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**UK-JAPAN COOPERATION ON RESEARCH ON
ENDOCRINE DISRUPTERS IN THE AQUATIC
ENVIRONMENT**

**ANNUAL SCIENTIFIC WORKSHOP 2005, at GLASGOW
CALEDONIAN UNIVERSITY**

SUNDAY, 16TH JANUARY

18.30 Workshop Reception at Langs Hotel

MONDAY 17th JANUARY

Deeprise Lecture Theatre (A005), Govan Mbeki Building.

09.00 – 10.30

SESSION 1: WELCOME AND INTRODUCTION

Chairs: Dr Fumi Irie, Ministry of the Environment and Dr Mike Roberts, Defra.

09.00 **Welcome Address**
Dr Ian Johnston, Vice-Chancellor, Glasgow Caledonian University

09.10 **Welcome Address**
Dr Kazuko Kamiya, Ministry of Environment, Environmental Health and Safety Division

09.15 **Housekeeping and Safety Arrangements**
Mrs Janet Pierotti

09.20 **UK Perspective on Endocrine Disruption (S1.1)**
Dr Jane Stratford, Defra CGMP Division

09.35 **Japanese Perspective on Endocrine Disruption (S1.2)**
Dr Fumi Irie, Ministry of Environment, Environmental
Health and Safety Division

10.00 **Scottish Perspective on Endocrine Disruption (S1.3)**
Dr John Redshaw, SEPA

10.20 **Discussion**

10.30 – 11.00 Tea/Coffee A303, Govan Mbeki Building.

11.00 – 12.30

SESSION 2: PROCESSES AND PATHWAYS

**Chairs: Prof Hiroaki Tanaka, Kyoto University and Dr Liz
McDonnell, Defra Water Quality Division.**

11.00 **Endocrine Disruption from Sewage Treatment Works
(S2.1)**
Dr Melanie Gross-Sorokin, Environment Agency

11.25 **Potential Steroid Oestrogen Contribution of Farm
Animals to UK Freshwaters (S2.2)**
Dr Andrew Johnson, Centre for Ecology and Hydrology

11.50 **Effective Reduction of Oestrogenic Activity in
Wastewater and Evaluation of Biological Significance
(S2.3)**
Prof Hiroaki Tanaka, Kyoto University, Paper 1
and
Dr Yutaka Suzuki, Public Works Research Institute, Paper 2

12.20 **Discussion**

15.00 Molecular Approach to Evaluate Androgenic and Anti-androgenic Effects of Chemicals in Stickleback (S4.4)

Dr Masaki Nagae, Nagasaki University

15.20 Molecular Cloning of the Sex-Differentiation Related Genes in Roach, *Rutilus rutilus* (S4.5)

Dr Yoshinao Katsu, National Institute for Basic Biology

15.40 **Discussion**

16.00– 16.30 Break

17.30 SIGNING CEREMONY FOR EXTENSION OF UK-JAPAN AGREEMENT AND RECEPTION

Hosted by Glasgow City Council at the Council Chambers

18.30 Depart for Dinner

TUESDAY 18th JANUARY

09.00 – 09.20

SESSION 5: A EUROPEAN PERSPECTIVE

Chair: Dr Jane Stratford, Defra CGMP

09.00 The EU Strategy for Endocrine Disrupters (S5.1)

Renate Paumann, European Commission

09.15 **Discussion**

09.20 – 10.30

SESSION 6: AMPHIBIANS

Chairs: Dr Minoru Takase, Hiroshima University and Prof Alex Scot, CEFAS Weymouth Laboratory.

**12.00 Morphological Basis on the Male Type of Genital Tracts
in Imposex-exhibiting Female Rock Shells, *Thais
clavigera* (S7.4)**

Prof Yasuhiko Ohta, Tottori University

12.20 Discussion

12.30 – 1300

SESSION 8: SUMMING UP THE WORKSHOP

**Chairs: Dr Fumi Irie, Ministry of the Environment and Dr Mike
Roberts, Defra**

12.30 Open Discussion

12.45 Summing Up and Future Steps
Prof Charles Tyler and Prof Taisen Iguchi (UK-Japan
National Supervisors)

**13.00 – 15.00 Lunch and Poster session with open discussion in each
group**

15.00 Tea and Depart

Talk Abstracts

(S1.1) UK Perspective on Endocrine Disruption

Dr Jane Stratford, Chemicals & GM Policy Division, Department for Environment, Food and Rural Affairs (Defra), UK

Endocrine disruption has been recognised as a potential environment problem since the 1950's. Since then, a great deal of research has been undertaken to try and address reported changes in the reproductive health of humans and the endocrinology of wildlife and their possible causal agent(s). This presentation will provide a review the scientific input to the development of policy on endocrine disruption from a UK perspective. It will also outline some of the priorities that have been identified for future research

(S1.2) Japanese Perspective on Endocrine Disruption

Fumi Irie

Ministry of the Environment, Japan

In May 1998, the Ministry of the Environment, Japan (MOE) announced its "Strategic Programs on Environmental Endocrine Disruptors '98" (SPEED '98). The Programs set forth the Ministry's basic perspectives on the problem, along with specific plans for action.

"SPEED '98" identified the need for progress on the following four items.

- (1) Promotion of a fact-finding study on detection in the environment and impact on wildlife
- (2) Promotion of testing and research, plus technology development
- (3) Assessment and management of environmental risk and provision of related information; and
- (4) Efforts for reinforcement of international networking on endocrine disrupter issues.

An environmental monitoring survey has been conducted since 1998 for 65 chemical substances, listed on the "SPEED '98", that were suspected of having endocrine disrupting effects then. Hazard assessment i.e the evaluation of effects that endocrine disrupters may have on human health and the ecosystem, has begun accordingly. The results of the study using rats for assessment of health impacts was that no clear endocrine disruption was observed for any of the 22 chemical substances tested at low dosage. Among the 26 chemical substances selected for tests using fish for assessment of ecological impacts, it was inferred that three of these substances, nonylphenol, 4-octylphenol and bisphenol A, have

endocrine disrupting effects on fish. In addition to conducting tests for hazard assessment, development of new testing methods was carried out along with basic research programs. International cooperation was reinforced through works with OECD, joint researches and annual international symposia.

With scientific knowledge accumulated and international assessment of the state-of-the-science developed, MOE commenced a two-year project to revise "SPEED'98" in 2003. A Working group which includes experts, representatives of consumers and of Industry was formed in October 2003. After 10 working-group meetings, the draft of the new plan was finalized in November 2004 and now we call for comments on this new plan from the public.

In the new plan, there are seven main themes;

- (1) Investigation of wild life
- (2) Exposure assessment
- (3) Basic Research
- (4) Hazard assessment
- (5) Risk assessment
- (6) Risk management
- (7) Risk communication

Though there are overlapping items between "SPEED'98" and the new plan such as hazard assessment, risk assessment/management, MOE will focus much more on other items which seem not to be fully explored by "SPEED'98". These new fields are investigation of wild life, exposure assessment, basic research and risk communication.

This presentation informs about the Japanese perspectives on next 5-year UK-J joint research under the framework of the new plan.

(S1.3) Scottish (Regulatory) Perspective on Endocrine Disruption.

Dr John Redshaw, Ecotoxicology National Centre Manager, Scottish Environment Protection Agency, 5 Redwood Crescent, East Kilbride, Glasgow G74 5PP.

As the guardian of Scotland's environment, SEPA's main aim is to *“provide an efficient and integrated environmental protection system for Scotland that will both improve the environment and contribute to the Scottish Ministers' goal of sustainable development”*.

To fulfil this aim, and help monitor its performance, SEPA is working towards the delivery of a series of long-term environmental outcomes (objectives). Sustainable development is the overarching principle for all of SEPA's work and we make all our regulatory decisions within the context of sustainable development, seeking synergies between social, economic and environmental needs. To this end, SEPA works closely and directly with a range of outside organisations to ensure that environmental policy and regulation are robust, proportionate and sustainable.

SEPA engages in national and international research to promote its understanding of the environment (ecosystems and human health), and the myriad of pressures on it, and to ensure that best practice is developed in managing these pressures to deliver a sustainable Scotland.

Within this context, this paper briefly reviews SEPA's involvement in research into endocrine disruption in the aquatic environment, identifies current gaps in our knowledge and understanding, and highlights some priorities for future research and development.

Session 2

(S2.1) Endocrine Disruption from Sewage Treatment Works

Melanie Gross-Sorokin, Environment Agency

Following an assessment of the available data on endocrine disruption in fish, in particular the feminisation of fish below sewage treatment works, the Environment Agency of England and Wales has concluded that the weight of evidence is sufficient to develop a risk management strategy for oestrogenically-active effluents. The Agency has been working in collaboration with the Department of Environment, Food and Rural Affairs (Defra) and the water industry to develop an 'Endocrine Disruption Demonstration Programme'. The main objective of this programme is to determine the effectiveness of existing and enhanced sewage technologies at reducing steroid concentrations and oestrogenic (feminising) activity in effluents. The proposed effluent testing programme will be a combination of chemical determinations and biological activity (*in vitro* and *in vivo*) monitoring, which will be conducted over different timescales, both short-term and long-term, within the sewage works and in the environment. The programme will also assess the costs and benefits of installing enhanced treatment technologies and will inform any future decisions on appropriate regulatory strategy with regard to oestrogenic biological activity of domestic STW effluents in England and Wales.

(S2.2) Potential steroid oestrogen contribution of farm animals to UK freshwaters

Andrew Johnson CEH Wallingford, Wallingford, OXON, OX10 8BB

Peter Matthiessen CEH Lancaster, Lancaster, Lancashire, LA1 4AP

Dave Arnold CEA, Boxworth, Cambridge, CB3 8NN

Given the strong links between steroid oestrogen water concentrations and endocrine disruption in fish, it would seem sensible to assess the quantities, and identify the sources, of steroid oestrogens that are entering our aquatic environment. While much current work has focused on steroidal oestrogens discharged from sewage treatment works, little attention has been paid to the potential contribution, via diffuse inputs, of sizeable farm animal population. From humans to sheep, and from Zebras to Ibexes, all vertebrates respond to, and excrete steroid oestrogens. Whilst the range of steroid oestrogens and their metabolites varies between the vertebrates, 17β -oestradiol ($E2\beta$) and oestrone appear to be common throughout.

The combined farm animal population is considerably larger than the human one in the UK. However, the majority of these animals are poultry, or more specifically broiler chickens which are not big oestrogen producers. To make comparisons on the amount of oestrogen produced per head by an animal (normalised) information needs to be gathered on the structure of that farm animal population (age and whether pregnant or not) and the amount of oestrogens produced by the animal at each of its life stages. These data are difficult to come by, and frequently incomplete. Nevertheless enough is available to make a preliminary assessment.

An individual dairy cow excretes two orders of magnitude more, and an average pig excretes more than one order of magnitude more oestrogens than an average human. In terms of excretion, the combined farm animal population (including sheep and poultry) generates around four times more oestrogens than the human population. The biggest contributor on the animal side is the relatively small dairy cow population (2 million head) followed by the pig population.

Unlike the human population, it is more difficult to predict how much of these farm-animal-derived oestrogens will reach the UK water courses. If we make an analogy with pesticides, in which a reasonable worst case loss to surface waters is around 1% of the applied active ingredient, then we would predict that farm animals are responsible for 18% of all the oestrogens in UK waters. The impact of these oestrogens will depend on the local conditions. If we use our excretion model for dairy cows we would predict that 100 head grazing on 1.2 ha would generate 80 mg E2 β equiv/ha/d. In a possible runoff scenario if 4 mm of water from a 10 mm rain event transported 1% of the oestrogens into a 'standard' ditch (1.0 m wide, 0.3 m deep) adjacent to the field, this would give rise to 11 ng/L E2 β equiv. concentration episode in the ditch. Using published data, if the recommended 50 tonnes/ha of dairy cow manure were spread on a 1 ha arable field, a similar runoff/ditch scenario could yield 223 ng/L E2 β equiv. concentration (assuming a worst case scenario in which all the oestrogen remained available for run-off). It is therefore plausible that some farms, particularly dairy, could generate transient oestrogen loadings in surface waters at levels of potential environmental concern.

(S2.3) Effective Reduction of Oestrogenic Activity in Wastewater and Evaluation of Biological Significance

Hiroaki Tanaka (Kyoto University, htanaka@biwa.eqc.kyoto-u.ac.jp)

Yutaka Suzuki (Public Works Research Institute, ysuzuki@pwri.go.jp)

The natural and synthetic oestrogens associated with domestic and livestock wastewater are potent endocrine disrupters and are strongly suspected to cause endocrine disruption in aquatic wildlife. They are unintentional pollutants present in urine and excreta, therefore to control this domestic source we must examine how the performance of sewage treatment plants (STPs) could be improved.

Our previous study demonstrated that oestrogenic activity in wastewater is considerably reduced, but not eliminated, during most of municipal STPs in Japan. The steroid oestrogen removal performance can vary with the type and management of the wastewater treatment facilities. Based on a bench-scale MBR experiment using municipal sewage we recently revealed that operation in a longer SRT (up to 60 days) results in elimination of free oestrogen and that higher MLDO exceeding 3 mg/L brings about effective reduction of free oestrogens in batch tests. However, remaining large amount of conjugated oestrogens in the treated effluents suggests to us the complexity in understanding of the fate of oestrogens in wastewater treatment. Further, still limited knowledge is available on their fates in the receiving waters although slow degradation is observed in the Tama River, an urbanized river where feminization of fish is highly concerned due to discharges from many STPs. Therefore, challenges are to examine whether existing wastewater treatment practice can be manipulated to further reduce the concentration of oestrogenic substances in wastewater effluent, and to understand the fate of the

oestrogenic substances in river water after discharge from wastewater treatment facility.

The prevalence of feminization of wild male carp in the Japanese rivers is frequently observed in terms of vitellogenesis. The vitellogenesis in the wild male carp occurs more frequently than the data on our exposure experiment of the male carp fed oestrogen-free food to secondary effluent in a STP. This suggests the importance of food-web route in river waters. However, knowledge is limited on how extent the concerned oestrogenic substances accumulate in food organisms. Therefore, to clarify the bioaccumulation of oestrogenic substances in their foods, estradiol (E2), estrone (E1), nonylphenol (NP) and its related compounds and oestrogenic activity were measured in water, periphytons, two groups of caddisflies (*Hydropsyche* and *Stenopsyche*) that are predator as well as collector-filterers, a specific stonefly (*Perlinae*) that are situated at top predators in benthic ecology, the other benthic invertebrates, detritus and sediment at the middle reaches of the Tama River. Bioaccumulation factor (BAF) defined is a ratio of the concentration of a target substance in the biota to that of water. Condensation factor (CF) of the sediment and the organic matter is also similarly defined. The BAFs of NP and oestrogenic activity reached 1000 to some hundreds for the periphytons, the caddisflies and the other invertebrates, and slightly decreased for the stonefly. The BAFs of E1 and E2 were some tens to hundreds for the caddisflies and the stonefly, which were slightly lower than those of NP. However, significant increase in the BAFs was not observed towards higher trophic level. Further, oestrogenic activity accumulated more in the benthic invertebrates than oestrogens. Benthic invertebrates may contain endogenous steroid hormones, but this alone cannot explain the increase in oestrogenic activities of the benthic invertebrates in the reaches

receiving STP discharge. These results suggest the importance of exposure route via food-web besides water itself on oestrogenic substances in the river environment particular for carp because they tend to eat organic matters and lower trophic biota.

Oestrogenic substances have been suspected to cause the feminization of wild fish in some rivers in Japan. To elucidate the influence of oestrogenic substances on fish in the rivers, we have developed an *in situ* fish exposure system using medaka, *Oryzias latipes*. The flow rate, the water temperature and the light-to-dark cycle are controlled in the system and can be set at the similar conditions of laboratory exposure. Then, we placed three sets of the systems in water quality monitoring stations along the River Tama. Adult male medaka were exposed for four weeks and their hepatic vitellogenin (VTG) concentrations were measured every two weeks. A commercial diet free from phytoestrogens was fed 4 times in a day using an automatic feeder. The exposure tests were repeatedly performed at an upstream location where no STP discharge is received and two downstream locations where some STP discharges are received. At the upstream site, VTG was not detected in any medaka, while at the most downstream in this test area, all the medaka produced VTG in the test performed. E1 was considered to be a major substance to cause oestrogenic activity because the induction of VTG in the medaka coincided with the occurrence of E1 and the oestrogenic activity of the river waters. This experimental system is expected to evaluate reduction of oestrogenic substances during wastewater treatment or of receiving waters from the biological viewpoint.

(S3.1) Population Impacts of Endocrine Disrupters on UK Seals - Effects on Juvenile Survival and Interactions with Disease

Ailsa Hall¹ and Gareth Thomas²

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²Department of Environmental Sciences, IENS, Lancaster University, Lancaster LA1 4YQ, UK

As top predators, seals can be exposed to high levels of persistent organic contaminants that are putative endocrine disrupters (EDs). The two objectives of this study were:

- (a) To determine the impact of polybrominated diphenyl ethers (PBDEs) and other organohalogenated EDs, such as polychlorinated biphenyls (PCBs) on the first year survival of grey seal pups. This has been carried out using a mark-recapture study in which the effect of blubber PBDEs and other EDs as individual covariates of survival are investigated.
- (b) To investigate the level of various EDs in harbour seals which were victims and survivors of the 2002 PDV epidemic. Five populations of harbour seals around the UK with differing mortality rates were studied.

Grey seal pups from the Isle of May (n=56) were fitted with instruments based on mobile phone technology. These 'phone tags' were programmed to regularly send text messages to us, which were received

whenever the animals were in coverage of a mobile phone mast (usually when they were hauled out ashore at coastal haulout sites). The text messages are equivalent to a recapture occasion in a conventional mark-recapture model. Blubber biopsy samples were also collected from the pups at the time the tags were deployed and these have been analysed for 21 PBDE congeners and 42 PCB congeners as well as other chlorinated pesticides that have been shown to be potential endocrine disrupters in vertebrates. These concentrations have been embedded in the mark-recapture model in order to determine their potential effect on the first year survival probability of grey seals from the Isle of May. In a preliminary analysis we found only weak evidence that blubber contaminant levels might be important in determining first year survival, after accounting for the effect of size, which we have found in previous studies is an additional important determinant of early survivorship in seals.

Harbour seals in the UK were affected by a second outbreak of phocine distemper virus in 2002 and blubber samples were collected from 18 freshly dead adults that died of PDV in the southeast of England. Blubber biopsies and blood samples were obtained from 20 surviving adult harbour seals in the Wash, southeast England. Samples were also collected from four populations in Scotland (Islay, Orkney, Moray Firth and St Andrews Bay, n=40). Preliminary results show that the levels of various EDs (PDBEs, PCBs and other pesticides) were higher in the blubber of the dead seals than the survivors. However, the condition (blubber thickness) of the animals at the time of sampling must be accounted for. Higher levels of PBDEs were found in surviving animals in the Wash population than at the four other locations and the highest levels of PCBs were found in animals from Islay. In a generalized linear model there was a significant relationship between circulating thyroid

hormones and blubber EDs when location and sex effects had been accounted for. This suggests that these contaminants could also be thyroid hormone EDs in harbour seals.

(S4.1) The three-spined stickleback (*Gasterosteus aculeatus*) as a sentinel organism for evaluating the effects of endocrine disruptors: a progress report

I. Katsiadaki and A.P Scott, CEFAS laboratory, UK

M. Nagae, Nagasaki University, Japan

We have previously shown that exposure to exogenous androgens causes female sticklebacks to produce the glue protein, spiggin, in their kidneys. This protein can be quantified by an enzyme-linked immunosorbent assay (ELISA), which was developed and validated by CEFAS.

In addition to the androgen test, we have developed a procedure for quantifying the vitellogenin in plasma, whole body, heart or liver extracts of the stickleback. The results of eight experiments in which sticklebacks were exposed to oestrogens indicated that the assay is sensitive for evaluating the effects of oestrogenic EDCs in the aqueous environment.

We have also developed a test for anti-androgens, a group of xenobiotics that is receiving increasing attention. The assay detected the anti-androgenic activity of flutamide, vinclozolin, linuron and fenitrothion. These results provided the first evidence of *in vivo* anti-androgenic activity of both linuron and fenitrothion in teleosts. We are currently designing an optimum system to evaluate the effects of anti-androgens on male stickleback reproductive behaviour and output.

CEFAS has recently organised a pilot ring test exercise following a draft OECD fish screening protocol for endocrine disruptors, which

resulted in a very good reproducibility so the stickleback was invited to participate in the next phase of the screening test validation programme.

Recent work on the development of quantitative PCR assays for both spiggin and VTG have produced positive results. The presence of a DNA-linked sex marker in this species together with the fact that the full genome will be sequenced by the end of 2005 are giving the species further advantages as a test and sentinel organism. Furthermore, the species is endemic and ubiquitous in Europe and possesses many ecological traits that make it more suitable for studying endocrine disruption in the field.

(S4.2) A search for evidence of endocrine disruption in a top predator fish.

Emma Vine (1), Jon Shears (2), Ronny Van Aerle (2), Charles Tyler (2) and John Sumpter (1)

1. Institute for the Environment, Brunel University
2. School of Biological and Chemical Sciences, University of Exeter

The high incidence of intersex roach (*Rutilus rutilus*) in some U.K. rivers that has been associated with exposure to sewage treatment works (STWs) effluent led us to hypothesize that top predator fish may also be affected by estrogenic chemicals, since they are likely to bioaccumulate lipophilic compounds through a predator-prey relationship. To investigate this possibility, pike (*Esox lucius*) were sampled upstream and downstream of STWs and examined for total estrogenic activity of their bile, measured using a yeast-based estrogen assay to determine the degree of recent exposure of the pike to estrogens, and vitellogenin induction and histological analysis of the gonads to assess possible disruption of sexual development. No evidence of severe disruption was found in the fish sampled (which came from 16 sampling sites along 'typical' English rivers). However, 14 % of pike were found to be intersex, of which fifteen out of sixteen showed patches of male germ cells amongst predominantly female gonadal tissue. The incidence of 'masculinisation' was found to be independent of whether the pike had been sampled upstream or downstream of STWs. Although pike are gonochoristic, it is not known if this 'masculinisation' of presumptive female pike is normal, or instead indicative of endocrine disruption. Vitellogenin concentrations were not elevated in male pike at sites either up or

downstream of STWs. The results suggest that sexual disruption is not common in pike, a fish at the top of the food chain in freshwaters in England.

(S4.3) Evidence derived from field surveys that indicates cod in the open sea are being exposed to oestrogenic endocrine disrupters.

Alexander P. Scott, CEFAS, Barrack Road, Weymouth, UK, DT4 8UB

The CEFAS laboratory in Lowestoft carried out the pioneering field surveys of rainbow trout in the late 1980s that provided the first proof that the freshwater environment was contaminated with estrogenic compounds. This proof came in the demonstration of elevated vitellogenin (VTG) levels in the blood plasma of caged trout that were placed in certain UK rivers (Purdom et al., 1994) – especially in the vicinity of sewage treatment works. In 1996, CEFAS, funded by Defra, turned their attention to estuaries – carrying out extensive collection and assay of VTG in blood plasma of a migratory flounder (*Platichthys flesus*). It was immediately discovered that several estuaries in the UK were heavily contaminated with estrogens. In 2002, CEFAS started to look for evidence of estrogenic endocrine disruption in the Atlantic cod (*Gadus morhua*), a species that lives its entire life cycle in the open sea. Collections of cod have now been made from several areas on the continental shelf. In at least three of these areas (off Iceland, the Shetland Box and the southern North Sea) male cod have been found with elevated levels of VTG. Furthermore, elevated VTG levels in males show a strong positive correlation with the size of the fish. These findings raise several questions, the main ones being: are elevated VTG levels a sign of endocrine disruption or of a natural aging process in males? Is endocrine disruption a problem for the cod (perhaps being linked to the sharp decline of this species in the North Sea)? Is it a problem for the

consumer? The synthesis of VTG by the liver requires oestrogen stimulation. Thus, if VTG in males were a natural process, we would expect to find 17β -oestradiol in the plasma of those males that also have elevated VTG. This we have not found (and nor have we found any of these males to be intersex). It thus seems probable that the causative agent is of external origin. We hypothesise that large cod pick up oestrogenic compounds via the food chain. This hypothesis is based upon the fact that the size of fish at which VTG levels rise sharply (ca. 5 kg) is also the size at which cod change their diet from mainly nektonic (free-swimming) to benthic (bottom-living) organisms. As also shown by Kevin Thomas at CEFAS, benthic organisms live in sediments that can contain very large amounts of oestrogenic compounds (at concentrations many times greater than those in the surrounding sea water). Further research is needed to prove the proposed link between elevated VTG levels and change in diet. This will involve: testing for the presence of oestrogenic compounds in the bile of large cod; direct cross-checking of VTG levels with gut contents; determining whether the dietary organisms are indeed also contaminated with oestrogens; identifying the oestrogens (in bile, benthic organisms and sediments) to determine whether they are natural or man-made.

I acknowledge the direct involvement of John Thain, Kevin Thomas and Ioanna Katsiadaki, and support from Defra and the CEFAS seedcorn fund.

(S4.4) Molecular Approach to Evaluate Androgenic and Anti-androgenic Effects of Chemicals in Stickleback

* Masaki Nagae, Fumie Kawasaki, Kiyoshi Soyano (Nagasaki University, Japan)

Akihiko Hara (Hokkaido University, Japan)

Ioanna Katsiadaki, Alexander P. Scott (Weymouth Laboratory, CEFAS, UK)

Spiggin is the glue protein produced by the kidney of breeding male sticklebacks and used for nest building. Its synthesis is strongly up-regulated by androgens. Currently this protein is used as a biomarker for androgenic chemical substances. However, details of spiggin protein molecule have not been well described. In UK-J joint research until now, we have isolated and characterized two different types of cDNAs encoding spiggin (SPG-I and SPG-II) in three-spined stickleback, *Gasterosteus aculeatus*, and also have analyzed their mRNA expression in kidney.

The deduced open reading frame of SPG-I and SPG-II cDNAs encode 616 and 639 amino acid residues, respectively. Although there was a marked structural difference (continuous 63 nucleic acids insertion (or deletion) in N-terminal side) between the two cDNAs, the deduced amino acid sequence of SPG-I showed high homology (80%) with that of SPG-II. In addition to the structural similarity, Northern blot hybridization using specific probes for each showed that both types of spiggin mRNA were clearly detected in breeding male kidney. Real-time PCR analysis also revealed that the level of both spiggin mRNA in kidney was almost same. Northern blot analysis also revealed that the expression of their mRNA was also induced only by the treatment of

androgens (methyltestosterone and 5α -dihydrotestosterone) for female stickleback, and that this induction of mRNA expression was completely inhibited by the co-treatment of flutamide, antagonist of androgen. The present results suggest that the stickleback glue protein for nest building is composed of two spiggin molecules and that both molecules are good biomarker for monitoring androgenic and anti-androgenic effect of chemicals. In this presentation, I will also talk about next research plan using stickleback in UK-J cooperation, in addition to above research data.

(S4.5) Molecular Cloning of the Sex-Differentiation Related Genes in Roach, *Rutilus rutilus*

Y. Katsu^{1,2}, A. Lange³, C.R. Tyler³ and T. Iguchi^{1,2}

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²Division of Molecular Endocrinology, National Institute for Basic Biology, Japan

³Environmental and Molecular Fish Biology, School of Biological and Chemical Sciences, University of Exeter, UK

A variety of natural and man-made chemicals can mimic hormonal activities by binding to hormone receptors and possibly modulate endocrine systems in wild animals, especially aquatic species. Hormonally active chemicals may cause disruption of sexual differentiation and development, which have impact on reproduction in fish species. In UK rivers, exposure of roach (*Rutilus rutilus* – a common cyprinid fish) to effluents from sewage treatment works containing complex mixtures of endocrine disrupting chemicals (EDCs) has been shown to alter sexual development and impact negatively on their reproductive capabilities. However, the molecular mechanisms underlying EDC-induced changes in roach have not yet been elucidated. The goal of this study is to identify genes undergoing altered expression in response to EDCs exposure in order to establish gene pathways which disrupt gonad sex differentiation. Using PCR-based cDNA cloning, we obtained the cDNA of steroid-hormone receptors (oestrogen receptor α and β , ER α and ER β ; androgen receptor; AR; mineralcorticoid receptor; MR and glucocorticoid receptor; GR), steroidogenic enzymes (aromatase, StAR, 3 β -HSD, 17 β -HSD, C17, 20-lyase, P450SCC) and sex

differentiation related genes (*vasa*, *DMRT-1*, *SOX-9*, *DAZ*, *Pumilio*, *SF-1*, *WT-1*, *Wnt4*). Analysis of amino acid sequences show that roach genes are classified into goldfish and zebrafish group, indicating that roach belongs to Cyprinidae. Now we have obtained some cDNA clones related to sex-differentiation and steroidogenesis, and started to make cDNA macro-array. We are going to analyze the expression pattern of these genes by EDCs exposure. The present results are helpful to understand and establish gene pathways which lead to disruption of gonad sex differentiation in roach. Furthermore, we have established the roach *ER α* and *ER β* reporter gene assays using mammalian expression system. We are now investigating the chemical specificity and species specificity and/or difference of ER functions.

(Supported by Research Grants from the Ministry of Environment, Japan and DEFRA, UK.)

Session 5

(S5.1) The EU Strategy for Endocrine Disrupters

Renate PAUMANN, European Commission

In order to address the potential environmental and health impacts of endocrine disruption, the European Commission in December 1999 adopted a Communication to the Council and to the European Parliament on a Community Strategy for Endocrine Disrupters (COM(1999)706). The Strategy sets out a number of short-, medium- and long-term actions relating to, inter alia, identification and prioritisation of substances, monitoring, research, international co-ordination, legislative action and communication to the public (see <http://europa.eu.int/comm/environment/endocrine>). A first progress report of the Strategy was published by the Commission in June 2001 and a second one was published in October 2004. The presentation will discuss some of the progress made to date.

(S6.1) Assessing the Impact of Endocrine Disrupters in the Aquatic Environment on British Frogs and Toads

Dr Dan Pickford, Brunel University

Recent concern over global declines in amphibian populations was predated by significant declines in UK amphibians from the middle of the last century. After some stabilisation in the 1970's there is renewed concern that populations of the Common toad, *Bufo bufo* are once again on the decline. While there are recent reports of endocrine disrupting effects of some industrial and crop protection chemicals, there is to date little or no robust evidence that environmental contaminants, and endocrine disrupters in particular, are to blame for amphibian extinctions globally or population declines in the UK. Patterns of extinctions and population declines globally suggest that these phenomena may have limited relevance to the situation in the UK. Nevertheless, both the UK and Japan may both present useful areas in which to examine the potential for endocrine disrupters to adversely affect amphibians at individual and population level. We are collating historical data on UK amphibian populations from various sources along with pesticide and fertilizer-use data in order to identify at-risk and reference breeding sites with differing potential exposure to endocrine disrupters. Extracts of water samples will then be tested in a suite of *in vitro* and *in vivo* tests for endocrine activity currently undergoing optimisation and validation. Preliminary screening of a water sample taken from a site of intensive agricultural activity was undertaken in collaboration with Japanese collaborators at University of Hiroshima and Towa Kagaku. Estrogenic

activity of the C18 and OASIS extracts was detected, as determined by significant induction of VTG secretion in primary monolayer cultures of *Xenopus* hepatocytes. There was no evidence of significant thyroid activity of the extracts in a short-term in vivo larval development assay using transgenic *Xenopus* carrying a thyroid response element-GFP reporter gene construct. Further sites will be selected for screening during spring 2005. It is expected that cooperation with Japanese collaborators on both bioassay development and GIS approaches to analysis of population/land use/pesticide data will generate parallel and complementary programs in UK and Japan to improve ecological impact assessment for native amphibians.

Land use changes have obviously been a major factor in historical declines in British amphibians, as the landscape of intensive agriculture is inimical to most UK amphibians. However, British amphibians are pond-breeding species and population data is available for several species which display different habitat requirements and different trends in population status over a lengthy period of potential exposure to environmental contaminants through agricultural activity.

(S6.2) Molecular Cloning and Expression Analysis of Genes Encoding Oestrogen Receptor, Thyroid Hormone Receptor, and Aromatase of the Frog *Silurana tropicalis*

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Some steroidal hormones are critical for physiological phenomena of the amphibian, such as gonadal differentiation with sex steroidal hormones, metamorphosis with thyroid hormone, and oocyte maturation with progesterone. The amphibian will be influenced by oestrogenic chemicals contained in river water and clay of the field, and organisms in a food chain, because endocrine disrupting chemicals affected gonadal differentiation of the amphibian. Thus, the amphibian will be a good environmental indicator in the field. End-point of our research in the UK-Japan cooperative work is to develop the method for ecological assessment using the amphibian. Genetic markers are necessary for development of the amphibian ecological assessment method. The present study is aimed to isolate genes encoding oestrogen receptor (ER), thyroid hormone receptor (TR), and aromatase of the frog *Silurana tropicalis* as candidates for genetic markers, and analyze their expressions.

First of all, we isolated full length of cDNAs encoding ER α and ER β of the frog using RT-PCR, RACE and oligo-capping methods. To know which tissue expresses *ER* mRNAs is very important to explore isolation of genetic markers. We analyzed the *ER* mRNA expressions in various

tissues of the frog using RT-PCR method. The RT-PCR analysis showed that both *ER* mRNAs were expressed abundantly in the brain, liver, and gonads with kidney of the frog, suggesting that those tissues express some genetic markers for ecological assessment. In addition, we isolated *TR α* , *TR β* and *aromatase* cDNAs of the frog. Both *TR* mRNAs were expressed in the brain, liver, and gonads with kidney, while *aromatase* mRNA was abundantly expressed in the brain and ovary with kidney. We analyzed expressions of the three genes, as well as the *ER* genes, in the brain, liver, and gonads with kidney during the development of the frog to know which stage is available for ecological assessment. Because gonadal sex differentiation occurred in the frog by stage 60, which is referred to the table of *Xenopus laevis* by P. D. Nieuwkoop and J. Faber (1956), we analyzed the gene expressions in the *ER* gene-expressing tissues at stage 51 to 2 months after completion of metamorphosis. The RT-PCR analysis showed that both *ER* mRNAs were expressed abundantly in the gonads with kidney during all examined stages. But the liver and brain expressed abundantly both *ER* mRNAs at stage 60 to 2-month of postmetamorphosis. Both *TR* mRNAs were expressed in the *ER* gene-expressing tissues at stage 60 to 2-month of postmetamorphosis, while *aromatase* mRNA was expressed abundantly in the developing brain and ovary with kidney. Thus, our results suggest that genetic markers expressed in the brain, liver, or gonad with kidney after gonadal sex differentiation will be available for amphibian ecological assessment.

This work was supported by a Grant-in-Aid from Ministry of the Environment.

(S6.3) Research on Amphibian Ecological Impact Assessment Method

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Amphibians represent the position from aquatic to land on the evolution. Concerning about the monitoring animals for environmental pollution, amphibians have been considered to be a sensitive and useful indicator, because they live both in water and on land, are vegetarians as the stage of larvae and carnivores as adults, and have permeable unprotected skin. In this research, we develop evaluating and assessing methods for amphibians affected by environmental chemicals at genetic, protein, tissue and population levels. For the development of the methods for evaluating and assessing amphibians, it is important to prepare a GIS database for ecological data, ecological assessment methods, biological testing methods, and model experiment systems.

The first year, development of database for field studies as well as development of biomarkers for genetic (hormone receptor genes) and protein (vitellogenin) levels will be started, centring on information collection and discussions for the overall plan.

By combining these studies, it is expected that the effective assessment methods for evaluating the ecological impact will be completed on amphibians.

Session 7

(S7.1) Gender-specific gene expression in common mussel (*Mytilus edulis*) and the effects of ethynyl oestradiol and copper.

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Mussel is a gonochoristic species with distinct male and female animals that spawn seasonally following development of sperm and ova. Gonad development in Scotland starts in late autumn (October-December) and culminates in spawning through spring and early summer (April-June). Gender identification has historically been dependent on histology but we have developed a PCR method based on expression of a male- and female-specific gene product. We have used this method to investigate gender distribution of mussel in the environment. Subsequently we have identified a wider range of gender-specific gene products assumed to be associated with gonadal development and studied the effects of laboratory exposure to ethynyl oestradiol (EE2) and copper on their expression.

Gender identification is based on expression of vitelline coat lysin (VCL) in male or vitelline coat receptor for lysin (VERL) in female. The

specificity of the method has been confirmed by standard cytology and histology. Surprisingly a proportion of the animals (up to 40 %) from environmental sites show production of both gene transcripts. We hypothesised that this was the result of intersex but can find no evidence for this by standard histological examination.

We have used Suppression Subtractive Hybridisation to prepare cDNA libraries enriched for gender-specific gene transcripts. The libraries were generated from separate, male and female, mantle cDNAs that were subtracted male from female and vice versa. The extent of gender specificity was determined by differential screening of clones with the starting cDNAs (male or female) and additionally with a second set of cDNAs from male or female mussel. In this way we have identified 23 male-specific, 110 female-specific, 45 male-selective and 127 female-selective clones. A selection of the gender-specific clones (33 in total) along with controls have been used on a macroarray to assess temporal changes (December-April) in gene expression in untreated animals and in animals treated in flow-through systems to EE2 (nominal 20 ng l⁻¹) or copper (nominal 5 µg l⁻¹). Characterization of the clones by sequence analysis and the changes in expression pattern will be described.

Supported by Defra and SEPA.

(S7.2) Endocrine Disruption in Aquatic and Terrestrial Invertebrates

P Kille (Cardiff University), I Johnson (WRc-NSF) and J Weeks (WRc-NSF)

In recent years major programmes of environmental research investigating the effects of endocrine disrupters on vertebrate species in aquatic environments have been conducted. However, less attention has been focussed on endocrine disruption effects on invertebrates and top predators. This is clearly an issue, particularly for the invertebrates, since these taxonomic groups account for approximately 95% of the known animal species, represent more than 30 different phyla and are key groups which need to be considered in hazard/ risk assessment strategies.

To address this data gap endocrine disruption in two key invertebrate taxonomic groups has been addressed. This involved

- Identification of sex-specific and/or sex determining genes in test species *Gammarus pulex* and *Eisenia* sp by homology with existing genome data. Genes associated with the process of moulting in *Gammarus* were also considered;
- Development of optimised procedures for measurements of biomarker gene expression in the test species;
- Generation of information on ‘normal’ background levels of biomarker gene expression during the life cycle of the test species;
- Evaluation of the biomarker responses (up/down regulation) following exposure of test species to a range of known or suspected endocrine disrupters in screening studies;

- Evaluation of the extent to which the biomarker responses are evidently linked to physiological effects in the test species.

The studies have expanded the current knowledge of the endocrinology of the sentinel species through the use of novel genomic techniques. The short-term screening and longer-term reproduction studies have also provided data on the effects that a range of known or suspected endocrine disrupters (oestradiol, ethinyloestradiol, testosterone, bisphenol A, nonylphenol, fenoxycarb, propoxur) have on the development and reproduction of the species tested. Hypotheses have also been developed regarding how endocrine disrupters may cause up or down regulation of gene expression of particular markers and subsequent physiological effects.

Key words

Gene expression, physiological effects, endocrine disrupters, invertebrates

(S7.3) Endocrinology and endocrine disruption of freshwater gastropods

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Within the field of endocrine disruption there is an increasing need for invertebrate tests to replace the widely used vertebrate tests. This need is driven both by an increasing pressure to reduce the volume of work being conducted on vertebrate species, and by a need for greater knowledge of the effects of endocrine disrupting chemicals (EDCs) on different environments. The gastropod molluscs present a promising group of candidate species for use in EDC research in the freshwater environment. Certain members of this group have already been shown to be affected by exposure to various EDCs. For example, some reproductive parameters of the neo-tropical snail, *Marisa cornuarietis*, such as egg production, are affected similarly to those of vertebrates in response to certain EDCs. Thus, *Marisa* holds promise as an alternative “surrogate” test species that could replace some existing vertebrate species. There is, however, a current fundamental lack of knowledge of gastropod endocrinology in general, and an increased understanding is required to elucidate the mechanisms by which EDCs may exert the observed responses. Work in our laboratory is investigating molecular mechanisms within the *Marisa* endocrine system, particularly those that may be involved in steroid and steroid-like pathways. The aim of this work is to increase our knowledge of the poorly-understood field of molluscan endocrinology, and perhaps provide insights into explaining observed responses to EDCs. This work will go some way to elucidating whether the observed gastropod responses to EDCs truly mirror those of

vertebrates and thus, whether the gastropods may provide good “surrogate” test species for vertebrates. Our lab is also conducting a considerable amount of research into the reproductive and developmental biology of European gastropod species and their responses to EDCs and treated sewage effluents. This work will enhance our basic understanding of molluscan biology, and may also highlight potential native test species that exhibit similar marked responses to EDCs to those observed in *Marisa*.

(S7.4) Morphological Basis on the Male Type of Genital Tracts in Imposex-Exhibiting Female Rock Shells, *Thais clavigera*

Prof. Yasuhiko Ohta, Tottori University and Prof. Taisen Iguchi, National Institute for Basic Biology

Gastropod imposex is well-known to be caused by organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), released from antifouling paints on ships and fishing nets, even at very low concentrations (Bryan et al., 1988; Horiguchi et al., 1997). During a few past decades, however, little evidence has been accumulated about imposex induction mechanisms, although two hypotheses are evaluated, the androgen theory (Spooner et al., 1991; Bettin et al., 1996; Ronis & Mason, 1996) and the neuropeptide theory (Féral & Le Gall, 1983; Oberdörster, & McClellan-Green, 2001, 2002). Recently, Nishikawa et al. (2004) elucidated in rock shells (*Thais clavigera*) that retinoid X receptor (RXR) bound to both 9-*cis* retinoic acid (9-*cis* RA) and to organotins (TBT and TPT), and that normal female rock shells receiving a single injection of 9-*cis* RA or TPT significantly developed imposex, suggesting that RXR is a key target of organotins and plays an important role in the induction and development of imposex (the differentiation and growth of male genital tracts) in females.

Histological examination revealed morphological similarity in penis and vas deferens between adult male and imposex-exhibiting female rock shells, based on light and electron microscopical observations. RXR gene expression in tissues of the rock shell demonstrated the highest expression in penis and ganglia in both male and imposex-exhibiting

females (Horiguchi et al., unpublished data). Immunohistochemical studies using a commercial antibody for RXR α supported the results from the gene expression, showing positive nuclear staining for certain cells in penis, vas deferens and ganglia of males and imposex-exhibiting females.

These results are in line with the hypothesis put forward by Nishikawa et al. (2004) that RXR is involved in differentiation and growth of penis and vas deferens in the rock shell.

Poster Abstracts

P1 Assessment and development of biomarkers of endocrine disruption in Crustacea. Dr Teresa F Fernandes, Dr Alex Ford and Professor Paul Read

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P6 Endocrine Disruption In The Shore Crab *Carcinus Maenas* - A Biomarker For Benthic Marine Invertebrates? C.M. Lye, M.G. Bentley, A.S. Clare, E.M. Sefton

P7 Effect of Ovarian Steroids on the Expression of Progesterone and Estrogen Receptors in the Uterus of Persistent Estrous Rats Given Androgen Neonatally. Ohta, Y. and Iguchi, T.

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P12 Food Standards Agency Funded Research And Surveys Of Known Or Potential Endocrine Disruptors. Barnes K.A., Benford D., Dowding A., Gem M., Rajapakse N., and Tahourdin C.S.M.

P13 Vitellogenin as an indicator of endocrine disruption in avian top predators. A Arch, I. Katsiadaki, Helen Thompson and A. Scott

P14 Sublethal effects of IGRs on honeybee colonies. Helen Thompson, Selwyn Wilkins, David Wilkinson

P15 Effects of an Androgenic Growth Promoter 17 β -Trenbolone on Masculinization of Mosquitofish (*Gambusia affinis affinis*). Sone, K., Hinago, M., Itamoto, M., Katsu, Y., Watanabe, H., Guillette, J. L. and Iguchi, T.

P16 Molecular cloning of sex hormone-binding globulin cDNA in carp, *cyprinus carpio*. M. Nagae, M. Okunaga, R. Hidaka, N. Ohkubo and T. Matsubara.

P17 Approach to analysis of the toxic action of estrogenic chemicals using two amphibian species, *Silurana tropicalis* and *Rana rugosa*. Minoru Takase and Taisen Iguchi

P18 Ontogenic gene expression of oestrogen receptors and aromatases in roach (*Rutilus rutilus*). A. Lange, Y. Katsu, R. Ichikawa, L. L. Chidgey, T. Iguchi, C. R. Tyler.

P1 Assessment and development of biomarkers of endocrine disruption in Crustacea.

Dr Teresa F Fernandes, Dr Alex Ford and Professor Paul Read,
School of Life Sciences, Napier University, Edinburgh
In collaboration with FRS, Marine Laboratory Aberdeen
(Dr Ian Davies and Craig Robinson)

Over the past decade there have been increasing concerns regarding the potential impact of a variety of anthropogenic chemicals on the endocrine systems of wildlife and humans. Whilst most effort has concentrated on vertebrates there is much interest in the overall assessment of how widespread endocrine disruption (ED) is and on the development of biomarkers of ED in invertebrates.

Since the late 1990s, work carried out at Napier University has focussed on the assessment of ED in marine Crustacea and the development of novel biomarkers for use in toxicity testing and bio-monitoring programmes. Research has centred on field based monitoring, laboratory exposures and bioassay development. Results from field based research have highlighted the following: (i) Feminisation (de-masculinisation) of the common shore crab (Brian, 2003), (ii) First time records of intersexuality in *Echinogammarus marinus*, with separate male and female intersex forms identified (Ford *et al.*, 2003a, 2004a); (iii) A higher incidence of intersex *E. marinus* at sites receiving chemical discharges (Ford *et al.*, 2004a, 2005a); (iv) Morphometric distinction of *E. marinus* intersexes, both male and female, ideally providing two separate forms of endocrine dysfunction for study (Ford *et al.*, 2004a); (v) A reduction of fecundity, fertility, pairing success, delayed maturity and possible sterility in intersex Crustacea (Ford *et al.*, 2003a, 2004a, 2005c);

(vi) A link between the degree of endocrine dysfunction and the degree of maleness in intersex females (i.e. number of genital papillae; Ford *et al.*, 2004b, 2005c); and (vii) High level of parasite incidence associated with intersex specimens as well as with specimens found in contaminated areas (Ford *et al.*, 2005a).

In our work we used intersex amphipods as models for the study of Crustacea with dysfunctional endocrine systems and assessed a variety of morphological and reproductive endpoints for their utility in ED studies. Results from morphological studies conducted on specimens collected from a variety of sites indicated that males from polluted sites show morphology similar to intersex males through reduced gnathopod size, indicating possible signs of anti-androgenic effects (Ford *et al.*, 2004a). In addition, normal females from impacted sites appear to mature at a larger size (and possibly age), and suffer reduced fecundity and fertility when compared to reference sites (Ford *et al.* 2003b; Ford, 2004). Chitobiase assays have been optimised for a range of British Crustacea (e.g. *Carcinus*, *Crangon*, *Echinogammarus*) and are currently being tested on a range of test compounds thought to promote and inhibit growth and moulting in crustaceans. This work is currently done in conjunction with Prof. Koji Arizono and fellow researchers at Kumamoto University, Japan, who are conducting similar research using Japanese models.

It is clear, therefore, that there are easily quantifiable effects, and costs, associated with intersex in a variety of Crustacea. These can be easily transferred to the study of ED both in the field and laboratory through the development of specific biomarkers. It is possible, however, that these might not prove complete evidence for ED. Nevertheless, we believe that through further research, intersex specimens may provide biochemical/receptor based biomarkers (possibly through differential

gene/protein expression) which might provide more conclusive evidence for ED. Even so, we suggest that field-collected specimens should be used with caution when assessing the effect of endocrine disrupting chemicals given that other factors can also affect intersex incidence (Ford & Fernandes, 2005b).

P2 The Environment Agency's Endocrine Disruption Programme of Research

Melanie Gross-Sorokin, Pete Simpson, Rachel Benstead and Danielle Ashton

Environment Agency Science Group

The Environment Agency of England and Wales has developed a programme of research (2004 – 2007) on endocrine disruption, which will be taken forward in collaboration with Defra and a number of academic research groups. The overall objective of the programme is to progress science in the area of endocrine disrupting chemicals (EDCs): ecological risk assessment of EDCs, ecologically-relevant measures of endocrine disruption and mitigation of adverse effects in support of the Agency's strategy on endocrine disruption, as well as the UK Endocrine Disruption Demonstration Programme. The programme consists of five separate packages of work: 1) Horizon scanning, 2) Ontogeny of sex determination and development in roach, 3) Application of the fathead minnow bioassay to the characterisation of sewage effluents, 4) Assessment of gastropod molluscs as indicator species of endocrine disruption and 5) Risk assessment of steroid oestrogens in effluents.

P5 Partitioning, bioavailability and effects of estrogens and xenoestrogens in estuarine sediments - EDAQ program (DEFRA): Project CTG0301

W. Langston, B.S.Chesman, G.R. Burt (Marine Biological Association, Plymouth) and C.H.Vane (British Geological Survey, Keyworth).

Details of the effects and scale of endocrine disruption in the aquatic environment are beginning to emerge from studies with fish (mainly freshwater), though characterization of responses in other organisms (particularly estuarine/marine benthic invertebrates) is much less advanced.

One of the earliest and most startling indications of the potential importance of endocrine disruption in the marine environment came in the 1980s with the recognition that extremely low concentrations of tributyltin (TBT) from antifouling paints was causing masculinization of female dog-whelks. Since then, the reduced viability and health of populations of a number of gastropods and bivalves (e.g. oysters, clams) has been attributed to TBT. Species such as dog-whelks appear to be highly sensitive because of the susceptibility of their steroidally-controlled reproductive systems to endocrine disrupting effects. This highlights the potential vulnerability of molluscs to the wider threats from endocrine disruption caused by elevated levels of natural and synthetic hormones and by compounds which mimic or antagonize the effects of endogenous hormones. These include xeno-oestrogenic chemicals such as alkylphenols, phthalates, polychlorinated and polybrominated compounds.

The broad threat of endocrine disruption and reproductive effects in sensitive benthic invertebrates represents a major area of uncertainty in

our assessment of risk from these chemicals. Another significant knowledge gap relates to the possibility that, particularly in estuaries, much of the oestrogenicity may reside in benthic sediments and, if bioavailable, may be transferred to benthic biota (and hence through the food chain). If this hypothesis is correct, it is important to understand the factors that control partitioning behaviour and bioavailability of oestrogens and xeno-oestrogens in estuarine sediments, in order to provide more accurate predictions of distribution, transport and impact. Other priorities include establishing the susceptibility of infaunal species, such as clams, to the effects of sediment-bound (xeno)oestrogens and the prognosis for impact at the population level.

Supported by Defra, we are embarking on a project to address these uncertainties. Emphasis is placed, initially, on interpreting the behaviour and effects of a small number of ‘model compounds’: the natural steroid 17 β -estradiol (E2) and the synthetic hormone 17 α ethinylestradiol (EE2), together with the alkylphenols octyl -and nonyl-phenol (OP, NP) as estrogen mimics. The weight of evidence suggests that these compounds could contribute to some of the most important oestrogenic impacts affecting freshwater and marine organisms, particularly if bioaccumulated.

To help fine-tune the new research component of the project we have recently completed a review¹ which provides a synthesis of current understanding and research gaps. Our goal over the next three years is to fill some of these gaps through studies on: sequestration of (xeno)oestrogens; accumulation rates and routes and bioavailability from sediments; and development of relevant assays and reproductive markers in the deposit-feeding clam *Scrobicularia plana*. In addition, we aim to establish the prognosis for impact in the field through histological and

population studies of clams from potentially contaminated and sensitive areas. Results will contribute toward better-informed risk assessments for endocrine disrupting substances in the estuarine environment and help to ensure that the most appropriate and cost-effective regulatory responses are made.

P6 ENDOCRINE DISRUPTION IN THE SHORE CRAB *CARCINUS MAENAS* - A BIOMARKER FOR BENTHIC MARINE INVERTEBRATES?

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A considerable amount of research has been conducted on the effects of certain contaminants that have the potential to impact the endocrine systems which regulates vital life processes in freshwater and marine fish. There is, however, a relative paucity, of information on aquatic and especially benthic marine invertebrate species, many of which could be seriously impacted by sewage effluent and industrial discharges. The present study used a combination of endpoints to assess possible endocrine disruption in a marine crustacean, the shore crab *Carcinus maenas*. These included pheromonally-mediated sexual behaviour, exoskeletal morphological measures, quantities of steroid moulting hormones (i.e. ecdysteroids) and the presence of egg yolk proteins, vitellin in male crabs. Crabs were collected from sites known to elicit high oestrogenic response in vertebrates and from coastal reference sites. The results suggest that shore crabs around the coast of the Great Britain are showing effects consistent with pollutant-mediated endocrine disruption. These include a reduced behavioural response to female sex pheromone, morphometric abnormalities such as retarded pleopod length ratios and enlarged abdomen width, enhanced steroid molting hormone (ecdysone equivalent) levels and the detection of vitellin-like proteins in

the hepatopancreas of male crabs. This multilevel approach may have significant potential for investigating endocrine disruption in marine crustaceans.

P7 Effect of Ovarian Steroids on the Expression of Progesterone and Estrogen Receptors in the Uterus of Persistent Estrous Rats Given Androgen Neonatally

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Neonatal treatment of female rats with androgen results in a permanent reduction of the uterine responsiveness to \square deciduogenic stimulus. Uterine response to progesterone (P) and estradiol-17 (E) injections supportive of the development of deciduomata was investigated in persistent estrous rats give 0.1 or 1.25mg testosterone propionate neonatally (PE rats) to evaluate the effect of persistent estrus on the uterus. A single injection of E in association with 3 daily injections of P lessened the P-induced reduction in P receptor (PR) immunostaining of the epithelial and stromal cells in the control rats. By contrast, in PE rats, the E treatment suppressed the appearance of PR in the epithelial cells. E receptor (ERa) expression in the endometrial cells was higher in PE rats than in the controls during the experimental period. In both PE and control rats, the expression of each receptor after a series of hormonal treatment was largely reflected in proliferation activity detected by BrdU labeling in the endometrial cells. Changes in expression of EGF and TGF-a in the endometrial cells after the steroid treatment were less evident compared with those in the receptor expression, although the expression of the GFs was lower in PE rats than in the controls.

From these results, it is concluded that uterine response to the steroids via their receptors is markedly affected in PE rats, which is responsible for the lowered uterine sensitivity for decidual response.

P8 Areas of research activity in endocrine disruption

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I am an aquatic ecotoxicologist with approximately 35 years of research experience. Since the late 1980s, I have focused much of my effort on the issue which is now known as endocrine disruption (ED), with emphasis on the effects of tributyltin (TBT) on molluscs, and oestrogens and their mimics on fish.

The work with TBT-based antifouling paints showed that they were causing masculinisation of a range of molluscs in UK estuaries and coastal waters, and that this ED activity (probably aromatase inhibition) was not only causing mollusc populations to decline or disappear, but was seriously damaging estuarine invertebrate communities. Widespread recovery of marine invertebrates has become evident since national and international bans on the use of TBT as an antifoulant (Matthiessen and Gibbs, 1998; Rees *et al.* 2001).

Subsequent research into the oestrogenic effects of sewage discharges on fish showed that many male fish in UK rivers, estuaries and coastal waters were becoming feminised to varying degrees, with effects including vitellogenin synthesis, ovotestis formation, and feminised secondary sexual characteristics (Matthiessen, 1998, 2003).

A major question raised by the work with fish concerns the possible impact of oestrogen-induced feminisation on reproductive success and population stability. We still do not know whether fish populations can withstand the degree of feminisation seen in some waters, although modelling suggests that impacts are to be expected (e.g. Hurley *et al.*, 2004). This is being investigated at CEH through a PhD project (Richard

Maunder) in which sticklebacks (*Gasterosteus aculeatus*) are being exposed to oestrogens as larvae and their reproductive capacity assessed as adults. Another issue being investigated at CEH, under the EU-funded EDEN programme, is how mixtures of different endocrine disrupting chemicals (EDCs) interact to produce effects in fish. This is an important issue for risk assessment, and DEFRA-funded contributions are also being made to the development of improved fish-based tests for EDCs via OECD's Validation Management Group for Ecotoxicity Tests (VMG-eco).

Finally, DEFRA-funded research is now in progress to investigate the severity of pollution in UK headwater streams by EDCs derived from intensive livestock farming. Theoretical calculations suggest that the oestrogen load contributed to surface waters by UK livestock is about 20% of the amount contributed by humans via sewage treatment plants, so the effects may be significant.

P9 UK-JAPAN COOPERATION ON RESEARCH ON ENDOCRINE DISRUPTERS IN THE AQUATIC ENVIRONMENT

Colin Moffat, Ian Davies, Craig Robinson and Matt Gubbins

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Fisheries Research Services is the Scottish centre for research on fisheries, aquaculture and the aquatic environment. It is an Agency of the Scottish Executive Environment and Rural Affairs Department (SEERAD) and supports policy and stewardship of living aquatic resources. To this end FRS monitors the quality of the Scottish aquatic environment and of aquaculture and fisheries products by undertaking a programme of monitoring and research in support of the provision of scientific advice and the enforcement of legislation. This includes monitoring of, and research into, endocrine disruption. FRS has a long-standing programme of monitoring the impact of TBT-specific effects in gastropods and has been instrumental in the development of assessment criteria for classification of water bodies, this includes harmonisation of the assessment criteria used by the Oslo and Paris Commission and that developed under the EU Water Framework Directive. Much of the research undertaken at FRS is funded through large-scale programmes, including EDMAR and EDIT, and has been of a collaborative nature with Prof John Craft at Glasgow Caledonian University. In the former programme, the effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success were investigated using the sand goby (*Pomatoschistus minutus*, Pallas) as a model. Effects of prolonged exposure of the sand goby to 4-

tert-octylphenol on biological indices of exposure have also been investigated as part of overall attempts to understand the ecological significance of oestrogenic biomarker responses. The research undertaken as part of EDIT has focussed on investigating the relationship between nuclear hormone receptors in the blue mussel (*Mytilus edulis*) and the effects of potential endocrine disrupting chemicals (copper and ethynyl oestradiol). The development of biomarkers of endocrine disruption in the amphipod crustacean *Echinogammarus marinus* has been undertaken in collaboration with Napier University, Edinburgh. The work has concentrated on physiological markers of endocrine disruption and consequential reproductive effects.

P10 Preliminary screening of water samples from an agricultural site in amphibian bioassays for endocrine disrupting activity

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Toxicity testing for endocrine disrupting effects of chemicals on wildlife can be costly, labour intensive, and lengthy, therefore, two short-term tests were developed to detect the estrogenic and/or thyroid disruptive properties of a water sample collected from an agricultural site. The sample was extracted using C₁₈ and Oasis cartridges, and eluted with methanol to a concentration x 10000 more concentrated than the original water sample. To assess the extract for estrogenic activity it was tested in primary monolayer cultures of *Xenopus* hepatocytes for its ability to stimulate VTG synthesis, detected by ELISA. Hepatocytes were harvested from anaesthetized *Xenopus* by liver perfusion with collagenase, cells were plated and treated with media alone (control), methanol (solvent control) or one of x 0.25, 0.5, 1, 2.5, 5, 10 concentrations of test sample. To assess the extract for thyroid agonist/antagonist activity it was tested in a short term *in vivo* test using transgenic *Xenopus laevis*, which carry a transgene containing a region of thyroid receptor gene and a green fluorescent protein gene (courtesy of University of Hiroshima/Towa-Kagaku). Stage 52 (Gosner) tadpoles were treated with aged tap water (control), methanol (solvent control), a thyroid agonist (T₄), a thyroid antagonist (PTU), or one of 0.25x, 0.5x or 1x concentrations of test extract. Parameters measured were total length, stage, hindlimb length (HLL), fluorescence, & hindlimb (HL) area taken at days 0, 5 & 10. Vitellogenin induction was significantly elevated in 2.5x, 5x, and 10x treatments in response to water samples from both types of cartridge, but not at lower concentrations. In the transgenic tadpoles, the only effect observed was in response to T₄, where stage,

HLL, and fluorescence were significantly elevated. It was expected that fluorescence would be a more sensitive measure of thyroid activity than HLL, but this was found not to be the case, perhaps due to the method employed for measuring fluorescence or perhaps because this parameter is in fact not more sensitive. Further investigation will clarify this. Finally, the lack of inhibition in response to PTU indicates that further modification of the experimental design is necessary, either through changing test concentrations or exposure duration.

P11 Histological observation of gonads in sex differentiation stages using all-male ZZ larvae of *Xenopus laevis*.

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We examined the gonadal differentiation from male to female in histological observation of gonads using all-male ZZ larvae. 17 β -Estradiol (E2) induces sex reversal from male to female in *Xenopus laevis* tadpoles. Then, cross breeding between wild males and feminized males having ZZ genotypes produces all ZZ male embryos. The all ZZ males are useful to study effects of estrogenic chemicals on sex reversal without bias of the initial embryo selection. In the morphological and histological observation of gonads, our previous studies have been shown that all ZZ male were sex reversed by exposure to as little as 1 nM E2 for approximately 4 weeks, but not by the vehicle alone without E2. In the present study, all-male ZZ larvae were exposed to 1 and 20 nM E2 from stage 49 to stage 57, including gonadal differentiation stages in *X. laevis*, then sections were prepared at each stage to observe the processes of feminization in male larvae. In addition, cDNA array was used to identify changes in gene expression in the gonad of larvae during E2-induced feminization.

P12 FOOD STANDARDS AGENCY FUNDED RESEARCH AND SURVEYS OF KNOWN OR POTENTIAL ENDOCRINE DIRUPTORS

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The poster will provide an overview of current and recently completed Food Standards Agency research and surveillance relating to known or suspected endocrine disruptors that may occur in food. It will also provide details of recent relevant reports and assessments carried out by the Committee on Toxicity of Chemicals in Food Consumer Products and the Environment and its subgroups. Listings include web addresses and details of where further information can be obtained. The Food Standards Agency is a member of the UK Interdepartmental Endocrine Disrupter Research Group and contributes to its website.

P13 Vitellogenin as an indicator of endocrine disruption in avian top predators

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The aim of this project is to provide a phased approach to the assessment of the use of direct and indirect vitellogenin assays to monitor the exposure of birds to estrogens, and evaluate the correlation of this with aromatase activity and reproductive capacity. Young male Japanese quail were injected with 17 β -oestradiol and blood samples taken for the vitellogenin (VTG) preparation. The vitellogenin was purified from plasma by FPLC and SDS-PAGE of E₂-treated plasma samples revealed the presence of at least three VTG sub-units (α , β , γ). A highly specific and sensitive ELISA was developed for the purified quail VTG with a detection limit of 0.02 μ g/ml of plasma VTG. Purification of cormorant vitellogenin was approached in the same way as that of the quail. However, the cormorant plasma did not demonstrate such dramatic induction of VTG as the quail irrespective of the length of time from treatment to sampling. An egg was obtained for development of an assay for vitellin in cormorants (a method shown to be sensitive for plasma VTG in Japanese quail), vitellin was purified, antibodies generated and an ELISA established with a detection limit of 0.01 μ g/ml.

Pilot studies were undertaken to determine appropriate dose levels for the full reproduction study. Batches of Japanese quail eggs were injected on Day 9 of incubation, one with a range of doses of estradiol (0.002 –20

µg/egg) and the other with a range of doses of, the more potent, ethynylestradiol (0.62- 620 ng/egg). The age of the eggs selected was based on previous published work which identified development days 10-12 as sensitive to the effects of estradiol when male sexual behaviour was assessed. There was no dose response in plasma VTG levels apparent after injection of estradiol or ethynylestradiol and background VTG levels were extremely variable.

Injection earlier in development (eg Day 3/4 of incubation) may result in morphological changes on sex organ development. A reproduction study was undertaken with male birds raised from the eggs injected at day 3/4 (0.2 and 20 µg estradiol/egg) and adult male birds injected with 0.0045mg estradiol/100g bodyweight and 0.000045 mg estradiol/100g bodyweight. The male birds were paired with untreated female birds, blood samples were taken weekly and reproductive parameters were measured. The fertility of eggs produced was significantly decreased in pairs in which in male birds were from eggs injected with 20 µg estradiol/egg. The hatchling weights of chicks were significantly lower from eggs produced by pairs where the male birds were injected with 0.0045 mg estradiol/100g bodyweight. There were no significant effects of treatment on VTG levels in the male birds.

P14 Sublethal effects of IGRs on honeybee colonies

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It is well established that effects on invertebrates can occur through interference with the endocrine system. This study aimed to assess the effects of exposure to a juvenile hormone analogue, fenoxycarb, a chitin synthesis inhibitor, diflubenzuron, an ecdysteroid synthesis inhibitor, azadirachtin and an ecdysteroid analogue, tebufenozide which are widely used as insecticides, on 1) the long term development of the honeybee colony; 2) viability of queens exposed immediately post-emergence; 3) sperm production in drones; and 4) integrate the data into a honeybee population model to assess the effects of the timing and level of exposure on the impact of honeybee colonies.

Groups of colonies were fed Insegar, Dimilin Flo, azadirachtin or tebufenozide in sucrose and a control group was fed untreated sucrose. There was significantly greater replacement/removal of marked eggs in the fenoxycarb and diflubenzuron treated colonies, but not in the azadirachtin or tebufenozide treated colonies, than in the control colonies in the first 2 weeks after treatment. Colonies treated with diflubenzuron resulted in a short term reduction in the numbers of adult bees and brood after treatment when compared with controls. There was no significant effect on development of brood the following spring but there did appear to be a slower increase in levels of brood when compared with controls. Colonies treated with fenoxycarb declined during the season earlier than the control or diflubenzuron treated colonies and started the season slower with one fenoxycarb treated colony failing to survive over the

winter. Although treatment with azadirachtin appeared to result in increased brood production during the season only one of the five colonies treated with azadirachtin overwintered successfully. Treatment with tebufenozide resulted in one colony failing to over-winter successfully, although one control colony also failed to develop in the spring.

Honeybee queens were reared and supplied with fondant containing the test item enabling the queens to be fed on the treated sucrose from emergence. The number of queens that successfully mated and laid eggs was 74% in the control, 71% in the diflubenzuron treated, 0% in the fenoxycarb treated, 100% in the azadirachtin treated and 92% in the tebufenozide treated. In the fenoxycarb treated queens were present but showed virgin queen characteristics, e.g. small abdomen, suggesting they had not been mated. A significant reduction in the numbers of eggs laid by queens treated with diflubenzuron was observed, no eggs were laid by any queens treated with fenoxycarb and no effects were observed on the numbers of eggs laid after treatment of the queens with azadirachtin or tebufenozide.

The colonies under study for the effects of IGRs on colony viability were also used to assess effects on drones raised in the colonies after treatment. There were no significant differences in the sperm counts between the colonies.

An existing honeybee population model was modified to include exposure to IGRs. A significant finding from the model was that application of IGRs in spring and early summer could have substantial long-term effects on colony size and viability. Whereas one might suppose that a colony would easily recover by the following winter, the model suggests this will not necessarily be the case. Sub-lethal effects such as premature ageing can have worse effects than massive brood

mortality, as it severely reduces the ability to rear the next generation of nurse bees, and there is a knock-on effect. The model showed that even if only those bees reared within two weeks of the IGR being applied are subject to premature ageing, such relatively short-term persistence of IGRs might nevertheless significantly reduce the size of over-wintering colonies, and increase the chance of the bee population dwindling and dying in late winter or early spring, as was shown for one fenoxycarb and four azadirachtin treated colonies.

P15 Effects of an Androgenic Growth Promoter 17 β -Trenbolone on Masculinization of Mosquitofish (*Gambusia affinis affinis*)

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Endocrine disrupting chemicals can affect normal hormone dependent processes through hormone receptors. Trenbolone acetate, a pharmaceutical, androgenic, anabolic steroid is a potent agonist of androgen receptors, and has been extensively utilized as a growth promoter for beef cattle in the United States. The effects of 17 β -trenbolone (TB), a hydroxylated active compound of trenbolone acetate, on adult and newborn mosquitofish (*Gambusia affinis affinis*), were examined. Two forms of mosquitofish androgen receptor (AR), ARa and ARb, were cloned. The expression levels of ARa and ARb were transiently increased in the anal fin of adult females at day 3 of TB exposure and gonopodium differentiation in the adult female anal fin was induced by 28 days of TB exposure. Gonopodium differentiation was also induced in mosquitofish fry by exposure for 28 days to 0.3 to 10 μ g/L TB. Furthermore, spermatozoa were observed histologically in the testes of male fry exposed to 1-10 μ g/L TB for 28 days; spermatozoa are normally observed only in the testis of mature males.

Spermatozoa were also found in the ovary of fry exposed for 28 days to 1-10 μ g/L TB. Thus, TB induced masculinization of the anal fin accompanied by a transient up-regulation of ARa and ARb in adult females. TB also induced differentiation of the anal fin into the

gonopodium in fry and stimulated precocious spermatogenesis in males and the formation of ovotestes in females.

P16 Molecular cloning of sex hormone-binding globulin cDNA in carp, *Cyprinus carpio*.

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Sex hormone-binding globulin (SHBG) is a plasma glycoprotein able to bind specifically sex steroids. It transports sex steroids in the blood, regulates their metabolic clearance and access to target cells. Therefore, SHBG is one of the key factors to control the effectiveness of sex steroids. Until now, only a few mammalian SHBG were isolated and characterized, non-mammalian SHBG has not yet. So, little is known about the details of SHBG (structure, function, expression, etc) in non-mammalian species. In the present study, for the initial step to understand the function of SHBG in teleosts, we isolated and characterized cDNAs encoding SHBG in Carp, *Cyprinus carpio*. Two types of fragment (SHBG-I and SHBG-II, 180 base pair (bp) in each) were amplified by the PCR using degenerate primers which was selected from the conserved region between mammalian SHBG cDNAs and SHBG like sequence in zebrafish genome. Both amino acid sequences are closely resemble (95%), also show high identity (about 60%) with the corresponding sequence of mammals. Using these fragments as a probe, SHBG-I and SHBG-II cDNAs contained whole open reading frame were isolated from liver cDNA library. They encode 401 and 388 amino acids respectively, and share high amino acid identity (90%). Although their amino acid sequence show low identity (about 35%) with those of mammalian SHBG, only steroid-binding site is conserved better than the

other part. In addition, the antibody against recombinant protein of these cDNAs bound specifically serum protein contained rich in sex steroids. These results suggest that present isolated two cDNAs encode carp SHBG. Northern blot analysis and RT-PCR revealed both types of SHBG mRNA were mostly transcribed in liver. The levels of their mRNAs in liver did not change by either exogenous estrogen or androgen. This result may indicate that SHBG synthesis is connected with steroidogenesis.

P17 Approach to analysis of the toxic action of estrogenic chemicals using two amphibian species, *Silurana tropicalis* and *Rana rugosa*

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To analyze the toxic action of estrogenic chemicals, we investigated two amphibian species, *Silurana tropicalis* and *Rana rugosa*.

[*Silurana tropicalis*]

The frog *S. tropicalis*, a species of clawed frog, is available for ecological assessment. We can easily obtain a lot of fertilized eggs by artificial insemination almost during the year. In addition, it is a diploid frog and matures by about half year after metamorphosis unlike the African clawed frog *Xenopus laevis*. Genetic markers are needed to develop assessment method using the amphibian for estrogenic chemical actions. We isolated the frog *estrogen receptor (ER)*, *thyroid hormone receptor (TR)* and *aromatase* cDNAs. RT-PCR analysis showed that the liver, gonad, and brain of the frog expressed abundantly *ER* mRNA, suggesting that these tissues respond strongly to estrogenic chemicals. The *TR* mRNA was expressed in the liver, gonad, and brain, while the *aromatase* mRNA was transcribed abundantly in the ovary and brain. We analyzed all the mRNA expressions in the liver, gonad, or brain during the development of the frog. The *ER*, *TR*, and *aromatase* mRNAs were transcribed abundantly in the liver, gonad, or brain of the tadpole at stage 60, when the gonad was sexually differentiated, and after that. We will explore to isolate genetic markers expressed in the three tissues of the tadpole at stage 60 and after that, and the frog after metamorphosis.

[*Rana rugosa*]

It is very important to investigate toxic actions of chemicals on the amphibian in the field. The frog *Rana rugosa* lives widely in Japan, and is easily sex-reversed by androgen and estrogen treatments. We analyzed effects of estrogenic chemicals on androgen-induced sex-reversal of the frog. First of all, we examined effective treatment for androgen-induced sex-reversal. All-female tadpoles were treated effectively with androgen to induce sex-reversal from female into male, and exposed to estrogen in breeding water. The estrogen treatment inhibited testicular differentiation in the androgen-primed tadpole. We analyzed genes differentially expressed during androgen-induced sex-reversal to isolate estrogen-responsive genes expressed during testicular differentiation in future. Ethynylestradiol and bisphenol A possessed also the inhibitory effect, suggesting that inhibition of masculinization of the amphibian may occur in the field. Analysis of the gonad of the frog *Rana limnocharis* showed the possibility that the masculinization was inhibited in the field near the town.

P18 Ontogenic gene expression of oestrogen receptors and aromatases in roach (*Rutilus rutilus*)

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Steroid oestrogens influence a wide range of physiological processes including growth, sex differentiation and reproductive activity. Formation of oestrogens from androgens is catalysed by the action of cytochrome P450 aromatase, a product of the *cyp19* gene. In fish two structurally and functionally distinct P450arom isoforms (P450aromB/*cyp19b*, representing the predominant neural form, and P450aromA/*cyp19a* as the predominant ovarian form) have been identified. Oestrogenic actions are mediated principally by ligand-activated transcription factors, oestrogen receptors (ERs) of which there are at least two subtypes, ER α and ER β in fish.

Full-length cDNAs encoding the four target genes *cyp19a*, *cyp19b*, ER α and ER β were isolated from roach (*Rutilus rutilus*) gonadal and brain tissues. Real-time RT-PCR was used to establish the ontogeny of expression for the four target genes in early life stages of roach (up to 250 days post hatch; dph). For this, head and body tissues were separated and analysed for target gene expression in fish up to 112 dph, and gonad and

brain tissues were dissected out and analysed in fish at 250dph. An increase of *cyp19a* expression was observed in both, head- and body-tissues during the development. Up to 112 dph, expression of *cyp19a* was generally higher in the head compared with the body tissue. At 250 dph, however, expression of *cyp19a* was 18 times higher in the gonads compared with the brain tissues. At this life stage, expression of *cyp 19a* was almost 6 times higher in testis compared to ovaries, but there were no gender-specific differences in *cyp19a* expression in the brain.

The expression of *cyp19b* was higher in head/brain samples compared to body/gonad tissues. No distinct changes in expression of *cyp19b* occurred in head tissue up to 112 dph, but this was followed by a very marked increase in expression at 250 dph.

Expression of the oestrogen receptors increased progressively in both tissues with increasing age (except for ER α in ovaries at 250 dph). There was no apparent tissue bias in the expression of ER α and ER β in head tissue (brains) or in body tissue (gonads) until 112 dph. At 250 dph, there were no differences in expression of ER α in the brain, but there was a significantly higher (more than 5 times) expression in the testis in males, compared with the ovary in females. For ER β , higher levels of expression were observed in bodies/gonads compared with heads/brains. At 250dph there were no significant differences in ER β expression between brains of the different genders. In contrast, at this life stage ER β expression was 2.5 times higher in ovaries compared with testes.

We are now investigating how exposure to environmental oestrogens during early life affects the normal patterns of expression of the gene targets and how this impacts subsequently on reproductive development and function in the roach.

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