Exploring Novel Endpoints, Exposure, Low-dose- and Mixture-Effects in Humans, Aquatic Wildlife and Laboratory Animals

Contract No. QLK4-CT2002-00603

Final Report

September 2007

PARTNER LIST

Partner 1 Co-ordinator	**Andreas Kortenkamp**, The School of Pharmacy, Centre for Toxicology, University of London, 29-39 Brunswick Square, London WC1N 1AX, United Kingdom Tel: +44-20-77535908, Fax: +44-20-77535908, andreas.kortenkamp@pharmacy.ac.uk
Partner 2 contractor	**Thomas Pottinger**, Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP, United Kingdom Tel: +44-1524-595854, Fax: +44-15394-46914, TGP@wpo.nerc.ac.uk
Partner 3 contractor	**Richard Sharpe**, MRC Human Reproductive Sciences Unit, Queens' Medical Research Institute, 47 Little France Crescent, Old Dalkeith Road, Edinburgh, EH16 4TJ United Kingdom Tel: +44-131-242-6387, Fax: +44-131-242-6231, r.sharpe@hrsu.mrc.ac.uk
Partner 4 contractor	**Niels Skakkebæk**, University Department of Growth and Reproduction, Rigshopitalet, Sect. GR 5064, Blegdamsvej 9, DK-2100 Copenhagen OE, Denmark Tel: +45-3545-5086, Fax: +45-3545-6054, nes@rh.dk
Partner 5 contractor	**Jorma Toppari**, Departments of Pediatrics and Physiology, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland Tel: +358-2-333-7297, Fax: +358-2-250-2610, jorma.toppari@utu.fi
Partner 6 contractor	**Anton Gerritsen**, Institute for Inland Water Management and Waste Water Treatment (RIZA), Department of Industrial Pollution Control, Zuiderwagenplein 2, PO Box 17, 8200 AA Lelystad, The Netherlands Tel: +31-320-298498, Fax: +31-320-298218, anton.gerritsen@tno.nl
Partner 7 contractor	**John Sumpter**, Brunel University, Department of Biological Sciences, Kingston Lane, Uxbridge, Middlesex, UB8 3PH, United Kingdom Tel: +44-1895-274000, Fax: +44-1895-203099, JPS@brunel.ac.uk
Partner 8 contractor	**Ian Mayer**, Department of Fisheries and Marine Biology, University of Bergen, PO Box 7800, 5020 Bergen, Norway Tel: +47-55-5844442, Fax: +47-55-584450, ian.mayer@ifm.uib.no
Partner 9 contractor	**Nicolas Olea**, Deptartment Radiologia y Medicina Fisica, Facultad de Medicina, Universidad de Granada, Granada 18071, Spain Tel: +34-9-58-242-864, Fax: +34-9-58-249-953, nolea@ugr.es
Partner 10 4contractor	**Terttu Vartiainen**, National Public Health Institute, Mannerheimintie 166, FIN 00300 Helsinki Finland Tel: +358-17-201395, Fax: + 358-17-201265, terttu.vartiainen@ktl.fi
Partner 11 contractor	**Rik I.L. Eggen**, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Department of Environmental Toxicology, Ueberlandstrasse 133, CH 8600, Duebendorf, Switzerland Tel: +41-1-8235320, Fax: +41-1-8235547, eggen@eawag.ch
Partner 12 contractor	**Nico Vermeulen**, Vrije Universiteit Amsterdam, Department of Pharmacochemistry – LACDR, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands Tel.: (+31) 020-4447590 Fax.: (+31) 020-4447610 npe.vermeulen@few.vu.nl
Partner 13 contractor	**Bertold Hock**, Technische Universitaet Muenchen, Department fuer Pflanzenwissenschaften, Lehrstuhl fuer Zellbiologie, Alte Akademie 12, D-85354 Freising, Germany Tel: +49-8161-713748, Fax: +49-8161-714403, Please contact Dr Karl Kramer kramer@wzw.tum.de
Partner 14 contractor	**Bernard Jegou**, Universite de Rennes, IFR 98 Reproduction, Developpment et Ecophysiologie, Campus de Beaulieu, Bat. 13, F 35042 Rennes, France Tel: +33-2-23236911, Fax: +33-2-23235055, bernard.jegou@rennes.inserm.fr
Partner 15 contractor	**Helmut Segner**, University of Bern, Institute of Animal Pathology, Centre for Fish and Wildlife Health, Laenggass-Str. 122, CH-3012 Bern, Switzerland Tel: +41-31-6312441, Fax: +41-31-6312611, helmut.segner@itpa.unibe.ch
Partner 16a contractor	**Stefan Hartung**, Universität Hamburg, Universitätsklinikum Eppendorf, Abteilung für Andrologie, Martinistr. 52, D 20246 Hamburg, Germany Present address: Universitätsklinikum Eppendorf, Klinik für Stammzelltransplantation (KMT), rest as above. Tel.: +49-40-42803-2391, Fax: +49-40-42803-3034, shartung@uke.uni-hamburg.de
Partner 16b contractor	**Andrea Salzbrunn**, Universität Hamburg, Universitätsklinikum Eppendorf, Abteilung für Andrologie, Martinistr. 52, D 20246 Hamburg, Germany Tel: +49-40-42803-4908, Fax: +49-40-42803-4875, salzbrunn@uke.uni-hamburg.de
Partner 17 contractor	**Manuel Tena Sempere**, Universidad de Cordoba, Department of Cell Biology, Physiology and Immunology, Faculty of Medicine, Menendez Pidal S/N, E 14071 Cordoba, Spain Tel: +34-957-218280, Fax: +34-957-218288, fi1tesem@uco.es
Partner 18 contractor	**Jean-Pierre Bourguignon**, Universite de Liege, Centre de Neurobiologie Cellulaire et Moleculaire, Institute of Pathology Bat. B23, C.H.U. Sart Tilman, Avenue de l'Hopital, 13, B 4000 Liege Belgium Tel: +32-4-3667247, Fax: +32-4-3667246, jpbourguignon@ulg.ac.be
Partner 19 contractor	**Alexander Scott**, Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, The Nothe, Barrack Road, Weymouth DT4 8UB, United Kingdom Tel: +44-1305-206637, Fax: +44-1305-206601, Please contact Ioanna Katsiadaki ioanna.katsiadaki@cefas.co.uk
Partner 20 contractor	**Ulla Hass, Jens-Jørgen Larsen**, National Food Institute, Technical University of Denmark, Dept of Toxicology, Morkhoj Bygade 19, DK-2860 Soborg, Denmark Tel: +45-33956544, Fax: +45-33956698, jjl@food.dtu.dk
Partner 21 contractor	**Leif Norrgren**, Sveriges Lantbruksuniversiteit, Department of Pathology, Box 7028, Travvaegen 12 D, S 75007 Uppsala, Sweden Tel: +46-18-671206, Fax: +46-18-673532, leif.norrgren@pat.slu.se

ABSTRACT

The EDEN project is an interdisciplinary effort conceived to address key issues that have hampered sound hazard and risk assessment for endocrine disrupting chemicals (EDCs) in the European Union. It has adopted an approach that fully integrates human, wildlife, exposures, mechanisms and low-dose/mixture evaluations. The project was structured into four interlocking themes focusing on EDC mixture exposures, mechanisms of action and novel endpoints, effects on male reproductive health and the evaluation of low-dose and mixture effects of EDCs.

For the first time, the simultaneous occurrence of nearly 150 different EDCs in human and wildlife tissues was measured. These studies revealed that numerous EDCs occur together in humans, yet differences in the levels of individual EDCs in specimens from boys with cryptorchidism and from women suffering from breast cancer and their respective controls did not become apparent. It appears that the likelihood of developing any of the above conditions cannot be attributed to any individual chemical at relevant exposure levels. However, there are signs that simultaneous exposure to many different EDCs may play a cumulative role in these disease processes. This means that efforts to develop biomarkers of cumulative EDC exposure should be re-doubled. In contrast, symptoms of endocrine disruption in fish could be explained largely in terms of exposure to estrogenic chemicals, but the possible role of antiandrogenic chemicals in disrupting sexual development in fish requires urgent attention. Fish caught in certain Dutch rivers exceeded the EU permissible levels for polychlorinated dioxins and furans.

Considerable progress has been made in establishing relevant modes of action of EDCs and in assessing new endpoints. The screening for EDCs, with its focus on steroid receptor interactions, has not kept up with the progress made in understanding rapid cellular signalling events that occur in the wake of receptor activation. In the interest of avoiding overlooking endocrine active agents it is necessary to expand screening tools to capture rapid signalling events. Substantial progress has been also achieved in characterising the role of the aromatase system in fish as a target of EDCs and the consequences of steroid synthesis disruption on sexual differentiation, reproduction as well as non-reproductive processes such as neurodifferentiation. Extensive investigations of the role of certain phthalates in disrupting male sexual development have revealed delays in germ cell differentiation and other molecular effects as key events underlying the induction of the testicular dysgenesis syndrome. The role of the InsL3 protein in promoting male sexual development proved to be more complex than thought previously. Although the development and validation of an assay for the measurement of InsL3 blood levels was successful, the differences in blood InsL3 levels in normal and cryptorchid boys were too small to exploit InsL3 as a biomarker indicative of disruption of testis descent. The hypothalamic pituitary unit proved to be exquisitely sensitive to the effects of several EDCs, and the effects may account for precocious sexual development observed after early EDC exposure. EDEN has advanced the study of endocrine disruption in fish with activities including the development of microarrays for assessing endocrine disruption in zebrafish and establishing sensitive screening tools for endocrine disruption in fish. The development of a transgenic fish model for the detection of EDC effects proved to be technically too demanding to be completed in time, but efforts continue to complete this project after conclusion of EDEN. An *in vivo* model in fish (the

three-spined stickleback) for the detection of antiandrogenic EDC was developed as a complement to the Hershberger assay.

Male reproductive health in Denmark and Finland showed a worrying declining trend. For the first time, it could be established that the same is true for young men in Germany. It is of concern that semen quality among young Germans is similar to the values found in young Danes, a group previously thought to show the lowest semen quality in Europe. Foetal exposure to smoking has been identified as one reason for these effects. Observations of a declining total natural conception rate among the young Danish cohorts imply that the current poor semen quality has an impact on the population fertility in the future – a situation which will be difficult to reverse in the short term. The current and projected widespread use of assisted reproductive technologies may be a sign of such an emerging public health problem which also adds to the load of medical costs in young population. It is of vital importance to continue surveillance of semen quality and all efforts should be made to identify the factors that may cause harm in order to prevent further deterioration.

Extensive low-dose studies with EDCs have shown that the conventional estimation of no-observed-adverse-effect-levels (NOAEL), with their reliance on hypothesis testing methods is inadequate for capturing low-dose effects of EDCs. Whenever possible, regression-based approaches with benchmark dose limits should replace NOAEL as the basis for establishing acceptable human exposure levels. A framework was developed to combine the strengths of both methodologies by making considerations of statistical detection limits and statistical power the starting point of testing procedures. Implementation of this framework will require a significant change in toxicological testing practice.

Determinants of additivity for EDC mixtures have been characterised and are now well understood for combinations of similarly acting EDCs. Experimental studies have produced evidence that EDCs of relatively low potency and at low exposure levels can still work together to produce significant combination effects when they are present in sufficient numbers. The perceived low potency of many EDCs alone is uninformative in anticipating possible risks stemming from these chemicals. Where EDCs act in concert with endogenous hormones, significant additional effects may result under certain circumstances. Uncertainty still exists in relation to the likelihood of synergistic mixture effects, and concerted efforts should be made to fill this gap. Another source of uncertainty that will hamper sound EDC mixture risk assessment is incomplete knowledge about the identity of EDCs, their exposure levels and number. This issue can only be resolved through the development of dedicated exposure assessment strategies that take account of cumulative exposures. Despite these uncertainties, knowledge about determinants and factors that govern the joint action of similarly acting EDCs is now sufficiently advanced to come to pragmatic risk assessment approaches that take mixture effects into consideration. A *modus operandi* for EDC mixtures was developed which includes the use of dose addition (including the toxic equivalency factor approach) to arrive at a "mixture no-observed-adverse-effect-level" (MNOAEL) for endpoints relevant to endocrine disruption. These are then combined with a safety factor to arrive at estimates of tolerable human exposure. "Data-poor" situations may require estimation of a crude MNOAEL by dividing individual NOAEL of certain prototype chemicals by the anticipated number of relevant similarly acting chemicals.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
ART	Assisted reproduction
BPA	Bisphenol A
CA	Concentration addition
DBP	Dibutyl phthalate
DRE	Dioxin-responsive elements
E1	Estrone
E2	17β-estradiol
EE2	17α-ethinylestradiol
EDC	Endocrine disrupting chemical
EEQ	Estradiol equivalent
ER	Estrogen receptor
ERE	Estrogen response element
FSH	Follicle stimulation hormone
GC	Germ cell
GFP	Green fluorescent protein
β-HCH	β-hexachlorocyclohexane
hEST	Human estrogen sulfotransferase
HP	Hypothalamic-pituitary
HPLC	High performance liquid chromatography
HRS	High resolution screening
IA	Independent action
InsL3	Insulin-like factor 3
LBD	Ligand binding domain
LC	Leydig cell
LH	Luteinising hormone
MNOAEL	Mixture no-observed-adverse-effect-level
MNG	Multinucleated gonocytes
NOEL	No-observed-effect-level
NOAEL	No-observed-adverse-effect-level
NR	Nipple retention
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCDD/F	Polychlorinated dioxins and furan
PND	Postnatal day
POPs	Persistent organic pollutants
SC	Sertoli cell
SHBG	Sex hormone-binding globulin
TDS	Testicular dysgenesis syndrome
TEF	Toxic equivalency factor
TEXB	Total effective xenoestrogen burden
TNCR	Total natural conception rate
VTG	Vitellogenin
YES/YAS	Yeast estrogen/androgen screens

INTRODUCTION

The EDEN project is an interdisciplinary effort conceived to address key issues that have hampered sound hazard and risk assessment for endocrine disrupting chemicals (EDCs) in the European Union (EU). It has adopted an approach that fully integrates human, wildlife, exposures, mechanisms and low-dose/mixture evaluations.

The EDEN project was structured into four parallel strands of work that centred on strongly inter-linked key issues (Figure 1):

- Complex EDC mixtures in human and fish tissues (Theme 1)
- Mechanisms of EDC action and novel endpoints and biomarkers (Theme 2)
- Indicators of impaired reproductive function in Europe (Theme 3)
- Low-dose and mixture effects of EDC (Theme 4)

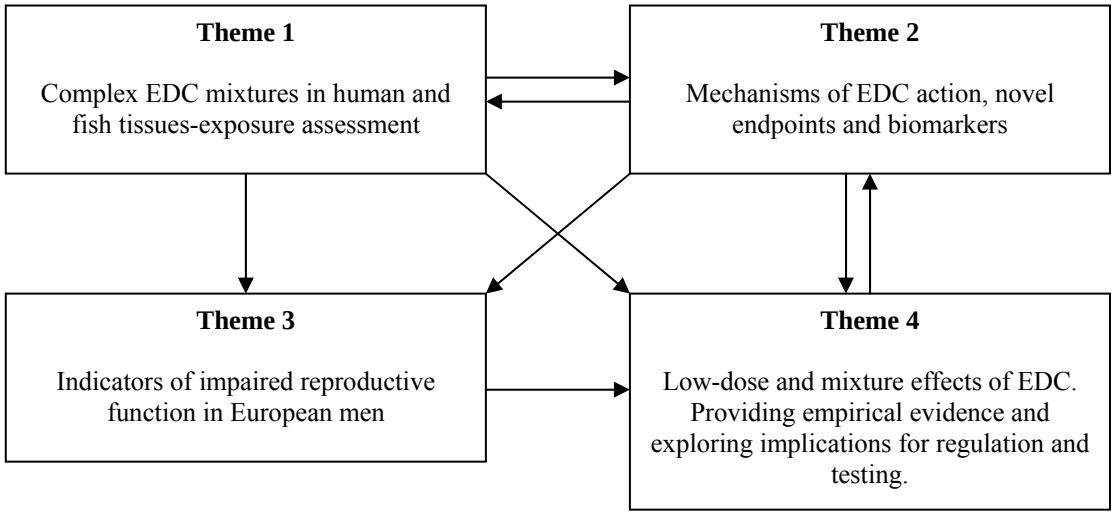

Figure 1 Four interlinking themes comprising EDEN

Complex EDC Mixtures in Human and Fish Tissues (Theme 1)

Humans and wildlife are exposed to a large number of EDCs, often individually present at low levels. Numerous measurements of individual chemicals in human tissues and environmental media have been documented (WHO, 2002) but comparatively little information is available on the cumulative occurrence of EDCs. However, to facilitate better assessments of the impact of EDC it is essential to carry out cumulative exposure assessment that provide a global view on many divergent and seemingly unconnected groups of chemicals. One aim of EDEN was to produce the foundations of better EDC exposure assessments by taking account of cumulative exposures. To realise this aim, specimens of human and fish adipose tissue and of fish bile were collected and analysed. These tissues were chosen because they are sinks for numerous EDCs.

Specimens from humans and fish with and without symptoms indicative of endocrine disruption were compared. In humans, this included samples from boys suffering from cryptorchidism, and fish with elevated levels of the egg yolk protein vitellogenin (VTG)

and with an intersex status, ovotestes. However, the aim of these studies was not to produce evidence that would allow inferences about factors at play during the genesis of these conditions. Much larger samples than could be realised in EDEN would have been necessary for such studies. Rather, the primary intention of EDEN was to gain an impression of the spectrum of EDCs that can be found in tissue specimens. Possible differences in the chemicals found in tissues from subjects with and without symptoms would develop leads for further studies.

Chemical analyses are essential in exposure assessment, but used alone there is a risk of overlooking agents that may also contributing to the overall load of EDCs. It was therefore necessary to take a global view on EDCs by making efforts to search for further, previously unrecognised EDCs. In the environmental arena, this issue has been tackled by using bioassay-directed fractionations of samples representative of complex mixtures. For example, extracts of sewage treatment effluents have been fractionated and the fractions interrogated with an *in vivo* assay for estrogenicity, the yeast estrogen screen (YES) (Desbrow *et al.*, 1998). This approach revealed the presence of steroidal estrogens, some of their conversion products and to a lesser degree, alkylphenols as contributing to the total estrogenic load in waste waters. However, human tissue samples have not been subjected to similar analyses, nor have other assays representative of EDC action been widely used in such studies. The EDEN project set out to fill this gap by analysing extracts from human tissues. Furthermore, attempts were made to analyse one and the same fractionated extracts with a battery of *in vitro* assays, including one for inhibition of a steroid metabolising enzyme, estrogen sulfotransferase.

Mechanisms of EDC Action and Novel Endpoints and Biomarkers (Theme 2)

A wide variety of assays are available for the identification and hazard characterisation of EDCs, ranging from *in vitro* assays to multi-generational and full life-cycle test (see for example OECD 2002). Nevertheless, EDC testing and screening faces a dilemma: Given that many EDCs act during specific developmental periods, an identification of the entire spectrum of EDC effects would require multi-generational studies and full life-cycle testing. However, to subject every suspect chemical to such exhaustive testing is not sustainable because it is extremely time consuming and cost intensive. In contrast, most of the available *in vitro* and *in vivo* screening assays for EDCs can be conducted rapidly, but they only encapsulate interactions with the estrogen, androgen and thyroid receptors and consequent down-stream events. Although positive test outcomes indicate the potential for endocrine disruption, the relevance of screening endpoints for risk assessment is often unclear:

For example, key processes of male sexual differentiation are regulated by the androgen receptor, and after *in utero* exposure, androgen receptor antagonists may lead to feminising effects among male offspring. However, not all androgen receptor antagonists identified in *in vitro* screens are capable of inducing such effects *in vivo*. At the time the EDEN project was conceived, there were suggestions that the spectrum of effects seen as a result of disruption of male sexual differentiation in rodent models (retained nipples, reduced anogenital distance etc) is distinct from testicular dysgenesis (misplaced Leydig cells, seminiferous tubules without gonocytes, etc), a condition of relevance to the human.

Thus, it was essential to further the understanding of the processes leading to the testicular dysgenesis syndrome (TDS) in the human.

Similar problems exist with the interpretation of positive results from estrogenicity screening, both *in vitro* and *in vivo*. The concern is that estrogens may contribute to breast cancer risks, but they also play a role in normal breast development. Although perhaps indicative of a potential for endocrine effects, it is difficult to equate estrogen receptor (ER) activation as such with adversity.

It is also unclear whether the appearance of ovotestes in fish is a phenomenon mediated by ER activation, or whether other processes such as antiandrogenicity, play a role (Jobling *et al.,* 2005), thus complicating the interpretation of positive results from estrogen screening assays.

Aromatase *cyp19* is an enzyme which converts androgens into estrogens, thus being the regulator of estrogen levels in the organism. Prediction of multiple transcription regulatory sites in the promoters of teleost aromatases suggested the sensitivity of this system to several EDC classes - a hypothesis that needed testing. More information was needed on the functional aspects of aromatase in fish as well.

EDEN has responded to some of these issues by conducting detailed research into the mechanisms that underlie EDC action. The focus was on the regulation of estrogen-dependent genes, on the mechanisms leading to TDS in humans, on searching for biomarkers predictive of male reproductive disorders such as cryptorchidism and on novel *in vivo* assays for the identification of antiandrogens.

Indicators of Impaired Male Reproductive Function (Theme 3)

In male adults, malformations that can be diagnosed shortly after birth (cryptorchidism, hypospadias) are predictive of poor semen quality and testis cancer risk later in life. Collectively, these conditions make up a cluster of disorders termed TDS (Skakkebæk *et al.,* 2001). During the last 5-7 years a number of studies have been carried out in different European countries (Andersen *et al*., 2000; Jørgensen *et al*., 2002; Jørgensen *et al*., 2006; Punjab *et al*., 2002; Richtoff *et al*., 2002) which revealed large regional differences in semen quality and the incidence of congenital malformations within Europe.

Consequences of disturbed Leydig cell function

In the past, poor reproductive health in men has mainly been explained in terms of disturbed Sertoli cell (SC) function. Compromised SC function is thought to lead to reductions in semen quality and development of testicular cancer (Skakkebæk *et al.*, 2001). Less attention has been given to the possible adverse effects of reductions in Leydig cell (LC) function. Disturbed LC function may have distinct effects on male reproductive health - mainly through altered testosterone production. Testosterone plays a key role in the development of the external male genitalia, and in the adult men drives spermatogenesis and libido. Longitudinal studies have shown a general decline of serum testosterone levels with age (Feldman *et al*., 2002; Morley *et al*., 1997) indicating a decrease in LC function with advancing age. However, cross-sectional surveys have demonstrated very different rates of decline, some even showing no decrease in testosterone levels with increasing age (Deslypere and Vermeulen, 1984; Drafta *et al*.,

1982; Gray *et al*., 1991; Gyllenborg *et al*., 2001; Nieschlag *et al*., 1982; Simon *et al*., 1992; Svartberg *et al*., 2003). This discrepancy in the age related decline in serum testosterone between cross-sectional and longitudinal studies might be due to confounders such as secular or cohort effects on hormone levels, which may hide or blunt the effect of aging. The EDEN project set out to investigate whether there has been a population level decline in male serum testosterone over the last decades.

Declines in semen quality in Central Europe

One of the first observations suggesting deteriorating male reproductive health was an apparent decline in sperm numbers of normal men reported 15 years ago (Carlsen *et al.,* 1992). After that meta-analysis it became obvious that prospective and international multi-centre studies are needed to assess temporal and regional differences in semen quality. It was soon found that there were rather large geographical differences in semen quality, and Finland was clearly contrasting Denmark with a better semen quality. On the other hand, very few data were available from Germany. The marked differences in industrial activities in East and West Germany after the Second World War offered the opportunity to investigate whether this had had any influence on semen quality. Thus, the objective was to expand an existing Scandinavian research database with data on clinical findings, semen quality, hormone levels, lifestyle factors and exposures to include also data on male reproductive health in the former East and West Germany.

Low-dose and Mixture Effects of EDCs (Theme 4)

The endocrine disrupter "low-dose" issue

There are claims that some EDC-mediated effects occur at dose levels lower than normally tested in toxicology (vom Saal and Hughes 2005). However, researchers have encountered difficulties in reproducing these "low-dose" claims (Ashby *et al.,* 2004) and this has provoked an unusually heated debate in the field.

Despite these disputes, observations of low-dose effects, coupled with non-monotonic dose-response curves continue to appear. A recent example is the estrogenic UV filter substance 3-benzylidene camphor which induces increased embryo production in aquatic snails at low, but not at high doses (Schlumpf *et al.,* 2004). An explanation for such effects may lie in dose-dependent changes in the mode of action of the chemical, such that estrogenic effects are masked by toxicity at higher doses. Similar conclusions have been drawn from experiments with phytoestrogens (Almstrup *et al.,* 2002). Thus, do EDC have special properties, in terms of their ability to induce effects at low-doses that disappear as doses are raised? Is there a danger of overlooking such effects during standard toxicity testing which is carried out at higher doses?

While these questions have attracted considerable attention in the endocrine disrupter field, another, less widely debated, perspective on the low-dose problem has emerged: Large-scale studies involving turtle eggs have suggested that EDCs may not exhibit effect thresholds when acting in concert with endogenous hormones (Shehan *et al.,* 1999). It has been argued that in such situations every quantum of external exposure will lead to additional effects, even if exposure is infinitesimally small.

The EDEN project has addressed these issues by assessing whether unusually shaped dose-response curves occur in a wide variety of assay systems relevant to EDC testing. Inevitably, this has made it necessary to confront the general problems that exist in estimating low-doses experimentally. For this reason, a second aim of the EDEN project has been to compare numerical low-dose estimates which were generated by employing two approaches currently used for the estimation of "safe" doses: hypothesis testing methods which arrive at no-observed-effect-levels (NOEL) and regression-based methodologies which yield so-called benchmark doses.

Mixture effects

EU chemicals risk assessment is carried out by dealing with single chemicals in isolation. The process does not take account of the possibility that combination effects might occur when humans or wildlife come into contact with several agents simultaneously. Considering that there are 30,000 – 50,000 chemicals marketed in the EU, with an estimated 50,000 chemicals present in surface waters (Matthiessen and Johnson 2007) and an unknown number prevalent in human tissues, the general potential for mixture effects is considerable, even if only a fraction of these chemicals have endocrine disrupting potential.

At the time the EDEN project was drawn up, comparatively little was known about EDC mixture effects. There was an emphasis on demonstrating synergistic effects, but when early reports of strong synergisms between estrogenic pesticides (Arnold *et al.,* 1996) could not be confirmed, the relevance of EDC mixture effects was called into question. However, given that exposure to EDCs may involve a large number of chemicals, even additivity is of concern. A key issue in risk assessment is whether combination effects with EDCs occur even when each individual chemical is present at low, ineffective exposure levels.

First multi-component mixture experiments conducted with *in vitro* assays showed that EDC acted together in an additive fashion (Payne *et al.,* 2000, 2001). While these studies were helpful in demonstrating principles, no data existed from *in vivo* assays with endpoints of toxicological relevance. There was great uncertainty whether *in vivo* endpoints would be sufficiently reproducible to be useful in experiments involving multi-component mixtures. Finally, information was missing about EDC mixture effects at environmentally relevant concentrations.

THEME 1 - COMPLEX EDC MIXTURES IN HUMAN AND FISH TISSUES

Overview and Objectives

EDEN has made a systematic attempt to analyse a wide range of multiple EDCs to gain an impression of the spectrum of contaminants simultaneously present in human and wildlife tissues. This has been achieved not only by analysing a wide range of multiple EDCs chemically, but also by newly developed high information content bioassays in order to characterise and profile low level exposures and effects of EDCs. Putative correlations between internal exposures derived from chemical analyses and conditions indicative of endocrine disruption have been integratively investigated. Using bioassay-directed fractionation, correlations have been made as well between measures of total EDC activity and chemicals analysed. With this new integrative strategy, this theme has provided new tools and insights into risk assessment procedures for low level mixture exposures of wildlife and humans. The specific objectives were to:

- Provide information on the spectrum of known EDCs in human and fish tissues.
- Search for new EDCs in human and fish sample extracts.
- Explore the usefulness of measures of total (anti)estrogenicity, (anti)androgenicity and estrogen sulfotransferase inhibition as predictors of unwanted outcomes in human and fish.
- Evaluate EDC bioassays for their suitability for bioassay-directed fractionations.

Materials and Methods

Adipose tissue samples from boys with undescended testes (cryptorchidism) and their controls (boys undergoing hernia operation or other abdominal surgery) were collected in Denmark (13:10, case:control) and Finland (35:30, case:control). Adipose tissue samples from ten breast cancer patients and ten women not suffering from breast cancer were collected in Spain. An additional 35 adipose tissue samples obtained from women in Southern Spain were provided for further chemical analyses. This data set is unique because it also involved the follow up of women who had undergone major surgery for breast cancer. Three successive tissue samples were obtained in an 18 month follow up period.

The bream (*Abramis brama*) was chosen as a fish species for tissue sampling as it occurs widely in European rivers. Analysis of pooled bile and adipose fat samples was based on fish sampled from the Dutch river Biesbosch stratified according to high VTG versus low VTG levels. Samples from the Dutch rivers Aa and Dommel were separated into fish with ovotestis, a condition indicative of early life stage exposure to EDC where male reproductive tissue is interspersed with female tissues - and fish with normal reproductive tissues. Of the captured bream, a large fraction (64-100%) showed elevated VTG levels which correlated to sewage effluent discharges into rivers. Ovotestes were less commonly found; in the river Aa 9 males (16%) showed ovotestis, whilst at the other impacted sites only two males with ovotestes were found. The selected fish in each set were of the same age.

All collected samples (human and fish) were analysed with GC/MS or LC/MS/MS for the following EDCs: Organochlorine pesticides (17 chemicals), bisphenol (Bisphenol A and 4 chlorinated derivatives), alkylphenols (2 chemicals), steroidal estrogens (4 chemicals), phytoestrogens, parabens (3 chemicals), phthalate monoesters (8 chemicals), or with GC/HRMS for polychlorinated dioxins and furans (PCDD/Fs) (17 congeners), polychlorinated biphenyls (PCBs) (37 congeners), polybrominated biphenyls (PBBs) (19 congeners), polybrominated diphenyl ethers (PBDEs) (14 congeners), and HO-PCBs according to methods described earlier (Kiviranta *et al*., 2002; Isosaari *et al*., 2006; Fernandez *et al*., 2006).

A new method for the analysis of phthalate monoesters in fish muscle has been developed and validated. Briefly, the method involves liquid extraction followed by 2 solid phase extractions with determination on LC/MS/MS. Fish tissue was extracted with ethyl acetate: cyclohexane (95:5) as described in detail for breast milk and placenta samples (Mortensen *et al.,* 2005). C-13 labelled monoesters were used as internal standards and added to all samples. For recovery experiments native standards were added to the sample.

For assessing the (anti)estrogenicity and (anti)androgenicity, adipose tissues from fish and humans and fish bile extracts underwent accelerated solvent extraction, liquid/liquid extraction or solid phase extraction (bile). The extracts were fractionated by using liquid chromatography and tested using a battery of *in vitro* assays, E- and A-Screen, yeast estrogen/androgen screens (YES/YAS), ER high resolution screening (HRS) technology combined with mass spectrometry and the human estrogen sulfotransferase (hEST) inhibition assay.

For bioassay directed fractionations, the total effective xenobiotic burden (TEXB) method extracts and separates the more lipophilic compounds (alpha fraction) from ovarian estrogens and most polar compounds (beta fraction). The alpha fraction was then examined in a bioassay (E- and A-Screen) for determination of the cumulative effect of the EDCs.

Results

Human and fish tissue samples collected as a part of EDEN were analysed for nearly 150 different EDCs. These analyses showed that a large number of EDCs occur together in human and fish tissue.

Considering single chemicals in human tissues, no differences were identified between the specimens collected from diseased subjects (breast cancer, cryptorchidism) and those without endocrine-related conditions. This may be due to two factors: (1) Many of the analytes are highly lipophilic and can accumulate through food chains. As a result, they occur everywhere, with the consequence that subjects free of these pollutants cannot be found. (2) The number of samples that could be collected for EDEN was too small to afford the statistical power that is needed to detect differences, if they exist. Even so, there were certain noteworthy trends:

Levels of EDCs in boys are similar to those found in adults

The small quantities of adipose tissue that could be obtained from cryptorchid boys and boys undergoing hernia operations did not permit analyses of a large spectrum of different EDCs. Instead, analytical work had to focus on persistent organic pollutants (POPs) only; the choice of POPs was based on results from another EU project (EXPORED). It was found that the body burden of POPs in boys can easily reach levels similar to those measured in adults (Kiviranta *et al*., 2005), likely as a consequence of exposure during the prenatal period and following lactation. The exposure data for EDCs in infants or very young children makes this study invaluable as little information is currently available worldwide.

POP profiles in cryptorchid boys from Denmark differed from those in Finland

An evaluation of the profiles of POPs in the tissues obtained from Danish and Finnish cryptorchid boys revealed interesting differences between the countries. The percentage profile of PCDD/F congeners from the sum of PCDD/Fs as well as percentages of each congener from $WHO_{PCDD/F}$-TEQs showed that boys in Denmark are more exposed to congeners with the highest toxic potency (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 2,3,4,7,8-PeCDF). Similarly, the percentage profiles for PCB and PBDEs indicated that boys in Denmark were more exposed to congeners with a higher degree of chlorination/bromination (Table 1). However, certain confounders must be addressed in order to confirm the validity of these findings (e.g. age of each child when operated, lactation history).

Table 1 Concentrations of sums of PCDD/Fs (17 congeners), PCBs (37 congeners), and PBDEs (14 congeners) as well as toxic equivalents of PCDD/Fs (WHOPCDD/F-TEQ) and PCBs (WHOPCB-TEQ) in adipose tissue of cryptorchid boys from Denmark and Finland.

Country	PCDD/F pg/g Mean (Stdev) Median Maximum	PCB ng/g Mean (Stdev) Median Maximum	PBDE ng/g Mean (Stdev) Median Maximum	WHO-$_{PCDD/F}$-TEQ pg/g Mean (Stdev) Median Maximum	WHO-$_{PCB}$-TEQ pg/g Mean (Stdev) Median Maximum
Denmark	74.9 (63.4)	203 (168)	5.4 (5.7)	8.7 (8.4)	7.8 (6.4)
	67.7	167	4.1	8.8	6.8
	248	697	30.3	27.9	27.9
Finland	125 (168)	136 (146)	11.5 (16.5)	7.8 (10.5)	5.5 (6.2)
	87.8	79.5	5.7	3.6	2.6
	1,270	600	85.3	48.6	31.1

Differential occurrence of EDC in fish showing signs of endocrine disruption

In contrast to the human samples, it was found that certain EDCs occurred at higher levels in tissues from some fish that showed signs of endocrine disruption. Notable were elevated levels of octylphenol in fish with ovotestes from the Dutch river Aa. Similarly, concentrations of the estradiol conversion product estrone (E1), and of the contraceptive pill ingredient 17α-ethinylestradiol (EE2) were significantly higher in fish with ovotestes compared with those that did not show this condition (Figure 2). However, the bile of fish with signs of recent exposure to estrogens, as judged by their VTG levels, did not show any differences in terms of steroid concentrations.

For a large number of predominantly lipophilic EDCs, including organochlorine pesticides such as *o,p'*-DDT, aldrin, dieldrin, HCB, lindane or endosulfan, statistically significant differences in fish with ovotestes and their controls, or those with elevated VTG and controls, could not be detected.

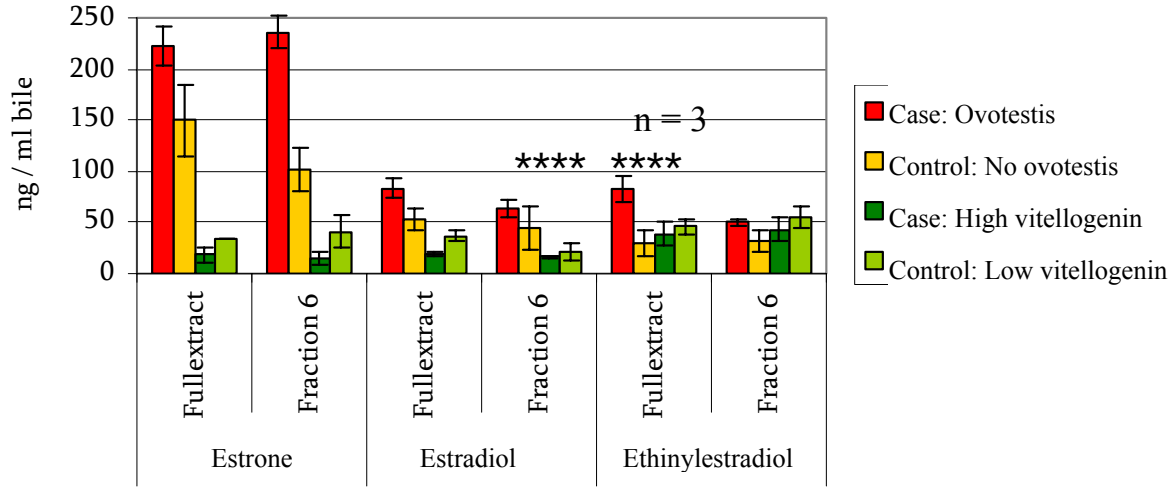

Figure 2 Concentrations of estrone, 17β-estradiol and 17α-ethinylestradiol in 1ml bile of bream from Dutch rivers. Shown are the results from two pools of fish: One stratified according to the presence of ovotestes (red/orange), the other according to the levels of VTG (high vs low) in the river Biesbosh (dark green/light green). For quality control reasons, the steroid levels found in the full extract were compared with those in the chromatographic fraction (Fraction 6) where the steroids elute.

The newly developed analytical method for phthalates in fish muscle was used to screen the fish. Of all the phthalates and their monoester breakdown products analysed, only mBP and mEHP were detected in fish. There were no significant differences in the levels of mBP and mEHP in muscle from fish with ovotestis and age-matched controls, neither when analysed for each different catch site nor for all three catch sites combined (Mann-Whitney test). The levels of mBP and mEHP in muscle from fish from the three catch sites Dommel, Aa and Biesbosh did not differ significantly (Table 2).

Table 2 Median levels of phthalate breakdown products in fish muscle from Dutch rivers. Ranges are shown in parentheses.

(µg/kg)	Dommel (n=6)	Aa (n=27)	Biesbosh (n=6)	P*
mBP	9.3 (6.7-11.0)	7.7 (2.5 – 15.0)	8.4 (2.9 – 13.0)	0.46
mEHP	10.4 (5.1-32.0)	9.8 (3.1 – 89.0)	5.9 (3.4 – 9.9)	0.14
*Kruskal-Wallis test				

Pollution levels of PCBs and PCDD/F in fish from the Dutch Biesbosch river exceed EU maximum permissible limits for fish meat

The levels of PCBs and PCDD/F that were found in the adipose tissues of Dutch fish revealed striking differences. The levels found in the rivers Biesbosh and Dommel were consistently higher than those in fish from the Aa. The PCDD/F and PCB levels in the

river Dommel and the river Biesbosch were elevated with the $WHO_{PCDD/F-PCB}$-TEQ concentrations (17-53 pg $WHO_{PCDD/F-PCB}$-TEQ/g fresh weight) exceeding the EU maximum limit value (8 pg $WHO_{PCDD/F-PCB}$-TEQ/g fresh weight) by three-fold an average (OJEU, 2006). The case for PCDD/F-TEQ levels in fish is illustrated in Figure 3.

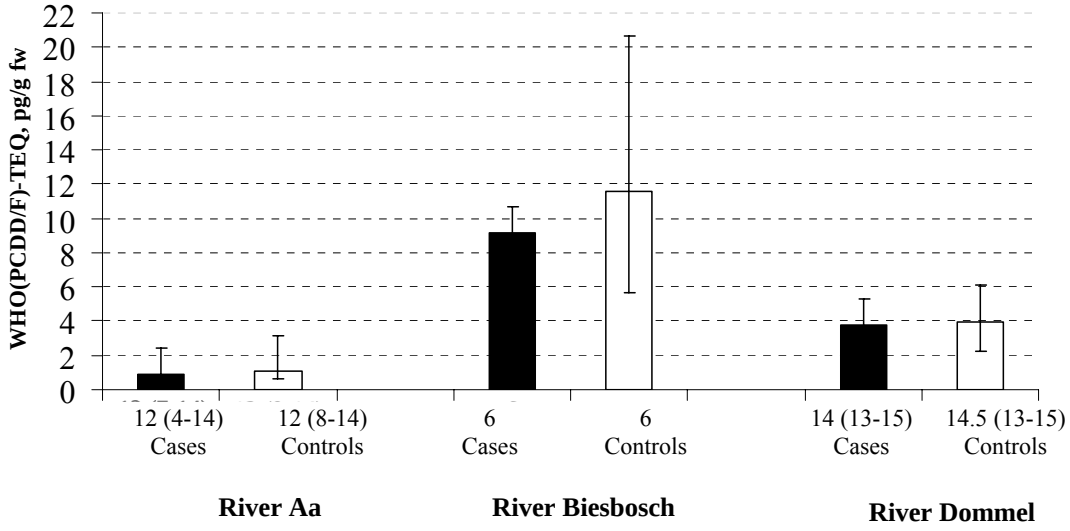

Figure 3 Median (and range) $WHO_{PCDD/F}$-TEQs in bream samples from different rivers in the Netherlands. The ages of the fish (ranges in parentheses) are shown below the bars. The dark blue bars are data from fish with ovotestes (Aa and Dommel) or with elevated VTG levels (Biesbosch), the light blue bars are from fish without any signs of EDC-related effects.

The fresh weight concentrations of flame retardants PBDEs in bream from the river Dommel were four times as high as levels in the other two rivers showing that river Dommel is clearly more polluted with those chemicals when compared with the other two rivers (Figure 4).

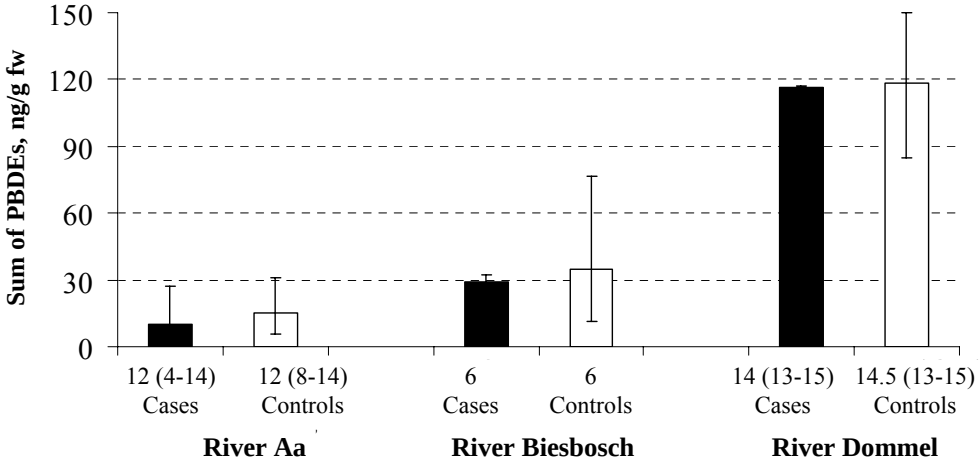

Figure 4 Sum of PBDEs in bream from Dutch rivers. Shown are median levels and their range (error bars). Numbers below bars are median ages of the fish and their range in parentheses. The black bars are data from fish with ovotestes (Aa and Dommel) or with elevated VTG levels (Biesbosch), the white bars are from fish without any signs of EDC-related effects.

Suitability of bioassays for bioassay-directed fractionation

A factor complicating the use of bioassays for bioassay-directed fractionations is toxicity arising from unidentified chemicals which may interfere with the assay and potentially mask biological effects arising from specific groups of chemicals which the assay is designed to respond to. Thus, it was important to assess the suitability of a variety of assays for bioassay-directed fractionations of complex mixtures from human and environmental samples. In order to determine the estrogenicity of human and fish samples following extraction and fractionation (high performance liquid chromatography - HPLC) the following assays were evaluated:

- MCF-7 breast cancer cell proliferation assay (E-Screen)
- YES, a reporter gene assay based on beta galactosidase
- HRS coumestrol-based ER affinity detection (HRS-ERα RAD) assay
- A HRS-androgen receptor affinity detection (HRS-AR RAD) assay was also undertaken, but too low expression levels of the androgen receptor necessitated postponement of ligand synthesis.

Both the E-Screen and YES bioassays are widely used for assessing estrogenicity. In general, similar responses were observed, however for the adipose tissues from fish with ovotestis, the E-Screen determined higher estrogenicity (~6.51 ng/g fat) than the YES (<LOQ = 1.08 ng/g fat). Possible reasons may be the different extraction methods which did not remove all agents that may inhibit the bioassays or the fact that the E-Screen also allows for stimulation of cell proliferation via cell signalling pathways not present in the YES. All chemicals were present at concentrations very close to the detection limits and with the small sample size concentration of the sample was not an option. In addition to the E-Screen and YES bioassays, two further bioassays were developed and evaluated.

A new bioassay called the hEST assay was developed and optimised to screen samples for unknown/new hEST-inhibitors. The substrate 1-hydroxy-pyrene is mixed with hEST which converts 1-hydroxy-pyrene into 1-sulphate-pyrene, a product which can be measured on HPLC with fluorescence detection. hEST inhibitors decrease the hEST-mediated product formation.

The HPLC-based hEST bioassay was applied to pooled fish bile samples and differences between the ovotestis cases and controls and the VTG cases and controls were observed. In particular, the ovotestis cases showed greater inhibition compared to the VTG cases and controls. However, the overall trend for inhibition in all case and control fractions was similar with high inhibition identified in Fractions 5-9 inclusive (Figure 5). EDCs correlating to these fractions are BPA and steroidal estrogens in Fraction 6 and nonylphenol in Fraction 8, with more polar compounds containing more hydroxyl or even acidic groups eluting prior to the Fraction 6. Whereas the YES found Fraction 6, to be responsible for all the estrogenicity identified in the fish bile samples, the hEST inhibition assay shows additional fractions possessing similar inhibition to that of Fraction 6 and determining the causative chemicals for this inhibition is for future study.

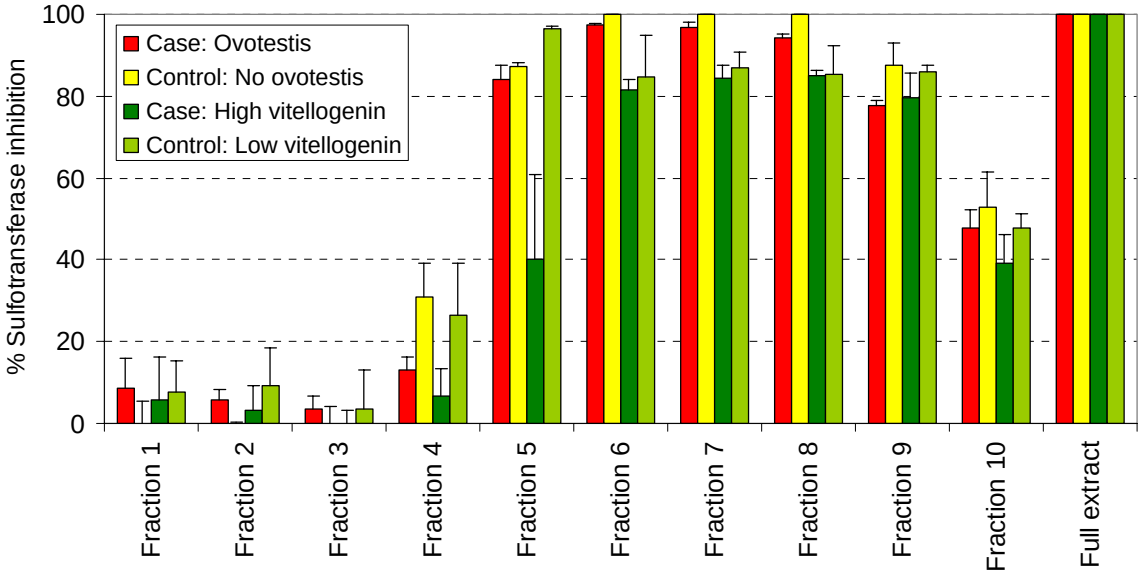

Figure 5 Results from the hEST inhibition screening of 10 fractions and the full extract from bile of bream from the pooled samples of two case/control studies: high versus low VTG levels and ovotestis versus normal fish.

Another bioassay called the coumestrol-based HRS ERα system was developed and applied. The HRS system consists of a HPLC system coupled on-line to an ERα bioaffinity detection system where an ERα ligand binding domain (LBD) is continuously mixed with coumestrol which shows fluorescence enhancement upon binding to the ERα LBD. With the HRS individual ERα binding components in mixtures can be identified. The detection limits for the ERα-binding response are strongly dependent of the intrinsic ERα-affinities of the individual components. The HRS ERα assay worked well: like the YES assay the HRS assay detected E1, 17β-estradiol (E2) and EE2 in fish bile samples. However, the HRS-assay appeared not suitable to detect low level EDCs with too low affinity for ERα and thus this assay was found not suitable for bioassay directed fractionation.

Novel EDCs were not identified by using bioassay-directed fractionations

In general, novel, previously unidentified EDC were not found in human or fish tissue samples. The use of *in vitro* estrogenicity assays on tissue extracts revealed significant estrogenicity, but correlations between the concentration of any single chemical or group of chemicals and the estrogenicity determined by the E-Screen bioassay could not be detected. However, in the multivariate analysis, both individual chemical residues and the estrogenicity of individual eluted fractions predicted the estrogenicity of the mixture with high accuracy.

Steroidal estrogens were largely responsible for the estrogenicity found in fish tissues

A different extraction methodology followed by a 10 part fractionation was applied to the pooled fish bile samples and then assessed with the YES and HRS assays. With the YES assay, the estrogenicity as estradiol equivalents (EEQs) was determined in the full extract and Fraction 6 which corresponded to elution of the steroid estrogens. Comparison of the two gave similar estrogenic responses, especially for the two cases, VTG and ovotestis

(Figure 6). The estrogenicity in the bile could be entirely explained by the steroid hormones E1, E2 and EE2.

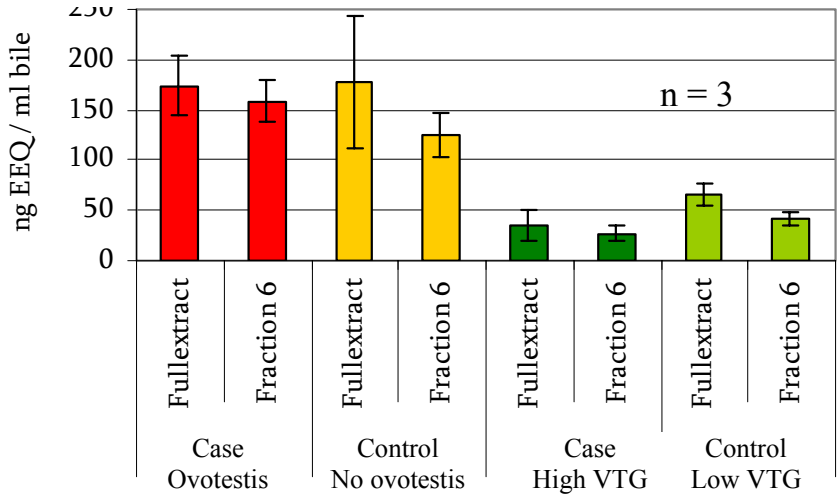

Figure 6 Comparison of the estrogenicity as measured by using the YES in 1 ml of bile for the full extract and Fraction 6 which corresponds to steroid estrogen elution [VTG; vitellogenin].

A significantly higher concentration of E1 and EE2 was found in the bile of the fish with ovotestis compared to those fish without ovotestis. Steroid concentrations were lower in the VTG case/controls than the ovotestis case/controls. No significant difference was found in estrogenicity or steroid concentrations between the fish with high and low plasma VTG levels.

Discussion

The EDEN project has made a concerted effort to analyse an array of nearly 150 EDCs from the same tissue samples, and this has revealed that numerous EDCs occur together in humans and fish. However, differences in the levels of individual EDCs in specimens from cryptorchid boys and from women suffering from breast cancer and their respective controls did not become apparent. Considering the size of the samples accessible to the EDEN project, and the ubiquitous character of the chemicals analysed this is not surprising. It appears that the likelihood of developing any of the above conditions cannot be attributed to any individual chemicals. On the other hand, there are signs that simultaneous exposure to many different EDCs may play a cumulative role. For example, Olea and associates (Ibarluzea *et al.,* 2004) have found significant associations between total xenoestrogenic load in blood serum and risk of breast cancer, and similar findings were made with estrogenicity in placenta and risk of cryptorchidism and hypospadias in young boys (Fernandez *et al.,* 2007). This means, that further efforts are required to develop meaningful biomarkers of EDC exposure that can encapsulate its cumulative nature.

In fish, signs of endocrine disruption could be attributed to exposure to steroidal estrogens, and in some cases there were elevations in certain alkylphenol levels. In general however, the picture emerging from analyses of the fish samples echoes that of the human situation: apart from steroidal estrogens, individual chemicals cannot be

identified as contributing significantly to endocrine disruption in fish. Again, this can be interpreted as demonstrating the need for developing and validating biomarkers representative of cumulative exposures.

In both cases, human and fish, the utilisation of bioassays sensitive to cumulations of certain classes of EDCs may be the way forward to resolve the issue.

Of concern are observations of elevated, an average three-fold, levels of dioxin-like pollutants in fish from certain Dutch rivers when comparing to the EU maximum limit of 8 pg $WHO_{PCDD/F-PCB}$-TEQ/g fresh weight). It is advisable that the Dutch authorities investigate how widespread this pollution of fish is.

Conclusions

A wide range of EDCs could be found in human tissues. There is considerable uncertainty about the full spectrum of EDCs in humans. Bioassay-directed fractionations producing several fractions where effects were observed but no known EDCs are yet attributable for these responses. Concerning estrogenic chemicals, the situation is different in fish. Here, steroidal estrogens accounted for the majority of estrogenicity found in complex mixtures extracted from tissue samples. However, bile extracts showed the ability to strongly inhibit hEST, and the identity of the causative inhibitory chemicals is largely unknown.

Literature Cited

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, and Waldock M, (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. *Environ. Sci. Technol.*, **32**: 1549-1558.

Fernandez MF, Araque P, Kiviranta H, Molina-Molina JM, Rantakokko P, Laine O, Vartiainen T, Olea N, (2006). PBDEs and PBBs in the adipose tissue of women from Spain. *Chemosphere*. **6**, 377-383.

Fernandez MF, Olmos B, Granada A, López-Espinosa MJ, Molina-Molina JM, Fernandez JM, Cruz M, Olea-Serrano F, Olea N, (2007). Human Exposure to Endocrine Disrupting Chemicals and Prenatal Risk Factors for Cryptorchidism and Hypospadias: A Nested Case-Control Study. *Environ. Health Perspect.* In Press.

Ibarluzea J, Fernández MF, Santa-Marina L, Olea-Serrano F, Rivas A, (2004). Breast cancer risk and the combined effect of environmental estrogens. *Cancer Causes Control* **15**, 591–600.

Isosaari P, Hallikainen A, Kiviranta H, Vuorinen PJ, Parmanne R, Koistinen J, Vartiainen T, (2006). Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, naphthalenes and polybrominated diphenyl ethers in the edible fish caught from the Baltic Sea and lakes in Finland. *Environmental Pollution* **141**, 213-225.

Kiviranta H, Vartiainen T, Tuomisto J, (2002) Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls in fishermen in Finland. *Environ Health Perspect* **110**: 355-361.

Kiviranta H, Tuomisto JT, Tuomisto J, Tukiainen E, Vartiainen T, (2005) Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls in the general population in Finland. *Chemosphere* **60** (7), 854-869.

Official Journal of the European Union (2006). Commission regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. OJ, **L 364** 5-24.

WHO (2002). Global assessment of the state-of-the-science of endocrine disrupters. Geneva

THEME 2 - MECHANISMS OF EDC ACTION AND NOVEL ENDPOINTS AND BIOMARKERS

Overview and Objectives

Although EDCs are suspected of being involved in inducing reproductive health problems in humans and wildlife the ways in which these effects are induced are largely unknown. To close important knowledge gaps, EDEN carried out studies with the following aims to:

- Further the understanding of the mechanisms of EDC action through identification of novel endpoints and biomarkers.
- Explore foetal exposure of rats to phthalates as a new model for the identification of mechanisms underlying the TDS in the human.
- Examine the validity of using insulin-like factor 3 (InsL3) levels in blood as indicator of endocrine disruption leading to male reproductive developmental disorders.
- Investigate the mechanisms that underlie disturbance of the hypothalamic-pituitary (HP) unit, and examine its biological consequences and relevance to man.
- Develop and utilise a transgenic, translucent fish model, based on the expression of the green fluorescent protein (GFP) to assess EDCs.
- Develop a standard operating procedure for the detection of antiandrogens *in vivo* in a fish model, the stickleback.

Materials and Methods

Gene expression profiling and cell signalling

Cultured mammalian cells (MCF-7 human breast cancer cells) were treated with EDC and RNA and protein extracts prepared using established procedures. The up- or down-regulation of certain gene products was assessed by using Western-blotting, and changes in mRNA levels were monitored by using real-time RT-PCR. Further, array technologies were developed for screening EDC effects in the zebrafish.

Foetal exposure of rats to phthalates

Studies directed at identifying new biomarkers of phthalate action on the foetal rat testis have involved treatment of time mated pregnant rats with daily doses of dibutyl phthalate (DBP) varying from 4 to 500 mg/kg/day with the treatment period ranging from embryonal day 13.5 (e13.5) to e21.5 (standard regimen used for most studies). In some studies it was restricted to particular shorter windows (e13.5-e15.5 or e19.5-e21.5). Exposed male offspring have then been sampled at foetal ages ranging from e15.5-e21.5 or postnatally at ages ranging from 4-90+ days (adulthood). At each age, testis morphology, cell numbers and cell function/maturation have been assessed and at some ages cell proliferation and/or apoptotic rates have been determined.

In vitro cell screening system of chemicals affecting InsL3 gene expression

Mouse tumour LCs (mLTC-1) were transfected with a reporter construct containing the Insl3 promoter in front of the luciferase reporter gene. This promoter possesses three SF-1 binding sites which have been shown to be important for regulation of the mouse Insl3 gene. Transfected cells were used to screen for the effects of E2, testosterone and human chorionic gonadotrophin (hCG) on Insl3 gene expression *in vitro*. mLTC-1 cells were seeded in 6-well plate wells in Waymouth's medium 24h prior to transfections. On the following day the transfections were performed in triplicate using 0.5µg, 1.0µg or 1.5µg of reporter construct, 0.25µg or 0.05µg mERa/empty construct and 6µl FuGENE6 transfection reagent per well. The amount of DNA/well was always set equal in every experiment by using empty pIRES-EGFP vector as a compensator. The day after the medium was replaced with fresh Waymouth's medium containing foetal calf serum, serum stripped of endogenous steroids and different concentrations of E2, testosterone or hCG in ethanol or ethanol only as control. Then, 24h after hormone stimulation the cells were lysed. The lysates were centrifuged and luciferase activity was measured from the supernatants. Tranfection efficiency was measured by co-transfecting the cells with Promega's phRL-SV40 vector that encodes Renilla luciferase.

Development of an ELISA for InsL3

The assay was built up using: 1. An anti-ratInsl3 antiserum (CR15) generated using genetic immunisation of rabbits. 2. A synthetic rat Insl3 peptide supplied by Richard Ivell (Adelaide) and Ross Bathgate (Melbourne) as a calibration standard. 3. The same peptide labelled with an Europium chelate to give fluorescence signals when used as a tracer to compete in the assay with calibration samples or with Insl3 molecules in actual rat blood samples, for binding sites on the anti-ratInsl3 antibodies attached to the measuring well. In the first experiments the use of antiserum CR15 resulted in disappointingly low uncompeted tracer binding (B0) values. Other antibodies were tried, but these turned out to lead to even worse values, both in terms of B0 values as well as in terms of sensitivity. After optimising buffer and incubation conditions, CR15 finally led to sufficiently high B0 values and gave reliable standard curves. The study materials comprised sera from normal adult female and male rats (as controls), sera from adult castrated rats, and blood samples from EDS-, and DBP- treated male rats. The sera from castrated rats should most presumably be free of Insl3 and thus served as blank sera, which were spiked with the synthetic calibration standard to generate calibration curves in the same matrix as the actual sample. The reliable measuring range for the assay encompassed 5 to 300 pg rat Insl3 per 100µl of sample. This was similar to the measuring range of the immunoassay for human Insl3. Specificity of the assay was controlled by spiking assay buffer with defined amounts of rat Insl3 and related peptides and comparing the respective competition curves. Only human Insl3, the closest relative to rat Insl3 in the peptides used, showed a slight reduction of tracer binding. Females, castrated and EDS-treated male rats (EDS destroys the Insl3-producing LC in the testes) were at the baseline, as expected.

Investigations of the sensitivity of the hypothalamic-pituitary (HP) unit to endocrine disruption

To investigate HP sensitivity to endocrine disruption, experimental (rat) studies, involving neonatal exposures (i.e., during critical period of brain sex differentiation) to different doses of synthetic estrogens, androgens or antiandrogens were conducted, and

brain (hypothalamus), pituitary and serum samples obtained at different age points of postnatal development. Analytical approaches involved the combination of gene expression and hormonal assays. Gene targets were selected on the basis of previous knowledge about their pivotal role in the control of key aspects of reproductive function as well as on high-throughput screening for differentially expressed genes after selected neonatal exposures. Selected gene targets were also screened in hypothalamic and pituitary samples from large-scale *in vivo* mixture experiments. In addition, *ex vivo* models (hypothalamic explants and measurements of gonadotropin releasing hormone – GnRH-) were used in combination with *in vivo* exposures to evaluate potential mechanisms for precocious activation of the HP unit by EDC compounds, such as DDT. The latter might be clinically relevant given the observation of precocious puberty in girls from underdeveloped countries migrating to Europe and USA for adoption.

Development of transgenic zebrafish for detecting EDC effects

A DNA constructs containing the estrogen-responsive promoter ERE coupled to the sequence coding for the GFP or different parts of zebrafish aromatase *cyp19b* promoter coupled to EGFP, with or without amplifying sequences, were prepared and injected into zebrafish embryos at the one cell stage. These were allowed to develop and were then used for breeding experiments. To ascertain successful incorporation of the transgene fish from successive generations were monitored.

Results

Environmental estrogens reveal molecular effect profiles and signalling cross-talk that might be missed in current screening exercises

Detailed investigations during the last few years have uncovered a surprising complexity of the mode of action of the sex steroid hormone, E2 and its interactions with the ER. The ER functions as ligand-dependent transcription factor which is able to directly regulate gene expression by binding to specific DNA sequences, estrogen response elements (ERE). In recent years it has become clear that E2 can also rapidly and transiently trigger a variety of second messenger signalling events, including the induction of cAMP and adenylate cyclase, of Src with consequent activation of the extracellular-regulated kinases Erk1 and Erk2 in the Src/Ras/Erk cascade, and many others. All these effects are believed to be mediated through membrane-associated or cytosolic ERs and have therefore been termed “non-genomic” or “extranuclear” actions of E2.

In what is referred to as transcriptional cross-talk, the “non-genomic” actions of E2 may indirectly influence gene expression, by activation of the ER through phosphorylations by Src/Erk signalling. To investigate whether environmental estrogens could induce rapid extranuclear phosphorylation events, the activation of the Src/Erk pathway in the human MCF-7 breast cancer cell line by endogenous and environmental estrogens was investigated. By utilising inhibitory agents such as the pure antiestrogen ICI 182,780 and the Mek 1 inhibitor PD 98059, the relevance of rapid phosphorylation events along the Src/Erk cascade for the expression of selected ER-target genes was analysed.

The organochlorines *o,p’*-DDT, *p,p’*-DDE and β-hexachlorocyclohexane (β-HCH) showed striking similarities to the effect profile seen with E2. They were able to promote

expression of the *TFF1* gene, the progesterone receptor (PR) and the cell cycle regulator *PRAD1* (coding for cyclin D1). In the case of β-HCH, this is surprising, given the inability of this chemical to bind the ER. Gene expression could be inhibited completely in the presence of the antiestrogen ICI 182,780, demonstrating that a functional ER is necessary to trigger these events. Inhibition of the Erk kinase led to suppressions of gene transcription in the case of *o,p'*-DDT and β-HCH, but not with *p,p'*-DDE. This suggested that *o,p'*-DDT and β-HCH should be capable of activating the Src/Erk signalling module, and that this activation should not occur with *p,p'*-DDE. Western blot analysis confirmed this prediction and showed that *o,p'*-DDT and β-HCH induced sustained activation of the kinases Src and Erk1/2.

These results are of relevance for the testing and screening of estrogenic agents. They show that ER-mediated cellular responses are influenced by the characteristics of the chemicals that interact with the ER. The screening and testing for estrogen-like activities, with its current focus on ER binding and associated events, urgently needs to be supplemented by assays that take the wider effect spectrum of xenoestrogens into account, especially their non-genomic responses. Otherwise, chemicals such as β-HCH, which are unable to bind the ER, but exhibit an otherwise "E2-like" molecular effect spectrum, will be missed during conventional screening.

The relevance of xenoestrogen-induced extranuclear signalling in breast cancer cells: genomic instability

Genomic instability is a hallmark of cancer cells and is thought to be the "breeding ground" for acquired resistance. The induction of micronuclei, small bodies of (cell) nuclear material ejected from the cell nucleus into the cytoplasm of the affected cells, is taken as an indicator of genomic instability. E2 is able to raise the frequency of micronuclei in ER-competent cells. Studies with MCF-7 cells were undertaken to analyse the importance of ER signalling in the induction of these events. A number of estrogenic chemicals, including bisphenol A (BPA), certain cosmetics ingredients such as parabens and synthetic musks also led to elevated micronucleus frequencies. To investigate whether the ability of the ER to promote gene expression is responsible for these effects, the influence of antiestrogens such as tamoxifen and ICI 182,780 on micronuclei induced by estrogens was investigated. The antiestrogens were without effect. However, inhibition of the kinases Erk 1/2 protected against the micronucleus-inducing effects of estrogenic chemicals.

These results suggest that it is the rapid extranuclear signalling events triggered in the wake of ER activation that have an influence on micronucleus frequency. Erk 1/2 is involved in a cell cycle checkpoint in mitosis. Unwarranted over-stimulation of these kinases through ER activation may lead to overriding of this checkpoint with disruption of the finely tuned events important in distributing equal chromosome numbers to daughter cells and consequent ejection of chromosomes into the cytoplasm where they appear as micronuclei. These events may have some relevance in explaining the role of estrogens during late stage breast carcinogenesis.

Identification and evaluation of new biomarkers of foetal phthalate exposure

The Western world is observing certain disorders of male reproductive health such as testicular cancer, cryptorchidism, hypospadias and low sperm counts. Though manifesting

at different life stages, there is strong evidence that these disorders may have a common origin in foetal life. Taken together these disorders have been hypothesised to indicate a TDS, which stems from abnormal development and function of SC and/or LC during male sexual differentiation (Sharpe & Skakkebæk, 2003). Thus, considerable interest exists in identifying the events in the foetal testis that might give rise to TDS.

Foetal exposure of rats to DBP offers a potential animal model in which to explore this syndrome (Mahood *et al.*, 2005). The following new endpoints (biomarkers) of DBP action on the foetal rat testis have been identified during the EDEN project. These biomarkers manifest at different foetal ages and affect one or more of the main cell types of the foetal testis.

Delays in germ cell (GC) differentiation

Foetal germ cell (GC) differentiation is delayed following exposure to high doses of DBP (Figure 7) with a ~40% reduction in foetal GC numbers at birth. These DBP effects are of particular interest because in human TDS, the carcinoma *in situ* cells from which testicular GC cancers arise in adulthood are themselves thought to arise because of failure of normal foetal GC differentiation. However, in DBP-exposed rats a failure of foetal GC differentiation has not yet been illustrated, only a transient delay. Postnatally (after cessation of DBP exposure), there is also a further delay in GC differentiation with delay in their migration to the basal lamina and resumption of proliferation. Consequently there is an even bigger deficit in GC numbers (~80% reduction compared with controls) by mid-puberty, though this is corrected by adulthood in normally descended (scrotal) testes. DBP-exposed animals exhibit a high rate (~75%) of infertility in adulthood, so the functional integrity of the adult GC may be compromised as a result of their impaired differentiation in foetal life. This will be important to investigate in future studies.

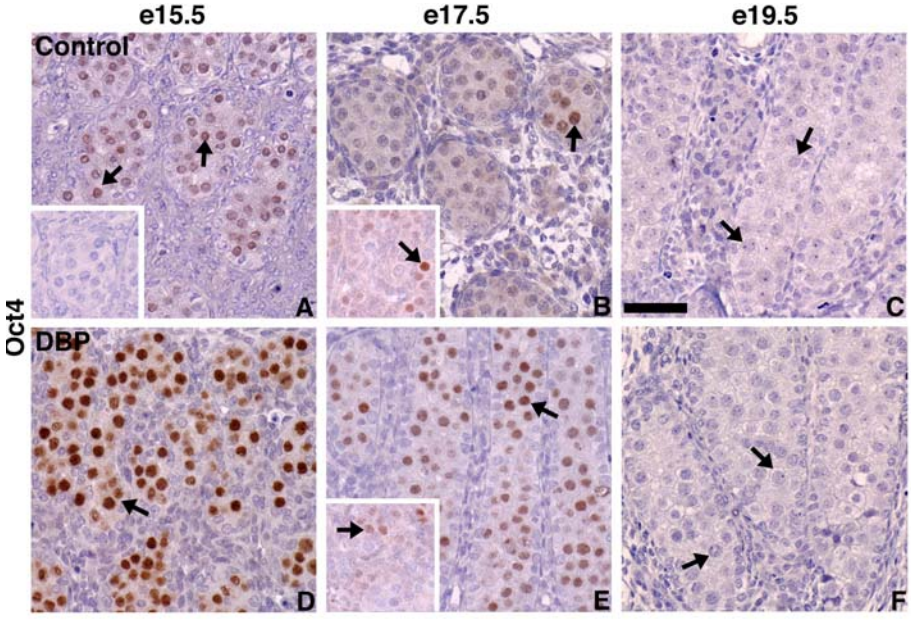

Figure 7 Delayed germ cell (arrows) differentiation in foetal testes of DBP-exposed animals as illustrated by prolongation of expression of the pluripotency factor Oct4 (brown staining). The stage of gestation is indicated at the top of each column.

Multinucleated gonocytes

Induction of gonocytes containing not one (as is normal), but several cell nuclei, so-called multinucleated gonocytes (MNG) is an indicator of reproductive abnormalities in male offspring. Exposure to DBP *in utero* results in dose-dependent induction of MNG throughout the foetal testis that persists postnatally until days 4-6. Studies showed that induction of MNG is unrelated to the aforementioned delayed GC differentiation. MNG thus occur only during the time window when foetal GC are quiescent and therefore has no relationship to proliferation. The mechanisms underlying MNG formation are unknown as are its consequences. It appears that MNG induction is a valid indicator of *in utero* phthalate exposure and is one of the most sensitive biomarkers of phthalate action as effects are evident at 20 mg/kg/day.

Foetal Leydig cell aggregation

Exposure to DBP *in utero* results in dose-dependent induction of LC aggregation in central regions of the testis. The number of LC is unaffected, though they are reduced notably in size (consistent with their reduced steroidogenic function). The aggregation is thought to arise because of migration of LC from more peripheral regions of the testis, but the trigger for this is unknown. The most important consequence of DBP-induced LC aggregation is that it interferes with the final stages of seminiferous cord formation in central regions, resulting in formation of focal dysgenetic areas in which the cords are malformed and which contain intratubular LC where the LC are in the wrong place (Figure 8). These focal dysgenetic areas persist throughout postnatal life and lack GC in adulthood. Similar focal dysgenetic areas are evident in testes of men with testicular GC cancer.

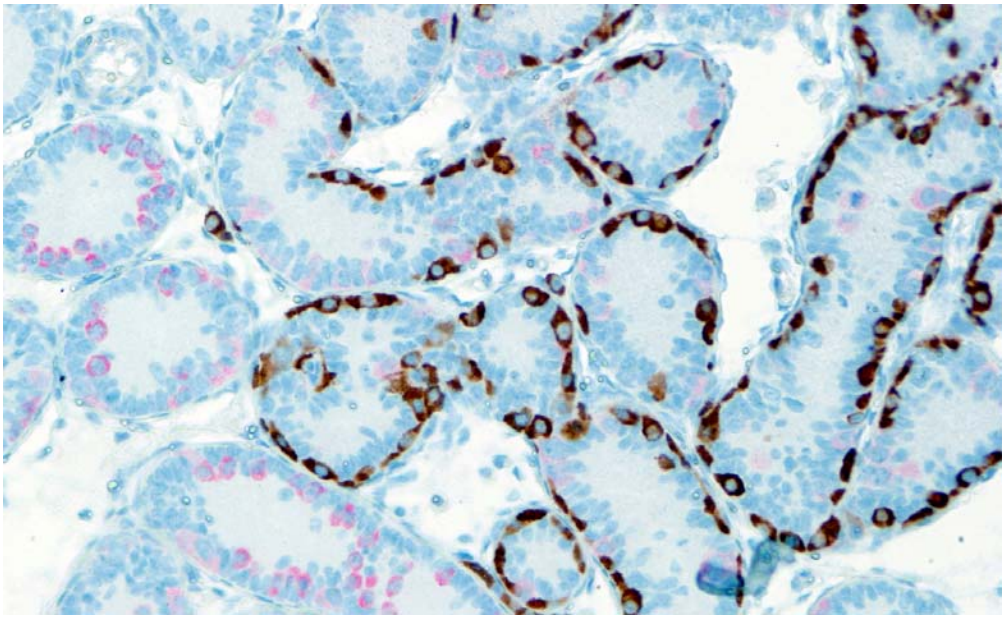

Figure 8 Abnormal location of adult Leydig cells (brown staining) inside of seminiferous tubules in the testis of a pubertal rat exposed *in utero* to DBP. Note the reduction in germ cell numbers (pink staining) where the intratubular Leydig cells occur.

Reduction in Sertoli cell number

Exposure to DBP *in utero* results in dose-dependent reduction in SC number at embryonal day 21.5 (e21.5), with the highest dose tested (500 mg/kg/day DBP) reducing SC number by ~45%. This decrease is probably secondary to the reduction in testosterone levels. It remains to be shown whether the DBP-induced reduction in SC number in rats persists through to adulthood. However, if this observation were to persist, then it would result in reduced capacity to make sperm in adulthood. In the human, this would lead to a reduction in sperm counts as is found in TDS.

Phthalates lead to suppression of Leydig cells in foetal testis

Leydig cells are important in synthesising steroid hormones during various phases of development. When pregnant rats were exposed *in vivo* to various doses of different phthalates, major suppression of LC hormone secretion was observed in the foetal testis. In males exposed to 500 mg/kg/day DBP administered to their mothers, testosterone levels within the testis were suppressed by 70-85% at embryonal day e19.5, when the peak of testosterone normally occurs. A similar pattern of suppression of Insl3 protein expression in LC is evident. InsL3 is thought to be important in regulating the descent of testes into the scrotal sack. Disruption of InsL3 function may be involved in the occurrence of undescended testes (cryptorchidism).

The observed hormonal changes are explained by reduced expression of genes involved in cholesterol uptake (scavenger receptor B1) and steroidogenesis (P450 side chain cleavage enzyme, C17-20 lyase) as well as Insl3. The adverse effects of DBP on LC gene expression are first evident *in vivo* at e17.5. The suppression of LC hormone secretion in studies *in vivo* is clearly important in explaining the high incidence of cryptorchidism and the occasional cases of hypospadias as well as the consistent reduction in anogenital distance (AGD) in DBP-exposed male offspring. Suppression of testosterone levels within the testis may also account for the major reduction in SC number and possibly for other changes in the foetal testis. The endpoint disorders related to suppression of foetal LC hormone production are directly relevant to human TDS. DBP exposure also causes abnormal migration/aggregation of the foetal LC in central regions of the testis. The cause is unknown but is presumed to result from altered production/action of cell attraction/motility factors. The most important effect of the DBP-induced LC aggregation is that it results in the focal formation of malformed seminiferous cords which may aberrantly trap foetal LC within them. These ‘focal dysgenetic areas’ containing “misplaced” intratubular LC, persist throughout postnatal life and always lack GC (Figure 8). Similar focal dysgenetic areas have been found in the testes of humans with testicular GC cancer.

At a later stage in gestation (embryonal days e19.5-e21.5) another effect of DBP treatment on GC becomes evident with the widespread occurrence of multinucleated GC (MNG) coupled with the abnormal aggregation of the foetal GC in the centre of the seminiferous cords. These effects only first become evident at e19.5 and can be induced by e21.5 even if DBP treatment is not started until e19.5. These GC effects are completely separate from, and unrelated to, the earlier effect of DBP on foetal GC differentiation above. It is likely that formation of MNG and GC aggregation reflect abnormal SC function and interaction with the GC. At the same time as MNG form there is also a big deficit in SC numbers in DBP-exposed males such that by e21.5, SC numbers

are reduced by ~45%. The most likely explanation for this decrease is that it is secondary to the reduction in testosterone levels in the testis as a similar reduction in SC numbers is observed in androgen receptor knockout mice.

Phthalate induced suppression of Insl3 and testicular descent

Following the identification of Insl3 as the key hormone regulating the first phase (trans-abdominal migration) of testis descent, EDEN has developed antibodies for measuring this protein. Insl3 immunoexpression in foetal LC is highly expressed in control males at e17.5 and e19.5 (during the period of trans-abdominal testis descent) and is notably lower at e21.5. In animals exposed *in utero* to 500 mg/kg DBP from e13.5, Insl3 immunoexpression was suppressed in most animals at e17.5-e19.5 but was not different from controls at e21.5. However, when Insl3 expression in LC was compared in normally descended and abnormally descended testes in DBP-exposed animals, there was no consistent relationship between the level of Insl3 immunoexpression and testis position. It was therefore concluded that there was no straightforward relationship between DBP-induced suppression of Insl3 in LC in the foetal testis and the normality or otherwise of testicular descent. Instead, failure of normal testis descent, as occurs with high frequency in animals exposed *in utero* to high doses of DBP, may be the consequence of combined suppression of Insl3 and testosterone production. Alternatively, it may be that Insl3 immunoexpression detected using the antibody generated may not accurately reflect changes in secretion (and thus the blood levels) of Insl3. The latter possibility is supported by the finding that neither *in utero* exposure to flutamide nor to DES resulted in consistent reduction in Insl3 immunoexpression in LC at e19.5, whereas similar treatment with flutamide did result in a significant reduction in Insl3 gene expression and a similar, and larger reduction in mRNA expression, has been reported by others after *in utero* exposure of mice to DES in which cryptorchidism was also induced.

InsL3 receptor expression in the gubernaculum

Various tissues were collected from rats at ages spanning from foetal life through to adulthood with a particular focus on the reproductive tract (testis, efferent ducts, epididymis, gubernaculum). Thus enabling an evaluation of the distribution and level of expression of receptors for Insl3 (the LGR8 receptor) using RT-PCR and immunohistochemistry. As it was previously shown that Insl3 protein expression was detectable in the gubernaculum, RT-PCR was utilised to detect whether Insl3 mRNA expression could also be shown. More limited evaluation of LGR8 immunoexpression in the testis and reproductive tract (including the gubernaculum) was also undertaken. It was shown that mRNA for LGR8 was expressed heavily in the gubernaculum throughout foetal and postnatal life, a finding confirmed at the protein level in more than one laboratory.

These findings confirm that the gubernaculum is a key target for Insl3 in foetal life when testis decent is occurring. It was also shown that no mRNA for Insl3 can be detected in the gubernaculum of the rat, despite localisation of the protein by immunohistochemistry. It was concluded that the Insl3 protein detected in the gubernaculum was protein bound to its LGR8 receptor. Another key finding was that LGR8 mRNA and protein expression is not confined to the gubernaculum but is evident in other reproductive tract tissues, notably in the efferent ducts and epididymis during foetal and postnatal development. This implies that Insl3 from the foetal LC may play a wider role in development of the

male reproductive tract, a process previously considered to be largely androgen-regulated (the androgens also emanating from the foetal LC). Unexpectedly, it was found that LGR8 protein is expressed in foetal GCs, implying a potential (but unknown) role for Insl3 in the development of these cells. Some of the abnormalities in developing foetal GCs found in rats exposed *in utero* to DBP, which coincide with suppression of Insl3, could be attributable to altered action of Insl3. It is concluded that, in the rat, the gubernaculum is an important target for the actions of Insl3 during the period when the testis descends through the abdominal cavity consistent with a role for Insl3 in testis descent. However, judged on the wider expression of Insl3 receptors throughout the foetal reproductive tract and in foetal GCs in the testis, it seems likely that there are other, unknown functions of Insl3 in development of the male reproductive system.

Relationship of Insl3 expression to size, number and androgen production by foetal Leydig cells in rats exposed to treatments *in utero*

These studies involved treatment of time mated pregnant rats with daily doses of DBP varying from 4 to 500 mg/kg/day or with vehicle (= controls) with the treatment period ranging from e13.5 to e21.5. Exposed male offspring have then been sampled at foetal ages ranging from e15.5-e21.5 and LC immunoexpression of Insl3 evaluated and compared with LC size (= cytoplasmic volume) and the expression of factors involved in different steps of steroidogenesis (SR-B1, P450ssc) or with other roles in LC (Inhibin-a, CRABP-II); at certain ages (e19.5, e21.5) intratestcular levels of testosterone have also been measured. Most attention has been focused on males exposed *in utero* to 500 mg/kg/day DBP because this is the only dose level that causes a high incidence (>70%) of (mainly unilateral) cryptorchidism and is the only dose to consistently reduce immunoexpression of Insl3 at certain foetal ages.

In addition, we have undertaken similar evaluations in foetal males from pregnant dams that have been treated with either 100 mg/kg/day flutamide from e15.5 until e21.5. These studies have shown that Insl3 immunoexpression in foetal LC appears to change more or less hand in hand with steroidogenic capacity and with the expression of steroidogenic enzymes that are highly regulated, such as P450ssc. Thus, in controls as testosterone levels and P450ssc immunoexpression increase from e15.5 to a peak at e19.5, so does the intensity of immunoexpression of Insl3. Furthermore, exposure to 500 mg/kg/day DBP results in a similar decrease in immunoexpression of Insl3 as in P450ssc, a change that also correlates with reduction in LC size.

In contrast, exposure *in utero* to flutamide does not affect immunoexpression of either Insl3 or P450ssc. However, it remains uncertain whether immunoexpression of Insl3 provides an accurate reflection of the level of secreted hormone, although for P450ssc there is clear evidence that reductions in immunoexpression (such as after treatment with DBP) correlate closely with reduction in testosterone production. With such fundamental reservations, and in the absence of data to correlate LC immunoexpression of Insl3 with levels of secreted Insl3, it was decided that further detailed evaluation of LC Insl3 immunoexpression in relation to other factors, would be pointless.

Association of InsL3 blood levels with reproductive disorders in humans and rats

Human studies

To characterise the relationship between serum Insl3 levels and male reproductive function, serum Insl3 levels were measured in blood samples collected from cryptorchid boys and from boys with normally descended testes. Insl3 levels were also measured in a large number of blood samples from male and female children of various ages from the neonatal period through puberty to adulthood. Additionally, blood samples from adult men during and after treatment-induced gonadotrophin suppression (which will suppress LC function) were evaluated for Insl3 levels.

The studies showed that serum InsL3 in normal men is not acutely sensitive to exogenous FSH or LH/hCG, but nevertheless is dependent on the stimulatory activity of gonadotrophins on LC. Suppression of endogenous gonadotrophins results in a marked decline in serum InsL3, and subsequently InsL3 is acutely sensitive to LH action. Subsequent to long-term gonadotrophin suppression, InsL3 does not recover to the same degree as does testosterone, suggesting that InsL3 may be more sensitive than testosterone to impaired LC function in adulthood. Reduced blood levels of InsL3 in cryptorchid boys suggests impairment of LC function in cryptorchid testes and supports the hypothesis that cryptorchidism is associated with a primary testicular disorder; however, the magnitude of difference between blood Insl3 levels in normal and cryptorchid boys when measured postnatally is modest (levels overlap for the two groups). This may imply that Insl3 alone is not the most critical determinant of testis descent.

Alternatively, it is possible that measurement of Insl3 levels postnatally does not accurately reflect levels in foetal life when Insl3 action on the gubernaculum plays its role in testis descent. Measurement of Insl3 levels in amniotic fluid and relating levels found to subsequent normality or not of testis descent may resolve this issue.

Rat studies

To characterise the relationship between Insl3 mRNA or protein expression levels in foetal life and testicular descent and non-descent (cryptorchidism), Insl3 expression in foetal testes and in the LC of such testes has been evaluated in rats exposed *in utero* to DBP, flutamide or to other EDCs (TCDD, p'p'-DDE). Immunoexpression of Insl3 in the adult generation of LC has also been evaluated in normal rats during progression through puberty into adulthood or after various treatments likely to affect LC function; blood samples from some of the latter treatment groups have been evaluated for Insl3 levels during development and validation of the rat Insl3 assay.

Finally, a transactivation assay for Insl3, which might serve to screen chemicals for Insl3-gene modifying activity was developed and tested. Results for blood levels of Insl3 in the rat have been very limited and the rat Insl3 assay has still not completed validation and optimisation. However, the results obtained are largely in tune with the findings from the human in showing that factors that suppress gonadotrophin secretion and/or LC development postnatally suppress Insl3 levels. In the foetus, it has been shown that a maternal treatment (DBP) that induces a high incidence of cryptorchidism in the male offspring (due primarily to impaired development of the gubernaculum), results in major suppression of Insl3 gene and protein expression. However, no consistent relationship

was found between cryptorchidism and the level of Insl3 expression, perhaps suggesting that the parallel suppression of testosterone production by DBP may also be an important factor in determining failure of testis descent.

Various pieces of evidence suggest that testosterone, or other androgens, might play a role in stimulating Insl3 expression, though this needs further investigation. The Insl3 reporter gene (transactivation) assay shows that there are no direct effects of estrogens in this system whereas androgens may be weakly stimulatory. The human and rat studies support a role for Insl3 in testis descent, but have also shown that there does not appear to be a simple, straightforward relationship between the normality of testis descent and Insl3 expression in the testis or when measured (postnatally) in blood. However, this conclusion is based on limited material. Postnatally, and particularly in adulthood, Insl3 levels may provide important information on the normality or not of LC function and more detailed comparison of testosterone and Insl3 levels should establish the relative utility and usefulness of these two markers. Transactivation assays for Insl3 probably have limited utility.

In vitro cell screening system for chemicals effecting InsL3 gene expression

Mouse tumour LC (mLTC-1) were transfected with a reporter construct containing the Insl3 promoter in front of the luciferase reporter gene. This promoter possesses three SF-1 binding sites which have been shown to be important for regulation of the mouse Insl3 gene. Transfected cells were used to screen for the effects of E2, testosterone and hCG on Insl3 gene expression *in vitro*. The Insl3 reporter gene assay showed that there were no direct effects by estrogens, whereas androgens slightly stimulated gene activity. It is therefore concluded that this Insl3 reporter gene assay cannot be used to screen effectively for chemicals likely to have an impact on Insl3 gene expression.

ELISA for the measurement of InsL3 in human and rat blood/testis extracts

An ELISA for the measurement of InsL3 in tissue extracts was developed. Extracts from female rats and from castrated and EDS-treated male rats (EDS destroys the Insl3-producing LC in the testes) did not produce signals, as expected. In male rats, there was an increase in Insl3 levels at around puberty, reaching a peak at day 43 and then declining somewhat in adulthood, a pattern that parallels that found for testosterone in numerous earlier studies. It can be assumed that Insl3 must have a function in the adult rat in the postpubertal age. Also, male rats appeared to have roughly ten times more Insl3 in their blood than male humans. Drastically reduced were Insl3 levels in blood of adult male rats which were exposed to DBP *in utero*, regardless of their actual testicular status. This has to be investigated in further detail and points towards an obstruction of LC or general testis function by DBP already in the foetal stage and lasting past puberty, also in rats in which the testes descend normally into the scrotum.

Hypothalamic-pituitary sensitivity to endocrine disruption

The HP unit plays a central role in the regulation of reproductive function. During early stages of development, the HP unit is highly sensitive to the organising effects of endogenous sex steroids. This raises the possibility that the developing HP unit might also be a potential target for the actions of EDCs. The aim was to explore the mechanisms, consequences and new endpoints of endocrine disruption at the HP unit, whose sensitivity to the organising effects of sex steroids (endogenous and synthetic) is expected to be

higher than that of the peripheral sex organs (internal and external genitalia). Most attention has focused on the characterisation of the deleterious effects of EDCs on reproductive health and analysis of direct effects at peripheral sex organs. Conversely, little was known about their potential actions on central reproductive systems. However, some of the putative 'clinical correlates' of endocrine disruption of reproductive function (e.g., disturbed timing of puberty and some forms of infertility) might theoretically stem from alterations at the HP unit.

Results have furthered knowledge on the physiological mechanisms controlling reproductive function at the HP unit and have set the basis for the identification of novel molecular mechanisms, end points and biomarkers for exposure/ action of EDCs at this site. Overall, the studies reinforced the notion that the developing HP unit is sensitive to the effects of synthetic estrogens, as monitored by molecular biomarkers, such as changes in expression of progesterone receptor (PR) and KiSS-1 genes at the hypothalamus and globin genes at the pituitary. Interestingly, some of these changes were tightly related to concomitant alterations in basal serum levels of luteinising hormone (LH) which is a putative marker for disruption of function of the gonadotropic axis and were also detected after neonatal exposure to the aromatisable androgen, testosterone propionate, the antiandrogen, flutamide, and BPA. The analyses so far conducted from *in vivo* mixture experiments, testing the effects of exposures to selected antiandrogenic EDCs alone or in mixtures, have failed to identify consistent changes in hypothalamic or pituitary expression of some of the above gene markers at early periods of postnatal development (postnatal day - PND 16). However, further analyses at later age points are required in order to define the potential impact at the HP unit of such exposures in terms of activation of gonadotropic axis at puberty and its function later in life.

Mechanistically, findings on the hypothalamic KiSS-1/GPR54 system have helped to define not only the fundamental role of KiSS-1 as a pivotal element in the central regulation of the gonadotropic axis in normal conditions but also its possible function as a target for endocrine disruption of reproductive function by exposure to estrogenic (and possibly androgenic) EDCs during critical periods of sexual differentiation of the HP unit (Figure 9). Also relevant from a mechanistic stand point, are results illustrating that selected EDCs, including DDT isomers can stimulate the GnRH secretion *in vitro* in the immature female hypothalamus through both rapid and/or slow effects and they involve both estrogen and dioxin receptor pathways. These effects can account for the precocious sexual development observed in female rats after early exposure to E2 or DDT *in vivo*, and might provide (at least partially) the molecular basis for the observation of precocious puberty in girls from underdeveloped countries migrating to Europe and USA for adoption. Thus, results have enlarged the basic knowledge on the consequences and potential modes of action EDCs (with estrogenic, androgenic or antiandrogenic activity) at different levels of the reproductive (or HP-gonadal) axis in mammals, which are likely to involve direct effects at the HP unit.

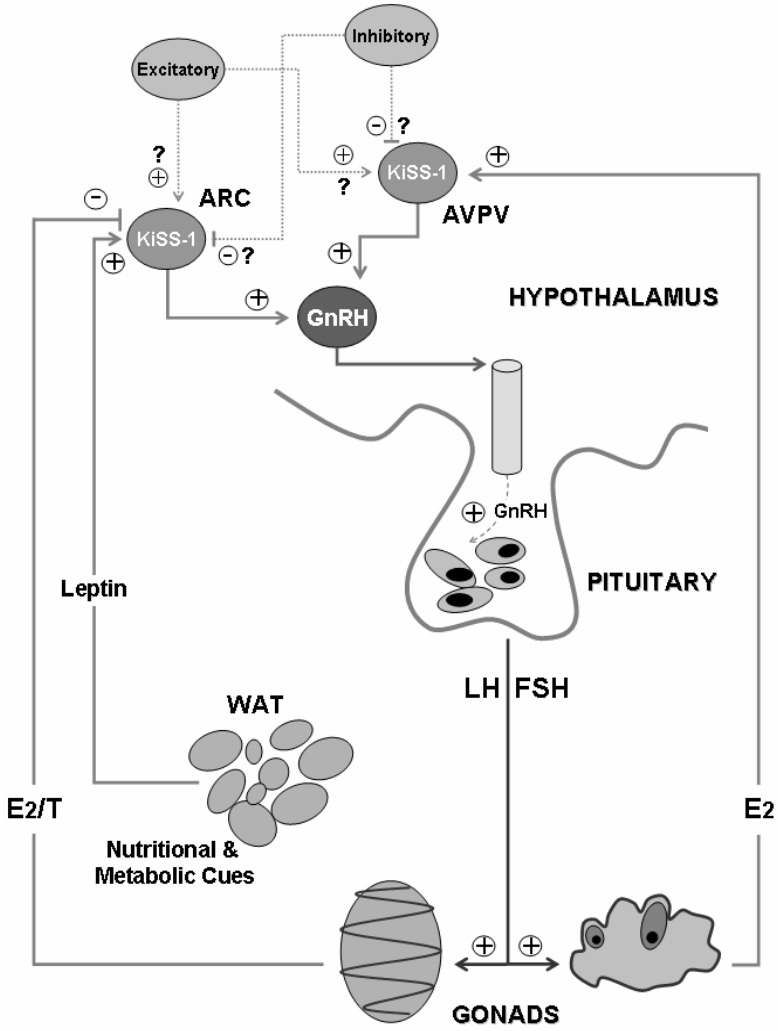

Figure 9 Pivotal role of KiSS-1 neurons in the hypothalamic circuitries controlling GnRH neurons and, thereby, reproductive function. Studies of neonatal exposure to synthetic estrogen and androgen point out that (i) functional organisation of neuronal circuits involving KiSS-1 neurons (so-called KiSS-1 system) is sensitive to the organising effects of sex-steroid acting compounds during critical periods of sexual differentiation of the brain; and (ii) reproductive defects linked to inappropriate exposures can be ameliorated by exogenous administration of kisspeptins. Altogether, these observations are the first to suggest that the hypothalamic KiSS-1 system might be a target for endocrine disruption of reproductive function at the HP unit.

Dose-response analyses showed that neonatal exposure to doses as low as 1-10 mg E2 benzoate per rat were able to induce significant changes in the relative mRNA levels of KiSS-1 (decrease) and PR (increase) at the hypothalamus, as well as in the expression levels (increase) of alpha- and beta-globin genes at the pituitary. The fact that these changes were tightly related to concomitant changes (decrease) in basal serum levels of LH, i.e. a conventional marker for disruption of function of the gonadotropic axis, strongly suggests that these might be regarded as genuine markers for potential endocrine disrupting events at the HP. Moreover, some of the above markers were also altered in

expression after neonatal exposure to the aromatisable androgen, testosterone propionate, the antiandrogen, flutamide, and/or the putative xenoestrogen, BPA, which reinforces the contention that expression of those genes (PR, KiSS-1, globins) at the HP unit might serve as reliable, highly sensitive, continuous biomarkers of exposure (and eventual disruption at this level) to sex steroid-like compounds during critical periods of sexual differentiation. From a mechanistic perspective, some of the identified changes in gene expression in the experimental models of exposure to synthetic estrogenic and related compounds might not only be relevant in terms of setting thresholds and sensitivity for endocrine disruption at the HP unit, but they may provide also novel information regarding end points and mechanisms for endocrine disruption at this level of the reproductive axis. The most salient example of this contribution is the definition of the hypothalamic KiSS-1 system not only as a pivotal element in the central regulation of the gonadotropic axis in normal conditions, but also as a putative target for endocrine disruption by exposure to estrogenic (and possibly androgenic) compounds during critical periods of sexual differentiation of the HP unit. Likewise, altered expression of PR at the hypothalamus, and eventually of globin genes at the pituitary, may prove mechanistically relevant to explain some of the alterations in development and/or function of the reproductive axis following early exposure to sex steroid-like acting compounds.

Rat studies were subsequently conducted to characterise the effects and threshold doses (sensitivity) of EDCs such as DDT, in terms of induction of precocious activation of the HP unit, using the rat hypothalamic explant incubation paradigm to investigate for novel molecular mechanisms for such a precocious activation. In addition, specific studies to define animal models for the *in vivo* characterisation of the above phenomenon were conducted. By a combination of *in vitro* and *in vivo* analyses, results show that some EDCs, including DDT isomers, are able to stimulate the GnRH secretion *in vitro* in the immature female hypothalamus through both rapid and/or slow effects and they involve both estrogen and dioxin receptor pathways. These effects can account for the precocious sexual development observed after early exposure to E2 or DDT *in vivo*, thus providing the molecular basis for the clinical observation of precocious puberty in foreign girls migrating for adoption in several European countries.

Development of new techniques and novel endpoints

A suite of advanced tools for diagnosing and assessing endocrine disruption in fish and mammals has been successfully developed. These tools include:

- Antibodies and real-time RT PCR protocols for quantifying EDC-inducible molecular responses.
- Customised microarrays to screen EDC-responsive genes in zebrafish.
- Sensitive screening assay for estrogenic EDCs using an aromatase reporter system.

Utilisation of these tools have led to the evaluation of the mechanisms by which EDCs interfere with sex steroid synthesis and disturb sexual development and reproduction of fish, and identifying novel targets of ED action beyond the reproductive axis.

Mechanisms of expression regulation and functional roles of aromatase *cyp19* in zebrafish

Aromatase is a key enzyme of steroidogenesis important in the regulation of endogenous levels of estrogens/androgens and thus directly influencing sexual differentiation and reproduction of vertebrates. Zebrafish have two aromatase forms which are expressed in the gonads (*cyp19a1*) and in the brain (*cyp19a2*). The brain form of aromatase is responsive to estrogens due to the presence of functional ERE in the promoter (Menuet *et al*., 2005). Prediction of dioxin-responsive elements (DREs) in the promoters of both *cyp19a1* and *cyp19a2* had suggested the direct regulation of these genes by aryl hydrocarbon receptor (AhR) and thus susceptibility to the dioxin exposure; however, *in vitro* study of these promoters with use of site-directed mutagenesis showed that these sites are probably not functional. Further reinforced by electrophoretic mobility shift assays and *in vivo* exposures of zebrafish larvae, the data proved that the *cyp19* aromatases in zebrafish are not directly regulated by AhR via DREs. Interestingly, the study revealed an important mode of crosstalk between ER and AhR, showing that dioxins are able to regulate the expression of the genes containing a functional ERE, not involving DREs on the promoter. These actions of dioxins may be either estrogenic (leading to stimulation of estrogen-receptor element-mediated transcription) or antiestrogenic (leading to attenuation of normal E2-induced upregulation of transcription), depending on the absence or presence of the estrogen receptor ligand, respectively. Thus, at the different stages of development, or in the different target organs, which might differ in estrogen content, the actions of dioxins may also be different. Similarly, the actions of dioxins in the environment can also be quite different depending on the presence or absence of estrogenic substances in the same exposure solution.

The specific sites of aromatase *cyp19a2* expression in the brain were identified, giving a hint towards the possible function of this enzyme in the brain. Presence and activities of the aromatases get disturbed (in time and total activity) in the presence of xenoestrogens. The previously assumed role of the brain aromatase in the sexual development could not be confirmed, pointing to the fact that sexual development in zebrafish is probably a multifactorial process.

Aromatase can also affect functions outside the reproductive system such as neurogenesis. Figure 10 illustrates the consequences of aromatase disruption in early life stages of zebrafish resulting in a statistically significant reduction of neuromast number.

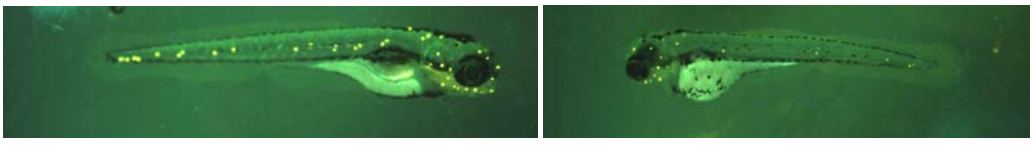

Figure 10 Embryos were injected with morpholinos to suppress *zfcyp19a* and *zfcyp19b* expression during early development. Left: control zebrafish larva, with neuromasts along the head and the lateral line (yellow dots). Right: morpholino-injected zebrafish with reduced number of neuromasts.

Developing and utilising transgenic zebrafish for detecting effects of EDCs

Candidate genes identified for the construction of transgenic zebrafish

From the gene expression experiments carried out using DNA-microarray techniques and RT-PCR, the ERE is recommended for constructing transgenic zebrafish. To enhance the signal, the use of a reporter gene under the control of 3 to 4 EREs is considered best practice. In addition, the regulation of aromatase by EDC renders aromatase an interesting target gene for the detection of EDC effects, as the expression of brain aromatase gene is significantly stimulated by estrogen. The promoter region of this gene (also containing an ERE) could therefore also be a good candidate for transgenic zebrafish.

Transgenic zebrafish model

Significant progress towards developing lines of useful transgenic fish has been made and efforts are continuing post EDEN. Injection of the gene construct into embryos at the one cell stage is shown in Figure 11.

Injection of the conventional vector consisting of z*fcyp19b* and the enhanced green fluorescent protein (EGFP) coding sequence into zebrafish embryos did not result in sufficiently high expression levels. This was irrespective of the different injection methods utilised and exposure to E2, which is known to activate z*fcyp19b* promoter in zebrafish embryos (Menuet *et al.*, 2005). The use of the previously recommended (Koester and Fraser, 2001) amplification system based on transcriptional activator Gal4VP16 increased the signal, but also introduced the problem of unspecificity, as the EGFP expression was frequently observed in the unexpected sites outside the brain. However, EGFP expression in the brain was observed most often and faded slower in the course of development than in other sites. Further, brain expression of EGFP, in contrast to expression in other tissues, was stabilised by exposure to estrogen and destabilised by exposure to ER antagonist, indicating its possible z*fcyp19b*-specificity. This work, although due to technical problems not leading to the production of transgenic zebrafish line suitable for EDC detection, had nonetheless provided the evaluation of different methods available for producing zebrafish expressing EGFP under the control of natural and artificial promoters.

For transgenic zebrafish possessing an estrogen-responsive promoter (an ERE) connected to the GFP gene, the current status is one close to establishing one or more lines. However, one further round of screening of the F1 fish is required, followed by breeding a further generation (F2 fish), which will need to be screened for the presence of the transgene. Unequivocal evidence of the presence of the transgene in some (probably 50%) of the F2 fish would indicate germline transmission of the transgene, and allow a transgenic line to be established.

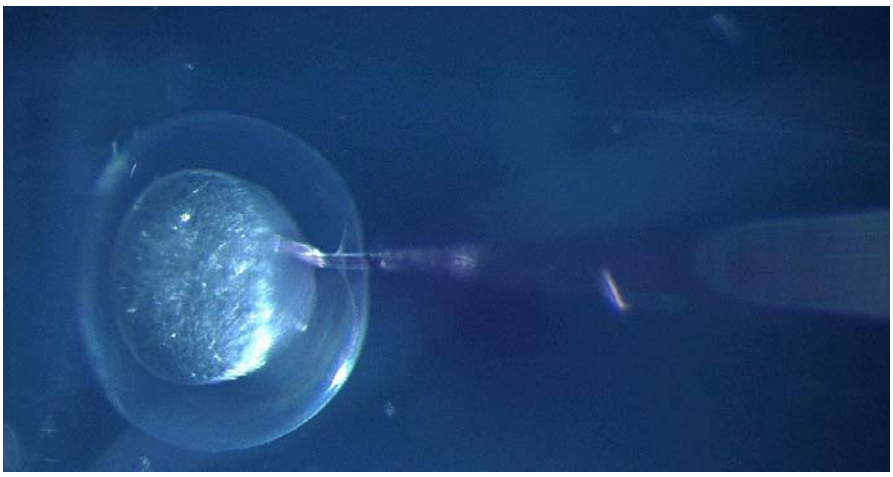

Figure 11 Injection of the 2 x ERE-EGFP vector into a zebrafish embryo at the one-cell stage.

Three factors have been identified as playing key roles in the production of transgenic fish, these being the number of eggs injected, the time involved in raising and screening offspring and luck! The more eggs that are injected with the transgene, the greater the chance of producing a useful transgenic line of fish. A great deal of time, and costs, are associated with rearing fish to adulthood, when they can reproduce, and produce the next generation. After a batch of eggs has been injected, it takes 4 to 6 months for the fish to reach sexual maturity. Only by screening the next generation can it be ascertained whether or not the transgene has been transmitted to the next generation. Even if it has, it will be to only a small proportion of the offspring, and these need to be identified, then reared to adulthood so that they in turn can reproduce. A minimum of 3 generations, and hence about 18 months, are required to unequivocally establish whether or not a transgene has been stably integrated into the germline. Efforts to study these fish will continue, in the hope that a useful line of transgenic fish can be obtained.

Utilising transgenic ZER-CALUX® zebrafish, an exposure study to the potent estrogen EE2 was carried out to determine the usefulness of transgenic reporter fish in detecting endocrine activities of chemicals and polluted environments. As expected, the fish synthesised VTG in response to EE2. Similar results would have been obtained if normal (not transgenic) fish had been used. All 3 concentrations of EE2 (up to 10 ng/L) produced significant responses, albeit only at the highest concentration was the response very pronounced. Also, as expected, luciferase was induced in the liver (the transgenic fish produce luciferase in response to exposure to estrogen). Given that VTG was synthesised in the liver in response to the EE2, it would have been extremely surprising if luciferase had not been induced in that organ. Luciferase induction was also investigated in the brain and testes. The results demonstrate that in the brain there was no luciferase induction above the control level in the fish exposed to EE2. This might imply that the brain is unresponsive to EE2, or responsive but less sensitive than the liver. Another explanation is that the EE2 could not reach the brain, possibly due to the existence of the blood:brain barrier. In complete contrast, exposure to the highest concentration of EE2 caused a major induction of luciferase in the testes (Figure 12). This can mean only that functional ERs exist in the testes. This tissue therefore becomes a prime candidate for effects caused by environmental estrogens. A more complete investigation of many different tissues may demonstrate that other tissues/organs are also estrogen-responsive.

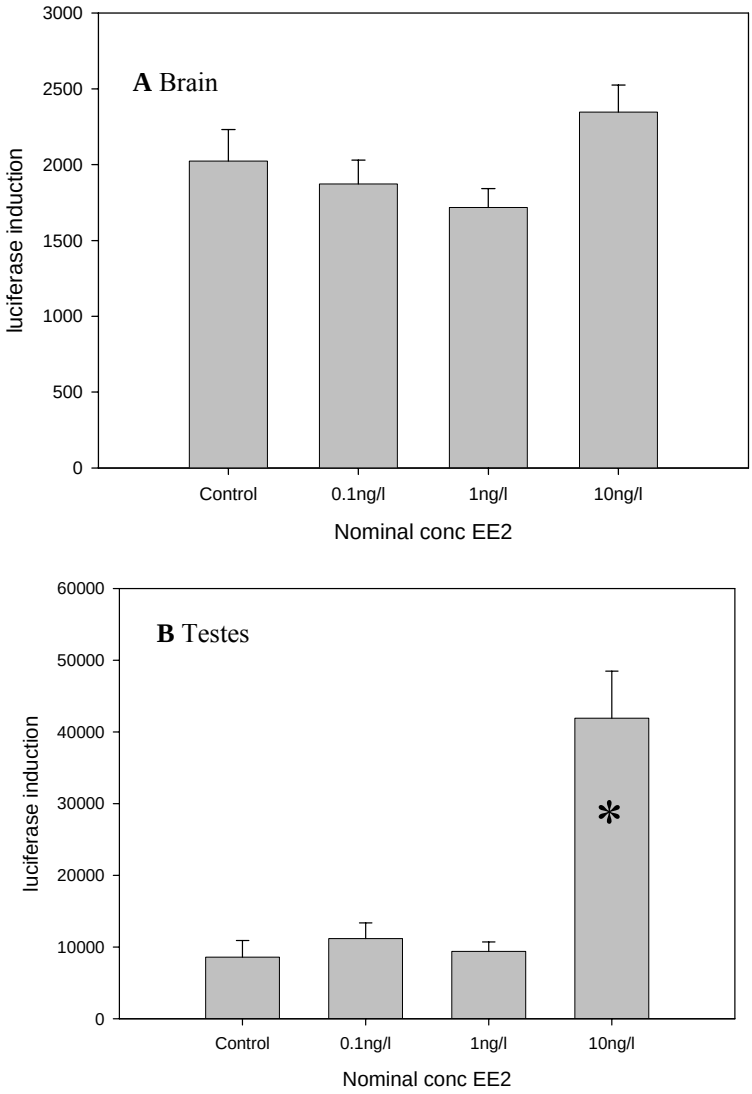

Figure 12 Luciferase induction in the brain (A) and testes (B) of transgenic zebrafish exposed to the potent estrogen ethinylestradiol (EE2).

Development of an *in vivo* model for the screening of antiandrogenic effects in fish

Until recently, there was no *in vivo* assay for the screening of antiandrogens available, apart from the Hershberger assay. An opportunity offered itself to develop an alternative to the Hershberger assay by exploiting a fish model based on the three-spined stickleback. Male stickleback synthesise large quantities of the glue protein spiggin during nesting. Spiggin induction is under the control of androgens. It is quite variable in male fish where it is influenced by light, social hierarchies and reproductive status.

Female sticklebacks exposed to androgens also induce the protein, but the response is much more uniform. Antiandrogenic activities of test chemicals can be detected as a downturn in kidney spiggin levels in female fish co-exposed to a stable androgen (dihydrotestosterone). A standard operating procedure for this assay was developed as part of EDEN Theme 2 work (Katsiadaki *et al.,* 2006). The protocol was applied to the low-dose and mixture studies in Theme 4.

Discussion

Considerable progress has been made in uncovering the mechanisms underlying EDC action at various different levels of biological organisation. The importance of signalling cross-talk in the effects of xenoestrogens on gene expression was highlighted. Foetal exposure to certain phthalates leads to a multitude of effects, with delays in GC differentiation, major disruption in cell migration during development and reductions in SC numbers being prominent features of DBP action.

The role of InsL3 in relation to testes descent and androgen production by foetal LC has been clarified and has revealed complex interrelations not previously anticipated.

The sensitivity of the HP unit to environmental and endogenous estrogens has been characterised and possible implications in relation to precocious puberty assessed.

Substantial progress has been made towards the study of endocrine disruption in fish with activities including the development of microarrays for assessing endocrine disruption in zebrafish, establishing sensitive screening tools for endocrine disruption in fish, evaluating the mechanisms by which EDCs interfere with sex steroid synthesis and disturb sexual development and reproduction of fish, and identifying novel targets of EDC action beyond the reproductive axis.

Candidate genes for developing transgenic zebrafish for the detection of EDC effects have been identified, but the technical difficulties in producing transgenic fish proved to be significant.

Conclusions

The work produced in Theme 2 of EDEN allows the following conclusions:

- The screening for EDC, which is mainly based on measuring interactions with steroid receptors, has experienced substantial advancement through the EDEN project by pointing to the importance of aromatase/steroid synthesis and of signal transduction events in endocrine disruption. To avoid the risk of overlooking endocrine active chemicals, existing screening tools should be expanded to take account these alternatives modes of action.

- Certain phthalates are able to induce effects in an experimental rat model that are of direct relevance to understanding the TDS in man. DBP essentially delays differentiation of GCs, interferes with the finely tuned events that govern the migration of various cell types during development, thereby leading to misplaced cells and aggregation of LC. It also reduces the number of SC in the developing testis, thereby limiting the number of GCs a testis can support.

- InsL3 secreted by foetal LC may play a wider role in the development of the male reproductive tract than thought previously. This process was thought to be largely controlled by androgens. There was no clear relationship between DBP-induced suppression of InsL3 in foetal LC and the normality or otherwise of testicular

descent. LC InsL3 immunoexpression did not provide accurate reflections of the levels of secreted InsL3.

- The development and validation of an assay for the measurement of InsL3 blood levels was successful, but the differences in blood InsL3 levels in normal and cryptorchid boys were too small to exploit InsL3 as a biomarker indicative of disruption of testis descent. It appears that InsL3 alone is not the most critical determinant of testis descent.

- The HP unit proved to be exquisitely sensitive to the effects of several EDC, and the effects may account for precocious sexual development observed after early EDC exposure.

- The development of a transgenic fish model for the detection of EDC effects proved to be technically too demanding to be completed in time, but efforts continue to complete this project after conclusion of EDEN.

Literature Cited

Katsiadaki I, Morris S, Squires C, Hurst MR, James JD, Scott AP (2006). Use of the three-spined stickleback (Gasterosteus aculeatus) as a sensitive *in vivo* test for detection of environmental antiandrogens. *Environ. Health Perspect*. **114** (Suppl. 1): 115-121

Koester RW, Fraser SE (2001). Tracing transgene expression in living zebrafish embryos. *Dev. Biology*, **233**(2), 329-346

Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, Kah O (2005). Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *J Comp. Neurol.* **485**(4), 304-320

Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS, Sharpe RM (2005). Abnormal Leydig Cell aggregation in the foetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* **146**: 613-623

Sharpe RM and Skakkebæk NE (2003). Male reproductive disorders and the role of endocrine disruption: Advances in understanding and identification of areas for future research. *Pure Appl. Chem*. **75**: 2023-2038

THEME 3 - INDICATORS OF IMPAIRED MALE REPRODUCTIVE FUNCTION

Overview and Objectives

One of the first observations suggesting deteriorating male reproductive health was an apparent decline in sperm numbers of normal men reported 15 years ago (Carlsen *et al.,* 1992). After that meta-analysis it became obvious that prospective and international multicentre studies are needed to assess temporal and regional differences in semen quality. Driven by the increased concern about deteriorating male reproductive health in several industrialised countries, a number of studies on reproductive health in men from the general population have been carried out in different European countries during the last 5-7 years. These studies have shown that large regional differences in male reproductive health exist within Europe. It was especially noted that Finland was clearly contrasting Denmark with a better semen quality. The recent findings of poor semen quality among at least 20 % of normal young men in Denmark raise the question whether it has reached a level where it may influence on pregnancy rates in the population.

The European studies on male reproductive health have provided a large data set that has been entered into a central research database in Copenhagen. This database contains data on clinical findings, semen quality, hormone levels, lifestyle factors, and exposures, which can be explored to search for causal factors. However, the previously conducted European studies on male reproductive health did not include data from Germany. The German population has experienced very different industrial history between East and West after the Second World War and although originating from the same genetic pool the young men of the former East and West Germany may have been exposed very different environment during their foetal development and childhood. Thus comparison of young men born in East and West Germany may provide additional clues to factors affecting male reproductive health.

It has been hypothesised that the observed adverse changes and regional differences in male reproductive health are attributable, at least partly, to altered/different exposures to endocrine disrupting agents during foetal development, leading to manifestation of the so called TDS. The focus has mainly been on SC function as disturbed SC function is suggested to cause the reduction in semen quality and development of testicular cancer. Less attention has been given to the possible adverse effects on LC function, although disturbed LC function has distinct effects on male reproductive health - mainly through altered testosterone production. Testosterone plays a key role in the development of the external male genitalia and in the adult man it drives spermatogenesis and libido. Based on longitudinal studies there is general agreement that male serum testosterone levels decline with age indicating a decrease in LC function with increasing age. However, cross-sectional surveys have demonstrated very different rates of decline, some even showing no decrease in testosterone levels with increasing age. The hypothesis is that this discrepancy in the age related decline in serum testosterone between cross-sectional and longitudinal studies might be due to confounders such as secular or cohort effects on hormone levels, which may hide or blunt the effect of aging.

The objectives of theme 3 are to:

- Expand the central database on male reproductive health to include also data on male reproductive health in the former East and West Germany and explore possible differences.
- Expand this database with new data on andrological parameters of currently 18-20 year old men in different European countries in order to follow secular trends in male reproductive health.
- Exploit data in the central database to identify environmental and lifestyle factors associated with affected male reproduction.
- Use unique Danish registers on births and abortions to evaluate a possible effect of the poor male fecundity on pregnancy rates among their presumed partners – the younger cohorts of women.
- Test the hypothesis that the discrepancy in the age related decline in serum testosterone between cross-sectional and longitudinal studies might be due to a population-level decline in male serum testosterone over the last decades.

Materials and Methods

Semen quality studies and hormone analyses

For expanding the data on male reproductive health in relation to exposure and lifestyle, a number of 1004 men at the age of 18-20 from the former West (Hamburg, 504 men) and East (Leipzig, 500 men) Germany were chosen from the compulsory medical examination for military eligibility. If both men and their mothers were born in the area where the men were currently residing, they were included in the final analysis (Hamburg, 334 men and Leipzig, 457 men). All participants were instructed to abstain from ejaculation for at least 48 hours prior to clinic attendance whereupon a physical exam and blood and semen samples were obtained. Prior to attendance, a questionnaire was completed including information on age and previous or current diseases, including any known history of fertility. From Finland, 18-20 year old men (n=150) were studied after invitation by letter as a reiteration of a study performed 3 years ago. Also the military conscripts born in 1982 and 1983 were invited to participate in the follow up study. 185 men (born 1982) were first examined in 2001 and 116 men re-examined in 2003. 195 men (born 1983) were first examined in 2002-2003 and the second round of re-examinations was completed 2004-2005. Participation rate in the second examinations was 57%. For the 5 year follow up, 100 men (born 1979-1981) and 51 men for the 2-year follow up (born 1983) of previously recruited cohorts were re-examined. By including some of men previously analysed (born between 1979-1981 and in 1983), secular changes in Finnish male reproductive health could be evaluated.

Clinical examination and semen analysis were standardised according to a rigid protocol established in previous European studies on male reproductive health. Laboratories participated in an international quality control program and morphological analysis of semen samples and hormone levels were analysed in centralised laboratories (Jørgensen *et al.,* 1997; Menkveld *et al.,* 1990). Serum levels of follicle stimulation hormone (FSH), LH and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay. Testosterone and E2 levels were determined using time-

resolved flouroimmunoassay and inhibin-B by a specific two-sided enzyme immunometric assay.

Correction for confounding factors

Numerous confounding factors (e.g. age, year of birth, duration of abstinence) affecting semen and hormone parameters, the effect of duration from ejaculation to assessment of motility for motility parameters and hour of blood sampling for effect on sex hormone levels were all tested in a multiple linear regressions analysis, taking possible interactions into account. To correct for the skewed distribution, semen volume, sperm concentration, total sperm counts and the hormone results were natural logarithmic transformed before analysis. Motility was logit-transformed before analysis. The quality control results were used to adjust sperm concentration and total sperm count to achieve results comparable with those previously reported from the Nordic-Baltic area. Data evaluation between group differences in self-reported previous diseases were statistically analysed by a Pearson Chi-Square test, and differences in age, years of school attendance, height, weight, BMI and testis sizes were tested by Mann-Whitneys tests. Data was entered into an existing SPSS database on male reproductive health, which was restructured to facilitate incorporation of exposure data.

Studies of conception rates

In order to evaluate trends in the number of pregnancies obtained without assisted reproduction (ART) rather than the number of births, the "total natural conception rate" (TNCR) was studied, defined here as:

Total natural conception rate = total fertility rate + total induced abortion rate – ART conception rate

where ART represents assisted reproductive techniques, which included IVF and ICSI (and excluded intrauterine inseminations due to incomplete data).

In Denmark, all citizens are registered with a unique personal identification number (CPR-number) from the Danish Civil Registration System. In order to estimate the TNCR we used the following five registers and data sources: The Medical Danish Birth Registry, The Registry of Legally Induced Abortions, National Patient Registry, The Danish IVF Registry and Statistics Denmark. For each entry in each of these registries, the CPR-number is recorded which allows an identification of all registered persons and thus also linking of data from different registries. From The Medical Birth Registry and The Registry of Legally Induced Abortions information about all births (live and stillbirths) and legally induced abortions with CPR number of mothers from 1973 to 1994 was obtained. From 1995, the National Patient Registry took over the registration from both registries and information was obtained through this registry. We obtained permission to link and store these data from the Danish Data Protection Agency.

Studies of birth cohort effects in hormone levels

In order to test the hypothesis that the discrepancy in the age related decline in serum testosterone between cross-sectional and longitudinal studies might be due to a population-level decline in male serum testosterone, serum samples were analysed from large population-based surveys conducted in Denmark and Finland. In total serum samples from 5350 Danish and 3270 Finnish men, who participated in large population

studies conducted in Denmark between 1983-1999 and in Finland between 1972-2002, were analysed for the concentration of testosterone. In addition samples were also analysed for LH and SHBG. LH secreted from the pituitary stimulates the production of testosterone in the LC. Testosterone on the other hand exerts a negative feed back regulation on the secretion of LH. Thus the ratio between testosterone and LH provides additional information about the function of the stimulated LC. In the circulation the majority of testosterone is bound to sex hormone binding globulin and only the few percent free testosterone are considered to be biological active. Thus strong feed back mechanisms operate to sustain a balance between testosterone, LH and free testosterone, and any change in one of the hormones and binding proteins will affect the others. The concentration of free testosterone were calculated using the method by Vermeulen *et al.,* (1999) and the T/LH ratio were calculated as testosterone (nmol/L)/LH (IU/L). A general linear model was used to investigate the effect of age and period of birth (cohort effect) on the individual hormones and calculated ratios.

Results

Comparing male reproductive health in the former West and East Germany

The former West and East Germany gives the unique situation of substantially different environmental exposure over the last 4-5 decades of genetically similar populations from which the state of male reproductive health can be assessed. In total 1,004 men were initially included into the study. However, 213 were excluded because either they or their mothers did not meet the eligibility criteria for place of birth. Thus, results of examination of 791 men are reported here; 334 from Hamburg, and 457 from Leipzig. The study period in Hamburg was February 2003 - July 2004, and in Leipzig July 2003 - April 2005.

Multivariate regression analyses were carried out to compare the two German groups of men and estimate the general level of each group for semen and hormone levels. In the final model duration of ejaculation abstinence was included as confounder for semen volume, sperm concentration and total sperm count, recent fever for all semen parameters and SHBG level, and hour of blood sampling in the analysis of hormone levels. The final models were subjected to standard checks of the residuals. Possible confounding factors affecting semen and hormone parameters were tested. The age of the man, his year of birth, season of year, recent fever and duration of abstinence were tested for confounding effects on semen and hormone parameters. Additionally, the effect of duration from ejaculation to assessment of motility was tested for effect on motility parameters and hour of day of blood sampling for effect on sex hormone levels. The effects of the possible confounders and effects of possible self-reported diseases and findings at physical examinations were tested in a multiple linear regressions analysis, and also taking possible interactions into account.

No significant differences were observed for sperm concentration and total sperm count, whereas the differences for semen volume, sperm motility and morphology were significantly different between men from the two areas; higher frequency of morphologically normal forms and higher semen volume in the Hamburg group and higher frequency of motile spermatozoa in the Leipzig group (Table 3).

Table 3 Semen parameters of young German men from Hamburg and Leipzig

	Observed		Adjusted	Difference between groups
	Mean (SD)	Median (5-95)	Median (95%CI)	
Semen volume (ml)				
Hamburg	3.4 (1.8)	3.2 (1.0-6.4)	3.4 (3.1-3.6)	p<0.0005
Leipzig	2.7 (1.5)	2.6 (0.8-5.4)	2.8 (2.6-3.0)	
Motile sperm (%)				
Hamburg	66 (12)	68 (4.-80)	67 (65-69)	p<0.0005
Leipzig	77 (19)	82 (36-95)	81 (80-81)	
Sperm concentration (mio/mL)				
Hamburg	63 (49)	49 (3-186)	46 (39-54)	p=0.4
Leipzig	65 (57)	45 (6-174)	42 (36-50)	
Total sperm count (mio)				
Hamburg	206 (231)	142 (10-609)	154 (129-184)	p=0.3
Leipzig	166 (166)	113 (10-476)	141 (118-168)	
Morphologically normal forms (%)				
Hamburg	9.3 (5.4)	8.5 (1.5-19.2)	9.4 (8.9-10.0)	p=0.005
Leipzig	8.3 (4.8)	8.0 (1.5-17.4)	8.4 (7.9-8.9)	

Observed: Results based on raw data. **SD:** Standard deviation. **5-95:** 5^{th}-95^{th} percentile.
Adjusted: Median and 95% CI (confidence interval) calculated for the individual groups by linear regression analysis. Sperm concentration and total sperm counts are adjusted to the reference laboratory level, and these variables and semen volume adjusted to a period of ejaculation abstinence of 96 hours. Additionally, recent fever is accounted for. See text for further explanation.

For the hormones the inhibin-B level was significantly higher for the men from Leipzig and E2 and LH higher for men from Hamburg, whereas there was no difference in the Testosterone level (Table 4). Increasing duration of ejaculation abstinence had an increasing effect on semen volume, sperm concentration and total sperm count (all p=0.001) up to 96 hours where after no further effect of increasing abstinence could be detected. Thus, the presented values for these semen parameters are adjusted to an abstinence period of 96 hours. Recent fever had a reducing effect on all semen parameters except semen volume, however, with varying significance; p=0.05 for motility, p=0.06 for sperm concentration, p=0.10 for total sperm count and p=0.02 for frequency of morphologically normal forms. Increasing hour of day had a decreasing effect on inhibin-B (p=0.001) and testosterone (p=0.04) and an increasing effect on FSH (p=0.01). The presented values for the adjusted hormone levels are thus corrected to a blood sampling time as 10 am. No statistically significant effect of hour of the day could be detected for LH, E2 or SHBG. Additionally, recent fever had a reducing effect on the level of SHGB (p=0.004).

This study shows that the semen quality of young men from the German general populations from Leipzig and Hamburg is at a low level, that there is no biologically relevant difference in semen parameters between the two groups and that their sperm counts are at the same level as young men from Denmark and Norway. The average German man did not reach the full fertile range as defined by Guzick *et al.*, 2001 (sperm concentration above 48.0 mio/mL, more than 12 % morphologically normal spermatozoa,

and more than 63 % motile spermatozoa) (Guzick *et al.*, 2001) or thresholds measured in fertile men (Slama *et al.*, 2002; Jensen *et al.*, 2001).

Table 4 Sex hormone levels of young German men from Hamburg and Leipzig

	Observed		Adjusted	Difference between groups
	Mean (SD)	Median (5-95)	Median (95% CI)	
FSH (U/L)				
Hamburg	3.4 (2.1)	2.8 (1.1-6.9)	2.6 (2.4-2.8)	p=0.4
Leipzig	3.2 (1.8)	2.8 (1.1-6.6)	2.7 (2.6-2.9)	
LH (U/L)				
Hamburg	4.2 (1.8)	4.0 (2.0-7.4)	3.9 (3.7-4.1)	p=0.001
Leipzig	3.9 (1.6)	3.6 (1.7-6.5)	3.5 (3.4-3.7)	
Testosterone (nmol/mL)				
Hamburg	23.2 (9.3)	22.0 (11.9-38.1)	22 (21-23)	p=0.5
Leipzig	23.6 (10.5)	22.4 (12.5-37.5)	23 (22-24)	
Estradiol				
Hamburg	87.8 (49.7)	82.0 (54.7-121.0)	83 (81-86)	p<0.0005
Leipzig	79.8 (22.6)	77.0 (52.0-115.2)	77 (75-79)	
SHBG (nmol/L)				
Hamburg	29.5 (11.5)	28.0 (12.0-51.0)	28 (26-29)	p=0.5
Leipzig	30.2 (11.7)	28.0 (13.9-53.0)	28 (27-29)	
Inhibin-B (pg/mL)				
Hamburg	184 (70)	178 (75-319)	176 (167-186)	p<0.0005
Leipzig	209 (73)	203 (98-334)	203 (195-213)	

Observed: Results based on raw data. **SD:** Standard deviation. **5-95:** 5^{th}-95^{th} percentile.
Adjusted: Median and 95% CI (confidence interval) calculated for the individual groups by linear regression analysis taking hour of blood sampling as confounder into consideration. SHBG additionally adjusted for recent fever.

Regional differences in male reproductive health and association with maternal exposure

Between 1996 and 1999, semen quality of young men from five European countries (889 from Denmark, 313 from Finland, 190 from Estonia, 157 from Lithuania, 221 from Norway) who were undergoing compulsory medical examination for possible military service were studied. Each man provided a semen sample, was examined by a physician, and, in collaboration with his mother, completed a questionnaire about general and reproductive health, current smoking habits, and exposure to smoking *in utero*. After adjustment for confounding factors, men exposed to smoking *in utero* had a reduction in sperm concentration of 20.1 % and a reduction in total sperm count of 24.5 % in comparison with unexposed men. Exposed men had a 1.15-ml (95 % CI: 0.66, 1.64) smaller testis size than unexposed men. The associations were present also when data from the study centres were analysed separately except for Lithuania, where only 1 % of mothers smoked during pregnancy. These findings strongly supported the hypothesis that foetal development is particularly vulnerable and important for adult reproductive health. Thus, maternal smoking may have long-term implications for the reproductive health of

the offspring and this further justifies the advice for pregnant women to avoid smoking (Jensen *et al.,* 2004b).

Secular trend in Finnish male reproductive health

Semen quality of the Finnish men born at 1983 and examined during 2002-2003 showed an adverse trend when compared to previous cohorts studied in 1998-1999 (Jørgensen *et al.,* in preparation). The median sperm concentration was only 47 million/mL that is similar to Danish figures while in the previous years the Finnish sperm concentrations were clearly higher (60) than Danish (47). The Finnish trend mirrors the increasing incidence of testicular cancer in the same age group of men, whereas in Denmark the increase in testicular cancer incidence appears to have levelled off and semen quality has remained unchanged over the last ten years. Thus, the difference in semen quality between Danes and Finns appears to vanish, and both share a serious problem in male reproductive health. Considering all cohorts of young men (n=4236) studied during the last ten years, semen quality in Finland (Turku) is still better than in Denmark (Copenhagen): median sperm concentration 46 million per ml in Copenhagen vs. 53 in Turku, total sperm counts 156 vs. 181 millions, respectively, and normal morphology (%) 6.5 vs. 8.8, respectively. However, the recent decline in Finnish semen quality gives a new warning signal that reproductive health can be endangered in all parts of Europe. Analysis of the background data revealed that exposure to maternal smoking during pregnancy was associated with poor semen quality corroborating our previous findings and indicating foetal development as a vulnerable period. The data of the three longitudinal examinations of the men born in 1979-1981 showed unchanged semen quality indicating that one can already at the age of 18 or 19 years have a good estimate of lifetime semen quality. These figures are lower than those of fertile Finnish men and those published from other Finnish study groups previously, suggesting a clear decline.

Declining trends in conception rates in recent birth cohorts of native Danish women

We have analysed data from the Danish birth and abortion registries as well as the Danish registry for ART and defined a TNCR = fertility rate plus induced abortion rate minus ART conception rate. A unique personal identification number allowed the linkage of these databases. The database included 706,270 native Danish women born 1960 to 1980. We used projections to estimate the fertility of the later cohorts of women who had not yet finished their reproduction. We found that younger cohorts had progressively lower TNCR and that in terms of their total fertility rate the declining TNCR is compensated by an increasing use of ART.

Assessing the validity of measured hormone levels and the influence of storage

The validity of the samples on measured hormone levels was explored in three different ways. Firstly, a different aliquot of 434 of the samples had been analysed in 1994 and by the same laboratory, which allowed a direct comparison of the obtained hormone levels. For FSH, LH and SHBG the same assay formats were used in both measurements whilst testosterone and E2 used different assay formats. With an average ratio between the two measurements close to 1, there was no change in FSH, SHBG and testosterone after 10 year period of storage (Table 5). The 12 % difference in LH levels was likely due to a long-term inter-assay variation, especially as the highest levels were measured in the aliquots that had been stored the longest. The mean ratio obtained for E2 is attributed to the different assay formats used.

Table 5 Correlation coefficient and mean ratio between the measurements analysed at the time of sampling in 1994 and after 10 years storage

Hormone	Correlation coefficient	Mean ratios
Follicle stimulation hormone (FSH)	0.991	1.04
Luteinising hormone (LH)	0.977	0.88
Sex hormone-binding globulin (SHBG)	0.980	1.04
Testosterone	0.830	0.97
Estradiol	0.680	1.18

Secondly, several of the reproductive hormones are interrelated and therefore certain biologically determined correlations can be expected. The correlations between the five reproductive hormones for each sampling period were separately tested, as hormone degradation due to storage will be independent. The expected correlations ($p<0.01$) between the five reproductive hormones were consistently found in all four studies.

Birth cohort effects for serum testosterone levels in individual regions of Europe

Evaluation of the cross-sectional data indicates that, in addition to the well known age effect of falling testosterone levels with increasing age, an age-independent population-level decline in testosterone levels seems evident with higher testosterone levels in the oldest cohort. A significant cohort effect was also observed for LH and SHBG with more recently born men having lower levels of all three hormones. Thus, Danish men born in the 1930ies had on average 19 % higher T, 28% higher LH, and 41 % higher SHBG levels than Danish men born between 1949 and 1954. Similar results were found for the Finnish men. In contrast, no cohort effect was evident for free T and T/LH levels suggesting that the cohort related changes in testosterone and SHBG were interrelated. We thus speculate that the secular decline in testosterone serum levels could be secondary to the decline in SHBG levels; simply adjusting the pituitary-gonadostat to a lower level in order to sustain the same level of free testosterone. The Danish and Finnish data could not be directly compared, as there was no overlap in the timing of collection of samples in the Danish and Finnish studies (Figure 13).

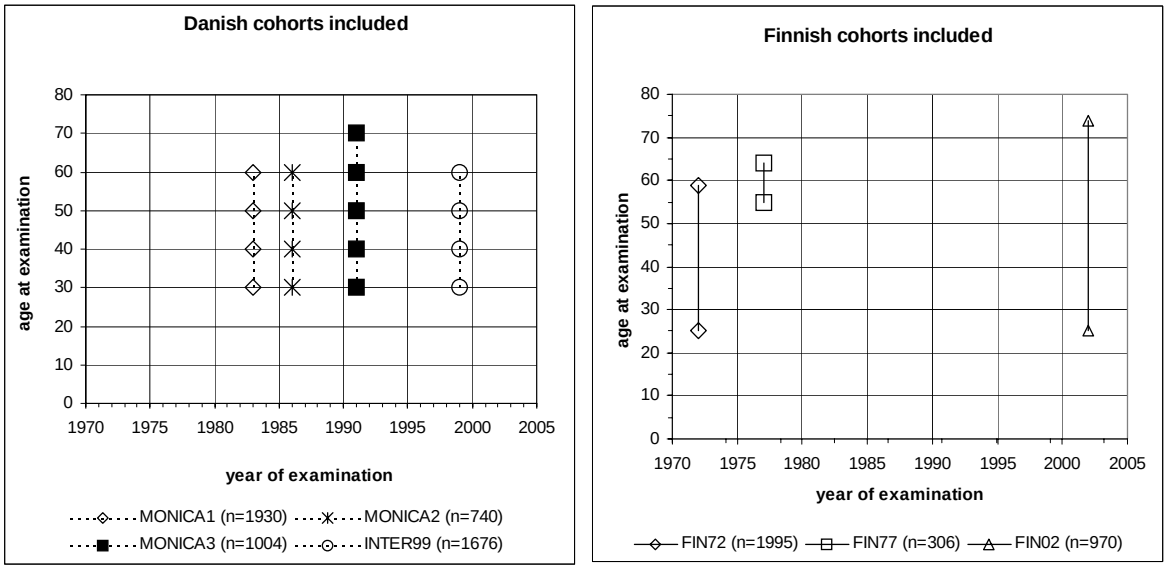

Figure 13 Number and distribution of Danish and Finnish samples

The most pronounced cohort effect observed was for SHBG. The regulation of SHBG is complex and is not only dependent on the serum levels of testosterone and estrogen; the level of SHBG is also regulated by insulin and is often decreased in obesity. However, although adjusting for a concurrent increase in body mass index with later year of birth reduced the observed changes in testosterone this adjustment had only a moderate effect on the observed cohort related changes in SHBG levels. The observed age-independent changes in SHBG and testosterone may be explained by an initial change in SHBG levels, which subsequently lead to adjustment of testosterone at a lower level in order to sustain free testosterone levels.

The aetiology behind the observed cohort effect remains to be resolved. The cohort effect was most pronounced for the oldest cohorts. Thus, SHBG levels in the men born between 1921-26>1931-36>1939-46>1949-56=1959-61=1969-70, indicating that whatever caused this cohort effect seems to have levelled out in the more recent cohorts. Large changes in lifestyle as well as in the environment occurred in Denmark during the 1900-century with a general significant increasing standard of life along with an increased industrialisation. A declining trend in male reproductive health manifested as an increase in testicular cancer and a declining sperm quality has been reported for the same period in many Western countries (Adami *et al.,* 1994; Andersen *et al.,* 2000; Carlsen *et al.,* 1995; Møller *et al.,* 1995; Swan *et al.,* 2000). These adverse trends in male reproductive health and the observed changes in male reproductive hormone levels presented here could be interrelated. The cohort related changes in SHBG and testosterone serum levels did not lead to a change in the level of free testosterone, which is believed to determine the androgen activity. Nevertheless, a general decreased testosterone production will lead to lower intra testicular testosterone levels and thus, the paracrine effects of testosterone within the testes may be blunted. This is in line with previous findings of decreased serum SHBG and testosterone levels as well as decreased sperm concentration in men with BMI> 25 kg/m^2 (Jensen *et al.,* 2004a).

Discussion

No significant difference in semen concentration or total sperm count was found between young men from former West and East Germany when the data were adjusted for abstinence time, fever and inter lab variation. The sperm quality of young men from the former West and East Germany are similar to that observed in the Danish population. Young men from five European countries who have been exposed to smoking *in utero* have a reduction in sperm concentration after adjustment for confounding factors. The strength of association varies with the European country. Deterioration in Finnish male reproductive health has been observed with testosterone decreasing and SHBG increasing with age within the birth cohort. The semen quality of young men from the Finnish general population is much worse than that of partners of pregnant women (fertile population) and the difference between Denmark and Finland appears to decline.

We found a progressively lower TNCR in the younger cohorts supporting our hypothesis of an ongoing birth cohort-related decline in fecundity. This hypothesis was also supported by our finding of increasing and substantial use of ART in the management of infertility of relatively young couples in the later cohorts. Furthermore, the lower rates of induced abortion among the younger birth cohorts, often viewed as a success of health education programs, may not be fully explained by improved use of contraception. It

seems more likely that decreased fecundity due to widespread poor semen quality among younger cohorts of otherwise normal men may explain some of the observed decline in conception rates.

Sample storage and its influence of hormone measurement validity is an important requirement prior to hormone analysis. In the Danish and Finnish samples, a non-linear drop in testosterone levels with age was observed as well as a cohort effect with early cohorts tending to have higher levels (at the same age). Results on testosterone levels are in accordance with the hypothesis that decreased male reproductive health, which seem to be more frequent and is believed in many cases to be related to the foetal/neonatal development of the testis, also is reflected in decreased testosterone levels. This is in line with recently published data showing also a population-level decline in male serum testosterone levels in American men (Travison *et al.,* 2007).

Conclusions

Danish and Finnish male reproductive health is in decline illustrating a secular trend. This also holds true for young men from the former West and East Germany, though a comparison between these two areas did not elicit significant differences. Foetal exposure to smoking has been highlighted as one reason for these observations in declining male reproductive health.

Our finding of a declining TNCR among the young Danish cohorts imply that the current poor semen quality have an impact on the population fertility in the future – a situation which is difficult to reverse in the short term. The current and projected widespread use of ART may be a sign of such an emerging public health problem which also add to the load of medical costs in young population. It is not surprising that Denmark and Finland with their public health supported ART practices have the highest rate of pregnancies achieved with either *in vitro* fertilisation or intracytoplasmic sperm injection (Nyboe Andersen 2006). It is of vital importance to continue surveillance of semen quality and all efforts should be made to identify the factors that may cause harm to be able to prevent further deterioration.

Literature Cited

Adami H-O, Bergström R, Möhner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Ziegler H, Rahu M, Gurevicius R, Stengrevics A (1994). Testicular cancer in nine Northern European countries. *Int. J. Cancer* **59**: 33-38

Andersen AG, Jensen TK, Carlsen E, Jørgensen N, Andersson A-M, Krarup T, Keiding N, Skakkebæk NE, (2000). High frequency of sub-optimal semen quality in an unselected population of young men. *Hum. Reprod.* **15**:366-372

Carlsen E, Giwercman A, Keiding N, Skakkebæk NE. (1992) Evidence for decreasing quality of semen during past 50 years. *Brit. Med. J.* **305**:609-613

Carlsen E, Giwercman A, Keiding N, Skakkebæk NE, (1995). Declining semen quality and increasing incidence of testicular cancer: Is there a common cause? *Environ. Health Perspect.* **103**:137-139

Deslypere JP, and Vermeulen A, (1984). Leydig cell function in normal men: effect of age, life-style, residence, diet, and activity. *J. Clinical Endocrinol. Metab*. **59**, 955-962

Drafta D, Schindler AE, Stroe E, and Neacsu E, (1982). Age-related changes of plasma steroids in normal adult males. *J. Steroid Biochem*. **17**, 683-687

Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviella AD, Bremner WJ, and McKinlay JB (2002). Age trends in the level of serum testosterone and other hormones in middle-aged man: longitudinal results from the Massachusetts Male Aging Study. *J. Clinical Endocrinol. Metab*. **87**, 589-598

Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL (2001). Sperm morphology, motility, and concentration in fertile and infertile men. *N. Engl. J Med*.**345**: 1388-1393

Gray A, Feldman HA, McKinlay JB, and Longcope C. (1991). Age, disease, and changing sex hormone levels in middle-aged men: results of the Massachusetts male aging study. *J. Clin. Endocrinol. Metab.* **73**, 1016-1025

Gyllenborg J, Rasmussen SL, Borch-Johnsen K, Heitmann BL, Skakkebæk NE, and Juul A (2001). Cardiovascular risk factors in men: The role of gonadal steroids and sex hormone-binding globulin. *Metabolism* **5**0, 882-888

Jensen TK, Andersson A-M, Jørgensen N, Andersen A-G, Carlsen E, Petersen JH, Skakkebæk NE, (2004a). Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil. Steril.* **82**:863-870

Jensen TK, Jørgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, Horte A, Andersen A-G, Carlsen E, Magnus Ø, Matulevicius V, Nermoen I, Vierula M, Keiding N, Toppari J, and Skakkebæk NE, (2004b). Association of *in utero* exposure to maternal smoking with reduced semen quality and testis size in adulthood: A cross-sectional study of 1,770 young men from the general population in five European countries. *Am. J. Epidemiol.,* **159**:49-58

Jensen TK, Slama R, Ducot B, Suominen J, Cawood EH, Andersen AG, Eustache F, Irvine S, Auger S, Jouannet P, Vierula M, Jørgensen N, Toppari J, Skakkebæk NE, Keiding N, Spira A. (2001) Regional differences in waiting time to pregnancy among fertile couples from four European cities. *Hum. Reprod*. **16**:2697-2704

Jørgensen N, Auger J, Giwercman A, Irvine DS, Jensen TK, Jouannet P, Keiding N, Le Bon C, MacDonald E, Pekuri A.-M, Scheike T, Simonsen M, Suominen J, and Skakkebæk NE, (1997). Semen analysis performed by different laboratory teams: an intervariation study. *Int. J. Androl*. **20**: 201-208

Jørgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen A-G, Andersson A-M, Haugen TB, Horte A, Jensen TK, Magnus O, Petersen JH, Vierula M, Toppari J, and Skakkebæk NE, (2002). East-West gradient in semen quality in the Nordic-Baltic area: a

study of men from the general population in Fenmark, Norwaym Estonia and Finland. Human Repro. **17** (8): 2199-2208

Jørgensen N, Asklund C, Carlsen E, and Skakkebæk NE (2006), Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. *Int. J. Andrology* **29**: 54–61

Jørgensen N, Askland C, Carlsen E, Holm M, Petersen JH, Jensen TK, Vierula M, Toppari J, and Skakkebæk NE, (2007). Surveillance of semen quality of Danish and Finnish populations: A recent downward trend from historically high semen quality of young Finnish men towards Danish level of poor semen quality. In Preparation

Menkveld R, Stander FS, Kotze TJ, Kruger TF, and Van Zyl JA, (1990). The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Human Reprod*. **5**:586-592

Møller H, Jørgensen N, Forman D, (1995). Trends in incidence of testicular cancer in boys and adolescent men. *Int. J. Cancer* **61**: 761-764

Morley JE, Kaiser FE, Perry HM, III, Patrick P, Morley PM, Stauber PM, Vellas B, Baumgartner RN, and Garry PJ (1997). Longitudinal changes in testosterone, luteinising hormone, and follicle-stimulating hormone in healthy older men. *Metabolism* **46**: 410-413

Nieschlag E, Lammers U, Freischem CW, Langer K, and Wickings EJ (1982). Reproductive functions in young fathers and grandfathers. *J. Clin. Endocrinol. Metab.* **55**: 676-681

Nyboe Andersen A, Erb K, (2006) Register data on assisted reproductive technology (ART) in Europe including a detailed description of ART in Denmark. *Int. J. Andrology*, **29**(1):12-16.

Punjab M, Zilaitiene B, Jørgensen N,Horte A, Matulevicius V, Peetsalu A and Skakkebæk NE, (2002). Regional differences in semen qualities in the Baltic region. *Int. J. Andrology* **25**: 243-252.

Richtoff J, Rylander L, Hagmar L, Malm, and Giwercman A, (2002). Higher sperm counts in Southern Sweden compared with Denmark. *Human Reprod*. **17** (9): 2468-2473

Simon D, Preziosi P, Barrett-Connor E, Roger M, Saint-Paul M, Nahoul K, and Papoz L (1992). The influence of aging on plasma sex hormones in men: the Telecom Study. *Am. J Epidemiol.* **135**: 783-791

Skakkebæk NE, Rajpert-De Meyts E, and Main KM, (2001). Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Human Reprod.* **16**: 972-978

Slama R, Eustache F, Ducot B, Jensen TK, Jørgensen N, Horte A, Irvine S, Suominen J, Andersen AG, Auger J, Vierula M, Toppari J, Andersen AN, Keiding N, Skakkebæk NE,

Spira A, Jouannet P. (2002) Time to pregnancy and semen parameters: a cross-sectional study among fertile couples from four European cities. *Hum. Reprod*.17:503-515

Svartberg J, Midtby M, Bonaa KH, Sundsfjord J, Joakimsen RM, and Jorde R (2003). The associations of age, lifestyle factors and chronic disease with testosterone in men: the Tromso Study. *Euro. J Endocrinol* **149**: 145-152

Swan SH, Elkin EP, Fenster L, (2000). The question of declining sperm density revisited:an analysis of 101 studies published 1934-1996. *Environ. Health Perspect.* **108**:961-966

Travison TG, Araujo AB, O'donnell AB, Kupelian V, McKinlay JB (2006) A population-level decline in serum testosterone levels in American men. *J. Clin. Endocrinol. Metab.* **92**:196-202

Vermeulen A, Verdonck L, Kaufman JM, (1999). A critical evaluation of simple methods for the estimation of free testosterone in serum. *J. Clin. Endocrinol. Metab.* **84**:3666-3672

THEME 4 - LOW-DOSE AND MIXTURE EFFECTS OF EDC

Overview and Objectives

The EDEN project was motivated by the need to address major knowledge gaps relating to the low-dose issue and the problem of assessing EDC mixture effects. There were the following specific aims:

Concerning EDC low-dose effects, EDEN aimed to

- Systematically compare low-dose estimates derived from two-point comparisons (NOEL) with those produced by regression-based approaches.
- Establish the shapes of dose-response curves in order to provide guidance for assessing the threshold issue.
- Develop criteria for well conducted EDC studies that permit better estimations of low-dose effects.

In relation to EDC mixture effects, the project aims were to investigate whether

- The joint effects of EDC on cells, rodents or fish can be predicted from knowledge of their individual potencies.
- Whether EDC produce observable mixture effects when combined at doses below their NOEL.
- How EDC with different modes of action work together.
- How knowledge about EDC mixture effects can be used productively in risk assessment.

Materials and Methods

A wide variety of assays, ranging from the cellular to the physiological level of biological complexity, was used to address the low-dose and mixtures issues:

Stimulation of cell proliferation in ER-competent cells

Induction of cell proliferation in estrogen-dependent tissues is considered to be the hallmark of estrogen action. Soto and Sonnenschein (Soto *et al.,* 1995) have developed an assay based on human MCF-7 breast cancer cells that enables the detection of estrogenic agents. The cells depend on estrogens for survival, and the potency of an estrogenic chemical can be determined in terms of the number of cells that have appeared after a defined period of treatment. Throughout EDEN, a protocol described previously (Rajapakse *et al.,* 2004; Soto *et al.,* 1995) was employed and adapted to a miniaturised format using 96-well microtiter plates (Silva *et al.,* 2007).

Extended developmental toxicity assay in the rat

Androgens are key regulators of male sexual differentiation during *in utero* and early postnatal development, both in humans and rodents, such as the rat. Exposure to chemicals that counteract androgen action at some stage in this period can permanently de-masculinise male foetuses and lead to malformations of the reproductive tract.

Reduced AGD, retention of nipples or areolas, hypospadias, agenesis of sex accessory tissues, and undescended testes in the male offspring have been described as consequences of disruption of androgen action in the developing rat. These effects are thought to arise through antagonism of androgens at the steroid receptor level and/or *via* suppression of testosterone synthesis in LC (Gray *et al.,* 2001).

To study the effects of antiandrogens and their mixtures in the rat, time mated nulliparous, young adult Wistar rats were dosed by gavage from early gestation through to the day before expected birth, and then from PND 1 until PND 16. The male offspring was examined for signs of disruption of male sexual differentiation by measuring changes in AGD, retained nipples and alterations in the weight of male accessory sex glands. Technical details concerning the quantitation of endpoints, dose-response analyses and assessment of mixture effects are described in Hass *et al.,* (2007) and Metzdorf *et al.,* (2007).

Vitellogenin induction in zebrafish and sticklebacks

In fish, synthesis of the egg yolk protein VTG is controlled by activation of the ER in the liver. Normally, male fish will not synthesise VTG, but it has been discovered that male fish exposed to estrogenic chemicals produce large quantities of the protein (Purdom *et al.,* 1994). This phenomenon has been exploited as a sensitive biomarker for exposure to estrogenic agents, and was used as an endpoint for EDEN low-dose and mixture studies with two fish species, the zebrafish, and the stickleback.

Fish were placed in tanks, and test chemicals delivered in continuous flow-through systems, as described by Brian *et al.,* (2005) and Katsiadaki *et al.,* (2006). VTG levels were determined by ELISA, and nominal concentrations of all tested chemicals were verified by chemical analysis.

Spiggin induction in the stickleback

Male stickleback synthesise large quantities of the glue protein spiggin during nesting. Spiggin induction is under the control of androgens. It is quite variable in male fish where it is influenced by light, social hierarchies and reproductive status. Female sticklebacks exposed to androgens also induce the protein, but the response is much more uniform. Antiandrogenic activities of test chemicals can be detected as a downturn in kidney spiggin levels in female fish co-exposed to a stable androgen (dihydrotestosterone) (Katsiadaki *et al.,* 2006).

Stickleback caught in European rivers were transferred to aerated aquaria and treated with test chemicals (flow-through systems) for 21 days. Spiggin analysis was performed by ELISA as described (Katsiadaki *et al.,* 2002). Nominal concentrations of test chemicals were analysed by solid phase extraction, followed by GC/MS for nonylphenol, vinclozolin and fenitrothion and LC/MS for flutamide and linuron.

Estimating low-dose effects: a methodological introduction

What is “low-dose”? – Definitions. The term “low-dose”, as used in the context of EDC is not precisely defined. It is variously used to mean “doses lower than used normally in toxicity testing”, “doses that approach, or are equal to, those encountered by humans” and “doses associated with low effects”. For the purposes of EDEN low-dose studies, the

latter definition was adopted. Low effect doses signify doses producing small effects. Regardless of their nominal value, the challenge is to measure low effects, sometimes with the aim of establishing doses at the threshold between "effect" and "no effect".

Approaches to estimating low effect doses. Two major approaches to estimating doses "without effect" can be distinguished: multiple comparison procedures and regression model-based approaches. Multiple comparison procedures aim to establish a no-observed effect level (NOEL) on the basis of statistical hypothesis testing. A NOEL is defined as the highest tested dose that does not induce effects significantly different from untreated controls. Multiple comparison procedures do not make meaningful statements about the statistical inference of the outcomes (e.g. confidence belts) and the NOEL can only be a dose or a concentration that was actually tested. Regression model-based approaches tackle the problem from a different angle. Instead of making statements about doses where no effects could be observed, regression modelling attempts to estimate confidence limits that indicate whether effects observed with a certain dose are different from untreated groups. These methods estimate a dose that corresponds to a predetermined response level that is deemed acceptable (benchmark dose). Both these approaches have inherent strengths and weaknesses.

Threshold concepts. NOEL are often wrongly equated with dose thresholds, toxicant doses that do not elicit any effects. It should be borne in mind, however, that NOEL are derived by hypothesis testing procedures. These examine whether the null hypothesis "controls and treated groups do not differ" can be rejected. A NOEL is the highest tested dose that did not produce a statistically significant effect. Only when the responses in the treated group exceed a certain limit (defined by significance criteria) can the hypothesis be rejected, and consequently, the tested dose is deemed as being larger than NOEL. However, this cannot be taken to mean that NOEL are devoid of effects. At and below NOEL, effects may either be truly absent or remain undetected, due to lack of statistical power. Therefore, rather than being a genuine reflection of zero effects, NOEL ("one of the most misunderstood notions in ecotoxicology"; Moore and Caux 1997) define a grey area, where it is impossible to distinguish whether effects are present or not.

Slob (1999) has clarified the issue by introducing three different threshold concepts:

- Seen from a *mathematical* point of view, a threshold is a dose associated with a zero response, and above which the response is larger than zero.
- In an experimental or *empirical* sense, a threshold can be a quantitative estimate of a dose below which no effects can be observed within a given experimental setup (this is similar to NOEL).
- The term can be taken to mean a *biological* threshold, in the sense of a dose below which the affected organism does not elicit any adverse effects.

These three concepts are depicted in Figure 14. Mathematical thresholds in the sense of true zero effect levels cannot be determined empirically, because the statistical detection limit of the chosen experimental setup will always be a barrier. The empirical threshold concept is problematic in terms of risk communication. Whether an effect is observable depends on the sensitivity of the experimental design. With expenditure on more resources, the sensitivity of experiments can be increased considerably, with a downward shift in terms of doses deemed to be without effect. The biological threshold concept

would need to define the effect size deemed to be adverse, but absolute criteria to resolve this question are not available for EDC relevant endpoints.

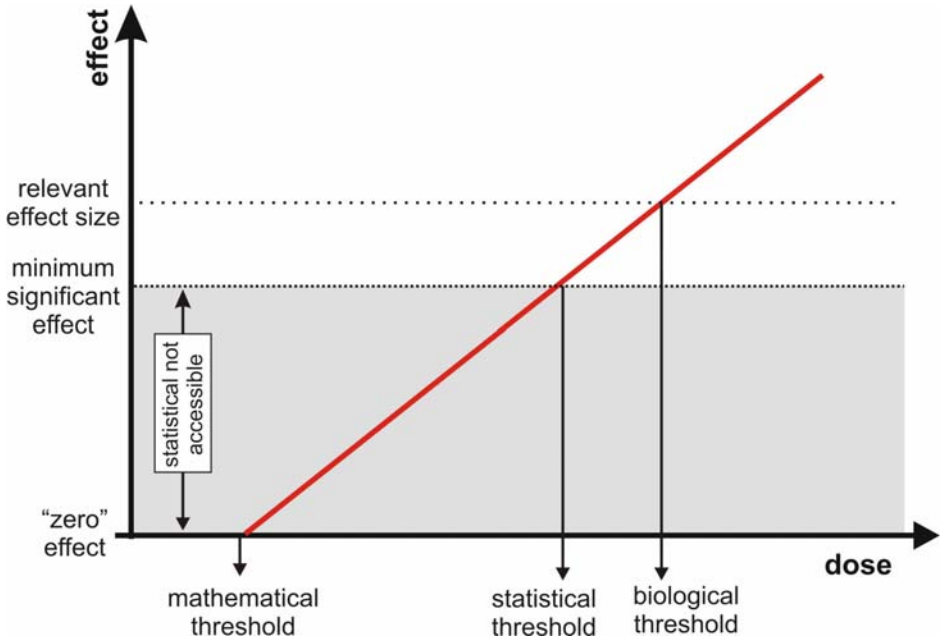

Figure 14 Three threshold concepts (adapted from Slob 1999). The shaded grey area defines the statistical detection limit of experiments used to derive estimates of doses "without effect". Effects within this area cannot be distinguished with reliability from those seen with untreated controls. The experimental design should be chosen such that the minimum significant effect level is below the relevant effect size.

Prediction and assessment of the effects of multi-component EDC mixtures

It is often thought that the combined additive effect of chemicals is the simple sum of their individual effects at specific doses. The fallacy of this assumption becomes clear by considering two chemicals combined at doses that each produce saturation effects. The expectation that the joint additive effect should then be the sum of the individual effect is unrealistic when the individual effects are near the biologically maximally possible response.

Thus, concepts better suited to dealing with chemicals that produce sigmoidal dose-response curves are needed in calculating sound additivity expectations. Two pharmacologically sound concepts for the calculation of expected additive effects have emerged and are widely used: *concentration addition* (CA) and *independent action* (IA). CA assumes that chemicals act in a similar fashion, IA posits that mixture components act on different sub-systems in organisms, even though they may produce the same observed effects. Often, but not always, the two concepts produce different additivity predictions.

Dealing with multi-component mixtures. Application of the two concepts to the assessment of multi-component mixtures requires that dose-response analyses of all individual mixture components are carried out to provide a basis for the calculation of expected additive effects for a mixture of specific composition (Fixed mixture ratio approach). The predicted mixture effects are then tested experimentally, and deviations

from expectation diagnosed as synergism or antagonism. EDEN has adopted this approach for all mixture studies.

Experimental requirements: quantifiable endpoints. Dose-response analyses for both individual mixture components and the mixtures themselves are therefore a prerequisite for sound assessments of mixture effects. Thus, the endpoints chosen for analyses have to be readily and reproducibly quantifiable. Especially for *in vivo* studies this excluded endpoints that relied e.g. on the histological scoring of tissue slides. Quantification of effects also had to be achievable in a fairly cost and time effective way. The endpoints chosen for EDEN mixture studies lend themselves to quantification. They were: Cell number as measured by cell staining and then back-extraction, (quantification by measurement of optical absorbance in plate readers); determination of protein levels (VTG, spiggin) in the fish studies, by using ELISA (quantification in plate readers); measurement of AGD, counting of retained nipples, determination of organ weights, and quantitative determination of gene expression levels in the rat studies.

Statistical and biometrical analyses. Statistical dose-response regression analyses were carried out by applying a best-fit approach (Scholze *et al.*, 2001). Various non-linear regression models (logit, probit weibull, generalised logit I and II), which all describe monotonic sigmoidal dose-response relationships, were fitted independently to the same data set and the best fitting model was selected on the basis of a statistical goodness-of-fit criterion. As much as possible, data analysis was performed on pooled data from all the repeat studies. To account for the intra- and inter-study variability associated with this nested data scenario, the generalised non-linear mixed modelling approach was used, in which both fixed and random effects are permitted to have a non-linear relationship with the effect endpoint (Vonesh and Chinchilli 1996). NOEC and LOEC values were derived by testing a trend in dose or concentration effects against controls by using nonparametric multiple contrast tests (Neuhaeuser *et al.*, 2000).

Results

The assays, chemicals and endpoints employed for low-dose studies in the EDEN project are listed in Table 6.

Table 6 EDEN low-dose mixtures

Assay	Partner	Chemicals	Endpoint
E-Screen	1	E2, β-HCH	cell proliferation
Developmental toxicity in the rat	20	DINP, DEHP	Changes in anogenital distance, nipple retention
Zebrafish	13, 21	E2, E1, nonylphenol	Vitellogenin induction
Stickleback	2, 8, 19	E1, nonylphenol	Vitellogenin induction
Stickleback	2, 8, 19	Fenitrothion, flutamide	Spiggin inhibition

Non-monotonic dose-response curves were not observed

None of the chemicals investigated in the endocrine disruption assays chosen for low-dose studies exhibited dose-response curves with non-monotonic curvatures (inverted-U)

in the low-dose range. In some instances, a down-turn of responses from doses associated with a plateau was observed which could be attributed to cytotoxicity (e.g. in the E-Screen assay). Concerns relating to the danger of overlooking effects at low-doses could not be confirmed.

Shallow gradients in the low-dose range were common

Instead, detailed low-dose response analyses revealed that many of the tested agents exhibited quite shallow curves in the low effect range, and this resulted in low-dose estimates with often surprisingly small numerical values. Figure 15 shows an example with E2 and the chlorinated hydrocarbon β-HCH in the E-Screen (Silva *et al.*, 2007).

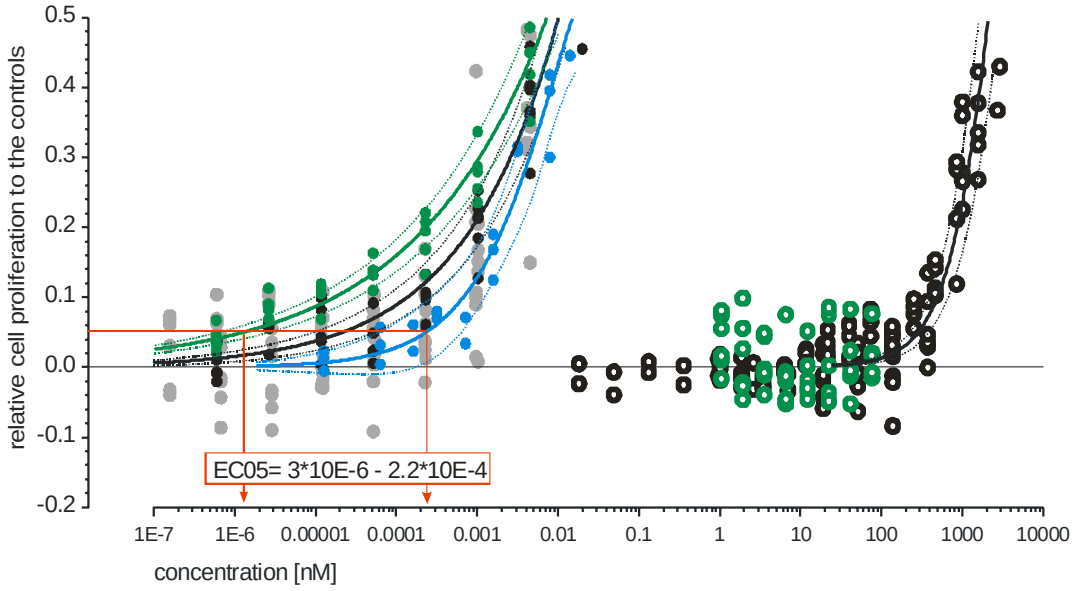

Figure 15 Concentration-response data and curves for E2 (closed circles, left plot) and β-HCH (open circles, right plot) in the E-Screen. For E2, the colours present data from different independent studies. The best-fitting regression models are shown as lines with the corresponding 95% confidence belt for the mean effect as dotted lines, with colours of lines corresponding to colours of data. The range of estimates for a concentration producing a 5% effect (EC05) is shown. For β-HCH (right), regression analysis (black open circles) yielded an estimate of 40 nM for a concentration producing a 1% effect. This was re-tested and confirmed directly with finely spaced concentrations between 1 and 100 nM (green open circles) (Silva *et al.*, 2007).

Dose-response curves with small gradients give rise to complications during the estimation of low effect doses. High statistical power is necessary to arrive at valid estimates, and in this sense, the E-Screen serves as an illustrative example for the resources that are needed for the demonstration of effects with small magnitudes. Through using large sample sizes and numerous repeats, the experimental power of the E-Screen was sufficiently high to measure effect magnitudes of around 1-2 % with reliability. However, such resources are usually not available for *in vivo* testing, with the consequence that the statistical detection limit is often considerably higher. If this coincides with shallow dose-response curves in the low effect range, the testing with only a few dose groups might fail to identify effects of a small size.

This dilemma is highlighted in Figure 16 with an example from an *in vivo* endpoint, suppression of spiggin induction by fenitrothion in female sticklebacks co-exposed to dihydrotestosterone. With the number of fish that could practically be used, and with the biological variation of the response, the statistical detection limit of the experiments did not allow reliable demonstrations of effects smaller than ca. 6% of the maximally inducible effect. Similar statistical detection limits were obtained with the other *in vivo* endpoints used for EDEN low-dose studies. This aspect of the EDC "low-dose" issue has not been appreciated sufficiently in the past.

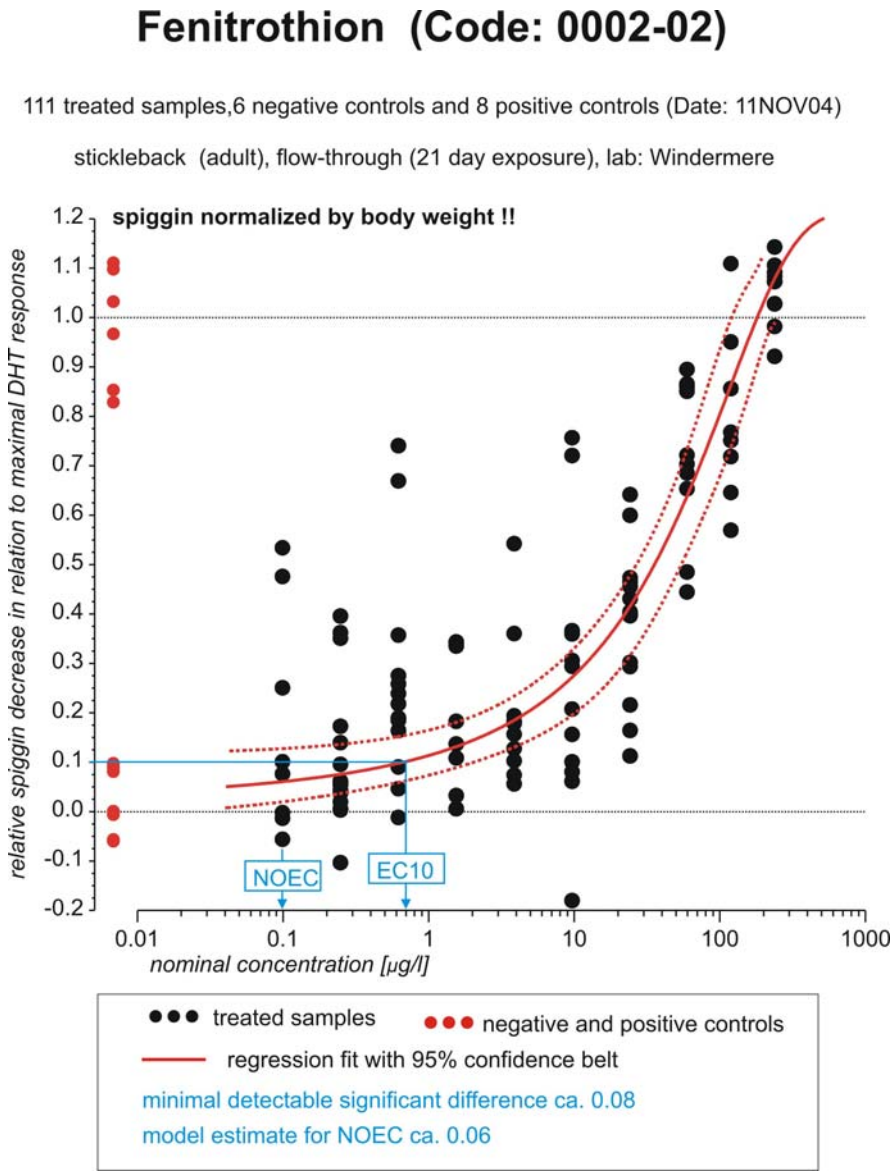

Figure 16 Reduction of maximally induced spiggin synthesis in the stickleback by fenitrothion. The shaded grey area highlights the statistical detection limit achievable within the experimental parameters. Effects of a magnitude of ca 6% cannot be distinguished reliably from control responses.

Comparison of low-dose estimates derived by hypothesis testing and regression-based approaches

The optimisation of the E-Screen as a high through-put assay offered a model system in which various low-dose estimates could be compared directly by using large numbers of repeat studies (Figure 15). In extensive studies with 23 xenoestrogens (Silva *et al.,* 2007) NOEL equated to effects of 1% in the E-Screen.

Due to the lower statistical power achievable with the *in vivo* tests, NOEL were associated with larger effects of around 10% of a maximally inducible effect. For example, hypothesis testing methods yielded a NOEL of 0.1 µg/L for antiandrogenic effects of fenitrothion in the stickleback spiggin assay. However, regression analysis estimated that this concentration is likely to be associated with an effect of 6% (Figure 16). Similar results, with often higher estimated responses corresponding to NOEL, were found with the other *in vivo* assays used in EDEN low-dose studies. These data showed very clearly, that NOEL cannot be equated with zero effect levels.

Criteria for well conducted low-dose studies

For each assay, detailed recommendations for low-dose studies were made, in terms of number of positive and negative controls, spacing and number of dose groups for regression analyses, and dose group sizes for studies based on hypothesis testing. Because a wide variety of different dose-response curves occurred, no uniform dose-response regression model could be found that was suitable for EDC in all cases. This means that the best fitting model should be chosen from a pool of regression models (Scholze *et al.,* 2001).

Combining both approaches

With the recognition that doses associated with zero effects cannot be determined empirically, the aim of low-dose EDC testing can only be to derive estimates of doses that correspond to a specific effect magnitude. Thus, the starting point of low-dose testing strategies should be a decision about the effect size a low-dose experiment should be able to demonstrate.

Only after such a choice has been made, can the strengths of hypothesis testing procedures (the ability to test certain doses with a large number of replicates) and those of regression-based approaches (the ability to assess effect trends) be exploited productively for low-dose testing. Key elements of an approach combining the strengths of hypothesis testing and regression methods were outlined (Scholze and Kortenkamp 2007). The proposed integrated procedure aims to (1) identify the minimum effective dose that is statistically significant and produces an effect that it is at least of the relevant effect size and (2) if reliable, to estimate the corresponding dose for this effect size (benchmark). Firstly, a power analysis is performed with the aim of assessing whether the suggested experimental design is of sufficient sensitivity to demonstrate effect sizes of relevance. This can be achieved by comparing the statistical detection limit that is achievable with the chosen experimental design with the magnitude of the effect of relevance.

The effects of multi-component EDC mixtures were predictable by using concentration addition

A large number of mixture studies were conducted as part of the EDEN project. The principal aim of these studies was to assess whether the joint effects of mixtures of EDC can be predicted accurately over a large effect range on the basis of dose-response data of the individual components. If there are demonstrable regularities between the potency of individual chemicals and the ways in which they act together, powerful tools for prospective risk assessment could become available. These tools could open the way to make productive use of existing single chemical data bases for the prediction of mixture effects. Table 7 gives an overview of the EDEN mixture experiments, the endpoints used and the chosen mixture components.

Table 7 EDEN mixture studies

Assay	Partner	Mixtures	Number of tested mixtures	Endpoint
E-Screen	1	Up to 22 estrogenic chemicals, combined with PCBs	10	cell proliferation
Developmental toxicity in the rat	20	Vinclozolin, flutamide, procymidone; DEHP, finasteride, vinclozolin, prochloraz	2	Changes in anogenital distance, nipple retention, reproductive organ weights
Zebrafish	13, 21	E2, E1, EE2, nonylphenol	1	Vitellogenin induction
Stickleback	2, 8, 19	E2, E1, EE2, nonylphenol	1	Vitellogenin induction
Stickleback	2, 8, 19	Fenitrothion, flutamide, linurone, vinclozolin, E2, E1, EE2, nonylphenol	6	Suppression of Spiggin induction Or spiggin inhibition

Xenoestrogens in the E-Screen

Development of a miniaturised format of the E-Screen (Silva *et al.*, 2007) allowed high throughput screening and this made it possible to conduct by far the largest mixture experiments of the EDEN project. The 22 chemicals listed in Table 8 were combined at a mixture ratio proportional to the individual potency of each agent.

Table 8 Chemicals included in E-Screen mixture studies

Groups	Compounds	Groups	Compounds
Endogenous hormones		**Plasticisers**	
	E2		Bisphenol A
	E1	**Preservatives**	
Phytoestrogens			Butylparaben
	Genistein		Propylparaben
Pesticides		**UV filters**	
	o,p'-DDT		3-BC (Unisol S-22)
	p,p'-DDT		4-MBC (Eusolex 6300)
	o,p'-DDD		OMC (Eusolex 22920)
	p,p'-DDE	**Musks**	
	Endosulfan I		AHTN (Tonalide)
	Endosulfan II		HHCB (Galaxolide)
	Methoxychlor		
	Dieldrin		
	Aldrin		
	β-HCH		

On the basis of best-fit regression models (Figure 17) for all chemicals, an expected dose-response curve was calculated by using CA, and this prediction was tested experimentally.

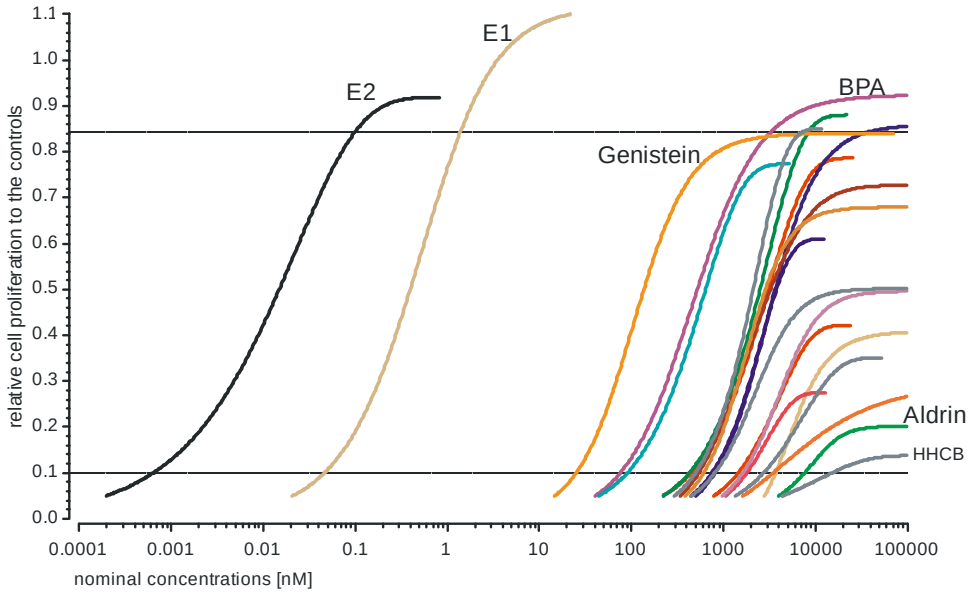

Figure 17 Concentration-response curves for 22 xenoestrogens and steroidal estrogens tested in the E-Screen assay. This information was used to calculate additivity expectations (see Figure 18).

The data shown in Figure 18 show that prediction and observation agreed very well demonstrating that the joint effect of the 22 estrogenic agents was (concentration) additive. When tested in combination with xenoestrogen mixtures dioxin-like PCBs suppressed cell proliferation in the E-Screen.

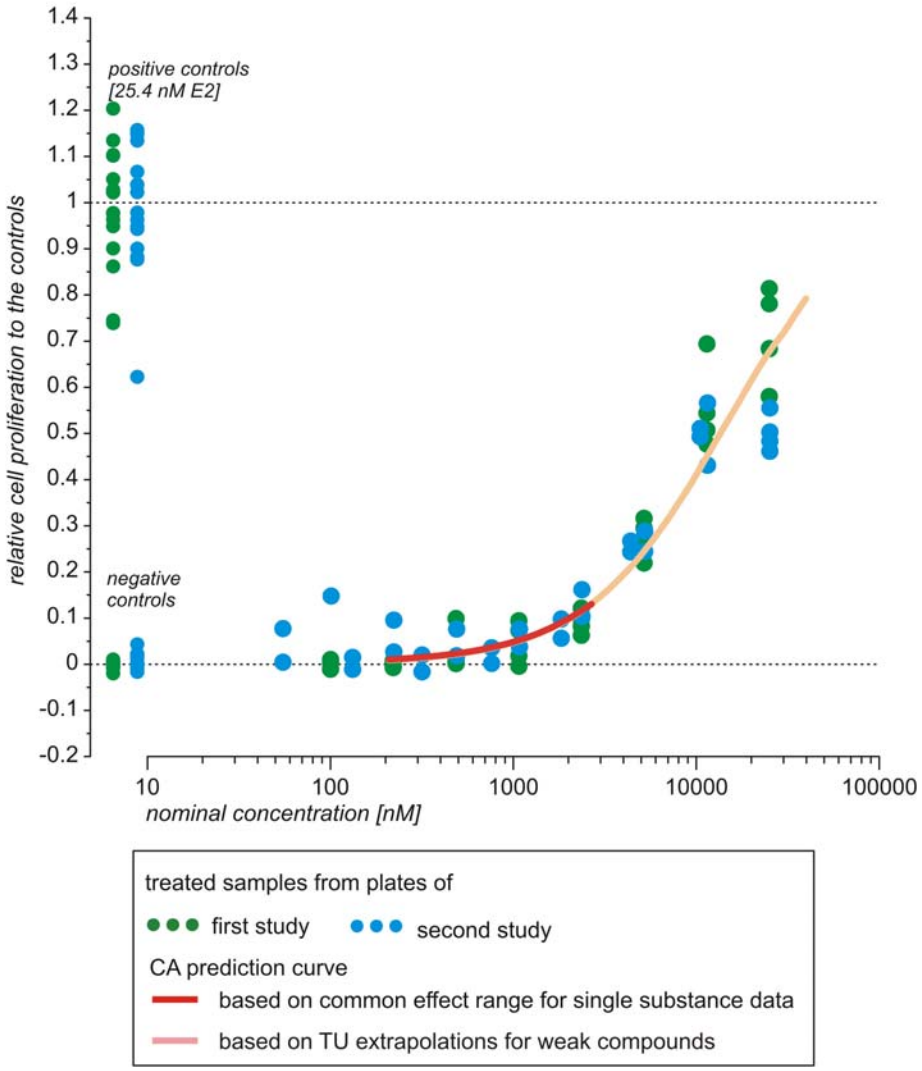

Figure 18 Predicted and observed effects of a mixture of 21 xenoestrogens in the E-Screen assay.

Antiandrogens in an extended rat developmental toxicity model.

The ability of a mixture of three androgen receptor antagonists to induce disruption of male sexual differentiation after *in utero* and postnatal exposure was investigated. Vinclozolin, flutamide and procymidone were administered by gavage to time mated nulliparous, young adult Wistar rats from gestational day 7 to the day before expected birth, and from PND 1 to 16. Changes in AGD, nipple retention (NR), and organ weights of accessory sexual glands in male offspring rats were chosen as endpoints for extensive dose-response studies with the individual mixture components (Figure 19).

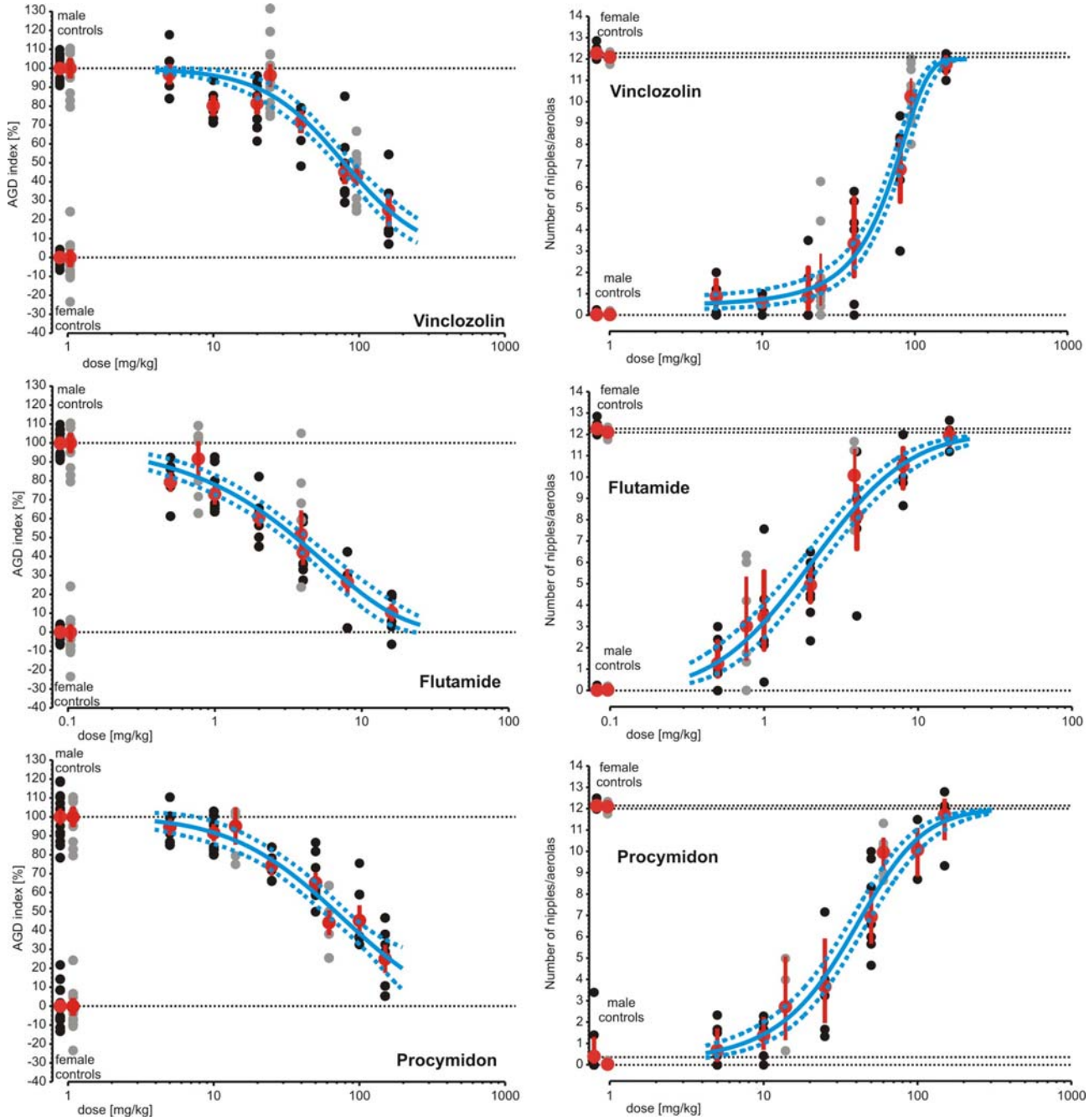

Figure 19 Effects of vinclozolin, flutamide and procymidone, given individually, on AGD (left) and NR (right). Results shown are group mean ± standard error (red), litter means from the dose-response studies (black), litter means from the single doses within mixture study (grey) and the mean dose-response curve ± 95% confidence belt based on regression analysis (blue) (Hass *et al.*, 2007).

Vinclozolin, flutamide and procymidone were combined at a mixture ratio proportional to their individual potencies for causing retention of 6 nipples in male offspring. With AGD as the endpoint, the joint of effects of the three antiandrogens were essentially dose-additive (Figure 20). However, the observed responses for NR were slightly higher than those expected on the basis of dose addition. A detailed account of these studies has been

published by Hass *et al.,* (2007). Joint effects were essentially dose-additive with changes in the organ weights of accessory sexual glands as the endpoint (Metzdorf *et al.,* 2007).

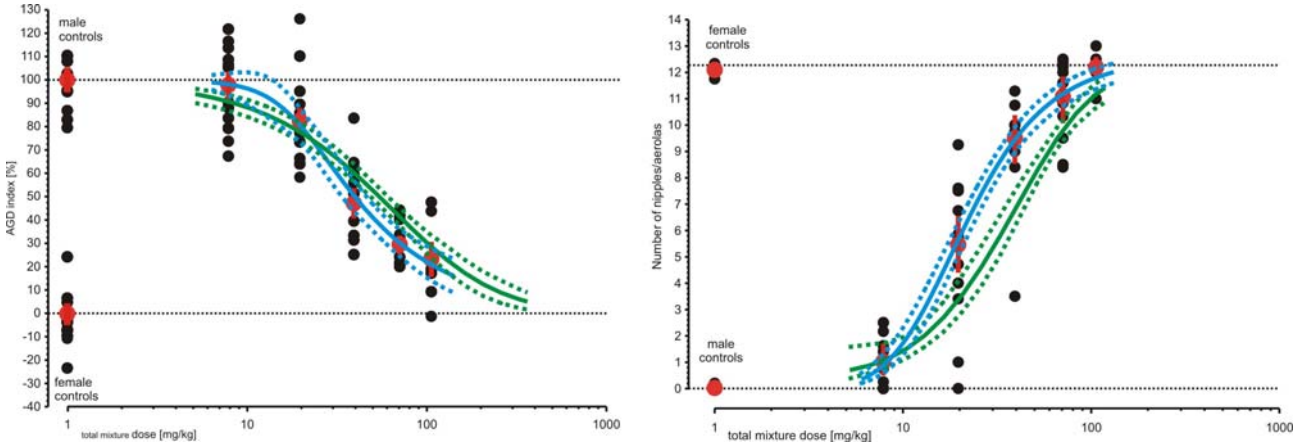

Figure 20 Effects of mixed exposure to vinclozolin, flutamide and procymidone on AGD (left) and NR (right). Results shown are group mean ± standard error (red), litter means (black), mean dose-response curve ± 95% confidence belt based on regression analysis (blue) and mean predicted mixture effect ± 95% confidence belt (green) (Hass *et al.,* 2007).

A second mixture composed of antiandrogens with differing modes of actions (inhibition of androgen action by interfering with steroid synthesis, inhibition of steroid-converting enzymes and androgen receptor antagonism) was also tested. The phthalate DEHP, vinclozolin, finasteride and prochloraz were subjected to indepth dose-response analysis, both singly and in combination. In this case, anticipated additive effects calculated by using IA and CA yielded very similar predictions. They agreed well with the observed effects on anogenital index and NR.

Estrogenic agents in zebrafish and sticklebacks

A combination of E2, E1, EE2 and nonylphenol was examined in zebrafish and sticklebacks with VTG induction as the endpoint. With both fish species, the joint effect of the estrogenic chemicals was predicted well by using the concept of CA.

Antiandrogens in the stickleback

The antiandrogens fenitrothion, flutamide, linuron and vinclozolin were able to suppress androgen-induced spiggin synthesis in female sticklebacks. When administered together, the four chemicals produced concentration-additive mixture effects in this *in vivo* model. From *in vitro* studies with antiandrogens in gene reporter assays, it has been known that many estrogenic chemicals also function as androgen receptor antagonists. Considering these effects, it became an attractive proposition to test estrogens for antiandrogenic responses in the stickleback model. E2, E1, EE2 and nonylphenol produced reductions in spiggin synthesis in female sticklebacks. In combination, their effects were predicted fairly accurately by using CA. Finally, the mixture of four antiandrogens was combined with the four estrogenic agents to produce a mixture of eight chemicals. The joint antiandrogenic effects of this mixture agreed well with the additivity expectation according to concentration addition.

EDC produce mixture effects when combined at low-doses, below NOEL

The extensive dose-response analyses conducted with vinclozolin, flutamide and procymidone allowed estimations of their NOEL with changes in AGD in the rat as the endpoint (Figure 21). When the three antiandrogens were combined at doses equivalent to their NOEL, significant reductions of AGD, equivalent to a 50% effect, were observed (Hass *et al.*, 2007). Similar effects were also seen with changes in the organ weights of accessory sexual glands as the endpoint (Metzdorff *et al.*, 2007).

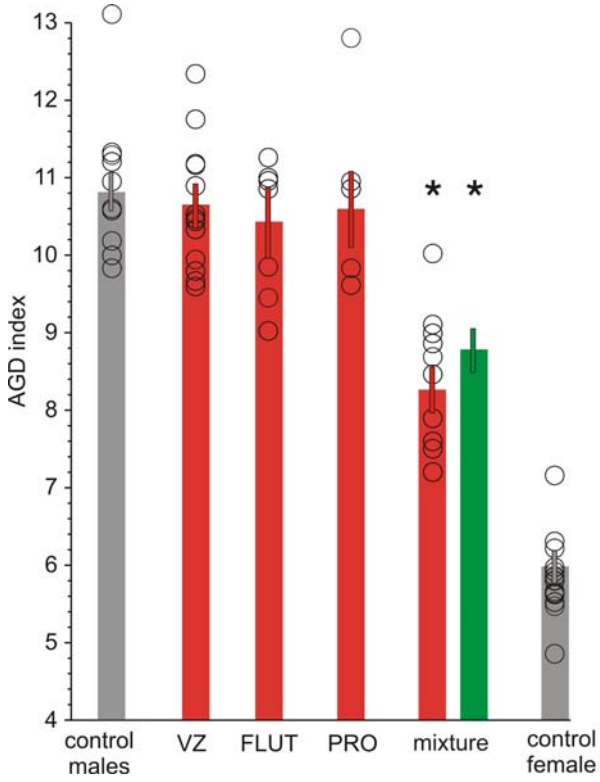

Figure 21 Mixture effects on AGD at low-doses of individual mixture components. Results shown are group means ± 95 % confidence intervals for control males and females (grey), individual doses of 24.5 mg/kg vinclozolin (VZ), 0.77 mg/kg flutamide (FLUT) and 14.1 mg/kg procymidone (PRO) (red), the combined mixture dose of 39.37 mg/kg (red) and the predicted mixture effect (green). Open circles represent litter means. * $p < 0.05$ compared to control (Hass *et al.*, 2007).

Suppressions of androgen-stimulated spiggin synthesis equivalent to a 30 % effect were found in the stickleback with vinclozolin, flutamide, linuron and fenitrothion at concentrations that by themselves did not produce responses statistically significantly different from controls.

Finally, information about blood serum and adipose tissue levels of xenoestrogens in European women was compiled (also using data emanating from EDEN Theme 1 work) to conduct a mixture experiment in the E-Screen at environmentally relevant concentrations. At the serum concentrations, the effects of most of the individual xenoestrogen were far too small to be measurable in the E-Screen. Combined at average European pollution levels, the impact of 19 xenoestrogens, for which exposure data were

available, on the effects of physiological levels of E2 and E1 was negligible. However, a composition modelled on more severe pollution levels found in certain South European countries revealed a significant additional combined effect load of the xenoestrogens.

Elements of an EDC mixtures risk assessment approach

Risk assessment approaches that take account of EDC mixture effects need to address three fundamental issues:

- What is the likelihood of synergistic combination effects and which parameters are important in increasing the chance of synergisms?
- Is it possible to adopt a default concept in predicting additive combination effects?
- What are determinants of additive mixture effects of EDC?

In principle, synergisms (or antagonisms) may occur when there are toxicokinetic interactions between mixture components. For example, one component may facilitate effective transport of another to target sites and in this way lead to greater effects than anticipated. Similarly, differential induction of toxifying (or detoxifying) steps will produce combination effects that differ substantially from additivity expectations. Although the available evidence suggests that EDC mixture effects are (dose) additive (Kortenkamp 2007), there are uncertainties about the likelihood of synergisms because mixtures where there is a reasonable chance of synergisms occurring have not been tested sufficiently. In the future, work should be undertaken to systematically explore the potential for synergisms, and this has to focus on the possibility that chemicals devoid of endocrine activity may lead to significant exacerbations of EDC mixture effects. A viable option of dealing with this uncertainty is to adopt an additional safety factor for EDC.

The published record in the literature shows that the effects of combinations of EDC belonging to a similar class do not deviate substantially from dose addition. Until evidence to the contrary emerges, dose (or concentration) addition can therefore be adopted as the default concept for risk assessment of EDC mixtures.

Four factors strongly determine the magnitude of combination effects to be expected from a mixture: The number of chemicals involved, their potency, their relative prevalence (mixture ratio) in the mixture and their total dose. Thus, mixtures composed of chemicals present at low-doses and with a low potency, can nevertheless induce significant combination effects if exposure is to a sufficiently high number simultaneously.

However, incomplete knowledge about the identity of chemicals with endocrine disrupting potential is one of the major sources of uncertainty in EDC mixtures risk assessment. Low-dose and low potency of EDC alone are uninformative in risk assessment. The other key determinant that decides whether a mixture can be deemed safe (or unsafe) is the sheer number of EDC that make up a particular human or wildlife exposure scenario, and this information is currently either incomplete or does not exist.

While the above data gaps should be filled as a matter of utmost urgency, satisfactory resolution of all uncertainties is not likely in the foreseeable future. On the other hand, the knowledge about determinants and factors that govern the joint action of similarly acting

EDC is sufficiently advanced to come to pragmatic risk assessment approaches. In the light of these considerations, a panel meeting with experts from EU environmental agencies and NGO's has proposed the following *modus operandi* for EDC mixtures:

- In particularly "data-rich" situations, where both exposure information and low-dose estimates are known, it is proposed to use the concept of dose addition (including the TEF approach) to arrive at a "mixture no-observed-adverse-effect-level" (MNOAEL) for endpoints relevant to endocrine disruption. These are then combined with a safety factor to arrive at estimates of tolerable human exposure.

- In "data-poor" situations, where there is fragmentary information about the number of chemicals with endocrine activities, their exposure levels, their potency, and their effects at low-doses, except for a few "prototype" chemicals, it is proposed to estimate a crude MNOAEL by dividing the individual NOAEL of the "prototypical" chemical by the anticipated number of relevant similarly acting chemicals. This will be based on chemicals where information about relevant *in vivo* effects is documented. If this information is not available, then additional chemicals should be "ruled in" on the basis of *in vitro* test results. If the number of chemicals contributing to mixture effects is unknown, it is suggested to choose a default number in the region of 10-20 until evidence to the contrary emerges. The resulting MNOAEL is then combined with the usual assessment factor (e.g. 100) to estimate tolerable levels for human exposure.

Discussion

The EDEN project has produced a wealth of new information about EDC low-dose effects and about the ways in which EDC act together in combination.

The experiments carried out to address the "low-dose" issue have not produced new examples of non-monotonic dose-response curves in the low-dose range. Instead, it was discovered that EDC exhibit dose-response curves with shallow gradients in the low-dose range. In these situations, the customary reliance of safety evaluations on estimating NOEL increases the likelihood of overlooking effects.

The mixture studies have shown that valid predictions of the effects of multi-component mixtures of EDC can be made in *in vivo* assays with endpoints of toxicological relevance. When sufficiently large numbers of EDC are combined, and when the statistical detection limit of the assay is sufficiently high, then joint effects of EDC can be demonstrated at doses below their individual NOEL. Such demonstrations heighten the need for taking mixture effects into consideration during risk assessment.

Conclusions

The application of hypothesis testing methods is inadequate for capturing low-dose effects of EDC. Whenever possible, regression-based approaches with their benchmark dose limits should replace NOEL as the basis for establishing acceptable human exposure levels. Ideally, hypothesis testing and regression methods should be combined in a framework that utilises the strengths of both methodologies by making considerations of statistical detection limits and statistical power the starting point of testing procedures.

Implementation of this framework will require a significant change in toxicological testing practice.

Determinants of additivity for EDC mixtures have been characterised and are now well understood for combinations of similarly acting EDC. EDC of relatively low potency and at low exposure levels can still work together to produce significant combination effects when they are present in sufficient numbers. In cases where EDC act in concert with endogenous hormones, this may result in significant additional effects under certain circumstances.

Uncertainty still exists in relation to the likelihood of synergistic mixture effects, and concerted efforts should be made to fill this gap. Another source of uncertainty that will hamper sound EDC mixture risk assessment is incomplete knowledge about the identity of EDC, their exposure levels and number. This issue can only be resolved through the development of dedicated exposure assessment strategies that take account of cumulative exposures.

Despite some knowledge gaps concerning exposure estimations and the likelihood of synergisms, knowledge about determinants and factors that govern the joint action of similarly acting EDC is now sufficiently advanced to come to pragmatic risk assessment approaches to take mixture effects into consideration. Elements of a *modus operandi* for EDC mixtures include the use of dose addition (including the TEF approach) to arrive at a MNOAEL for endpoints relevant to endocrine disruption. These are then combined with a safety factor to arrive at estimates of tolerable human exposure. “Data-poor” situations may require estimation of a crude MNOAEL by dividing individual NOAEL of certain prototype chemicals by the anticipated number of relevant similarly acting chemicals.

Literature Cited

Almstrup K, Fernandez MF, Petersen J, Olea N, Skakkebæk NE, Leffers H, (2002). Dual effects of phytoestrogens result in U-shaped dose-response curves. *Environ. Health Perspect.* **110**, 743-748

Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette Jr. LJ, McLachlan JA, (1996). Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **272**, 1489-1492

Ashby J, Tinwell H, Odum J, Lefevre P, (2004). Natural variability and the influence of concurrent control values on the detection and interpretation of low-dose or weak endocrine toxicities. *Environ. Health Perspect.* **112**, 847-853

Brian JV, Harris CA, Scholze M, Backhaus T, Booy P, Lamoree M, *et al.,* (2005). Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environ. Health Perspect.* **113,** 721-728

Gray LE Jr., Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, Guillette L (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum. Reprod. Update* **7**, 248-264

Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, Broeng Metzdorff S, Kortenkamp A (2007). Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environ. Health Perspect*. doi:10.1289/ehp.9360 (available at http://dx.doi.org/), Online 8 June 2007

Katsiadaki I, Morris S, Squires C, Hurst MR, James JD, Scott AP (2006). Use of the three-spined stickleback (Gasterosteus aculeatus) as a sensitive *in vivo* test for detection of environmental antiandrogens. *Environ. Health Perspect*. **114**, 115-121 Suppl. 1

Katsiadaki I, Scott AP, Hurst MR, Matthiessen P, Mayer I (2002). Detection of environmental androgens: A novel method based on ELISA of spiggin, the stickleback (Gasterosteus aculeatus) glue protein. *Environ. Toxicol. Chem*. **21** (9), 1946-1954

Kortenkamp A (2007) Ten years of mixing cocktails – a review of endocrine disrupter mixture effects. *Environ. Health Perspect*. doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007 (In press)

Matthiessen P, Johnson I (2007). Implications of research on endocrine disruption for the environmental risk assessment, regulation and monitoring of chemicals in the European Union. *Environ. Pollution* **146**, 9-18

Metzdorff SB, Dalgaard M, Christiansen S, Axelstad M, Hass U, Kiersgaard MK, Scholze M, Kortenkamp A, Vinggaard AM (2007) Dysgenesis and histological changes of genitals and pertubations of gene expresssion in male rats after *in utero* exposure to antiandrogen mixtures. *Toxicol. Sci.* [Epub ahead of print])

Moore CRJ, Caux PY (1997). Estimating low toxic effects. *Environ. Toxicol. Chem.* **16**, 794-801

Payne J, Rajapakse N, Wilkins M, Kortenkamp A (2000). Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environ Health Perspect* **108**, 983-987.
Payne J, Scholze M, Kortenkamp A (2001). Mixtures of four organochlorines enhance human breast cancer cell proliferation. *Environ. Health Perspect.* **109,** 391-397

Rajapakse N, Silva E, Scholze M, Kortenkamp A (2004). Deviation from additivity with estrogenic mixtures containing 4-nonylphenol and 4-tert-octylphenol detected in the E-SCREEN assay. *Environ. Sci. Technol.* **38,** 6343-6352

Schlumpf M, Lichtensteiger W, Jarry H, Seidlova-Wuttke D, Wuttke W, Oetken M, Bachmann J, Schulte-Oehlmann U, Oehlmann J (2004) Effects of UV filters on aquatic wildlife. *CREDO Newsletter* **3**, 1-3 (ISSN 1744-1978)

Scholze M, Bödeker W, Faust M, Backhaus T, Altenburger R, Grimme LH (2001). A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. *Environ. Toxicol. Chem.* **20**, 448-457

Scholze M and Kortenkamp A (2007). Statistical power considerations show the endocrine disrupter low-dose issue in a new light. *Environ. Health Perspect.* doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007

Sheehan DM, Willingham E, Gaylor D, Beregeron JM and Crews D (1999) No threshold dose for E2-induced sex reversal of turtle embryos: How little is too much? *Environ. Health Perspec.t* **107**, 155-159

Silva E, Scholze M, Kortenkamp A (2007) Activity of xenoestrogens at nanomolar concentrations in the E-Screen. *Environ. Health Perspect*. doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007

Slob W (1999). Thresholds in Toxicology and Risk Assessment. *Int. J. Toxicol.* **18**, 259-268

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103** (Suppl 7), 113-122

vom Saal FS, Hughes C (2005). An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ. Health Perspect.* **113**, 926-933

Vonesh E, Chinchilli VM. 1996. Linear and nonlinear models of the for the analysis of repeated measurements. New York: Marcel Dekker.

EXPLOITATION AND DISSEMINATION OF RESULTS

Peer Reviewed Publications

Complex EDC mixtures in human and fish tissues (Theme 1)

Araque P, Soto AM, Olea-Serrano MF, Sonnenschein C, Olea N, (2006). Pesticides in human fat and serum samples versus total effective xenoestrogen burden. In: *Pesticide Protocols*. Vidal JLM, Frenich AG (Eds). Methods in Biotechnology. Humana Press, New York

Botella B, Crespo J, Rivas A, Cerrillo I, Olea-Serrano MF, Olea N, (2004). Exposure of women to organochlorine pesticides in Southern Spain. *Environ. Res.* **96**, 34–40

Cerrillo I, Granada A, Lopez-Espinosa MJ, Olmos B, Jimenez M, Araque P, Olea N, Olea-Serrano MF, (2005). Endosulfan and their metabolites in fertile women, placenta, cord blood and human milk. *Environ Res*. **98**, 233-239

Cerrillo I, Olea-Serrano MF, Ibarluzea J, Exposito J, Torne P, Laguna J, Pedraza V, Olea N (2005). Environmental and lifestyle factors for organochlorine exposure among women living in southern Spain. *Chemosphere*, **62** (11), 1917-1924

Fernandez M, Rivas A, Olea-serrano, F, Cerrillo I, Molina-Molina JM, Araque P, Martinez-Vidal JL, Olea N, (2004). Assessment of total effective xenoestrogens burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. *Anal. Bioanal. Chem*. **379**, 163-170

Fernandez MF, Araque P, Kiviranta H, Molina-Molina JM, Rantakokko P, Laine O, Vartiainen T, Olea N, (2007). PBDEs and PBBs in the adipose tissue of women from Spain. *Chemosphere*. **66**, 377-383

Fernandez MF, Olmos B, Granada A, López-Espinosa MJ, Molina-Molina JM, Fernandez JM, Cruz M, Olea-Serrano F, Olea N, (2007). Human Exposure to Endocrine Disrupting Chemicals and Prenatal Risk Factors for Cryptorchidism and Hypospadias: A Nested Case-Control Study. *Environ. Health Perspect.* (In press)

Fernández MF, Olmos B, Olea N (2006). Endocrine disrupter chemicals as prenatal risk factors for cryptorchidism and hypospadias. In Congenital annomalies. Nicolopoulo-Stamati P, Hens L, Howard CV, (Eds). Kluwer Academic Publishers

Fernandez M, Santa-Marina L, Ibarluzea JM, Exposito J, Aurrekoetxea JJ, Torne P, Laguna J, Rueda AI, Pedraza V, Olea N (2007). Analysis of population characteristics related to the total effective xenoestrogen burden, a biomarker of xenoestrogen exposure in breast cancer. *Euro. J. Cancer* **43**(8):1290-1296

Fernandez MF, Arrebola JP, Taoufiki J, Navalón A, Ballesteros O, Pulgar R, Vilchez JL, Olea N (2007). Bisphenol-A and chlorinated derivatives in adipose tissue of women. *Repro. Toxicol*. **24(**2): 259-264

Houtman CJ, Booij P, Van der Valk KM, Van Bodegom PM, Ven den Ende F, Gerritsen AAM, Lamoree MH, Legler J, Brouwer A, (2007). Biomonitoring of estrogenic exposure and identification of responsible compounds in bream from Dutch surface waters. *Environ. Toxicol. Chem.* **26** (5), 898-907

Ibarluzea J, Fernández MF, Santa-Marina L, Olea-Serrano F, Rivas A, (2004). Breast cancer risk and the combined effect of environmental estrogens. *Cancer Causes Control* **15**, 591–600

Kool J, Ramautar R, van Liempd SM, Beckman J, de Kanter FJ, Meerman JH, Schenk T, Irth H, Commandeur JN, Vermeulen NP, (2006). Rapid on-line profiling of estrogen receptor binding metabolites of tamoxifen. *J. Med. Chem*., **49,** (11) 3287-3292

Lopez-Espinosa MJ, Granada A, Carreno J, Salvatierra M, Olea-Serrano F, Olea N, (2006). Organochlorine Pesticides in Placentas from Southern Spain and Some Related Factors. *Placenta.* **28**(7): 631-638

Olea N, Fernández Cabrera MF, (2006). Xenoestrógenos y cáncer de mama. In: *Cancer de mama*. Diaz Faes J, Rubial Morell A (Eds). Fundación de Estudios Mastológicos (FEMA) Madrid, Spain. Pg 319-333

Reinen J, Vriese E, Glatt H, Vermeulen NP, (2006). Development and validation of a fluorescence HPLC-based screening assay for inhibition of human estrogen sulfotransferase. *Anal. Biochem*., **357**(1): 85-92

Ribas-Fitó N, Ramón R, Ballester F, Grimalt JO, Marco A, Olea N, Posada M, Rebagliato M, Tardón A, Torrent M, Sunyer J, (2006). Child Health and the Environment: The INMA Spanish Study. *Paed. Perinatal Epidemiol.* **20** (5): 403-410

Ribas-Fito N, Ramon R, Ballester F, Grimalt J, Marco A, Olea N, Posada M, Rebagliato M, Tardon A, Torrent M, Sunyer J., (2006) Child health and the environment: the INMA Spanish Study. *Paed. Perinatal Epidemiol*. **5**: 403-410

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV, (2007). Human exposure to bisphenol-A (BPA). *Repro. Toxicol.* **24**(2): 139-177

Mechanisms of EDC action and novel endpoints and biomarkers (Theme 2)

Alberti M, Kausch U, Haindl S, Seifert M. (2005) Gene expression analysis for exposure to estrogenic substances. *Acta Hydrochim. Hydrobiol*. **33**, 38-44

Alberti M, Kausch U, Haindl S, Leibiger R, Budczies J, Seifert M, Hock B. (2005) Gene expression patterns – a tool for bioanalysis. *Intern. J. Environ. Anal. Chem*. **85**, 589-608

Barreiro ML, Pineda R, Gaytan F, Archanco M, Burrell MA, Castellano JM, Hakovirta H, Nurmio M, Pinilla L, Aguilar E, Toppari J, Dieguez C, Tena-Sempere M. (2005) Pattern of orexin expression and direct biological actions of orexin-a in rat testis. *Endocrinology* **146**: 5146-5175

Castellano JM, Navarro VM, Fernandez-Fernandez R, Nogueiras R, Tovar S, Roa J, Vazquez MJ, Vigo E, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M. (2005) Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology* **146**:3917-3925

Castellano JM, Gaytan M, Roa J, Vigo E, Navarro VM, Bellido C, Dieguez C, Aguilar E, Sanchez-Criado JE, Pellicer A, Pinilla L, Gaytan F, Tena-Sempere N. (2006) Expression of KiSS-1 in the rat ovary: Putative local regulator of ovulation? *Endocrinology* **147**:4852-4862

Castellano JM, Navarro VM, Fernandez-Fernandez R, Castaño JP, Malagon MM, Aguilar E, Dieguez C, Magni P, Pinilla L, Tena-Sempere M. (2006) Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. *Mol. Cell. Endocrinology* **257-258**:75-83

Castellano JM, Navarro VM, Fernandez-Fernandez R, Roa J, Vigo E, Pineda R, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M. (2006) Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. *Diabetes* **55**:2602-2610

Cheshenko K, Brion F, Le Page Y, Hinfray N, Pakdel F, Kah O, Segner H, Eggen RIL (2007). Expression of zebrafish aromatase *cyp19a* and *cyp19b* genes in response to the ligands of estrogen receptor and aryl hydrocarbon receptor. *Tox. Sci.,* **96**(2), 255-267

Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RIL. (In press). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen. Comp. Endocrinol*. (Epub 2007 Mar 21)

Dominé F, Parent AS, Rasier G, Lebrethon MC, and Bourguignon JP (2007). Assessment and mechanism of variations in pubertal timing in .internationally adopted children: a developmental hypothesis. *Euro. J. Endocrinology* **155**: S17–S25

Fernandez-Fernandez R, Martini AC, Navarro VM, Castellano JM, Dieguez C, Aguilar E, Pinilla L and Tena-Sempere M. (2006) Novel signals for the integration of energy balance and reproduction. *Mol. Cell. Endocrinology* **254-255**:127-132

Ferrara D, Hallmark N, Scott HS, Brown R, McKinnell C, Mahood IK, Sharpe RM (2006) Acute and long-term effects of *in utero* exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. *Endocrinology* **147**: 5352-5362

Hallmark N, Walker M, McKinnell C, Mahood IK, Scott HS, Bayne R, Coutts S, Anderson RA, Greig I, Morris K, Sharpe RM, (2007). Effects of monobutyl- and di (n-butyl) phthalate *in vitro* on steroidogenesis and Leydig cell aggregation in foetal testis explants from the rat: comparison with effects *in vivo* in the foetal rat and neonatal marmoset and *in vitro* in the human. *Environ. Health Persp.* (In press)

Hutchison G, Scott HM, Walker M, McKinnell C, Mahood IK, Sharpe RM (2007) Sertoli cell development and function in an animal model of testicular dysgenesis syndrome. *Biology of Reproduction* (In press)

Hutchison G, Sharpe RM, Mahood IK, Jobling M, Walker M, McKinnell C, Mason JI, Scott HM (2007) The origins and time of appearance of focal testicular dysgenesis in an animal model of testicular dysgenesis syndrome: evidence for delayed testis development? *Int. J. Andrology* (In press)

Kallivretaki E, Eggen R, Neuhauss S, Alberti M, Kausch U, Segner H. (2006). Aromatase in zebrafish: a potential target for endocrine disrupting chemicals. Marine *Environ. Res.* **62**: S157-160

Kallivretaki E, Eggen RIL, Neuhauss SCF, Kah O, Segner H (2007). The zebrafish brain-specific aromatase, *cyp19a2,* is neither expressed nor distributed in a sexually dimorphic manner during sexual differentiation. *Dev. Dyn*. (In press)

Kallivretaki E, Eggen R, Neuhauss S, Segner H. (2007). Knockdown of the *cyp19a1* aromatase gene decreases neuromast numbers in the lateral line organ of zebrafish embryos. *Mol. Cell. Endocrinol.* (In review)

Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, and Tena-Sempere M (2007). Sexual differentiation of KiSS-1 gene expression in the brain of the rat. *Endocrinology* (Epub ahead of print Jan 4)

Kausch U , Alberti M, Haindl S, Budczies J, Hock B, (2007). Biomarkers for exposure to estrogenic compounds: Gene expression analysis in zebrafish (Danio rerio). *Environ Tox.* (In press)

Leffers H, Navarro VM, Nielsen JE, Mayen A, Pinilla L, Malagon M, Castaño JP, Skakkebaek NE, Aguilar E, Tena-Sempere M.(2006) Increased expression of α- and β-globin mRNA at the Pituitary following exposure to estrogen during the critical period of neonatal sex differentiation in the rat. *J. Steroid Biochem. Mol. Biol.* **99**:33-43

Le Page Y, Scholze M, Kah O, Pakdel F (2006) Assessment of xenoestrogens using three distinct estrogen receptors and zebrafish brain aromatase gene in a highly responsive glial cell system. *Environ. Health Perspect.* 114, 752-758.

Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS, Sharpe RM (2005). Abnormal Leydig Cell aggregation in the foetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* **146**: 613-623

Martini AC, Fernandez-Fernandez R, Tovar S, Navarro VM, Vigo E, Vazquez MJ, Davies JS, Thompson NM, Aguilar E, Pinilla L, Wells T, Dieguez C, Tena-Sempere M. (2006). Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinising hormone secretion in male rats. *Endocrinology* **147**:2374-2382

McKinnell C, Sharpe RM, Ivell R, Staub C, Jégou B, Hartung S (2005) Expression of Insulin-like factor 3 (Insl3) protein in the rat testis during foetal and postnatal

development and in relation to cryptorchidism induced by *in utero* exposure to Di-(*n*-butyl)phthalate. *Endocrinology* **146**: 4536-4544

Menuet A, Le Page Y, Torres O, Kern L, Kah O, Pakdel F. (2004) Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. *J. Mol. Endocrinol*. **32**, 975-986

Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, Kah O (2005). Expression and the estrogen-dependent regulation of the zebrafish brain aromatase gene. *J. Comp. Neurol.* **485**, 304-320

Menuet A., Adrio F., Kah O. and Pakdel F. (2005) Regulation and Function of Estrogen Receptors: Comparative Aspects (Review). In Hormones and their receptors in fish reproduction (edited by Philippa Melamed and Nancy Sherwood), Chapter 7, pp: 224-253

Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, Gaytan F, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M. (2004) Advanced Vaginal Opening and Precocious Activation of the Reproductive Axis by KiSS-1 Peptide, the Endogenous Ligand of GPR54. *J Physiol.* **561**:379-386

Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. (2004) Characterisation of the Potent LH Releasing Activity of KiSS-1 peptide, the Natural Ligand of GPR54. *Endocrinology* **146**:156-163

Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. (2005) Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology* **146**:1689-1697

Pellegrini E, Menuet A, le Lethimonier C, Adrio F,Gueguen MM, Tascon C, Anglade I, Pakdel F, Kah O (2005). Relationships between aromatase and estrogen receptors in the brain of teleost fish. *Gen. Comp. Endocrinol.* **142**, 60-66

Pellegrini E, Mouriec K, Anglade I, Menuet A, Le Page Y, Gueguen MM, Marmignon MH, Brion F, Pakdel F, Kah O (2007) Identification of aromatase-positive radial glial cells as progenitor cells in the ventricular layer of the forebrain in zebrafish. *J. Comp. Neurol*. **501**, 150-167

Rasier G, Toppari J, Parent AS, Bourguignon JP (2006). Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: A review of rodent and human data. *Mol. Cell. Endocrinology* **254-255**:187-201

Rasier G, Parent AS, Gérard A, Lebrethon MC, and Bourguignon JP (2007). Early maturation of gonadotropin-releasing hormone secretion and sexual precocity after exposure of infantile female rats to estradiol or dichlorodiphenyltrichloroethane. *Biol. Reprod*. **77**:734-742

Rasier G, Parent AS, Gérard A, Denooz R, Lebrethon MC, Charlier C, and Bourguignon JP (2007). Mechanisms of interaction of endocrine disrupting chemicals with glutamate-evoked secretion of gonadotropin-releasing hormone. *Toxicol. Sci*. (In press)

Roa J, Vigo E, Castellano JM, Navarro VM, Fernandez-Fernandez R, Casanueva FF, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M. (2006). Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female rat. *Endocrinology* **147**:2864-2878

Sánchez-Criado JE, Martín de las Mulas J, Bellido C, Navarro VM, Aguilar R, Garrido-Gracia JC, Malagón MM, Tena-Sempere M, Blanco A. (2006) Gonadotropin secreting cells in ovariectomized rats treated with different oestrogen receptor (ER) ligands: a modulatory role for ERb in the gonadotrope? *J. Endocrinology* **188**:167-177

Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, de Gendt K, Verhoeven G, O'Shaughnessy PJ, Sharpe RM (2007) Role of androgens in foetal testis development and dysgenesis. *Endocrinology* **148**: 2027-2036

Sharpe RM and Skakkebæk NE (2003). Male reproductive disorders and the role of endocrine disruption: Advances in understanding and identification of areas for future research. Pure *Appl. Chem*. **75**: 2023-2038

Sharpe RM (2006). Pathways of endocrine disruption during male sexual differentiation and masculinisation. In: Endocrine disruptors (Ed. P Darbre) *Bailliere's Best Practice and Research in Clinical Endocrinology and Metabolism* **20**: 91-110

Sharpe RM, Skakkebæk NE (2007) Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. *Fert. Steril.* (In press)

Tena-Sempere M, Navarro VM, Mayen A, Bellido C, Sanchez-Criado JE. (2004) Regulation of estrogen receptor (ER) isoform messenger RNA expression by different ER ligands in female rat pituitary. *Biol. Reprod.* **70**:671-678

Tena-Sempere M, Barreiro ML, Aguilar E, Pinilla L, (2004). Mechanisms for altered reproductive function in female rats following neonatal administration of raloxifene. *Euro. J. Endocrinology* **150**:397-403

Tena-Sempere M, Dalgaard M, Hass U. (2004) Joining forces to target endocrine disrupting events at the hypothalamic-pituitary unit. *CREDO Newsletter* (**3**). ISSN 1744-1878

Tena-Sempere M, Aguilar E. (2005) Biological effects and markers of exposure to xenosteroids and selective estrogen receptor modulators (SERMS) at the hypothalamic-pituitary unit. In: Kordon C, Gaillard R, Christen Y (eds): Hormones and the Brain. Research and Perspectives in Endocrinology 3. New York, Berlin, Heidelberg, Berlin, Springer, pp 79-98

Tena-Sempere M. (2005) Hypothalamic KiSS-1: the missing link in gonadotropin feedback control? *Endocrinology* **146**:3683-3685

Tena-Sempere M. (2006) GPR54 and kisspeptin in reproduction. *Human Reprod. Update* **12**:631-639

Tena-Sempere M. (2006) Novel roles of kisspeptins and GPR54 in pubertal development. *Curr. Opin. Ped.* **18**:442-447

Tena-Sempere M. (2006) KiSS-1 and reproduction: Focus on its role in the metabolic regulation of fertility. *Neuroendocrinology* **83**:275-281

Tovar S, Vazquez MJ, Navarro VM, Fernandez-Fernandez R, Castellano JM, Vigo E, Roa J, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M. (2006) Effects of single or repeated intravenous administration of kisspeptin upon dynamic LH secretion in conscious male rats. *Endocrinology* **147**:2696-2704

Indicators of impaired male reproductive function (Theme 3)

Andersson AM, Jensen TK, Juul A, Petersen JH, Joergensen T, Skakkebæk NE (2007). Secular decline in male testosterone and sex hormone binding globulin serum levels in Danish population surveys. *J. Clin. Endocrinol. Metab.* (In press)

Jensen TK, Andersson A-M, Jørgensen N, Andersen A-G, Carlsen E, Petersen JH, Skakkebæk NE, (2004). Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fert. Steril.* **82**:863-870

Jensen TK, Jørgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, Horte A, Andersen A-G, Carlsen E, Magnus Ø, Matulevicius V, Nermoen I, Vierula M, Keiding N, Toppari J, and Skakkebæk NE, (2004). Association of *in utero* exposure to maternal smoking with reduced semen quality and testis size in adulthood: A cross-sectional study of 1,770 young men from the general population in five European countries. *Am. J. Epidemiol.,* **159**:49-58

Jensen TK, Sobotka T, Hansen MA, Pedersen AT, Lutz W, Skakkebæk NE (2007). Declining trends in conception rates in recent birth cohorts of native Danish women: A possible role of deteriorating male reproductive health. *Int. J. Androl.*(In press)

Jensen TK, Andersson A-M, Jørgensen N, Andersen A-G, Carlsen E, Petersen JH, Skakkebæk NE, (2004). Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil. Steril.* **82**:863-870.

Jørgensen N, Asklund C, Carlsen E, and Skakkebæk NE (2006), Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. *Int. J. Andrology* **29**: 54–61

Jørgensen N, Askland C, Carlsen E, Holm M, Petersen JH, Jensen TK, Vierula M, Toppari J, and Skakkebæk NE, (2007). Surveillance of semen quality of Danish and Finnish populations: A recent downward trend from historically high semen quality of young Finnish men towards Danish level of poor semen quality. In Preparation.

Nyboe Andersen A, Erb K, (2006) Register data on assisted reproductive technology (ART) in Europe including a detailed description of ART in Denmark. *Int. J. Andrology*, **29**(1):12-16

Skakkebæk NE, Jørgensen N, Main KM, Rajpert-De Meyts E, Leffers H, Andersson AM, Juul A, Carlsen E, Mortensen GK, Jensen TK, and Toppari J, (2006). Is human fecundity declining? *Int. J. Andrology* **29**(1):2-11

Virtanen HE, and Toppari J, (In press). Testicular dysgenesis syndrome as a congenital disease. In: Congenital Anomalies and the Environment 1-8. Nicolopoulos-Stamati P (Ed.). Springer, Netherlands

Low-dose and mixture effects of EDCs (Theme 4)

Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, Broeng Metzdorff S, Kortenkamp A (2007). Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environ. Health Perspect*. doi:10.1289/ehp.9360 (available at http://dx.doi.org/), Online 8 June 2007

Katsiadaki I, Morris S, Squires C, Hurst MR, James JD, Scott AP (2006). Use of the three-spined stickleback (Gasterosteus aculeatus) as a sensitive *in vivo* test for detection of environmental antiandrogens. *Environ. Health Perspect*. **114**, 115-121 Suppl. 1

Katsiadaki I, and Scott AP, (2006). The stickleback model in endocrine disruption research: an essential tool in the laboratory and field. *Marine Environ. Res*. **62**(Supp 1): S228-S229

Katsiadaki I, (2006). The use of the stickleback as a sentinel and model species in ecotoxicology. Chapter 10, in 'The Biology of the three-spined stickleback', edited by Sara Östlund-Nilsson, Ian Mayer, and Felicity Huntingford, pp 319-352

Kortenkamp, A. (2006) Breast cancer, estrogens and environmental pollutants: a re-evaluation from a mixtures perspective. *Int. J. Androl.* **29**, 193-198

Kortenkamp A (2007) Ten years of mixing cocktails – a review of endocrine disrupter mixture effects. *Environ. Health Perspect*. doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007 (In press)

Kortenkamp A, Faust M, Scholze M, Backhaus T (2007) Low level exposure to multiple chemicals – reason for human health concerns? *Environ. Health Perspect*. doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007

Metzdorff SB, Dalgaard M, Christiansen S, Axelstad M, Hass U, Kiersgaard MK, Scholze M, Kortenkamp A, Vinggaard AM (2007) Dysgenesis and histological changes of genitals and pertubations of gene expresssion in male rats after *in utero* exposure to antiandrogen mixtures. *Toxicol. Sci*. [Epub ahead of print])

Scholze M and Kortenkamp A (2007). Statistical power considerations show the endocrine disrupter low-dose issue in a new light. *Environ. Health Perspect.* doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007

Silva E, Scholze M, Kortenkamp A (2007) Activity of xenoestrogens at nanomolar concentrations in the E-Screen. *Environ. Health Perspect*. doi: /ehp. (available at http://dx.doi.org/), Online 8 June 2007

Conference Presentations

For a full listing of presentations given at conferences and meetings, please refer to the Scientific Annual Reports. To illustrate the wide representation EDEN has had at numerous conferences over the past 4.5 years, a selection of conferences attended by several of the Partners is given below:

Bourguignon JP The impact of endocrine disrupting chemicals on female puberty. Presented at the Annual Endocrine Society meeting in Toronto, June 2007 (By Invitation).

Cheshenko K, Molecular Research in Environmental Medicine, 2nd International Conference, Paris, France. September 2006

Christiansen S & Hass U (2006) Mixture effects of antiandrogens in rat male offspring: Effects on anogenital distance, nipple retention, external and internal male reproductive organs. Oral presentation at EDEN forum meeting, Granada, Spain

Eggen R, Cheshenko K, Neuhauss,S, and Segner H. The production of transgenic zebrafish that respond to EDC. Prague, Czech Republic 10-12 May 2005.

Eggen R, Kallivretaki E, Cheshenko K, Neuhauss S, and Segner H. Mechanisms of endocrine disruption in zebrafish. Cadro, Switzerland, 2005.

Gomes RL, Kortenkamp A, 2006. Progress in the garden of EDEN. Presented at the *Gordon Research Conference Environmental Endocrine Disruptors,* Il Ciocco, Italy. 4-9 June 2006 (By Invitation)

Sharpe RM, 'Animal models for testicular dysgenesis syndrome'1st International Conference on Urogenital Disorders. Malmo, Sweden, September 2005 (By Invitation)

Sharpe RM, 'Use of an animal model to study the foetal origins of the commonest reproductive disorders in newborn and young adult males'. Gordon Research Conference on 'Environmental endocrine disruptors'. Il Ciocco, Italy June 2006 (By Invitation)

Sharpe RM, 'Foetal and early postnatal origins of environmental reproductive health effects in the male'. UCSF-CHE Summit on Environmental Challenges to Reproductive Health and Fertility. January, San Francisco, USA January 2007 (By Invitation)

Hass U, Filinska M, Kledal TSA (2003). Antiandrogenic effects of diisononyl phthalate in rats. Poster at 31st Conference of European Teratology Society, Denmark. Abstract in Reproductive Toxicology 2003, 17, 493-494.

Hass U (2003). Effects on sexual dimorphic behaviour in rats after developmental exposure to anti-androgens. Invited speaker at 31st Conference of European Teratology Society, Denmark. Abstract in Reproductive Toxicology 2003, 17, 482.

Hass U (2003). How do current regulatory test methods for chemicals cope with late effects and vulnerable windows in reproductive toxicology? Invited speaker at European Environmental Agency Meeting "Reproductive Toxicology and Chemicals: a matter of timing?", Copenhagen, October 2003.

Hass U (2004). The role of *in vitro* and *in vivo* screening assays in prioritising testing strategies – are we using the right tools? Speaker at EURISK/CREDO Cluster Workshop on "Multiorganic Risk Assessment of Endocrine Disruptors", Mallorca, Spain, March 2004.

Hass U (2004). Mixture and low-dose effects in rats after pre- and postnatal exposure to EDCs. Speaker at EDEN Planning Meeting, Mallorca, Spain, March 2004

Hass U, Filinska M, Pedersen S, Dalgaard M, Kledal TSA (2004). Effect of finasteride and DEHP on anogenital distance and nipple retention after perinatal exposure in rats. Poster presented at the European Teratology Society Conference, September 2004, Greece. Abstract in Reprod Toxicol 2004.

Hass U, Christiansen S, Axelstad M, Scholze M and Kortenkamp A (2007). Combined exposure to dissimilarly acting antiandrogens causes markedly increased frequency of hypospadias in the rat. Poster at 4th Copenhagen Workshop on Endocrine Disrupters, Denmark

Jolly C, Katsiadaki I, Le Belle N, Mayer I and S. Dufour (2006) Development of a stickleback *in vitro* assay for the rapid screening of (anti-)androgenic Environmental contaminants. 23rd Conference of European Comparative Endocrinologists, Manchester, UK, September 2006

Katsiadaki I, Sebire M, Scott AP, Geoghegan F, Katsu Y, Iguchi T, Nagae M (2005) The stickleback model in endocrine disruption research: a progress report within the UK-Japan co-operation. The 7th Scientific Workshop of the UK-J Research on Endocrine Disrupters, Okinawa, Japan, 2nd-3rd December 2005

Katsiadaki I, and Scott AP, (2005). The stickleback model in endocrine disruption research: an essential tool in the laboratory and field. PRIMO 13, Alessandria, June 2005

Katsiadaki I, Allen Y, and Scott AP, (2005) The three-spined stickleback as a sentinel organism for evaluating the effects of endocrine disrupters: a progress report.. SETAC Europe, 15th annual meeting, Lille, France, May 22-26,
2005

Katsiadaki I, and Scott AP, (2004). Is the humble stickleback one of the best model species to study the effects of xenobiotics with endocrine modulating activity?. The 5th International Symposium on Fish Endocrinology, Castellon, Spain, September 5-9, 2004

Katsiadaki I, and Scott AP, (2004) The stickleback model: From lab to field. CREDO workshop on Endocrine Disruptors, Exeter University, July 2004

Katsiadaki I, (2004). Simultaneous assessment of oestrogenic and (anti)-androgenic effects using the three-spined stickleback. UK-Japan Workshop on endocrine disrupters, 19 February 2004, Kumamoto, Japan.

Katsiadaki I, Mayer I, and Scott AP, (2003) The three-spined stickleback as the European sentinel and a model species for endocrine disruption research. The 4th International conference on Stickleback Behaviour and Evolution, Sweden

Katsiadaki I, Scott AP, and Mayer I, (2003) The three-spined stickleback as a model for the detection of (anti)-androgenic and oestrogenic xenobiotics. SETAK UK conference, York, April 2003

Sanders MB and Katsiadaki I (2006). Stickleback biomarkers and behaviour as sensitive indicators of exposure to, and effects of, steroids and pharmaceuticals. Risk Assessment of Pharmaceuticals & Veterinary Medicines in the Environment Conference, 26-27 September 2006, Amsterdam, The Netherlands

Sanders M, Katsiadaki I (2006) The stickleback model in ecotoxicology: an essential tool in the laboratory and the field. Fifth International Conference on Stickleback Behaviour & Evolution, Anchorage, Alaska 30th July - 4th August 2006

Sebire M and Katsiadaki I (2006) The effect of endocrine disrupting chemicals that interfere with androgen action on the reproductive behaviour of the three-spined stickleback. The 5th International Conference on Stickleback Behaviour and Evolution, Anchorage, Alaska, 30th July - 4th August 2006

Toppari J, Regional and temporal trends in the prevalence of cryptorchidism and hypospadias. In the International Symposium on Endocrine Disrupters, 25.-28.11.2002, Hiroshima, Japan.

Toppari J, Trends in hypospadias and cryptorchidism in Europe. In the 2nd Copenhagen Workshop on Endocrine Disrupters: A possible role of mixed exposures for reproductive failures and malignancies, 7.-9.12.2002, Copenhagen, Denmark.

Toppari J, Timing of puberty, In the Nordic Network of Pediatric Endocrinologists, 20-23.11.2003, Reykjavik, Island

Toppari J, Prevalence of genital malformations in male newborns: in Finland. In the Environmental Disrupters and Pediatric Endocrine Diseases, 12-13.12.2003, Montpellier, France

Toppari J, Cryptorchidsm as a part of testicular dysgenesis syndrome. In the CREDE (Turku)-IRDB meeting, 21-22.4.2004, London, U.K.

Toppari J, Nordic differences in testis development and its disorders. In the 13th European Testis Workshop, 24-28.4.2004, Dunblane, Scotland
Toppari J, Trends of male reproductive health in Nordic countries. In the 6th Congress of the Hellenic Andrology Society, 5-6.11.2004 Thessaloniki, Greece

Toppari J, Regional and temporal differences in the prevalence of cryptorchidism and hypospadias: ecological differences in exposure to organochlorines and pesticides. In the Copenhagen Workshop on Environment, Reproductive Health and Fertility, 15-18.1.2005, Copenhagen, Denmark

Toppari J, Endocrine disrupters and sexual precocity. In the 2nd European Congress on Diagnosis and Treatment of Central Precocious Puberty, 1st International Symposium on Growth Hormone Treatment in Short Children: State of the Art, 17-18.3.2005 Pisa, Italy

Toppari J, Differences in male reproduction health between Denmark-Finland: effects of neonatal exposures. In the CREDO Workshop on Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects, 10-12.5.2005, Prague, Czech Republic

Toppari J, Incidences, regional differences and temporal changes in occurrence of hypospadias. In the NAFA Workshop on Hypospadias and Leydig Cell, 30.9-1.10.2005, Copenhagen, Denmark

Toppari J, Male reproductive health in Europe - Cryptorchidism and hypospadias. In the Workshop on the Development and Function in Adulthood of the Male Reproductive System Potential Chemical-induced Effects, 15.2.2006, York, UK.

Toppari J, Mechanisms of male undermasculinisation. In the 20th Summer School of the European Society for Pediatric Endocrinology, 26-30.6.2006, Nunspeet, Holland

Toppari J, Trends in congenital abnormalities of male reproductive system: possible role of endocrine disrupters. In the 45thAnnual meeting of the European Society for Paediatric Endocrinology (ESPE) on Pediatric Endocrinology: The impact of programming, 30.6-3.7.2006, Rotterdam, The Netherlands

Toppari J, The Finnish-Danish gradient in male reproductive health. In the Life Cycle of the Gonad symposium in honour of professor Niels E. Skakkebæk, 25.10.2006, Copenhagen, Denmark

Toppari J, Biological and toxicological mechanisms leading to altered semen quality, with a focus on mechanisms relevant to environmental contaminants. In the International Workshop on Air Pollution and Human Reproduction. 9-11.5.2007 Munich, Germany

Toppari J, Cryptorchidism up-date. In the Centre of Reproductive Medicine, Scanian Andrology Centre, Malmö University Hospital, Lund University. 15.5.2007 Malmö, Sweden

Toppari J, Male reproductive disorders in Nordic-Baltic area. In the 2nd Workshop on Reproductive Biomedicine. 17-19.5.2007, Tartu, Estonia

Toppari J, Early signs of problems in male reproductive helth. In the XVIII annual meeting of Sociedad Chilena de Reproduccion y Desarrollo, 16-18.8.2007 Chillan, Chile.

Toppari J, Endocrine disruption intruders. In the 1st Workshop on Environmental and Health. Capacity building for decision making, 17-21.9.2007 Kos, Greece.

Press and Magazines

Outcomes from EDEN have been widely publicised in the media and popular press. This has included television appearances on endocrine disruption and the mixture issue (Kortenkamp A, ITV, United Kingdom) and *in vivo* test methods used for assessing antiandrogenic effects in rats (Hass U, Arte, France).

Newspapers throughout Europe have reported on many results arising from EDEN. Examples include:

- Utilising the TEXB approach for EDCs pioneered by Nick Olea and colleagues (Spain).
- Poor sperm quality and male reproductive health in Denmark and Finland and comparisons between these two countries as led by Niels Skakkebæk and colleagues (Denmark) and Jorma Toppari and colleagues (Finland).
- Rat mixture studies showing marked adverse mixture effects at dose levels where the individual chemicals alone show no effects carried out by Ulla Hass and colleagues (Denmark).

The popular scientific magazine, the New Scientist has often cited EDEN outcomes with two features referring to the work carried out by Manual Tena Sempere, Ulla Hass and Andreas Kortenkamp.

As part of the International Conference entitled 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects' organised by the EDEN and FIRE consortia, a press conference held in Brussels was organised and reported in the European press.

Cluster of Research on Endocrine Disruption in Europe (CREDO)

Brochure, Newsletters and Website

A network of research called the Cluster of Research on Endocrine Disruption in Europe (CREDO) was established under the jurisdiction of the EDEN project (http://www.credocluster.info/intro.html). CREDO would facilitate effective research collaborations across a core of 4 projects (EDEN, COMPRENDO, EURISKED and FIRE) and a further 8 projects funded in the last round of the Fifth Framework programme. A brochure profiling CREDO was first available detailing the aims of the cluster and research programme (http://www.credocluster.info/docs/credobrochure.pdf).

Eight newsletters were published from September 2003 to November 2006 communicating research features from the projects and information on workshops. The final double issue (7&8) provided summaries of the findings from these projects, along with the perspectives for environment and health research activity in the Seventh Framework Programme (FP7).

EDEN Website

A website was developed to inform the public and scientific community about the EDEN project and its progress. The site introduces the research area and the projects aims and research themes. The 'Achievements' section gives the publications arising from the EDEN project which number >100. The 'what's new' section is continually updated including a section on the Prague Declaration on Endocrine Disruption which arose from the Workshop held in Prague 2005 and there is also a separate header with the complete document. As well as the summaries for each of the four yearly reports, a poster presented to the GRC on the activities arising from EDEN is available on the 'what's new' section of the website. All eight issues of the CREDO Cluster newsletter are also available as pdfs on the main site, along with a synopsis of each issue. A separate part of the website is only accessible to EDEN members to aid communication.

International Workshop 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects'

International experts and scientists representing many different disciplines came together in Prague on 10 – 12 May 2005 for a workshop on chemicals that interfere with hormone systems, so-called endocrine disrupters. The workshop 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects' was organised by the EDEN and FIRE consortia and was convened to discuss recent European research on the health risks associated with these chemicals. At this international workshop, scientists from across Europe presented the latest research findings in endocrine disrupter research. Much of this work emanated from large research projects funded by the European Union, and joined together in the cluster for research on endocrine disrupters, CREDO. There were discussions to promote the transfer of know-how between scientists on the issues of exposure assessment, epidemiology, low-dose and mixture effects. The programme included sessions on male reproductive health in Europe, human and wildlife exposure to endocrine disrupters, novel endpoints and biomarkers, as well as on low-dose and mixture effects of endocrine disrupters and their assessment. The results presented at the Prague workshop have reinforced concerns over the long-term consequences of exposure to endocrine disrupters to humans and wildlife. Outcomes from the workshop resulted in a Special Issue in Environmental Health Perspectives which was available online from June 2007. The workshop also gave rise to the 'Prague Declaration on Endocrine Disruption' which has been signed by over 200 scientists (http://www.edenresearch.info/public/PragueDeclaration%2026%20May%202006.pdf).

Prague Declaration on Endocrine Disruption

The Prague Declaration which to date has been signed by more than 200 scientists active in the field of endocrine disruption was unveiled at a press conference held in Brussels 2005, the press release is given below:

"EMBARGOED UNTIL 20 JUNE 2005

PRESS RELEASE

LEADING SCIENTISTS CALL ON EU TO TAKE ACTION ON HARMFUL CHEMICALS

Brussels, 20 June 2005. Leading scientists today called on the EU to take precautionary measures to protect humans and wildlife from chemicals that interfere with the hormone system, known as endocrine disrupters.

More than 100 scientists actively working in research in this area in Europe and in the US have now signed the Prague Declaration on Endocrine Disruption which sets out latest research results in the area, as well as highlighting the shortcomings of the EU's proposed REACH regulation for dealing with chemicals.

"*Recent research that has been carried out in Europe and in the US indicates that existing rules to protect babies and young children from certain chemicals are targeting the wrong life stage*, " said Dr Andreas Kortenkamp, coordinator for the EU-funded EDEN research project into endocrine disruption. "*We need to protect pregnant women from exposure to these chemical substances so that we prevent genital abnormalities occurring in the developing foetus*."

Endocrine disrupters are a diverse group of chemicals in everyday use, including some pesticides, flame retardants, pharmaceuticals and certain plasticisers or phthalates found in soft vinyl plastic toys and cosmetic ingredients, for example.

At today's press conference, Professor Niels E. Skakkebæck M.D, who coordinates EDEN's research into human male reproductive health presented research detailing the high prevalence of reproductive disorders in European boys and young men and the rise in cancers of reproductive organs, such as breast and testis. "*We have identified an extremely disturbing trend that shows a substantial rise in genital disorders in boys and young men in Europe*," said Professor Skakkebæck. "*We need to make absolutely sure that research is constantly updated in this area*."

Professor Dr Jörg Oehlmann is coordinating European research into wildlife effects produced by endocrine disrupters, as part of the EU-funded COMPRENDO project into comparative research on endocrine disrupters. "*The severity of the endocrine disrupting effects we have observed in wildlife as a direct consequence of exposure to certain chemicals is a cause for concern amongst scientists around the world. We should remember that, while wildlife represents a protection target in its own right, it also provides early warnings of effects produced by endocrine disrupters which may as yet be unobserved in humans*."

Dr Kortenkamp warned that current proposals by the EU to regulate chemicals do not cover endocrine disrupting chemicals. "*At the moment, REACH does not specify endocrine disrupting properties nor does it include clearly defined criteria to objectively identify substances with endocrine disrupting properties. This could mean that endocrine disrupters will fall outside of the EU authorisation process which would be a major obstacle to the efficient regulation of chemicals, and its intended role to protect humans and wildlife from harm*."

ENDS

NOTES FOR EDITORS

At a recent meeting in Prague, European scientists working in this area agreed the Prague Declaration on Endocrine Disruption which now has over 100 signatories, including leading scientists from the US.

The text of the Prague Declaration is accessible at http://www.edenresearch.info/ under the 'what's new' section. The names of the scientists are also listed.

In the past month, the results of more research into the area of endocrine disrupters have shown that exposure to certain chemicals in everyday use pose serious health concerns. These published scientific studies include research linking pregnant women's exposure to phthalates and adverse effects on the genital development of their male children. A summary of recent scientific studies is included in the press pack.

REACH is the proposed European regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals."

The Prague Declaration can be accessed from the EDEN website (http://www.edenresearch.info/declaration.html). It has had wide dissemination including a journal and broadsheet:

- Europa research. http://europa.eu.int/comm/research/environment/newsanddoc/article_2826_en.htm
- EPHA Environment Network. http://www.env-health.org/a/1821
- Our Stolen Future. http://www.ourstolenfuture.org/Consensus/2005-0620praguedeclaration.htm
- ESPR – *Environ. Sci. Poll. Res*. **12** (4) 188 (2005). http://www.scientificjournals.com/sj/espr/Pdf/aId/7527
- EMHF European Men's health forum. http://www.emhf.org/index.cfm/item_id/291
- Guardian June 20 2005 http://www.safecosmetics.org/newsroom/the_guardian_6_20_05.cfm
- EUROPEAN WATER MANAGEMENT NEWS, 3 august 2005 http://www.nwp.nl/objects/EWMN%203%20August%20-%202005.doc
- PAN issue no 23 July – august 2005 http://www.pan-europe.info/newsletter/news23.shtm

Expert Panel Workshop on the Options for Incorporating Knowledge and Low-dose and Mixture Effects in Testing Strategies and Regulatory Efforts

An Expert Panel Workshop was convened on 15-19 May 2006 in Granada to discuss the options for incorporating knowledge on low-dose and mixture effects in testing strategies and regulatory efforts. Invited attendees represented national regulatory agencies, NGOs and research organisations. A representative from Schering AG, Berlin, Germany was contacted and invited, but his company was uninterested and did not grant permission to attend. The workshop itself was very productive. The panelists worked on a stepped procedure for mixtures regulation and risk assessment.

First considerations were to which chemicals should be included for EDC mixture regulation, and a decision on this issue depends on an answer to the question as to how to define what an EDC should be. It was felt that it is important to distinguish "mode of action" from "effects". "Endocrine disruption" is a mode of action relevant to developmental and reproductive toxicity, but not an effect. Instead, this mode of action can give rise to a plethora of different effects. Mechanistic considerations open the way for grouping EDCs according to test results in "mode of action" screens, as with the customary classification into "estrogens", "(anti)androgens", "thyroid-actives" (EAT). However, the panelists recognised that this classification, with its emphasis on steroid and thyroid receptor interactions, does not capture all known endocrine effects and therefore suggested to include as additional category "others", thus: EATO. The "mode of action" screen classification leads into a dilemma: The EATO grouping is of limited relevance for risk assessment, because the predictive value of such screening outcomes for the occurrence of (adverse) effects is questionable. For example, not all androgen receptor antagonists produce responses typical of disruption of male sexual development (such as NR, altered AGD etc). For estrogens, *in vivo* effect models capturing the conditions of concern in the human (e.g. breast cancer) are not available. Furthermore, screens are imperfect, as exemplified by the case of certain phthalate esters which disrupt male sexual development by interfering with steroid synthesis, and not by receptor interactions, yet many antiandrogen screening tools do not detect these phthalates. On the other hand, a grouping of EDCs in a phenomenological fashion according to effects is not currently possible, because too few chemicals have been tested in relevant toxicity studies, or because the appropriate tests are not yet available. This situation is not likely to change in the foreseeable future.

An all-encompassing solution is not on the horizon, however, for specific chemicals, effects and exposure scenarios it is now well established that EDCs can act in an additive fashion. Therefore, there are possibilities for utilising existing knowledge and data for making progress with risk assessment and regulation that takes account of mixture effects. Several examples and exposure settings were discussed including: a) vitellogenin induction in fish from cumulative exposure to estrogenic chemicals (so-called mixture maps); b) NR and changes in AGD in the rat; c) Spiggin induction in the stickleback and d) internal exposure to estrogens in women. Conclusions were that CA is a powerful risk assessment tool for cumulative exposure in these examples. The panelists highlighted that knowledge about realistic exposure scenarios for EDC mixtures is fragmentary. In most cases, we simply do not know enough about the identity of EDC that co-occur in food,

environmental matrices or human tissues, let alone their levels. This presents a formidable bottleneck to rational risk assessment for combined exposures – some participants even thought that this lack of data is the obstacle to making progress. Thus, there is a need to deal with knowledge gaps by making informed assumptions, and a step-wise approach, depending on the quality of data available, was discussed.

The following situations could be distinguished: a) In some cases, sufficient knowledge about the identity and levels of relevant EDCs is available – in these situations dose addition can be applied; b) More often, however, there is uncertainty as to whether specific EDCs are able to induce the effects under consideration, but there may be some reason for concern. In these cases, a "mixture assessment factor" could be applied for such EDC, so as to enable risk assessors to proceed and c) If no data or information is available, it was considered to apply a default mixture assessment factor, making certain assumptions about the likely number of chemicals in the mixture.

POLICY RELATED BENEFITS

The results of EDEN are of considerable importance in providing better chemicals risk assessment and regulation in the European Union. The following project findings have policy relevant benefits:

- The observation that no individual EDC at relevant exposure levels could be shown to be associated with endocrine-related disorders in the human highlights the importance of developing better biomarkers that capture cumulative exposure to EDC. Very likely, this should involve biological measures of cumulative internal exposure. This finding also presents a major challenge to risk assessment and risk reduction measures: If the contribution of single chemicals to possible risks stemming from endocrine disruption is small, then regulatory approaches have to be developed that adopt a holistic approach and capture the entirety of endocrine active chemicals, across various regulatory arenas, including pesticides, non-pesticide contaminants, and chemicals used in consumer products and personal care products.

- The realisation that the testing and screening for EDC effects has not kept up with advances in our understanding of rapid signalling events triggered in the wake of steroid receptor activations should stimulate the development and validation of additional screening tools that are able to capture these signalling events. This is to avoid that endocrine active agents are overlooked.

- Of particular concern are the observed declines in male reproductive health in parts of Europe, especially in Germany and in relation to the narrowing of the differences between Finland and Denmark. These findings are policy relevant in as far as they highlight the need to take urgent action to avoid personal suffering and an elevation of public health costs.

- The EDEN project has provided evidence that the application of hypothesis testing methods which aims at estimating no-observed-adverse-effect-levels (NOAEL) is inadequate for capturing low-dose effects of EDC. Regression-based approaches with their benchmark dose limits should replace NOAEL as the basis for establishing acceptable human exposure levels. Ideally, mandatory risk assessment procedures in the EU should be amended to combine hypothesis testing and regression methods in a framework that utilises the strengths of both methodologies by making considerations of statistical detection limits and statistical power the starting point of testing procedures. Implementation of this framework will require a significant change in toxicological testing practice and regulatory policy in Europe, but will have considerable benefits in providing better protection for European citizens.

- The extensive experimental mixture studies carried out in the EDEN project have significantly advanced knowledge about determinants and factors that govern the joint action of similarly acting EDC. Pragmatic risk assessment approaches that take mixture effects into consideration are now a viable proposition. Implementation of such approaches in relevant procedures should be pursued with

vigour, because the customary chemical-by-chemical approach to risk assessment will likely lead to underestimations of risk when exposure is to a large number of similarly acting EDC.