Female reproduction and endocrine disrupting chemicals

(FEMREP 2013)

November 5-6, 2013
Uppsala, Sweden

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ISSN: 1404-5915
ISBN: 978-91-576-9057-9

Tryck: SLU Service/Repro, Uppsala 2013

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Foreword

The conference Endocrine disrupting chemicals and female reproduction (FEMREP 2013) is arranged by the Centre for Reproductive Biology in Uppsala (CRU), November 5-6, 2013, Uppsala, Sweden. CRU is a multidisciplinary network of about a hundred scientists working in the field of animal and human reproduction at the Swedish University of Agricultural Sciences (SLU) and Uppsala University (UU).

The risk of endocrine disrupting chemicals (EDC) to the reproductive health in wildlife and humans is currently a matter of great world-wide concern. Epidemiological and experimental data suggest that EDCs are involved in some of the observed female reproductive disorders. There are however often a lack of data or contradictive findings which makes it difficult to establish cause-effect relationships. FEMREP 2013 aims to 1) gather current evidence for EDC involvement in female reproductive disorders in humans, wildlife, and domestic animals, 2) to identify data gaps and research needs, and 3) to facilitate interdisciplinary knowledge transfer and collaboration. The conference will address the following questions: *What is known about the effects of EDCs on female reproduction in humans, wildlife and domestic animals? What are the trends for female reproductive disorders and their links to EDCs and how do we find them?*

The organizing committee consists of Associate Professor Cecilia Berg, Department of Environmental Toxicology, UU, Associate Professor Katrin Lundstedt-Enkel, Department of Environmental Toxicology, UU, Professor Matts Olovsson, MD, PhD, Department of Women´s and Children's Health, UU, and Sara Persson, PhD, Department of Clinical Sciences, SLU.

Financial support for the conference is provided by the CRU and the Swedish research council Formas.

We hope that you will find opportunities for learning and networking and wish you a warm welcome!

Cecilia Berg

On behalf of the organizing committee

Programme

Endocrine disrupting chemicals and female reproduction 2013

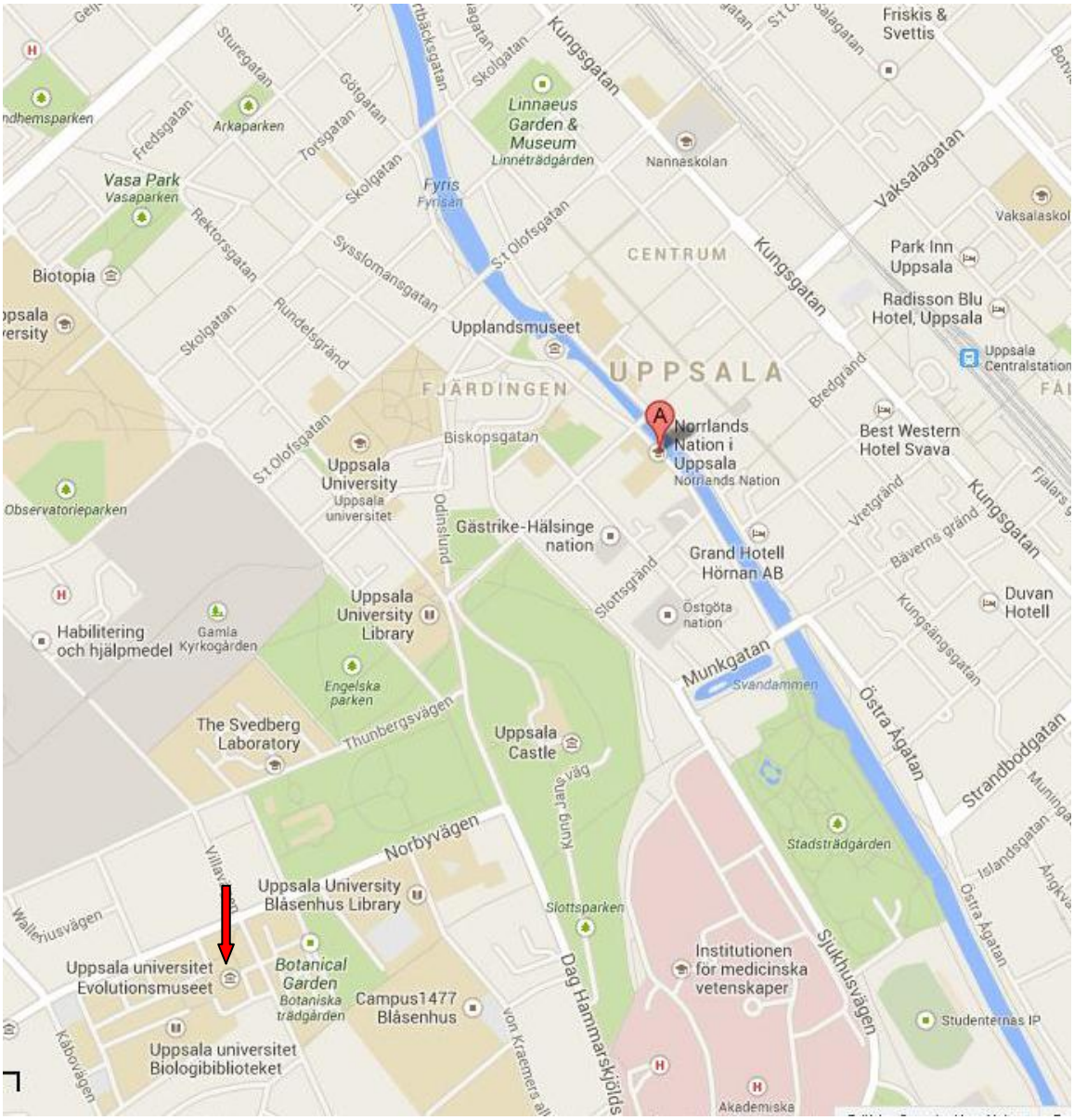
Ekman lecture hall, Norbyvägen 14, Evolutionary Biology Centre (EBC), Uppsala University, Sweden.

5th November		
Session I. ***Chair: Assoc. Prof. Cecilia Berg***		
08.00-09.00	***Registration and coffee***	
09.00-09.15	Welcome	Assoc. Prof. Cecilia Berg *Organizing committee*
09.15-10.00	Environmental contaminants and a wildlife sentinel species: gene expression and epigenetic regulation of gonadal development	Prof. Louis Guillette, USA *Key note speaker*
10.00-10.30	***Coffee, poster session***	
10.30-11.15	Female sexual maturation and reproduction after developmental exposure to endocrine disrupting chemicals	Dr. Anne-Simone Parent, Belgium *Invited speaker*
11.15-11.30	Mammary gland development: sensitive markers of endocrine disruption?	Dr. Karen Mandrup, Denmark *Selected abstract*
11.30-11.50	EDC – Policy development and regulatory needs	Prof. Christina Rudén, Sweden *Invited speaker*
11.50-12.00	Discussion	
12.00-13.15	***Lunch at the EBC restaurant (included in fee)***	
Session II. ***Chair: Assoc. Prof. Katrin Lundstedt-Enkel***		
13.15-14.00	EDC involvement in reproductive dysfunction in rodent studies and the occurrence of non-monotonic dose-response relationships	Dr. Julie Boberg, Denmark *Invited speaker*
14.00-15.00	***Coffee, poster session***	
15.00-15.15	Risk to all or none? The Bisphenol A risk controversy	Dr. Anna Beronius, Sweden *Selected abstract*
15.30-15.45	Science in Risk Assessment and Policy (SciRAP) – an online resource for evaluation and reporting of (eco)toxicity studies	Linda Molander, Sweden *Selected abstract*
15.45-16.15	Discussion	
19.00 –	***Social activity, DINNER at Norrlands Nation, Västra Ågtan 14 (Included in fee)***	

6th November		
Session III. ***Chair: Prof. Matts Olovsson***		
09.00-9.45	The evidence for EDC involvement in reproductive dysfunction in women	Prof. Paul Fowler, UK *Invited speaker*
09.45-10.30	***Coffee, poster session***	
10.30-11.15	Early breast development in girls after prenatal exposure to non-persistent pesticides	Dr. Christine Wohlfahrt Veje, Denmark *Invited speaker*
11.15-11.30	Bisphenol A affects human endometrial endothelial cell angiogenic activity *in vitro*	Dr. Malin Helmestam, Sweden *Selected abstract*
11.30-11.45	Female developmental reproductive toxicity of estrogen and progestin in the model frog *Xenopus tropicalis*	Assoc. Prof. Cecilia Berg, Sweden *Selected abstract*
11.45-12.00	Discussion	
12.00-13.15	***Lunch at the EBC restaurant (included in fee)***	
Session IV. ***Chair: Dr. Sara Persson***		
13.15-14.00	Evidence for EDC involvement in reproductive dysfunction in female domestic animals	Prof. Ulf Magnusson, Sweden *Invited speaker*
14.00-14.15	Maternal exposure to estradiol and endocrine disrupters cause malformations in eelpout fry	Dr. Jane Ebsen Morthorst, Denmark *Selected abstract*
14.15-14.30	Hormone disruption by cadmium–a risk for reproductive health?	Dr. Pauliina Damdimopoulou Sweden *Selected abstract*
14.30-15.00	***Coffee, poster session***	
15.00-15.15	Circulating levels of PCBs and sex hormones in a population-based sample	Dr. Johanna Penell *Selected abstract*
15.15-15.30	Discussion of all presentations Concluding remarks	Assoc. Prof. Cecilia Berg *Organizing committee*

Map

The venue for the conference is the Evolutionary Biology Centre (EBC), Norbyvägen 18 (arrow). The conference dinner will take place at Norrlands nation, Västra Ågatan 14 (marked "A"). Dinner starts at 19.00



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Oral presentation abstracts

Environmental contaminants and a wildlife sentinel species: gene expression and epigenetic regulation of gonadal development

Louis J. Guillette Jr.

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A growing list of environmental contaminants has been shown to alter the development of the reproductive system. Many of these compounds act by altering endocrine signaling and are known as endocrine disrupting compounds (EDCs). Coupled with exposure to EDCs, is an alteration in phenotype that is related to modified gene expression. Recent studies in mammals indicate that DNA methylation can be the basis for altered patterns of gene expression. DNA methylation is a highly conserved epigenetic modification that influences gene expression and is a key mediator between an organism's genome and its environment. The presence of methylated gene promoters is generally thought to result in transcriptional repression. We have examined gene expression in embryos exposed to agricultural-based pollutants as well as during temperature-mediated sex determination in the American alligator embryo and identified altered gene expression profiles. We have examined promoter methylation patterns for several key genes, such as Sox9, AMH and Cyp19arom and observed sexual dimorphic patterns. Further, we have examined alterations in promoter methylation for these genes in embryos naturally exposed to a mixed of agriculturally-based contaminants (embryos from Lake Apopka, FL). Finally, global genome methylation has been determined for the testis and blood cells from adult male alligators from three sites: Lake Woodruff (reference site), Lake Apopka (pesticide contamination) and Merritt Island Wildlife Refuge (metal contamination). Testicular tissue from adult male alligators from Merritt Island exhibited elevated global methylation when compared to the other two sites. Future work is expanding our understanding of gene specific and age specific methylation patterns in this sentinel species.

Female sexual maturation and reproduction after developmental exposure to endocrine disrupting chemicals

Dr. Anne-Simone Parent

Developmental Neuroendocrinology Unit, GIGA-Neurosciences, University of Liège, Belgium

The timing of puberty has been mainly studied in females for several reasons including the possible evaluation of a precise timer i.e. menarcheal age and concerns in the high prevalence of precocity in females as opposed to males. Human evidence of altered female pubertal timing after exposure to endocrine disrupting chemicals (EDCs) is equivocal. Among limiting factors, most studies evaluate exposure to single EDCs at the time of puberty and can hardly assess the impact of lifelong exposure to mixtures of EDCs. Some rodent and ovine studies indicate a possible role of fetal and neonatal exposure to EDCs, along the concept of early origin of health and disease. Such effects are possibly involving neuroendocrine mechanisms since the hypothalamus is a site where homeostasis of reproduction as well as control of energy balance are programmed and regulated. In our previous studies, pulsatile Gonadotrophin Releasing Hormone (GnRH) secretion control through oestrogen, glutamate and aryl hydrocarbon receptors (AhR) was shown to be involved in the mechanism of sexual precocity after early postnatal exposure to the insecticide dichlorodiphenyltrichloroethane (DDT). Very recently, we have shown that neonatal exposure to the potent synthetic oestrogen diethylstilbestrol (DES) or Bisphenol A is followed by early or delayed puberty depending on the dose, with consistent changes in developmental increase of GnRH pulse frequency. Moreover, DES results in reduced leptin stimulation of GnRH secretion *in vitro*, an effect that is additive with prenatal food restriction. Thus, using puberty as an endpoint of EDC effects, it appears necessary to consider pre- and perinatal exposure to low doses and to pay attention to the other conditions of prenatal life such as energy availability, keeping in mind the possibility that puberty could be not only advanced but also delayed through neuroendocrine mechanisms.

Mammary gland development: sensitive markers of endocrine disruption?

Mandrup K.R., Boberg J., Hass U.

National Food Institute, Technical University of Denmark, Division of Toxicology and Risk Assessment, Mørkhøj Bygade 19, 2860 Søborg, Denmark

Introduction
The incidence of precocious breast development and breast cancer is increasing in USA and Europe. Mammary gland examination in toxicological studies may be useful to improve knowledge on influences of endocrine disrupting compounds (EDCs) on human mammary glands and for detection of endocrine disrupting effects of chemicals as part of safety testing.

Aims
The aims were to investigate the effects of EDCs on mammary gland development and to investigate the sensitivity of the methods.

Material and Methods
Rat dams were exposed during pregnancy and the lactation period to EDCs with estrogenic or anti-androgenic modes of action, including ethinyl estradiol, mixtures of phytoestrogens, mixtures of pesticides, and mixtures of environmental chemicals. Changes in mammary gland development were examined in prepubertal and adult female offspring.

Results
Estrogenic compounds appeared to increase outgrowth in prepubertal mammary glands and ethinyl estradiol increased the density. Changes in epithelial morphology were observed in adults exposed perinatally to phytoestrogens. No effects of anti-androgens were observed in female mammary glands.

Discussion
Estrogenic compounds appeared to enhance female mammary development, and in studies on estrogenic chemicals marked effects on prepubertal female mammary glands were observed at lower levels than those affecting other endpoints studied.
Conclusions: The present findings suggest that EDCs may affect mammary development and estrogenic chemicals may contribute to precocious breast development in girls. Histological examination of adult mammary glands are included in the extended one-generation OECD guideline studies, however, risk assessment of estrogenic chemicals may overlook effects on mammary glands if effects on prepubertal mammary glands are not investigated.

EDC – Policy development and regulatory needs

Christina Ruden

Stockholm University, Stockholm, Sweden

This presentation gives an overview of how EDC are managed in current EU chemicals legislation, the outstanding issues and ongoing policy development.

EDC involvement in reproductive dysfunction in rodent studies and the occurrence of non-monotonic dose-response relationships

Julie Boberg, Louise Krag Isling, Pernille Jacobsen, Karen Mandrup, Sofie Christiansen, Marta Axelstad, Ulla Hass.

This presentation will cover two issues of importance to female reproduction, namely the influence of environmental chemicals on female reproduction in a rodent model and the issue of non-monotonic dose-response curves described in toxicological studies.
In recent years, observed changes in the female reproductive system have been proposed to constitute an "Ovarian dysgenesis syndrome". Impaired placental function, early pregnancy loss, breast cancer, altered pubertal timing and premature ovarian failure are suspected to be causally linked and to be influenced by exposure to endocrine disrupters. Rodent studies have indicated that mammary development, ovarian folliculogenesis, fertility and onset of puberty and menopause can be altered by perinatal exposure to environmental contaminants including endocrine disrupting chemicals.
In a study on perinatal exposure of Wistar rats to a mixture of human relevant endocrine disrupting chemicals, the influence on the female reproductive system was examined. The mixture consisted of 13 anti-androgenic or estrogenic chemicals, including phthalates, pesticides, cosmetic ingredients, bisphenol A and paracetamol, and the mixture ratio reflected high-end human intakes. Throughout gestation and lactation time-mated rats (n=16-20 dams) were exposed to the total mixture (TotalMix) at 100, 150, 200 and 450 times high-end human intake, or to mixtures of only the anti-androgens (AAMix) or only the estrogens (EMix) at 200 or 450 times human intake, or to paracetamol.
In prepuberty, increased growth of female mammary glands was seen in the groups exposed to estrogenic compounds (EMix and TotalMix groups). At 1 year of age, signs of earlier reproductive senescence (menopause) and decreased ovary weights were seen in groups exposed to antiandrogens (AAMix and TotalMix groups). Histological examination of one ovary section from each 1-year old female revealed an increased incidence of rats with complete lack of corpus lutea in the AAMix group and the paracetamol group.
This study showed that developmental exposure of rats to endocrine disrupters induced early as well as long-lasting effects on female reproduction. Adverse reproductive effects were observed at mixtures reflecting 100-200 times high end human exposure. Thus, a safety margin of 100 usually required for regulatory purposes may not be obtained for highly exposed women, suggesting that highly exposed human population groups may not be sufficiently protected against endocrine disrupting effects of environmental chemicals.
The second part of the talk focuses on non-monotonous dose-response curves. The issue of non-monotony is of current debate, as emerging data on particularly estrogenic compounds such as Bisphenol A have indicated that effects seen at low dose levels differ from those seen at higher dose levels. This is a well-described feature in clinical situations, where e.g. hormonal treatment of mammary cancer leads to growth-stimulating effects at low dose levels ("flare") and growth inhibition at higher dose levels. This phenomenon also occurs in toxicological studies, and a few examples and explanations will be presented. The issue of possible consequences for interpretation of toxicological studies and for risk assessment will be raised for discussion.

”Risk to all or none? The Bisphenol A risk controversy”

Anna Beronius[1,2]

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Bisphenol A (BPA) is an endocrine disruptor that has received much attention and for which risk assessment has proven particularly complicated. Several different authorities and expert groups have evaluated BPA during the last decade but have come to different conclusions regarding the human health risks posed at current exposure levels (Beronius et al., 2010). In regulatory toxicity studies, conducted according to standardized and internationally validated test guidelines, effects of BPA have only been observed at relatively high doses, with a no observed adverse effect level (NOAEL) of 5 mg/kg bw/day. These standard studies have generally been used as key evidence in regulatory risk assessments of BPA. In contrast, over the last decade a large number of non-standard research studies, i.e. studies not conducted according to standardized methods, have reported effects at very low doses of BPA, often in the µg/kg bw-range and within or close to estimated human exposure levels. These research studies provide data that may be important to fill information gaps and improve risk assessment conclusions in the BPA-case (Beronius et al., 2013). However, research studies are often criticized for having methodological limitations or being too insufficiently reported to serve as evidence in regulatory risk assessment. To bridge the gap between academic research and regulatory risk assessment we need to promote a level of reporting that fulfill the requirements for risk assessment, as well as develop tools that can be used to facilitate systematic and transparent evaluation of research studies.

References

Beronius A, Johansson N, Rudén C and Hanberg A. 2013. The influence of study design and sex-differences on results from developmental neurotoxicity studies of bisphenol A, implications for toxicity testing. Toxicology. Published online ahead of print: http://dx.doi.org/10.1016/j.tox.2013.02.012.

Beronius A, Rudén C, Håkansson H and Hanberg A. 2010. Risk to all or none? A comparative analysis of controversies in the health risk assessment of Bisphenol A. Reprod Toxicol 29:132-46.

Science in Risk Assessment and Policy (SciRAP) – an online resource for evaluation and reporting of (eco)toxicity studies

Linda Molander[1], Marlene Ågerstrand[1], Anna Beronius[1,2], Annika Hanberg[2], Christina Rudén[1]

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Risk assessment of endocrine disrupting compounds (EDC) is often hampered by large scientific uncertainties. In part, this stems from the complex toxicity of these compounds, which challenges many assumptions and principles traditionally applied in (eco)toxicity testing and risk assessment. Toxicity studies conducted according to internationally standardized test guidelines, such as the OECD test guidelines, are often considered reliable by default and preferred as key evidence in regulatory risk assessment. However, there is a large amount of academic research studies available in the peer-reviewed literature. Many of these use novel methods argued to be more sensitive and relevant than current standardized methods for the evaluation of the (eco)toxicity of EDCs. There is thus an identified need to facilitate the use of non-standard academic research studies in risk assessment of EDCs, as well as for other groups of chemicals.
We propose a framework for study evaluation that would facilitate systematic and transparent evaluation of the reliability and relevance of (eco)toxicity studies for health and environmental risk assessment. The framework includes specific criteria to guide study evaluation, as well as a color-coding tool developed to aid the application of these criteria and conclusions regarding study reliability and relevance. In addition we provide guidelines intended for researchers on how to report such studies. These resources, including the color-coding tool, are publically available on-line and free of charge at www.scirap.org. The intention is to increase the usability of all relevant data that may fill information gaps and thereby reduce scientific uncertainty in chemical risk assessment conclusions. This could especially contribute to reducing scientific uncertainty in risk assessment and policy decisions for EDCs.

The evidence for EDC involvement in reproductive dysfunction in women

Prof. Paul Fowler

University of Aberdeen, UK

Early breast development in girls after prenatal exposure to non-persistent pesticides

Dr. Christine Wohlfahrt Veje

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American and European girls today develop breasts earlier than 15-20 years ago. Alterations in BMI alone cannot account for these changes. Several currently used pesticides possess endocrine disrupting properties and may interfere with reproductive development, but human data are sparse.

We examined girls whose mothers worked in greenhouses in the first trimester of pregnancy to assess the long-term effects of prenatal pesticide exposure on puberty. Mothers were prenatally categorized as exposed or unexposed to pesticides. We studied the offspring of these greenhouse workers, and evaluated the anthropometry, pubertal staging in the girls, and blood samples were drawn at three months of age (n=90) and again once at school age (6 to 11 years, n= 83).

No clinical and biochemical differences were found between the exposed and unexposed girls at three months of age. Mean onset of B2+ was 8.9 years (95% CI: 8.2; 9.7) in prenatally exposed girls, compared to 10.4 years (9.2; 17.6) in the unexposed (p=0.05), and 10.0 (9.7-10.3) years in a Danish reference population (p=0.001). Exposed girls had higher serum androstenedione levels (geometric means: 0.58 vs. 0.79 nmol/l, p=0.046), and lower Anti-Müllerian Hormone (AMH) compared with the unexposed (geometric means: 16.4 vs.21.3 pmol/l, $p<0.05$) and the reference group (20.2 pmol/l, p= 0.012). Levels of testosterone, estradiol, prolactin, FSH, LH, SHBG, DHEAS, DHT, Inhibin A, and Inhibin B did not differ between the groups.

In conclusion, our findings suggest that prenatal exposure to currently approved pesticides may cause earlier breast development in girls. This association appeared not to be due to changes in gonadotropins, but rather to higher androgen levels, which indirectly may increase estrogens through aromatisation. In addition, lower serum AMH levels indicated a reduced pool of antral ovarian follicles. The long-term consequences of our findings in regard to establishment of future reproductive function are yet unknown.

Bisphenol A affects human endometrial endothelial cell angiogenic activity *in vitro*

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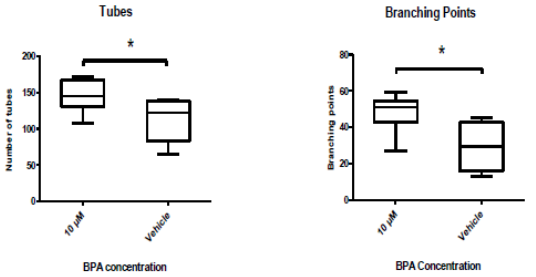
Introduction
The widespread use of bisphenol A (BPA) and the high content in consumables has led to ubiquitous exposure in human populations. Bisphenol A is classified as an endocrine disrupting chemical (EDC) with estrogenic properties. Human endometrial endothelial cells (HEECs) cells play a key role in the control of endometrial angiogenesis.

Aim
In this study we sought to examine whether or not there are any effects of environmentally relevant concentrations of BPA on co-cultured HEEC proliferation, viability, gene and protein expression and angiogenesis *in vitro*.

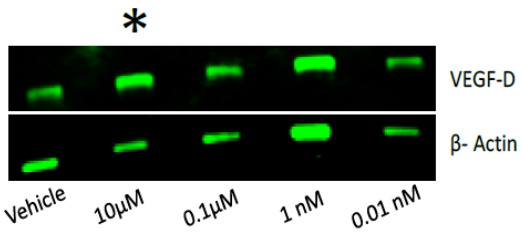
Materials and Methods
Primary HEECs were co-cultured together with primary endometrial stromal cells, and exposed to BPA (10 M, 0.1 M, 1 nM, 0.01 nM). The effects of BPA were evaluated by way of proliferation and viability assays, tube-formation assays, quantitative PCRs (qPCRs), Western blots and VEGF ELISAs.

Results
Bisphenol A induced increased HEEC tube formation and VEGF-D mRNA and protein expression compared with vehicle, without affecting HEEC viability or proliferation.

Discussion and Conclusions
Here we have shown that exposure to BPA increases HEEC tube formation and also induce VEGF-D protein and mRNA expression. Bisphenol A might therefore disturb endometrial vascular development and function, and subsequently endometrial actions important for normal function and successful implantation and placentation.



Tube formation assay with cells exposed to 10 µM BPA showed increased angiogenic activity as seen by increased numbers of tubes and branching points *p<0.05.



VEGF-D protein expression was significantly increased in HEEC with vehicle when exposed to 10µ BPA.

Female developmental reproductive toxicity of estrogen and progestin in the model frog *Xenopus tropicalis*

Cecilia Berg

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Introduction
The present project aims to develop methods to investigate developmental and reproductive effects of endocrine disrupting chemicals using the model frog *Xenopus tropicalis*. Developmental reproductive toxicity was characterised using the estrogen ethinylestradiol (EE_2) and the progestin levonorgestrel (synthetic progesterone). They are used as model compounds as well as environmental contaminants as they are ubiquitously found in the aquatic environment. Both progestins and estrogens are extensively used in human and veterinary medicine.

Aim
Here I review our findings regarding impacts of EE_2 and levonorgestrel on the differentiation of the gonads and Müllerian ducts. The Müllerian ducts are precursors of the female reproductive tract in vertebrates (except teleost fish).

Material and Methods
Xenopus tropicalis were exposed to EE2 or levonorgestrel via the water during the larval period encompassing the period of sex differentiation. Exposure concentrations were verified by chemical analysis. Exposure was discontinued at metamorphosis, whereafter the frogs were raised to adult age for evaluation of fertility and reproductive organ morphology.

Results
Low environmental concentrations of EE_2 caused infertility by disrupting the differentiation of Müllerian ducts and testicles. Developmental levonorgestrel exposure inhibited oogenesis and caused a complete lack of developed oviducts rendering the females sterile. No effects of levonorgestrel were seen in the males.

Discussion and conclusions
In summary, 1) egg development and Müllerian duct differentiation are targets for developmental progestin exposure, 2) testicular and Müllerian duct differentiation are sensitive targets for developmental estrogen exposure, and 3) development of the female reproductive system is more susceptible to progestin toxicity than the male system.

Evidence for EDC involvement in reproductive dysfunction in female domestic animals

Ulf Magnusson, DVM, PhD

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At large our data on endocrine disruption emerge from experimental *in vivo* or *in vitro* studies, field or epidemiological studies or clinical case reports. This presentation will deal with non-experimental data in domestic animal, primarily with data from livestock. Notably, wildlife (or humans) with endocrine-disrupted reproductive systems is mostly on a much higher trophic level in the food web than herbivorous livestock. The omnivorous pig, depending on feeding regimen, can, however, be on a higher trophic level, and biomagnification of endocrine disruptors may thus be more prominent. Still, several endocrine-disrupting chemicals have been reported in pigs, cows and sheep from European countries.

A possible example of endocrine disruption from man-made chemicals in farm animals is heifers that were drinking water in direct contact with a sewerage overflow in the Netherlands and then showed increased age at first calving. In general though, where the practice of spreading sewage sludge on pasture occurs, the concentrations of analyzed endocrine disrupting chemicals in cattle and sheep are regarded to be too low to impair the reproductive performance.

In contrast, endocrine disruption by phyto oestrogens, i.e. oestrogens produced by plants, is a well known clinical issues in farm animal reproduction. A classical example of such a disruption is the so-called sweet clover disease caused by formononetin and genistein with prolapsed uterus and embryonic death in sheep. There are also reports about pigs suffering from various signs of hyperoestrogenism such as vaginal prolapse, abortions and stillbirths, owing to phytoestrogen produced by the fungi Fusarium that is growing in poorly stored feed stuff. Recently, it has also been reported that feed rich in coumestrol caused lack of ovulation and uterine fluid accumulation in mares.

In conclusion, the current data show that there are evidence of EDC involvement in reproductive dysfunction in female domestic animals under natural conditions. These EDCs are phytoestrogens. Man-made EDCs seems to be associated with reproductive dysfunction just in the case of accident like point-contaminations.

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Maternal exposure to estradiol and endocrine disrupters cause malformations in eelpout fry

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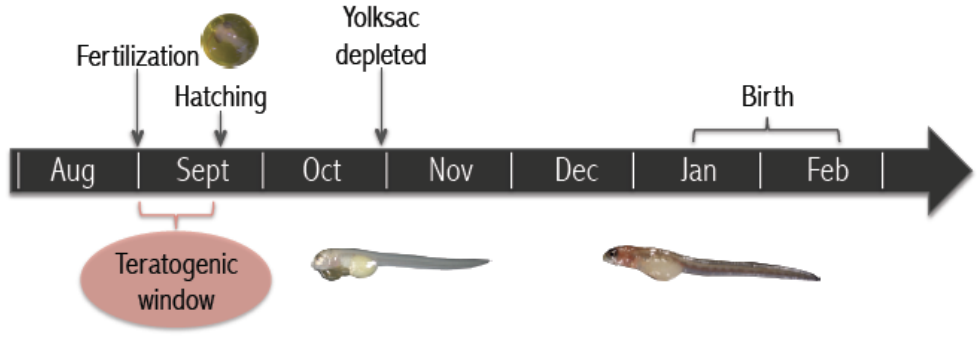
Introduction
Recently malformations among eelpout fry living in North European coastal areas with high anthropogenic input have been observed. The eelpout (*Zoarces viviparous*) is a viviparous fish and maternal exposure to chemicals including endocrine disrupters might explain the observed fry malformations as those compounds have caused similar effects when eggs of oviparous fish are exposed.

Aim
To investigate mother-offspring interactions e.g. teratogenic effects upon maternal exposure to 17β-estradiol (E2) and endocrine disrupters and to investigate if a teratogenic window for E2 could be established.

Materials and methods
Wild pregnant eelpout with newly fertilized eggs were exposed to 17β-estradiol (E2) during different weeks of pregnancy or exposed continously to 17β-estradiol, 17α-ethinylestradiol, 4-t-octylphenol or pyrene from week one to six of pregnancy.

Results
The chemicals did not have an influence on the survival of females. Plasma levels of estradiol and the yolk protein precursor vitellogenin were increased in mothers exposed continously to E2 and an increased abundance of fry malformations was observed. A teratogenic window for induction of malformations by E2 in eelpout fry was established.

Discussion and conclusion
Maternal axposure of 17β-estradiol and endocrine disrupters are able to cause severe malformations in eelpout fry however the first two to three weeks of pregnancy seems to be the most sensitive period. As eelpout are fairly stationary during their pregnancy individual populations could be differentially influenced as the local exposure scenarios are different and most likely vary from year to year.



Hormone disruption by cadmium –a risk for reproductive health?

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Introduction
EDCs are considered as potential risk factors for the reproductive health. Exposure to cadmium is unavoidable due to its ubiquitous presence in the environment. High exposure leads to well known toxic effects to the skeletal system and kidney as well as to increased risk of lung cancer. However, even low, dietary exposure levels associate to increased risk of hormonal cancers possibly due to cadmium's suggested estrogenicity.

Aim
To elucidate the mechanisms behind hormone disruption by low level cadmium exposure.

Material & Methods
Cadmium (CdCl2) was studied in 1) in estrogen receptor (ER) binding assays, 2) immature female estrogen reporter ERE-Luciferase mice, and 3) cell based assays, and 4) association between blood Cd and steroid hormones was assessed in postmenopausal women.

Results
Cadmium binds with high affinity to ERα without inducing agonist conformation. In immature mice, 3-day s.c. exposure to 5 ug/kg/day CdCl2 reduces ERE-Luciferase expression in uterus and ovary, does not promote uterine growth or precocious puberty, but induces thickening of uterine epithelium and ERK1/2 phosphorylation in liver. In cell culture, 10-8M CdCl2 activates Raf/MEK/ERK1/2 via epidermal growth factor receptor (EGFR) ultimately leading to increased pMdm2/p53 ratio. Cadmium significantly associates to lower estradiol/testosterone ratio in women.

Discussion and Conclusions
Low level exposure to cadmium leads to repression of estrogen signalling and activation of mitogenic kinases. The stimulation of EGFR-Raf/MEK/ERK1/2-pMdm2/p53 pathway may contribute to carcinogenicity of cadmium. Lower estradiol/testosterone ratio may be indicative of aromatase inhibition. As estrogen signalling, EGFR pathway and steroidogenesis are central to reproductive functions, the effects of cadmium in this context merit addressing.

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Circulating levels of PCBs and sex hormones in a population-based sample

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Background
High levels of PCBs are still found in humans. PCBs are mainly regarded as Ah-receptor agonists, but effects on reproductive organs have been reported, suggesting effects of PCBs on the biosynthesis of steroids.

Aim
To evaluate the relationships between circulating levels of PCBs and sex hormones in men and women.

Materials and methods
Plasma samples from 1000 70-year-olds were analyzed as part of a large population-based study (Prospective Investigation of the Vasculature in Uppsala Seniors). Eleven sex steroids were quantified, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and PCBs (PCB118, 126, 156, 169, 170 and 206) were measured with high-resolution GC/MS. Women with current/previous menopausal hormone therapy were excluded from the data analysis.

Results
Concentrations of two dioxin-like PCB (PCB118 and PCB156) and one non-dioxin-like PCB (PCB206) were inversely related to levels of testosterone in women only ($p<0.05$). Two of the non-dioxin-like PCBs (PCB170 and 206) were furthermore inversely related to estradiol levels in women only ($p<0.005$).

Discussion
Physiological effects of PCBs likely vary with the congener/-s involved. Therefore the association of PCBs with metabolism and biotransformation of steroids should be studied for relevant individual PCBs, or by using marker PCBs for the general exposure or assess mixture exposure.

Conclusions
In samples of elderly, concentrations of circulating PCBs were associated with concentrations of sex hormones.

Poster abstracts

Heavy metals in female mink in Sweden

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Introduction
Heavy metals interfere with a large range of biological processes and cause reproductive abnormalities in the exposed organisms. Well known heavy metals include mercury (Hg), cadmium (Cd) and lead (Pb). The use of all of these substances has been increasingly regulated in the last decades, but they are present in the biosphere, both naturally and as a result of anthropogenic activities. Currently, the use of silver (Ag) as an antibacterial substance in different consumer products is increasing, but little is known about the fate of the silver in the environment.

Aim
The aim of this investigation was to describe the concentrations of four heavy metals in a top predator in the Swedish environment.

Material and Methods
Livers from ninety-five female mink were analysed for Hg, Cd, Pb and Ag by inductively coupled plasma atomic emission spectroscopy.

Results
Mean concentrations and range, expressed in nanograms per gram liver (wet weight):
Hg: 706 (28-5945)
Cd: 99 (2.4-541)
Pb: 192 (12-2735)
Ag: 43 (0.4-280)

Discussion and Conclusions
Previously, the concentrations of heavy metals in mink livers have been examined in Norway and North America. Apparently, the concentrations of mercury were slightly higher in Norwegian mink. The mercury concentrations in liver varied in North American studies, mainly depending on where the animals had been caught, but were generally higher than in the present study. Mean concentrations of lead and cadmium were generally similar, or slightly lower than previously reported concentrations from mink caught in different locations in Canada. No previous studies of silver concentrations in mink have been identified. The mercury concentrations were lower than those associated with lower whelping percentage in farmed mink.

Combined Effects of Progestin and Estrogen on Androgen Receptor Transcription during Sex Differentiation

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Introduction
Progestins and estrogens are two groups of synthetic hormones widely used in human and veterinary medicine. Considering that these hormones are frequently found in surface and ground water and that they both are potent developmental and reproductive toxicants data on the toxicity of combined exposure to these hormones is surprisingly scanty.

Aim
We tested the hypothesis that the progestin levonorgestrel (LNG) antagonises estrogenic effects of ethinylestradiol (EE_2) on gonadal differentiation and mRNA levels of vitellogenin (*Vtg)* and hormone receptors.

Material and Methods
Xenopus tropicalis larvae were exposed to LNG (0.1 nM) alone, or EE_2 (0.1 nM) alone or in combination with LNG (0.01, 0.1, 1.0 nM) via the water until metamorphosis. The gonadal sex was determined histologically and the levels of hepatic *Vtg*, and the receptors for estrogen (*ERα, ERβ*), androgen (*AR),* and progesterone (*iPR)* were quantified.

Results
All groups exposed to EE_2 showed female-biased gonadal sex ratios and strikingly increased *Vtg* mRNA levels. Co-exposure to LNG did not counteract the *Vtg* induction but an antagonistic effect of LNG on gonadal differentiation was implied.The LNG exposed females had increased *AR* transcript levels compared with the controls as well as the co-exposed groups, indicating an antagonistic effect of co-exposure to EE_2 on AR transcription.

Discussion and conclusions
Antagonistic effects of EE_2 and LNG were implied at the mRNA level and on gonadal differentiation in juvenile *Xenopus tropicalis* tadpoles. The present study indicates that up-regulated hepatic AR transactivation is involved in the mode of action of LNG-induced female developmental reproductive toxicity.

The progestin levonorgestrel is a potent androgen in the three-spined stickleback (*Gasterosteus aculeatus*)

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Aim
Progestins from contraceptive pharmaceuticals are widespread aquatic contaminants, and can impair fish reproduction at low ng L^{-1} concentrations. The mechanisms behind the reproductive toxicity of progestins are largely unknown. Certain progestins, like levonorgestrel, have androgenic properties and seem orders of magnitude more potent in terms of reproductive impairment than non-androgenic progestins. The contribution of the androgenicity of progestins to their reproductive toxicity should be determined. To determine the androgenic activity of levonorgestrel in fish, we utilized three-spined stickleback (*Gasterosteus aculeatus*), which has the only known molecular biomarker for androgens in fish. Male stickleback kidneys produce a glue-like glycoprotein, spiggin, which is used in nest building and whose production is directly governed by androgens. Spiggin is normally absent in females but is induced by exposure to exogenous androgens.

Material and methods
Female sticklebacks were exposed to levonorgestrel at 5.5, 40 and 358 ng L^{-1} for 21 days. The effects of levonorgestrel were evaluated from kidney morphology and on mRNA transcription of *spiggin* and *vitellogenin* genes.

Results
Spiggin transcription, kidney size and kidney epithelium height were all increased at concentrations $\geq$ 40 ng L^{-1}, and *vitellogenin* transcription was reduced at 358 ng L^{-1}.

Discussion and Conclusions
The results show strong androgenic effects of levonorgestrel at concentrations $\geq$ 40 ng L^{-1}. These are the first in vivo quantitative data showing that levonorgestrel acts as a potent androgen in fish, supporting to the contention that the androgenicity of certain progestins may contribute to their reproductive toxicity. Furthermore, its low effective concentration establishes levonorgestrel as one of the most potent androgenic contaminants known.

The frog model for developmental reproductive toxicity: Anti-Müllerian hormone mRNA expression during sex differentiation in *Xenopus tropicalis*

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Introduction
The study is part of a project aiming at the development of methods to investigate developmental reproductive toxicity of endocrine disrupting chemicals (EDCs) using the African clawed frog (*Xenopus tropicalis).* The AMH (anti-Müllerian hormone) is a key hormone for the differentiation of the Müllerian ducts (precursors of the female reproductive tract). AHR is interesting both as a target for EDCs and as a potential marker of gonadal sex. AMH and its receptors, AMHR1 and AMHR2 have been identified and characterized in most vertebrates but not in anuran amphibians.

Aim
The specific objectives were (i) to partially clone *amh*, (ii) to quantify *amh* and *amhr2* mRNA levels during sex differentiation; and (iii) to compare these to that of *cyp19a1*, which is much higher in the ovary than in the testis.

Material and Methods
PCR primers for predicted gene sequences were optimized for *amh* and *amhr2* based on phylogenetic analyses. mRNA levels in the gonad-mesonephros complexes from animals (n= 3-7 per developmental stage) at developmental stage 50 to 66 (metamorphosis) and 6 days post-metamorphosis were determined by qPCR and normalized to total RNA content.

Results
The *amh* mRNA levels were higher in males than in females starting at the beginning of gonadal differentiation (stage 55/56). The level of *amhr2* mRNA was higher in females than in males from stage 65.

Discussion and conclusions
This study demonstrates for the first time *amh* and *amhr2* expression in anuran amphibians. The results indicate that both *amh* and *cyp19a1* mRNA expression may be used as early markers of gonadal sex in *Xenopus tropicalis*.

The significance of oxytocin and progesterone in canine mammary gland tumorigenesis

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Introduction
Oxytocin is a hormone that targets the mammary gland during lactation. Oxytocin receptors have recently been described also in breast cancer cell lines. Despite that oestrogens have been most studied in breast cancer the interest of progesterone is also increasing.

Aim
The aims were to find out if the cell proliferation in the canine mammary carcinoma cell line CMT-U27 was affected upon oxytocin and progesterone stimulation, and to investigate the presence of oxytocin and progesterone receptors respectively.

Material and Methods
Cell viability and proliferation was analysed with Colorimetric Cell Viability Test. Oxytocin and progesterone receptors were detected by immunocytochemistry. The progesterone receptor and progesterone content were evaluated with ELISA-kits.

Results
The results showed that oxytocin significantly inhibited the cell proliferation. Based on the location of the oxytocin receptors it seemed to be at least two groups within the cell line. The tumour cells contained a higher rate of progesterone than the conditioned medium.

Discussion
The cellular location of the oxytocin receptors show that they have been activated and further studies are encouraged to find out if the cells produce oxytocin. The fact that the cells contained a higher rate of progesterone than the conditioned medium might indicate that they produce their own progesterone.

Conclusions
Our results demonstrate that the studied canine mammary carcinoma cells have receptors for both oxytocin and progesterone. The results also indicate that the cells can produce progesterone. However, there is a need for further studies to confirm this and to further explore the underlying mechanisms if these hormones act as endocrine disruptors.

Environmental concentrations of norethindrone and progesterone inhibit egg development in amphibians

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Introduction
Several different progestogens are present in the aquatic environment. We recently showed that environmental concentrations (1 ng/L) of the progestin levonorgestrel disrupt egg development (oogenesis) by inhibiting vitellogenesis in the model frog *Xenopus tropicalis*. Information on the effects, potency and modes of action of progestogens in the environment is needed in order to understand the risk for mixture effects of these compounds.

Aims
To 1) determine the effects of environmental concentrations of norethindrone (NET) and progesterone (P4) on oogenesis, 2) elucidate a potential mode of action by analyzing aromatase activity as an indicator of estrogen synthesis.

Material and Methods
Female *Xenopus tropicalis* were exposed to environmental concentrations of NET (0, 1, 10, 100 ng/L), or P4 (10, 100 ng/L) via the ambient water for 28 days, after which the full cycle of oogenesis, aromatase activity in brain and ovaries, and the morphology of the reproductive organs were analyzed.

Results
Both test substances caused reduced relative gonadal weight, increased proportions of previtellogenic oocytes and reduced proportions vitellogenic oocytes compared with the controls. The effects were ascertained also at the lowest tested concentrations. None of the test substances impacted on aromatase activity significantly.

Discussion and conclusions
Both substances impaired oogenesis by inhibiting vitellogenesis, at environmental concentrations. The results indicate that the mode of action did not involve reduced estrogen synthesis. Considering the crucial role of oogenesis in female fertility our results indicate that progestins in the environment pose a risk to reproduction in exposed wild frog populations.

CRU Publication series

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Proceedings of the Inauguration of the Centre for Reproductive Biology, 1997, by *Andersson H, Kindahl H, and Neil M (editors)*

Report 2 (In Swedish)
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Report 27
Lactation research in mammals and humans: the mammary gland in health and disease with particular focus on milk ejection and emptying of the mammary gland. Proceedings from a symposium in Uppsala, Sweden December 4-5, 2012, *by Sigrid Agenäs (editor).*