values of $p < 0.05$ were considered as statistically significant. Normality was checked using the Kolmogorov-Smirnov test. Because of the relative low number of samples in the study and the detection of outliers, non-parametric tests were used. Differences in compound concentrations between adipose and liver tissue from the same cow were tested using the Wilcoxon matched pairs signed rank test. Regional differences in tissue concentrations were checked with the Mann Whitney U-test. Finally, correlations were investigated using the Spearman rank test.

RESULTS

Concentrations of EDCs in tissue and body fluid samples from slaughterhouse dairy cows in Belgium

Dairy cows selected in the slaughterhouse were 9.0 ± 1.6 yrs. Average lipid percentages with standard deviations were 88.6 ± 8.0 %, 4.6 ± 0.6 %, 3.0 ± 1.6%, 2.7 ± 0.3 % and 1.4 ± 0.7 % for adipose tissue, liver, muscle, kidney and ovary, respectively. Several compounds (CBs 28/31, 74, 128, 132, 167, 194, 196/203, 199, *p,p'*-DDD, PBDEs and chlordanes) could not be detected above their LOQs, ranging from 0.5-1.0 ng/g lipid weight (lw). Other compounds had a detection frequency < 50 %, so they were excluded from statistical analysis (CBs 52, 95, 99, 101, 105, 110, 118, 149, 156, 170, 183, 187, HCHs, *p,p'*-DDT). On the other hand, CB 153 and *p,p'*-DDE were present in all tissue samples. Compounds selected for statistical analysis were CB 138, CB 153, CB 180, sum PCBs, HCB and *p,p'*-DDE (Table 1 and 2). The highest PCB-concentrations were found in liver samples [median sum PCBs: 11.7 (1.1 – 112.8) ng/g lw] (Table 1). CB 153 and CB 138 were the most abundant PCB-congeners in all tissue samples, with a contribution to the total amount of PCBs of respectively 44 % and 33 % for adipose tissue and of 41 % and 39 % for liver (Figure 1).

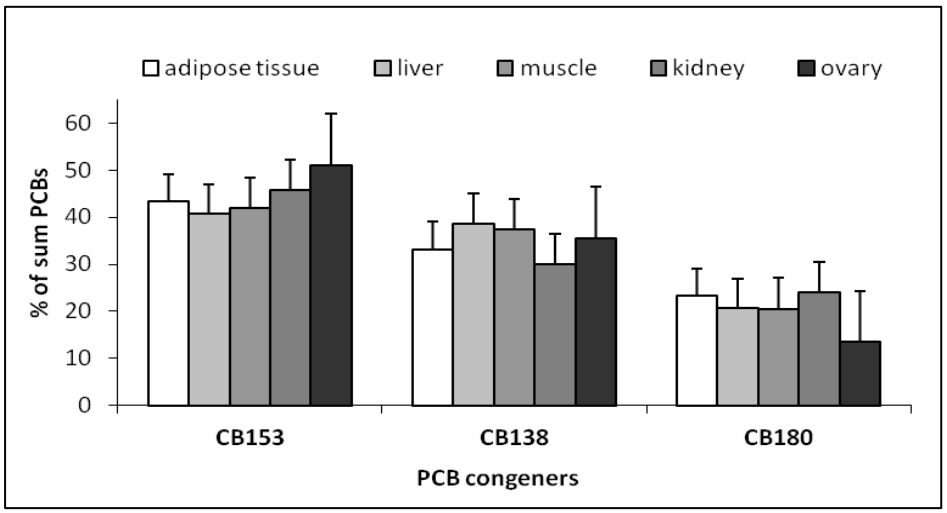

Figure 1 Contribution of PCB-congeners + SD (%) to the total amount of PCBs in tissue samples (adipose tissue and liver: n = 20; muscle, kidney and ovary: n = 3), calculated by defining the average detected concentration of a CB relative to the average total amount of detected PCBs in the different tissue samples.

Table 1 Median concentrations (min – max) (ng/g lw), limits of quantification LOQ (ng/g) and detection frequencies f (%) of different organohalogenated compounds in adipose tissue and liver samples from 20 Belgian dairy cows.

		Adipose tissue		Liver	
Lipids (%)		88.6 ± 8.0		4.6 ± 0.6	
Compound	LOQ	f	Median (min – max)	f	Median (min – max)
CB 95	1.0	0		15	1.0 (1.0 – 1.4)
CB 99	1.0	5	1.6	15	1.5 (1.2 – 3.9)
CB 101	1.0	0		10	1.3 (1.1 – 1.5)
CB 110	1.0	0		15	1.0 (1.0 – 4.0)
CB 118	1.0	45	1.1 (1.0 – 3.8)	55	1.5 (1.0 – 4.6)
CB 138 [a]	0.5	90	1.2 (0.6 – 9.4)	95	3.1 (1.0 – 37.7)
CB 149	0.5	15	1.0 (0.6 – 1.2)	25	1.1 (0.9 – 1.8)
CB 153 [a]	0.5	100	1.6 (0.8 – 10.3)	100	3.3 (1.1 – 33.7)
CB 156	0.5	5	1.2	0	
CB 170	0.5	40	0.7 (0.5 – 1.7)	60	1.0 (0.5 – 5.8)
CB 180 [a]	0.5	85	0.9 (0.5 – 5.5)	90	1.7 (0.7 – 19.4)
CB 183	0.5	15	0.6 (0.5 – 1.3)	80	0.8 (0.5 – 7.7)
CB 187	0.5	5	0.5	10	0.6 (0.5 – 0.7)
Sum PCBs [a]			4.0 (0.8 – 33.8)		11.7 (1.1 – 112.8)
HCB [a]	1.0	80	1.4 (1.0 – 2.9)	60	1.2 (1.1 – 3.8)
***p,p'*-DDE** [a]	0.5	100	5.0 (0.5 – 13.5)	100	3.8 (1.1 – 12.7)
***p,p'*-DDT**	0.5	10	0.8 (0.5 – 1.1)	50	3.2 (0.9 – 4.5)
β-HCH	1.0	0		15	11.7 (8.4 – 46.0)
γ-HCH	0.5	0		0	

[a] Compound selected for statistical analysis

Table 2 Mean concentrations ± standard deviations SD (ng/g lw), limits of quantification LOQ (ng/g) and detection frequencies f (%) of different organohalogenated compounds in muscle, kidney and ovary tissue samples from 3 Belgian dairy cows.

		Muscle		Kidney		Ovary	
Lipids (%)		3.0 ± 1.6		2.7 ± 0.3		1.4 ± 0.7	
Compound	LOQ	f	Mean ± SD	f	Mean ± SD	f	Mean ± SD
CB 118	1.0	67	1.6 ± 0.5	33	1.7	33	1.6
CB 138 [a]	0.5	100	3.0 ± 2.1	100	1.8 ± 1.1	67	3.4 ± 1.5
CB 153 [a]	0.5	100	3.3 ± 2.5	100	2.7 ± 1.6	100	3.4 ± 1.5
CB 170	0.5	33	2.7	0		0	
CB 180 [a]	0.5	67	2.2 ± 1.7	67	1.9 ± 1.5	33	1.9
CB 183	0.5	33	0.5	33	0.7	0	
Sum PCBs [a]			9.9 ± 9.1		6.5 ± 5.6		6.8 ± 5.2
HCB [a]	1.0	100	1.5 ± 0.4	67	1.2 ± 0.3	33	1.7
***p,p'*-DDE** [a]	0.5	100	4.8 ± 3.0	100	5.2 ± 3.0	100	5.5 ± 5.1
β-HCH	1.0	100	4.6 ± 5.7	100	9.7 ± 7.5	33	0.6
γ-HCH	0.5	0				33	2.2

[a] Compound selected for statistical analysis

All compounds were normally distributed except for the PCB-congeners in liver samples. The lipid percentage was significantly lower in liver samples originating from Flanders than from Wallonia. In contrast, HCB and CB 153 levels were significantly higher in liver samples originating from Flanders (Table 3).

Table 3 Median lipid content (%), CB 153 and HCB concentrations (ng/g lw, min – max) in liver samples originating from 2 different regions in Belgium ($p < 0.05$).

	Flanders	Wallonia
n	10	8
Lipids	4.1 (3.8 – 4.9)	4.9 (4.1 – 5.9)
CB 153	3.4 (2.7 – 33.7)	2.4 (1.1 – 5.0)
HCB	1.5 (0.7 – 3.8)	0.7 (0.7 – 1.2)

Compound concentrations were significantly different in paired liver and adipose tissue samples, except for *p,p'*-DDE. Highly significant correlations were found between the levels of individual PCB-congeners and sum PCBs in liver and adipose tissue (Table 4). Also, both in adipose tissue and liver samples, HCB and *p,p'*-DDE levels were positively correlated (Table 4). Age was only positively correlated with *p,p'*-DDE in adipose tissue. Lipid percentage in liver samples was negatively correlated with CB 153 and HCB (Table 4).

Table 4 Correlations between different variables in adipose tissue and liver samples. A = adipose tissue, L = liver

Correlation between	r	p <
Single CB congeners L – Sum CBs L	> 0.95	0.001
Single CB congeners A – Sum CBs A	> 0.87	0.001
HCB A – *p,p'*-DDE A	0.75	0.001
HCB L – *p,p'*-DDE L	0.66	0.01
Age – *p,p'*-DDE A	0.49	0.05
Lip% L – CB 153 L	- 0.53	0.05
Lip% L – HCB L	- 0.53	0.05

Levels of the organohalogenated compounds were below the LOQ in nearly every serum sample (n = 20); for example CB 153 (LOQ: 10 pg/ml) was only detected in 2 samples. Moreover, no compound was detected above the LOQ (ranging from 10-30 pg/ml) in the follicular fluid samples. Because contamination was very low or even absent in serum and follicular fluid, no statistics could be performed on these samples. In contrast, compounds could be detected in milk samples (n = 7) with a median sum PCBs of 5.9 ng/g lw (1.8 – 35.3 ng/g lw) (Table 5). The lipid percentage of the milk samples was 2.0 ± 1.0 %. All compounds were normally distributed.

Concentrations of EDCs in serum and milk samples from high producing dairy cows taken 2 – 3 weeks post-partum in Belgium

In serum samples, almost no compounds were detected above the LOQs (10-30 pg/ml), except for CB 153 (average: 10 pg/ml). In milk samples, average lipid percentage was 4.4 ± 2.2 %. Milk samples exhibited detectable levels of organohalogenated compounds which were normally distributed (Table 5). No correlation was found for CB 153 between serum and milk samples ($r = 0.35$, $p > 0.05$).

Table 5 Median concentrations (min – max) (ng/g lw), limits of quantification LOQ (ng/g) and detection frequencies f (%) of different organohalogenated compounds in milk samples from slaughterhouse and high producing dairy cows.

	Slaughterhouse			High producing		
N	7			8		
Lipids (%)	2.0 ± 1.0			4.4 ± 2.2		
Compound	LOQ	f	Median (min – max)	LOQ	f	Median (min – max)
CB 99	1.6	0		0.7	13	1.6
CB 118	0.5	71	1.8 (0.6 – 6.0)	0.2	100	3.0 (1.6 – 10.8)
CB 138	0.5	100	2.3 (0.6 – 12.5)	0.2	100	4.5 (2.9 – 17.5)
CB 153	0.5	100	1.8 (0.6 – 10.9)	0.2	100	4.4 (2.7 – 16.7)
CB 156	0.5	0		0.2	50	0.4 (0.2 – 0.8)
CB 170	0.5	29	0.6 (0.5 – 0.8)	0.2	100	0.8 (0.5 – 2.9)
CB 180	0.5	71	0.8 (0.5 – 6.0)	0.2	100	1.8 (1.1 – 6.9)
CB 183	0.5	0		0.2	75	0.6 (0.2 – 1.3)
CB 187	0.5	0		0.2	25	0.2
Sum PCBs			5.9 (1.8 – 35.3)			15.5 (9.2 – 57.0)
HCB	0.5	100	3.6 (0.8 – 24.8)	0.2	100	4.7 (2.4 – 18.0)
***p,p'*-DDE**	1.6	14	23.3	0.7	100	19.0 (7.1 – 66.8)
***p,p'*-DDT**	1.6	0		0.7	13	0.9
***p,p'*-DDD**	1.6	0		0.7	13	0.9
α-HCH	0.5	0		0.2	75	0.4 (0.3 – 1.1)
β-HCH	0.5	0		0.2	13	0.3
γ-HCH	1.6	86	2.4 (1.1 – 12.3)	0.7	100	1.2 (0.9 – 6.7)
Oxy-chlordane	1.1	29	2.7 (2.1 – 3.3)	0.5	38	1.0 (0.7 – 1.2)
BDE 47	0.5	0		0.2	25	0.8 (0.2 – 1.4)
BDE 99	0.5	0		0.2	25	0.5 (0.1 – 0.9)

DISCUSSION

Fertility of high producing dairy cows showed a worldwide decline during the last decades. The presence of endocrine disrupting chemicals (EDCs) in the environment has already been argued as one of the possible causes of the reproductive problems of these animals. Because data about the possible contamination of dairy cow tissues and body fluids are rare, the present study aimed to obtain an indication of the contamination of Belgian dairy cows with different EDCs (PCBs, PBDEs and

OCPs), known for their environmental abundance in Belgium (Jaspers *et al.*, 2006; Covaci *et al.*, 2008b). The levels found in the tissue and body fluid samples of this study were all in concordance or even lower than the scarcely reported EDC-concentrations in bovine tissue and/or body fluids (Thomas *et al.*, 1999; Kamarianos *et al.*, 2003; La Rocca and Mantovani, 2006; Blanco-Penedo *et al.*, 2008; Glynn *et al.*, 2009). Similarly to these cow studies and in other animals, CB 153 was found at the highest concentration compared to other PCB-congeners (Blanco-Penedo *et al.*, 2008; Glynn *et al.*, 2009). Although average HCB-concentrations in liver samples from Flanders were 2 times higher than in Wallonia (presumably caused by a different use of HCB in the past), concentrations are still low compared to other species (Jaspers *et al.*, 2006; Covaci *et al.*, 2008b). In addition, total PCB-concentrations in dairy cow livers were about 25 times lower compared to the levels found in human livers originating from the same region (Covaci *et al.*, 2008b).

These low levels can be largely explained by the position of cows on the bottom of the food chain. The process of bioaccumulation is therefore very low or even not applicable in cows. One study has even reported about a 'biodilution' effect of PCBs through the agricultural food chain (McLachlan, 1996). Moreover, Regulation EC No 999/2001 of the European Parliament (EC, 2001) prohibits the feeding of animal derived proteins to ruminants, with an exception for the production of milk replacers for young animals (EC, 2008). The background levels of EDCs detected in our study can most likely be attributed through the ingestion of contaminated grass or through the accidental uptake of polluted soil during grazing (Rhind, 2005). Furthermore, sewage sludge, often applied to land as fertilizer, contains a whole set of EDCs (O'Connor, 1996; Hale *et al.*, 2001). Although levels of EDCs in this sludge are low, disruption of fetal testis and ovarian development has already been observed in sheep reared on sewage sludge contaminated pasture (Paul *et al.*, 2005; Fowler *et al.*, 2008). Sewage sludge can also contaminate surface water, which is often used as drinking water during the grazing season and has already been linked to reproductive problems in cows (Meijer *et al.*, 1999). The toxicity of these low levels can be related to mixture toxicity. Experiments already showed that when adding mixtures, the toxic concentrations are much lower than when individual compounds are being applied (Rajapakse *et al.*, 2002). Therefore, when interpreting the low levels observed in our study, the aspect of mixture toxicity has to be taken into account.

In this study, contamination was almost absent in serum samples from slaughterhouse dairy cows. In concordance to the results from tissue samples, milk samples exhibited low contamination levels. After calving, high producing dairy cows experience a state of negative energy balance (NEB) which is most severe around two weeks post-partum (Butler, 2003). The NEB-period is accompanied by a

massive lipolysis induced by a low insulin status which safeguards milk production. To investigate if this massive lipolysis can be accompanied with a release of EDCs from the adipose tissue, serum and milk samples were taken from high producing dairy cows 2 - 3 weeks post-partum. As in serum samples from slaughterhouse dairy cows, almost no compounds could be detected, with an exception of CB 153. EDCs are capable of transferring through the placental barrier in cattle (Hirako *et al.*, 2005). Because embryos, fetuses and new-born animals are known to be more susceptible to EDCs than adult animals (Boerjan *et al.*, 2002; Sweeney, 2002), awareness must be present in more heavily polluted areas where serum levels of EDCs can be detected.

Our results concerning milk samples suggest that PCB concentrations are higher in milk from high producing dairy cows compared to slaughterhouse dairy cows. However, more exposure studies controlling feed and management practices are necessary to evaluate the possible role of massive lipolysis in the amount of EDCs present in milk and serum. Suckling calves are thus exposed to (low) concentrations of EDCs. Studies have shown that neonatal calves are able to absorb EDCs added to the colostrum already 1 h post-partum (Keller *et al.*, 2001). Therefore, it cannot be excluded that EDCs located in the colostrum and milk can interfere with the physiological status of the growing calf. On the other hand, despite the presence of EDCs in human milk samples (Colles *et al.*, 2008) in even higher concentrations than those found in cow milk samples, breastfeeding is still recommended by the WHO (WHO and UNICEF, 2003).

Domestic ruminants have been suggested as a valuable model to study reproductive events in human. Due to a greater similarity between bovine reproductive physiology and human compared to laboratory rodents and human, the bovine model has already been used to study the effect of hyperlipidaemia on embryo quality, reproductive aging, uterine infection and immunity and to elucidate the mechanisms controlling ovarian follicle development (Campbell *et al.*, 2003; Malhi *et al.*, 2005; Herath *et al.*, 2006; Leroy *et al.*, 2010). Next to these large similarities in reproductive physiology between bovines and humans, the accessibility of bovine ovaries in the slaughterhouse and the reduction of the number of laboratory animals needed are also important advantages of the bovine model. Moreover, farm animals have already been proposed as an alternative species to investigate the effects of endocrine disruption (Magnusson, 2005). The bovine ovarian follicle has already been applied in *in vitro* studies to examine the effects of different toxic substances like PCBs, OCPs and alkylphenols on oocyte maturation and subsequent embryo development (Alm *et al.*, 1998; Krogenaes *et al.*, 1998; Pocar *et al.*, 2001; Pocar *et al.*, 2006); other groups use porcine follicles (Campagna *et al.*, 2006). Furthermore, exposing bovine oocytes to chemicals during *in vitro*

maturation and fertilization is already been used as a test within the EU funded 'ReProTect' project (Lazzari *et al.*, 2008). The harmful influence of these substances seen in the majority of these studies can only be a valuable representation of the real life situation if *in vivo* bovine oocytes have direct contact with those substances in the follicular fluid. What's more, in these experimental settings, nothing is known about the level of contamination with EDCs in the follicles. It is possible that the follicular cells already encountered negative influences due to EDC-exposure in the follicular fluid, before *in vitro* exposure even started.

Therefore, we investigated the possible contamination of bovine follicular fluid with EDCs. In contrast to the levels found in Greece, (e.g. *p,p'*-DDE: 1.50 ng/ml, sum PCBs 3.05 ng/ml) (Kamarianos *et al.*, 2003), not one of our investigated samples showed levels of EDC above their LOQs (10 - 30 pg/ml). Unlike the results obtained by Kamarianos and co-workers (2003), follicular fluid concentrations detected in our study are therefore lower than the toxic concentrations observed in the previously mentioned *in vitro* experiments, where negative effects of the added substances were not seen below 100 pg/ml. The only exception is the study of (Krogenaes *et al.*, 1998), where they found a significant decrease in blastocyst development after the addition of 1 pg/ml CB 126, the most toxic PCB congener. Although analysis was performed at a random time-point, the fact that we screened older animals from diverse locations in Belgium makes us conclude that bovine follicles in Belgium are relatively free of contamination with PCBs, PBDEs and OCPs and therefore Belgian dairy cow ovarian follicles can be used as an experimental *in vitro* model for reproductive toxicology research concerning these substances.

In conclusion, our study is the first to give an overview of the *in vivo* levels of EDCs in bovine tissues and body fluids. Levels are extremely low compared to other species. Considering this and other well-known causes of the failing reproductive performance of high producing dairy cows, like the metabolic changes during the period of NEB post-partum, the presence of PCBs, PBDEs and OCPs is probably not one of the major causes of this declining fertility. However, the influence of possible interactions between EDCs and bovine fetuses and (neonatal) calves through blood and milk respectively, may not be neglected as well as the role of mixture toxicity. Secondly, our results confirmed that the bovine ovarian follicle can be applied to study the possible effects of EDCs on human reproduction. Due to regional differences however, it is opportune to check for contamination in the follicular fluid before starting *in vitro* experiments.

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ENDOCRINE DISCRUPTING CHEMICALS IN HUMAN FOLLICULAR FLUID IMPAIR *IN VITRO* OOCYTE DEVELOPMENTAL COMPETENCE

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SUMMARY

BACKGROUND: Increased global industrial activity has exposed humans to a wide variety of chemical substances some of which, called 'endocrine disrupting chemicals' (EDCs) can disrupt the endocrine system in the body. The ovarian follicle is a very fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. *In vitro* experiments suggest that EDCs can disturb this finely tuned balance, but very scarse *in vivo* data are available to confirm this assumption. Therefore, we have investigated if the presence of EDCs in human follicular fluid is a risk factor for the developmental competence of an *in vivo* exposed oocyte. Furthermore, because of the limited access to human follicular fluid, we verified if follicular fluid contamination can be predicted based on EDC-levels in serum.

METHODS: Follicular fluid (n = 40) and serum (n = 20) samples from women undergoing Assisted Reproductive Technology (ART) were analyzed by means of gas chromatography combined with mass spectrometry (GC/MS) to examine the presence of different EDCs, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs). Statistical models were used to investigate the relation between the characteristics and ART results of the patients and the contamination status of their follicular fluid, and to assess the capacity of serum samples to predict follicular fluid contamination.

RESULTS: Chlorinated biphenyl (CB) 153 (72 ± 44 and 201 ± 106 pg/ml) and *p,p'*-DDE (392 ± 348 and 622 ± 406 pg/ml) were the compounds found in the highest concentrations in follicular fluid and serum samples, respectively. A new variable Principal Component 1 (PC1), representing the overall contamination status of the follicular fluid samples, is strongly associated with fertilization rate ($p < 0.00001$) and the proportion of high quality embryos relative to the amount of retrieved oocytes ($p < 0.05$), even when the analysis is adjusted for age, [E2], BMI, fertilization procedure and male subfertility as explanatory variables. The strong correlations between the EDC-concentrations in serum and follicular fluid ($r \geq 0.93$) allowed us to build regression models which accurately predict EDC-concentrations in follicular fluid based on serum samples.

CONCLUSION: An overall higher EDC-contamination in the follicular micro-environment was associated with a decreased fertilization rate and consequently with a lower chance of an oocyte to develop into a high quality embryo. In addition, EDC-concentrations in serum were reliable predictors of the contamination status of the follicular micro-environment.

INTRODUCTION

Since the Industrial Revolution, the release of chemicals into the environment has significantly increased. However, it was only after the publication of Rachel Carson's book 'Silent Spring' in 1962 that attention was drawn to the possible toxic effects of these chemicals on our ecosystem (Carson, 1962). This growing awareness resulted in a ban of multiple pesticides (e.g. dichlorodiphenyltrichloroethane, DDT) and other chemicals (e.g. polychlorinated biphenyls, PCBs) in many developed countries during the seventies (Colborn *et al.*, 1993). Nevertheless, various banned substances are still detected in our environment because of their long half-lives (Covaci *et al.*, 2002; Jaspers *et al.*, 2006; Josefsson *et al.*, 2011). Additional research revealed that some of these chemicals were able to interfere with the synthesis, function, storage and/or metabolism of hormones (Sweeney, 2002). As a result of this ability to interact with the endocrine system, these substances were denominated as 'endocrine disrupting chemicals' (EDCs) (Colborn *et al.*, 1993). Furthermore, due to their lipophilic and persistent characteristics, EDCs undergo the process of bioaccumulation, with the highest concentrations found in species at the top of the food chain (e.g. human) (Jaspers *et al.*, 2006; Wang and Needham, 2007; Covaci *et al.*, 2008).

Wildlife observations in highly contaminated geographical areas revealed that EDCs can cause reproductive abnormalities (Vos *et al.*, 2000; Bernanke and Kohler, 2009; Hamlin and Guillette, 2010). Alligator ovaries for example exhibited increased numbers of multi-oocyte follicles and polynuclear oocytes after a large pesticide spill with primarily dicofol and DDT-derivatives (Milnes and Guillette, 2008). Subsequently, laboratory animal experiments confirmed the ability of EDCs to induce reproductive disorders (Diamanti-Kandarakis *et al.*, 2009). Although it remains unclear whether EDCs can execute comparable effects in humans (Ross, 2004; Vandenberg *et al.*, 2009), they have already been suggested as a potential key player in human subfertility (Toft *et al.*, 2004; Diamanti-Kandarakis *et al.*, 2009). Within the reproductive system, the ovarian follicle can be considered as a very fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. Disruption of this finely tuned (endocrine/paracrine) balance can lead to anovulation (Mumford *et al.*, 2011), cystic deformation (Baptiste *et al.*, 2010) or a diminished oocyte quality which jeopardizes further embryo development (Leroy *et al.*, 2005). Although *in vitro* experiments suggest a role for EDCs in disturbing the tightly regulated endocrine and paracrine signaling in the different cells of the ovarian follicle (Brevini *et al.*, 2004; Pocar *et al.*, 2006; Kwintkiewicz *et al.*, 2010), these exposure experiments can only be related to the *in vivo* situation if environmental relevant

EDC-concentrations are considered. Moreover, almost no *in vivo* data are currently available about the association of a women's *in vitro* fertilization (IVF) record and the presence of EDCs in her follicular fluid, i.e. the *in vivo* micro-environment in which the female gamete grows and matures. Knowledge about the contamination status of human follicular fluid is thus indispensable, which implies the continuous need for monitoring EDC-concentrations in the follicular fluid (Trapp *et al.*, 1984; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010). In addition, due to the complicated sampling procedure and to ethical requirements, follicular fluid can only be obtained on a regular basis in Assisted Reproductive Technology (ART) settings. The possibility to predict EDC-concentrations in follicular fluid based on serum measurements would therefore be of a great value.

In the present study, we investigate if the presence of EDCs in human follicular fluid is a risk factor for a reduced developmental competence of the *in vivo* exposed oocyte. Consequently, the aims of this study were 1) to assess the presence of the most common EDCs in follicular fluid from ART-patients; 2) to examine a potential link between these concentrations and the patient's characteristics and ART-outcome and 3) to examine the option of using serum samples to predict EDC-concentrations in follicular fluid.

MATERIALS AND METHODS

Sample collection

During two identical sampling periods, human follicular fluid samples (n = 20, March 2008) and paired serum and follicular fluid samples (n = 20, March – May 2009) were collected in the fertility unit of ZNA Middelheim Hospital, Antwerp, Belgium, following the approval of the ethical committees from the University of Antwerp and ZNA Middelheim Hospital. All patients signed informed consent papers. During routine ART-procedures, patients were stimulated daily with human menopausal gonadotropins (Menopur®, Ferring Pharmaceuticals, Copenhagen, Denmark) or follicle stimulating hormone (FSH) (Gonal-F®, Merck Serono, Geneva, Switserland or Puregon®, MSD, Oss, The Netherlands) at a dose of 150-225 IU/day for approximately 14 days. Patients received a long GnRH-agonist protocol whereby busereline (0.1 mg/dose, Suprefact®, Sanofi-Aventis, Frankfurt, Germany) was administered six times a day intranasally from 3 weeks before the beginning of the stimulation protocol until the moment of ovum pick-up (OPU). When at least three follicles were larger than 17 mm, 10000 IU human chorionic gonadotropin (hCG, Pregnyl®, MSD, Oss, The

Netherlands) was administered. Oocytes were retrieved 37 h later by means of ultrasound-guided follicular aspiration. To prevent contamination with blood or flushing medium, only follicular fluid from the first punctured follicle was collected. Venous blood samples were taken immediately following the ART-procedure. Follicular fluid and coagulated blood samples were centrifuged (1500 g, 20 min and 1400 g, 30 min respectively) and the supernatant was stored in polypropylene tubes at -20° C until analysis.

Patient information and ART-characteristics

In our study group, age, height, weight, occupation and residence were collected from the patients and consequently Body Mass Index (BMI, kg/m²) was calculated. Furthermore, the following cycle parameters were collected: serum estradiol concentration at the day of hCG administration [E2] (i.e. 37h before OPU), number of retrieved oocytes, number of zygotes with 2 pronuclei (2PN-zygotes, 18h post insemination/injection), number of high quality embryos (Table 1), fertilization procedure (IVF or Intracytoplasmic Sperm Injection, ICSI), initial subfertility problem, ART- and pregnancy outcome. Embryos were characterized as a high or top quality embryo in the absence of multinucleated blastomeres, 4-5 blastomeres on day 2, ≥7 blastomeres on day 3 and ≤20% anucleated fragments (Van Royen *et al.*, 1999). With these data, the fertilization rate (number of 2PN-zygotes / number of retrieved oocytes) and the proportion of top quality embryos of a patient relative to the amount of retrieved oocytes as well as to the amount of 2PN-zygotes (number of high quality embryos / number of retrieved oocytes and number of high quality embryos / number of 2PN-zygotes respectively), could be calculated. An ART-procedure was defined as successful if a gestational sac with positive heart beat was detected around 7 weeks amenorrhea. The birth of one or more healthy babies was considered as a successful pregnancy.

Chemical analysis of body fluids

In all samples, 26 PCB-congeners, 7 polybrominated diphenyl ether (PBDE) congeners, and the pesticides hexachlorobenzene (HCB), chlordanes (cis-chlordane, trans-chlordane, trans-nonachlor and oxychlordane), hexachlorocyclohexanes (α-, β-, γ-HCHs), *p,p'*-DDT and its metabolites (*p,p'*-DDD and *p,p'*-DDE) were analyzed. Serum and follicular fluid samples were treated as previously described following a slightly adjusted method (Covaci and Schepens, 2001). All standards were obtained from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). All solvents were of pesticide-grade purity and

were, together with concentrated sulfuric acid (analytical grade) and silica gel 60 (63 – 230 mesh), available from Merck (Darmstadt, Germany). In short, samples (3 - 4 ml) were spiked with internal standards, mixed with deionized water and formic acid (used for protein denaturation) and, after ultrasonication, loaded on C18 Empore™ solid-phase disk extraction cartridges (3M Company, St. Paul, MN, USA). After elution, the extracts were cleaned on acidified silica and analytes were further eluted with hexane and dichloromethane. The cleaned eluate was then concentrated to near dryness, redissolved in iso-octane and analyzed by gas chromatography coupled to mass spectrometry (GC/MS). The GC/MS was operated in electron-capture negative ionization (ECNI) mode, using a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm).

Multi-level calibration curves ($r^2 > 0.999$) in the linear response interval of the detector were created for the quantification. The quality control was performed by regular analyses of procedural blanks, by random injection of standards, spiked samples, and solvent blanks. Procedural blanks were consistent (RSD < 15%) and therefore the mean procedural blank value was used for subtraction. After blank subtraction, the method limit of quantification (LOQ) was set at 3 x standard deviation (SD) of the blank (which ensures >99% certainty that the reported value is originating from the sample) taking into consideration the volume of the analyzed sample. The quality control scheme was also assessed through regular participation to inter-laboratory comparison exercises organized by the Arctic Monitoring and Assessment Program (AMAP) (persistent organic pollutants in human serum) (AMAP, 2010). Obtained values were deviating with less than 20% from the consensus values.

The total amount of lipids (TL) in the serum samples was calculated using following formula: TL (g/l) = 2.27 x total cholesterol + triglycerides + 0.62 (Phillips *et al.*, 1989).

Data treatment

Statistical analysis was performed using SPSS 15.0 for Windows (Chicago, IL, USA) and R version 2.13.0 (R Development Core Team, 2008). Values of $p < 0.05$ were considered as statistically significant. Samples with levels below the LOQ were assigned a value of f x LOQ with f being the detection frequency or the ratio between the number of samples detected above the method LOQ and the total number of analyzed samples (Voorspoels *et al.*, 2002). By doing so, data below LOQ can still be used in the statistical data treatment. However, compounds with < 50% of the measurements above their LOQs were excluded from statistical analysis.

Data are presented as mean with standard deviation (SD). Serum samples are expressed both per volume (/ml) and lipid normalized (per g lipid weight; /g lw). Normality was checked using the

Shapiro-Wilk test. Because of the relative low number of samples in the study and the detection of outliers, the Wilcoxon matched pairs signed rank test was used to examine the difference in compound concentrations between serum and follicular fluid. Correlations between follicular fluid and serum concentrations and patient characteristics were investigated using the Spearman rank test. Regression analysis was carried out on both untransformed (linear) as well as log-transformed concentrations to examine how accurately EDC-concentrations in follicular fluid can be estimated based on serum concentrations. Principal Components Analysis (PCA), a data reduction technique that re-organizes the 9 original variables into 9 new variables, called Principal Components (PCs), was performed on the correlation matrix. Subsequently, scores were calculated for the first PC. These PC scores were then entered in logistic regression analysis to test their association with the fertilization rate, proportion of top quality embryos relative to both the number of retrieved oocytes and the number of 2PN-zygotes, ART- and pregnancy outcome. For all these outcomes, significance of the PC scores was tested by a likelihood ratio test, comparing a model with the PC-scores, age, [E2], BMI, fertilization procedure and the fact if male subfertility was the initial reason to start with ART, to a null model only containing these latter 5 variables. This procedure was executed for all the variables. When the outcome was a proportion (fertilization rate, and proportion of high quality embryos relative to both the number of retrieved oocytes and 2PN-zygotes), logistic regression was performed as described in Venables and Ripley (Modern Applied Statistics in S, 4th Edition, pp191-192, Springer). Basically, the outcome variable is ordered into a two-column matrix, with the first column giving the number of successes, and the second column the number of failures for each individual. For instance, to perform the analysis on the proportion of high quality embryos relative to the total number of ova, the first column would contain the number of high quality embryos (success), with the second column containing the number of ova that failed to become a high quality embryo.

RESULTS

Patients (n = 40) were on average 34.5 ± 4.4 yrs old, had an average BMI of 23.1 ± 4.8 kg/m² (Table 1) and they all lived in urbanized area. None of the patients from which information about their occupation was available (n = 14) worked in a high risk exposure environment. IVF was used in the majority of patients (58 %), the others were treated with ICSI. Of all ART-procedures in this study, 30% achieved an ongoing pregnancy and 25 % achieved a live birth.

Table 1 Characteristics and IVF record of the 40 participating patients

Characteristics	**Mean**	**SD**	**Median**	**Min – Max**
Age (years)	34.5	4.4	34.1	25.2 – 42.8
Height (cm)	167	6.6	168	155 – 182
Body weight (kg)	64.8	15.4	62.0	45 – 117
BMI [a] (kg/m²)	23.1	4.8	22.1	16.9 – 38.2
[E2] [b] (pg/ml)	1847	1129	1677	253 - 4572
# retrieved oocytes	9	5	8	2 – 28
# 2PN-zygotes [c]	6	4	5	0 – 24
Fertilization rate [d]	0.72	0.26	0.76	0 – 1
# top quality embryos [e]	2	3	1	0 – 11
Proportion top quality embryos [f]	0.22	0.21	0.17	0 – 0.71
Outcomes	**%**			
Clinical pregnancy [g]	30			
Live birth [h]	25			

[a] BMI = Body Mass Index; [b] [E2] = serum estradiol concentration on the day of hCG administration (i.e. 37h before ovum pick-up); [c] # zygotes with 2 pro nuclei; [d] # zygotes with 2 pro nuclei / # retrieved oocytes; [e] Van Royen *et al.*, 1999; [f] # top quality embryos / # aspirated oocytes; [g] detection of gestational sac with positive heart beat (after 7 weeks amenorrhea); [h] birth of one or more healthy babies

Several compounds (CBs 118, 138, 153, 170, 180, HCB, β-HCH and *p,p'*-DDE) were present in nearly every serum and follicular fluid sample, whereas other chemicals had a detection frequency < 50% and were excluded for statistical analysis (Table 2). Other compounds could not be detected above their respective LOQs in both serum and follicular fluid samples (CBs 28/31, 52, 74, 95, 99, 101, 110, 128, 132, 149, 156, 167, 194, 196/203, 199; BDEs 28, 100, 153, 154, 183; cis- and trans-chlordane; α-HCH and *p,p'*-DDD). CB 153 was the most abundant PCB-congener in both body fluids (serum: 201 ± 106 pg/ml, follicular fluid: 72 ± 44 pg/ml). The average lipid content of serum samples was 5.04 ±

0.74 g/l. Compound concentrations were significantly different in serum and follicular fluid, with higher levels found in serum (Table 2).

Table 2 Mean concentrations with standard deviations (SD) [pg/ml and ng/g lipid weight (lw)] and detection frequencies DF (%) of different endocrine disrupting chemicals in serum and follicular fluid samples from women undergoing assisted reproductive technology. LOQs ranged from 2 – 30 pg/ml and 0.4 – 4 ng/g lw.
Compounds with DF > 50% were selected for statistical analysis.

		Serum (n = 20)			**Follicular fluid (n = 40)**
Compound	DF	pg/ml	ng/g lw	DF	pg/ml
CB 105	75	8 ± 5	1.5 ± 0.9	0	/
CB 118	100	42 ± 28[a]	8.1 ± 4.8	68	15 ± 8[b]
CB 138	100	120 ± 57[a]	23.5 ± 9.2	98	49 ± 32[b]
CB 153	100	201 ± 106[a]	39.2 ± 17.6	98	72 ± 44[b]
CB 170	100	44 ± 23[a]	8.7 ± 4.4	85	21 ± 13[b]
CB 180	100	111 ± 61[a]	21.9 ± 10.9	93	51 ± 33[b]
CB 183	100	12 ± 7	2.4 ± 1.1	5	13 ± 3
CB 187	100	25 ± 16	4.9 ± 2.8	30	18 ± 10
Sum PCBs		562 ± 294[a]	110.1 ± 48.9		213 ± 136[b]
HCB	100	59 ± 31[a]	11.3 ± 4.7	93	32 ± 19[b]
OxC	100	21 ± 11	4.0 ± 1.6	0	/
TN	100	15 ± 10	2.9 ± 1.8	0	/
***p,p'*-DDE**	100	622 ± 406[a]	119.8 ± 68.8	98	392 ± 348[b]
***p,p'*-DDT**	70	16 ± 12	3.4 ± 2.0	8	35 ± 5
β-HCH	95	35 ± 22[a]	6.9 ± 3.7	78	34 ± 35[b]
γ-HCH	45	7 ± 2	/	5	34 ± 1
BDE 47	83	3 ± 3	0.7 ± 0.7	3	12
BDE 99	5	2 ± 1	0.5 ± 0.1	3	14

CB = chlorinated biphenyl, HCB = hexachlorobenzene, OxC = oxychlordane, TN = *trans*-nonachlor, DDE = dichlorodiphenyldichloroethylene, DDT = dichlorodiphenyltrichloroethane, HCH = hexachlorocyclohexane, BDE = brominated diphenyl ether
Data marked with different superscripts in a row differ significantly among groups ($P<0.05$)

For every compound, strong correlations were detected between serum and follicular fluid concentrations ($r \geq 0.93$), except for HCB ($r = 0.631$) (Figure 1). Moreover, for the compounds detected in nearly every follicular fluid and serum sample, linear regression models to predict follicular fluid EDC-concentrations from serum were statistically significant (Figure 1). The same result was obtained using log-transformed values (data not shown). Age was positively correlated with nearly every compound in both follicular fluid and serum, whereby CB 170 ($r = 0.628$) and oxychlordane ($r = 0.783$) showed the highest and β-HCH ($r = 0.424$) and CB 118 ($r = 0.468$) the lowest correlations with age in follicular fluid and serum respectively. No correlations were found in follicular fluid between BMI and the detected chemical compounds. In serum samples, BMI showed moderate positive correlations with only 2 compounds: HCB ($r = 0.520$) and β-HCH ($r = 0.477$). The number of retrieved oocytes did not correlate with any compounds in both follicular fluid and serum. Low negative correlations were found between [E2] and several compounds in both body fluids ($-0.337 \leq r \leq -0.522$). A correlation table of the patient and cycle characteristics and the detected compounds in follicular fluid and serum can be found in the supplement.

To test the relationship between the contamination status of the follicular fluid and the proportion of high quality embryos, we first summarized the 9 original variables into new summary values (PC scores) using PCA. Next, we found that the first PC score (PC1) encompasses 64 % of all the information contained in the 9 original variables, characterizing the follicular fluid contamination using the following formula: PC1 = – 0.296 x [CB 118] – 0.359 x [CB 153] – 0.380 x [CB 138] – 0.339 x [CB 180] – 0.339 x [CB 170] – 0.366 x [sum PCBs] – 0.326 x [HCB] – 0.162 x [β-HCH] – 0.378 x [*p,p'*-DDE]. This means that the lower the PC1 score, the higher the overall contamination status of the follicular fluid in that patient, and that this PC1 score can be used as a summary statistic for the overall contamination status of follicular fluid. Logistic regression models with fertilization rate, proportion of high quality embryos relative to both the number of retrieved oocytes and 2PN-zygotes, ART- or pregnancy results as outcome, and age, [E2], BMI, fertilization procedure and male subfertility as explanatory variables, showed a significantly improved fit upon adding PC1 as a covariate for fertilization rate ($p < 0.00001$) and the proportion of high quality embryos relative to the number of retrieved oocytes ($p < 0.05$). This suggests that a lower PC1 score, meaning higher EDC-concentrations in follicular fluid, leads to a lower fertilization rate and subsequently, to a lower proportion high quality embryos. These significances remain if only those patients are considered in which the original fertility problem was attributed to a male factor ($n = 27$). Furthermore, BMI was shown to have a significant effect on the proportion of high quality embryos relative to both the number of retrieved oocytes ($p < 0.01$) and 2PN-zygotes ($p < 0.001$). In both cases, a higher BMI leads

to a lower proportion of top quality embryos. No associations were found between clinical pregnancy and live birth rates and the contamination status of follicular fluid. Full logistic regression results are shown in Supplementary Table 3 and the goodness of fit of the models with significant associations is shown in Supplementary Figure 1. The addition of PC2, which explains only 24 % of the information of the 9 original variables, to this logistic regression model did not improve the fit of the model ($p > 0.05$).

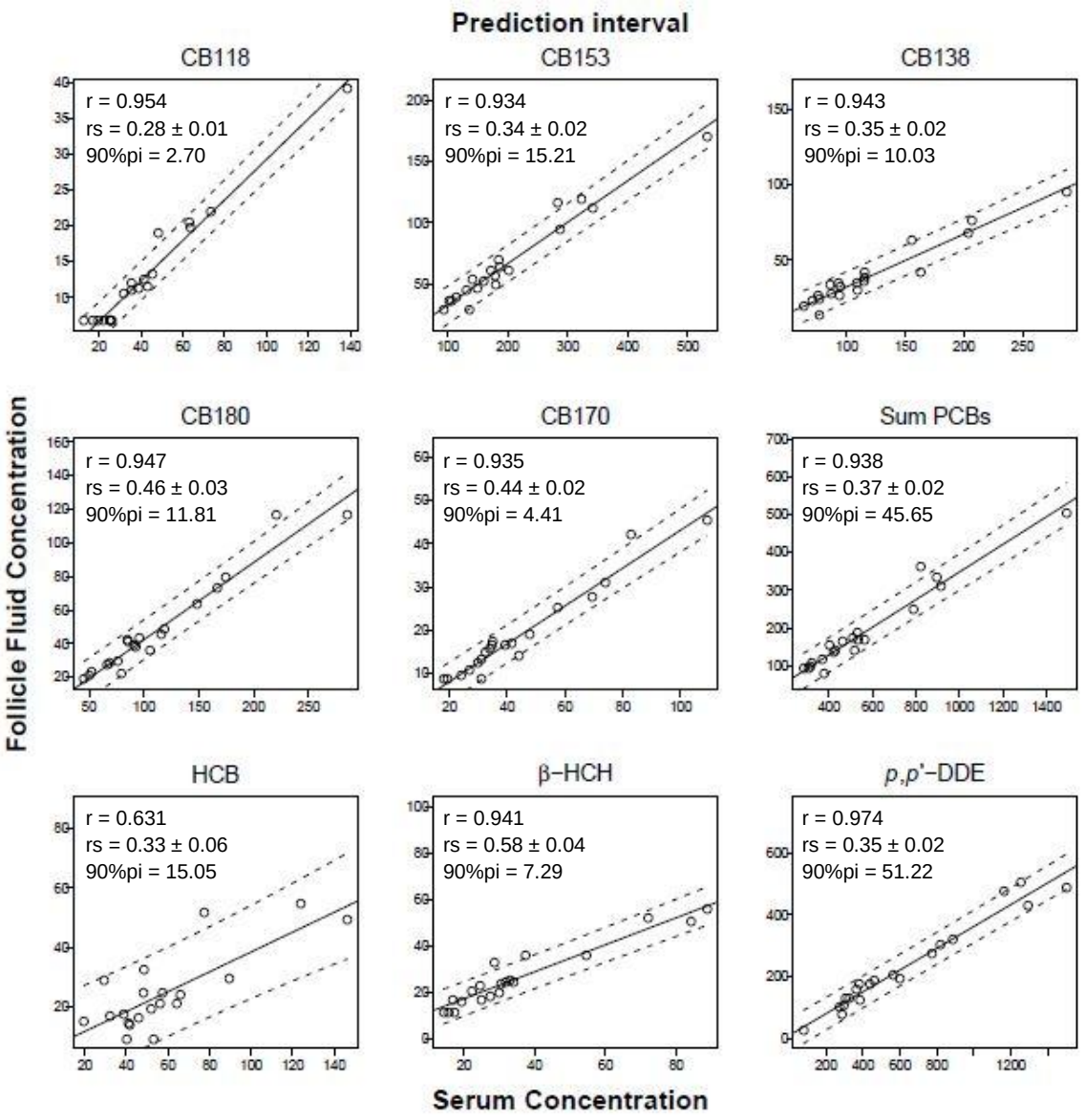

Figure 1 Spearman correlation coefficients (r, P<0.01), linear regression model (regression slope, rs) and 90% prediction interval (90%pi, pg/ml) with serum as independent and follicular fluid concentrations as dependent variable.

DISCUSSION

In this study, we hypothesized that the presence of EDCs in the follicular micro-environment may be a risk factor for impaired oocyte development. Therefore, we aimed first to determine EDC-concentrations in the follicular fluid of women undergoing ART-procedure. Consequently, we related these concentrations to the patient's profile and ART characteristics. Finally, because follicular fluid can only be obtained in clinical settings, we verified if EDC-levels in follicular fluid could be predicted based on serum measurements.

To our knowledge, our results are the first to report a significant *in vivo* association between higher EDC-concentrations in the follicular fluid of a patient and reduced developmental competence of her oocytes which were directly exposed to these EDCs *in vivo*. Using PCA, we demonstrate that patients with lower PC1 scores, which reflect higher EDC-contamination of the follicular fluid, have a highly significant drop in fertilization rate and a significant lower proportion of top quality embryos, independent of the age, BMI, E2 levels of the patient, fertilization procedure or the presence of male subfertility. The strong influence of the follicular fluid contamination status on the fertilization rate is likely to be the most important factor to explain the observed lower proportion of high quality embryos. It is clear that if fewer oocytes are fertilized, less can develop into high quality embryos. This conclusion is supported by the observation that the proportion of 2PN-zygotes that develop into top quality embryos is not affected by the PC1 score; in other words, if an oocyte has the potential to develop into a zygote, its chance to further develop into a high quality embryo is not hampered by the EDC-concentration in the follicular fluid. On the contrary, a higher BMI was shown to have a profound negative effect on the proportion of 2PN-zygotes that produce high quality embryos. Because fertilization rate is not influenced by BMI and the BMI-effect is most clear on the number of top quality embryos relative to the amount of 2PN-zygotes, it could be speculated that BMI particularly impairs the first steps of embryonic development. Because embryo quality is an important determinant of successful implantation (Cakmak and Taylor, 2011), the observed lower proportion of high quality embryos in our study, paralleled by decreased fertilization rates, may thus (partially) explain the lower implantation rates described recently in women with higher PCB-concentrations in their serum (Meeker *et al.*, 2011), which however could not be confirmed by our data. Until our study, the only available information about follicular fluid contamination with EDCs and IVF-outcome was the suggestion of a possible link between the presence of *p,p'*-DDE in follicular fluid and serum and an impaired fertilization rate (Younglai *et al.*, 2002; Weiss *et al.*, 2006). Our study significantly confirms this negative effect of EDC-contamination in follicular fluid on fertilization

rates. However, cleavage rate seems not to be affected by the presence of EDCs (Jarrell *et al.*, 1993). In a Czech study, only non-significant trends were found between PCB-levels in the follicular fluid and ART results. However, they did not measure the most frequently detected PCB-congeners (CB 138, CB 153, CB 180) so certain associations were maybe underestimated (Jirsova *et al.*, 2010). In our study, no associations were found between ART- and pregnancy outcome and the EDC-contamination status of follicular fluid. This can be explained by the fact that although fertilization rate and embryo quality are imperative factors, there are undoubtedly other aspects later in embryonic development (f.e. uterine environment) which are also essential for the establishment of a successful pregnancy (Singh *et al.*, 2011).

In antral follicles, oocytes and follicular somatic cells are in close contact with the EDCs present in the follicular fluid, which is why EDCs can directly interact with these cells and influence the processes of folliculo- and/or oögenesis with a potential diminished oocyte quality as a result. A substantial amount of *in vitro* studies already reported on the inability of oocytes to fulfill both nuclear and cytoplasmic maturation after exposure to generally high EDC-concentrations during *in vitro* maturation (Campagna *et al.*, 2001; Pocar *et al.*, 2001; Lenie *et al.*, 2008). Furthermore, oocytes that were exposed to a PCB-mixture, which approaches the PCB-levels in follicular fluid of our study, were able to successfully complete maturation, while a significant number of them failed to reach the blastocyst stage (Pocar *et al.*, 2001). This observation underpins the finding that the initial quality of the oocyte is not only imperative for its own maturation, but that it is also fundamental for its further developmental competence (Sirard *et al.*, 2006). Also, cumulus and granulosa cells were vulnerable to the action of EDCs (Campagna *et al.*, 2001; Younglai *et al.*, 2004; Mlynarczuk *et al.*, 2009; Kwintkiewicz *et al.*, 2010). Although we recognize the possible existence of other confounders which were not taken into account in our study, such as the smoking habits of the parents (Cooper and Moley, 2008; Calogero *et al.*, 2009), the presence of undefined compounds in the follicular fluid, the metabolic state of the mother (Van Hoeck *et al.*, 2011) or the lack of paternal exposure data, we point out that our *in vivo* study indicates that the harmful influence of relatively low EDC-concentrations on embryo development observed *in vitro* indeed characterizes what can happen *in vivo*. Another possible source of bias might be the choice of fertilization procedure. However, assignment of patients to ICSI was decided based on the local clinical criteria of the participating hospital before PCB-exposure was known. There was no evidence of an association between male subfertility and fertilization procedure. The use of varying criteria between hospitals and the relatively small sample size preclude investigation of whether the effect of PCBs on fertilization differed between IVF and ICSI.

This research also contributes to the crucial global monitoring of EDCs in follicular fluid (Caserta *et al.*, 2011). Our results confirm the worldwide declining trend of well-known EDC-levels in follicular fluid (Trapp *et al.*, 1984; Baukloh *et al.*, 1985; Schlebusch *et al.*, 1989; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; De Felip *et al.*, 2004; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010). More specifically, the concentration of CB 153 (the most abundant PCB congener) in our study is more than 2 times lower compared to studies executed around the turn of the century (Pauwels *et al.*, 1999; Younglai *et al.*, 2002; De Felip *et al.*, 2004) and comparable with CB 153 levels in a more recent report (Meeker *et al.*, 2009). In the Czech study, the total PCB-concentration was more than 4 times lower, which can again be explained by the fact that they studied rather rarely detected PCB-congeners (Jirsova *et al.*, 2010). HCB-levels dropped considerably as compared to the first measurement of pollutants in follicular fluid (Trapp *et al.*, 1984) with a tendency for stabilization the last decade (De Felip *et al.*, 2004; Meeker *et al.*, 2009). The levels of *p,p'*-DDE are more variable between countries which can be caused by a different use of DDT in the past (Jarrell *et al.*, 1993; De Felip *et al.*, 2004; Weiss *et al.*, 2006; Meeker *et al.*, 2009). Nonetheless BDE 47 and BDE 49 were only detected in one sample, to the best of our knowledge, it is the first time that PBDE-congeners are detected in follicular fluid. In case of our serum samples, EDC-concentrations are in the same order of magnitude with recent studies in other Western countries (Porta *et al.*, 2010; Herrick *et al.*, 2011; Kalantzi *et al.*, 2011), with a distinct decline in contamination levels compared to older studies (Pauwels *et al.*, 1999; Voorspoels *et al.*, 2002; Needham *et al.*, 2005). Also, the bioaccumulation potential of these EDCs is once more demonstrated by the positive correlation between the age of the patients and the contamination levels in both serum and follicular fluid.

Based on our results, we also confirm that for compounds, regularly detected in both serum and follicular fluid samples (Pauwels *et al.*, 1999; Younglai *et al.*, 2002; Meeker *et al.*, 2009), the screening of serum is an easy and reliable approach to predict EDC-contamination of follicular fluid, as was previously suggested (Pauwels *et al.*, 1999; Meeker *et al.*, 2009). This can be largely attributed to the very high correlations between the contaminant concentrations in follicular fluid and serum observed in every study except for the study of Jarrell *et al.* (1993). This inconsistency can be explained by the rather high limits of detection in the latter study which resulted in a substantial number of 'blank' samples, which implicitly may have influenced statistical analysis. On the contrary, the correlations calculated in our study are similar to the ones in a previous Belgian study (Pauwels *et al.*, 1999) and slightly higher than in a US study (Meeker *et al.*, 2009). Roughly, concentrations of PCB-congeners, *p,p'*-DDE and HCB were around 2 - 3 times higher in serum than in follicular fluid and

in the middle of the range described in other studies (Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Weiss *et al.*, 2006; Meeker *et al.*, 2009). Because the vast majority of EDCs are lipophilic substances, the fact that serum contains more lipids than follicular fluid is the main reason for the difference in contamination status of both body fluids (De Felip *et al.*, 2004; Browne *et al.*, 2008). To improve precision, regression models were built allowing to predict with 90% accuracy the follicular fluid EDC-contamination -and thus the direct *in vivo* exposure of human oocytes and follicular somatic cells to EDCs- when only serum samples are available. Because our patient group shares several characteristics like weight, height and BMI with pregnant volunteers from larger biomonitoring studies and the serum levels of EDCs from these volunteers are in the same range as those in our study (Koppen *et al.*, 2009; Darnerud *et al.*, 2010; Llop *et al.*, 2010), we are confident that our study group matches the profile of the Western (pregnant) female population in terms of EDC-contamination. Keeping this in mind and given the strong correlations between serum and follicular fluid concentrations of the most frequently detected EDCs, and because it is ethically impossible to obtain follicular fluid samples from women who are not undergoing ART, we recommend our regression models as useful tools to estimate the EDC-contamination of the follicular micro-environment of these women outside an ART-setting.

In conclusion, our study is the first to document overall higher EDC-contamination in the follicular micro-environment being associated with a lower chance of an oocyte to develop into a top quality embryo, mainly due to a reduced fertilization rate. Further research is needed to explore this interesting link. Also, this study gives an update on and contributes to the essential continuous monitoring of the contamination status of follicular fluid with well-known persistent EDCs. In general, the levels of these substances are declining and stabilizing to background concentrations, a finding which puts forward the need to further investigate the presence of some other 'newly' emerging contaminants in the follicular micro-environment. PBDE-levels are detected for the first time in follicular fluid, however only in one sample. Serum levels of EDCs are comparable with other recent studies. Furthermore, the strong correlations between serum and follicular fluid EDC-concentrations, enabled us to build, for the most detected compounds, linear regression models that predict with 90% accuracy follicular fluid concentrations of EDCs based on serum levels. In this way, follicular fluid samples are not crucial anymore to gain knowledge about the contamination status of the follicular micro-environment with these EDCs.

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SUPPLEMENTARY DATA

Supplementary Table 1 Correlation coefficients between the patient and cycle characteristics and the detected compounds in follicular fluid (FF)

FF	CB 118	CB 138	CB 153	CB 170	CB 180	Sum PCBs	HCB	*p,p'*-DDE	β-HCH
Age	0.578	0.602	0.576	0.628	0.591	0.593	0.578	0.547	0.424
[E2]	-0.358	-0.348	-0.337	-0.369	/	-0.360	-0.406	/	/

CB = chlorinated biphenyl, HCB = hexachlorobenzene, DDE = dichlorodiphenyldichloroethylene, HCH = hexachlorocyclohexane
[E2] = Estradiol concentration (pg/ml) in serum on the day of hCG administration

Supplementary Table 2 Correlation coefficients between the patient and cycle characteristics and the detected compounds in serum (S)

S	CB 118	CB 138	CB 153	CB 170	CB 180	CB 183	CB 187	Sum PCBs	HCB	*p,p'*-DDE	β-HCH	*p,p'*-DDT	OxC	TN
Age	0.468	0.618	0.671	0.767	0.755	0.695	0.675	0.732	0.559	0.543	0.552	/	0.783	0.774
BMI	/	/	/	/	/	/	/	/	0.520	/	0.477	/	/	/
[E2]	-0.445	/	/	/	/	/	/	-0.489	-0.456	-0.522	/	/	/	/

CB = chlorinated biphenyl, HCB = hexachlorobenzene, OxC = oxychlordane, TN = *trans*-nonachlor, DDE = dichlorodiphenyldichloroethylene,
DDT = dichlorodiphenyltrichloroethane, HCH = hexachlorocyclohexane, BDE = brominated diphenyl ether
BMI = Body Mass Index (kg/m²), [E2] = Estradiol concentration (pg/ml) in serum on the day of hCG administration

Supplementary Table 3 Regression tables including regression coefficients (first column), their standard error, Wald Z-statistic, p-value (Wald test) and the estimate of the odds ratio, along with the boundaries of the 95% confidence interval (lowlim(OR) and uplim(OR)).

Supplementary Table 3a Fertilization rate

	Estimate	Std.err	Z	pvalue	OR	lowlim(OR)	uplim(OR)
(Intercept)	-1,64	1,55	-1,06	0,29			
BMI	0,01	0,03	0,42	0,67	1,01	0,95	1,08
Age	0,10	0,05	1,88	0,06	1,11	1,00	1,23
E2max	-0,41E-03	0,14E-03	-2,90	3,71E-03	1,00	1,00	1,00
PC1	0,60	0,14	4,42	1,00E-05	1,82	1,40	2,38
ICSI=Yes	-0,90	0,27	-3,38	0,72E-03	0,40	0,24	0,68

Supplementary Table 3b High quality embryos relative to the amount of retrieved oocytes

	Estimate	Std.err	Z	pvalue	OR	lowlim(OR)	uplim(OR)
(Intercept)	-1,32E+00	1,55E+00	-0,86	0,39			
BMI	-1,10E-01	3,85E-02	-2,86	0,42E-03	0,90	0,83	0,97
Age	8,01E-02	5,33E-02	1,50	0,13	1,08	0,98	1,20
E2max	8,31E-06	1,29E-04	0,07	0,95	1,00	1,00	1,00
PC1	3,03E-01	1,35E-01	2,25	0,02	1,35	1,04	1,76
ICSI=Yes	-2,41E-01	2,75E-01	-0,88	0,38	0,79	0,46	1,35

Supplementary Table 3c High quality embryos relative to the amount of 2PN-zygotes

	Estimate	Std.err	Z	pvalue	OR	lowlim(OR)	uplim(OR)
(Intercept)	-0,02	1,76	-0,01	1,00			
BMI	-0,15	0,04	-3,37	0,75 E-03	0,86	0,79	0,94
Age	0,06	0,06	1,03	0,30	1,06	0,95	1,20
E2max	0,29 E-03	0,17 E-03	1,74	0,08	1,00	1,00	1,00
PC1	-0,01	0,16	-0,04	0,97	0,99	0,72	1,36
ICSI=Yes	0,21	0,31	0,70	0,49	1,24	0,68	2,27

Supplementary Table 3d Clinical pregnancy

	Estimate	Std.err	Z	pvalue	OR	lowlim(OR)	uplim(OR)
(Intercept)	8,55	5,70	1,50	0,13			
BMI	-0,04	0,13	-0,29	0,77	0,96	0,75	1,24
Age	-0,22	0,17	-1,28	0,20	0,80	0,57	1,13
E2max	-0,71E-03	0,48 E-03	-1,49	0,14	1,00	1,00	1,00
PC1	-1,00	0,52	-1,92	0,06	0,37	0,13	1,02
ICSI=Y	0,89	0,95	0,94	0,35	2,44	0,38	15,71

Supplementary Table 3e Live birth

	Estimate	Std.err	Z	pvalue	OR	lowlim(OR)	uplim(OR)
(Intercept)	8,57	6,30	1,36	0,17			
BMI	-0,11	0,14	-0,79	0,43	0,89	0,67	1,18
Age	-0,18	0,19	-0,95	0,34	0,84	0,58	1,21
E2max	-0,97 E-03	0,57 E-03	-1,70	0,09	1,00	1,00	1,00
PC1	-0,77	0,53	-1,45	0,15	0,46	0,16	1,31
ICSI=Y	1,56	1,00	1,56	0,12	4,77	0,67	34,02

PERFLUOROALKYL ACID CONTAMINATION OF FOLLICULAR FLUID AND ITS CONSEQUENCE FOR *IN VITRO* OOCYTE DEVELOPMENTAL COMPETENCE

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SUMMARY

BACKGROUND: Perfluoroalkyl acids (PFAAs) are detected in several human tissues and body fluids. Although considered for a long time as being biologically inactive, PFAAs have been shown to induce negative effects in laboratory animals and in *in vitro* experiments.

The ovarian follicle constitutes a very fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. *In vitro* experiments suggest that PFAAs can influence this finely tuned balance, but very scarce *in vivo* data are available to confirm this assumption; in fact, the distribution pattern of PFAAs in the follicular micro-environment is still unknown.

OBJECTIVES: We investigated if the potential PFAA-presence in human follicular fluid could be a risk factor for the developmental competence of an *in vivo* exposed oocyte. Furthermore, we also compared the distribution characteristics of PFAAs within serum and follicular fluid.

METHODS: Follicular fluid (n = 38) and serum (n = 20) samples from women submitted to Assisted Reproductive Technology (ART) were analyzed by liquid chromatography - mass spectrometry (LC/MS) to examine the presence of different perfluoroalkyl acids (PFAAs). Statistical models were used to investigate PFAA-distribution in both body fluids, compare this behavior with the distribution of previously detected Persistent Organic Pollutants (POPs) in follicular fluid and to explore the relationship between patient characteristics, ART results and follicular fluid contamination status.

RESULTS: Perfluoro-octane sulfonate (PFOS) was the PFAA-compound found in the highest concentration in follicular fluid [7.5 (0.1 – 30.4) ng/mL] and serum samples [7.6 (2.8 – 12.5) ng/mL]. A new variable, Principal Component 1 (PC1), representing the overall PFAA-contamination status of the follicular fluid samples, showed to be associated with a higher fertilization rate ($P < 0.05$) and a higher proportion of high quality embryos relative to the amount of retrieved oocytes ($P < 0.05$), even after adjusting for age, [estradiol], BMI, male subfertility and the presence of other POPs as explanatory variables.

CONCLUSION: An overall higher PFAA-contamination in the follicular micro-environment was associated with a higher chance of an oocyte to develop into a high quality embryo. Also, PFAAs have different distribution patterns between serum and follicular fluid compared to previously reported POPs.

INTRODUCTION

Over the past century, the awareness of the harmful effects of environmental pollution on humans and wildlife has increased enormously, mainly due to the physiological abnormalities observed in species living in highly polluted areas (Bernanke and Kohler, 2009; Hamlin and Guillette, 2010). Thousands of new chemicals are being introduced onto the market, some of which have proven to induce negative effects on laboratory animals and in *in vitro* experiments (Zhao *et al.*, 2012).
From this group of recently discovered toxicants, perfluoroalkyl acids (PFAAs), considered to be biologically inactive for a long time, are detected in human at concentrations exceeding the levels found in many other species (Jensen and Leffers, 2008). PFAAs consist of a carbon backbone occupied by fluoride atoms, where the C-F bond is the strongest in organic chemistry, which make them exceedingly stable and almost non bio-degradable. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), used in fire fighting foams (Paul *et al.*, 2009) and as processing aid (Trudel *et al.*, 2008) respectively, and both end-metabolites of several PFAAs (Lau *et al.*, 2004), are most frequently detected, whereby PFOS concentrations are in general the highest (Lau *et al.*, 2007). Due to their combined unique hydrophilic and hydrophobic properties, PFAAs have broad applications in consumer and industrial fields (‘Baygard’, ‘Scotchgard’, ‘Gore-Tex’) (Jensen and Leffers, 2008). In humans, PFAAs are readily absorbed, poorly eliminated and distributed primarily in serum and liver (Lau *et al.*, 2004), with long half-lives of around 4 and 5 years for PFOA and PFOS, respectively (Olsen *et al.*, 2007). Humans are mainly exposed to PFAAs through drinking water, food, indoor air and dust particles contaminated with PFAAs, via the anti-adhesive surface layer on cookware and a whole range of water- and/or oil-resistant PFAA-coated consumer products (e.g. clothes, carpets) (Jensen and Leffers, 2008; D'Hollander *et al.*, 2010a; D'Hollander *et al.*, 2010b; Haug *et al.*, 2011).

Conflicting results are reported when investigating the relationship between the presence of PFAAs in human serum and subfecundity (Fei *et al.*, 2009; Whitworth *et al.*, 2012). In rat and mice, prenatal exposure to high PFOS concentrations (5-20 mg/kg) led to subsequent sudden neonatal mortality, postnatal growth and developmental retardation among offspring (Lau *et al.*, 2004). In addition, female mice neonates showed increased body weight and elevated serum insulin and leptin concentrations after *in utero* exposure to low (0.01 mg/kg) and medium (0.3 mg/kg) PFOA levels (Lau *et al.*, 2009). While the ability of PFAAs to interact with the nuclear Peroxisome Proliferator-Activated Receptor family (PPARs) has been put forward as an explanation for the observed metabolic

disturbances, mainly through PPARα-activation (Rees *et al.*, 2008; Wolf *et al.*, 2012), PPARs have also been suggested to be involved in gamete function and embryo development (Huang, 2008; Minge *et al.*, 2008). For example, PPARγ-expression increases in granulosa cells during folliculogenesis and drops following the LH-surge (Froment *et al.*, 2006), thereby possibly playing an important role in cell steroidogenesis (Huang, 2008). Within the ovarian follicular micro-environment, PFAA-binding to PPARs could thus possibly interfere with steroidogenesis (i.e. estradiol, testosterone and progesterone) and subsequently influence oocyte and embryo development. In adult male rats, PFAAs are shown to affect the endocrine system by decreasing testosterone and increasing estradiol levels (Jensen and Leffers, 2008). These estrogenic effects of PFAAs were also observed in PFAA-exposed MCF-7 cell cultures (Maras *et al.*, 2006).

Thus, it can be hypothesized that PFAAs are capable of interfering with the tightly regulated endocrine processes taking place in the ovarian follicle, where well-balanced interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. Subsequently, it is important to demonstrate the presence of PFAAs within the follicular fluid, as it may further substantiate their potential direct effects on oocyte, cumulus and granulosa cells. While different chemicals, such as polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE), have already been detected in human follicular fluid (Trapp *et al.*, 1984; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010; Petro *et al.*, 2012), to the best of our knowledge, no detailed information is currently available regarding the presence and distribution of PFAAs in human follicular fluid. Only one study mentions the presence of PFAAs in human follicular fluid, but no exact information regarding the identity and concentrations of the detected PFAAs is given (Governini *et al.*, 2011).

Therefore, the aim of the current study is to investigate whether the (possible) presence of PFAAs could be a risk factor for *in vivo* oocyte and embryo development by examining these items: 1) to detect and quantify individual PFAAs in human follicular fluid obtained through transvaginal follicular aspiration in the course of assisted reproductive therapies; 2) to detect and quantify the same set of PFAAs in human blood serum samples obtained at the moment of transvaginal oocyte retrieval; 3) to assess the distribution characteristics of the detected PFAAs in follicular fluid and serum; and 4) to link follicular fluid PFAA-concentrations to the final reproductive outcome in terms of fertilization rate and the amount of *in vitro* produced high quality embryos.

MATERIAL AND METHODS

Patient information, cycle characteristics and sample collection

Patients undergoing an assisted reproductive technology (ART) treatment were invited to participate after ethical committee approvals of the University of Antwerp and ZNA Middelheim Hospital were obtained. All patients signed informed consent papers. During two periods, human follicular fluid samples (n = 18, March 2008) and paired serum and follicular fluid samples (n = 20, March – May 2009) were collected in the fertility unit of ZNA Middelheim Hospital, Antwerp, Belgium.

Data were collected on female age, height, weight, occupation and residence of each woman and consequently Body Mass Index (BMI, kg/m²) was calculated. Furthermore, the following cycle parameters were collected: serum estradiol concentration [E2] at the day of human chorionic gonadotropin (hCG) administration (37h before ovum pick-up), number of retrieved oocytes, number of zygotes with 2 pronuclei (2PN-zygotes, 18h post insemination/injection), number and quality of the embryos (Table 1), fertilization procedure (*In vitro* Fertilization, IVF or Intracytoplasmic Sperm Injection, ICSI), subfertility cause and ART-outcome. Embryos were characterized as a high or top quality embryo in the absence of multinucleated blastomeres, 4-5 blastomeres on day 2, ≥7 blastomeres on day 3 and ≤20% anucleated fragments (Van Royen *et al.*, 1999). With these data, the fertilization rate (number of 2PN-zygotes / number of retrieved oocytes, 2PN/ova) and the proportion of top quality embryos relative to the amount of retrieved oocytes (number of high quality embryos / number of retrieved oocytes, HQemb/ova) as well as relative to the amount of 2PN-zygotes (number of high quality embryos / number of 2PN-zygotes, HQemb/2PN) could be calculated. An ART-procedure was defined successful if a gestational sac with positive heart beat was detected around 7 weeks amenorrhea. The birth of one or more healthy babies was considered as a successful pregnancy.

An overview of the sample collection is described in Petro et al. (2012). Follicular fluid and coagulated blood samples were centrifuged (1500*g*, 20min and 1400*g*, 30min respectively) and the supernatant was stored in polypropylene tubes at -20°C until analysis.

Chemical analysis of body fluids

Sample preparation and analysis

Serum and follicular fluid were extracted using a procedure based on Taniyasu et al. (2005) and Kärrman et al. (2007). Briefly, internal standards ($^{13}C_8$-PFOS, $^{18}O_2$-PFHxS, $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_8$-PFOA, $^{13}C_9$-PFNA, $^{13}C_6$-PFDA, $^{13}C_7$-PFUnDA) and 2 mL formic acid/water (1/1) were added to approximately 0.5 mL sample. The samples were extracted on Oasis WAX cartridges and analytes were eluted with ammonium hydroxide in acetonitrile. Analysis was performed using an ACQUITY UPLC coupled to a tandem quadrupole mass spectrometer (ACQUITY, TQD, Waters, USA) with an electrospray interface operating in negative ion mode (ESM-MS/MS). Separation was performed on an ACQUITY BEH C18 column (2.1 x 50 mm; 1.7µm, Waters, USA). A pre-column (ACQUITY BEH C18 column 2.1 x 30 mm; 1.7µm, Waters, USA) preceded by a guard column containing the same stationary phase and particle dimensions (Van Guard, Waters) was inserted between the solvent mixer and the injector to retain any PFAAs originating from the UPLC. Detailed information concerning sample preparation, analysis and instrumental parameters can be found in the Supplemental Material (SM, Table SM-1).

Quality assurance/control

Native standards of PFBS, PFHxS, PFOS, PFDS, PFBA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoA, PFTrA and PFTeA (acronym list: Table SM-2) were used to construct ten-level calibration curves (r^2>0.98) encompassing the entire linear range. Labeled $^{18}O_2$-PFHxS, $^{13}C_8$-PFOS, $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_8$-PFOA, $^{13}C_9$-PFNA, $^{13}C_6$-PFDA, $^{13}C_7$-PFUnDA were used as internal standards to spike the samples prior to extraction. Results are corrected for matrix effects and recovery of each analyte was based on the response (area) of the corresponding internal standards. Sulfonate concentrations are based on the ion, not the salt.

Quality control was performed by regular analysis of procedural blanks (1 per batch of 6 samples). Triple extractions and analysis of spiked samples were performed to evaluate repeatability (multiple analyses on same day) and reproducibility (multiple analyses on different days) (results: Table SM-3). Spiked and natural contaminated serum samples were extracted and analyzed following the procedure described above. Coefficients of variation were between 2-6%. Quality control was assessed through participation in interlaboratory comparison exercises for PFAAs in serum (AMAP, 2007; Fluoros, 2009) (results: Table SM-4).

The limits of quantification (LOQs) were calculated as ten times signal to noise for each compound and ranged between 0.1 – 1.35 ng/mL (except for PFUnDA with a LOQ of 19.20 ng/mL). Individual LOQs are given in the SM (Table SM-2).

Data treatment

Statistical analysis was performed using SPSS 20.0 for Windows (Chicago, IL, USA) and R version 2.13.0 (R Development Core Team, 2008). Values of $p<0.05$ were considered statistically significant. Samples with concentrations below the LOQ were assigned a value of f x LOQ with f being the detection frequency or the ratio between the number of samples detected above the method LOQ and the total number of analyzed samples (Voorspoels *et al.*, 2002). By doing so, data below LOQ can be used in the statistical data analysis. However, compounds with <50% of the detected measurements above their LOQs were excluded from statistical analysis.

Data are presented as median with min – max range. Normality was checked using the Shapiro-Wilk test. Because of the relative low number of samples and the detection of outliers, the Wilcoxon matched pairs signed rank test was used to examine the difference in compound concentrations between serum and follicular fluid. Correlations between follicular fluid and serum concentrations and patient characteristics were investigated using the Spearman rank test. Principal Components Analysis (PCA) was performed on the correlation matrix for follicular fluid samples. PCA is a data reduction technique, whereby the information contained in a large number of original variables, is re-ordered in a number of independent variables (Principal Components, PCs), that are linear combinations of the original variables. PC scores from the first PC were then entered as covariates into logistic regression models to test their association with ART-outcomes. PC score significance was tested by a likelihood ratio test, comparing a model with the PC scores, age, [E2], BMI and male factor subfertility to a null model only containing these latter 4 variables.

RESULTS

Patients (n = 38) were on average 34.6 ± 4.4 yrs old, had an average BMI of 23.2 ± 4.9 kg/m² (Table 1) and all lived in urbanized areas. Four PFAAs, i.e. PFOS, PFOA, PFNA and PFHxS, were detected in each serum sample. For follicular fluid samples, PFOS was detected in every sample, except one and PFOA, PFNA and PFHxS showed detection frequencies above 75% (Table 2). PFOS was the compound found

in the highest concentrations in follicular fluid [7.5 (0.1 - 30.4) ng/mL] and serum samples [7.6 (2.8 - 12.5) ng/mL] (Table 2). The remaining 10 PFAAs were below their detection limit in each serum and follicular fluid sample. PFOS and PFOA concentrations were significantly different in serum and follicular fluid, with slightly higher concentrations in serum, whereas PFNA and PFHxS concentrations showed no difference between the two matrices (Table 2).

Table 1 Characteristics and ART outcomes of the 38 participating patients

Characteristics	**Average**	**SD**	**Median**	**Min – Max**
Age (years)	34.6	4.4	34.1	25.2 – 42.8
Height (cm)	167	6	168	155 – 176
Body weight (kg)	64.9	15.9	61.0	45 – 117
BMI [a] (kg/m²)	23.2	4.9	22.1	16.9 – 38.2
[E2] [b] (pg/ml)	1885	1141	1740	253 - 4572
# retrieved oocytes	9	5	8	2 – 28
# 2PN-zygotes [c]	6	4	5	0 – 24
Fertilization rate [d]	0.72	0.26	0.76	0 – 1
# top quality embryos [e]	2	3	1	0 – 11
Proportion top quality embryos [f]	0.21	0.20	0.17	0 – 0.63
Outcomes	**%**			
Clinical pregnancy [g]	26			
Live birth [h]	21			

[a] BMI = Body Mass Index; [b] [E2] = serum estradiol concentration on the day of hCG administration (i.e. 37h before ovum pick-up); [c] # zygotes with 2 pro nuclei; [d] # zygotes with 2 pro nuclei / # retrieved oocytes; [e] Van Royen *et al.*, 1999; [f] # top quality embryos / # aspirated oocytes; [g] detection of gestational sac with positive heart beat (after 7 weeks amenorrhea); [h] birth of ≥1 healthy baby

Table 2 Median concentrations with range (min – max) [ng/mL] and detection frequencies (DF, %) of PFAAs in serum and follicular fluid samples from women undergoing ART.

		Serum (n = 20)		**Follicular fluid (n = 38)**
Compound	DF	ng/mL	DF	ng/mL
PFOA	100	2.1 (1.0 - 3.2)*	92	1.8 (0.3 - 3.3)**
PFOS	100	7.6 (2.8 - 12.5)*	97	7.5 (0.1 - 30.4)**
PFNA	100	0.5 (0.2 - 1.0)*	76	0.4 (0.2 - 2.1)*
PFHxS	100	0.3 (0.1 - 0.7)*	76	0.3 (0.1 - 1.4)*

Data marked with different superscripts (*) in a row differ significantly among groups ($p < 0.05$)

All PFAAs showed strong correlations with each other within the follicular fluid (Table SM-5). In contrast, only a few weak to moderate correlations were detected between PFAA concentrations in follicular fluid and serum (Table SM-5). Also, PFAAs were not correlated with patient characteristics, except PFNA which showed a moderate negative correlation with the patient's BMI for serum and follicular fluid concentrations (r = -0.532, r = -0.345 respectively). In addition, PFAAs showed no correlation with other POPs present in the follicular fluid, such as PCBs, *p,p'*-DDE and HCB (Petro *et al.*, 2012). This absence of correlation was visualized using PCA on the follicular fluid concentrations of PFAAs and the previously detected POPs together (Figure 1) (Petro *et al.*, 2012). When all PFAA concentrations and all previously detected POPs were used as input variables, we observed that the first PC score is a size component, documenting the overall sample contamination status. Furthermore, we could notice a contrast for PC2 between PFAAs (positive sign) and the other POPs (negative sign) (Table SM-6). The PC2 score will be high in case of high PFAA and low previously detected POP concentrations and the PC2 will be negative in case the previously detected compound concentrations are high and PFAA-levels low. This contrast can be observed in the PCA-biplot, showing the correlations between the original compounds (Figure 1). All PFAAs are closely associated, indicating high correlations between these compounds. Almost perpendicular to the PFAA-cluster, a second cluster was formed by the previously detected POPs in these follicular fluid samples. These POPs are thus also strongly correlated to each other, but relatively independent of the PFAA-compounds.

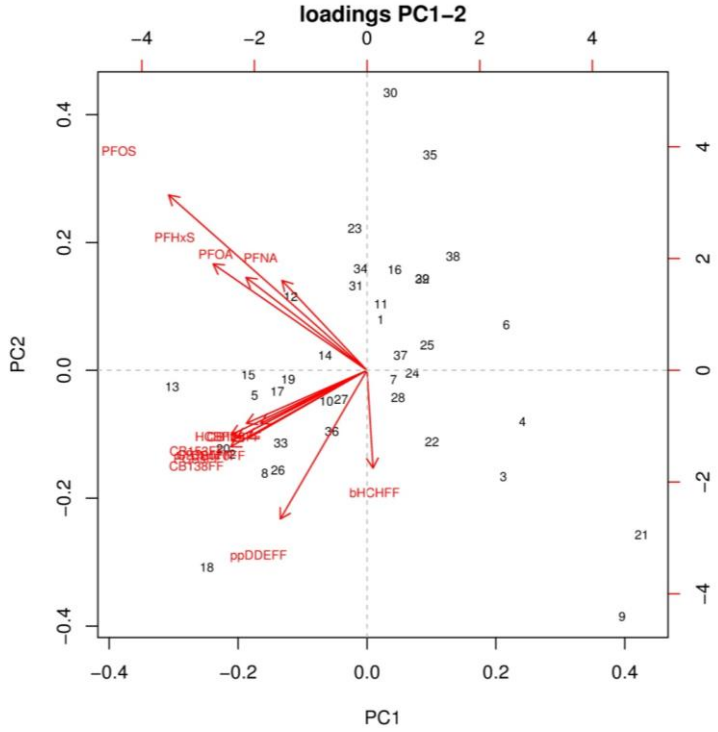

Figure 1 Principal Component Analysis biplot of the correlations between the original PFAAs and POPs in human follicular fluid samples (n = 38).

To generate separate summary statistics for PFAAs and previously detected POPs in follicular fluid, PCA was performed separately generating different PC scores for PFAAs and POPs. The concentrations of the previously detected POPs in follicular fluid were summarized by the first PC of these compounds ($PC1_{other}$), as outlined in (Petro *et al.*, 2012). The first PC of the PFAA variables ($PC1_{PFAA}$) encompasses 81% of all the information contained in the 4 original variables, meaning that this $PC1_{PFAA}$ score can be used as a valid summary statistic for the overall PFAA-contamination status of follicular fluid. $PC1_{PFAA}$ is a linear combination of the four original PFAA-variables, using the following formula: $PC1_{PFAA}$ = – 0.39 [PFOA] – 0.69 [PFOS] – 0.33 [PFNA] – 0.51 [PFHxS]. The minus signs indicate that lower $PC1_{PFAA}$ scores reflect higher PFAA-concentrations in the sample and vice versa.

To test the relationships between the presence of PFAAs in the follicular fluid and ART-outcomes (i.e. fertilization rate and the proportion top quality embryos relative to the number of retrieved oocytes or to the number of 2PN-zygotes), $PC1_{PFAA}$ was added as a covariate to logistic regression models, with the ART-results as outcome, and age, [E2], BMI, male factor infertility and $PC1_{other}$ as explanatory variables. Adding $PC1_{PFAA}$ significantly improved the fit of the fertilization rate model, and of the model predicting the ratio of top quality embryos relative to the number of retrieved oocytes. A higher fertilization rate and a higher number of high quality embryos relative to the number of retrieved oocytes were observed in samples with lower $PC1_{PFAA}$ scores, indicative of higher PFAA-concentrations. The raw data, whereby for each follicular fluid sample the individual PFAA-concentrations are plotted against the observed fertilization rate of that patient confirmed this statistical observation (Figure 2). The PFOS-outlier detected in these raw follicular fluid data plots did not influence the observed statistical outcomes, because the same associations were observed when the outlier was removed from the dataset (data not shown). No significant effect of the addition of $PC1_{PFAA}$ was observed on the model predicting the proportion top quality embryos relative to the amount of 2PN-zygotes ($p>0.05$).

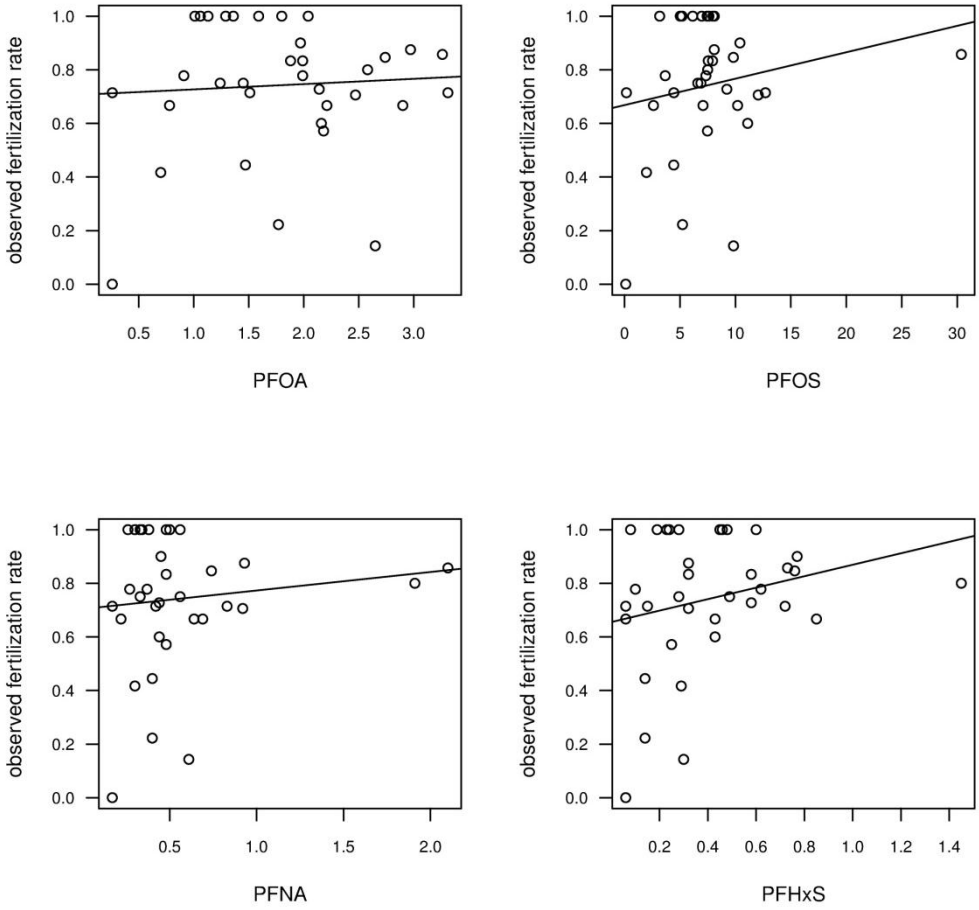

Figure 2 Raw data plot whereby the original PFAA-concentrations detected in a human follicular fluid sample are plotted against the observed fertilization rate of that patient.

DISCUSSION

This study was designed to investigate if the presence of PFAAs in the ovarian follicular micro-environment could be considered a risk factor for oocyte development. Firstly, we demonstrated that PFAAs are indeed present in the ovarian follicles of women undergoing ART. Moreover, we also report that PFAAs display a different distribution pattern in serum and follicular fluid compared to already detected POPs in follicular fluid. Finally, a positive association could be observed between PFAA-concentrations in follicular fluid on the one hand and ART-results such as a higher fertilization rate and a higher rate of top quality embryos relative to the amount of retrieved oocytes on the other hand.

PFAA-presence in human follicular fluid

To our knowledge, this is the first study giving a detailed overview on the follicular fluid contamination with PFAAs. Although PFAA-biomonitoring started around the turn of the century (Giesy and Kannan, 2001), it took more than a decade before the PFAA-presence in follicular fluid was documented. It is therefore currently impossible to discuss the PFAA-contamination pattern in follicular fluid over time and between studies. Recently, one study briefly reported the PFAA-presence in follicular fluid, however no detailed information was available regarding the identity and concentration of the detected PFAAs (Governini *et al.*, 2011). Besides the now confirmed presence in follicular fluid, PFAAs were already detected in other human body fluids, such as umbilical cord serum and serum (Lau *et al.*, 2007; Monroy *et al.*, 2008; Fei *et al.*, 2009; von Ehrenstein *et al.*, 2009; Roosens *et al.*, 2010; Stein *et al.*, 2012), amniotic fluid (Stein *et al.*, 2012), human milk (von Ehrenstein *et al.*, 2009; Roosens *et al.*, 2010; Croes *et al.*, 2012) and semen (Toft *et al.*, 2012; Joensen *et al.*, 2013). The PFAA-contamination pattern in follicular fluid shows to be very similar with these other biological fluids: PFOS is detected in the highest concentration, followed by PFOA, PFNA and PFHxS, whereby PFOS and PFOA are present in almost every follicular fluid sample. Next to these 4 PFAA-compounds, no other PFAA-compounds could be detected in the follicular fluid samples.

Further serum sample analysis from the same women showed that also in serum, only the above mentioned PFAA-compounds could be detected. PFOS-concentrations in our serum samples are lower than the levels observed at the beginning of the century (Kannan *et al.*, 2004; Karrman *et al.*, 2006; Fei *et al.*, 2009), and in the same range as more recent samples (Olsen *et al.*, 2012; Stein *et al.*, 2012). In Flanders, PFOS-concentrations decreased compared to samples collected between 2002 and 2005 (Roosens *et al.*, 2010). This confirms the decline of serum contamination with PFOS in Europe, which can be mainly attributed to the phase out of PFOS and related products in 2002 (EPA, 2000) and the almost complete ban of PFOS use in 2006 by the European Union (EC, 2006). In contrast to the PFOS ban, PFOA production has decreased but is not yet completely phased out worldwide (EPA, 2013). This can be an explanation why PFOA serum concentrations (including our samples) fluctuate already more than a decade around the same concentrations (Kannan *et al.*, 2004; Karrman *et al.*, 2006; Fei *et al.*, 2009; Olsen *et al.*, 2012; Stein *et al.*, 2012).

PFAA-distribution in human serum and follicular fluid

Following our observation that PFAAs are present in human follicular fluid, we verified the behavioral characteristics of these substances in follicular fluid and serum and compared them to POPs which were already detected several times in follicular fluid over the last 30 years (e.g. PCBs, *p,p'*-DDE, HCB) (Trapp *et al.*, 1984; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; Weiss *et al.*, 2006; Jirsova *et al.*, 2010; Meeker, 2010; Petro *et al.*, 2012). Overall, PFAAs distribute differently than POPs in follicular fluid and serum. This could already be assumed by the fact that no correlations were observed between PFAA and POP-concentrations in both matrices. The detected PFAAs are present in concentrations exceeding those of the already detected POPs in the same follicular fluid samples. It turns out that the PFOS-concentration (in ng/mL) is around hundred times higher than the CB 153 concentration (also in ng/mL). Also, PFHxS, the PFAA-compound present in the lowest concentration, was detected at more or less the same range as *p,p'*-DDE, being the major lipophilic POP in follicular fluid. Furthermore, opposite to the high correlations between POP-concentrations in follicular fluid and serum, almost no correlations were found for the PFAA-concentrations between both matrices. In addition, there was almost no difference in PFAA-contamination status between serum and follicular fluid, whereas for POPs, a clear concentration gradient could be observed, with lower concentrations found in follicular fluid (Petro *et al.*, 2012).

The most logical explanation for these divergent behavioral patterns lies in the fact that PFAAs and POPs possess completely different properties. While PCBs, *p,p'*-DDE and HCB are lipophilic, PFAAs are both lipophilic and hydrophilic (Jensen and Leffers, 2008). In contrast to POPs, PFAAs are not stored in adipose tissue, but undergo extensive enterohepatic circulation and are predominantly found in liver, serum and kidney (Lau *et al.*, 2007; Jensen and Leffers, 2008). Because PFAAs are able to bind to proteins (specifically B-lipoproteins), albumin and liver fatty acid-binding proteins, PFAAs are most probably being transported in serum bound to these carrier proteins (Jones *et al.*, 2003; Wu *et al.*, 2009; Luo *et al.*, 2012). Due to the ability of albumin to pass the blood follicle barrier (Hess *et al.*, 1998; Schweigert *et al.*, 2006), it can be assumed that PFAAs are easily being transported from the blood stream into the growing follicle. This can explain why, in contrast with the lipophilic POPs, no concentration gradient was observed between PFAAs in serum and follicular fluid. Moreover, follicular fluid has a higher protein content than lipids, which can also explain the higher PFAA-concentrations compared to POPs in follicular fluid.

PFAAs in follicular fluid and ART-outcome

The differences between PFAAs and POPs were even more profound when comparing their associations with the ART-outcomes. In this study, follicular fluid samples with higher PFAA-contamination were associated with a higher fertilization rate and a subsequent higher amount of top quality embryos relative to the amount of retrieved oocytes, while in our previous study, an overall higher contamination status with PCBs, *p,p'*-DDE and HCB in the same follicular fluid samples showed to have a significant negative effect on the fertilization rate and amount of top quality embryos (Petro *et al.*, 2012). Although our sample size is rather small and our results could not yet be confirmed by literature, this remarkable association cannot be neglected. The ability of PFAAs to bind to PPAR-receptors could be a plausible explanation (Bjork and Wallace, 2009; Ren *et al.*, 2009; Wolf *et al.*, 2012). PPARs are suggested to be crucial for a successful oocyte development (Froment *et al.*, 2006; Dupont *et al.*, 2008; Minge *et al.*, 2008; Rees *et al.*, 2008). PPARγ can for example potentially play a role during cell steroidogenesis in granulosa cells (Froment *et al.*, 2006), although it is also suggested that PPARγ can act as a negative regulator of folliculogenesis (Lovekamp-Swan and Chaffin, 2005; Rees *et al.*, 2008). Therefore, hypothetically, if PFAAs are able to influence PPARs in such an extremely subtle way, without disturbing the well-balanced follicular micro-environment, it is possible that PFAAs can 'enhance' this micro-environment in such a way which is beneficial for the oocyte to develop into a fully competent, mature and fertilizable oocyte. More in depth fundamental research is however obviously essential before definite causality conclusions can be drawn about this possible positive PFAA-effect on folliculogenesis and oocyte maturation.

In conclusion, our study provides the first, detailed overview of the contamination status of follicular fluid with PFAAs. It became clear that the different characteristics of PFAAs compared to POPs are being reflected by a different distribution pattern in follicular fluid and serum. Also opposite findings were found between the presence of PFAAs and POPs in relation to the ART-outcomes: higher PFAA-contaminated follicular fluid samples were associated with a better fertilization rate and consequently with a higher chance of an oocyte to develop into a high quality embryo. Further research is however strongly needed to expand our knowledge regarding the presence, distribution and action of PFAAs in follicular fluid.

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SUPPLEMENTAL MATERIAL

Sample preparation

Internal standards ($^{13}C_8$-PFOS, $^{13}O_2$-PFHxS, $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_8$-PFOA, $^{13}C_9$-PFNA, $^{13}C_6$-PFDA, $^{13}C_7$-PFUnDA) were added to or 0.5 mL serum in a polypropylene tube and mixed thoroughly. After adding 2 mL formic acid/water (1/1) to the samples, the solution was sonicated and centrifuged. The samples were extracted on Oasis WAX cartridges (60 mg, 3 mL) which were conditioned with acetonitrile (2 mL) and water (2 mL). After loading, columns were washed with 40% acetonitrile in water. Elution was performed with 1 mL 2% NH_4OH in acetonitrile. Before injection, a volume of 70 µL eluate was mixed with 130 µL water and transferred into polypropylene vials with septa-less polyethylene screw caps (pre-slit; Waters). Concentrations are expressed in ng/mL.

Instrumental Analysis

Analysis was performed using an ACQUITY UPLC coupled to a tandem quadrupole mass spectrometer (ACQUITY, TQD Waters, USA) with an electrospray interface operating in negative ion mode (ES-MS/MS). Separation was performed on an ACQUITY BEH C18 column (2.1 x 50 mm; 1.7µm, Waters, USA) kept at 60°C. A pre- column (ACQUITY BEH C18 column 2.1 x 30 mm; 1.7µm, Waters, USA) was inserted between the solvent mixer and the injector to retain any PFAAs originating from the UPLC system. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The starting condition was 65% A and 35% B and reduced to 0% A at 3.4 min before returning to the original condition at 4.7 min. The total run was 6.7 min. The injection volume was 10 µL and the flow rate was 450 µL/min. Acquisition was performed in the multiple reaction monitoring (MRM). Transitions (precursor ion (m/z) → product ion (m/z)) used for determination are given in Table SM-1.

Table SM-1 List of analytes and internal standards with mass transitions used for detection.

Compound	Isotopically labeled standards*	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
PFBA	$^{13}C_4$-PFBA	213	169	19	19
PFHxA	$^{13}C_2$-PFHxA	313	296	19	21
PFHpA	$^{13}C_2$-PFHxA	363	319	24	40
PFOA	$^{13}C_8$-PFOA	413	369	22	13
PFNA	$^{13}C_9$-PFNA	463	419	28	17
PFDA	$^{13}C_6$-PFDA	513	469	25	29
PFUnDA	$^{13}C_7$-PFUnDA	563	519/269	16	47
PFDoA	$^{13}C_7$-PFDoA	613	569/319	22	21
PFTrA	$^{13}C_7$-PFDoA	663	619	26	21
PFTeA	$^{13}C_7$-PFDoA	713	669/369	28	21
PFBS	$^{13}O_2$-PFHxS	299	99	46	45
PFHxS	$^{13}O_2$-PFHxS	399	99	22	30
PFOS	$^{13}C_8$-PFOS	499	80/99	60	58
PFDS	$^{13}C_8$-PFOS	599	80	29	63
$^{13}C_4$-PFBA	/	217	172	19	19
$^{13}C_2$-PFHxA	/	315	270	19	21
$^{13}C_8$-PFOA	/	421	376	22	13
$^{13}C_9$-PFNA	/	472	427	28	17
$^{13}C_6$-PFDA	/	519	474	25	29
$^{13}C_7$-PFUnDA	/	570	525	16	47
$^{13}C_7$-PFDoA	/	615	169/570	22	21
$^{13}O_2$-PFHxS	/	403	103	22	30
$^{13}C_8$-PFOS	/	507	80	60	58

* isotopically labeled internal standards used for quantification of the recoveries

Table SM-2 Limits of Quantification (LOQ, ng/ml) for the PFASs analyzed in this study.

Compound	Abbreviation	LOQ (ng/ml)
Perfluorobutanoic acid	PFBA	1.35
Perfluorohexanoic acid	PFHxA	0.73
Perfluoroheptanoic acid	PFHpA	1.39
Perfluorooctanoic acid	PFOA	0.28
Perfluorononanoic acid	PFNA	0.22
Perfluorodecanoic acid	PFDA	1.26
Perlfuoroundecaonoic acid	PFUnDA	19.2
Perlfuorododecanoic acid	PFDoA	0.57
Perfluorotridecanoic acid	PFTrA	0.53
Perfluorotetradecanoic acid	PFTeA	0.91
Perfluorobutane sulfonic acid	PFBS	0.78
Perfluorohexane sulfonic acid	PFHxS	0.08
Perfluorooctane sulfonic acid	PFOS	0.10
Perfluordecanoic sulfonic acid	PFDS	0.57

Table SM-3 Results of repeatability (multiple analyses on same day) and reproducibility (multiple analyses on different days) of serum samples spiked with PFOS and PFOA (ng/ml)

Compound	Sample 1	Sample 2	Sample 3	Mean	SD	CV
Repeatability						
PFOS (35.0)	35.23	36.17	33.41	34.9	1.4	4.0
PFOA (3.5)	3.64	3.48	3.45	3.52	0.1	3.0
Reproducibility						
PFOS (50.0)	50.03	52.84	56.46	53.1	3.2	6.1
PFOA (3.5)	3.64	3.69	3.79	3.71	0.1	2.2

SD: standard deviation, CV: coefficient of variation

Table SM-4 Z-scores of the University of Antwerp (UA) for samples of 2 interlaboratory studies (IL)

IL study	PFOS (ng/ml			PFOA (ng/ml)		
	CV	UA	Z-score	CV	UA	Z-score
Fluoros A	4.9	4.4	-0.72	0.6	0.0	-1.9
Fluoros B	22.9	26.5	0.8	6.0	8.4	-0.8
AMAP 1	10.21	9.1	-0.4	/	/	/
AMAP 2	67.2	67.7	0.06	/	/	/
AMAP 3	27.9	25.3	-0.42	/	/	/

CV: consus value

Table SM-5 Significant Spearman correlation coefficients between compound concentrations detected in serum (S), follicular fluid (FF) and patient characteristics ($p < 0.05$)

	PFOA FF	PFOS FF	PFNA FF	PFHxS FF	PFOA S	PFOS S	PFNA S	PFHxS S	Age	BMI	E2 max
PFOA FF	1	0.783	0.853	0.638	0.529	/	/	/	/	/	/
PFOS FF	0.783	1	0.763	0.653	/	0.513	/	/	/	/	/
PFNA FF	0.853	0.763	1	0.594	0.508	/	0.649	/	/	-0.345	/
PFHxS FF	0.638	0.653	0.594	1	/	/	/	/	/	/	/
PFOA S	0.529	/	0.508	/	1	/	0.704	/	/	/	/
PFOS S	/	0.513	/	/	/	1	/	/	/	/	/
PFNA S	/	/	0.649	/	0.704	/	1	/	/	-0.532	/
PFHxS S	/	/	/	/	/	/	/	1	/	/	/
Age	/	/	/	/	/	/	/	/	1	0.548	-0.377
BMI	/	/	-0.345	/	/	/	-0.532	/	0.548	1	/
E2 max	/	/	/	/	/	/	/	/	-0.377	/	1

Table SM-6 Principal Components (PCs) after Principal Component Analysis using the concentrations of PFAAs and previously detected POPs in human follicular fluid as input variables

	PC 1	**PC 2**
CB 118	-0,23	-0,15
CB 153	-0,30	-0,19
CB 138	-0,30	-0,22
CB 180	-0,29	-0,20
CB 170	-0,26	-0,20
PCBs	-0,30	-0,20
HCB	-0,27	-0,15
β-HCH	0,01	-0,28
***p,p'*-DDE**	-0,19	-0,43
PFOA	-0,27	0,27
PFOS	-0,44	0,51
PFNA	-0,19	0,26
PFHxS	-0,34	0,31

THE INFLUENCE OF ENVIRONMENTALLY-RELEVANT PCB-, *P,P'*-DDE AND PFOS-CONCENTRATIONS ON GRANULOSA CELL VIABILITY AND FUNCTION USING A SERUM-FREE BOVINE GRANULOSA CELL CULTURE MODEL

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***Toxicology In vitro**, submitted.*

SUMMARY

BACKGROUND: Within the female reproductive system, the ovarian follicle is, because of its strict endocrine control, one of the main targets on which endocrine disrupting chemicals (EDCs) can potentially exert their actions. In particular, the somatic theca and granulosa cells can be considered as possibly sensitive for EDC-interference since they are responsible for the steroidogenesis in the follicle. The already documented presence of several EDCs in human follicular fluid, such as polychlorinated biphenyls (PCBs), dichloordiphenylethylene (*p,p'*-DDE) and perfluoroalkylated substances (PFASs), only underpins this assumption. To verify this hypothesis, we investigated if environmentally-relevant concentrations of a PCB-mixture (A-1254), p'p-DDE and PFOS could be cytotoxic and/or interfere with the normal steroidogenesis in the ovarian follicle, using a serum-free bovine granulosa cell culture.

METHODS: Primary bovine granulosa cells were purified from slaughterhouse ovaries and cultured for 48 h in serum-free conditions and exposed to low, environmentally-relevant and high concentrations of A-1254, *p,p'*-DDE and PFOS. After 48 h of exposure, cytotoxicity was tested using colorimetric cell count analysis while both culture and exposure media were analyzed on their content of estradiol (E2), progesterone (P4), testosterone and androstenedione using a chemiluminescent microparticle immunoassay.

RESULTS: Environmentally-relevant A-1254, *p,p'*-DDE and PFOS-concentrations (1 ng/ml) were not cytotoxic for granulosa cells after 48 h of exposure ($p > 0.05$), but they showed to have a significantly stimulating effect on E2-production ($p < 0.05$). High concentrations (10 µg/ml) of *p,p'*-DDE and PFOS decreased and increased P4-production respectively ($p < 0.05$).

CONCLUSION: Environmentally-relevant concentrations of A-1254, *p,p'*-DDE and PFOS are not to be considered cytotoxic for granulosa cells, but each substance at environmentally-relevant concentrations is capable of interfering with the normal steroidogenic processes in granulosa cells.

INTRODUCTION

Environmental pollution has been put forward as one of the causes of the increase in reproductive disorders observed nowadays. Human and wildlife observational studies, as well as *in vivo* and *in vitro* experiments, show that environmental toxicants are able to interfere with different processes of both male and female reproductive system. These toxicants can act genotoxic, mutagenic and/or have the ability to interact with the (neuro)endocrine system, whereby these substances are better known as 'Endocrine Disrupting Chemicals' (EDCs).

Within the female reproductive system, the ovarian follicle can be considered as a very fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte (Petro *et al.*, 2012b). Within the growing follicle, somatic theca and granulosa cells are considered indispensable key players for normal folliculogenesis, oocyte growth and development and the communication of the oocyte and its surroundings (Tanghe *et al.*, 2002; Scaramuzzi *et al.*, 2011). Not only are they responsible for the delivery of nutrients to the oocyte, they are also mainly accountable for the crucial steroidogenesis in the ovarian follicle [Chapter 1B, Figure 2 (Jones, 2005)]. In short, theca cells, establishing the outside cell layer of the ovarian follicle, easily take up cholesterol, the substrate for steroidogenesis, which is converted by multiple enzymes into androgens (i.e. testosterone) in a Luteinizing Hormone (LH) dependent way (Young and McNeilly, 2010). Then, testosterone diffuses through the basal lamina and enters the granulosa cells. Granulosa cells possess the enzyme aromatase which, under influence of Follicle Stimulating Hormone (FSH), converts testosterone into estradiol (E2), the hormone ultimately responsible for the appearance of the LH-surge, which is in turn a requirement for successful ovulation (Edson *et al.*, 2009). It is obvious that disruption of this finely tuned (endocrine/paracrine) balance can lead to anovulation (Mumford *et al.*, 2011), cystic deformation (Baptiste *et al.*, 2010) or a diminished oocyte quality which jeopardizes further embryo development (Leroy *et al.*, 2005). After ovulation, granulosa cells transform into luteinizing cells and produce progesterone (P4), which is responsible for decidualization of the endometrium, essential for successful implantation and maintaining pregnancy (Large and DeMayo, 2012).

Keeping this in mind, it is evident that concerns arise when several studies show that well-known EDCs, such as PCBs and *p,p'*-DDE were found in the follicular fluid of women undergoing Assisted Reproductive Technology (ART) procedures (Trapp *et al.*, 1984; Baukloh *et al.*, 1985; Schlebusch *et al.*, 1989; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; De Felip *et al.*, 2004; Weiss *et*

al., 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010; Petro *et al.*, 2012a). Also newly emerging EDCs, such as PerFluorinated Alkylated Substances (PFASs) with perfluoro-octane sulfonate (PFOS) in the highest concentrations, have recently been found in human follicular fluid (Petro *et al.*, unpublished). This presence of EDCs in human follicular fluid implicates that, during *in vivo* folliculogenesis, granulosa cells are in direct contact with different EDCs and so it is reasonable to assume that EDCs could interfere with granulosa cell function, thereby compromising the oocyte's survival and normal follicle growth. There are two main action mechanisms how EDCs can potentially exert their actions on granulosa cells within the growing ovarian follicle. First, in case environmental relevant EDC-concentrations are found to be cytotoxic, the total amount of viable (and functional) granulosa cells producing E2 in the growing follicle would decrease. In theory, it would thus be possible that the total E2-concentration in the follicular fluid would be too low to generate the crucial LH-surge, with anovulation as final outcome. Secondly, if environmental relevant EDC-concentrations are not directly cytotoxic, they can still jeopardize the critical hormonal balance within a follicle by interfering with the granulosa cell function, thereby again resulting in anovulation or in ovulated oocytes with diminished quality. While several *in vitro* studies already confirmed this assumption for PCBs and *p,p'*-DDE (for review: see Petro *et al.*, 2012), to our knowledge, there are currently no data available regarding the potential influence of PFASs on granulosa cells in the growing follicle. In addition, next to these *in vitro* experiments, our recent observational studies indicate that higher concentrations of EDCs in follicular fluid of women undergoing ART are associated with altered fertilization rates and changes in the proportion of high quality embryos during ART-procedures (Petro *et al.*, 2012a; Petro *et al.*, unpublished).

The majority of the studies performed to understand the mechanisms through which EDCs can influence granulosa cell function uses primary human luteinized granulosa cells obtained during ART-procedures (Moran *et al.*, 2000; Younglai *et al.*, 2004; Wu *et al.*, 2006) or primary serum-supplemented granulosa cell cultures of different origin (e.g. rat, porcine, bovine, …) (Lovekamp-Swan and Chaffin, 2005; Grasselli *et al.*, 2010; Mlynarczuk *et al.*, 2013). However, because serum cultured granulosa cells only have limited physiological resemblance to granulosa cells of an *in vivo* follicle reaching dominance (Gutierrez *et al.*, 1997b), a serum-free primary granulosa cell culture would be more suitable to mimic the follicular micro-environment more closely. In addition, the bovine ovarian follicle, including its individual components, has already been put forward as a promising *in vitro* reproductive toxicology model (Petro *et al.*, 2012b), due to the fact that it might not be historically contaminated (Petro *et al.*, 2010) and the striking similarities in early reproductive

physiology between human and the bovine species (Neuber and Powers, 2000; Campbell *et al.*, 2003). Therefore, a serum-free primary bovine granulosa cell culture can be considered as an exquisite model to study the effects of EDCs on granulosa cell function. Furthermore, most of these studies also use EDC-exposure concentrations with no environmental relevance.

Consequently, the aims of the present study can be defined as follows: 1) to establish a bovine serum-free granulosa cell culture *in vitro*, 2) to assess the cytotoxicity of environmentally high, relevant (i.e. female follicular fluid concentrations) and low PCB, *p,p'*-DDE and PFOS-concentrations on these granulosa cell cultures after 48 h of exposure and, 3) to verify if the direct contact with PCBs, *p,p'*-DDE and PFOS modifies the hormonal balance within the granulosa cell cultures by measuring hormone concentrations (i.e. androstenedione, testosterone, E2 and P4) in culture and exposure medium.

MATERIALS AND METHODS

Granulosa cell collection and purification

Bovine ovaries were collected immediately after slaughter at nearby abattoirs, and transported to the laboratory where they were washed 3 times in warm saline solution supplemented with 0.5 % kanamycin. Subsequently, small to medium sized follicles (< 8 mm in diameter) were punctured and follicular fluid was repeatedly aspirated to disrupt the granulosa cell layer with a 21G needle connected to a 10 ml syringe. The collected follicular fluid was transferred into a 15 ml tube containing 1 ml culture medium (McCoys 5a with 20 mM Hepes, 100 U/ml + 100 µg/ml penstrep, 4 µg/ml glutamine, 2.5 µg/ml apotransferrin, 4 ng/ml So-selenite, 1 ngl/ml FSH, 1 mg/ml BSA, 1 ng/ml LR-IGF1, 10 ng/ml insulin and 1 µg/ml androstenedione) and 50 µl heparin to prevent clotting. The follicular fluid was then centrifuged (800 g, 10 min), after which the supernatant was discarded except ±1 ml in which the cell pellet was resuspended. Subsequently, the suspension was loaded on a 45 % Percoll solution and centrifuged at 720 g for 30 min to separate the red blood cells from the granulosa cells. The pellet on top of the Percoll gradient was aspirated, washed and after centrifugation (800 g, 10 min) resuspended in culture medium. Cell count and viability were assessed using trypan blue, whereby 7 µl of the granulosa cell suspension was mixed for 2 min with 7 µl of trypan blue and subsequently the cell density and percentage of viable granulosa cells were determined in a Bürker counting chamber. A product origin overview can be found in the Supplementary Material (Table SM-1).

Granulosa cell culture

The granulosa cell culture system was adapted from previously described serum-free granulosa cell cultures (Gutierrez *et al.*, 1997a; Vanholder *et al.*, 2005). Briefly, granulosa cells (±70 x 10^3/well) were cultured in a total volume of 250 µl/well culture medium, vehicle control culture medium (containing 0.1 % EtOH) or exposure medium in 96-well tissue culture plates (Figure 1). Exposure media were prepared by further dilution of stock concentrations of Aroclor 1254 (A-1254, PCB-mixture, in 100 % EtOH), *p,p'*-DDE (in 100 % EtOH) and PFOS (in McCoys 5a) in culture medium to obtain final exposure concentrations, containing max 0.1 % EtOH (Table SM-2). Per replicate (3 in total), 6 wells were used per treatment (Figure 1). For each culture plate, the outer wells as well as the space between the wells were filled with sterile water to prevent evaporation of the culture and exposure media. The cells were incubated for 48 h in a humidified incubator at 38.5 °C (95 % CO_2, 5 % O_2). Furthermore, to perform cell count analysis after 48 h of exposure, granulosa cells were also seeded in 24-well tissue culture plates in 500 µl culture medium at the same cell/surface ratio as the 96-well plates (±420 x 10^3 cells/well) and cultivated under the same conditions as the exposed granulosa cells (controls).

A-1254 (green) + *p,p'*-DDE (red) + PFOS (blue) exposure

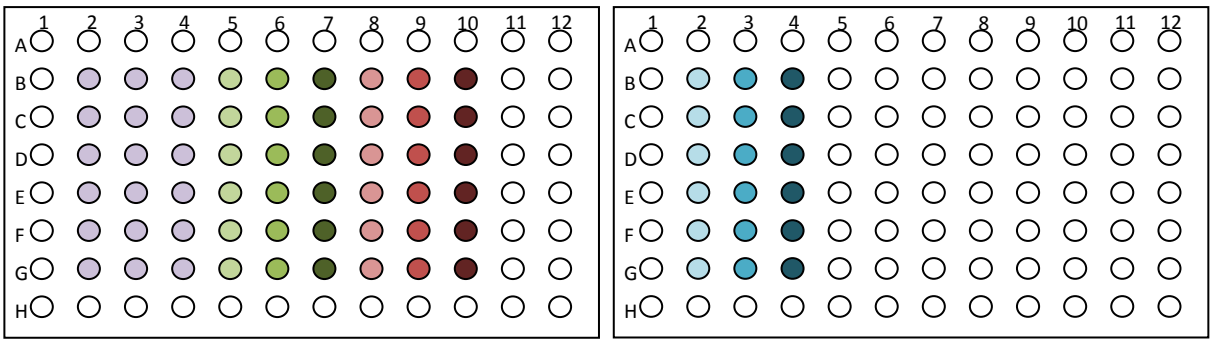

Figure 1 Example of the experimental exposure set-up in 96-well plates.
Purple wells are unexposed wells containing only culture medium (column, C, 2), control granulosa cells (C3) or vehicle (0.1 % EtOH) control granulosa cells (C4). For the exposed granulosa cells, exposure concentration increases with increasing color intensity (see Table SM-2 for exact exposure concentrations).
After 48 h, 125 µl medium/well was pooled per 3 wells per treatment (i.e. B3 to D3 and E3 to G3 for control granulosa cells).
Space between the wells as well as the wells surrounding the 'experimental' wells were filled with sterile water to prevent media evaporation. The other wells were left empty.

Medium and cell count analysis

After 48 h of exposure, the granulosa cell cultures were checked morphologically upon their resemblance to the typical tight clumps of spherical granulosa cells, which is the characteristic cellular morphology of a serum-free granulosa cell culture as described by Gutierrez *et al.* (1997a, b). Subsequently, culture and exposure media wells (125 µl/well) were pooled (3 wells per treatment, Figure 1), centrifuged for 5 min (200 g) and the supernatant transferred to a cryotube, and stored at -80 °C until medium analysis. Thus, per experiment 2 cryotubes (2 x 3 pooled wells) were available for medium analysis per treatment. Medium was analysed with a Chemiluminescent Microparticle Immunoassay (CMIA, Architect, Abott). To make sure that the detected hormone concentrations could be calculated using the standard calibration curves of the system, medium was first diluted when appropriate.

Cell count analysis was performed after 48 h of exposure using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay System, a colorimetric method to determine the number of viable cells. Living cells are able to reduce the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), into the colored, soluble formazan product. The quantity of formazan is thus directly proportional to the number of living cells in culture. After removal of the 125 µl medium/well for medium analysis, 25 µl of the MTS-solution was added to each well and incubated for 4 h at 38.5 °C, 95 % CO_2, 5 % O_2. After incubation, the 96-well plates were read (3 times and average absorbance calculated) using a multiwell plate reader (Tecan, Männedorf, Switzerland) at 492 nm.

To calculate the amount of cells per well, a standard curve was determined using the 24-well cultured granulosa cells. After removal of the culture medium, cells were washed with PBS without Ca^{2+} and Mg^{2+} and trypsin (200 µl) was added and directly removed. After 10 min, 500 µl of culture medium was added, aspirated and transferred into 15 ml tubes. The tubes were centrifuged (125 g, 5 min), redundant culture medium discarded, the cell pellet re-suspended in culture medium and cell concentration was determined using trypan blue. Next, cells were seeded in 96-well plates in a total volume of 125 µl culture medium at different plating densities to obtain a known cell range (0, 10 x 10^3, 30 x 10^3, …, 150 x 10^3 cells/well) and 25 µl MTS-solution was added to each well. As with the exposed cells, the MTS-solution was incubated at 38.5 °C in 95 % CO_2, 5 % O_2 and the absorbance was read at 492 nm (3 times and average absorbance calculated). A standard curve could be created linking the known cell numbers/well with the observed absorbances, whereby a linear relationship between the number of cells/well and the absorbance values was observed ($r > 0.95$). Finally, the

number of granulosa cells in the exposed wells could be estimated using their absorbance values in the resulting regression equation from the standard curve.

Statistics

To test whether the response of the granulosa cells on an increasing concentration of toxic compound is the same across all compounds, a mixed model was fitted with the cell count as outcome, and concentration and compound as predictor variables. To adjust for the batch effect (i.e. non-independence between observations within the same batch), a random intercept term for batch was added to the model. The blank observation was entered as concentration zero. Hence, the analysis corresponds to a two-way ANOVA with correction for the batch effect. To test whether the change in cell count with concentration differed between the toxic compounds, an interaction term compound*concentration was added to the model. As this term was significant, we subsequently fitted 3 separate models for A-1254, *p,p'*-DDE and PFOS, respectively, testing the influence of concentration on cell count, adjusting for the batch effect.

The influence of the toxic compounds on the measured hormone concentrations E2, P4, testosterone and androstenedione was studied using linear mixed model analysis for each of the 4 products separately. The hormone concentration was entered as outcome variable. The number of cells per well was accounted for by including this variable as a fixed effect in the regression model. Other predictor variables included the concentration of the toxic compound, the toxic compound itself (A-1254, *p,p'*-DDE or PFOS) as well as the interaction between them. Concentration was entered as a categorical variable. A random intercept term for batch was entered to account for the relatedness between observations within the same batch. If the interaction term was significant, we subsequently fitted separate models for each of the compounds, testing the influence of compound concentration of the measured hormone concentration.

For all linear mixed regression models, an overall significance of the concentration effect was calculated using a likelihood ratio test, comparing the fitted model to a null model only including the batch effect. A posthoc-test was performed on all pairwise comparisons using a Tukey correction for multiple testing. Mixed models were fitted using the lme function in the nlme package in R (R Development Core Team, 2008). Posthoc tests were performed using the multcomp package in R.

RESULTS

In general, granulosa cell viability and the observed hormonal changes in the granulosa cell cultures as a response to increasing concentrations of A-1254, *p,p'*-DDE and PFOS were not uniform over the three toxic compounds and therefore, for both endpoints, results are described per compound.

Cell count

Exposure to A-1254 resulted in a significant higher number of viable granulosa cells if exposed to 1 pg/ml and 1 ng/ml A-1254 concentrations compared to no exposure ($p = 0.01$); 1 µg/ml A-1254 did not have a significant effect on the number of living granulosa cells (Figure 2a). The number of granulosa cells exposed to 10 µg/ml *p,p'*-DDE for 48 h showed to be significantly lower than cells exposed to 1 ng/ml and 10 pg/ml *p,p'*-DDE ($p = 0.002$). Remarkably, there was no significant difference in cell number between unexposed granulosa cells and cells exposed to 10 µg/ml *p,p'*-DDE (Figure 2b). Finally, a higher cell count was found for granulosa cells exposed to 10 µg/ml PFOS than compared to granulosa cells exposed to the lower PFOS-concentrations and unexposed granulosa cells ($p = 2.0 \times 10^{-4}$) (Figure 2c).

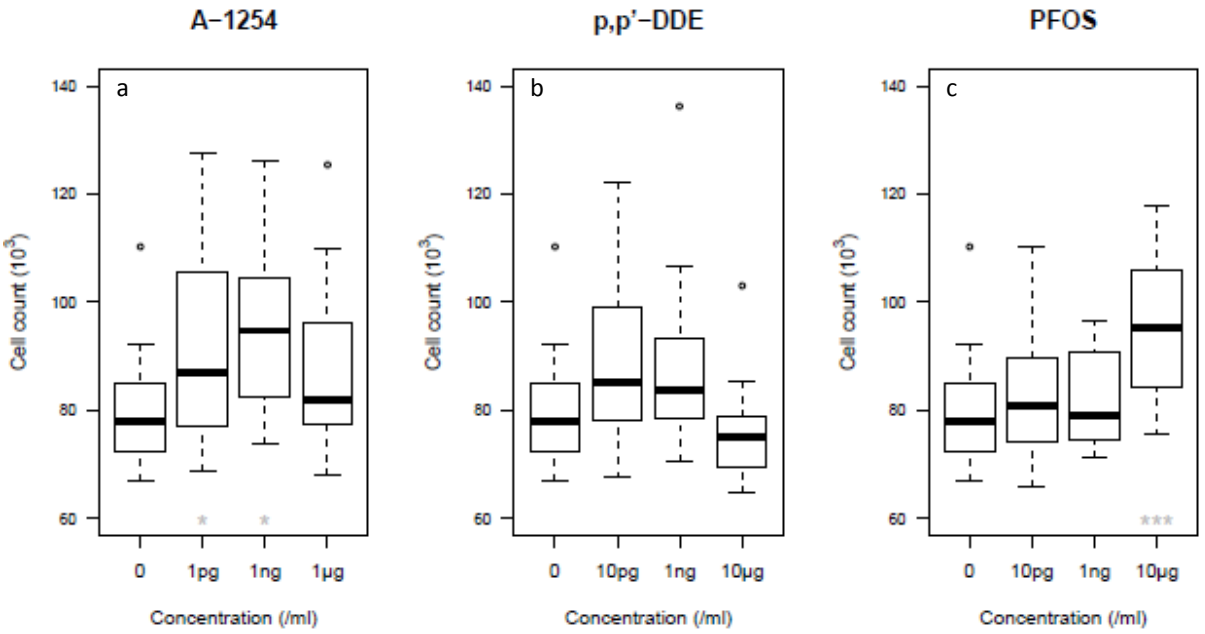

Figure 2 Granulosa cell count analysis after 48 h of exposure to different concentrations of A-1254, *p,p'*-DDE and PFOS

Medium analysis

After 48 h of A-1254 exposure, E2-concentrations were significantly higher in culture medium of granulosa cell cultures exposed to 1 ng/ml and 1 µg/ml A-1254 (p = 0.013) (Figure 4a), whereas P4-concentration was only increased when granulosa cells were exposed to 1 µg/ml A-1254 (p = 0.03) (Figure 4b). For *p,p'*-DDE, exposure to 10 pg/ml and 1 ng/ml *p,p'*-DDE resulted in significantly higher measured E2-concentrations in culture medium compared to both unexposed granulosa cells and cells exposed to 10 µg/ml *p,p'*-DDE (p = 0.003) (Figure 4c). Exposure to 10 µg/ml *p,p'*-DDE resulted in significantly lower P4-concentrations compared to the other exposure concentrations and unexposed cells (Figure 4d). Exposure to 10 pg/ml and 1 ng/ml *p,p'*-DDE resulted in increased P4-production compared to unexposed cells (p = 9.1 x 10-7) (Figure 4d). For PFOS, an increase of E2-production after 48 h exposure to 1 ng/ml and 10 µg/ml PFOS was noticed (p = 0.04) (Figure 4e). Higher P4-concentrations were observed after exposure to 10 µg/ml PFOS (p = 0.01) (Figure 4f). None of the 3 compounds had a significant effect on the testosterone concentration in the granulosa cell cultures. For androstenedione, a difference in reaction could be observed between unexposed and A-1254 exposed cells on the one hand and *p,p'*-DDE and PFOS-exposed cells on the other hand, regardless of the exposure concentration (p = 0.005). On average, cells exposed to *p,p'*-DDE and PFOS show average androstenedione concentrations that are respectively 359 and 346 ng/ml higher compared to non-exposed cells. The cells exposed to A-1254 do not show higher androstenedione concentrations compared to nonexposed cells.

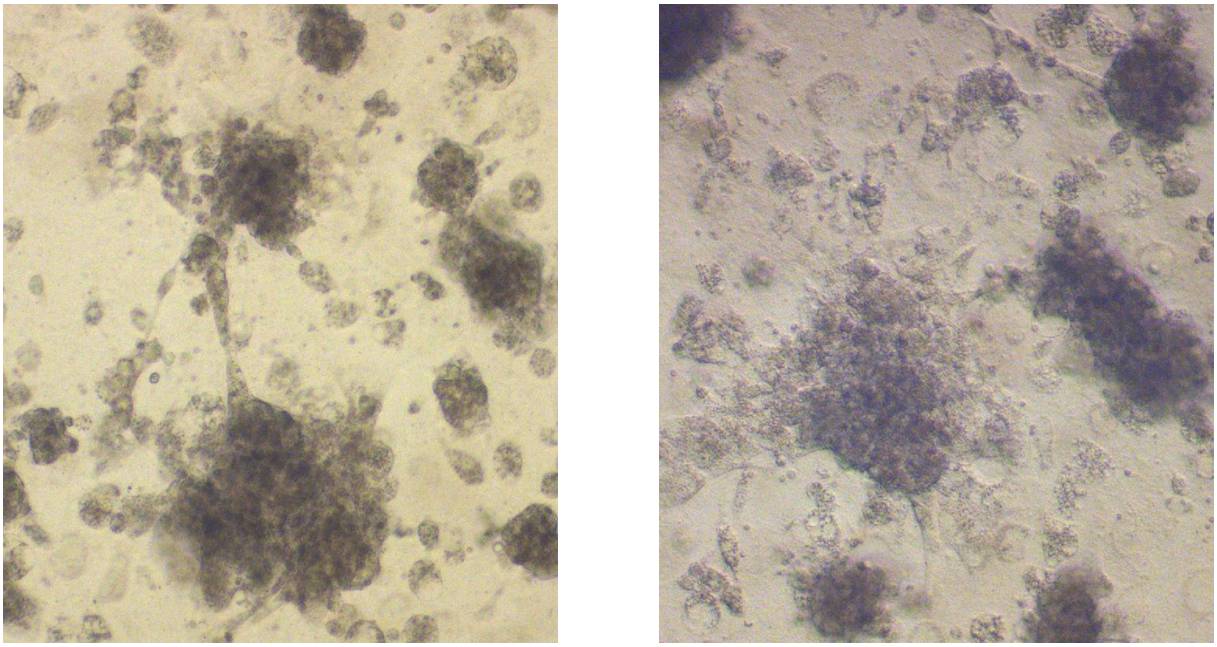

Figure 3 Two examples of typical granulosa cell cultures after 48 h, cultured in serum-free conditions: tight clumps of spherical granulosa cells attached to the culture plate by enlarged, flattened fibroblast-like cells.

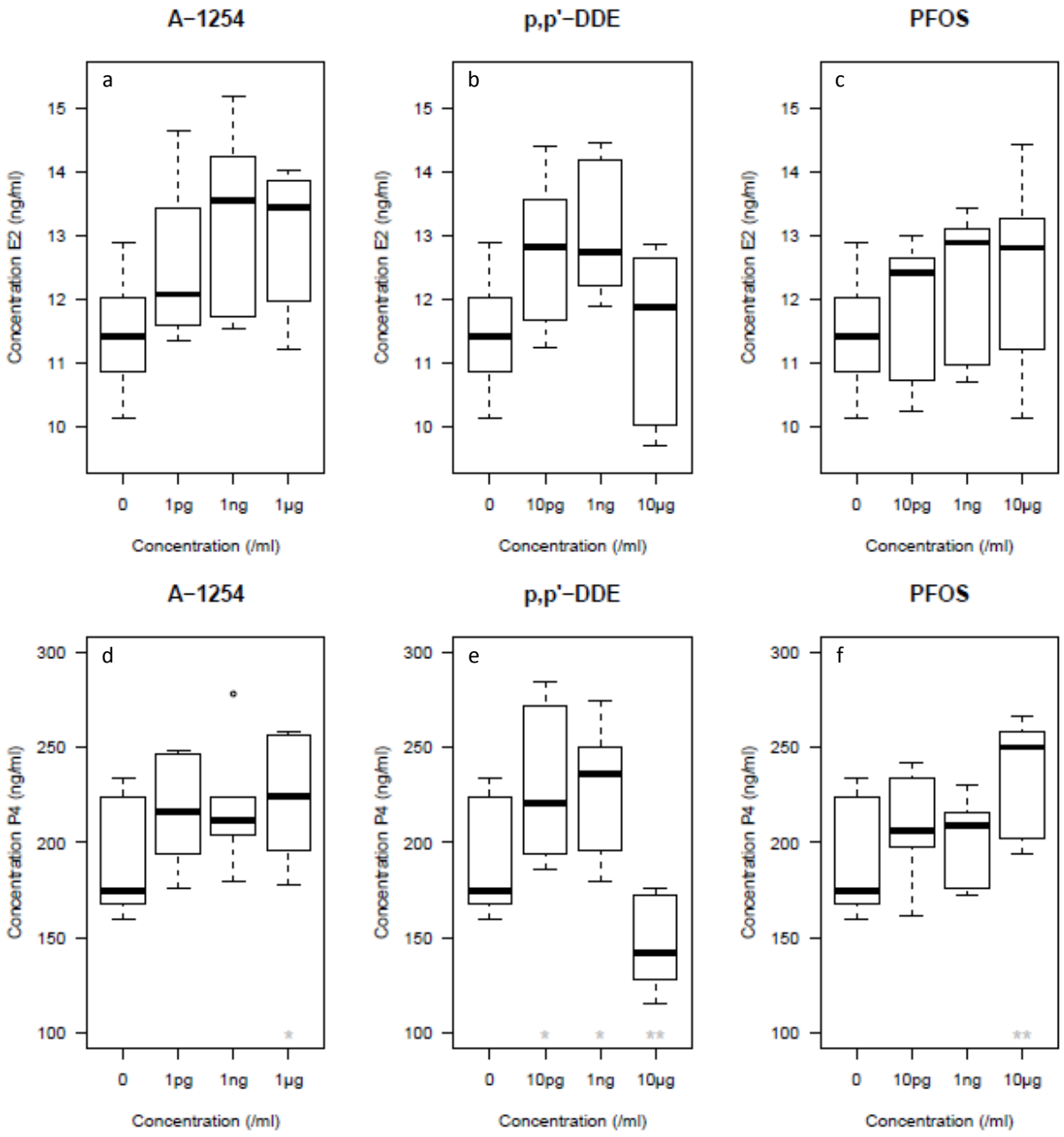

Figure 4 Estradiol (E2) and Progesterone (P4) concentrations measured in culture and exposure medium of granulosa cells exposed for 48 h to different concentrations of A-1254, *p,p'*-DDE and PFOS in a serum-free granulosa cell culture

DISCUSSION

This study was set-up to investigate if environmentally-relevant concentrations of PCBs, *p,p'*-DDE and PFOS are able to influence granulosa cell viability and function; thereby potentially disturbing the endocrine balance in a growing ovarian follicle. Firstly, we succeeded in developing a serum-free bovine granulosa cell culture. Using this culture, surprisingly more granulosa cells were present after 48 h exposure to environmentally-relevant and high concentrations of PCBs and PFOS respectively. Our results further demonstrate that the exposure to each compound at environmental relevant concentrations gives rise to higher E2-concentrations compared to unexposed cells. Also, high *p,p'*-DDE and high PFOS-concentrations have respectively a significant negative and positive effect on the P4-production and therefore on luteinization.

In the past, several studies were already performed to examine the effects of different EDCs on granulosa cells from several species (Lovekamp-Swan and Chaffin, 2005; Grasselli *et al.*, 2010; Mlynarczuk *et al.*, 2013). In each of these experimental set-ups, serum was added to facilitate cell attachment and to assist *in vitro* growth of the granulosa cells. However, granulosa cells spontaneously luteinize in serum-supplemented cultures, possibly under the influence of the LH present in serum (Murphy, 2000). During this luteinization process, granulosa cells undergo ultrastructural and morphological changes (Gutierrez *et al.*, 1997b), and as a result, serum-cultured granulosa cells only have limited physiological resemblance to non-luteinized granulosa cells of an *in vivo* growing follicle. Next to this, E2-production can only be maintained in granulosa cells cultured in serum-free conditions (Gutierrez *et al.*, 1997a). As a consequence, the previously reported EDC-exposure experiments using serum-supplemented granulosa cells can be of value when examining the effect of EDCs on the corpus luteum. However, the use of these cultures as a model to study the effect of EDCs on a growing follicle and its cell populations is disputable.

In our experiments, we have decided to use bovine granulosa cells due to the striking similarity of early reproductive physiology between bovine and humans, in contrast to major differences in ovarian physiology and reproductive function between human and rodents or other domestic animals (Neuber and Powers, 2000; Campbell *et al.*, 2003; Petro *et al.*, 2012b). Moreover, because bovine ovaries are waste products during slaughter, they can easily be obtained at nearby abattoirs and in this way, no additional animals need to be sacrificed. To create our serum-free bovine granulosa cell cultures, we used granulosa cells from small to medium antral follicles (<8 mm in diameter), because E2-production is better sustained in these follicles compared to larger follicles.

Also, because cell plating density can influence E2-production, granulosa cells cultured in 96-well and 24-well plates were seeded in the same cell to surface ratio (Portela *et al.*, 2010). To replace serum in cell culture systems, supplements need to be added to basal culture media in order to sustain cell growth and proliferation (van der Valk *et al.*, 2010). In addition to commonly supplied components such as ITS (insulin, transferrin and selenium), antibiotics, Hepes, BSA and glutamine, the addition of cell type specific supplements were essential for maintaining granulosa cell function: FSH for aromatase stimulation, androstenedione as a precursor molecule of E2 and the LR IGF-1 growth factor, which is more potent than native IGF-1 (Gutierrez *et al.*, 1997a), to sustain aromatase expression and activity (Silva and Price, 2002). During each experiment, granulosa cell cultures were checked upon their resemblance to the characteristic cellular morphology of a serum-free granulosa cell culture, i.e typical tight clumps of spherical granulosa cells attached to the culture plate by enlarged, flattened fibroblast-like cells (Gutierrez *et al.*, 1997a; Gutierrez *et al.*, 1997b) (Figure 3).

In our experiments, we exposed granulosa cells to EDC-concentrations which were in line with recently detected concentrations in human follicular fluid (Petro *et al.*, 2012a; Petro *et al.*, unpublished). Next to these environmentally-relevant concentrations, granulosa cells were also exposed to lower and higher concentrations of each compound (Table 1). With our experiments, we wanted to gain insight if it is possible that *in vivo* detected EDC-concentrations in follicular fluid could influence granulosa cell viability and function *in vitro*. Overall, granulosa cell viability was not hampered after 48 h of exposure. On the contrary, surprisingly more granulosa cells were found in cultures exposed to environmentally-relevant PCB (1 ng/ml A-1254) and high PFOS (10 µg/ml) concentrations compared to unexposed cells; whereby the effect of the high PFOS-concentration was the strongest. This suggests a kind of trophic effect of these concentrations on the granulosa cell populations. In literature, high PFOS-concentrations are mainly documented as being cytotoxic by inducing apoptosis and oxidative stress (Mao *et al.*, 2013), although one recent study also reports that the viability of H259R cells was not affected by high PFOS-concentrations (Kraugerud *et al.*, 2011). Literature documenting about positive effects after PCB-exposure on cell viability is also extremely scarce.

Remarkably, the environmentally-relevant concentrations of A-1254, *p,p'*-DDE and PFOS all showed to have a stimulating effect on E2-production, because significantly higher E2-concentrations were measured in these cultures compared to unexposed granulosa cells after 48 h exposure (Figure 3). Regarding PFOS, the ability of PFASs to interact with PPAR-receptors could be a plausible explanation (Bjork and Wallace, 2009; Ren *et al.*, 2009; Wolf *et al.*, 2012). The expression of PPARγ in granulosa

cells, can potentially explain this higher E2-production because PPARγ is linked to steroidogenesis in granulosa cells (Froment *et al.*, 2006). This can be an explanation for the surprisingly positive association that was found between higher PFASs-concentrations in follicular fluid and higher fertilization rates of women undergoing ART (Petro *et al.*, unpublished). Both *p,p'*-DDE and PCBs (individual congeners as well as mixtures) have been shown to have both estrogenic and anti-estrogenic effects on granulosa cells cultured in serum-supplemented conditions (Younglai *et al.*, 2004; Wu *et al.*, 2006; Kwintkiewicz *et al.*, 2010). It is important to point out that the increase in E2-production does certainly not imply that these chemicals only have a positive effect within the follicular micro-environment. Under normal circumstances, both folliculogenesis and oogenesis are strictly controlled and progress simultaneously whereby in the end, a dominant follicle contains an oocyte arrested in metaphase II. During folliculogenesis, the amount of E2 within the follicle is of extreme importance, because finally, there should be sufficient E2 present at the plasma level to trigger the GnRH peak center in the hypothalamus to produce GnRH, which results in the LH-surge essential for ovulation. If however, under the influence of these chemicals, the E2-production increases, the E2-concentration necessary to trigger the LH-surge will be reached much sooner. In this way, the presence of EDCs could create an imbalance between folliculogenesis and oogenesis resulting in premature ovulation of an immature oocyte ultimately leading to failing fertilization.
The high concentrations of *p,p'*-DDE (10 µg/ml) and PFOS (10 µg/ml) showed to induce strong effects on P4-production, whereby the interaction of *p,p'*-DDE decreased and PFOS increased P4-production in the exposed granulosa cells. No effects were noticed after A-1254 exposure, which can possibly be explained by the fact that A-1254 is a mixture of different PCB-congeners, whereby each individual congener could have the same, opposite or synergistic effect compared to another congener, which may result in no net effect. The negative effect of *p,p'*-DDE on P4-production is also described in literature using the stable JC-140 granulosa cell line (Crellin *et al.*, 2001). For PFOS, no literature is currently present, but our results indicate that a high PFOS-concentration in the follicle favors premature luteinization of the granulosa cells within a growing follicle. We want to emphasize that this high PFOS-concentration far exceeds the recently detected PFOS levels in human follicular fluid (Petro *et al.*, unpublished).

In conclusion, we were able to closely mimic the *in vivo* conditions by establishing a serum-free bovine granulosa cell culture. The exposure of these cultures to environmentally-relevant concentrations of A-1254, *p,p'*-DDE and PFOS showed that the tested concentrations are not cytotoxic, but we did notice that each substance at environmentally-relevant concentrations could

interfere with the normal steroidogenesis processes in the granulosa cells. Further research is needed to examine the action mechanisms behind this interference. For future experiments, we plan to further optimize our serum-free bovine granulosa cell culture, so that it can be used for longer term exposure experiments.

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SUPPLEMENTARY MATERIAL

Table SM-1 Overview of the products used in the serum-free bovine granulosa cell exposure experiments

Product name	Product number	Company	City	Country
McCoys 5a medium	26600023	Life Technologies	Merelbeke	Belgium
Hepes	15630056	Life Technologies	Merelbeke	Belgium
Penstrep	15140122	Life Technologies	Merelbeke	Belgium
Glutamine	G7513	Sigma-Aldrich	Diegem	Belgium
Apotransferrin	T1428	Sigma-Aldrich	Diegem	Belgium
Na-selenite	S5261	Sigma-Aldrich	Diegem	Belgium
FSH	F19715.02	Merck-Serono	Overijse	Belgium
BSA	A6003	Sigma-Aldrich	Diegem	Belgium
LR-IGF1	I1271	Sigma-Aldrich	Diegem	Belgium
Insulin	I1882	Sigma-Aldrich	Diegem	Belgium
Androstenedione	46033	Sigma-Aldrich	Diegem	Belgium
Aroclor-1254	48586	Sigma-Aldrich	Diegem	Belgium
p,p-DDE	PS696	Sigma-Aldrich	Diegem	Belgium
PFOS	77282	Sigma-Aldrich	Diegem	Belgium
CellTiter 96 Aqueous One Solution Cell Proliferation assay	G3582	Promega	Leiden	The Netherlands

Table SM-2 Overview of final A-1254, *p,p'*-DDE and PFOS exposure concentrations (after dilution of stock concentrations). Environmental relevant concentrations are based on the published substance concentrations detected in human follicular fluid (Petro *et al.*, 2012; Petro *et al.*, unpublished).

Compound	Low exposure conc	Environmentally-relevant exposure conc	High exposure conc
A-1254	1 pg/ml	1 ng/ml	1 µg/ml
p,p'-DDE	10 pg/ml	1 ng/ml	10 µg/ml
PFOS	10 pg/ml	1 ng/ml	10 µg/ml

GENERAL DISCUSSION

Environmental pollution has become a major side effect of our industrialized world. Each day, a tremendous amount of different chemicals are globally released into the environment, without sufficient knowledge if these compounds can be considered safe for the environment, animals and humans. Some of these substances can act as endocrine disrupting chemicals (EDCs) by interfering with the synthesis, function, storage and/or metabolism of endogenous hormones. Due to its tight endocrine control, both the male and female reproductive systems are considered targets for EDC action. In this research, the emphasis was put on the interactions of EDCs with the female reproductive system in general and with the ovarian follicular micro-environment in specific.

HIGH PRODUCING DAIRY COW SCREENING ON EDC-PRESENCE

For more than 30 years, a significant decrease of high producing dairy cow fertility has been observed. This decline in reproductive performance is caused by a combination of different factors such as the use of high energetic and protein rich diets (Butler, 1998), larger herd sizes (Fahey *et al.*, 2002), genetic selection in favor of the milk yield (Snijders *et al.*, 2000) and the negative influence of metabolic disorders occurring shortly after parturition on the oocyte and subsequent embryo development (Leroy *et al.*, 2005). Next to these causes, the influence of EDCs has been suggested in the last years as a potential additional factor contributing to the dairy cow fertility problems. This suggestion becomes obvious if we take a closer look on the complicated metabolism of these 'industrialized' animals. First of all, it was already documented in 1960 that an excessive intake of natural phytoestrogens by ruminants, due to grazing on clover rich pastures, could lead to various reproductive disorders (Adler and Trainin, 1960). Attention only really increased after it was noticed that the drop in dairy cow fertility of the past 3 to 4 decades, largely corresponds with the time period during which the EDC-concentrations in the environment increased significantly. Interestingly, the dairy cow's lactation peak, around 6 to 8 weeks after calving, coincides with the ideal moment of establishing a new gestation (Leroy *et al.*, 2008). This intensive lactation is accompanied by a massive lipolysis, whereby it is more than plausible that EDCs, which are mostly stored in adipose tissue due to their highly lipophilic nature, are simultaneously released in the bloodstream. At the same time, folliculogenesis and follicle recruitment take place within the ovary. Inside antral follicles, the follicular fluid derives from blood flowing through the thecal capillaries (Rodgers and Irving-Rodgers, 2010). It is thus possible that the mobilized EDCs, now present in blood, are further transferred to the follicular fluid fairly easily due to their lipophilicity. As a result, individual components of the antral follicle (i.e. theca cells, granulosa cells and the oocyte) come in direct contact with these EDCs.

In this way, it becomes more clear why the presence of EDCs, such as PCBs and several organochlorine pesticides, was suggested as an additional explanation for the deteriorating reproductive performance of the high producing dairy cow.

Our research however suggests that environmental EDCs are probably not a major additional cause for the declining fertility of high producing dairy cows (Petro *et al.*, 2010). We analyzed several tissues (liver, adipose tissue, kidney, muscle and ovary) and body fluids (serum, milk and follicular fluid) from slaughtered dairy cows older than 6 years old to maximize the detection of the bio-accumulation effect of these substances (Petro *et al.*, 2010). These analyses showed that the detected EDC-concentrations in the tissues and body fluids (for overview: see Chapter 3A) were all in accordance or even lower than the scarcely earlier reported EDC-concentrations in bovine tissue and/or body fluids (Thomas *et al.*, 1999; Kamarianos *et al.*, 2003; La Rocca and Mantovani, 2006; Blanco-Penedo *et al.*, 2008; Glynn *et al.*, 2009), and certainly much lower than the levels found in human tissues from the same geographical region (Covaci *et al.*, 2008). In case of follicular fluid samples, not one EDC-compound was detected above its limit of quantification (LOQs, 10 – 30 pg/ml range). However, these results cannot exclude that follicular cells of these dairy cows were never in contact with EDCs, but it does indicate that if EDCs were present in follicular fluid at the time of sampling, the follicular cells were only exposed to EDC-concentrations lower than 10 - 30 pg/ml.

Since we were only able to analyze tissues and body fluids from a limited amount of animals (n = 20), we compared our results to the yearly reported results of the Federal Agency for the Safety of the Food Chain (FASFC, Federaal Agentschap voor de Veilighed van de Voedselketen, FAVV) of Belgium, which are in agreement with our low contamination results. During the period of our research (2006-2012), between 3000 and 4000 samples of meat products and fat from slaughterhouse animals were tested by the FASFC/FAVV for the presence of dioxins or PCBs, whereby only 10 samples exceeded the maximum regulatory concentration limit set for dioxins/dioxin-like PCBs (FAVV, 2007, 2008, 2009, 2010, 2011, 2012, 2013). Furthermore, both in our postmortem serum and in serum samples taken from dairy cows 2 - 3 weeks post-partum (the period in which the massive lipolysis, and thus potential EDC-release is at its maximum), no EDCs could be detected above their LOQs, except for CB 153, the globally most detected PCB-congener (Petro *et al.*, 2010).

These low EDC-contamination levels can be largely explained by the herbivorous character of cows and thus, their lower position in the food chain, meaning that the process of bio-accumulation, in which the EDC-concentrations in animals increases through the food chain, is very low or even not relevant. One study has even reported on a 'biodilution' effect of PCBs through the agricultural food

chain (McLachlan, 1996). This is even more true following an almost complete ban of animal derived products in ruminant nutrition, with an exception for the production of milk replacers for young animals (EC, 2001, 2008). Therefore, EDC-concentrations found in dairy cow tissues and body fluids (Petro *et al.*, 2010) are much lower than in human or wildlife species (Covaci *et al.*, 2002; Jaspers *et al.*, 2006; Weijs *et al.*, 2010; Belpaire *et al.*, 2011) and well below the levels that disrupt the endocrine system in wildlife (Vos *et al.*, 2000; Hamlin and Guillette, 2010). The intake of the EDCs by dairy cattle can most likely be attributed to the ingestion of contaminated grass or to the accidental uptake of polluted soil during grazing (Rhind, 2005). Although we conclude that EDCs are probably not a major contributor to the declining dairy cow fertility, we want to point out that caution is advised when animals are reared on sewage sludge treated pastures, because high EDC-concentrations can be detected in sewage sludge (Clarke and Smith, 2011). Although EDC-levels in the applied sewage sludge were relatively low, disruption of fetal testis and ovarian development and disturbances of the hypothalamic-pituitary function have been observed in sheep reared under these conditions (Paul *et al.*, 2005; Fowler *et al.*, 2008; Bellingham *et al.*, 2009; Bellingham *et al.*, 2010).

The Belgian dairy cow thus has a low contamination status. More specific, the follicular micro-environment seems even relatively free of contamination, although the presence of EDCs (i.e. PCBs and organochlorine pesticides) at lower concentrations that the method LOQ cannot be ruled out completely. However, if EDCs would be present, this will only be in concentrations that are not considered as being endocrine disrupting. As a consequence, we can state that it is highly unlikely that *in vivo*, the individual components of the bovine ovarian follicle already have encountered potential harmful effects of EDCs. This makes us to feel comfortable to use these primary cells for *in vitro* exposure experiments. The bovine ovary and its individual components have already been successfully used as a model for the human ovarian follicular micro-environment due to the striking similarities between bovine and human early pre-implantation embryo and reproductive physiology (for overview: see Chapter 1B). Therefore, we conclude and even recommend the bovine ovarian follicle and its individual components as a promising *in vitro* reproductive toxicology model.

HUMAN FOLLICULAR FLUID SCREENING ON EDC PRESENCE AND IVF-OUTCOME

In contrast to dairy cows, humans are positioned higher in the food chain and thus, the bioaccumulation process of EDCs is more likely to occur. Also, humans are more exposed to EDC-

containing materials. Therefore, it seems plausible that the human body is more contaminated with EDCs as compared to the dairy cow.

This is confirmed by our analyses of serum and follicular fluid of women undergoing Assisted Reproductive Technology (ART)-procedures. While in dairy cows, (almost) no contamination was detected in both matrices, each of our analyzed human serum and follicular fluid samples contained the targeted EDCs. They most probably enter the human follicular micro-environment in the same way as was proposed for the dairy cow, via blood flowing through the thecal capillaries (Rodgers and Irving-Rodgers, 2010). This means that since EDCs are being detected in serum from children (Marek *et al.*, 2013), as from the onset of puberty, during each menstrual cycle, follicular cells of antral follicles will probably come in direct contact with EDCs present in the follicular fluid. We decided to screen our samples on the presence of Persistent Organic Pollutants (POPs) with endocrine disrupting properties, such as PCBs, PBDEs and a whole set of organochlorine pesticides including DDT and metabolites (DDTs), and the newly emerging group of PerFluorinated Alkylated Substances (PFASs). For the first group of contaminants, we made an inventory of the contamination status of human follicular fluid since the first report was published back in 1984 (Trapp *et al.*, 1984). Since then, only a handful of papers were published regarding this topic (for overview, see Chapter 1B). In these articles, almost no information is available regarding the possible effects of the EDC-presence in follicular fluid on the ART-outcome of the patients. On our opinion, this is as least as important as the contamination status of the follicular fluid itself and therefore, next to the biomonitoring aspect, the following question turned out into being a major goal of this part of our research: Can we observe -if present- any associations between the EDCs in human follicular fluid and the outcome of the ART-procedures of the participating patients?

To the best of our knowledge, we are the first to report a detailed overview of the presence of PFASs and PBDEs in human follicular fluid, although the latter group of substances (BDE 47 and BDE 49) was only present in 1 out of 40 analyzed samples (Petro *et al.*, 2012a). For PFASs however, the contamination pattern in follicular fluid shows to be very similar to the pattern in other biological fluids, such as (umbilical cord) serum (Lau *et al.*, 2007; Monroy *et al.*, 2008; Fei *et al.*, 2009; von Ehrenstein *et al.*, 2009; Roosens *et al.*, 2010; Stein *et al.*, 2012), amniotic fluid (Stein *et al.*, 2012), human milk (von Ehrenstein *et al.*, 2009; Roosens *et al.*, 2010; Croes *et al.*, 2012) and semen (Toft *et al.*, 2012; Joensen *et al.*, 2013). PFOS is detected in the highest concentration, followed by PFOA, PFNA and PFHxS, whereby PFOS and PFOA are present in almost every follicular fluid sample. Next to these 4 PFAS-compounds, no other PFASs could be detected in the investigated follicular fluid

samples. Together with the detection of bisphenol-A (Ikezuki *et al.*, 2002) and phthalate metabolites (Krotz *et al.*, 2012), our results clearly contribute to the mapping of the total contamination status of human follicular fluid. We could also confirm the worldwide declining trends of PCBs and p,p´-DDE levels in follicular fluid (Trapp *et al.*, 1984; Baukloh *et al.*, 1985; Schlebusch *et al.*, 1989; Jarrell *et al.*, 1993; Pauwels *et al.*, 1999; Younglai *et al.*, 2002; De Felip *et al.*, 2004; Weiss *et al.*, 2006; Meeker *et al.*, 2009; Jirsova *et al.*, 2010). For both the POPs and PFASs, the contamination level of the investigated serum samples is in the same order of magnitude as reported by recent studies in other Western countries (Porta *et al.*, 2010; Herrick *et al.*, 2011; Kalantzi *et al.*, 2011; Olsen *et al.*, 2012; Stein *et al.*, 2012), with a distinct decline in contamination levels compared to older studies (Pauwels *et al.*, 1999; Voorspoels *et al.*, 2002; Kannan *et al.*, 2004; Needham *et al.*, 2005; Karrman *et al.*, 2006; Fei *et al.*, 2009).

If we however compared both EDC-groups with regard to their distribution patterns in serum and follicular fluid, some striking differences were noted. First of all, PFASs are present in concentrations exceeding those of POPs in the same follicular fluid and serum samples. While we could observe high correlations between serum and follicular fluid concentrations of POPs, almost no correlations were found for the PFAS-concentrations between both matrices. For POPs, we noticed a concentration gradient between serum and follicular fluid concentrations, whereby PCB-, *p,p'*-DDE- and HCB-concentrations showed to be approximately 2 - 3 times higher in serum than in follicular fluid. In contrast, for each individual PFAS-compound, the concentration detected in either serum or follicular fluid was more or less in the same range (Chapter 3C, Table 2). Also, no correlations were observed between PFAS- and POP-concentrations in either serum or follicular fluid.

We suggest that the differences in physico-chemical properties of both groups and the different composition of serum and follicular fluid are the main contributing factors for these opposing patterns. Due to their lipophilic nature, POPs predominantly reside in a lipid-rich environment, such as adipose tissue, while PFASs are both lipophilic and hydrophilic and are mainly found in blood, liver and kidney. In blood, PFASs are mostly transported bound to carrier proteins, such as B-lipoproteins and albumin (Jones *et al.*, 2003; Wu *et al.*, 2009; Luo *et al.*, 2012). Since albumin is capable of passing the blood-follicle barrier quite easily (Hess *et al.*, 1998; Schweigert *et al.*, 2006), it can be assumed that PFASs are being transferred from the blood stream into the growing follicle without too much problems. This could be an explanation why no concentration gradient is observed between PFAS-concentrations in serum and follicular fluid. Next to this observation, blood contains more lipids than follicular fluid and this further explains why the concentrations of the lipophilic POPs are higher in

blood than in follicular fluid. Also, the combination of the protein-binding capacity of PFASs and the fact that follicular fluid contains higher protein than lipid levels (Valckx *et al.*, 2012) is another reason why PFAS-concentrations are higher as compared to POPs in follicular fluid.

More remarkable are the opposite results found for PFASs and POPs with regards to their potential influence on the oocytes developmental competence as determined by the ART-procedures. The overall outcomes showed to be independent of the woman's age, BMI, E2 levels of the patient, fertilization procedure or the presence of male subfertility. Whereas an *in vivo* higher total contamination level of follicular fluid with POPs was associated with a highly significant drop in fertilization rate and a significant lower proportion of top quality embryos, higher *in vivo* PFAS-concentrations in follicular fluid were associated with a surprisingly higher fertilization rate and a subsequent higher amount of top quality embryos. Our results indicate that both groups of contaminants most likely influence the fertilization process rather than the first steps of embryonic development. If an oocyte has the potential to develop into a zygote, its chance to further develop into a high quality embryo is not hampered/further stimulated by the follicular fluid contamination.
Our results suggest that POPs can interrupt the normal folliculo- and/or oögenesis resulting in an immature or lower quality oocyte that is unable to undergo successful fertilization. This underpins the finding that the initial quality of the oocyte is not only imperative for its own maturation, but that it is also fundamental for its further developmental competence (Sirard *et al.*, 2006; Swain and Pool, 2008). Because embryo quality is an important determinant of successful implantation (Cakmak and Taylor, 2011), the observed lower proportion of high quality embryos, paralleled by decreased fertilization rates in the patients studied, may thus (partially) explain the lower implantation rates described recently in women with higher PCB concentrations in their serum (Meeker *et al.*, 2011). Our results also confirm the suggested relationship between the presence of *p,p'*-DDE in follicular fluid and blood and an impaired fertilization rate (Younglai *et al.*, 2002; Weiss *et al.*, 2006). We also want to stress that these *in vivo* results agree with the *in vitro* observed harmful influence of relatively low concentrations of POPs on embryo development (Campagna *et al.*, 2001; Pocar *et al.*, 2001; Lenie *et al.*, 2008).
The ability of PFASs to bind to PPAR-receptors (Bjork and Wallace, 2009; Ren *et al.*, 2009; Wolf *et al.*, 2012) could be a plausible explanation for the intriguing positive association that we found between higher PFAS-contamination in follicular fluid and a higher fertilization and subsequent higher amount of top quality embryos. PPARs are suggested to be crucial for a successful oocyte development (Froment *et al.*, 2006; Dupont *et al.*, 2008; Minge *et al.*, 2008; Rees *et al.*, 2008). Hypothetically, if

PFASs are able to influence PPARs in such a subtle way, without disturbing the well-balanced follicular micro-environment, it is possible that PFASs can 'enhance' this micro-environment in a way that is beneficial for the oocyte to develop into a fully competent, mature and fertilizable oocyte.

Biomonitoring of human follicular fluid is essential, because it is the only way to acquire information about the exact contaminant concentrations to which follicular cells, including the oocyte, are directly exposed to. Defining environmental relevant concentrations is of significant importance if one wants to perform *in vitro* research whereby the *in vivo* situation is to be mimicked as closely as possible. However, human follicular fluid is not easy to obtain and the only ethically approved setting where you can collect follicular fluid samples from different women over a relatively short time period is in a hospital's ART-unit. To (partially) overcome this problem, we created, based on the strong correlations between serum and follicular fluid concentrations of POPs, regression models that can predict with 90% accuracy the POPs contamination in follicular fluid, when only blood samples are available. Our patient group shares several characteristics like bodyweight, height and BMI with pregnant volunteers from larger biomonitoring studies and the serum levels of POPs from these volunteers are in the same range as in our serum samples (Koppen *et al.*, 2009; Darnerud *et al.*, 2010; Llop *et al.*, 2010). Therefore, we are confident that our study group matches the average profile of the Western (pregnant) female population in terms of contamination with these POPs. As a result, we promote these regression models as useful tools to estimate the EDC contamination of the follicular micro-environment of women outside the ART-setting. Our models can also be used to estimate environmentally-relevant concentrations of EDCs for *in vitro* exposure experiments when only blood is available.

EXPOSING SERUM-FREE BOVINE GRANULOSA CELL CULTURES TO PCBs, *p,p'*-DDE AND PFOS

After screening for EDCs in human follicular fluid, we have built knowledge on the current contamination of follicular fluid with these substances. In this way, we knew to which EDC-concentrations the individual components of the human ovarian follicle are currently exposed *in vivo*. In our opinion, these environmentally-relevant EDC-concentrations are necessary to conduct *in vivo* mimicking based on *in vitro* experiments; however, they are often neglected. Many of these exposure experiments use completely irrelevant (too high) EDC-concentrations, and therefore, their relevance is debatable.

During the last part of our research (Chapter 3D), we verified if these environmentally relevant EDC-concentrations would be able to interfere with the endocrine balance within the ovarian follicle. More specifically, we examined if EDCs were cytotoxic for the bovine granulosa cells and/or able to interfere with the functionality of these cells. We choose to focus on PCBs, *p,p'*-DDE and PFOS, because these substances were found in the highest concentrations in human follicular fluid (Petro *et al.*, 2012a; Petro *et al.*, unpublished). Within the ovarian follicle, granulosa cells are, together with theca cells, mainly responsible for the steroidogenesis and therefore, as being target cells for EDC-interference, are selected as research cells. We decided to use bovine granulosa cells for two main reasons: as already mentioned, the bovine ovary is the recommended model for the human ovarian follicular micro-environment and, when using bovine ovaries, no additional animals need to be sacrificed since bovine ovaries are considered waste products in slaughterhouses (Petro *et al.*, 2012b). Before the exposure experiments could be executed, we first needed to establish a serum-free bovine granulosa cell culture. The elimination of serum in our culture medium was essential to stay as close as possible to the *in vivo* ovarian follicular micro-environment, because the granulosa cells spontaneously luteinize after contact with serum, which can possible be attributed to the presence of LH in serum (Murphy, 2000). As a consequence, these granulosa cells undergo ultrastructural and morphological changes (Gutierrez *et al.*, 1997b), resulting in granulosa cells which have only limited physiological resemblance to granulosa cells of an *in vivo* growing follicle. Although the exclusion of serum in culture medium seems obvious considering the above explanation, all currently reported exposure experiments that investigated the potential influence of EDCs on granulosa cells use serum-supplemented culture media. On our opinion, these experiments would better serve as a model to examine the effect of EDCs on the corpus luteum.

Our serum-free bovine granulosa cell culture is based on previously described cultures (Gutierrez *et al.*, 1997a; Vanholder *et al.*, 2005), whereby next to the commonly supplied components such as ITS (insulin, transferrin and selenium), antibiotics, Hepes, BSA and glutamine, also cell type specific supplements essential for maintaining granulosa cell function were added to the culture medium. In our culture medium, these cell type specific supplements are FSH for aromatase stimulation, androstenedione as a precursor molecule of E2 and the LR IGF-1 growth factor, which is more potent than native IGF-1 (Gutierrez *et al.*, 1997a), to sustain aromatase expression and activity (Silva and Price, 2002). Next to this, we used granulosa cells from small to medium follicles (> 8 mm), because these granulosa cells can better sustain E2-production and we made sure that in each experiment,

granulosa cells were plated within the same cell to surface ratio to rule out the fluctuations in E2-concentrations related to a different plating density (Portela *et al.*, 2010).

Our results indicated that environmentally-relevant concentrations of PCBs, *p,p'*-DDE and PFOS are not cytotoxic for granulosa cells after 48 h of exposure. Surprisingly, a higher number of granulosa cells were found in cultures exposed to environmentally-relevant PCB mixture (1 ng/ml A-1254) and high PFOS (10 µg/ml) concentrations compared to unexposed cells, whereby the effect of the high PFOS-concentration was the strongest. Because of these unexpected results, additional research is definitely needed to further explore this suggestive trophic effect of environmentally-relevant PCB-concentrations. Environmentally-relevant concentrations of PCBs, *p,p'*-DDE and PFOS showed to have a stimulating effect on E2-production, because significantly higher E2-concentrations were measured in these cultures compared to unexposed granulosa cells after 48 h exposure.

Before our experiments, no data were available regarding the possible influence of PFOS on granulosa cells. PFOS has been shown to interact with PPARγ, which in turn is expressed in granulosa cells and which has been suggested to being involved in steroidogenesis in granulosa cells (Froment *et al.*, 2006). This can be an explanation for our PFAS-screening results in human follicular fluid, whereby a remarkably positive association was found between higher PFAS-concentrations in follicular fluid and higher fertilization rates of women undergoing ART (Petro *et al.*, unpublished). In literature, both estrogenic and anti-estrogenic effects have been attributed to *p,p'*-DDE and PCBs (individual congeners as well as mixtures) on serum-supplemented granulosa cell cultures (Younglai *et al.*, 2004; Wu *et al.*, 2006; Kwintkiewicz *et al.*, 2010). The observed stimulatory effect on E2-production in our experiments does certainly not directly imply a positive effect of these substances on the follicular micro-environment. Under normal circumstances, both folliculogenesis and oogenesis are strictly controlled and progress simultaneously whereby in the end, a dominant follicle contains an oocyte arrested at metaphase II. During folliculogenesis, the amount of E2 within the follicle is responsible for triggering the LH-surge essential for ovulation. If however, based on our experiments, the E2-production increases more rapidly under influence of the present EDCs, the E2-concentration necessary to generate the LH-surge will be attained (much) earlier. In this way, the presence of PCBs, *p,p'*-DDE and PFOS within the human follicular micro-environment could create an imbalance between folliculogenesis and oogenesis resulting in premature ovulation of an immature oocyte ultimately leading to failing fertilization. Environmentally-relevant PCB-, *p,p'*-DDE or PFOS-concentrations could not influence progesterone (P4) production in the bovine granulosa cell cultures. The high *p,p'*-DDE-concentration (10 µg/ml) decreased and the high PFOS-concentration (10 µg/ml) increased P4-production in exposed granulosa cells, meaning that high *p,p'*-DDE levels

postpone and high PFOS-levels favor (premature) luteinization. We do want to clearly point out that these high levels used in the *in vitro* experiments exceeds the recently *p,p'*-DDE and PFOS-concentrations detected levels in human follicular fluid (Petro *et al.*, 2012a; Petro *et al.*, unpublished).

To summarize, with our research, we succeeded in obtaining a better understanding of the impact of EDC-contamination on the physiology of both the bovine and human ovarian follicle. We gained more knowledge on the contamination status of both the human and bovine follicular micro-environment, whereby the human ovarian follicle is unmistakably more contaminated than the bovine follicle. We are convinced that the bovine ovarian follicle and its individual components are a true asset for EDC-research, due to its relative 'cleanliness' regarding EDC-contamination (for PCBs and several organochlorine pesticides in specific), and the many similarities between bovine and human ovarian physiology. In fact, we believe that the bovine species is the preferred species when examining the ovarian follicular micro-environment. We could also demonstrate an association between EDCs present *in vivo* in the human ovarian follicle and the outcome of patients undergoing ART, whereby the fertilization process seems to be mostly affected. Next to this, our results also show that the different characteristics of EDCs are being reflected in a different *in vivo* distribution pattern of these substances between serum and follicular fluid. Although we strongly encourage further biomonitoring of human follicular fluid, we created regression models to predict the concentration of POPs with endocrine disrupting properties in follicular fluid, when only serum samples are available. With our final *in vitro* exposure experiments using a serum-free bovine granulosa cell culture, we could indicate that environmentally-relevant EDC-concentrations are able to interact with granulosa cells, thereby possibly influencing the normal steroidogenesis in a growing ovarian follicle. For future research, we hope that the results described in this thesis can contribute in unraveling the action mechanisms of EDCs in the ovarian follicle.

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CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS

The research presented in this thesis investigated crucial factors with regards to the potential influence of EDCs on both the human and bovine follicular micro-environments and showed that:

1) The presence of PCBs, PBDEs and OCPs is not one of the major causes of the failing reproductive performance of high producing dairy cows.

2) The bovine ovarian follicle and its individual components can be applied as a valuable model to study the possible effects of EDCs on the human ovarian follicular micro-environment.

3) The levels of POPs with endocrine disrupting properties, such as PCBs and *p,p'*-DDE, in human follicular fluid are declining.

4) Next to POPs, other newly emerging EDCs, such as PFASs and PBDEs, are also present in the human follicular fluid and, in the case of PFASs, in concentrations exceeding those of the POPs.

5) When no follicular fluid samples are available, the contamination of human follicular fluid with POPs with endocrine disrupting properties can be predicted based on human serum concentrations using linear regression models.

6) The presence of EDCs in human follicular fluid is associated with altered Assisted Reproductive Technology (ART) outcomes: higher concentrations of POPs are linked to a lower chance and higher PFAS-concentrations to a higher chance of an oocyte to develop into a top quality embryo in ART-settings, mainly due to an impact on the fertilization rate.

7) The different properties and characteristics of PFASs compared to POPs are being reflected in a different distribution pattern between human follicular fluid and serum.

8) For *in vitro* exposure experiments, *in vivo* steroidogenesis in a human antral follicle can be closely mimicked by establishing a serum-free bovine granulosa cell culture.

9) Environmentally-relevant concentrations of a PCB-mixture (A-1254), *p,p'*-DDE and PFOS are, after 48 h of exposure
    - not cytotoxic for granulosa cells.
    - able to interfere with the normal steroidogenesis processes in the granulosa cells.

PERSPECTIVES FOR FUTURE RESEARCH

We concluded that the bovine ovarian follicle is a valuable model to study the influence of EDCs on the human follicular micro-environment. We also demonstrated that the presence of EDCs in human follicular fluid *in vivo* is associated with altered ART-outcomes, whereby fertilization seems mostly affected. For future research, we therefore suggest to use the bovine model to explore the mechanisms how EDCs can interact with the fertilization process.

Within EDC-research, the investigation of chronic exposure to EDCs remains an imperative problem. Therefore, in the light of ovarian follicular physiology, the prolongation of the serum-free bovine granulosa cell culture in time and the establishment of a long term bovine ovarian follicle culture from primordial until ovulatory stage follicles would surely enhance the research on chronic EDC-effects.

Next to prolonging the serum-free bovine granulosa cell culture in time, expanding it into a serum-free co-culture of theca and granulosa cells would further improve the ultimate goal of establishing an *in vitro* follicular somatic cell culture which resembles the *in vivo* situation as closely as possible.

Further research is definitely required to fully characterize, optimize and eventually valorize the bovine ovarian model in general and its different research set-ups in specific, such as the serum-free bovine follicular somatic cell cultures, the individual culture of bovine cumulus oocyte complexes and the bovine ovarian follicle culture. Notwithstanding this, we already want to propose the bovine ovarian follicle as a promising, inexpensive, high throughput *in vitro* model with broader reproductive toxicology research applications than only EDC-research. Not only can the bovine ovarian model be useful for the required *in vitro* toxicity testing of the REACH-project (Lazzari *et al.*, 2008), we think it can also be of interest in early pharmaceutical research, when for example several new drug molecules need to be tested on their potential reprotoxic nature.

Finally, we clearly encourage further biomonitoring of human follicular fluid. Our research showed that also new emerging chemicals are present in human follicular fluid. The analysis of human follicular fluid is the only way to know exactly to which contaminants the components of the human ovarian follicle are directly exposed to and to what extent this contamination fluctuates over time.

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SUMMARY

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SAMENVATTING

SUMMARY

The overall aim of this thesis was to gain more insight on the possible impact of endocrine disrupting chemicals (EDCs) on both the human and bovine ovarian follicular micro-environment.

Chapter 1A gives an overview of the chemicals currently identified as EDCs and describes the evolution of EDC-related research and the typical characteristics of these chemicals. An addition, we formulated the hypothesis that EDCs could be at least a partial, potential explanation for the declining reproductive performance observed in high producing dairy cows over the last decades.
EDCs can be subdivided in synthetic and natural EDCs. The group of synthetic EDCs consists of industrial chemicals (e.g. PCBs, dioxins, flame-retardants and PFASs), pesticides (e.g. DDT and metabolites), detergents and plastic additives (e.g. phthalates, bisphenol-A and alkylphenol polyethoxylates), synthetic hormones and preservatives, such as parabens. Phytoestrogens and mycoestrogens are examples of natural EDCs. We further describe that, although EDC-related research has been neglected for a long time, EDCs possess specific properties which makes more in depth research on the possible harmful effects of these compounds highly recommended. Several EDCs have long half-lives, are lipophilic and undergo bioaccumulation through the food chain, EDCs can have a higher bioavailability in the body compared to endogenous hormones and they can induce transgenerational effects. Furthermore, EDCs are always present as a mixture (occasionally also including their metabolites), whereby these substances can influence each other's action in an additive, adverse or synergistic way.
Over the last years, the presence of EDCs in the environment has been suggested as an additional factor contributing to the fertility problems observed in dairy cows nowadays. Because the lactation peak coincides with the best moment of establishing a new gestation, the lipophilic EDCs, such as the POPs, possibly released during lipolysis accompanying lactation, can have an adverse impact on the quality of the (ovulated) oocyte and embryo with a hampered initiation of pregnancy as a result. Different *in vitro* experiments already underpin this hypothesis. However, we point out that before definite conclusions can be drawn, reliable and recent data on the presence and concentration of EDCs in different tissue types and body fluids of dairy cows, specifically including the contamination status of the ovarian follicular micro-environment is truly essential, but currently only scarcely available.

Since our research focuses on ovarian follicular physiology, **Chapter 1B** reviews the current knowledge regarding the possible influence of EDCs on each component of this well-balanced follicular micro-environment, from the earliest stage of the folliculogenesis (e.g. primordial follicle) until ovulation. Chemicals that are able to interfere with the normal growth of primordial and primary follicles through a general cytotoxic action, can induce irreversible and indirect endocrine disrupting effects on the reproductive system. EDCs can influence the somatic cells of the ovarian follicle, thereby deregulating the well-timed local steroidogenesis, essential to generate a fully competent oocyte for ovulation. We also describe how EDCs can influence the final stages of folliculogenesis before ovulation. In addition, we also propose in **Chapter 1B** the bovine ovarian follicle and its individual components as a promising *in vitro* reproductive toxicology model due to the striking similarities between human and bovine ovarian physiology and because no additional (laboratory) animals need to be sacrificed.

Chapter 2 summarizes the main concerns which led to the aims of this thesis and summarizes the specific aims.

Chapter 3 describes the actual experiments and results of this thesis whereby each experimental set-up is being discussed in a specific subchapter.
In **Chapter 3A**, we investigated the status of contamination with EDCs of high producing dairy cows, by analyzing different tissues and body fluids of Belgian Holstein dairy cows, including follicular fluid on their content of well-known EDCs (PCBs, PBDEs and OCPs). Overall, contamination was very low in both tissue and body fluid samples, with follicular fluid samples showing no detectable levels of investigated EDCs. These (very) low levels can be largely explained by the position of the cow in the lower part of the food chain, whereby the process of bioaccumulation is very low or not even present. Therefore, EDCs can hardly be considered as a (major) cause of declining reproductive performance of high producing dairy cows in Belgium. We however could conclude that since the follicular micro-environment seems relatively free of contamination and the striking similarities between human and bovine follicular physiology, the bovine ovarian follicle and its individual components can be considered as a valuable model for EDC-related research.

In **Chapter 3B**, we investigated if the presence of EDCs in human follicular fluid could be a risk factor for a reduced developmental competence of the *in vivo* exposed human oocyte. Therefore, we analyzed follicular fluid and serum samples of women undergoing Assisted Reproductive Technology

(ART)-procedures on their content of EDCs (PCBs, PBDEs and OCPs) and linked the contamination status of the follicular fluid with the patient's characteristics and ART-outcomes. Our results demonstrated that, although the levels of the investigated EDCs are declining compared to earlier biomonitoring studies, an overall higher EDC-contamination in the follicular micro-environment was associated with a decreased fertilization rate and consequently with a lower chance of an oocyte to develop into a high quality embryo, independent of the age, BMI, E2 levels of the patient, fertilization procedure or the presence of male subfertility. Next to this, we could also document the presence of PBDEs in human follicular fluid for the first time, although only in one sample. Furthermore, very high correlations ($r > 0.93$) were found between serum and follicular fluid concentrations of EDCs, whereby the levels are around 2 – 3 times higher in serum than follicular fluid. Due to these high correlations, we were able to create regression models to predict EDC-levels in follicular fluid based on serum levels. These models can prove to be very useful when only serum samples are available.

In **Chapter 3C**, we investigated if the (potential) presence of PFASs, a group of emerging toxicants, in human follicular fluid could be a risk factor for the developmental competence of an *in vivo* exposed oocyte. Next to this, we also compared the distribution characteristics of PFASs within serum and follicular fluid. We could identify different PFASs within human follicular fluid, with PFOS in the highest concentrations, and could thus provide the first, detailed overview of the contamination status of follicular fluid with PFASs. It became clear that PFASs have a completely different distribution pattern in follicular fluid and serum than the lipophilic EDCs (**Chapter 3B**) which can mainly be attributed to the different characteristics of both chemical groups. Also opposite findings were found between the presence of PFASs and the lipophilic EDCs in relation to the ART-outcomes: unexpectedly, high PFAS-contaminated follicular fluid samples were associated with a better fertilization rate and consequently with a higher chance of an oocyte to develop into a high quality embryo.

The analyses of human follicular fluid described in **Chapters 3B & 3C** have resulted in knowledge about environmentally-relevant concentrations of different EDCs in the human follicular micro-environment. Within the ovarian follicle, granulosa cells can be considered as potentially sensitive for EDC-interference, as being mainly responsible for the steroidogenesis in the follicle. EDC-related research with granulosa cells is mainly executed using primary granulosa cells cultured in the presence of serum. Since granulosa cells spontaneously luteinize under the influence of serum, we suggest in **Chapter 1B** and **Chapter 3D** that a serum-free granulosa cell culture would be more

valuable to examine the EDC-effects on a growing follicle. This was investigated in **Chapter 3D**, where we exposed bovine granulosa cells, cultured *in vitro* in serum-free conditions for 48 h to environmentally-relevant concentrations of a PCB-mixture, *p,p'*-DDE and PFOS. We verified if these substances could be cytotoxic and/or interfere with the normal steroidogenesis in the ovarian follicle. Our results showed that the environmentally-relevant concentrations of this PCB-mixture, *p,p'*-DDE and PFOS were not cytotoxic for granulosa cells, but that for each compound, these levels interfered with the normal steroidogenic processes in granulosa cells

The overall results are thoroughly discussed in **Chapter 4**. **Chapter 5** summarizes the conclusions of this thesis and provides suggestions for future research. In the end, both an English and Dutch summary are provided in **Chapter 6**.

SAMENVATTING

De algemene opzet van deze doctoraatsthesis was om meer inzicht te verwerven over de mogelijke impact van endocriene verstoorders (EVs) op zowel het boviene als humane ovariële micromilieu.

Hoofdstuk 1A geeft een overzicht van de chemicaliën die momenteel als EVs werden geïdentificeerd en beschrijft de geschiedenis van EV-onderzoek en de typische eigenschappen van deze verbindingen. Verder wordt ook geopperd dat EVs ten minste deels een verklaring zouden kunnen zijn voor de dalende vruchtbaarheidscapaciteit van hoogproductief melkvee.

EVs kunnen onderverdeeld worden in synthetische en natuurlijke EVs. The groep van synthetische EVs bestaat uit industriële chemicaliën (bv. PCBs, dioxines, vlamvertragers en perfluoralkylverbindingen), pesticiden (bv. DDT en metabolieten), detergenten en plastiek additieven (bv. ftalaten, bisphenol-A en alkylphenol polyethoxylaten), synthetische hormonen en bewaarmiddelen zoals parabenen. Fyto-estrogenen en myco-estrogenen zijn voorbeelden van natuurlijke EVs. Verder beschrijven we dat, alhoewel onderzoek naar EVs lange tijd werd verwaarloosd, EVs specifieke eigenschappen bezitten, waardoor onderzoek naar de mogelijk schadelijke effecten van deze verbindingen ten zeerste aan te raden blijkt. Zo hebben verscheidene EVs een lange halfwaardetijd, zijn sommige EVs lipofiel en bioaccumuleren ze doorheen de voedselketen. Bepaalde EVs hebben bovendien een grotere biobeschikbaarheid in het lichaam in vergelijking met endogene hormonen en EVs zijn ook in staat om transgenerationele effecten te induceren. EVs zijn grotendeels aanwezig als mengsels (occasioneel ook met inbegrip van hun metabolieten), waarbij deze verbindingen elkaar op een additieve, tegengestelde of synergetische manier kunnen beïnvloeden.

De afgelopen jaren werd meermaals gesuggereerd dat de aanwezigheid van EVs in het milieu een additionele factor kan zijn die bijdraagt tot de huidige vruchtbaarheidsproblemen van melkkoeien. Aangezien de lactatiepiek van de koe samenvalt met het ideale tijdstip om opnieuw drachtig te worden, kan het zijn dat de lipofiele EVs, zoals de POPs, die mogelijks worden vrijgesteld tijdens de lactatie lipolyse, een negatieve impact hebben op de kwaliteit van de (geovuleerde) oocyt en embryo met een verstoorde start van de dracht als resultaat. Verschillende *in vitro* experimenten onderbouwen reeds deze stelling. Wij benadrukken echter in dit hoofdstuk dat voordat definitieve conclusies kunnen getrokken worden, betrouwbare en recente gegevens noodzakelijk zijn aangaande de aanwezigheid en concentraties van EVs in verschillende weefsels en lichaamsvloeistoffen van dit

melkvee, waarbij kennis over de contaminatiegraad van het ovariële folliculaire micromilieu essentieel is. Echter zijn deze resultaten momenteel zeer weinig beschikbaar.

Aangezien dit onderzoek focust op de ovariële folliculaire fysiologie, handelt **Hoofdstuk 1B** over de huidige kennis omtrent de mogelijke invloed van EVs op elk onderdeel van dit evenwichtige ovariële folliculaire micromilieu, van de eerste stadia in de folliculogenese (primordiale follikel) tot de ovulatie. Chemicaliën die via hun cytotoxiciteit in staat zijn om te interfereren met de normale groei van primordiale en primaire follikels, kunnen irreversibele en indirecte endocrien verstorende effecten veroorzaken op het reproductieve systeem. EVs kunnen ook de somatische cellen van de ovariële follikel beïnvloeden, waardoor ze de uitgekiende steroidogenese in de follikel, essentieel om een competente oocyt te genereren, ontregelen. We beschrijven ook hoe EVs de laatste stadia in de folliculogenese kunnen beïnvloeden. Bovendien stellen we in **Hoofdstuk 1B** de boviene ovariële follikel tezamen met zijn individuele componenten voor als een veelbelovend *in vitro* model voor reprotoxicologisch onderzoek, wegens de opmerkelijke overeenkomsten tussen de humane en boviene ovariumfysiologie en omwille van het feit dat geen proefdieren moeten opgeofferd worden bij het gebruik van dit model.

Hoofdstuk 2 vat de belangrijkste kwesties samen die tot de verschillende doelen van deze thesis geleid hebben en overloopt ook deze specifieke doelen.

Hoofdstuk 3 beschrijft de uitgevoerde experimenten en resultaten van deze thesis, waarbij elke experimentele set-up wordt belicht in een eigen hoofdstuk.
In **Hoofdstuk 3A** onderzochten we de contaminatiegraad van hoogproductief melkvee met EVs, door verscheidene weefsels en lichaamsvloeistoffen, inclusief follikelvocht, van Belgische Holstein melkkoeien te analyseren op de aanwezigheid van reeds bekende EVs (PCBs, PBDEs en OCPs). Algemeen kunnen we stellen dat de contaminatie zeer laag ligt in zowel weefsels als lichaamsvloeistoffen waarbij in de follikelvochtstalen zelfs geen detecteerbare concentraties van de onderzochte EVs werden teruggevonden. Deze zeer lage concentraties kunnen voornamelijk verklaard worden doordat koeien zich laag in de voedselketen bevinden, waardoor bioaccummulatie niet van toepassing is bij deze dieren. Daarom kunnen we besluiten dat EVs niet beschouwd kunnen worden als een belangrijke oorzaak van de dalende vruchtbaarheid van hoogproductief melkvee in België. We kunnen echter wel concluderen dat aangezien het boviene folliculaire micromilieu relatief ‘vrij’ blijkt te zijn van contaminatie en de opmerkelijke overeenkomsten tussen de humane en

boviene ovariumfysiologie, de boviene ovariële follikel kan beschouwd worden als een waardevol model voor EV-onderzoek.

In **Hoofdstuk 3B** werd onderzocht of de aanwezigheid van EVs in humaan follikelvocht een risicofactor kan zijn voor een verminderde ontwikkelingscapaciteit van de *in vivo* blootgestelde humane oocyt. Hiervoor werd follikelvocht en serum van vrouwen die een vruchtbaarheidsbehandeling ondergaan, onderzocht op de aanwezigheid van EVs (PCBs, PDBEs en OCPs). De contaminatiegraad van het follikelvocht werd vervolgens gekoppeld aan de patiëntengegevens en de vruchtbaarheidsresultaten. Deze resultaten toonden aan dat, hoewel de concentraties van de EVs dalen ten opzichte van vroegere biomonitoringstudies, een hogere contaminatie van follikelvocht met EVs geassocieerd was met een gedaalde fertilisatiegraad en als gevolg, ook met een lagere kans van de eicel om zich tot een embyro van hoge kwaliteit te ontwikkelen, onafhankelijk van de leeftijd, BMI, estradiolwaarden van de patiënt, bevruchtingsprocedure of de aanwezigheid van mannelijke subfertiliteit. Bovendien werden in dit onderzoek ook voor de eerste maal PBDEs aangetroffen in humaan follikelvocht, weliswaar enkel in 1 staal. Verder werden ook grote correlaties ($r > 0.93$) aangetoond tussen serum- en follikelvochtconcentraties van EVs, waarbij de waarden in serum ongeveer 2 tot 3 keer hoger lagen dan in follikelvocht. Door deze hoge correlaties konden regressiemodellen gecreëerd worden, zodat de EV-waarden in follikelvocht kunnen voorspeld worden op basis van serumconcentraties. Deze modellen kunnen dus van belang zijn indien enkel serumstalen beschikbaar zijn.

In **Hoofdstuk 3C** werd onderzocht of de (mogelijke) aanwezigheid van perfluoroalkylverbindingen (PFASs), een groep van vrij recente toxicanten, in humaan follikelvocht een risicofactor kan zijn voor een verminderde ontwikkelingscapaciteit van de *in vivo* blootgestelde humane oocyt. Daarnaast werden ook de distributie-eigenschappen van PFASs in serum en follikelvocht nagegaan. Verschillende PFASs konden geïdentificeerd worden in humaan follikelvocht, met PFOS in de hoogste concentratie. Deze resultaten kunnen dus beschouwd worden als het eerste, gedetailleerde overzicht van de contaminatiegraad van humaan follikelvocht met PFASs. Bovendien werd duidelijk dat PFASs een volledig verschillend distributiepatroon vertonen in follikelvocht en serum dan de lipofiele EVs (**Hoofdstuk 3B**), wat voornamelijk te verklaren valt door de verschillende eigenschappen van beide chemische groepen. Ook werden tegengestelde resultaten bekomen betreffende de uitkomst van de vruchtbaarheidsbehandeling: vrij onverwacht waren hogere PFAS-concentraties in follikelvocht

geassocieerd met een betere bevruchtingsgraad met als gevolg, ook met een hogere kans van een eicel om zich te ontwikkelen tot een hoogkwaliteitsembryo.

Door de analyseresultaten van humaan follikelvocht beschreven in **Hoofdstukken 3B & 3C** beschikten we over essentiële informatie aangaande de huidige *in vivo* contaminatie van het humane folliculaire micromilieu met EVs. In de ovariële follikel zijn granulosacellen kwetsbaar voor de invloed van EVs, aangezien deze cellen verantwoordelijk zijn voor de steroidogenese in de follikel. Onderzoek naar de invloed van EVs op granulosacellen wordt voornamelijk uitgevoerd met behulp van primaire granulosacellen gecultiveerd in de aanwezigheid van serum. Aangezien granulosacellen echter spontaan luteïniseren onder invloed van serum, wordt in **Hoofdstuk 1B** en **Hoofdstuk 3D** het gebruik van een serumvrije granulosacelcultuur voorgesteld als een meer toepasselijk model om de effecten van EVs in de groeiende follikel na te gaan. Dit werd onderzocht in **Hoofdstuk 3D**, waar boviene granulosacellen, serumvrij *in vitro* gecultiveerd, werden blootgesteld aan relevante *in vivo* concentraties van PCBs, *p,p'*-DDE en PFOS. Daarbij werd nagegaan of deze verbindingen cytotoxisch zijn voor granulosacellen en/of ze kunnen interfereren met de normale steroidogenese in de ovariële follikel. Onze resultaten toonden aan dat deze relevante *in vivo* concentraties van PCBs, *p,p'*-DDE en PFOS niet cytotoxisch bleken te zijn voor granulosacellen, maar dat de verbindingen in deze concentraties wel in staat zijn om de normale steroidogenese te Fkunnen beïnvloeden.

In **Hoofdstuk 4** worden alle resultaten onderworpen aan een grondige discussie. **Hoofdstuk 5** vat de conclusies van deze thesis samen, en bespreekt ook meerdere invalshoeken voor toekomstig onderzoek. Finaal zijn zowel een Engelse als Nederlandse samenvatting beschikbaar in **Hoofdstuk 6**.

CURRICULUM VITAE

Evi Monique Laszlo Petro werd geboren op 1 januari 1985 te Merksem. Na het behalen van het diploma hoger secundair onderwijs (Latijn-Wiskunde) aan het Onze Lieve Vrouw van Lourdesinstituut te Ekeren, begon ze haar studies Biomedische Wetenschappen aan de Universiteit Antwerpen. Ze behaalde het diploma van Licentiaat in de Biomedische Wetenschappen met onderscheiding in 2006.

Op 1 november 2006 trad Evi in dienst als Dehousse doctoraatsbursaal op het Labo Fysiologie van de Huisdieren aan de Universiteit Antwerpen, waar ze onderzoek verrichtte naar de invloed van endocriene verstoorders op de ovariële follikel. Tijdens dit doctoraatsonderzoek werd Evi geselecteerd voor het Belgische nationale bobsleeteam, waardoor haar doctoraat tweemaal werd onderbroken (Nov 2008 – Mar 2009 en Oct 2009 – Mar 2010) om zich volledig te kunnen focussen op haar bobsleecarrière. Dit resulteerde in 2010 in een selectie voor de Olympische Winterspelen te Vancouver als reserveremster. In 2011 behaalde ze het getuigschrift van de doctoraatsopleiding.

Evi Petro is auteur van verschillende publicaties in nationale en internationale tijdschriften, en was spreker op verschillende nationale en internationale congressen.

Momenteel is ze werkzaam als Senior Clinical Research Associate bij genae associates nv, de CRO gespecialiseerd in het begeleiden van klinisch onderzoek voor ‘medical devices’ (medische hulpmiddelen).

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