Project Progress Summary Year 3

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Section 2: Project Progress Report **NOT CONFIDENTIAL**

Objectives:

EDEN is a multidisciplinary effort designed to address key issues that currently hamper hazard- and risk-assessment for endocrine disrupting chemicals (EDCs) in the European Union. EDEN will fully integrate human, wildlife, exposures, mechanisms and low-dose/mixture-evaluations, with the following specific objectives:

▪To gather data about the composition of complex mixtures of endocrine disrupting chemicals (EDCs) in human and fish tissues from within the European Union.

▪To investigate the mechanisms underlying the action of EDCs to evaluate current experimental models for wildlife and human hazard assessment and to develop novel endpoints and biomarkers for the early detection of effects.

▪To provide new insights into indicators of impaired reproductive function and to extend and improve existing European databases.

▪To gather data about low-dose effects of EDCs, and to develop criteria for low-dose studies by evaluating various bio-statistical approaches for estimating low responses.

▪To assess the effects of multi-component mixtures of EDCs and to investigate whether EDCs produce joint effects when combined at doses below their individual effect thresholds

▪To assess how low dose- and mixture effects ca be considered in testing guidelines and risk assessment procedures for wildlife and humans

Results and Milestones:

In order to understand how EDCs produce effects, tissues from young boys, women and fish have been collected for cumulative analysis for endocrine disrupting chemicals (EDCs). Extensive analyses of numerous EDCs are well underway utilising several procedures developed specifically for EDEN. For fish samples, two issues currently being addressed are a) whether the range of chemical contaminants associated with the presence of ovotestis (intersex) differs from that in unaffected fish and b) do tissue chemical contamination profiles explain divergent plasma VTG levels in bream from a site not known to be impacted by STW effluent?

The effects of EDCs have been shown to involve cross talk between signalling pathways in addition to the classical ER pathway. Further, aromatases are important factors in EDC action. Effects of dioxin and phthalates on the testis in fetal life are more dose sensitive than in the adult which may be explained by impaired Leydig cell function. An assay to measure Leydig cell function in humans (insl3) has been developed and validated. Cryptorchid boys have lower Insl3 levels than normal boys, indicating defects in Leydig cell function. EDCs have been postulated to modulate GnRH secretion in immature female hypothalamic explants *in vitro*, through both rapid and/or slow effects that likely involve both estrogen and dioxin receptor pathways.

Clinical examinations, sample collection and laboratory analyses have been completed in the German study on reproductive health of young men, with all data held on the central European database on male reproductive health. The follow-up study of young Finnish men has been extended, supported by national funding. The first report on the relationship between male reproductive health and maternal exposures during pregnancy has been published, but further associations may be unveiled as the questionnaire and clinical data are further examined.

Experiments designed to assess the effects of low doses of EDC in cultured cells, fish and rodents are completed. Unusually-shaped dose-response curves were not observed, but the work highlighted the weaknesses of customary approaches for estimating low dose effects. Significant progress has been made

with mixture studies. The effects of a four-component mixture on zebra-fish and of a three-component mixture on male sexual differentiation in the rat could be successfully predicted by using the concept of dose addition. These observations are of great importance for the regulation of EDC. Preliminary organisation for expert panel meeting and identification of participants has been undertaken which is scheduled to be held in May 2006.

EDEN continues to play a key role in fostering collaborations among EU-projects on endocrine disruption within the CREDO cluster. Extensive publicity has been achieved for the Prague Declaration on Endocrine Disruption which over 200 scientists have signed. The EDEN project continues to attract attention worldwide.

Benefits and Beneficiaries:

New assays have been developed and molecular tool utilised by research scientists to allow the clinicians and epidemiologists to test for novel biomarkers in human population studies. The relationship between EDCs and their effects in a range of test systems are being explored which will feed into risk assessment strategies. A database of information pertaining to human male reproductive health in European countries will allow further investigation into the relationship between issues currently affecting human health and causative compounds.

EDEN will strive to determine whether the current hazard and risk assessment strategies currently in place in the European Union are sufficient to deal with the issues of EDCs or require revising. The results of exposure assessment and mixture studies undertaken will help EDEN to assess how this data can be taken into consideration in testing guidelines and risk assessment procedures for wildlife and humans.

All of the information gained from this study is of direct relevance to the research community, the citizens of the European Community and industry.

Future Actions (if applicable):

Progress Report

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Contents

1. OBJECTIVES AND EXPECTED ACHIEVEMENTS

EDEN is an interdisciplinary effort designed to address key issues that currently hamper sound hazard- and risk assessment for endocrine disrupting chemicals (EDC) in the European Union. It adopts an approach that fully integrates human, wildlife, exposures, mechanisms and low-dose/mixture-evaluations, with the following specific objectives;

1. To gather data about the composition of complex mixtures of endocrine disrupting chemicals (EDC) in human and fish tissues from different areas of the European Union.

Systematic exposure assessments that provide a global view on EDC, and take the mixtures issue into account, are missing. EDEN will bridge this gap by analysing tissue specimens from humans and fish that show reproductive disorders. In this way, EDEN will optimise the chances of detecting potential causal relationships and will provide a rational basis for wildlife and human risk assessment. As much as possible, the data gathered in these studies will also inform experimental mixture studies.

2. To investigate the mechanisms underlying the action of EDC in order to evaluate the relevance of existing experimental models for wildlife and human hazard assessment and to develop novel endpoints and biomarkers for the early detection of effects.

The relevance of many currently used *in vivo* EDC assays to real existing effects in humans and wildlife is often unclear. EDEN will focus on endpoints in laboratory animals that have a clear relationship to real existing disorders in humans and fish. The project will identify key mechanisms and common pathways and will lead to the development of new cell-based, rodent and fish models, as well as clinical assays for the early detection of EDC effects.

3. To provide new insights into indicators of impaired reproductive function in European citizens and to extend and improve existing European databases.

Disruption of hormone production in foetal life leads to disorders of the male reproductive system. Some of these effects are evident at birth; others become obvious only later in life. Lifelong lowering of testosterone levels may link these perinatal and adult events. Using unique and extensive collections of longitudinal human blood samples, EDEN aims to test this hypothesis. The potential link between foetal and adult disorders will also be evaluated in animal mechanistic studies by studying the effects of EDC that induce lowering of testosterone levels in foetal life. Existing databases on sperm quality will be improved by extending the analyses to European countries not previously studied. This work will open new possibilities for proving/disproving links between health and environmental exposure.

4. To gather data about low-dose effects of EDC, and to develop criteria for well-conducted low-dose studies by evaluating the usefulness of various bio-statistical approaches for estimating low responses.

There is a dearth of appropriately conducted studies for the detection of low-dose effects of EDC. In order to address this controversial issue scientifically, it is necessary to carry out studies on the basis of bio-statistical considerations already at the stage of experimental design. EDEN aims to improve our knowledge about low-dose effects in cell-based assays, fish and laboratory rodents and will specifically address claims that the action of EDC is not coupled to thresholds. This work will produce scientific data that provide the basis for guidance about ways of regulating EDC and will be invaluable for the development of appropriate testing regimens. As much as possible, these studies will make use of EDEN's development of novel endpoints.

5. To assess the effects of multi-component mixtures of EDC and to investigate whether EDC produce joint effects when combined at doses below their individual effect thresholds

Statistically valid low-dose effect data will form the basis for evaluations of the joint effects of multi-component mixtures of EDC. Assessments in terms of synergism or antagonism will have to rely on pharmacologically sound concepts for the calculation of additive mixture effects. EDEN aims to produce information about the joint effects of EDC at low concentrations in cell-based assays, fish and rodents. A key issue of these studies will be to explore whether combination effects are predictable from information about the potency of individual mixture components. As much as possible, exposure assessments in environmental media and tissues will guide the selection of suitable test chemicals and will enable EDEN to make links to real exposure scenarios in wildlife and humans.

6. To assess how low dose- and mixture effects should be taken into consideration in testing guidelines and risk assessment procedures for wildlife and humans

Existing testing regimens are not designed for the identification of low-dose- and mixture effects. The experience gathered with low-dose- and mixture studies will enable EDEN to address the question as to how testing- and risk assessment procedures should be modified to take account of these issues.

2. PROJECT WORKPLAN

2.1 Introduction

EDEN will proceed in four parallel strands centred on the following themes:

Theme 1: **Complex EDC mixtures in human and fish tissues - exposure assessment** (WP1-4)
Theme 2: **Mechanisms of EDC action - novel endpoints and biomarkers** (WP5-10)
Theme 3: **Indicators of impaired reproductive function in European men** (WP11-12)
Theme 4: **Low-dose- and mixture effects of EDC - providing empirical evidence and exploring implications for regulation and testing** (WP13-16)

As shown in Figure 1 below, the strands of the project are strongly interlinked: The work aimed at establishing the composition of EDC mixtures in human and fish tissues (WP1-4) will provide valuable guidance for experimental mixture studies (WP14-15). The identification of key mechanisms and novel endpoints of EDC action (WP5-10) will assist the work on human reproductive health (WP11-12) and on low-dose- and mixture effects (WP13-15). The latter studies will feed into work considering how testing- and risk assessment procedures should be modified to take account of low-dose and mixture effects of EDC.

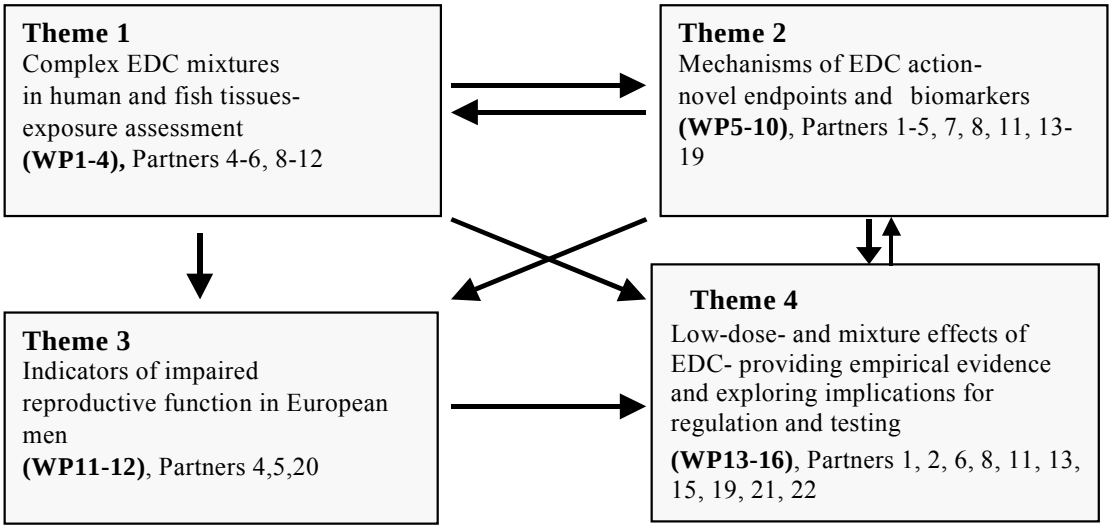

Figure 1 Structure of the workplan and management structure for EDEN

2.2 Project Structure, Planning and Timetable

Theme 1: **Complex EDC mixtures in human and fish tissues - exposure assessment** (WP1-4) (Partners 4-6, 8-12)

Numerous scientific papers have shown that humans and wildlife in the European Union come into contact with EDC, often from quite unexpected sources. It appears that typical

exposure scenarios for humans and wildlife involve exposure to a very large number of EDC, with individual EDC present at rather low levels. This situation requires integrated approaches to exposure assessment that provide a global view on many divergent and seemingly unconnected groups of chemicals. However, such data are completely missing. Instead, the literature is full of reports that focus on particular groups of EDC. In closing this gap, WP1-4 will produce the foundations for EDC exposure assessments that take account of the mixtures issue. Furthermore, the results of WP1-4 will provide valuable guidance for the selection of mixtures to be tested in WP14 and 15.

To realise these aims, EDEN will in the first instance focus on tissues that are sinks for a large number of EDC. Many EDC accumulate in adipose tissue of humans and fish. In fish, bile often shows extraordinarily high concentrations of a large variety of xenobiotics and their metabolites. For these reasons, human and fish adipose tissue and fish bile will be selected for in-depth analyses.

Meaningful exposure assessments have to rely on comparisons with appropriate control samples. However, with many EDC this is complicated by the fact that they are widely dispersed and consequently found everywhere in the biosphere. EDEN will deal with this problem by carrying out comparative analyses on specimens from humans and fish with and without symptoms indicative of endocrine disruption.

In establishing the spectrum of EDC present in tissue specimens, EDEN will adopt a two-pronged approach:

(1) Chemical analyses - Tissue specimens will be analysed for a large number of different groups of chemicals with known endocrine-disrupting potential. Not only will this provide useful data in relatively short periods of time, but also - due to the small sample amounts required - allow exploratory studies aimed at establishing exposure-effect relationships. However, comprehensive exposure assessments of EDC require an alternative approach, since analyses for chemicals with known endocrine-disrupting potential may prove limiting. For this reason, EDEN will adopt the more open-ended search for EDC explained in (2):

(2) Bioassay-directed extractions and fractionations - Specimens will be extracted and extracts interrogated with appropriate bioassays in order to provide summary biological measures of endocrine-disrupting potential (total “estrogenicity”, “anti-androgenicity” etc.). Further fractionation and biotesting, followed by chemical analyses, have the potential to reveal previously unknown EDC. In contrast to approach (1), these studies require large amounts of tissue specimens for preparative scale extractions and fractionations. Therefore, pooled specimens will be used.

Work will proceed in three steps: (i) Collection of tissue specimens from humans (WP1) and wild fish (WP2) and storage and distribution to Partners responsible for analyses; (ii) chemical analyses (WP3); and (iii) bioassay-directed extractions and fractionations (WP4).

Theme 2: Mechanisms of EDC action - novel endpoints and biomarkers (WP5-10) (Partners 1-5, 7, 8, 11, 13-19)

The ability of EDC to modulate gene expression is thought to be the initial step in effector chains that lead to differing phenomenological effects. Thus, the application of modern molecular biology technologies to the study of EDC offers the opportunity to discover novel endpoints that are very sensitive to EDC exposure. WP5 aims to:

- search for new EDC-responsive marker genes in MCF-7 cells and zebra fish by applying DNA array technology, differential display, RT-PCR, and proteomics;
- carry out detailed analyses of the mechanisms underlying gene expression and of relevant signalling pathways, in order to aid the construction of transgenic fish in WP10, and;
- evaluate novel biomarkers for use in low-dose and mixture studies (WP13-15)

MCF-7 cells and zebra fish obtained in WP13-15 will be utilised for comparative gene expression studies by using RT-PCR and differential display and DNA microarrays, proteomics as well as immunohistochemistry and in situ hybridisations. Cell lines will be transfected with expression vectors containing zf ER (ERa and ERb) and reporter gene constructs will be used to evaluate the impact of low-dose mixtures on the transcriptional activity of each ER subtype.

<u>New models for the study of male reproductive disorders - an introduction to WP6-8</u>
Human male reproductive disorders (germ cell cancer, hypospadias, cryptorchidism, low sperm counts) can all arise because of abnormalities in testis development. Although disturbances of hormone production by the foetal testis and of hormone action or balance ('endocrine disruption') are accepted as a major factor in these reproductive developmental disorders, it is unknown what causes this disruption. The mechanistic pathways leading to these effects are ill-defined and there are no endpoints that reliably signal this disruption. This lack of basic understanding and the absence of endpoints present considerable obstacles to rational assessments of whether or not EDC might contribute to human male reproductive disorders. These knowledge gaps have also meant that researchers searching for EDC effects in animal studies, including 'low dose' effects, have resorted to undefined endpoints (e.g., uterine and prostate weight), that show no clear relationship to foetal testis function or to the human reproductive disorders of interest. Motivated by these deficiencies, the animal experimental studies in this proposal are comprised of three workpackages (WP6-8) that will:

- define the pathways of endocrine disruption that lead to the reproductive developmental abnormalities of interest in the human and evaluate new endpoints arising from these studies, and
- define the sensitivity to endocrine disruption of the hypothalamic-pituitary unit.

Work will be broken down into the following WP: (i) Foetal exposure of rats to phthalates - a new model for the identification of mechanisms underlying the "testicular dysgenesis syndrome" in human (WP6); (ii) InsL3 levels in blood as an indicator of endocrine disruption leading to reproductive developmental disorders (WP7); and (iii) hypothalamic-pituitary (HP) sensitivity to endocrine disruption: Mechanisms, consequences, new endpoints and relevance to man (WP8).

Novel effect markers for the study of endocrine disruption in fish - introduction to WP9 and 10

To date, most of the effects on aquatic wildlife attributed to EDC are of the feminising type and are thought to be mediated by estrogens. Knowledge about the action of oestrogenic chemicals on fish is fairly advanced. However, information about EDC that interact with different hormonal systems is comparatively sparse. The need to focus more on non-oestrogenic chemicals found in surface waters is highlighted by recent observations of androgenic discharges by pulp mills, and of severely masculinised fish downstream of these mills. There is also a lack of methods for the detection of the effects of anti-androgenic compounds in surface waters. Furthermore, a truly non-invasive assay for the identification of endocrine disruption in fish is missing. This part of EDEN comprises two WP that aim to improve this situation by

- optimising the stickleback assay as a method for the simultaneous screening of oestrogenic and (anti)androgenic chemicals, and
- developing a transgenic, translucent fish model, based on the expression of the green fluorescent protein (Gfp).

The corresponding WPs are: (i) Vitellogenin- and spiggin induction in the stickleback as biomarker for the simultaneous identification of oestrogenic and (anti)androgenic EDC in surface waters (WP9); and (ii) a transgenic fish model based on the green fluorescent protein (Gfp) as a marker for oestrogen exposure in translucent fish (WP10).

Theme 3: Indicators of impaired reproductive function in European men (WP11-12) (Partners 4, 5, 16)

It has been suggested that the adverse changes and regional differences in male reproductive health in Europe are associated with exposure to EDC during foetal development, leading to manifestation of a Testicular Dysgenesis Syndrome (TDS). In the past, the focus of study has been on disturbed Sertoli cell function, as this is suggested to be associated with declines in semen quality and the development of testicular cancer. Possible adverse effects on Leydig cell function have received less attention, although disturbed Leydig cell function - through altered testosterone production - may have distinct effects on male reproductive health. Recent unpublished results indicate that significant regional differences in serum testosterone levels in young men exist throughout Europe.

When comparing the age-related decline in serum testosterone observed in cross-sectional studies with individual age-related declines based on longitudinal samples, a discrepancy becomes evident. The individual decline in serum testosterone levels seems to be much larger than the population based age-related decline in testosterone. This suggests that a birth cohort effect is present, with lower serum testosterone levels in younger cohorts. It is the objective of WP11 to test this hypothesis. Both cross-sectional and longitudinal serum samples available through population-based health surveys in Europe during the last decades will be used.

Studies on male reproductive health in Europe have revealed large regional differences; however, the majority of this work has focused on North European countries. WP12 aims to extend such studies to Central Europe and to improve an existing database on male

reproductive health. Studies will be carried out in the former East and West Germany, in order to take advantage of the fact that environmental exposures in East and West Germany differed substantially in the past, while the populations are genetically similar. This situation offers the unique possibility to investigate the role of environmental factors in male reproductive health. Better knowledge of regional differences in male reproductive health may maximise the chances of identifying environmental factors that affect reproduction.

Recent European studies have revealed a secular trend, with poorer sperm quality observed in the later-born men. The proposed work will establish whether similar trends exist in Central Europe and will continue in North Europe. Information on lifestyles, occupation, eating habits etc. will also be gathered and analysed.

Theme 4: Low-dose- and mixture effects of EDC - providing empirical evidence and exploring implications for regulation and testing (WP13-16) (Partners 1, 2, 6, 8, 11, 13, 15, 19, 20, 21)

Many studies that have explored low-dose effects of EDC were not designed to biometrical and statistical standards that would allow a conclusive resolution of the low-dose problem. Connected to this issue is a debate about the limitations of the methods used to derive no-observed-effect-levels (NOEL).

A second perspective on the low-dose problem has emerged: EDC may not exhibit effect thresholds when acting in concert with endogenous hormones. It has been argued that in such situations every quantum of external exposure will lead to additional effects, even if exposure is infinitesimally small. In view of the immense implications of these findings for the regulation of EDC, it is urgently required to carry out studies in other experimental models, with a view to probe the existence of hyperbolic dose-response curves. WP13 aims to make a contribution to resolving these issues by

- systematically comparing low-dose estimates derived from two-point comparisons (NOEL) with those produced by regression-based approaches,
- establishing the shape of dose-response curves in order to provide guidance for assessing the threshold issue, and
- developing criteria for well-conducted EDC studies that permit better estimations of low-dose effects

Work on mixtures will proceed in two steps: (i) Assessment of combination effects of similarly acting EDC (WP14); and (ii) combination effects of dissimilarly acting EDC (WP15).

Focusing on EDC that induce phenomenologically similar effects, WP14 aims to investigate whether

- the joint effects of EDC on cells, rodents and fish can be predicted from knowledge of their individual potencies, and
- whether EDC produce observable mixture effects when combined at doses below their NOEL

As in WP13, Partners will make optimal use of the specimens generated here by making it available for the study of novel endpoints (WP6-8). WP14 will yield valuable data about mixture effects of EDC and are a unique attempt to extend mixture assessments to *in vivo* assays.

It is unclear how EDC that induce differing effects will act in combination. This is a question of great importance for hazard and risk assessment. For instance, it has been suggested that the possible adverse effects of oestrogenic EDC may be neutralised by the presence of anti-oestrogenic chemicals, such as dioxins or coplanar PCB, but conclusive data are missing. Furthermore, it is at present unforeseeable how oestrogenic and (anti)androgenic EDC act in concert. WP15 aims to address these questions by

- assessing the impact of antioestrogenic PCB on cell proliferation, oestrogen receptor expression and oestrogen-inducible genes, caused by oestrogenic EDC and elucidating underlying mechanisms,
- exploring how oestrogenic, androgenic and anti-androgenic EDC act in combination on the stickleback, and
- investigating possible antagonistic effects of anti-oestrogenic EDC on the action of anti-androgens on male rats exposed *in utero*.

EDEN will produce a large amount of scientific data about low-dose- and mixture effects of EDC. The question arises as to the possible implications of all this information for testing strategies and for risk assessment procedures. Is it necessary to amend existing testing guidelines to take account of low-dose- and mixture effects, and if yes, how can this be achieved? Is it necessary to alter current risk assessment paradigms to incorporate combination effects? Can the concepts used in WP14 and 15 for the calculation of expected mixture effects be utilised for prospective assessments of combination effects of EDC, thereby making use of existing data on their potency? What are the options for regulating EDC - should they be treated like genotoxic carcinogens or can they be regulated using procedures that assume effect thresholds? WP16 will address these issues by evaluating the data produced in WP 13-15, taking account of the exposure assessments in WP1-4.

These complex issues will be debated with the participants of other EU projects on endocrine disruption which together will form the **Cluster for Research on Endocrine Disrupters in Europe** (CREDO), and with a panel of experts from national regulatory agencies, industry and NGO's at a series of annual workshops. WP16 will produce a guidance document on options for the regulation of chemicals able to protect European wildlife and people.

Table 1 Project timetable

Workpackage	Year 1	Year 2	Year 3	Year 4
WP1	▬▬▬	▬▬▬		
WP 2	▬▬▬			
WP 3	▬▬▬	▬▬▬	▬▬▬	
WP 4		▬▬▬	▬▬▬	▬▬▬

WP 5				
WP 6				
WP 7				
WP 8				
WP 9				
WP 10				
WP 11				
WP 12				
WP 13				
WP 14				
WP 15				
WP 16				
WP 17				

Table 2 Deliverables

Deliverable No.	Deliverable title	Delivery date	Delivered	Delayed
D1.1	Annual report on the progress of human adipose tissue sample delivery	12, 24	✓ breast tissues	3 control (boys) due month 30
D1.2	Delivery of human adipose tissue samples	6, 12, 18, 24	✓ breast tissues	as above
D2.1	Delivery of fish adipose tissue and bile samples for chemical analyses	6, 12	✓	
D3.1	Preliminary results of chemical analyses in human and fish samples	18	✓	
D3.2	Database on EDC in human and fish tissues	36		in progress
D3.3	Levels of EDC in human and fish samples	36		in progress
D4.1	Report on suitability of EDC assays for screening of fractions	24		in progress
D4.2	Report on preliminary results with bioassay-directed fractionations	36		delayed until mth 40 (P9)
D4.3	Report about bioassay-directed fractionations of human and fish tissue samples	48		
D4.4	HRS technology for androgens	48		
D5.1	Candidate genes for construction of transgenic zebrafish	12	✓	
D5.2	Report on preliminary results with novel endpoints and tools	18	✓	

D5.3	Novel endpoints and tools for assessment of EDC effects in mammalian cells and fish	36	✓	
D5.4	Recommendations for utilisation of genomic responses in testing guidelines with fish	36	Preliminary report	
D6.1	Progress report on identifying endpoints of phthalate action on fetal testes	12	✓	
D6.2	Report on preliminary data concerning short-term and long-term effects of phthalates	24	✓	
D6.3	Report on comparative sensitivity of rat and human foetal testes to phthalates in vitro	36	* (see WP)	
D6.4	Report on new biomarkers of foetal phthalate exposure	36		delayed until month 42
D6.5	Final report on pathways of phthalate action on foetal testes	36		delayed until month 42
D7.1	Report on relative incidence of disorders of InsL3 expression by foetal LC in rats	36		delayed until month 46
D7.2	Report on InsL3 receptor expression in the gubernaculum	36		delayed until month 48
D7.3	Report on relation of InsL3 expression to characteristics of foetal Leydig cells in the rat	36		delayed until month 48
D7.4	Report on predictivity of InsL3 for reproductive disorders in humans and rats	36		delayed until month 48
D7.5	Report on in vitro screening systems for agents affecting InsL3 expression	36		delayed until month 48
D7.6	ELISA for the measurement of InsL3	36		delayed until month 46
D8.1	Progress report of HP unit work	18	✓	
D8.2	Report on comparative sensitivity of HP unit	36	✓	
D8.3	Report on dose levels of EDC able to activate HP	36		delayed until month 48
D8.4	Report on ability of EDC to induce changes in HP unit	36		delayed until month 48
D9.1	Standard operating procedure for use of the stickleback for EDC detection	12	✓	
D10.1	Progress report on production of transgenic fish	24	✓	
D10.2	Report on production of transgenic reporter fish	48		in progress
D10.3	Use of transgenic fish for the detection of EDC	48		in progress
D11.1	Birth cohort effects for serum testosterone	24	✓	
D11.2	Comparison of birth cohort effects for serum testosterone in different regions	24		* (see WP)
D11.3	Regional and temporal differences on birth cohort effects for serum testosterone	24		* (see WP)
D12.1	Report on male reproductive health in Germany	24	✓	

D12.2	Secular trends in male reproductive health in Europe	24	✓	
D12.3	European male reproductive health database accessible	36	✓	
D13.1	Report on preliminary low-dose testing results in cell-based EDC assays	12	✓	
D13.2	Report on preliminary low-dose testing results in fish assays (stickleback, zebrafish)	12	✓	
D13.3	Report on preliminary low-dose testing results in female rats	12	✓	
D13.4	Criteria document for low-dose testing	24	✓	
D14.1	Preliminary report about predictability of mixture effects in cell-based EDC assays	24	✓	
D14.2	Preliminary report about predictability of mixture effects in fish assays (stickleback, zebrafish)	24	✓	
D14.3	Preliminary report about predictability of mixture effects in female rats	24	✓	
D14.4	Mixture effects of EDC at levels below NOEL	48		
D15.1	Half-term progress report on dissimilarly acting EDC in cell-based assays	24	✓	
D15.2	Half-term progress report on dissimilarly acting EDC in fish assays (stickleback)	24	✓	
D15.3	Half-term progress report on dissimilarly acting EDC in female rats	24	✓	
D15.4	Joint effects of dissimilarly acting EDC	48		
D16.1	First considerations of data requirements for adequate risk characterisations of EDC mixtures	36	✓	
D16.2	Workshop with expert panel	36		organised for May 2006
D16.3	Guidance notes on the implications of knowledge about low-dose- and mixture effects for testing strategies and risk assessment procedures	48		
D17.1	Planning and coordination of CREDO cluster workshops	0 +	✓	
D17.2	CREDO cluster website	3	✓	
D17.3	Cluster newsletters	6, 12, 18, 24, 30, 36, 42, 48	✓ (6,12,18, 24)	5th due month 37
D17.4	Cluster brochure	3	✓	
D17.5	Cluster press releases	0, 12, 24, 36	✓ (0, 32)	
D17.6	Representation of cluster at policy meetings	0 +	✓	
D17.7	CREDO cluster workshop on dose-response analysis and mixture effects, testing guidelines, epidemiology	18	✓ (30)	

D17.8	Policy-relevant report on cluster workshops	42		
D18	EDEN project website	6	✓	
D19	Mid-term review of CREDO cluster work	18		

Table 3 Milestone List

Milestone No.	Milestone title	Delivery date	Delivered	Delayed
M1.1	Delivery of adipose tissue from human breast cancer cases and controls for chemical analyses	1 +	✓	
M1.2	Delivery of adipose tissue samples from cryptorchid boys and controls (I)	12	✓	
M1.3	Delivery of adipose tissue samples from cryptorchid boys and controls (II)	24	✓	
M2.1	Delivery of fish adipose and bile specimens	12	✓	
M3.1	Analytical data about the occurrence of EDC in human and fish tissue specimens	36	✓	
M4.1	Insights into the usefulness of EDC bioassays for bioassay-directed fractionations	24	✓	
M4.2	Information about the predictive power of total measures of endocrine disrupting potential of extracts	48		in progress
M4.3	Information about novel EDC in human and fish samples	48		in progress
M5.1	Identification of some marker genes involved in EDC action	12	✓	
M5.2	Recognition of signalling pathways in EDC action	36		dependent on WP14-15
M6.1	Identification of pathways of phthalate action on testis	36		due month 42
M6.2	Relationship of EDC pathways to reproductive disorders	36		due month 42
M6.3	New endpoints of phthalate action using proteomics	36		due month 42
M6.4	Comparison of phthalate action on rat and human testis	36		due month 42
M7.1	Relationship of InsL3 expression to reproductive disorders	36		due month 46
M7.2	ELISA for InsL3 in blood and testis extracts	36		due month 46
M7.3	InsL3 levels in boys and rats with reproductive disorders	36		due month 46
M8.1	Dose thresholds for disruption of HP unit	36		due month 48
M8.2	New endpoints of EDC disruption of HP unit using differential display	36		due month 48
M8.3	Precocious activation of HP unit by EDC	36		due month 48
M8.4	Effect of sub-threshold EDC levels on HP disruption	36		due month 48

M9.1	Standard operating procedures for tests in the stickleback	12	✓	
M10.1	Knowledge about the production of transgenic reporter fish	48		in progress
M10.2	Information about the specificity and sensitivity of transgenic reporter fish	48		in progress
M10.3	Information about the usefulness of transgenic reporter fish to detect endocrine activities of chemicals and effluents	48		in progress
M11.1	Retrieval of serum samples A from biobanks	8	✓	
M11.2	Retrieval of serum samples B from biobanks	12	✓	
M11.3	Serum testosterone levels in all samples	15	✓	
M11.4	Serum testosterone data incorporated in data base	18	✓	
M11.5	Serum testosterone data analysed within each region	24	✓ (P4)	on going (P5)
M11.6	Regional differences in serum testosterone levels analysed	24		begin month 40
M12.1	Collection of data and tissue specimens from men in Germany	6	✓	
M12.2	Collection of data and tissue specimens from men in a northern country	6	✓	
M12.3	Analysis of hormones in blood samples from Germany	12	✓	
M12.4	Analysis of hormones in blood samples from a northern country	12	✓	
M12.5	Data on German reproductive health entered in database	18	✓	
M12.6	Data on Northern European reproductive health entered in database	18	✓	
M12.7	European databank on male reproductive health	36	✓	
M13.1	Information about data requirements for low-dose effect estimations	24	✓	
M14.1	Insight into predictive power of concentration and independent action	48		in progress
M14.2	In vivo data on EDC mixture effects	48		in progress
M14.3	Insights into suitability of novel endpoints for mixture studies	48		in progress
M15.1	Information about the ability of EDC to oppose the actions of other classes of EDC	48		in progress
M15.2	Insights whether these antagonistic EDC effects occur at levels that are relevant in real existing exposure scenarios	48		in progress
M16.1	Insights into adequate risk assessment procedures for EDC	48		in progress

3. PROGRESS SUMMARY DURING THIRD REPORTING PERIOD

The following specific coordination actions were undertaken during the third year of the EDEN project:

3.1 Theme 1: Complex EDC Mixtures in Human and Fish Tissues

Problems with sample collection experienced in previous years had resulted in delays in completing WP1. Sample collection is now completed (bar three further cryptorchid samples due in month 39) and extensive chemical analyses of mothers' breast milk samples and placentas from cryptorchid boys and their controls have been carried out. Analysis for polybrominated diphenyl ethers will be undertaken during the fourth year by Partner 10, due to the small sample size extensive analysis is not possible. As an extension to the original breast samples collected, a significant number of new breast cancer patients have been recruited and clinical variables including response to treatments have been collected.

The delivery of fish tissue samples to respective Partners have been completed with fish having been examined for symptoms of endocrine disruption. As a deviation from the Technical Annex to avoid duplication of results, an analytical strategy that targets endpoints has been adopted. The samples will now be assessed to determine if the range of chemical contaminants associated with the presence of ovotestis (intersex) differ from that in unaffected fish and whether tissue chemical contamination profiles explain divergent plasma VTG levels in bream from a site not known to be impacted by STW effluent.

Methodologies have been established as part of WP3 for phthalate monoesters in fish muscle, bisphenol-A and chloro derivatives and benzophenone in human tissues and the assessment of the total effective xenoestrogen burden in fish tissues. Preliminary results indicate detectable levels of the phthalate monoesters MBP and MEHP in fish tissue. Using the method to assess for the total effective xenoandrogen burden, data on EDCs in mammary and abdominal adipose tissues from human breast cancer patients and in fish tissue have been produced and included in the database of exposure.

As part of WP4, the YES and E-Screen were evaluated and gave similar responses for extracted adipose tissue. MCF-7 AR transfected cells have been compared with PALM PC-3 cells in an assay for (anti)-androgenicity. The hyperfractionation methodology is sufficient to effectively separate chemicals extracted from human and fish tissues. No correlation was observed between the concentration of any single chemical in human adipose tissue and the estrogenicity determined in the E-Screen bioassay. Possible reasons for this lack of concordance are that the estrogenic effects depicted in the E-Screen bioassay are a consequence of the combined effect of several organohalogens and/or the proliferative effect is due to other unmeasured chemicals. The fractionation method developed by Partner11 that was initially validated with fish from a reference site (Lake Constance) also proved applicable to fish from polluted sites. Pooling, fractionation and analysis (YES, chemical) of bile and visceral fat samples is well underway. The hEST assay has been optimised and validated and used

to determine the IC_{50} values of known estrogenic compounds. The hEST is ready to be used for the measurement of E-screen positive bile and tissue fractions from other partners. The on-line HRS-ERα platform has been optimised by developing a detection method based upon fluorescence polarisation which will also enable detection of fluorescent phytoestrogens. This method can now be used to measure breast tissue samples (α- and β-fractions and E-screen positive fractions) and fish bile samples (pooled fractions and E-screen positive fractions). Partner 12 have obtained the pET15b hAR LBD plasmid and begun expression experiments in *E. coli* and a radio-labelled receptor binding assay is available for checking expression levels.

3.2 Theme 2: Mechanisms of EDC Action

Three major achievements have resulted from WP5, a) Cell-based screening assays have been developed for high-throughput screening on EDC actions and the potential of modern molecular tools (real-time RT-PCR, in situ hybridisation and microarrays) was evaluated. The information obtained has been compared in EDC exposure experiments, and responses related to tissue accumulation of EDCs, b) Results from the identification of novel signalling pathways involved in the genomic action of EDCs demonstrate that the effects of estrogenic chemicals are not only mediated via the classical ER pathway, but additional signalling pathways as well as cross talks to other receptor pathways play an important role and c) Aromatases and ERs as important factors in EDC action. They highlight that the effects of estrogenic substances are not restricted to reproductive processes, but involve multiple targets; in particular neuronal/glial systems can be impacted by xenoestrogens. From the mixture context, marker gene responses have been analysed in the experiments involving MCF-7 cells. As part of WP6, altered fetal Leydig cell function following DBP exposure may be held accountable for most of the observed changes that are evident in fetal life and at later ages. These changes may also explain in part the occurrence of Sertoli cell-only tubules in adulthood following fetal exposure to DBP. Data from WP6 lends further evidence that DBP exposure of the fetal rat provides a model for studying the development and consequences of testicular dysgenesis that may be of relevance to testicular dysgenesis syndrome in the human. In terms of monitoring of adverse effects of phthalates, it appears that effects on the testis in fetal life are more dose-sensitive than are the endpoint effects that are evident in adulthood. Dioxin causes clear adverse effects on the axis at this time-point, whereas DDE and DINP do not seem to affect either the testis or adrenals strongly. The rat E14.5 fetal gonad assay (FEGA) provides a new model system in which to investigate the direct effects of phthalates *in vitro*. Its initial application has revealed potential time-, cell- and metabolite-specific effects of DEHP/MEHP on the fetal testis.

The recently developed antibody for rat Insl3 has been validated, which will then be utilised for Insl3 assay development in rat blood. It has been demonstrated that there is no straightforward relationship between DBP-induced suppression of Insl3 and the occurrence of cryptorchidism in individual testes. Fetal germ cells are a potential target for Insl3 from the Leydig cells during fetal development and serum Insl3 in normal men is not acutely sensitive to exogenous FSH or LH bioactivity (hCG), but nevertheless is dependent on the stimulatory activity of gonadotrophins on Leydig cells. Subsequent to long-term gonadotropin suppression, Insl3 does not recover to the same degree as testosterone, suggesting that Insl3 may be more sensitive than testosterone to impaired Leydig cell function. Reduced Insl3 in cryptorchid boys suggests impairment of Leydig cell function in cryptorchid testes and supports the

hypothesis that cryptorchidism is associated with a primary testicular disorder. Insl3 is a target for anti-androgen action during fetal development and may contribute to adverse effects, such as cryptorchidism. The development of transactivation assays for mInsl3 has proved more complex than first envisaged with the key components of the assay now having been tested. The sites of expression of LGR8 during development in the reproductive tract of the male rat have been demonstrated. The gubernaculum is a key target for Insl3 and there is evidence that Insl3 may have several target sites outside of the gubernaculum, namely in the fetal germ cells and efferent ducts/epididymis during fetal and postnatal development. As part of WP8, data has been obtained on the sensitivity of the HP unit to exposure to synthetic estrogens and a detailed evaluation of the potential use of several markers, such as PR, KiSS-1, GPR54, and α- and β-globin genes as endpoints/biomarkers of endocrine disruption has been conducted. The analysis of selected gene targets (e.g. KiSS-1 and GPR54) and differentially expressed genes have paved the way for identification of novel mechanisms of endocrine disruption at the HP unit. Experimental data has concluded that E2 and *o,p*'-DDT can evoke a time-dependent increase in glutamate-induced GnRH secretory response, the latter of which also conducted slow effects in the hypothalamic explant model. An increase in GnRH pulse frequency by either E2 or *o,p*'-DDT was prevented by DNQX and ICI 182,780 with β-naphtoflavone inhibiting *o,p*'-DDT accelerated GnRH pulse frequency only. GnRH-secreting neurons can be directly activated by glutamate and E2 and the model allows study directly at the GnRH neuron level the interactions between glutamate, E2 and EDCs. For coumestrol, data infers that it possesses both estrogenic and (possibly) anti-estrogenic effects on GnRH secretion which could involve, respectively, ERα and ERβ which are both expressed in the hypothalamus.

3.3 Theme 3: Indicators of Impaired Reproductive Function in European Men

All samples have been analysed for the reproductive hormones testosterone, LH, FSH, SHBG and estradiol. A database has been constructed, which contains the hormone data as well as information on age, birth year, body mass index and a range of other clinical and lifestyle factors that have been retrieved from databases of the original population studies from which the samples originate. The analysis of the Danish cross-sectional data has been completed with the analysis of the Finnish cross-sectional data to be initiated in the fourth reporting period. A direct comparison of hormone levels in the Danish and Finnish cohorts will not be possible due to the lack of a temporal overlap in the cohorts from the two countries. In the German study on reproductive health of young men all the clinical examinations, sample collections and laboratory analyses have been completed (D12.1) and all the data has been entered into the central European database on male reproductive health (D12.3). From this database, information has been extracted to a SPSS dataset for data evaluation, which is currently ongoing. A manuscript is also under preparation. The follow-up study of young Finnish men was completed (D12.1 and D12.2), but has also been extended supported by national funding. The first report on the relationship between male reproductive health and maternal exposures during pregnancy has been published, but further associations may be unveiled as the questionnaire and clinical data are further examined.

3.4 Theme 4: Low-dose- and Mixture Effects of EDC

The work on low dose effects (WP13) has been completed. The main observation from these studies is that unusually shaped dose-response curves, such as those reported by some authors in the literature did not become apparent. However, this work has highlighted the difficulties associated with estimating low effect doses and concentrations with reliability. Estimates produced by hypothesis testing using the customary "no-observed-effect-level" (NOEL) approach are clearly associated with small effects, as evidenced in studies with human cells, fish and rodents. Regression-based methods for the estimation of low effects are an alternative to hypothesis testing. Both these approaches have complementary strengths and weaknesses, and therefore a framework was developed that combines the strengths of these methods.

Significant progress has been made with the mixture studies carried out in WP14 and 15. Following on from the weak antagonism observed with a 10-component mixture in the E-Screen assay, steps have been taken to explain this in terms of interactions between mixture components on steroid-metabolising enzymes of the CYP family. Furthermore, a reference mixture for additivity in the E-Screen has been defined. The impact of EDC with modes of action dissimilar from estrogenic chemicals has been explored, both in terms of cell proliferation and expression of key regulatory genes.

The combined effects of three androgen receptor antagonists on male sexual differentiation in the rat were assessed. The three chosen chemicals, vinclozolin, flutamide and procymidone, acted additively in terms of changes in anogenital distance of male offspring. With nipple retention as the endpoint, the joint effects were slightly stronger than those anticipated by using the concept of dose addition. All in all, these results show that dose addition provides a good basis for the prediction of developmental effects in the rat, and these findings are deemed to be of major relevance for the regulation of mixtures of EDC.

A first mixture experiment involving the three steroidal estrogens E1, E2 and E3, as well as NP has been completed successfully. With induction of vitellogenin (vtg) as the endpoint, the results did not deviate significantly from anticipated concentration additivity. Considerable efforts went into the screening of further chemicals, not usually tested in fish. This included the fungizide Cyproconazole, and the UV-filter agents 4-MBC and OMC, none of which induced vtg production in juvenile zebrafish. Thus, none of these chemicals can be included in further mixture experiments in zebrafish or sticklebacks.

In the stickleback, eight exposure tests (2 range-finding and 6 definitive) have been completed. Two model estrogens (E2 and EE2) also exert anti-androgenic activity that can be detected as a reduction of androgen-dependent spiggin production in the kidney of exposed sticklebacks. This observation is very significant and will be exploited in the mixture studies to be carried out during the next reporting period.

All these observations have been fed into the preparations for exploring options for mixture regulation (WP16).

3.5 Discussion/Conclusions

Progress for the 3rd reporting period has been substantial in all four themes. For WP1 and WP2, collection and delivery is completed bar three cryptorchid controls due in month 39. Extensive analyses of numerous EDCs are in progress using several procedures developed specifically for EDEN. Results obtained from Theme 2 are numerous, with the development of screening assays and evaluation of modern molecular tools. The effects of EDCs have been shown to involve additional signalling pathways and cross talks in addition to the classical ER pathway and that aromatases and ERs are important factors in EDC action. Effects from dioxin and phthalates on the testis in fetal life are more dose sensitive than in the adult which may be explained by the altered Leydig cell function. The FEGA provides a new model system for investigating direct effects of phthalates *in vitro*. Cryptorchid boys have lower Insl3 levels than normal boys, indicating defects in Leydig cell function. WP8 postulates that EDCs are able to modulate GnRH secretion in immature female hypothalamic explants *in vitro*, through both rapid and/or slow effects that likely involve both estrogen and dioxin receptor pathways. Clinical examinations, sample collection and laboratory analyses have been completed in the German study on reproductive health of young men (D12.1), with all the data entered into the central European database on male reproductive health (D12.3). The follow-up study of young Finnish men (D12.1 and D12.2) has been extended, supported by national funding. The first report on the relationship between male reproductive health and maternal exposures during pregnancy has been published, but further associations may be unveiled as the questionnaire and clinical data are further examined. Low dose testing in all systems has been completed. Similar mixtures studies have been undertaken in the E Screen, rat and zebrafish and the planning for dissimilar mixtures have been concluded. Preliminary organisation for expert panel meeting and identification of participants has been undertaken which is scheduled to be held in May 2006.

3.6 Future Action

Breast tissue analysis is ongoing and analysis of fish samples will be completed by month 40. The small sample size of the cryptorchid boys and their controls limits EDC analysis. Fractionation and distribution of samples will continue to be tested with the HRS-AR platform based receptor affinity detection assay to be finalised. Microarray and RT-PCR analyses of tissues from rats and fish exposed to EDC mixtures will be executed as part of Theme 2 after completion of mixture studies in Theme 4. Changes in the working environment and personnel for Partner 16a have led to a delay in answering the deliverables for WP6 and WP7, being dependent on the final validation and extensive application of the rat Insl3 assay. This assay will then be utilised to characterise the normal developmental profile of Insl3 secretion into blood, and its hormonal regulation at various life stages. In addition, there is the need to correlate whether or not treatment-induced cryptorchidism results in altered levels of Insl3 in blood at any life stage. Insl3 measurements in different target groups will be continued and association between Insl3 levels and exposure data from the cohort boys will be analysed. Definitive studies on LGR8 expression in the gubernaculum and elsewhere in the reproductive tract will be undertaken together with evidence for how expression may vary according to age and/or hormone or chemical exposures. In WP8, the molecular biomarkers evaluated will allow a pertinent assessment of the sensitivity of the HP unit to the exposure to potential EDCs during the 4th reporting

period, with special attention to the effects of low-dose and mixture-effects (as indicated in D8.4 and M8.4). Evaluation of Danish longitudinal data and complete evaluation of Finnish cross sectional data will be completed for WP11. From the database constructed as part of WP12, data has been extracted to a SPSS dataset for data evaluation, which is currently ongoing. The mixture studies will continue, more or less as planned originally. The next reporting period will see a very large mixture study, involving more than 20 components, in the E Screen. The final definitive study will be completed for the stickleback, after which a 4 component similar and 8 component dissimilar mixture exposure studies will be initiated. In the rat, evaluation of the dose-response curve for the mixture of four dissimilarly acting antiandrogens will be carried out. A further similar mixture study will be undertaken in the zebrafish. A workshop debate with a panel of experts from national regulatory agencies, industry and NGO's is to be held in Granada month 42 (D16.2). The outcome could find entry into the proposed "Weybridge +10" conference in Helsinki, November 2006. The production of guidance notes on the implications of knowledge about low-dose- and mixture effects for testing strategies and risk assessment procedures is currently nearing completion (D16.3)

3.7 Action Requested from the Commission

None requested.

1. WORKPACKAGES

WP1	Collection of adipose tissue specimens from human subjects with diseases indicative of endocrine disruption, and their disease-free controls						
Phase							
Start date	0						
Completion Date	12 months						
Current Status	active						
Partner(s) responsible:	5	4	9				
Person-months per Partner:	52 (24)[1]	27 (14)	47 (32)				
Already devoted person months per Partner and total:	40	15	47				

[1]given are the total person months contributed by each Partner to complete the WP; the numbers in parenthesis are the person months for which support from the EU is requested.

Objectives for the Reporting Period

- Complete collection of adipose tissue samples from boys with undescended testes (cryptorchidism) and their controls (boys undergoing hernia operation)
- Complete the delivery to the laboratories performing the chemical exposure analyses
- Complete the recruitment of patients with breast cancer

Methodology and Study Materials for the Reporting Period

1. *Samples from cryptorchid boys and controls*

Partner 5 has collected 33 fat samples from the cryptorchid cases and 26 samples from the controls. Four of the cases, however, had unilateral anorchia and therefore have to be separated from the cryptorchid group. It proved to be particularly difficult to get samples from controls, i.e., boys undergoing herniotomy or other abdominal surgery in Turku University Central Hospital, and therefore Partner 5 did not reach 30 which was the aim.

For similar reasons it was already earlier decided (detailed in the 2nd Annual Report) that the number of samples in Copenhagen (10 + 10) will be smaller than in Turku. This goal has now been achieved and there are 13 samples from cryptorchid boys and 10 from controls in Copenhagen (Partner 4). There will be three more control samples obtained by month 38. Part of the fat samples has already been sent to Kuopio (Partner 10), while part of them is currently stored in Turku and Copenhagen, and they will be delivered to Partner 10 by month 38.

2. *Cohort study of breast cancer patients*

A total of 56 women who underwent surgical treatments for breast cancer were recruited by Partner 9 in order to increase the information regarding chemical content and TEXB activity in patients with malignant disease of the breast. The study consists of a follow up of breast cancer patients during 18 months, with five points of performance: surgery (0 months), period 1 (1 to 5 months), period 2 (6 to 11 months), period 3 (12 to 17 months) and period 4 (18 to 22 months).

Scientific Achievements

Partner 4 contribution

13 fat samples collected from cryptorchid boys and 10 control samples.

Partner 5 contribution

33 fat samples collected from cryptorchid boys (4 of which turned out to have unilateral anorchia) and 26 control samples.

Partner 9 contribution

Fat and blood samples have been processed in WP3 and WP4 according to the planned protocols.

Progress Summary

Sample collection from cryptorchid boys and controls is near completion (3 more samples scheduled to come in month 39). Extensive chemical analyses of mothers' breast milk samples and placentas from cryptorchid boys and their controls have been carried out. Dioxins and furans, PCBs, selected organochlorine pesticides, phthalates and polybrominated diphenyl ethers were analysed as a part of another EU project, EXPORED. On the basis of those analyses it was decided that the small fat samples that should be analysed for polybrominated diphenyl ethers will be done by Partner 10 during the fourth year. A significant number of new breast cancer patients have been recruited and clinical variables including response to treatments have been collected.

Plan and Objectives for the Next Reporting Period

- Samples from the cryptorchid boys and their controls will be analysed for polybrominated diphenyl ethers in Kuopio by Partner 10. It was decided early on that the amount of the samples is too small to be divided to more than one analytical laboratory.

- For the breast cancer patients, the recruitment process has been finished and the follow up of patients is in course.

Deliverables and Milestones

Deliverables	
✓ delays (12) ✓ delays (24) near completion (36)	**D1.1:** Annual report on the progress of human adipose tissue sample delivery (month 12, 24)
✓ (P9) delays (P4 + P5) delivery on-going to P10 (36)	**D1.2:** Delivery of human adipose tissue samples to Partners 4, 9, 10, 11, 12 (month 6, 12, 18, 24)
Milestones	
✓ except to P12 (P9) ✓ (36)	**M1.1:** Samples from human breast cancer cases and controls delivered (month 1 and onwards)
ongoing (P5) delays (P4) ✓ (36)	**M1.2:** 15 adipose tissue samples collected in area A and B from both cryptorchid cases and controls (60 samples) (Month 12)
✓ (P5) ongoing (P4) adjusted numbers completed (36)	**M1.3**: Next 15 adipose tissue samples collected in area A and B from both cryptorchid cases and controls (Month 24)

WP2 Collection of bile and adipose tissue from wild fish collected at freshwater pollution hot-spots and control sites in the European Union							
Phase							
Start date	0						
Completion Date	12 months						
Current Status	completed						
Partner(s) responsible:	6	8	11				
Person-months per Partner:	12	4	1				
Already devoted person months per Partner and total:	12	4	1				

Objectives for the Reporting Period

- To deliver adipose tissue and bile samples from fish showing signs of endocrine disruption, and from controls
- To examine fish for symptoms of endocrine disruption

Methodology and Study Materials for the Reporting Period

Six polluted river sites in different member states of the EU and six control sites will be selected to catch wild male fish (roach and bream). The sample sites will be chosen to include a wide variety of emission sources that release different classes of EDC into surface waters, including sewage treatment plants (Partners 6, 11) and pulp mills (Partner 8). The fish (ca 50 per site) will be examined for symptoms of endocrine disruption by carrying out histopathological examinations of the reproductive tracts. Bile and fat will be prepared. To provide sufficient material for analyses, samples from each site will be pooled, stored and distributed to Partner laboratories.

Scientific Achievements

This workpackage has incurred further delays due to the late distribution of samples. The delivery of samples of adipose tissue, bile and muscle to the labs of Partners working on WP3 and WP4 was completed in June 2005 (D2.1 and M2.1).

For Partner 6, the distribution of samples (D2.1 and M2.1) was delayed due to a miscommunication with one of the sub-contractors and due to uncertainties over the definite scheme for sample distribution. The latter issue was solved at the EDEN meeting in Prague (May, 2005) forum meeting of EDEN. At this meeting the selection scheme for fish tissue samples to be used in WP3 and WP4 was discussed in detail. It was concluded that:

- A technical report from Partner 6 provides extensive information on the range of contaminants present in fish from the Dutch EDEN study sites

- This, it was agreed, renders any unfocused analytical effort by the EDEN partners an unnecessary duplication of effort

- Following discussion by the participating partners, the previous strategy for selection of samples for analysis, as outlined in the EDEN Technical Annex, was abandoned

- Instead, an analytical strategy that targets endpoints was adopted

This inturn has repercussions on WP3 and WP4 and two issues that will be addressed based on these samples collected will be:

1. *Does the range of chemical contaminants associated with the presence of ovotestis (intersex) differ from that in unaffected fish?*

The factors responsible for the occurrence of ovotestis in wild fish remain uncertain but are likely to be chemical. The fish tissue samples collected in WP2 provide a unique opportunity to seek evidence to support this possibility. The chemical profile of tissues from fish whose gonadal structure was characterised as intersex (n = 13) will be contrasted with the chemical profile present in fish not presenting with ovotestis but matched for site, age, and plasma VTG level (n = 26; ratio of case:control - 1:2). All ovotestis positive fish were caught at three sites: Dommel, Aa and Biesbosch.

2. *Do tissue chemical contamination profiles explain divergent plasma VTG levels in bream from a site not known to be impacted by STW effluent?*

Fish from the Biesbosch study site display a wide range of plasma VTG levels but no clearly identifiable STW inputs exist. This element of WP4 will seek to determine whether the range of VTG levels evident within this population can be linked to estrogenic activity isolated from bile. Two pools of bile will be derived by combining samples from fish with high (n = 11; VTG = 382,683 – 9,147,062 ng/ml; B11, B12, B18, B24, B27, B3, B30, B33, B37, B59, B95) and low (n = 15; VTG < 800 ng/ml; B14, B38, B41, B43, B53, B60, B61, B67, B70, B73, B74, B82, B87, B90, B102) plasma VTG levels. In the first instance these pools will be subject to fractionation and screening for estrogenic activity. Depending on the outcome of this initial screening, further direct analysis of additional tissues may be undertaken.

The final scheme for fish tissue samples distribution is given in the minutes for the EDEN 4th meeting (Annex II).

Progress Summary

Delivery of tissue samples to respective Partners have been completed with fish having been examined for symptoms of endocrine disruption. Due to a technical report by Partner 6 on the range of contaminants present in fish from the Dutch EDEN study it was agreed that any unfocused analytical effort by the EDEN partners warrants unnecessary duplication of effort. Consequently, the previous strategy for selection of samples for analysis as outlined in the EDEN Technical Annex was abandoned and an analytical strategy that targets endpoints has been adopted. The samples will now be assessed to

determine if the range of chemical contaminants associated with the presence of ovotestis (intersex) differ from that in unaffected fish and whether tissue chemical contamination profiles explain divergent plasma VTG levels in bream from a site not known to be impacted by STW effluent.

Plan and Objectives for the Next Reporting Period

This workpackage has been completed with examination of the fish for endocrine disruption and sample delivery finalised in month 31.

Deliverables and Milestones

Deliverables	
completed	**D2.1**: Delivery of fish adipose and bile specimens to Partners 4, 9, 10, 11, 12 (months 6, 12)
Milestones	
completed	**M2.1**: Delivery of fish adipose and bile specimens (month 12)

WP3	**Analyses of human and fish tissue specimens for chemicals with endocrine-disrupting potential**						
Phase							
Start date	0						
Completion Date	36 months						
Current Status	active						
Partner(s) responsible:	9	4	10				
Person-months per Partner:	75 (53)	27 (14)	117 (59)				
Already devoted person months per Partner and total:	38	16	26				

Objectives for the Reporting Period

- Develop a new method for analysis of phthalate monoesters in fish muscle and bisphenols and organochlorine bisphenols,
- Analyse fish samples (male breams) from the Netherlands as planned in Prague (month 30), positive and/or negative fish eluent fractions from YES- and YAS-assays and the fat samples from cryptorchid boys from Denmark and Finland
- Analyse fat samples (mammary and abdominal) from breast cancer patients from Spain for polychlorinated dioxins and furans (PCDD/Fs), polychlorinated biphenyls (PCBs), their hydroxylated metabolites (HO-PCBs), polybrominated biphenyls (PBBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OC).
- Develop a new method for the assessment of the total effective xenoestrogen burden (TEXB) in fish tissues and assess the TEXB in human and fish tissues.

Methodology and Study Materials for the Reporting Period

The samples collected in WP1 and WP2 will be analysed for the following groups of chemicals:

Partner 9: organochlorine pesticides, bisphenol A and its halogenated derivatives, alkylphenols, steroidal estrogens, phytoestrogens, parabenes
Partner 10: polychlorinated dioxins and furans (PCDD/F), polychlorinated biphenyls (PCB) and their hydroxylated metabolites, polybrominated biphenyls (PBB), polybrominated diphenyl ethers (PBDE)
Partner 4: phthalates

Scientific Achievements

The development of high specificity and sensitivity methods to quantify known EDCs using techniques such as GC/MS and LC/MS are given below:

1. *Bisphenol, chloro BPA and phthalates*

In addition to the chromatographic methodologies developed by Partner 9 to quantify ED pesticides in different specimens, the last months have been devoted to the development of two new techniques. Firstly, the analysis of Bisphenol A and chlorinated derivatives mono, di, tri, tetrachloro (BPA-CL, BPA-CL2, BPA-CL3, BPA-CL4) Bisphenol A in human samples. This approach utilised BSTFA as the silyating agent for GC/MS detection. Secondly, method development of Bisphenol A and its chlorinated derivatives and the phthalates (DMP, DEP, DBP, DEHP, DOP and BBP) in food and water. Solid phase extraction (LiChroluet C18) was followed by either derivatisation with BSTFA/TMCS (GC/MS) or HPLC-DAD.

2. *Benzophenone analysis*

Due to the interest in benzophenone analysis, Partner 9 is developing a new method for the determination of 4-hydroxybenzophenone (HBZF), 2-hydroxy-4-metoxibenzophenone (2H-4MBZF), 2,4-dihydroxybenzophenone (2,4-DIHBZF), 2,3,4-trihydroxybenzophenone (2,3,4-TRIHBZF) and 2,2',4,4'-tetrahydroxybenzophenone (22',44'-TETHBZF).

3. *Phthalate monoesters analysis*

A new method for analysis of phthalate monoesters in fish muscle has been developed and validated by Partner 4 using pooled samples from bream slices not selected for chemical analysis. The method consists of a liquid extraction followed by 2 solid phase extractions with determination on LC/MS/MS. The monoesters included are: monomethyl phthalate (mmp), monoethyl-phthalate (mep), mono-n-butyl phthalate (mbp), monobenzyl phthalate (mbzp), mono-(2-ethylhexyl) phthalate (mehp), mono-(2-ethyl 5-hydroxyhexyl) phthalate (mehhp), mono-(2-ethyl 5-oxohexyl) phthalate (meohp) and monoisononyl phthalate (mnp).

Prior to analysis, fish samples were thawed and the muscle tissue homogenised. Three grams was extracted with 15 mL 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. Ten mL of the extract was evaporated to dryness and dissolved in 1.0 mL basic buffer for further clean-up by SPE with on-line injection (Aspec XL, Gilson Inc, Middleton, USA). Solid phase extraction cartridges (Strata XL, 200 mg / 3 mL, Phenomenex, Germany) were utilised for both clean-up – first a basic SPE step, where hydrophobic compounds were retained on the column followed by pH adjustment to acid conditions and a new SPE step was included where the phthalate monoesters were retained at the column. The phthalates were eluted with 1.5 mL acetonitrile followed by 1.5 mL ethyl acetate and then evaporated to dryness under a gentle stream of nitrogen at 42.5 ºC. The residue was finally dissolved in 500 µL water: acetonitrile (9:1) for detection by LC/MS/MS.

The analyis of phthalate monoesters was accomplished using high pressure liquid chromatography (Surveyor, ThermoFinnigan, San Jose, California) with a GEMINI column (100 x 2.1 mm x 3 µm) (Phenomenex, Anschaffenburg, Germany). The column temperature was 25 ºC, the injection volume was 20 µL and the flow rate was 300

µL/min. Detection was by a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer in combination with Xcalibur software programme (Thermo Electron Corporation, San Jose, California). The instrument was run in negative mode using the electrospray source (ESI). Figure 1 show a calibration curve for detection of mehp and chromatograms of a standard, a fish sample and a spiked fish sample.

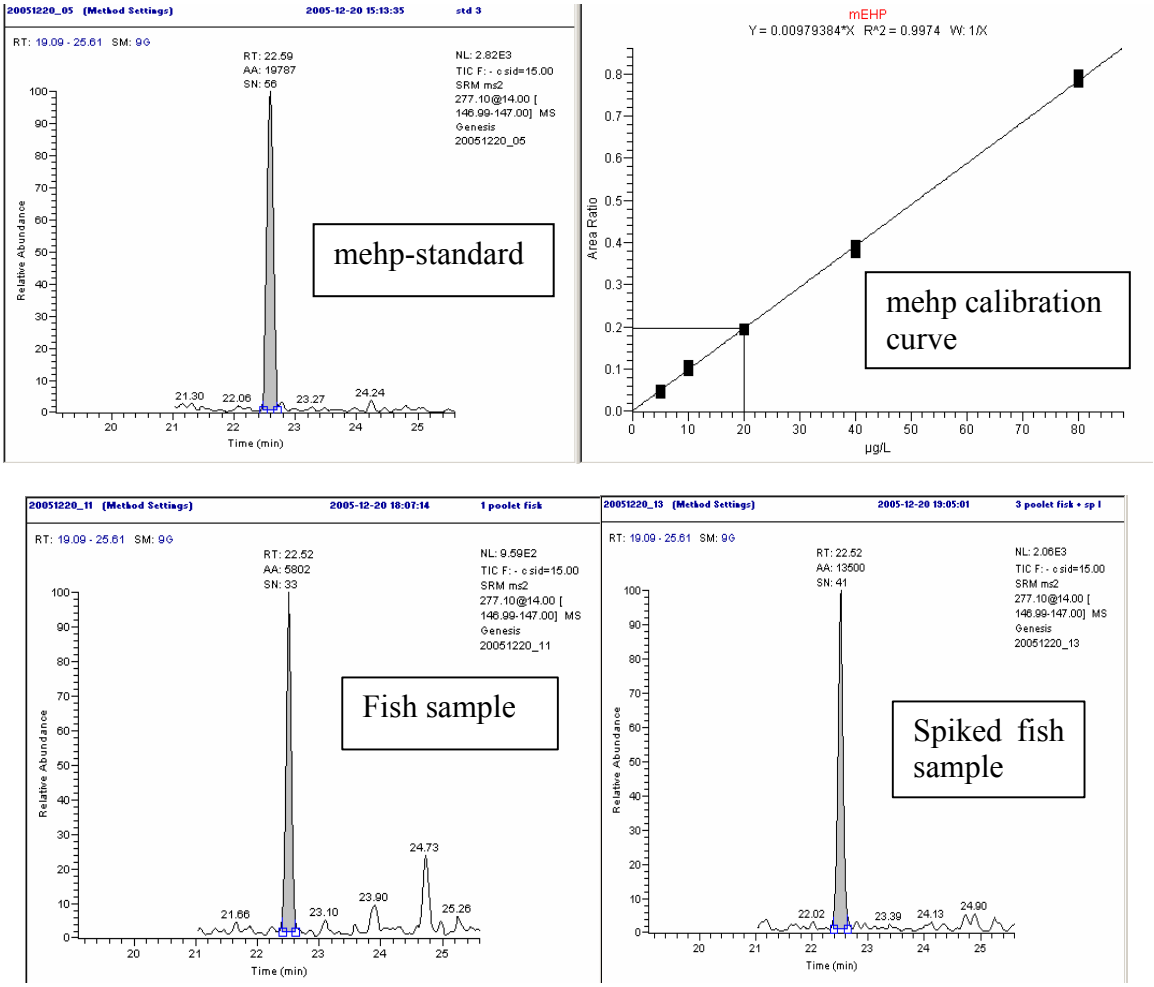

Figure 1 Calibration curve for mehp and chromatograms of a standard, a fish sample and a spiked sample

4. *Analysis of human breast cancer and control tissues*

Twenty samples (the same that were sent to Kuopio, Copenhagen and Eawag, 10 breast cancer cases and 10 controls) have been processed with the appropriate methodology in order to investigate the presence of 18 organochlorine pesticides. These eluted in the HPLC-alpha fraction: *o,p*-DDT, *p,p*'-DDT, *o,p*-DDD, *p,p*'-DDE, Methoxychlor, Mirex, Lindane, Aldrin, Endrin, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan ether, Endosulfan lactone, Endosulfan diol, and Endosulfan sulfate. Partner 9 incorporated hexaclorobenzene (HCB) and vinclozolin to the chemicals of interest because of the extensive use of the later as a fungicide in Europe. In addition, PBDEs, PBB, and TCDD/Fs were investigated by Partner 10. Results of the chemical content on these samples have been analysed and processed and included in the database of exposure.

5. *Cohort study of breast cancer patients*

A total of 56 women who underwent surgical treatments for breast cancer were recruited by Partner 9 (WP 1) in order to increase the information regarding chemical content and TEXB activity in patients with malignant disease of the breast. The study consists of a follow up of breast cancer patients during 18 months, with five points of performance: surgery (0 months), period 1 (1 to 5 months), period 2 (6 to 11 months), period 3 (12 to 17 months) and period 4 (18 to 22 months). Organochlorine pesticides eluted in the HPLC-alpha fraction have already been analysed in both mammary and abdominal adipose tissues. A selected group of samples is now ready to be sent to Partner 10 for further analysis (Table 1).

Surgery mamary adipose tissue)	**3 months (abdominal adipose tissue)**	**18 months (abdominal adipose tissue)**
1a	1b	1c
2a	2b	2c
3a	3b	3c
4a	4b	4c
5a	5b	5c
6a	6b	6c
7a	7b	7c
8a	8b	8c
9a	9b	9c
10a	10b	10c
11a	11b	11c
12a	12b	-
-	13b	13c

Table 1 Samples from breast tissue patients and those selected for further analysis by Partner 10

6. *Adipose tissue samples from cryptorchid boys in Denmark and Finland*

Partner 10 received 21 tissue samples of cryptorchid boys and their controls from Partner 4 in Copenhagen. Unfortunately the amount of tissue in each of the samples is very small (and the amount of fat is even smaller) and it is highly unlikely that any satisfying results will be available from those samples. The issue has been discussed between Partner 10 and Partner 5 and forwarded to Partner 4 and it was decided that extensive EDC analysis is not possible and these will be sent to Kupio for PBDE analysis. Samples from Turku are in the process of delivery.

7. *Fish samples from the Netherlands and Switzerland*

Partner 10 has received bream samples from the Netherlands and Switzerland. At the thematic meeting in month 30, the entire fish analyses were rearranged. This meant that the analysis was no longer based on geographical distribution of fish according to the pollution level of each study location. Instead it was decided that the analysis of bream was based on the case-control setting in which the cases were male breams with ovotestis and controls were breams with no ovotestis. The selected fish in each case-control set were equally old and caught from the same location. The analysis of those selected fish (number is 39) is currently ongoing by Partner 10. Chemical analysis will be ready by the

end of January 2006 (month 38). To date, no positive or negative fractions in YES or YAS assays have been received in Kuopio. Partner 9 has received bream samples which have been analysed for organochlorine pesticides (HCB, lindane, endosulfan and metabolites, ppDDT and metabolites) given in Table 2.

Pesticide	Median (ng/g)	Frequency	time (min)	max
HCB	11.62*	94.7	-	84.61
Lindane	11.53	94.7	-	80.09
Endo-ether	6.02	78.9	-	24.75
Aldrin	20.49	94.7	-	85.50
Endo-lactone	18.22	94.7	-	149.45
Endo-I	14.13	89.5	-	48.05
DDE	64.31	100	19.16	728.72
DDD	74.26	100	14.60	748.92
Dieldrin	26.47	100	2.06	124.65
Endrin	92.29	94.7	-	423.54
Endo-II	32.44	89.5	-	121.99
opDDT	153.32	78.9	-	728.83
ppDDT	76.13	84.2	-	489.65
Endo-sulfate	8.19	68.4	-	271.46
Metoxychlor	23.54	100	-	117.82
Mirex	0	5.3	-	23.36

Table 2 Organochlorine pesticides in fish tissue samples analysed by Partner 9

8. *NetTotal effective xenoestrogen burden (TEXB) assessment of fish tissue samples*

To date, 39 fish tissue samples have been analysed in the E-Screen bioassay. Samples selected for extraction and TEXB determination and chemical content were the same as in OC pesticide residues experiments, these being D59, A82, A24, A105, D69, A01, A32, A29, D32, A100, A111, B38, D27, A51, A19, B97, D60, A21, A11, B95, D67, A50, A55, B45, A65, A44, A23, B21, A02, A13, A40, A66, A113, A73, A71, A77 and A46. Two hundred mg of each sample was extracted using hexane and the extracts then passed through HPLC in order to separate more polar xenoestrogens from lipophilic compounds (alpha and beta fractions). The total effective xenoestrogen burden (TEXB) has been measured in all alpha and beta fractions.

9. *Total effective xenoandrogen burden (TEXB-A) assessment*

Adipose tissues from breast cancer patients and controls (original 20 mammary and abdominal samples) were extracted and proccesed by HPLC in order to obtain alpha and beta fractions. Both fractions have been assayed in the A-Screen, based on MCF-7 androgen receptor transfected cells (AR) and the data on androgenicity (TEXB-A) is now being analysed.

10. *Preliminary results for phthalate monoesters*

Only few samples have been measured, but for all samples there have only been measurable contents of mbp and mehp. The content of mbp has been from 2 - 7 µg/kg and the content of mehp between 6 - 19 µg/kg. The recoveries at levels between 10 and 50 µg/kg fish sample were generally between 90 and 110 %. During the first months of 2006 the bream samples selected for chemical analysis will be analysed for phthalate monoesters.

Progress Summary

A method for of phthalate monoesters in fish muscle has been developed and validated and preliminary results indicate detectable levels of the phthalate monoesters MBP and MEHP in fish tissue. Method for bisphenol-A and chloro derivatives and benzophenone in human tissues and the assessment of the total effective xenoestrogen burden in fish tissues have both been developed and validated. The method for the assessment of the total effective xenoandrogen burden has been applied to adipose tissue samples from breast cancer patients and controls. Data on ED content in mammary and abdominal adipose tissues from human breast cancer patients and in fish tissue have been produced and included in the database of exposure. The milestone M3.1 regarding the data about EDCs in human and fish samples that guide the mixture experiments in WP14 and WP15 has already been delivered prior to the 3rd reporting period.

Plan and Objectives for the Next Reporting Period

- Complete the analysis of the selected fish samples. Analysis of samples is currently ongoing and expected to be completed by month 40.
- Follow with the development of protocols of high specificity and sensitivity to quantify selected EDCs (BPA, benzophenone, phthalates) using GC/MS and HPLC methodologies.
- Apply the protocol for the identification of active compounds in the extracts and successive fractions after HPLC hyperfractionation, using GC/MS and LC/MS methodologies.
- Finalise the analysis of current bream samples
- Analyse the fat samples from cryptorchid boys depending on whether there is a common consensus regarding the analysing scope of those tiny samples

Workpackage Related Appendices

Annex I (D3.2) Database on EDC found in human adipose tissue and in fish

Annex I (D3.3) Report about the chemical analyses of human and fish samples

Deliverables and Milestones

Deliverables	
✓	**D3.1:** Preliminary results of chemical analyses of human and fish samples (month 18)
in progress (24) on going (36)	**D3.2:** Database on EDC found in human adipose tissue and in fish (month 36)
on going (36)	**D3.3:** Report about the chemical analyses of human and fish samples (month 36)
Milestones	
completed (36)	**M3.1**: Data about EDC in human and fish samples that guide the mixture experiments in WP14, 15 (month 36)

WP4	**Extraction and bioassay-directed fractionation of human and fish tissue specimens**						
Phase							
Start date	12						
Completion Date	48 months						
Current Status	active						
Partner(s) responsible:	11	9	12				
Person-months per Partner:	33 (26)	75 (53)	50 (36)				
Already devoted person months per Partner and total:	22	35	17				

Objectives for the Reporting Period

- Explore the usefulness of measures of total (anti)estrogenicity, (anti)androgenicity and estrogen sulfotransferase inhibition as predictors of unwanted outcomes in humans and fish
- Evaluate EDC bioassays for their suitability for bioassay-directed fractions
- Search for new EDCs in human and fish sample extracts
- Develop an on-line androgen receptor high resolution screening bioassay

Methodology and Study Materials for the Reporting Period

Partner 9 compiled a tissue bank and a database on total effective xenoestrogen burdens (TEXB) in human tissue with reproductive disorders as well as in human tissue controls free of symptoms covering a wide range of Spanish population ages. Partner 9 is also in the process of producing a database on TEXB of fish adipose tissue.

Partner 11 has validated and adapted the fractionation method for higher sample volume. By increasing the injection volume, higher sample throughput could be achieved. This fractionation method was validated using one fish with a high vitellogenin level from the River Dommel. Pooling and extraction of the adipose tissue from selected bream was carried out by Partner 11 according to the decisions taken at the Prague meeting in month 30. Quantification of the steroid hormones (E1, E2 and EE2) and fractionation of the pools is currently underway. Pooling of the bile from bream selected at the Prague meeting has been completed, while extraction is still underway.

Partner 12 has optimised the HPLC-based hEST-assay using hSULT1E1, expressed in Salmonella bacteria.

Scientific Achievements

Partner 9 contribution

The YES and the E-Screen bioassays have been utilised to assess the total effective xenoestrogenic xenobiotic burden (TEXB) of selected groups of human samples. This work has been carried out in collaboration with Partner 1. Table 1 and 2 depict TEXB estimations using both methods.

	Alpha fraction		**Beta Fraction**	
E-Screen	pM/gpla	pM/lip	pM/gpla	pM/lip
Mean	28.38	1093.03	43.49	1936.25
Median	9.78	347.66	15.05	693.88
Standard deviation	36.45	1556.69	68.88	3672.43
Range	0.77-129.21	9.78-6017.50	0.20-290.83	8.92-18291.86
Frequency	62.50% (n=25/40)		87.2% (n=35/40)	

Table 1 TEXB assessment in the E-Screen bioassay

	Alpha fraction		**Beta Fraction**	
YES	pM/gpla	pM/lip	pM/gpla	pM/lip
Mean	22.43	961.38	119.40	4391.00
Median	14.38	474.07	67.08	2410.00
SD	29.37	1263.50	134.47	4654.81
Range	1.53-116.23	62.32-4761.39	12.87-578.40	373.83-20800.00
Frequency	50.00% (n=20/40)		72.5% (n=29/40)	

Table 2 TEXB assessment in the YES assay

Bioassays based on gene expression modulation via the androgen receptor (AR) and other steroid receptors are being utilised to study a group of selected human tissue samples for hormonal activities alternative to estrogenicity or antiestrogenicity. The objectives are:

- To study the potential (anti)androgenic activity via AR we have used PALM cells, the PC3 cell line stably transfected with human AR and a luciferase gene under transcriptional control of MMTV. The antagonistic activity was tested against the synthetic androgen R1881.

- To determine PR agonist/antagonist activity we have used HG_5LN Gal4-PR cells. The PR reporter cell line was done in two steps, the Gal4 responsive reporter gene, was firstly stably transfected into HeLa cells, generating HG_5LN cell line. In a second step, these HG_5LN cells were transfected with the Gal4-PR plasmid construct to obtain the HG_5LN Gal4-PR.

- To study the potential activity via GR we used HG_5LN Gal4-GR cells. The GR reporter cells were obtained in the same manner as the PR cell line. HG_5LN cells were transfected with Gal4-GR plasmid construct to obtain the HG_5LN Gal4-GR.

The EC_{50} value for Dex was 5 nM. Antagonist activity was determined by co-incubation with 5 nM Dex.

- Mineralocorticoid and antimineralocorticoid activity has been investigated by using HG_5LN Gal4-MR cells. The MR reporter cells were obtained in the same manner as PR and GR cell lines. HG_5LN cells were transfected with the Gal4-MR plasmid construct to obtain the HG_5LN Gal4-MR.

HPLC-hyperfractionation of human adipose tissues was addressed by developing and standardising a method to assess the total estrogenic xenobiotic burden (TEXB) in human adipose tissue, including more tissue samples from breast cancer patients and improving the fractionation method, in order to investigate bioaccumulated xenoestrogens that are candidates for estrogenicity and to assess their combined estrogenic effect. This was achieved by extensive HPLC separation of xenoestrogens from endogenous hormones followed by testing of individual fractions in the E-Screen test for estrogenicity. Organochlorine pesticides, PCBs and OH-PCBs, and halogenated bisphenols and alkylphenols, together with PBDEs, PBBs, dixoins PCDD/Fs were collected in the most lipophilic fractions; followed by progestins, androgens, and estradiol esters, and then by steroidal estrogens; phyto- and myco-estrogens were collected around the end of the run. These results were confirmed by exhaustive chemical analysis by Partners 9 and 10 as part of WP3. Figure 1 gives an example TEXB profile for each one of the 32 HPLC fractions collected using mammary adipose tissue from a breast cancer patient.

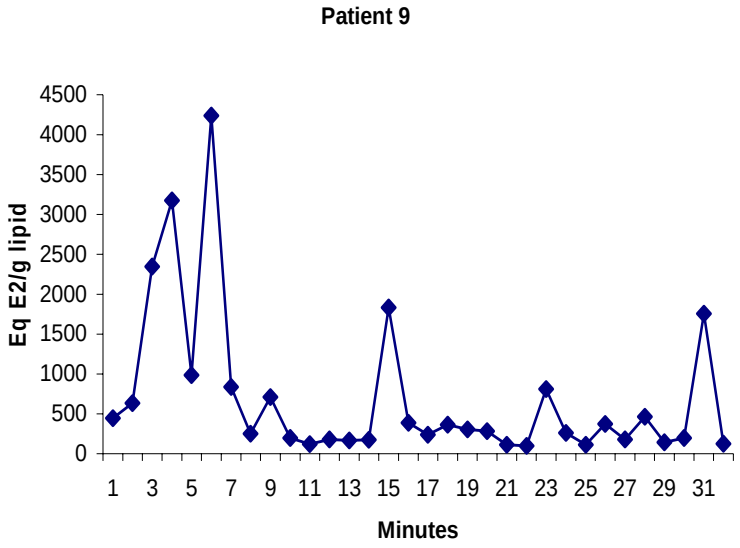

Figure 1 TEXB profile for each of the 32 HPLC fractions colleted using mammary adipose tiisue from a breast cancer sample.

The total effective xenoestrogen burden of the original 20 human adipose tissue samples and a summary of the individual fractions and alpha and beta fractions are summarised in Tables 3 and 4 respectively.

	N	%	Mean	± SD	25th percentile	50th percentile	75th percentile	95th percentile
α-Fraction	20	100	91.70	87.40	9.00	62.70	176.50	230.00
β-Fraction	20	100	152.64	181.37	6.23	90.95	279.50	583.10

Table 3 Mean and median values of the total effective xenoestrogen burden of the 20 human adipose tissue samples (pM EEQ/g lipid).

Patient Number	1-11 minutes EEQ/g lipid (pM E2)	12-32 minutes EEQ/g lipid (pM E2)	Fraction α EEQ/g lipid (pM E2)	Fraction β EEQ/g lipid (pM E2)
1	36.98	50.68	3.15	1.91
2	174.33	155.30	7.74	4.80
3	2812.51	2936.73	4.40	2.55
4	837.68	869.61	47.20	26.50
5	3612.47	3325.99	11.40	12.50
6	2244.70	3297.79	23.30	10.50
7	171.27	954.43	8.20	292.00
8	665.95	243.60	22.80	3.28
9	13952.40	8574.15	12.20	16.10
10	2576.05	3736.07	6.46	3.77
11	176.42	659.08	212.00	141.00
12	378.29	1780.27	149.00	241.00
13	2020.94	4639.81	89.00	84.60
14	106.09	812.37	160.00	97.30
15	1669.42	2657.34	178.00	586.00
16	83.68	1135.49	78.20	101.00
17	3100.02	2626.31	189.00	364.00
18	82.69	129.40	230.00	528.00
19	634.30	1657.22	230.00	242.00
20	687.09	1057.91	172.00	294.00

Table 4 TEXB of the sum of individual fractions eluted between minutes 1 and 11, and 12 and 32, as well as the TEXB of the alpha and beta fractions.

Partner 11 contribution

After discussions held at the thematic meeting in month 30 and summarised in the minutes (Annex II), pooling of bile and gonadal fat was carried out based on two

considerations using case-control studies 1) high versus low vitellogenin levels and 2) ovotestis versus normal fish.

log vtg 5,6:	B03,11,12,18,24,30,33,37,59,95	Biesbosh
log vtg 1,2:	B14,38,41,43,53,60,67,70,73,74,82,87,90,102	Biesbosh
ovotestis score 1-3:	A11,23,29,40,46,55,73,77,105	Aa
	B21,45	Biesbosh
	D60,67	Dommel
ovotestis score 0:	A01,02,08,13,15,19,21,32,33,50,51,65,66,71,82,100,	
	A111,113	Aa
	B97	Biesbosh
	D27,32,59,69	

Changes in the fish number for the ovotestis control were due to missing samples: A24, A44, and A109 were replaced by A08, A15, and A33. B37, B38 and B95 were used up for another experiment and could not be replaced. No viable alternatives were available for the replacement of B27 and B61. The pooling of bile samples has been completed and extraction is still underway. The pooling, extraction and fractionation of visceral fat and target chemical analysis (E1, E2, and EE2) have been accomplished. Determination of estrogenicity (YES) is still underway; hence the determination and identification of unknowns that may potentially exist in the fractions have not been carried out as yet.

Bioassay-directed fractionation

The suitability of the YES for screening of fractions has been demonstrated during the previous reporting period (D4.1), together with the validation of the bioassay-directed fractionation method. In this reporting period, the sample throughput was increased by changing to 4.6 mm ID columns and 100 µL injection volumes which resulted in a 10-fold decrease in time for the bioassay directed fractionation.

The methodology was tested with bream number D24 from the River Dommel. The selection was based on the fact that it was not part of any pools, possessed a high vitellogenin level (log vtg 7 ng/mL) and contained high amounts of bile (0.7 mL) and visceral fat (74.8 g). Recoveries were slightly lower for spiked bile and oil due to matrix suppression effects and usually increase the limits of quantitation (Table 5).

Steroid	**Bile**		**Visceral Fat**	
	Recovery		Recovery	
	Standard	Spiked Sample	Standard	Spiked Sample
E1	80 ± 4 %	69 ± 3 %	98 ± 5 %	68 ± 4 %
E2	94 ± 9 %	77 ± 6 %	88 ± 14 %	69 ± 2 %
EE2	67 ± 11 %	61 ± 12 %	67 ± 22 %	28 ± 5 %

Table 5 Quality control

The following amounts of steroids were found in bile from D24:

E1 452.5 ± 31.0 ng/mL bile

E2 136.8 ± 0.8 ng/mL bile
EE2 < LOQ (1.2 ng/mL bile)

After separating the sample into 10 fractions, all steroids were identified in fraction 6. Estradiol equivalents (EEQs) were calculated to be 308.7 ng/mL bile for fraction 6, using relative potencies determined in the YES (E1 0.38, E2 1, EE2 1.19) and data obtained with LC/MS/MS. Figure 2 depicts the EEq for the bile fractions and full extract.

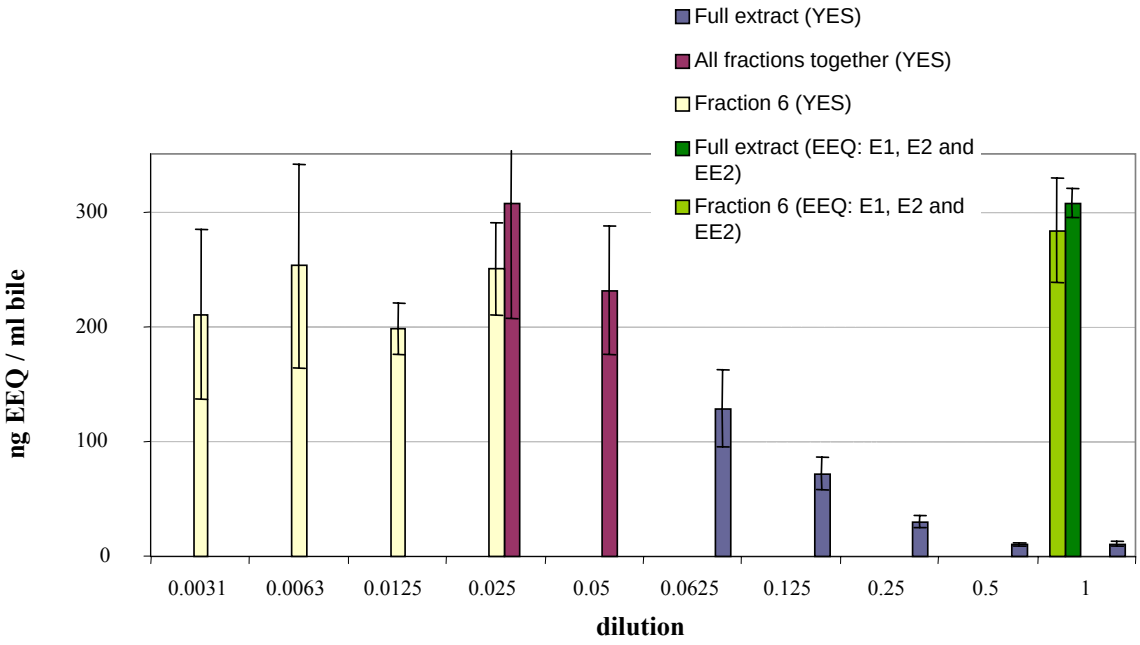

Figure 2 Estradiol equivalents (EEQ) in 1 mL bile from D24 (dilution series and comparison of LC/MS/MS and YES results).

The same EEQs were found for the full extract (dark green in Figure 2). The only estrogenic activity was identified in fraction 6 (yellow bars in Figure 2), when screening the fractions with the YES. Most of the estrogenicity was suppressed, when analysing the full extract with the YES (blue bars in Figure 2) and further dilution was not possible, due to the limited amount of sample available. This is surprising, because no toxicity was observed (no reduction of cell density) and no anti-estrogenicity detected when spiking the sample with standards. Another factor must be responsible since estrogenicity increased with dilution (blue bars in Figure 2). EEQs for the full extract and fraction 6 only matched the YES results for fraction 6 and the reconstituted full extract (red bars in Figure 2).

The following amounts of steroids were found in visceral fat from D24:

E1 0.56 ± 0.13 ng/mL oil
E2 0.68 ± 0.04 ng/mL oil
EE2 < LOQ (0.63 ng/mL oil)

After separating the sample into 10 fractions, all steroids were found in fraction 6. The calculated estrogenicity of the full oil extract based on the chemical data corresponded to 0.89 ng EEQ/mL oil which matched the estrogenicity determined with the YES (0.67 ng

EEQ/mL oil). The entire estrogenicity was again found in fraction 6 whilst no anti-estrogenicity could be observed.

The fractionation of bile and visceral fat resulted in no losses in estrogenicity (YES) or steroid hormones (chemical analysis). All estrogenicity determined was found in fraction 6 and could be explained by E1 and E2. No other estrogenic compounds could be found in significant amounts. However, a minimal dilution of 0.05 was necessary for bile in order to obtain similar estrogenicity as found in fraction 6 and with chemical analysis.

Partner 12 contribution

The HPLC-based hEST-assay using hSULT1E1, expressed in Salmonella bacteria has been optimised. The assay procedure and conditions are indicated in Figure 3. Figures 4-6 and Table 6 depict further results from inhibition studies.

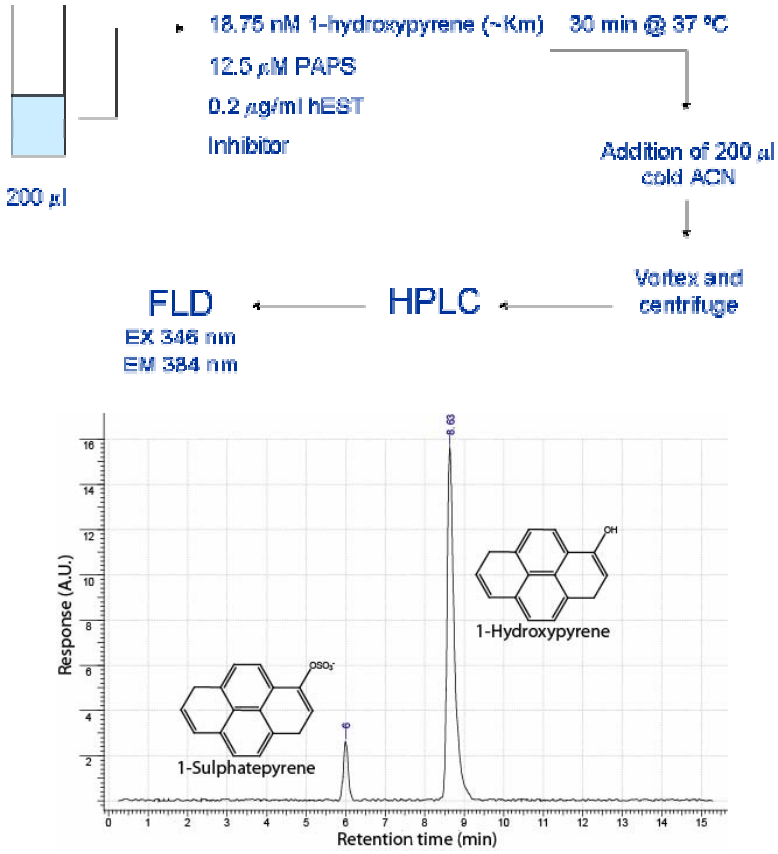

Figure 3 Typical HPLC fluorescence chromatogram after baseline subtraction showing the substrate and the product of the hEST-reaction to be inhibited. Normal incubation conditions are 40 ng hEST protein, 30 min incubation with 18.75 nM substrate 1-hydroxypyrene and 12.5 µM cofactor PAPS at 37 °C at pH 7.4.

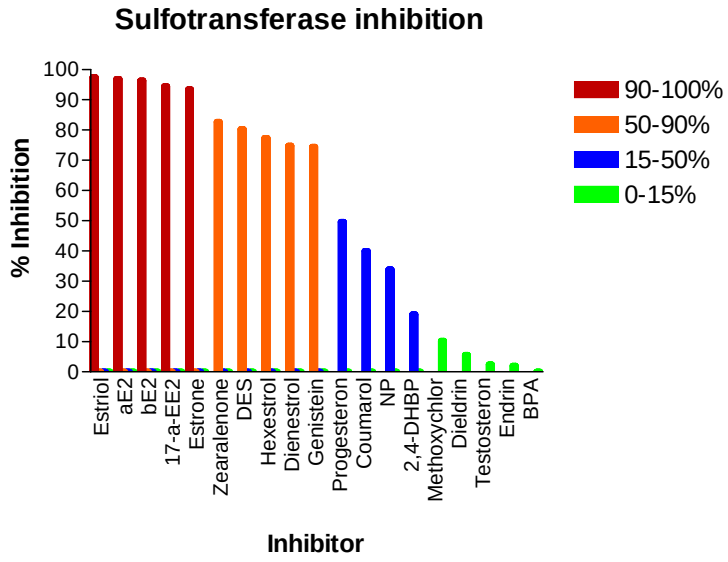

Figure 4 Typical results for inhibition screening of 19 compounds with known estrogenic properties at one concentration. Incubations were performed in the presence of 40 ng hEST, 18.75 nM 1-hydroxypyrene, and 2.5 µM inhibitor for 30 min at 37 °C at pH 7.4. The inhibitors are classified in 4 ranges: high, high- intermediate; intermediate-low and low potency inhibitors.

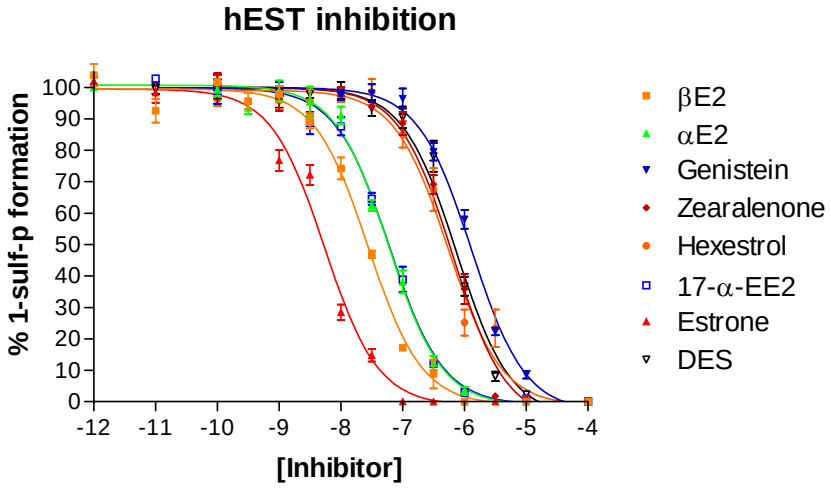

Figure 5 Typical results of IC_{50} measurements using the HPLC-based hEST inhibition assay of selected estrogenic compounds (i.e. with high and high-intermediate inhibitory potency). Incubations were performed in the presence of 40 ng hEST, 18.75 nM 1-hydroxypyrene, and varying inhibitor concentrations for 30 min at 37 °C at pH 7.4.

Compound	IC_{50} (M)
Genistein	$1.30 \pm 0.12 * 10^{-6}$
DES	$7.46 \pm 0.83 * 10^{-7}$
Zearalenone	$6.42 \pm 0.79 * 10^{-7}$
Hexestrol	$5.43 \pm 0.87 * 10^{-7}$
17α-Ethynylestradiol	$6.07 \pm 0.58 * 10^{-8}$
17α-Estradiol	$5.91 \pm 0.61 * 10^{-8}$
Estriol	$3.62 \pm 0.41 * 10^{-8}$
17β-Estradiol	$2.77 \pm 0.25 * 10^{-8}$
Estrone	$5.45 \pm 0.49 * 10^{-9}$

[a] Values are the mean ± S.D. (n = 6)

Table 6 Summary of the IC_{50} values for the inhibition of 1-hydroxypyrene hEST sulfonation obtained for a set of estrogenic compounds [a]

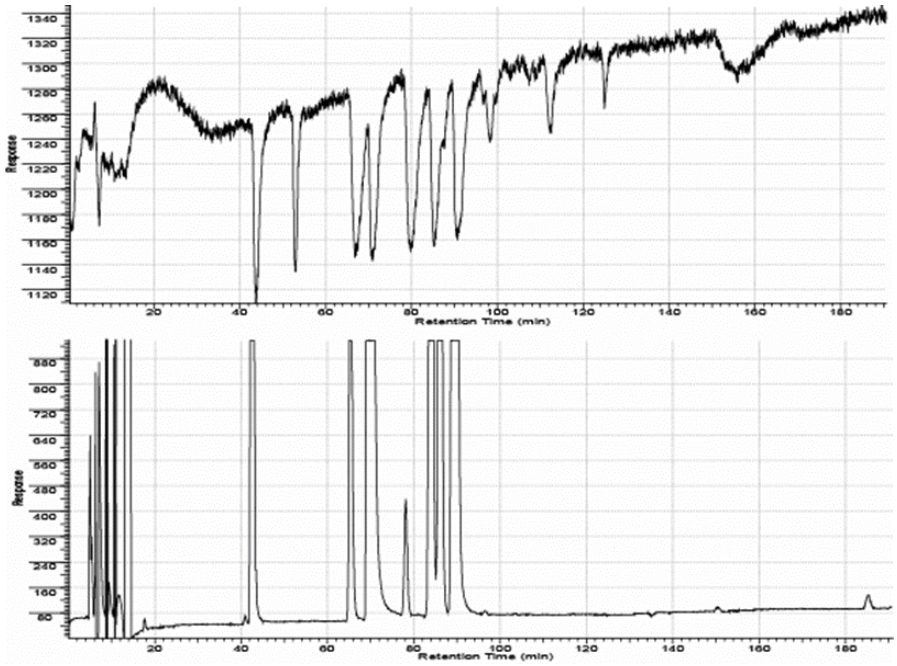

Figure 6 Bioaffinity (upper panel) and UV (lower panel) trace of a mixture of 19 known estrogenic compounds separated using the on-line HRS-ERα platform. The estrogenic compounds are: ethinylestradiol, bisphenol A, nonylphenol, genistein, tert-octylphenol, 17β-estradiol, 17α-estradiol, 2,4-dihydrobenzophenone, DES, 4,4'-dihydrobenzo-phenone, dienestrol, estriol, estrone, hexestrol, mestranol, n-butylparaben, n-propyl-paraben, resorcinol monobenzoate, and zearalenone.

For the development of a novel on-line HRS-androgen receptor affinity (HRS-AR-AD) assay, Partner 12 performed an extensive literature survey. Concurrently, the following was also undertaken:

- Creation of an appropriate, genetically engineered expression system (in *E. coli* bacteria) for the ligand binding domain (LBD) of the androgen receptor, rather then the complete receptor

- Synthesise an appropriate fluorescent ligand for the androgen receptor. For both items preliminary steps have been undertaken

Progress Summary

Extraction of adipose tissue by Partner 9 gives a similar response in both the YES and E-Screen. MCF-7 AR transfected cells have been compared with PALM PC-3 cells in an assay for (anti)-androgenicity. The hyperfractionation methodology is sufficient to effectively separate chemicals extracted from human and fish tissues. No correlation was found between the concentration of any single chemical in human adipose tissue and the estrogenicity determined in the E-Screen bioassay. There may be several reasons for this lack of concordance:

- The estrogenic effects depicted in the E-Screen bioassay are a consequence of the combined effect of several organohalogens and/or

- The proliferative effect is due to other unmeasured chemicals

As additive, synergistic or antagonistic mechanisms may account for the final effect observed in the pooled fractions, the approach proposed seems more appropriate for exposure assessment in epidemiological studies compared with the determination of individual chemicals in human samples.

The fractionation method developed by Partner11 that was initially validated with fish from a reference site (Lake Constance) also proved applicable to fish from polluted sites. Pooling, fractionation and analysis (YES, chemical) of bile and visceral fat samples is well underway.

Partner 12 has optimised and validated the hEST assay and used it to determine the IC_{50} values of known estrogenic compounds. The hEST is ready to be used for the measurement of E-screen positive bile and tissue fractions from other partners. The on-line HRS-ERα platform has been optimised by developing a detection method based upon fluorescence polarisation which will also enable detection of fluorescent phytoestrogens. This method can now be used to measure breast tissue samples (α- and β-fractions and E-screen positive fractions) and fish bile samples (pooled fractions and E-screen positive fractions). Partner 12 have obtained the pET15b hAR LBD plasmid and begun expression experiments in *E. coli* and a radio-labelled receptor binding assay is available for checking expression levels.

Difficulties/Delays

Due to delays in reception of bream samples there will be a delay by Partner 9 for completion of D4.2 until month 40. An extension of the contract would be opportune. Partner 11 has been affected by time delay caused by problems with obtaining male bream in reasonable numbers form the sites of interest and some missing samples that had to be replaced. For Partner 12, a delay in the measurement of tissue samples has occurred, because until now no samples had been received from other partners. It is very important for Partner 12 to know the exact nature of the samples in order to optimise the HPLC

methods. Partner 12 has also experienced a delay in the development of the HRS AR platform. Expression experiments have just recently been started due to the difficulties with obtaining an appropriate hAR LBD plasmid. The LBD is preferred over the complete androgen receptor, because of possible stability issues.

Plan and Objectives for the Next Reporting Period

- Partner 9 plans to finalise the hyperfractionation data analysis and to continue with the bioassay-directed fractionation and analysis of samples.

- Partner 11 will identify active fractions in bile and visceral fat from fish, distribute those to Partners 9, 10, and 12 and analyse them for unknown endocrine disrupting compounds.

- Partner 12 will measure samples from other partners with the validated hEST assay and the new on-line HRS-ERα FP technology, optimise the hAR LBD expression in *E. coli*, construct a fluorescent ligand for the on-line HRS-AR platform and develop a HRS-AR platform based receptor affinity detection assay. Partner 12 will also measure cryptorchid samples and other samples possibly containing androgenic chemicals.

Workpackage Related Appendices

Annex I (D4.1) Report on suitability of assays for screening fractions

Deliverables and Milestones

Deliverables	
✓ on going (36)	**D4.1:** Report on suitability of assays for screening of fractions (month 24)
in progress (24) delayed until month 40 P9 (36)	**D4.2:** Report on preliminary results with bioassay-directed fractionations of human and fish samples (month 36)
in progress (36)	**D4.3:** Report about results of bioassay-directed fractionations of human and fish samples (month 48)
in progress (36)	**D4.4:** HRS technology for detection of androgens (month 48)
Milestones	
✓	**M4.1:** Insights into the usefulness of EDC bioassays for bioassay-directed fractionations (month 24)
in progress (36)	**M4.2:** Information about the predictive power of total measures of endocrine disrupting potential of extracts (month 48)
in progress (36)	**M4.3:** Information about novel EDC in samples (month 48)

WP5	**The application of genomics and proteomics to the development of sensitive endpoints of EDC action**					
Phase						
Start date	0					
Completion Date	36 months					
Current Status	active					
Partner(s) responsible:	13	1	4	11	14	15
Person-months per Partner:	35.5 (24)	15.6 (12)	26 (20)	20 (16)	26 (13)	30 (20)
Already devoted person months per Partner and total:	18	10	16	12	11	15

Objectives for the Reporting Period

- Understand the effects of EDCs on cell signalling pathways and the cross-talk between cell signalling pathways and ER, using MCF-7 cells
- Apply genomic tools including arrays to assess EDC effects in rats
- Detect novel EDC-responsive genes in zebrafish by DNA microarrays
- Elucidate mechanisms involved in EDC genomic action in fish using transient assays in cell lines, zebrafish eggs and juvenile and adult zebrafish *in vivo*
- Utilise established and novel markers to assess low-dose effects of EDC in zebrafish

Methodology and Study Materials for the Reporting Period

1. The experimental study material used to understand EDC effects on signalling pathways and their cross-talk was the i*n vitro* mammary cell proliferation assay (E-Screen). The effects of EDCs were assessed through molecular (PCR, western blot) analysis of Src/Erk pathway activation and through assessing EDC effects on cell proliferation, in the presence or absence of inhibitors of the signalling pathway.

2. To study EDC-responsive gene expression in testes of mouse and rats, a range of quantitative and qualitative molecular methods were used, including DNA arrays, real-time RPCR and in situ hybridisation.

3. Cell lines transiently transfected by reporter gene plasmids were used to study mechanisms involved in EDC genomic action and to explore the potential of such systems as bioassays. The reporter gene plasmid consisted of inducible promoter and luciferase coding sequence, with or without different over-expression vectors containing coding sequences of studied receptors. Plasmid expressing β-galactosidase under control of constitutive promoter was co-transfected in the same wells to normalise for differences between transfection efficiency and cell numbers from well to well. Transfected cells were treated with chemicals or vehicle control, allowed to grow for 48 hours, then lysed and assayed for luciferase and β-galactosidase activity.

4. The ER-negative glial cell line (U251-MG) was used for transfection experiments to express each ER subtype and the endogenous zebrafish Aro-B promoter as reporter genes. With these recombinant cells, the impact of low doses and mixtures of EE2, E1, α-zeralenol and genistein, parallel to E2 (positive control) and ethanol (solvent, negative control) was investigated.

5. *In vivo e*xposure experiments were used to study gene activation in zebrafish by exposure to low doses of xenoestrogens. During the reporting period the following exposures were carried out (a) 17ß-estradiol (E2) (adult male zebrafish, concentrations: 0/5/50/100/200 ng/L); (b) Ethinylestradiol (EE2) 30ng/L (adult male zebrafish, as a positive control); (c) Bisphenol A (BPA) 0.1 µg/L, 2 µg/L, 20 µg/L, 200 µg/L, 400 µg/l, 1 mg/L and 2 mg/L (adult male zebrafish). Samples were taken from the fish and analysed for previously established marker genes (vtg- Partner 13, aromatase – Partner 15) by means of real time RT-PCR, an for novel marker genes by microarray technology.

6. To evaluate the role of aromatase in early life of zebrafish, and to identify aromatase-mediated adverse effects of EDCs in the embryo stage, morpholino knockdown experiments were performed. Antisense morpholinos of the aromatase genes were designed and zebrafish eggs were injected between the 1-4 cell stage with 300, 500 or 700nM of either zf-aromatase morpholinos or control morpholino. As an additional control, morpholinos with 5 mismatches were designed and injected in zebrafish. The effects were assessed until the 5th dpf using FM1-43 live staining of neuromasts, acridine orange live staining for apoptosis, alcian blue staining for assessment of cartilage development. Control experiments were performed either by adding E2, EE2 ("rescue experiment") or by using the ER antagonist, ICI 182780.

7. To evaluate the role of aromatase in sexually differentiating zebrafish and to identify aromatase-mediated adverse effects of EDCs in the sexual differentiation stage, the role of aromatase in sexually differentiating fish were studied. RNA was extracted from the heads and was further used in real time RT-PCR in order to evaluate *zfcyp19a2* expression levels during that stage and whether these expression levels are linked to gonadal sex. Gonads were histologically sexed for assigning gonadal sex to each individual. Additional fish were exposed to BrdU and these fish were used for immunohistochemistry of CYP19A2 and BrdU. Gonads were again used for assigning gonadal sex to each individual

Scientific Achievements

Partner 1 contribution

The objective of Partner 1 during the reporting period was to examine the role of different signalling pathways in mediating the effects of xenoestrogens. The experiments performed during the 3rd year-period showed that the mitogenic effects of estrogenic compounds, both natural estrogens and xenoestrogens is indeed mediated not only through the classical ER pathway, but involves other, non-genomic signalling pathways such as the Src/Erk pathway. This implies that the mitogenic effects of endocrine disrupters can be regulated not only by the classical receptor-binding activity, but also by activation of rapid cell signalling pathways and ligand-independent ER activation. These

findings highlight the need to take these short term effects into account (rather than simply measuring direct ER-binding) when evaluating the full estrogenic potency of chemicals.

Experimentally, the role of the Src/Erk pathway was studied by treating MCF-7 cell with a single concentration (which was found to induce maximum cell proliferation response) of the xenoestrogens (E2, *o,p*'-DDT, *p,p'*-DDE, β-HCH), in combination with increasing concentrations of a specific inhibitor of the Src/Erk cascade (PD98059). As exemplified in Figure 1, a clear decrease in cell number was detected when concentrations of 10, 25 and 50 µM PD98059 were added to E2, *o,p*'-DDT and β-HCH. A minimal reduction in cells treated with *p,p*'-DDE was also observed. This clearly indicates that the proliferation of MCF-7 cells in response to estrogenic compounds is not only regulated by the direct binding and activation of the ER, but also by the indirect activation of the receptor via cell signalling pathways.

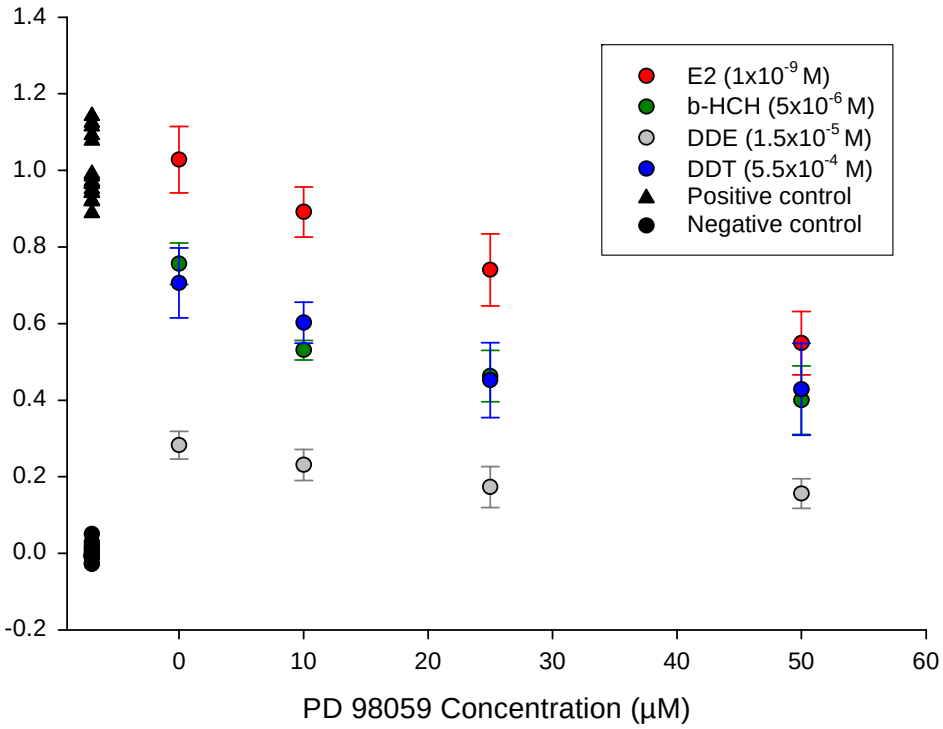

Figure 1 Effect of the MAPK cascade inhibitor PB98059 on the mitogenic activity of E2, *o,p*'-DDT, β-HCH and *p,p*'DDE in the E-Screen

Partner 4 contribution

The objective was to validate a new rat testis microarray and to apply this tool to tissue samples of rats which had been exposed to EDC mixtures. Partner 4 has previously constructed and validated a mouse testis DNA oligo array. Validation (i.e. expression in distinct germ-cell types) by in situ hybridisation has been done for almost 200 genes (Almstrup *et al.*, 2004). This technology has now been transferred to the rat, i.e. the corresponding rat genes were identified, and a rat testis micro array was constructed.

During the reporting period, major efforts were devoted to validate the rat-array for use in analysing gene expression changes in rat testis. This includes also the subsequent verification of putative differentially expressed genes. For that purpose, Partner 4 has

transferred the in situ hybridisation technology developed originally for mouse samples to rat testis. The results showed that the mice technology works on rat testis as well. A range of fixatives were tested in order optimise the in situ hybridisation methodology. Best results are obtained on testis fixed in both paraformaldehyde- and Stieves-fixative. With these findings, Partner 4 is ready to perform the gene expression study on tissues of rats exposed to EDC mixtures. However, as a result of the slight delays in the animal part of the study, the study material is not yet available.

Almstrup K, Nielsen JE, Hansen MA, Tanaka M, Skakkebaek NE, and Leffers H, (2004). Analysis of cell-type-specific gene expression during mouse spermatogenesis. *Biol. Repro.*, **70** (6) 1751-1761.

Partner 11 contribution

The objective during the reporting period was to study the regulation the two aromatase genes (*cyp19a* and *cyp19b)* of zebrafish. Aromatases regulate systemic and local estrogen levels, and moreover their promoters were shown to contain multiple putative transcription regulation elements, which could make them susceptible to exposure to different classes of EDCs. Thus, the two aromatase genes are potential targets of EDC genomic action and EDC effects on signalling pathways. The experimental models used for the studies were transiently transfected cell lines and zebrafish eggs.

In close collaboration with Partner 15 and Partner 14, the following progress was made:

- Reporter gene constructs were generated for *in vivo* studies (microinjection in zebrafish eggs), composed of *zfcyp19a* and *zfcyp19b* promoter parts, "enhancing sequences" Gal4-VP16-UAS-E1b and EGFP coding sequence (for more details, see report on WP10).

- Luciferase reporter gene constructs were generated for *cyp19a* (short and long versions, containing 600bp and 1800bp of the *zfcyp19a* promoter region in plasmid pGL2 basic).

- The dependency of the *zfcyp19a* promoter activity on the cell context was evaluated. It was found that the promoter is much more active in an ovarian cell context (CHO cells) than in a glial cell context (U251). (also compare with the findings of Partner 14 on the cell context dependency of *zfcyp19b* expression).

- Since putative CRE elements were predicted in both *zfcyp19a* and *zfcyp19b*, the effects of CREB pathway mediators on the activity of these promoters *in vitro* were examined. The role of the CREB pathway was studied using a range of activating chemicals, as well as experiments with different promoter constructs (short and long version, mutation in the CRE element). From the results of these experiments it can be concluded that predicted CRE elements in the *zfcyp19a* and *zfcyp19b* promoters do not seem to be functional.

- The *zfcyp19a* promoter response to E2 treatment *in vitro* was studied. Investigations showed that the *zfcyp19a* promoter is responsive to E2 treatment although it does not contain a full ERE sequence. The working hypothesis was that the upregulation possibly goes via SF1 and 0.5 ERE sites. During the course of the studies, it became

evident that E2 responsiveness results already from transfection with the empty vector, although the overall luciferase activity produced by empty pGL2 was about 50 times lower than that produced with vector containing *zfcyp19b* promoter. Thus, it remains to be clarified if the observed upregulation of *zfcyp19b* promoter by estrogen treatment was dependent on the promoter or was simply an artefact produced by empty vector sequences.

- The response of *zfcyp19a* and *zfcyp19b* promoters to treatment with AhR ligands *in vitro* was analysed, in order to evaluate whether AhR ligands , which include a wide range of important environmental contaminants such as dioxins, PCBs, and PAHs, may be able to modulate the aromatase system. The results indicate that the two promoters do not respond significantly to treatment with AhR ligands. It is worth to mention here that the predicted AhREs in *zfcyp19* promoters are poorly conserved in comparison to AhRE consensus sequence. These observations allowed us to conclude that the putative AhREs predicted in the *zfcyp19a* and *zfcyp19b* promoters do not function via classical AhR pathway.

- Finally, possible interaction of ER and AhR and their ligands and the resulting effect on *zfcyp19a* and *zfcyp19b* promoters' activity *in vitro* was studied. It was found that the AhR and its ligands affect the activity of *zfcyp19b* promoter *via* interaction with ER. This mechanism involves ERE site but is independent of putative AhRE site located on the promoter. Taken together, the results suggest that the predicted AhRE in the *zfcyp19b* promoter does not function via classical AhR/ARNT mechanism, rather, the effects of AhR ligands on its activity are due to unspecific interaction of ER and AhR/ARNT, functioning via the ERE site independent of AhRE. This *in vitro* data offers a plausible explanation to some contradicting in vivo data, pointing to the importance of estrogen availability in the system, which causes estrogenic or anti-estrogenic effects of dioxins that can be observed.

Partner 13 contribution

The objective for Partner 13 was to further evaluate the value of microarrays in assessing EDC genomic action in fish. To this end, zebrafish were exposed to various concentrations of E2, BPA and NP and tissue samples were analysed using array technology. For comparison, the response of an established marker gene, vitellogenin, was measured using real time RT-PCR. The arrays were self-spotted arrays containing 40 genes selected on the basis of the results from the previous experiments of Partner 13 (given in the 2nd Annual Report) and a list of the 40 genes is presented in D5.3 (Annex I).

The real time RT-PCR analysis showed that there is a significant increase in the *vtg1* expression at 200 ng/L E2 and 2000 µg/L BPA. Due to the individual variation in the responses to NP no significant increase was observed with this compound. The array experiments revealed that with increasing concentration of E2 the number of regulated genes/sequences and the level of expression rises. An induction of gene expression is observable at concentrations higher than 100 ng/L E2. At a concentration of 200 ng/L for E2, only the *vtg1* and *vtg3* are up-regulated with a significance of 95 %. At a concentration of 500 ng/L E2 the *vtg1* is up-regulated 832-fold and the *vtg3* is up-regulated 6-fold. In addition 25 further genes/sequences are expressed at a concentration of 500 ng/L with a significance of 95%.

In addition to the microarray experiments on E2-exposed zebrafish, analyses were also carried out with samples from fish exposed to BPA. To detect genes/sequences responding to BPA, whole genome arrays were used to analyse samples from fish exposed to a concentration of 1000 µg/L, since the real time RT-PCR results indicated significant induction of *vtg1* at that concentration. 38 genes/ sequences were found to be regulated. These 38 genes/sequences plus the 40 genes/ sequences regulated by E2 were spotted on epoxy slides. Hybridisation experiments with all concentrations (0.1, 2, 20, 200, 400, 1000, 2000 µg/L BPA) are currently in progress.

Partner 14 contribution

The objectives for Partner 14 during the reporting period were to evaluate the utility of the zebrafish brain aromatase (Aro-B) gene as biomarker in cell-based assays for detecting the exposure to estrogenic chemicals, and to analyse the impact of low-dose and mixture EDC on three distinct ERs. Initially, the dependency of *zfcyp19b* induction on the cell context was studied. Transfection experiments with the promoter-luciferase reporter in different cell contexts showed that, similar to the *in vivo* situation, full E2-upregulation of the *zfcyp19b* gene is restricted to glial cell lines, such as the human glial cell line U251-MG. Then, a glial cell-based assay using *zfcyp19b* as the target gene was developed in order to investigate the impact of environmental estrogenic chemicals on distinct ER activity. To this end, the ER-negative glial cell line (U251-MG) was transfected with the three zebrafish ER subtypes and the *zfcyp19b* promoter linked to luciferase reporter gene. E2-treatment of U251-MG glial cells co-transfected with zebrafish ER and the *zfcyp19b* promoter-luciferase reporter resulted in a 60 to 80-fold stimulation of luciferase activity. The detection limit was below 0.05 nM and the EC50 was 1.4 nM. Interestingly, in this glial cell context, maximal induction achieved with the *Aro-B* reporter was 3 times more than that observed with a classical Estrogen-Response-Element-reporter gene (ERE-tk-Luc). Dose-response analyses with ethinylestradiol (EE2), estrone (E1), α-zeralenol and genistein showed that estrogenic potency of these agents markedly differed depending on ER subtype in the assay. Moreover, the combination of these agents showed an additive effect according to the concept of concentration addition (CA). This confirmed that the combined additive effect of the xenoestrogens leads to an enhancement of the estrogenic potency, even when each single agent might be present at low effect concentrations. In conclusion, Partner 14 demonstrate that the bioassay provides a fast, reliable, sensitive and efficient test for evaluating estrogenic potency of endocrine disruptors on ER subtypes in a glial context.

To evaluate the impact of EDCs on molecular and cellular aspects of endocrine disruption, it is necessary to develop cell-based transcription assay systems that could reflect different cellular contexts. However, very little effort has been made to investigate the impact of environmental estrogenic chemicals in the glial cells, although they may represent an important *in vivo* target for adverse effects of EDCs. With the work carried out by Partner 14 during the reporting period, a glial cell model is now available that enables the analysis of the impact of environmental estrogenic chemicals on transcriptional activity of all three ER subtypes characterised to date in a vertebrate species. In this glial cell model, the strong E2-stimulation of luciferase activity under the control of E2-sensitivity *Aro-B* reporter construct enables to obtain accurate results in 96-well plates making the assay suitable for sensitive and reliable high throughput screening.

Partner 15 contribution

The objectives were to a) understand the role of the aromatase system in various life stages of zebrafish, b) study the responsiveness of the aromatase system to EDC exposure and c) to associate any consequences on developmental or reproductive parameters of the zebrafish. In order to examine the role of aromatase in early life stages of zebrafish, embryos were injected with morpholinos to suppress *zfcyp19a* and *zfcyp19b* expression during early development. Microinjections with *cyp19a* morpholinos resulted in a statistically significant reduction of neuromast number, as illustrated on Figure 2. This finding highlights the fact that estrogen-active chemicals may affect much more targets than sexual differentiation and reproduction, although research to data has been focused almost exclusively on the latter two targets.

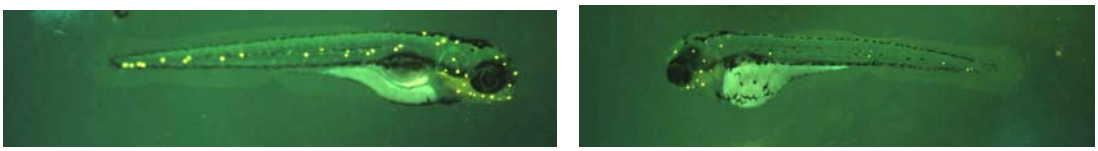

Figure 2 Left figure: control zebrafish larva, with neuromasts along the head and the lateral line (yellow dots). Right figure: morpholino-injected zebrafish with reduced number of neuromasts.

Previously, Partner 15 has shown that inhibition of aromatase in developing zebrafish results in complete and irreversible masculinisation. In this reporting period, the expression change of aromatase in zebrafish during sexual differentiation was studied, as a basis to analyse in future experiments how EDCs modulate this expression, and how this relates to altered sexual differentiation. While *zfcyp19a* was displaying sexual dimorphism, no sexual dimorphism was evident for *zfcyp19b* expression. However, there was a pronounced, apparently not sex-linked individual variation in the expression levels of *zfcyp19b* in both males and females, the physiological meaning of which is not understood. This variation was not only expressed during the period of sexual differentiation but also in adults.

The question was if such individual variation of *zfcyp19b* expression would be modified by exposure to estrogenic substances. To answer this question, Partner 15 joined exposure experiments performed by Partner 14 and analysed the *zfcyp19b* expression levels in the brains of the experimental animals. Exposure of adult male zebrafish to BPA, EE2 or E2 did not alter the variation of *zfcyp19b* expression, but 200 ng E2/L led to a significant increase of the average expression level of *zfcyp19b.*

Progress Summary

The work carried out by the involved partners under WP5 led to four major innovations:

1. Identification of candidate genes for construction of transgenic zebrafish (completed in the first year of the project).

2. Identification of novel diagnostic tools to assess exposure to and effects of EDCs. Cell-based screening assays were developed for high-throughput screening on EDC actions. Further, the potential of modern molecular tools such as real-time RT-PCR,

in situ hybridisation and in particular microarrays was evaluated. Arrays were constructed both for mammalian and fish tissues. Information extractable from the various methodological approaches was compared in EDC exposure experiments, and responses were related to tissue accumulation of EDCs. Both the identification of candidate genes for transgenic zebrafish and the development of new tools and bioassays provide important technologies for EDC hazard assessment.

3. Identification of novel signalling pathways involved in the genomic action of EDCs. The results from this work demonstrate that the effects of estrogenic chemicals are not only mediated via the classical ER pathway, but additional signalling pathways as well as cross talks to other receptor pathways play an important role.

4. Identification of novel targets of EDC action was primarily achieved by studying the mechanisms involved in the action of EDCs. The results from this work pointed to aromatases and ERs as important factors in EDC action, and they highlighted that the effects of estrogenic substances are not restricted to reproductive processes, but involve multiple targets; in particular neuronal/glial systems can be impacted by xenoestrogens. Both the findings on the new signalling pathways and the new targets of EDC action are of high relevance for the risk assessment of EDCs.

With these results, WP5 has achieved its objectives with the exception of the application of the new molecular tools in analysing tissues from rat and fish treated with EDC mixtures. The mixture experiments with rats and zebrafish are delayed and consequently, the results from the genomic analyses of these samples are not available yet. These studies will be executed during the forthcoming project year. Contrary to rat and fish studies, mixture experiments with MCF-7 cells have been completed and marker gene responses have been analysed (see report from Partner 1 in WP14).

Plan and Objectives for the Next Reporting Period

As indicated above, for most of the objectives in WP5 the experimental work has been completed. Research during the 4th reporting period will focus on completion of ongoing studies, and on the analysis of the mixture experiments:

- Microarray and RT-PCR analyses of tissues from rats and fish exposed to EDC mixtures.

- Completion of hybridisation experiments with whole genome arrays on tissues of BPA-exposed zebrafish, to examine whether BPA induces partly different gene than does E2.

- Further analysis of ER/AhR interaction and the regulation of *zfcyp19a*.

- Search for glial factors which confer estrogen-regulation of *zfcyp19b* gene.

Workpackage related appendices

Annex I (D5.3) Novel endpoints (markers) and tools (including DNA arrays) for the assessment of EDC effects in mammalian cells, fish and rats

Annex I (D5.4) Recommendations for the utilisation of genomic responses in testing guidelines with fish (preliminary report given)

Deliverables and Milestones

Deliverables	
✓	**D5.1:** Identification of candidate genes for construction of transgenic zebrafish (WP 10) (month 12)
✓	**D5.2:** Report on preliminary results with novel endpoints and tools (month 18)
✓	**D5.3:** Novel endpoints (markers) and tools (including DNA arrays) for the assessment of EDC effects in mammalian cells, fish and rats (month 36)
preliminary report (36)	**D5.4:** Recommendations for the utilisation of genomic responses in testing guidelines with fish (month 36)
Milestones	
✓	**M5.1:** Identification of some marker genes by the various methodological approaches (month 12)
dependent on WP14-15 (36)	**M5.2:** Recognition of signalling pathways involved in EDC action, characterisation of marker responses under low dose and mixture exposure (month 36)

WP6	Foetal exposure of rats to phthalates - a new model for the identification of mechanisms underlying the "testicular dysgenesis syndrome" in the human					
Phase						
Start date	0					
Completion Date	36 months					
Current Status	active					
Partner(s) responsible:	3	5	14			
Person-months per Partner:	68 (34)	41 (18)	34 (17)			
Already devoted person months per Partner and total:	28	17	15			

Objectives for the Reporting Period

- Further elucidate the cellular mechanisms via which DBP treatment *in utero* induces focal dysgenetic areas in the rat testis, which persist throughout life (Partner 3).
- Evaluate and characterise changes in fetal germ cell proliferation and differentiation in fetal life and in early postnatal life following *in utero* exposure to DBP: primary emphasis on evaluating whether altered germ cell differentiation occurs that might have analogies to the origin of CIS cells in the human (Partner 3).
- To compare the effect on fetal testis parameters of short (E19.5 – E20.5) or long (E13.5 – E20.5) term dosing with DBP in the rat (Partner 3).
- To compare and contrast the dose sensitivity to DBP of fetal and adult testicular 'dysgenetic' endpoints (Partner 3).
- To compare the effects of endocrine-disrupting chemicals (EDC) known to affect the fetal testis, namely 4,4'-DDE, Di-isononyl phthalate (DINP) and dioxin (TCDD) on testicular and adrenal structure and steroidogenesis in the rat (Partner 5).
- To identify new potential targets for phthalates and other EDC in the fetal rat testis, with emphasis on factors able to affect development of the Leydig cells (Partner 5).
- To use the rat Fetal Gonad Assay (FEGA) to screen for the effects of DEHP and MEHP *in vitro*, with the view of identifying new endpoints and mechanisms (Partner 14).
- To undertake proteomic mapping of adult rat efferent ducts as a prelude to identification of potential target genes for androgens, estrogens and Insl3 during development (Partner 14).

Methodology and Study Materials for the Reporting Period

1. Pregnant rats will be administered dibutyl phthalate (DBP) or diethyl hexyl phthalate (DEHP) on days E13-E21 at doses (100-500 mg/kg/day) that induce reproductive

disorders in the male offspring. Animals will be sampled during foetal life (E15, E19) for studies of pathways of phthalate action, and on postnatal days 5, 15, 25, 35 and 90 to identify short- and long-term consequences. Latter to include hypospadias, testis weight/morphology, testis descent, hormone levels (testosterone, estradiol, inhibin B, FSH, LH), numbers of Leydig, Sertoli and germ cells, cell proliferation and apoptotic rates. At E15-19, additional endpoints will be testis testosterone + expression (mRNA/protein) of factors likely to inform on pathways of phthalate action: steroidogenic enzymes (P450scc, 3b-HSD, 17a-hydroxylase, aromatase), LH receptor, SF-1, StaR , ERK-1 & 2, c-kit, GDNF, AR, ERα, ERβ, plus new genes/proteins (from 2). Pathways activated by phthalate exposure will be contrasted to those activated by other pregnancy treatments that induce male reproductive disorders, e.g., DES, flutamide. Partners 3 and 5 have all methods established and will exchange tissue specimens.

2. Isolation and short-term culture of foetal rat testis explants on E15-E19 ± monoester phthalates and specified hormones, or isolation of such explants from animals in 1 and their culture. A proteomics approach, established by Partner 14, will be used to identify proteins affected by phthalates. By reference to databases, cloning/sequencing, mRNA probes and/or antibodies to the new genes/proteins will be generated and used to identify the pathways in 1.

3. Human foetal testis explants (obtained from abortuses during the period of sexual differentiation) will be cultured ± monoester phthalates and hormones, using methods established by Partners 3 and 14, and the pathways and sensitivity to phthalates compared to the rat. Ethical permission for such studies has been obtained.

Scientific Achievements

Partner 3 contribution

In addition to the methods detailed previously which have centred around the administration by gavage of DBP in various doses (500, 100, 20 or 4mg/kg/day) to time-mated pregnant female rats followed by the collection of testicular and other materials at various fetal and postnatal ages for evaluation of numerical and functional changes in testis cell parameters and the relationship to disorders of testis development and function and position. A further approach has been adopted, the comparative evaluation of the effect of short versus longer term dosing with 500mg/kg/day DBP on testis parameters at E21.5 and also on day 4 postnatally and in adulthood. The primary approaches that have been used for evaluating altered cell function in the testis is to use light microscopy, stereology (to establish cell numbers and size) and immunohistochemistry, and in particular confocal microscopy, to establish the location and development of testicular cells using a battery of cell-specific protein markers. In excess of 20 different cell functional markers have been used or evaluated in the past year in pursuit of these goals and new endpoints are constantly being evaluated as understanding advances. In all postnatally sampled animals, blood samples have also been obtained and used for measurement of testosterone, FSH and hopefully soon Insl3.

The following details the main areas in which work has moved forward over the past 12 months:

1. Evidence has shown that the most likely primary site of action of DBP on the fetal testis is disruption of Leydig cell distribution and function. The latter is evident from expression of testosterone levels and Insl3 expression as well as evidence from other studies by Partner 3 which have shown marked suppression of expression of genes involved in cholesterol uptake, transport and metabolism, including several steroidogenic enzymes, as well as expression of other genes/proteins such as inhibin-α and CRABP2 (cellular retinoic acid binding protein-2). The suppression of the latter 2 proteins are of particular interest as they are also expressed in Sertoli cells within the seminiferous cords but their expression is unaffected in this compartment whilst being markedly suppressed in the Leydig cells (Figure 1). This and other findings suggest that at least some of the effects of DBP treatment are Leydig cell-selective. This recognition contrasts with Partner 3's starting hypothesis which suggested that the Sertoli cell was the most likely primary sight of action of DBP and its effects on the Leydig cells might be secondary to such effects; this appears not to be the case.

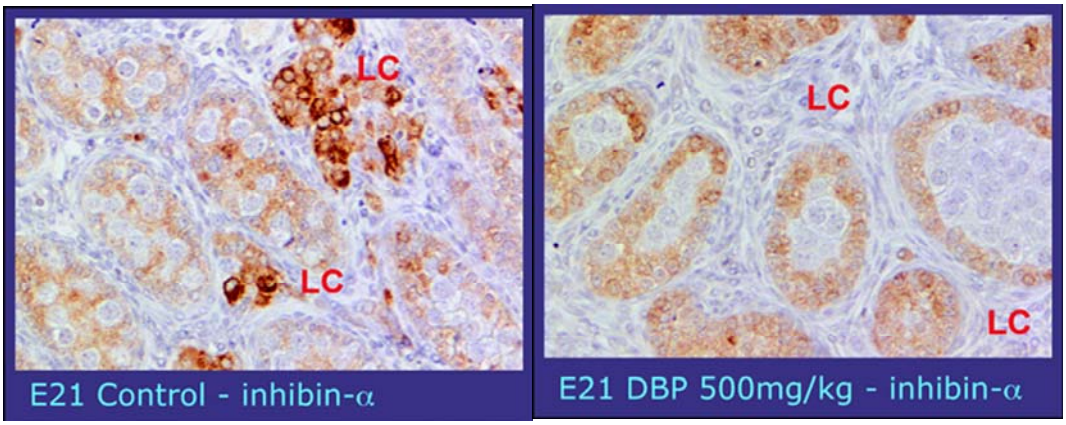

Figure 1 Effect of fetal exposure to DBP on immunoexpression of inhibin-α in the fetal rat testis. Note the DBP-induced loss of expression in Leydig cells (LC) but the lack of effect on expression in Sertoli cells in the seminiferous cords.

2. Comparative data on the dose-sensitivity of endpoint effects of DBP treatment that is evident either in fetal or adult life. Partner 3 have evaluated several endpoints at each of these time points which have shown are affected by DBP exposure *in utero* and for which there are reasonable ideas about their ontogeny, mechanistic cause and relevance to suppression of hormone production in fetal life. The endpoints evaluated in fetal life are: occurrence of multinucleated germ cells, aggregation of fetal Leydig cells and testicular testosterone levels. The endpoints evaluated in adulthood are: cryptorchidism, hypospadias, fertility and the occurrence of focal dysgenetic areas in the testis. These data are nearing completion and evaluation but it is already apparent that there is greater dose sensitivity to DBP for the endpoints evaluated in fetal life compared with those evaluated in adulthood. In the latter, for instance, it is only really with the highest tested dose of DBP (500mg/kg/day) that consistent and significant effects are found, whereas for the fetal endpoints, clear effects are found with doses as low as 20mg/kg/day.

3. Evidence for alteration of the timing of fetal germ cell differentiation and proliferation following *in utero* exposure to DBP. What has been found is that the entry of gonocytes into a state of quiescence, which in essence involves loss of expression of

stem cell-like characteristics (e.g. expression of OCT4) is delayed in DBP-exposed animals. Partner 3 have recently found that this delayed differentiation also extends to a delay in resumption of germ cell proliferation after birth, such that a reduction in proliferation rate and germ cell numbers is evident between the period 4-8 days postnatal. Whilst the mechanistic basis for these changes is still under investigation, these findings are of particular importance for 2 reasons. First, the prolonged expression of OCT4 is reminiscent of what is proposed to happen with the origin of CIS cells in fetal life which give rise to testicular germ cell cancer in adulthood in the human. Second, the age at which alteration in germ cell development is first evident is at E15.5, an age at which no other effects of DBP on the testis have so far been discerned by this Partner or by other groups working in this area. The germ cell effects are therefore the earliest known adverse effects that result from DBP exposure. The other important observation is that this particular effect of DBP on germ cell proliferation and functional differentiation appears to be completely independent of the effects that DBP has in inducing the appearance of multinucleated gonocytes. The latter only become evident at E19.5 and are at its most pronounced at E21.5 and shortly after birth. The induction of multinucleated gonocytes occurs even with short-term DBP treatment (see above) which commences at E19.5, whereas this treatment has no effect on the differentiation status of the germ cells as far as we can detect.

4. New evidence to suggest that development and/or function of the adult population of Leydig cells may be affected by *in utero* exposure to DBP. This is preliminary data based on analysis of blood testosterone levels, but it has prompted Partner 3 to undertake analysis of Leydig cell numbers and function at different postnatal ages and in different DBP dose groups in order to establish whether or not this is the case.

Partner 5 contribution

Time-mated pregnant rats were subjected to one of the following treatment regimens: (i) TCDD (0, 0.3 or 1.0 μg/kg maternal weight) administered as a single treatment on E11.5; (ii) 4,4'-DDE (0, 50 or 100 mg/kg) administered daily from E13.5 - E17.5; (iii) DINP (0, 250 or 750 mg/kg) administered daily from E13.5 - E17.5; (iv) Flutamide (25 mg/kg) administered daily from E10.5. Foetuses from treatments (i) – (iii) were recovered on E19.5 and testicular testosterone and progesterone levels measured, and the expression of StAR, steroidogenic enzyme, ERα and androgen receptor then analysed by RT-PCR and Western analysis. Fetuses from treatment group (iv) were recovered on E14.5, 15.5, 17.5, 19.5 or postnatal day 1 and the expression of the following regulatory molecules then assessed using real-time PCR: Desert hedgehog (dhh) and its receptor patched (Ptc-1), the steroidogeneic enzymes P450scc and 3β-HSD type 1, steroidogenic factor-1 (SF-1) and Insl3 (see WP7).

1. *Fetal effects of TCDD*

The highest dose of TCDD caused a small decrease in weight of the fetuses. Pituitary levels of LH decreased, whereas corticosteroid levels increased. Levels of androgen receptor and oestrogen receptor-α decreased in the testis. Analysis of blood hormone levels will be undertaken in the first few months of 2006.

2. *Fetal effects of DINP*

The highest dose of DINP caused a tendency to increased mRNA levels of steroidogenic enzymes in both adrenals and testes, but no change was seen at the protein level. Plasma corticosteroid levels were increased in fetuses that were exposed to the lower dose (250 mg) of DINP.

3. *Fetal effects of DDE*

Effects of DDE were analysed particularly in adrenals. With the doses used, no significant effects on steroidogenesis or steroidogeneic enzyme expression were found. These studies indicate that dioxin is a potential endocrine disrupter in the fetal testis and adrenal gland, whereas DINP and DDE at the doses used do not cause any striking adverse effects. These findings are in marked contrast to the major effects found on the fetal testis, but not adrenal, after exposure of male rats to DBP *in utero* as shown by Partner 3. The results above are accepted for publication in Toxicology and Applied Pharmacology.

4. *Fetal effects of exposure to flutamide*

Flutamide exposure *in utero* resulted in significant suppression of mRNA levels for dhh, Ptc-1, Insl3, and P450scc and 3β-HSD type 1 from E17.5 onwards, though the magnitude of effect was generally not large. Nevertheless, the observation that flutamide, which is an androgen receptor antagonist, can alter expression of factors such as dhh and Ptc-1, which are known to influence differentiation of fetal Leydig cells, illustrates new mechanisms via which environmental chemicals can potentially alter testis development and downstream targets dependent on normal fetal Leydig cell function.

Partner 14 contribution

Partner 3 has been unable to identify effects of DBP, or its presumed active metabolite MBP, *in vitro* on testosterone production by explants of rat fetal testes at age E19.5-E21.5. This finding raised several key questions. First, is age E19.5 too late for showing phthalate effects *in vitro* because the testis must be cut into several pieces at this age to facilitate culture? Second, could metabolites other than the monoester phthalates be involved *in vivo* in the effects of DBP and DEHP on the fetal rat testis? Third, can other endpoint effects of phthalates on the early fetal rat testis be identified?

To address these questions, studies investigated the effect of DEHP and MEHP at 10^{-5}M on intact testis explants from E14.5 rat fetuses in culture for different periods of time. Partner 14 also investigated the effect of the further metabolite of DEHP/MEHP, namely 5-hydroxy-MEHP (metabolite IX). Four main endpoints were studied in priority:

1. The general anatomy of the gonad (light microscopy).
2. The number of gonocytes (light microscopy).
3. The apoptosis of gonocytes (TUNEL).
4. Testosterone production measured by radioimmunoassay.

1. *General anatomy of the fetal testis*

The methods for culture of E14.5 intact fetal rat testes was previously developed and validated by Habert *et al*. (1991) and Lassurguère *et al*., (2003). At the concentration of DEHP used (10^{-5}M) and for the time-period of culture explored (24 to 72 h) the effects seen were discrete and the different compartments of the testis were in general unaffected. However, DEHP did have an unequivocal effect on the number of gonocytes, and for some of these cells the nuclei appeared pyknotic. In contrast, MEHP had no such effect, whereas metabolite IX of MEHP induced the same negative effect as did DEHP.

In order to assess the distribution of DEHP in the culture system, ^{14}C-DEHP was also added to the culture wells in the presence or not of the fetal testis. Partner 14 found that only 0.2 % of DEHP was present within the gonads, the vast majority of it being distributed in the nitrocellulose filter (17 %) or in the culture medium (17 %), the rest being absorbed by the plastic of the culture system. This may indicate that one potential reason why DEHP (and possibly MEHP) exerts only minimal effects *in vitro* on the fetal testis is because the majority of the added test compound is absorbed into the matrix of the culture system as opposed to being free to act within the explanted testis.

2. *Numbers of gonocytes and their apoptosis*

As DEHP and metabolite IX both caused a reduction in the number of gonocytes in cultured E14.5 fetal testes, the effect of these two phthalates and of MEHP on gonocyte apoptosis was assessed using TUNEL methodology. Results have shown that both DEHP and metabolite IX of MEHP (10^{-5}M) induced a significant increase in gonocyte apoptosis, thus indicating that the decrease in number of fetal germ cells caused by these two compounds results at least in part from the induction of apoptosis. In contrast, MEHP consistently failed to increase gonocyte apoptosis. Further experiments are underway to verify this observation which may indicate that the direct effect of DEHP seen in culture may be mediated by metabolite IX.

2. *Testosterone production*

Another surprise was that after 72h of culture a significant stimulation of testosterone production was observed in the culture media when the gonads were exposed to DEHP (10^{-5}M). In contrast, MEHP inhibited testosterone production and the same trend was observed when metabolite IX was tested (one experiment only). These observations need to be repeated several times, as earlier studies by Partner 3 using E19.5 fetal rat testis explants also illustrated an overall small but significant decrease in testosterone production after addition of MBP, but there was considerable variability in the magnitude of such effects. The present findings may also indicate that the direct effect of phthalates on the fetal Leydig cells is not necessarily mediated by the same metabolites of DEHP as those that are active on gonocytes. Partner 14 are pursuing these experiments by increasing the number of gonads cultured, the number of endpoints studied (Sertoli cells also) and the number of phthalate metabolites tested (5 oxo-MEHP and 5 carboxy-MEHP in addition to 5 OH-MEHP).

Habert R, Devif I, Gangnerau MN, and Lecerf L, (1991). Ontogenesis of the *in vitro* response of rat testis to gonadotropin-releasing hormone. *Mol. Cell. Endocrinol.*, **82** (2-3) 199-206.

Lassurguère J, Livera G, Habert R, and Jégou B, (2003). Time- and dose-related effects of estradiol and diethylstilbestrol on the morphology and function of the fetal rat testis in culture. *Toxicol. Sci*., **73** 160 - 169.

Progress Summary

1. Altered fetal Leydig cell function following DBP exposure probably accounts for most of the observed changes that are evident in fetal life and at later ages. The occurrence of cryptorchidism, hypospadias and focal dysgenetic areas are probably all explained by changes in Leydig cell function or distribution in fetal life. These changes may also explain in part the occurrence of Sertoli cell-only tubules in adulthood following fetal exposure to DBP.

2. The effects of DBP exposure on fetal germ cells are evident at a very early age (E15.5) and so far no obvious mechanisms via which these effects occur have been identified. Other germ cell effects (multinucleated gonocytes) are evident towards the end of the fetal period and appear to be induced by a completely separate mechanism. The possibility that Insl3 may be involved in the latter effects is under investigation as gonocytes express the receptor for Insl3 (see WP7).

3. The delay in entry of gonocytes into quiescence (=delayed differentiation) after fetal exposure to DBP, as typified by prolongation of Oct4 expression, has parallels in CIS cells in the human, from which testicular germ cell cancer develops in young adulthood. This finding therefore provides further evidence that DBP exposure of the fetal rat provides a model for studying the development and consequences of testicular dysgenesis that may be of relevance to testicular dysgenesis syndrome in the human.

4. In terms of monitoring of adverse effects of phthalates, it appears that effects on the testis in fetal life are more dose-sensitive than are the endpoint effects that are evident in adulthood.

5. E19.5 is a suitable time point in fetal life to analyse effects of potential endocrine disruptors on steroidogenic activity, because the complete pituitary-gonadal axis is active at this age. Dioxin causes clear adverse effects on the axis at this time-point, whereas DDE and DINP do not seem to affect either the testis or adrenals strongly.

6. The rat E14.5 fetal gonad assay (FEGA) provides a new model system in which to investigate the direct effects of phthalates in vitro. Its initial application has revealed potential time-, cell- and metabolite-specific effects of DEHP/MEHP on the fetal testis.

Plan and Objectives for the Next Reporting Period

To complete all delayed studies and achieve the cited deliverables and milestones. Completion of D6.3 is not feasible due to effects of MBP/DBP unable to be reproduced in vitro using fetal rat testis explants. Thus, it appears unlikely that comparative sensitivity of rat and human fetal testes to MBP can be compared. D6.4 and D6.5 have been delayed

by until month 42 because of 6-month delay in commencement of WP6 studies by Partners 3 & 14. As this involves key elements in this programme, it is considered better to delay than to report prematurely, though no problems are anticipated in delivering the final report.

Deliverables and Milestones

Deliverables	
✓	**D6.1:** Report on progress with identifying molecular, biochemical and hormonal endpoints of phthalate action on the fetal testis (month 12)
✓	**D6.2:** Report on preliminary data concerning relevance of molecular, biochemical and hormonal endpoints to short-term (hypospadias, cryptorchidism, gonocyte abnormalities) and long-term (impaired spermatogenesis, Sertoli/Leydig/germ cell abnormalities, low testosterone levels) effects of phthalates (month 24)
*	**D6.3:** Report on the comparative sensitivity of rat and human foetal testis to phthalates in vitro. Comparison of the cell types and mechanistic pathways affected (month 36)
delayed until month 42 (36)	**D6.4:** Report on identification and evaluation of new biomarkers of foetal phthalate exposure and/or that are indicative of specific reproductive disorders (month 36)
delayed until month 42 (36)	**D6.5:** Final report on pathways of phthalate action on foetal testes (month 36)
Milestones	
delayed until month 42 (36)	**M6.1**: Identification of the pathways of phthalate action on the foetal testis and new endpoints (month 36)
delayed until month 42 (36)	**M6.2**: Relationship of identified pathways/endpoints to short- and long-term reproductive disorders (month 36)
delayed until month 42 (36)	**M6.3**: New pathways/endpoints of phthalate action using proteomics (month 36)
delayed until month 42 (36)	**M6.4**: Comparison of phthalate action on rat and human foetal testis explants (month 36)

* From the present perspective it appears unlikely that objective 3 will be met unless there are new and unforeseen developments. As there is no obvious reason why the effects of MBP/DBP cannot be reproduced in vitro using fetal rat testis explants, it appears unlikely that comparative sensitivity of rat and human fetal testes to MBP can be compared. The only viable alternative approach envisaged is to use in vivo treatments of a no-human primate, such as the marmoset, to evaluate the likelihood that DBP/MBP exerts similar effects in rat and primates (human). However, such studies will take considerable time and resources that are not available from the present programme of work.

WP7	InsL3 levels in blood as an indicator of endocrine disruption leading to reproductive developmental disorders					
Phase						
Start date	0					
Completion Date	36 months					
Current Status	active					
Partner(s) responsible:	3	5	14	16a	4	
Person-months per Partner:	34 (17)	27 (12)	24 (12)	61 (36)		
Already devoted person months per Partner and total:	12	11	10	27	3	

Objectives for the Reporting Period

- To apply the assay for human Insl3 (developed in the last reporting period) to a wider variety of serum samples from men and boys so as to enable more accurate assessment of the role, regulation and importance of Insl3 in male reproductive development and function (Partners 4, 5).

- To develop and validate an antibody to rat Insl3 for use in detection of Insl3 protein by immunohistochemistry and Western blotting, and to apply this antibody to study of the role, regulation and importance of Insl3 in male reproductive development and function in rats (Partner 3).

- To develop and validate an assay for rat Insl3 using the validated antibody just described (Partner 16a).

- To develop a reporter gene assay for detection of chemicals with the ability to affect Insl3 gene expression (Partner 5).

Methodology and Study Materials for the Reporting Period

1. Expression of InsL3 (mRNA, protein) will be measured (Partners 3, 5, 14, 16) in foetal and neonatal LC, and InsL3 receptor expression in the gubernaculum, in rats exposed *in utero* to treatments affecting LC development/function and that cause reproductive disorders; treatments will include DES, phthalates (WP6), other EDC and mixtures (WP 13-15). InsL3 expression will be related to testosterone levels and LC numbers in foetal life through to adulthood and to occurrence of reproductive disorders (WP 6).

2. Development of an ELISA for measurement of InsL3 in blood/testis extracts. This will use peptide antibodies already generated by Partner 16 and synthesis of peptide fragments specific for InsL3 and generation of new antibodies. Workup and validation of an ELISA for both rat and human samples.

3. Application of InsL3 ELISA to blood and testis extracts from: (i) boys diagnosed with crytorchidism or hypospadias, and their controls (WP 1); (ii) rats exposed *in utero* to

EDC, including compounds established to adversely affect reproductive development (1 above and WP 6); (iii) foetal gonad explants from rat and human exposed in vitro to EDC such as phthalates (WP 6), (Partners 3, 5, 14, 16).

4. Partner 5 has generated a 408 bp InsL-3 promoter fragment and subcloned it in front of the luciferase reporter gene to generate a reporter construct with full transactivation activity, dependent on steroidogenic factor-1 (SF-1) action. Mouse tumour LC (mLTC-1), which expresses SF-1, will be transfected with InsL-3-luciferase reporters. Transfected cells will be used to screen for effects of putative EDC on InsL-3 transactivation *in vitro*.

Scientific Achievements

Partner 3 contribution

Blood samples and testicular tissue samples used for the studies were obtained from Wistar rats that had been subjected to a variety of treatments that are known to alter either the development or the function of Leydig cells in fetal and postnatal life. For example, blood and testis tissue samples were obtained from adult rats that had been treated with EDS (ethane dimethane sulphonate) 6 days earlier which results in complete loss of all Leydig cells and thus removal of all Leydig cell products from the testis. Other postnatal samples were from animals which had been treated neonatally with compounds that have been shown previously to alter development of the adult population of Leydig cells whilst other samples were obtained in fetal life from animals that had been exposed *in utero* to DBP or to the anti-androgen, flutamide. Immunohistochemistry was used to establish the cellular site and level of expression of Insl3 in fixed testis tissue from these samples and frozen testis tissue extracts were used for Western analyses. Blood samples were similarly provided for Partner 16A to run in the rat Insl3 assay that is being developed. To enable Partner 3 to establish the relationship between the level of Insl3 immunoexpression in fetal Leydig cells and the normality of testis descent, rats at various fetal ages from mothers that have been treated with vehicle (control) or with DBP or flutamide were accurately assessed for testicular position on both left and right sides in order that potential correlations between Insl3 expression and testis position could be established.

The studies above resulted in 3 main achievements in the past year. The most important has been the extensive validation of the new antibody to rat Insl3 using immunohistochemistry and testis tissues from animals subjected to various treatments. This has validated the specificity and usefulness of the antibody and has resulted in a key publication (McKinnell *et a*l., 2005). This analysis also enabled Partner 3 to establish that there was no straightforward relationship between DBP-induced suppression of Insl3 in Leydig cells in the fetal testis and the normality or otherwise of testicular descent. This led to the conclusion that failure of normal testis descent, as occurs with high frequency in animals exposed in utero to high doses of DBP, is most likely the consequence of combined suppression of Insl3 and testosterone production (McKinnell *et al*., 2005). Finally, Partner 3 have shown in preliminary studies that the receptor for Insl3 (LGR8) is expressed in fetal germ cells in the rat and it is therefore possible that Insl3 production by fetal Leydig cells targets the germ cells for as yet unknown functions during fetal development. This has been one of the findings that have stimulated more detailed

investigation of the effects of DBP exposure on germ cell development, as outlined in WP6.

McKinnell C, Sharpe RM, Ivell R, Staub C, Jégou B, and Hartung S, (2005). Expression of Insulin-like factor 3 (Insl3) protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by *in utero* exposure to Di-(*n*-butyl)phthalate. *Endocrinol.* **146** 4536-4544

Partner 4 contribution

To further characterise the relationship between serum Insl3 levels and male reproductive function, serum Insl3 levels were measured in adult men during and after gonadotrophin suppression. The study material included longitudinal serum samples from 15 men enrolled in a short-term study on gonadotrophin suppression and induced recovery, and 11 men enrolled in a long-term study on gonadotrophin suppression and spontaneous recovery. Information on LH and testosterone levels was also available. The short-term study had 3 phases: (i) acute gonadotrophic stimulation, (ii) gonadotrophin suppression (12 weeks), and (iii) selective gonadotrophin replacement (12 weeks). In the long-term study, endogenous gonadotrophin levels were suppressed by using combined androgen (800 mg T implants every 4-6 months) and progestin (DMPA 300 mg im. every 3 months). The interval between the first and last hormone treatment was 8.5 months, following which the men were followed for spontaneous recovery of their gonadal function

In both studies, Insl3 levels declined markedly from 6 weeks after initiation and throughout the suppression phase (6-13.5% baseline, $P<0.05$). In the short-term study, hCG and hCG+FSH partially reversed the suppression of Insl3 levels by 4 days after the first administration (7.5% to 38.3% baseline, $P<0.05$ for hCG and 13.5% to 52.8% baseline, $P=0.068$ for hCG+FSH). These increases in Insl3 correlated significantly with the corresponding increases in serum pro-αC ($r=0.82$, $P<0.01$ for both groups). FSH alone did not reverse the suppression of Insl3. In the long-term study, serum testosterone recovered significantly better (80% baseline) when compared to serum Insl3 (38.9% baseline) ($P<0.01$), in the presence of fully recovered serum LH, suggesting that Insl3 is more sensitive to Leydig cell impairment than is testosterone. A manuscript reporting these results has been prepared and submitted for publication.

A manuscript on the results of the measurements of Insl3 serum levels in 193 children described in the report for the 2nd reporting period [8 newborn girls at 0 (cord blood) and 3 months; 13 prepubertal girls (4-9 yrs); 105 normal 3-month-old boys, 34 of these were also measured at birth; 27 3-month-old boys with cryptorchidism at birth and at 3 months; 26 normal prepubertal boys (4-10 yrs.); and 14 cryptorchid boys (5-13 yrs.) measured before and after hCG treatment (1500 IU i.m. twice a week for 3 weeks)], has been prepared in collaboration with Partners 5 and 16a and submitted for publication.

Partner 5 contribution

The main role in WP7 has been (1) to provide blood samples for Insl3 measurement, and (2) to develop a reporter gene assay for detection of chemicals with the ability to affect Insl3 gene expression. However, as part of Partner 5's contribution to WP6, it has been shown that *in utero* exposure to flutamide can reduce expression of the Insl3 gene in the

fetal rat testis, though findings by Partner 3 using Insl3 immunoexpression have not confirmed this effect at the protein level (McKinnell *et al*., 2005).

1. Partner 5 have provided Partner 16a with cord blood samples for assay development and after the assay was transferred to the laboratory of Partner 4, the samples from the Finnish cohort at 3 months of age were analysed together with parallel Danish samples. Normative data for Insl3 levels in childhood were established. The levels in cryptorchid boys were lower than in normal boys, suggesting deficient Leydig cell action in the cryptorchid boys. This may be either a cause or a consequence of cryptorchidism. A manuscript reporting these results is under review in the Journal of Clinical Endocrinology and Metabolism.

2. The development of a stably-transfected cell line containing an Insl3 promoter-reporter construct suitable for screening potential endocrine disruptors necessitated testing and development of the following key components: (i) four different mammalian cell lines (Hela, HEK-293, MLTC-1, and TM3) by transient transfection with (ii) Insl3 reporter constructs containing different sized fragments from the promoter region of the Insl3 gene, in combination with (iii) different constitutive-expression constructs of a reporter gene to normalise for varying transfection efficiencies, and co-transfected with (iv) constitutive-expression constructs of different oestrogen receptor isotypes.

3. Transient transfection assays indicated that the MLTC-1 host provided the most promising sensitivity, but inconsistent results attributable to the other components of our system have necessitated the engineering of IRES-reporter constructs for isolating stably-expressing transfected cells by FACS sorting and cloning. As both IRES-reporter constructs are now ready, transfection, sorting, culture and screening of stably transfected MLTC-1 cells should develop when the researcher in charge returns from maternity leave in April 2006.

Partner 14 contribution

The main contributions made by Partner 14 to this workpackage have centred on 3 aspects. (i) Collection of various tissues from rats at ages spanning from fetal life through to adulthood, especially from the reproductive tract (testis, efferent ducts, epididymis, gubernaculum), to enable evaluation of the distribution and level of expression of receptors for Insl3 (the LGR8 receptor) using RT-PCR; (ii) To use some of these same tissues to confirm restriction of Insl3 expression to the testis using RT-PCR; (iii) Provision of rat serum samples for validation of the rat Insl3 assay being developed by Partner 16a.

The main advances made by these studies in the past year have been as follows. In collaboration with Partners 3 and 16a, Partner 14 has established that no mRNA for Insl3 can be detected in the gubernaculum of the rat, despite localisation of the protein here by immunohistochemistry. It was concluded that the Insl3 protein detected in the gubernaculum was protein bound to its LGR8 receptor. In this regard, we showed that mRNA for LGR8 was expressed heavily in the gubernaculum throughout fetal and postnatal life, a finding confirmed at the protein level by ourselves as well and by Partner 3. These findings confirm that the gubernaculum is a key target for Insl3 in fetal life when testis decent is occurring. However, another key finding has been 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 fetal and postnatal development. This implies that Insl3 from the fetal Leydig cells 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 fetal Leydig cells). Similar to partner 3, we have also shown that LGR8 protein is expressed in fetal germ cells, implying a role for Insl3 in development of these cells as well.

Partner 16a contribution

Having established the immunoassay for human Insl3 (described in the previous reports) and made available this assay to Partner 4, who performed the measurements on human samples in cooperation with Partner 5, the main goal for Partner 16a in the past year was to establish a rat Insl3 assay. This assay was built up using:

- An anti-ratInsl3 antiserum (CR15) generated using genetic immunisation of rabbits (see previous reports).

- A synthetic rat Insl3 peptide (see Kawamura et al. 2004, PNAS 101, 7323-28) supplied from Richard Ivell (Adelaide) and Ross Bathgate (Melbourne) as calibration standard.

- The same peptide labelled by us with a 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 (B_0) values. So other antibodies were tried (2-8F, a monoclonal against synthetic bovine Insl3, cross-reacting both with human and rat Insl3, a gift from Erika Büllesbach and Chris Schwabe; CR 14, made by Partner 16a through genetic immunisation of rabbits against human Insl3; and 3/3A, the antiserum that Partners 16a and 4 use for the human Insl3 assay), but these turned out to be even worse, both in B_0 values as well as in sensitivity. After optimising buffer and incubation conditions, CR15 finally led to sufficiently high B_0 values and gave reliable standard curves.

The study materials so far, aside from the antisera and synthetic Insl3 peptides mentioned above, comprised sera from normal adult female and male rats (as controls) gathered in Hamburg, sera from adult castrated rats sent by Partners 17 and 14, and blood samples from EDS-, DBP- and otherwise treated as well as control male rats from Partner 3. The sera from castrated rats should most presumably be free of Insl3 and thus serve as blank sera, which can be spiked with the synthetic calibration standard to generate calibration curves in the same matrix as the actual sample. This allows more accurate measurements than would be possible with calibration curves run in buffer alone and circumvents the extraction of Insl3 from the samples.

The reliable measuring range for the assay encompasses 5 to 300 pg rat Insl3 per well (which takes 100µl of sample, values in Figure 1 are calculated for 1 ml). This is similar to the measuring range of Partner 16a immunoassay for human Insl3. Samples can be measured either directly, as undiluted 100µl units of serum or plasma in the case of low Insl3 content, or can be diluted 1:5 or 1:10 in assay buffer when the amount of Insl3 exceeds the measuring range in 100µl serum/plasma. 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 as shown in Figure 1. Only human Insl3, the closest relative to rat Insl3 in the peptides used, shows a slight reduction of tracer binding.

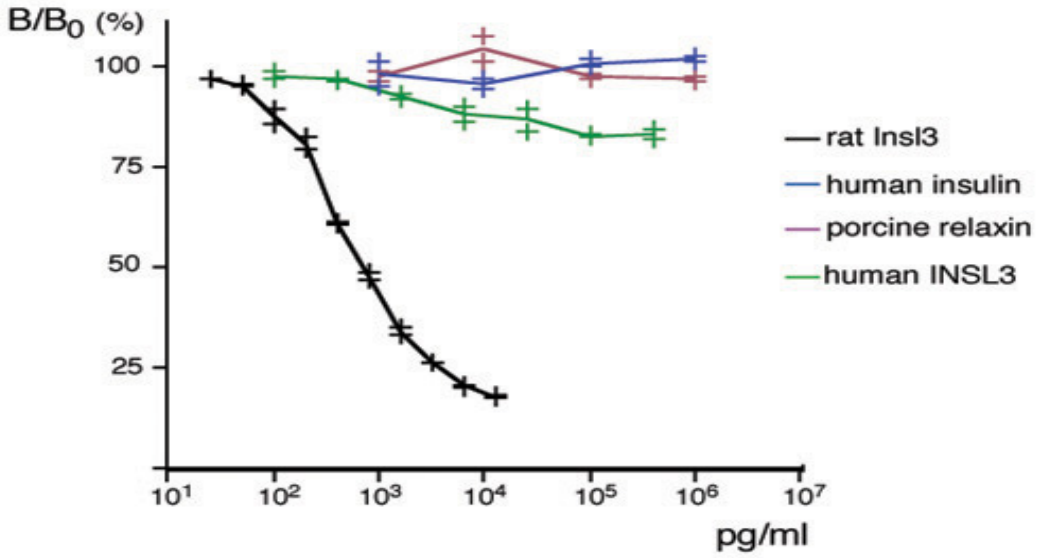

Figure 1 Specificity of the rat Insl3 immunoassay

The first measurements are summarised in Figure 2. Females, castrated and EDS-treated males (EDS destroys the Insl3-producing Leydig cells in the testes) are at the baseline, as expected. In male rats, there is an increase in Insl3 levels at around puberty (d 20/21), 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. The decline with age agrees with previous studies (Paust *et al*., 2002), and also the increase in hormone levels between day 30 and day 43 (Boockfor *et al*., 2001). However, Partner 16a measurements are about ten times higher than in the Boockfor study and suggest more pronounced dynamics. This disagreement has to be resolved. Also, male rats appear to have, according to the preliminary studies, roughly ten times more Insl3 in their blood than male humans, as measured by Partner 4 with the assay for human Insl3.

Drastically reduced are Insl3 levels in blood of adult male rats having been 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 Leydig cell or general testis function by DBP already in the fetal stage and lasting past puberty, also in rats in which the testes descend normally into the scrotum.

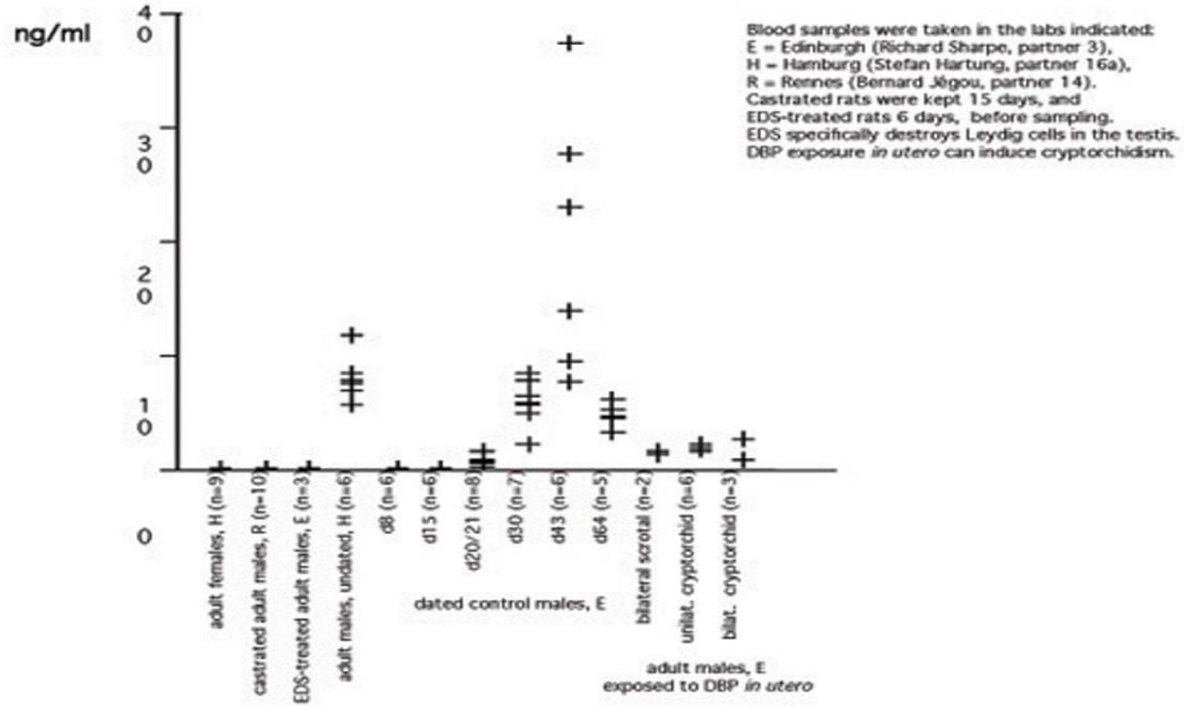

Figure 2 Insl3 in rat blood

Paust HJ, Wessels J, Ivell R, and Mukhopadhyay AK, (2002). The expression of the RLF/INSL3 gene is reduced in Leydig cells of the aging rat testis. *Exp. Gerontol*. **37** (12) 1461-1467.

Boockfor FR, Fullbright G, Bullesbach EE, and Schwabe C, (2001). Relaxin-like factor (RLF) serum concentrations and gubernaculum RLF receptor display in relation to pre- and neonatal development of rats. *Reprod.* 1**22** 899-906.

Progress Summary

Partner 3 has validated the recently developed antibody for rat Insl3 as a prelude to its further use for development of an assay for Insl3 in blood for the rat. It can be demonstrated that DBP exposure *in utero* results in suppression of Insl3 immunoexpression whilst flutamide exposure *in utero* has no effect on Insl3 immunoexpression when administered at 50 mg/kg/day but reduces immunoexpression in a proportion of animals when administered at 100 mg/kg/day. Thus there is no straightforward relationship between DBP-induced suppression of Insl3 and the occurrence of cryptorchidism in individual testes. In addition, it can be shown that fetal germ cells are a potential target for Insl3 from the Leydig cells during fetal development.

The studies on insl3 serum levels in humans by Partner 4 have altogether resulted in 3 manuscripts describing Insl3 levels in males at different ages and developmental stages and the interaction of insl3 with other reproductive hormones. It can be shown that serum Insl3 in normal men is not acutely sensitive to exogenous FSH or LH bioactivity (hCG), but nevertheless is dependent on the stimulatory activity of gonadotrophins on Leydig cells. Suppression of endogenous gonadotrophins thus results in a marked decline in

serum Insl3, following which Insl3 is acutely sensitive to LH action. Subsequent to long-term gonadotropin suppression, Insl3 does not recover to the same degree as testosterone, suggesting that Insl3 may be more sensitive than testosterone to impaired Leydig cell function. Reduced Insl3 in cryptorchid boys suggests impairment of Leydig cell function in cryptorchid testes and supports the hypothesis that cryptorchidism is associated with a primary testicular disorder.

Partner 5 illustrates that Insl3 is a target for anti-androgen action during fetal development and most probably contributes to adverse effects, such as cryptorchidism. Normative Insl3 levels in children have now been established. Cryptorchid boys have lower Insl3 levels than normal boys, indicating defects in Leydig cell function. The development of transactivation assays for mInsl3 proved to be much more complex than originally envisioned. Key components of the assay system have now been tested.

Partner 14 has demonstrated sites of expression of LGR8 during development in the reproductive tract of the male rat. Confirmation that the gubernaculum is a key target for Insl3 and that the latter is not produced locally within the gubernaculum whilst there is evidence that Insl3 may have several target sites outside of the gubernaculum, namely in the fetal germ cells and efferent ducts/epididymis, during fetal and postnatal development.

The immunoassay for rat Insl3 has been basically established by Partner 16a, but requires further refinements (e.g, intra- and interassay variance have not yet been measured, also the discrepancy between my measurements and those in the Boockfor study have to be clarified). First measurements have been performed and suggest a malfunction of Leydig cell function in rats exposed to DBP *in utero*, which is apparently lasting into adulthood and is not dependent on testicular position. However, these are preliminary results which have to be verified by increasing the accuracy of the assay and by including more rat blood samples of different treatment.

Due primarily to late commencement of the studies in this workpackage, and changes in the working environment and personnel for Partner 16a, there will be a delay in timing of the final deliverables. As many of the deliverables are dependent on final validation and extensive application of the rat Insl3 assay by Partner 16a, the timing of final delivery will be determined by this partner, who estimates completion by week 46 (part-time work only is possible, hence the rather lengthy delay). Nevertheless, from the present perspective, it is anticipated that all deliverables will be achieved.

Plan and Objectives for the Next Reporting Period

- The main objective for Partners 3 and 16a for the remainder of the period is to provide sufficient and varied blood samples from rats to enable thorough validation of the recently developed assay for blood Insl3 in the rat. This assay will then be utilised to characterise the normal developmental profile of Insl3 secretion into blood, and its hormonal regulation at various life stages. In addition, there is the need to correlate whether or not treatment-induced cryptorchidism results in altered levels of Insl3 in blood at any life stage.

- The objectives of Partner 4 for this workpackage have been completed

- Partner 5 will report the Insl3 levels in normal and cryptorchid boys. Insl3 measurements in different target groups will be continued and association between Insl3 levels and exposure data from the cohort boys will be analysed.

- Transfection of MLTC-1 cells with the new Insl3 reporter constructs, and sorting, culture and screening of stably transfected MLTC-1 cells will take place when the researcher in charge returns from maternity leave in April.

- Definitive studies by Partner 14 on LGR8 expression in the gubernaculum and elsewhere in the reproductive tract together with evidence for how expression may vary according to age and/or hormone or chemical exposures.

- The completion of deliverables which have been delayed until month 46. D7.1 and D7.6 are both delayed due to contractual and re-siting issues regarding Partner 16b and the time taken to hire experienced postdoc to assist in research. D7.2, D7.3, D7.4 and D7.5 are subsequently delayed as results are required from the D7.1 and D7.6 before reports can be formalised.

Deliverables and Milestones

Deliverables	
delayed until month 46 (36)	**D7.1:** Report on the relative incidence of disorders of InsL3 expression by foetal LC in rats exposed to treatments in utero (month 36)
delayed until month 46 (36)	**D7.2:** Report on InsL3 receptor expression in gubernaculum (month 36)
delayed until month 46 (36)	**D7.3:** Report on the relationship of InsL3 expression to size, number and androgen production by foetal LC in rats exposed to treatments *in utero* (month 36)
delayed until month 46 (36)	**D7.4:** Report on the evaluation of whether blood levels of InsL3 are associated with, and predictive of, cryptorchidism and other reproductive disorders in humans and rats (month 36)
delayed until month 46 (36)	**D7.5:** Report on the in vitro cell screening system for chemicals that affect InsL3 gene expression (month 36)
delayed until month 46 (36)	**D7.6:** ELISA for the measurement of InsL3 in blood/testis extracts from humans and the rat (month 36)
Milestones	
delayed until month 46 (36)	**M7.1**: InsL3 expression related to reproductive disorders, androgens, estrogens in male rats after foetal exposures (month 36)
delayed until month 46 (36)	**M7.2**: ELISA for InsL3 in blood/testis extracts (month 36)
delayed until month 46 (36)	**M7.3**: InsL3 levels in boys with cryptorchidism or hypospadias and in rats with similar disorders, screening system for detection of chemicals that directly alter expression of InsL3 gene (month 36)

WP8	Hypothalamic-pituitary (HP) sensitivity to endocrine disruption: Mechanisms, consequences, new endpoints and relevance to man					
Phase						
Start date	0					
Completion Date	36 months					
Current Status	active					
Partner(s) responsible:	17	3	4	18		
Person-months per Partner:	107.5 (36)	8 (4)	7 (1)	27.8 (18)		
Already devoted person months per Partner and total:	36	4	3	18		

Objectives for the Reporting Period

This WP aims are to explore mechanisms, consequences and new endpoints of endocrine disruption at the hypothalamic-pituitary (HP) unit, based on the proven sensitivity of the developing HP system to the organising effects of sex steroids (endogenous and synthetic), which is expected to be higher than that of the peripheral sex organs (internal and external genitalia). To accomplish this general goal, the work to be conducted by Partner 17 within WP-8 included: (1) Neonatal treatment of rats with potent synthetic estrogen, androgen and anti-androgen in a limited range of doses, to establish thresholds for disruption at the HP unit, as well as potential mechanisms of action; (2) Transfer of relevant tissue samples to Partner 4 for conduction of DOP-PCR differential display (DD) analysis to identify new endpoints and mechanisms for endocrine disruption at the HP unit; (3) Detailed analyses of gene candidates selected on the basis of DD-assays, mainly at the mRNA level, by means of RT-PCR and (eventually) in situ hybridisation; and (4) Analysis of expression of selected endpoints (markers and mechanistic factors) at the HP unit of rats exposed to EDCs, alone or in mixtures, obtained and transferred by partner 21 (WP-13-15). In addition, the work to be conducted by Partner 18 involved: (5) Testing of the activational effects of EDCs upon the HP unit by culturing hypothalamic explants isolated from immature rats, with various doses of EDCs and monitoring spontaneous pulsatile GnRH secretion and glutamate-evoked GnRH secretion; and (6) Analysis of whether exposure of immature female rats to E2 (or EDCs) *in vivo* could result in sexual precocity and premature acceleration of pulsatile GnRH secretion *ex vivo*, in order to identify the basis for advanced puberty onset observed in foreign girls migrating for adoption. Finally, the task of Partner 3 was mostly related with coordination of interactive studies between partners involved in WP8-Theme2-EDEN.

From these global objectives, the specific aims for the 3rd reporting period included:

- To complete evaluation of the expression profiles of progesterone receptor (PR) gene at the hypothalamus as marker for exposure to estrogenic/androgenic compounds during critical periods of sex differentiation of the brain.

- To further analyse of the physiological role of the KiSS-1/GPR54 system in the control of reproductive axis, and its potential involvement as putative mechanistic target for endocrine disruption at the HP unit following exposure to

estrogenic/androgenic compounds during critical periods of sex differentiation of the brain.

- To finalise the characterisation of globin gene expression at the pituitary as putative biomarker of exposure to estrogenic (and eventually anti-androgenic) compounds during critical periods of sex differentiation of the HP unit.

- To advance the analysis of selected end-points (nuclear sex steroid receptor –NSR-, KiSS-1 and globin genes) at the HP unit of rats exposed to EDCs, alone or in mixtures, during the gestational and/or perinatal periods, obtained and transferred by Partner 20 (WP-13-15).

- To complete the elucidation of some of the mechanisms for the effects of estradiol (E2) and dichlorodiphenyltrichloroethane (*o,p*'-DDT) on gonadotropin-releasing hormone (GnRH) secretion by hypothalamic explants *in vitro*, and to investigate whether selected phytoestrogens (coumestrol and genistein) could also have hypothalamic effects in this explant model.

- To evaluate whether a prolonged exposure of female rats (from neonatal to postpubertal period) to E2 or *o,p*'-DDT *in vivo* could result in a delay of central sexual maturation (estrous cycling). Of note, previous experiments had already shown that transient exposure of immature female rats to *o,p*'-DDT may account for sexual precocity. The present work intended to complete those observations, aiming at mimicking the situation of children living and staying in a country contaminated by DDT versus the internationally adopted children moving to a foster European country. In the latter group, withdrawal from exposure due to migration to a setting not contaminated with DDT should result in sexual precocity whereas sexual precocity is not seen following persisting exposure in the countries of origin.

Methodology and Study Materials for the Reporting Period

The standard methodology has included (mostly) tissue sampling after gestational and/or perinatal manipulation of the sex steroid milieu (by administration of potent synthetic estrogens, androgens, anti-androgens, or treatment with different EDCs -transferred by partner 21-), gene expression analysis by final-time and real-time RT-PCR (optimised for semi-quantitative detection) and (when relevant) in situ hybridisation, protein expression analysis by Western blot (in selected samples), differential display by means of DOP-PCR, and hormone measurements by specific radioimmunoassays. All these techniques are described in detail in the scientific publications produced by the laboratory of Partners 17 and 4 during this reporting period. In addition, the methodology employed by Partner 18 included protocols of administration of estradiol and EDCs *in vivo*, as well as incubation of rat hypothalamic explants and measurement of GnRH release *in vitro.* These procedures are described in detail in the scientific publications produced by Partner 18 during this and previous reporting periods. The study materials employed by Partners 17 and 18 during the 3rd reporting period included rat brain (hypothalamic), pituitary and serum samples from the experimental groups described above.

Scientific Achievements

Partner 17 contribution

The scientific achievements and progress of work of Partner 17 in EDEN WP8 will be summarised into four major sections (following the major goals for this reporting period):

1. *Progesterone receptor (PR) gene expression at the hypothalamus: Physiologic profiles and alteration by neonatal exposure to estrogenic and androgenic compounds.*

Expression of PR gene is proven highly sensitive to the activational (acute) regulatory actions of endogenous and synthetic estrogen in a wide variety of tissues. However, its potential persistent modulation by exposure to estrogenic (or eventually androgenic) compounds during critical periods of brain sex differentiation remained ill defined.

Considering its sensitivity to estrogen, and the proven role of PR in key aspects of development and function of the HP unit within the reproductive axis (for both the male and female), Partner 17 have completed a detailed characterisation of the physiologic pattern of PR gene expression at the hypothalamus of male and female rats (postnatal development, estrous cycle), and assessed the levels of PR mRNA after neonatal manipulation of the sex steroid milieu by means of administration of the potent estrogen estradiol benzoate (EB) or the synthetic androgen testosterone propionate (TP). Neonatal exposure to high doses of EB evoked a persistent increase in relative steady-state levels of PR mRNA at the hypothalamus of male and female rats; a phenomenon which was detected at doses as low as 10 μg/rat and persisted up to early adulthood in males. These observations have been extended to neonatal androgenisation of the female, where administration of high doses of the aromatisable androgen TP evoked a similar, persistent elevation in hypothalamic PR mRNA levels. Expression analyses are under way to assess PR mRNA expression at the hypothalamus of pubertal and adult rats neonatally exposed to the suspected estrogenic EDC, bisphenol A (BPA).

The above data, which have been gathered during whole WP8 life-span, are now in the stage of final completion and discussion, in order to release a scientific publication on this matter that is anticipated could be published during the 4th reporting period of EDEN.

2. *Hypothalamic KiSS-1/GPR54 system: Physiologic role in the control of the reproductive axis and potential target for endocrine disruption at the HP unit.*

Since the pioneering observations that inactivating mutations of the G protein coupled receptor GPR54 are linked to hypogonadotropic hypogonadism (published in late 2003), an enormous research activity has been conducted by a number of laboratories (including that of Partner 17) in order to define the crucial role of the ligand-receptor system, KiSS-1/GPR54, in the control of development and function of the reproductive axis.

In the context of EDEN-WP8, the objectives of the work conducted by Partner 17 have been two-fold. First, Partner 17 have aimed to contribute to expanding their (originally narrow) knowledge on the physiological role of GPR54 and its ligands, the kisspeptins, in the control of the gonadotropic axis along lifespan. This step was considered as absolutely mandatory in order to safely proceed to the second aim, i.e. to define whether alterations in expression and/or function of this system at the hypothalamus might be mechanistically relevant in the

disruption of the development and/or function of the reproductive axis after exposure to estrogenic (and eventually androgenic) compounds during the critical periods of sex differentiation of the HP unit.

In relation to the first aim, the research activities conducted by Partner 17 have significantly contributed to define the physiologic role of the KiSS-1/GPR54 system in the hypothalamic control of gonadotropin secretion. The most salient contributions reported during this period included: (i) the first demonstration of the ability of the agonist of GPR54, kisspeptin-10, to advance puberty onset in the female rat; (ii) the complete characterisation of the potent luteinising hormone (LH) releasing activity of kisspeptin-10, including dose-response and time-course analyses, as well as the study of its interactions with other relevant regulators of gonadotropin secretion; (iii) the comparative evaluation of the releasing activity of kisspeptin-10 upon follicle-stimulating hormone (FSH) and LH secretion, which proved that FSH secretion is ~100-fold less sensitive to kisspeptin than LH; and (iv) the demonstration that hypothalamic KiSS-1 is sensitive to the feedback effects of estradiol and androgen, and is also sensitive to the metabolic state of the organism; observations that pointed out that central KiSS-1 system may operate as final integrator of a wide diversity of regulatory signals that modulate the function of the reproductive axis. All the above observations have been already published in international peer-reviewed journals, as described in Section 6.1. In addition, results of this work have been presented in National and International meetings by Partner 17 as invited speaker.

In addition, within this first objective, additional experimental work, which is mostly completed and presently under evaluation, but still unpublished, has been conducted. The most interesting findings include: (i) the characterisation of the ontogeny of the LH-releasing effects of kisspeptin-10, as well as its actions on hypothalamic gonadotropin-releasing hormone (GnRH) secretion; (ii) the analysis of the gonadotropin releasing effects of kisspeptin-10, as well as the pattern of KiSS-1 gene expression, in different functional states of the female reproductive axis (e.g. estrous cycle, pregnancy, lactation); (iii) the assessment of the effects of peripheral (single or repeated) administration of kisspeptin-10 and its longer variant, kisspeptin-52, on dynamic gonadotropin secretion; and (iv) the evaluation of the expression patterns of KiSS-1 gene and the effects of kisspeptin administration in models of pathological disruption of the gonadotropic axis, such as undernutrition and diabetes. As indicated above, this set of work is contained in (at present) four independent manuscripts, which are presently under evaluation for publication in international peer-reviewed journals.

Regarding the second aim; KiSS-1 as Target for Endocrine Disruption, the physiological data generated at our laboratory (specially those related with the role of hypothalamic KiSS-1 as integrator for signals governing the gonadotropic axis and sensitive to acute regulation by estrogenic and androgenic compounds) led to the proposal that alterations in the expression and/or function of the KiSS-1 system might be mechanistically relevant for some of the perturbations of the reproductive system suspected to take place after exposure to sex steroid-like compounds during critical periods of maturation. This hypothesis was supported by the original observation that neonatal exposure to the synthetic estrogen, estradiol benzoate, was able to induce a persistent decrease in hypothalamic expression of KiSS-1 gene, detectable in adulthood. The experimental work conducted by Partner 17 during the 3rd reporting period, which included both functional hormonal tests and expression analyses, have provided further basis for such hypothesis. As summary of the major findings during this reporting period:

- Inhibition of hypothalamic expression of KiSS-1 mRNA by neonatal exposure to synthetic estrogen was confirmed in different experimental settings. This phenomenon has been proven strictly dose-dependent (both in male and female rats) and inversely correlated with serum LH levels.

- Defective gonadotropin secretion after neonatal estrogenisation was reverted by exogenous administration/replacement of KiSS-1 peptide. Indeed, the potent LH-releasing effect of kisspeptin was conserved in neonatally estrogenised animals, despite clearly decreased basal gonadotropin secretion.

- Defective gonadotropin responses to gonadectomy (as index of altered feedback control of pituitary LH and FSH secretion) were normalised by exogenous administration/replacement of KiSS-1 peptide. Hypothalamic responses (in terms of increased KiSS-1 gene expression) to gonadectomy were altered in neonatally estrogenised male rats.

- Persistently altered hypothalamic expression of KiSS-1 gene was observed after neonatal administration of the synthetic androgen, testosterone propionate, to female rats, as well as after neonatal exposure to BPA in male and female rats.

The above data are now in the stage of final completion and discussion, in order to release a scientific publication on this matter that is anticipated to be published during EDEN 4th reporting period.

3. *Pituitary globin gene expression: Putative biomarker for early exposure to estrogenic compounds.*

This work has been conducted in close collaboration with Partner 4. Research activities conducted during the first two reporting periods by partners 4 (DD assays by DOP-PCR and in situ hybridisation) and 17 (extensive RNA analysis by final- and real-time RT-PCR) allowed us to postulate that exposure to synthetic estrogen during critical periods of sexual maturation resulted in a persistent increase in the expression of α- and β-globin genes at the pituitary (see previous reports). These original observations were included in a manuscript that was submitted (at the end of the 2nd reporting period) for publication to an international journal. Interaction with the reviewers forced further elaboration on this finding (including addition of new experimental work). After such amendments, this manuscript has been now accepted for publication in the *Journal of Steroid Biochemistry and Molecular Biology*. As summary, the most salient finding of this study is the identification of persistent up-regulation of α- and β-globin mRNA expression at the pituitary following neonatal estrogenisation; a phenomenon which was confirmed by combination of RT-PCR analyses and in situ hybridisation. Induction of α- and β-globin mRNA expression at the pituitary by neonatal exposure to estrogen was demonstrated as dose-dependent and it was persistently detected up to puberty. In contrast, durable up-regulation of α- and β-globin genes was not detected at the hypothalamus, cortex, cerebellum, liver and testis. Interestingly, enhanced levels of α- and β-globin mRNAs at the pituitary were also demonstrated after neonatal administration of the anti-androgen flutamide. Overall, α- and β-globin genes may prove as sensitive, pituitary-specific biomarkers of exposure to estrogenic (and/or anti-androgenic) compounds at critical periods of sex development, whose potential in the assessment of endocrine disrupting events at the HP unit merits further investigation. In this context, during the 4th reporting

period, we plan to screen pituitary globin gene expression in the samples transferred by Partner 21, obtained after gestational and/or perinatal exposure to suspected EDCs.

4. *Expression analyses at the HP unit following exposure to EDCs, alone or in mixtures.*

Relevant tissue samples (brains and pituitaries) from rats treated with different EDCs have been transferred by Partner 21 (WP13-15) along EDEN lifespan. Samples from five independent studies have been received so far at the laboratory of Partner 17: Study 03-10 (Effects of pre- and post-natal exposure to DINP in rats); Study 03-28 (Effects of pre-natal and postnatal exposure to Vinclozolin, Flutamide and Prochloraz in rats), Study 04-10 (Effects of pre-natal and postnatal exposure to Finastiride, DEHP, and Procymidone in rats), Study 04-30 (Dose-response study of Procymidone and low-dose study of DEHP and Procloraz), and Study 05-10 (First mixture study of similarly acting anti-androgens, i.e. Procymidon, Vinclozolin and Flutamide, in the rat). One additional set of samples (corresponding to the second mixture study, to be conducted by Partner 21 during the first half of 2006) is expected to be delivered to Partner 17 lab by June-July 2006.

Considering the large amount of samples to be processed and screened (which will not be completed until mid-2006), and the need to define a restricted set of markers to be evaluated in those samples (on the basis of the research work conducted at other lines of WP8), during this reporting period Partner 17 prioritised the completion of the rest of the research activities included in WP8 (see points 1-3 of this section). Accordingly, gene expression analyses in tissue samples from animals exposed to EDCs, alone or in mixtures are still at an early stage. Nonetheless, during this reporting period, extensive RNA isolation from most of the samples from Studies 03-10, 03-28, 04-10 and 04-30 has been performed. In addition, final-time RT-PCR assays have been initiated in a sub-set of these samples. In detail, most of the initial efforts have been directed towards the analysis of potential changes in hypothalamic expression of PR and KiSS-1 genes following early exposure to suspected anti-androgens (Studies 03-28 and 04-10). Since some in-group variability for the expression levels of gene targets has been detected, and given that some of the compounds under analysis were included in more than one study, it was decided to complete the stage of RNA isolation of samples from the different experimental designs (to obtain the largest possible n for each group) before proceeding with exhaustive RNA expression analyses. Partner 17 expects that this stage of RNA isolation will be completed in the coming weeks.

Overall, Partner 17 are confident that the information gathered already in WP8 concerning the expression profiles of selected genes (NSR, globins, KiSS-1, GPR54), and the efforts in terms of processing and isolation of RNA samples conducted to date, will allow Partner 17 to considerably speed up the process of evaluation of such samples during the final reporting period of EDEN. In this sense, it is to be stressed that all the research activities of Partner 17 during the 4th reporting period, as well as all the remaining funds will be devoted to this specific aim.

Partner 18 contribution

A significant effort was undertaken by Partner 18 to complete the comparative analysis of the effects of estradiol and selected EDCs using the *in vitro* paradigm of 15-day-old female rat hypothalamic explants incubated for 4h. When E2 (10^{-8}M) was repeatedly applied during 15 min only, the GnRH release evoked by glutamate every 37.5 min was

similarly potentiated by E2 during a 4-h study period, irrespective of time. In contrast, when E2 (10^{-8}M) was continuously incubated with the explants, the glutamate-evoked GnRH release showed a significant potentiation that increased with time after 3.5h of incubation. Similar observations were made using *o,p*'-DDT. This indicated that beyond the rapid effects of E2 and o,p'-DDT, an additional less rapid effect was taking place and evidenced only after few hours of incubation with the steroid or the endocrine disrupting chemical.

The effect of receptor antagonists on GnRH pulse frequency was also studied. The reduction in GnRH interpulse interval (IPI; mean ± standard deviation), caused by 10^{-7}M of E2 (45.7 ± 2.2 min) or 10^{-4}M of *o,p*'-DDT (49.8 ± 3.8 min), was significantly prevented by DNQX, an antagonist of the kainate subtype of glutamate receptor (58.3 ± 3.3 min) and ICI 182,780, an antagonist of the estrogen receptors (ERs; 56.7 ± 3.9 min). When β-naphtoflavone, an antagonist of the orphan dioxin aryl hydrocarbon receptor was used, only the reduction in IPI caused by *o,p'*-DDT (10^{-4}M) was partly but significantly prevented (54.2 ± 3.3 min).

Preliminary data obtained in electrophysiological experiments (whole cell patch-clamp) using a new model of immortalised rat GnRH neuronal cell line (Gnv-4) indicated that application of glutamate (2.10^{-3}M) for 1 min resulted in change in the curve of voltage-intensity (-100 to +40mV). E2 (10^{-9}M) also accounted for some changes. Coumestrol and genistein caused a significant increase in glutamate-evoked GnRH release when used at 10^{-4}M. When E2 (10^{-7}M) was applied with increasing concentrations of coumestrol (10^{-7} to 10^{-4}M), the increase in glutamate-evoked GnRH secretory response was prevented in the presence of 10^{-4}M of coumestrol. However, when GnRH IPI was studied in the presence of coumestrol (10^{-6} to 10^{-4}M), no effect was observed.

In addition to extensive *in vitro* work, protocols of *in vivo* administration were implemented to determine whether a prolonged exposure of female rats (from neonatal to postpubertal period) to E2 or *o,p*'-DDT *in vivo* could result in a delay of central sexual maturation (estrous cycling). This piece of work is presently on-going and, although no conclusive results can be drawn yet, expected completion is by the end of the 4th reporting period. Of note, previous experiments had already shown that transient exposure of immature female rats to *o,p'*-DDT may accounts for sexual precocity. The present work intended to complete those observations, aiming at mimicking the situation of children living and staying in a country contaminated by DDT versus the internationally adopted children moving to a foster European country. In the latter group, withdrawal from exposure due to migration to a setting not contaminated with DDT results in sexual precocity whereas sexual precocity is not seen following persisting exposure in the countries of origin.

Partner 3 contribution

The task of Partner 3 in this workpackage has been mostly related with coordination of interactive studies between partners involved in WP8-Theme2-EDEN.

Partner 4 contribution

The primary focus has been the conduction of work related to identification of differentially expressed genes at the pituitary and hypothalamus following neonatal

exposure to estrogenic compounds by means of DD DOP-PCR, and the characterisation of the phenomenon of persistently elevated globin mRNA levels at the pituitary following neonatal estrogenisation. Description of these activities is given in detail in point 3 of Partner 17 contribution section. This work has resulted in a recently accepted article to be published in the *Journal of Steroid Biochemistry and Molecular Biology*.

The research work conducted in points 1 (PR expression at the hypothalamus), 2 (KiSS-1 and GPR54 expression at the hypothalamus) and 3 (Globin expression at the pituitary) of Partner 17 contributions, including dose-response analyses of the effects of early (neonatal) exposure to synthetic estrogens upon the expression levels of these selected targets, has help us to define the thresholds for potential disruption of the HP unit, using molecular markers (M8.1) and to determine the sensitivity of the HP unit to potential disruption by estrogenic compounds (D8.1). In this sense, neonatal exposure to doses as low as 10μg estradiol benzoate per rat was 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 of α- and β-globin genes at the pituitary. Interestingly, these changes were tightly related to concomitant changes in basal serum levels of LH, i.e. a conventional marker for disruption of function of the gonadotropic axis.

Similarly, the research work conducted in points 1-3 of Partner 17 contributions might not only be relevant in terms of setting thresholds and sensitivity for endocrine disruption at the HP unit, but it may provide also novel information regarding end-points and mechanisms for endocrine disruption at this level of the reproductive axis (M8.1 and D8.1). 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. In addition, the work described in Partner 18 contributions will help to elucidate the molecular basis for the precocity in pubertal activation observed in immigrated children, and its eventual link to changes in exposure to EDCs with estrogenic activity, such as DDT derivatives (M8.3 and D8.3).

Finally, the on-going work, including analysis of expression of selected targets in hypothalamic and pituitary samples from animals exposed (during the gestational and/or perinatal periods) to EDCs, alone or in mixtures, will be crucial to define the effects of sub-threshold doses of EDC on disruption of the HP unit (M8.4) and to determine the ability of these compounds to induce transient or durable changes at this level of the reproductive axis (D8.4). This work is expected to be completed by the end of the 4th (and final) reporting period of EDEN.

Progress Summary

The work conducted during the present and previous reporting periods has allowed Partners to cover most of the goals planned for WP8 in the corresponding workplan. Concerning activities conducted by Partner 17, such a research work was first directed towards the generation of a relatively large amount of tissue samples from selected

experimental models of neonatal exposure to estrogenic and androgenic compounds where to conduct detailed analyses in the search of novel endpoints, biomarkers and mechanisms of action for endocrine disruption at the HP level. Once generated, data on the sensitivity of the HP unit to exposure to synthetic estrogens were obtained, and detailed evaluation of the potential use of several markers, such as PR, KiSS-1, GPR54, and α- and β-globin genes as endpoints/biomarkers of endocrine disruption was conducted. These molecular, continuous biomarkers will allow a pertinent assessment of the sensitivity of the HP unit to the exposure to potential EDCs during the 4th reporting period, with special attention to the effects of low-dose and mixture-effects (as indicated in D8.4 and M8.4). Similarly, progress in the analysis of selected gene targets (e.g. KiSS-1 and GPR54) and differentially expressed genes have paved the way for identification of novel mechanisms of endocrine disruption at the HP unit (as indicated in D8.2 and M8.2). Both sensitivity to low-dose and mixtures and mechanistic data will help to enlarge our knowledge on the effects and health risks associated to exposure to EDC during critical periods of sex differentiation in mammals.

In addition, the experimental data obtained by Partner 18 can be summarised into the following major conclusions: (i) E2 and *o,p'*-DDT can evoke a time-dependent increase in glutamate-evoked GnRH secretory response; observations that reinforce previous data obtained in studies of the frequency of pulsatile GnRH secretion. In addition to the rapid effects occurring within 7.5 to 15 min, *o,p*'-DDT was able also to conduct slow effects in the hypothalamic explant model; (ii) DNQX and ICI 182,780 prevented the increase in GnRH pulse frequency caused by E2 or o,p'-DDT. In addition, β-naphtoflavone inhibited the acceleration of GnRH pulse frequency caused by *o,p*'-DDT but did not affect the acceleration caused by E2. These results confirm previous observations based on glutamate-evoked GnRH release; (iii) The electrophysiological properties of the GnRH neuronal cell line, Gnv-4, were affected by glutamate and E2, suggesting that GnRH-secreting neurons could be directly activated by such surrounding neurotransmitters and steroids. Moreover, this model would enable to study directly at the GnRH neuron level the interactions between glutamate, E2 and EDCs; and (iv) Coumestrol and genistein caused a rapid increase in GnRH release evoked by glutamate, while coumestrol prevented the potentiation of GnRH secretory response caused by E2. This suggests that coumestrol has both estrogenic and (possibly) anti-estrogenic effects on GnRH secretion. These opposite effects could involve, respectively, ERα and ERβ which are both expressed in the hypothalamus. Overall, taken together with previous data obtained by Partner 18 during the first two years of WP8, the present observations allow to postulate that EDCs are able to modulate GnRH secretion in immature female hypothalamic explants *in vitro*, through both rapid and/or slow effects that likely involve both estrogen and dioxin receptor pathways.

Plan and Objectives for the Next Reporting Period

As indicated in previous sections, the research work conducted so far during the first three reporting periods of WP8 has allowed to cover most of the of the goals originally included in the corresponding workplan, except for the analysis of the impact of early exposure to EDCs, alone or in mixtures, at the HP unit, using selected molecular end-points, which remains to be completed for the reasons stated above. Similarly, some experimental work to be conducted by Partner 18, including completion of protocols of continuous administration of estradiol and selected EDCs *in vivo*, as well as some

electrophysiological studies using Gnv-4 GnRH cells and pharmacological tests in hypothalamic explants (e.g. testing the pathways for coumestrol and genistein effects), are yet to be fully completed.

Accordingly, the major aims for the next reporting period will be to carry out expression analyses of selected gene candidate, at hypothalamic and pituitary tissues from pubertal and/or adult animals exposed during early stages of development (*in utero* and lactation) to suspected EDCs. These samples (that have been or will be generated and collected by Partner 20) include protocol of exposure to single compounds (at different doses) or mixtures of similarly acting or dissimilarly acting EDCs. As indicated in previous sections, the panel of genes selected for analysis include PR (and, eventually, other NSR), KiSS-1, GPR54, and globins.

Likewise, pending *in vivo* and *in vitro* experiments, to be implemented by Partner 18, will be conducted and completed during the 4th reporting period.

Workpackage Related Appendices

Annex I (D8.2) Report detailing the comparative sensitivity of HP unit to disruption by EDC during sexual differentiation in male and female rats and information about molecular, biochemical or hormonal endpoints that signal these changes

Deliverables and Milestones

Deliverables	
✓	**D8.1**: Report detailing progress with work (month 18)
✓	**D8.2**: Report detailing the comparative sensitivity of HP unit to disruption by EDC during sexual differentiation in male and female rats and information about molecular, biochemical or hormonal endpoints that signal these changes (month 36)
delayed until month 48 (36)	**D8.3**: Report on doses of EDC able to activate hypothalamic-pituitary function in a precocious manner, analogous to puberty (month 36).
delayed until month 48 (36)	**D8.4**: Report on the ability of selected EDC, alone or in mixtures, to induce any of the changes above (month 36).
Milestones	
delayed until month 48 (36)	**M8.1**: Dose thresholds for disruption of the HP unit based on perinatal hormone/EDC treatments (month 36)
delayed until month 48 (36)	**M8.2**: New endpoints of hormone/EDC disruption of HP unit using differential display (DD) (month 36).
delayed until month 48 (36)	**M8.3**: Precocious activation of the HP unit by EDC (month 36).
delayed until month 48 (36)	**M8.4**: Effect of sub-threshold dose of EDCs, alone or in mixtures, on disruption of HP unit (month 36).

WP9	Vitellogenin- and spiggin induction in the stickleback as biomarkers for the simultaneous identification of estrogenic and (anti)androgenic EDC in surface waters					
Phase						
Start date	0					
Completion Date	12 months					
Current Status	complete					
Partner(s) responsible:	19	2	8			
Person-months per Partner:	16 (8)	8.5 (5)	9 (9)			
Already devoted person months per Partner and total:	12	8.5	9			

Objectives

- To deliver standard operating procedures for performing studies with the stickleback assay, for single compounds and mixtures
- To carry out range-finding studies as a basis for low-dose- and mixture experiments in WP13 and 15

Methodology and study materials

The advantages and disadvantages of using whole body homogenates (speedy, but perhaps leading to a loss of resolution) versus the sampling of specific tissues (a high signal-to-noise ratio, but perhaps too time-consuming) will be examined in the stickleback, using vitellogenin- and spiggin-induction as the endpoints (Partners 19, 2, 8). This information will form the basis of a standard operating procedure that will be developed.

Once an optimal operating procedure is established, all Partners will jointly conduct range-finding-studies for ethinyl estradiol and nonylphenol applied as single agents, using vitellogenin induction as the endpoint. Similarly, concentration ranges for flutamide suppression of spiggin synthesis will be established. These studies will underpin the detailed low-dose- and mixture studies in WP13 and 15.

This workpackage has been completed.

WP10	A transgenic fish model based on the green fluorescent protein (GFP) as a marker for oestrogen exposure in translucent fish					
Phase						
Start date	0					
Completion Date	48 months					
Current Status	active					
Partner(s) responsible:	7	11	13	14	15	
Person-months per Partner:	127.5 (48)	4 (3)	35.5 (24)	22 (11)	5 (2)	
Already devoted person months per Partner and total:	24	3	18	16	5	

Objectives for the Reporting Period

- Screening and crossing of zebrafish (Partner 7)
- Production of transgenic zebrafish that respond to EDCs by Partner 11
- Partner 14 has made reporter constructs with ERα (PA 1,8kb-GFP) and Aro-B (*Cyp19b* 0.5kb-GFP & *Cyp19b* 3kb-GFP) promoters linked to the Green fluorescent protein (GFP) gene
- Partner 13 carry out hybridisation experiments for E2 to determine exposure level for induction of gene expression

Methodology and Study Materials for the Reporting Period

Based on the recommendations from last reporting periods, Partner 11 focused on the aromatase genes (*cyp19a* and *cyp19b*) promoters, because they were shown to contain multiple putative transcription regulation elements, which could make them susceptible to exposure to different classes of EDCs. A reportergene construct was made composed of the *zfcyp19b* promoter region and EGFP coding sequence. Microinjection of this construct in zebrafish egss failed to produce a stable and reproducible EGFP expression pattern, even upon exposure to estrogens. To overcome this obstacle, Partner 11 have cloned so called "enhancing sequences" Gal4-VP16-14UAS-E1b between the studied promoter and reportergene regions (Figure 1), since it was shown to increase the expression levels of the weak promoters while sustaining their tissue-specificity.

Figure 1 Schematic representation of reporter vector structure

Several constructs containing "enhancing sequences" were produced, namely proAroB-GVP-UG, containing -759/+44 promoter/exonI region of *zfcyp19b,* proAroA(short)-GVP-UG and proAroA(long)-GVP-UG, containing -536/+37 and -1734/+37 promoter/exonI

regions of *zfcyp19a*, respectively, and negative control GVP-UG, containing no promoter in front of enhancer sequence. Embryos were injected in the cytoplasm at 1-2 cell stage with 30ng/ul of plasmid DNA up to 1/5 of the total cell volume, raised and observed up to 8 days at the latest, using fluorescent microscope or, in some cases, confocal microscope.

Scientific Achievements

Partner 7 contribution

The basic approach of the research is shown in a very simplified manner in Figure 2, fertilised eggs are injected with the transgene, after which it is a waiting game to see if, once sexually mature, any of the fish express the transgene (even if any do, these fish are likely to be mosaic for the transgene). To screen the mature fish, pairs (one male, one female) are allowed to reproduce, and the fertilised eggs they produce screened for the presence of the transgene.

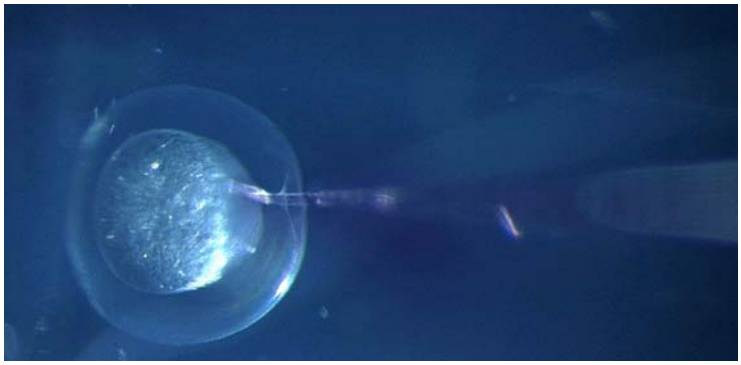

Figure 2 Injection of the transgene into the fertilised eggs of a zebrafish

Before any of the putative transgenic fish were screened, it was important to determine the sensitivity of the PCR techniques to be used to detect the transgene among extracted genomic DNA from the embryos (the F1 generation). As it is not known how many of the offspring within the 200 eggs/embryos screened per cross (pair of fish) were likely to be transgenic (as the parent fish, even if transgenic, will probably be mosaic), it was essential to determine the lowest number of copies of the transgene that could be detected. Five different primer pairs were used, in case one pair proved to better at detecting the transgene than the others. A serial dilution (from 10^8 down to 10^1 copies) of template plasmid DNA, was PCR-amplified in the presence of 200ng of wildtype zebrafish genomic DNA using each set of primers in an attempt to amplify the transgene prior to its detection on gels.

It was determined that 10 copies of the transgene could be detected using most of the primer sets. This level of detection is thought to be suitable for determining the presence of a fish with the transgene incorporated mosaically within its germ line. All genomic DNA from batches of eggs/embryos was also screened using primers specific to the EF1α gene (Figure 3). This additional PCR step is to show that the genomic DNA from each extraction is readily PCR-amplified. This is further to check that a negative result in the transgene is not due to poor quality DNA.

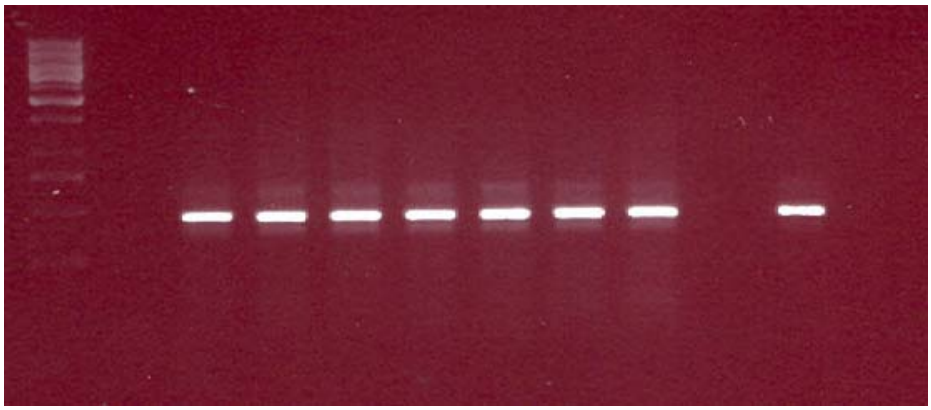

Figure 3 Screening of genomic DNA from ~ 200 eggs/embryos using EF1α primers, to check that the DNA was of the appropriate quality to detect single genes. It can be seen that the EF1α gene is readily detectable, demonstrating the high quality of the genomic DNA.

In general, 24-hour embryos were used in preference to younger ones, because these should (if transgenic) contain a higher number of copies of the transgene (one or more per cell), making it easier to detect. From Batch 1 (sexually mature fish produced from eggs microinjected in Year 2), a total of 30 fish produced eggs that were screened by PCR. A total of 44 fish (of both sexes) matured, but not all of these spawned (for example, some females became 'egg bound', and so did not spawn), and of those that did, some did not produce enough viable eggs to enable enough genomic DNA to be extracted for PCR. None of these 30 fish have to date shown any evidence of germline inheritance of the transgene (Figure 4). These fish from Batch 1 which produced 'extra' (over 200) viable eggs above those required for PCR were also screened by exposure of the 24 hour-old offspring to 1 µg E2/litre, to see if any fluoresced. Seven Batch 1 fish had offspring screened in this way. None showed any fluorescence, providing no evidence of incorporation of the transgene into the germline. Analysis of fin clips from all adult fish from Batch 1 will shortly be carried out, and another way of assessing whether any transgene did get integrated into the genome of any Batch 1 fish.

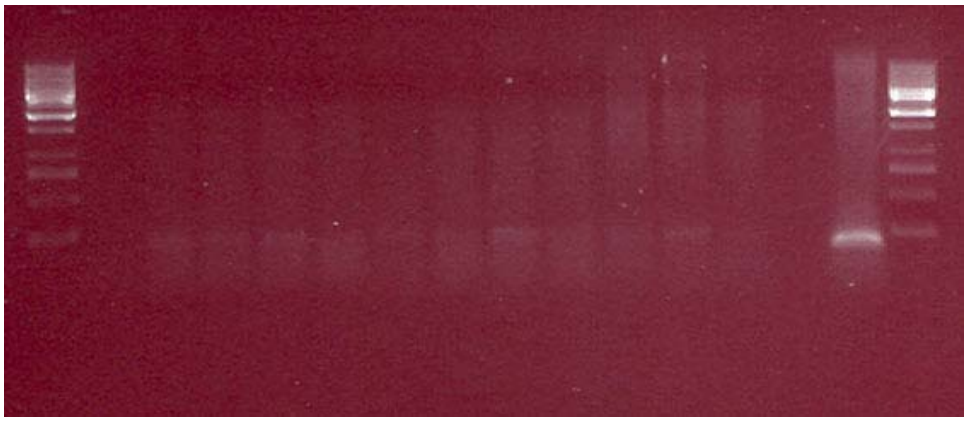

Figure 4 PCR analyses of the eggs/embryos of putative transgenic fish from Batch 1. At both sides of the gel are 1Kb DNA ladders. A positive control (plasmid and wild-type genomic DNA) was run in the penultimate lane (next to a ladder), and produced the expected band. All other samples showed no evidence of a band corresponding to the transgene.

It appears that none of the first batch of fish derived from microinjected eggs is transgenic. Thus, further batches of eggs were microinjected, and the viable offspring from these (there are about 50) are currently being reared to sexual maturity. The most likely explanation for the failure to produce a transgenic zebrafish to date (in Batch 1) is that the incidence of germline integration is low (lower than 1 in 30), and hence the chances of having one or more stably-transgenic fish in Batch 1 were low. Some of Batch 2 has now reached sexual maturity, and crossing has begun. The eggs/embryos from the crossing of Batch 2 will be screened as before. Partner 7 are endeavouring to continue increasing the number of putative transgenic fish, through continual microinjection of batches of eggs, to increase our chances of producing one or more stable transgenic lines of estrogen-responsive transgenic fish.

Partner 14 has tested Partner 7s transgene construct in their transient cell transfection assay with zfERa, b1 and b2, in order to determine their functionality. The results of transfection assays with the ERE-egfp in CHO cells (ER negative ovary cell line) are shown in Figure 5. The reporter ERE-egfp was transfected with and without different zfER subtypes in CHO cells. Cells were treated with ethanol (solvent) and E2. The expression of GFP was analysed in living cells at different conditions and also compared to the PEGFP vector used as positive control in these experiments. According to these results, the ERE-egfp construct works and the expression of GFP was significantly E2-stimulated with all the three zfERs.

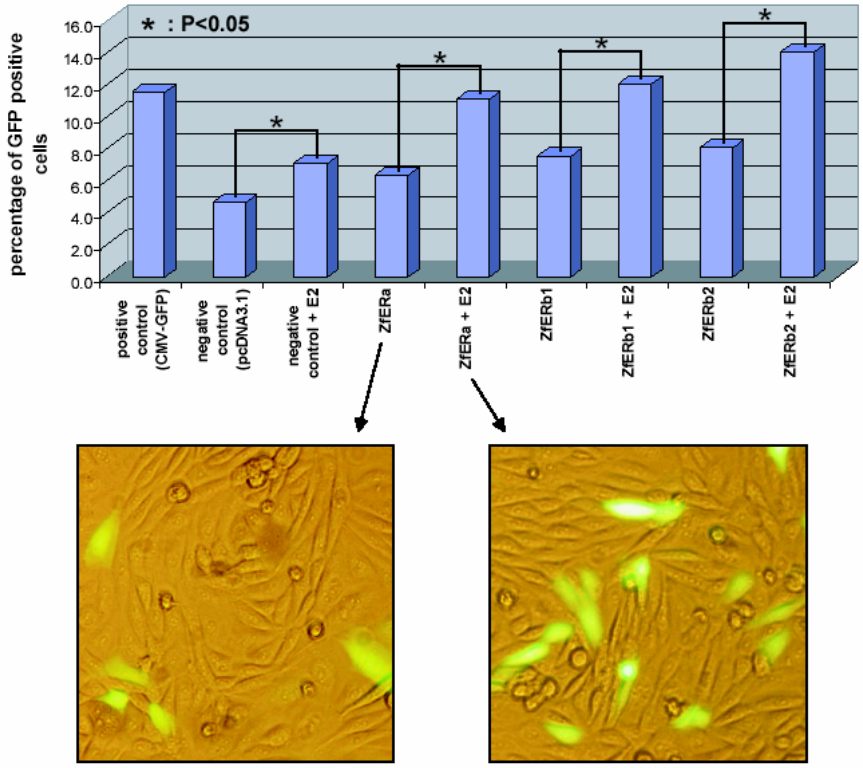

Figure 5 Estrogenic regulation of the ERE-TATAbox-GFP vector. CHO cells were co-transfected with the reporter vector ERE-TATAbox-GFP and the expression vectors of the three zebrafish estrogen receptors. CMV-GFP vector is used as a positive control and the empty expression vector pcDNA3.1 is co-transfected with ERE-TATAbox-GFP as a negative control. Cells were exposed to ethanol or to E2 10-8M during 48 hours. Statistical differences are shown by asterisks.

Partner 11 contribution

The work undertaken was related to M10.1 (knowledge about the production of transgenic reporter fish), M10.2 (information about the specificity and sensitivity of transgenic reporter fish), M10.3 (information about the usefulness of transgenic reporter fish to detect endocrine activities of chemicals and effluents) and deliverable D10.2 (production of transgenic reporter fish).

1. *Control plasmids pFRMwg and EF-GVP-UG*

To gain sufficient experience in microinjection, the plasmids pFRMwg (containing carp β-actin promoter which drives expression in muscle cells) and EF-GVP-UG (containing "enhancing sequences" and frog *ef1a* promoter, which produces ubiquitous expression) were injected as a positive control (Figures 6 and 7). The efficiency of injection was evaluated by comparing the death rate of injected embryos to that of control ones, and by calculating the expression rate of injected embryos (data not shown). The results allowed Partner 11 to conclude that the microinjection technique is well established and thus can be reliable for characterisation of new constructs.

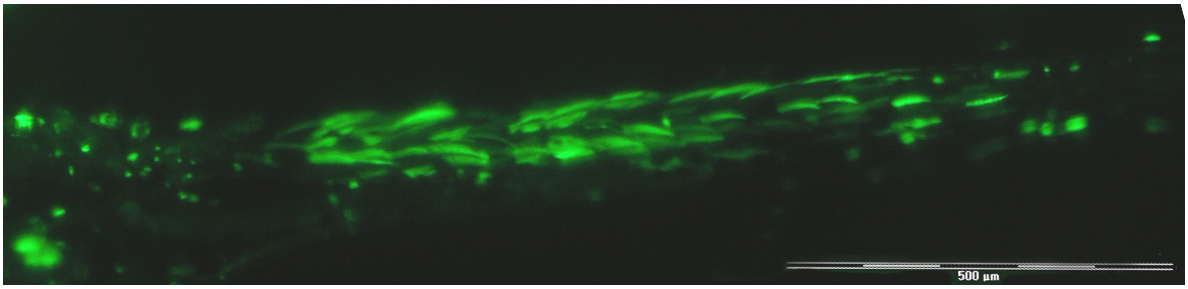

Figure 6 dpf embryo injected with pFRMwg. Abundant EGFP expression can be observed in muscle cells

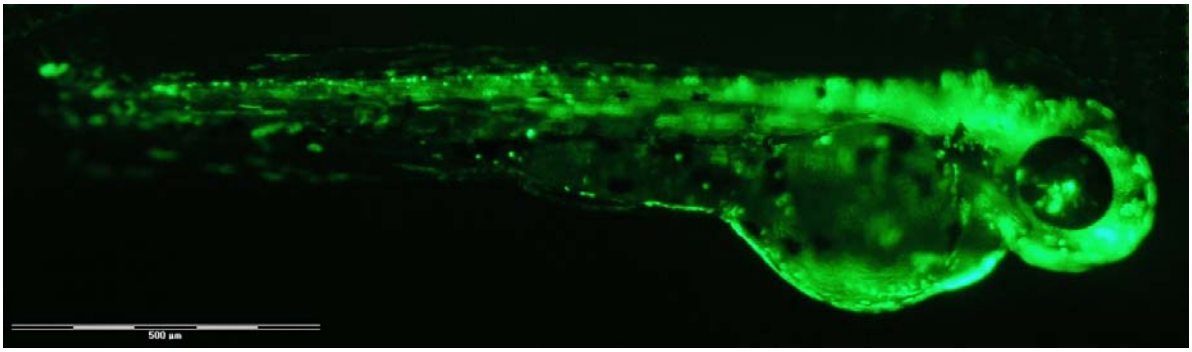

Figure 7 dpf embryo injected with EF-GVP-UG. EGFP expression can be observed in most cell types.

1. *proAroB-GVP-UG*

EGFP expression in proAroB-GVP-UG injected embryos could be observed starting at 1dpf, was most intensive at 2-3 dpf and mostly faded by 8dpf completely. It was mainly found in the brain, but was also often observed in muscle cells, ectodermal cells and yolk (Figure 8), and rarely in different cellular structures along the body and in the eye. At 2 dpf the

expression in the brain (presumably specific site) and other sites (presumably unspecific) was very high and mostly equally intensive. At later stages the EGFP expression starts fading generally (as it happens in any transient microinjection approach), however, presumably unspecific expression faded faster than the supposedly specific one (in the brain).

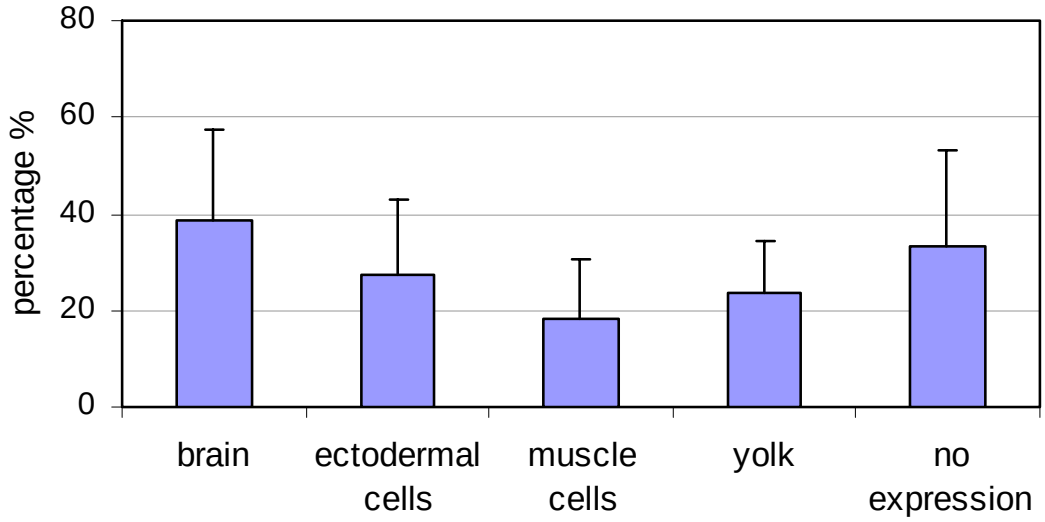

Figure 8 Frequency of appearance of main expression patterns in embryos injected with proAroB-GVP-UG. Expression patterns were divided into main categories such as brain, ectodermal cells, muscle cells and yolk, percentage of non-expressing embryos is also shown. The rate of expression is calculated as number of embryos exhibiting the pattern normalised to total number of survivors. The expression pattern often overlapped in one embryo therefore the expression rates summarise to more than 100%

Estrogen was shown to induce expression of *cyp19b* and it was assumed that exposure to this compound will increase the rate of specific expression in the brain or will extend the expression in time. In an experimental setup, half of the proAroB-GVP-UG injected embryos were exposed to E2 and the other half was kept as untreated or vehicle-treated control, and the expression patterns as well as the rate of EGFP expression fading over time were compared within groups (Figure 9). There was no difference between the groups in the number of embryos expressing EGFP in the brain up to 3 dpf. At 4dpf the signal in the brain was sustained in E2-treated embryos but not in unexposed ones. E2 treatment had no effect on the rate of unspecific expression occurrence and fading in both groups.

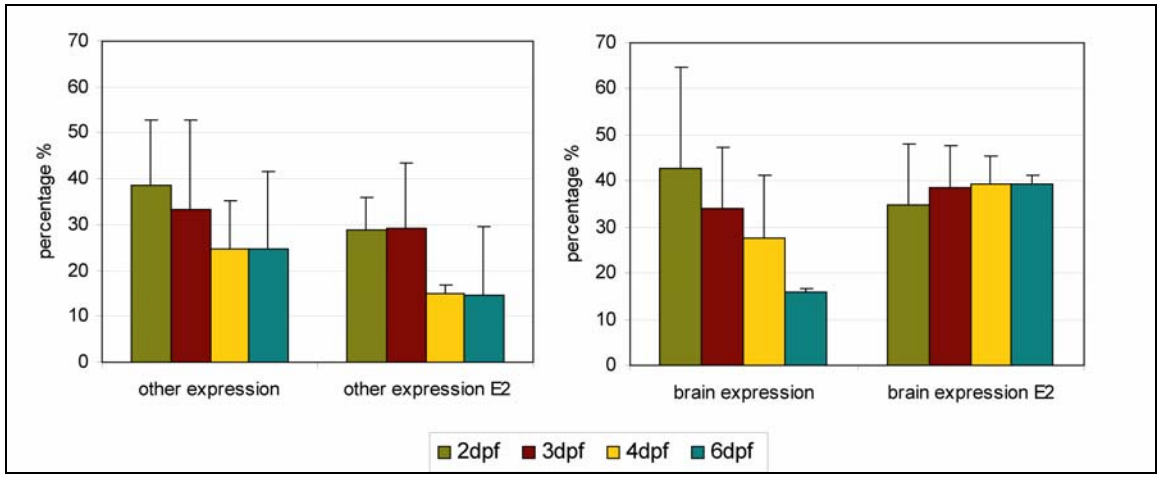

Figure 9 Comparison of EGFP expression between E2-exposed (10^{-8} M) and unexposed proAroB-GVP-UG injected embryos. Other expression refers to the total number of embryos showing any expression pattern but not only in the brain (A). Brain expression is the total number of embryos showing expression in the brain (B).The rate of expression is calculated as number of embryos exhibiting the pattern normalised to total number of survivors. The expression pattern often overlapped in one embryo therefore the expression rates summarise to more than 100%.

Partner 14 has shown by means of IHC that *cyp19b* in zebrafish seems to be expressed only in radial glial cells, and exposure to E2 increases *cyp19b* abundance and is needed to detect *cyp19b* in embryos. Embryos injected with Partner 11 constructs exhibited EGFP expression in the brain even without E2-exposure, which can be explained by the strength of enhancer sequences. The brain regions exhibiting EGFP expression occasionally corresponded with Partner 14 data (compare Figure 10 and 11). However, many embryos exhibited neuronal-like projections expressing EGFP, which was unexpected. For better characterisation of expressing cells, embryos were observed under confocal microscope, however, there were some difficulties with setup (inability to observe one embryo several times at diferent stages) and statistical interpretation of the results, since both the neuronal and supposedly radial glial cells expressing EGFP were seen (Figure 12), and the number of examined embryos was not high enough to make significant conclusions.

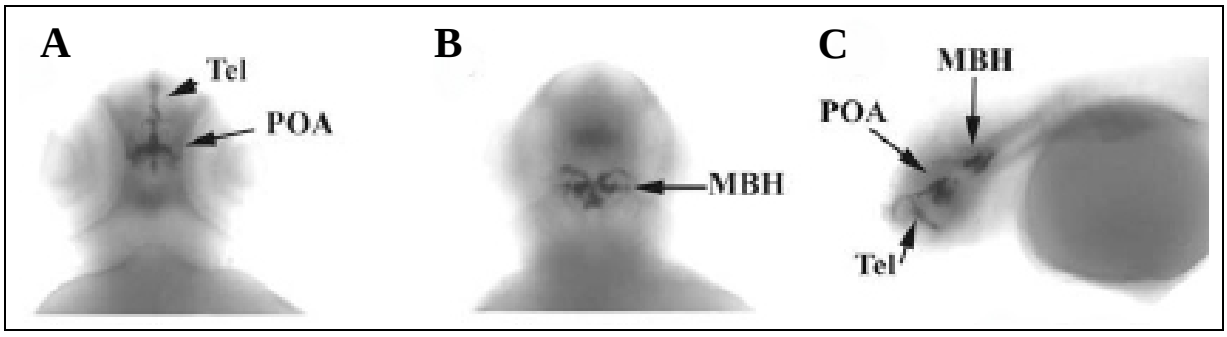

Figure 10 *Cyp19b* mRNA detected with whole-mount in situ hybridisation in 48dpf E2 (10^{-8}M) exposed embryos (Menuet et al, 2005). A, B: dorsal views focused on (A) telencephalon (Tel) and preoptic area (POA) or (B) the mediobasal hypothalamus (MBH). C: Side view of embryo showing *cyp19b* mRNA in the Tel, POA, MBH.

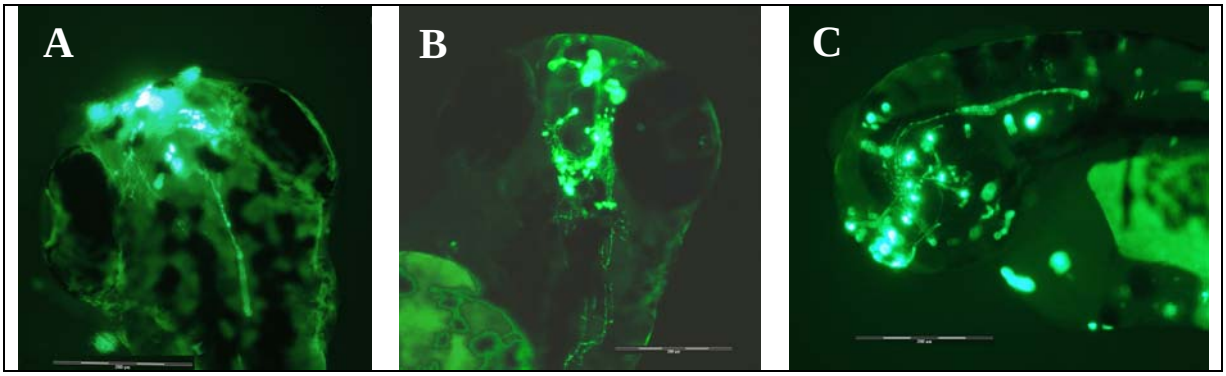

Figure 11 Brain expression sites in proAroB-GVP-UG-injected embryos. A: 3dpf, dorsal view. B: 3dpf, ventral view. C 2dpf, lateral view.

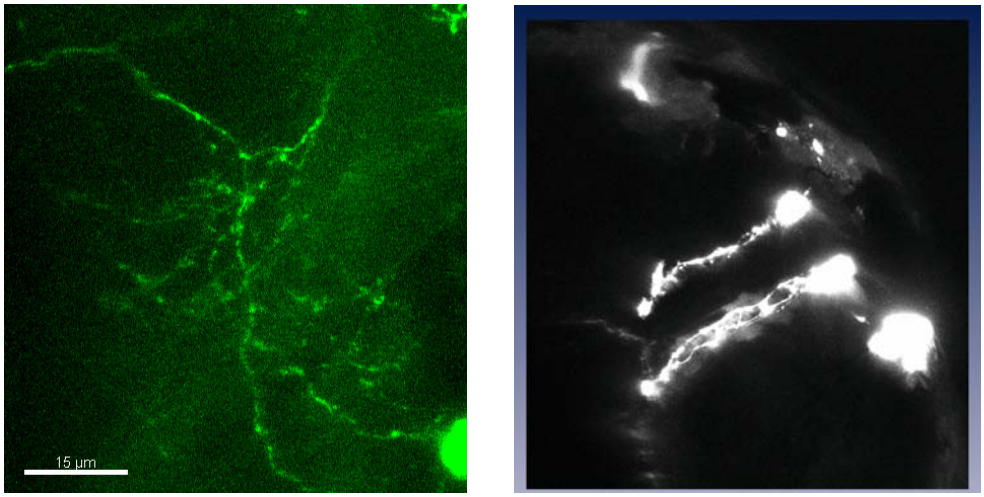

Figure 12 Different patterns of EGFP expression observed under confocal microscope in proAroB-GVP-UG injected embryos (3dpf)

Partner 11 used anti-EGFP to further characterise EGFP-expressing cells in 1-5dpf proAroB-GVP-UG-injected embryos to investigate the hypothesis that EGFP is initially expressed in the radial glial cells, and then retained in neurons which originate from these cells, leading to more neuronal-like looking pattern in the older embryos. Another hypothesis was that aromatase in zebrafish could be expressed in both neurons and radial glia, however, in neurons much weaker and only possible to see with the construct, but not with conventional anti-CYP19b IHC. It was supposed that E2-exposure should bring up the number of radial glial cells expressing EGFP, since exposure to E2 leads to detection of *cyp19b*-expressing radial glial cells in zebrafish embryos as shown by Rennes group. Research towards both assumptions is in the process at the moment, since large numbers of embryos and conditions should be examined in order to be able to draw statistically relevant conclusions.

Double-staining experiments have also begun in collaboration with Farzad Pakdel and Olivier Kah from Partner 14 to investigate co-localisation of EGFP with *cyp19b*, neuronal and glial markers in proAroB-GVP-UG injected embryos. Yet another hypothesis could be that the *cyp19b* promoter part used is not enough to produce strict *cyp19b*-tissue-specificity, but simply leads to EGFP expression in the cells which express normally express estrogen receptors (neurons), since the ERE site is present in this promoter. To

investigate this, it is planned to introduce a mutation in the ERE site in the construct and/or suppress ER expression in the injected embryos (by means of either ER antagonist ICI or morpholino knockdown of ERs). In the mean time, Partner 11 is raising some proAroB-GVP-UG-injected embryos and plan to start examination of F1 generation in February 2006. Even if not totally *cyp19b*-specific, this construct could still potentially be used for production of transgenic line responding to estrogenic compounds.

3. *proAroA(short)-GVP-UG and proAroA(long)-GVP-UG*

The main expression pattern categories observed in proAroA(long)-GVP-UG and proAroA(short)-GVP-UG injected embryos were the same as in proAroB-GVP-UG injected ones (Figure 13). However, the most frequent expression pattern observed this time exhibited unevenly distributed ectodermal cells (Figure 14). The expression rate in proAroA(short)-GVP-UG injected embryos was much lower than in proAroA(long)-GVP-UG injected ones.

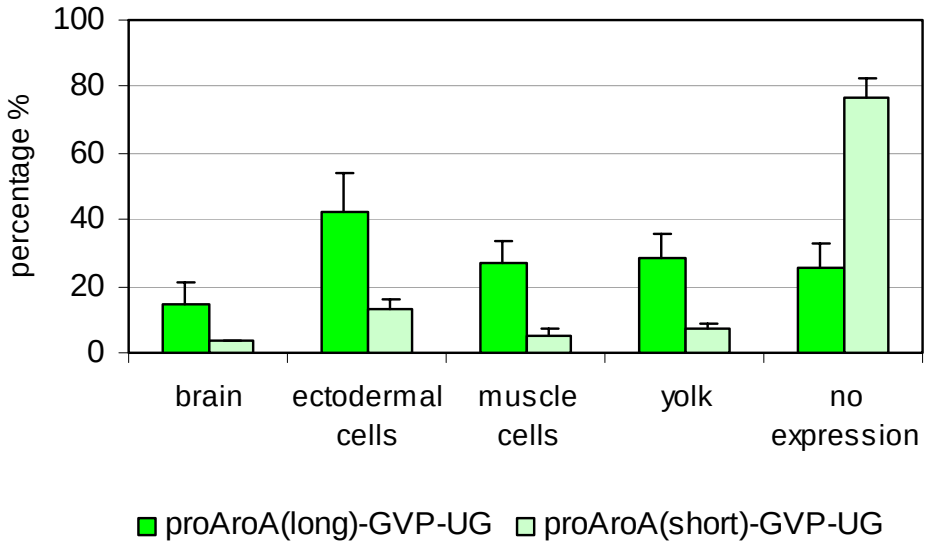

Figure 13 Frequency of appearance of main expression patterns in embryos injected with proAroA(short)-GVP-UG and proAroA(long)-GVP-UG. Expression patterns were divided into main categories such as brain, ectodermal and muscle cells and yolk, percentage of non-expressing embryos is also shown. The rate of expression is calculated as number of embryos exhibiting the pattern normalised to total number of survivors. The expression pattern often overlapped in one embryo therefore the expression rates summarise to more than 100%.

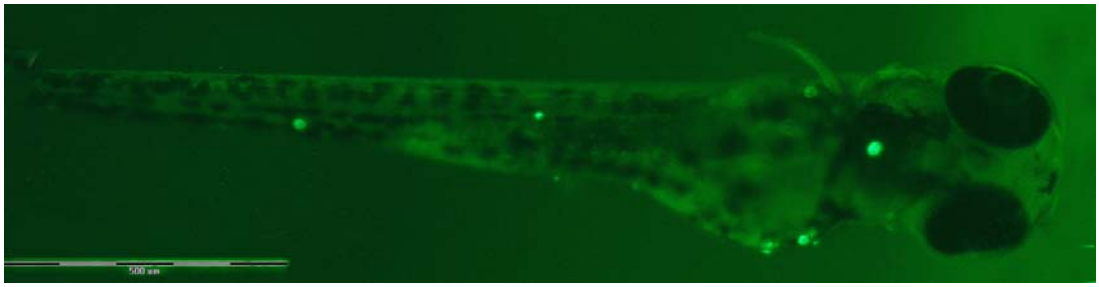

Figure 14 3dpf embryo injected with proAroA(long)-GVP-UG. EGFP expression can be observed in ectodermal cells.

Cyp19a was shown to be expressed in gonads of adult fish, and also in primordial germ cells of larvae, however, the transient EGFP expression was not sustained long enough to be able to examine these later stages of development. In addition, the lab of Partner 11 has observed *cyp19a* expression in neuromasts with in situ hybridisation, and have further confirmed its importance for neuromast development by examining *cyp19a*-morphants(unpublished data), thus we supposed that these EGFP-expressing cells could correspond to neuromasts. To reveal neuromasts pattern, 6dpf embryos were stained with DASPEI. Different concentration and incubation times were used to stain control embryos to find the best staining conditions (Figure 15). All subsequent stainings were done with 0.005% DASPEI and 10 min incubation.

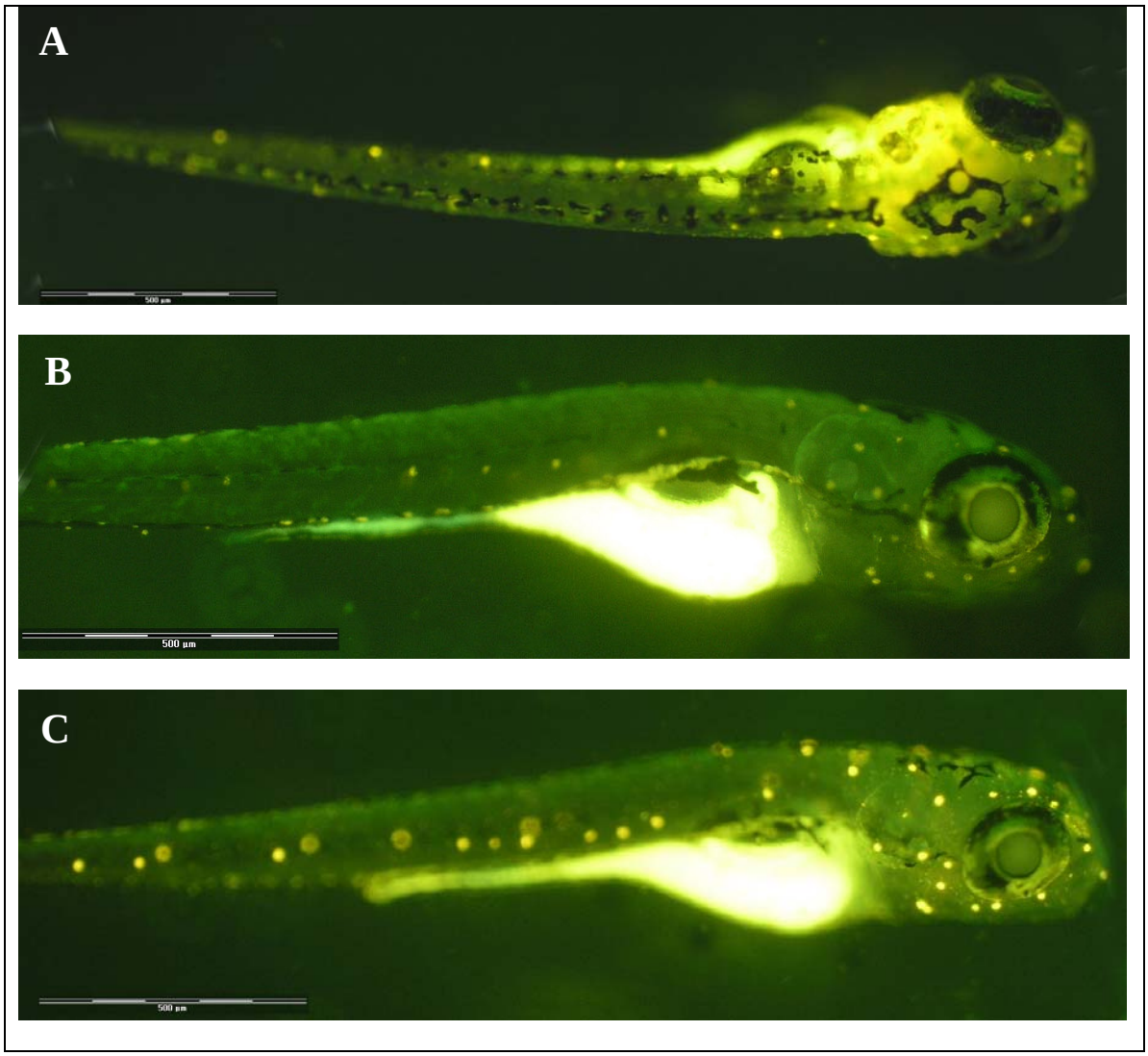

Figure 15 6dpf control embryos stained with DASPEI. A: overstained (0.01%, 15min); B: weak staining (0.002%, 10min) C: perfect staining (0.005%, 10min)

Injected embryos with expression pattern possibly corresponding to neuromasts were stained with DASPEI to see if the localisation of both siganls would overlap. However, a correlation between the DASPEI staining and EGFP expression was not observed (Figure 16).

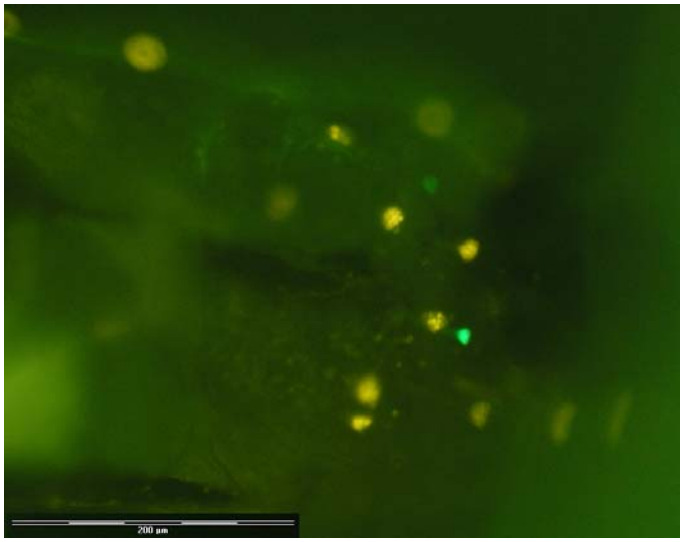

Figure 16 Staining with DASPEI of an proAroA(long)-GVP-UG injected embryo (6dpf) with EGFP-expressing ectodermal cells.

Neuromasts can be also recognised morphologically, by specific flower-like structure of supporting cells (Figure 17). However, EGFP expressing ectodermal cells did not show apparent neuromast-like structure.

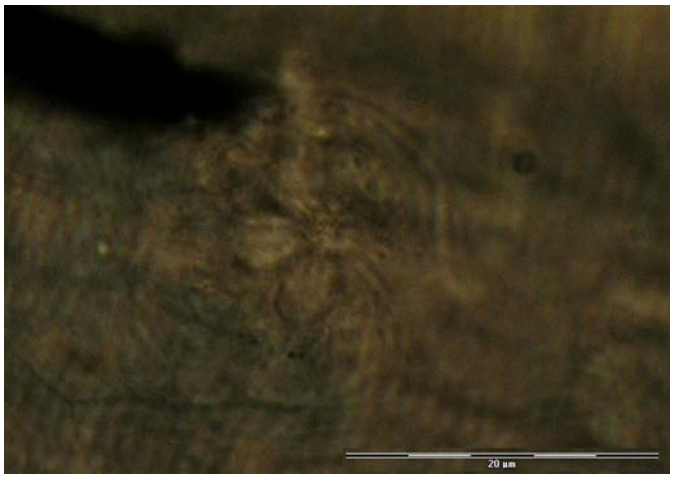

Figure 17 Flower-like structures of neuromasts visible at magnification 100x in 6dpf control embryo

4. *Negative control GVP-UG*

GVP-UG was constructed as vector containing only enhancer sequences without promoter, to use as a negative control. The main expression pattern categories observed in GVP-UG-injected embryos were the same as with the promoter-containing vectors and included brain, ectodermal and muscle cells and yolk (Figure 18). The expression rate of GVP-UG injected embryos was lower than in the embryos injected with proAroB-GVP-UG and proAroA(long)-GVP-UG, but still relatively high. However, this EGFP expression faded very quickly and was practically absent already at 4dpf, pointing to the general unspecificity of observed pattern. Expression rates observed in proAroA(short)-GVP-UG injected embryos were even lower than those for GVP-UG, but, again, the

EGFP expression itself was more stable over time in proAroA(short)-GVP-UG-injected embryos than in GVP-UG-injected ones.

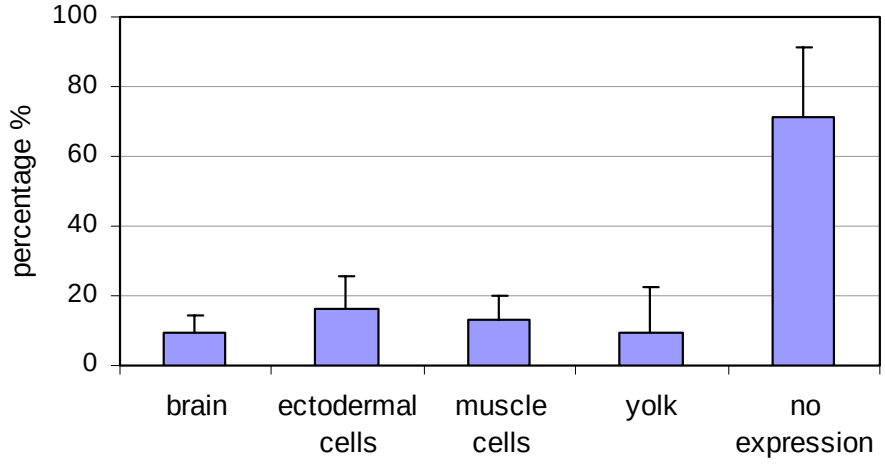

Figure 18 Frequency of appearance of main expression patterns in embryos injected with GVP-UG. Expression patterns were divided into main categories such as brain, ectodermal cells, muscle cells and yolk, percentage of non-expressing embryos is also shown. The rate of expression is calculated as number of embryos exhibiting the pattern normalised to total number of survivors. The expression pattern often overlapped in one embryo therefore the expression rates summarise to more than 100%.

Frequency of appearance of different expression patterns in embryos injected with different plasmids containing "enhancing sequences" is shown in Figure 19. Brain expression pattern was occurring most frequently in proAroB-GVP-UG injected embryos. Ectodermal cells expression pattern was occurring most frequently in proAroA(long)-GVP-UG injected embryos. Thus, introduction of "enhancer sequences" in the classical promoter-EGFP vectors indeed has brought up the signal levels, easing the observation obstacles. However, it also led to appearance of presumably unspecific expression patterns. Unspecific "leaking" of very strong promoters in addition to specific expression has been reported before however, to say with certainty in our case, if our constructs produce any specific pattern at all, thorough investigation is needed.

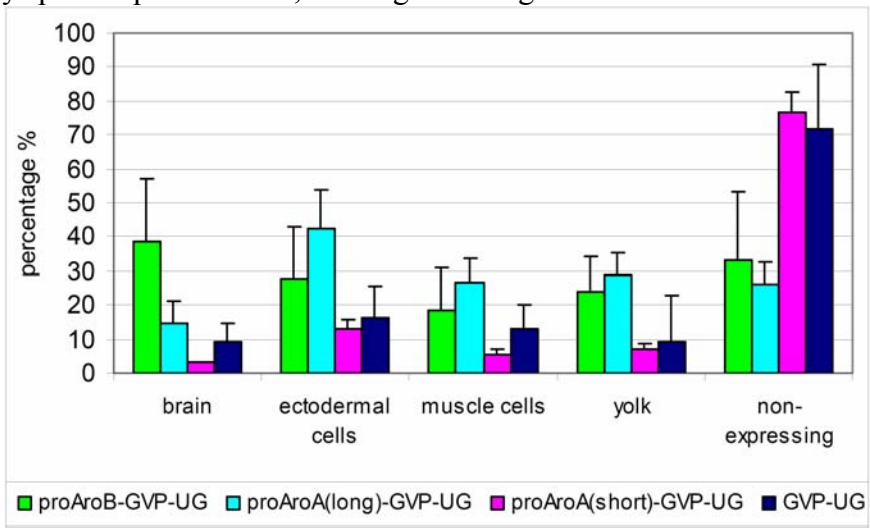

Figure 19 Frequency of occurrence of different expression patterns in injected embryos (2dpf).

Partner 13 contribution

1. *Zebrafish exposure*

During the reporting period the following exposures were carried out:

- 17β-estradiol (E2) (adult male zebrafish, concentrations: 0/5/50/100/200 ng/L)
- Ethinylestradiol (EE2) 30ng/L (adult male zebrafish, as a positive control)

Water samples were taken from E2 and EE2 tanks and measured by CEFAS. Twelve adult male zebrafish were exposed in each tank and the duration of exposure was 11 days. Liver tissue was dissected from each fish after the end of exposure and its total RNA was extracted. The mRNA was then reversely transcribed into cDNA.

2. *DNA microarray experiments*

DNA microarray experiments with samples exposed to E2 were carried out on self-spotted arrays. For this purpose, 40 genes/sequences were selected from the whole-genome arrays (cf. 2nd Annual Report). The corresponding oligonucleotides were immobilised on epoxy glass slides. The genes selected for self-spotted arrays (of which the grey marked genes are zebrafish genes) are given in D5.3 and were obtained after statistical analysis using a specificity of 95 % for the selection procedure.

During the reporting period, liver samples of all exposed and control fish were dissected, total RNA extracted and reversely transcribed. For microarray experiments self-spotted arrays were produced. Each array contains 40-50 mer-oligonucleotide probes coding for genes/sequences regulated by E2 (500 ng/L). In addition, 10 probes coding for housekeeping genes that were not regulated by E2 were immobilised for data normalisation.

Hybridisation experiments for all concentrations (5, 50, 100, 200, 500 ng/L E2) were carried out. Data normalisation and statistical analyses were performed to determine exposure level for induction of gene expression. Table 1 gives the regulation of spotted genes, the numbers in the first column indicating the genes listed in the Table given in D5.3. Figure 20 shows the foldchange in the expression of *vtg1* and *vtg3*.

No.	5 ng/L E2	50 ng/L E2	100 ng/L E2	200 ng/L E2	500 ng/L E2
1	n.s.	n.s.	n.s.	27.2	832.1
2	n.s.	n.s.	n.s.	n.s.	9.7
3	n.s.	n.s.	n.s.	n.s.	6.0
4	n.s.	n.s.	n.s.	n.s.	7.3
5	n.s.	n.s.	n.s.	n.s.	n.s.
6	n.s.	n.s.	n.s.	n.s.	2.9
7	n.s.	n.s.	n.s.	n.s.	n.s.
8	n.s.	n.s.	n.s.	n.s.	3.0
9	n.s.	n.s.	n.s.	n.s.	28.7
10	n.s.	n.s.	n.s.	n.s.	3.2
11	n.s.	n.s.	n.s.	n.s.	3.3
12	n.s.	n.s.	n.s.	n.s.	2.5
13	n.s.	n.s.	n.s.	n.s.	4.0

14	n.s.	n.s.	n.s.	n.s.	3.5
15	n.s.	n.s.	n.s.	n.s.	n.s.
16	n.s.	n.s.	n.s.	2.3	5.7
17	n.s.	n.s.	n.s.	n.s.	4.1
18	n.s.	n.s.	n.s.	n.s.	4.2
19	n.s.	n.s.	n.s.	n.s.	8.7
20	n.s.	n.s.	n.s.	n.s.	19.2
21	n.s.	n.s.	n.s.	n.s.	n.s.
22	n.s.	n.s.	n.s.	n.s.	2.9
23	n.s.	n.s.	n.s.	n.s.	n.s.
24	n.s.	n.s.	n.s.	n.s.	2.8
25	n.s.	n.s.	n.s.	n.s.	1.7
26	n.s.	n.s.	n.s.	n.s.	8.2
27	n.s.	n.s.	n.s.	n.s.	2.6
28	n.s.	n.s.	n.s.	n.s.	n.s.
29	n.s.	n.s.	n.s.	n.s.	n.s.
30	n.s.	n.s.	n.s.	n.s.	n.s.
31	n.s.	n.s.	n.s.	n.s.	2.8
32	n.s.	n.s.	n.s.	n.s.	n.s.
33	n.s.	n.s.	n.s.	n.s.	n.s.
34	n.s.	n.s.	n.s.	n.s.	n.s.
35	n.s.	n.s.	n.s.	n.s.	3.0
36	n.s.	n.s.	n.s.	n.s.	n.s.
37	n.s.	n.s.	n.s.	n.s.	n.s.
38	n.s.	n.s.	n.s.	n.s.	n.s.
39	n.s.	n.s.	n.s.	n.s.	n.s.
40	n.s.	n.s.	n.s.	n.s.	n.s.

Table 2 Regulation of spotted genes in fold-change. n.s.: not significant

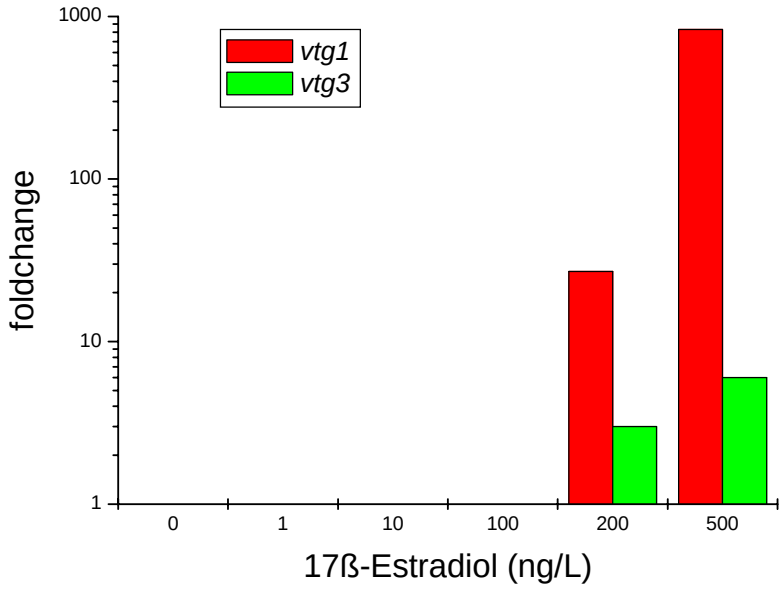

Figure 20 Foldchange in the expression of *vtg1* and *vtg3* at different E2 concentrations.

The results of the microarray experiments show that a significant induction of gene expression is observable at concentrations higher than 100 ng/L E2. At a concentration of 200 ng/L E2 just the *vtg1* (27-fold up-regulated) and *vtg3* (2-fold up-regulated) are expressed with a significance of 95%. At a concentration of 500 ng/L E2 the *vtg1* is up-regulated 832-fold and the *vtg3* is up-regulated 6-fold. In addition 22 further genes/sequences are expressed at a concentration of 500 ng/L with a significance of 95%. It is clear that with increasing concentration of E2 the number of regulated genes/sequences and the level of expression rises. In addition these results correspond very well with the data from the LightCycler experiments. These show a significant induction of *vtg1* at concentrations higher than 100 ng/L E2, too. Figure 21 shows the confidence interval for *vtg1* with a significance of 95%. A significant increase of expression at 200 and 500 ng/L E2 is observable. Figure 22 shows the confidence interval for the housekeeping gene ß-actin at a specificity of 95%. There is no significant change in gene expression observable. The normalised median fold-change is less than +/- 1.5 at all concentrations. Therefore it is well suitable for normalisation.

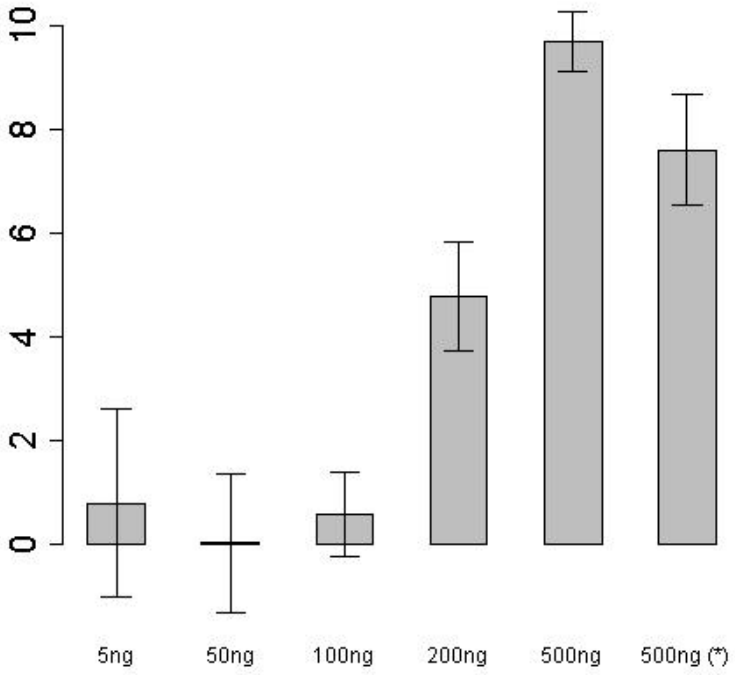

Figure 21 Confidence interval for *vtg1* at a specificity of 95%. The logarithmic fold-change is plotted. Data were obtained from whole genome arrays.

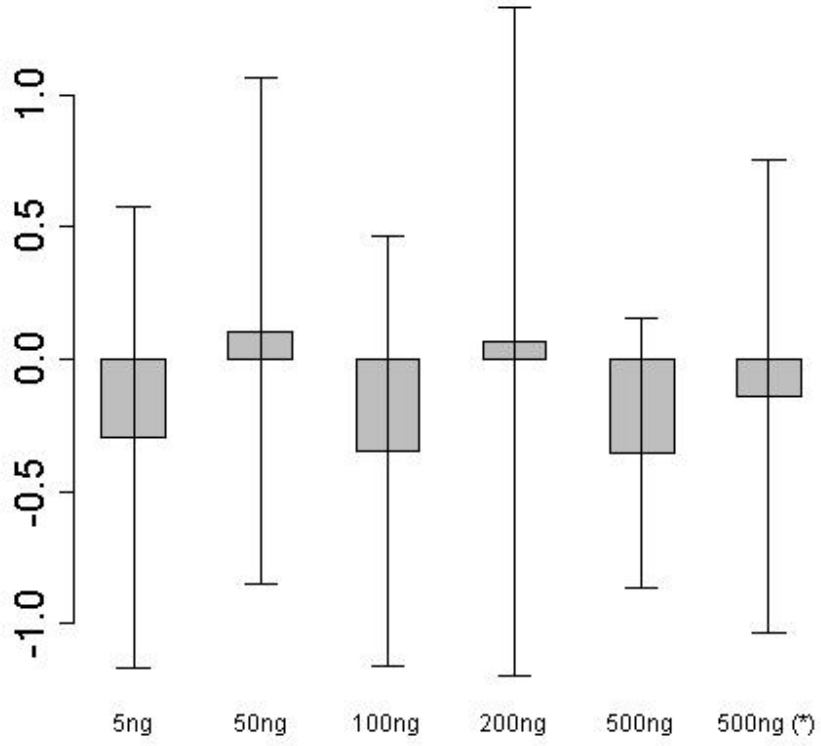

Figure 22 Confidence interval for ß-*actin* at a specificity of 95%. The logarithmic fold-change is plotted. *: Data were obtained from whole genome arrays.

In addition microarray experiments with samples exposed to BPA are carried out. To detect genes/sequences responding to BPA whole genome arrays were used for samples exposed to a concentration of 1000 µg/L, since an induction of *vtg1* was observed by LightCycler experiments. 38 genes/sequences were found to be regulated. These 38 genes / sequences plus the 40 genes / sequences regulated by E2 were spotted on epoxy slides. Hybridisation experiments with all concentrations (0.1, 2, 20, 200, 400, 1000, 2000 µg/L BPA) are currently in progress.

Details of the Light Cycler experiments are given in WP13

Partner 14 contribution

Previous cell-based transfection assays with both zebrafish ERα and Aro-B promoter regions demonstrated that the E2-dependent regulation of these genes involves a transcriptional action of ER that requires an ERE and a half site ERE. However, the data obtained on different cell lines demonstrated that a glial cell context is necessary for full E2 induction of Aro-B but not for ERα. Partner 14 has made reporter constructs with these promoters linked to the Green fluorescent protein (GFP) gene. Both promoters, ERα (PA 1,8kb-GFP) and Aro-B (*Cyp19b* 0.5kb-GFP & *Cyp19b* 3kb-GFP), were able to direct the expression of GFP in cell cultured assays in the presence of estrogens. It can be concluded that these promoters could therefore be used in the transgenic zebrafish. Three constructs were microinjected into the zebrafish eggs in the presence of meganuclease. As positive controls, CMV-GFP and Neurotubuline-GFP vectors were used. After microinjections, embryos were exposed with and without 10-8M to 10-6M E2. While high number of GFP-positive embryos (30 to 50%) was obtained with CMV-GFP or

Neurotubuline-GFP vectors, none of embryos injected with aromatase promoter or with ERα promoter showed fluorescence. These results indicated that the activity of Aro-B and ERα promoters are very weak for GFP analysis *in vivo*. In collaboration with Partner 11, another aromatase-GFP vector (proAroB-GVP-UG) was used, possessing enhancer sequences to amplify GFP expression. Further detail on this vector is given by Partner 11 in this WP10.

Twenty four to 48h after microinjection, GFP-positive embryos showed heterogeneously high expression of GFP in different tissues such as brain, muscle, ectoderms and yolk body. Moreover, the expression of GFP was not affected by E2 treatment. However, 5 days after microinjection, the expression of GFP was observed only in the brain (Figure 23). These results suggest that non-specific and high expression of GFP during the first days (1-2) after microinjection become more tissue-specific and localised after 3 to 5 days. This could be explained by integration of the plasmid into the zebrafish genome. Nevertheless, the brain specific expression of proAroB-GVP-UG was not localised in the glial cells but in the neuronal cells. The expression of GFP in these cells may correlate with the presence of ER. To test this hypothesis, Partner 14 are planning to treat embryos after microinjection with the antiestrogen, ICI182,780. This experiment will show whether the expression of GFP in these neurons is ER-dependent. If this is the case, Partner 14 will confirm that by mutating the ERE element within the promoter.

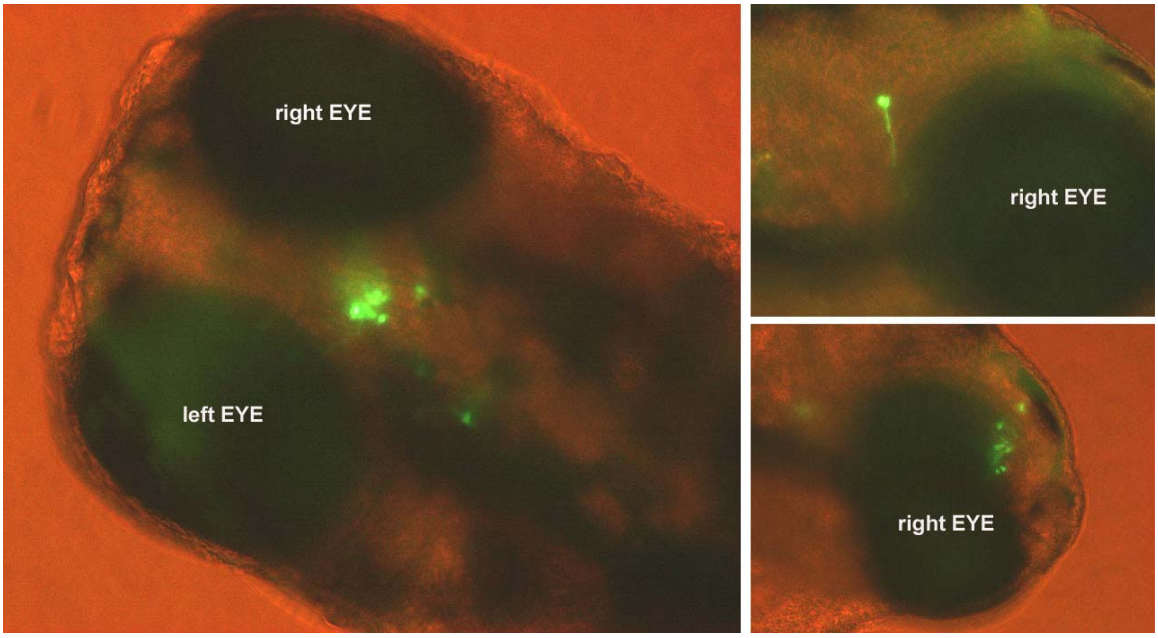

Figure 23 Expression of GFP in the brain of zebrafish embryo 5 days after microinjection of proAroA(long)-GVP-UG.

1. *Plasmid constructs used for microinjections*

Cyp19b 0.5kb - GFP : correspond to 500pb of Aromatase promoter described previously (Menuet *et al.*, 2005) inserted into BamH I site of the plasmid Sce-pEGFP1-Sce. Sce-pEGFP1-Sce from the plasmid pEGFP-1 (Clontech) in which were added two sites of Meganucléase (Thermes *et al*., 2002).

Cyp19b 3kb - GFP : correspond to 3000 pb of Aromatase promoter that were amplified by PCR from the zebrafish genomic DNA and inserted into BamH I site of the same plasmid Sce-pEGFP1-Sce. The oligonucleotides for the PCR were:

5'-CCCGGGATCCGGAGTGTTCCGGG-3' et 5'-GGATCCCCGTCCTCAGGCTTCC-3'.

proAroB-GVP-UG : is Partner 11 which contains the promoter of aromatase *Cyp19b*, described in Menuet *et al* 2005, inserted in the vector Gal4-VP16 (Koster and Fraser, 2001).

PA 1,8kb-GFP correspond to 1800pb of zebrafish ER-alpha gene promoter (Menuet *et al*., 2004) inserted into BamH I site of the plasmid Sce-PEGFP1-Sce.

2. *Microinjection, treatment and observation*

Zebrafish eggs were injected 15 minutes after laying with 20 ng/µL of each plasmid, 0.1 µg/µL of BSA and 20 UI of Meganuclease in the presence of 0.02% of phenol red. Notice that for the plasmid proAroB-GVP-UG, BSA was not used. Embryos were then incubated at 26°C in 50ml of embryonic media with or without E2. The expression of GFP was analysed by microscope with epifluorescence light in embryos injected (1 to 5 dpf). GFP-positive embryos were introduced into 1.2% agarose and observed using microscope multiphotonic with camera and program that allow making three-dimensional reconstitution of the pictures.

Koster RW, and Fraser SE, (2001). Tracing transgene expression in living zebrafish embroyos. *Dev. Biol*. **233** (2) 329-346.

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

Menuet A, Le Page Y, Torres O, Kern L, Kah O, and 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.

Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, and Joly JS, (2002). I-SceI meganuclease mediates highly efficient transgenesis in fish. *Mech. Dev.* **118** (1-2) 91-98

Partner 15 contribution

The role of Partner 15 in WP10 was to deliver candidate genes for the development of transgenic fish (from WP5). This contribution has been finalised during the first project year.

Progress Summary

To date, a transgenic (construct) had been designed and created by Partner 7. This consists of two estrogen response elements (ERE) attached to the sequence for green fluorescent protein (GFP). A few hundred early embryos had been injected with this

transgene, and these were being grown on until they matured, at which stage they could be crossed, and eggs (the F1 generation) screened for possible integration of the transgene into the genome.

Micro-injections of zebrafish eggs have been established and can be performed reproducibly by Partner 11. Several constructs containing zebrafish *cyp19a/b* promoters and "enhancing sequences" were produced and the EGFP expression patterns produced by microinjection of these constructs in zebrafish embryos were characterised. The constructs without enhancer elements did not give visible GFP signals though the construct containing *cyp19b* promoter region with enhancer elements produces high rates of EGFP expression in the brain. Expression from these enhanced *cyp19b* promoter constructs responded to E2 exposure, EGFP expression was sustained. The EGFP expression produced by the constructs containing *cyp19a* promoter regions did not fit with any specific cellular structure known before to express *cyp19a* in fish. Hence Partner 11 considers expression from this construct aspecific and will not continue with this. ProAroB-GVP-UG-injected embryos are being raised to investigate the possibility of producing transgenic line and its possible application for estrogen exposure

From Partner 13's studies, there is a significant increase in the *vtg1* expression at 200 ng/L E2 and 2000 µg/L BPA. Due to the individual responses to NP there was no significant increase observed. The results of the E2 microarray experiments show that an induction of gene expression is observable at concentrations higher than 100 ng/L E2. At a concentration of 200 ng/L E2 just the *vtg1* and *vtg3* are up-regulated with a significance of 95%. At a concentration of 500 ng/L E2 the *vtg1* is up-regulated 832-fold and the *vtg3* is up-regulated 6-fold. In addition 25 further genes/sequences are expressed at a concentration of 500 ng/L with a significance of 95%. First analysis of the BPA microarray experiments suggest that BPA takes effect in a completely different way than E2 but these results have to be confirmed by further analysis that are in progress. These results and the results from the LightCycler experiments confirm earlier recommendations to use *vtg1* for producing a transgenic zebrafish to detect the endocrine activities of chemicals (cf. D10.3 and 2nd Annual Report of Partner 13).

Partner 14 has linked the promoter regions of zebrafish ERα and Aro-B to the Gfp reporter gene. The reliability of these constructs was tested by transient expression in vitro by cell-based assays. The results showed that these two promoters can be directly activated by estrogens. These promoters were used *in vivo* by microinjecting zebrafish embryos but GFP could not be detectable probably because the activity of Aro-B and ERα promoters are too weak for GFP analysis *in vivo*, proAroB-GVP-UG possessing enhancer sequences was therefore used. GFP-positive embryos showed fluorescence in neuronal cells but not in glial cells. The expression of GFP seems to be constitutive and not E2-dependent. . However, the expression of GFP in neuronal cells may correlate with the presence of ER. To test this hypothesis, it is planned to treat embryos after microinjection with the antiestrogen, ICI182,780. If this is the case, these transgenic zebrafish may be used for *in vivo* screening of chemicals with anti-estrogenic potency.

Plan and Objectives for the Next Reporting Period

- Partner 7 will analyse fin clips of Batch 1 fish to conclusively determine whether or not the transgene is in the germ line. Further screening will be undertaken to

determine presence (or otherwise) of transgene in the germ line. To increase fish numbers in Batch 3, there will be a further microinjection of eggs. There remains the possibility of using luciferase transgenic zebrafish in exposure studies.

- Partner 11 will further characterise of EGFP expression produced by proAroB-GVP-UG injected embryos by means of IHC, promoter mutation and exposure to ER-affecting compounds.

- Analysis of F1 generation of proAroB-GVP-UG-injected embryos and possible production of F2 generation for transgenic line establishment (Partner 11).

- Partner 11 will test whether the expression of GFP in neuronal cells of zebrafish embryos injected with proAroB-GVP-UG is ER-dependent. If this is the case, make transgenic zebrafish with this construct in order to be used for *in vivo* screening of chemicals with anti-estrogenic potency.

- The experiments with BPA samples on self-spotted arrays are completed, statistic analysis are in progress and Partner 13 will complete in the 4th reporting period

- To test the hypothesis that the expression of GFP in these cells may correlate with the presence of ER, Partner 14 are planning to treat embryos after microinjection with the antiestrogen, ICI182,780. This experiment will show whether the expression of GFP in these neurons is ER-dependent. If this is the case, Partner 14 will confirm that by mutating the ERE element within the promoter.

Difficulties/Delays

Partner 7 has experienced technical difficulties and unpredictability in producing transgenic fish. Thus, they have looked into possibilities of obtaining an established transgenic zebrafish from Amsterdam. These fish have a luciferase reporter gene attached to an estrogen receptor binding sequence integrated into their genomes. It is likely that our colleagues in Amsterdam will supply us with these fish, which would then allow us to conduct some physiological experiments with them, while at the same time trying to produce our own line of transgenic, fluorescent fish. These zebrafish will provide a potential tool for rapidly determining the estrogenic and anti-estrogenic potency of chemicals or water bodies using a flow through dosing system.

Partner 11 - Embryos injected with classical *cyp19b* promoter-EGFP construct did not exhibit strong enough, stable and reproducible EGFP expression pattern, probably due to the general weakness of the promoter. Introduction of “enhancer sequences” in the classical promoter-EGFP vector indeed has brought up the signal levels, easing the observation obstacles. However, it also led to appearance of presumably unspecific expression patterns. To say with certainty if the construct containing the studied promoter and “enhancing sequences” produces any specific pattern that can be further used for transient or transgenic reportergene studies, thorough investigation is needed, requiring additional characterisation of expressing cells and conditions altering the EGFP expression.

Deliverables and Milestones

Deliverables	
✓	**D10.1:** Report on progress with production of transgenic zebrafish (month 24)
in progress (36)	**D10.2:** Report describing the production of transgenic reporter fish (month 48)
in progress (36)	**D10.3:** Report describing the use of transgenic reporter fish to detect the endocrine activities of chemicals (month 48)
Milestones	
in progress (36)	**M10.1:** Knowledge about the production of transgenic reporter fish (month 48)
in progress (36)	**M10.2:** Information about the specificity and sensitivity of transgenic reporter fish (month 48)
in progress (36)	**M10.3:** Information about the usefulness of transgenic reporter fish to detect endocrine activities of chemicals and effluents (month 48)

WP11	**Regional and secular trends in Leydig cell function – serum testosterone levels of European men as a new marker of male reproductive health?**					
Phase						
Start date	6					
Completion Date	24 months					
Current Status	active					
Partner(s) responsible:	4	5				
Person-months per Partner:	43 (22)	3 (3)				
Already devoted person months per Partner and total:	28	7				

Objectives for the Reporting Period

The objectives of this workpackage are to: 1) explore the presence/scale of a possible birth cohort effect on male serum testosterone levels in two different European regions, that have significantly different semen quality among young men; 2) if possible, to give an estimation of the onset of this birth cohort effect in the two regions; and 3) to relate data on birth cohort effects on male serum testosterone levels to known regional differences and temporal changes in exposure. Specifically for this reporting period the objectives have been:

- To complete the analysis of the Danish cross sectional data and prepare a manuscript for publication
- To initiate the analysis of the Finnish cross sectional data

Methodology and Study Materials for the Reporting Period

An overview of the distribution and number of samples already analysed for the reproductive hormones testosterone luteinising hormone (LH), follicle stimulation hormone (FSH), sex hormone binding globulin (SHBG) and estradiol is shown in Figure 1. Hormone levels were analysed by well established and commercially available immunoassays in the laboratory of Partner 4 during the first reporting period. The hormone data has been entered into an SPSS database. Information on birth year and age as well as a range of clinical and lifestyle data (e.g. BMI) has been extracted from the databases of the various population studies from which the samples originated and merge with the hormonal data.

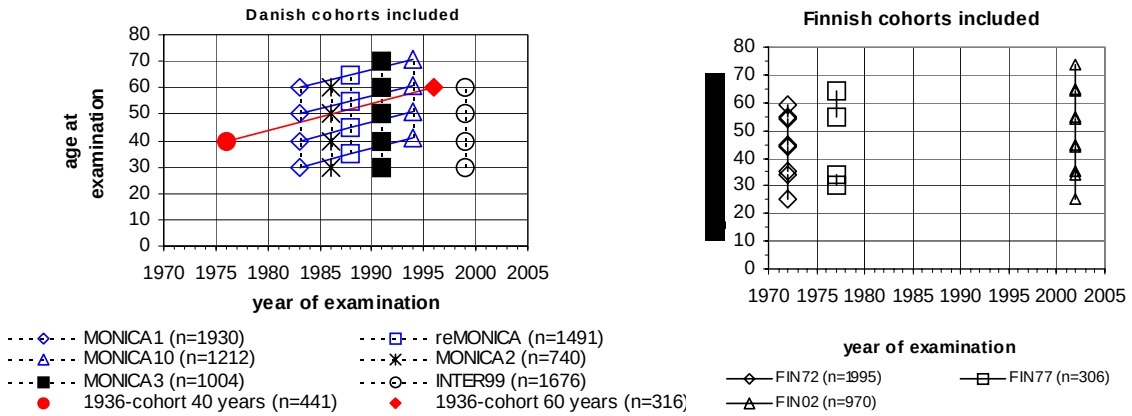

Figure 1 Overview of the distribution and number of samples already analysed for the reproductive hormones testosterone luteinising hormone (LH), follicle stimulation hormone (FSH), sex hormone binding globulin (SHBG) and estradiol

Evaluation of the Danish cross-sectional data was initiated during the 2nd reporting period and continued during this reporting period using the SPSS 13.0 statistical package software. Free testosterone was calculated from the testosterone and SHBG concentrations following a published method (Vermeulen *et al.*, 1999), with the assumption of an average serum albumin concentration of 43 g/L. Hormone levels were natural logarithm transformed in order to obtain approximate homoscedacity and an approximate normal distribution of residuals. Initially, hormone levels of the different age groups were compared using one-way ANOVA with Bonferroni post hoc test. To disentangle the effects of age, calendar time (period), and year of birth (cohort), age-period-cohort modelling was used (Clayton *et al.*, 1987). The following general linear model was used:

$$\ln\left(\text{hormone}_{ijk}\right) = \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$$

Where α_i represents the age effect in the i^{th} age group, β_j the period effect in the j^{th} period, and γ_k the cohort (birth year) effect of the k^{th} cohort. The remainder term ε_{ijk} is the residual representing the noise. The model included age (30, 40, 50, 60, and 70 years), period (1982-83, 1986-87, 1991-92, and 1999-2000), and year of birth (1921-26, 1931-39, 1940-46, 1949-52, 1956-61, and 1969-70) as categorical variables. In all but two analyses, the age-cohort model was accepted as adequate. In the analysis of SHBG the period effect was also statistically significant and the full age-period cohort model was necessary. To account for the problem that the linear component of the period effect cannot be estimated (Holford, 1992) period was entered in the model with the restriction that the first and last category are identical. To estimate an overall age effect, expressed as a percent change in the hormone level for each year, age was included in the above models as a linear effect instead of as a categorical variable. The same was then done to estimate an overall effect of cohort. The significance of the effects of age was the same irrespective of whether age was included in the model as a linear term or as a categorical variable. The same was true for the significance of the effect of year or birth for SHBG and free testosterone. For total testosterone there was a significant decrease when year of birth was included in the model as a covariate. As a categorical explanatory variable,

testosterone was significant when including data from the Inter99 survey, but non-significant when excluding data from this survey. This difference in results is taken as an indication that the statistical power to detect a change is lower when using a categorical variable than with a linear trend. In subsequent analyses, body mass index (BMI) was included in the statistical model as an additional explanatory variable to investigate whether the age- and cohort-related hormonal changes could be explained by a change in BMI. The final models were objected to standard checks of the residuals. Similar statistical methods will be used in the evaluation of the Finnish data.

Clayton D, and Schifflers E, (1987). Models for temporal variation in cancer rates. I: Age-period and age-cohort models. *Stat. Med*. **6** 449-467.

Holford TR, (1992). Analysing the temporal effects of age, period and cohort. *Stat. Meth. Med.Res.* **1** 317-337.

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.

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.

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

Scientific Achievements

Partner 4 contribution

The evaluation of the Danish cross sectional data was initiated during the 2nd reporting period and continued during this 3rd reporting period The validity of the samples has been closely examined in order to role out that storage of the samples (up to 33 years) might have affected the hormone results. This examination has revealed that the 1936-cohort 40 years samples most likely have been affected by storage condition or previous handling and results on this subset of samples will be excluded from subsequent analyses. The validity of the remaining samples has been verified by different approaches. As higher hormone levels were generally measured in samples stored the longest, there was concern about whether any evaporation of samples had occurred during storage/handling, which could lead to concentration of the samples.

During the third reporting period, Partner 4 therefore measured serum Na^+ in 25 randomly selected samples from each of the surveys (total n=100 samples) as an estimate of the concentration of samples. A significant negative correlation between year of sampling and the Na^+ concentration was observed indicating that some evaporation of samples during storage or handling may have occurred (Figure 2a). The mean Na+ concentration in the samples from the MONICA I, MONICA II, MONICA III, and Inter99 surveys

were respectively 172, 169, 162, and 154 mmol/L which all were above the normal range for serum Na+ concentrations.

In order to normalise serum concentration to a normal mean serum Na+ concentration of 140 mmol/L, hormone levels measured in samples from the MONICA I, MONICA II, MONICA III, and Inter99 surveys were multiplied with a correction factor of 0.81, 0.83, 0.86, and 0.91, respectively (and thereby adjusted for any effect of evaporation) (Figure 2b). The variation in Na+ levels were larger than the normal biological variation for Na+ indicating that samples from the same sampling period were not identically affected by storage. Thus, by using a general correction factor for all samples from the same sampling period some samples may be undercorrected and some may be overcorrected. However, due to the large number of samples available and the fact that Partner 4 in this study were interested in general trends rather than individual levels it can be justified to use a general correction factor for each sampling period.

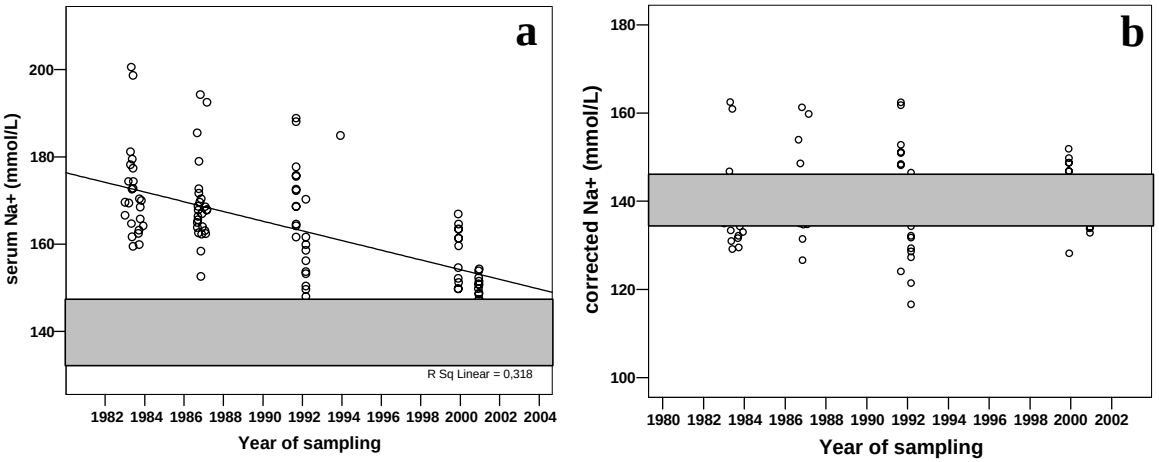

Figure 2 Measured serum Na^+ in 25 randomly selected samples from each of the surveys. a) significant negative correlation between year of sampling and the Na^+ concentration; b) use of correction factor to adjust for any effect of evaporation.

Evaluation of the Danish cross sectional data (adjusted for evaporation effects) is now almost completed and a manuscript for publication in a peer-reviewed scientific journal is currently being prepared and is soon ready for submission. Data evaluation of the Danish results indicate that, in addition to the well-known age effect of falling testosterone levels with increasing age, a cohort effect seem evident with higher testosterone levels in the oldest cohort. However, the most pronounced cohort effect observed was for SHBG with significantly decreasing levels in the more resent cohorts. In contrast, no cohort effect was observed for free testosterone levels. Adjusting for a concurrent increase in body mass index (BMI) with later year of birth reduced the observed changes in testosterone but had only a moderate effect on the observed birth cohort related changes in SHBG levels. The observed birth cohort related 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.

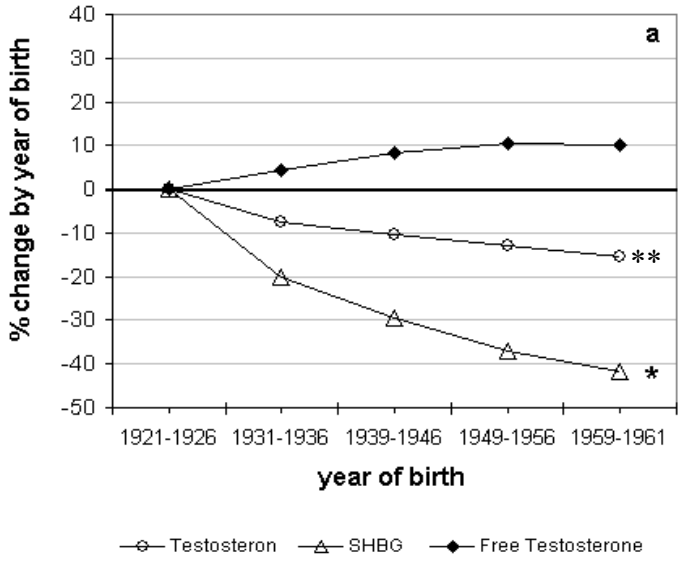

Figure 3 Percent change in hormone levels with later birth cohorts (level in the 1921-26 birth cohort as reference) after adjustment for the effect of age and period of sampling. *p<0.01, **p=0.02

Partner 5 contribution

The analysis of the Finnish results was initiated during this third reporting period. Preliminary analyses indicate that the Finnish data illustrate similar type of age-related decline in testosterone values as in Denmark. Birth cohort effect is also apparent, showing a decline, but these results need further analysis to account for all confounding factors.

The first results of this study have been presented at the Copenhagen workshop on "Environment, reproductive health and fertility" in January, 2005, at the CREDO workshop in Prague, May 2005, the 21th Annual meeting of the ESHRE, Copenhagen, Denmark in June 2005, and at the Athena Workshop on Environmental impact congenital diseases, June.2005, Kos, Greece.

Progress Summary

All samples have been analysed for the reproductive hormones testosterone, LH, FSH, SHBG and estradiol. A database has been constructed, which contains the hormone data as well as information on age, birthyear, bodymass index and a range of other clinical and lifestyle factors that have been retrieved from databases of the original population studies from which the samples originate. The analysis of the Danish cross-sectional data has been completed and a manuscript is under preparation. The analysis of the Finnish cross-sectional data has been initiated.

A direct comparison of hormone levels in the Danish and Finnish cohorts will not be possible due to the lack of a temporal overlap in the cohorts from the two countries.

Plan and Objectives for the Next Reporting Period

- To complete the manuscript on results from Danish cross-sectional samples for submission and initiate evaluation of Danish longitudinal data.

- To complete evaluation of Finnish cross sectional data and initiate preparation of a manuscript. During the project it was recognised that, due to the necessity of a close collaboration with the coordinators of the original populations studies from which the samples originated, Finnish speakers need to be involved in the analysis of data. Once Finnish data evaluation is completed, will then be able to see if trends observed in the two is similar which will go towards D11.2.

Workpackage Related Appendices

Annex I (D11.1) Estimation of extent and course of birth cohort effects resulting in lower serum testosterone levels in young men in individual regions of Europe

Deliverables and Milestones

Deliverables	
✓ (P4) completed	**D11.1:** Estimation of extent and course of birth cohort effects resulting in lower serum testosterone levels in young men in individual regions of Europe (month 24).
delays, expected month 30 (24) * (36)	**D11.2:** Comparison of birth cohort effects on male serum testosterone levels in different regions of Europe (month 24).
delays, expected month 36 (24)	**D11.3:** Relation of regional and temporal differences of birth cohort effects on male serum testosterone levels in two different regions of Europe to regional and temporal differences in known exposures (month 24).
Milestones	
✓	**M11.1**: Retrieval of country A serum samples from biobanks and shipping to analysing laboratory (month 8).
✓	**M11.2**: Retrieval of country B serum samples from biobanks and shipping to analysing laboratory (month 12).
✓	**M11.3**: Serum testosterone levels measured in all samples (completed by month 15)
✓	**M11.4**: Serum testosterone data incorporated into database (completed by month 18)
✓ (P4) on going P5	**M11.5**: Serum testosterone data analysed within each region (completed by month 24)
delays, begin month 40 (24)	**M11.6**: Regional differences in serum testosterone data analysed (completed by month 24)

***** A direct comparison will not be possible because of a lack of temporal overlap between the available Danish and Finnish samples.

WP12	**Expansion of data on European male reproductive health in relation to exposure and lifestyle and creation of an European database on male reproductive health accessible for the European research community**					
Phase						
Start date	0					
Completion Date	48 months					
Current Status	active					
Partner(s) responsible:	4	5	16b			
Person-months per Partner:	69 (47)	41 (18)	94 (72)			
Already devoted person months per Partner and total:	45	16	70			

Objectives for the Reporting Period

The objectives of this workpackages are to expand an existing European database on male reproductive health with data on andrological parameters of 18-20 year old men born and raised in the former East and West Germany and with new longitudinal data on andrological parameters of young Finnish men in order to follow secular trends in male reproductive health.

Methodology and Study Materials for the Reporting Period

In several European countries all young men, except those suffering from chronic severe diseases (<15 %), are required to attend a compulsory medical examination before they may be considered for military service. Such an attending group of men may be considered representative of the general population of young men in their country. This opportunity has previously been exploited in studies of the reproductive health in Copenhagen and Aalborg in Denmark, Turku in Finland, Oslo in Norway, and Tartu in Estonia and now in the EDEN project also in Hamburg and Leipzig in Germany through collaborate with the military health authorities in the respective cities.

Partner 16b enrolled young men from Hamburg and Leipzig into the present study when they attended the compulsory medical examination, irrespective of whether they were declared fit for military service. Additional criteria of eligibility were that the men and their mothers were born in the area where the men were currently living. An appointment was made for attendance at the clinic in respectively Hamburg or Leipzig. All participants were instructed to abstain from ejaculation for at least 48 h before attendance at the clinic. The current recommendation (World Health Organisation, 1992) is that semen samples are collected after a minimum abstinence from ejaculation of 48 h, but not more than 7 days, in order to standardise the influence of this factor. In the present study, no upper time limit was given, as a reduction in the number of participants was anticipated if such a limit were to be introduced. On the day of attendance at the clinic, each man returned a completed questionnaire (see below), underwent a physical examination, and provided both blood and semen samples. Participants received financial compensation for their

participation. Prior to the study, a standardised questionnaire was developed in the English language and translated into German. These translated questionnaires were back-translated to control for translation errors. In order to assure the quality of the information regarding previous conditions, the questionnaire was sent to participants before their attendance at the hospital/laboratory, and they were asked to complete it (if possible) in collaboration with their parents. The questionnaire included information on age and previous or current diseases, including any known history of fertility.

The physical examination of each participant was performed on the day of delivery of his semen sample. An evaluation of the Tanner stages of pubic hair was performed. For the assessment of testicular size, all examiners used the same type of wooden orchidometer (Pharmacia & Upjohn, Denmark). A blood sample was withdrawn from a cubital vein of each participant, centrifuged, and the serum was separated and frozen. Serum was sent frozen to Denmark for a centralised analysis in the laboratory of Partner 4. Serum levels of FSH, LH and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). Testosterone and estradiol levels were determined using time-resolved flouroimmunoassay (Delfia, Wallac, Turku, Finland) and inhibin-B by a specific two-sided enzyme immunometric assay (Serotec, UK). Intra- and inter-assay coefficients of variation (CV) for measurements of both FSH and LH were 3 and 4.5% respectively. CV for testosterone and SGBG were <8 and <5% respectively. The intra- and inter-assay CV for estradiol and inhibin-B were 7.5 and 13%, and 15 and 18% respectively.

The semen samples were obtained by masturbation and ejaculated into a clean collection tube in the privacy of a room adjacent to the laboratory. The semen samples were maintained at 37°C until taken for analysis. The analysis of semen samples was performed according to WHO guidelines (World Health Organisation, 1992), but was further specified following a study of inter-laboratory variation previously published by some of the involved centres (Jørgensen *et al*., 1997). The period of ejaculation abstinence was calculated as the time between current and previous ejaculation as reported by the men. Ejaculate volume was estimated by weighing the collection tube. Phase-contrast microscopy (positive phase-contrast optics) was used for the examination of fresh semen. For the assessment of sperm motility, 10 µL of well-mixed semen was placed on a clean glass slide (which had been kept at 37°C) and covered with a 22x22 mm coverslip. The preparation was placed on the heating stage of a microscope (37°C), and immediately examined at x400 magnification. The sperm were classified as either motile (WHO motility classes A+B+C) or immotile (WHO motility class D), in order to report the percentage of motile sperm. The motility assessment was performed in duplicate and the average value was calculated for both samples. For assessment of the sperm concentration, the samples were diluted in a solution of 0.6 mol/L $NaHCO_3$ and 0.4% (v/v) formaldehyde in distilled water. The sperm concentration was assessed using a haemocytometer. Only sperm with tails were counted. Smears were prepared for morphological evaluation, Papanicolaou stained and finally sent to Finland for assessment of sperm morphology according to strict criteria (Menkveld *et al*., 1990). All morphology assessments were performed in random and blinded. Part of ejaculate where embedded in epon for electron microscopical examination of spermatozoa by Partner 16b. After cutting the epon pad photos were taken and scanned for morphological analysis.

Both German centres participated in an external quality control programme for sperm concentration assessment conducted by Partner 4. Briefly, each month five blinded samples were sent from the Danish laboratory to the laboratories in Hamburg and Leipzig (as well as to other participating centres). Fresh samples from normal semen donors were preserved by addition of 10 µL of a 3 mol/L sodium azide solution per 1 mL of the ejaculate after liquefaction. Each centre received 600 µL of semen sent by mail in 1 mL cryotubes. Thus, all centres performed counting according to their techniques, 4–8 days after the semen preparation. The results were reported to Partner 4 for statistical analysis.

Standardised questionnaires, record forms for physical examination and semen analyses were labelled with ID-numbers. The information linking ID-numbers to personal data was kept separately at each centre in order to preserve confidentiality. Questionnaires, results of physical examination and results of semen analysis were sent to Partner 4 and entered into a centralised database. Additionally, the results of hormone analyses were also entered into the database.

Similar studies on the reproductive health of young men have been performed in Turku, Finland following the same protocols as in the German study. In order to study the secular changes in male reproductive health a 5-year follow-up study of some of the men originally included in these studies have been performed. This 5-year follow-up includes men born between 1979-1981. Also a 2-year follow-up of young Finnish men born in 1983 has been performed.

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.

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.

Scientific Achievements

Partner 4 contribution

All received data from the German and Finnish studies on male reproductive health have been entered into the European Database on Male Reproductive Health (D12.3). Testosterone, LH, FSH, estradiol and SHBG have been measured in all the German samples. Finnish samples have not yet been analysed as hormone analysis will not be performed until all samples have been collected. Partner 4 has in collaboration with Partner 20 initiated the evaluation of the German data.

Partner 5 contribution

During the third year Partner 5 studied 100 men for the 5-year follow-up (men born 1979-1981) and 51 for the 2-year follow-up (men born 1983) of previously recruited cohorts. Serum samples were sent to Partner 4 for hormone analyses. Clinical and

questionnaire data and results from semen analysis were also sent to Partner 4 for entering into the European database. In addition, Partner 5 carried out morphological analysis of the samples from Germany and Denmark.

The aim is to complete the five-year follow-up before reporting the results. However, semen quality of young men from general population remains much worse than that of partners of pregnant women (fertile population). In the interim analysis of the follow-up we saw that semen quality does not improve when the men grow older. In the long run these studies will be the basis for follow-up surveys of male reproductive health.

An abstract submitted to the Endocrine Society is given below: A clear birth cohort effects in serum testosterone and SHBG levels in Finnish men by Perheentupa A, Laatikainen T, Vierula M, Skakkebæk NE, Andersson A-M, Toppari J.

It is well established that serum testosterone (T) values decrease with age. In this study we wanted to compare the T and sex hormone binding globulin (SHBG) levels in men of different ages born in different decades in order to evaluate the possible birth cohort effect on the T levels, which may confound age-dependent changes observed in cross sectional studies.

T and SHBG were analysed in 3271 male serum samples from three Finnish population surveys conducted in 1972, 1977 and 2002 by the National Public Health Institute. Men were divided into six age groups, birth cohorts were divided into seven groups (Table 1). T and SHBG were both measured by time-resolved fluoro-immuno assays (DELFIA®, Wallac Oy, Turku, Finland).

Significant birth cohort related changes in T (Table 1) and SHBG as well as calculated free T serum levels were observed with lower levels in more recently born men of the same age. T decreased and SHBG increased with age within the birth cohort. The etiology behind the observed cohort effect remains to be resolved. The described birth cohort effect partly blunts the age-related decrease of T levels and exaggarates the age-related change in SHBG levels in cross-sectional analysis. Our data supports the hypothesis that male reproductive health has deteriorated during the study period.

Birth year	Age	1970-77	1960-69	1952-59	1942-51	1933-41	1923-32	1913-22	ALL
	27	19,1 A, a (8,7-27,3)			26,4 A, b (15,1-44,0)				25,3 A (13,3-43,5)
	35	17,2 A, a (7,7-37,6)	16,1 A, a (8,6-28,3)		20,5 B, b (11,1-38,6)	22,0 A, b (11,6-38,6)			20,5 B (10,3-37,6)
	45		15,7 A, a (7,5-25,8)	14,6 A, a (8,5-29,8)			22,6 A, b (11,2-39,6)		20,9 B (9,5-38,4)
	55			17,4 A, a (8,7-30,9)	15,3 C, a (7,5-30,4)			22,6 A, b (11,3-40,9)	19,9 B (8,7-39,9)
	65				13,8 C, a (7,7-27,8)	14,4 B, a (8,0-31,3)		21,9 A, b (10,3-40,9)	17,0 C (8,1-36,4)
	72						16,9 A (8,4-28,3)		16,9 C (8,4-28,3)

Table 1 Serum testosterone concentrations in nmol/l (median, 5-95 percentiles, nmol/L) in birth cohorts of Finnish men. Capital letters depict statistically significant difference down each column, while small letters depict differences across each row (P<0.05, ANOVA).

Partner 16b contribution

The recruitment and clinical examination of 500 young men born and raised in Hamburg (former West Germany) and additional 500 young men born and raised in Leipzig (former East Germany) was completed during the third reporting period. Serum samples were sent to Partner 4 for hormone analyses. Clinical and questionnaire data and results from semen analysis were also sent to Partner 4 for entering into the European database. Evaluation of the German data has been initiated and a manuscript is under preparation.

In both Hamburg and Leipzig the young men's sperm concentration was low (median value of 48.5 and 38.5 mill/ml in Hamburg and Leipzig, respectively) and comparable to the previously published values from Denmark and Norway and lower than observations from Finland and Estonia. There did not seem to be any significant difference in sperm concentration between the two German centres, but due to a lower volume of ejaculate in Leipzig, the total sperm count was lower in the East German centre and indeed seem to be the lowest reported in Europe for young men. The reason for the low ejaculate volume in Leipzig remains unclear but in future analysis confounder like abstinence time will be

adjusted for. It has, however, been hypothesised, that not only the sperms but also the accessory sex glands may be harmed by toxicants.

The area of Leipzig has a high degree of environmental pollution; however, there are no specific environmental influences in the area of Hamburg compared to other German conurbations. Nevertheless the sperm concentrations were low in both German centres. It is possible that different factors are responsible for the low sperm counts in the two centres. Further evaluation of questionnaire data may reveal associations between exposures, particularly during fetal development, and adult reproductive health. Thus, there seemed to be a higher incidence of maternal smoking during the pregnancy in Hamburg.

Progress Summary

In the German study on reproductive health of young men all the clinical examinations, sample collections and laboratory analyses have been completed (D12.1) and all the data has been entered into the central European database on male reproductive health (D12.3). From this database data has been extracted to a SPSS dataset for data evaluation, which is currently ongoing. A manuscript is also under preparation.

The follow-up study of young Finnish men was completed (D12.1 and D12.2), but has also been extended supported by national funding. The first report on the relationship between male reproductive health and maternal exposures during pregnancy has been published, but further associations may be unveiled as the questionnaire and clinical data are further examined.

Plan and Objectives for the Next Reporting Period

- Evaluation of the German data will continue and a manuscript on German data will be completed and submitted for publication

- The tasks of the clinical part of the Finnish study were completed (D12.2 and D12.1) and the later action has been an extension. The follow-up study is on-going and the final report will be given when the follow-up is completed. Funding for the extension has come from the Academy of Finland and Turku University Central Hospital.

Workpackage Related Appendices

Annex I (D12.1) Report on male reproductive health of men the general population in different regions of Germany (month 24)

Annex I (D12.3) European male reproductive health database accessible for the European research community

Deliverables and Milestones

Deliverables	
✓ (36) ✓ (24)	**D12.1:** Report on male reproductive health of men the general population in different regions of Germany (month 24); Regional differences in male reproductive health and their association with maternal exposures (month 24)
✓	**D12.2:** Report on secular trend in male reproductive health of young men from the general population in Finland (month 24)
✓	**D12.3:** European male reproductive health database accessible for the European research community (month 36)
Milestones	
✓	**M12.1**: Collection of clinical data, semen samples and blood samples from men from the general population in different regions of Germany (completed by month 6).
✓	**M12.2**: Collection of clinical data, semen samples and blood samples from young men from the general population in a Northern European country (completed by month 6).
✓	**M12.3**: Analysis of reproductive hormones in blood samples from Germany (completed by month 12)
✓	**M12.4**: Analysis of reproductive hormones in blood samples from Finland (completed by month 12)
✓	**M12.5**: Data on German male reproductive health entered into database (completed by month 18)
✓	**M12.6**: Data on male reproductive health of young men from Finland entered into database (completed by month 18)
✓	**M12.7**: Creation of European databank on male reproductive health in order to make data available for the European research community (completed by month 36).

WP13 Low-dose effects of EDC in cell-based assays, fish and rodents								
Phase								
Start date	0							
Completion Date	24 months							
Current Status	completed*							
Partner(s) responsible:	1	2	8	11	13	15	19	20
Person-months per Partner:	18.2 (14)	14 (8.2)	15 (10)	4 (4)	44 (30)	8 (5)	8 (4)	80 (40)
Already devoted person months per Partner and total:	18.2	14	15	4	44	8	8	80

* Though the objectives and deliverables for this workpackage have been completed, several Partners are continuing to undertake experiments in order to further knowledge in this area.

Objectives for the reporting period

- Partner 15 to assess the effects of low-dose exposure of zebrafish to xenoestrogens on brain aromatase expression.

- Further analysis by Partner 20 of the results of the 2nd DEHP study and a combined analysis of the two DEHP studies using a NOEL and a benchmark approach. The goal is to establish the shapes of the dose-response curves in order to a) provide guidance for assessing the threshold issue and to b) develop criteria for well-conducted EDC studies which will permit improved estimation of low dose effects.

Methodology and study materials for the reporting period

Zebrafish studies: Under laboratory conditions, adult male zebrafish were exposed for 3 weeks to BPA (2mg/L), ethinylestradiol (30ng/L) or estradiol (5, 50, 100, 200 ng/L). From these experiments, Partner 15 obtained brain samples, extracted RNA, and real-time RT-PCR was performed in order to assess the regulation of *cyp19a2*.

Rat studies: During the first half of the 2nd reporting period, a dose-response study of DEHP with a benchmark approach was performed. The study included a control group of 16 time-mated animals and 6 dose groups with 8 time-mated animals per group. The dose levels of 10, 30, 100, 300, 600 and 900 mg/kg were selected based on earlier studies on DEHP and covered the whole dose-response curve for DEHP effects on anogenital distance and nipple retention in male offspring. During the later half of the 2nd reporting period, a DEHP study aimed at the NOEL approach was initiated. This study included a control group of 16 time-mated animals and 3 dose groups with 8 time-mated animals per group. The dose levels were similar to the lowest dose levels in the first DEHP study, i.e. 10, 30 and 100 mg/kg. The study also included an additional group of 16 time-mated animals exposed to 3 mg/kg DEHP, because the results of the first study showed statistically significant effects on anogenital distance and nipple retention at 10 mg/kg. Consequently, a lower dose level was needed in order to find a clear NOEL for DEHP. During this reporting period, further analysis of the results of the 2nd DEHP study and a

combined analysis of the two DEHP studies using a NOEL approach have been performed.

Scientific Achievements

Partner 1 contribution

The low dose experiments undertaken in the E Screen were completed during the second reporting period. During the present reporting period, Partner 1 had continuing commitments to this WP in terms of finalising statistical analyses.

Partner 2, 8 and 19 contributions

At the beginning of the 3rd reporting period, the stickleback low dose experiments were ongoing for nonylphenol using VTG induction as the endpoint and pending for flutamide using spiggin induction as the endpoint. These have now been completed as described in the minutes taken from the local meeting on sticklebacks (Annex II) and the information is being used to derive predictions of anticipated mixture effects in the mixture experiments described in WP14 and WP15. For this reason, the experimental data of the low dose experiments are reported as part of WP14, Figures 5-10.

Partner 13 contribution

The zebrafish low dose experiments were completed during the second reporting period. However, Partner 13 has continued to obtain data to further knowledge relating to this workpackage.

1. *Zebrafish exposure*

During the reporting period the following exposures were carried out:

- 17ß-estradiol (E2) (adult male zebrafish, concentrations: 0/5/50/100/200 ng/L)
- Ethinylestradiol (EE2) 30ng/L (adult male zebrafish, as a positive control)
- Bisphenol A (BPA) 0.1 µg/L, 2 µg/L, 20 µg/L, 200 µg/L, 400 µg/l, 1 mg/L and 2 mg/L (adult male zebrafish)

Water samples were taken from E2 and EE2 tanks and measured by CEFAS. Twelve adult male zebrafish were present in each tank with an exposure duration of 11 days. At the end of the exposure period, liver tissue was dissected from each fish and its total RNA was extracted. The mRNA was then reversely transcribed into cDNA using SIGMA TRI Reagent.

2. *RT-PCR experiments*

All RNA samples of zebrafish exposed to BPA (0.1 µg/L, 2 µg/L, 20µg/L, 200 µg/L, 400 µg/L, 1 mg/L, 2 mg/L and 30 ng/L EE2 as positive controls) were reversely transcribed into cDNA. To control the quality of the transcription, a PCR was performed for each cDNA sample. The primers used for this reaction are complementary to parts of the vitellogenin 1 gene (*vtg1*) and the ß-Actin gene.

vtg1 forward: 5' – GCC AAA AAG CTG GGT AAA CA – 3'
vtg1 reverse: 5' – AGT TCC GTC TGG ATT GAT GG – 3'
ß-actin forward: 5' – AAG CAG GAG TAC GAT GAG TCT G – 3'
ß-actin reverse: 5' – GGT AAA GGC TTC TGG AAT GAC – 3'

Figure 1 shows the electrophoresed amplificates of the samples exposed to EE2 (positive control) and non-exposed fish. The positive controls show a strong expression of *vtg1*, the negative controls show no expression at all. Figure 2 shows the electrophoresed amplificates of a PCR with ß-actin primers. This PCR was used to check the quality of the cDNA. Figure 3 gives the electrophoresed amplificates of a PCR with *vtg1* primers. Figure 4 shows the electrophoresed amplificates of the samples exposed to 1 mg/L BPA.

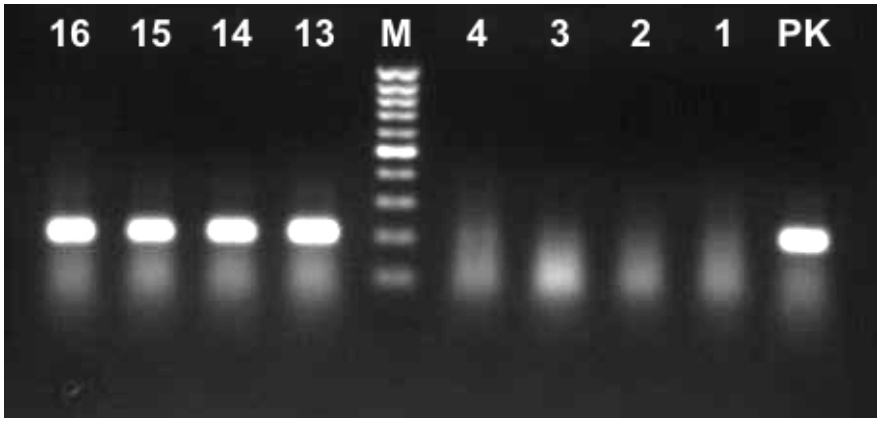

Figure 1 Electrophoresed PCR amplificates. The *vtg1* amplificates are 210 bp at size. 16, 15, 14, 13: EE2, 30 ng/L, positive controls; M: ladder; 4, 3, 2, 1: non-exposed negative controls; PK: internal positive control (non-exposed female).

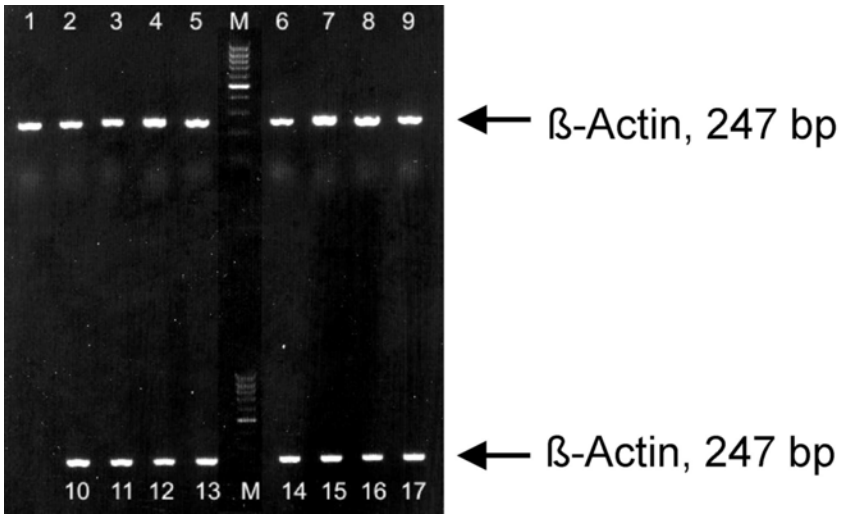

Figure 2 Electrophoresed PCR amplificates. 1: Internal positive control; 2-5: Four different non-exposed males (negative controls); 6-7: Two different males exposed to 30 ng/L EE2 (positive controls); 8-9: Two different males exposed to 1 mg/L BPA; 10-11: Two different males exposed to 400 µg/L BPA; 12-13: Two different males exposed to 20 µg/L BPA; 14-15: Two different males exposed to 2 µg/L BPA; 16-17: Two different males exposed to 0.1 µg/L BPA; M: Marker;

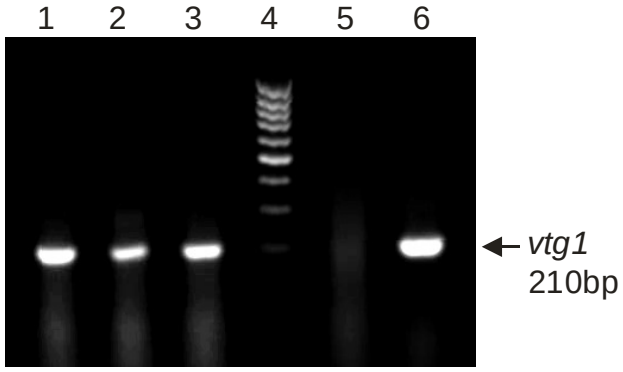

Figure 3 Electrophoresed PCR amplificates. 1-3: Three different males exposed to 2 mg/L BPA; 4: Marker; 5: Non-exposed male (negative control); 6: Internal positive control (non-exposed female); *vtg1*: vitellogenin1 PCR amplificates, 210 bp at size.

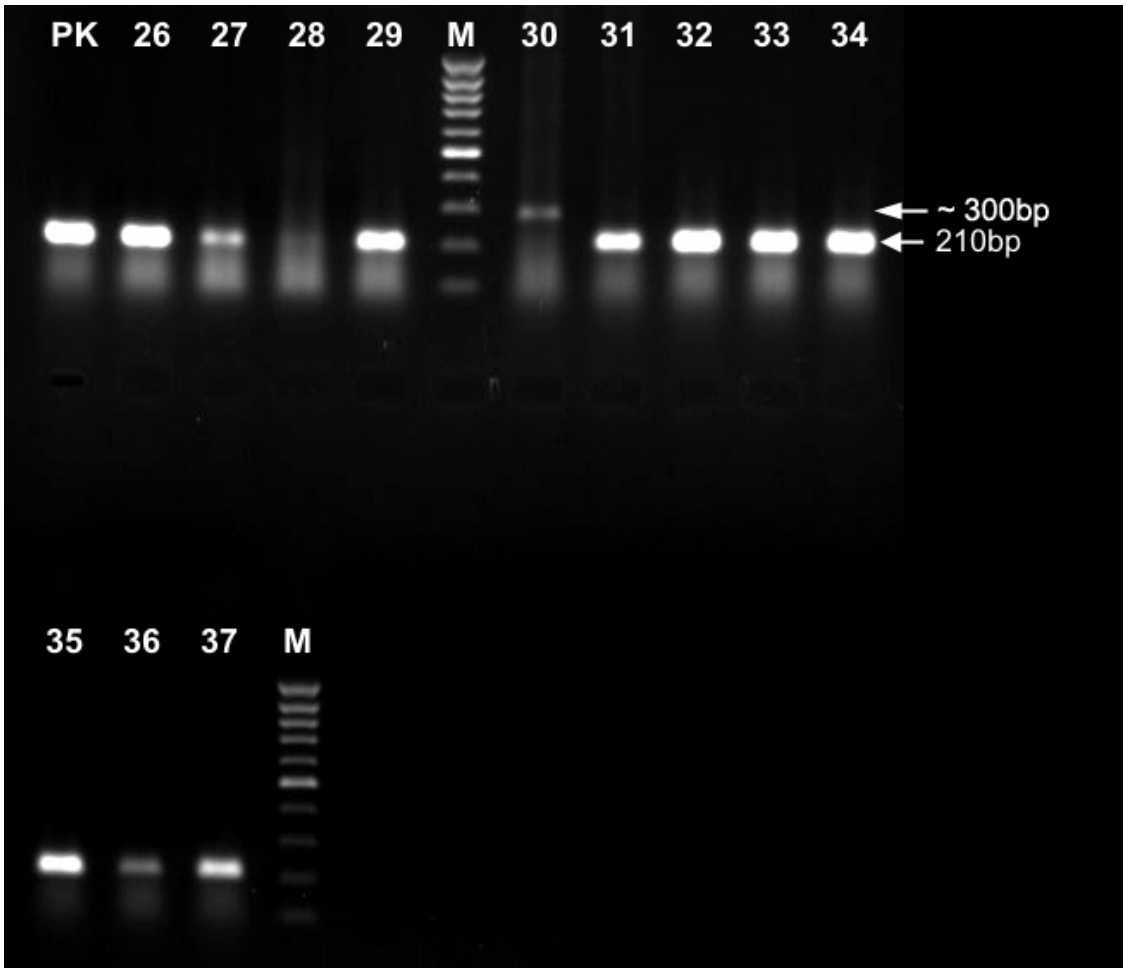

Figure 4 Electrophoresed PCR amplificates. The *vtg1* amplificates are 210bp at size. PK: internal positive control (non-exposed female); 26-37: male zebrafish exposed to 1 mg/L BPA. M: ladder.

There is a weak band at around 300 bp in sample 30 which is likely to be an artefact, since the comparison of the used primers to the whole zebrafish genome (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=euk), using the blast

function resulted in only one match: the *vtg1* gene. Almost all male fish show an expression of *vtg1* after exposure to 1 mg/L BPA. The samples 28 and 30 show no response to the chemical, likely a consequence of the variability of the response among the individuals.

3. *Light Cycler experiments*s

cDNA was used for qPCR experiments performed with a LightCycler. The expression of the gene coding for *vtg1* was examined using primers complementary to this gene.

vtg1 forward: 5' – GCC AAA AAG CTG GGT AAA CA – 3'
vtg1 reverse: 5' – AGT TCC GTC TGG ATT GAT GG – 3'
ß-actin forward: 5' – AAG CAG GAG TAC GAT GAG TCT G – 3'
ß-actin reverse: 5' – GGT AAA GGC TTC TGG AAT GAC – 3'

For the comparison of different individuals, all crossing points of the LightCycler experiments were normalised to the expression of the gene coding for ß-actin ("housekeeping-gene"). The efficiency of the PCR reaction was calculated for the *vtg1* and the ß-actin amplification while amplifying several different dilutions of a sample (non-diluted, 1:10, 1:100, 1:1,000, 1:10,000). The efficiency of 1.81 is used for comparing the expression rates of *vtg1* in the different samples. To normalise the different samples, it is necessary to determine the efficiency of the PCR for the ß-actin gene, too. Figure 5 details the results of the qPCR experiments with the BPA samples with each dot representing one sample (one zebrafish).

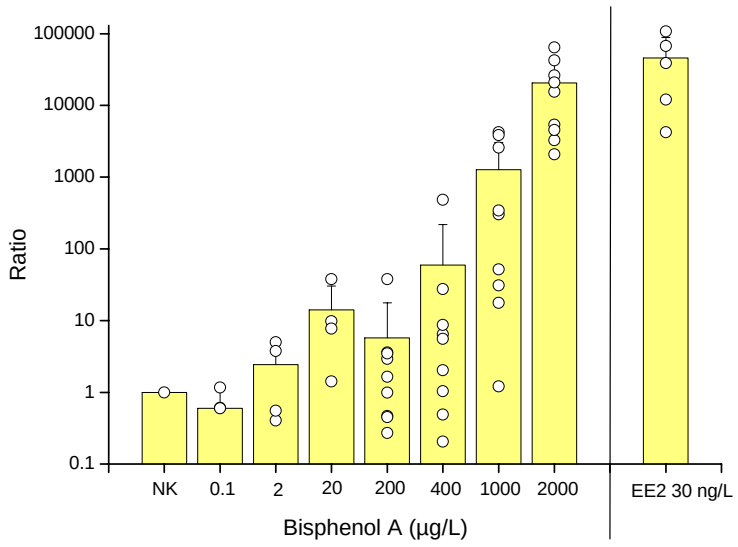

Figure 5 Expression of the *vtg1* gene as a response to the exposure to BPA. NK: negative control (non exposed male), this value is set to 1. Each dot represents one sample.

There is an increase in the expression of *vtg1* with the increase of the BPA concentration in the tanks. Due to the variability of the individual responses, only the 2 mg/L samples show a significant increase in the *vtg1* expression. For microarray experiments, samples exposed to 1 mg/L were used for hybridisation on the whole genome arrays to avoid the measurement of possible toxic effects. Samples of all other concentrations will be used

for hybridisation to the self-spotted arrays. Figure 6 shows a comparison of the expression level of *vtg1*. The fish were exposed to E2, NP, BPA and EE2.

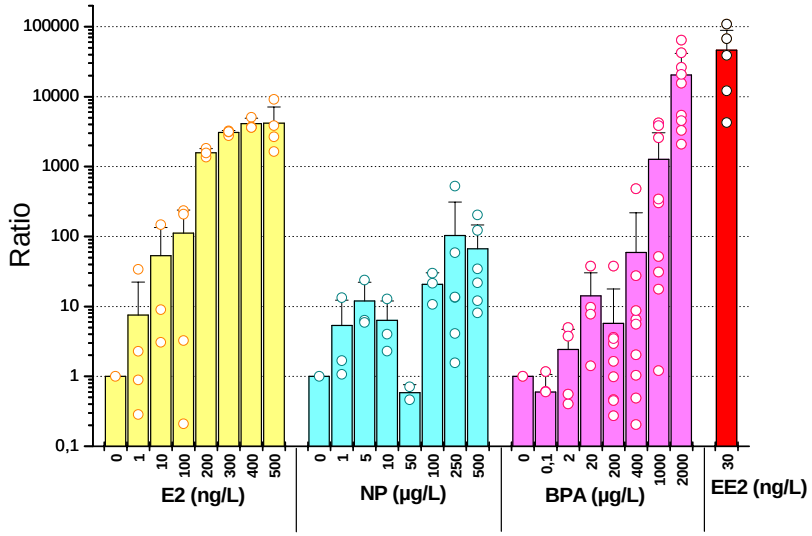

Figure 6 Comparison of the effects of several EDCs on the expression of *vtg1*. Each dot represents one sample.

Partner 15 contribution

Complete results are presented in WP5 however in short the exposure of adult male zebrafish to BPA, EE2 or E2 did not alter the expression of *cyp19a2* levels in the brain, except for the 200ng/L E2 concentration (P>0.001) as illustrated in Figure 7. The expression levels of the gonad form of aromatase, *zfcyp19a*, were not affected by the estrogen treatment. This result was expected since *zfcyp19b* does not posses an ERE.

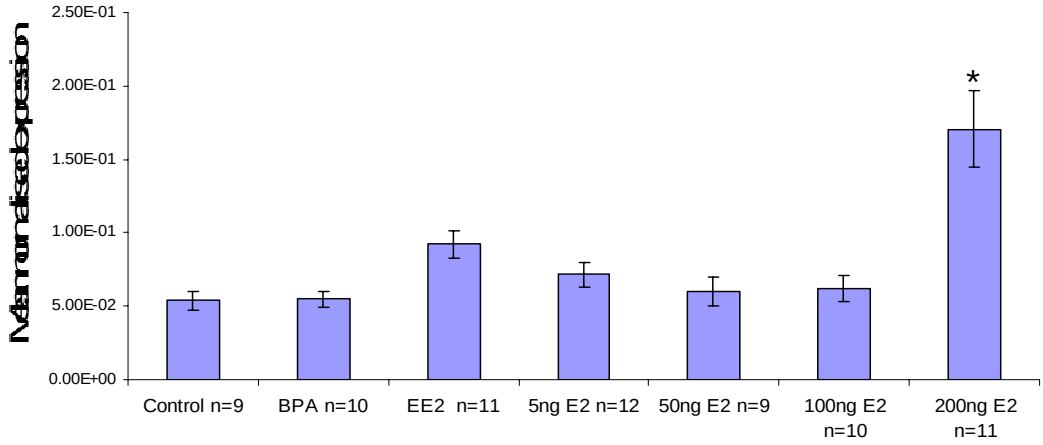

Figure 7 Mean normalised expression levels of *cyp19a2* in the brains of adult male zebrafish after exposure to xenoestrogens. Gene expression levels were normalised against 18S.

Partner 20 contribution

The results of the 1st DEHP study showed dose-related anti-androgenic effects on anogenital distance and nipple retention from the lowest dose assessed (10 mg/kg). In

addition, dose-related decreases of reproductive organ weight were identified on postnatal day 16 in the male offspring, with the weight of muscular *levator ani* and prostate significantly decreased from 10 and 30 mg/kg respectively. The dose-response curve for nipple retention was unusual using the 3 lowest doses of DEHP (10, 30, 100 mg/kg), as the 10 mg/kg actually appeared to induce the most marked effects (Figure 8).

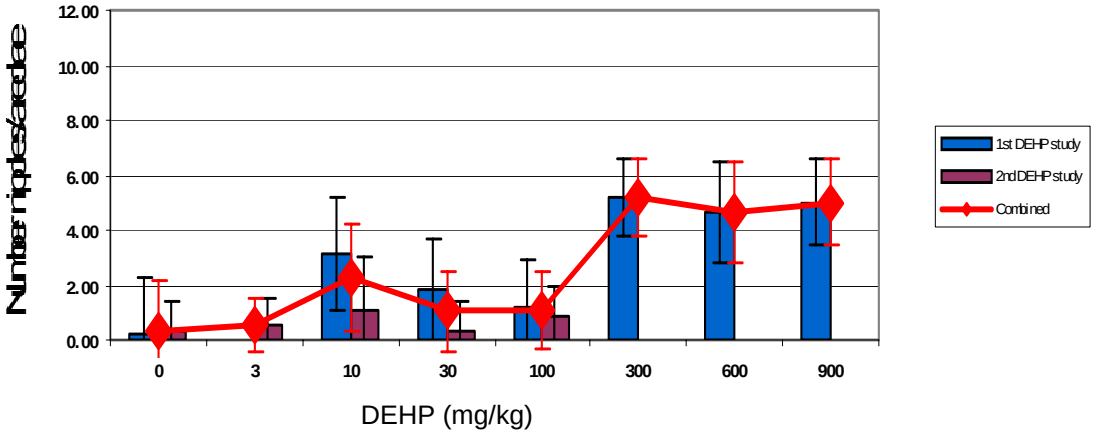

Figure 8 Nipples/areolae in male offspring in the 1st and 2nd DEHP study plus combined, mean + standard deviation.

The results of the 2nd DEHP study indicate that the lowest dose level in this study (3 mg/kg) is a NOEL for effects on anogenital distance and nipple retention in male offspring. The effects at 10, 30 and 100 mg/kg were not as marked as in the first study and statistically significant differences were mainly found at 100 mg/kg. The dose-response curve for nipple retention was unusual in this study, as the 10 mg/kg seemed to induce more marked effects than 30 mg/kg.

Statistical analysis performed during this reporting period including the results from both DEHP studies (with due consideration of study number as a factor in the statistical analysis) shows significant effects at 10, 30 and 100 mg/kg. These results indicate that the use of a NOEL approach for studies with relatively small groups (8 per group) may lead to false-negative results. As such, this supports the recommendations in the OECD Test Guidelines for Reproductive Toxicity Testing (e.g. TG 416 two-generation study), where the group size is at least 20 litters per group.

The data from the two studies separately or combined have at present not been analysed using a benchmark approach, because this requires a monotonic dose-response curve. This has not been found for nipple retention so far. During the next reporting period, further data on the effects of DEHP in dose-ranges from 3 –30 mg/kg will be recorded in the mixture study in WP15. Depending on the results of this, the feasibility and relevance of performing benchmark analysis will be reconsidered.

Progress Summary

The RT-PCR experiments and the LightCycler experiments undertaken by Partner 13 show that male zebrafish begin to express *vtg1* at a BPA concentration of 1mg/L. There is a significant increase in the *vtg1* expression at 200 ng/L E2 and 2 mg/L BPA. Due to the individual responses to NP there was no significant increase observed. The exposure to 30

ng/L EE2 resulted in the strongest *vtg1* expression in male fish. This fact is particularly important for water pollution from active ingredients of contraceptives.

The results from Partner 15 demonstrate for the first time for adult zebrafish that the brain aromatase is inducible by environmental estrogens. To understand the functional significance of this effect, more must be learnt about the physiological role of brain aromatase in sexual reproduction which will be undertaken in WP5.

From the data obtained by Partner 20, the use of a NOEL approach for studies with relatively small groups (i.e 8 per group) may lead to false-negative results. As such, this supports the recommendations in the OECD Test Guidelines for Reproductive Toxicity Testing (e.g. TG 416 two-generation study), where the group size is at least 20 litters per group.

Plan and Objectives for the Next Reporting Period

- For Partner 15, the responsiveness of brain aromatase will be characterised for a xenoestrogen (DEHP) and for a non-estrogenic EDC, fadrozole (aromatase inhibitor). The molecular analysis of the tissue sample is underway.

- Partner 20 will undergo analysis of organ samples from the 2nd DEHP study and undertake further analysis including the DEHP data obtained in WP15.

Workpackage Related Appendices

Annex I (D13.4) Criteria document for low-dose testing

Deliverables and Milestones

Deliverables	
✓	**D13.1:** Report on preliminary low-dose testing in cell-based EDC assays (month 12)
✓	**D13.2:** Report on preliminary low-dose testing in fish (stickleback and zebra fish) (month 12)
✓	**D13.3:** Report on preliminary low-dose testing in female rats (month 12)
✓	**D13.4:** Criteria document for low-dose testing (month 24)
Milestones	
delayed until month 28 (24) ✓ (36)	**M13.1**: Information about the number of data needed to establish low-dose effects with confidence, knowledge about the comparative performance of NOEL versus regression-based approaches for estimating low effects (month 24).

WP14 The assessment of combination effects of similarly acting EDC								
Phase								
Start date	0							
Completion Date	48 months							
Current Status	Active							
Partner(s) responsible:	1	2	8	11	15	19	20	21
Person-months per Partner:	47.5 (36)	13.4 (8.2)	16 (12)	4 (4)	6 (5)	9 (5)	144 (72)	111 (77.2)
Already devoted person months per Partner and total:	31	5.5	8	1	1	6	36	36

Objectives for the reporting period

- In conjunction with WP5, Partner 1 is to evaluate the regulation of EDC-responsive genes by single compounds and mixtures of EDCs, assess the mixture effects and predictability of a reference mixture containing E2, E1 and E3, combined at two mixture ratios and understand the deviation from additivity observed with the 10 component mixture reported in the previous year.

- Partner 20 is to perform a mixture study on similarly acting antiandrogens based on the dose-response studies performed during 1st and 2nd period. The objective is to evaluate whether the antiandrogens produce joint effects that are predictable according to the concept of dose addition (concentration addition).

- Complete the pre-mixture test schedule for sticklebacks by providing first range-finding and then definitive dose response data for all the chemicals identified as appropriate for formulating the mixture exposures (Partners 2, 8 and 19) working in close collaboration.

- Partner 19 objectives for the year were related to dose-response studies for single agents, which will form the basis for the mixture studies. These single agent data will allow it to establish how many chemicals need to be combined to conclusively confirm or refute the hypothesis that there are joint effects below NOEL. Using single agent data, mixture effects will be predicted for a specific multi-component mixture with known mixture ratio. The predictions will be tested experimentally.

- Partner 21 is to perform range finding and definitive studies of candidate compounds to be included in mixture experiments, to validate concentration effects obtained from the range findings and definitive studies and finally to conduct the first mixture experiment.

Methodology and Study Materials for the Reporting Period

The procedures evaluated in WP13 for the evaluation of low effects will be used to estimate low effect doses of EDC in the following assays:

MCF-7 cell proliferation assay, EROD assay, real-time PCR (Partner 1), vitellogenesis in the zebrafish (Partner 21), spiggin and vitellogenin induction in the stickleback (Partners 2, 8, 19) and reproductive effects in female pregnant rats (Partner 20). Work will focus exclusively on EDC that induce phenomenologically similar effects. The dose-response studies for single agents (chosen from the BHK list) will form the basis for the mixture studies. These single agent data will allow it to establish how many chemicals need to be combined to conclusively confirm or refute the hypothesis that there are joint effects below NOEL.

On the basis of single agent data, mixture effects will be predicted for a specific multi-component mixture with known mixture ratios, using the concepts of concentration addition and independent action. The predictions will be tested experimentally. Data from WP1-4 will be fed into WP14 to inform selection of test agents for work with MCF-7 cells. The outcome of WP5-8 (novel endpoints) will be evaluated critically for their suitability for mixture studies. Partner 1 will be responsible for the experimental design and statistical evaluation of all studies.

Scientific Achievements

Partner 1 contribution

1. *Effects of xenoestrogens and mixtures of xenoestrogens on the expression of target genes, as evaluated by real-time PCR.*

As presented in the 2nd Annual Report, a number of primer pairs were evaluated and amplification reactions optimised. These were the reference genes: *β-actin, GAPD and HPRT* and the genes of interest: *TFF1; ER; PRAD1; BRCA1*. These primers were optimised for use in quantitative real-time PCR, which allows an accurate and very sensitive evaluation of gene regulation.

The regulation of target genes by selected EDCs was determined as follows. MCF-7 cells were seeded in 75 cm^2 culture flasks at a density of 500,000 cells per flask and allowed to attach for 24 h. Media was then replaced with phenol red free media containing 10% CDFBS and the test compound at appropriate concentrations. Control cells were treated with vehicle (ethanol 0.5%). Following 24 h treatment, total RNA was extracted, purified and reverse transcribed into cDNA. Cell treatments were as follows: E2 (1×10^{-8} M); *o,p'*-DDT (1×10^{-5} M); β-HCH (1×10^{-5} M) and PCB 126 (1×10^{-8} M).

Amplification profiles were obtained by real-time PCR and the resulting data was analysed using the Pfaffl method. This allows determining the change in expression of genes (up or down-regulation) caused by treatments in relation to untreated controls. The comparison between gene expression in treatment and controls takes into account the amplification efficiency for each gene and is normalised against a reference gene (whose expression remains constant independently of treatment). Following preliminary experiments with three reference genes, β-actin was selected for normalisation. Figure 1 show the effects of the compounds of interest over the expression of well known estrogen regulated genes: *TFF1*; *ER*; *BRCA1* and *PRAD1*.

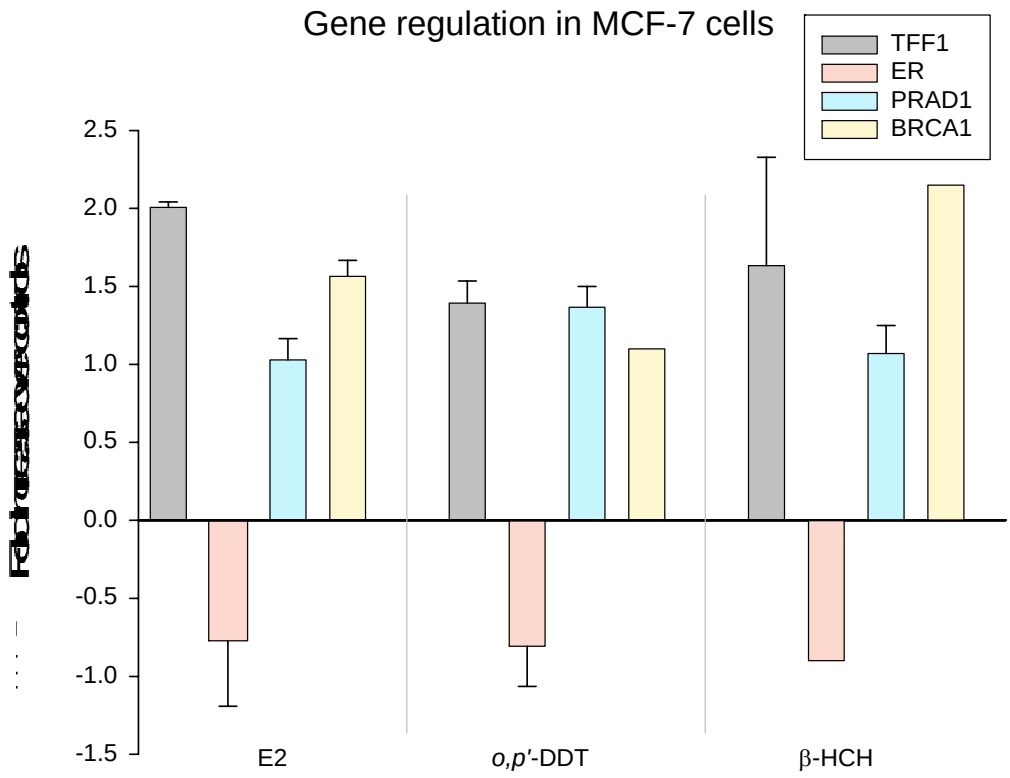

Figure 1 Expression of target genes in MCF-7 cells measured by real-time PCR. Cells were treated for 24 hours with E2 (1 x 10^{-8} M), *o,p'*-DDT (1 x 10^{-5} M) and β-HCH (1 x 10^{-5} M). Following treatment, RNA was isolated and reverse-transcribed into cDNA. Amplification analysis was performed using an icyler, real-time PCR system and Sybr Green detection. *β-actin* was used as the reference gene for normalisation. Results are presented as the fold increase of normalised gene expression in relation to untreated controls and represent the mean ± SD of three independent experiments.

All three compounds produced a similar pattern of gene regulation. It is not surprising that the results with *o,p'*-DDT are comparable to those with E2, as the pesticide activates the ER in a similar way to the endogenous hormone. However, contrary to E2 and *o,p'*-DDT, β-HCH does not directly bind and activate the ER. It is plausible that the strong non-genomic effects (such as the reported activation of the Src/Erk cascade) seen with β-HCH lead to the indirect activation of the ER and consequent activation of its transcriptional targets, such as *TFF1* and *PRAD1*. Similarly, this indirect activation of the ER would lead to a feed-back reduction in the levels of the receptor's mRNA, similarly to what happens in the presence of classical ER activators.

2. *Effects of a reference mixture composed of three steroidal hormones, estradiol, estriol and estrone.*

In the previous report, Partner 1 reported the results from the first mixture experiment containing 10 estrogenic compounds, selected due to their similar mitogenic effects in the E-Screen assay. A slight deviation from addititivity expectations was observed according to *concentration addition*. This observation necessitated to define a reference case for additivity, according to strict similarity criteria. To this end, a reference mixture of similarly acting compounds (all compounds were steroids with similar chemical structure) was prepared. The mixture components were: estradiol, estrone and estriol, combined at equipotent concentrations proportional to their EC50s. As can be observed in Figure 2, the *concentration addition* model accurately predicted the effects of the reference mixture composed of similarly acting compounds. It is clear, from this data, that the E-Screen can be applied for determination of mixture effects and that the deviations reported previously are due to genuine interactions between the mixture components. The nature of such interactions is being currently studied by Partner 1.

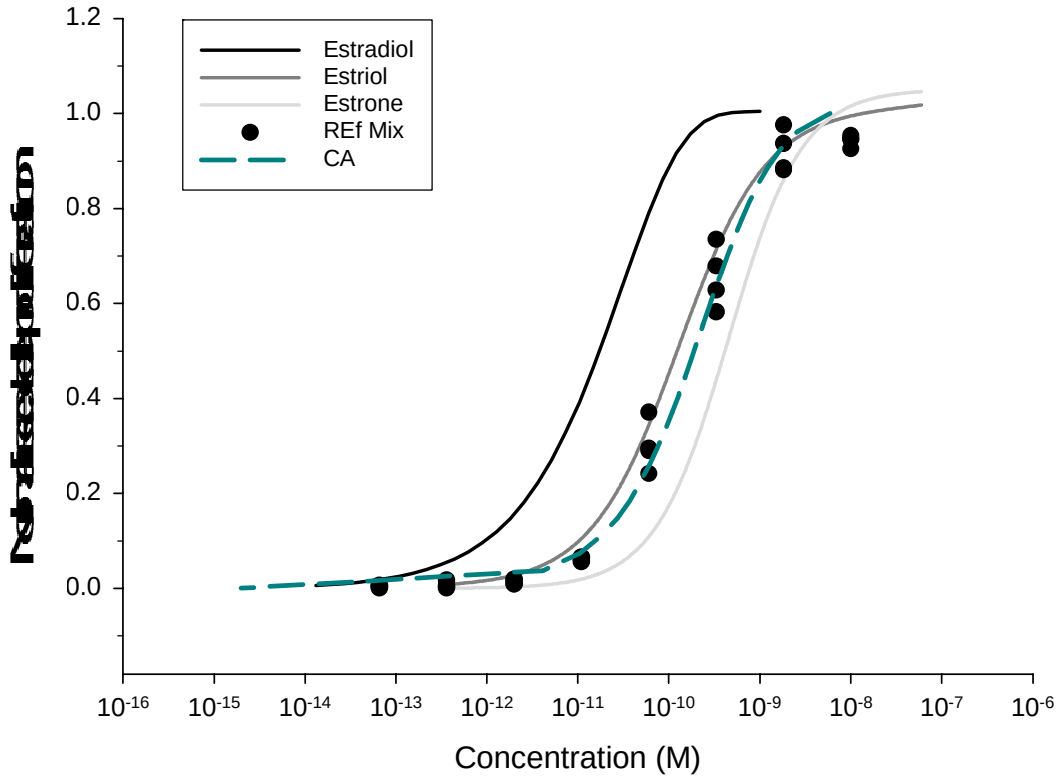

Figure 2 Predicted and observed mixture effects of a reference mixture composed of three steroidal hormones. Black, dark grey and light grey lines represent best-fit regression models for estradiol, estriol and estrone respectively. Observed mixture effects (black circles) are from two independent experiments run in duplicate. Predicted effects were calculated using the model of concentration addition (blue dashed line).

3. *Effects of EDCs and mixtures of EDCs on the steroid metablising enzymes (CYP1A1, CYP1B1 and CYP3A4)*

The slight deviations from predicted concentration additivity reported with the already tested 10-component mixture (see 2nd Annual Report) triggered work aimed at understanding the mechanisms behind this. It was hypothesised that some of the chemicals in the mixture interfere with steroid metabolising enzymes (specifically *CYP1A1*, *CYP1B1* and *CYP3A4*), thus increasing elimination of the steroidal estrogens present in the mixture. It is plausible that this effect may lead to a deviation from the additivity expectations, with a slight shortfall from anticipated effects. In order to clarify this matter, Partner 1 has determined the effects of a series of compounds and mixtures on the activity of *CYP1A1* using the EROD assay (Figure 3). Due to the known activation of *CYP1A1* by the co-planar PCB 126, this chemical was chosen as the positive control for the EROD assay.

For the EROD assay, MCF-7 cells were seeded in 96 black flat bottomed plates at a density of 20,000 cells/well. Cells were allowed to attach for 24 h and changed into assay media (supplemented with 5% charcoal/dextran treated FCS) containing the test compounds at the desired concentrations. Following 24 h incubation, media was washed and replaced with fresh assay media containing ethoxyresorufin (EROD) and dicumarol. Fluorescence was measured using 535 nm excitation and 590 nm emission filters for 30 min at 5 min intervals at 37 °C. Finally, the cell number was determined by fixing the cells with trichloroacetic acid and staining with sulfurhodamine B. Absorbance was read at 510 nm.

It was observed that after 24 h incubation, the positive control (PCB 126) induced a dramatic increase of the EROD activity at the concentration of 2×10^{-8} M. This effect remained constant up to 1×10^{-5} M. On the other hand, none of the single agents tested (E2, EE2, *o,p'*-DDT, *p,p'*-DDE, Methoxychlor, Kepone, Endosulfan I, Endosulfan II, propylparaben and butylparaben) was able to induce significant EROD activity in relation to untreated controls after 24h incubation. A similar effect was seen with 72 h treatments. A lack of effect was also observed when cells were incubated for 24 and 72h with a wide range of concentrations of the 10 component mixture previously described (Annual report from 2004, WP14). These observations indicate that the compounds present in the 10 component mixture are unable to activate *CYP1A1*. It is, therefore, unlikely that the increase in steroidal estrogens metabolism by *CYP1A1* is the reason for the deviation from additivity reported previously.

Partner 1 has also designed and optimised 2 sets of primers in order to evaluate the effects of mixtures and single agents on the expression of *CYP1B1* and *CYP3A4* by real-time PCR. PCB 126 was used as a positive control for *CYP1B1* activation, whereas rifampicin (1×10^{-8} M) was used as the positive control for *CYP3A4* activation. As expected, PCB 126 and rifampicin induced an up-regulation of *CYP1B1* and *CYP3A4* expression, respectively after 24 h incubation.

The effects of the test chemicals were more subtle than the positive controls. β-HCH clearly up-regulated *CYP 1B1* and down-regulated *CYP3A4*. Conversely, E2 and *o,p'*-DDT induced a down-regulation of *CYP 1B1* and an up-regulation of *CYP3A4*. However the effects of these two chemicals were quite small, when compared to the positive controls. Work on the differential regulation of these genes by components of the 10-component mixture is ongoing.

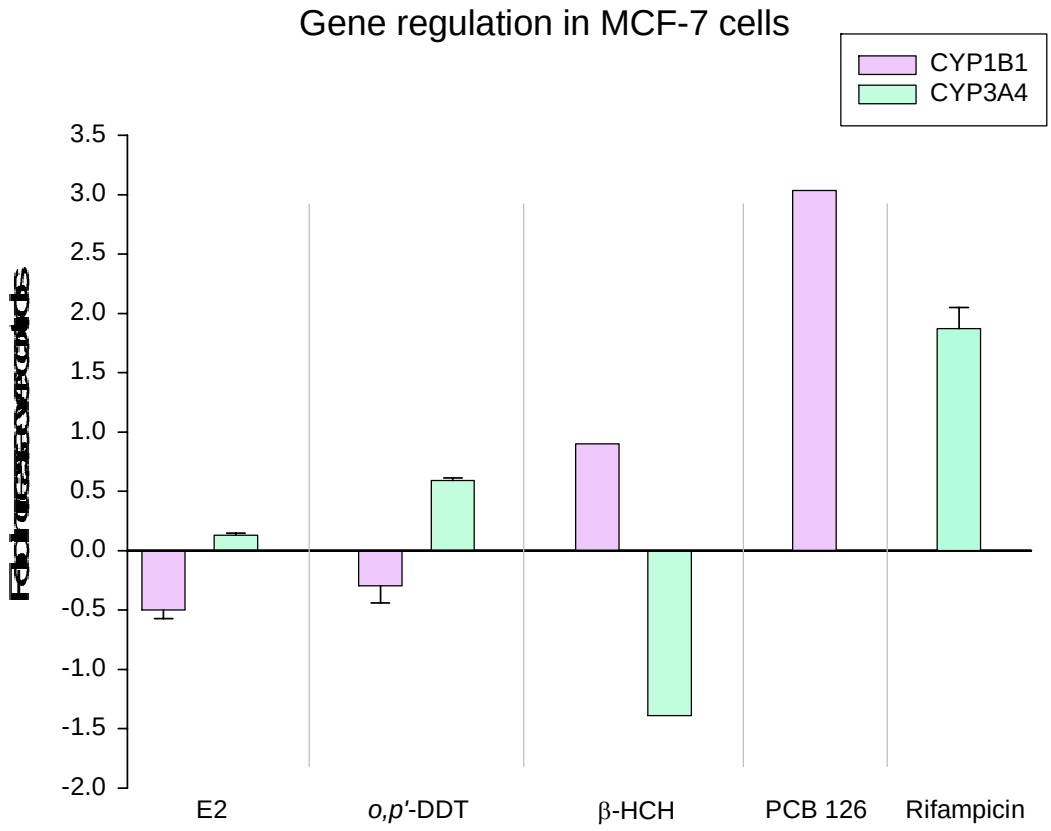

Figure 3 Regulation of *CYP1B1* and *CYP3A4* genes by EDCs in MCF-7. Cells were treated for 24h with E2 (1 x 10^{-8} M), *o,p'*-DDT (1 x 10^{-5} M), β-HCH (1 x 10^{-5} M), PCB 126 (2 x 10-8 M) and Rifampicin (100 µM). Determination of relative gene expression was was performed by real-time PCR, using Sybr detection. Data was analysed using the Pfaffl method and normalised to *β-actin*. Data results from two independent experiments and is presented as mean ± SD.

Partner 2 contribution

The stickleback groups (Partner 2, 8 and 19) have agreed to change the original mixture design by testing single substances as long as 6 estrogens have been selected for the final 6-compound mixture studies. Instead, due to the clear outcomes from the ACE project (mixture studies on VTG induction for two fish species) and in order to reallocate remaining resources more optimal, it was decided to cease the testing of more estrogens. This means that information of 4 estrogenic compounds for the final mixture study is available and can be used for a mixture study. The free resources are used to conduct 4 more tests on the concentration response of these 4 estrogens (E1, E2, EE2, NP) on spiggin inhibition in female fish that are simultaneously treaded with 5µg/L DHT (in exactly the same way as in the anti-androgens test).

Some analyses remain incomplete for the studies listed above. All tank-based fish exposures listed have been carried out, and fish tissue and water sample extracts have been delivered to the appropriate Partner/sub-contractor. Tissue analyses are pending for 3 studies, water chemistry for 5 studies. For all the studies for which a complete date set is available, Partner

1 (Martin Scholze) has provided a statistical analysis of the outcomes. These studies represent necessary preparation for the mixture exposure tests that will take place starting early 2006 and for which the Partners are now well positioned. Results of range-finding and definitive studies undertaken within this reporting period follow.

1. *Anti-androgenic effects of fenitrothion – definitive study*

Figure 4 depicts the data plotted against nominal concentrations (the figure and interpretative analysis is supplied by Martin Scholze, Partner 1). Figure 5 shows the same data plotted (mean ± SEM) against the measured concentrations of fenitrothion.

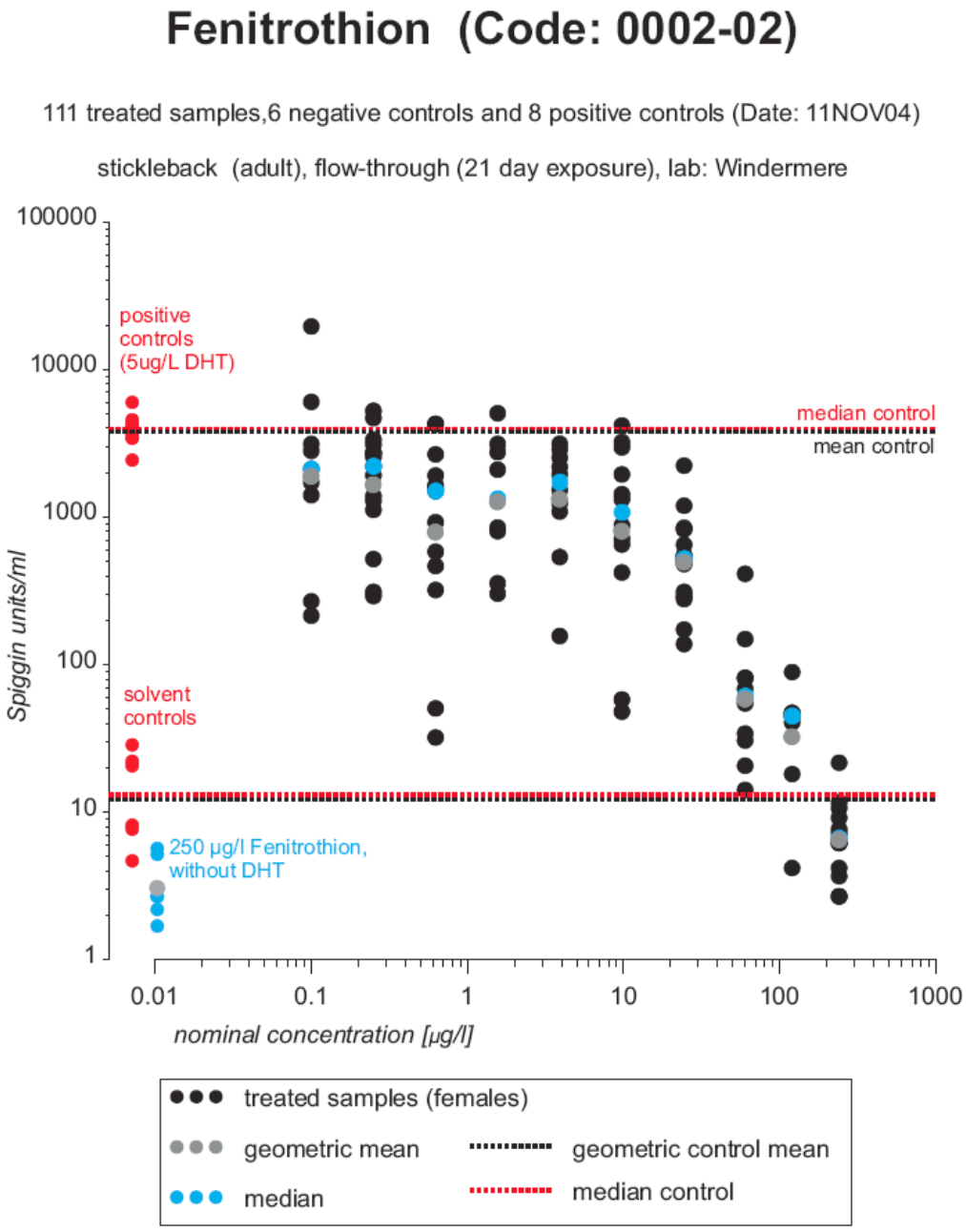

Figure 4 Effects of fenitrothion on spiggin production in the kidneys of sticklebacks exposed to dihydrotestosterone (DHT). Definitive study, fenitrothion concentrations are nominal.

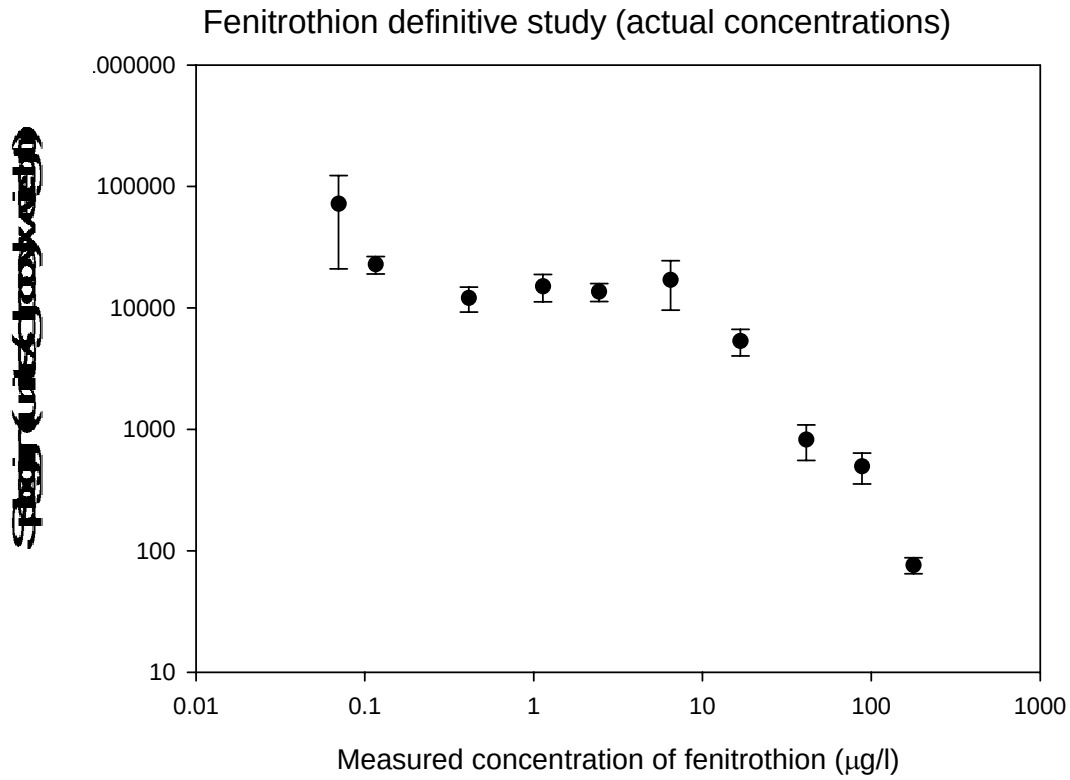

Figure 5 Fenitrothion definitive study, concentrations of fenitrothion are presented here as the means of the measured concentration in each tank on 4 occasions during the study (spiggin data: mean ± SEM)

2. *Estrogenic effects of 17β-estradiol - definitive study*

Figure 6 depicts the data plotted against nominal concentrations. Figure 7 shows the same data plotted against the measured concentrations of E2. E2 was detected in the control, solvent only, samples. That is, in the radioimmunoassay employed to measure E2 in the cartridge extracts, there was displacement of the labeled ligand relative to the assay blank. Whether this is an artifact, perhaps arising from interference in the assay from elements washed from the extraction cartridge, or is indicative of contamination of the samples or tanks, is unresolved. The latter seems unlikely given the results of other chemical analyses in this series of studies and the practical likelihood that low levels of E2 could be introduced into the exposure tanks.

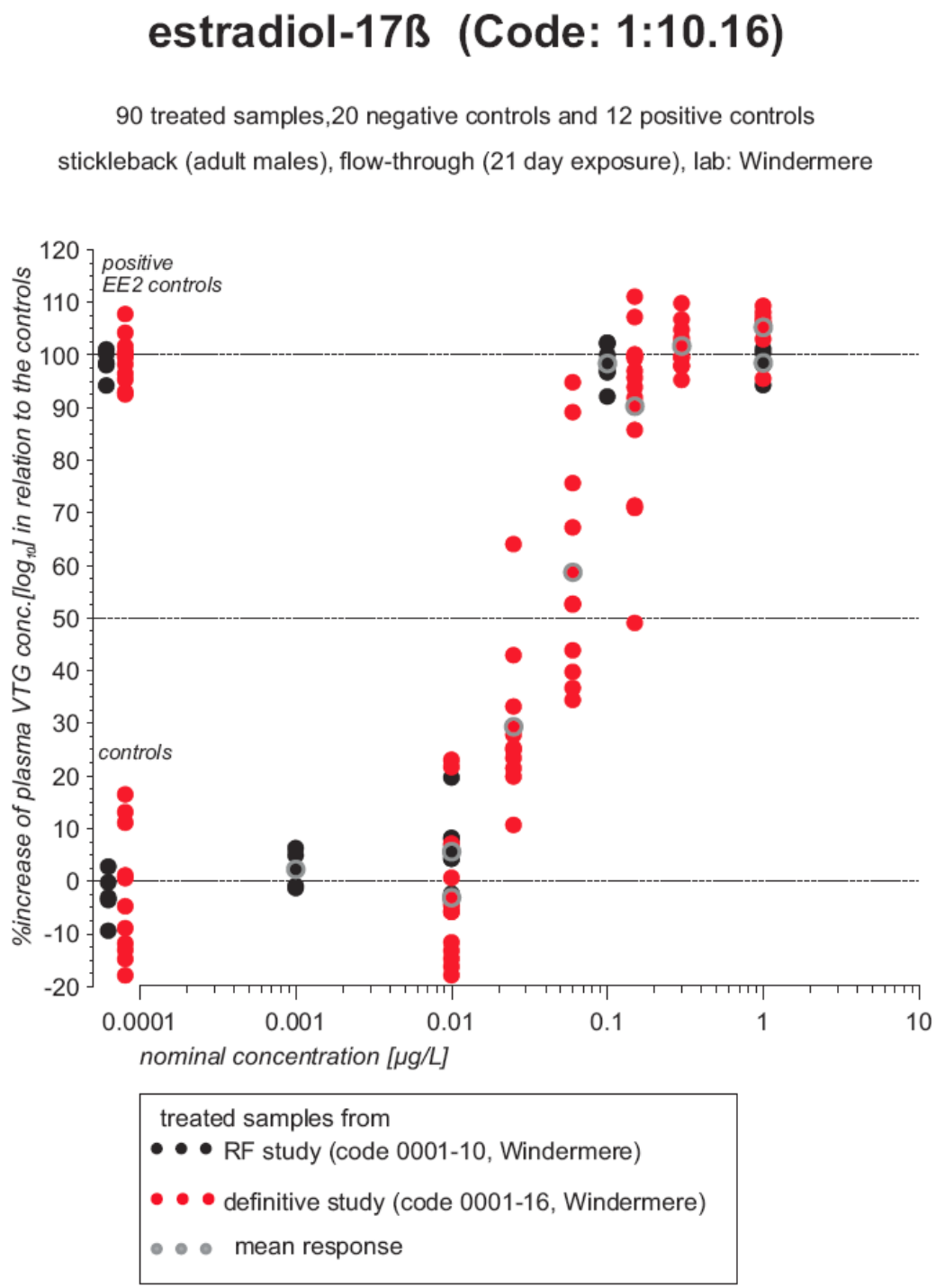

Figure 6 The effects of estradiol-17β on VTG concentrations in the blood (heart contents) of sticklebacks. Definitive study - nominal concentrations of E2.

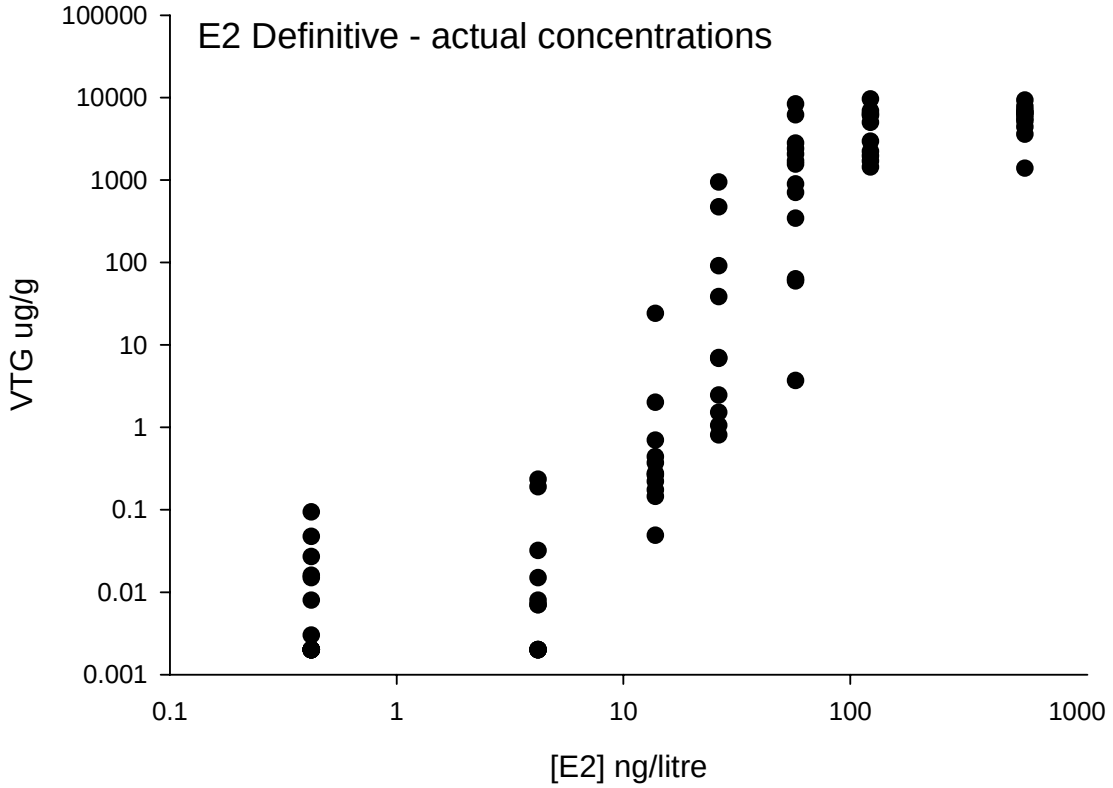

Figure 7 Estradiol-17β definitive study, concentrations of E2 presented are means of the measured concentration in each tank on 4 occasions during the study

3. *Estrogenic effects of Ethinylestradiol - definitive study*

The effects of EE2 on VTG concentrations in the blood (heart contents) of sticklebacks are given in Figure 8. The figure and interpretative analysis has been supplied by Partner 1. For the EE2 definitive study, the concentrations of EE2 presented in Figuere 8 are the means of the measured concentration in each tank on 4 occasions during the study. As was the case for E2, EE2 was detected in samples from the control, solvent only, exposure tanks. Again, whether this is an artifact or indicative of contamination of the samples or tanks, is unresolved.

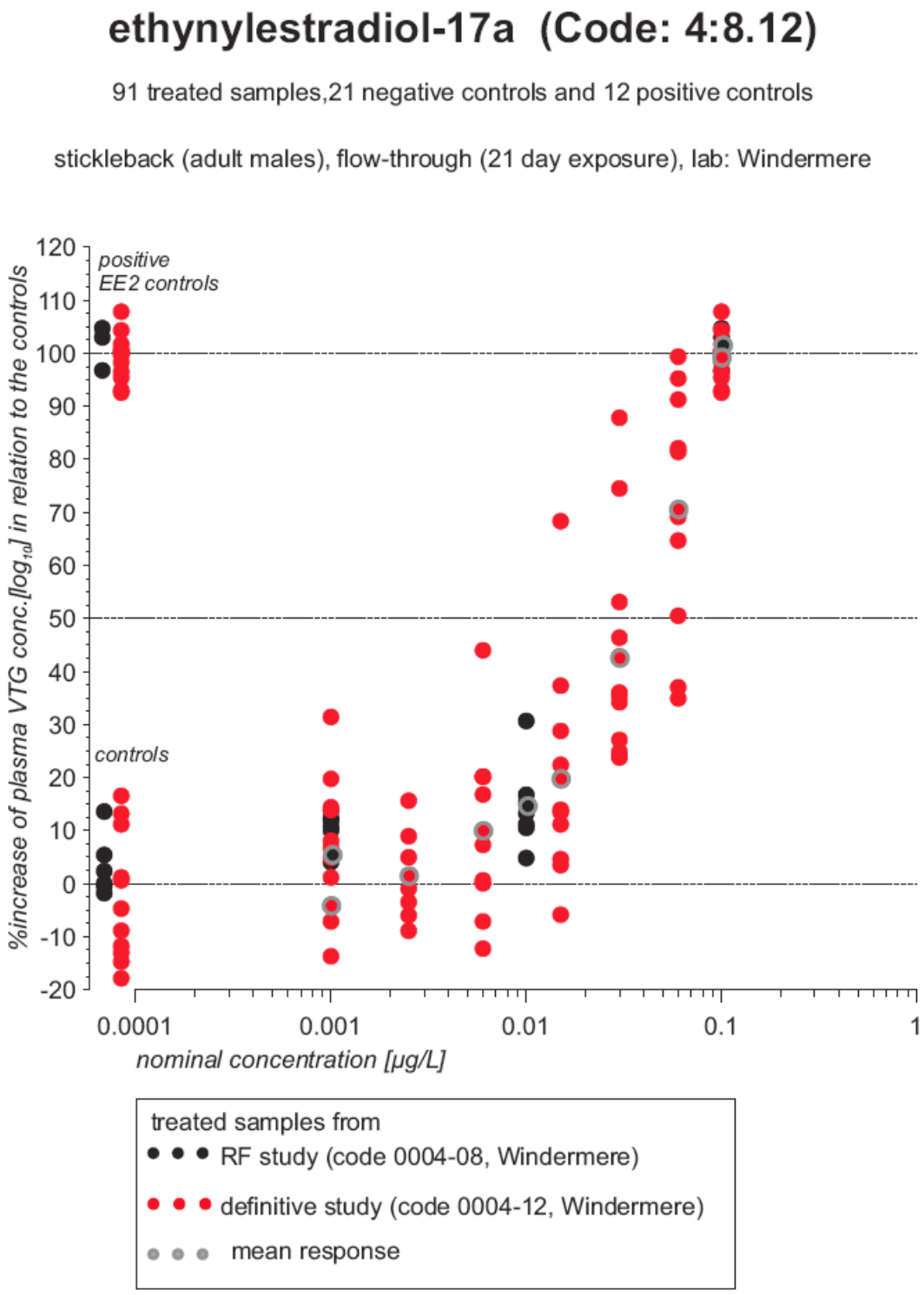

Figure 8 Definitive study - nominal concentrations of EE2

4. *Anti-androgenic activity of estradiol-17β (spiggin endpoint) – range-finding study*

The effects of E2 on spiggin production by the kidney in sticklebacks exposed to DHT are given in Figure 9. Concentrations of E2 are nominal and the water chemistry analysis is currently pending and will be due by month 38.

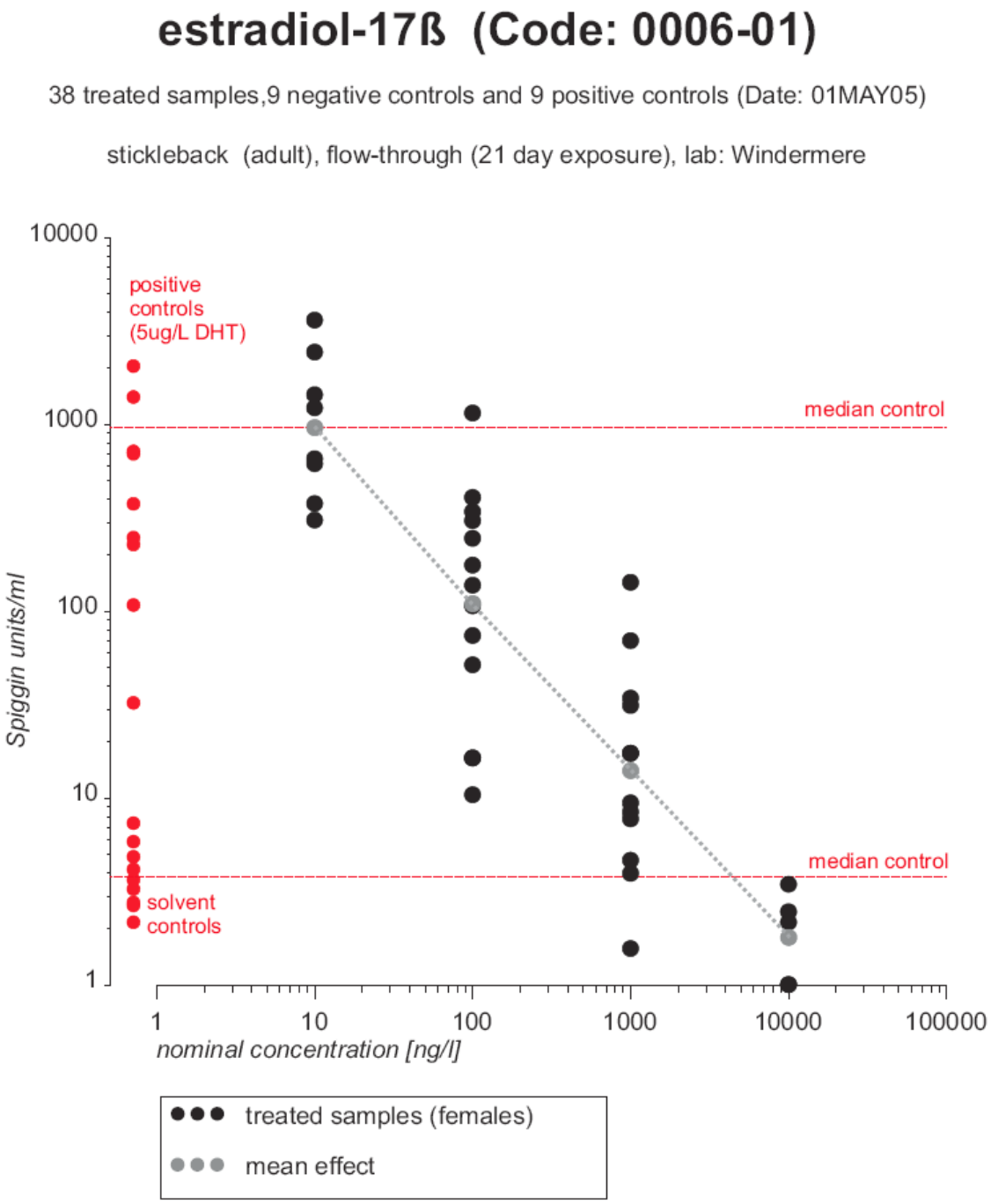

Figure 9 Range finding of E2 on spiggin production

5. *Anti-androgenic activity of ethinylestradiol (spiggin endpoint) – range-finding study*

The effects of EE2 on spiggin production by the kidney in sticklebacks exposed to DHT are given in Figure 10. Concentrations of E2 are nominal and water chemistry analyses are pending, due month 38.

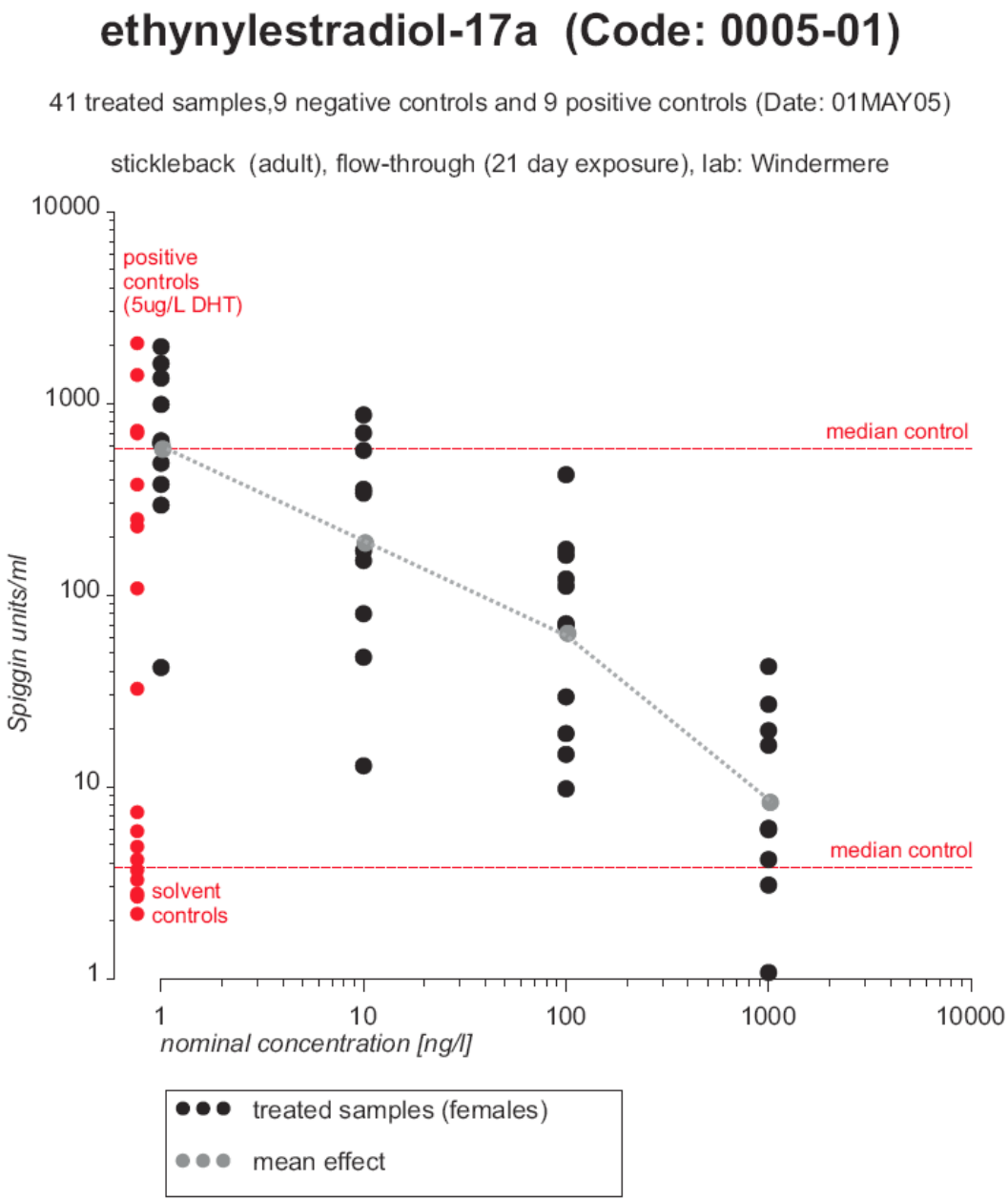

Figure 10 Range finding of EE2 on spiggin production

Partner 8 contribution

This study period has been concerned with completing the remaining conventional range-finding definitive dose-response studies and executing range-finding and definitive studies for two of the estrogen anti-androgenic endpoint studies. Details of the conditions for individual studies performed by Partner 8 during year 3 are given in Table 1.

Test compound	RF / DEF	Nominal concentrations	Controls	Endpoint
Nonylphenol	DEF	0, 5, 10, 20, 35, 50, 100, 250 µg/L	2 solvent, positive control (EE_2)	VTG
Flutamide	DEF	0, 5, 25, 50, 75, 100, 150, 200 µg/L	Solvent, flutamide , DHT	Spiggin
Vinclozolin (+ DHT)	RF	0, 0.25, 2.5, 25, 250, 500 µg/L	Solvent, DHT, Vinclozolin	Spiggin
Linuron	RF	0, 0.25, 2.5, 25, 250, µg/L	Solvent, DHT, Linuron	Spiggin
Nonylphenol - repeat	DEF	0, 100, 200, 300 µg/L	Solvent, EE_2	VTG
Flutamide -repeat	DEF	0, 2, 5, 10 µg/L	Solvent, DHT	Spiggin
Linuron	DEF	0, 2,10, 25, 100, 250 µg/L	Solvent, DHT, Linuron	Spiggin
Vinclozolin	DEF	0, 10, 100 µg/L	Solvent, DHT, Vinclozolin	Spiggin
NP (+ DHT)*	RF	0, 25, 100, 500 µg/L	Solvent, DHT	Spiggin + VTG
Estrone (+ DHT)*	RF	0, 1, 5, 10 µg/L	Solvent, DHT	Spiggin + VTG

Table 1 Summary of studies undertaken during the 3rd reporting period

Some analyses remain incomplete for the studies listed in Table 1 (illustrated by asterisk). All tank-based fish exposures listed have been carried out, and fish tissue and water sample extracts have been delivered to the appropriate Partner/sub-contractor. Tissue analyses are pending for 2 studies, water chemistry for 4 studies. For all the studies for which a complete date set is available, Partner 1 (Martin Scholze) has provided a statistical analysis of the outcomes. Two final exposure studies have to be completed prior to starting the planned mixture studies, these being the NP/DHT and Estrone/DHT definitive tests. It is projected that these will be finished by mid-February, 2006.

These studies represent necessary preparation for the mixture exposure tests that will take place starting early 2006 and for which we are now well positioned. Results of range-finding and definitive studies undertaken within this reporting period follow.

1. *Estrogenic properties of 4-nonylphenol - definitive study*

Figure 11 depicts the data plotted against nominal concentrations, interpretative analysis has been supplied by Partner 1.

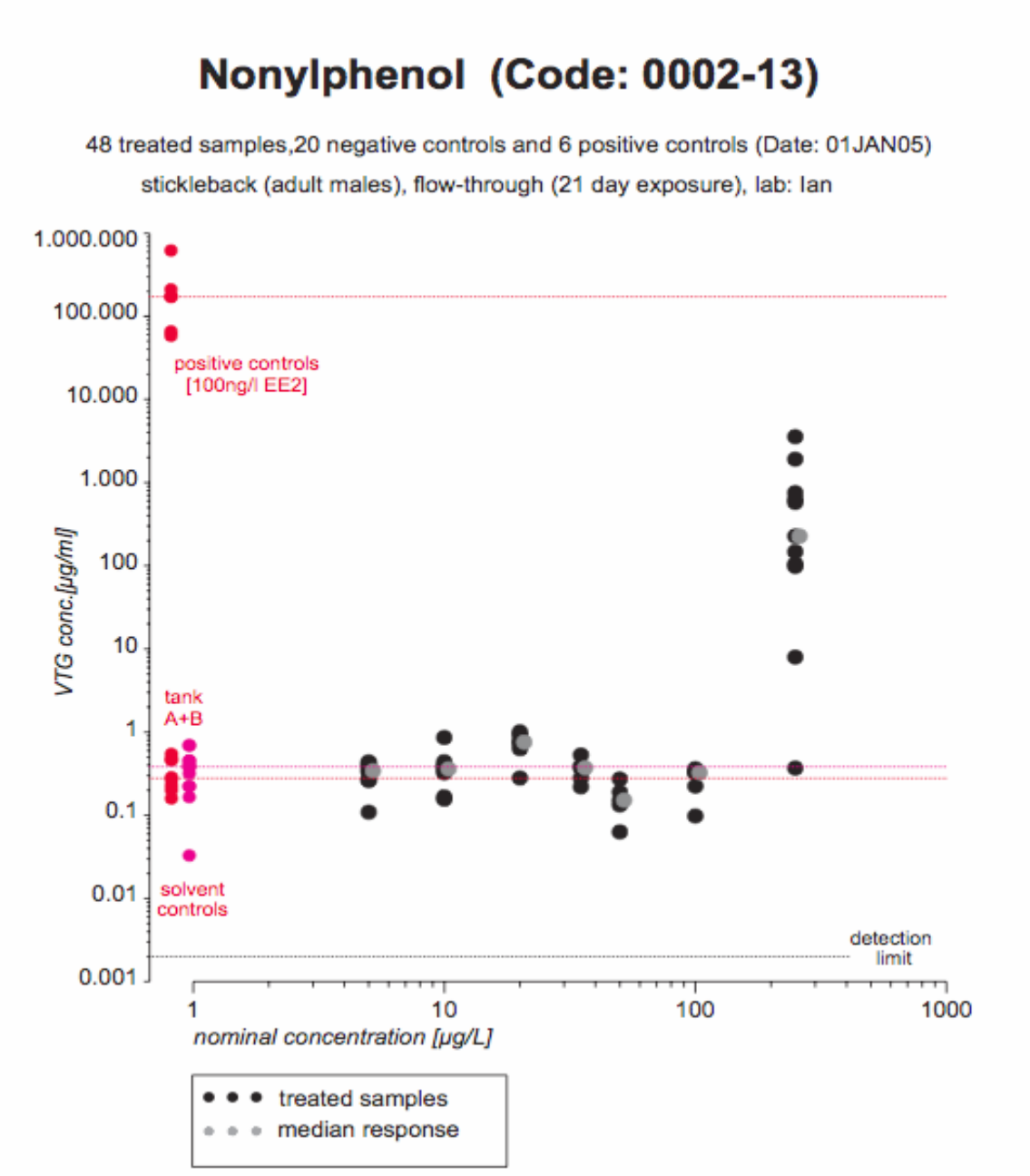

Figure 11 Effects of 4-nonylphenol on VTG induction in male sticklebacks. EE2 (100 ng/L) was employed as a positive control.

2. *Anti-androgenic properties of flutamide - definitive study*

Figures 12 depicts the effects of flutamide on spiggin induction by kidneys of female sticklebacks co-exposed with DHT (5 µg/L) from combined definitive studies (original and repeated study).

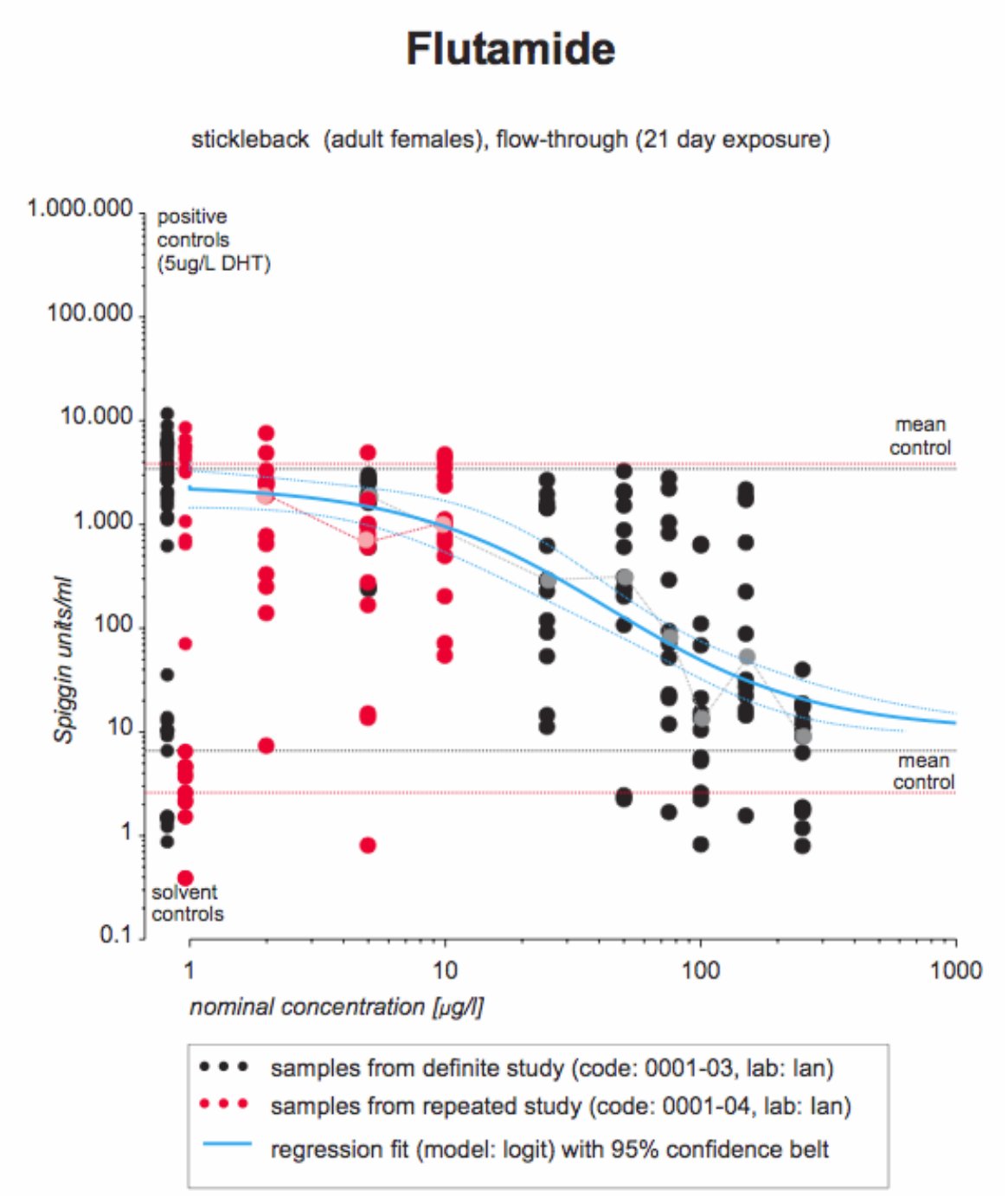

Figure 12 Effects of flutamide on spiggin induction by kidneys of female sticklebacks co-exposed with DHT (5 µg/L). Regression fit showing 95% confident belt shown.

3. *Anti-androgenic properties of Linuron*

Figures 13 gives the effects of linuron on spiggin induction by kidneys of female sticklebacks co-exposed with DHT (5 µg/L) from range finding and combined definitive studies.

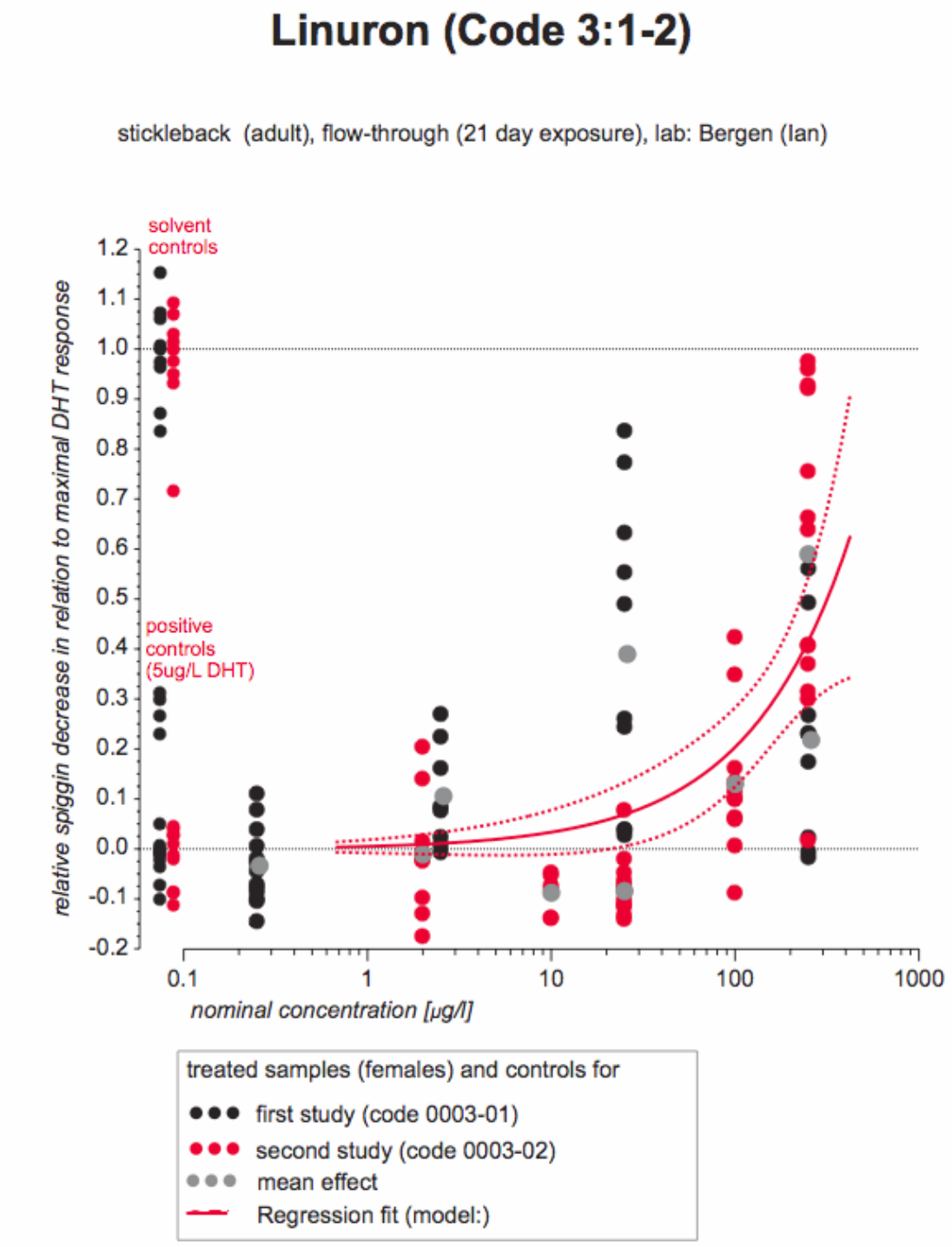

Figure 13 Effects of linuron on spiggin induction by kidneys of female sticklebacks co-exposed with DHT (5 µg/L) from the combined range-finding and definitive exposure studies. Regression fit showing 95% confident belt shown.

4. *Anti-androgenic properties of Vinclozolin*

Figures 14 depict the effects of vinclozolin on spiggin induction by kidneys of female sticklebacks co-exposed with DHT (5 µg/L) from range finding and combined definitive studies.

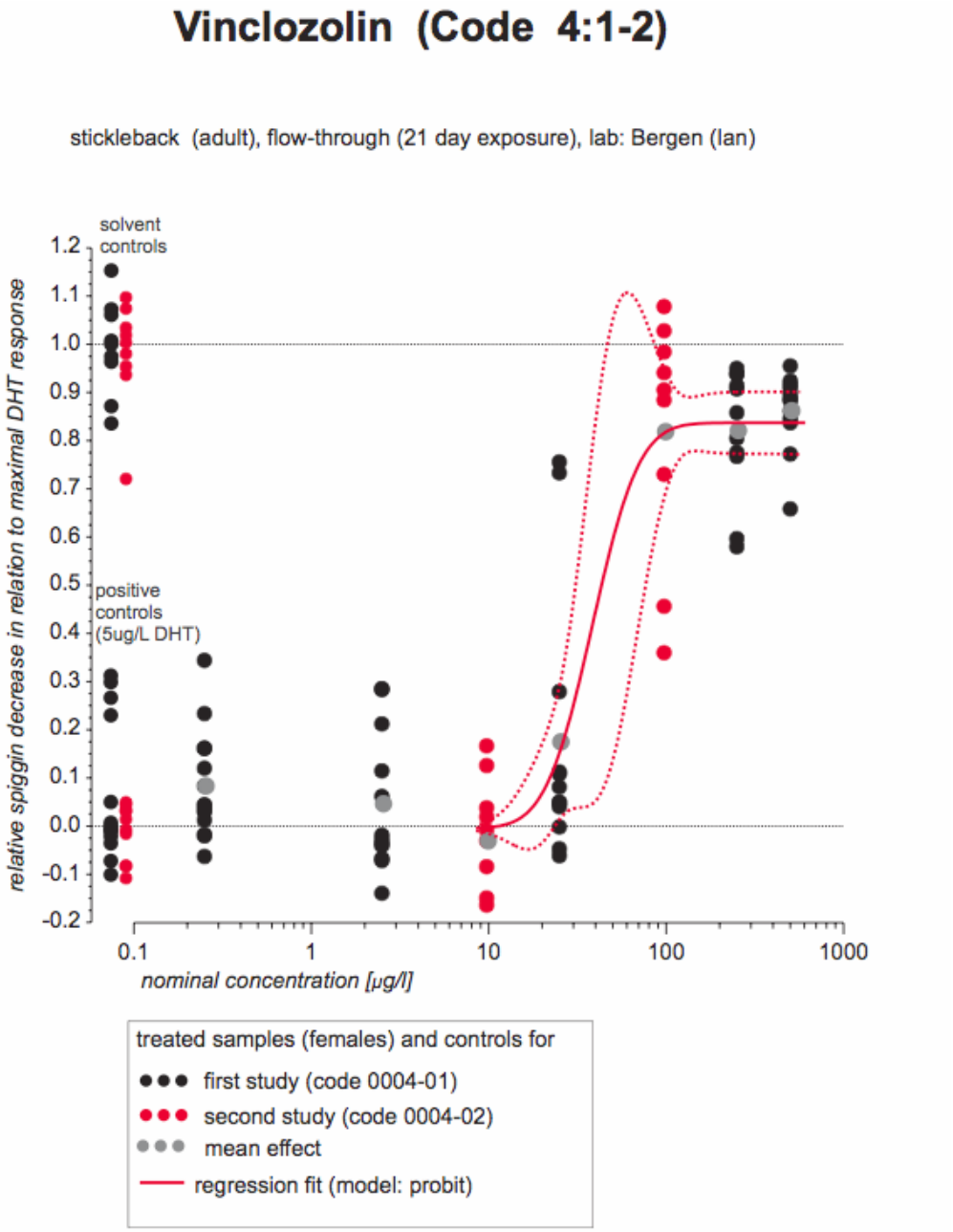

Figure 14 Effects of vinclozolin on spiggin induction by kidneys of female sticklebacks co-exposed with DHT (5 µg/L). Combined exposure studies, showing regression fit showing 95% confident belt shown.

For the anti-androgenic activity of 4-nonylphenol and estrone (spiggin endpoint) – range-finding study, exposures are finished and both tissue and water samples have been distributed to respective Partners for analysis. Definitive exposures will follow shortly (mid-January, 2006).

Partner 11 and 15 contributions

Tools have been developed to detect estrogen induced gene expression in zebrafish. Due to the delay experienced with the mixture experiments, Partners 11 and 15 have yet to receive tissue samples for analysis from mixture-exposed fish. Partner 21 is now performing such mixture experiments with zebrafish, and Partner 11 and 15 will be able to undertake the genomic analyses in the forthcoming project period.

Partner 19 contribution

In collaboration with Partners 2 and 8, activities related to estrogenic compounds have included a definitive study (full dose response curve) for E2, EE2, E1 and NP, where NP proved to be more of a challenge for providing reproducible effect data. The first definitive study did not match the expected response from the range finding studies so Partner 8 repeated it. However, due to delays during transport the samples arrived in Partner 19s laboratory in sub-optimum condition and although analysis was attempted, the marker protein were in decay and unsuitable for analysis. Partner 2 volunteered to repeat the exposure once more and samples are due for analysis in December 2005. Activities related to antiandrogenic androgenic compounds are range finding studies for Linuron (LN) and Vinclozolin (VZ) and definitive studies (full dose response curve) for flutamide (FL), fenitrothion (FN), LN and VZ. The graphs of which have been given in the sections under Partner 2 and 8 and Table 2 summarises the experiments for which Partner 19 has carried out analyses (tissues provided by Partners 2 and 8).

Compound	Lab	Date tested	VTG	Spiggin	Statistical analysis
E1 Definitive	CEH	09/2004, (Sent 10/04)	10/04 & 02/05	NR	March, 2005
FN definitive	CEH	11/2004 (Sent 12/04)	NR	02/2005	February, 2005
NP definitive	Bergen	01/2005 (Sent 02/05)	02/2005	NR	March, 2005
FL Definitive	Bergen	02/2005 (Sent 02/05)	NR	03/2005	March, 2005
E_2 Definitive	CEH	02/2005 (Sent 04/05)	05/2005	NR	June, 2005
EE_2 Definitive	CEH	02/2005 (Sent 04/05)	05/2005	NR	June, 2005
Vinclozolin (VZ) RF	Bergen	March 05, (Sent 04/05)	NR	04/2005	April, 2005
Linuron (LN) RF	Bergen	March 05, (Sent 04/05)	NR	04/2005	April, 2005
NP and FL repeats	Bergen	May 2005, (Sent 06/05	08/2005	07/2005 (FL)	September, 2005
LN Definitive	Bergen	09-10/2005 (Sent 11/05)	NR	11/2005	December, 2005
VZ Definitive	Bergen	09-10/2005 (Sent 11/05)	NR	11/2005	December, 2005
NP-Second Repeat	CEH	09-10/2005 (Sent 10/05)	11/2005 (Reassay)	NR	Not available

Table 2 Summary of exposures and analyses during the 3rd reporting period for the stickleback. The laboratory in which they were tested (CEH=partner 2;Bergen University = partner 8), the date on which they were tested, the date on which VTG assays were completed, the date on which spiggin assays were completed and the date on which statistical analyses were completed by Partner 1.

Partner 20 contribution

1. *Anogenital distance (AGD) in male offspring*

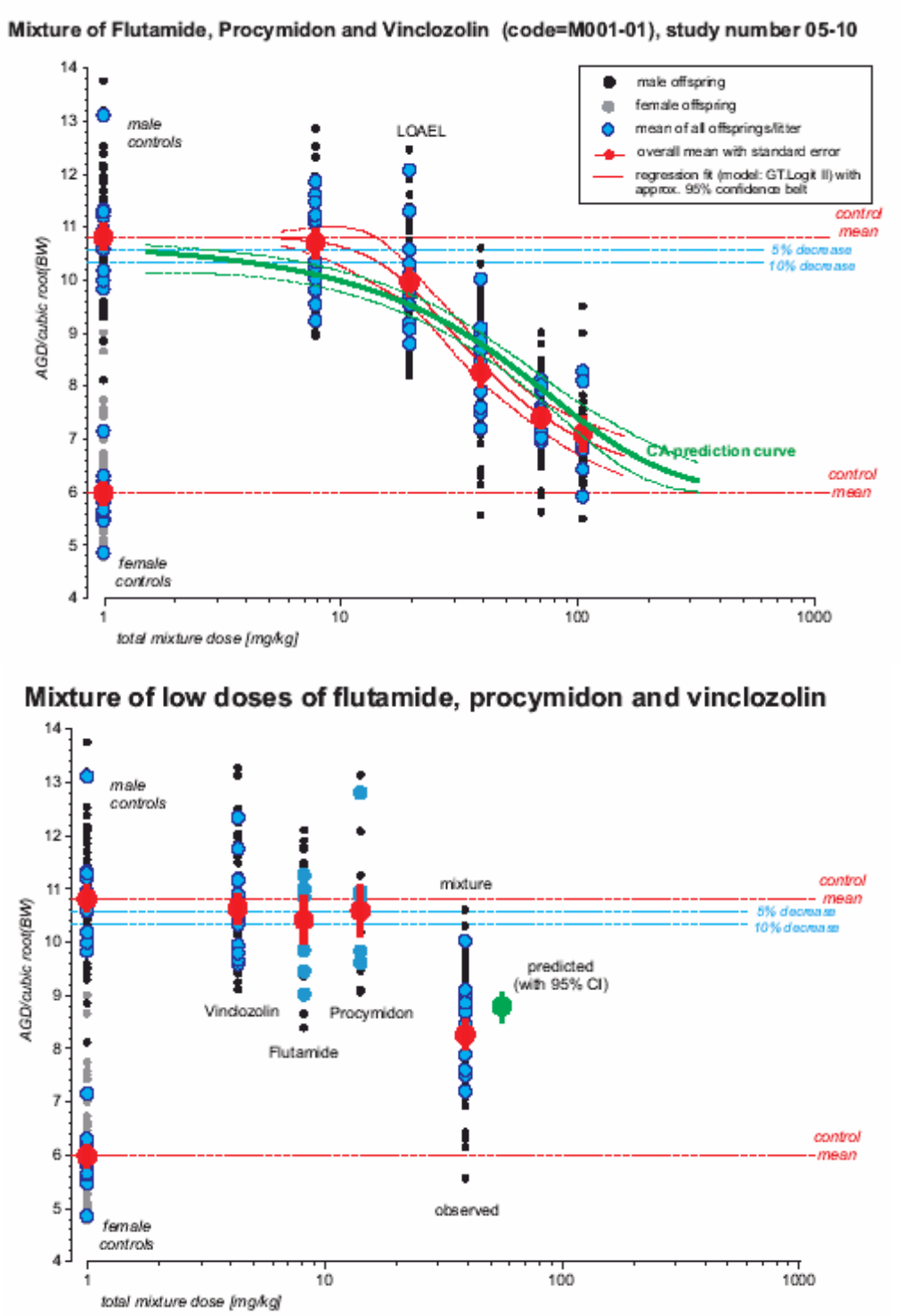

Figure 15 Effects of flutamide, vinclozolin and procymidon component mixture on anogenital distance (AGD) in male offspring.

A mixture experiment involving the androgen-receptor antagonists vinclozolin, flutamide and procymidone was carried out. The dose-response curve for changes in anogenital distance of male offspring based on the control and the 5 mixture doses covered the effect range from (almost) no effect to almost complete feminisation of this endpoint (Figure 15).

Based on comparison with the expected results, i.e. the CA-prediction curve, the mixture effects appears additive. Comparison of the mixture effects of low doses of the three chemicals with the single effects of these chemicals shows that the single chemicals only induce a rather small decrease of the AGD, while the mixture effects is marked (Figure 15).

2. *Nipple/aerola rentention in male offspring*

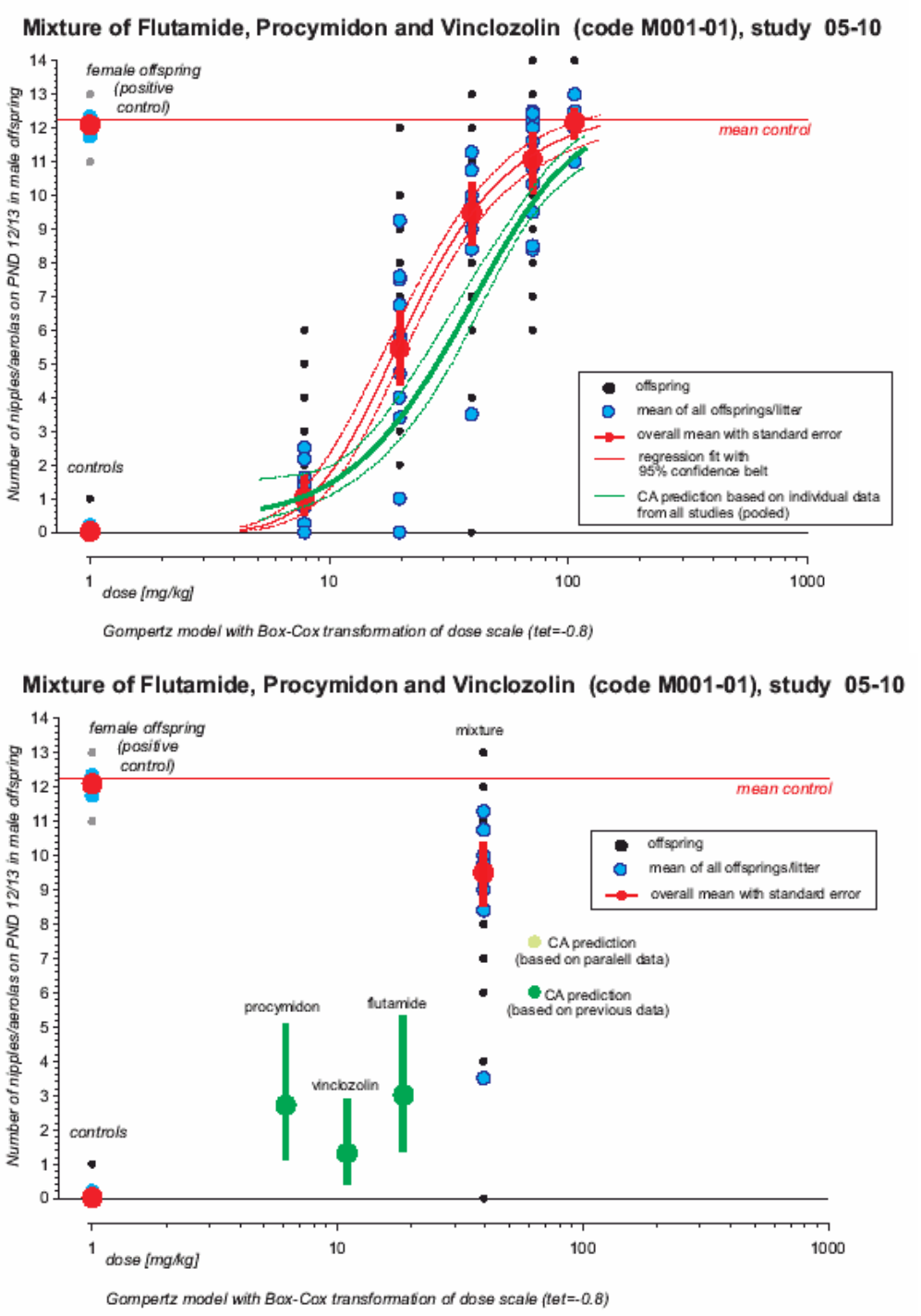

Figure 16 Effects of flutamide, vinclozolin and procymidon component mixture on nipple/aerola rentention in male offspring.

The dose-response curve for nipple retention in male offspring based on the control and the 5 mixture doses covered the effect range from (almost) no effect to a complete feminisation of this endpoint (Figure 16). Based on comparison with the expected results, i.e. the CA-prediction curve, the mixture effects appears additive at the low end of the dose-response curve. In the middle part of the dose-response curve, the mixture results indicate synergism. The results at the high end of the dose-response curve could seem to indicate additive effect, because the results are similar to the CA-prediction. However, synergism cannot be ruled out, because the nipple retention in the males cannot be higher than the number of nipples normally developed, i.e. 12. Comparison of the mixture effects of low doses of the three chemicals with the single effects of these chemicals shows that the single chemicals induce a modest nipple retention, i.e. 1-3, while the mixture effects is marked, i.e. 9-10. These results of these studies have been submitted for publication in a peer-reviewed journal.

3. *Malformations of male external reproductive organs on PND 47*

For some of the groups in the mixture study, animals were kept after weaning in order to investigate sexual dimorphic behaviour, sexual maturation and semen quality (Figure 17). The groups included for this were controls, mixture group 1-4 and the two groups dosed with vinclozolin. The highest mixture group, i.e. mix-5, were not kept after weaning for animal welfare reasons because the effects on the male reproductive organs were expected to be very marked based on the effects seen on AGD and nipple retention.

Malformations of the male external organs were investigated on postnatal day 47 around the time of sexual maturation. The results of this showed a high frequency of serious malformations in the males (split penis, vaginal opening) in the groups dosed with mix-3, mix-4 and the high dose of vinclozolin. Based on animal welfare considerations, these animals were killed. Also, further results for these animals would not contribute significantly to a risk assessment. The very high frequency of malformations in the group dosed with mix-3 was somewhat unexpected and shows that combined dosing with the 3 AR-antagonist induces a very high frequency of serious effects that are not seen after dosing with the single chemicals alone.

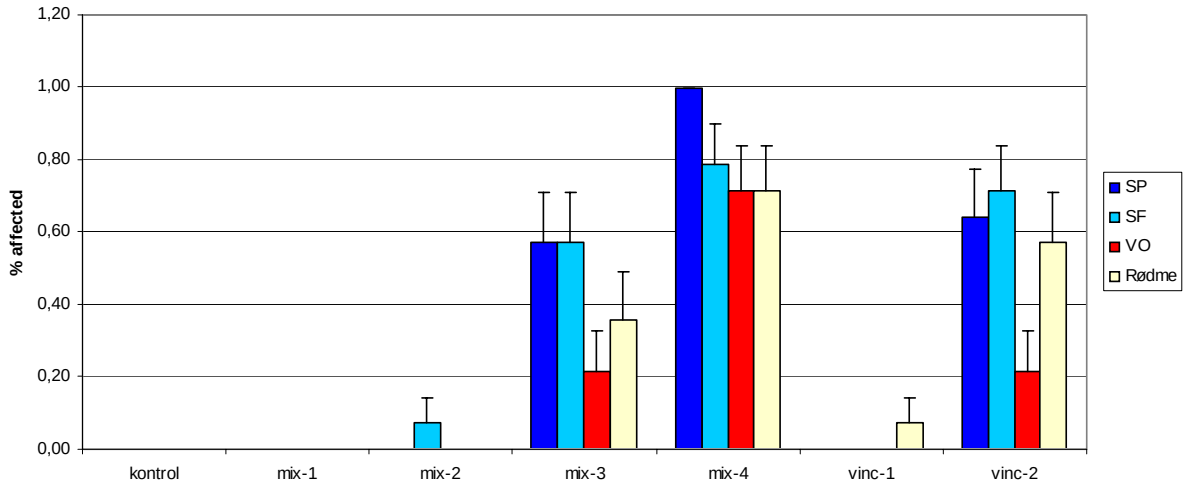

Figure 17 Malformations of male external reproductive organs on PND 47. SP: split penis, VO: vaginal opening.

Partner 21 contribution

In order to incorporate further chemicals into a mixture of estrogenic substances, the fungizide cyproconazole and the UV-filter agents OMC and 4-MBC were screened for vitellogenin induction in zebrafish. For the range finding studies, exposure to 1000 ng/L of 4-MBC resulted in 100% mortality. High mortalities were also observed after exposure to 500 µg/L of 4-MBC, and 100, 500 and 1000 µg/L of OMC. No effects on vitellogenin concentrations were measured for any of the chemicals (Figure 18). Unfortunately, these results preclude the use of any of these substances in further mixture experiments.

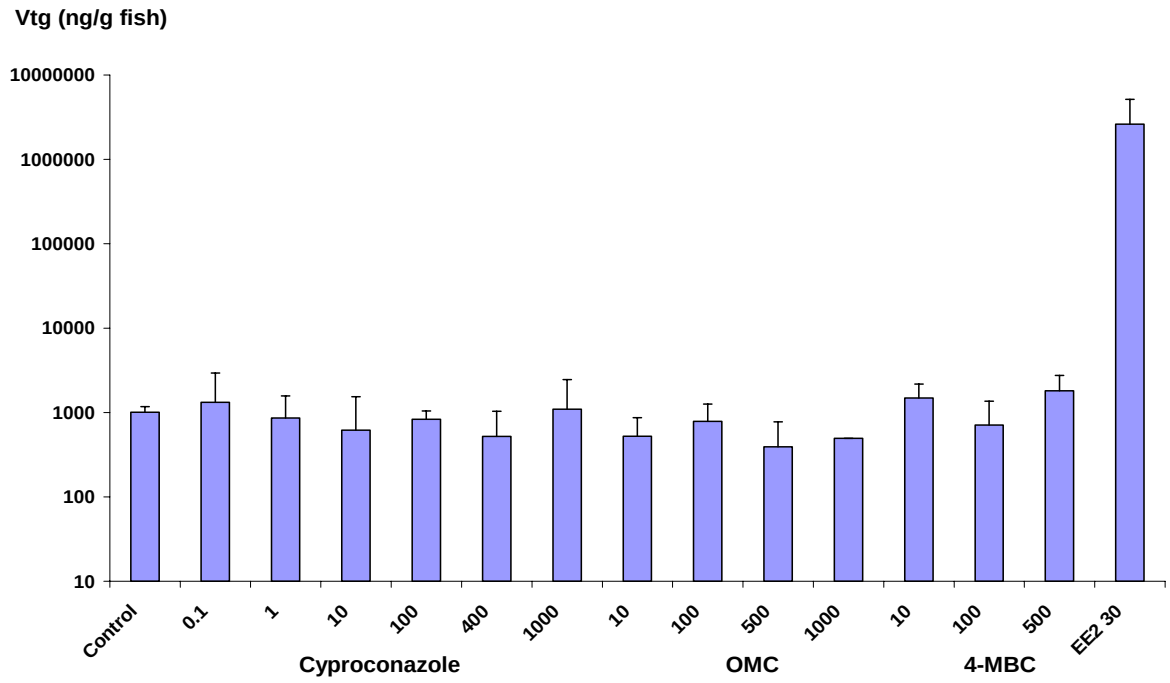

Figure 18 Whole-body vitellogenin concentrations in juvenile zebrafish exposed from days 17 to 38 post-hatch to Cyproconazole, OMC, and 4-MBC, as well as the positive control EE2.

During the reporting period, definitive concentration-response studies and repeat studies were carried out with E1, E2, EE2 and NP. Exposure to 400 µg/L of NP resulted in 100% mortality. In the study repeats for E1 and E2 the vitellogenin concentrations matched exactly those of previous exposures. Repeated exposures to NP, however, resulted in slightly lower effects than in previous studies. For EE2 slightly higher effects were observed. The data on vitellogenin production in fish exposed to NP and EE2 are presented together with the repeated effect studies in Figure 19.

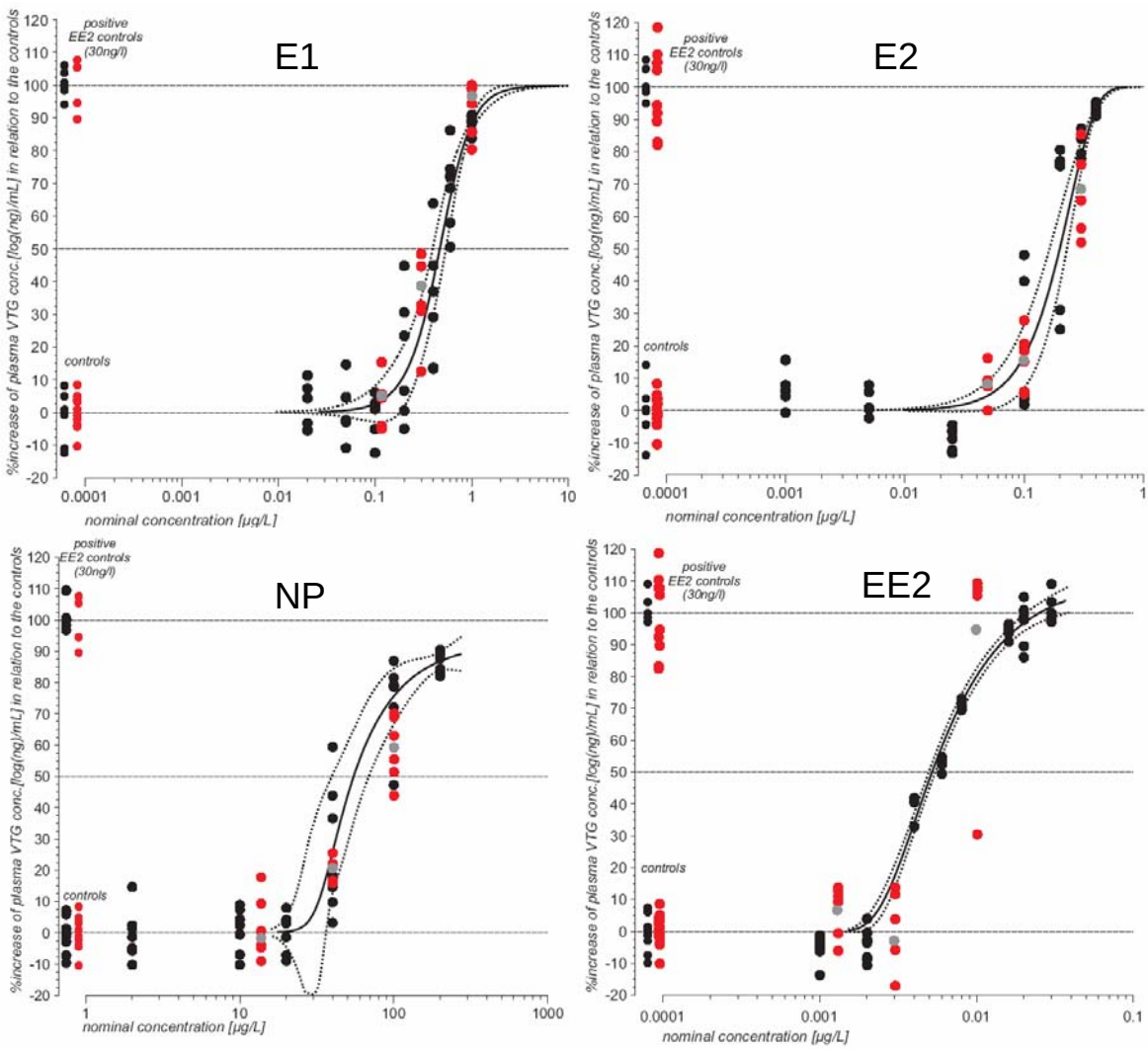

Figure 19 Repeated concentrations effects on vitellogenin production of the four estrogens, estrone (E1), estradiol (E2), nonylphenol (NP) and ethinylestradiol (EE2). The level of effect is compared with EE2 (30 ng/L) as positive control. Repeat effects of each estrogen (red dots) are compared with previously obtained results (black dots).

The data shown in Figure 19 were used to compute predicted mixture effects of a combination of all four estrogenic chemicals. The observed mixture effects were generally in good agreement with the expected, although the predicted effects were found to be slightly overestimated (Figure 20).

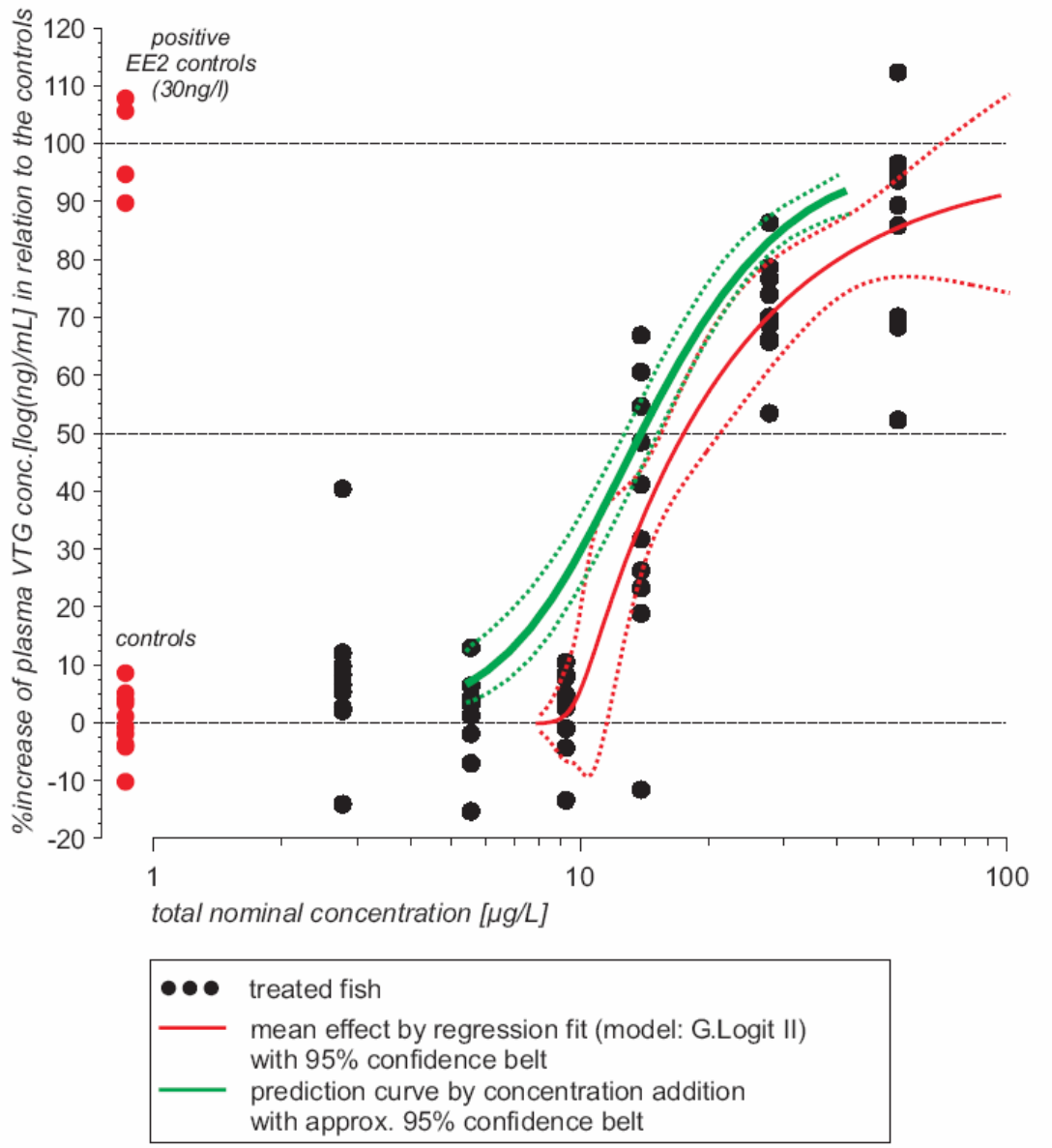

Figure 20 Results from the mixture study including the four estrogens E1, E2, NP and EE2. The predictive effect curve (green) compared with observed effect curve (red).

Progress Summary

Significant progress has been made with mixture testing during this reporting period.

- Partner 1 has tested the effects of xenoestrogens on the expression of a number of genes by real-time PCR. This preliminary data will serve as the basis for future work with mixtures of the tested agents, and comparison between single agents and mixture effects. It could be demonstrated that concentration addition accurately predicts the effects of a reference mixture composed of E2, E1 and E3, combined at equi-effective concentrations. In order to test the hypothesis that the deviation from additivity observed with the 10 component mixture was due to an increased metabolism of steroidal hormone, Partner 1 has begun testing the effects of the mixture components on *CYP1A1*, *CYP1B1* and *CYP3A4*. Neither the single agents

nor the 10 component mixture significantly activated the E2 metabolising enzyme *CYP1A1*. Work on *CYP1B1* and *CYP3A3* has revealed that these genes can be up-regulated and down-regulated, respectively, by β-HCH. Further work on the remaining mixture components and the mixture itself is underway.

- Eight exposure tests (2 range-finding and 6 definitive) have been completed with the stickleback. Collectively, the Partners (2, 8 and 19) have been able to demonstrate that two model estrogens (E2 and EE2) also exert anti-androgenic activity that can be detected as a reduction of androgen-dependent spiggin production in the kidney of exposed sticklebacks. This is a novel, previously unreported finding and will contribute significantly to the design of the mixture exposure studies to follow during the final reporting period. In conclusion, we are well placed to begin the final phase of these studies.

- For Partner 20, the mixture study in the rat has been performed as planned and has produced results considered of great relevance for interpretations of mixture effects and evaluation of mixed exposure scenarios. These being that the combined effects seems additive for AGD, but may not be additive for nipple retention at higher doses and in general probably not for malformations of the male reproductive organs. Exposure to single chemicals caused only small or modest effetcs on AGD and nipple retention, while combined exposure to 3 chemicals at these doses induced marked effects on these endpoints as well as a a very frequency of serious malformations of the male reproductive organs.

- To aid the selection of potentially estrogenic chemicals for zebrafish mixture studies Partner 21 tested three different chemicals. The substances Cyproconazole, 4-MBC and OMC did not induce vtg production in juvenile zebrafish. Dose-response studies have been finalised for EE2 and NP. The repeated effect studies of the four single estrogens E1, E2, EE2 and NP showed generally a good agreement with previous conducted studies. The first mixture study performed indicates a slight overestimation of predicted effects.

Plan and Objectives for the Next Reporting Period

- Partner 1 will carry out further gene expression experiments in order to test the hypothesis that estrogenic chemicals might interact in differentially promoting the expression of steroid metabolising CYP isoforms. The effects of the test chemicals on further genes including progesterone receptor, APOD and cyclin D3 will be evaluated in order to substantiate (or otherwise) the presumption of "similar action". The effects of mixtures on selected target genes will be tested. The mixtures tested will be a reference mixture (E2, E1 and E3) and the already evaluated 10 component mixture.

- Partner 2, 8 and 19 will complete the final definitive study (anti-androgenic properties of estrone and 4-nonylphenol) which requires completion of some outstanding analytical chemistry and tissue analyses. Following this work, mixture exposure studies will be initiated, following discussion and agreement with the workpackage participants on mixture ratios and further experimental details.

- Partner 20 is to undertake further analysis of organ samples and will gather results of behavioural testing and semen quality investigations.

- Partner 21 will undertake the planning and performance of further mixture studies.

Difficulties/delays

Some minor delays for Partner 2 were incurred consequent to the refurbishment and commissioning of a new aquarium at CEH Lancaster, following the relocation of CEH Windermere. The new aquarium has since functioned without problems. Some delays have inevitably been encountered because of the dispersed nature of the laboratories engaged in what is a tightly integrated series of studies. Inevitably, timetabling of exposures and analyses, and staff and facility availability did not always coincide. Partner 8 has experienced a major delay, due to the difficulty in catching sticklebacks in the field, encountered during the early summer of 2005. In the first two years of EDEN, a number of sites were identified where large numbers of sticklebacks could be caught by means of traps or beach seine during the early summer. Unfortunately, the south coasts of Norway (including the Oslo fjord) experienced an unusually cold early summer, which resulted in very few sticklebacks migrating into the shallow coastal waters. Repeated sampling trips during the period May-July failed to catch many sticklebacks. Sufficient numbers of sticklebacks were finally caught in the late autumn (August-September). High fish mortality was experienced in the highest dose in the exposure study involving the co-exposure of NP and DHT (Anti-androgenic activity of 4-nonylphenol and estrone with spiggin endpoint – range-finding study). The original NP dose range was decided as 0, 100, 500 and 1000 µg/L. However, very high fish mortality (> 80%) was experienced after only 5 days of exposure in the 1000 µg/L tank. This was presumed to be a toxic effect of this high NP dose. For this reason, the 1000 µg/L dose tank was terminated and an additional low NP dose (25 µg/L) started. This resulted in the final NP dose range of 0, 25, 100 and 500 µg/L.

Deliverables and Milestones

Deliverables	
✓	**D14.1:** Preliminary report about predictability of mixture effects in cell-based EDC assays (month 24)
✓	**D14.2:** Preliminary report about predictability of mixture effects in fish assays (month 24)
✓	**D14.3:** Preliminary report about predictability of mixture effects in female rats (month 24)
on time (36)	**D14.4:** Report about mixture effects of EDC at levels below NOEL (month 48)
Milestones	
on time (36)	**M14.1:** Insights into the predictive power of concentration addition and independent action for multi-component mixtures of EDC (month 48)
on time (36)	**M14.2:** *in vivo* data on EDC mixture effects (month 48)
on time (36)	**M14.3:** Insights into the suitability of novel endpoints for mixture studies (month 48)

WP15	Combination effects of dissimilarly acting EDC						
Phase							
Start date	0						
Completion Date	48 months						
Current Status	active						
Partner(s) responsible:	1	2	8	11	15	19	20
Person-months per Partner:	47.5 (36.5)	14 (8.2)	15.5 (11)	4 (3)	6 (4)	8 (4)	143 (72)
Already devoted person months per Partner and total:	24	6	6	1	1	2.5	17

Objectives for the Reporting Period

- Evaluate the influence of an anti-estrogenic compound (co-planar PCB126) on the effects of a mixture of 10 mitogens in the E-Screen. (Partner 1)

- Detailed planning of the mixture study based on the results of dose-response studies for single chemicals and the experience from the first mixture study on similarly acting EDCs (WP14) for stickleback (Partners 2, 8, 20).

- Complete range finding and definite studies for mixture undertaking in the rat and prepare for dissimilar mixture experiments in year 4 (Partner 19).

Methodology and Study Materials for the Reporting Period

Unlike WP14, this WP focuses on EDC that do not induce the same effects or can be assumed to act via different mechanisms. Partner 1 will carry out studies with MCF-7 cells (E-Screen) and will utilise quantitative RT-PCR and Western blotting to evaluate the influence of anti-estrogenic EDC on the actions of EDC that are mitogenic to MCF-7 cells. Endpoints studied will include modulations of estrogen receptor levels and induction of cell division. Partners 2, 8 and 19 will be responsible for work with the stickleback (induction of vitellogenein and spiggin), and Partners 3 and 20 for work with rats (endpoints: hypospadias and testis weight/descent, anogenital distance in male offspring). Partner 1 will provide the biometrical input required for experimental design and data analysis.

Scientific Achievements

Partner 1 contribution

The 10 component mixture studied as part of WP14 (Annual report 2004) was used to study the influence of an anti-estrogen on the effects of the previously tested mixture. The chemical selected for the first experiment was the co-planar PCB 126, in view of the levels reported in human tissues (WP3 – Annual report 2004) and its known anti-estrogenic effect.

Firstly, the possible mitogenic effect of PCB126 on its own was evaluated in the E-Screen. The co-planar PCB failed to produce any significant increase in MCF-7 cell number, when tested at a wide range of concentrations.

For the combination experiments, MCF-7 cells were treated with increasing concentrations of the ten component mixture combined with three different fixed concentrations of PCB 126. The components of the mixture were: 17β-estradiol, 17α-ethynylestradiol, *o,p'*-DDT, Endosulfan I, Endosulfan II, Methoxychlor, Kepone, β-hexachlorocyclohexane (β-HCH), butylparaben and propylparaben. The concentrations of PCB 126 tested were: 1 x 10^{-12} M, 1 x 10^{-8} M and 1 x 10^{-6} M.

Partner 1 observed that at concentrations of 1 x 10^{-12} M and 1 x 10^{-8} M, PCB126 did not have any significant influence on the mitogenic effect of the ten component mixture (Figure 1). However, at a concentration of 1 x 10^{-6} M, PCB 126 greatly decreased the effect of the mixture.

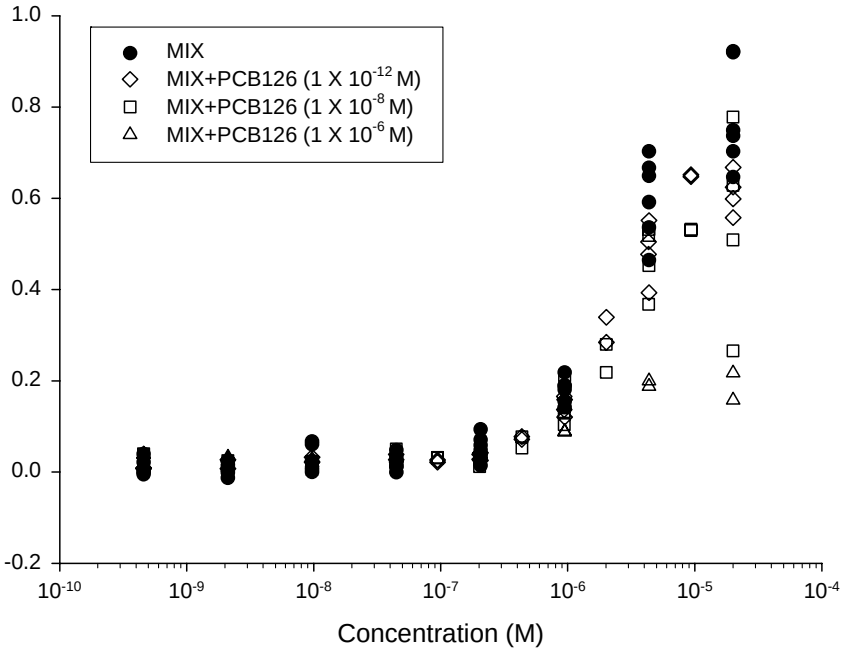

Figure 1 Ten component mixture study illustrating that concentrations of 1 x 10^{-12} M and 1 x 10^{-8} M, PCB126 did not have any significant influence on the mitogenic effect

Partner 2 and 8 contributions

The similar mixture experiments will allow the design of a mixture's test with dissimilarly acting (i.e. dissimilar mechanism of action) components where the 4 estrogens will be mixed with the 4 anti-androgens and 5µg/L DHT to assess the similarities between predicted and observed response. This approach appears to be far more interesting than testing only similarly acting compounds and maximises the potential of the stickleback as a model organism for EDCs.

Joint discussions between all partners involved (1, 2, 8, 19) in May 2005 conluded that the number of anti-androgens to be tested should be 4 (FL, LN, VZ, FN) and testing of

these compounds was completed midway through the 3rd reporting period. All 4 compounds have been re-tested in at least two concentrations (EC10-15 and EC60-70) for inter lab comparison. At the same time, Partners 2 and 8 began conditioning female fish to a low photoperiod and temperature to participate in the evaluation of anti-androgenic effect of estrogens (i.e. variability of endogenous estrogens should be of minimum to allow a dose response curve).

Partner 11 and 15 contribution

Zebrafish tissue specimens from low-dose exposures were collected for use to assess gene expression studies with the tools developed in WP5. Partner 15 has assessed the suitability of *cyp19a2* for the assessment of EDC effects, and these results are described as part of WP5.

Partner 19 contribution

Activities were related to the potential anti-androgenic effect of estrogens. The following tests were conducted:

1. Range finding study for estradiol (E_2)
2. Range finding study for ethinylestradiol (EE2)
3. Definitive study (full dose response curve) for E_2
4. Definitive study (full dose response curve) for EE2
5. Range finding study for estrone (E_1)
6. Range finding study for nonylphenol (NP)

The results so far have been very impressive and results for nonylphenol and estrone will be obtained in early January. Partner 1 is providing all figures related to work from Partners 2, 8 and 19. Table 1 summarises the experiments for which Partner 19 have carried out analyses (tissues provided by Partners 2 and 8).

Compound	Lab	Date tested	VTG	Spiggin	Statistical analysis
E2/DHT RF	CEH	05/2005	NR	08/2005	September, 2005
EE2/DHT RF	CEH	05/2005	NR	08/2005	September, 2005
NP/DHT RF	Bergen	10/2005 (Not sent)	NR	Not available	Not available
E1/DHT RF	Bergen	10/2005 (Not sent)	NR	Not available	Not available
E2/DHT Definitive	CEH	10/2005 (Sent 11/05)	NR	Pending	Not available
EE2/DHT Definitive	CEH	10/2005 (Sent 11/05)	NR	Pending	Not available
Planned tests for 2006					
NP/DHT Definitive	Bergen				
E1/DHT Definitive	Bergen				
Mixture of similarly acting chemicals	Bergen				
Mixture: dissimilarly acting chemicals	CEH				

Table 1 Summary of exposures and analyses undertaken so far and planned for 2007. The table shows the name of the compound, the laboratory in which they were tested (CEH=Partner 2; Bergen University = Partner 8), the date on which they were tested, the date on which VTG assays were completed, the date on which spiggin assays were completed and the date on which statistical analyses were completed by Partner 1.

Partner 20 contribution

Further discussions on the chemicals to be included in the dissimilar mixture study in this WP were needed in order to perform the most relevant study. The possibilities were:

- Include only 4 "dissimilarly acting" chemicals, i.e. DEHP (reduces the testosterone surge in male foetuses), vinclozolin (AR antagonist) , finasteride (alfa-reductase inhibitor) and prochloraz (various possible mechamisms including reduced testosterone surge and AR antagonism)

- Include all the antiandrogens for which we have performed dose-response studies, i.e. both "similarly acting" and "dissimilarly acting" chemicals, i.e. DINP, DEHP, Flutamide, Vinclozolin, Finasteride, Procymidone and Prochloraz.

The latter possibility may seem more attractive for demonstrating low-dose effects of mixtures as inclusion of 7 chemicals instead of only 4 probably increases the possibility to evaluate whether there may be a combined effect at low doses, where the individual chemicals alone do not induce antiandrogenic effects. However, inclusion of three AR antagonists (flutamide, vinclozolin and procymidone) in the mixture will lead to an overrepresentation of this mechanisms, especially as the first mixture study indicates additivity of these three chemicals (see WP14). This might potentially lead to problems with the interpretation of the results and consequently the first possibility was chosen.

A detailed planning of the mixture study on dissimilarly acting antiandrogens has been developed and the animals and chemicals have been ordered. The number of animals and dose levels were discussed at a meeting in Copenhagen with Partner 1. In order to maintain comparability with historical data, two doses of each single chemical were considered relevant (similarly as in the first mixture study in WP14). For vinclozolin and finasteride, the dose levels chosen were the dose included in the low and high mixture doses. As the dose-response curves for prochloraz and especially for DEHP were less clear than those for finasteride and vinclozolin, it was decided to include three doses of DEHP and prochloraz, i.e. the same doses as those included the three mixtures (Table 2).

Group and doses (mg/kg)	**Block 1**	**Block 2**	**Block 3**	**Block 4**	**Total**
1: Control	4	4	4	4	16
2: Mix-13.1	4	4	4	4	16
3: Mix-65.05	4	4	4	4	16
4: Mix-130.1	4	4	4	4	16
5: Finasterid-0,01	2	2	2	2	8
6: Finasterid-0,1	2	2	2	2	8
7: Vinclozolin-5	2	2	2	2	8
8: Vinclozolin-50	2	2	2	2	8
9. DEHP-3	2	2	2	2	8
10. DEHP-15	2	2	2	2	8
11: DEHP-30	2	2	2	2	8
12: Prochloraz-5	2	2	2	2	8
13: Prochloraz-25	2	2	2	2	8
14: Prochloraz-50	2	2	2	2	8
Total, mated (ca. litters)	**36 (32)**	**36 (32)**	**36 (32)**	**36 (32)**	**144 (126)**

Table 2 Mixture experiments for rat studies

The choice of dose levels was also based on modelling from Partner 1. The dose-response curves for the four chemicals are dissimilar and attempts were made to find doses where each chemical would contribute equally to the effect. In addition, the dose levels of the mixture were chosen in order to enhance the possibility for discriminating between concentration addition, independent action or synergism of the chemicals. The expected dose-response curves for the four chemicals are shown in the Figure 2 together with the dose-response curve for the mixture based on concentration addition and independent action of the chemicals in the mixture.

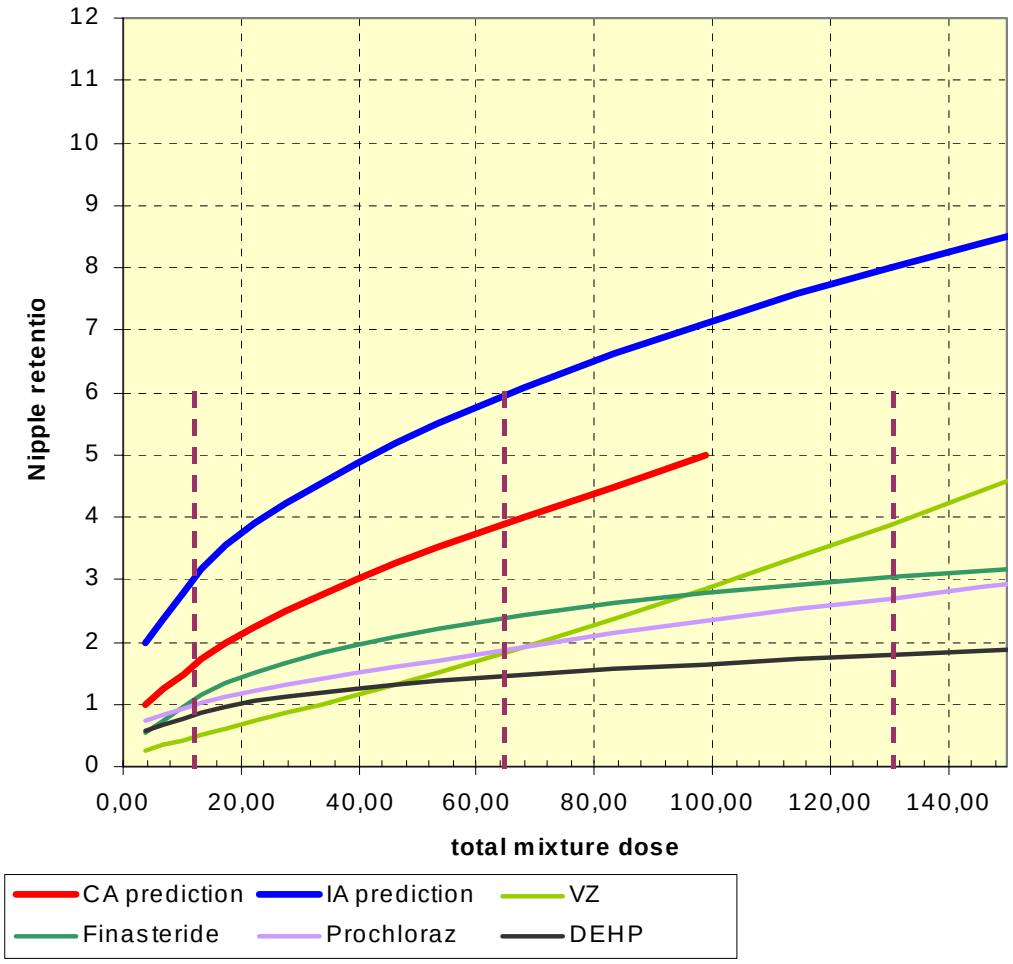

Figure 2 Prediction curve for nipple retention for the mixture and the four chemicals in the mixture. (CA = concentration addition; IA = independent action; VZ = vinclozolin)

Progress Summary

- Partner 1 has shown that at a concentration of 1 x 10^{-6} M PCB 126 significantly decreases the effect of a mixture of 10 mitogenic compounds in the E-Screen. This provides evidence that an anti-estrogen has the ability to oppose the actions of estrogenic chemicals. However, this effect is only seen at relatively high concentrations of the PCB.

- The main findings from the stickleback group were the confirmation of the hypothesis that estrogens acts as anti-androgens at high concentrations by inhibiting spiggin induction after DHT treatment of female sticklebacks. Both estradiol and ethinylestradiol inhibited the androgen-induced protein at high concentrations and resulted in reduced spiggin production in lower concentrations tested.

- For the rat studies undertaken by Partner 20, the term 'dissimilarly acting' has been clearly defined and the mixture study has been carefully planned in detail.

Plan and Objectives for the Next Reporting Period

- Partner 1 will test a large mixture (containing over 25 components) that emulates real-life exposures in the E-Screen. This will be composed of a wide variety of agents, such as steroidal hormones, organochlorine pesticides, phytoestrogens, PCBs, PBDEs, parabens, UV filters and heavy metals. Data emanating from the exposure studies in Theme 1 will be used to construct this mixture.

- Based on the optimisation of real-time PCR and primers for the estrogen receptor, the effect of anti-estrogens, such as PCB 126 on ER levels will be evaluated in MCF-7 cells.

- Partner 1 will also evaluate how the co-planar PCB influences the effects of mitogens and mixtures of mitogens on the same endpoint.

- The plan for Partner 20 is to perform the rat dissimilar mixture study and evaluate the results. The objectives are to evaluate if dose-response curve for the mixture of four dissimilarly acting antiandrogens indicate: concentrations addition, independent action or synergism, mixture effect at dose levels where the chemicals alone do not cause antiandrogenic effects on the male offspring.

- The stickleback group (Partner 2, 8 and 19) will undertake the 8 component dissimilar mixture study. An alternative method for water sampling is proposed that, instead of spot testing, an integrated sampling procedure should be used for the mixture experiments. However, it was pointed out that the sampling method for mixtures and single agents should remain the same. This will assure that the differences between nominal and measured concentrations are the same for single agents and mixtures. If the partners wish to use an integrated sampling procedure for the mixtures, they still need to do spot testing.

Deliverables and Milestones

Deliverables	
✓	**D15.1:** Report outlining half-term work progress with cell-based EDC assays (month 24)
✓	**D15.2:** Report outlining half-term work progress with fish assays (month 24)
✓	**D15.3:** Report outlining half-term work progress with female rat assays (month 24)
in progress (36)	**D15.4:** Report describing the joint effects of dissimilarly acting EDC (month 48)
Milestones	
in progress (36)	**M15.1:** Information about the ability of EDC to oppose the actions of other classes of EDC (month 48)
in progress (36)	**M15.2:** Insights whether these antagonistic effects occur at levels that are relevant in real existing exposure scenarios (month 48)

WP16	Options for incorporating knowledge about low-dose and mixture effects in testing strategies and regulatory efforts					
Phase						
Start date	25					
Completion Date	48 months					
Current Status	active					
Partner(s) responsible:	1	6	11			
Person-months per Partner:	14.2 (10)	10.5 (5.5)	5 (4)			
Already devoted person months per Partner and total:	3.5	0	0			

Objectives for the Reporting Period

- To explore the need for incorporating knowledge about low-dose- and mixture effects of EDC into testing strategies.
- To evaluate the implications of knowledge about low-dose- and mixture effects for risk assessment strategies.
- To carry out a workshop with a panel of experts.

Methodology and Study Materials for the Reporting Period

Jointly, Partners 1, 6 and 11 will carry out:

- Significance analyses: The significance of risks arising from realistic exposure scenarios for multiple EDC at low concentrations (see WP1-4) will be evaluated.
- Uncertainty considerations: The uncertainties of risk estimates for mixtures derived from experimental effect data and from the use of mixture effect prediction models (WP14, 15) will be evaluated.
- Evaluations of data requirements: The demands that adequate risk characterisations place on the quality and quantity of experimental data will be considered.
- Analyses of standard risk assessment procedures: In the light of low-dose- and mixture data from WP 13-15, standard risk assessment methods (PEC/PNEC, safety/uncertainty factors) will be evaluated in relation to their ability to appropriately handle estimated risks, and with respect to their ability to provide adequate protection levels.

These complex issues will be debated with a panel of experts from national regulatory agencies, industry and NGO's at a workshop. WP16 will produce a guidance document on options for the regulation of chemicals able to protect European wildlife and people.

Scientific Achievements

Partner 1 contribution

In collaboration with Partners 6 and 11, Partner 1 has developed a concept for a discussion document that will be tabled at the workshop with experts from industry, regulation and NGO's. This draft document will form the basis for the workshop, and after incorporation of the experts' comment, will be edited to become the final deliverable D16.3. In preparation of the draft document, Partner 1 has prepared a review of data of EDC mixture effects, as well as a review of data of combination effects at low doses, including data from non-EDC studies. Both papers have been submitted to peer-review journals and will be distributed to the experts taking part in the workshop.

To enable meaningful EDC mixtures risk assessments to be carried out, it was crucial to wait for further exposure data emanating from Theme 1 workpackages. Furthermore, important mixture studies are currently underway, and some will be completed in early 2006. For this reason, it was decided to delay the workshop until month 42. It will be held in Granada (Spain), directly following the EDEN Forum meeting in May 2006. The initial organisation for holding the expert panel workshop has begun and a list of experts to invite has been prepared.

Partner 6 and 11 contributions

Partner 6 has initiated collation of exposure data relevant to EDC mixtures in aquatic systems. This collation will directly feed into the document to be prepared for the expert workshop. Partner 11 has begun to review the literature concerning mechanistic approaches to mixture assessment (PBPK modelling etc).

Progress Summary

- A discussion paper in preparation for the expert panel workshop is in preparation, and the organisation of the workshop has begun.

Plan and Objectives for the Next Reporting Period

- Workshop debate with a panel of experts from national regulatory agencies, industry and NGO's to be held in Granada month 42 (D16.2). The outcome could also find entry into the proposed "Weybridge +10" conference in Helsinki, November 2006.

- Production of guidance notes on the implications of knowledge about low-dose- and mixture effects for testing strategies and risk assessment procedures (D16.3)

Workpackage Related Appendices

Annex I (D16.1) First considerations of data requirements for adequate risk characterisations for EDC mixtures

Deliverables and Milestones

Deliverables	
✓ (36)	**D16.1:** First considerations of data requirements for adequate risk characterisations for EDC mixtures (month 36)
delayed til month 42 (36)	**D16.2:** Workshop with a panel of experts (month 36)
in progress (36)	**D16.3:** Guidance notes on the implications of knowledge about low-dose- and mixture effects for testing strategies and risk assessment procedures (month 48)
Milestones	
in progress (36)	**M16.**1: Insights into risk assessment procedures and testing regimens that will provide adequate protection of European citizens and wildlife from exposure to EDC (month 48)

WP17	Cluster for Research on Endocrine Disrupters in Europe					
Phase						
Start date	0					
Completion Date	48 months					
Current Status	active					
Partner(s) responsible:	1	ALL				
Person-months per Partner:	74 (57)	0.2-1 (0)				
Already devoted person months per Partner and total:	34					

Objectives for the Reporting Period

- To establish a focus for ED research in Europe by forming a Network of research.
- To facilitate the development of methods, techniques and know-how on EDC research by exchanging information and data between Network members.
- To co-ordinate the collection of data and it's interpretation and to disseminate information for the public, policy makers, end-users and stakeholders on endocrine disrupters and their possible impacts on the environment and human health

Approaches for the Reporting Period

- Regular exchange of research plans and results within the European Endocrine Disrupter Cluster (CREDO).
- Exchange and dissemination of scientific know-how by visits of scientific and/or technical staff of Partner institutes (expertise and training).
- Co-ordination of sampling programmes and databases within the European Endocrine Disrupter Cluster.
- Linked annual project progress meetings.
- Organisation of annual Cluster workshops on identified topics
- Co-ordinated approach to IPR within the Endocrine Disrupter cluster
- Common cluster and linked project web sites

Scientific Achievements

During the 3rd reporting period, the following meetings have been organised and attended to aid communication within the consortium. On 01.12.04, a planning meeting for the CREDO workshop on 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects' was held. The third and forth thematic workshops focusing on exposure assessment (organised by the FIRE project) and low dose, and mixture effects, as well as epidemiology (organised by the EDEN project) were held in Prague, Czech Republic during month 30. Publication of the thematic workshop will be as a Special Issue of Environmental Health Perspectives. Partner 1 is currently collecting manuscripts from those invited to take part and proceedings will be published by the end of 2006, beginning of 2007.

In terms of cluster activities, the most significant achievement has been the finalisation of the "Prague Declaration on Endocrine Disruption". Partner 1 has drafted this document, and initiated a wide-ranging discussion of this document across the projects participating in the CREDO cluster, including the seven projects that were later loosely associated with the cluster. The declaration summarizes and assesses recent advances in European endocrine disrupter research, including CREDO cluster work, and formulates recommendations for policy action and research. There was a very lively response, and the declaration was finalised during the thematic workshops held in Prague. By October 2005, more than 200 scientists actively engaged in endocrine disrupter research have signed the declaration. This includes prominent scientists from outside the European Union, not directly involved with the CREDO cluster. The declaration has been circulated to the international press and continues to find significant resonance worldwide.

In view of the fact that 2006 will see the 10th anniversary of the 1996 Weybridge meeting on Endocrine Disruption, the idea was conceived to hold a follow-on meeting in the later parts of 2006. Partner 1 was involved in taking steps to initiate this meeting, called "Weybridge+10". It will be organised under the auspices of the Finnish Government who will have the EU presidency in the second half of 2006. Support from DG Research, the European Environment Agency, the Finish Academy of Sciences, and others has been secured.

The website of the CREDO cluster has been continuously updated to include information about the forthcoming cluster workshops, and other issues of relevance to the cluster. The website has developed into an important information resource for cluster members and beyond. Four CREDO newsletters are abilable from both the CREDO and EDEN websites with articles for the fifth currently being collated and edited.

Partner 1 has responded to numerous invitations to present information about the CREDO cluster at international meetings in Europe. Invited talks were given at the Copenhagen Workshop on Environment, Reproductive Health and Fertility, Rigshospitalet in January 15-18th 2005, the 1st International Workshop on Modifiers of Chemical Toxicity (sponsored by DG JRC) in Poros/Athens, Greece, June 12-15, 2005. A presentation 'Latest research and recommendations regarding endocrine disrupting chemicals' was given at the Pesticides Action Network Europe Annual Conference 'Towards better environment, health and rural economies', November 7-9, 2005, Krakow, Poland. A poster presentation 'Is the regulation of endocrine disrupters within REACH?' was

presented at the Copenhagen Workshop on Environment, Reproductive Health and Fertility, Rigshospitalet in January 15-18th 2005.

Progress Summary

Communication between Partners and interested parties has continued along with dissemination of activites by inviteds talks and via the CREDO and EDEN websites. Preparation and finalisation of the Prague Declaration has provided a focus for, as well as a resumee of, CREDO cluster activities.

Plan and Objectives for the Next Reporting Period

- Remaining 4 CREDO newsletters to be completed and website maintained.
- Activities will focus on preparing the Weybridge+10 meeting in Helsinki, November 2006. To this end, four thematic working groups have already been set up by the Finish Academy of Sciences, and Partner 1 is involved as the rapporteur for a working group on risk assessment. In preparation of the Weybridge+10 conference, it is planned to hold a preparatory meeting in May 2006, likely on the island of Kos, Greece. To objective of this meeting will be to agree on a framework document for the main meeting in Helsinki, with a view to ratifying an updated document at the end of 2006.

Workpackage Related Appendices

Annex I (D17.3) Production of Cluster newsletter (8 editions)

Annex I (D17.5) Preparation of coordinated Cluster press releases

Annex I (D17.6) Representation of ED research priorities at relevant EU policy meetings

Annex I (D17.7) Thematic workshop on 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects'

Deliverables and Milestones

Deliverables	
✓ ongoing	**D17.1:** To lead CREDO cluster workshops and to provide planning, co-ordination, logistic support and facilities
✓	**D17.2:** Setting-up and maintaining of a dedicated Cluster website (month 3 onwards)
✓ (6,12,18,24) 5th in progress	**D17.3:** Production of a Cluster newsletter (8 editions) (months 6, 12, 18, 24, 30, 36, 42, 48)
✓	**D17.4:** Production of a Cluster brochure (month 3)
✓ (0-24)	**D17.5:** Preparation of coordinated Cluster press releases
✓	**D17.6:** Representation of ED research priorities at relevant EU policy meetings (to a maximum of 2 meetings per year)
✓	**D17.7:** Thematic workshop on "dose-response analysis and mixture effects, testing guidelines, epidemiology (month 18)
in progress (36)	**D17.8:** Preparation of a policy relevant final report of the cluster workshop(s) (month 42)
Milestones	
✓	• Common entry/information point for EU Endocrine Disrupter research information (website)
✓	• Focus for EU Endocrine Disrupter research through the formation of an ED Cluster open to related existing and future EU supported RTD projects (month 6 onwards)
✓	• Determination of priorities, strengths and weaknesses of/for EU Endocrine Disrupter research (month 18)
✓ ongoing	• Acknowledgement of the EU Endocrine Disrupter cluster in all scientific reports and other dissemination activities
✓ (30) publication due end 2006 (36)	• Thematic workshop on "dose-response analysis and mixture effects, testing guidelines, epidemiology and related topics" (month 18) and published proceedings (month 24)

5. PROJECT MANAGEMENT AND COORDINATION

The following specific coordination actions were undertaken during the third year of the EDEN project:

5.1 EDEN Project Meetings

1. On 01.12.04, a planning meeting for the CREDO workshop on 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects' was held in Brussels. The minutes for this meeting are available in Annex II (1).

2. On 18.01.05, a local EDEN meeting regarding rat studies between Partner 1 and Partner 20 was held in Copenhagen. Minutes are given in Annex II (2).

3. On 21.03.05, the School of Pharmacy was the meeting place for a local meeting on stickleback studies. Partners 1, 2, 8 and 19 were present in addition to Steve Morris who is subcontractor for the analytical work at CEFAS. Annex II (3) has the minutes for this meeting.

4. On 09.05.05, the Mid Term review for EDEN was held at Hotel Praha in Prague. The examiners were Lou Guillette and Carlos Sonnenschein and the meeting was chaired by Tuomo Karjalainen. Thematic presentations were given by the Theme coordinators with lively discussions throughout. The minutes are given in Annex II (4).

5. On the 10th and 13th 05.05, the EDEN forum meeting and thematic meetings took place. Annex II (5) details the minutes on a theme by theme basis.

6. On 15.11.05, the Steering Board convened in Brussels. This was to review current work progress for EDEN and discuss dates and venue for the EDEN Forum and Thematic meetings. The format for the scientific report has also been modified following negotiations with Tuomo Karjalainen. The minutes are given in Annex II (6).

7. Partners 2, 3 and 4 were active in facilitating interactions between all partners involved with work for Themes 1-3. Many of these exchanges utilised email and/or telephone to avoid excessive travelling.

5.2 Contribution of EDEN to CREDO Cluster Activities

Management of the Cluster of Research into Endocrine Disrupters in Europe (CREDO) has continued throughout the third year by undertaking the following co-ordination actions:

1. Partner 1 has maintained a dedicated CREDO cluster website which serves as a portal for all participating cluster projects, provides information about cluster activities and gives an introduction into the ED problem targeted at interested lay people. The site was continuosly updated. It can be found at

www.credocluster.info. In order to highlight the forthcoming Prague workshop in May 2005, the look of the portal on the CREDO website was modified.

2. During the 3rd reporting period a further newsletter has been produced and circulated in May 2005. Research news from several of the projects within CREDO are presented including the latest in glowing fish, the completion of the pre-screening phase within FIRE and a focus of the issues relating to male reproductive health. From a regulatory perspective of endocrine disrupters, two articles looked at the implementation of the European Commission strategy and the proposed European regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). A summary of the COMPRENDO workshop on the ecological relevance of chemically induced endocrine disruption in wildlife, which was held in July 2004 in Exeter along with further details regarding the publication of the workshop proceedings was made available. All four issues can be downloaded from the EDEN website (http://www.edenresearch.info/news.html) and also the CREDO website (http://www.credocluster.info/resources.html).

3. The fifth issue is currently in preparation and is anticipated to go to print in January 2006. Included is a report on the completion of the ACE project and research news from GENDisrupt and FIRE. Details for the final COMPRENDO project workshop to be held in March 2006 are given as well as a summary of the CREDO workshop organised by EDEN and FIRE which was held in Prague in May 2005.

4. Partner 1 has represented the cluster at international meetings in Europe by responding to invitations to present information about the CREDO cluster. Invited talks were given at numerous meeting, all detailed in the report about WP17. Partner 1 has instigated the Prague Declaration on Endocrine Disruption which summarizes ongoing research and research achievements in the CREDO cluster and makes suggestions concerning better chemicals regulation and about further research activities.

5.3 Other Management Activities

1. Partner 1 has continued to maintain the EDEN website (http://www.edenresearch.info/). The ‘what’s new’ section has been continually updated including a section on the Prague Declaration on Endocrine Disruption and there is also a separate header with the complete document. The overview for the 2nd scientific report is available for all with the complete report accessible to EDEN members on the ‘members only’ site. All four issues of the CREDO Cluster newsletter are also available as pdf on the main site along with a synopsis of each issue.

2. Helmet Segner (Partner 15) has replaced Bernard Hock (Partner 13) as the Responsible Partner/WP Collator for Workpackage 5.

3. Meetings scheduled for the next reporting period are detailed below.

4. The 5th CREDO Cluster newsletter is currently in press with the 6th newsletter in progress with articles being received. The 5th newsletter holds several articles from the FIRE project and a report on the EDEN/FIRE workshop that was held in May 2005. The Prague Declaration on Endocrine Disruption is also highlighted.

5. Partner 16 has changed addresses which have been updated in both the 3rd Annual report and on the EDEN website.

5.4 Meetings Scheduled for the Next Reporting Period

In summary, the following meetings have taken place or are scheduled for EDEN in the forthcoming reporting period:

The Steering board meeting for month 30 was cancelled due to the Mid Term Review and Forum and Thematic meetings taking place at the same time.

The Forum and Thematic meetings proposed for month 42 will be held in May 2006 and the venue is currently being finalised (expected to be Granada or Malaga) with organisation to begin in early 2006.

The WP16 workshop with a panel of experts due month 36 (D16.2) is scheduled for May 2006 to follow on from the EDEN Forum and Thematic meetings. Early meetings have already been undertaken and experts are currently being reviewed for the panel.

Cluster meetings are planned for July 2006 provisionally to be held in Greece to appraise the cluster research and other cluster related activities.

Organisational level	Date (month)	Purpose	
Steering board	0	Preparation of "kick-off" forum meeting	✓
Forum	3	"Kick-off" meeting	✓
Thematic groups	3	Co-ordination of research activities within themes 1 - 4	✓
Steering board	9	Progress review	✓
Thematic groups	9	Review of research, planning, collation of materials for deliverables D1.1, D1.2, D5.1, D6.1, D9.1, D13.1, D13.2, D13.3,	✓
Steering board	15	Preparation of forum meeting in month 18, preparation of EDEN cluster workshop in month 18	✓
Forum	18	Review of data, research and progress	✓
Thematic groups	18	Review of research, planning, collation of material for deliverables D3.1, D5.2, D8.1, D17.7	✓
Steering board	24	Progress review, preparation of forum meeting in month 24	✓
	24	Midterm review by external experts	✓ (mth 30)
Thematic groups	24	Review of research, planning, presentation of deliverables D4.1, D6.2, D10.1, D11.1-11.3, D12.1, D12.2, D13.4, D14.1-14.3, D15.1-15.3,	✓
Steering board	30	Progress review	cancelled
Forum	30	Discussion of general issues relevant to	✓

		work in progress	
Thematic groups	30	Review of data, research and progress, collation of materials for deliverables D3.2, D3.3, D5.3, D5.4, D6.3-6.5, D7.1-7.6, D8.2-8.4, D12.3, D16.2	✓
Steering board	36	Progress review, preparation of forum meeting in month 42	✓
Forum	42	Discussion of issues relevant to completion of project	on schedule
Thematic groups	42	Review of data, research and progress, collation of materials for deliverables D4.3, D4.4, D10.2, D10.3, D14.4, D15.4, D16.3	on schedule
Steering board	45	Preparation of final report to European Commission	on schedule
Forum	48	Workshop with experts from industry, government agencies, NGO's, EC officials	

To ensure effective management of the cluster, EDEN will organise the following meetings:

Organisational level	Date (month)	Purpose	
Cluster coordinators	0	Preparation of a cluster "kick-off" meeting	✓
All cluster participants	3	Cluster "kick-off" meeting	✓
Cluster coordinators	15	Evaluation of cluster "kick-off" meeting, preparation of workshops on wildlife and dose-response analysis and mixture effects, testing guidelines, epidemiology and related topics in month 18	✓
All interested cluster participants	18	Cluster workshop on dose-response analysis and mixture effects, testing guidelines, epidemiology and related topics	✓ (mth 30)
Cluster coordinators	24	Evaluation of cluster workshops and preparation of further cluster workshops	✓
Cluster coordinators	30	Preparation of a final cluster meeting	✓
All cluster participants	36	Presentation and appraisal of cluster research	in progress delayed till mth 44

6. EXPLOITATION & DISSEMINATION ACTIVITIES

Below is a list of publications for each Partner followed by presentations given for the 3rd reporting period.

6.1 Scientific Publications

Partner 1

Arrhenius Å, Backhaus T, Grönvall F, Junghans M, Scholze M, and Blanck H, (2005). Effects of three antifouling agents on algal communities and algal reproduction: Mixture toxicity studies with TBT, Irgarol, and Sea-Nine. *Arch. Environ. Contam. Toxicol*. In Press.

Rajapakse N, Butterworth M, and Kortenkamp A, (2005). Detection of DNA strand breaks and oxidized DNA bases at the single-cell level resulting from exposure to estradiol and hydroxylated metabolites. *Env. Mol. Mutagen.* **45**, 397-404.

Kortenkamp A, Silva E, Rajapakse N, and Scholze M, (2005). Are there risks from low dose mixture effects of endocrine disrupters? In: *Chemikaline in der Umwelt mit Wirkung auf das endocrine System*. 3. UBA Statusseminar. Fraunhofer IRB Verlag Stuttgart, Germany.

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

Partner 5

Wang Y, Suominen JS, Parvinen M, Rivero-Muller A, Kiiveri S, Heikinheimo M, Robbins I, Toppari J, (2005). The regulated expression of c-IAP1 and c-IAP2 during the rat seminiferous epithelial cycle plays a role in the protection of germ cells from Fas-mediated apoptosis. *Mol. Cell Endocrinol*. **245** (1-2) 111-120.

Myllymaki SA, Haavisto TE, Brokken LJ, Viluksela M, Toppari J, Paranko J, (2005). In utero and lactational exposure to TCDD; steroidogenic outcomes differ in male and female rat pups. *Toxicol Sci.* **88** (2) 534-44.

Myllymaki SA, Karjalainen M, Haavisto TE, Toppari J, and Paranko J, (2005). Infantile 4-tert-octylphenol exposure transiently inhibits rat ovarian steroidogenesis and steroidogenic acute regulatory protein (StAR) expression. *Toxicol Appl Pharmacol*. **207** (1) 59-68.

Ahtiainen P, Rulli SB, Shariatmadari R, Pelliniemi LJ, Toppari J, Poutanen M, and Huhtaniemi IT, (2005). Fetal but not adult Leydig cells are susceptible to adenoma formation in response to persistently high hCG level: a study on hCG overexpressing transgenic mice. *Oncogene.* **24** (49) 7301-7309.

Santti H, Mikkonen L, Anand A, Hirvonen-Santti S, Toppari J, Panhuysen M, Vauti F, Perera M, Corte G, Wurst W, Janne OA, and Palvimo JJ, (2005). Disruption of the murine PIASx gene results in reduced testis weight. *J. Mol. Endocrinol*. **34** (3) 645-54.

Myllymaki S, Haavisto T, Vainio M, Toppari J, and Paranko J, (2005). In vitro effects of diethylstilbestrol, genistein, 4-tert-butylphenol, and 4-tert-octylphenol on steroidogenic activity of isolated immature rat ovarian follicles. *Toxicol. Appl. Pharmacol*. **204** (1) 69-80.

Partner 9

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, and Olea N, (2005). Environmental and lifestyle factors for organochlorine exposure among women living in southern Spain. *Chemosphere*. In Press.

Araque P, Soto AM, Olea-Serrano FM, Sonnenscheinm C, and Olea N, (2006). Pesticides in human fat and serum samples versus total effective xenoestrogen burden. In *Pesticide Protocols*. JL Martinez Vidal, A Garrido Frenich eds. Methods in Biotechnology. Humana Press, NJ, USA.

Ribas-Fitó N, Ballester F, Grimalt JO, Marco A, Olea N, Posada M, Rebagliato M, Torrent M, Sunyer J. Environment and Child's Health: The INMA Spanish Study. *Paed. Perinatal Epidemiol.* In Press.

Fernández MF, Olmos B, and Olea N, (2005). Endocrine disrupter chemicals as prenatal risk factors for cryptorchidism and hypospadias. In *Congenital annomalies*. Nicolopoulo-Stamati, P., Hens, L., Howard, C.V. eds. Kluwer Academic Publishers, NJ, USA. In Press.

Nicolás Olea y Mariana F. Fernández Cabrera. Xenoestrógenos y cáncer de mama. In *Cancer de mama*. J. Diaz Faes, A. Rubial Morell eds. Fundación de Estudios Mastológicos (FEMA) Madrid, Spain. In Press.

Partner 13

Alberti M, Kausch U, Haindl S, Seifert M, (2005). Gene expression analysis for exposure to estrogenic substances. *Acta Hydrochim. Hydrobiol*. **33** (1) 38-44.

Alberti M, Kausch U, Haindl S, Leibiger R, Budczies J, Seifert M, Hock B, (2005). Gene expression patterns - a tool for bioanalysis, *Int. J. Environ. Anal. Chem*. **20** (20) 589-608.

Partner 19

Katsiadaki I, Morris S, Squires C, Hurst MR, James JD, and Pickering Scott A, (2005). A sensitive, in vivo test for the detection of environmental anti-androgens, using the three-spined stickleback (*Gasterosteus aculeatus*). *Environ. Health Persp.* In Press.

Partner 20

Vinggaard AM, Hass U, Dalgaard M, Andersen HR, Bonefeld-Jørgensen E, Christiansen S, Laier P, and Poulsen ME, (2005). Prochloraz – an imidazole fungicide with multiple mechanisms of action. *Int. J. Andrology*. In Press.

Borch J, Axelstad M, Vinggaard AM, and Dalgaard M, (2005). Diisobutyl phthalate has comparable anti-androgenic effects to di-n-butyl phthalate in fetal rat testis. *Toxicol. Letters*. In Press.

Laier P, Metzdorff SB, Borch J, Hagen ML, Hass U, Christiansen S, Filinska M, Kledal T, Dalgaard M, McKinnell C, Brokken L, and Vinggaard AM, (2005). Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol. Appl. Pharmacol.* In Press.

Partner 1 and 7

Mihaich EM, Borgert C, Brighty GC, Kortenkamp A, Laenge R, Snyder SA, Sumpter J (2005). Evaluating simple and complex mixtures containing pharmaceuticals in the environment. In: *Human Pharmaceuticals: Assessing the Impacts on Aquatic Ecosystems* Williams RT, (Ed). SETAC press ISBN 1-880611-82-1.

Brian JV, Harris CA, Scholze M, Backhaus T, Booy P, Lamoree M, Pojana G, Jonkers N, Runnalls T, Bonfà S, Marcomini A, and Sumpter JP, (2005). Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environ. Health Persp.* **113** (6) 721-728.

Partner 1 and 9

Silva, E., Molina-Molina, J.M., Fernández, M., Lopez-Espinoza, M.J., Olea, N. and Kortenkamp, A. (2006). Inactivity of cadmium in a variety of *in vitro* estrogenicity assays. *Tox. Appl. Pharmacol.* In Press.

Partner 1 and 14

Le Page Y, Scholze M, Kah O, and Pakdel F, (2005). Assessment of xenoestrogens using three distinct estrogen receptors and zebrafish brain aromatase gene in a highly responsive glial cell system. *Environ. Health Persp*. In Press.

Partner 5 and 17

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. *Endocrinol.* **146** (12) 5164-5175.

Partner 5 and 14

Dorval-Coiffec I, Delcros JG, Hakovirta H, Toppari J, Jegou B, and Piquet-Pellorce C, (2005). Identification of the leukemia inhibitory factor cell targets within the rat testis. *Biol. Reprod*. **72** (3) 602-11.

Partner 9 and 19

Cerrillo I, Granada A, MJ Lopez-Espinosa, Olmos B, Jimenez M, Araque P, Olea N, Katsiadaki I, (2005). Using the stickleback to monitor androgens and anti-androgens in the aquatic environment. In *Techniques in Aquatic Toxicology Volume II*, edited by Gary K. Ostrander, pp 339-356. (The publication was after invitation by the editor.)

6.2 Scientific Workshops/Presentations

Partner 1

Kortenkamp A, Multi-component mixtures of endocrine disrupters - experimental requirements and recent test results. "Environmental Endocrine Disrupters 2004"**,** a meeting organised by the Japanese Ministry of the Environment, 18 December 2004, Nagoya, Japan

Kortenkamp A, Estrogens, environmental pollutants and breast cancer – a re-evalaution from a mixture perspective. 3rd Copenhagen Workshop on Environment, Reproductive Health and Fertility, Copenhagen, Copenhagen, Denmark 15-18 January 2005

Kortenkamp A, Breast cancer and environmental pollutants, INMA workshop, 4 February 2005, Granada, Spain.

Kortenkamp A, CREDO – Research on endocrine disrupters and their effects on human health and the environment, EC DG Research Workshop on Internatioanl Collaboration in EDC Research, 26 January, Brussels, Belgium.

Scholze M. Quantitative considerations of low-dose testing. Invited speaker, CREDO Workshop, Prague, Czech Republic 10-12 May 2005.

Kortenkamp A, Are there risks from low dose mixture effects of endocrine disrupters? 3rd UBA Status seminar on chemicals with endocrine activity, 2 June 2005, Berlin, Germany

Kortenkamp A, Endocrine disrupters - mixture effects at low doses. 1st International workshop on modifiers of chemical toxicity - implications for human health risk assessment, a workshop sponsored by the European Commission and the Environmental and Occupational Health Sciences Institute, 12-15 June 2005, Poros, Athens, Greece

Pedersen R, Latest research and recommendations regarding endocrine disrupting chemicals. PAN European Annual Conference, 7-9 November 2005, Krakow, Poland

Kortenkamp A, Prediction and assessment of mixture effects, SENSPESTI workshop, 25-26 November 2005, Parma, Italy

Partner 4

Skakkebæk NE, Andersson AM, Juul A, Jørgensen N, Leffers H, Main KM, Rajpert-De Meyts E, Mortensen GK, and Toppari J. Is human fecundity declining? In the 3rd Copenhagen Workshop on Environment, Reproductive Health and Fertility, Copenhagen, Copenhagen, Denmark 15-18 January 2005.

Andersson AM. Trends in reproductive hormone levels in Danish men. In the CREDO Pregue Workshop on endocrine disrupters: exposure assessment, epidemiology, low-dose and mixture effects. Prague, Czech Republic 10-12 May 2005.

Andersson AM. Trends in reproductive hormone levels in Danish men. 21st Annual meeting of the European Society of Human Reproduction and Embryology. Copenhagen, Denmark June 2005.

Andersson AM. Trends in male reproductive health and ecological exposure to persistent organic pollutants in Nordic countries. In the Finnish Society of Toxicology Annual Symposium on Risk, Reproduction and Cell Signalling-Multidisciplinary Collaboration in Toxicology". Turku, Finland 19-20th May 2005.

Partner 5

Adamsson A, Brokken L, Paranko J, and Toppari J. In utero and in vitro effects of flutamide and diethylstilbestrol on prenatal testicular testosterone production in rat. In the 3rd Copenhagen Workshop on Environment, Reproductive Health and Fertility. Copenhagen, Denmark 15-18 January 2005.

Haavisto TE, Myllymäki SA, Adamsson NA, Viluksela M, Toppari J, and Paranko J. The effects of the maternal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on testicular testosterone production in infantile male rats. In the 3rd Copenhagen Workshop on Environment, Reproductive Health and Fertility, Copenhagen, Denmark 15-18 January 2005.

Nurmio M, Toppari J, Paranko J, Söder O, and Jahnukainen K. Effects of tyrosine kinase inhibitor Imatinib mesylate (Glivec®) on testicular development in rat. In the 3rd Copenhagen Workshop on Environment, Reproductive Health and Fertility, Copenhagen, Denmark 15-18 January 2005.

Paranko J, Brokken L, Adamsson A, and J. Toppari. In utero exposure to the anti-androgen flutamide interferes with foetal Leydig cell function. In the CREDO Pregue Workshop on endocrine disrupters: exposure assessment, epidemiology, low-dose and mixture effects. Prague, Czech Republic 10-12 May 2005.

Nurmio M, Toppari J, Zaman F, Paranko J, Söder O, and Jahnukainen K. The tyrosine kinase inhibitor imatinib mesylate affects postnatal testicular development in the rat. In the European Congress of Endocrinology 2005 on Expanding Endocrinology, Göteborg, Sweden 3-7 September 2005.

Toppari J. Physiology of sexual determination and differentiation. Suomen lastenendokrinologien satelliittisymposium. Helsinki, Finland 19 October 2005.

Toppari J. Differences in male reproductive health between Denmark and Finland: effects of perinatal exposures. In the CREDO Pregue Workshop on endocrine disrupters: exposure assessment, epidemiology, low-dose and mixture effects. Prague, Czech Republic 10-12 May 2005.

Partner 11

Brandenberger C, Cheshenko K, and Eggen R. Construction of *cyp19a/b*-EGFP reportergene vectors and examination of their expression in zebrafish embryos. Diploma thesis, Federal Institute of Technology Zurich (ETHZ). 2005.

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.

Partner 13

Hock, B. IAEAC Workshop Meeting. Rome, Italy 2005

Hock, B. EMCO Workshop Meeting. Dubrovnik, Croatia 2005.

Hock, B. Frontiers in Environmental Science. Kyoto, Japan 2005

Partner 19

Katsiadaki I, (Invited speaker). SETAC (Society for Environmental Toxicology and Chemistry), Europe, Lille, France, May 2005.

Katsiadaki I, (Invited speaker). PRIMO (Pollutant responses in Marine Organisms) 13, Allessandria, Italy, June 2005.

Katsiadaki I, (Invited speaker). UK-Japan Co-operation on Endocrine Disruptors, Workshop, Okinawa, Japan, December 2005.

Partner 20

Hass U, Christiansen S, Dalgaard M, Filinska M, Borch J, Vinggaard AM, Metzdorff SB. Low-dose effects of anti-androgens in male rat offspring after perinatal exposure. Presentation by Ulla Hass as invited speaker at the CREDO Cluster Workshop on Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects, Prague, May 2005.

Christiansen S, Hass U, Filinska M, Dalgaard M, and Vinggaard AM. Pre- and postnatal exposure to the imidazole fungicide prochloraz feminizes the development and behaviour of the male rat offspring. Poster presented at the European Teratology Society Conference, September 2005, Netherland. Abstract in Reprod Toxicol 2005.

Hass U. Investigations of reproductive behaviour in current reproductive toxicity test guidelines. Invited speaker at Symposium on Reproductive Behaviour and Environmental Pollutants, Stockholm, September 2005.

7. ETHICAL ASPECTS AND SAFETY PROVISIONS

No ethical issues/concerns were raised during the third reporting period.

ANNEX I: DELIVERABLES

Contents

D1.1 Annual report on the progress of human adipose sample delivery

Responsible Partner: 5 **Participating Partners: 4, 9**

Objectives

- Collection of adipose tissue samples from boys with undescended testes (cryptorchidism) and their controls (boys undergoing hernia operation)
- Delivery to the laboratories performing the chemical exposure analyses
- Complete the recruitment of patients with breast cancer

Research Progress

Samples from cryptorchid boys and controls: Partner 5 has collected 33 fat samples from the cryptorchid cases and 26 samples from the controls. Four of the cases, however, had unilateral anorchia and therefore have to be separated from the cryptorchid group. It proved to be particularly difficult to get samples from controls, i.e., boys undergoing herniotomy or other abdominal surgery in Turku University Central Hospital, and therefore we did not reach 30 that was the aim. For similar reasons it was already earlier decided that the number of samples in Copenhagen (P4) (10 + 10) will be smaller than in Turku. This goal has now been achieved and there are 13 samples from cryptorchid boys and 10 from controls in Copenhagen. There will be three more control samples due in month 38. Part of the fat samples has already been sent to Kuopio (Partner 10), while part of them is currently stored in Turku and Copenhagen, and they will be delivered to Kuopio by month 38.

Cohort study of breast cancer patients: A total of 56 women who underwent surgical treatments for breast cancer were recruited by partner 9 in order to increase the information regarding chemical content and TEXB activity in patients with malignant disease of the breast. The study consists of a follow up of breast cancer patients during 18 months, with five points of performance: surgery (0 months), period 1 (1 to 5 months), period 2 (6 to 11 months), period 3 (12 to 17 months) and period 4 (18 to 22 months).

Discussion and Conclusions

Samples from cryptorchid boys and controls: The sample collection has now been almost completed (3 more samples scheduled to arrive in month 38). Partner 9 has done extensive chemical analyses of mothers' breast milk samples and placentas from cryptorchid boys and their controls. Dioxins and furans, PCBs, selected organochlorine pesticides, phthalates and polybrominated diphenyl ethers were analysed as a part of another EU project, EXPORED. On the basis of those analyses it was decided that the small fat samples that should have been analysed for polybrominated diphenyl ethers will be undertaken in Kuopio by Partner 10 during the fourth year.

Breast cancer patients: A significant number of new breast cancer patients have been recruited. Clinical variables including response to treatments have been collected.

D1.2 Delivery of human adipose tissue samples to Partners 4, 9, 10, 11, 12

Responsible Partner: 5 **Participating Partners: 4, 9**

Objectives

- Delivery to the laboratories performing the chemical exposure analyses
- Complete the recruitment of patients with breast cancer

Research Progress

Samples from cryptorchid boys and controls: Part of the fat samples have already been sent to Partner 10 in Kuopio by Partner 4, while part of them are currently stored in Turku and Copenhagen, and they will be delivered to Kuopio in January 2006 (month 38).

Cohort study of breast cancer patients: Partner 9 delivered the planned samples already during previous reporting periods, and further work is an extension with a follow-up of patients.

Discussion and Conclusions

Samples from cryptorchid boys and controls: The sample collection has now been almost completed (3 more samples scheduled for month 38). Extensive chemical analyses have been undertaken with mothers' breast milk samples and placentas from cryptorchid boys and their controls. Dioxins and furans, PCBs, selected organochlorine pesticides, phthalates and polybrominated diphenyl ethers were analysed as a part of another EU project, EXPORED. On the basis of those analyses it was decided that the small fat samples that should be analysed for polybrominated diphenyl ethers will be done in Kuopio by Partner 10 during the fourth year.

Breast cancer patients: A significant number of new breast cancer patients have been recruited. Clinical variables including response to treatments have been collected.

Interaction and Collaboration

In line with the original intentions of the Workpackage plan, active interaction with other concerned Partners has been implemented:

- *Partner 4 – Partner 5* have actively collaborated in studies characterising the cryptorchid cohort and published several joint studies on that. Extensive chemical analyses of breast milk samples and placentas guided the decision making to restrict the chemical analyses of the new fat samples to analysis of polybrominated diphenyl ethers.

D2.1 Collection of bile and adipose tissue from wild fish collected at freshwater pollution hot-spots and control sites in the European Union

Responsible Partner: 6 **Participating Partners: 8, 11**

Objectives

- To deliver adipose tissue and bile samples from fish showing signs of endocrine disruption, and from controls
- To examine fish for symptoms of endocrine disruption

Research Progress

The deliverable incurred further delays due to the late distribution of samples. The delivery of samples of adipose tissue, bile and muscle to the labs of partners 4, 9 10, 11 and 12 was completed during the 3rd reporting periodas well as the examination of the fish for endocrine disruption.

For Partner 6, the distribution of samples (D2.1 and M2.1) was delayed due to a miscommunication with one of the sub-contractors and due to uncertainties over the definite scheme for sample distribution. The latter issue was solved at the EDEN meeting in Prague (May, 2005) forum meeting of EDEN.

At this meeting the selection scheme for fish tissue samples to be used in WP3 and WP4 was discussed in detail. It was concluded that:

- A technical report from partner 6 provides extensive information on the range of contaminants present in fish from the Dutch EDEN study sites
- This, it was agreed, renders any unfocused analytical effort by the EDEN partners an unnecessary duplication of effort
- Following discussion by the participating partners, the previous strategy for selection of samples for analysis, as outlined in the EDEN Technical Annex, was abandoned
- Instead, an analytical strategy that targets endpoints was adopted

Two issues will be addressed a) does the range of chemical contaminants associated with the presence of ovotestis (intersex) differs from that in unaffected fish and b) do tissue chemical contamination profiles explain divergent plasma VTG levels in bream from a site not known to be impacted by STW effluent.

D3.2 Database on EDC in human and fish tissues

Responsible Partner: 9 **Participating Partners: 4, 10**

Objectives

- To provide information on the spectrum of known EDC in human and fish tissues and to produce data about their levels.
- To establish whether tissue specimens from humans and fish with reproductive disorders show a spectrum of EDC that is qualitatively and quantitatively different from that found in controls free of symptoms.
- To add the exposure information from the boy samples to a European database on male reproductive health and to create a new database on breast adipose tissue samples and on fish tissue specimens.

Research Progress

Data base of of human breast cancer and control tissues: Partner 9 is currently making a database and tissue bank to store data on known EDC levels in a wide range of the Spanish population. Data and samples are stored from human tissues (from tissue samples from patients with reproductive disorders, as well as from human tissue from controls free of symptoms). Data of twenty samples (the same that were sent to Kuopio (Partner10), Copenhagen (Partner 4) and EAWAG (Partner11), 10 breast cancer cases and 10 controls) investigated for the presence of 18 organochlorine pesticides (*o,p*-DDT, *p,p*'-DDT, *o,p*-DDD, *p,p*'-DDE, Methoxychlor, Mirex, Lindane, Aldrin, Endrin, Dieldrin, Endosulfan I, Endosulfan II, Endosulfan ether, Endosulfan lactone, Endosulfan diol, and Endosulfan sulfate, Hexaclorobenzene (HCB) and Vinclozolin as well as PBDEs, PBBs, and TCDD/Fs are now included in the database. In addition, data on xenoestrogen content of a total of 56 women who underwent surgical treatments for breast cancer recruited by Partner 9 (WP1) is being produced. Organocholorine pesticides are already analysed. Samples are ready to be delivered to Partner 10 for further analysis. The study consists of a follow up of breast cancer patients during 18 months, with five points of performance: surgery (0 months), period 1 (1 to 5 months), period 2 (6 to 11 months), period 3 (12 to 17 months) and period 4 (18 to 22 months).

Data base on the total effective xenoestrogen burden (TEXB) assessment of human tissue samples: Human tissue samples (56) have been studied so far in the E-Screen bioassay. Eight hundred mg of each sample was extracted using hexane. Extracts of both mammary and abdominal adipose tissues were passed through HPLC in order to separate more polar xenoestrogens from lipophilic compounds (alpha and beta fractions). The total effective xenoestrogen burden (TEXB) has been measured in all alpha and beta fractions. Data are stored in the database under construction.

Data base of fish samples from the Netherlands and Switzerland: Partners 10 has received bream samples from the Netherlands and Switzerland. In Prague, the whole fish analyses were rearranged. This meant that the analysis was not any longer based on geographical distribution of fish according to the pollution level of each study location. Instead it was decided that the analysis of bream was based on the case-control setting in which the cases were male breams with ovotestis and controls were breams with no ovotestis. The

selected fish in each case-control set were equally old and caught from the same location. The analysis of those selected fish (numbering 39) is currently ongoing by Partner 10. Chemical analysis will be ready by the end of January 2006 (month 38). To date they have not received any positive or negative fractions in YES or YAS assays in Kuopio (Partner 10).

Partner 9 received bream samples. Organochlorine pesticides (HCB, lindane, endosulfan and metabolites, *p,p*'-DDT and metabolites) have already been analysed.

Organochlorine pesticides in fish tissue simples

Pesticide	**Median**	**Frequency**	**min**	**max**
HCB	11.62*	94.7	-	84.61
Lindane	11.53	94.7	-	80.09
Endo-ether	6.02	78.9	-	24.75
Aldrin	20.49	94.7	-	85.50
Endo-lactone	18.22	94.7	-	149.45
Endo-I	14.13	89.5	-	48.05
DDE	64.31	100	19.16	728.72
DDD	74.26	100	14.60	748.92
Dieldrin	26.47	100	2.06	124.65
Endrin	92.29	94.7	-	423.54
Endo-II	32.44	89.5	-	121.99
o,p-DDT	153.32	78.9	-	728.83
p,p'-DDT	76.13	84.2	-	489.65
Endo-sulfate	8.19	68.4	-	271.46
Methoxychlor	23.54	100	-	117.82
Mirex	0	5.3	-	23.36

*ng/g tissue

Total effective xenoestrogen burden (TEXB) assessment of fish tissue samples: Fish tissue samples (39) have been studied so far in the E-Screen bioassay. Samples selected for extraction and TEXB determination and chemical content were the same as in OC pesticide residues experiments: D59, A82, A24, A105, D69, A01, A32, A29, D32, A100, A111, B38, D27, A51, A19, B97, D60, A21, A11, B95, D67, A50, A55, B45, A65, A44, A23, B21, A02, A13, A40, A66, A113, A73, A71, A77 and A46

Two hundred mg of each sample was extracted using hexane. Extracts were passed through HPLC in order to separate more polar xenoestrogens from lipophilic compounds (alpha and beta fractions). The total effective xenoestrogen burden (TEXB) has been measured in all alpha and beta fractions. Data are stored in the data base under construction.

Discussion and Conclusions

- Data on EDC content in mammary and abdominal adipose tissues from human breast cancer patients have been produced and is now being included in the database of exposure.
- Data on EDC content in fish tissues has been produced for organochlorine pesticides and is included in the database of exposure.

D3.3 Levels of EDC in human and fish tissues

Responsible Partner: 9 **Participating Partners: 3, 9, 10**

Objectives

- To provide information on the spectrum of known EDC in human and fish tissues and to produce data about their levels.
- To establish whether tissue specimens from humans and fish with reproductive disorders show a spectrum of EDC that is qualitatively and quantitatively different from that found in controls free of symptoms.
- To add the exposure information from the boy samples to a European database on male reproductive health and to create a new database on breast adipose tissue samples and on fish tissue specimens.

Research Progress

Development of high specificity and sensitivity methods to quantify known EDCs:

1. Bisphenol A, chloro BPA and phthalates: In addition to the chromatographic methodologies developed by Partner 9 to quantify ED pesticides, including vinclozolin, in different specimens they have devoted the last months to the development of the new techniques described below: First, fousing on the analysis of bisphenol-A and chlorinated derivatives mono, di, tri, tetrachloro (BPA-CL, BPA-CL2, BPA-CL3, BPA-CL4) bisphenol-A in human samplesusing BSTFA prior to GC-MS injection. Secondly, a methodology was developed for bisphenol-A and chlorinated derivatives, as well as phthalates (DMP, DEP, DBP, DEHP, DOP and BBP) in food and environmental samples (water). This method utilised solid phase extraction (SPE, LiChroluet C18) followed by either derivatisation with BSTFA/TMCS (GC/MS) or HPLC-DAD

2. Benzophenone analysis: Due to the interest in benzophenone analysis Partner 9 is developing a new method for the determination of 4-hydroxybenzophenone (HBZF), 2-hydroxy-4-metoxibenzophenone (2H-4MBZF), 2,4-dihydroxybenzophenone (2,4-DIHBZF), 2,3,4-trihydroxybenzophenone (2,3,4-TRIHBZF) and 2,2',4,4'-tetrahydroxybenzophenone (22',44'-TETHBZF).

3. Phthalate monoesters analysis: A new method for analysis of phthalate monoesters in fish muscle has been developed and validated by Partner 3 using pooled samples from bream slices not selected for chemical analysis. The method consists of a liquid extraction followed by 2 solid phase extractions (SPE clean-up) and a final determination on LC/MS/MS. The monoesters included are: monomethyl phthalate (mmp), monoethyl-phthalate (mep), mono-n-butyl phthalate (mbp), monobenzyl phthalate (mbzp), mono-(2-ethylhexyl) phthalate (mehp), mono-(2-ethyl 5-hydroxyhexyl) phthalate (mehhp), mono-(2-ethyl 5-oxohexyl) phthalate (meohp) and monoisononyl phthalate (mnp).

4. Adipose tissue samples from cryptorchid boys in Denmark and Finland: Partner 10 received in Kuopio 21 tissue samples of cryptorchid boys and their controls from

Copenhagen. Unfortunately the amount of tissue in each of the samples is very small (and the amount of fat is even smaller). The amount of fat in samples is so small that it is highly unlikly that analysis would come up with any satisfying results with those samples. Aft discussion, it was decided that Partner 9 would not start the analysis with these samples but will instead wait if there will be other usage for these samples. Samples from Turku are still in Turku.

5. Fish samples from the Netherlands and Switzerland**:** Partners 10 has received bream samples from the Netherlands and Switzerland. In Prague in the midterm review meeting the whole fish analyses was rearranged. This meant that the analysis was not any longer based on geographical distribution of fish according to the pollution level of each study location. Instead it was decided that the analysis of bream was based on the case-control setting in which the cases were male breams with ovotestis and controls were breams with no ovotestis. The selected fish in each case-control set were equally old and catched from the same location. The analysis of those selected fish (number is 39) is currently ongoing by Partner 10. Chemical analysis will be ready by the end of January 2006. To date they have not received any positive or negative fractions in YES or YAS assays in to Kuopio. Partner 9 has processed selected bream samples. Organochlorine pesticides (HCB, lindane, endosulfan and metabolites, ppDDT and metabolites) have already been analysed.

5. Total effective xenoestrogen burden (TEXB) assessment of fish tissue samples: Fish tissue samples (39) have been studied so far in the E-Screen bioassay. Samples selected for extraction and TEXB determination of the alpha and beta fractions were the same as in OC pesticide residues experiments: D59, A82, A24, A105, D69, A01, A32, A29, D32, A100, A111, B38, D27, A51, A19, B97, D60, A21, A11, B95, D67, A50, A55, B45, A65, A44, A23, B21, A02, A13, A40, A66, A113, A73, A71, A77 and A46. Two hundred mg of each sample was extracted using hexane. Extracts were passed through HPLC in order to separate more polar xenoestrogens from lipophilic compounds (alpha and beta fractions). The total effective xenoestrogen burden (TEXB) has been measured in all alpha and beta fractions.

6. Total effective xenoandrogen burden (TEXB-A) assessment: Adipose tissues from breast cancer patients and controls (mammary and abdominal. Original 20 samples) were extracted and HPLC proccesed in order to give alpha and beta fractions. Both fractions have been assayed in the A-Screen, based on MCF-7 androgen receptor transfected cells (AR). Data on androgenicity (TEXB-A) is now being analysed.

7. Phthalate monoesters: Preliminary results: Only few samples have been measured until now, but for all samples there have only been measurable contents of mbp and mehp. The content of mbp has been from 2 to 7 µg/kg and the content of mehp between 6 and 19 µg/kg. The recoveries at levels between 10 and 50 µg/kg fish sample were generally between 90 and 110 %. During the first months of 2006 the bream samples selected for chemical analysis will be analysed for phthalate monoesters. Figures show calibration curve for mehp and chromatograms of a standard, a fish sample and a spiked sample.

Discussion and Conclusions

- A method for of phthalate monoesters in fish muscle has been developed and validated. Preliminary results indicate detectable levels of the phthalate monoesters MBP and MEHP in fish tissue.

- A method for bisphenol-A and chloro derivatives and benzophenone in human tissues has been developed and validated.

- A method for the assessement of the total effective xenoestrogen burden in fish tissues has been developed and validates. Preliminary results indicate the utility of the method in TEXB assessment of fish adipose/muscle tissue.

- The method for the assessment of the total effective xenoandrogen burden has been applied to adipose tissue samples from breast cancer patients and controls

D4.1 Report on suitability of assays for screening of fractions

Responsible Partner: 11 Participating Partners: 9, 12

Objectives

The objective was to test various assays for their suitability for screening of fractions obtained from bile and visceral fat from wild bream.

Research Progress

D4.1 was already discussed in the 2nd Annual Report which at that time it was stated that Partner 9 has developed a method to assess the total estrogenic xenobiotic burden (TEXB) in human tissue using the E-Screen. Optimisation of the A-Screen has been accomplished. Partner 11 has established and validated the YES and used it for bioassay-directed fractionation. The YAS has been established but proofed to be less rugged than the YES. Partner 12 has evaluated and validated the HRS-hERα technology (formerly called HRS-E-RAD). The hEST was also validated and ready for screening purposes.

During the 3rd reporting period, Partner 12 has used the hEST assay to determine the IC_{50} values of known estrogenic compounds. A publication is being prepared. Partner 12 also has optimised the HRS-hERα bioaffinity assay by developing a detection method based upon fluorescence polarisation, allowing the detection of fluorescent phytoestrogens. A publication is underway. Partner 12 obtained the pET15b hAR LBD plasmid and has started expression experiment in *E. coli* for the HRS-hAR bioaffinity assay.

Discussion and Conclusions

- The hEST is ready to be used for the measurement of E-screen positive bile and tissue fractions from other partners.
- The on-line HRS-hERα bioaffinity assay can be used to measure breast tissue samples (α- and β-fractions and E-screen positive fractions) and fish bile samples (pooled fractions and E-screen positive fractions).
- Work on the on-line HRS-hAR bioaffinity assay has started.

Any deviations/delays and justification

Delay in the measurement of tissue samples with the hEST inhibition and the on-line HRS-ERα bioaffinity assay has occurred, because until now no samples have been received from Partners 9 and 11. Some delay in the development of the on-line HRS-hAR platform. Expression experiments have just recently been started due to the fact that we could not get hold of the appropriate hAR LBD plasmid. The LBD is preferred over the complete androgen receptor, because of possible stability issues.

D5.3 Novel endpoints (markers) and tools (including arrays) for the assessment of EDC effects in mammalian cells, fish, and rats

Responsible Partner: 15 **Participating Partners: 1, 4, 11, 13, 14**

Objectives

This document provides information on progress with both novel endpoints and novel tools for the assessment of EDC effects in vertebrates. As study models, mammalian cells (MCF-7 cells), fish (zebrafish) and mammals (rats) were used. The findings reported here emanated from WP 5 "The application of genomics and proteomics to the development of sensitive endpoints of EDC action".

The objectives of Deliverable 5.3 are a) to develop novel tools for studying mechanisms of EDC action, b) to exploit the mechanistic knowledge to derive novel endpoints for the asssessment of EDC effects and c) to utilise the novel tools for the assessment of EDC effects. The research carried out for D5.3 generated insight into the scopes and limits of genomic technologies in searching for novel endpoints and marker genes, in establishing their responsiveness and dose-response relationships and in exploring mechanisms and signalling pathways involved in adverse effects of EDCs.

Research Activities

To date, most of the effects on aquatic wildlife attributed to EDC are of the feminising type and are thought to be mediated by the estrogen receptor pathway (ER). Knowledge about the action of estrogenic chemicals on fish is fairly advanced. However, information about EDC that act through alternative signalling pathway or interact with endocrine functions other than estrogen signalling is comparatively sparse. This narrow focus is related to two current limitations of research on endocrine disruption, that are a) the limited knowledge on the diversity of possible modes of EDC action, and b) the limited avialability of tools and endpoints to assess those other modes of action. The limited set of tools and endpoints in no way reflects the pleiotropic nature of hormone actions and the multiple potential targets of EDCs.

Given this situtaion, work carried out as part of D5.3 aimed to broaden current understanding of targets and mechanisms of EDC action, to idnetify key mechanisms and common pathways, and to utilise such knowledge to develop novel tools and endpoints for the assessment of EDC effects. The ability of EDCs to modulate gene expression is thought to be the initial step in effector chains that lead to differing phenological effects. Thus, the research tools used relied strongly on the application of modern molecular biology technologies to study EDC mechanisms and to discover novel endpoints that are sensitive to EDC exposure.

I Establishment of a series of novel tools for assessing EDC efffects in fish and mammals

First characterisation of homologous antibodies to the brain isoform of zebrafish aromatase (*CYP19B*)

Up to now, specific antibodies for *CYP19B* were not available. Partners 11, 14 and 14 succeeded in establishing two polyclonal antibodies with high specificity and comparable sensitivity (Figure 1). The properties of the antibodies have been presented in two publications (Menuet *et al*., 2005, Kallivretatki *et al*., 2006). This tool is essential to study possible disrupting effetcs of environmental xenoestrogens on brain function, neuroendocrine regulation of reproduction and sexual development, and on neurogenesis.

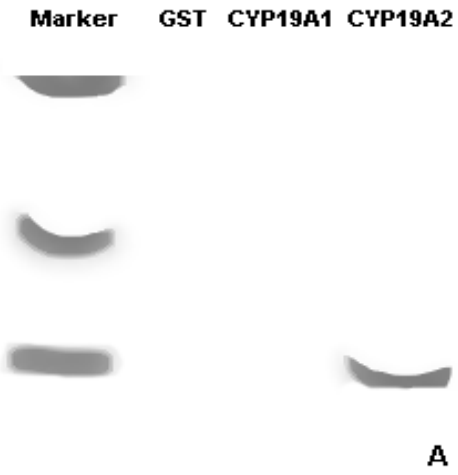

Figure 1 Western blots using polyclonal anti-*CYP19a2* antibody. The antibody reacts with *CYP19A2*, but shows no cross-reactivity to *CYP19A1*.

Validation of a mouse oligo array for assessing EDC effects in rat tissues.

The genes on the mouse array were selected from a differential display study of gene expression during development, with the aim of identifying genes that were specific for the almost 30 different germ-cell differentiation steps present in adult testis. 386 genes were selected and arrayed. Validation (i.e. expression in distinct germ-cell types) by in situ hybridisation has been done for almost 200 genes (Almstrup *et al*., 2004). The mouse array has then been transferred to the rat by identifying the corresponding rat genes and constructing a rat testis array. By searches in different expression and genomic databases, the rat genes corresponding to 294 of the mouse genes were identified. Arrays were printed and used in hybridisation experiments, especially, for comparisons of the expression of the genes in testis and liver. The results demonstrated that most genes are either higher expressed in testis that in liver or exclusively expressed in testis (Figure 2). Thus, this array is suitable for detecting tissue-specific EDC effects in rats.

An important aspect addressed in the studies on genomic responses of mamamls is the question how gene induction relates to internal concentrations of EDCs. To study this question, the uptake of estradiol, testosterone, and two phthalates (dibuthyl- (DBP) and diethyl-phthalate (DEP) into MCF7 cells was studied. In brief, the uptake of estradiol was found to be only dependent on the estrogen receptor expression level in the cells, when all receptors are occupied the cells are saturated with estradiol and the level inside the cells

remains constant. In contrast, the up-take of the two phthalates (DBP & DEP) showed that the uptake was different: there was no saturation effect, in stead the concentration inside the cells was at all tested media-concentrations about 5 fold higher than in the media.

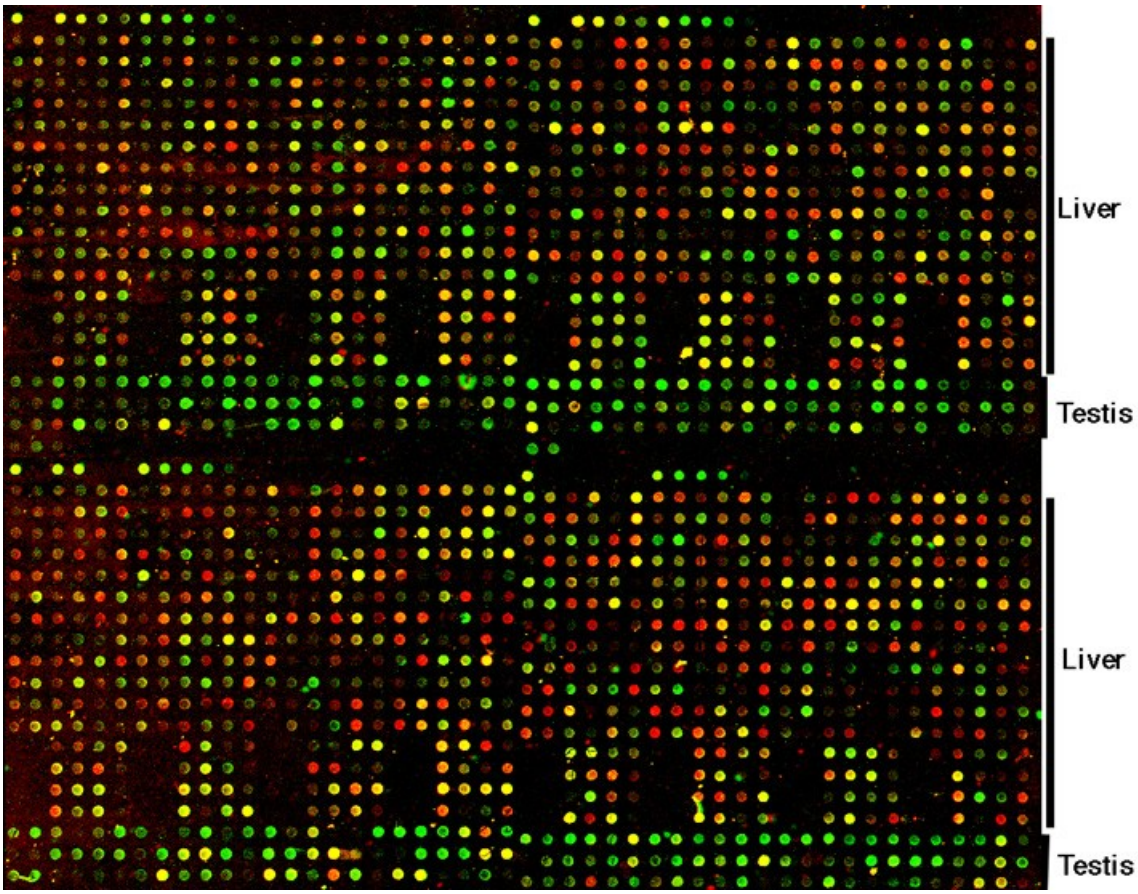

Figure 2 Slide with oligoes derived from genes highly expressed in the liver (MWG-Biotech Rat-liver array) and highly expressed in the testis (our Differential-display derived testis array), the additional apots are control spots. Two probes were hybridised to the array, one derived from RNA from rat testis (green) and one derived from rat liver (red).

Determination of the uptake of different compounds into MCF7 cells is a key question that has never been looked at properly. The above mentioned findings suggest that the amount of media and metabolism of the compounds (DBP and DEP) in the media changes the exposure experienced by the cells during the assay period. Thus, the cells are not exposed to the amount of the compounds that would be expected from what was added to the media. For estradiol the levels in the cells reach a plateau, at low media concentrations, which results in a much higher concentration inside the cells than in the media, whereas at high media concentrations the level inside the cells is lower than in the media. For phthalates the metabolism changes the media concentrations significantly during the assay period. These aspects will have a large impact on the sensitivity and reliability of all analyses performed using MCF7 cells, including cell proliferation assays (E-screen).

1. *Development of real-time RT-PCRs to quantify EDC-induced gene expression.*

As mentioned above, the initial step in EDC action often is the modulation of gene expression, and thus, the ability to quantitatively measure such effects is an essential tool for the assessment of EDC exposure and effects. Within the work done for D5.3, real-time RT-PCRs have been developed for a range of genes, including vitellogenin and aromatase. In the following, vitellogenin is taken as an example.

Vitellogenin is a biomarker for estrogenic exposure of fish, but up to now it has been measured almost exclsuively at the protein level, usually by means of ELISA. The ELISA technique depends strongly on the availability of specific antibodies, but for many fish species, specific antibodies are not vailable. Then, the measurement of vitellogenin mRNA by means of RT-PCR can be a valuable alternative. In addition, sensitivity and kinetics of the vitellogenin induction response are known to differ between the mRNA and protein levels, and this can be important in assessing low-dose exposures, as it has been recently shown by Burki *et al*., (2006). Thus, Partner 13 developed a real-time RT-PCR method for quantifying vitellogenin induction in zebrafish.

To develop the method, adult zebrafish were exposed to BPA (0.1 µg/L, 2 µg/L, 20µg/L, 200 µg/L, 400 µg/L, 1 mg/L, 2 mg/L and 30 ng/L EE2 as positive controls). Liver RNA was extracted and was reversly transcribed into cDNA. To control the quality of the transcription, a PCR was performed for each cDNA sample. Figure 3 shows the electrophoresed amplificates of smples from control fish and exposed fish. Almost all male fish show an expression of vtg1 after exposure to 1 mg/L BPA. The samples 28 and 30 show no response to the chemical. Most probably this is because of the variabilty of the response among the individuals.

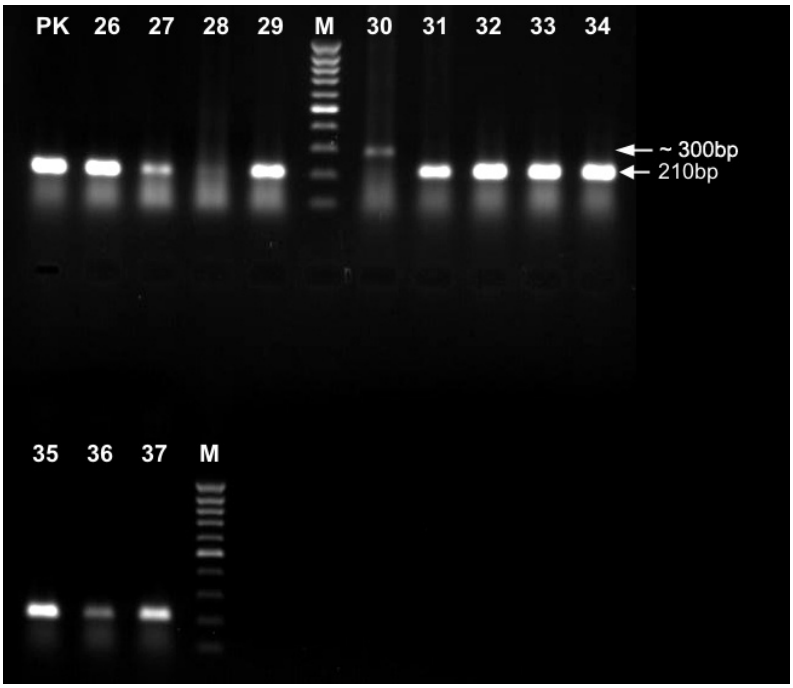

Figure 3 Electrophoresed PCR amplificates. The *vtg1* amplificates are 210bp at size. PK: internal positive control (non-exposed female); 26-37: male zebrafish exposed to 1 mg/L BPA. M: ladder.

After having established the specificity of the primers with the experiments shown above, the next step was to establish the real-time RT-PCR protocol. These experiments were performed with a LightCycler. For the comparison of different individuals, all crossing points of the LightCycler experiments were normalised to the expression of the gene coding for ß-actin ("housekeeping-gene"). The efficiency of the PCR reaction was calculated for the *vtg1* and the ß-actin amplification and used (1.81) for comparing the expression rates of *vtg1* in the different samples. To normalise the different samples, it is necessary to determine the efficiency of the PCR for the ß-actin gene, too.

With the method thus having been established, it was applied to measure dose-dependent induction of VTG mRNA in zebrafish to E2, NP, BPA and EE2 (Figure 4). The results clearly demonstrate that the method is specific, sensitive and dose-dependent.

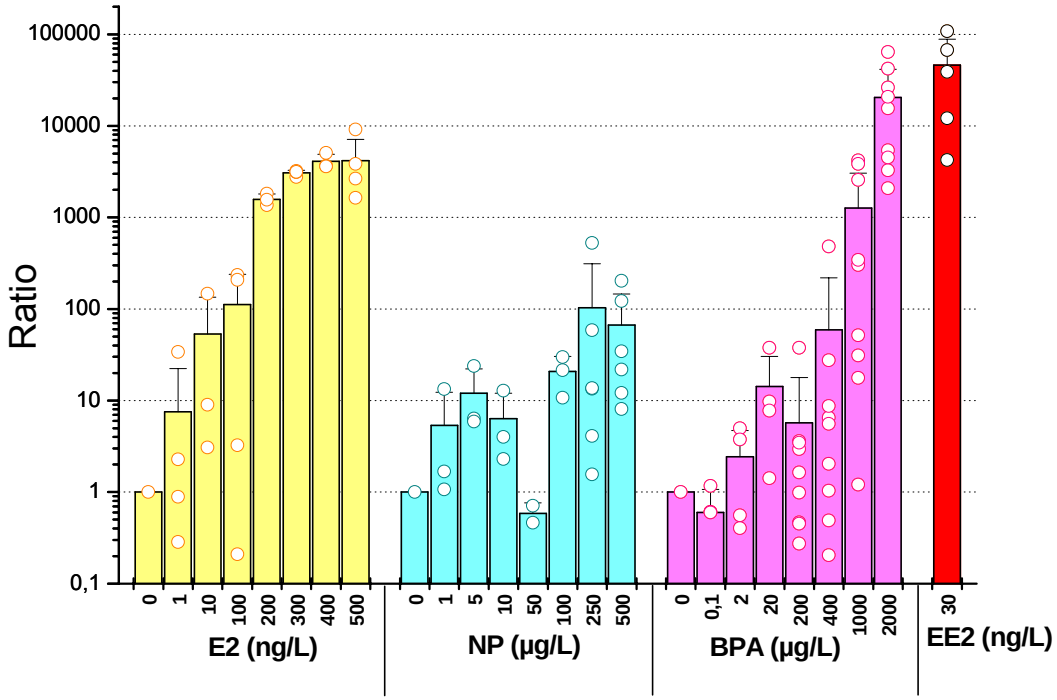

Figure 4 Comparison of the effects of several EDCs on the expression of *vtg1*. Each dot represents one sample (one fish).

2. *Establishing microarrays to identify EDC-responsive genes in sebrafish*

During the first reporting period, whole genome scale arrays were used to select candidate genes for an EDC-targeterd array. Based on the results from those experiments, self-spotted arrays containing 40 genes/sequences were produced and DNA microarray experiments with samples of zebrafish exposed to E2 were carried. Table 1 shows the genes selected for self-spotted arrays. They were obtained after statistical analysis using a specificity of 95% for the selection procedure. Rows with a grey background show zebrafish genes. In addition to the 40 E2-responsive genes, 10 probes coding for housekeeping genes were immobilised for data normalisation.

No.	Gene name	p value	Fold change	Gene description
1	similar to genpept|af406784|af406784_1 vitellogenin 1 - danio rerio	9.23E-09	442.4	vitellogenin, egg yolk protein, lipid transporter activity
2	vitellogenin 1	3.18E-07	128.9	vitellogenin, egg yolk protein, lipid transporter activity
3	similar to genpept|z49204|z49204_1 nadp transhydrogenase - mus musculus	7.72E-07	94.1	proton-pumping nicotinamide nucleotide transhydrogenase, which catalyzes the reversible reduction of NADP+ by NADH; investigated in all mammalian tissues
4	similar to genpept|af068286|af068286_1 hdcmd38p - homo sapiens	8.95E-05	63.6	RIPK5 (receptor interacting protein kinase 5), inducer of cell death, apoptosis
5	similar to genpept|bc016428|bc016428_1 phenylalanine-trna synthetase-like - mus musculus	3.58E-06	43.5	protein biosynthesis
6	similar to genpept|d86970|d86970_1 kiaa0216 - homo sapiens	1.30E-04	33.4	DNA repair, damaged DNA binding
7	\homeo box a3a; hoxa3a\""	1.51E-06	31.0	homeobox gene, embryonic development (A. Amores, 1998)
8	similar to genpept|ak017839|ak017839_1 sap domain containing protein data source:pfam, source key:pf02037, evidence:iss putative - mus musculus	9.68E-06	28.8	DNA binding, involved in chromosomal organization
9	similar to genpept|u41744|u41744_1 pdgf associated protein - rattus norvegicus	1.89E-05	24.9	phosphorylated by casein kinase II and binds to PDGF with low affinity
10	\similar to genpept|ab071402|ab071402_1 chondroitin synthase; chsy - homo sapiens\""	9.86E-05	20.7	chondroitin synthase, CHSY1 synthesizes chondroitin sulfate, a glycosaminoglycan expressed on the surface of most cells and in extracellular matrices. Glycosaminoglycan chains are covalently linked to a wide range of core protein families and regulate many biologic processes, including cell proliferation and recognition, extracellular matrix deposition, and morphogenesis.[supplied by OMIM]
11	similar to genpept|m12105|m12105_1 aspartate aminotransferase precursor - gallus gallus	8.98E-05	18.5	similar to aspartate aminotransferase
12	\similar to genpept|m77013|m77013_1 beta-catenin; beta-catenin - xenopus laevis\""	3.60E-06	17.8	in zebrafish dorsoventral patterning; mediation of cell-cell interactions and possible central role in intracellular signaling
13	\similar to genpept|u80741|u80741_1 cagh44; cagh44 - homo sapiens\""	4.88E-05	17.7	This gene encodes an evolutionarily conserved transcription factor expressed in fetal and adult brain. This transcription factor is a member of the forkhead/winged-helix (FOX) family of transcription factors, and contains a FOX DNA-binding domain and a large polyglutamine tract. Members of the FOX family of transcription factors are

				regulators of embryogenesis. The product of this gene is thought to be required for proper development of speech and language regions of the brain during embryogenesis. Although a point mutation in this gene has been associated with the KE pedigree segregating developmental verbal dyspraxia, no association between mutations in this gene and another speech disorder, autism, has been found. Four alternative transcripts encoding three different isoforms have been identified.
14	\similar to genpept|u67156|u67156_1 mitogen-activated kinase kinase kinase 5; mapkkk5 - homo sapiens\""	4.35E-05	17.1	MAP/ERK kinase kinase 5; MAPK/ERK kinase kinase 5; apoptosis signal regulating kinase; Mitogen-activated protein kinase (MAPK) signaling cascades include MAPK or extracellular signal-regulated kinase (ERK), MAPK kinase (MKK or MEK), and MAPK kinase kinase (MAPKKK or MEKK). MAPKK kinase/MEKK phosphorylates and activates its downstream protein kinase, MAPK kinase/MEK, which in turn activates MAPK. The kinases of these signaling cascades are highly conserved, and homologs exist in yeast, Drosophila, and mammalian cells. MAPKKK5 contains 1,374 amino acids with all 11 kinase subdomains. Northern blot analysis shows that MAPKKK5 transcript is abundantly expressed in human heart and pancreas. The MAPKKK5 protein phosphorylates and activates MKK4 (aliases SERK1, MAPKK4) in vitro, and activates c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) during transient expression in COS and 293 cells; MAPKKK5 does not activate MAPK/ERK.
15	\similar to genpept|al030996|al030996_1 dj1189b24.4 novel putative protein similar to hypothetical proteins s. pombe c22f3.14c and c. elegans c16a3.8; dj1189b24.4 - homo sapiens\""	5.15E-04	16.1	Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. Nat Genet. 2002 Jun;31(2):135-40.
16	\vitellogenin 3 precursor; vg3\""	4.43E-06	15.9	vitellogenin, egg yolk protein, lipid transporter activity
17	\similar to genpept|af026527|af026527_1 zipcode-binding protein; zbp1 - gallus gallus\""	4.31E-04	14.6	Characterization of a beta-actin mRNA zipcode-binding protein. Mol Cell Biol. 1997 Apr;17(4):2158-65.
18	similar to genpept|af250920|af250920_1 12cc4 - homo sapiens	1.75E-04	13.1	Regulation of gene expression by integrin engagement
19	similar to	5.59E-04	13.0	excision repair cross-complementing

	genpept|m31899|m31899_1 ercc3 - homo sapiens			rodent repair deficiency, complementation group 3 [Danio rerio]; nucleotide-excision repair
20	\similar to genpept|af084461|af084461_1 type i cytokeratin; cyt1 - danio rerio\""	1.59E-05	10.2	type I cytokeratin, enveloping layer [Danio rerio]; structural molecule activity
21	\similar to genpept|u53922|u53922_1 dnaj-like protein; rdj1 - rattus norvegicus\""	9.08E-05	9.9	interacts with the N-terminal fragment of hsc70; involved in DNA damage repair [RGD]
22	\vitellogenin 3 precursor; vg3\""	3.43E-04	9.8	vitellogenin, egg yolk protein, lipid transporter activity
23	\nothepsin; nots\""	2.97E-05	6.8	similar to mammalian cathepsin E and cathepsin D, liver specific aspartic protease, Role: post-translational processing of vitellogenin in the liver prior to its secrection into the blood stream
24	\estrogen receptor; er\""	3.59E-04	6.7	nuclear receptor, ligand-inducible transcription factor
25	rev-erb beta 2	6.70E-05	6.1	nuclear receptor, "orphan receptor" - no ligand has been yet identified, regulation of transcription, DNA-dependent; Euteleost fish genomes are characterized by expansion of gene families.
26	\putative mature peptide; dvr1\""	3.30E-04	5.7	decapentaplegic and Vg-related 1; dvr1; growth factor activity, regulation of cell cycle, growth, cell proliferation
27	similar to genpept|af224337|af224337_1 beta-1 integrin - ictalurus punctatus	1.83E-05	5.1	Molecular characterization and leukocyte distribution of a teleost beta1 integrin molecule
28	\similar to genpept|x86969|x86969_1 beta 1 subunit of heterotrimeric gtp-binding protein; xgbeta1 - xenopus laevis\""	8.89E-05	3.9	The mRNA encoding a beta subunit of heterotrimeric GTP-binding proteins is localized to the animal pole of Xenopus laevis oocyte and embryos
29	\mesoderm posterior b; mespb\""	8.05E-04	3.4	nucleus, transcription factor activity, anterior/posterior pattern formation, somitogenesis
30	\kallmann syndrome 1b sequence; kal1b\""	8.63E-04	1.9	Kallmann syndrome 1b sequence; prior pubertal development; congenital, isolated, idiopathic hypogonadotropic hypogonadism (IHH) and anosmia
31	\similar to genpept|l10911|l10911_1 splicing factor; cc1.4 - homo sapiens\""	2.33E-04	1.5	Other Designations: RNA-binding region containing protein 2; coactivator of activating protein-1 and estrogen receptors; hepatocellular carcinoma protein 1; splicing factor CC1.3; splicing factor HCC1; The protein encoded by this gene is an RNA binding protein and possible splicing factor. The encoded protein is found in the nucleus, where it colocalizes with core spliceosomal proteins. Studies of a mouse protein with high sequence similarity to this protein suggest that this protein may act as a transcriptional coactivator for JUN/AP-1 and estrogen receptors. Multiple transcript variants encoding several different isoforms have been found for this gene.

32	\similar to genpept|af275309|af275309_1 transcription factor foxp1; foxp1 - homo sapiens\""	1.55E-04	-1.8	Integrin engagement regulates monocyte differentiation through FOXP1.
33	\ephrin b2a; efnb2a\""	4.34E-04	-1.9	The ephrinB2a may signal a subpopulation of RGC axons that they have reached their target neurons in the tectum. Integral to membrane, development, neurogenesis, synaptic target recognition; EphrinB2a in the zebrafish retinotectal system. J Neurobiol. 2004 Apr;59(1):57-65.
34	similar to genpept|af151886|af151886_1 cgi-128 protein - homo sapiens	2.12E-04	-2.0	possibly involved in hematopoietic development and differentiation
35	\similar to genpept|af057146|af057146_1 putative deubiquitinating enzyme ubpy; ubpy - mus musculus\""	8.25E-04	-2.0	1. Results indicate that Nrdp1 is a specific target for the USP8 deubiquitinating enzyme and are consistent with a model where USP8 augments Nrdp1 activity by mediating its stabilization. 2. deubiquitinating enzyme specifically expressed in testis, associates with the acrosome and centrosome in mouse germ cells
36	similar to genpept|bc003745|bc003745_1 similar to dead/h asp-glu-ala-asp/his box polypeptide 15 - mus musculus	6.37E-04	-2.0	DEAD box protein ?
37	zg2	1.57E-04	-2.1	GATA-binding protein 2; zg2; regulation of transcription, DNA-dependent
38	similar to genpept|m14644|m14644_1 alpha-tubulin 2 - drosophila melanogaster	6.01E-04	-2.3	The Drosophila SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. Cell. 2004 Mar 19;116(6):817-29.
39	similar to genpept|af283813|af283813_1 cytochrome p450 monooxygenase cyp2k6 - danio rerio	3.00E-04	-2.6	Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquat Toxicol. 2004 Apr 14;67(2):105-26. Segner/Fenske
40	\similar to genpept|ab052623|ab052623_1 warm-temperature-acclimation-related-65 kda-protein; wap65 - cyprinus carpio\""	5.04E-04	-4.8	cDNA cloning and characterization of the warm-temperature-acclimation-associated protein Wap65 from carp, Cyprinus carpio

Table 1 40 selected genes for self-spotted microarrays. The grey marked genes are zebrafish genes.

Using the self-spotted array, hybridisation experiments were carried out on liver samples of zebrafish expsoed to various concentrations of E2. Data normalisation and statistical analyses were performed to determine exposure level for induction of gene expression. The results from these experments showed that the number of induced genes increases with increasing exposure concentration. The sensitivity of the array is comparable to that of the real-time RT-PCR. For instance, *vtg1* induction was found to be significant at >100 ng E2/L both with PCR and array. This finding on zebrafish contradicts the results of joint experiments of Partners 1 and 4 comparing RT-PCR and arrays to assess genomic

responses in MCF-7 cells; the results of that comparison showed that the sensitivity of the arrays was several fold lower than PCR-based methods.

In addition to the microarray experiments with E2-exposed fish, the array technology was also applied to BPA-treated zebrafish. To detect genes/sequences responding to BPA whole genome arrays were used for samples exposed to a concentration of 1000 µg/L, since an induction of *vtg1* was observed by LightCycler experiments. 38 genes/sequences were found to be regulated. These 38 genes/sequences plus the 40 genes/sequences regulated by E2 were spotted on epoxy slides. Hybridisation experiments with all concentrations (0.1, 2, 20, 200, 400, 1000, 2000 µg/L BPA) are still in progress, but first results suggest that BPA takes effect in a completely different way than E2. This means that although both substances bind to the estrogen receptors and induces the biomarker vitellogenin; their biological effects still may differ because the overall pattern of genomic activation is not identical. If this still preliminary finding would be confirmed, this would have important implications for risk assessment.

Glial -cell-based reporter assay to assess low-dose and mixture effects of EDCs on the three types of estrogen receptors in zebrafish

The brain cytochrome P450 aromatase (*Aro-B*) in zebrafish is expressed in radial glial cells, strongly stimulated by estrogens and thus can be used, *in vivo*, as a biomarker of xenoestrogens effect on the central nervous system. By quantitative real time PCR, it was first confirmed that the expression of *Aro-B* gene is robustly stimulated in juvenile zebrafish exposed to several xenoestrogens. To investigate the impact of environmental estrogenic chemicals on distinct ER activity, a glial cell-based assay was developed using *Aro-B* as target gene. To this end, the glial cell line (U251-MG) was transfected with the three zebrafish ER subtypes and the *Aro-B* promoter linked to luciferase reporter gene (Figure 5). E2-treatment of U251-MG glial cells cotransfected with zebrafish ERalpha and the *Aro-B* promoter-luciferase reporter resulted in a 60 to 80-fold stimulation of luciferase activity (Figure 6). The detection limit was below 0.05 nM and the EC50 was 1.4 nM. Interestingly, in this glial cell context, maximal induction achieved with the *Aro-B* reporter was 3 times more than that observed with a classical Estrogen-Response-Element-reporter gene (ERE-tk-Luc). Dose-response analyses with several xenoestrogens showed that estrogenic potency of these agents markedly differed depending on ER subtype in the assay. Moreover, the combination of these agents showed an additive effect according to the concept of concentration addition (CA). This confirmed that the combined additive effect of the xenoestrogens leads to an enhancement of the estrogenic potency, even when each single agent might be present at low effect concentrations. In conclusion, this demonstrates that the bioassay provides a fast, reliable, sensitive and efficient test for evaluating estrogenic potency of endocrine disruptors on ER subtypes in a glial context (Le Page *et al*., 2005).

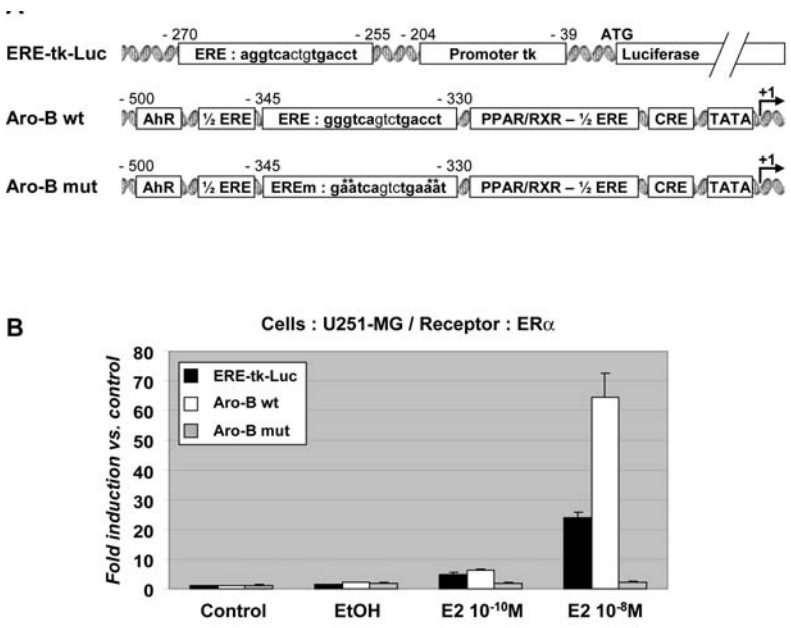

Figure 5 *Aro-B* reporter gene is highly upregulated by E2 in the glial cell line U251-MG. (a) schematic representation of the three luciferase reporter constructs used. The ERE-tk-Luc contains a consensus ERE oligonucleotide linked to thymidine kinase-luciferase. The *Aro-B* luciferase reporter genes correspond to 500 bp of the proximal promoter of zebrafish cytochrome p450 19b gene, containing ERE (*Aro-B*) or mutated ERE (*Aro-B* mut). (b) U251-MG cells were transfected in 24-well plates with empty expression vector (control) or zfERα expression vector and together with either ERE-tk-Luc or *Aro-B* or *Aro-B* mut constructs. Cells were treated with ethanol (EtOH), 0.1 nM E2 and 10 nM E2. Data are expressed as fold induction relative to empty vector (control). Each experiment was repeated at least twice in triplicate.

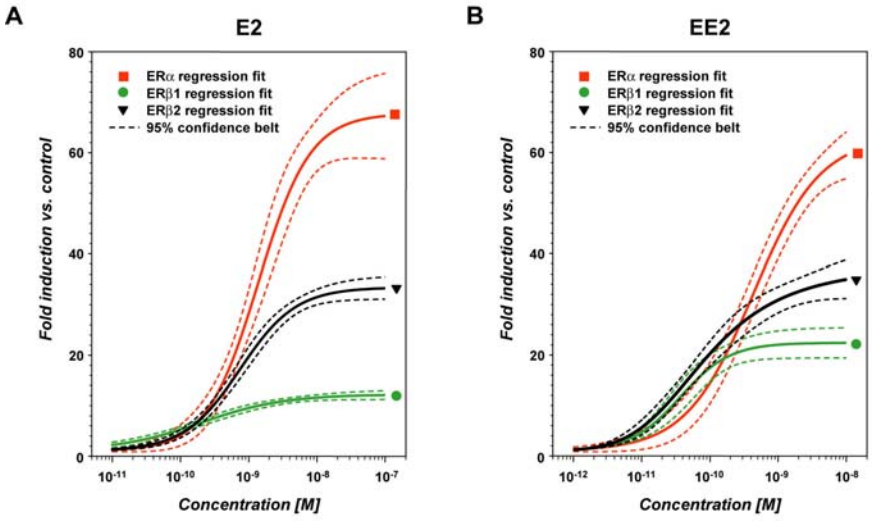

Figure 6 Dose-dependent effect of E2 and EE2 on the transcriptional activation of zfERs. U251-MG cells were transfected with the *Aro-B* reporter gene and either zfER expression vector. Cells were treated with increasing concentration of E2 (10^{-11} M to 10^{-7} M) (**A**) and EE2 (10^{-12} M to 10^{-8} M) (**B**). Data are expressed as fold induction relative to empty vector (control) from at least three experiments.

II Identification of novel endpoints for the assessment of EDC exposure and effects.

Signalling pathways being involved in EDC action

Endogenous estrogens and growth factors, such as the epidermal growth factor (EGF) act as mitogens promoting cell proliferation in the breast and the reproductive tract. Many EDCs also induce mitogenicity. In recent years, it has become clear that the EGF and estrogen-mediated signalling pathways are intertwined (Figure 7).

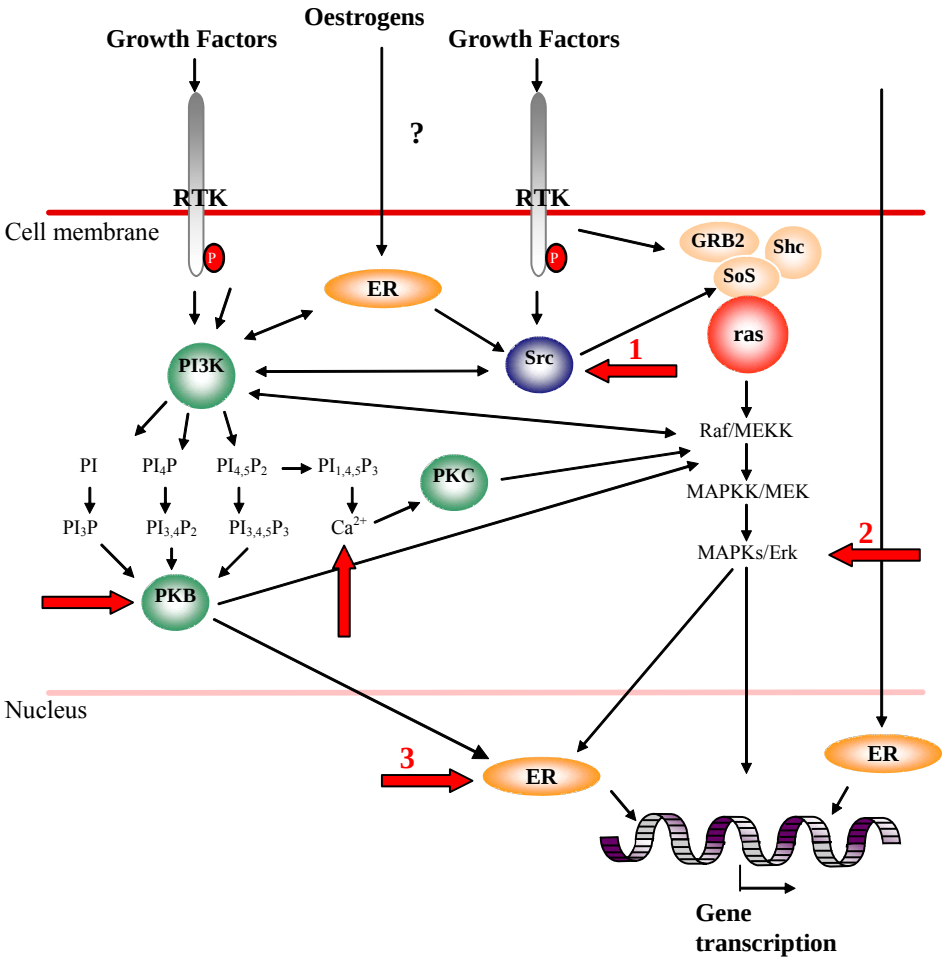

Figure 7 Possible cross-talks between growth factors and ER cell signalling pathways. Activation of RTKs by growth factors such as EGF, IGF-I and TGF-α leads to Ras-Raf-MEK-Erk1/Erk2 mediated phosphorylation of the ER. The same growth factors may also lead to activation of Src, which also results in activation of the ER by the MAPK cascade. The PI3-K/PKB cascade has also been associated with the ligand-independent activation of the ER. Oestradiol, and possibly other oestrogens, rapidly activate the Src-Ras-Erk1/Erk2 and PI-3K/PKB cascades. Abbreviations are: ER: oestrogen receptor; Erk: extracellular signal-regulated kinase; GRB2: growth factor receptor-binding protein 2; IP: inositol phosphate; MAPK: mitogen-activated protein kinase; MAPKK: MAPK kinase; MEK: Erk kinase, MEKK: MEK kinase; PI3-K: phosphotidylinositol-3-kinase; PKB: protein kinase B; PKC: protein kinase C; RTK: receptor tyrosine kinase; ShC: SH2-containing protein; SoS: son of sevenless. Red arrows show our current areas of interest.

17β-estradiol (E2) can induce the rapid activation of growth factor signalling pathways, such as Src/Ras/Erk cascade in mammalian cells. This leads to the phosphorylation and ligand-independent activation of the ER. In turn, EGF is able to induce the expression of estrogen-dependent genes, even in the absence of E2, by phosphorylation of the ER. Thus, it is clear that the action of E2 at the molecular level involves not only the classical binding and activating of the nuclear ER (genomic effects), but also the activation of short-term cytoplasmic signalling pathways (non-genomic effects). Xenoestrogens are frequently classified on the basis of their affinity to the ER. The effects of these chemicals are often analysed in receptor-based assays, where only the affinity to the nuclear ER is evaluated. The observations suggesting that E2 exerts its multiple biological effects not only by direct activation of the ER, but also by alternative growth factor pathways, raised the possibility that xenoestrogens are also able to promote a similar range of effects.

The involvement of Partner 1 for D5.3 was to address this issue and evaluate whether it could be exploited with a view to develop new markers of biological effect. The compounds used in this work were selected due to the reported differences in estrogenic activity and ER activation. The pesticide *o,p'*-DDT is a well characterised xenoestrogen, which binds and activates the ER leading to an increase in its transcriptional activity and consequent mitogenicity. *p,p'*-DDE, the metabolite of *o,p'*-DDT also activates the ER, but in much lesser extent than its parent compound. Finally, β-hexachlorocyclohexane (β-HCH) is a chlorinated hydrocarbon that, in spite of inducing estrogenic effects such as activation of ER target genes and proliferation of estrogen-responsive cell lines, is not able to bind the ER. E2 and EGF were selected as positive controls for genomic and non-genomic effects, respectively.

1. *Activation of the Src/Erk pathway (arrows 1 and 2 in figure 7)*

Direct evidence for the activation of the Src/Erk pathway was obtained by treating MCF-7 cells with the test compounds and probing for phosphorylated Src, Erk1 and Erk2 by Western Blotting. Significant increases in Erk1/Erk2 phosphorylation were observed after 5 min treatment with E2, EGF and β-HCH (Figure 8). However, *p,p'*-DDE failed to induce Erk phosphorylation under these experimental conditions. Similarly, E2, EGF and β-HCH induced phosphorylation of the Src kinase, whereas *p,p'*-DDE produced no observable effect. With EGF and β-HCH the effects was strong and became apparent after 2 min incubation (Figure 9). These results clearly indicate that, in a similar way to E2, β-HCH is able to rapidly activate the Src/Erk pathway.

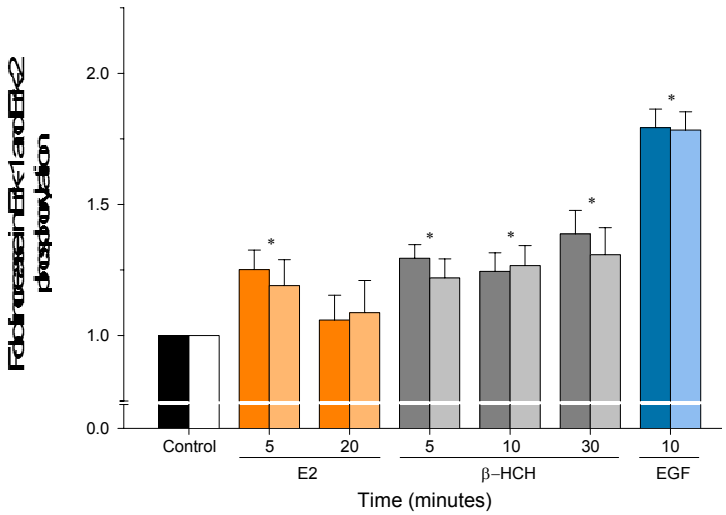

Figure 8 Activation of Erk-1/Erk-2 by the test agents in the MCF-7 cell line. MCF-7 cells were either left untreated (control) or treated with 10 nM E2, 10 μM β-HCH or 100 ng/ml EGF for the lengths of time indicated. Cells were then lysed and probed by Western Blotting for phosphorylated Erk-1 (dark bars) and Erk-2 (light bars). The data shown above are representative of three independent experiments. Band intensities from these individual experiments were quantified and results represented as fold increase over untreated controls. Data plots are representative of mean ± SEM. * indicates Erk-1/Erk-2 activation significantly ($p < 0.05$) by paired *t* test) greater than that in untreated cells.

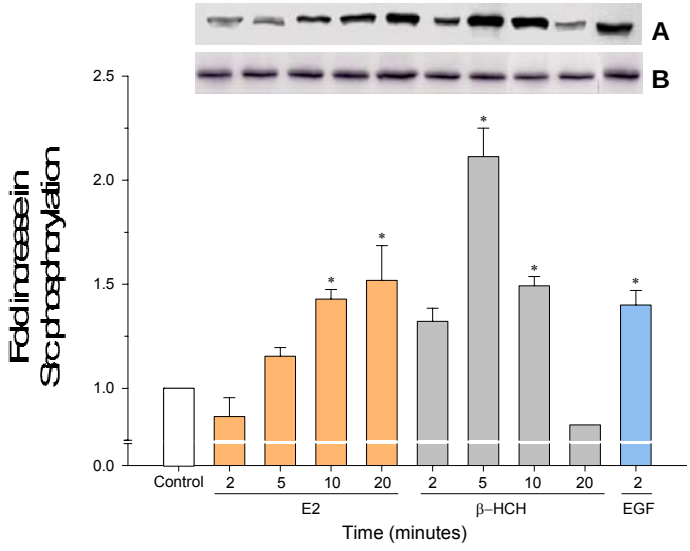

Figure 9 Activation of the Src kinase by the test agents in MCF-7 cells. Cells were left untreated (white bar) or treated with 10 nM E2 (orange bars), 10 μM β-HCH (grey) or 100 ng/ml EGF (blue) for the indicated times. Total Src was then immunoprecipitated. The resulting immunoprecipitates were resolved on SDS-acrylamide gels, transferred to nitrocellulose and probed for phosphorylated Src (Tyr 416) (blot A). The nitrocellulose membrane was then stripped and reprobed with an antibody that recognises total Src (blot B). Bars represent the fold increase in Src phosphorylation relative to untreated controls. Data plots correspond to mean + SEM ($n = 3$). * indicates samples where Src activation is significantly ($p<0.05$, paired *t* test) greater than untreated controls.

2. *Effects on gene expression (arrow 3 in figure 1)*

These results raised the possibility that the activation of the Src/Erk pathway by E2 and β-HCH could lead to more sustained effects, such as ER activation and consequent increase in gene expression. In order to test this, the expression of the *TFF1* gene in MCF-7 cells was monitored by reverse –transcription competitive PCR. All test agents induced up-regulation of the *TFF1* gene and the effect could be inhibited by the anti-estrogen ICI 182,780 (Figure 10). This indicates that the transcriptional regulation of TFF1 by the test compounds is mediated by the ER. Partial inhibition of the gene expression was seen upon administration of the Erk cascade inhibitor, PD98059, suggesting that the growth factor signaling events have an impact on the genotropic effects of estrogen-like environmental chemicals. An exception was *p,p'*-DDE, where Erk cascade inhibitors failed to affect the xenoestrogen's induction of TFF1. This data also shows that, similarly to E2 and β-HCH, *o,p*'-DDT has the ability to activate the Src/Erk cascade, which in turn leads to an increase in the transcriptional activity of ER.

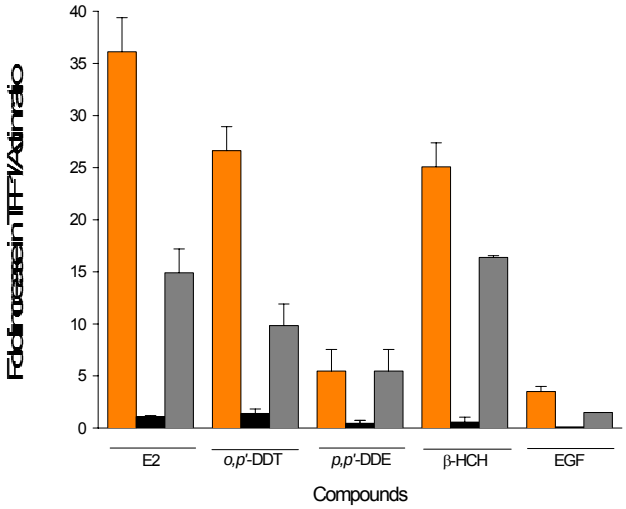

Figure 10 Fold increases in TFF1 mRNA levels in MCF-7 cells in relation to untreated controls. Cells were individually treated with 100 pM E2, 10 μM *o,p'*-DDT, 10 μM *p,p'*-DDE, 10 μM β-HCH or 100 ng/ml EGF (orange bars) and TFF1 expression analysed by RT-competitive PCR. The black and grey bars show the effects of each chemical after the addition of 0.1 μM of the pure ER antagonist ICI 182,780 or 50 μM of the MAP kinase cascade inhibitor PD 98059, respectively. The results are represented as the mean ± SEM of three individual experiments.

3. *Effects on cell proliferation (Arrow 3 in Figure 1)*

Finally, Partner 1 was interested in assessing whether, in a similar way to gene regulation, the effect of xenoestrogens on the Src/Erk cascade could contribute to the proliferation of MCF-7 breast cancer cells. By treating MCF-7 cells with ther test compounds in combination with the Src/Erk cascade inhibitor PD98059, Partner 1 was able to block the activation of the ER via this cells signaling pathway and evaluate how this affected the proliferation of the cells. Cells were treated with a constant concentration of each chemical, which produced a maximal effect, in combination with increasing concentrations of PD98059.

A clear decrease in cell number was detected when concentrations of 10, 25 and 50 µM PD98059 were added to E2, *o,p'*-DDT and β-HCH. A minimal reduction was also observed with *p,p'*-DDE (Figure 11). This clearly indicates that the proliferation of MCF-7 cells in response to estrogenic compounds is not only regulated by the direct binding and activation of the ER, but also by the indirect activation of the receptor via cell signaling pathways.

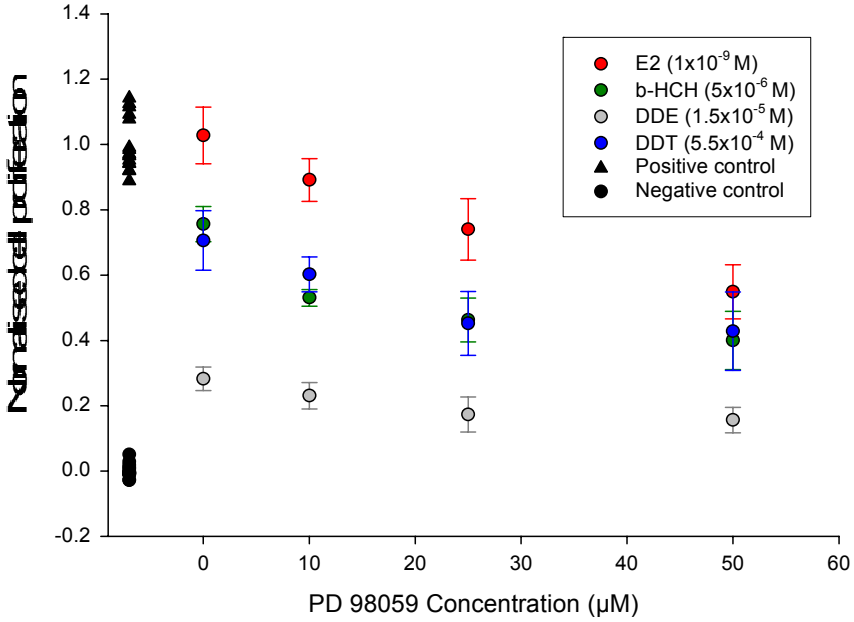

Figure 11 Effect of the MAPK cascade inhibitor PB98059 on the mitogenic activity of E2, *o,p'*-DDT, β-HCH and *p,p'*-DDE in the E-Screen.

An overview of the characteristics of cell signalling observed with the test chemicals is shown in Table 2.

Agent	Mitogenicity	ER binding	TFF1 up-regulation	Src/Erk activation
E2	+	+	+	+
o,p'-DDT	+	+	+	+
β-HCH	+	-	+	+
p,p'-DDE	+	+	+	-
EGF	+	-	+	+

Table 2 Cell signalling characteristics observed with the test chemicals studied

Steroidogenesis (aromatase) as EDC endpoint

Aromatase is a key enzyme of steroidogenesis, deciding on endogenous levels of estrogens/androgens, and thus directly governing sexual differentiation and reproduction of vertebrates. Therefore, emphasis in D5.3 was given on this endpoint of EDC action, addressing a) the regulation of the aromatase genes and the responsiveness of aromatase gene expression to EDC exposure, and b) the consequences of disruption of the aromatase system. As experimental model, the zebrafish was used.

1. *Regulation of aromatase*

The brain isoform of zebrafish aromatase, *zfcyp19b*, possesses ERE and is upregulated by estrogenic substances. This has been shown within D5.3 for zebrafish embryos (Menuet *et al*., 2005) as well as in adult zebrafish (Figure 12). This responsiveness makes the functions influenced by cerebral aromatase activity – likely functions involved in the neuroendocrine regulation of reproduction and reproductive behaviour, but possibly other functions like brain growth as well - principally susceptible to the action of EDCs. It is important to further investigate this aspect because of it would have important implications on the assessment of EDCs.

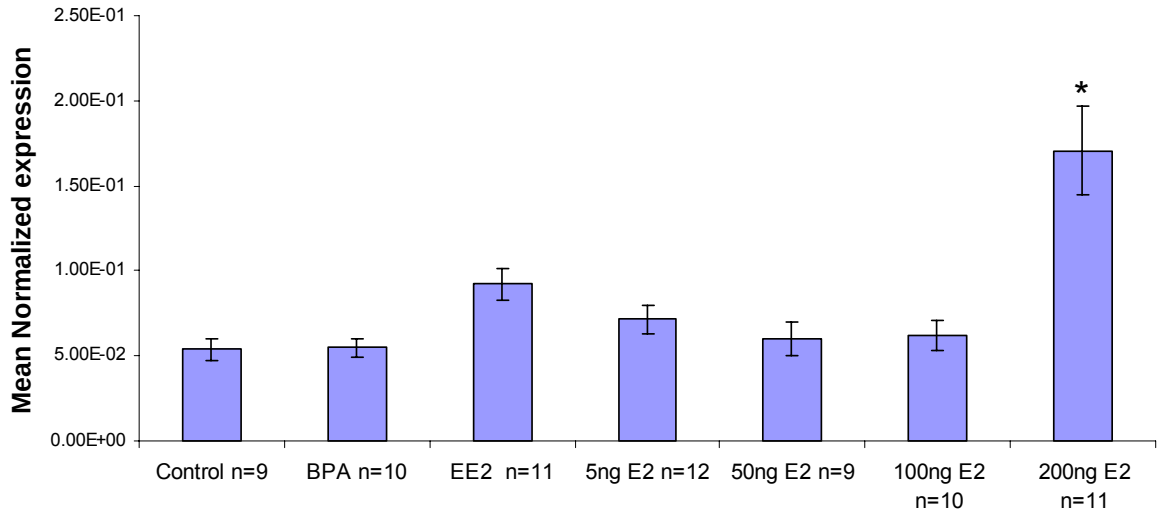

Figure 12 Mean normalised xpression levels of *cyp19a2* in the brains of adult male zebrafish after exposure to xenoestrogens. Gene expression levels were normalised against 18S

The work done for D5.3 strongly advanced the understanding of the regulation of the gonadal isoform of zebrafish aromatase, *zfcyp19a*. Studies using *in vitro* cell systems as wella s transiently transfected zebrafish embryos have demonstrated:

- *zfcyp19a* promoter is active only in the ovarian cell (CHO cells) context but not in an glial cell context. This is exactly the opposite to what ahs been observed for the brain aromatase (see above). Both isoforms need some unknown factors of the original cell environment for their regulation.

- *zfcyp19a* promoter is upregulated by estrogen treatment *in vitro*, however, it is still unclear whether the effect seen is fully due to the promoter or is just caused by the empty vector sequences

- *in silico* predicted CRE sites in both the *zfcyp19a* and the *zfcyp19b* promoter are not functional, hence no cross-talk with cAMP signalling pathways (Figure 13)

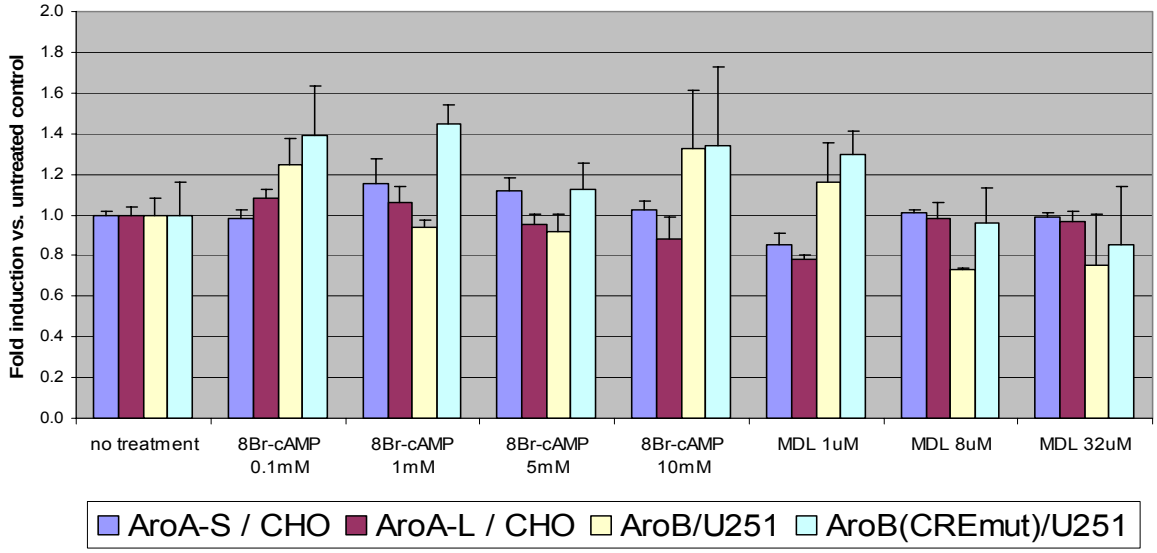

Figure 13 Responsiveness of *zfcyp19a* promoters to mediators of the CREB pathways

The results shown in Figure 14 suggest that predicted AhRE sites in the *zfcyp19a* and *zfcyp19b* obviously do not function via a classical AhR/ARNT pathway. Rather, the effects of AhR ligands on its activity are due to unspecific interaction of ER and AhR/ARNT, functioning via the ERE site independent of AhRE. This *in vitro* data offers a plausible explanation to some contradicting *in vivo* data, pointing to the importance of estrogen availability in the system, which causes estrogenic or anti-estrogenic effects of dioxins:

ER + AhR/ARNT + TCDD: induction of *cyp19* expression
ER + E2 + AhR/ARNT + TCDD: downregulation of normal ER + E2 response

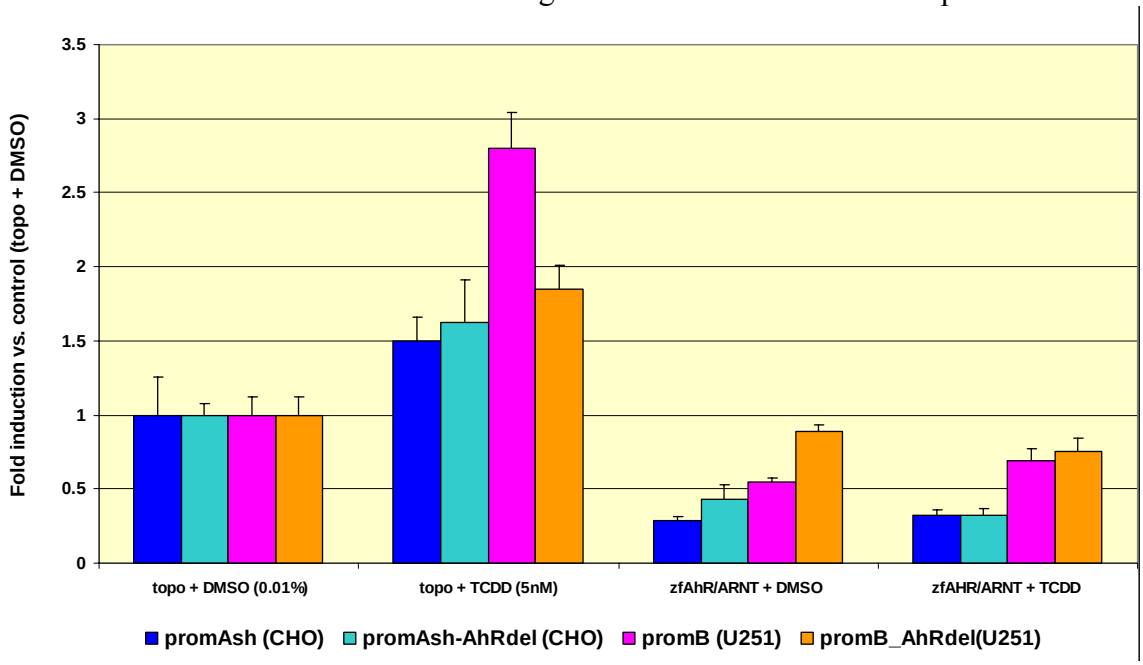

Figure 14 Response of differently modified *zfcyp19a* or *zfcyp19b* promoters to TCDD

2. *Consequences of disruption of the aromatase system*

To examine the consequences of aromatase disruption in early life stages of zebrafish, embryos were injected with morpholinos to suppress *zfcyp19a* and *zfcyp19b* expression during early development. Microinjections with *cyp19a* morpholinos resulted in statistically significant reduction of neuromast number, as illustrated on Figure 15. This finding highlights the fact that estrogen-active chemicals may affect much more targets than sexual differentiation and reproduction, although research to data has been focused almost exclusively on the latter two targets.

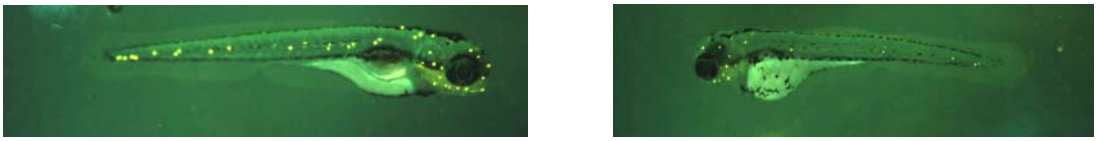

Figure 15 left: control zebrafish larva, with neuromasts along the head and the lateral line (yellow dots). right: morpholino-injected zebrafish with reduced number of neuromasts.

To examine the consequences of aromatase disruption in sexually differentiating zebrafish, this life stage was exposed to aromatase inhibitors. Zebrafish develops as a non-fucntional juvenile hermaphrodite that is all individuals go though a non-functional ovarian stage of gonad development, before they transform into individuals with either functional testis or ovary. Whn zebrafish is treated with aromatase inhibitors during the ontogenetic period of gonadal differentiation; the results in complete and irreversible masculinisation of the exposed cohort (see Figure 16). Obviously, the inhibitor leads to low endogenous production of estrogen and the absence of elevated e2 levels direct gonad differentiation into a testis. These findings indicate that environmental chemicals with aromatase inhibiting activity, scuh as tributyltin or various imidazol pesticides may have profound effects on fish sexual development. .

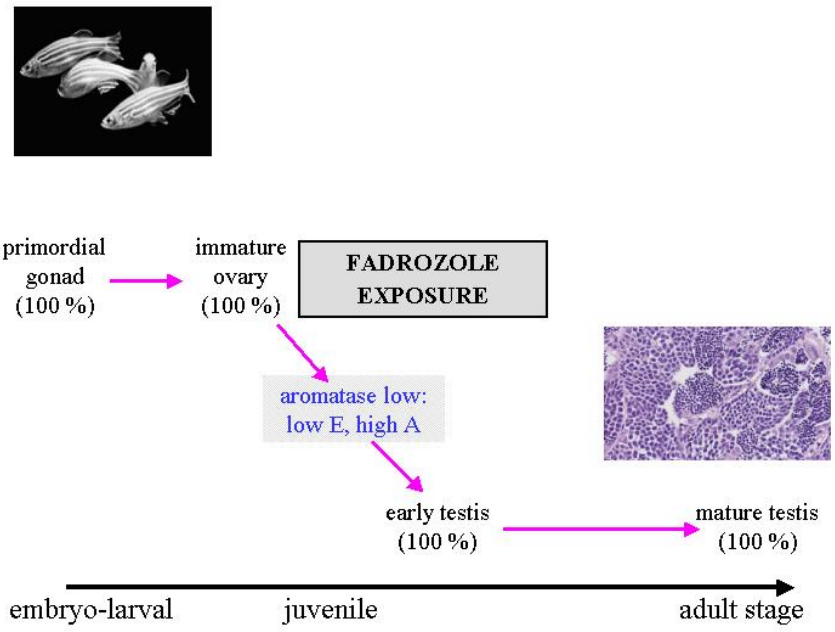

Figure 16 Masculinisation of zebrafish exposed during the period of sexual differentiation to an aromatase inhibitor (fadrozole).

Finally, to examine the consequences of aromatase disruption on mature, adult zebrafish, this life stage was exposed to aromatase inhibitors. The short-term (3 weeks) treatment resulted in a significant reduction of fecundity, what is related to a clearly increased oocyte atresia in the ovaries of the exposed females (Figure 17). The males appeared to be not affected by the treatment.

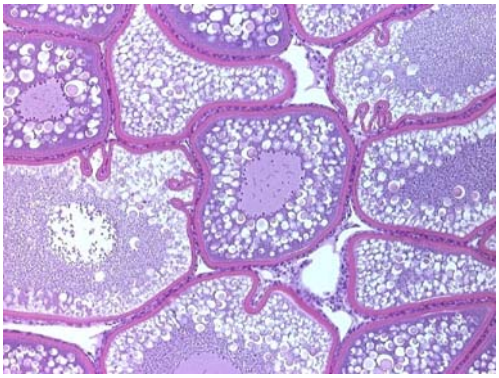

Figure 17 Oocyte atresia in aromatase inhibitor (fadrozole) exposed female zebrafish.

Overall, the results form this comparative assessment at three different life stages of zebrafish clearly indicate that compounds which are able to modulate aromatase in particular and steroidogenesis in general can lead to profound changes of neuronal, developmental and reproductive functions of zebrafish.

Conclusions

The research done for completion of D5.3 led to the following major achievements:

- A suite of advanced tools for diagnosing and assessing endocrine disruption in fish and mammals has been successfully developed. These tools include antibodies, PCR protocols, cell-based screening assays for high-throughput screening of EDCs, as well as microarray.

- By means of microarray technology, novel genomic endpoints were found for the assessment of EDC effects. Nevertheless, vitellogenin was found to be the more sensitive marker gene than the new candidate genes. The microarray experiments are especially useful for the comparison of the effects of different EDCs in order to reveal to what extent the mode of action of the various compounds agree or disagree.

- The investigations on signalling pathways have clearly that the genomic effects (gene regulation and mitogenicity) effects of endocrine disrupters can be regulated not only by the classical receptor-binding activity, but also by interfering with rapid cell signalling pathways that ultimately lead to the ligand-independent ER activation. This highlights the need to take these short term effects into account (rather then simply measuring direct ER-binding) when evaluating the full estrogenic potency of chemicals.

- Novel targets of EDC action have been identified. Particular emphasis has been given to the steroidogenesis/aromatase system. It was shown that the brain isofom

of aromatase is responsive to estrogens, and the role of the estrogen receptors involved has been identified. Further, evidence was provided that the brain isofom needs a glial context to become activated. With respect to the gonadal form, an ovarian context was found to be essential for gene activation, and that predicted CRE sites in the *zfcyp19a* promoters are not functional. Gonadal aromatase is responsive to dioxin-like compounds, but predicted AhRE sites in the *zfcyp19a* do not function via classical AhR/ARNT pathway. Environmental inhibition of the aromatase system can lead to profound changes of neuronal, developmental and reproductive functions of zebrafish.

With these results, D5.3 provides researchers and testing laboratories with a suite of novel tools for diagnosis of EDC exposure and effects. Further, the results summarised herein have important implications for risk assessment of EDCs since they point to new mechanisms of action and new targets at risk by EDCs.

Deviations/Delays and Justification

In the studies on regulation of *zfcyp19a,* it was discovered that luciferase expression in an empty luciferase expression vector (pGL2basic) was induced upon E2 treatment in CHO cells context. This caused ambiguity and subsequent delays in interpreting the results obtained on the effects of estrogen treatment on *zfcyp19a* promoter activity.

The start of the micro array-based gene expression analysis of mixture effects in rat testis was delayed, because of delays with the animal work, caused by unexpected effects at low doses. These observations required additional single-compound testing, and thus, the experiments are some months behind schedule. It is expected to receivee the first material in the spring of 2006 and we do not expect that the delay will further affect the work. We will allocate additional resources to complete this work if necessary.

Interaction/Collaboration with other Partners

The substantial work progress achieved under D5.3 was possible only by the intensive collaboration an interaction of the involved partners. For instance, samples from experiments with zebrafish done by Partner 13 were provided to Partner 15 to perform analyses on aromatase responsiveness. Partners 11, 14 and 15 closely interacted, including exchange of materials, protocols as well as PhD students, in their studies on the aromatase system of zebrafish. Partner 1 and Partner 4 had a close collaboration on genomic responses of MCF-7 cells. Further, Partner 14 collaborated with Partner 1 for the assessment of mixture effects. Further collaborations exist beyond the WP5 Partners, for instance, Partner 15 cooperates with Partner 21 on mixture experiments in fish, and Partner 4 cooperates with Partner 20 on mixture experiments in rats.

D5.4 Recommendations for the utilisation of genomic responses in testing guidelines with fish

Responsible Partner: 15 Participating Partners: 11, 13, 14

Objectives

This document provides recommendations on the the utilisation of novel genomic endpoints and tools in toxicity testing with fish. The conclusions reported here emanated from WP 5 "The application of genomics and proteomics to the development of sensitive endpoints of EDC action".

The existing toxicity test guidelines using fish rely on the endpoints survival, growth and reproduction. Although EDCs will ultimately affect growth and reproduction, these endpoints due to their apical nature are not able to reveal the underlying endocrine-disrupting mode of action. This problem has been recognised and as one consequence, OECD has initated the development of "enhanced" fish test protocols which incorporate endpoints diagnostic of an endocrine-disrupting mode of action. The endpoints currently considered by OECD are vitellogenin and gonad histopathology. While the latter is not absolutely specific for EDCs – for instance, oocyte atresia may be caused by generally toxicity as well as by endocrine toxicity – the former is indicative of one specific endocrine action, i.e. the ligand-dependent activation of the estrogen receptor. However, hormone systems other than the estrogen receptor pathway can be targets of environmental chemicals, and such actions will be not or hardly detected by the currently considered endpoints. Thus, there is an urgent need to broaden our abilities to screen for specific modes of action by developing appropriate diagnostic endpoints. Here, WP5 has made a susbtantial contribution by identifying:

- Novel targets and mechanisms of EDC action
- Novel endpoints to detect endocrine-disrupting activities of chemicals
- Novel diagnostic tools for use in EDC screening and assessment.

The ED case illustrates how essential mechanism-related information on toxicity pathways is to understand and to predict adverse effects of chemicals. For example, knowing that a chemical acts by mimicking endogenous hormones is necessary to understand why this compound may show a U-shaped concentration-response curve, since hormones can act differently at low and high doses. Knowing that a chemical acts by mimicking endogenous hormones is necessary to understand why this compound can induce quantitatively and qualitatively different effects in different life stages, for instance, permanent organisational effects during the period of sexual differentiation and transient activational changes during the adult reproductive cycle. Knowing that a chemical acts by mimicking endogenous hormones is essential to understand complex toxicity profiles since hormones act in homeostatic feedback loops and have pleiotropic effects so that one and the same initial event may lead to alterations in a range of animal functions, for instance, a xenobiotic ligand of the ER may affect gonadal function directly through binding to gonad ER, but also indirectly through modulation of ER-regulated pathways in the brain and in the neuro-endocrine axis regulating gonad function.

The importance of mechanism-related information is already evident from the fact that wildlife effects of EDCs have been not predicted by conventional risk assessment. This

failure is at least partly due to the absence of mechanism-related endpoints in the existing testing procedures. Withouth questioning the value of apical endpoints in predicting potential ecological effects, the ED lesson teaches us that the exclusive focus on them can result in shortcomings in both diagnostic and predictive risk assessment and that the inclusion of mechanism-related endpoints is valuable or even inevitable to identify ecologically relevant toxic properties of chemicals. Knowing, for instance, that a chemical is able to bind to the ER, and knowing that the ER pathway is involved in regulation of reproduction, indicates a potential hazard of this chemical to the reproductive capabilities of the organism. One may argue that the ecologically relevant effect of the ER-interacting chemical is not the disruption of the receptor signaling pathway but the associated disruption of reproductive performance, and that this apical effect will be detected in the standard toxicity tests. With other words: the hazard arising from an ER-binding xenobiotic will be detected even without knowing that this substance acts through an endocrine-disrupting patway, and without incorporating a mechanism-related endpoint into the testing procedure. However, the argument does not hold true for mainly two reasons. First, prolonged or life cycle tests usually are performed for an only limited number of substances, with the vast majority of available ecotoxicity test data originating from acute lethality tests. Second, measuring exclusively apical endpoints, and focusing on high dose effects such as lethality informs only on a part of a chemical's toxicity, whereas inclusion of mechanism-related endpoints helps to reveal the multiple toxicity profile of a chemical, particularly in the environmentally relevant low concentration range. By including mechanism-related endpoints at an early stage of testing, priorities could be set for further hazard evaluation, and, instead of executing the standard testing routine, a tailor-made testing scheme could be applied. For instance, the information that a test agent is able to activate the ER signaling pathway indicates a potential of this compound to adversely affect development and reproduction of exposed organisms and this would prioritise the test substance for a life cycle test. In a knowledge-based testing scheme, the trigger for chronic testing could be production volume (exposure information) plus the experimentally or computationally determined ability of the compound to activate a specific toxicity pathway (mechanistic hazard information), because the activation of this pathway implies the risk of long-term developmental and reproductive changes. Thus, a lesson learned from ED is that genomic endpoints indicative of toxicity mechanisms can act as a "signpost" to guide further hazard evaluation and to identify ecologically relevant functions at risk.

Although potentially useful for predicting possible adverse effects of chemicals at the population level, an exclusive focus on apical (whole animal) endpoints can result in shortcomings in both diagnostic and predictive risk assessments. Specifically, apical endpoints (e.g. growth, development and reproduction) lend little insight as to causative mechanisms or modes of action, which can only be assessed by integrating genomic responses in addition to the apical endpoints. Work done in WP5 has illustrated how molecular biology, specifically studies on single specific gene transcripts, can assist unravelling the pathways and mechanisms of estrogenic disruption in fish and can helpin establishing novel endpoints for toxicity assessment (see D5.3). Given the pleiotropic activity of hormones, more comprehensive molecular approaches (beyond assessing effects at single gene targets) are needed to identify pathways and mechanisms of endocrine disruption. The potential for 'omics' technologies to help unravel how EDCs interact in fish and mediate their effects is now well recognised and work done in EDEN has highlighted the important potential of genomic tools and mechanistic understanding to enhance and improve current test guidelines with fish. However, the practical science is

still in its infancy and it is too early yet to include such tools into routine testing protocols.

Conclusions

Genomic response have a high potential to improve and enhance existing tets guidelines with fish, since they can unravel if an adverse effect of a chemical on growth, development and reproduction of fish is based on an endocrine mode of action. In addition, genomic responses can be used at an early stage of testing to direct further hazard assessment and to make it more targeted. Finally, genomic tools offer the possibility to detect multiple actions of one substance as well as interactions of multiple substances. To conclude, genomic responses clearly increase the ability to detect biological and ecological functions at risk and render both diagnostic and predictive hazard assessment more reliable. Currently, the major drawback in the utilisation of genomic responses is that these technologies are still at the beginning of their application in fish systems, and require further knowledge and experinece before they can be incorporated into standard testing protocols.

D8.2 Report detailing the comparative sensitivity of HP unit to disruption by EDC during sexual differentiation in male and female rats and information about molecular, biochemical or hormonal endpoints that signal these changes

Responsible Partner: 17 **Participating Partners:** 3, 4

Objectives

Specific aims of D8.2 are to:

- To define the sensitivity of the HP unit to the disrupting effects of estrogenic (and eventually androgenic) compounds after exposure during critical periods of sexual development.

- To identify novel molecular end-points (biomarkers and mechanistic factors) involved in these disrupting events.

Male and female rats were neonatally exposed to different doses of the synthetic estrogen, estradiol benzoate, and brain (hypothalamus), pituitary and serum samples were obtained at different age-point of postnatal development. In addition, protocols of neonatal exposure to the synthetic androgen, testosterone propionate, the anti-androgen, flutamide, and the suspected xeno-estrogen, bisphenol A, have been implemented. Of note, brain sexual differentiation in rodents takes place perinatally (between E17.5 and d-10 postpartum) and is mainly a hormonally-driven phenomenon (estrogen being the major signal). Thus, the selected model of neonatal exposure is especially suitable for targeting endocrine disrupting events at the developing HP unit, which might result in durable (organising-like) effects that manifest later in life.

Research Progress

Different research activities have been undertaken towards D8.2 during the three year life-span of EDEN WP8. In brief summary:

- Neonatal treatments of male and female rats with potent synthetic estrogen, androgen or anti-androgen have been conducted by Partner 17, and relevant tissue samples (hypothalamus, pituitary and gonads) have been collected over a wide range of age-points after neonatal exposure. Exposure protocols have included treatments over a limited range of doses.

- Analyses of potential gene markers of exposure to estrogen at the HP unit have been carried out by Partner 17 in order to characterise potential mechanisms of action of estrogenic and related compounds as well as to determine thresholds of disruption. Among gene candidates for analysis, and on the basis of scientific background, most of our attention has been focused in evaluation of expression of progesterone receptor (PR) gene at the hypothalamus. In addition, expression of PR and ER variant TERP-1 at the pituitary in different experimental models was also conducted. Finally, such analyses were extended to seek for ER-α and ER-β specific effects (by means of the use of ER-subtype specific ligands), as well as for

the evaluation of the effects of neonatal exposure to synthetic compounds with combined estrogenic and anti-estrogenic actions as tamoxifen and raloxifen (globally termed as selective ER modulators or SERMs).

- The recent identification of the relevant role of KiSS-1/GPR54 system in the control of the reproductive function at the HP unit led to carrying out expression analyses of KiSS-1 and GPR54 genes at the pituitary and hypothalamus in different models of neonatal exposure to synthetic steroids; a work that was mainly conducted by Partner 17. Initially, the physiological role of the KiSS-1 system in the control of gonadotropic axis and puberty onset was characterised. In addition, analyses to define the effects of neonatal exposure to different doses of the synthetic estrogen estradiol benzoate upon the expression levels of KiSS-1 and GPR54 genes at the hypothalamus were undertaken. Upon initial characterisation of the effects (in terms of gene expression) and sensitivity (threshold doses), further analyses were implemented in additional experimental groups to define the putative role of the hypothalamic KiSS-1/GPR54 system as mechanistic target for endocrine disruption at the HP unit following exposure to estrogenic/androgenic compounds during critical periods of sex differentiation of the brain.

- Transfer of relevant tissue samples to Partner 4 was conducted in order to carry out screening of differentially expressed genes at the HP unit after neonatal exposure to estrogenic and anti-androgenic compounds by means of DOP-PCR differential display (DD). Such analyses conducted by Partner 4 allowed us to identify potential novel biomarkers and endpoints of endocrine disruption at the HP unit. Among these, most of the efforts have been focused in the characterisation of globin gene expression at the pituitary as putative biomarker of exposure to estrogenic (and eventually anti-androgenic) compounds during critical periods of sex differentiation of the HP unit (see below).

- Initial identification by DD of persistent enhancement of expression of α- and β-globin genes at the pituitary after neonatal treatment with estrogen was followed by detailed characterisation of such a phenomenon. Thus, real-time RT-PCR assays were optimised for these targets, and age- and dose-response analyses were conducted. These fully confirmed the initial observations on the induction of expression of both genes at the pituitary by neonatal estrogen exposure. Using the same methodological approach, induction of globin genes in other tissues (as hypothalamus, liver, brain and testis) was explored, and the responses to neonatal treatments with a potent anti-androgen or an aromatase inhibitor in terms of α- and β-globin gene expression were evaluated. In addition, in situ hybridisation assays were conducted in collaboration between Partners 4 and 17.

In this scheme, Partner 3 has carried out a central role in providing coordination of interactive studies. Likewise, harmonisation of standard operational procedures has been conducted through Partner 3. Those contributions which are considered as the most salient, and closely related to D8.2 have been summarised in the report by Partner 17.

Discussion and Conclusions

The major conclusions drawn from the research activities conducted towards D8.2 can be summarised as follows:

(i) The developing HP unit appears to be highly sensitive to the effects of synthetic estrogens, as monitored by molecular biomarkers, such as expression of PR and KiSS-1 genes at the hypothalamus and that of globin genes at the pituitary. Dose-responses curves allowed Partner 17 to define that neonatal exposure to doses as low as 10μg estradiol benzoate per rat was 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 α- and β-globin genes at the pituitary. Interestingly, 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, suggesting that these might be regarded as genuine markers for potential endocrine disrupting events at the HP. Moreover, the fact that some of the above markers were also altered in expression after neonatal exposure to the aromatisable androgen, testosterone propionate, the anti-androgen, flutamide, and/or the putative xeno-estrogen, BPA, 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 exual differentiation.

(ii) 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.

Interaction and Collaboration

In line with the original intentions of the Workpackage plan, active interaction with other concerned Partners has been implemented in order to cover the goals included in D8.2 and related deliverables:

1. **Partner 17 - Partner 4:** Pituitary and hypothalamic samples from different experimental models of neonatal exposure were collected by Partner 17 and transferred to Partner 4 for conduction of DD-analyses. Upon initial identification of putative biomarkers, detailed expression analyses (involving semi-Q and real-time

RT-PCR and ISH) have been conducted in a collaborative manner between these to laboratories.

2. **Partner 21 - Partner 17:** Pituitary and brain samples from different models of in utero/perinatal exposure to suspected EDCs (range of doses) generated by Partner 21 have been transferred to Partner 17 for processing and expression analyses. Active collaboration between the two groups (exchange of ideas and fruitful discussions) has also focused in selection of suitable gene markers at the HP unit. This collaboration will be mostly included in D8.4.

3. **Partner 17 - Partner 18:** Although not overlapping in terms of contents, contacts between these two partners have allowed exchange of information concerning progress of research work at both laboratories.

4. **Partner 17 - Partner 3:** Partner 3 has carried out a central role in providing coordination of interactive studies between partners involved in WP8-Theme2-EDEN. Likewise, harmonisation of standard operational procedures has been conducted through Partner 3. More recently, exchange of tissue samples (not primarily related with D8.2, but within the general scope of EDEN) has been initiated between Partner 17 and Partner 3 laboratories.

5. **Partner 17 - Partner 5 / Partner 17 - Partner 9:** Active collaboration between Partners 5 and 17 is ongoing in studies on Reproductive Biology, not directly related with D8.2 has taken place during EDEN life-span. In addition, scientific contacts between Partners 9 and 17 have been conducted in order to explore potential interactions within EDEN.

D11.1 Birth cohort effects for serum testosterone

The validity of the measured hormone levels have been explored in three different ways and are detailed in the 2nd reporting period Annex II (8). Results are in accordance with Partner 4's hypothesis that decreased male reproductive health, which seem to be more frequent and which it is believed in many cases is related to the fetal/neonatal development of the testis, also is reflected in decreased testosterone levels. The change in hormone levels with later birth cohorts (level in the 1921-26 birth cohorts as reference) after adjustment for the effect of age and period of sampling is given in Figure 1.

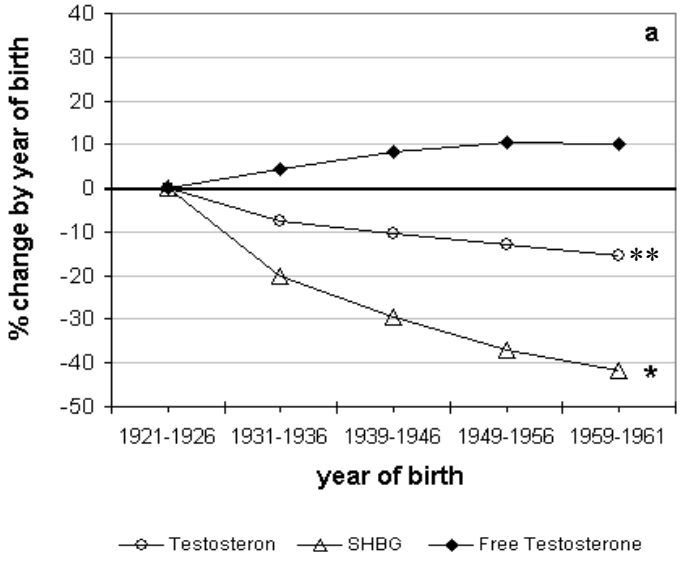

Figure 1 Percentage change in hormone levels (*p<0.01, **p=0.02)

In concordance with the Danish results, preliminary analyses indicate that the Finnish data illustrates a similar type of age-related decline in testosterone values. Birth cohort effect is also apparent, showing a decline. It is well established that serum testosterone (T) values decrease with age and the testosterone and sex hormone binding globulin (SHBG) levels in men of different ages born in different decades were compared in order to evaluate the possible birth cohort effect on the testosterone levels, which may confound age-dependent changes observed in cross sectional studies.

Testosterone and SHBG were analysed in 3271 male serum samples from three Finnish population surveys conducted in 1972, 1977 and 2002 by the National Public Health Institute. Men were divided into six age groups, birth cohorts were divided into seven groups (Table 1). T and SHBG were both measured by time-resolved fluoro-immuno assays (DELFIA®, Wallac Oy, Turku, Finland).

Significant birth cohort related changes in testosterone (Table 1) and SHBG as well as calculated free T serum levels were observed with lower levels in more recently born men of the same age. T decreased and SHBG increased with age within the birth cohort. The etiology behind the observed cohort effect remains to be resolved. The described birth cohort effect partly blunts the age-related decrease of T levels and exaggarates the age-related change in SHBG levels in cross-sectional analysis. The Finnish data supports the hypothesis that male reproductive health has deteriorated during the study period.

A manuscript has been prepared based on the Finnish data entitled 'A clear birth cohort effects in serum testosterone and SHBG levels in Finnish men' by Perheentupa A, Laatikainen T, Vierula M, Skakkebæk NE, Andersson A-M, Toppari J. However, these results need further analysis to account for all confounding factors though in essence this D11.1 is completed.

Birth year	**Age**	**1970-77**	**1960-69**	**1952-59**	**1942-51**	**1933-41**	**1923-32**	**1913-22**	**ALL**
	27	19,1 A, a (8,7-27,3)			26,4 A, b (15,1-44,0)				25,3 A (13,3-43,5)
	35	17,2 A, a (7,7-37,6)	16,1 A, a (8,6-28,3)		20,5 B, b (11,1-38,6)	22,0 A, b (11,6-38,6)			20,5 B (10,3-37,6)
	45		15,7 A, a (7,5-25,8)	14,6 A, a (8,5-29,8)			22,6 A, b (11,2-39,6)		20,9 B (9,5-38,4)
	55			17,4 A, a (8,7-30,9)	15,3 C, a (7,5-30,4)			22,6 A, b (11,3-40,9)	19,9 B (8,7-39,9)
	65				13,8 C, a (7,7-27,8)	14,4 B, a (8,0-31,3)		21,9 A, b (10,3-40,9)	17,0 C (8,1-36,4)
	72						16,9 A (8,4-28,3)		16,9 C (8,4-28,3)

Table 1 Serum testosterone concentrations in nmol/L (median, 5-95 percentiles, nmol/L) in birth cohorts of Finnish men. Capital letters depict statistically significant difference down each column, while small letters depict differences across each row ($P<0.05$, ANOVA).

D12.1 Report on male reproductive health in Germany; Regional differences in male reproductive health and their association with maternal exposures

Report on male reproductive health in Germany

A preliminary report on the male reproductive health in Germany was given in the 2nd year reporting period Annex I (D12.1). Electron microscopial analysis of all semen samples has now been completed and Table 1 shows all the key parameters for male reproductive health in Leipzig and Hamburg.

valid%		**Hamburg**	**Leipzig**
sexual abstinence, h	n	331	456
	mean	94.88	81.41
	median	73.0	65.0
	std. deviation	174.064	160.788
	5%-95%	19.6-160.4	15.0-160.0
semen volume, ml	n	332	456
	mean	3.382	2.721
	median	3.155	2.55
	std. deviation	1.7724	1.451
	5%-95%	1.027-6.404	0.785-5.415
motility, %	n	328	455
	mean	65.744	76.544
	median	67.667	81.667
	std. deviation	11.7799	18.8684
	5%-95%	43.233-80.183	36.333-95.0
sperm concentration, mill/ml	n	326	454
raw data	mean	62.524	64.496
	median	48.5	45.4
	std. deviation	61.1297	57.2714
	5%-95%	3.37-186.43	5.5-174.375
sperm concentration, mill/ml	n	326	454
quality control adjusted*	mean	62.524	54.198
	median	48.5	38.151
	std. deviation	61.1297	48.1272
	5%-95%	3.37-186.43	4.622-146.534
total sperm count, mill	n	327	454
quality control adjusted*	mean	205.87	139.63
	median	142.49	95.05
	std. deviation	230.842	139.538
	5%-95%	9.51-609.45	8.18-400.22

* adjusted for differences in counting between centres based on quality control samples

Table 1 Comparison of reproductive health for German men in Hamburg and Leipzig.

Clear differences in the spermatozoa counts of the two German centres can be observed. Though D12.1 is completed, in order to publish in a peer reviewed journal, several confounders need to be considered and adjusted. Most of this data analysis has been

completed by Niels Jørgensen and the statistician from Partner 4 in close collaboration with Partner 16b.

Regional differences in male reproductive health and their association with maternal exposures

The regional differences in male reproductive health and association with maternal exposures were addresssed in the 2nd year reporting period Annex I (D12.1) and have been published.

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, Skakkebaek, 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.

D12.3 European male reproductive health database accessible for the European research community

The database is accessible to the European researchers that have contributed to the dataset. The data gathered in the database represents an investment of many millions and it was never the intention that this database should be made public for all to use. Statistics on the number of researchers who have access to it and other information can be provided on request.

D13.4 Criteria document for low-dose testing

Responsible Partner: 1 Participating Partners: 2, 8, 13, 19, 20, 21

Document written by Martin Scholze and Andreas Kortenkamp, Partner 1 of University of London, School of Pharmacy.

Introduction

This document was prepared as part of the EDEN project (QLRT-2001-00603). It provides information about outcomes from low-dose testing in a variety of bioassays relevant to endocrine disrupting chemicals (EDC). It emanated from WP 13 "Low-dose effects of EDC in cell-based assays, fish and rodents". The following partner laboratories were involved with experimental studies:

Partner 1: Dr Kortenkamp, London, UK
Partner 2: Dr Pottinger, Lancaster, UK
Partner 8: Prof Mayer, Bergen, Norway
Partner 13: Prof Hock, Muenchen, Germany
Partner 19: Dr Katsiadaki and Prof Scott, Weymouth, UK
Partner 20: Dr Hass and Dr Larsen, Copenhagen, Denmark
Partner 21: Prof Norrgren, Uppsalla, Sweden

Why is "low dose" an issue in endocrine disruption?

A number of specific properties and effect profiles are commonly thought to set endocrine disrupting chemicals (EDC) apart from other hazardous substances. For example, the effects provoked by EDC that interfere with androgen action may be irreversible, because key steps of sexual differentiation of males during development in the womb may be altered in ways that do not become apparent until later in life. Many of the abnormalities of concern cannot be predicted from changes in hormone levels that may occur as a result of exposure to EDC in adulthood. Furthermore, some EDC-mediated effects were shown to occur at dose levels lower than normally tested in toxicology. Further, unusually shaped dose-response curves were observed by some laboratories (vom Saal and Sheehan 1998) but could not be replicated by others *(Ashby et al*., 1999). This has provoked an unusually heated controversy in the field.

Thus, are EDC "special", both in terms of their toxic profiles and their ability to induce effects at low doses that disappear as doses are raised and may therefore be overlooked during testing at higher doses? The aim of the studies carried out as part of the EDEN project was to assess whether such unusually shaped dose-response curves occur in a wide variety of assay systems for the testing of EDC. 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 using different approaches.The following table gives an overview of low-dose studies carried out in the EDEN project.

Assay	Partner	Chemicals	Endpoint
E-Screen	1	E2, β-HCH	cell proliferation
Female rats and offspring	20	DINP, DEHP	Changes in anogenital distance, nipple retention
Zebra fish	13, 21	E2, E1, NP	Vitellogenin induction
Stickleback	2, 8, 19	E1, NP	Vitellogenin induction
Stickleback	2, 8, 19	Fenitrothion, flutamide	Spiggin induction

Table 1 Low dose studies

What is "low dose"? – Definitions

The term "low dose" has been coined to describe the fact that some effects reported by certain laboratories occurred at doses that were perceived to be lower than expected. However, there is a controversy concerning the validity and reproducibility of these observations.

The term "low dose", however, as used in the context of EDCs, is not precisely defined, nor does it describe a new phenomenon. 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" to "doses associated with low effects".

The first definition, "doses lower than used normally in toxicity testing", is ambiguous because the range of doses employed in toxicity testing is very large, and depends not least on the potency of the chemicals under investigation. The second definition, i.e. doses in the range of exposures encountered by humans, may be problematic in many cases, because many bioassays lack the power and sensitivity to demonstrate effects in such dose ranges. Thus, it becomes apparent that the term "low dose" is difficult to use in a manner divorced from any measure of biological effect or potency.

For the purposes of EDEN, it was therefore necessary to operationalise the term and to adopt a definition in the sense of "low effect dose". "Low effect doses" or "concentrations" signify doses or concentrations that are associated will low effects, regardless of the nominal value of these measures. In the toxicological assessment of chemicals, the aim is to define threshold doses between "no-effect" and "effect".

Thresholds and zero effect doses

The idea that chemicals cause (non-cancer) effects only above a certain dose, the so-called dose threshold, is a key concept in toxicology. It forms the basis of chemicals safety assessment and underpins much of regulatory and legislative activities. Although the assumption of dose thresholds has been described as one of the strongest dogmas in toxicology (Slob 1999), the issue continues to provoke debates, most recently in the endocrine disruption arena, and clearly cannot be regarded as settled.

A "threshold" is usually equated with a toxicant dose that does not elicit any effects. However, in this formulation, the idea of a "threshold dose" is too vague to be useful as a basis for regulatory activities. Slob (1999) has clarified the problem by highlighting three different aspects of a "threshold dose":

(1) 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.
(2) 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 set-up.
(3) 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.

In the strict quantitative (mathematical) sense, thresholds do not exist in dose-response relationships. If they did exist, then one molecule should already make a difference between response and lack of response.

The empirical definition of thresholds is problematic, because the question as to whether an effect is observable or not depends on the sensitivity of the experimental set-up used for measuring effects. With expenditure on more resources, the sensitivity of experiments can be increased, with a consequential down-ward shift in the effects that are resolvable and therefore observable. Thus, the existence (or otherwise) of a dose threshold cannot be proven empirically.

The biological, more qualitative notion of thresholds can only be translated into quantitative terms by defining thresholds in terms of the effect size of continuous endpoints. Thus, the question arises as to what size of an effect an organism can cope with and when the effect should be deemed adverse (biological relevance). In the context of endocrine disrupter research, this question is extremely difficult to decide, and there are almost no rational criteria that would help to decide whether an effect is adverse or otherwise.

Against the background of these conceptual and fundamental difficulties, it is of relevance to assess what size of an effect can be detected in a given experimental system with a certain statistical power. The low dose studies carried out in EDEN were aimed to evaluate reliable statistical effect thresholds for typical experimental designs.

Approaches to estimating low effect doses

There are two major strategies used for estimations of doses “without effect”: multiple comparison procedures and regression model-based approaches. Multiple comparison procedures aim to establish a no-observed adverse 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 approaches estimate a dose that corresponds to a predetermined response level that is deemed acceptable (benchmark dose).

Both approaches have inherent limitations, but also have particular advantages and strengths.

Hypothesis testing approaches

Multiple comparison procedures have been the focus of extensive criticism, because the statistical procedures employed to make comparisons between (untreated) controls and exposed groups all too often state that there is no effect, when in fact there is one. As a result, effects that in reality exist are frequently overlooked (Moore and Caux 1997). However, this is strongly dependent on the statistical concept used: small effects can only be detected as statistically significant up to a certain degree (power); otherwise no statistical conclusions are possible. Under this concept, the situation becomes further complicated by the fact that there are numerous statistical tests available for the estimation of NOEL. This may lead to widely differing numerical values, depending on which method is used, even when the same biological data are analysed. Furthermore, the poorer the data quality and the sloppier the experimental designs, the higher the doses tend to be that are estimated as being without effect. Another weakness has been described as the multiplicity dilemma which arises when multiple comparisons have to be made. In such situations, the overall significance level (family-wise error rate α) that is deemed acceptable (e.g. the customary 5%) as well as the number of multiples tested determine whether a significant effect can be detected as significant or not; if the number of multiples grows, the significance level associated with each individual tested dose becomes ever smaller (adjustment of p-values), thus making it more likely to accept that there are no effects when in fact there are ("penalty for doing a job better"). On the other hand, hypothesis testing procedures do not necessarily require any assumptions about underlying dose-response relationships and can deal with very different testing situations. With well chosen dose levels and a high number of replicates, it may be possible to identify small effects reliably.

Regression-based approaches

The weaknesses of hypothesis testing methods have motivated a search for alternative methods in estimating low effect doses, and regression-based approaches are increasingly promoted as a viable solution to replace NOELs (Crump 1995). The rationale is to carry out dose-response analyses to construct a regression model. The model is then used to estimate low effect doses either by interpolation or by extrapolation. The advantage of these methods lies in the fact that the entire information and statistical power contained in experimental observations is used to derive doses associated with small effects. Furthermore, poor data quality will increase the confidence interval of dose estimates. This will tend to decrease low effect estimates if these are based on the lower confidence limit, as is customary. However, the validity of conclusions from modelling approaches depends highly on the correct choice of a regression model. Unfortunately, there are no *a priori* criteria for choosing an appropriate model, especially in the low dose range, and this leads to a dilemma for planning experimental studies. Finally, there are no universally accepted criteria for choosing a predetermined effect level that is deemed to be safe.

Modelling and fitting of concentration-effect data

The concentration response relationships for the chosen effect endpoints in the EDEN project are not known from first principles, and a universal model which allows the description of every possible concentration response pattern does not exist. Thus, empirical regression models have to be employed, in order to smooth the observed effect data in the best possible way. These models do not have any mechanistic meaning. It is

therefore not an option to use pre-existing knowledge about the mode of action of test chemicals as a selection criterion for a suitable regression model.

However, it may be possible to say that one model is "better" than another in the sense that it produces more accurate effect predictions. But generally we cannot say that it is more probable (therefore it is inappropriate to assign probabilities to models). A way out of this dilemma is to use as many models as possible to minimize the chance of biased curve estimations. This can be achieved by fitting different suitable models independently from each other to the same set of data. Based on appropriate goodness of fit criteria it is then possible to decide the "best-fitting" model in a relative sense, i.e. by comparing the fits of different models to each other. For so-called nested-models, this may be tested statistically (e.g., by the likelihood ratio test), i.e. when one model parameter contains no relevant information and can be dropped from the model. Unfortunately, the models are usually not nested to each other, thus no formal comparison is possible by statistical testing. Different models that are found to fit the data equally well do not necessarily result in different curve estimates, at least within the interpolative data range. In most cases, the estimation of median effects is model-independent. Thus, the data themselves influence the outcome of assessments much more strongly than the specific model chosen for analysis, and model comparisons are only useful for extremely "good quality" data outcomes which allows very precise estimations. However, in the case of low effects or extrapolations, the outcome can be highly dependent on the model selected. Therefore, the choice of the "best" model is extremely important.

Models for non-nested endpoints

Numerous models are theoretically possible, but in practice only a limited number is applied, mostly determined by historical conventions in various specialist fields. In order to select suitable parametric models for the description of the concentration effect data produced in EDEN, the following criteria were used, and assumptions made:

- The model describes continuous, non-negative measurements (for that reason all polynomial models and derivates are excluded).
- Exclusion of threshold values: it is highly unlikely that all animals (or cells) have exactly the same concentration-threshold for an endpoint chosen for analysis. Furthermore, the estimation of a threshold is always problematic because of the non-mechanistic use of threshold models and its dependence of the chosen start values within the fitting process and its dependence of the chosen concentration regime.
- The model includes a model parameter θ_{min} which describes the lower effect asymptote, i.e. the mean negative control if data is not normalised to zero.
- The model should include a model parameter θ_{max} which equals the upper effect asymptote (if data is not normalised to one).
- The model includes a maximum of three model parameters (+ maximal two model parameters for both asymptotes) for the description of the sigmoidal curve shape in order to avoid the trap of overgenerality, i.e. the nonlinear regression models should be parsimonious ("simple" models, i.e. models that are likely to exhibit good estimation behaviour, are those that generally have a simple form and few parameters).
- "Commonly" used models should be included.

- The model should be "best" reparameterised to enhance the numerical fitting behaviour, i.e. all models are excluded that are really the same basic model, but appears in different forms, and, whenever possible, the model parameters giving raise to poor behaviour of their least-squares estimators should be replaced with better expected-value parameters.
- Exclusion of all models for which the statistical properties in estimation always tend to be poor and cannot be reduced by any reparameterisations.

One advantage is that by setting the asymptotic model parameters θ_{min} and θ_{max} to a fixed default value, e.g. in order to model fractional effect data ($\theta_{min} = 0$, $\theta_{max} = 1$), not only effect data can be modelled which assume a non-zero control, but also effect parameter from different studies with slight control responses. However, in this way the control means are assumed as fixed values out of uncertainty, i.e. all statistical inference statements about low dose estimates are then underestimated. But usually only effect estimates near the control levels are affected.

Thus, a pool of maximal twelve parametric regression models was created. All these models are *a priori* suitable for descriptions of the expected concentration response patterns, for the non-nested endpoints used in the EDEN project (i.e. all *in vivo* fish endpoints and *in vitro* parameters). When data from repeated studies were pooled, it was necessary to account for the intra- and inter-experimental variability associated with this nested data scenario. To achieve this, we used a generalised non-linear mixed modelling approach in which both fixed and random effects are permitted to have a non-linear relationship with the effect endpoint. As random effect, we always included a shift parameter in the non-linear regression model for *in vivo* data from fish studies, which accounts for a shift of the whole curve based on the log10-transformed concentration scale. In case of the E-Screen data, we included a random effect accounting for different curve maxima, and in some cases, if fitting improvement was tested as relevant, also an additional parameter for variations between curve slopes was embedded in the model. The best-fit approach was followed, although the pool of suitable models was reduced to five.

Models for nested endpoints

Special considerations apply to dose response models for nested endpoints such as those for the *in vivo* studies with rodents. In these studies, pregnant females ("dams") are given several doses of a substance, and the offspring ("pups") is examined for signs of abnormal development (e.g. anogenital distance and nipple retention). In such studies it is usual for the responses of the pups in the same litter to be more similar to each other than the responses of pups of different litters ("intra-litter correlation", or "litter-effect"). The variance among the proportions of pups affected in individual litters is greater than would be expected if the pups were responding completely independent of each other. This is comparable to a typical modelling situation in developmental toxicology studies, which allows adopting models developed in this field for the project. For example, the US-EPA recommends three models for the description of monotonic dose response relationships for nested endpoints: the logistic, the NCTR model and the Rai and Van Ryzin Model (the two latter ones are modifications of the Weibull model). Common to all these models is that they include a litter-specific covariate, which is expected to account for at least some of the extra inter-litter variance. Another common approach is to use models that

provide for extra inter-litter variance of the proportion of pups affected (beta-binomial probability model, overdispersion models etc.).

As the effect endpoints used in the rat studies are of different statistical nature - anogenital index is continuous, but number of nipples typically a counting variable – two different model approaches were used: dose-response data for the AGD index were analyzed by a generalized non-linear mixed modelling approach, with the litter as random effect for individual AGD data in order to account for the nested litter effect. The corresponding statistical analysis was performed on basis of the SAS procedure PROC NLMIXED. The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and 12, corresponding to the average number of nipples for the females. Correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method (GEE). All statistical analysis was performed on basis of the SAS procedure PROC GENMOD.

Combining both approaches

Since both approaches to estimating low effects, multiple comparison methods, and regression model-based approaches, have complementary strengths and limitations, it would be desirable to develop a framework for an integrated approach that combines the strengths of multiple comparison techniques with those of regression model-based techniques for the analysis of dose response data (Bretz *et al*., 2005).

The approach proposed has the following features (Figure 1), as it is impossible to determine a zero effect dose in the strict quantitative sense, the aim must be to derive estimates of doses that correspond to a critical effect size. The dose or concentration associated with the critical effect size will then be regarded as the maximal acceptable dose. This should prepare the ground for devising experimental designs that are more reliable, and lead to statistical tools that are more suitable. Generally, the data set should only be accepted when sufficient power is guaranteed, i.e. type I and type II errors are controlled. One way to ensure that statistical significance can be used in a meaningful way for planning purposes is to determine the minimum significant different (MSD) on the basis of many studies (Phillips *et al*., 2001). MSD is a statistic that indicates the difference between two means (the mean of the sample and control replicates) that will be considered statistically significant given the observed level of among-replicate variation and the alpha level chosen for the comparison, e.g. the detectable difference inherent in a bioassay protocol can be determined by identifying the magnitude of difference detected by the protocol in 90% of all studies. Measurements such as the MSD should therefore be used as a quality control tool. Furthermore, low effect estimates are only justified when a significant trend in the dose response data pattern is present. However, this trend does not necessarily have to be monotonic.

From a pool of *a priori* selected candidate parametric models, the most significant one in terms of advanced contrast tests should be chosen (Hothorn 2004; Bretz and Hothorn 2001; Neuhaeuser *et al*., 2000). These multiple comparison tests make certain assumptions about the underlying shape of a dose-response curve. In case of non-significance, no statistically significant effective doses are present. In all other cases, the most significant model is chosen for the modelling of the dose response relationship in order to provide inference on desired doses. By using an appropriate multiple testing test, additionally either the lowest tested dose can be determined which is significantly higher than the critical effect, or the traditional NOEL.

The aim was to analyze the limitations of this approach and to outline possible critical effect sizes with regard to common testing designs and statistical consequences. The estimated MSD are always based on a error rate α=5% and a power of 80%.

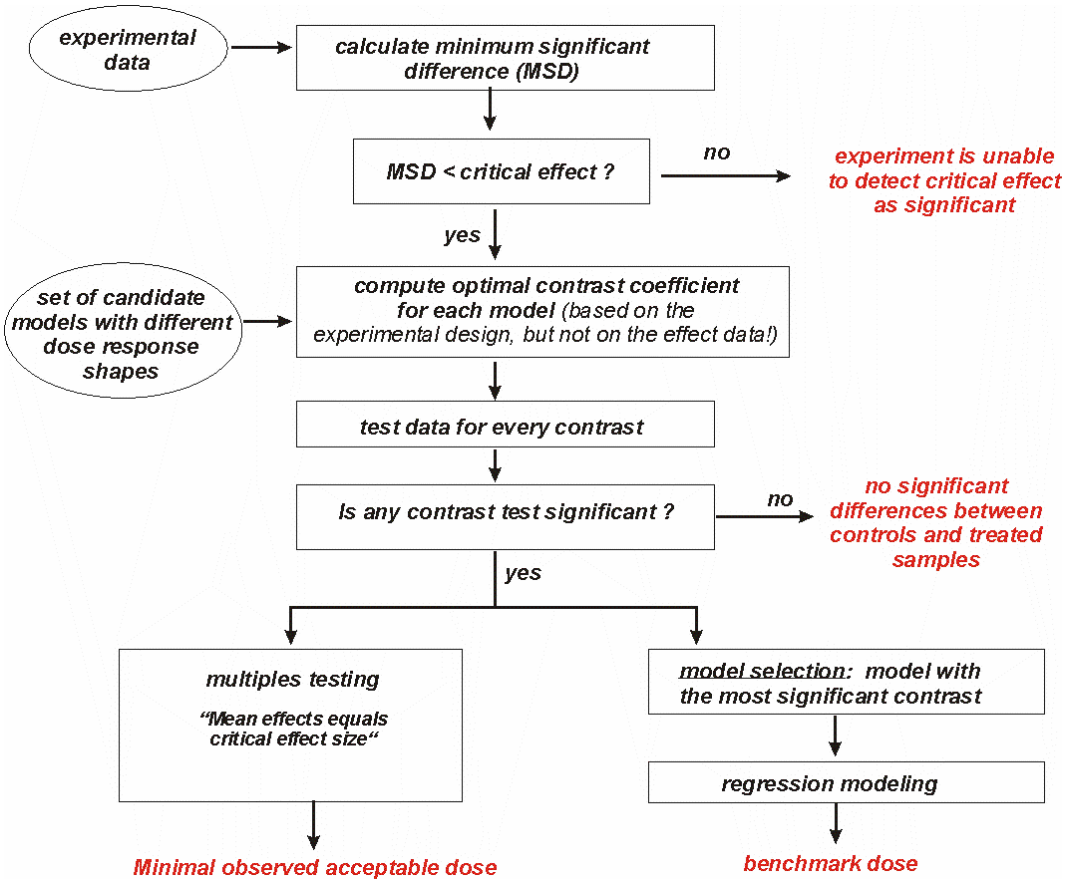

Figure 1 Integrated approach for the estimation of acceptable doses

Planning tool – Power and sample size considerations in multiple testing situations

The adequacy of an experimental design and the statistical test used to analyse study results are often evaluated in terms of the power of the statistical test. Power is defined as the probability that a false null hypothesis will be rejected by the statistical test in favour of a true alternative. Power therefore expresses the likelihood of detecting a significant effect. That power depends on the alternative hypothesis. Generally, the larger the effect, the higher the power becomes to detect that effect. Thus, if a test compound has had some effect on the organisms in an assay, power is the probability that a difference between treatment groups and untreated controls will be detected. The power of a test can be calculated if the size of the effect to be detected, the variability of the endpoint, the number of treatment groups, and the number of replicates in each treatment group are all known. The primary use of power analysis in low-dose studies is in the design stage. By demonstrating that a study design and test method has adequate power to detect effects that are large enough to be deemed important, we can have some confidence that there is no effect of concern at that dose if we can demonstrate that, at this dose, there is no statistically significant effect. However, power does not quantify this confidence. Failure to adequately design or control an experiment so that statistical tests have adequate power

can result in large effects to be statistically insignificant. On the other hand, a test can be so powerful that it will find statistically significant effects of little biological importance.

To perform a power calculation, the condition "null hypothesis is false" must be specified precisely. For example, when testing the null hypothesis "control mean equals exposure mean", i.e. H_0: $\mu_0=\mu_1$, the condition "null hypothesis is false" must be specified by giving a particular nonzero value for the difference $\mu_1-\mu_0$, for example $\mu_1-\mu_0=5$. The power is, therefore, a function of the size of the difference, and the usual approach for designing experiments is to ensure that a "meaningful" difference will be detected with sufficient high probability. For example, if $\mu_1-\mu_0=5$ is considered to be a "meaningful" difference, then it is possible to design the experiment such that the power is, say, 80 percent when $\mu_1-\mu_0=5$. However, when testing more than one group against the control, as in a multiple testing situation or multiple comparison application, it becomes more complicated to define power. This is because there are now multiple parameters with multiple individual null hypotheses H_{0i}. In this case usually two different kinds of power have to be considered:

- *Complete power*, i.e. the probability to reject all individual null hypotheses that are false, and
- *Individual power*, i.e. the probability to reject a particular individual null hypothesis that is false. Usually the minimal significant difference is based on the individual power.

Complete power

This power appears almost the most attractive, since obviously it is from a statistical point of view the aim to reject all false hypotheses. However, it is usually very difficult to obtain rejections for all false hypotheses, since, according to this definition; reasonable designs very often have low power. For example, with 7 groups tested against a control, all with individual power of 0.8, the complete power is approximately $(0.8)^7=0.21$. Exact computations are usually not feasible and only specific computer simulations produce results that are sufficiently accurate for design purposes. With such methods the control of the global type I error α (familywise error rate) is guaranteed.

Individual power

Individual power is most closely related to the ordinary definition of power which is found in the basic statistical literature. The disadvantage of individual power is that, from the multiple statistical standpoint, it presumes a particular interest in just one of the multiple hypotheses. However this does not correspond to the testing situation: if interested only in a particular hypothesis, this can be tested solely without any multiplicity adjustments. The interpretation of the power is then "the probability of detecting a false null for, say, test number three is 0.8", but not "the probability of detecting all false nulls is 0.8" or "the probability of detecting at least one false null is 0.8". We therefore operated always with this power, in order to use existing tools like the MSD.

Example – In vivo testing with vitellogenin induction in zebrafish

While individual power refers to a particular hypothesis, complete power involves several hypotheses simultaneously about individual mean comparisons. As such, their calculations are complicated as they depend on the alternative settings for all non-null hypotheses, not just the alternative setting for one particular test of interest (usually the joint non-central distribution of the test statistic is required). Usually simulations are used in order to provide useful, slightly imprecise results.

In the following the usefulness of both power considerations as a planning tool is demonstrated on the basis of the results of the vitellogenin induction in the zebrafish (see statistical descriptors in Table 3). Dunnett's test is used as a test statistic for a balanced experimental design (exception: number of controls). This test is only valid for a continuous, normal distributed effect endpoint and it belongs to one of the most popular tests for the comparison of the mean control values to many group means. However, it is well known that for many applications the power of this test is not optimal, especially when some additional assumptions about the expected concentration response pattern can be made, for example monotony. However, as non-monotonic curves are still under debate in the endocrine field, we decided not to exclude this possibility already at the planning stage, but instead to use the more conservative Dunnett test.

In Figure 2 the number of replicates needed to detect a vitellogenin induction as significant from the negative control is shown. This is an example for using individual power as a tool for the experimental planning. As test statistics the one-sided Dunnett test was used, and this implied that we excluded the possibility that vitellogenin levels significantly lower than in controls will be observed. The familywise error rate is set to 0.05 (α=5%) and the power is assumed as 80%. The test design is chosen as 7 different concentrations, whereas the number of replicates (*a*) is equal for each treated samples, but the number of controls is set optimal in relation to the number of treated replicates (7*square root of *a*). An average vitellogenin concentration 40 ng/ml was assumed for the controls, and two scenarios of data variability considered: firstly, the experimental data follows an average uncertainty (Variance=0.07, black line), and secondly, as a worst-case scenario, the variability is increased (red line). Thus, with the given number of replicates, and associated variability, any observed mean vitellogenin induction below the level described by each line cannot be detected as statistically significant.

The usefulness of a complete power consideration is illustrated in Figure 3. Here, the aim is to estimate how many replicates are needed to detect all true effects as significant for a given uncertainty scenario. The assumption is that seven test concentrations are used, and that the resulting effects (vitellogenin induction) should all be statistically significantly different from the control. The underlying concentration response curve can be characterised as relatively flat (see inlet). In this case, a minimum of 11 replicates is needed to guarantee a global power of at least 80%.

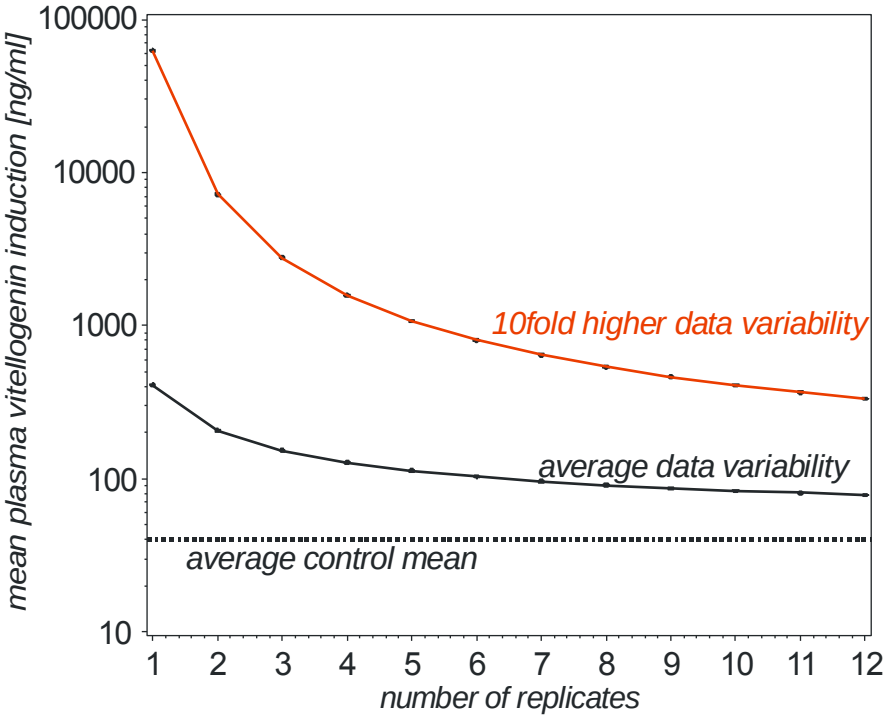

Figure 2 Number of replicates needed to detect a vitellogenin induction as significant from the negative control

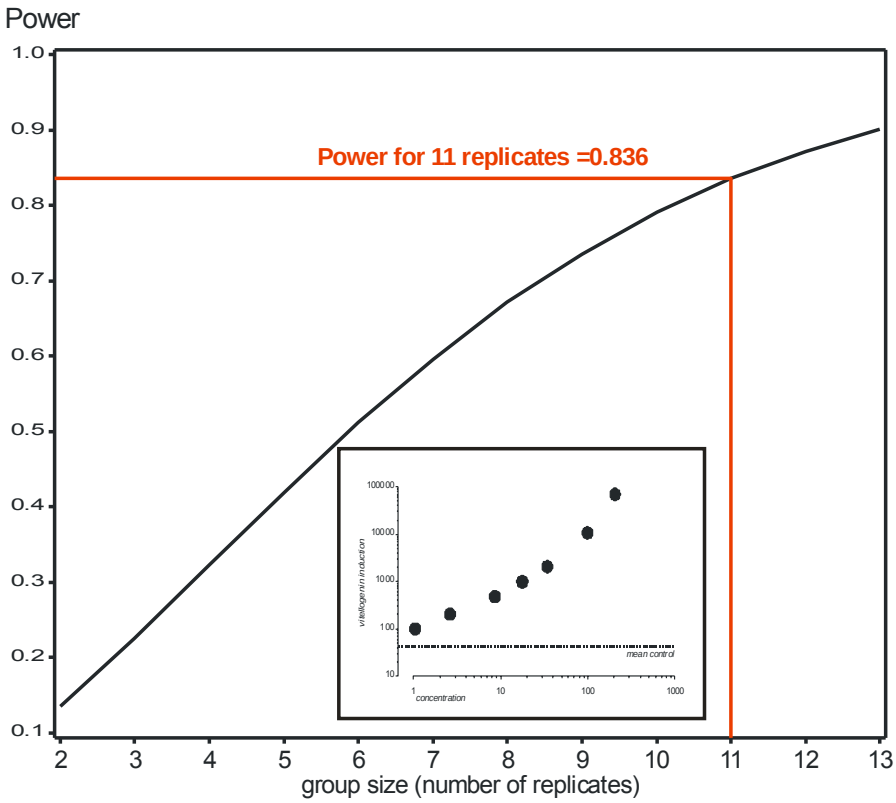

Figure 3 Number of replicates needed to detect the vitellogenin induction of all tested concentrations as significant from the negative control

Experimental data

E-Screen

To facilitate full implementation of the E-Screen (96-well format) for routine screening, Partner 1 (Andreas Kortenkamp) has carried out data-variance analyses, and parts of these analyses have found entry in recent publications (Rajapakse *et al*., 2004), Silva *et. al.* 2006 submitted). Extensive low dose studies were completed with 17ß-estradiol (E2) and β-hexachlorocyclohexane (β-HCH). The latter agent was chosen because its mode of action differs from that of E2 (the chemical exhibits all the biochemical effects of E2, but fails to bind to the estrogen receptor). Due to the large data volume that could be produced using the 96-well format of the E-Screen, it was possible to analyse the predictive power of low dose effect estimates from experiments carried out over a long time period and to consider the possible impact of confounding factors. Furthermore, both compounds worked as reference cases for the planning of further studies with different compounds. Although the experiments with these additional chemicals were performed using fewer replicates, their aim were to establish optimised experimental designs.

17β-estradiol and β-HCH (selected for extensive low dose studies)

The concentration-response data for both compounds yielded the familiar sigmoidal curve shapes; anomalies such as inverted U-shapes were not observed in the low concentration ranges (Figure 4). However, the low dose estimations for both compounds showed different patterns with respect to shape and inter-study variation: three independent low-dose studies were performed for 17β-estradiol and compared with historical data obtained from the same lab. Two of the three low-dose studies were performed using the same cell passage number. The second agent, β-HCH, were tested twice. Results show that low effect estimates for E2 depend strongly on the study and the cell passage; the lower the effect level X, the higher the inter-study variation of corresponding ECx estimates, which was higher than the intra-study variation. For example, when EC5 estimates for E2 are compared, the maximal difference observed for the three studies is nearly 200-fold. This variability has a strong influence on the curve shape, featuring a very shallow slope in the low concentration range, even down to concentrations in the fmolar range (green curve!). Despite the large variation in the low concentration/low effect range, variations in terms of general potency (EC50) were not apparent, when compared to two year-old historical data from our lab. This extreme shallowness of the low end of the concentration-response curve was so far only observed with E2. Whether this is specific for E2, or holds true also for other steroids remains to be seen.

With β-HCH, the situation was different. The data obtained from a repeat low-dose study (blue circles) were in excellent agreement with previous outcomes (black circles). A further study was carried out to analyse the predictive value of very low effect estimates: the EC01 (40.2nM β-HCH) obtained from first study (black line) was retested with a high and equal number of replicates and controls. A statistically significant degree of cell proliferation (n=16, p=0.007, t-test, adjusted for lane) corresponding to a relative effect of 1.22% [95% CI: 0.06-1.8] was observed. Thus it can be concluded that for this compound the inter-study variation did not influence the predictive power of very low effect estimates. Although other xenoestrogens were not tested with comparable high data power, all revealed a similar good reproducibility for EC estimates of low effect levels.

Further agents

Table **2** gives an overview of low dose effect estimates from further 11 xeno-oestrogens: it reports the NOEC as well as LOEC values obtained from statistical multiple comparison procedures and shows the corresponding empirical mean ("observed") and the effects estimated by regression modelling. As with the two extensively tested chemicals E2 and β-HCH, the concentration-response curves for all agents did exhibit the familiar sigmoidal pattern. Anomalies, such as inverted U-shapes were not observed in the low concentration ranges. Based on these outcomes it can be concluded that already eight test concentrations tested on eight different lanes might be sufficient to identify NOEC values having evoked non-significant cell proliferations of maximal 1%. However, the statistical power for identifying an effect as significant depends highly on the test agent, as some are showing a typically reproducible higher data variation (e.g., o,p'-DDT) and leading to NOEC values which correspond to estimated regression effects of around 5% cell proliferation.

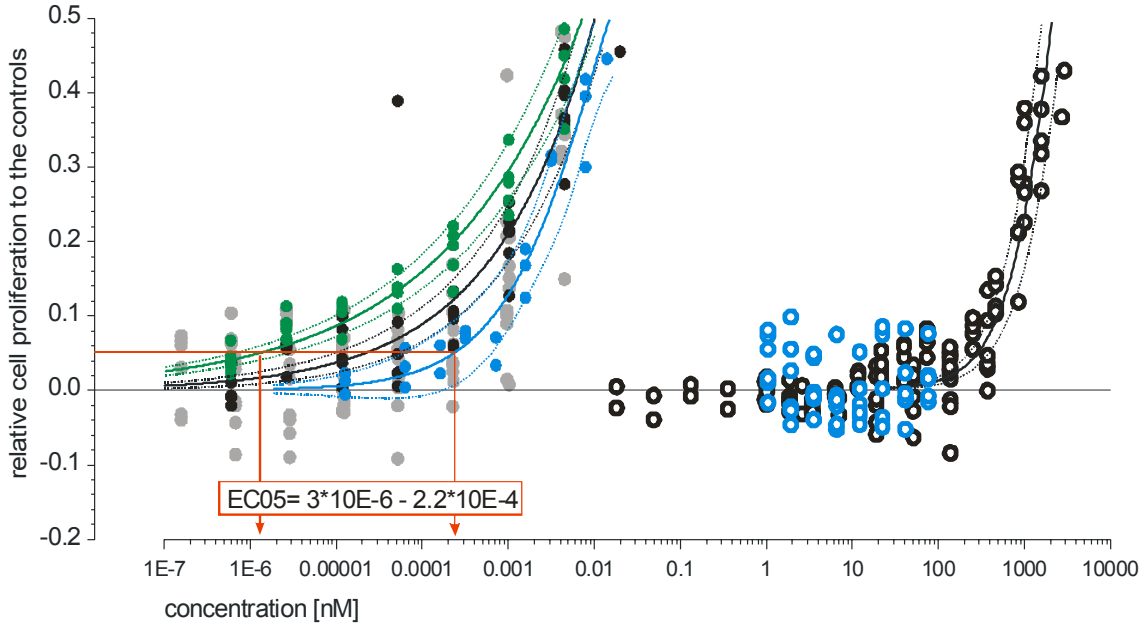

Figure 4 Concentration-response data and curves for 17β-estradiol (dots) and β-HCH (open dots) in the E-Screen assay with MCF-7 BUS cells. Colours present data from different independent studies: cells from the same passage (black and green) or different passages (blue), and historical data from studies carried out two years ago (grey dots). The best-fitting regression models (see Table 1) are shown as lines with the corresponding 95% confidence belts for the mean effect as dotted lines. For E2, the range of EC05 values is pictured, as obtained from data from different studies.

agents (in order of increasing NOEC)	experimental design: number of tested			NOEC[d] nmol/L	observed mean effect	estimated regression effect	LOEC[d] nmol/L	observed mean effect	estimated regression effect
	conc.[a]	lanes[b]	N[c]						
Genistein	8	10	80	2.29	0.6 %	0.6 %	9.18	3.6 %	3.8 %
Bisphenol A	8 (+2)	8	68	5.29	0.3 %	0.6 %	16.53	3.0 %	2.4 %
Butyl paraben	7 (+6)	8	68	32.6	0.7 %	0.2 %	104.3	1.5 %	1.2 %
o,p'-DDT	8	12	96	51.6	4.4 %	5.5 %	139.4	16.0 %	14.9 %
Methoxychlor	8 (+4)	10	88	81.9	0.0 %	0.5 %	204.8	2.4 %	1.8 %
Endosulfan a (I)	8/4 (+1)	6/4	66	90.4	-1.4 %	1.4 %	234.8	4.9 %	4.3 %
Kepone	8	10	80	97	1.5 %	1.5 %	213	3.9 %	5.3 %
Propyl paraben	8 (+6)	4	44	104	-0.3 %	0.4 %	333	2.9 %	2.5 %
Endosulfan b (II)	8	12	96	153	0.3 %	0.8 %	406	4.6 %	4.0 %
Dieldrin	8 (+4)	12	104	680	2.8 %	1.9 %	1666	9.6 %	9.6 %
p,p'-DDE	8	8	64	1501	3.1 %	1.0 %	3227	7.5 %	7.9 %
reference agents									
β-HCH	12 (+8)	8	196	51.6	-0.4 %	1.3 %	74.2	4.3 %	1.8 %
17β-estradiol									
study 1	8	6	48	5.97E-07	0.8 %	1.3 %	2.65E-06	5.4 %	2.3 %
study 2	8	5	40	--	--	--	5.97E-07	4.1 %	4.1 %
study 3	16	3	40	1.28E-05	1.1 %	1.0 %	0.000064	3.2 %	2.6 %

[a] number of concentrations used for the hypothesis testing, in brackets additional concentrations used for regression modelling (duplicates); [b] number of replicates used for the hypothesis testing (number of controls corresponds to 8 fold numbers of lanes); [c] absolute number of data (not included: controls); [d] NOEC, LOEC – No Observed Effect Concentration and Lowest Observed Effect Concentration, determined by nonparametric contrast test;

Table 2 Low effect estimates of single agents (normalised cell proliferation, E-Screen)

Recommendations

In general, the protocol used for the 96-well format of the E-Screen was proven to be a very sensitive tool for the detection of low effects, however also sensitive against small deviations from the protocol. As an important source for biased small effect measurements, the allocation of exposures and controls on the 96-well plate were identified: the positive control might influence the cell proliferation of neighbouring wells and lanes ("creeping effect"), thus it is highly recommended to leave blank lanes between controls and exposures. Furthermore, the between-plate variability was higher than the within-plate variability, whereas the between-stock and -dilution variability was negligible (exception: E2). Therefore, "replicates" should be defined as units between plates, and optimally "independent study" as a study based on a different cell passage (to capture between-stock/between-dilution variability).

The minimum number of controls should be at least three for the negative, and seven for the positive controls. As abnormal dose-response pattern were never observed or reported for the E-Screen, eight concentrations should be sufficient to cover the low effect range with high certainty. The testing of too high concentrations should be avoided, as a downturn of effects can be observed and this may complicate the data analysis. However, the detectable effective concentration range might become small: in **Error! Reference source not found.** the concentration-response curve for endosulfan II is shown, demonstrating that only a relatively small concentration range of two magnitudes of order

falls into the statistically detectable effective range (assuming that only effects higher than 5% can be detected). Weaker agents with a much smaller maximal possible proliferation in the E-Screen might even have a smaller "effective window", setting thus high demands to the dose regime in order not to overlook effects. The number of replicates (=lanes) should be at least 6 to detect a minimum effect size of 5%. However, in certain cases the determination of low effect estimates can still fail (example *o,p*'-DDT). Furthermore, it remains unclear whether for steroids a higher between-study variation is common.

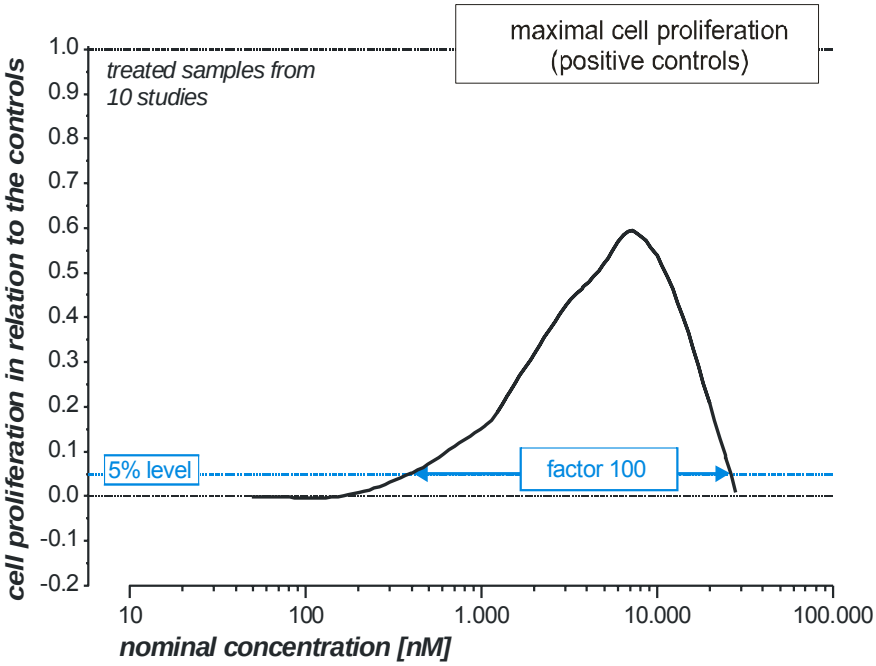

Figure 5 Concentration-response curve for endosulfan II in the E-Screen assay with MCF-7 BUS cells

Heterogeneity of data (increasing data variation with increasing effect levels) is present and may violate the assumption of equal data variation for all effect levels for most common multiple testing methods which are based on ANOVA approaches (Dunnett's test, Bartholomew test, etc.), for raw data as well as normalised data. To eliminate heterogeneity, either an appropriate data transformation should be selected (e.g., Box-Cox transformation), or alternatively non-parametric methods considered. Especially non-parametric contrast tests were proven to be a good option, with different scores - powerful for different distributions - and different contrasts - powerful for different shapes (Neuhaeuser *et al.*, 2000). With at least 6 replicates it was in most cases possible to identify effects higher than 5%. Instances may be found where a reversal or downturn at higher doses is likely to occur. How to deal with them? Either delete these data from analysis, or use more simple testing methods (e.g. Dunn's test). However, these methods have less power.

No uniform concentration-effect model could be identified which allowed for all tested agents a reasonable good description of the observed data. Thus we recommend a best-fit approach, based on at least three models which are able to describe different curve patterns. For instance, if using the logit (or Hill) model, which can describe only sigmoidal shapes symmetrically to the median effect, additionally a model form should be selected which is able to describe non-symmetrical concentration-effect data.

Transformation of the concentration scale might be also an option for improving the data fitting, which would allow the use of the same regression model to the effect data (e.g. by using Box-Cox transformation). This might be useful when the software program provides only a limited number of regression models for the data analysis.

High effect patterns can hamper low effect estimations, especially when complex downturn effect patterns are present. Then a correct description of all observed effect data requires a more advanced regression model, which means that more model parameters are necessary which all have to be fitted simultaneously to the data. Apart from the difficulty to find a suitable model form and to fit the data to this model, it increases the likelihood of an overparameterization. But as the estimation of low effects is the main purpose, this violates clearly the principle of parsimony. Thus the data analysis should be restricted only to the relevant effect data, i.e. high effect data as well as concentration ranges leading to downturn effects should be excluded from any regression analysis. Estimation method should reflect plates as nested factor in order to guarantee correct statistical inference statements (std error, CIs), at least when data for mean effect levels above 30% are included in the modelling.

Studies in fish

The main statistical descriptors are shown in Table 3, together with the fish number in the control and exposure tanks which were analysed for vitellogenin or spiggin. The coefficient of variation for the controls as well the pooled standard deviation indicates whether relevant data heterogeneity exists. The LOEC and NOEC were derived by statistical multiple testing approaches from non-normalised vitellogenin and spiggin data; parametric and non-parametric testing methods were favoured with assuming certain curve shapes by appropriate contrasts. In case of spiggin, the organ weight was included as co-factor in data analysis. Corresponding effects for the NOEC and LOEC were estimated by the empirical mean and the regression model fit. Generally, a model fit provides a better estimation than the empirical mean, as it uses all data and smoothes the data to the expected model shape. However, it assumes always that the correct model is selected. If both mean estimates deviate strongly from each other, than it provides some indications that either the underlying unknown low effect pattern is too complicated to be described by a simple model, or, which is mostly the case, that the data is too uncertain to be described well by the model. This might occur when the number of fish per exposure is too low, but the individual effect variation huge. A flow-through testing system was used in all cases, with relatively low water flows. This might be a factor explaining cases where the exposures measured in the water tanks during the testing duration differed from the nominal concentrations. Deviations between nominal and measured concentrations of the test agents are expressed as average recovery rate on the basis of water samples measured at t_0, t_7, t_{14} and t_{21}.

Zebra fish

Partner 13 (Bertold Hock) has carried out low-dose exposures with estrone (juvenile zebrafish, 21 day exposure) and nonylphenol (adult zebrafish, 11 day exposure, study B), additionally Partner 21 (Leif Norrgren) performed a confirmatory study with 21 day nonylphenol exposures on juvenile zebrafish (study A). Whole body homogenates of exposed zebrafish were analysed for vitellogenin (VTG) by using ELISA.

Exemplarily effect data is shown only for nonylphenol exposures, but from both studies (

Figure **6**). The corresponding statistical low effect estimates are given in Table 3. In most cases the observed data variability was lower for the controls than for the exposures ($STD_{control}$ vs. STD_{Pooled}), even after a log10-tranformation of the vitellogenin data. However, data from the positive controls showed a similar data variation as for the negative controls. Thus, it is very likely that the observed data variation expresses an increased biological intra-individual variability due to exogenous exposures. In all studies, a monotonic increasing dose-response relationship was observed. Although the data might indicate different potencies of the same compound with regard to different life stages, all low effect estimates became similar when based on the measured water exposures during the testing period: the nominal LOEC derived from study A (500 µg/L) approximately equals a measured LOEC of 75 µg/L, whereas the nominal LOEC of 40 µg/L equals a measured LOEC of 77 µg/L. As the corresponding effect estimates are of similar magnitude, the outcomes from both studies can be considered as comparable, despite different life stages and exposure durations. However, the obtained number of fish in study A resulted in a design of low statistical power – 7 control fish vs. 9 exposure fish – which allowed only the detection of effects larger than 15%. As a consequence, the observed effects of the NOEC could not be detected as statistically significant although an average 13% effect increase was observed. The study with estrone exposures was performed with a higher number of fish per tank and controls, which allowed the detection of 5% effect differences to the controls as statistically significant. In

Figure **6**, additionally the estimated EC10s are pictured (32 µg/L in study A, 140 µg/L in study B), with the width of the boxes indicating their 95% confidence belts.

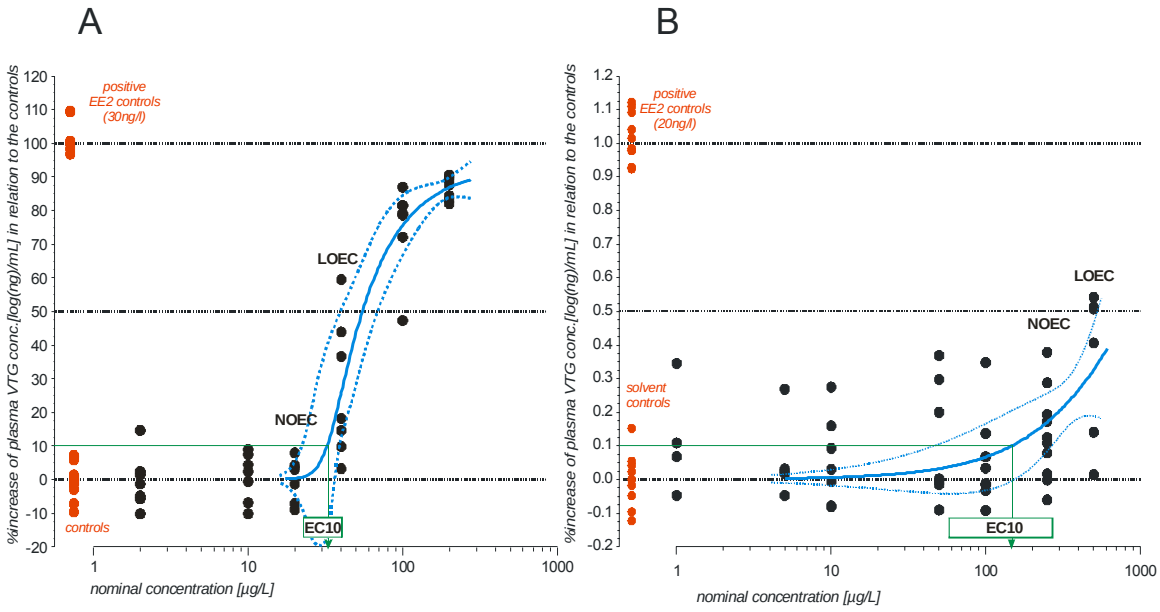

Figure 6 Relative vitellogenin induction in juvenile zebra fish after 21 day nonylphenol exposures (A) and in adult zebra fish after 11 day nonylphenol exposures (B)

Stickleback

Partners 2 (Tom Pottinger), 8 (Ian Mayer) and 19 (Ioanna Katsiadaki, Alex Scott) were involved with low dose studies using vitellogenin- and spiggin-induction in the stickleback. As estrogenic chemicals, estrone (E1) and nonylphenol (NP) were selected. Fenitrothion and flutamide were chosen as (anti)androgenic compounds. Earlier range-

finding studies were duplicated at the facilities of Partners 2 (Tom Pottinger) and 8 (Ian Mayer) in order to determine how reproducible the results of the exposure system were when identical chemicals were evaluated at two sites by two different teams, for both vitellogenin and spiggin induction. This proved to be the case, with good agreement between the results from both groups. In addition, we compared the results obtained from the assay of whole-body homogenates with the results of organ specific analysis. Again, agreement between the two methods was good and organ specific measurements were adopted routinely on the basis of target tissue relevance and sensitivity.

Estrogenic compounds: Two well-known estrogenic chemicals, estrone (E1) and nonylphenol (NP) were chosen for the low-dose studies. Figure 7 shows the VTG results in male sticklebacks after 21day-estrone exposures. The observed dose-response pattern is increasing, with a NOEC of nominal 0.2 µg/L and an effect mean of 6.2% (estimated by regression as 9.3%). As the MSD is ≈ 8.5% for this experimental design, the effect data could not be detected as statistically significant. Due to the very steep curve shape for median effects, the next higher tested concentration (LOEC with a nominal 0.5 µg/L) produced a strong VTG induction (80%). Therefore these outcomes are a good example for the dependence of NOEC determinations on the tested dose ranges: the estimated EC10 (nominal 0.21 µg/L) is only slightly higher than the NOEC. Had this concentration been tested effects would have been detected, due to the high power of the study (19 controls, at least 10 fish per exposure tank). Analytical chemistry confirmed a significantly lower recovery of estrone exposures, with stable measurements corresponding to ca. 32% of nominal values.

The results for nonylphenol (NP) showed a similar clear monotonic concentration-response pattern as observed for estrone, with an NOEC of 100 µg/L nominal NP and an estimated regression effect of 2%. The LOEC was nominal 250 µg/L, with a mean effect of 45%. The MSD for this study was around 7%. The effect variability for the exposures was on average the same as observed in the estrone study (pooled standard deviation of ca. 10%), although the variation in the controls was significantly lower. Referring the effect estimates to measured concentrations, the outcomes with the stickleback are still comparable with those obtained from studies with NP on zebra fish: the LOEC equals an EC45 of 67.5 µg/L, and for studies with zebra fish the LOECs corresponding an EC26 of 77 µg/L (study A) and an EC32 of 75 µg/L (study B) were determined, i.e. all concentrations are similar and yielded VTG inductions of similar magnitude.

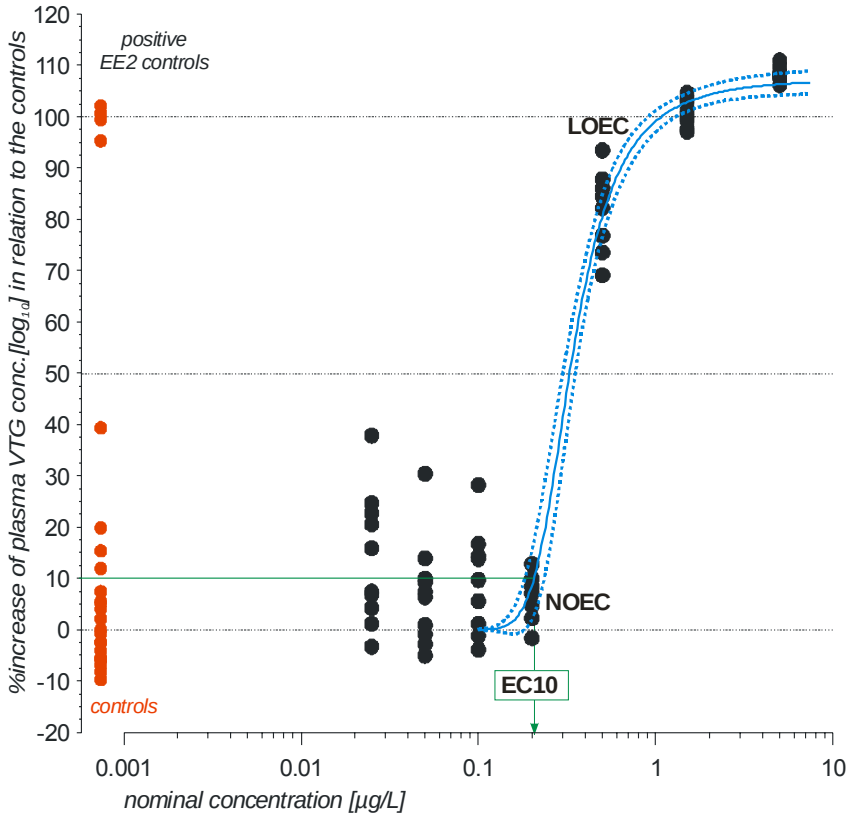

Figure 7 Relative vitellogenin induction in male stickleback after 21 day exposures to estrone

Flutamide:

Figure **8** shows the inhibitory influence of flutamide exposures on maximal spiggin levels in female sticklebacks. The maximum spiggin production is always induced by 5 µg/L DHT, and the effects of the test agents is described relative to the effect of DHT. Different colour were used to depict data from two different studies (black and red dots), with corresponding controls on the left side of the graph; no significant differences could be identified between both control groups, confirming the outcomes from previous studies that the within-tank variability is relatively small. Analytical data for the control tanks (not shown) confirms the comparability with similar DHT exposure over the whole testing period of 21 days. The dose-response data show a clear decreasing monotonic dose-response pattern. In the first study (20 controls, 10 fish per exposure), the lowest tested concentration of 5 µg/L already produced a response statistically significantly different from the controls. Thus, a second study with three low concentrations (2, 5 and 10 µg/L) was performed. To our surprise, a significant response was detected for the lowest tested exposure. Consequently, it was not possible to determine a NOEC in a strict quantitative way. These outcomes provide a clear indication that the dose-response relationship for low effects might be very flat for (anti)androgens, and that thus a testing with only a few exposures covering a small dose range might fail to identify the low effect range of interest. The corresponding regression fit for the pooled data resulted in an EC10 estimate that was slightly higher than 2 µg/L. This demonstrates that the power of the chosen data design was able to detect effects smaller than 10%. Indeed the MSD for the second study was estimated to be around 7.5%, whereat for the first study around 10% (higher data variation was compensated by higher data amount!).

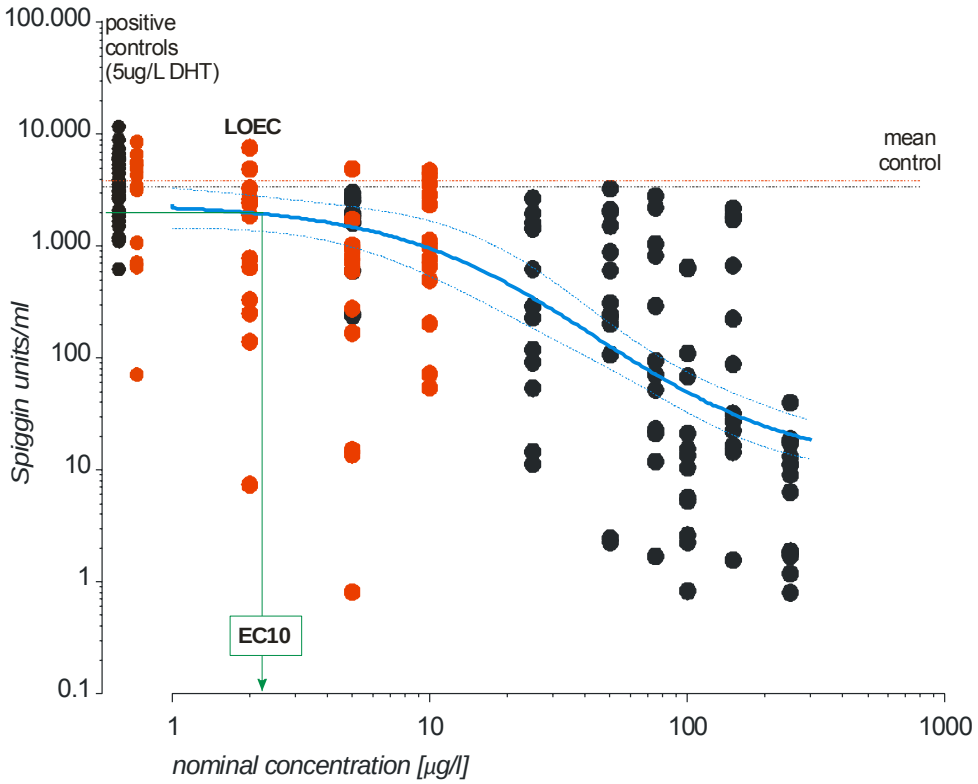

Figure 8 Reduction of maximal induced spiggin by flutamide exposures

Fenitrothion:

Figure **9** shows the inhibitory impact of fenitrothion exposures on maximal spiggin levels in female sticklebacks, again induced by 5 µg/L DHT. In contrast to the previous figure, effect data is shown as values normalised to fully induced controls (equalling zero on the effect scale) and the solvent controls (equalling one), with a value of 0.1 meaning an 10% decrease in mean Spiggin induction in relation to the controls. As for flutamide, the data show a clear monotonic dose-response pattern. The lowest nominal concentration for which a significant response could be detected (LOEC) was estimated as 0.25 µg/L, resulting in a NOEC of nominal 0.1 µg/L (corresponding measured 0.05 µg/L). A mean effect of 11.3% (7.5% according regression fit) was observed for the LOEC, well above about the estimated MSD of 9.5%.

The shape of the concentration-response pattern for low effects is again relatively flat, spanning nearly two orders of magnitude on the concentration axis between the EC50 and EC10, and confirming therefore the flutamide outcomes.

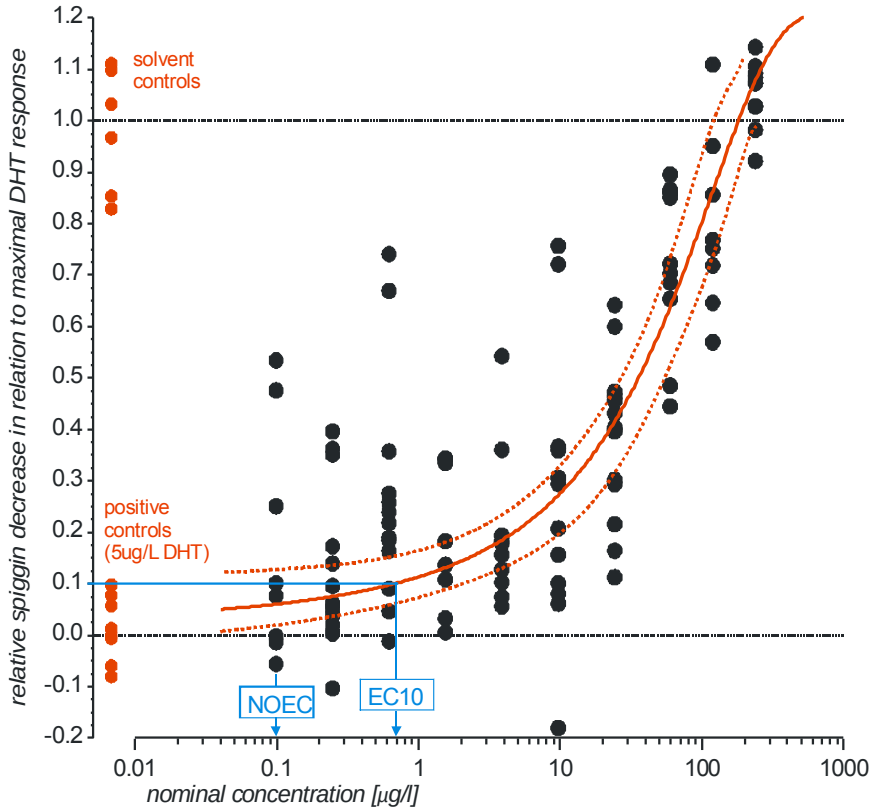

Figure 9 Reduction of maximal induced spiggin by fenitrothion exposures

agents	data descriptors				NOEC[d] [µg/L]	observed mean effect for NOEC	estimated effect for NOEC	LOEC [µg/L]	observed mean effect for LOEC	estimated effect for LOEC	average recovery rate[f]
	conc. numbers	fish number	$CV_{control}$[a]	STD_{pooled}[b]							
normalised vitellogenin induction in the zebrafish assay [e]											
estrone	5	10(21)	12.3%	13.5%	0.09	0.8%	0.1%	0.15	5.2%	3.1%	32%
nonylphenol (B)	7	7(9)	8.3%	15.6%	250	13.0%	16.9%	500	35.4%	32.5%	15%
nonylphenol (A)	6	7(14)	4.7%	11.3%	20	0.3%	0.3%	40	26.6%	25.7%	192%
normalised vitellogenin induction in the stickleback assay [e]											
estrone	7	10(19)	11.8%	10.4%	0.2	6.2%	9.3%	0.5	81.7%	81.7%	31%
nonylphenol	7	8(20)	5.3%	10.6%	100	-4.4 %	1.7%	250	45.7%	45.4%	27%
normalised spiggin induction in the stickleback assay [e]											
fenitrothion	10	>8 (8)	6.4%	14.9%	0.1	0.2%	3.8%	0.25	11.3%	7.5%	52%
flutamide [c]	7	12(20)	17.4%	30.6%	n.d.	--	--	2	8.0%	8.3%	43%
	3	12(12)	7.5%	11.5%							

[a] CV=coefficient of variation for controls, equals the standard deviation for normalised control data; [b] STD_{pooled}= standard deviation, pooled over all exposure groups (excluded: concentrations>EC90); [c] pooled data from repeated studies; [d] NOEC, LOEC – No Observed Effect Concentration and Lowest Observed Effect Concentration, based on nominal concentrations; [e] Only regression is based on normalised values, NOEC/NOAEL determination is based always on non-normalised data. [f] average recovery rate is based on arithmetic mean from water samples measured at t_0, t_7, t_{14} and t_{21} for the NOEC and LOEC exposures.

Table 3 Low effect estimates of single agents in fish assays

Results

For the two tested anti-androgens (fenitrothion, flutamide) no unusual dose-response pattern were observed, always clear monotonic sigmoidal curve shapes, with very flat curve shapes for low effects. Usually the variability of effect data from negative controls was much lower than observed from exposures (data heterogeneity), for the rough as well normalised data. Normalised data can be assumed to be normally distributed, although often hampered by data outliers who require robust statistical estimation methods for the model fitting (e.g. winsorization techniques). Untransformed VTG and spiggin data can be easily normalised by log10-Transformation. The minimal statistical detection limit (MSD) was in most cases below 10% (normalised endpoints), at least when the information of ten fish per dose were available. In case of a much higher number of controls (optimal according factor k-rule), even lower effect differences could be detected as statistically significant (5.5% VTG in zebra fish for estrone, 8% spiggin in stickleback for flutamide). Effects for the NOEC concentrations were estimated to be between 0% and 13%, for flutamide the lowest tested concentration (2 µg/L) showed a significant effect, corresponding to an estimated 8% effect spiggin decrease.

Always differences between nominal and measured exposures were identified, with recovery rates down to 15% (nonylphenol, study B with zebra fish). However, the measured exposures were stable over exposure time (data not shown).

Recommendations

Measured concentrations: There is a special problem with the test design of the spiggin induction assay: fish are always exposed to two agents, the androgen DHT to stimulate a maximal spiggin induction, and the test agent in order to measure an anti-androgenic effect. Consequently, the sole application of a maximal dose of DHT (5 ug/L DHT) to the stickleback works as control reference, and all spiggin measurements observed from the test agents are compared to that reference. This requires that the fish are always exposed to an identical and constant DHT concentration in the study. A lower DHT concentration would lead to a lower spiggin induction and thus compromise direct comparability with the reference. However, usually only 40-80% of the nominal DHT concentration could be detected in the water samples during the testing duration. These recovery rates were constant over exposure time and tanks, but differed between studies. As a consequence, the observed control spiggin values from different studies varied significantly. Thus it is strongly recommended that within a study the acceptable maximal variation of DHT concentrations measured during the study is set to a relatively small scale, in order to guarantee comparability of effect data from different tanks with respect to a common data analysis. Otherwise this source of uncertainty can become a serious confounding factor, which does not allow the comparison of effect data on the concentration scale of the test agent.

Controls: Although no relevant between-tank variation could be detected, a minimum of two control tanks should always be used. Good for cases when the proposed DHT concentration is outside the quality control window (spiggin), good in case of an unforeseen event (not the whole study is lost), good for a better power (multiple testing), and always good for a more accurate and precise low effect regression estimate.

Body weight: In none of the studies, normalisation of VTG or spiggin units to the organ or body weight of the fish (either by a simple ratio or as co-factor in the analysis) yielded

any relevant differences for the statistical low-dose descriptors, neither when based on multiple testing nor on regression fitting (in terms of mean, p-values and confidence intervals).

Data below detection limit: Biometrical regression analysis becomes challenging if some of the effect measurements are below the technical detection limit of spiggin analysis, in statistical terminology called censored data. The difficulty with censored data is that they are not missing in a random pattern, but they are all missing at one end of the effect scale. Often in such a situation, regression is carried out naively by taking the censored values equal to (i) detection limit, (ii) half of the distance between control background level and detection limit, (iii) background control level, or simply (iv) by ignoring the censored observations. It well known that in all these cases common regression approaches results in a biased estimation of the parameters in a nonlinear model, leading in worst case to completely erroneous low dose estimates. Several methods exist for handling regression with censored data for linear models, including iterative least squares (ILS) methods and maximum likelihood (ML) methods. However, methods to estimate the parameters of a nonlinear model for censored data are sparse. We applied the general ML method for parameter estimations of non-linear models developed by Sharma et al (2003) in combination with the best-fit approach (Scholze et al 2003) and compared it with multiple imputation methods, which provides a useful strategy for dealing with censored data as data sets with missing values. Instead of filling in a single value for each missing value, multiple imputation procedure replaces each missing value with a set of plausible values that represent the uncertainty about the right value to impute. These multiply imputed data sets are then analyzed by using standard procedures for complete data and combining the results from this analysis. In our case, these values are replaced with most likely values between zero and the detection limit on the basis of the whole data set of measured data. To our experience this approach resulted into similar low dose estimates, but was much easier to implement. Thus we decided to use this approach. However, it assumes that a sufficient number of effect data could be determined above the technical limit, according to out experience at least 60%. Otherwise it is recommended to categorise the low effect data according to their measurability and using corresponding statistical methods. However, this approach has usually a lower statistical power.

Multiple Testing: Heterogeneity of data (controls showed lowest data variation) favours nonparametric methods. We decided for powerful contrast tests, based on a pool of different scores to handle different distributions and different contrasts to deal with different dose-response shapes. In case of vitellogenin as effect endpoint, additionally parametric approaches can be used (Likelihood ratio test according Bartholomew, Williams test), at least when the heterogeneity of data is only weak.

Regression benchmark: No uniform concentration-effect model could be identified which allowed always a reasonable good description of the observed data. Thus we recommend a best-fit approach, based on at least three different models. In case of repeated studies, appropriate estimation methods should take this into account (nested random factor, correlation structures) in order to guarantee correct statistical inference statements (std error, CIs).

Design: To guarantee general relative effect sizes for the benchmark approach, it is strongly recommended to have sufficient information available about negative and positive controls. It is more important to achieve accurate estimates from the negative

controls than from the positive, therefore the corresponding number of fish should reflect this: in case of VTG, a minimum of four positive controls is recommended, in case of spiggin induction at least 6 fish. The minimal number of negative controls (solvent for VTG, DHT for spiggin) should be 10 per tank, with at least two replicated tanks. As abnormal dose-response pattern were never observed, a minimum of 5 concentrations should guarantee a reliable low effect regression modelling. Depending on the desired statistical detection limit, 10 fish per tank should allow a MSD of 10% for both endpoints. Furthermore, in order to avoid masking downturn effects which might complicate unnecessarily low effect estimations, too high concentrations should not be tested. When working with juveniles, another important planning aspect is that these numbers refer to final measurements: as only females are considered for the spiggin induction and only males for the vitellogenin analysis, but the final sex determination is possible only after the study, the study has to deal with this uncertainty in the same as with unforeseen mortalities. As consequence for practise it is therefore unrealistic to expect effect data which follows a perfect balanced design with equal numbers of units per exposure.

One-generation studies in the rat

The anti-androgenic phthalates DINP and DEHP were selected for in-depth studies (partner 20, Ulla Hass and Jens-Jorgen Larssen), with the aim to compare estimates of low dose effects on anogenital distance and nipple retention derived from NOEL and benchmark approaches. The low dose-response study on DINP included 60 time-mated rats (control plus 4 dose levels) dosed by gavage from gestation day 7 to postnatal day 16. Dose-response data for anogenital distance (normalised by body weight) are shown in

Figure **12**.

The study design for DEHP has been considered and developed based on the experiences obtained from the DINP study. The minimum requirement for the study with a NOEL approach is 3 dose levels and 12 animals per group, while the study with a benchmark approach were decided to be based on 6 dose levels and 6 animals per group. The optimal strategy for a comparative assessment of both approaches would be to perform these two studies at the same time. However, this would be a very large study and instead it was considered feasible to compare the results from two separate studies including appropriate control groups and performed half a year apart.

Firstly, a dose-response study of DEHP with a benchmark approach was performed. The study included a control group of 16 time-mated animals and 6 dose groups with 8 time-mated animals per group. The dose levels of 10, 30, 100, 300, 600 and 900 mg/kg were selected based on our earlier studies on DEHP and with the aim of covering the whole dose-response curve for DEHP effects on anogenital distance and nipple retention in male offspring.

Secondly, a DEHP study aimed at the NOEL approach was initiated. This study included a control group of 16 time-mated animals and 3 dose groups with 8 time-mated animals per group. The dose levels were similar to the lowest dose levels in the first DEHP study, i.e. 10, 30 and 100 mg/kg. The group size was not increased to 12 animals per group as originally planned, but instead we decided to pool the results of the first and second study for the NOEL approach evaluation (with due consideration of study number as a factor in the statistical analysis). The study also included an additional group of 16 time-mated animals exposed to 3 mg/kg DEHP, because the results of the first study showed

statistically significant effects on anogenital distance and nipple retention at 10 mg/kg. Consequently, a lower dose level was needed in order to find a clear NOEL for DEHP.

The results of the 1st DEHP study showed dose-related antiandrogenic effects on anogenital distance and nipple retention from the lowest dose included, i.e. 10 mg/kg. In addition, dose-related decreases of reproductive organ weight were found on postnatal day 16 in the male offspring, as the weight of muscular levator ani and prostate were significantly decreased from 10 and 30 mg/kg, respectively. The finding of antiandrogenic effects of DEHP at 10 mg/kg supports the NOAEL of 5 mg/kg in the EU Risk Assessment report of DEHP. The dose-response curve for nipple retention was unusual at the three lowest doses of DEHP (10, 30, 100 mg/kg), as the 10 mg/kg actually seemed to induce the most marked effects. In addition, the study (unexpectedly) did not provide a NOAEL for the DEHP effects. Consequently, it was decided to investigate the three low dose levels as well as a lower dose level in a 2nd DEHP study. Statistical analysis of the results shows that the lowest dose level in this study, i.e. 3 mg/kg, is a NOEL for effects on anogenital distance and nipple retention in male offspring. The effects at 10, 30 and 100 mg/kg were not as marked as in the first study and statistically significant differences were mainly found at 100 mg/kg. However, statistical analysis including the results from both studies (with due consideration of study number as a factor in the statistical analysis) shows significant effects at 10, 30 and 100 mg/kg (see Figure 10 and Figure 10 Anogenital distance for DEHP exposures (pooled data, normalised to the control mean values and body weight)

). These results indicate that the use of a NOEL approach for studies with relatively small groups (i.e. 8 per group) may lead to false-negative results. As such, this supports the recommendations in the OECD Test Guidelines for Reproductive Toxicity Testing (e.g. TG 416 two-generation study), where the group size is at least 20 litters per group

Together with the data outcomes from studies with five other compounds (vinclozolin, flutamide, procymidone, finasteride and prochloraz) we can conclude that:

- Unusual dose-response pattern never occurred, at when for non-toxic dose ranges (otherwise downturn effects).
- Dose-response relationships vary extremely with respect to shape (sigmoidal, sublinear, and linear), steepness and maximal response.
- Extent of nipple retention was for the majority of compounds the better endpoint with respect to sensitivity, minimal statistical detection limit and statistical power.
- A minimal number of 8 litters per group might be not sufficient for a reliable regression modelling (see DEHP), even when data for six doses is available. However, this depends heavenly on the selected compound.
- Estimations of effect levels below 10% are not recommended for AGD, even when they are interpolative, based on 12 litters per group and a clear dose-response relationship is present. The estimations of the corresponding lower confidence belt for the IC10 might become too sensitive, depending heavenly on the chosen model and estimation method.
- For nipple retention, the estimation of low effect levels depends heavenly on the observed average number of nipples in the control reference group: in most studies it was more or less not possible to detect any nipples in the male control pubs,

which consequently lead to statistical low effect estimates of extreme high power and low uncertainty, enabling reliable estimates up to one nipple. However, in one exceptional case a higher control baseline with an increased data variation was measured, leading to a significant higher minimal statistical effect level.

- Our recommendations for benchmark testing are therefore a minimum of 5 doses for AGD together with 12 litters per group to ensure reliable effect estimates of 10%. For the NOAEL determination, i.e. testing whether the exposed pubs responded significantly different from the controls, even a higher litter number should be chosen. At least one dose should be below the IC10 (no extrapolation), and at least one tested doses should be higher than the estimated IC10.
- For nipple retention, its average number in the male controls determines heavenly the statistical testing power and certainty of low effect estimates. In optimal case the control pubs have no nipples/aerolas at all, which would guarantee the detection of one nipple in average in the exposed pubs even for small sample sizes such as 6 litters per group.
- We recommend the use of a higher number of control litters than litters per exposure group, and to monitor also the female pubs as positive control (essential for the AGD normalisation).

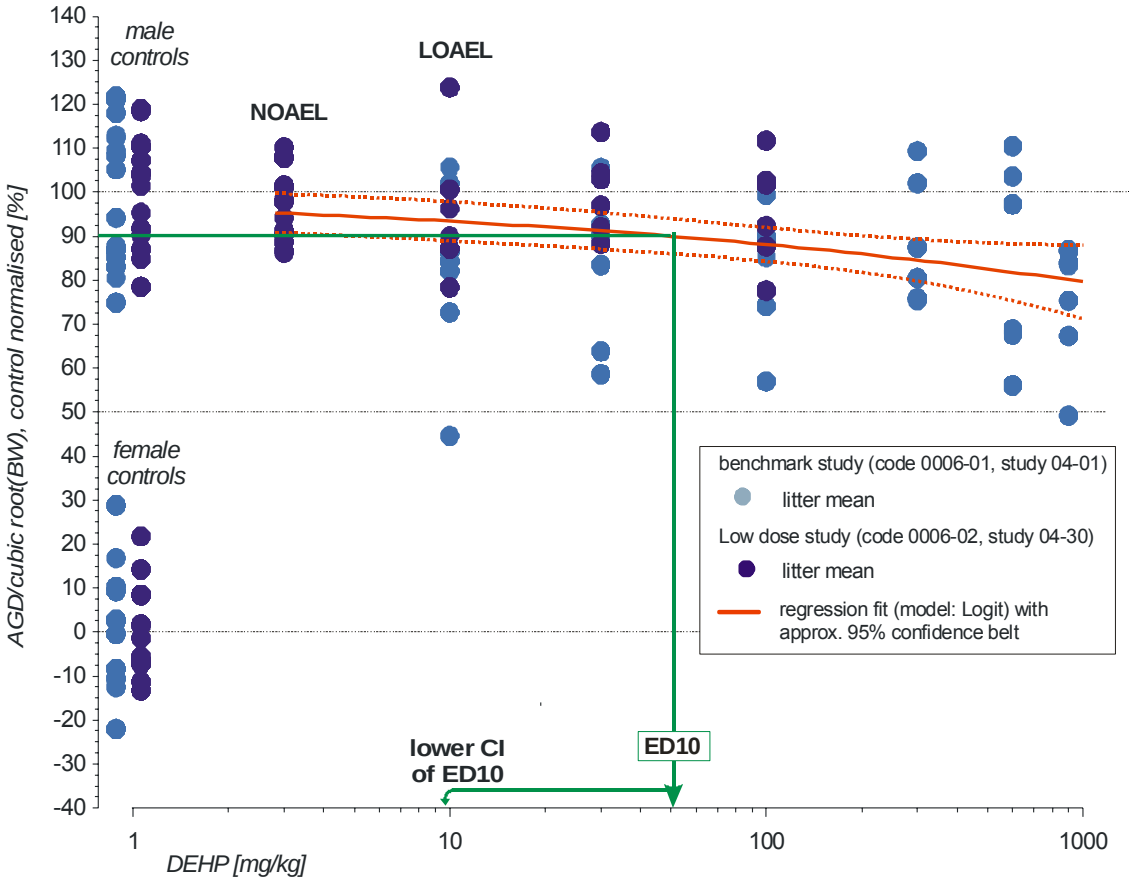

Figure 10 Anogenital distance for DEHP exposures (pooled data, normalised to the control mean values and body weight)

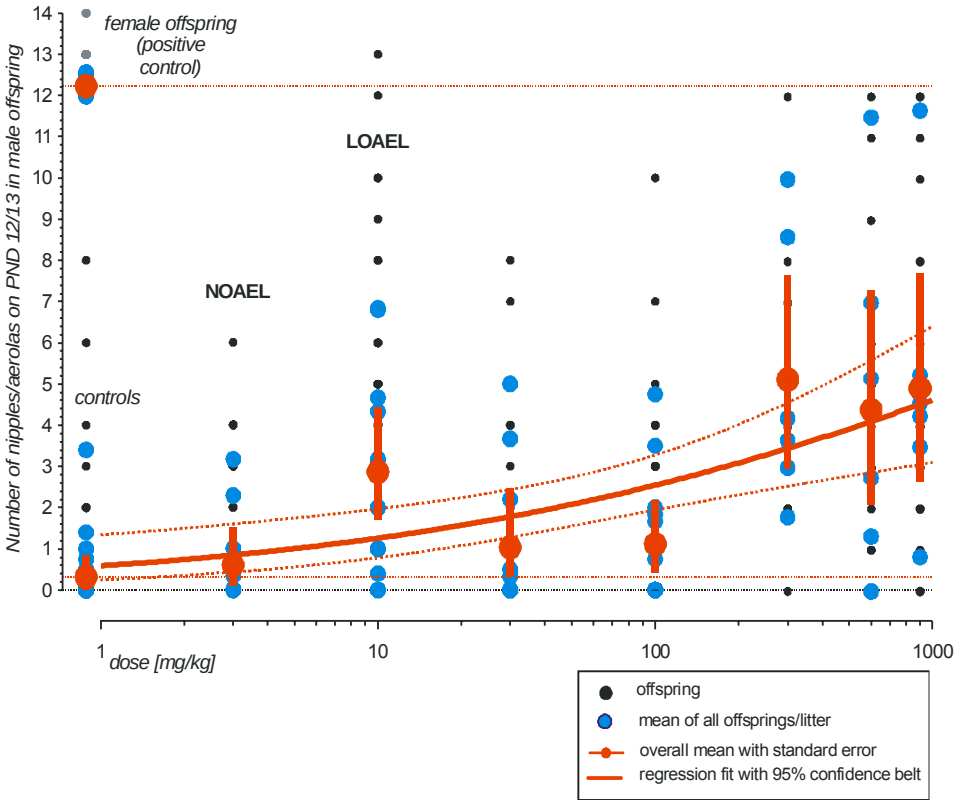

Figure 11: DEHP related responses to the number of nipples/aerolas in male offspring

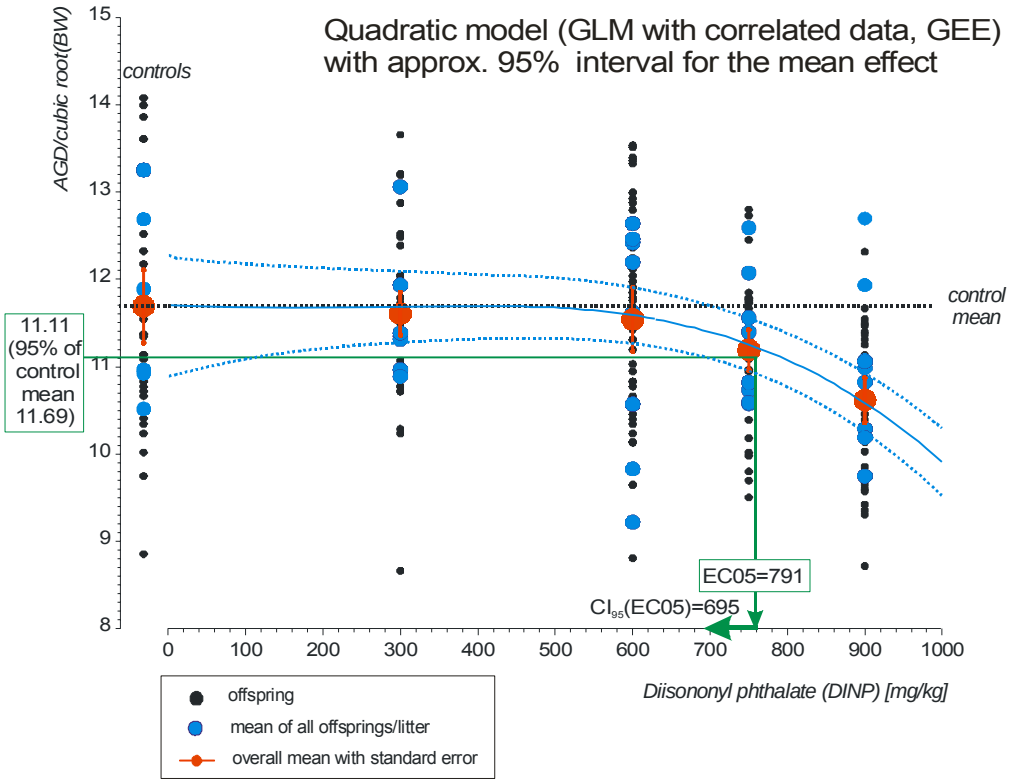

Figure 12 DINP related responses to the individual anogenital distance (normalised by body weight)

Requirements for low effect studies

There are a number of experimental requirements which have to be fulfilled for low-dose studies:

- The endpoint chosen for analysis must be quantifiable.
- Data have to be representative, i.e. randomised experimental design has to be adopted to avoid systematic errors.

Hypothesis testing: NOEL

- A statistical multiple comparison test of high power should be chosen.
- There has to be at least one dose which produces no significant effect.
- At least one dose should have produced an effect significantly different from untreated controls.
- Suitably high numbers of replicates should be chosen, depending on data variability (increased power).
- The number of dose groups used for comparisons with untreated controls should be as low as possible in order to deal with the multiplicity dilemma.

An optimum format would be to choose 3-4 concentrations (doses), with the lowest tested concentration corresponding to the NOEL. The effect of the LOEL should fall within the effect range defined by the minimal detection limit of the statistical test. This effect should be only slightly higher as the acceptable effect ("critical effect size").

Benchmark

- A dose –response relationship must be present for the tested dose range.
- An "optimal" model for the dose-response relationship must be available (high accuracy).
- There has to be a statistical estimation method for the calculation of the statistical uncertainty.
- It must be possible to define *a priori* a "critical" effect size.
- A large number of the doses chosen for analysis should give responses in the range between lowest and highest effect level (data requirement for the model fitting).
- The relation between the effect range necessary for the model estimation and the average data variability should be large.

Ideally, the model chosen for the description of the dose response data should correspond to the true dose-response relationship, i.e. there should be no systematic error due to an erroneously selected model. At least 5 doses should have produced responses between the minimum and maximal possible effect for the chosen endpoint. The benchmark should fall within the tested dose ranges, such that the need for extrapolations is obviated. The number of replicates should be sufficiently high to provide precise model estimates, especially for the estimation of the benchmark concentrations, resulting in a narrow confidence belt.

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D16.1 First considerations of data requirements for adequate risk characterisations for EDC mixtures

Responsible Partner: 1 **Participating Partners:**

Introductory considerations

Before attempting to approach the topic of risk characterisations for EDC mixtures, it is necessary to reflect on whether there is actually a need to deal with this topic. This can be decided by considering the following two questions:

1) Is there scientific evidence that EDC can act together to produce combination effects?
2) Is there evidence that EDC act together at low doses?

Only when answers to these questions are "yes" is there a need to consider risk characterisations for EDC mixtures.

In the last few years considerable evidence has accumulated to show that EDC can act jointly to produce combination effects. Published data support this notion for estrogenic chemicals *in vitro* (Payne *et al*., 2001, Silva *et al*., 2002, Rajapakse *et al*., 2002, Rajapakse *et al*., 2004, Heneweer *et al*., 2005) and *in vivo* (Tinwell and Ashby 2004, Brian *et al*., 2005), for chemicals with anti-androgenic properties *in vitro* and *in vivo* (Nelleman *et al*., 2003, Birkhoj *et al*., 2004) and for thyroid hormone-disrupting agents *in vivo* (Crofton *et al*., 2005).

The papers by Silva *et al*. (2002), Rajapakse *et al*. (2002), Tinwell and Ashby (2004), Brian *et al*. (2005) and Crofton *et al*. (2005) also provide evidence that EDC produce joint effects when combined at doses that individually did not produce observable effects. Taken together, therefore, risk assessment for EDC merits serious consideration.

Elements of risk assessment

Risk assessment for chemicals relies on exposure assessment, hazard assessment and risk characterisation. When applied to the topic of EDC mixtures risk assessment, the following conceptual needs and data requirements become obvious:

- There is a need to arrive at a definition as to which chemicals should be considered to be "endocrine disrupters". This issue is fundamental to risk assessment, because a consensus has to be reached about which chemicals should be included in risk assessment.
- Linked to the first issue is the question as to which combinations of EDC should be regarded as being of concern. Data and criteria regarding effect profiles of individual chemicals are needed to decide whether specific chemicals should be considered as part of EDC mixtures.
- Data on EDC exposures are essential to judge what relevant doses or concentrations present in combinations are.

Also important are reflections on settings for which mixture risk assessment might be needed. It is possible to identify the following relevant settings for EDC mixtures:

- Medium- or population-specific risk assessment (RA) (e.g. human exposure via food, air, water, or specific highly exposed human populations, considering vulnerable windows of exposure; wildlife, especially aquatic wildlife).
- Process-specific RA (this is relevant for sewage treatment works or pulp mills and emissions into the aquatic environment).
- Product-specific RA (for EDC this could include certain plastics which leach EDC, cosmetics, air fresheners, etc).
- Generic RA for certain groups of chemicals (e.g. PCB's and dioxins, steroidal hormones, etc).

Exposure assessment

For humans, knowledge about the total exposure via food, air, water, and consumer products is necessary. Attention should be paid to the identification of specific highly exposed sub-populations. In terms of data requirements, this task will be greatly aided by data about ***internal exposure*** to EDC, e.g. data about tissue levels etc. Some of these data are currently gathered as part of the EDEN project, but literature searches are essential to supplement this. Data about combined exposures of one and the same human population would be particularly valuable here, but are very hard to obtain. A comprehensive exposure assessment for humans is complicated by the fact that there is still considerable uncertainty about the nature of EDC humans may have contact with. Exposure scenarios for wildlife are comparatively better defined.

Hazard identification for mixtures

The task of hazard assessments for EDC mixtures can be sub-divided into two main elements, each with differing data requirements:

- How should mixture effects be determined?
- How should mixture effects be assessed and predicted?

Three main approaches have been used for mixture effect determination: Complex mixture testing, sometimes also termed "whole mixture testing", as in whole effluent testing, etc.; the use of reconstituted laboratory mixtures, variously termed "similar mixture testing" or "simple mixture testing"; and finally mechanistic approaches that consider the mechanisms underlying the action of mixture components, with regard to endpoints of toxicological relevance.

The data requirements of each of these approaches differ considerably. Whole mixture approaches have to rely on fairly well-defined sampling protocols that take account of the possibility that the precise composition of the mixture to be studied (which remains unknown in whole mixture approaches!) may vary depending on sampling conditions. Whole mixture approaches may help characterising whether there are joint effects at all, but without knowledge about its chemical composition, and the effects of individual mixture components this type of approach is only useful on a case-by-case basis.

To make the testing of reconstituted mixtures ("similar mixture approaches", or "simple" mixtures tested in the laboratory) viable, data about the effects of each individual mixture component are required. In some cases, knowledge about specific effect doses (e.g.

ED50) may be sufficient, but often information about entire dose-response relationships is essential if judgements in terms of additivity, synergisms or antagonisms are to be made.

Mechanistic approaches have to rely on data about the underlying mechanisms of action of chemicals in the combination. Often, this information is only fragmentary, and in such cases, knowledge about sites of action may be sufficient.

Two main concepts exist for the quantitative assessment of mixture effects in terms of additivity, synergism or antagonism. These are *concentration or dose addition* and *independent action*, sometimes also referred to as *response addition*. Often, but not always, these two approaches yield the same quantitative mixture effect prediction. Thus, the question arises as to whether it is possible to select one of the two concepts as a default approach for mixture effect assessment.

In the ecotoxicological arena, systematic comparative studies of the mixture effect predictions produced by *dose addition* and *independent action* have shown that *dose addition* yielded the more conservative predictions, but that overall, the quantitative differences between both concepts were relatively small (Backhaus *et al.* 2000; Faust *et al.* 2003). Here, the case can be made for using *dose addition* as the default approach for mixture assessments. This would avoid lengthy and largely fruitless discussions about establishing modes of action. Such a modus operandi would have two advantages: First, the data requirements for proper use of *dose addition* are less stringent than those for *independent action*. While the former works well on the basis of effect doses, the use of *independent action* usually requires knowledge of entire dose-response curves, particularly in the low effect range. Second, prospective mixture effect assessments should be compliant with the precautionary principle. This favours the concept that typically yields the more conservative predictions, i.e. *dose addition*.

While the case for *dose addition* is validated in ecotoxicology, the situation is not so clear-cut in human toxicology. Here, the relevant information is largely missing and research efforts are currently directed into conducting studies to fill these gaps.

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D17 Cluster related activities

Responsible Partner: 1 Participating Partners: All

D17.3 Production of a Cluster newsletter

The forth issue was published in May 2005 and research news from several of the projects within CREDO are presented including the latest in glowing fish, the completion of the pre-screening phase within FIRE and a focus of the issues relating to male reproductive health. From a regulatory perspective of endocrine disrupters, two articles look at the implementation of the European Commission strategy and the proposed European regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). A summary of the COMPRENDO workshop on the ecological relevance of chemically induced endocrine disruption in wildlife, which was held in July 2004 in Exeter along with further details regarding the publication of the workshop proceedings. All four issues can be downloaded from the EDEN website (http://www.edenresearch.info/news.html) and also the CREDO website (http://www.credocluster.info/resources.html).

The fifth issue is in progress and due to be published in January 2006. Included is a report on the completion of the ACE project and research news from GENDisrupt and FIRE. Details for the final COMPRENDO project workshop to be held in March 2006 are given as well as a summary of the CREDO workshop organised by EDEN and FIRE which was held in Prague in May 2005.

D17.5 Preparation of coordinated Cluster press releases

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, 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 disruptors.

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 disruptors, as part of the EU-funded COMPRENDO project into comparative research on endocrine disruptors. "*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 disruptors 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 disruptors 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 & Pollut 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

D17.6 Representation of ED research priorities at relevant EU policy meetings

Kortenkamp A, Multi-component mixtures of endocrine disrupters - experimental requirements and recent test results. "Environmental Endocrine Disrupters 2004", a meeting organised by the Japanese Ministry of the Environment, 18 December 2004, Nagoya, Japan

Kortenkamp A, Estrogens, environmental pollutants and breast cancer – a re-evalaution from a mixture perspective. 3rd Copenhagen Workshop on Environment, Reproductive Health and Fertility, Copenhagen, Copenhagen, Denmark 15-18 January 2005

Kortenkamp A, Breast cancer and environmental pollutants, INMA workshop, 4 February 2005, Granada, Spain.

Kortenkamp A, CREDO – Research on endocrine disrupters and their effects on human health and the environment, EC DG Research Workshop on Internatioanl Collaboration in EDC Research, 26 January, Brussels, Belgium.

Scholze M. Quantitative considerations of low-dose testing. Invited speaker, CREDO Workshop, Prague, Czech Republic 10-12 May 2005.

Kortenkamp A, Are there risks from low dose mixture effects of endocrine disrupters? 3rd UBA Status seminar on chemicals with endocrine activity, 2 June 2005, Berlin, Germany

Kortenkamp A, Endocrine disrupters - mixture effects at low doses. 1st International workshop on modifiers of chemical toxicity - implications for human health risk assessment, a workshop sponsored by the European Commission and the Environmental and Occupational Health Sciences Institute, 12-15 June 2005, Poros, Athens, Greece

Pedersen R, Latest research and recommendations regarding endocrine disrupting chemicals. PAN European Annual Conference, 7-9 November 2005, Krakow, Poland

Kortenkamp A, Prediction and assessment of mixture effects, SENSPESTI workshop, 25-26 November 2005, Parma, Italy

D17.7 Thematic workshop on 'dose-response analysis and mixture effects, testing guidelines, epidemiology'

The workshop 'Endocrine Disrupters: Exposure Assessment, Epidemiology, Low-dose and Mixture Effects' was organised by the EDEN and FIRE consortia and held on the 10-12th May 2005 in Prague, Czech Republic. The event was publicised on the CREDO website (http://www.credocluster.info/workshop1.html), the text of which si given below:

At this international workshop, scientists from across Europe will present the latest research findings in endocrine disrupter research. There will be discussions to promote the transfer of know-how between scientists on the issues of exposure assessment, epidemiology, low-dose and mixture effects.

The programme includes 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.

We invite scientists who are not members of cluster projects as well as policy makers and PhD students working in these areas to register. Workshop proceedings will be published in a special journal issue. We encourage submission of abstracts for the poster sessions.

The CREDO workshop on endocrine disrupters: exposure assessment, epidemiology, low-dose and mixture effects is organised by the EDEN and FIRE research consortia. CREDO is funded by the European Commission's Fifth framework programme for research, technological development and demonstration activities in the European Community. A joint project funded by two Thematic Programmes: Quality of Life and Management of Living Resources Programme, and Energy, Environment and Sustainable Development Programme.

The programme can be viewed on the CREDO website at http://www.credocluster.info/docs/ws_prague_final_programme.pdf.

ANNEX II: PROJECT MANAGEMENT

Contents

1. Mini Minutes: Planning Meeting for CREDO workshop 'Exposure assessment, low-dose and mixture effects, testing guidelines and epidemiology', Brussels, Belgium. 1st December 2004

Present: Jeff Vos, Pim Leonards, Andreas Kortenkamp, Ragnor Pedersen

1. At Prague workshop:
    a. Speakers from CREDO projects will pay their own travel and hotel costs, but registration fees will be waived
    b. Speakers from out side CREDO – conference will pay all costs.
2. Advertising will be through CREDO newsletter, societies, email and journals. Leaflets/posters will not be used.
3. RP to enquire if meeting is cancelled for unforeseen reason, what will EDEN/FIRE have to pay?
4. RP to request contract for workshop venue 'Masarykova kolej' (MK).
5. FIRE and EDEN (non CREDO) room requirements at MK will be booked and billed through EDEN.
6. Budget from FIRE is 5000 euro. Budget from EDEN is 9000 euro.
7. Any profits from workshop will be split 5/14 to FIRE and 9/14 to EDEN.
8. The deadline for poster abstract submission is 1 April.
9. The web application form will ask 'Do you intend to submit a poster?'
10. Registration will state 'Abstract book included' in registration fee.
11. Manuscripts from speakers to be handed in at the meeting.
12. First choice journal is *EHP*. Other possibility is *Toxicology*.
13. Hotel Praha reception:
    a. There will be a welcome address/talk
    b. Wine/beer/soft drinks
    c. Welcome drink and drink with meal included
14. Registration support staff in Prague and IT support staff in Prague will be organised by FIRE
15. Suggested 'Consensus statement' issues to drive workshop sessions. In style of statement on global warming.
    a. FIRE suggest strong text on exposure and brominated flame retardants
    b. EDEN suggest text on mixtures / male health
    c. Format to be considered
    d. Possibly to address the 7th Framework
    e. Taking stock of where ED research/knowledge is now.
    f. State of the art?
    g. Statements on Reach.
16. Discussion on Scientific Program as updated in the programme document.

2. **Minutes: Local EDEN Meeting: Rat studies,** Rigshospitalet, Copenhagen, Denmark. 18th January 2005

Present: Julie Borch (JB), Sophie Christiansen (SC), Majken Dalgaard (MD), Marta Filinska (MF), Rachel Gomes (RG), Ulla Hass (UH), Andreas Kortenkamp (AK), Jens-Jørgen Larsen (JJL), Steine Metzdorff (SM), Martin Scholze (MS), Elisabete Silva (ES)

Agenda:
- Histopathology
- Low dose mixture studies
- Future activities for next 2 years

Histopathology

- Started in Summer Jane Fisher teaching ES using postnatal day 6 cutting blocks starting with highest dose and working down
- DEHP 900 mg/kg < 8-9 nuclei - MF says multinuclei gonacytes undergoing degeneration. Observe this at lower doses when no hydroplasia but also observe in controls and also in gestational stage 21. Haven't done tunnel apoptosis staining but might be funny. Smooth muscle actin and haemoglobin stainin
- Flutamide 16 mg/kg - do not see multinuclei gonadcytes but see funny thing in cell. Could this be an effect – investigate further if see more. Sometimes can be in a state of mitosis but MF doesn't think this is the case
- MF says finasteride is comparable to control. Use diameter of tubules as estimate. If cluster of leydig cells larger than tubules then consider it as hypoplasia
- AK motivation that damage at microscopic level link with other endpoints which UH aggress seems to make sense. MF says testis not main target. External genitals and accessory glands, AGD and nipples are more obvious, androgenic. DEHP is very interesting.
- JB markers for cytokinesis worth looking at to give any answers as separation not occurring through cytoplasm.
- AK see in context of mixed modes of action, get mixed picture? Main motivation.
- ES what to expect in testis using mixtures? Single agents at different concentrations compare with mixture at different concentrations. MF: testis weight goes down but histology? 4 AR antagonist at PND6 would reckon mix effects. ES just observing hypopla. MF sees Sertoli vacuation, germ cell shading, leydig cell hypoplasia, infiltration with inflammatory cells. Go to morphometrics but not enough samples.
- Discussion between AK and ES as to whether histology is worth it and decided that it is. ES and MF will keep in touch so don't repeat work.
- MF has looked at AR expression in AR receptor antagonists flutamide and dES in adults but nothing observed. Suggestion that epididymis/protrate would be better than testis.

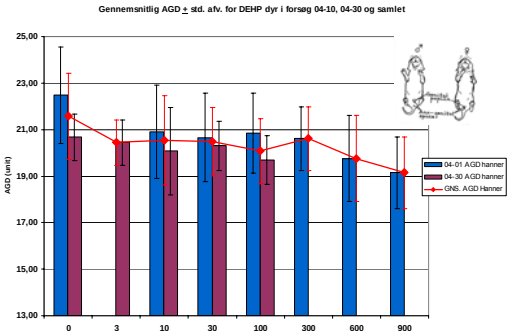
Low dose mixture studies

- Possibility of MS to combine 2 studies and analyse together
- Significant effect between 10 and 100mg/kg with 10 giving more effects, don't know why?
- 3mg/kg compare with control – worth while to do more to see if effect
- What would be results if used benchmark approach?

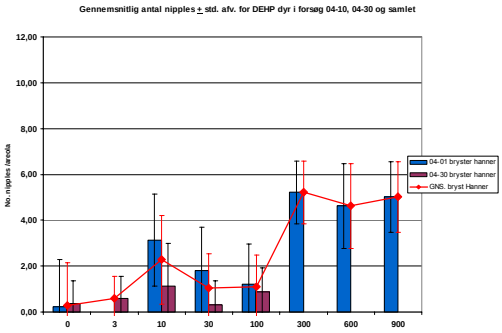
Low dose effects
NOAEL vs. Benchmark approach

Spring 2004
- 0, 10, 30, 100, 300, 600 og 900 mg/kg
- Ca. 6 litters per DEHP-group, ca 12 in control group
- Effect på AGD and nipples from 10 mg/kg

Winter 2004
- 0, 3, 10, 30 og 100 mg/kg
- Ca. 12 litters in control and 3 mg/kg groups, 6 litters at 10, 30, 100 mg/kg
- Total ca. 24 litters in control, 12 in DEHP low dose



DEHP-1: LOAEL 10 mg/kg; DEHP-2: 100 mg/kg
Combined: LOAEL 10 mg/kg; Interaction study*group



DEHP-1: 10 mg/kg; DEHP-2: 'borderline' at 10 og 100 mg/kg
Combined: LOAEL 10 mg/kg; No interaction study*group

Preliminary conclusions

AGD
- **Combined LOAEL: 10 mg/kg DEHP**
- **NOAEL: 3 mg/kg?**
- **Interaction study*group**

Nipples
- **Combined LOAEL: 10 mg/kg DEHP**
- **NOAEL: 3 mg/kg (?)**
- **No interaction study*group**

6 litters per group: not useful for low dose studies and NOAEL approach - risk of false negative/positive

Benchmark approach?

12 litters per group – much better

OECD TG: 8 or 20 litters per group

Mixture study 1
- Definition of similar and dissimilar
- Flutamide, Vinclozolin, Procymidone similar acting
- Vincolzolin and Procymidone very similar, Flutamide similar but to the left
- Groups and animals: change from control/dose 16/8 as difficult to have animals testing and behaviour. Need >6. Proposal stick to 16 control and 16 mix apart from the low ones so take behaviour and semen quality.
- 8 animals because have behaviour data from before for other chemicals.
- Handle s 2 x 8 animals when using 16 which will say about ability to replicate effects.
- UH says either have sufficient group size or don't do behaviour. SC: see if pronounced effects then wouldn't let them live to see behavioural studies. Won't keep animals for behaviour work is severely malformed.
- Suggestion of dose levels for mixture given in slide 4.
- Conclusions: Base study of mixtures in AGD and nipples/16 animal size

Mixture study 1
Similarly acting EDCs

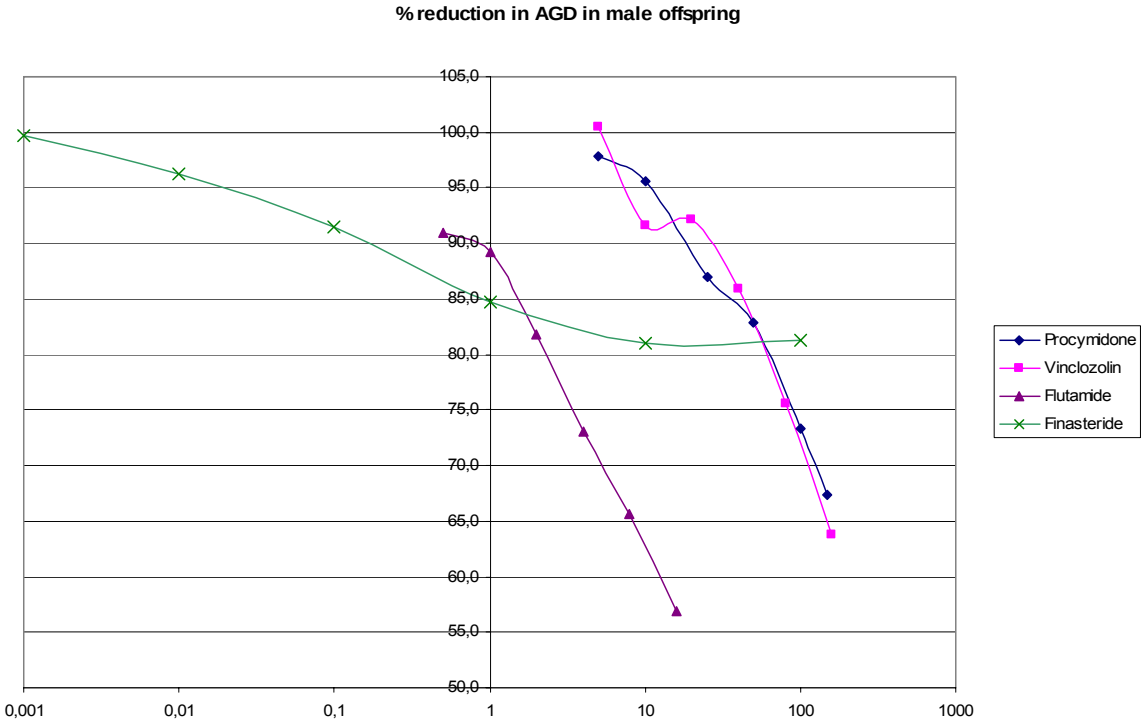
"Similarly" = induce marked feminization of AGD and/or nipple retention in the abscence of marked maternal toxicity or unspecific pup toxicity

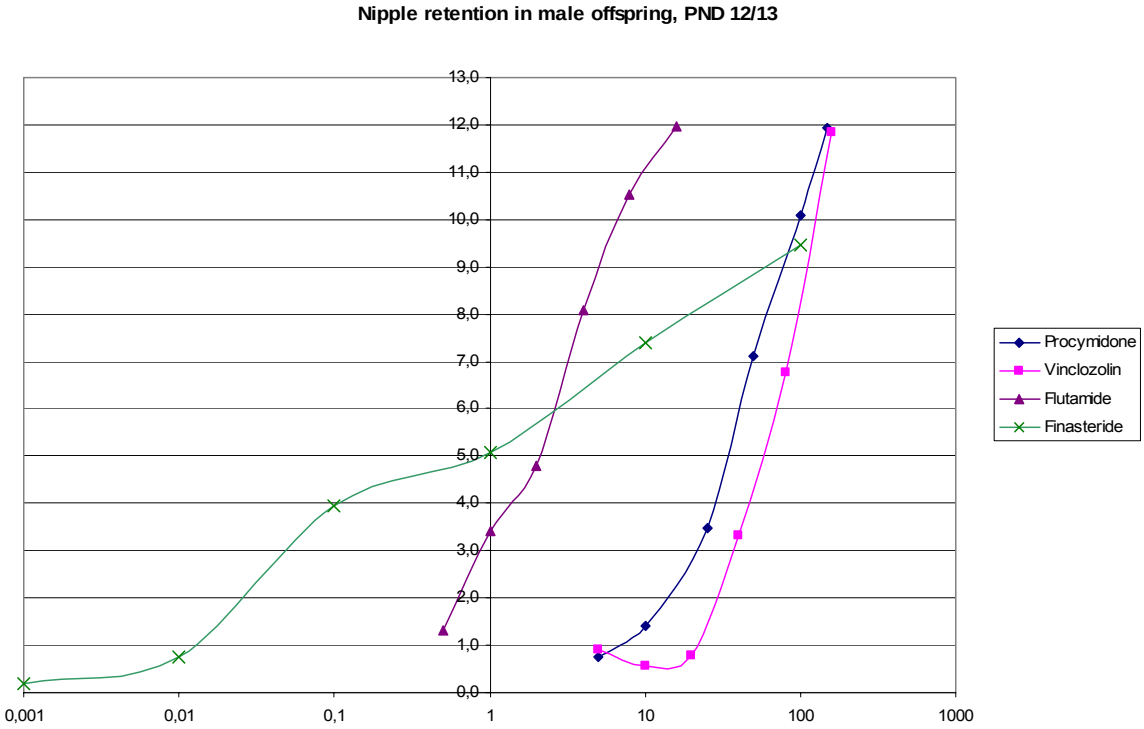
"Dissimilarly" = not similarly acting!

Chemicals and time scale
- Flutamide
- Vinclozolin
- Procymidone
- Finasteride

- Animals: April/May
- PND 0 & 6: May
- PND 16: Medio May to medio June
- Behaviour: June to November
- Semen quality: November and December

Prague Meeting OK!


% reduction in AGD in male offspring
105,0
100,0
95,0
90,0
85,0
80,0
75,0
70,0
65,0
60,0
55,0
50,0
0,001
0,01
0,1
1
10
100
1000
Procymidone
Vinclozolin
Flutamide
Finasteride


Nipple retention in male offspring, PND 12/13
13,0
12,0
11,0
10,0
9,0
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0,001
0,01
0,1
1
10
100
1000
Procymidone
Vinclozolin
Flutamide
Finasteride

Groups and animals

Group	Mated	Litters ex	Behav-M	Behav-F	Comments
Control	16	14	12	12	
Mix-very low	8	6	0	0	16?
Mix-low	16	14	12	12	
Mix-medium	16	14	12	12	
Mix-high	16	14	12	12	
Flut-very low	8	6	0	0	
Flut-medium	8	6	0	0	Behaviour 2000
Fin-very low	8	6	0	0	
Fin-medium	8	6	0	0	Behaviour 04-01
Procy-very lo	8	6	0	0	
Procy-mediur	8	6	0	0	Behaviour 04-30
Vin-very low	16	14	12	12	Behaviour?
Vin-medium	16	14	12	12	Behaviour?
Total	**152**	**126**	**72**	**72**	

Dose levels - mixture

Mixture of four compounds (in brackets the expected single effects)

dilution	Vinclozolin [mg/kg]	Procymidine [mg/kg]	Finasteride [mg/kg]	Flutamide [mg/kg]	mixture dose	CA effect (number of nipples)
1 (sum of ED6s)	73.42 (NR=6)	73.42 (NR=6)	1.684 (NR=6)	2.322 (NR=6)	150.84	max
0.6	44.05 (NR≈4)	44.05 (NR≈4)	1.011 (NR≈5.5)	1.393 (NR≈4.5)	90.50	9.5
0.25	18.35 (NR≈1.5)	18.35 (NR≈1.5)	0.421 (NR≈4.5)	0.581 (NR≈1.5)	37.71	6
0.06	4.41 (NR<1)	4.41 (NR<1)	0.101 (NR≈3)	0.139 (NR<1)	9.05	3.75
0.01	0.73 (NR<1)	0.73 (NR<1)	0.017 (NR≈2)	0.023 (NR<1)	1.51	≈2

Dose levels – individual EDCs

Vinclozolin	Procymidine	Finasteride	Flutamide	comment
95.94 mg/kg	95.94 mg/kg	17.43 mg/kg	3.86 mg/kg	ED8, just for modeling purposes
18.35 (NR≈1.5)	18.35 (NR≈1.5)	0.421 (NR≈4.5)	0.581 (NR≈1.5)	low effect dose, present in test mixture dose

Comments/discussion

- Recalculate, including procymidone data
- Nipples or AGD?
- Animals per group at lowest mixture dose?
- More practical doses (fewer decimals)

- AK: Definition of dissimilar is not a simple negation of similar
- Toxicology and pharmacology can't agree on complexity level
- Molecular definition similar is identical molecular, interaction with same receptor. Personal opinion: will model on CA
- UH: have 3 chemicals which are similar, finasteride completely different and different mechanism. Need minimum of 4 chemicals.
- Discussion follows on number required: JJL says first study with 3 compounds not 4 (as 3 are similar). MF feels 3 not challenging situation and to use 4 or 5 especially relate to real world. AK comments are Earl Gray did just binary so don't know number as never been done.
- AK: What endpoint to base design mixture on?
- Flutamide's long flat curve is worrying as limits CA prediction

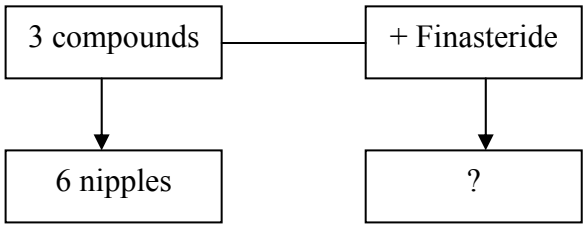
Presentation by MS

- AK: What endpoint to base design mixture on?
- Vinclozolin: AGD classical approach or benchmark approach. First define range (relative effects range) where are the limits. Nearly impossible ti jusif EC5 because falls outside tested range. Never trust a valie based on extrapolation alone.
- Finasteride: very serious problems because basic comparitive assessment on high range of effects not small. Thus has lmited effect range. Not good basis as limits effct range at least for this endpoint.
- Reason MS prefers other endpoint: nipple retention

1. Like this very much because variability is very good even with such small data set
2. Very easy as adjustmemnt for other covariants not needed e.g. body weight

3. Not needed to normalise the scales as 0 is control level and 12-13 is the other end.

- In model, litter effect is corrected. Fluatmide illustrates beautiful dose reposnse curve. Finasteride have confidence at higher effect levels.
- Need for prediction: need to know reproducibility so favour repeating and allow to correct these models.
- Therefore base planning on NR endpoint but both dose effect range are very similar for both endpoints.
- Starting point is mixture ratio: Classical approach defining 1 effect level when expects each compound contributes the same way. Hencem reason decided on level 6 (may be 7-5 though).
- Second step: calculate for each compound the doses for producing the effect. Cakculate 'toxic unit' using fraction p.
- Need to decide which mixture dose to use.
- Whether to tes nigher mixture levels and what about room for very low dose (<1.5)?
- MF: Large variability observed in control group for AGD. If choose dose at 1.5 – could it be representative of variability between studies. Very low dose would not be significant. Would argue for next dose up (2.5).
- Number of chemicals: for 4 chemicals might remove finasteride. UH: Expected to induce 1.5 nipples in itself. AK: Only at 6 would all 4 contribute at the same level. So not something from 'nothing' as low dose, any effects would be from finasteride.
- Waiting on data from last compound before deciding on how many compounds.
- AK suggests going for 4 but MS thinks 3 as may not see the nice agreement we would expect and then what?
- MS: Test 3 compounds together with finasteride and use spare to spot check with finasteride.



- UH: leaves room for antagonism and synergism if add finasteride so expect 9 nipples.

3. **Minutes: Local EDEN Meeting: Sticklebacks,** London School of Pharmacy, London, UK. 21st March 2005

Present: Rachel Gomes (RG), Ioanna Katsidaki (IK), Andreas Kortenkamp (AK), Ian Mayer (IM), Steve Morris (SM), Tom Pottinger (TP),), Martin Scholze (MS), Alex Scott (AS).

Brief overview with data analysis by MS for wild fish caught in Norway and UK.

Spiggin Fenitrothion

- effect shape, very flat curve was not expected from range finding studies
- not statistically significant effects expect low effect
- defined dose/response: lowest tested concentration still significant but when normalised to body weight the lowest tested concentration is no longer significant
- Flat curves usually means the need to test more concentrations (higher and no effects) and Fenitrothion is the only tested compound to act like this
- Outliers observed with spiggin endpoint, extra sensitive or not sensitive?
- MS does not see outliers when using VTG as endpoint
- Possible evidence of bimodal distribution?
- TP raises possibility that subordinate animals may be responsible for the outliers due to hierarchy influence
- AK genetic influence as very diverse
- Bimodal doesn't work for medium effect in second study
- Problem with statistical interpretation: have to be sure DHT is positive, saturating concentration of DHT 5µg/L
- Needs to be high effect levels but below the maximum effect
- IK 5µg/L looks like left hand side/AK and MS think it is optimal
- IK: if give more DHT will make more spiggin but close to maximum effect spiggin induction
- Lowest tested concentration on majority of fish have significant effect
- Problem with benchmark
- Not possible to identify 10% increase on log scale, realistic 15-20% as endpoint
- Stickleback sentient rather than testing, relevant to population studies (lab stickleback have low fecundity so prefer wild caught)
- MS: good news for mixtures because based on this data have high confidence because effect range between 10-90%

Flutamide

- AK: see bimodal distribution with solvent controls
- IK: is artefact with assay e.g. pump failures therefore use 2 tanks for this exposure to exclude one tank is need be (MS)
- MS positive control seeing possible bimodal in tank B
- Reproducibility important issue for mixture study. IM's range finding (RF) very different to definite study, TP's RK good
- IM: due to soluability problems at high doses, comes out of solution in stock tank
- IK: could be seasonal influence for IM's RF being higher than TP's RF
- Looking at chemistry to see if influence but doesn't explain, DHT lower than should be, flutamide 20% of expected
- Little difference between observed flutamide in IM's or TP's labs expect at 250

- Normalised data – problem with positive controls. Have two different populations responding in different ways
- IK normalises using weight
- Mixture possible but as low effect study is dodgy because lowest tested concentration has produced effect
- AK to IM: is it feasible to rerun selected concentrations of flutamide, start in 2 weeks time, finish end April and then have to analyse
- AK: low dose deliverable is ½ year behind and Tuomo will chase. Use IK own data for stickleback deliverable and do IM's concentration and send
- SM: prioritise flutamide? Option 1: not do anything and just use IK data or option 2 IM so extra repeat concentrations

VTG

- E1 detection limit problem. If up against defn limits of analytical methodology, is a problem in estimating low effects at low dose. If see nothing, is it then sue to LOD or because no effect?
- How to estimate the mean, use different method probability distribution
- First 4 concentrations no effect concluded or due LOD problem
- E1 pooled studies RF. Unlikely that chemistry can answer this
- 2 tanks observe differences between controls
- TP/SM: artefact on extraction or assay as to why control higher showing E1
- E2 and EE2 awaiting analysis

NP

- IM tanks A and B are similar. Not expected results based on RF results from other species – waiting on analytical data
- MS thinks something wrong with exposure side, so need analytical chemistry to check concentration in water at 7, 14 and 21 days

- Priority is flutamide and NP chemistry. SM: extracting NP last week so in system and process flutamide and NP in next two weeks
- AK says to redo flutamide and some MP but IK says no as can't compare with previous. Must be full dose, not the odd concentration
- IM must do spot checks pronto on flutamide and NP, 100µg/L need to do it again before mixture study for confirmation
- Plan last week – IM and TP both estrogens and androgens – carry on with this. This time on lab does steroids and other spiggin because of lab-lab variability

Mixture studies

- Windemere E2 and EE2 finished, NP Bergen done (1st one). Test 2 or 3 UV filters at the same time
- Traizoles designed to inhibit esterase that lies on anabolic pathway of sterol synthesis
- Receptor mediated detection via an intermediate – also happens in fish
- Shortly before mixture study, just repeat 1 or 2 concentrations to be sure
- CEFAS has no analytical methods for UV filters, can trawl the literature but would need development
- IK find which are responsive and then choose most estrogenic to then develop methods
- IM doing 2 UV filters, TP doing one 1

- Need 2 of 4 (cyproconazole and 3 UV filters) to get response in stickleback – would be new data
- ACE project has 5 component mixture in seabass
- Best case mixture 3 steroids
- Can we afford to be economic with anti-androgens because is novel and would give time to do 6 estrogenic mixture
- MS: test at 2 different mixture ratios
- TP: money available for chemical analysis for CEFAS subcontractor – possible budget anxiety
- SM: will incur higher costs when get further into mixture and use different analyses
- AK: use €70,000 from Munich to offset analysis costs
- SM to contact Martin and Ulf about using their money

Mixture planning: VTG
Compounds: **estrone**, nonylphenol, estradiol, ethynylestradiol, cyproconazole and UV filter agent [3-benzylidene camphor (3-BC), 3-(4-methylbenzylidene) camphor (4-MBC) or octyl-methoxycinnamate (OMC)]

	Windermere	**Bergen**	
☑	estrone	nonylphenol	
april	estradiol, EE2	confirmation: nonylphenol, flutamide	june
end may	cyproconazole (RF), UV filter agents (RFs)	UV filter agents (RFs), UV filter agent	end july
end june	cyproconazole, UV filter agent, unforeseen + retesting of nonylphenol	unforeseen + retesting (estrone, EE2, estradiol, cyproconazole)	end september
	mixture	mixture	

Mixture planning: spiggin
Compounds: **flutamide**, linuron, **fenitrothion**, vinclozolin (procymidon, cemetidine)

	Windermere	**Bergen**	
☑	fenitrothion	flutamide	
	linuron	linuron (RF), vinclozolin (RF)	end april
	vinclozolin		
	unforeseen + retesting (flutamide, linuron, vinclozolin)	unforeseen + retesting (fenitrothion, procymidon, cemetidine)	
	mixture	mixture	

- Need mixture studies completed (fish and water chemistry) by certain point to allow statistical interpretation – May 2006
- Schedule in Majorca has slipped considerably. What the prospects that the timeplan today will be more realistic?
- Communication must be improved, keep everyone in the loop. IK suggests using stronger leadership with a recap every 2 months

- Anti-androgens study is more time consuming. Could double up on RF in parallel and do 4 compounds and not 6 (fen, flu, lin and vincl)
- IM: Lin and vincl will be completed in ten days. When will spiggin data be available? Need Sam to dissect beforehand. Send to IK in first week of April (3 weeks time) and end of April have results
- TP and IM – send sources UV filters in next few days. TP will begin first and decide which to use. Not worrying about chemical analysis until have response for UV filters
- AS: stop anti-androgens and concentrate on estrogens and mixture

4. Minutes: EDEN Mid Term Review, Hotel Praha, Prague, Czech Republic. 9th May 2005

Present: Anna-Maria Andersson (A-MA), Rachel Gomes (RG), Louis J. Guillette Jr. (LG), Niels Jorgensen (NJ), Tuomo Karjalainen (TK), Ioanna Katsidaki (IK), Hannu Kiviranta (HK), Andreas Kortenkamp (AK), Kim Mahood (KM), Ian Mayer (IM), Nicolás Olea (NO), Jorma Paranko (JP), Tom Pottinger (TP), Matthew Sanders (MS), Richard Sharpe (RS), Niels Skakkebaek (NS), Carlos Sonnenschein (CS), Jorma Toppari (JT),

10:00 -10:15	**Andreas Kortenkamp** (Project coordinator) Welcome address
10:15 - 10:45	**Tuomo Karjalainen** (Scientific project officer/co-chair) Introduction and role of mid term review
10:45 - 11:00	**Andreas Kortenkamp** (Project coordinator) Overview of the EDEN project, management issues
11:00 - 11:30	Refreshments
11:30 - 12:00	**Tom Pottinger** (Theme 1 coordinator) Complex EDC mixtures in human and fish tissues - exposure assessment
12:00 - 12:15	Discussion and reviewer queries
12:15 - 12:45	**Richard Sharpe** (Theme 2 coordinator) Mechanisms of EDC action - novel endpoints and biomarkers
12:45 - 13:00	Discussion and reviewer queries
13:00 - 14:00	Lunch
14:00 - 15:30	**Niels Skakkebæk** (Theme 3 coordinator) Indicators of impaired reproductive function in European men **Niels Skakkebæk** Introduction **Niels Jørgensen** Semen quality data **Anna-Maria Andersson** Hormone analysis
15:30 - 15:45	Discussion and reviewer queries 5 minute break to refresh drinks/snacks
15:45 - 16:25	**Andreas Kortenkamp** (Theme 4 coordinator) Low-dose- and mixture effects of EDC - providing empirical evidence and exploring implications for regulation and testing
16:25 - 16:40	Discussion and reviewer queries
16:40 - 17:10	**Louis J. Guillette Jr. & Carlos Sonnenschein** (Expert reviewers/co-chairs) Concluding discussion and comments

Several presentations for the Mid Term Review are also available on the EDEN member's site. The minutes detail the discussions and issues raised by the examiners.

10:15 – Introduced changes to FP7 with endocrine disrupters to be in three themes;

In the FP5 there was integration between human and wildlife which EDEN benefited from. FP6 lacked this but Tuomo says that FP7 environment and health will allow this.

10:45 – Presentations will be data driven in encourage scientific discussion. Theme 1 can be difficult as the definition of EDCs is divergent. For Theme 2, research is expanding the male data from Denmark/Finland to Germany as well.

Introduced ourselves to the examiners, as requested by CS.

11:30 – Theme 1 (TP): Fish most appropriate wildlife donor and much is known about problems and causes in this organism. Boys are the most appropriate and accessible for human sampling. Two approaches:

- Targeted analysis: generation of data but may overlook chemicals
- TIE: makes no assumption about chemicals but is slow and requires a large amount of sample

There has been a revision of expectations, in terms of the total number of samples collected and subsequently the delivery for analysis. It is expected to complete analysis deliverables within the timeframe.

Issue: agreement on individual samples for analysis which is currently unresolved. Discussions are ongoing regarding the selection of appropriate samples for chemical analysis, dependent on age, extent of endocrine disruption, amount of tissue.

- The amount of tissue is likely to be a limiting factor for some assays
- Ages of fish are different, should they be the same to avoid the issue of bioaccumulation?

CS: *was this anticipated?*
TP: *when this was planned, the main question was whether we could get the fish*
LG: *so no collection now going on as only have 14 fish from the reference site?*
TP: *collection has finished and there are also 8 fish from a further reference site as well*

Issue: Partner 9 have analysed human samples and initial interpretation found that levels of pesticides are not different between control and case samples.

CS: *lack of difference attributed to?*
TP: *the initial female samples were low with 10 cases and 10 controls. In addition the controls were diseased albeit not with breast cancer. Hence, a further 56 women were recruited*
LG: *not casual but associative. The fact that chemicals are detected and the fact that there is little data on exposure, still makes this significant. Am concerned that the deliverable may not be possible to be delivered*
AK: *the aim of Theme 1 was not to carry out a case control but document pollutants. The dilemma is that people will ask about controls. Statistical power of 20 specimens is not possible to link to pollutant exposure*
LG: *wanted to know the causation as not possible*
NO: *regarding the 20 samples, wanted to know the human exposure to feed into Theme 4. This work was based on 200 cases and 300 controls which was published previously*

LG: *target chemical analysis aimed at lipophilic. Why no assessment of fungicides and herbicides?*
AK: *didn't consider this actively with respect to low levels*
LG: *bothers me and not directed at this programme but at the field as to why we concentrate on these lipophilic compounds only*
NO: *wine and breast milk are sinks and not included in this project but included in EXPORED*
AK: *went for adipose tissue but realise that this would cut out a certain group of pollutants. Build on adipose and them move onto blood and wine*
JP: *considered but banned so thought levels would be very low especially as amount of sample was lower than anticipated, would be good focus for Spanish though*
NO: *need to place emphasis on biomarkers*
LG: *likes JP's comment that what may be inappropriate in one area may be appropriate in other areas (infers Spain)*

LG: *for fish sampling, the age of the female will be critical*
TP: *only make fish being used due to complications being raised by LG*
LG: *what hormone analysis is being carried out?*
TP: *VTG and have scope for doing additional testing as have kept the samples such as 11 keto*
LG: *testosterone evry important*
TP: *information from sticklebacks is for E2, testosterone and 11 keto*

CS: *one of our concerns was the size of the project representing half of the funds, could anything of this size be managed. From what is seen, it is manageable. There is no experience of this in the States. Is a great experience for Europe to test their scientific might. The fact that not every single aspect is considered doesn't mean it won't be considered*
LG: *YES and E Screen – very important*
AK: *ACE and collaborate with NO comparing the YES and E Screen. EC50 values for xenoestrogens for YES and E Screen do not vary but does vary with steroids. YES very sensitive but E Screen less so. May have to do with specificity of the yeast cell wall. However why is it only with steroids?*
A-MA: *May also be the metabolism*
CS: *prepared to make recommendation on not wasting effort and resources on bioassays that are not so sensitive*
AK: *not the scope of EDEN but is for ACE but there will be knowledge transfer*
TK: *REACH endocrine disrupter testing need in vitro assays*
CS: *not identified method of testing. Comment: the EU is wise in following their own timetable instead of waiting for US to produce anything*
AK: *EQUALM are about to make their mind regarding assays to use but ACE and EDEN (to a lesser extent) have a lot of relevant information*
NO: *A Screen everything directed to MCF cells (solvent, analytical approach). If use a different assay, then will have to redevelop the approach as you develop and adapt the extraction to the endpoint utilised*

12:15 – Theme 2 (RS): Selected highlights as has been lots of progress in this large theme.

- For microarray studies in WP10, BPA shown to down regulate whilst E2 up regulates. However, preliminary results show that reproducibility may be a problem
- In the glowing fish work, they are currently crossing 47 glowing fish with a wildtype fish, only a small number are likely to be germline transgenic
- Development of assay and antibodies for Insl3 has been very difficult however Insl3 may be a potentially useful biomarker for Leydig cells
- Issue: Currently developing the rat assay which still has to be validated
- KISS-1/GRP54 system is a new focus of interest and possible new target for endocrine disruption. At present, not all mechanisms and pathways have been worked out yet

CS: *Girls puberty precocious, has Bourguignon looked at where they are from?*
RS: *Can anyone answer this?*
NS: *Our own study illustrated it was country specific*
LG: *So malaria versus non-malaria, use of DDT?*
IK: *Has that been correlated with amount of DDT?*
NS: *No*
CS: *This should be done*

LG: *What's the highest dose of phthalate use - 500?*
RS: *Highest dose and dose responsive though still effects with 30mg/kg. fetal endpoints are more sensitive to post endpoints*

TK: *What is the length of time for studying the rats in WP6?*
RS: *21 days, may look later as leydig cell not functionally normally. There is 20-25% infertility at 100mg/kg*
LG: *Are their reproductive lifetimes shorter? Would be interested to know*
RS: *We have moved so it would be expensive to continue*
NS: *What is the LOEC?*
RS: *LOEC is 20mg/kg*
AK: *It is alarming that with only 6 animals, disruption is observed at 20mg/kg*
RS: *May be other effect where you see LOEC. Anogenital distance and nipple endpoints are insensitive – is that correct?*

CS: *Location of leydig cells not much different to Sertoli cells, could this be Sertoli cells that have changed?*
RS: *Not sure we can definitively exclude that as there is no precedent. We used a battery of markers which tick them as being leydig cells and not Sertoli cells*

14:00 – Theme 3 (NJ): Interlaboratory variation assessment with 5 samples per month to assess the sperm concentration. Around July/August 2003, a change in the stability leading to an increased was observed in Leipzig. However, based on the preliminary data the sperm concentration is similar after adjustment. The semen quality over time for the same male does not vary. The data was adjusted to the period of abstinence and inter-technician variation.

- Regarding D12.2, the data is being collected and analysis is be undertaken in August 2005
- For discerning an effect of *in utero* smoking exposure form the mother, needed >1500 samples in order too see an effect

- BMI and semen quality seems related with the slimmer the individual, the more reduced the semen quality

TK: *Is the database in a format available for everyone?*
NJ: *Unsure as to how to use it formally*
NS: *Important to discuss the continuity of the studies and the funding issues. Need 5-10 years to make sense of this data and extra time to further evaluate and understand*
CS: *Is the period in which there is no funding, for example 1 year, will this hurt the registry?*
NS: *Needed support of the Danish industry*
TK: *FP7 added long-term follow up of cohorts but the first call will be the end of next year*
CS: *Could the EU do bridging funding?*
TK: *Yes*

14:00 – Theme 3 (A-MA): Have to account for storage issue and effect on the results. Inter99 different to MONICA because this was an intervention study (e.g. try to stop smoking). This may therefore introduce some bias into the choice of men in the study. Denmark have a central person registry so could study breast cancer. If there is a cohort effect then suggesting that breast cancer stems back to *in utero*.

CS: *Perhaps window of susceptibility to each type of yumour*
LG: *Have you carried out regression to look at individual males?*
A-MA: *Programme can't cope and crashes. Ned a statistician and different programme*
LG: *How did you do the inter- and intra-validation?*
A-MA: *Waited until all samples (Denmark and Finland) and ran mixed so any variation is spread and also ran internal controls which demonstrated 8% assay variation*

15:45 – Theme 4 (AK): Low dose is linking the dose with effect because dealing with agents which vary a lot with effect. Have to ensure that toxicity doesn't mean the endocrine effect is missed as is capable of masking it. Low dose for E2 can be 1 molecule in 40 cells but seems to be only the case with steroidal estrogens.

CS: *In bacteria have co-metabolism*
LG: *My own studies have observed sex reversal at* 10^{-16}

Also power considerations, number of animals. Comparing the number of animals used of 10 versus 20 fish, this shifts the NOEC and LOEC down one tested concentration. Crump benchmark approach is an alternative to the NOEC, LOEC and its use lowers the confidence limit:

- Uses all dose-effect information therefore increasing statistical power
- Not limited to tested doses because dealing with regression models
- Benchmark problem is the difficulties defining a critical effect level (arbitrarily set at 50%)

Favouring the approach of using both concepts side by side, however is thresholds cannot be assumed then just use the benchmark.

Mixture approach is by fixed ratio design so does not let one chemical 'override' the others in terms of effect. Have observed deviation with the E Screen, which may be due

to p450 induction which removes the steroidal estrogens form the effect equation. In zebrafish will be mixing 6 compounds with results expected end of 2005.

TK: *Make's the life of the regulator very difficult!*
CS: *Fortunate to consider AK being involved and to the depth*
LG: *Can semen quality be looked at in fish, especially as human serum levels have been looked at*
AK: *Have to look at the prediction tools and endpoint*
LG: *Have concern that some of the endpoints are relatively insensitive. What is the number of VTG in blood considered to give an effect? ...difficult*
AK: *Can debate the relevance (though not in the rat) but we are at stage 1*
CS: *Caution of the E Screen - the process of proliferation not necessarily linked to nuclear ER and when mixtures contain certain candidates, some may work in a totally different way*
AK: *have chosen the blackbox approach – mechanism free approach. Don't need to make underlying mechanistic theories. This doesn't mean we're not interested in mechanisms. The deviation raises possibility p450 which is mechanistic*
LG: *"Supermix" Theme information use human as E Screen*
AK: *Fish would be easier. There are two experimental approaches – mix all in proportion of low effect concentration e.g. EC1 or mix prevalence to human tissue*
LG: *Take the medium of all analytical human results*
AK: *Distinction between similar and dissimilar - would be ideal to fuse WP14 and WP15 because the label is becoming meaningless. The deviation may be similar because induce cell proliferation but dissimilar because mechanistically different*
CS: *Tamifox is a weak agonist of estrogen and not an anti estrogen but people think it is - comes back to mechanism of action.*
NS: *Mixtures in breast milk would be very interesting as exposed from 0 age*
LG: *10-15 years ago, we were told that mixtures can't be done. Several people have now made a lot of progress*
TK: *If had to predict 10 years from now when REACH is functioning?*
AK: *Still not mixtures! Very resistant response and linking to regulation is difficult*
CS: *Not ruling out dialogue with regulators*
AK*: No*
CS: *Perhaps be more proactive and foster relations*
TK: R*egulators are very afraid*
AK: *WP16 which started this year will look at options for regulation and present a working document to these people for comment*

CS: *I would like to encourage the members of EDEN in what way that running of the programme would be improved?*
- no comments raised by those present
CS: *Like to propose to TK that may of benefit to the programme and add something. Have part time historian, sociologist or philosopher of science to look at historical perspective to see how issues like this should be treated by people in the EU*
TK: *Looking for company to do this for us and look at the impact of the project*
LG: *Been fairly significant findings and am very impressed by the reality of the shared data and coordination. Seldom are there mechanisms to really work together, US, Japan, EU. Good to hear that EU will take leadership role. I have no reservations in hearing some of the delays, having been a field biologist*
TK: *Thanks to CS and LG and reports to be sent in within one month*

5. Minutes: EDEN 4th Forum Meeting, Masarykova kolej, Prague, Czech Republic. 10th and 13th May 2005

Present: Martin Alberti (MA), Anna-Maria Andersson (A-MA), Sofie Christiansen (SC), Majken Dalgaard (MD), Rik Eggen (RE), Anton Gerritsen (AG), Rachel Gomes (RG), Stefan Hartung (SH), Ulla Hass (UH), Yann Jacques Le Page (YJLP), Bernard Jegou (BJ), Niels Jorgensen (NJ), Ioanna Katsidaki (IK), Ulf Kausch (UK), Hannu Kiviranta (HK), Andreas Kortenkamp (AK), Henril Leffers (HL), Kim Mahood (KM), Piedad Martin-Olmedo (PM-O), Ian Mayer (IM), Stine Metzdorff (StineM), Steve Morris (SM), Gerda Krog Mortensen (GKM), Lief Norrgren (LN), Farzad Pakdel (FP), Jorma Paranko (JP), Ragnor Pedersen (RP), Jorgen Petersen (JorgenP), Tom Pottinger (TP), Stefan Örn (SO), Jelle Reinen (JR), Ana Rivas (AR), Edwin Routledge (ER), Tamsin Runnalls (TR), Matthew Sanders (MatS), Martin Scholze (MS), Helmut Segner (HS), Richard Sharpe (RS), Elisabete Silva (ES), Niels Skakkebaek (NS), John Sumpter (JS), Marc Suter (MarcS), Manuel Tena Sempere (MTS), Jorma Toppari (JT), Frank van den Ende (FVDE), Terttu Vartiainen (TV), Nico Vermeulen (NV), Anne Marie Vinggaard (AMV), Christiane Vöegeli (CV),

Tuesday 10th May
Due to the positive Mid Term Review and the need to discuss further funding and the Prague Declaration, the agenda was modified to look at these issues.

Mid Term Review

- Disseminated yesterdays activities
- Concluded that do not expect many alterations due to the positive feedback from the examiners

Follow-on Funding
There is an opening at the end of this year, last round of calls for FP6. AK is willing continue coordinating if no others wish to do so. However, the call may only be suitable for a fraction of the EDEN partners. Need to find out more and AK will be meeting with Tuomo in the next few days.

A-MA raised possibility of joint application between call for human and environment and wildlife. Make 2 applications but linked, however if one is not successful then the other will automatically fail regardless.

Prague Declaration

- At present there are 70 signatures
- AK has received feedback from several members of FIRE who have issues with signing this so want to encourage friendly discourse. One of the issues referred to precautionary action, this is not the same as the precautionary principle so doesn't have to be straight out banning but can start with labelling etc. RS suggests placing examples in the text to clarify the reasons/approach
- UH asked to explain the declaration and AK replied that the reasons for doing this are because
  1. The Cluster is halfway through, this position paper will forma lasting piece of text from the Cluster

2. It explains to the European taxpayer who pays for this research what is happening
3. Political motivation with respect to FP7
4. Gives suggestions for the future

- Where to be distributed and who is this targeting?
  1. General public
  2. Decision makers
  3. Press conference to be held in Brussels with subcontractors, Analytica arranging this who specialise in strategic communication
  4. Publish as part of the conference proceedings, NS proposes Nature as a possibility and important that signatories are limited to scientists. AK replies that signatures will be scientists in the field but not limited to CREDO. Jan Ake Gustafsson has circulated this throughout CASCADE and other FP6 funded projects. NS suggests keeping to Europe for now and in the future can be EU and US
- In answer to A-MA enquiry as to how to handle this at the workshop, specific points will be highlighted that require discussion. Alex Scott wanted to say that endocrine effects in sea water fish which are remote from emission sources have been identified illustrating signs of endocrine disruption. IK thinks this should be included but could exclude people from signing it. Suggestions about placing references at the end of text though not citing or including a glossary to explain certain things. NS comments that it is risky to put forward the Prague Declaration. AK suggests having Ragnor Pedersen and Louise Gale (from Analytica) as a contact point to create small group discussions for people to contribute, breakout meetings. They can then summarise the issues and then raise them in the discussion.
- AK doesn't want to engage in exercise of small changes so during the break, RS, A-MA, NS, NJ and IK construct to points regarding reproductive disorders in humans. Then go through the declaration , point by point with those present

Friday 13th May
Initial group meeting to disseminate following:

- Mid Term Review meeting
- Follow-on funding which may not be available for all such as fish people. Will obtain a text asap so can easier to plan. RS commented that unlikely get something like EDEN of that breadth
- 20/5/05 Brussels research priorities in FP7, AK to present as CREDO coordinator. What issues would group like to be raised?
  1. JS: what does endocrine disruption mean to wildlife and almost rank it on a list of other threats to wildlife
  2. AK: there needs to be a home for endocrine disruption as block rather than spread other themes
  3. UH: chemicals affecting thyroid haven't been looked at (not in EDN)
  4. AK: propose to roll breast and testis cancer into one and look at the relationship between?
  5. LN: impact of population levels in wildlife
  6. HL: behavioural, autistic, attention deficit may be linked to endocrine disruption
  7. PM-O: human health linking especially to diseases
  8. NV: metabolism, bioactivation and formation of metabolites. Model estrogenic compounds and secondly ub relation with the mixture issue. Concept of mixtures and metabolism
  9. RE: Basis of physiology understanding – gaps

- Prague Declaration – entered 3rd round of consultation and is gaining focus and not being watered down which was a fear. Both Tuomo and Kirsi have a very positive reaction to the Prague Declaration
- Weybridge Statement 1996: David Gee proposed the idea during the CREDO Workshop of following up with a statement for 2006

Information pertaining to each Theme is given below:

Theme 1
Revised selection scheme for fish tissue samples to be used in WP3 and WP4.

- A new report provides extensive information on the range of contaminants present in fish from the EDEN study sites
- This, it was agreed, renders any unfocused analytical effort by the EDEN partners an unnecessary duplication of effort
- Following discussion by the participating partners, the previous strategy for selection of samples for analysis, as outlined in the EDEN Technical Annex, was abandoned
- Instead, an analytical strategy that targets endpoints was adopted

Two issues will be addressed:

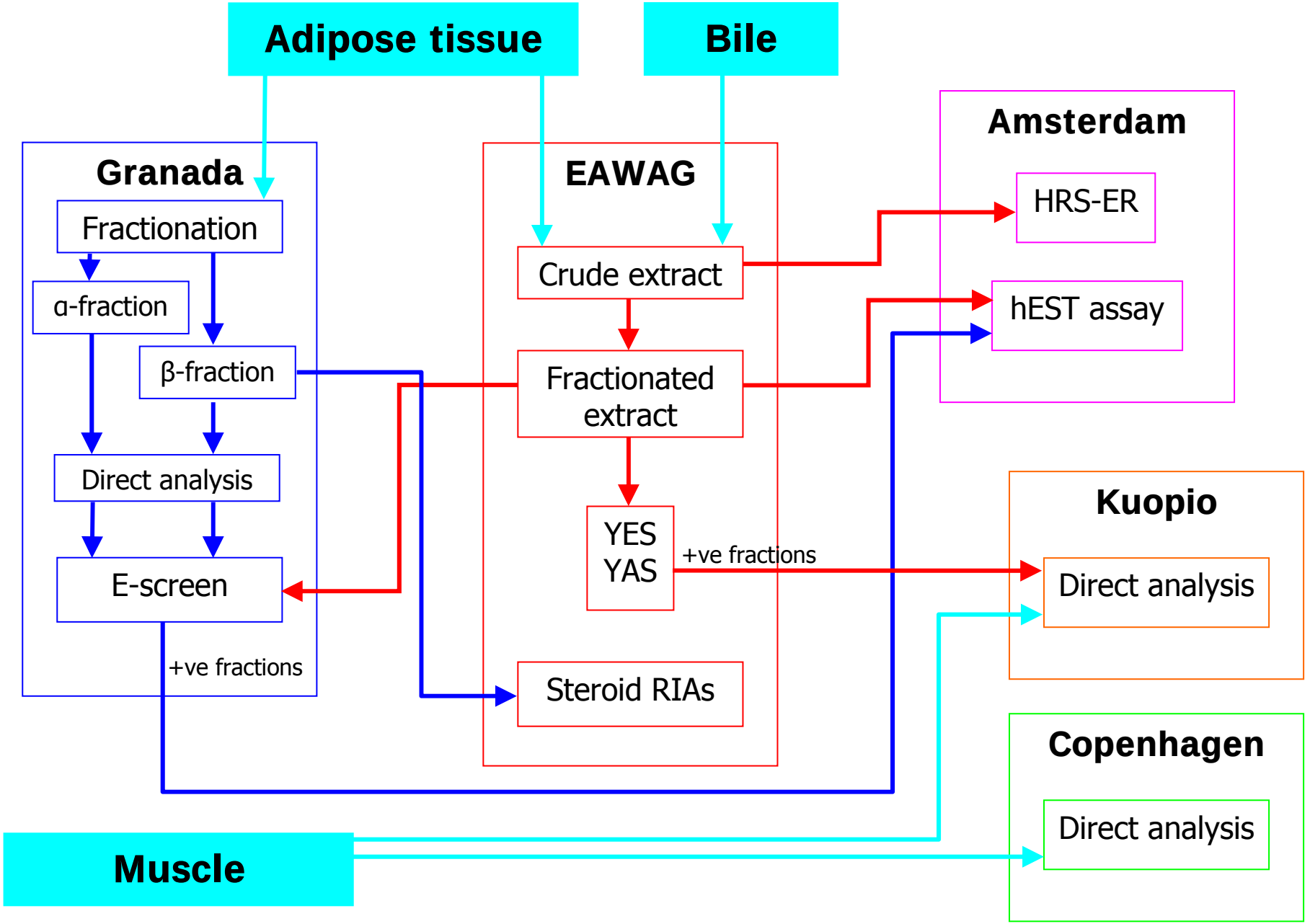
1. Does the range of chemical contaminants associated with the presence of ovotestis (intersex) differ from that in unaffected fish?

The factors responsible for the occurrence of ovotestis in wild fish remain uncertain but are likely to be chemical. The fish tissue samples collected in WP2 provide a unique opportunity to seek evidence to support this possibility. The chemical profile of tissues from fish whose gonadal structure was characterised as intersex (n = 13) will be contrasted with the chemical profile present in fish not presenting with ovotestis but matched for site, age, and plasma VTG level (n = 26; ratio of case:control - 1:2).

All ovotestis positive fish were caught at three sites: Dommel, Aa and Biesbosch..

2. Do tissue chemical contamination profiles explain divergent plasma VTG levels in bream from a site not known to be impacted by STW effluent?

Fish from the Biesbosch study site display a wide range of plasma VTG levels but no clearly identifiable STW inputs exist. This element of WP4 will seek to determine whether the range of VTG levels evident within this population can be linked to estrogenic activity isolated from bile. Two pools of bile will be derived by combining samples from fish with high (n = 11; VTG = 382,683 – 9,147,062 ng/ml; B11, B12, B18, B24, B27, B3, B30, B33, B37, B59, B95) and low (n = 15; VTG < 800 ng/ml; B14, B38, B41, B43, B53, B60, B61, B67, B70, B73, B74, B82, B87, B90, B102) plasma VTG levels. In the first instance these pools will be subject to fractionation and screening for estrogenic activity. Depending on the outcome of this initial screening, further direct analysis of additional tissues may be undertaken.


Adipose tissue
Bile
Granada
Fractionation
α-fraction
β-fraction
Direct analysis
E-screen
+ve fractions
EAWAG
Crude extract
Fractionated extract
YES
YAS
+ve fractions
Steroid RIAs
Amsterdam
HRS-ER
hEST assay
Kuopio
Direct analysis
Copenhagen
Direct analysis
Muscle

Theme 2

Research presentations were given with summaries below and the ppts can be obtained from the member's site.

P14b: objectives to identify and characterise estrogen-sensitive genes

- Cloned 3 ERs (α, β1, β2) give 3 distinct genes. All 3 are functional, bind to E2 with high affinity, activate on ERE-reporter gene exhibit distinct expression in adult tissue and during development of the embryo
- Now looking at aromatase B (Aro-B) - biomarker of xenoestrogens effect on the CNS. Aro-B is only expressed in radial glial cells and strongly stimulated by E2 (E2 treatment over 8 hours). Developed Q-PCR for aromatase B in zebrafish. Three day exposure followed by RNA extraction form whole body, then Q-PCR. EE2 at 1nM - highest effect
- Cloned the reporter of Aro-B linked to LCF gene. Tested tissue specificity of Aro-B reporter. Used Hela for uterin and CHO for ovary (also other tissues)
- Compared to ERE reporter gene, Aro-B is 3x more efficient than ERE-tk-Luc (E2 at 10nm)
- ERE is necessary but not enough for the full E2-stimulating of Aro-B gene in glial context
- 3 constructs made: control: full response; minus ERE: no response, minus CoE: this caused 80% decrease in Aro-B stimulation.
- Cooperation between ER and glial specific factors is necessary for the full E2 stimulation of Aro-B in the glial context
- Which ER recepters regulate Aro-B in zebrafish? All can regulate, α more potent, then β2, then β1
- Evaluated receptor subtypes using Luciferase (glial cell based assay) following 48 hour exposure to different concentrations of estrogens. Observe a close effect on all receptors of E2 and EE2. β1 and β2 more sensitive to EER
- Working on bioassays with xenoestrogens: bioassays provide a fast, reliable, sensitive and efficient test for evaluating estrogenic potency of ED's on ER subtypes in glial context

Q&A: Aromatase is there but have no direct evidence of a substrate in the embryo. Problem with seeing what has embryo origin as mother provides androgens - could be source?

P11 (RE): aromatases have role in sex differentiation, are also regulated. Looking at promoter genes in zebrafish.

- Reporter gene studies: *in vivo*: EGFP as a reporter gene, transient approach – microinjection in zebrafish; *in vitro*: luciferase as reporter gene and transfections in cell culture; RT-PCR studies on zebrafish
- EGFP as an *in vivo* reporter gene: have added in new element to plasmid construct to enhance expression levels
- 3x aromatase constructs + negative control used in injection

1. ProAro-B-GVP-UG: EGFP expression was mainly observed in brain (40%), muscle cells, ectodermal cells and in the yolk (30% no expression). All but brain expression thought to be non-specific as other studies point to glial-cell expression. E2 exposure: it is regulated in brain or other sites? Fading of brain expression over time, when exposed to E2 get no fading of expression in brain. IHC could not correspond expression to the glial cells using confocal – will do IHC at different stages of development

2. ProAroA-GVP-UG: got much higher expression rate using longer construct. Localised expression to neuromasts. Expression in ectodermal cells not co-localised with neuromasts
3. GVP-UG: no expression

- Luciferase assay: test responsiveness of *cyp19a* promoter regions to different lengths in different cell contexts. Get dose response of both reporters, more defined in the long-reporter compared to short reporter
- SF1 and ERE sites can possibly interact with liganded ERs site-directed mitogenesis will be employed to investigate if this is true
- Responsiveness to mediators of cAMP pathway: *cyp19a* is decreased while *cyp19b* is increased
- Promoter responsiveness to AhR ligands – investigation is under way. There are putative binding sites for this receptor
- *In vivo* reporter gene studies: possibility of using transgenic line *cyp19b* but not *cyp19a*
- *In vitro*: luciferase used with *cyp19a* and *cyp19b*. Transfection experiments in cell culture work.
- RT-PCR studies: exposure studies in zebra fish to be done

P11 (HS): aim is to search for potential new EDC marker genes in zebrafish gonad aromatase *CYP19a* and brain aromatase *CYP19b* (first year).

- Ongoing: Detailed analysis of expression of aromatase in zebrafish and EDC induced alterations. Low dose and mixture studies using aromatase response
- *CYP19a* – ERE; *CYP19b* no ERE (but may be controlled in some way)
- Exposure to EE2 (<10ng/L), embryo/larval stage: no alteration of sex ratio/reproductive function at adult stage when exposed at larval stage but no effect in adulthood
- When exposed for whole life span, get complete sex reversal. No lasting effect on aromatase expression in the adult
- In embryo, aromatase has role in neural development looked at neuromasts. Decreased *CYP19a* expression in neuromasts with knockdown
- Juvenile stage 30-60 days: develops morphologically distinct gonads, think aromatase involved. Exposure to inhibitors – complete masculisation
- Exposure to E2 – arrestment of male differentiation (stops feminization), stops at immature ovary. Remove EDC and continues development as male
- What happens to brain aromatase expression during this time? – PCR of brain aromatase compared to histological stage of gonad (ongoing work). Seems to be related to developmental stage of gonad
- Adult stage: exposure to EE2 results in alteration of reproductive function (do not get sex reversal as fish fully developed), get decreased feminization
- Think there are high and low expressors of both brain and gonad aromatase in male zebrafish (has been reported before) – needs to be checked thoroughly
- Conclusions – stage dependent aromatase expression, must be careful about what stage you are studying and not talk about aromatase expression per se

P13: WP5 exposure of zebra fish to EDCs - E2, NP, BPA, EE2 and E1 tested

- E2 – dose response in vitellogenic expression from 100-500 ng/L (Q PCR and RT PCR)

- NP – response at 1, 5, 10, not 50, 100, 200, 500 ng/L. Huge standard deviation observed but backed up with ELISA for VTG. Appears to be individual fish expression of VTG
- BPA – VTG expression at 2 mg/L
- Microarray experiments – 14k zebrafish arrays: E2 at 500ng/L and BPA undecided concentrations; self spotted arrays: ranges of E2 and BPA. 121 genes up-regulated and 89 genes down-regulated. A lot of developmental genes are up-regulated and a lot of embryonic development genes also down-regulated. Need 8-10 microarray experiments to get specificity – high costs!
- BPA seems to act differently than E2 according to array result using 2mg/L BPA
- Further experiment: E2 and BPA on self-spotted arrays; VTG induction QPCR, whole genome arrays (BPA)

Q&A: HL suggests checking all array (throw genes might never have thought of) results with QPCR (need to get mRNA expression)

P3 (RS): WP6 aim to compare phthalate action of rat/human foetal testis explants and assess the value of new biomarkers comparing pathways activated and sensitivity to phthalates

- Rat/human fetal cultures not giving any results – stopped
- MBP studies on male marmosets 500mg.kg.day for 14 days to neonates. Chose neonates as there is a testosterone (T) surge at 2 weeks. Co-twin study (control and treated) with 5 of each – no effect on testosterone
- Fetal T in rat is LH-independent therefore when DBP suppresses T there is no LH to compensate
- Neonate marmosets T production is LH-driven therefore if MBP treatment decreases T then LH should compensate to bring it up to normal levels
- No LH assay for marmoset!
- Might get increase in LH number/size due to LH surge. Looks like there may be an effect on the LC – stereology (cell counting) in process. Looking at LH-regulated genes e.g. $P450_{scc}$

P5 (JP): WP6 *in utero* exposure to flutamide-interferences with fetal LC function

- Fetal LC: autoregulation by T. LH independent but what other factors are involved? Looking at this and not endpoint effects in target tissues
- May be regulated by AR – used flutamide to study this
- Block AR then should block autoregulation of T
- T production led by flutamide. No effect on StAR, AR may be increased – not significant (more animals needed)
- With AR IHC it looks like protein is decreased in flutamide animals
- *In utero* exposure to flutamide gives significant decrease in $P450_{scc}$, 3βHSD type 1, Insl3 mRNA level. No effect on StAR, AR may be increased
- Conclusions: Steriodogenic autoregulation may have some role in fetal LC

Q&A: RS doesn't think fetal LC has AR. AR expressed in interstitial cells, need to colocalise with 3βHSD to see if is actually is LC expressing AR

- Future work: resolve mechanisms of anti-androgenic mechanisms and look at effect of DDE in model
- Also looked at the adreno-testicular axis and chemical stress. Looking at corticosteroids. Very preliminary but may have role in endocrine disrupter effects

P14 (BJ): WP6 Effect of phthalates on fetal endpoints

- Development and validation of fetal gonad assay (FEGA)
- Use proteomics to assess phthalate assay
- E2/DES exposure of FEGA
- Gonocytes: decrease number, decrease mitotic index, increase apoptosis
- SC: decrease number, decrease mitotic index, no change in apoptosis and cAMP/FSH production
- LC: decrease number, decrease basal and LH-induced T production
- Gonocyte effect earlier, then SC, then LC
- 5 experiments with FEGA performed - DEHP 10^{-5} M decrease in gonocyte number 48 and 72 hrs: Not due to increased apoptosis, likely proliferation effect. NO effect on basal of LH induced T production
- 4 experiments with MEHP 10^{-8} + 10^{-6}M – no significant effect of MEHP, even on gonocyte number, seems to agree with literature. May be increase in apoptosis at highest MEHP concentration

P14 (BJ): WP7 Insl3

- No ERβ expression in efferent ducts, no aromatase expression, AR and ERα are expressed. Same 5α-reductase II expression therefore efferent ducts do not convert T to E2 but do convert T to 5α androgen
- Have done a lot of proteomics on the efferent ducts 43% of proteins expressed at highest levels were primary metabolism proteins, 15% protein synthesis and processing

P16 (SH): WP7 Immunoassay for Insl3 human and rat

- Human assay accomplished. No difference in Insl3 levels between control and infertile men
- Insl3 production appears to be dependent on LH (long term rather than acute)
- All systemic Insl3 produced by testes
- Boys cryptorchid at 3 months significantly decreased Insl3 compared to control
- Normal boys - Insl3 levels correlated with T and LH – this correlation did not hold for cryptorchid boys
- Ray assay – done by genetic immunization – successful
- Also performed classical boost step with transfected cells
- Decreased Insl3 expression in embryonic day (e)17.5 DBP treated testes
- Insl3 gubernaculum expression at e20.5 – similar pattern
- Protein not synthesized there but arrives in the blood stream

P17 (MTS): WP8 Testing endocrine disrupters on hypothalamic-pituitary; identification of mechanisms and biomarkers and look at mixtures and low dose effects

- All seems to be going on schedule but wants to extend one deadline in order to match collection of samples by UH in Copenhagen this Summer and next Summer
- 2 strategies: literature based and high throughput technologies
- PR expression upregulated in male brain following estrogen exposure
- KISS-1 system important area of research and seems hormonally and developmentally regulated

Theme 3

None available - for further information contact NJ

Theme 4

Fish testing action plan: Zebrafish

In order to receive first mixture results and to maintain comparability with stickleback VTG work (see changes for stickleback groups later), Leifs' group will proceed asap with the first mixture study of 4 chemicals (E2, EE2, E1 and NP). This includes a confirmatory testing of two well-defined effect concentrations for each compound (totally 8 concentrations). For the mixture at least 5 concentrations should be tested, leading together with the 3 control tanks to an overall set of at least 16 tanks.

Afterwards, as soon as the UV filters are received by Steve Morris, the search for an additional 2 estrogens is continued by conducting range finding tests for all 3. If positive, select one and add to the list as the 5th oestrogen for mixture testing. Additionally, again the testing of cyproconazole (CZ) is aimed at higher concentrations (100, 500 and 1000 µg/L, confirmation of negative results from first run). If positive on VTG induction, then CZ can be included as the 6th estrogen to be tested in mixtures. If negative, then we have two options: to proceed with 4,tetra-pentaphenol (PP) at 100, 500 and 1000 µg/L in a range finding study or to test a couple of parabens (Elisabete to suggest the exact chemicals and potential suppliers). Advantages of PP are (1) it is an OECD suggested model compound (weak estrogen), (2) it is easier to quantify chemically (no further delays or money burnt for analytical method development). However, parabens might be considered as more interesting novel estrogens.

Fish testing action plan: Stickleback

The stickleback groups have agreed to change the original mixture design by testing single substances as long as 6 estrogens have been selected for the final 6-compound mixture studies (hint: in London was already decided to test only a mixture of four anti-androgens). Instead, due to the clear outcomes from the ACE project (mixture studies on VTG induction for two fish species) and in order to reallocate remaining resources more optimal, it was decided to stop testing of more estrogens. This means that information of 4 estrogenic compounds for the final mixture study is available and can be used for a mixture study asap. The free resources are used to conduct 4 more tests on the concentration response of these 4 estrogens (E1, E2, EE2, NP) on spiggin inhibition in female fish that are simultaneously treaded with 5µg/L DHT (in exactly the same way as in the anti-androgens test).

These experiments will allow the design of a mixture's test with dissimilarly acting (i.e. dissimilar mechanism of action) components where the 4 estrogens will be mixed with the 4 anti-androgens and 5µg/L DHT to access the similarities between predicted and observed response. This approach appears to be far more interesting than testing only similarly acting compounds and maximises the potential of the stickleback as a model organism for EDCs.

In any case it was accepted by all that the number of anti-androgens to be tested should be 4 (FL, LN, VZ, FN). As these compounds have been tested now, the stickleback group has reached the end of the first testing phase and both labs can proceed with the first mixture on similarly acting anti-androgens. It was suggested that all 4 compounds should be re-tested in at least two concentrations (EC10-15 and EC60-70) at the same time as the mixture's concentrations (if tank numbers allow, Martin to give details on levels of treatment required for the mixture), at least those compounds which definite study was not performed in the same lab. At the same time, Ian and Tom can start conditioning

female fish to a low photoperiod and temperature to participate in the evaluation of anti-androgenic effect of estrogens (i.e. variability of endogenous estrogens should be of minimum to allow a dose response curve).

Once the first responses have been evaluated and the hypothesis can be confirmed that a concentration response pattern of estrogens on the inhibition of the DHT induced spiggin exists, then the second mixture study can be conducted with all four estrogens (including re-testing of all components at EC10-15 and EC60-70).

Upon completion of the second mixture of similarly acting compounds, the only test remaining to be completed (and perhaps the most interesting one) is mixture of all eight dissimilarly acting EDCs.

If possible, the last step for mixture testing will be to use the 6 estrogens previously tested on zebrafish to be tested in stickleback. However, to analyse the potential of inter-species extrapolations on the predictive power of concentration addition, expected VTG response for the 4th mixture are predicted partly on effect information obtained only in zebrafish (i.e. for the two remaining estrogens).

Expected number of studies:

- 4 range finding and 4 definitive for antiandrogenic activity of E1, E2, EE2, NP (2 and 2 in each laboratory). Ioanna suggest starting by using one level of magnitude higher concentrations to the RF used for the evaluation of estrogenic activity for each chemical
- 4 mixture studies for each lab, including re-testing of 2 concentrations for each chemical. This means that each mixture is tested twice, but in different labs (confirmation step). The comparative mixture assessment should be based on a minimum of 5 mixture concentrations plus one negative and one positive control, which equals 7 tanks for the mixture, plus for each compound 2 concentrations, which equals 8 tanks. Thus totally at least 15 tanks should be available for testing simultaneously all compounds as well as the mixtures
- If all goes well, then this means that each lab needs to run 8 more studies

<u>Analytical Chemistry:</u>
IMPORTANT: The partners were informed of the revised standard operating procedures for water extraction. They should contact Steve Morris for the updated SOPs as a percentage of methanol is now added during the extraction procedure.

Steve Morris highlighted some problems with the samples being sent from the different labs:

- Some cartridges, when they reach Steve, are broken (split). This is because they still contain some residual water when frozen and transported. Steve advised the different groups to push out all the residual water from the cartridges before freezing and shipping
- Some partners have failed to inform Steve on the water volumes extracted. It was requested that all partners send a datasheet to Steve with the exact volume of water extracted, when they send their samples to be analysed
- Steve has requested that all partners keep him informed of their next moves. It is important that he knows in advance which chemicals are being tested and when to

expect samples from each partner. This will allow Steve to prepare for the arrival of the samples and will avoid delays

The costs of the chemical analysis were discussed. Due to the need to analyse new compounds, new methods have to be developed. This will increase the costs. Each partner will have to negotiate the costs with Steve, in view of the allocated budget.

Finally, an alternative method for water sampling was discussed. It was proposed that, instead of spot testing, an integrated sampling procedure should be used for the mixture experiments. However, it was pointed out that the sampling method for mixtures and single agents should remain the same. This will assure that the differences between nominal and measured concentrations are the same for single agents and mixtures. If the partners wish to use an integrated sampling procedure for the mixtures, they still need to do spot testing.

6. **Minutes: EDEN Steering Board Meeting,** Brussels, Belgium. 15th November 2005

Present: Anna-Maria Andersson, Andreas Kortenkamp, Ragnor Pedersen, Richard Sharpe,

Theme 1
WP 1 and 2
Sample collection is currently ongoing. Leydig cell function - all samples from cryptorchid boys collected (Jorma Toppari). Andreas Kortenkamp showed flow diagram for samples of adipose tissue, muscle and bile and Present results of assay for inhibition of sulfation.

WP 3
D 3.3 probably delayed from month 36 to month 39 – and related M 3.1 correspondingly delayed. All other deliverables are on course.

WP 4
High resolution screening of ER affinity detection and results – query over detection limit by Nico Vermeulen.

Theme 2
WP 5
All on track.

WP 6 to 8
Action to be taken: Richard Sharpe to contact partners and ask when deliverable reports will be finished? To enquire where they are, whether they are going to be delayed on delivering, staffing situation and the form of final delivery. It needs to be decided when completion of work packages 6 to 8 will be.

D 6.4 Where is "Report on new biomarkers of foetal exposure" (month 36) ?

WP 9
Finished.

WP 10
Delay in glowing fish.

Theme 3
WP 11
D 11.1
MS 11.1 – 11.4 finished
MS 11.4 Birth cohort effects are seen on Danish data. Finish data collected but needs analysing.

WP 12
Data collected but there is a need to analyse data for scientific publications.

Theme 4
WP 13
Data all there
D 13.1 to 13.3 Final delivery report on low dose testing will be written by Martin Scholze and Andreas Kortenkamp.

Format for Annual Report
Format for Annual Report changed to be as projects under EESD and as agreed by Tuomo Karjalainen. Sections for each work package are to be written by work package coordinators and gathered in by theme coordinators. Andreas Kortenkamp will write detailed instructions. Last date to deliver next report to the European Commission is end of January 2006.

EDEN Extension
Any extension would probably need to be applied for in spring 2006. This could be discussed at the EDEN forum.

Date and venue for EDEN forum meeting
Suggested venues: Malaga or Granada, Majorca, or Canary Islands.