

# PROJECT FINAL REPORT

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## 4.1 Final publishable summary report

The sections of the report are on the following pages with each new section starting on a new page:

## **EXECUTIVE SUMMARY: layperson friendly**

Many of the chemicals that are present in the environment (environmental chemicals: ECs) enter the mother by routes such as eating, drinking and skin contact. Some of these chemicals are broken down by the mother's liver and by the placenta, but significant quantities pass to the fetus in the womb. Once in the fetus, some of these environmental chemicals may cause damage. Such chemicals include endocrine-disrupting compounds (EDCs), which either mimic or block the effects of hormones in the fetus. Resulting reproductive damage has been seen in wildlife, domestic animals and those used to test toxic chemicals and in humans themselves. Although the reproductive system in females is sensitive to the damaging effects of ECs, most focus has been on the male or has relied on a toxicological approach, which uses short-lived rodent species exposed to high doses of single ECs for short periods of time. While these studies have generated important findings, they are not necessarily a good way of understanding what is occurring in humans and domestic animals who are inadvertently exposed to ECs for long periods of time to low doses of complex "cocktails" of chemicals in the environment.

The REEF project has used three models to investigate the effects of ECs on the female reproductive system. Firstly, sheep exposed to cocktails of chemicals present in processed sewage sludge used to fertilise pasture according to agricultural guidelines. This is a "real-life" model. Secondly, mice and sheep exposed to selected chemicals, which we have shown are increased in the livers of sheep on sewage-sludge fertilised pastures. This model is designed to investigate mechanisms and pathways that lead from exposure to damage. Thirdly, normal human fetuses obtained by elective terminations from women in the second trimester of pregnancy, with a second "real-life" exposure of the fetus to chemicals imposed when the women smoked cigarettes.

The objectives of the REEF project were to:

- Examine the effects of sewage sludge exposure during specific periods of fetal ovarian development in the sheep.
- Examine the effects of environmental concentrations of specific chemicals on female reproductive development and health in the sheep and mouse. Both cell culture and whole animal approaches were used.
- Investigate the effects of these chemicals on to subsequent generations.
- Examine the effects of cigarette smoke on the human fetus.
- Integrate human and animal models in order to better understand the processes leading to reduced fertility and other problems such as increased obesity.

*Main conclusions of the REEF project:*

- Exposure to sewage sludge chemicals has different effects on the female fetus than the male, with the fetal ovary most sensitive to ECs late in pregnancy.
- Different individual chemicals have different effects from each other and from sewage sludge and have more damaging effects on the mouse ovary than the sheep.
- In the mouse, the damaging effects can pass through to the great-grandchildren of the animal exposed to ECs while pregnant.
- In sheep, grand-daughters of exposed animals did not have abnormal ovaries.
- DEHP and PCBs act as "obesogens" making mice fatter.
- A women who smokes while pregnant increases the quantity of ECs reaching her fetus and causes fetal ovary changes likely to reduce fertility in adulthood.

In conclusion, the REEF project has shown complex effects of ECs on the female reproductive system, with differences in the nature and degree of damage varying depending on the stage of pregnancy during exposure, the sex of the fetus and the species being investigated. Since some findings were also not predicted, REEF suggests that caution must be used to extrapolate EC exposure findings to humans.

## **SUMMARY OF PROJECT CONTEXT AND OBJECTIVES**

During the last decade, concerns have emerged about the increasing incidence of abnormalities in reproductive function and other aspects of health and wellbeing in humans, farm animals and some wildlife species and the possible correlation with exposure to environmental compounds (ECs), including endocrine disrupting compounds (EDCs), heavy metals and other potentially toxic elements (PTEs) and other pollutants (Toppari et al, 1996 *Environ Health Perspect* 104 (Suppl 4) 741-803). Moreover, it is increasingly recognised that animals are most vulnerable to such adverse effects during early stages of development (Rhind, 2005. *Reprod Dom Anim* 40 282-290) and there is evidence that effects of exposure in one generation can be expressed in the unexposed offspring of later generations.

In humans, problems in gametogenesis, oocyte transport, hormonal preparation of the uterine lining, implantation and viability of the conceptus all produce difficulty in achieving a recognised pregnancy, but an estimated 30% of infertility cases are unexplained (Evers, 2002. *Lancet* 360 151-159). Premature ovarian failure (POF) is a primary ovarian defect characterized by absent menarche (primary amenorrhea) or premature depletion of ovarian follicles before the age of 40 years (secondary amenorrhea). Amongst the possible causes, environmental pollutants, as well as genetic defects, drugs and autoimmunity have been implicated. Populations of numerous wildlife species are in decline and in many cases exposure to ECs and EDCs, in particular, have been implicated in this process. The proposed effects have been detected in various components of reproductive systems (Rhind, 2009. *Phil Trans Royal Soc* 364 3391-3401).

Major stages of ovarian development (oogenesis, folliculogenesis and steroidogenesis) take place during fetal life and it is becoming increasingly clear that that female pathologies result from alterations in fetal ovary development. Indeed, oocyte production in adulthood depends on fetal ovary development since it is during this period that the germ cell stock is established, that meiosis initiates and follicles form and begin to differentiate. By breaking gestation down into discrete biologically relevant periods (i. sex determination and early ovary development; ii. around primordial follicle formation; iii. secondary and then antral follicle formation) and comparing sewage sludge exposed to non-exposed animals, we aimed to investigate the impact of real-life, environmentally relevant EC cocktails on critical events during ovary genesis. Studying maternal and fetal genes, proteins, hormones and ovarian morphology we will unravel key effects of such exposure and identify candidate genes involved in the mechanism of action of EC cocktails.

The involvement of ECs in the perturbation of female reproductive tissue development in the fetus and on subsequent adult reproductive function has been postulated (Hruska et al. *Clin Obstet Gynecol* 43 821-829, 2000 ). EDCs include a wide range of groups of chemicals, primarily anthropogenic in origin, such as dioxins, polychlorinated biphenyls (PCB), organochlorine pesticides and plasticizers that have been/are used extensively in manufacturing and agriculture. Together with heavy metal pollutants, EDCs are ubiquitous in the environment, and domestic animals, as well as wildlife species, are potentially exposed to them (Colborn et al., 1993. *Environ Health Perspect* 101 378-384; Fries, 1995. *J Anim Sci* 73 1639 -1650). Through the consumption of meat and dairy products, and probably through many other routes, such as inhalation and absorption through skin, humans are also exposed. However, target tissue concentrations are poorly defined and their mechanisms of action are poorly understood.

Associations between tissue concentrations of EDCs and expression of variables

relevant to reproductive dysfunction cannot, in isolation, demonstrate a causal relationship. This problem is compounded by the fact that mixtures of pollutants of different classes can operate in an additive (or even contrary) manner, possibly in conjunction with inorganic metal pollutants. A further difficulty arises when addressing effects on humans since deliberate contamination of human subjects to investigate possible causal relationships is ethically unacceptable. Thus, there is a need to study animal models with real-world rates of exposure, focussing on likely components of relevant reproductive circuits and mechanisms.

To date, much of the work concerning effects of ECs, EDCs and other pollutants has focussed on rodent models, using single compounds administered for short periods, often at pharmacological doses. While such studies enhance understanding of the mechanisms of action of individual compounds, they do not elucidate risks to animal production, ecosystem wellbeing and human health. These depend on prolonged exposure to low real-world levels of a mixture of pollutants including low concentrations of multiple compounds. The work of this project was designed to take account of these limitations and to address effects of prolonged, low level exposure to a mixture of pollutants.

Chemicals of many classes have been shown to perturb reproductive function in animals and humans (IEH, 1999) and they are known to act additively (Rajapakse et al., 2002 *Environ Health Perspect* 110 917-921). Accordingly, some of our studies were based on sheep exposed to sewage sludge-treated pastures to simulate real-world exposure to a mixture of ECs. Clearly, it is impossible to measure every individual chemical, or even every class of chemical, partly because insufficient material is available in some cases. A range of chemicals representative of a number of key classes known to exert adverse biological effects was therefore selected for study. Soil and animal and human tissues were analysed in the Macaulay Institute's accredited laboratories.

The environmental burden of ECs and EDCs comprises something like 100,000 different chemicals and since it is clearly logistically impossible to measure them all, ubiquitous, environmentally-persistent chemicals with diverse chemical properties, all of which are known to exert endocrine disrupting effects, were selected for study:

(a) Diethylhexyl phthalate. Phthalates are high-production, synthetic chemicals that are ubiquitous environmental contaminants because of their use in plastics and other common consumer products. Di-2-ethylhexyl phthalate (DEHP) is the most abundant phthalate in the environment. Humans are exposed to these compounds through ingestion, inhalation and dermal exposure for their whole lifetime including intrauterine life. It has been suggested, previously, that tissue accumulation of phthalates was likely to be of little biological significance because they are readily degraded, either in the environment (Fries, 1996. *Sci Total Environ* **185** 93 -108) or in the digestive tract (Heindel et al., 1989. *Fundam Appl Toxicol* 12 508-518) but they nevertheless they are present in sheep tissue (Rhind et al., 2005. *Environ Health Perspect* 113 447-453).

(b) Selected polychlorinated biphenyls (PCB); the ICES 7, a set of congeners (28, 52, 101, 118, 138, 153, 180) that is internationally recognised as an appropriate measure of PCB pollution, was used. These are also of special interest due to their known ubiquitous environmental persistence, tissue accumulation and reproductive toxicity. They are known to accumulate in the tissue and milk of many species of animals and humans (Bachour et al., 1998. *Arch Environ Contam Toxicol* 35 666-673). While concentrations of most PCB were found to be higher in maternal than fetal tissue, the reverse was found for PCBs 101 and 118 (Rhind et al., 2010; *J Environ Monit* 12

1582-1593). PCB reach the embryo as early as shortly after conception by uterine fluid and while concentrations are low, it is known that early embryos are potentially sensitive to such low levels of PCB. Furthermore, in-utero exposure to PCB mix during pregnancy reduces the number of preantral and antral follicles of certain size classes in rats, indicating that the developing ovary is a sensitive target organ to PCB exposure, an important finding may account for reduced fecundity and fertility in adult offspring.

(c) Polybrominated diphenyl ethers (PBDE) congeners covering a range of bromination (28, 47, 99, 100, 153, 154, 183). While environmental levels of PCBs are generally declining, those of PBDEs have increased in recent years owing to their extensive use as fire retardants (Darnerud et al., 2001. *Environ Health Perspect* 109 (Suppl 1) 49-68). They are chemically related to PCBs and may exert similar effects.

(d) Polycyclic aromatic hydrocarbons (PAH); 16 compounds that have been defined by the US EPA as priority pollutants. Many of these are derived from the combustion of fossil fuels, as well as from forest fires and volcanoes and so they are highly ubiquitous in the environment.

(e) Metals – potentially toxic elements as well as important elements in nutrition were measured Cd; Cr; Cu; Ni; Pb; Zn (“toxic 6”) and Hg; As, Mo, Mn, Na, K, Mg, Ba, Ti, V, W, Fe, Co, B, Al, Sn, P, Sb, Se, S. The toxicity of some metals at relatively high concentrations has long been recognized but they may also act at low concentrations, perhaps in conjunction with other chemicals.

We already know that DEHP and two PCBs preferentially accumulate in the sheep fetus following exposure of their mothers. We therefore investigated, in separate studies, effects of combinations of these chemicals on both the fetal sheep and mouse ovaries. EC-sensitive genes and proteins identified in our animal studies were used as signposts for studies of normal second trimester human fetal ovaries, to better understand the risks of ECs on human female reproductive development in the womb. The mouse was used to study the mechanisms by which ECs interfere with reproduction. We used both models to study whether or not the effects of chemicals on a developing fetus are passed on to her offspring in turn.

We hypothesised that altered female reproductive function and health could be due to the reprotoxic effects of environmental factors acting mainly during fetal life. Since our preliminary data in the sewage sludge ovine model indicated that DEHP and PCBs preferentially accumulate in the fetus, we aimed to focus on the impact of these chemicals, as well as a real-life cocktail of environmental chemicals (ECs) in sewage sludge, on the developing female gonad. Through imprinting/methylation analyses, the importance of transgenerational effects of developmental insults on the fetus was investigated.

Our overall objective was to link measurements of tissue EC concentrations and associated physiological indices in animal models to parallel measurements of EC concentrations and physiological indices in human fetuses derived from mothers with different patterns of EC exposure (according to relevant indices: cigarettes/day, maternal age, weight, height, on-going illness and medication). By measuring human and animal tissue EDC concentrations and associated physiological changes in parallel, it was aimed to extrapolate animal responses to humans in a controlled manner and thereby improve understanding of relationships between exposure and effect in humans. Despite the best efforts of many research teams, this remains a critical blind-spot in our current understanding.

The objectives of the studies were:

1. To assess rates of accumulation of selected EDCs in animal tissues and, in particular, to determine:
  - a. Relative concentrations in maternal and fetal tissues,
  - b. Differences with chemical class in tissue burdens and
  - c. Effects of exposure on rates of accumulation stages of fetal development.
2. To assess impacts of real-life exposure at low concentration for a prolonged period of cocktail of chemicals on mammalian female reproduction (sheep).
3. To investigate molecular mechanisms of ovarian development affected by exposure to environmental chemicals (alone or in mixture) in-utero in sheep and mice.
4. To conduct a functional analysis of pollutant effects on female reproductive function in two animal models (sheep and mouse).
5. To investigate transgenerational transmission of reproductive defects in F2 and F3 animals. Our ultimate objective was to determine how the developing human female fetus might be affected by exposure to ECs during development. One of our key objectives, therefore, was to integrate the mouse and sheep exposure studies with others on the human. Accordingly, we aimed to determine the expression and localisation of EC-sensitive genes in the human fetal ovary itself. This is based on the rationale that electively terminated, normal, fetuses from women who either did or did not, smoke cigarettes during pregnancy are an excellent model for the study the effects of complex mixtures of ECs on fetal human development (Fowler et al., 2012 Mol Cell Endocrinol in press).

***The expected outcomes of this study will allow the assessment of the nature and intensity of the reproductive responses to both selected compounds and complex mixtures.***

## DESCRIPTION OF THE MAIN S&T RESULTS/FOREGROUNDS

### TECHNICAL ADVANCE: PRODUCTION AND OPTIMISATION OF A CUSTOMIZED OVINE MICROARRAY

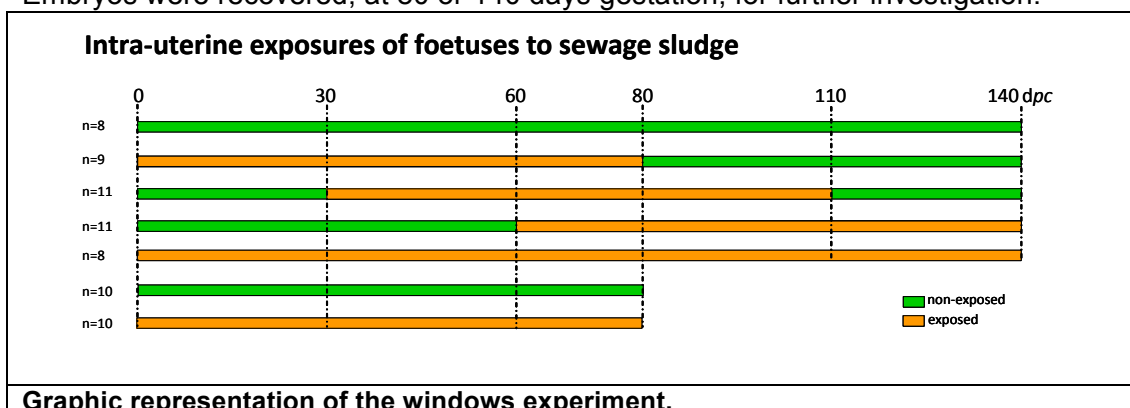
REEF required the development of a specific tool: the sheep microarray produced by Agilent (commercially available since July 2008). This array contained fifteen thousand 60-mer oligos with eight arrays by slide allowing the study of eight samples by slide. The oligos were designed from transcripts present in different sheep libraries available in databases and very few involve reproductive function. Consequently, we decided to improve this tool by adding ovarian transcripts isolated by Partner 3 (Baillet et al. BMC Genomics. 2008; 9:436). Moreover, in collaboration with the INRA bioinformatics service (<http://www.sigenae.org>) we have greatly improved the annotation of this array. The last version of annotation is linked to about 7000 different genes. In addition, we have added about a hundred genes known to be involved in gonad differentiation and gametogenesis. This array has been validated and optimised for studies of the sheep gonad.

### WHEN DURING GESTATION IS FETAL SHEEP REPRODUCTIVE DEVELOPMENT MOST SUSCEPTIBLE TO DAMAGE BY EXPOSURE TO EDCS?

By breaking gestation down into discrete biologically relevant periods (i. sex determination and early ovary development; ii. around primordial follicle formation; iii. secondary and then antral follicle formation) and comparing sewage sludge-exposed and non-exposed animals, we aimed to investigate the impact of real-life, environmentally relevant EC cocktails on critical events during ovary genesis. Groups of 14 ewes were synchronised in oestrus and mated at different times so that the groups were exposed to sludge-treated pastures during broadly the same calendar period but at the different stages of gestation:

- (i) unexposed during 0-80 days of gestation (control) and slaughtered at 80 days gestation;
- (ii) exposed during 0-80 days and slaughtered at 80 days gestation;
- (iii) exposed during 0-80 days and slaughtered at 140 days gestation;
- (iv) exposed 30-110 days and slaughtered at 140 days gestation;
- (v) exposed 60-140 days and slaughtered at 140 days gestation;
- (vi) exposed 0-140 days of gestation and slaughtered at 140 days gestation;
- (vii) unexposed during 0-140 days of gestation (control) and slaughtered at 140 days gestation.

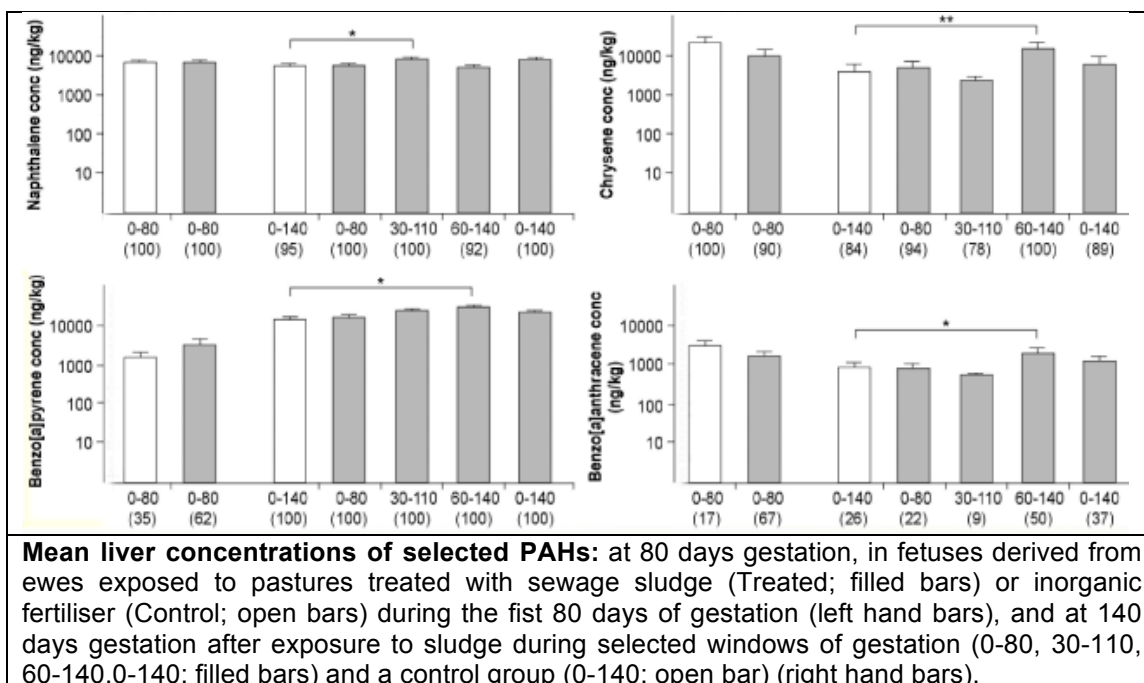
Embryos were recovered, at 80 or 140 days gestation, for further investigation.



### Effects on fetal and maternal EDC load

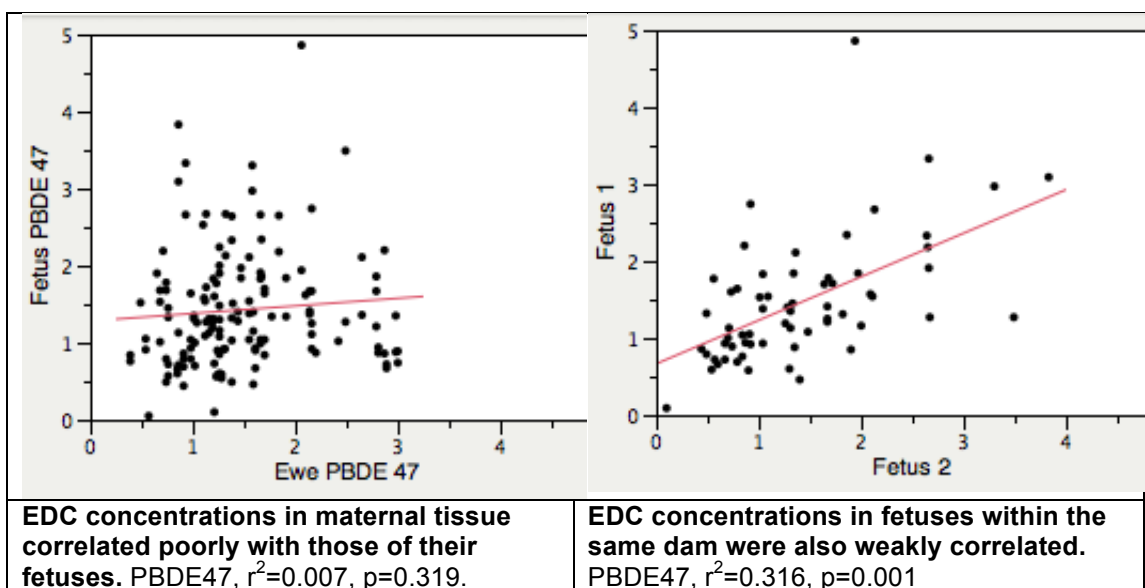
The objective was to understand the efficiency of transfer to the fetus of ECs of

different classes. Maternal and fetal tissue EDC determinations of selected PCBs (28,52, 101, 118, 138, 153, 180), PBDEs (28, 47, 99, 100, 153, 154, 183), PAHs (naphthalene, acenaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene and benzo[ghi]perylene) and DEHP) were determined. Concentrations of some individual congeners were below the limit of detection in the majority of tissues and so there were insufficient data for meaningful statistical analysis; these data are not reported. Tissue concentrations of all EDC classes were highly variable across individual animals, including animals on the same experimental treatment and from the same window of gestation. Maternal liver concentrations were higher in the sludge-exposed animals for DEHP (0-80; 30-110 days) and PCBs 153 and 180 (30-110 days). PBDEs were generally very low but the direction of differences with window of exposure was not consistent. There were differences with window of exposure in PAH concentrations but the direction of differences was not consistent. There were no differences between sludge-exposed and unexposed groups in tissue pollutant concentrations at 80 days of pregnancy.



Exposure of ewes from 30-110 or 60-140 days gestation was found to be associated with lower ( $P=0.001$ ) fetal liver concentrations of PCB congeners 28 and 118 than in unexposed controls. Concentrations of polybrominated diphenyl ether (PBDE) congener 47 were lower ( $P=0.02$ ) in animals exposed from 60-140 days. On the other hand, there were higher mean fetal liver concentrations of naphthalene ( $P<0.05$ ), chrysene ( $P<0.01$ ), benzo[a]pyrene ( $P<0.05$ ) and benzo[a]anthracene ( $P<0.05$ ). Similarly, fetal DEHP was elevated in those exposed at 60-140 days ( $P<0.05$ ) and 30-110 days ( $P<0.05$ ). One example of the variance in tissue burdens with chemical and stage of gestation is illustrated below:





These data suggest that tissue burdens are influenced by many different factors, perhaps including rates of uptake by the dam, efficiency of transfer across the placenta and metabolism and excretion rates in the fetus.

**Conclusion** The effects of exposure to elevated environmental pollutant levels on fetal tissue concentrations of pollutants differed, greatly, with a) individual, b) the type of pollutant (both within and between chemical classes) and c) the stage of development at which the fetuses were exposed. Although adverse effects of exposure on fetal reproductive function were found, maternal exposures to sludge-treated pastures were not necessarily associated with higher fetal tissue concentrations, which showed minimal relationships between dam and fetus or between fetuses of the same litter.

#### **Effects on fetal and ovarian morphology and endocrinology**

Morphologically the day 60-140T fetuses were the most affected. However, AGD was increased in this group but this was not due to elevated testosterone at day 140 since only the 0-80T group increased testosterone.

<b>Effects of exposure to sewage sludge applied to pasture, at different times of gestation, on the morphology and endocrinology of day 140 ovine female fetuses.</b> Bold indicates significant differences vs Control (0-140C) at p values <0.05. Paired organs are combined into a single weight.					
<b>Exposure Groups</b>	0-140C Control	0-140T Continuous	0-80T Early	30-110T Intermediate	60-140T Late
<b>Morphology</b>					
No of female fetuses	9	8	9	11	10
Fetal weight (kg)	<b>5.01±0.20</b>	4.59±0.32	4.29±0.25	4.29±0.27	<b>3.59±0.33</b>
<i>Body weight-normalised parameters (kg)</i>					
AGD <sup>B</sup> (mm/kg)	<b>2.0±0.2</b>	1.7±0.1	1.9±0.2	<b>3.3±0.2</b>	<b>3.1±0.3</b>
Ovary (mg/kg)	16.6±2.0	20.1±3.2	19.4±2.6	14.3±0.9	17.5±1.2
Thyroid (mg/kg)	<b>221±18</b>	223±20	227±17	265±17	<b>308±26</b>
Adrenals (mg/kg)	<b>125±12</b>	115±3	110±8	128±9	<b>156±14</b>
Uterus (mg/kg)	<b>212±10</b>	233±16	244±19	217±15	<b>162±11</b>
Liver (g/kg)	<b>24.3±0.4</b>	<b>20.5±1.1</b>	24.2±1.2	24.9±1.1	<b>28.4±1.7</b>
<b>Endocrinology</b>					
Testosterone (nmol/l)	<b>2.7±0.1</b>	3.2±0.3	<b>3.5±0.3</b>	2.7±0.3	2.3±0.1
Progesterone (nmol/l)	119±11	102±20	136±18	90±11	132±13
Free T3 (pmol/l)	<b>2.7±0.2</b>	3.4±0.4	3.0±0.3	3.2±0.2	<b>1.7±0.2</b>
Free T4 (pmol/l)	<b>25.3±1.3</b>	26.3±2.2	26.7±1.4	27.1±1.4	<b>29.5±1.0</b>

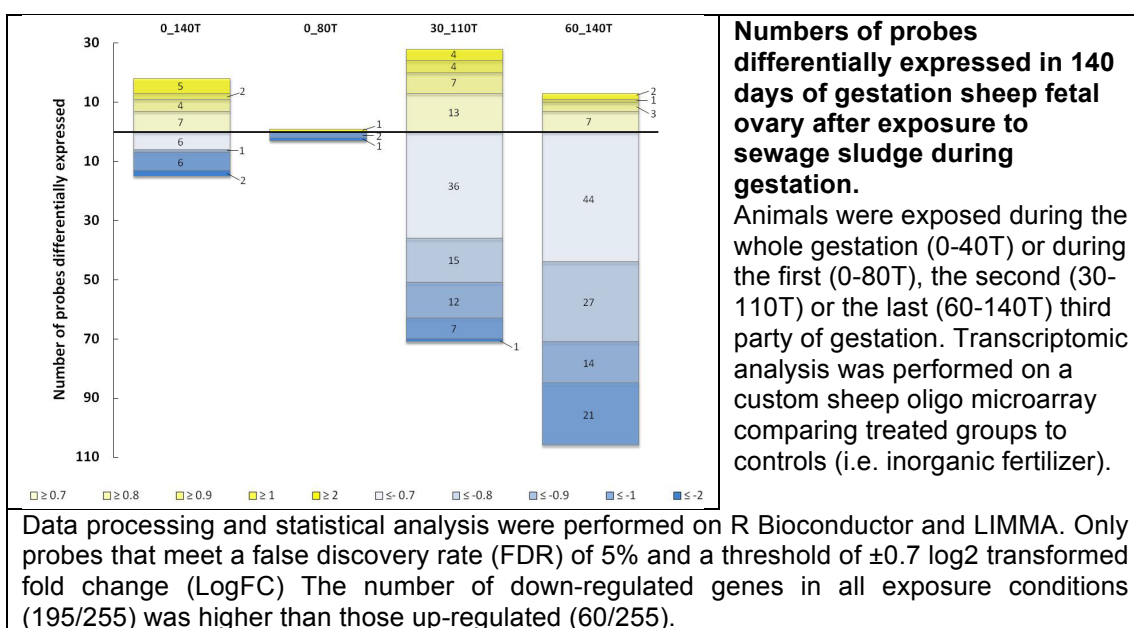
Numbers of Type1 and also Type 4 and larger follicles showed no differences, but follicle characteristics of the day 60-140T group were most affected by exposure, especially in terms of numbers of atretic follicles and type 2 follicles, suggesting increased activation.

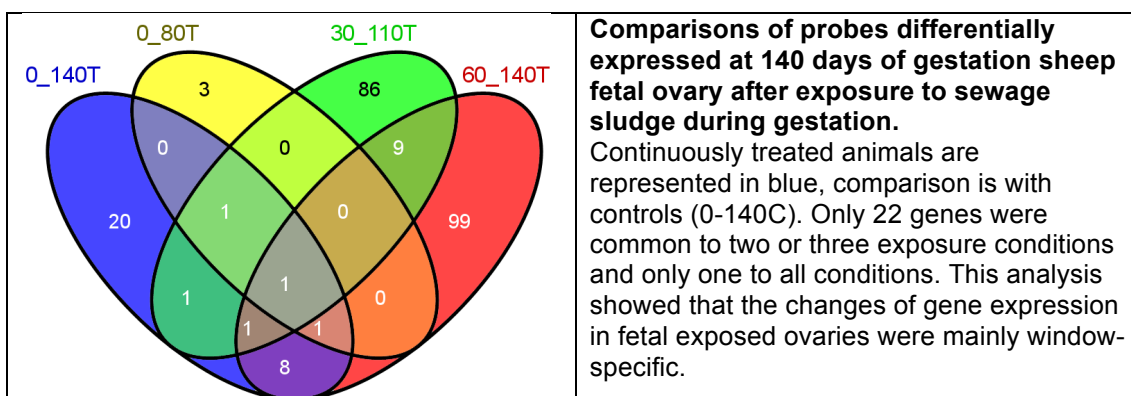
**Effects of sewage sludge chemicals on ovarian oocyte/follicle density of day 140 fetuses exposed at different times of gestation.** Bold indicates statistically significant differences vs Control (0-140C) at p values <0.05.

Exposure Groups	Oocyte/follicle density (no/mm <sup>2</sup> )				
	0-140C Control	0-140T Continuous	0-80T Early	30-110T Intermediate	60-140T Late
All types	18±7	19±4	20±5	30±4	29±6
Healthy	17.26±7.02	17.69±3.46	17.59±4.07	26.14±3.89	26.25±5.48
Atretic	<b>0.46±0.19</b>	1.05±0.35	1.24±0.43	<b>2.45±0.49</b>	<b>2.05±0.5</b>
Type 0	0.35±0.20	1.02±0.42	1.05±0.36	1.57±0.51	0.56±0.27
In nests	<b>0.15±0.11</b>	0.85±0.44	0.56±0.28	<b>1.46±0.49</b>	0.48±0.27
Healthy	0.25±0.14	0.90±0.39	0.85±0.30	1.35±0.44	0.42±0.24
Atretic	0.07±0.05	0.04±0.03	0.10±0.04	0.13±0.07	0.07±0.04
Type 1a	11.86±4.57	11.67±1.98	11.53±2.79	18.24±2.77	17.13±2.96
Healthy	11.56±4.45	10.83±1.87	10.14±2.36	16.11±2.65	15.61±2.63
Atretic	<b>0.19±0.09</b>	0.57±0.18	0.94±0.39	<b>1.58±0.30</b>	0.98±0.28
Type 2	<b>1.66±0.29</b>	1.85±0.54	1.11±0.21	2.29±0.37	<b>4.54±0.89</b>
Healthy	1.59±0.27	1.60±0.50	1.05±0.21	1.81±0.29	3.84±0.74
Atretic	<b>0.06±0.02</b>	0.24±0.15	0.04±0.01	0.39±0.15	<b>0.65±0.33</b>

### Effects on the fetal ovarian transcriptome

We identified 255 probes as differentially expressed according to the window of exposure. 3.4% (255/7500) of genes were significantly differentially expressed between controls and exposed fetal ovaries. Thirty-three genes (0.4%) were significantly differentially expressed at 0-140T (exposed continuously) against only eight genes in the 0-80T group. A total of 99 genes (1.32%) and 119 genes (1.58%) were significantly differentially expressed in the 30-110T and 60-140T groups respectively. These groups were exposed in the period of follicle formation (75-140dpc in sheep). It is noteworthy that the continuous exposure (0-140T) produced less change in ovarian transcriptome than shorter (30-11T and 60-140T) windows of exposure.





### Effects on the fetal ovarian proteome

Numbers of ovarian protein spots differentially altered in comparison to control fetuses.	
Exposure groups	Total
0-140T	4
0-80T	7
30-110T	15
60-140T	13
0-140T & 0-80T	1
0-140T & 60-140T	2
30-110T & 60-140T	8
0-80T & 30-110T	4
0-80T & 60-140T	2
0-80T & 30-110T & 60-140T	5
All groups	2

Overall 63 protein spots showed at least one EDC exposure effect. While the patterns of changes were complex, two conclusions emerged: (i) that the 30-110T and 40-160T groups were the most affected and that the majority of proteins were up-regulated (51 vs 12), opposite to the transcriptome data.

The identified proteins fell into functional categories: (i) Structural/cytoskeleton (*LMNA*, *TAGLN*, *DPYSL2*) (ii) Transcription/signal transduction/immune response (*HMGB1*, *SARNP*, *COPS4*) (iii) Chaperone (*PDIA3*, *ALDH1A2*, *UCHL1*) (iv) Enzyme activity/ multiple functions (*PHGDH*, *BLVRA*, *AKR1B1*, *MTHFD1*, *PPP1CB*, *TALDO1*, *ME1*, *SUCLG2*, *SEPHS1*, *IDH3A*, *ACADSB*, *ACSF2*) (v) Molecular binding/transport (*HBB*, *TF*). Most of these proteins were either oocyte-enriched or oocyte-specific.

### Conclusions

IPA (<http://www.ingenuity.com>) functional analysis tools were employed to explore the distribution of differentially expressed genes. This analysis indicated that differentially expressed transcripts were associated with cellular growth and differentiation, cell cycle, cell death, cellular development and cell movement. These cellular functions are particularly active during follicle formation when pre-granulosa cells proliferate and move to surround oocytes and were particularly affected during the 60-140 window, which was most affected morphologically. Among the biological pathways mostly disturbed, we founded ERK/MAPK and PI3K/AKT signalling, growth hormone and actin cytoskeleton signalling pathways involved in early folliculogenesis. During this process a massive apoptosis occurs and we observed increased BAX in the 60-140T group. Transcriptome analysis of fetal ovaries revealed predominantly a decreased level of gene expression. This could be due either to a repressive action of ECs on the promoters of numerous genes (or of some key regulators) in each cell type of the ovary either to a decreased of one or more cell type within the gonad. As the number of type 2 follicles increased in 60-140T, we can hypothesise that genes repressing the type1-type 2 transition were under expressed inducing a large activation of type 1 follicles. The fact that the ovarian pro-apoptotic Bax protein was significant reduced in 60-140T ovaries could explain a decreased apoptosis in the exposed ovaries. Indeed these ovaries presented a total

number of follicles significantly increased in the 60-140Tvs control. Ovaries from Bax<sup>-/-</sup> mice present an excess of follicles consequently to a default of apoptosis.

Surprisingly, the continuous exposure (0-140T) produced less change in ovarian transcriptome than the 30-110T and 60-140T exposure windows. One explanation could be the setting up of an adaptative mechanism when the exposure becomes chronic. In contrast, when the exposure is acute, the disturbances are more intense. The changes in exposed ovaries were mainly window-specific. The period of follicle formation and differentiation is a critical period for the ovary.

# WHAT EFFECTS DO SPECIFIC EDCS (ELEVATED IN TISSUES OF SEWAGE SLUDGE-EXPOSED SHEEP) HAVE ON IN-VIVO FETAL SHEEP REPRODUCTIVE DEVELOPMENT?

The effects of DEHP, PCBs (101+118) and a mixture of both, on fetal reproductive development were investigated in the same way as the WINDOWS study above.

## FEMALES

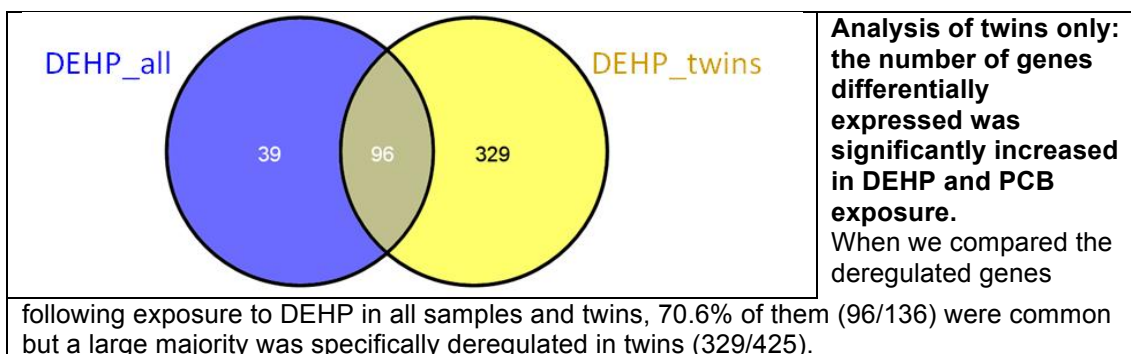
### Effects on fetal and ovarian morphology and endocrinology

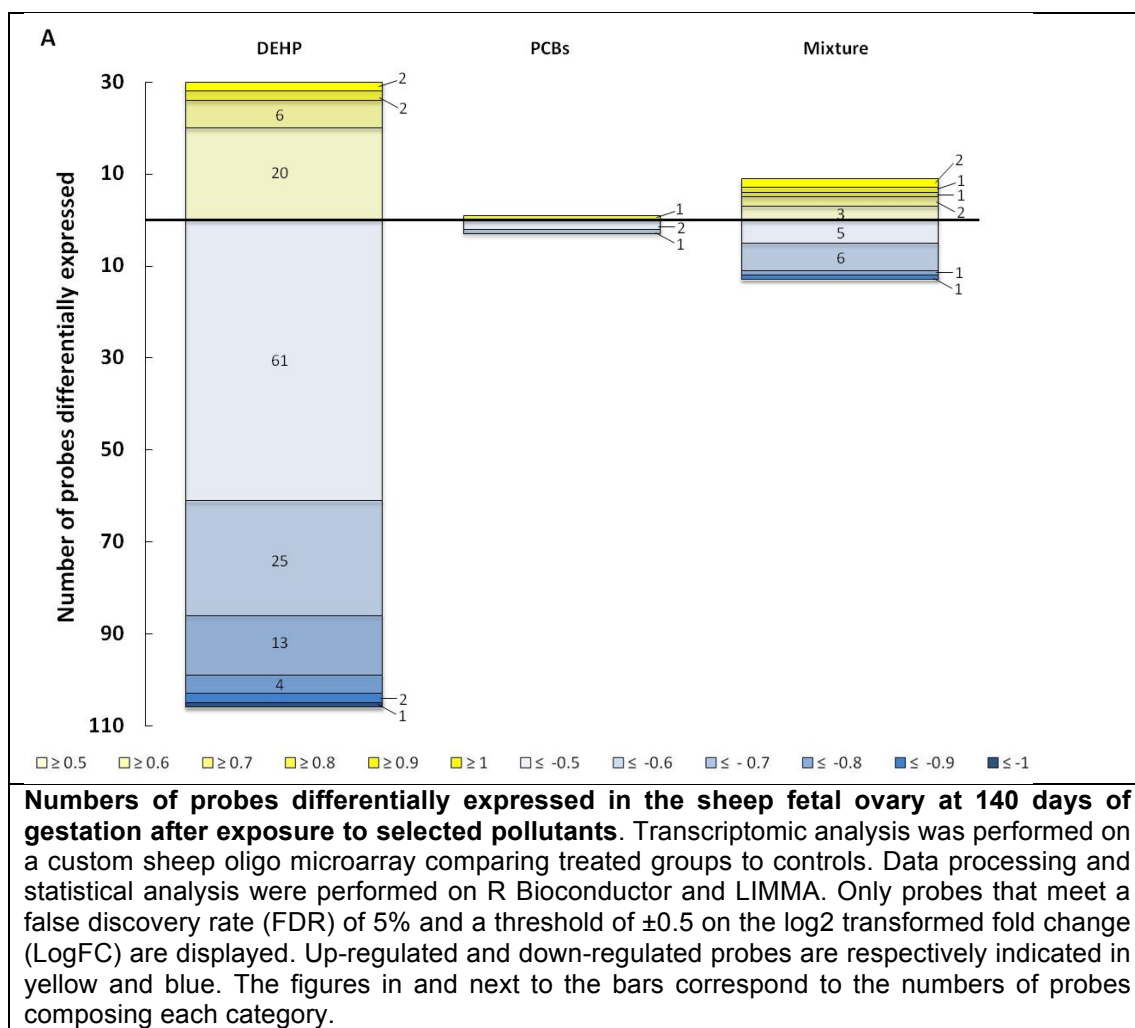
There were no overall significant effects of the exposures on fetal ovarian follicle dynamics at day 140 compared with control fetuses (vehicle only).

### Effects on the fetal ovarian transcriptome

In DEHP exposure, a total of 136 genes were differentially expressed between Controls and exposed fetuses (FDR 5% and absolute fold-change (aFC) > 1.5) Figure 2.5). A greater number of genes were down-regulated (106/136) as compared to those that were up-regulated (30/136), and this observation was in accordance to the data obtained in "Windows" experiments. A much smaller number of genes were significantly affected by PCB exposure; only 4 genes were differentially expressed. When considering the effect of mixture DEHP+PCBs, only 24 genes were differentially expressed (9 up, 13 down). It is surprising that the number of affected genes was fewer in mixture condition than in DEHP alone. The effects of both compounds were not additive.

The most affected functions by DEHP exposure appeared to be RNA Post-transcriptional modifications, protein synthesis and both cell cycle and cell death. The functions commonly affected both in all samples and in twins were cell death, cell cycle and cell morphology. The most regulated pathways were EIF2 signalling and protein ubiquitination pathway (see below).



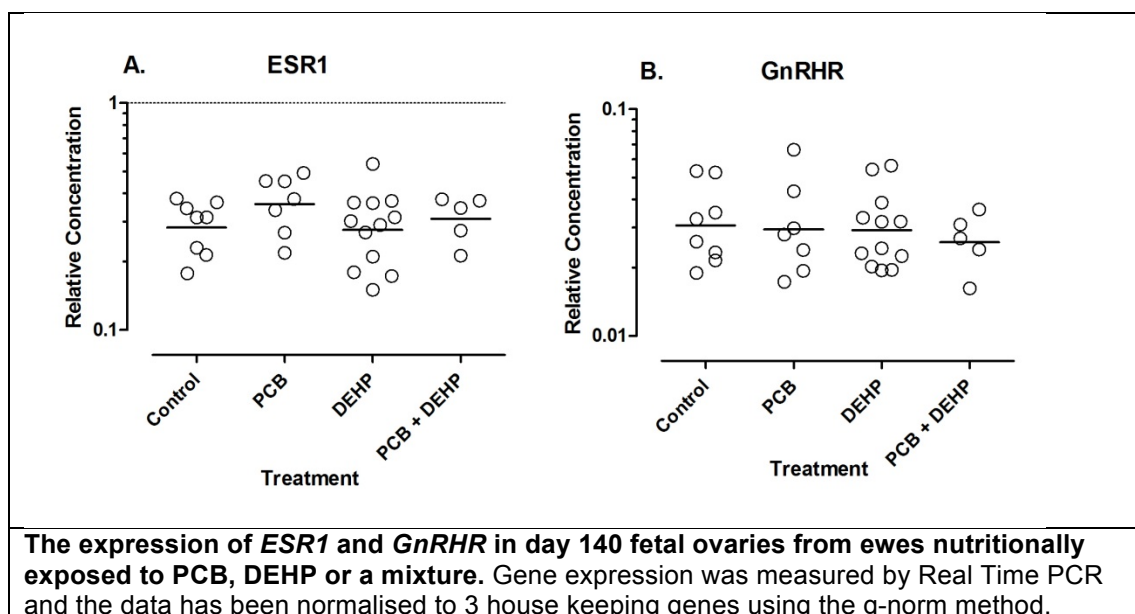


### ***Effects on the fetal ovarian proteome***

94 of 776 protein spots were differentially expressed ( $P < 0.05$ ). The largest single group, 27 spots  $\geq 1.2$ -fold,  $p < 0.05$ , were different between the DEHP+PCB mixture and the controls, which is startlingly different to the transcriptomic findings. A large proportion of identified proteins had metabolic or detoxification functions (ME1, GSTM1, GSTM3, LTA4H, DDAH2, MPST, RBP4), and a number had structural and signalling (LMNA, DPYSL2, CFL1, ANXA5, TGM2, SEP11, RPLPO), replication (MCM), or transport (HBA1, HBB, TF).

### ***Effects on pituitary gene expression***

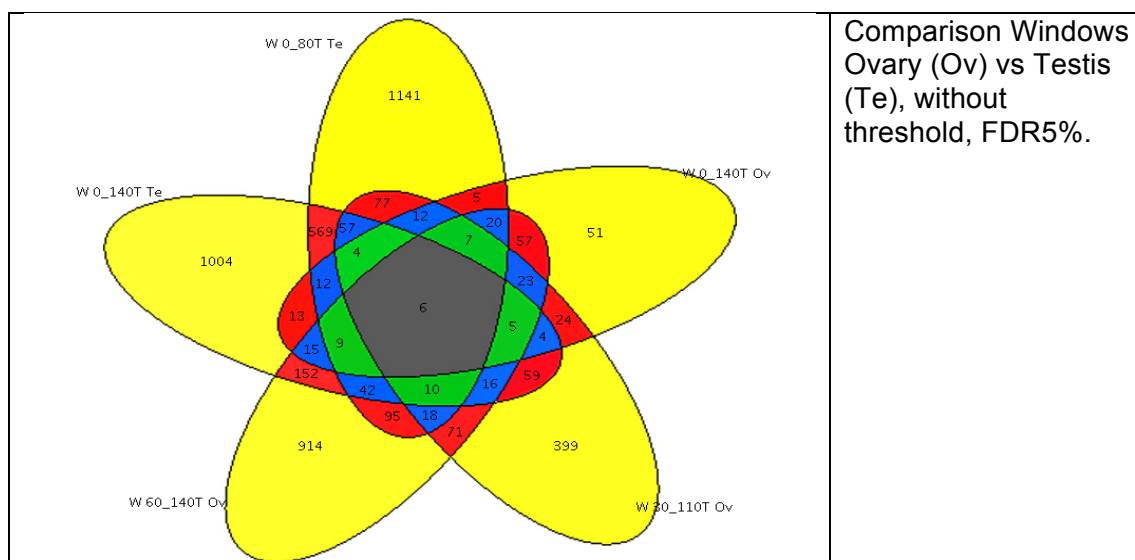
There were no significant treatment effects on *ESR1* or *GnRHR*. It is uncertain if this reflects reduced sensitivity of the pituitary to these chemicals compared with the sewage sludge model since this awaits studies of additional genes.



### Effects of DEHP, PCBs and DEHP+PCBs on the fetal testis

When we compared male and female gonads exposed continuously to sewage sludge during the period 0-140days of pregnancy, 13 genes were common among these: prostaglandin F2 receptor negative regulator (PTGFR), Multidrug resistance-associated protein 5 (ABCC5), Rho/Rac guanine nucleotide exchange factor 2 (ARHGEF2), and Progesterone receptor Fragment (PGR). The comparison between all the female data (0-140, 30-110, 60-140) with the testis (0-140) pointed out 5 genes differentially expressed (SETDB1, SMARCA4, SHMT1 FAM40A DLST).

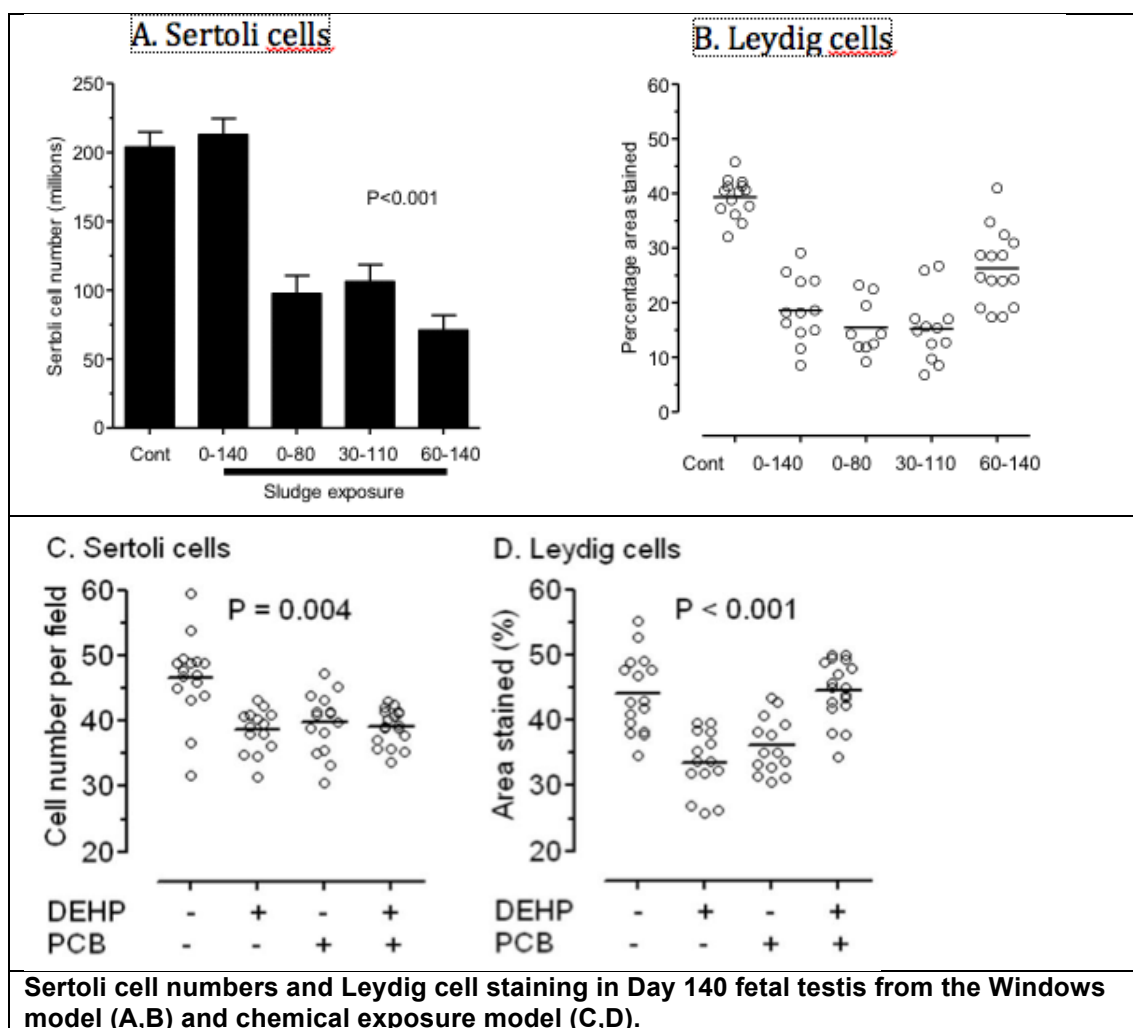
Comparison of all the transcriptomic data from male and female gonads exposed in utero to sewage sludge showed that 6 genes were differentially expressed in all conditions. They correspond to: Tyrosine-protein kinase receptor (DDR1), Werner helicase interacting protein 1 (WRNIP1), vacuolar protein sorting 4 homolog A (VPS4A), MAP kinase-interacting serine/threonine-protein kinase 1 (MKNK1), Deoxyribonuclease-1 Precursor (DNASE1) and adiponectin receptor 1 (ADIPOR1). This latter was also common in all female *in vivo* data; it mediates increased AMPK, PPARA ligand activity, fatty acid oxidation and glucose uptake by adiponectin.



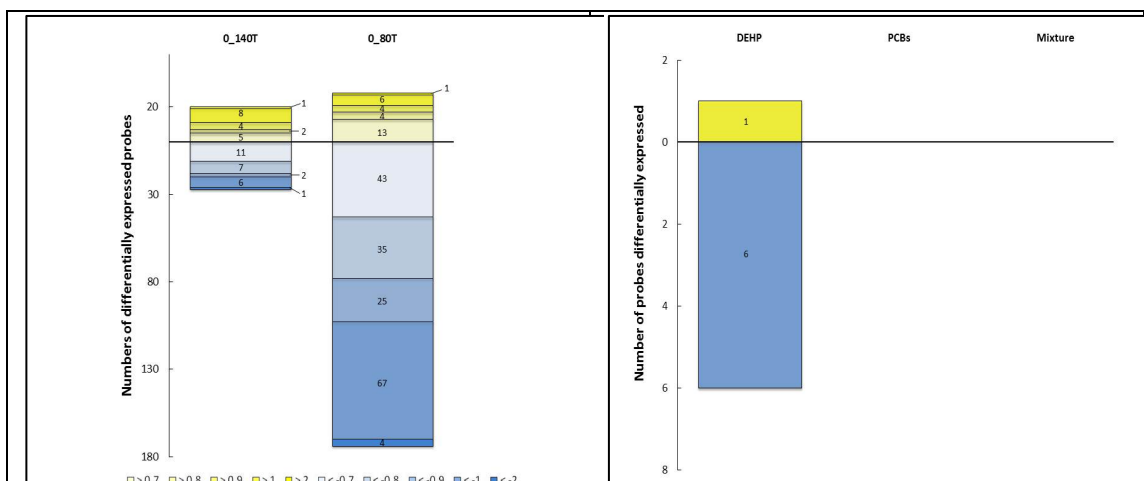


### MALES: WINDOWS AND SELECTED EDC STUDIES

In the Windows of exposure study, Sertoli cell numbers were significantly reduced in the intermediate groups (0-80, 30-110, 60-140:  $P < 0.001$ ) but not in animals exposed all through gestation. Leydig cell CYP17A1 staining was reduced in all sludge exposed ewes ( $P < 0.001$ ). Interestingly, at day 80, both Sertoli cell numbers ( $P < 0.01$ ) and Leydig cell staining ( $P < 0.05$ ) was reduced. In the chemical exposure model, Sertoli cell numbers were reduced in all treatment groups (DEHP, PCB and Mix). Leydig cell staining was reduced in animals exposed to DEHP and PCB individually but not in animals exposed to the mixture.



Windows: Analysis revealed that 249 probes were differentially expressed according to the window of exposure. More genes were differentially expressed in testes from ewes exposed from 0-80 days (202) than in ewes exposed throughout gestation (47). Interestingly, 22 differentially expressed genes are common to both the 0-80 and 0-140 exposure groups. Chemical exposure: Only 7 genes were differentially expressed. Despite the small number of differentially expressed genes, these data parallel our previous observations on the fetal ovary in 2 ways: (1) the DEHP exposed gonads exhibit the largest number of differentially expressed genes and (2) most differentially expressed genes are down regulated. One possibility is that the surprisingly low number of genes reflects a bias in the array towards ovarian rather than testicular genes. However, since 249 differentially regulated genes were identified in the sewage sludge exposed fetal testes, this may simply reflect a lack of sensitivity specific to this model.



**Numbers of probes differentially expressed in the sheep fetal testis at 140 days of gestation after exposure to a cocktail of pollutants (A) and specific pollutants (B).** Transcriptomic analysis was performed on a custom sheep oligo microarray - as previously described. Up-regulated and down-regulated probes are respectively indicated in yellow and blue. The figures in and next to the bars correspond to the numbers of probes composing each category. A: testes from the sewage sludge model, B: testes from the chemical exposure model.

## WHAT EFFECTS DO SPECIFIC EDCS (ELEVATED IN TISSUES OF SEWAGE SLUDGE-EXPOSED SHEEP) HAVE ON IN-VIVO MOUSE REPRODUCTIVE DEVELOPMENT?

We examined the effects in mice of exposure to di(2-ethyl-hexyl) phthalate (DEHP), polychlorinated biphenyls (PCBs) or DEHP/PCB mixture throughout pregnancy and lactation on reproductive health of female offspring at adulthood. CD-1 and C3H/N mouse dams were exposed to contaminants with the diet from gestational day 0.5, and in a second study from the age of 4 weeks on, until the end of lactation. The doses employed were within the range of environmental exposure levels in humans: DEHP (0, 0.05, 5 and 500 mg DEHP/kg/day); PCB 101+118 (proportion of 1:1, 0,1, 10 and 100 µg PCB/kg/day); DEHP/PCB (mixture of the above).

### ***In-vivo exposure to DEHP***

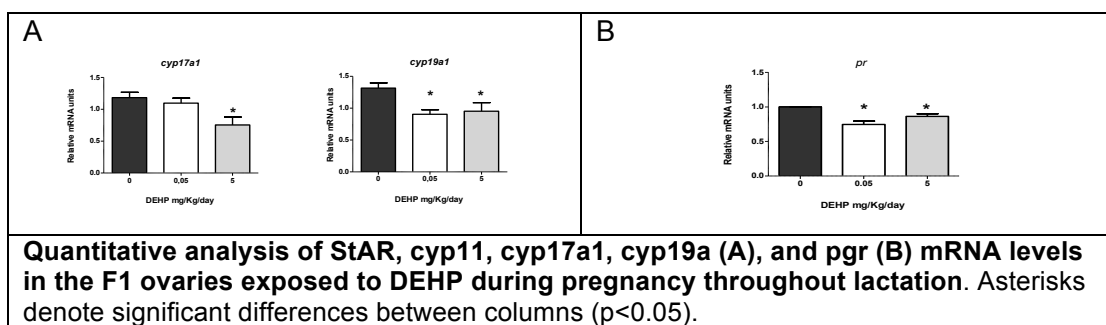
Treatment of pregnant CD-1 and C3H/N dams with 500 mg DEHP/kg/day induced complete pregnancy failure with embryonic resorption starting between dpc 10.5 and 11.5. In addition, in the 5 mg/Kg/day group a significant reduction in mean litter size was observed. No differences in mean litter size were observed in the 0.05 mg/Kg/day. However, in CD-1 F1 offspring in both the 0.05 and 5 mg/Kg/day group, significantly reduced body weight and abdominal fat content were observed at PND +21 through PND +84. In C3H/N dams and F1 offspring all DEHP dosages led to a significant increase in abdominal fat and body weight. The C3H/N-dams also showed a significantly higher food-intake. Histological sections of the visceral adipose tissue were performed. In a double blank test we found that the adipocytes of DEHP exposed mice were larger (hypertrophied) compared to the controls (control:  $567 \pm 0.02$  adipocytes per unit area; 0.05 mg DEHP:  $285 \pm 0.02$  adipocytes per unit area; 5 mg DEHP:  $250 \pm 0.04$  adipocytes per unit area, mean  $\pm$  SEM, N=3) ( $p < 0.05$ ). After a feeding period of eight weeks DEHP treated C3H/N mice had significantly elevated absolute liver weights in an exposure but not dose dependent manner (control:  $1128.98\text{mg} \pm 21.27\text{mg}$ ; 0.05mg DEHP:  $1297.08\text{mg} \pm 15.52\text{mg}$ ; 5 mg DEHP:  $1431.97\text{mg} \pm 30.75\text{mg}$ ; 500 mg DEHP:  $1672.11\text{mg} \pm 36.36\text{mg}$ , mean  $\pm$  SEM, N=4) ( $p < 0.05$ ). Additionally to this elevation we detected a significant increase of the



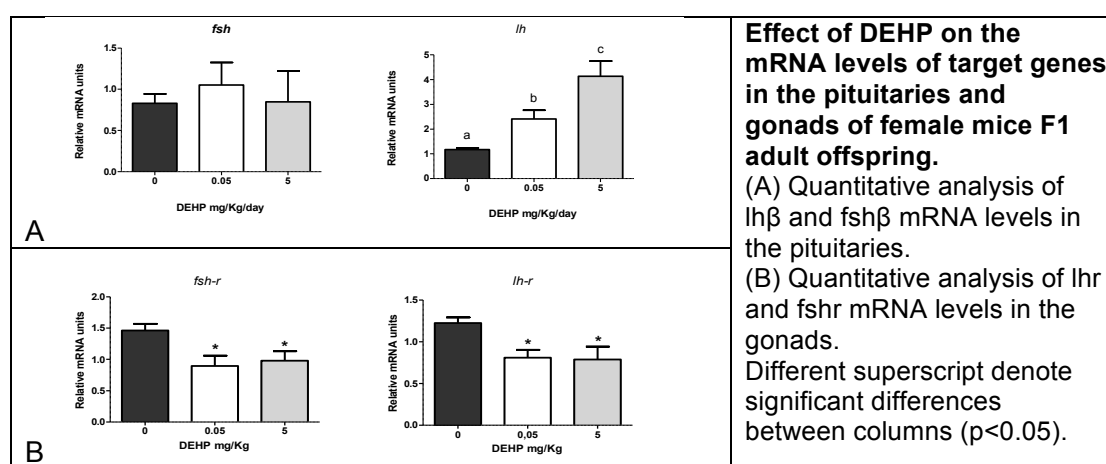
relative liver triglycerides in the lowest and middle DEHP dosage group (0.05 mg DEHP: 1.9876 %  $\pm$  0.15% fold change of control; 5 mg DEHP: 2.09 %  $\pm$  0.06 % fold of control; mean $\pm$ SEM, N=3) ( $p < 0.05$ ). DEHP did not change the plasma triglyceride concentrations in treated C3H/N mice.

<b>Effects of DEHP exposure on in-vitro embryo production from oocytes derived from F1 offspring.</b> Different superscripts show significant differences among rows ( $p < 0.05$ )			
	DEHP (mg/Kg/day)		
	0 (control)	0.05	5
Oocytes/animal	35.20 $\pm$ 3.1	36.80 $\pm$ 2.3	32.80 $\pm$ 5.1
Cleavage rate (%)	59.63 $\pm$ 4.5 <sup>a</sup>	34.48 $\pm$ 4.2 <sup>b</sup>	63.61 $\pm$ 4.6 <sup>a</sup>
Blastocyst rate (%)	42.46 $\pm$ 5.6 <sup>a</sup>	9.03 $\pm$ 2.4 <sup>b</sup>	48.17 $\pm$ 3.6 <sup>a</sup>

DEHP exposure significantly altered developmental capacity of oocytes in F1. It is noteworthy to notice that, major adverse effects were observed in the lowest investigated dose, suggesting non-monotonic response curve and low-dose effects, similar to what observed in recent studies reporting U-shape dose response curves upon treatment with phthalates. Analysis of transcription profiles in ovaries revealed a significant decrease of *Cyp17a1*, *Cyp19a1* and progesterone receptor gene expression, suggesting a persistent alteration of the estrogen synthesis.

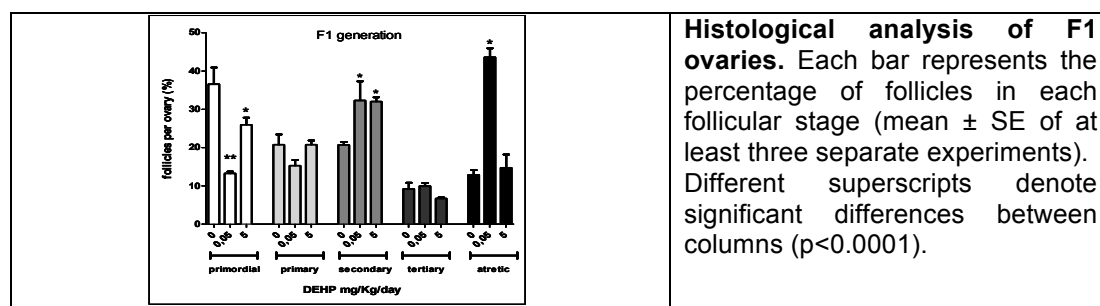


Low estrogen levels may affect the pituitary-gonadal feedback mechanisms. Therefore, we investigate possible long-lasting damage of the pituitary-gonadal axis in female adult offspring maternally exposed to DEHP. Our results indicated that in pituitaries from treated offspring a significant up-regulation of the expression levels of *lh- $\beta$*  subunit mRNAs occurred, together with a down-regulation of *fshr* and *lhr* mRNAs in the ovaries, thus suggesting a dysregulation in gonadotropin signalling.



In ovaries, there was a significant decrease in the relative number of primordial follicles, together with an increased number of growing pre-antral follicles (primary

and secondary) in both DEHP doses. There was no statistically significant difference in the density of follicles in ovarian tissue.



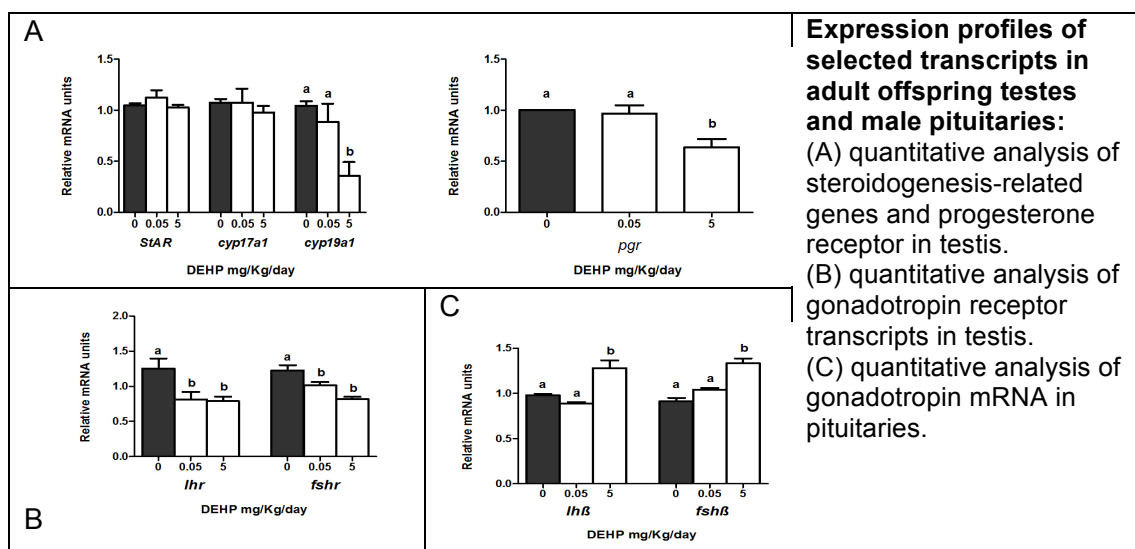
The relative distribution of follicular classes observed in DEHP treated F1 females suggests that increased follicular recruitment may contribute to the depletion of the ovarian reserve. In agreement with this hypothesis, a significant up-regulation of *gd9* transcript in DEHP-treated ovaries was observed. Indeed, recent studies in neonatal rats have shown that GDF-9 promotes follicular recruitment, increasing the number of growing preantral follicles, concomitantly decreasing the complement of primordial follicles. Furthermore, the DEHP-induced ovarian phenotype observed in the present study also corresponds to the histological appearance of FSH-R insufficient mice, a result that nicely correlates with the disruption of pituitary-gonadal axis observed. Taken together, our findings suggest that in maternally exposed female mice, DEHP may act on multiple pathways involved in estrogen biosynthesis and lead to imbalance of pituitary-gonadal cross-talk, which in turn would impair gonad function and gamete quality when the offspring reaches adulthood.

**MALES** - As previously described for female offspring, treatment of dams with 0.05 and 5 mg DEHP/kg/day significantly reduced the body weight of F1 male pups at weaning and at adult age. Besides, in sexually matured males, both DEHP doses significantly reduced testis and seminal vesicle weight. Furthermore, in both DEHP doses treated F1 offspring presented a significantly decrease of sperm concentration (of about 50%) and viability (nearly 20% less viable). Finally, in *in vitro* fertilization protocols using oocytes from untreated females, the sperm from both the 0.05 and the 5 mg DEHP/kg/day groups resulted in zygotes with a significantly reduced capacity to reach the blastocyst stage.

**Effect of pre- and perinatal DEHP exposure on developmental capacity of sperm from adult male offspring.** (superscripts =  $p < 0.05$ )

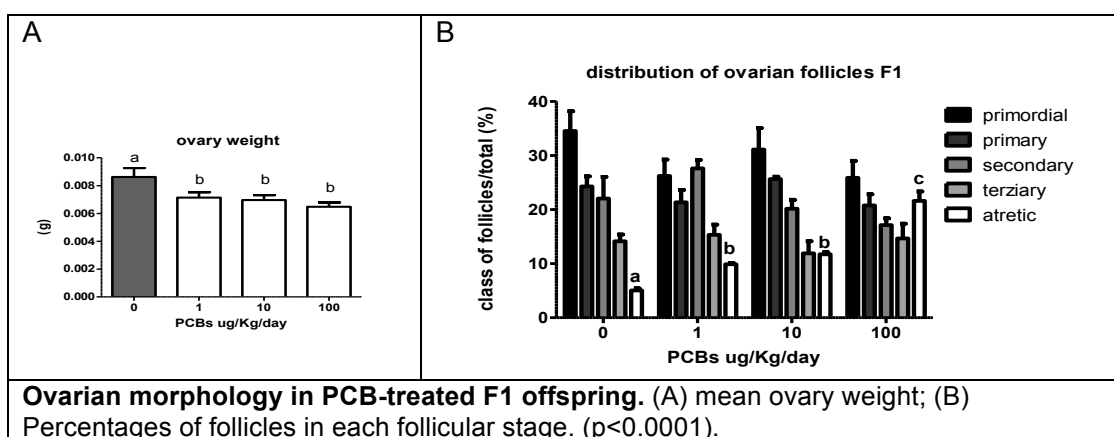
	DEHP mg/kg/day		
	0	0.05	5
Cleavage rate (%)	65.2 $\pm$ 9.3	65.0 $\pm$ 10.2	47.4 $\pm$ 18.3
Blastocyst rate (%)	43.9 $\pm$ 11.5 <sup>a</sup>	13.50 $\pm$ 6.5 <sup>b</sup>	4.4 $\pm$ 0.8 <sup>b</sup>

At molecular level, in testis, as in ovaries, a significant down-regulation of both *cyp19a1* and *pgr* transcript was observed, concomitantly with a decreased expression level of both *fshr* and *lhr* mRNAs at all doses investigated. In the pituitary, expression of both *lh $\beta$*  and *fsh $\beta$*  mRNA was significantly up-regulated in the 5 mg DEHP/kg/day group. It is therefore possible to speculate that also in male offspring maternal DEHP exposure have long-lasting adverse effects on pituitary-gonad crosstalk, which, in turn, results in disturbances of sperm quality at adult age. This conclusion is supported by recent studies in men linking defective aromatase activity to decreased sperm concentration and motility, and to increased sperm damage.



### ***In-vivo exposure to PCBs 101+118 - FEMALES***

Concentrations of total PCBs in tissues from dams were consistent with doses administered however, despite PCB mixture was composed of equal amounts (1:1), PCB 118 shows a higher accumulation rate than PCB 101 in both dams and offspring. This difference is likely related to differences in clearance and redistribution rates, being these processes more rapid for PCB 101 than for PCB 118. Of interest is the fact that PCB accumulation was significantly higher in offspring than in dams, confirming the progeny as a target of maternal exposure. Treatment with PCBs did not affect CD-1 and C3H/N F0 dams' health, duration of gestation, litter size, or viability index. In the case of C3H/N mice, exposure to PCBs did alter the number of alive pups 3 weeks after birth in the highest treatment group. We observed, that 44% of pups did not survive the first 3 weeks after birth in this group. An alteration of the sex ratio of *in utero* and lactationally exposed pups was found in the middle concentration group (control: 45% females: 55% males; 10 µg PCBs: 75% females: 25% males (N=2; p < 0.05). A significant elevation of adipose tissue and thyroid gland weight was found in male offspring compared to control. In CD-1 F1 adult females a significantly reduced ovarian weight was observed in all PCB doses. In contrast C3H/N F1 female offspring had significantly higher ovary weights compared to control. Furthermore, histopathological analysis of the ovaries of CD-1 mice indicated that PCB treatment increased the incidence of follicular atresia in a dose-dependent manner, without affecting the number of pre-antral or antral follicles.



This observation is peculiar, however, it has been previously reported that maternal exposure to coplanar (mono-ortho PCB 118) and non coplanar (di-ortho PCB 101)

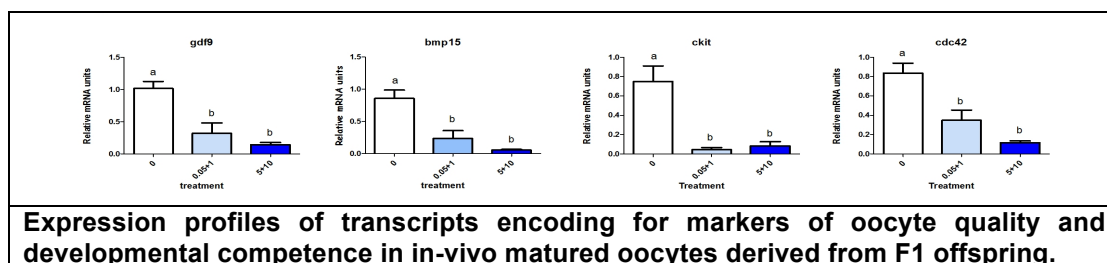
congeners may exert opposite effects on follicular dynamics. We, therefore, postulate that the interaction of the two congeners could have a stimulatory effect on follicle count that would be masked by increased rate of follicular atresia. This hypothesis is supported by the significant down-regulation of *pten* transcript observed in PCB-treated ovaries. PTEN-deficiency in oocytes leads to premature activation of the pool of primordial follicles thereby advancing infertility, whereas granulosa cells lacking Pten induces ovulation and enhanced fertility. It is therefore possible to hypothesize that the exposure to the 101+118 PCB mixture might act on follicular dynamics differentially acting on PTEN signalling in a cell-specific manner. In view of the pattern of follicle populations in F1, no alteration in fertility rate was expected. Nevertheless, a significant reduction in mean litter size was observed in PCB-treated F1 parental animals. This result may be explained by the reduced ability of oocytes to reach blastocyst stage observed in the present study in both the 10 and 100 µg PCB/kg/day groups compared to control (0 µg PCBs: 66.87±4.4%; 1 µg PCBs: 56.20±6.4%; 10 µg PCBs: 43.71±6.5%; 100 µg PCBs: 31.36±6.6%;  $p < 0.05$ ). These results are in agreement with other studies indicating that PCBs affect oocyte maturation, fertilization and blastocyst development in a variety of animal models. Taken together, our data indicate that developmental exposure to selected PCB congeners, showing preferential accumulation in offspring, directly affects reproductive health and performance of female offspring in mice. Further analyses are necessary to investigate the cellular and molecular mechanisms involved.

#### ***In-vivo exposure to DEHP/PCB mixture - FEMALES***

Several effects caused by exposure to DEHP/PCB mixture during pregnancy and lactation on female F1 reproductive health were similar to those caused by DEHP or PCBs alone, whereas other consequences were significantly different from what observed upon exposure to parent compounds, thus suggesting synergistic and additive effects. Similarly to what observed with DEHP, the highest dosage of the mixture induced pregnancy failure around mid-gestation. In addition in CD-1 mice significantly reduced and in C3H/N mice significantly increased body weights and abdominal fat were observed in F1 females of both low and medium doses from weaning through adult age. The combined exposure of C3H/N mice to DEHP and PCBs led to an increase in absolute liver weight in the highest dosage group (control: 1111.36 ± 69.9 mg; Mix III: 1585.89 ± 37.41 mg (mean ± SEM, N =2) ( $p < 0.05$ ). At adult age, significantly reduced ovarian weight was observed in CD-1 females from both treated groups, however without evident alteration in follicle distribution, a phenotype which differs from both DEHP and PCBs exposure. However, analysis of gene expression profiles indicated a decreased expression of *pten* transcript, as observed upon exposure to PCBs. Furthermore, in treated F1 ovaries a significant down-regulation in both *star* and *cyp17a1* and *fsh-r* mRNAs occurred, suggesting a possible alteration in steroid synthesis and pituitary-gonadal cross-talk, however with different molecular mechanisms compared to single compounds. This hypothesis is further supported by the observed down-regulation in the expression levels of *fsh* and *lh* mRNAs in pituitaries from in the highest dosage. In-vitro fertilization and embryo culture of oocytes from DEHP/PCB-treated F1 adult offspring indicated a significant decrease in the developmental capacity of oocytes in both dosages compared to control, suggesting a possible additive effect of DEHP and PCBs.

<b>Effects of DEHP/PCBs mixture exposure on in-vitro embryo production from oocytes derived from F1 offspring. (superscripts = <math>p &lt; 0.05</math>)</b>			
	DEHP + PCB (mg-µg/Kg/day)		
	0-0 (control)	0.05-1	5-10
Cleavage rate (%)	87.5 ± 3.4	87.0 ± 4.1	74.4 ± 11.0
Blastocyst rate (%)	41.7 ± 4.7 <sup>a</sup>	18.3 ± 4.3 <sup>b</sup>	20.7 ± 6.5 <sup>b</sup>

Preliminary data on oocytes expression profiles nicely correlate with altered oocyte developmental capacity observed. In fact, a significant down-regulation in genes related to oocyte quality (gdf9, bmp15, c-kit, and cdc42) was detected in both treated groups. This result may, at least partially, explain the adverse changes observed in resultant adult offspring.



## DO THE EFFECTS OF EDC EXPOSURE PERSIST IN EITHER A MULTI-GENERATIONAL OR TRANSGENERATIONAL MANNER?

Multi-generational extends a study to the F2 generation. If the Pregnant F0 generation is exposed, then the germ cells that will form the F2 generation when the F1 generation is exposed inside its pregnant mother. In contrast, by extending our studies to the F3 generation (only possible in mice within the lifespan of REEF) then true transgenerational effects would occur if the F3 generation (which is not itself, even as germ cells, directly exposed to the chemicals received by the F0 pregnant dams) showed differences compared with the F3 generation from unexposed F0 pregnant dams.

### **Limited multi-generational effects in sheep**

In total, fifteen F2 female fetuses were recovered (8 controls and 7 from mothers exposed to sewage sludge themselves as fetuses in-utero). There was no significant differences in fetal morphology, endocrinology or in the oocyte density between two groups: C=338±144; T=304±145 oocytes/section. Out of 1100 spots that were selected for analysis, 273 were significantly different (fold change ≥1.2; p<0.05) between the treatment groups: 269/273 were decreased as a result of the exposure to sewage sludge. Of the identified proteins, the majority had binding, detoxification & metabolic functions and especially cell cycle, cell division & transcription functions. In contrast to the proteome, there was no difference at the transcriptome level between F2 control and exposed ovaries.

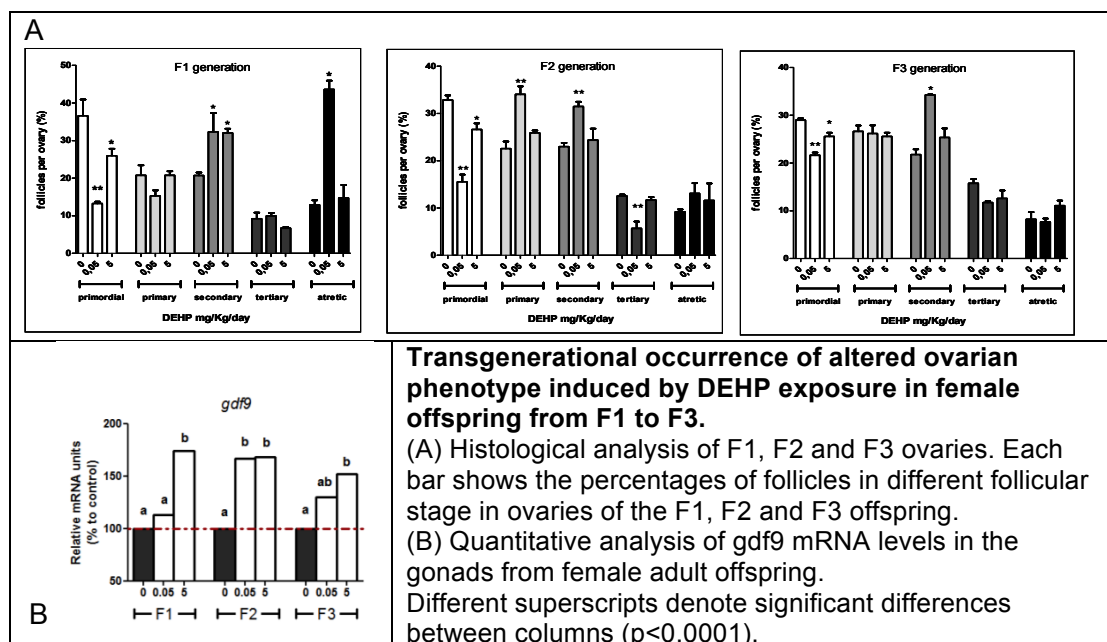
### **Marked trans-generational effects in mice**

In order to investigate transgenerational transmission of adverse reproductive effects in female mouse offspring upon exposure to DEHP or PCBs, single or in combination, a cohort of F1 female offspring was mated to unexposed males to obtain F2 animals. The experimental protocol was then repeated with F2 animals, in order to produce F3 offspring. Exposure to PCBs or DEHP was limited to F0 dams.

### **FEMALES**

No evidences of transgenerational transmission of reproductive adverse effects were observed upon exposure to PCBs in females. Conversely, DEHP administration to pregnant mice caused alterations of the reproductive health of female offspring not only in the first generation, but also in the two subsequent generations. Similarly to what observed in F1 females, both F2 and F3 female mice showed altered ovarian histology with a significant decrease in the percent number of primordial follicles and an increased number of growing pre-antral follicles. Up-regulation of gdf-9 transcript expression profile was also maintained up to the third generation, suggesting that a

common molecular mechanism of action might account for the observed altered ovarian phenotype in all three generations. Our data further indicate that the 0.05 mg DEHP/Kg/day dose reduced the ability of the oocytes to undergo first mitotic division and to develop to the blastocyst stage in F2 and F3 generations, as already observed in the F1 generation.



**Effects of DEHP+PCB exposure on *in vitro* embryo production from oocytes derived from F1, F2 and F3 offspring.** (superscripts =  $p < 0.05$ )

	DEHP (mg/Kg/day)		
	0 (control)	0.05	5
<b>F1 offspring</b>			
Cleavage rate (%)	59.63 ± 4.5 <sup>a</sup>	34.48 ± 4.2 <sup>b</sup>	63.61 ± 4.6 <sup>a</sup>
Blastocyst rate (%)	42.46 ± 5.6 <sup>a</sup>	9.03 ± 2.4 <sup>b</sup>	48.17 ± 3.6 <sup>a</sup>
<b>F2 offspring</b>			
Cleavage rate (%)	80.88 ± 6.9 <sup>a</sup>	55.46 ± 2.1 <sup>b</sup>	96.77 ± 1.9 <sup>a</sup>
Blastocyst rate (%)	74.46 ± 7.0 <sup>a</sup>	39.37 ± 3.1 <sup>b</sup>	79.92 ± 13.7 <sup>a</sup>
<b>F3 offspring</b>			
Cleavage rate (%)	82.09 ± 4.1 <sup>a</sup>	69.62 ± 1.9 <sup>b</sup>	84.38 ± 3.5 <sup>a</sup>
Blastocyst rate (%)	79.78 ± 4.5 <sup>a</sup>	26.91 ± 7.7 <sup>b</sup>	57.61 ± 10.6 <sup>a</sup>

The mechanisms of transmission of DEHP-mediated reproductive adverse effects in females along multiple generations are still to be clarified. However, the fact that adverse, DEHP-mediated, reproductive effects were highly consistent, between individuals and amongst litters, strongly suggests epigenetic variations in the germ-line could be involved. As for DEHP, also the adverse effects of the treatment with the DEHP/PCBs mixture were transmitted from F1 to subsequent generations. However, the effects were limited to a reduced oocyte developmental capacity in F2, which may suggest that adverse effects may be transmitted only multigenerationally. Whether such effects would involve epigenetic variations or is the results of direct exposure of F2 germ-line it's a matter which deserves further investigation.

## MALES

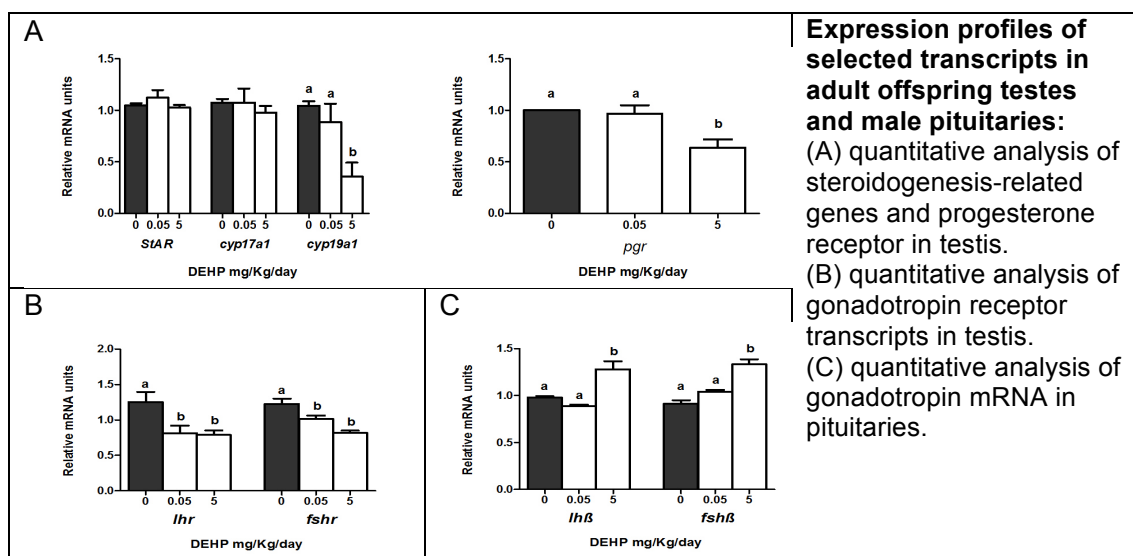
**PCBs:** Treatment of dams with all doses of PCBs 101+118, during pregnancy and lactation induced in F1 and F2 male pups at adult age a significant reduction in testis weight together with decreased tubule diameter and increased sperm-depleted tubules at the expenses of normal ones. This latter effect was also observed in F3

animals. Furthermore, PCB treatment significantly reduced the sperm viability of adult offspring up to the third generation, being sperm 20-30% less viable than in controls of the same generation. This result correlates with decreased developmental capacity of sperm from PCBs groups, observed upon in-vitro fertilization of unexposed oocyte. Sperm from the 10 and the 100 µg/kg/day groups resulted in zygotes with a significantly reduced capacity to reach the blastocyst stage.

**Effect of pre- and perinatal DEHP exposure on developmental capacity of sperm from adult male offspring.** (superscripts =  $p < 0.05$ )

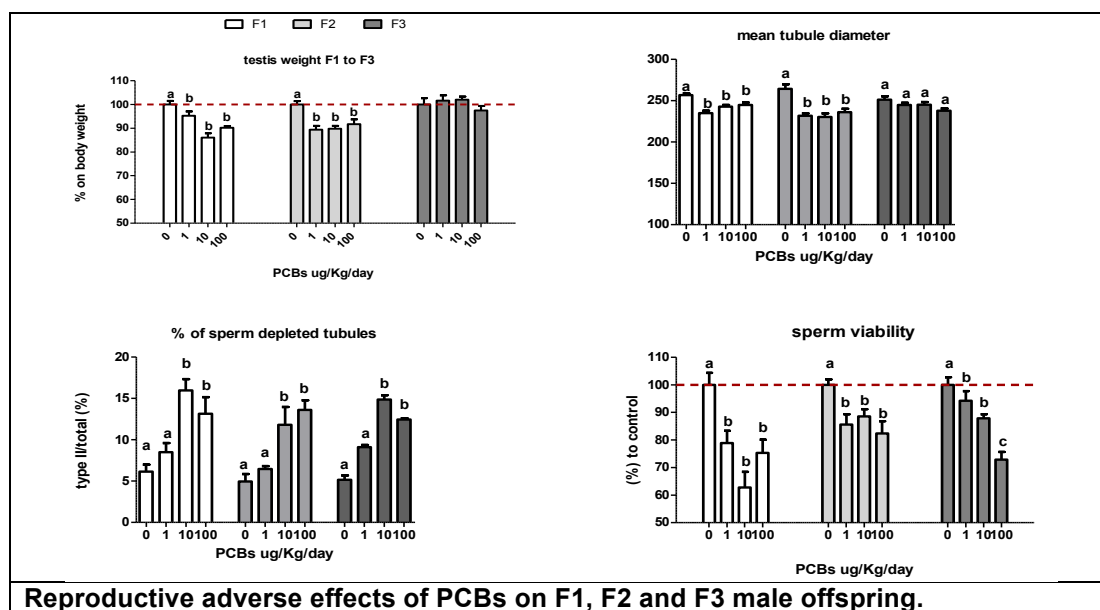
	DEHP mg/kg/day		
	0	0.05	5
Cleavage rate (%)	65.2 ± 9.3	65.0 ± 10.2	47.4 ± 18.3
Blastocyst rate (%)	43.9 ± 11.5 <sup>a</sup>	13.50 ± 6.5 <sup>b</sup>	4.4 ± 0.8 <sup>b</sup>

At molecular level, in testis, as in ovaries, a significant down-regulation of both *cyp19a1* and *pgr* transcript was observed, concomitantly with a decreased expression level of both *fshr* and *lhr* mRNAs at all doses investigated. In the pituitary, expression of both *lhβ* and *fshβ* mRNA was significantly up-regulated in the 5 mg DEHP/kg/day group. It is therefore possible to speculate that also in male mouse offspring maternal DEHP exposure have long-lasting adverse effects on estrogen biosynthesis and gonadotropin signalling, which, in turn, results in disturbances of sperm count and viability at adult age. This conclusion is supported by recent studies in men linking defective aromatase activity to decreased sperm concentration and motility, and to increased incidence of sperm DNA damage.



**PCBs:** Treatment of dams with all doses of PCBs 101+118, during pregnancy and lactation induced in F1 and F2 male pups at adult age a significant reduction in testis weight together with decreased tubule diameter and increased sperm-depleted tubules at the expenses of normal ones. This latter effect was also observed in F3 animals. Furthermore, PCB treatment significantly reduced the sperm viability of adult offspring up to the third generation, being sperm from F1, F2 and F3 male offspring about 20-30% less viable than in controls of the same generation. This result nicely correlates with the decreased developmental capacity of sperm from PCBs groups, observed upon in vitro fertilization of unexposed oocyte. In fact, sperm from the 10 and the 100 µg/kg/day groups resulted in zygotes with a significantly reduced capacity to reach the blastocyst stage, compared to controls.





## WHAT EFFECTS DO SPECIFIC EDCS HAVE ON IN-VITRO DIFFERENTIATION OF MURINE EMBRYONIC STEM CELLS?

### DEHP

During the differentiation of P19 cells to cardiomyocytes, mRNA was isolated at specific time points (d0, d2, d5, d5+5, d5+10). The P19 cells were exposed to three different concentrations of DEHP [5, 50, 100 µg/ml]. The expression analysis of the key metabolic markers PPARgamma, PPARalpha, GLUT4 and FABP4 revealed significant changes especially during the last stages of differentiation. Functional parameters in the differentiating cardiomyocytes were analysed by mRNA levels of Cx43 and alpha-MHC. Both markers showed altered expression patterns in all dosage groups. Analyses of the beating rate of differentiated cardiomyocytes with the help of a multielectrode array (MEA), showed significantly faster beating in cardiomyocytes early exposed to 50 and 100 µg/ml DEHP. Additionally the differentiation into beating cardiomyocytes began much more earlier in the middle and the highest dosage group compared to the control. Due to alterations of gene expression in later stages of differentiation, epigenetic modulation seemed to be relevant. Analyses of the expression of DNA-methyltransferases (DNMTs) revealed significant changes in all DNMTs, especially an elevation of DNMT3a. Due to this the methylation status of 30 CpGs were determined in selected regions within the promoter associated CpG island of PPARgamma1. Here methylation was low with 2.2% of CpGs being methylated. There was a significant interaction between DEHP treatment and CpG site. Within this region the percentage of methylated CpGs was increased at 5 of the 30 CpG sites. Finally, the methylation status of 10 CpGs were determined in selected regions within the promoter associated CpG island of Slc2a4 (GLUT4) and PPARalpha. Methylation was low with just 3.1% of CpGs being methylated. As for PPARalpha, the percentage methylated CpGs was not affected by DEHP treatment, but did vary between individual CpGs.

### PCB exposure

The P19 cells were exposed to three different concentrations of PCB 101+118 [1, 10, 100 ng/ml]. The expression analysis of the key metabolic markers PPARgamma, PPARalpha, GLUT4 and FABP4 revealed significant changes especially during the first stages of differentiation. Functional parameters in the differentiating cardiomyocytes were analysed by mRNA levels of Cx43 and alpha-MHC. Both markers showed altered expression patterns in all dosage groups. Analyses of the



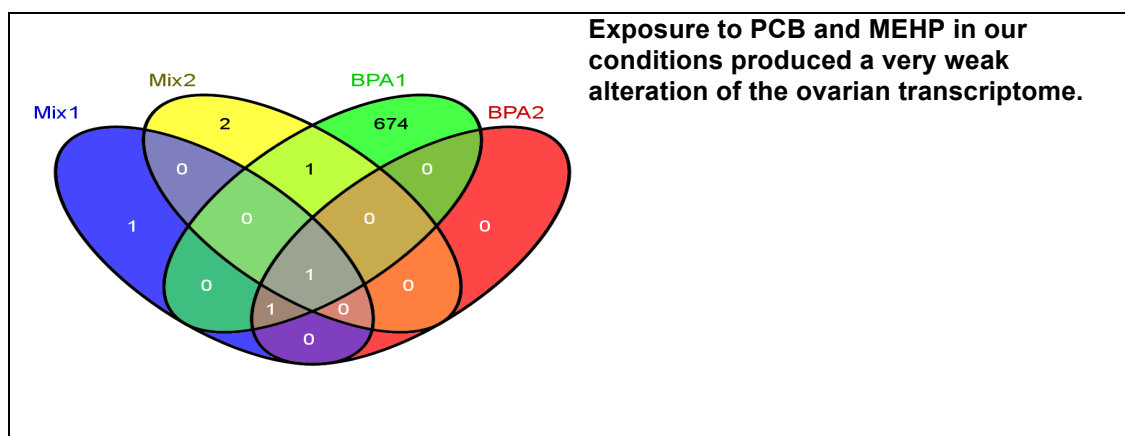
expression of DNA-methyltransferases (DNMTs) revealed significant changes in all DNMTs. Additionally significantly altered HDAC1 expression patterns occurred under PCB [100 ng/ml] exposure.

#### **Exposure to DEHP/PCB mixture**

Exposure of P19 cells to DEHP and PCB (101+118) also led to significant alterations in the key marker gene expression patterns of GLUT4, FABP4 and PPARalpha but not in PPARgamma. The most effective dosage of DEHP was the combination of the lowest concentrations of the single compounds. In contrast to the single exposures only alpha-MHC showed altered expression patterns, again in the lowest concentration group. The results of our in-vitro P19 stem cell studies showed, that DEHP was the more potent EC. DEHP disturbed the differentiation process into cardiomyocytes and led to significant long lasting changes in metabolic and functional as well as epigenetic markers. Those expression changes also led to quantifiable effects like higher beating rates and CpG-methylation. PCB effects have been less potent under the investigated aspects. The mixed exposures could not confirm any additive effects, but rather compensatory effects.

#### **DOES DEHP AFFECT THE FETAL OVARY IN-VITRO? Development and use of an ovine fetal ovary culture system**

Multiple culture conditions at two developmental stages (50-70dpc and 90- 120dpc) were tested to develop an in-vitro sheep fetal ovary culture system to test EDCs. (i) 50-70dpc = meiotic division of oogonia (11-22 wks in human). (ii) (90-120dpc = ovarian follicle formation (early folliculogenesis, 18-28 wks in human). 4-6 ovarian fragments per fetus were placed on an insert floating at the surface of the culture medium. Cultured explants were compared with ex-vivo organs. The conditions providing the best results both for the gene expression and stable morphology during the meiosis period included Am580+kit-L. For the 90-110dpc period, follistatin was required while VEGF was necessary for the 110-130dpc period of follicle development. Cultures were maintained for 20 days. This long-term organ culture system was used to investigating the effects of selected ECs (DEHP, MEHP and PCB110-118) and mixtures on early ovarian development. MEHP and PCB 101-118 at  $10^{-6}$  M (MEHP1 and PCB1 respectively) or combinations of both (Mix1) and MEHP and PCB 101-118 at  $10^{-8}$  M (MEHP2 and PCB2 respectively) or combination of both (Mix 1 and Mix2) were used. These concentrations correspond to environmental concentrations found in human umbilical cord samples. Very few genes were differentially expressed between controls and PCB, MEHP and mixture exposures. Only the highest BPA exposure (positive control) resulted in a large deregulation of ovarian gene expression.



*Stage 1: Meiotic division of oogonia (57-70dpc)* Only 3 genes were up regulated in the highest concentration of the mixture (Mix1). In Mix 2, 4 genes were differentially expressed, one was down-regulated and 3 up regulated. Only one gene was common between Mix 1 and 2: TMEM167A. When we compared the mixture exposures (Mix 1 and Mix2) with BPA exposures, three genes were deregulated in at least two conditions: TMEM167A, COUP-TFI/NR2F1 and Dual-specificity phosphatase 6 (DUSP6, mitogen-activated protein kinase (MAPK) phosphatase 3 or PYST1). COUP-TFI represses the transcription of genes involved in steroidogenesis, FABP5 functions to deliver ligands to and enhance the transcriptional activity of the nuclear receptor peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta). Target genes of this receptor include genes involved in cell growth and survival and DUSP6 dephosphorylates phosphotyrosine and phosphothreonine residues on extracellular signal-regulated kinase (ERK1/2; MAPK1/2) to inactivate the ERK1/2 kinase.

*Stage 2: Follicle transition (90-110dpc)* MEHP and DEHP (DEHP1=10<sup>-6</sup>M, DEHP2=10<sup>-8</sup>M) in addition to PCB and mixtures were tested with BPA as positive control. The transcript up-regulated in Mix 1 and 2 and DEHP1 was homologous to Bos taurus synaptotagmin XI (SYT11) mRNA. Synaptotagmin proteins are calcium sensor proteins; their functions are unknown in ovaries.

*Stage 3: Follicle transition (110-130dpc)* Only one gene was deregulated in PCB1 and Mix1 exposures, it corresponded to Cytochrome P450, family 1, subfamily A, polypeptide 1, CYP1A1 gene, previously described as encoding a phase I cytochrome P450 enzyme, involved in the oxidative metabolism of estrogens.

There were no significant differences in morphology or in oocyte or follicle numbers. The hormonal production appeared normal. Different hypotheses can be formulated for explaining the limited effects of the exposures: the low concentrations of ECs tested, the lack of sensitivity or the timing of analysis. As shown by the BPA exposure, the cultured fetal ovaries could react to low doses of chemicals and the techniques used were enough sensitive for detecting transcriptome and hormonal dosages differences after 20 days of culture. Consequently, we can conclude that PCB (10<sup>-6</sup> and 10<sup>-8</sup> M) and MEHP (10<sup>-6</sup> M and 10<sup>-8</sup> M) and DEHP and mixtures produced very subtle alterations of the ovarian transcriptome after in-vitro culture of fetal sheep ovary. It is interesting that data emerging from the DEER project and elsewhere suggest that DEHP has little effect on human gonad development.

## CONCLUSIONS DERIVED BY COMPARING IN-VIVO AND IN-VITRO STUDIES

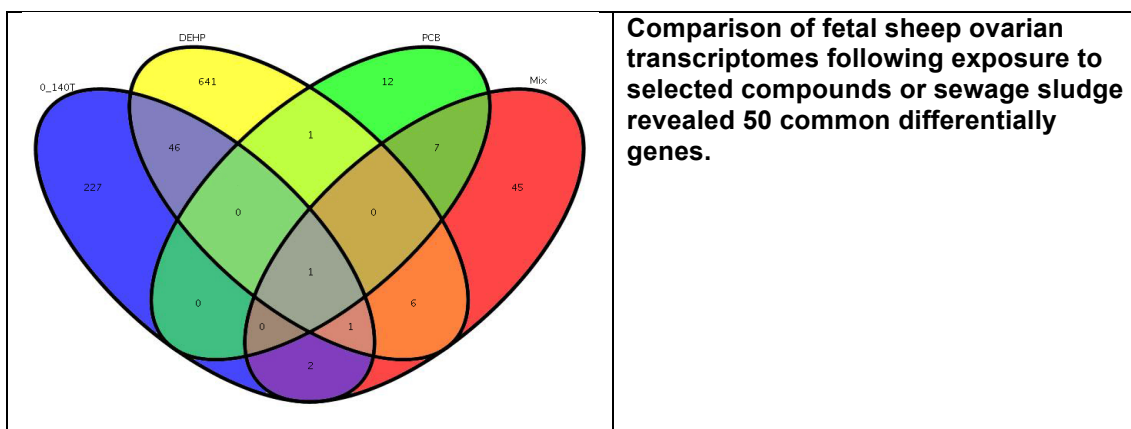
**SHEEP:** In spite of the fact that survival and differentiation of the different ovarian cell types “normally” occurs in our in vitro culture system, it produced discordant results with in-vivo experiments. More disturbances were observed in-vivo than in-vitro for the same compounds. For explaining discordances between in-vivo and in vitro results, we can hypothesise that the period of cultures was too short for visualising long-term effect observed in-vivo. In the in-vivo protocol, fetuses were exposed from 0 to 140 dpc. Another main difference with in-vivo experiments lies in the fact that PCB and DEHP/MEHP metabolites were not tested in-vitro. PCB biotransformation has been shown to lead to two classes of PCB metabolites that are present as contaminant residues in the tissues: hydroxylated (HO) and methyl sulfone (MeSO<sub>2</sub>) PCBs. For DEHP, five metabolites, namely mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), and mono[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) have been isolated. Moreover, other chemicals or physiological factors present in-vivo could act in synergy with PCB or DEHP/MEHP themselves or their metabolites.

**MICE:** Results that have been observed in-vivo differ from those obtained in-vitro. Marker gene expression that had been changed in-vivo mostly also changed in-vitro, but the direction of change was often discordant. This result is due to the fact that exposure windows were different and endpoints, of course differed too. Cell culture experiments lasted for only 21 days while F1 offspring were 4 weeks or older when they were processed and samples were collected. Besides this, metabolism of the substances is different in whole animals than in cell culture (see “sheep” above).

## INTEGRATION OF HUMAN AND ANIMAL DATA

### ***Are there common effects of sewage sludge exposure (complex mixture of EDCs) compared with focused exposures (DEHP, PCBs, DEHP+PCBs) on the fetal sheep ovary?***

Evidence increasingly emerging in the literature supports the concern that outcomes of exposures to single or focused selections of ECs, including EDCs, are not necessarily the same as those obtained with more complex, real-life, mixtures. REEF data strongly supports this concern. For example, at the morphological level exposure to real-life mixtures alters follicle development in the fetal ovary whereas exposure to selected chemicals (DEHP and PCBs 101, 118) or a combination of the two had no such effect. A further example of this is evident from the REEF transcriptomic studies:



1. EZH2, the catalytic subunit of Polycomb repressive complex 2 (PRC2) is a highly conserved histone methyltransferase that targets lysine-27 of histone H3. This methylated H3-K27 chromatin mark is commonly associated with silencing of differentiation genes. Studies on human tumours show that EZH2 is frequently over-expressed in a wide variety of cancers. Functional links between EZH2-mediated histone methylation and DNA methylation suggest partnership with the gene silencing machinery implicated in tumor suppressor loss. The increase of EZH2 transcripts could explain the under-expression of multiple ovarian genes following EDC exposures. This shows that epigenetic mechanisms could be affected by ECs and consequently globally affect the expression of multiple genes involved in cell differentiation.
2. Adiponectin Receptor 1. Adiponectin exerts its action by binding at least to two seven-transmembrane domain-containing specific receptors, Adiponectin Receptors 1 and 2 (AdipoR1 and AdipoR2). These two receptors activate various intracellular signaling pathways including AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), protein kinase B (Akt), p38 and p44/42 (ERK<sub>1/2</sub>) mitogen activated protein kinase (MAPK). These pathways are implicated in the regulation of energy metabolism. Recently, AdipoR1 and AdipoR2 expression has been reported in ovary, and other reproductive tissues, in human, pig, rodents, and cow. Within the ovary,

adiponectin and its receptors were present in primary and antral follicles. The mechanisms involved in different effects of adiponectin on steroid secretions and cell proliferation of ovarian cells are not yet known but alterations of its action by a deregulation of its receptor could negatively affect female fertility.

### ***What has been translated from the animal studies to the human fetus?***

While it is not possible to outline our findings in the human fetus in detail, we have taken the information generated by the REEF animal and in-vitro studies and made a number of unique and important findings in the human, some of which have not yet been published. In addition, because we have had insurmountable problems producing a functional long-term in-vitro human fetal ovary culture system, we have utilised the complex mixture exposure model presented by maternal cigarette smoking while pregnant. Examples of key translation and integration of human and animal studies are listed below:

### **Endocrine signalling**

Alterations in oestrogens or oestrogenic signalling were found following exposure to selected and complex mixtures of EDC. As a result we have:

1. Identified that the fetal human ovary, especially the oocyte, possesses all the necessary cellular machinery to synthesis and detect steroid signalling, especially oestrogenic signalling.
2. Exposure to complex EDC mixtures (maternal smoking) dysregulates steroidogenic, proliferative, apoptotic and hormone signalling pathways in the fetal human ovary. Furthermore, the intra-ovarian balance between inhibin and activin signalling is shifted towards activin signalling. These pathways equate with those identified in ovine fetuses exposed to real life mixtures i.e., transcriptomic studies have revealed that exposure dysregulates pathways associated with follicle formation including apoptosis, cellular growth and differentiation, cell cycle, cellular development, cell movement and steroidogenic pathways. It is notable that these pathways are similar to many of the pathways also affected by EDC exposure in the sheep fetus.
3. Confirmed that very high levels of total oestrogens are present in the human FEMALE fetal circulation and that they are significantly increased if the mother smokes cigarettes (NB this data is NOT derived from cord blood)
4. Demonstrated that very high levels of total oestrogens are present in the human MALE fetal circulation, but, unlike the female, not significantly affected by maternal smoking. Unconjugated oestrogens, while <10% of the total, tended to be a larger proportion in males exposed to maternal cigarette smoking. Furthermore, oestrogen receptor expression was reduced in the fetal testis by maternal smoking. The membrane-bound oestrogen receptor GPER is expressed in most fetal human testis cell types.
5. Shown that there are sex differences in circulating AFP levels and that maternal smoking disrupts *SHBG* and *APF* transcript levels. Throughout the second trimester the fetus possesses the machinery necessary to transport steroid hormones throughout the circulation and reach target tissues. There are also sex specific differences in steroid transport protein expression in both the gonad and liver, which are affected by maternal cigarette use during pregnancy. However, members of the fetal steroid-binding system are significantly disrupted by in-utero exposure to cigarette smoke metabolites in male fetuses only.

These findings suggest that mildly oestrogenic EDCs are very unlikely to have any effect on the human fetal gonad unless they also operate via different mechanisms. Furthermore, the probable high level of potential oestrogenic signalling in the human fetus during normal in-utero development has serious implications for the use of model species, such as rats and mice, in which fetal oestrogen levels are very low.

Consequently, this may not yield relevant data for the human. Suggestions that some EDCs may act via oestrogen-triggered mechanisms, rather than directly via oestrogenic actions, is interesting since we have shown the presence of the non-classical GPER oestrogen receptor in the fetal testis.

### **EDC burden**

We have shown that the female fetal human liver preferentially accumulates several PAHs and overall has markedly increased (5.7-fold) PAH burden. This is important because we have also shown in collaborative studies that activating AhR signalling in the human fetal ovary reduces germ cell proliferation by 25%. This shows a highly likely mechanism by which maternal smoking reduces fecundity of female offspring. Furthermore, there is a clear species and systems similarity in that AhR is widely implicated across species and organs in terms of exposure-induced deficits. We also investigated potential toxic elements in the human fetal liver and found that levels of cadmium were very low and although arsenic was readily detectable, neither was affected by maternal smoking. The same findings were made for another 16 elements, although lithium, magnesium and cobalt showed sex-specific effects of maternal cigarette smoking. These support the widely variable, unpredictable and sometimes surprising effects of exposure to complex chemical cocktails in sewage sludge on fetal EDC burdens. Indeed patterns of EDC accumulation were complex following exposure in the *in vivo* animal models. These are issues that need to be addressed with regulators since it is likely the combinations of chemicals that pose a developmental risk rather than concentrations of specific chemicals.

### **EDCs affect obesity and gonadal metabolic/detoxification genes/proteins**

The animal data collected during REEF indicated major EDC effects on metabolism/metabolic pathways in the developing fetus and post-natally. As a result we have:

1. Observed clear sex differences in CNTF and clear correlation with weeks of gestation in the case of ACTH. Maternal cigarette smoking increases circulating fetal AGRP only but both TSH and ACTH show an interaction between maternal smoking sex or stage of gestation, indicating that cigarette smoke chemicals disturb sex and gestational changes or differences in these two critical hormones. It is highly likely that this will have profound developmental effects of fetal programming of health and reproduction.
2. Investigated thyroid hormone signalling in the fetal gonad. Fetal ovarian expression of *THRA* and *THRB* increased across the second trimester while in the testis, only *THRA* increased significantly. Testicular *THRA* was localised to Sertoli cells. In control (non-exposed) fetuses, expression of *DIO3* was significantly higher in males. In smoke-exposed fetuses *DIO1* and *DIO3* were both significantly higher in females. Seminiferous tubule area was greater in smoke-exposed fetuses compared with controls and seminiferous tubule area correlated to the number of cigarettes smoked per day. The thyroid hormone signalling system is present in the second trimester human fetal gonad, with gender-specific expression differences, and is subtly altered by exposure to maternal cigarette smoke, likely partly directly on the gonad and partly via dysregulation of fetal TSH.

Pollutants and toxicants passing from the mother to the fetus may damage developing organ systems. The human fetal liver is both a potential target organ and a critical defence against exposure to such xenochemicals.

3. We therefore determined the effects of human fetal toxicant exposure, via maternal smoking, on metabolic enzyme transcripts in the fetal liver. Eight transcripts showed significant sex-specific differences in expression levels (*EPHX1*, *GSTA1*, *GSTT1*, *AHR*, *AS3MT*, *GLRX2*, *GGT1*, *CAR*). In male fetuses, maternal smoking was associated with a decrease in expression of

three transcripts (*GGT1*, *CYP2R1*, *CAR*) and an increase in eight transcripts (*CYP1A1*, *EPHX1*, *NQO1*, *GSTP1*, *GSTT1*, *AHR*, *AS3MT*, *GLRX2*). In the female, *CYP3A7* and *EPHX1* were increased in smoke-exposed fetuses. The human fetal liver expresses a wide array of metabolic enzymes, with sex differences apparent in 44% of the transcripts measured. Exposure of the fetus to pollutants/toxicants is associated with significantly altered transcript expression with the more marked response in the male potentially affecting levels of endogenous factors involved in fetal growth.

Our data show further species differences since in the rodents commonly used for EDC testing, fetal liver activity is virtually non-existent until either very late in gestation or post-natally.

### **Transgenerational effects of EDCs**

The literature and REEF findings suggest strong epigenetic effects of exposure to EDCs on gene methylation and thus epigenetic, transgenerational disturbance of fetal reproductive development. Similarly, cigarettes smoking and EDCs have been associated with epigenetic changes in the human. We have demonstrated the following:

1. Shown that hepatic cobalt and vitamin B12 concentrations were significantly higher in female fetuses than in males and significantly reduced in females by exposure to cigarette smoke. Fetal hepatic transcript expression for the two cobalt-dependent enzymes, *MTR* and *MUT*, showed gender differences and *MTR* was significantly increased in females and decreased in males by maternal smoking.
2. Investigated DNA methyltransferases DNMT1, DNMT3a and DNMT3b. Hepatic transcript levels for *DNMT1*, but not *DNMT3a* and *DNMT3b*, expression was affected by maternal smoking (males only) although all 3 showed significant sex differences in hepatic expression levels.
3. Show that methylation levels of 9 CpGs increased across the second trimester. Maternal cigarette smoking affected methylation levels: decreased 1 GR and 3 IGF CpG in females and 2 GR and 1 HSD11B2 CpG. Maternal smoking increased 1 H19 CpG in male fetuses. Overall sex differences were up to 7.09-fold and smoking effects up to 2.25-fold.

The disturbance of the cobalt/vitamin B12, cobalt-dependent enzymes and DNMTs that we report here represent a previously unsuspected way in which in-utero exposure to pollutants may perturb fetal development and methylation. In addition, methylation of key growth-related CpGs were significantly different between sexes and differentially affected by maternal smoking thereby likely disturbing health and function in the resulting adults and subsequent generations. These findings may also explain, at least partially, why the transcriptomic and proteomic findings in the various studies performed as part of REEF did not necessarily agree in terms of direction of effects and identity of affected pathways: changes in methylation of genes would be likely to have disconnected some of the gene/protein expression ratios.

### **Ano-genital distance**

Anogenital distance (AGD) provides a read-out of fetal androgen exposure and is reduced by in-utero exposure to harmful chemicals in rodents. In our sheep model AGD was increased in female fetuses exposed to sewage sludge chemicals later in gestation, although, paradoxically, testosterone was not different from controls in the affected fetuses. We therefore examined 83 electively-terminated, normally-progressing, second trimester fetuses between 11 and 20 weeks of gestation. A gender difference in AGD (1.4-fold longer in males) was already apparent at 11-13 weeks, rising to 2.00-fold longer in males at 17-20 weeks of gestation. In males, AGD and AGD normalized against ponderal index (a measure of fetal leanness) were

significantly and unexpectedly, increased by maternal smoking (1.19-fold and 1.31-fold respectively). The difference between smoke-exposed and non-exposed male AGD was greatest at 11-13 weeks (1.25-fold) but had declined to 1.01-fold by 17-20 weeks of gestation. AGD was not affected by smoke-exposure in females. Androgen programming of masculinization occurs before 11-13 weeks gestation in the human since AGD is already significantly longer in male fetuses by that stage. AGD reaches the two-fold difference reported for the neonate by 17-20 weeks gestation. Significantly longer AGD values in smoke-exposed males was surprising and may indicate increased androgen exposure in the early programming window. Convergence of AGD by late second trimester suggests, however, that by birth male AGD may be shorter in smoke-exposed individuals. The lack of effect of maternal smoking on female AGD is similar to the always-exposed sheep fetuses in the REEF windows experiment although we do not yet know if AGD would be different in smoke-exposed human female fetuses late in gestation.

#### **Example of follow-up and integration of a EDC-sensitive ovarian**

The reduction of major vault protein (MVP) at both 110 and 140 days of gestation in fetal ovine ovaries exposed to sewage sludge was confirmed by 2D-WB and was validated by 1D-WB. In ovine fetal ovaries (days 110, 140), and in the ewe, MVP localised to the oocyte cytoplasm. However, prior to follicle formation (day 55) MVP staining was hardly detectable. Similarly, in human fetal ovaries, at 17-19 weeks staining of the cytoplasm of oocytes that had recruited granulosa or pre-granulosa cells was very intense, whereas earlier in gestation (13-16 weeks) staining was weak. MVP transcript in human fetal ovaries increased significantly in weeks 17-21 vs 11-13 (13-fold) and 14-16 (6-fold). However, it was not significantly altered by maternal cigarette smoking. Our data indicate that MVP is expressed in the cytoplasm of oocytes engaged in folliculogenesis, its expression increases with gestation and is decreased by environmental chemicals, although not by maternal smoking in the human fetal ovary. On potentially difficult concept to carry over in this respect is the finding in our sheep studies that intermittent/windowed exposure to sewage sludge may cause more damage to the fetus than continuous exposure. Therefore, a question to be addressed is whether women who smoke irregularly while pregnant, especially during specific windows of development, cause more damage to their fetuses than those who smoke steadily?

### **KEY CONCLUSIONS**

- EC Effects on the developing fetus and ovary are window-specific during gestation.
- Selected ECs (DEHP±PCBs [101 & 118]) had different effects on fetal development than the complex mixture of ECs (sewage sludge).
- Responses of developing fetuses to exposure to both selected and complex mixtures of EDCs were markedly different between males vs females.
- Differences between species in terms of the in-vivo responses to EC exposures were marked.
- Differences between sexes in terms of the in-vivo responses to EC exposures were marked.
- The window of exposure producing most changes in fetal gonads differs between males and females. The testes are more affected by EC exposure during the first third of pregnancy while ovaries are more disturbed during the last third of pregnancy.
- Continuous exposure to sewage sludge during pregnancy produces fewer alterations in fetal gonads than short-term exposure for a part of gestation. The effects on gonad function are window specific. Similarly, gene expression

studies of the fetal gonad suggest that the fetus is more sensitive to a short exposure period than to continuous exposure throughout gestation.

- Most of the differentially expressed genes following sewage sludge exposure are down-regulated.
- Expression of key genes coding for proteins involved in metabolic and epigenetic processes is affected in fetal gonads after in-utero EC exposures. Dietary exposure of pregnant ewes to environmental concentrations of DEHP alters the expression of more fetal ovarian genes than exposure to PCBs or a mixture of DEHP+PCBs.
- In-utero exposure to DEHP±PCBs differentially effects the developing gonad: fetal ovarian morphology is not affected whereas testicular cell types are reduced in number.
- The developing fetal ovary is more sensitive to DEHP than PCBs or both combined, the latter is indicative of an interaction between the two chemical types. Under DEHP+PCB exposure most of the effects of DEHP exposure alone showed up again.
- In some mouse strains, DEHP is an obesogen producing elevated food intake, weight gain and increase in visceral adipose tissue mass in dams and their F1 offspring following dietary exposure of pregnant mice to environmental concentrations of DEHP.
- Key markers of the fatty acid metabolism (PPARs, Leptin, FABP4, Aco) are up-regulated by DEHP.
- In-utero and lactational exposure to DEHP in a range of doses relevant to human exposure induced functional and molecular dysregulation of pituitary-gonadal cross-talk in male and female mouse F1 offspring leading to reduced reproductive performance.
- In the mouse, transgenerational transmission, over three generations, of affected ovarian morphology characterized by early depletion of primordial follicular reserve occurs. This would lead to premature ovarian failure upon in utero and lactational exposure to DEHP at doses relevant to human exposure.
- In-utero and lactational exposure of mice to PCBs, showing preferential accumulation in offspring compared to their dams, induced decreased reproductive performance in both male and female mouse offspring. In males, adverse effects on sperm quality and testis morphology were observed up to the third generation.
- In-vitro exposure of sheep fetal gonads to DEHP±PCBs produces fewer alterations in the ovary than in-vivo exposure.
- Genes differentially expressed following in-vitro and in-vivo EDC exposures are different
- Extrapolation from in-vitro to in-vivo may be highly inaccurate.
- EDC exposure via cigarette smoke chemicals have multiple and sometimes sex-specific effects in the human fetus, including:
  - EDC burden (PAHs, PTEs)
  - Gene methylation, supporting concerns about epigenetic consequences of EC exposure
  - Multiple endocrine signalling mechanisms, including gonadal, pituitary and other organ systems and steroid hormone binding proteins
  - Gonadal gene expression
  - Anogenital distance
  - Liver function, including growth factors, xenosensors and biotransforming enzymes (e.g. steroid synthesis and
  - Vitamins B12 and members of the 1 Carbon cycle enzymes
  - Retinoic acid signalling system
  - Proliferation of ovarian germ cells, likely via AhR activation



**SOCIO-ECONOMIC IMPACT/IMPLICATIONS***Sludge and recycling*

Sewage sludge recycling to land is practised in many parts of the world including many countries in Europe. As the cost of fossil fuels and rock phosphate increases and availability declines, the cost of production of artificial fertilisers will increase, in real terms, while the population and demand for food continues to increase. Thus, it becomes increasingly **important to recycle waste products such as sewage sludge** and composts to land in order to recycle the valuable nutrients that they contain. However, the results of the present studies indicate that while sludge is a valuable fertiliser which can be used to maintain good pasture growth and associated meat production, there may be **adverse consequences for the reproductive development of the animals exposed** and, indeed, for later generations. It should be noted that this practice may result in small increases in the EDC load in the meat produced. However, the significance of the findings to human health probably lies not in increased exposure to EDCs through meat consumption but in the fact that a small increase in level of exposure and/or tissue burden is associated with reproductive perturbations. If these can occur in sheep exposed through sewage sludge, they may also occur in humans exposed through other mechanisms. The work reported here provides information concerning the effects of recycling practices and will underpin future policy decisions. Such decisions will clearly have significant socio-economic implications because elimination of potentially damaging EDCs from the recycled products will incur major costs. However, these have to be weighed against the potential costs in terms of animal and human health and in terms of threats to ecosystem sustainability, e.g. **damage to soil organisms and soil fertility as a result of application of wastes to land may adversely affect food production. Policy makers will be able to use information provided by this work to make more informed decisions about the regulatory regime surrounding applications to land, including amounts of sludge applied, withdrawal periods for livestock and restrictions on the classes of livestock allowed to graze treated land (e.g. breeding animals might not be permitted to graze sludge treated pastures).**

*Tissue concentrations of selected EDCs*

Although EDCs have been studied extensively, there are surprisingly few reports of tissue concentrations in target species. This reflects the high cost of such determinations and in some cases the difficulties in obtaining sufficient material for analysis. This series of studies provides a significant body of information concerning **EDC concentration in human tissue and in tissues of two model species** (sheep and mice); this will **underpin future studies of the effects of EDCs on animal physiology**.

A number of important observations have been made which will contribute to future experimental designs:

- a) Following exposure to the same experimental treatment, **individual animals exhibited very different rates of tissue accumulation**; this is probably a reflection of differences in uptake, metabolism and excretion, all of which are a function of genotypic differences between individuals. Similar variation in the capacity of animals to respond to a given EDC insult contributes to further variance in measurements. The high level of individual variation means that

relatively large numbers of animals must be studied in order to achieve statistically meaningful data.

- b) Rates of accumulation of chemicals of different groups were often different i.e. it was **not possible to extrapolate the results obtained with one chemical group to another**.
- c) The relationships between chemical burdens and responses were examined in some detail but it was **seldom possible to identify any one compound that was a major determinant of observed physiological changes**. An example from REEF is DEHP. There is increasing evidence that DEHP may not have a major effect on human reproduction and REEF shows that DEHP has marked effects on ovarian morphology in the mouse, but not the sheep, both in-vivo and in-vitro. This is despite the fact that pasturing on sewage sludge fertilised fields increased sheep tissue DEHP levels.
- d) **Correlations between maternal and fetal concentrations were very low**, making it difficult to extrapolate the concentration found in the mother to that of the fetus. Similarly, **correlations between fetuses of the same litter were very low**.

**Understanding of the importance of these factors in the determination of tissue burdens and associated risk to the target species will aid the formation of policies concerning human fetal and neonatal health and effects of environmental EDCs on wildlife populations.**

#### *Physiological effects with low concentrations of a mixture of compounds*

Until recently, it has often been argued that environmental concentrations of EDCs are much too low to be of concern because the measured concentrations are generally well below concentrations known to induce biological responses. However the results of the sheep studies in REEF have highlighted the potential for ***prolonged exposure to low concentrations of a complex mixture of EDCs, to induce adverse physiological responses, particularly in the developing fetus***. While it may be argued that the significance of the findings is limited to sheep exposed to sewage sludge, it is likely that the findings have very much wider implications. The tissue levels of EDCs in animals in these studies was minimally increased in response to exposure, indicating that **physiological systems could be perturbed by very small changes in EDC burden** and this suggests that other animal species, including humans, may also be affected by similar subtle changes. This hypothesis is further supported by controlled exposure studies performed in mouse model. Results of this study indicated that maternal exposure to selected EDCs during fetal gonadal development, at doses ranging from background to occupational human exposure, induced a variety of biological and molecular alteration in the reproductive system of offspring at adulthood. Of particular concern is the observation that some EDCs induced the most severe **effects at low doses, in the range of human background exposure levels**. Data from animal models may be difficult to extrapolate to humans. However, considering the substantial conservation of endocrine and reproductive processes across species, and the similarity of the transfer of EDCs from mother to fetus in mammals, it is reasonable to assume that human fetal EDCs exposure during critical point in development is a major reason of concern. Moreover, fetal gonad development in rodents does not complete until after birth. In contrast, in humans, ovarian and testicular development occurs entirely pre-natally. This in turn, makes effects of exposure to EDCs in the womb potentially more marked in the human than in the mouse. **A direct and important consequence is that more investigations on long lasting effects of EDCs exposure during fetal life are of a high priority.**

Furthermore, results of our studies in animal models, indicated that developmental exposure to a technical **mixture of EDCs induced synergistic or additive adverse effects of the single compounds, magnifying subtle effects on reproductive performance at very low doses**. This aspect is of particular relevance considering that in human individuals and populations are exposed to environmental compounds as mixtures, rather than single chemicals.

Animals and humans are likely to be exposed to such subtle increases in exposure under many different circumstances. Wildlife species may be exposed through pollution of air, water or soil and, particularly, through the consumption of contaminated prey if they are near the top of the food chain and effects of environmental pollution are magnified. Humans may be exposed through working in factories or in agriculture where specific EDCs are produced or used, through exposure in the home to cleaning products, fire retardants and food contaminants and through use of cosmetics and absorption of EDCs through skin. **Knowledge derived from the current studies of the EDC “insult” and associated effects on animal physiology will underpin future experimental design by identifying the most important phenotype and gene expression changes so that future research can focus on these.** We have shown that phthalate or PCBs (101,118) at low environmental concentrations differentially affect the developing ovine gonad in a sex dependent manner: testicular morphology was perturbed but there was no effect on follicle development in the fetal ovary. Nevertheless, DEHP in particular impacted on both the ovarian and testicular transcriptome and ovarian gene pathways associated with detoxification, structure, replication and transport have been highlighted for future research.

#### Effect of stage of development

Animals are known to be most sensitive to EDCs during early developmental stages but there is very little information in the literature concerning rates of tissue accumulation of EDCs in fetuses or how they may change according to the timing and duration of the exposure. The REEF studies have shown that patterns of **fetal tissue EDC accumulation differ with the stage of gestation** at which the dams are exposed and with the **duration of exposure**. Interestingly, **change in exposure patterns were apparently more disruptive than continuous exposure to sludge**. The window of exposure producing most changes in the ovaries corresponds to the last third of pregnancy when follicles form and differentiate. It is noteworthy that **the most sensitive period is not the same for male and female fetuses**. In males, early differentiation of testis (0-80dpc in sheep) appears more affected than late processes taken place in the end of pregnancy. This differs with the females where greater numbers of genes are affected during mid and late gestation. **This difference between genders must be taken in account in risk assessment, particularly in the choice of the tested exposure period.**

For both sexes, pregnancy represents the period of life during which, the women must make the greatest efforts to limit the exposure to EDCs. The identification of a specific period of gestation where sensitivity is maximal is complicated further by our ovine studies showing that a change in exposure during pregnancy is more disruptive for the developing gonad than continuous exposure. Indeed in terms of testicular Sertoli cell numbers, exposure during early, mid or late pregnancy is equally disruptive. Nevertheless in terms of both the phenotypic and transcriptomic impact of mixtures of chemicals, it would appear that the early gestation testis and mid plus late gestation fetal ovary are particularly at risk.

### Human and animal health

While the primary objective of the work was to address effects on the female reproductive system, we have taken the opportunity to harvest tissues to study many other aspects of EDC effects. We have shown perturbation, not only of the fetal female and male reproductive systems, but also in the bone structure of adult ewes, and various physiological or structural changes in the fetal hypothalamus, pituitary, thyroid gland, uterus and adrenal glands, as well as obesogenic effects in mice. Bearing in mind the fact that the increases in the rate of exposure or in tissue burden were modest, we suggest that the low levels of environmental EDCs to which humans and other animals are routinely exposed may be causing subtle, adverse effects. Such effects may result in similarly sub-acute adverse effects on human health. The observed (and decreasingly disputed) reduction in the human male sperm count and associated increases in the incidence of testicular cancer, and the increased rate of breast cancer in women may be examples of such effects. We have demonstrated in the human fetus that EC exposure affects and induces responses in the fetal liver and other tissues. It is important to bear in mind that polymorphisms in many genes, including detoxification genes, will affect to what degree an individual is affected by exposure to chemicals in-utero. The consequence is that exposures alter the incidences of abnormalities, such as we have shown for the sheep testis exposed in-utero and post-natally to sewage sludge. Such effects would be much less likely to be identified in highly inbred laboratory species and yet this is exactly what we see with human health; i.e. changes in incidences of reduced reproductive function, not global abnormal development. Therefore, much more research is required to understand the real implications of EC exposure for human health and wellbeing.

While there are many human health issues that have been linked, at least tentatively, to EDC exposure, little is known of effects on other species. Associations between high environmental / dietary levels of exposure to EDCs and health problems have been reported, particularly in some top predators such as polar bears and seals, effects of low level, chronic exposure are unknown. The results of the REEF studies suggest that **subtle adverse effects on organ function and animal health are likely and because they provide evidence of the developmental stages and physiological mechanisms that are likely to be most important they will inform both research and policy decisions**. Multiple signalling pathways are affected and timing and duration of exposure during pregnancy, together with fetal sex and species result in significant differences in the molecular mechanisms that are affected.

### Transgenerational effects

Studies involving mice have shown clearly that some effects on EDCs on animal physiology can be expressed in second, third and fourth generations, even although there has been no additional exposure after the first generation. Interestingly, the results of the current studies suggest that the patterns of **EDC accumulation in tissue may differ with treatment in later generations** but there are surprisingly **few changes in gene expression**.

In controlled studies in mice, current studies evidenced that selected EDCs, besides affecting reproductive health of *in utero* and lactationally exposed offspring, may also induce adverse effects in both male and female reproductive systems in later generations, despite not being additionally exposed. This observation is consistent with observations concerning other physiological system and environmental toxicants. Due to the nature of the compound investigated (rapidly metabolized vs. persistent), the mechanisms underlying the multigenerational transmission of

reproductive effects may involve epigenetic modifications in germ-lines and/or persistent body burden in *in utero* exposed offspring through sexual maturity. Nevertheless, independently of the mechanism underlying the inheritance of adverse effects along several generations, the observations that an **endocrine disruptor can cause a multigenerational defects on male and female reproduction have a significant impact on our understanding of the hazards of these compounds in mammals**. Elucidation of the mechanism involved in multigenerational endocrine disruptor actions will undoubtedly provide insights into diagnostics and therapeutics for environmental exposures, risk assessment and adult-onset disease.

Contrasting results were observed in sheep, in which F2 animals presented very subtle changes compared to mice. These differences are probably due to the difference of gonad-differentiation timing in these two species. The fact that continuous exposure produces fewer changes than exposures during short specific periods, indicates that some of the changes could be reversible and our findings are consistent with the data showing that epigenetic modifications could be involved in observed effects. Imprinting or DNA methylation, in gametes, occurs at various periods of development among mammals, in a species-specific manner. Several points merit consideration in risk assessment: a) the time separating the end of exposure and the analysis of effects, b) the gestational timing and gestational duration, of the exposure and c) the species. Additional research is required into large mammals (including humans) on the mechanisms and timing of apposition and erasure of epigenetic marks in gametes.

**The maternal cigarette-smoking model used in REEF has provided a conclusive link between human and animal: clear methylation changes in the human fetal liver.** These changes have occurred in genes involved in growth and it is striking that the male was the more affected since male fetal growth is reduced to greater extent by maternal indices, such as cigarette smoking, in many of the studies of birthweight in humans. However, female fetuses also exhibited methylation changes in some of these genes and it is likely that complex mixtures of ECs and EDCs cause epigenetic changes in the human.

Changes in gene methylation can have another consequence: a greater disconnect between gene expression profiles and expression levels of the proteins for which they are coded. The observation, in REEF, of many differences between transcriptomic and proteomic expression patterns is likely to reflect EC effects on levels gene activation and repression, thus modulating protein production in complex and unpredictable ways. This would also reduce the accuracy of extrapolation from effects of single chemicals to real-life situations. **Given the significant differences of timing of in-utero development between species, especially between human and mouse or rat, these findings also encourage greater caution in extrapolation from these rodents to the human.**

#### Attitudes to EDCs

In a study led by Dr Kathryn Mearns of the School of Psychology, University of Aberdeen, the attitudes of 211 participants (67 men and 144 women; research scientists (24), psychology students (72), biology students (105) and chemistry students (10)) were investigated. The results suggested that people are generally ignorant about EDCs and their proposed effects but once information is provided perceptions change significantly in certain groups.

Based on these findings it was considered of interest to determine how an informed group of participants perceived EDCs; the group selected were the research

scientists of the NECTAR cluster working on a range of issues associated with EDCs. A questionnaire was designed using a well-established psychological model of how people think about and behave in relation to specific health threats. Peoples' intentions to behave in a particular way are dependent upon three major factors : their attitudes toward the issue under investigation; the perceived level of control individuals feel they have when responding to the issue and how people who are important to them perceive how they should respond to the issue (subjective norms). The questionnaire asked for demographic information (gender, scientific discipline and age group); it included 18 items designed to tap the participants' attitudes, perceived behavioural control and subjective norms. Other questions related to the individuals' intentions to avoid EDCs and the final item was an open-ended question which allowed participants to give feedback and recommendations about the questionnaire and EDCs. All items, except the last, asked participants to select one number from a 7-point Likert scale to indicate the response that most closely matched their own views from '1 strongly agree' to '7 strongly disagree'. The questionnaire is shown in Appendix 1. Mean scores were calculated for each group of questions (attitude, perceived behavioural control, subjective norms and intentions) thus creating scale scores. These scale scores were then entered into SPSS for analysis. The results indicated that most scientists were scoring in the mid-range of the scale 'Slightly agree' to 'Neutral', with little deviation around the mean and the fullest range of responses being given for 'Perceived Behavioural Control'. A regression analysis was run to determine which variable: 'Attitude'; 'Perceived Behavioural Control' or 'Subjective Norm' predicted 'Intention to Avoid EDCs' but the model was not significant indicating that other factors outside those measured in this study were probably contributing to intended behaviour regarding EDCs. The small sample of informed scientists surveyed in this study had fairly neutral attitudes to EDCs and did not perceive that they had high levels of behavioural control. Neither were they influenced unduly by the expectations of significant others (family, friends and colleagues) regarding exposure to EDCs. The feedback from the scientists focussed on the education of both producers and consumers with particular emphasis on organizations related to prenatal and antenatal care. It is concluded that understanding risk perceptions and attitudes is not straightforward, significant disagreement being found amongst scientists with respect to the threats that EDCs pose and the impact they may have on the environment and human and animal health.

## **DISSEMINATION ACTIVITIES AND EXPLOITATION OF RESULTS**

The group involved in this work has been very active in disseminating their findings. Thirty two refereed scientific papers, concerning original data, and review papers, have already been published (<http://www.abdn.ac.uk/reef/publications/> ). However, it must be pointed out that there are at least a further **21** publications in-press, submitted or in preparation for peer-reviewed publication. These include the largest, highest impact papers reporting the major key findings of the REEF project.

In addition, a large number of conference talks and posters (<http://www.abdn.ac.uk/reef/presentations/conference/> ) have also been generated with the work of the group being highlighted, in particular, at two Copenhagen Workshops on Endocrine Disrupting Compounds and at a Gordon Conference on Environmental Endocrine Disruptors but also at numerous additional conferences.

A number of the PIs have presented invited review papers at international conferences (<http://www.abdn.ac.uk/reef/presentations/conference/> ) and much of the recent work of the REEF group has been publicised to the scientific and medical communities through these presentations and the published papers associated with

many of them. Through these meetings and other, less formal meetings (<http://www.abdn.ac.uk/reef/presentations/seminars-talks/>), efforts have been made to present the findings in an appropriate to various specialised interest groups, concerned with aspects of policy (e.g. French Ministry of Health (one PI was involved in a collective expertise on Environment and Reproduction asked to INSERM by the Ministry of Health (a report has been written and is available on line: <http://www.inserm.fr/thematiques/sante-publique/expertises-collectives>), waste recycling practice (Chartered Institute of Waste Managers), veterinary issues (Nordic Committee for Veterinary Scientific Cooperation), students (Lectures as part of courses), school children (Presentation at Satrosphere, Aberdeen) and the public (Macaulay Institute Open Day). In addition, the work of REEF was presented at the Covance Environmental Risk Assessment Symposium, held in Brussels on the 8-9<sup>th</sup> March, 2011, which was attended by a broad range of stakeholders (<http://www.covance.com/products/environmental/index.php>). REEF has also worked to reach wider audiences, including the European Commission itself, e.g. by press-releases (<http://www.abdn.ac.uk/reef/presentations/press-releases/>) and by articles in dissemination magazines (<http://viewer.zmags.com/publication/78fc1aa2#/78fc1aa2/36>).

Several PIs are Professors or give seminars to students at several University levels. Data from REEF have been included and disseminated through their courses. Several Master and PhD students have contributed to REEF results, these data figured in their reports and have been presented in European Universities and Institutes.

***Papers in press, submitted or in preparation for publication in peer-reviewed journals:***

1. Rhind SM. (2012) Anthropogenic pollutants – an insidious threat to animal health and productivity. *Acta Veterinaria Scandinavica* In press.
2. O'Shaughnessy P.J., Monteiro A., Bhattacharya S. & Fowler P.A. (2012) Expression of enzymes involved in alternative ("Back-door") androgen synthesis in the human fetal gonad and effects of maternal cigarette smoking. Intended for *J. Clin. Endocrinol. Metab.*
3. O'Shaughnessy P.J., Monteiro A., Bhattacharya S. & Fowler P.A. (2012) Effect of gender and maternal smoking on enzymes linked to steroid synthesis and metabolism in the human fetal liver. Intended for *J. Clin. Endocrinol. Metab.*
4. Amezcua M.R., O'Shaughnessy P.J., Monteiro A., Bhattacharya S. & Fowler P.A. (2012) oocyte and gestation stage-specific expression of major vault protein (MVP) in the ovary. Intended for *Reproduction*.
5. Flannigan S., Monteiro A., Fraser M.J., Stoney P.N., O'Shaughnessy P.J. & Fowler P.A. (2012) Maternal smoking disturbs sex-steroid transport protein expression in the male fetus. Intended for *J. Clin. Endocrinol. Metab.*
6. Fowler P.A., Drake A.J., O'Shaughnessy P.J., Kerrigan D., Bhattacharya S., Sinclair K.D., Monteiro A., Goetz S., Raab A., Rhind S.M., Meharg A. & Feldmann J. (2012) In-utero exposure to cigarette chemicals induces gender-specific fetal epigenetic modifications and disruption of one-carbon metabolism. *J. Clin. Invest.*
7. Fowler P.A., Childs A.J., Courant F., Rhind S.M., McIlwain L., Coutts S., Kinnell H., Antignac J-P., Le Bizet B., Maheshwari A., Bhattacharya S., Monteiro A., Anderson R.A., & O'Shaughnessy P.J. (2012) How does maternal cigarette smoking reduce fertility of human female offspring? *J. Clin. Invest.*
8. Huuskonen P., Storvik M., Keski-Nisula L., Heinonen S., Monteiro A., Fraser M.J., Stoney P.N., O'Shaughnessy P.J., Fowler P.A., Pasanen M. (2012)

- Proteomic analysis of placentas from cigarette smoking mothers. Intended for *Mol. Cell. Endocrinol.*
10. Hombach-Klonisch S., Danescu A., Begum F., Amezaga M.R., Rhind S.M., Sharpe R.M., Cotinot C., Mandon-Pepin B., Evans N., Bellingham M., Fowler P.A., Klonisch T. (2012) Periconceptional changes in maternal exposure to sewage sludge affects fetal thyroid gland development in sheep. Intended for *Mol. Cell. Endocrinol.*
  11. Amezaga M.R., Bellingham M., Mandon-Pepin B., Speers C.J.B., Kyle C.E., Evans N.P., Richard Sharpe R.M., Cotinot C., Rhind S.M., & Fowler P.A. (2012) Exposure to chemical cocktails before or after conception - the effect of timing on ovarian function. *Mol. Cell. Endocrinol.*
  12. Amezaga M.R., Mandon-Pepin B., Loup B., Sinclair K.D., Lea R.G., Rhind SM, Cotinot C., Fowler PA (2012) In-utero exposure to chemical cocktails via the mother : the fetal ovary has specific windows of sensitivity. Intended for *Environ. Health. Perspect.*
  13. Amezaga M.R., Mandon-Pépin B., McNeilly A.S., Evans N.P., Bellingham M., Sharpe R.M., Rhind S.M., Cotinot C. & Fowler P.A. (2012) Fetal and lactational exposure to a complex mixture of environmental chemicals reduces the quality of the ovarian reserve in young adults. Intended for *PLoS Biology*.
  14. Schmidt J.-S., Schaedlich K., Fiandanese N., Pocar P., Fischer B. (2011) Di(2-ethylhexyl) phthalate (DEHP) impairs female fertility and promotes adipogenesis in C3H/N mice. *Environ. Health. Perspect.* Final revision
  15. Loup, B., Amezaga M.R., Rhind S.M., Lea R., Sinclair K., Fowler PA., Cotinot C., Mandon-Pépin B. Development of an ex-vivo ovine fetal ovary culture for assessing chemical effects on female meiosis and early folliculogenesis.
  16. Loup, B., Amezaga M.R., Rhind S.M., Lea R., Sinclair K., Fowler PA., Cotinot C., Mandon-Pépin B. Effect of low doses of environmental chemicals on fetal ovaries ex vivo.
  17. Sinclair KD, Loup B, Mandon-Pépin B., Amezaga M.R., McKinlay R., Rhind S.M., Cotinot C. Evans N.P., Bellingham M., Fowler P.A., Lea RG. (2012) Environmental concentrations of Di(2-ethylhexyl) phthalate (DEHP) and PCBs (101,118) perturb development of the fetal ovine HPG axis. Intended for *Environ. Health. Perspect.*
  18. Lea RG., Loup B., Mandon-Pepin B., Amezaga M.R., Rhind SM, Cotinot C., Fowler PA Sinclair K.D. (2012) The effects of a cocktail of chemicals contained in sewage sludge on ovine fetal testis development during different periods of gestation. Intended for *Environ. Health. Perspect.*
  19. Aralla M., Mobasheri A., Rhind SM., Fowler PA., Marples D., Sinclair KD., Arrighi S., Lea RG. (2012). The expression of aquaporin 7 in the ovine fetal epididymis is perturbed by in-utero exposure to a cocktail of chemicals contained in sewage sludge fertiliser. Intended for *Reproduction*.
  20. McLaggan D, Lagido C, Amezaga MR, Petra E, Frost A, Duff EI, Rhind SM, Fowler PA, Glover A, Lagido C. 2012. Effect of an Environmentally-Relevant Mixture of Pollutants on *Caenorhabditis elegans* as a Model Biosensor. Intended for *PLoS One*.
  21. Fowler P.A., Courant F., Antignac J-P., Le Bizec B., Bhattacharya S., Monteiro A., Sharpe R.M. & O'Shaughnessy P.J. (2012) The male human fetus has very high circulating oestrogen levels: implications for oestrogenic endocrine-disrupting compounds. Intended for *Nature*.
  22. Transgenerational effects of DEHP on the mouse: being planned, final bioinformatics analyses underway. Should achieve very high impact.



## PROJECT WEBSITE AND RELEVANT CONTACT DETAILS



<http://www.abdn.ac.uk/reef/>

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