



8th SCIENTIFIC WORKSHOP 2006

UK-J Co-operation for Research on Endocrine Disruptors
in the Aquatic Environment

Dartington Hall, Totnes, Devon
9-11th October 2006



The 8th UK-Japan Annual Scientific Workshop
on Research on Endocrine Disrupters in the Aquatic
Environment



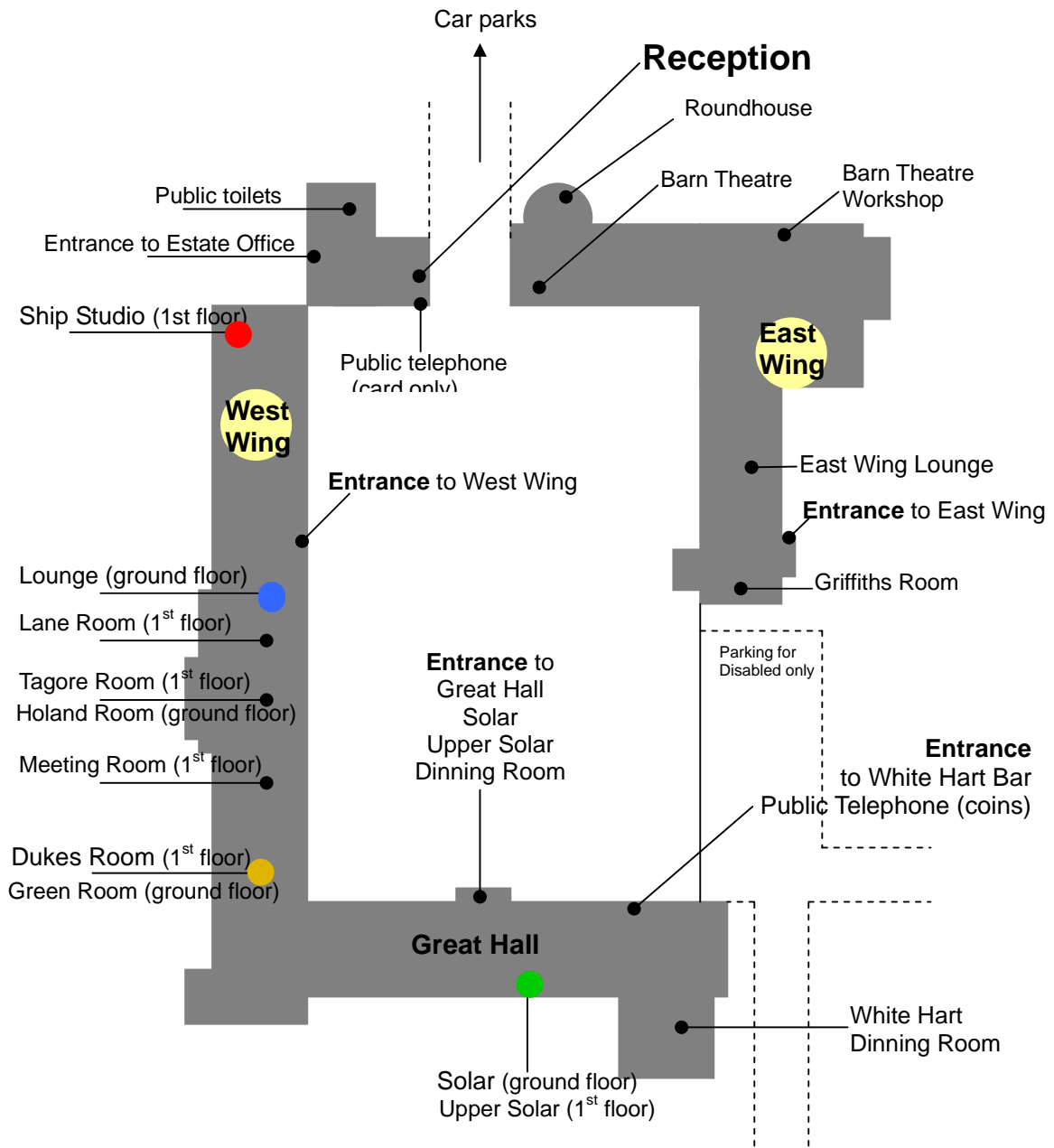
Dartington Hall
Totnes, Devon
9th – 11th October 2006




Abstracts and Programme Information



Dartington Hall Estate

Medieval Courtyard



Key	
 Bedrooms	 East Wing Lounge - Coffee breaks & Lunch
 Ship Studio – Conference Room	 Dukes Room – Breakout discussions
	 Solar – Reception (Mon) & Dinner (Tue)



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CORE PROJECT 2: Evaluation of endocrine disrupting potency of chemicals using stickleback model

9:40 - 10:00 Powerful new additions to the stickleback 'toolbox'. **(C2.1)**
Prof. Alexander Scott

10:00 - 10:20 Detection and assessment of androgenic potency of endocrine-disrupting chemicals using the stickleback. **(C2.2)**
Dr. Masaki Nagae

10:20 -10:40 Coffee Break (West Wing Lounge)

CORE PROJECT 3: Mechanism of testis-ova induction in fish

10:40 - 11:00 Investigating the molecular mechanisms of oestrogenic disruption in the roach, *Rutilus rutilus* **(C3.1)**
Dr. Anke Lange

11:00 - 11:20 Study of ligand-specificity of fish estrogen receptors. **(C3.2)**
Dr. Yoshinao Katsu

CORE PROJECT 4: Development of partial life cycle methods for investigating effects of endocrine disrupters on reproductive function in the emerging amphibian model, *Silurana tropicalis*

11:20 - 11:40 Expression of estrogen-responsive genes during development of the frog *Xenopus (Silurana) tropicalis* and Construction of Standard Database for the Estrogen-exposed tadpole **(C4.1)**
Dr. Minoru Takase

11:40- 12:00 Development of partial life cycle methods to assess reproductive impacts of aberrant gonadal differentiation in the emerging amphibian model *Silurana (Xenopus) tropicalis*. **(C4.2)**
Dr. Daniel Pickford

12:00 - 12:30 Discussion

12:30 - 2:30 Buffet Lunch, Poster presentations, and Breakout discussion for core project groups

15:00 – Excursion: Dartington Estate – Guided walk of the Gardens

19:30 – Conference Dinner at Dartington Hall, Solar room



Wednesday, 11th October

Ship Studio

INVITED PRESENTATIONS

Chairs: Prof. Taisen Iguchi and Prof. Charles Tyler

9:00 - 9:30 Hormone Disruption and the Developing Reproductive System. **(S5.1)**
 Prof. Louis J. Guillette

9:30 - 10:00 Genetic and epigenetic sex reversal in medaka, *Oryzias latipes*, with
special reference to the impact of endocrine disruptors on wild life. **(S5.2)**
 Prof. Satoshi Hamaguchi

10.00 - 10:30 EDCAT - A UK-based research programme to investigate Endocrine
Disruption in a CATchment. **(S5.3)**
 Prof. Peter Matthiessen

10:30 -11:00 Coffee Break (West Wing Lounge)

11:00 - 12.30 Summing up and further directions for Core Projects

Objectives of each core project for 2007

One presenter for each core project (max 2 slides per project!)

Synopsis and Discussion

Prof. Charles Tyler and Prof. Taisen Iguchi

12:30 - 12:45 Closing remarks

Dr. Mike Roberts

Dr. Tatsuya Aoki

12:45 - 14:00 Lunch (West Wing Lounge)

15:00 - 17:00 Excursion - Steam train from Buckfastleigh to Totnes (Devon cream
tea included!)

19:00 Depart Dartington for Dinner at Riverford Farm (Organic Farm).

Thursday, 12th October

Departure - room checkout by 9.30am



POSTERS

P1 Immunohistochemical demonstration of androgen receptor in the kidney of three-spined stickleback (*Gasterosteus aculeatus*)

Yasuhiko Ohta, Masaki Nagae & Taisen Iguchi

P2 Evaluation of effect of atrazine on developing *Xenopus laevis*

T. Oka, N. Mitsui, M. Miyahara, O. Tooi, N. Santo & T. Iguchi



Abstracts



(C1.1) Reduction of Estrogenic Activity in Wastewater and Evaluation of Biological Significance

Hiroaki Tanaka¹, Yutaka Suzuki² and Yuji Okayasu²

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²Public Works Research Institute, 1-6 Minamihara, Tsukuba, Ibaraki 305-8516, Japan

Behavior of Estrogens in Sewage Treatment Process

This paper describes observed behavior of free estrogens in simulated one type of biological nitrogen removal process (BNRP). In the simulated BNRP, an aeration tank was divided into an aerobic zone and an anoxic zone. Hydraulic retention time (HRT) of the aerobic section was set at 6 hours and HRT of the anoxic section was set at 2 hours. Aerobic solids retention time (A-SRT) was set at about 7.5 days. Target substances were 17beta-estradiol (E2), estrone (E1), 17alpha-ethynylestradiol (EE2). Concentrations of dissolved target substances in primary effluent, mixed liquor in aerobic zone, mixed liquor in anoxic zone and secondary effluent were determined by Solid Phase Extraction (SPE) and LC-MS/MS Method. Concentrations of free estrogens declined in the aerobic zone, while they increased in the anoxic zone and secondary sedimentation tank. Moreover, in order to obtain further information about anoxic degradation of estrogens, a batch anoxic degradation experiment using activated sludge in the anoxic zone of the simulated BNRP was conducted. We found that concentration of dissolved E1 drastically increased, and that of dissolved E2 increased to some extent. This result indicates that considerable source of estrogens still remain in treated effluent and/or activated sludge and some fermentation reactions under anoxic condition are related to estrogen transformation.

Behavior of Estrogens in Receiving Waters

In the Yodo River, the estrogen load from wastewater treatment plants, tributaries and upstream on the main rivers were surveyed in March and November in 2005. The results demonstrate that in this region where sewage system is provided in most of the area, about 80% of the total influent load comes from wastewater treatment plants, and greatly influence estrogens in the receiving waters. The results of the survey showed that in March when the water temperature was low, the load discharged into the river basin was high and E1 load was almost unchanged as it flowed down the river. While that in November when the water temperature was relatively high, the load discharged into the river basin was low, and the E1 load decreased by about 1/3 as it flowed down the river. The flow rate in November was lower than 2/3 of that in March, and it is assumed that in addition to the water temperature, the change of the flow time also impacted its load change.



(C1.2) Comparing the role of sewage treatment in the overall impact of endocrine disruption between the UK and Japan

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Data is emerging on typical effluent concentrations of steroid oestrogens, and sewage treatment works (STW) removal rates in Japan and the UK. But steroid oestrogen concentrations in effluent are only one part of the story of endocrine disruption of wild fish. We have made a preliminary survey of the key factors that will have a bearing on the endocrine disruption impact between the two countries. These include such diverse issues as the geography of the country, the distribution of the human population, their consumption of the contraceptive pill and the sensitivity of the indigenous wild fish of the respective countries.

We will present some preliminary findings and offer some unconventional approaches to assess how critical STW performance might be to the overall impact of endocrine disruption in the UK and Japan.



(C2.1) Powerful new additions to the stickleback ‘toolbox’

Ioanna Katsiadaki, Marion Sebire & Alex Scott*

Cefas, The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, UK

The three-spined stickleback has played an important role in the evaluation of EDCs due to the presence of an endpoint for androgenic/antiandrogenic compounds (spiggin). This has been developed into both in vivo and in vitro assays using immunoassay or RT-PCR to quantify spiggin production. Oestrogens can also be monitored in this species (as in many others) using an immunoassay for vitellogenin (VTG). Genetic sex can also be tested and tools for genomics and proteomics (as previously reported) are in an advanced stage of development.

We will report on 1) the development and application of behavioural tests for anti-androgens in sticklebacks and 2) the development of a procedure for non-invasive measurement of sex steroids in sticklebacks. These new tools are powerful additions to the stickleback toolbox – allowing the assessment of endocrine disrupters at the important level between physiological and population effects.

* Dr Scott will present this work

We acknowledge the support of Defra, UK.



(C2.2) Detection and Assessment of Androgenic Potency of Endocrine-disrupting Chemicals using the Stickleback

Masaki Nagae¹, Kiyoshi Soyano¹, Yoshinao Katsu², Yasuhiko Ohta³, Akihiko Hara⁴,
Ioanna Katsiadaki⁵, Alexander P. Scott⁵

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³Tottori University, Japan

⁴Hokkaido University, Japan

⁵Cefas, Weymouth Lab, UK

The use of specific biomarker enables quick and precise evaluation of the hormonal potency of environmental chemicals. Male three-spined stickleback (*Gasterosteus aculeatus*) produces a glue protein, spiggin, which is used in the building of the nest that females enter in order to spawn. The synthesis of spiggin is strongly controlled by androgen. Last year, cloning of two types of spiggin (SPG-I and SPG-II) was completed, and quantification system of spiggin mRNA was established using real-time quantitative RT-PCR technique. In this presentation, the effectiveness of real-time quantification system of spiggin mRNA coupled with an in vivo chemical exposure system was investigated. In addition, we also performed cDNA cloning of stickleback androgen receptor (AR α and AR β) as an initial step to understand the mechanism by which androgens induce spiggin synthesis.

Immature male and female sticklebacks were treated for 1 week with 17 α -methyltestosterone (MT) at various concentrations (10¹ to 10⁻⁵ μ g/L, nominal concentration) under semi-static water conditions. Kidney RNA samples were extracted for spiggin mRNA quantification. The real-time quantification system detected a significant increase of spiggin mRNA in both males and females treated with concentrations of MT between 10¹ to 10⁻¹ μ g/L. This result showed the high sensitivity of the current evaluation system for environmental monitoring of androgens using spiggin induction. The detection sensitivity of this system will be certainly improved by the introduction of continuous flow-through system for exposure.

By the combination of the screening of the stickleback kidney cDNA library and 5'-RACE PCR, full-lengths of two AR cDNAs were isolated. Stickleback AR α and AR β cDNAs were 3001 and 3076 bps long (648 and 728 amino acid residues), respectively. By the alignment of deduced amino acid sequences with other teleost ARs (Mosquitofish, Nile tilapia, Japanese eel and rainbow trout), it was shown that the two critical domains, for DNA-binding and ligand-binding, are highly conserved among teleosts.



(C3.1) Investigating the molecular mechanisms of oestrogenic disruption in the roach, *Rutilus rutilus*

Anke Lange¹, Yoshinao Katsu², Taisen Iguchi² and Charles R. Tyler¹

¹School of Biosciences, University of Exeter, United Kingdom

²Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Japan

The aim of this core project is to get a more detailed understanding of the gene pathways affected by oestrogenic exposure that subsequently result in the disruption of sexual differentiation and the induction of testis-ova in fish. This presentation will focus on some of the collaborative work conducted between Japan and the UK for one of the study species, the roach. In this work, a suite of 42 genes known to be involved in reproductive function of fish has been cloned from roach. These genes include cDNAs encoding for steroid hormone receptors, steroidogenic enzymes, genes involved in sex differentiation and gonad development, growth and hormone activity and three control genes. QPCR assays have been established for a suite of these gene targets including ER α , ER β , *cyp19a*, *cyp19b*, *vasa*, *sox9*, *amh*, *dmrt1* and *sf1* and a macroarray has been developed and validated for use containing all 42 roach cDNAs. This has provided us with a molecular toolbox to investigate both the normal mechanisms controlling sexual development and function in the roach and how oestrogenic chemicals disrupt these processes.

Here we present how the genes cloned and the macroarray have been used to investigate oestrogenic disruption and intersex in roach. In this work fish were exposed to the synthetic pharmaceutical oestrogen 17 α -ethinyloestradiol (EE₂) in the laboratory and effects on gonadal gene expression and the associated gonadal phenotype investigated at various sampling life stages. In the initial part of this presentation we will show some of the differences in gonadal gene expression patterns between control males and females, control intersex males and control and oestrogen-exposed females. In the second part of this presentation, we will detail the gonadal gene responses in fish exposed to environmental concentrations of EE₂ during early life (until 120 days post hatch, dph), subsequently maintained in clean water (until 518 dph) and then re-exposed to a single concentration of EE₂ for ten days. The gene responses were investigated using the qPCR assays established. In fish re-challenged to EE₂ there was an enhanced vitellogenic response in fish exposed to the higher concentrations of EE₂ during early life. This enhanced vitellogenic response was also seen for ER α , ER β and *cyp19a* in the ovary. This indicates that exposure to oestrogen during early life may sensitise the responsiveness of fish to oestrogen subsequently in later life. Tissue samples from roach in our long-term exposures (lab-based EE₂ and effluent) are now being analysed to further unravel the mechanisms underlying sexual disruption in wild roach in UK rivers.



(C3.2) Study of Ligand-Specificity of Fish Estrogen Receptors

Y. Katsu¹, A. Lange², C.R. Tyler² and T. Iguchi¹

¹Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Japan

²School of Biosciences, University of Exeter, United Kingdom

A variety of natural and man-made chemicals can mimic hormonal activities by binding to hormone receptors and in turn this may modulate endocrine systems. This is especially true for aquatic species where exposure to such chemicals can be considerable and continuous. Hormonally active chemicals have been shown to cause disruption of sexual differentiation and development, and to impact on reproduction in various 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. Estrogens and estrogenic compounds, acting via estrogen receptors, have been shown to play a major role in the disruption of sexual differentiation and development of wild roach.

In this study, we developed ER α reporter gene assays for two fish species (medaka and roach) to investigate the ability of a range of environmental estrogenic chemicals to bind and activate them. In this work, we used a reporter gene transcriptional assay using a GAL4 system with Chinese Hamster Ovarian K-1 (CHO-K1) cells. We modified the two-hybrid system using mammalian cells that can be used to study protein-protein interactions. The pBIND vector employed contains the yeast GAL4 DNA-binding domain. The pG5-luc vector contains five GAL4 binding sites upstream of a minimal TATA-box, which in turn, is upstream of the firefly luciferase gene. Using two vectors, we examined the luciferase expression in the CHO-K1 cells. Estrogenic activities of various steroid hormones and chemicals were tested and as for the conventional ERE-luciferase reporter assay system, estrone (E1), 17 β -estradiol (E2), estriol (E3), 17 α -ethinylestradiol (EE2), diethylstilbestrol (DES) all induced transcriptional activity via GAL4-ERs. Thus we have established reporter gene assays for both roach and medaka using a GAL4-system. We found that E2-induced luciferase activity by more than 100-fold, which appears to be more sensitive than the ERE-luciferase system and thus may be more suitable for assays of ER-transactivation. Concentration-dependent stimulation of transcriptional activity was found at concentrations of E2 higher than 10⁻⁹M for both the medaka and roach ER assays, but the medaka ER α was responded at lower concentrations of EE2 and DES compared with roach ER α . These results indicate that the medaka ER α and roach ER α differ in their relative sensitivities to different estrogens.



(C4.1) Expression of Estrogen-responsive Genes during Development of the Frog *Xenopus (Silurana) tropicalis* and Construction of Standard Database for the Estrogen-exposed Tadpole

Minoru Takase

Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Japan

It appears that the amphibian is a good environmental indicator, because it occupies distinctive habitats, depending upon the developmental phase (frog or tadpole), and shows unique responses to some hormones, such as sex-reversal induced by sex steroid hormones and metamorphosis induced by thyroid hormone. In this project, we aimed to develop and evaluate methods for characterizing amphibians affected by environmental chemicals at genetic, protein, tissue and population levels. *Xenopus laevis* has been commonly used in developmental biology due to the ease of laboratory husbandry and breeding by hormone treatment throughout the year. Because of relatively shorter generation time (about 5 months) and diploidy (20 chromosomes) in addition to advantages shared with *X. laevis*, *X. (Silurana) tropicalis* now provides a more popular model system for genetics studies as well as developmental biology studies.

In the workshop in 2005, I presented isolation of vitellogenin (*Vtg A* and *Vtg B* cDNAs from the liver of the frog *X. (S.) tropicalis* as estrogen-responsive genes employing a macroarray technique. In this workshop, I will present our recent work on expression of the estrogen-responsive genes during development, and isolation of more estrogen-responsive genes. *X. tropicalis* larvae were exposed to 2 μ M estradiol-17 β (E2) in breeding water from stages 51 or 55 for 21 days. They developed to stages 55 or 66 (when metamorphosis is completed) respectively. After treatment, total RNA was extracted from the liver, and used for RT-PCR analysis. Exposure to E2 from stage 51 did not influence expression of *Vtg A* and *Vtg B* genes in the liver. On the other hand, exposure to E2 from stage 55 enhanced and induced expression of *Vtg A* and *Vtg B* transcripts in the liver, respectively. Thus, *Vtg A* and *Vtg B* will be good gene markers for estrogenic chemicals in the tadpole at metamorphosing stages and after that. In addition to the two *Vtg* genes, I tried to isolate more estrogen-responsive genes using differential display (DD) method. Adult male *X. tropicalis* were exposed to 20 μ M E2 in breeding water for 21 days, or solvent control. After treatment, total RNA was extracted from the liver, and used for DD using 72 primer pairs as an initial template. I isolated 25 cDNAs differentially expressed in the liver of the adult frog after E2 treatment using DD method. I will show results on sequence analyses of them and their expression in *X. tropicalis* larvae after E2 treatment.



Recently, several laboratories, including Prof. Iguchi's lab, have reported methods for detecting and assessing impacts of chemicals on amphibians, while the fish is still a major experimental model for toxicological research. The amphibian and the fish share some unique responses to estrogenic chemicals with each other. Thus, we need to compare sensitivity of amphibians as experimental models for detection of effects of estrogenic chemicals, with that of fish. Finally, I will show current work in progress. Standard database including histology of thyroid and gonad, body length, and developmental stage has been constructed during normal development of *X. (S.) tropicalis* so far. We are going to investigate histological differentiation and development of thyroid and gonad of the tadpole exposed to ethynylestradiol (EE2) to construct standard database for the estrogen-exposed tadpole in the UK-J project. Exposure of EE2 will be started before initiation of differentiation of the thyroid and gonad. Every 7 days external appearance (size and developmental stage) will be recorded and thyroid and gonadal tissues will be collected from subsamples, for histological analysis. The database will help with comparison of toxicological responses in amphibian and fish models.



(C4.2) Development of partial life cycle methods to assess reproductive impacts of aberrant gonadal differentiation in the emerging amphibian model *Silurana (Xenopus) tropicalis*.

Daniel Pickford and Severine Larroze

Institute for the Environment, Brunel University, UK

Concerns over the potential impact of endocrine disrupting environmental contaminants on amphibian populations persist, and have stimulated numerous studies on the effects of chemicals on larval growth, development and gonadal differentiation. Extrapolating from results of single-chemical studies, measuring effects at individual level, to population-level impacts is problematic. Ultimately, information from ecoepidemiological studies of real amphibian populations will be needed to assess the impacts of environmental exposure to complex mixtures. However, useful interim steps in assessing the risk posed by endocrine disrupters are likely to include characterisation of the baseline incidence of aberrant sexual differentiation of the gonad; and linking developmental and reproductive endpoints at individual level in (partial) life-cycle studies. *Silurana (Xenopus) tropicalis* is an emerging amphibian model that offers some advantages for these type of studies, in having a short life cycle. In particular, *S. tropicalis* exhibits a relatively short period between completion of metamorphosis and sexual maturation (4-5 months), compared to *Xenopus laevis* (18 months or more). We aim to develop a partial life cycle method for assessing the impacts of endocrine disrupters on reproductive function, wherein histological and biomarker data collected at completion of metamorphosis can be linked to measures of reproductive fitness upon attainment of sexual maturity in control and exposed cohorts. These data should provide a more secure basis for estimating population-level consequences of disruption of gonadal differentiation, subsequent to larval exposure to endocrine disrupting chemicals.



(S5.1) **Hormone Disruption and the Developing Reproductive System**

Louis J. Guillette Jr., Brandon Moore and Dieldrich Bermudez

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Over the last four decades, we have spent billions of dollars and millions of research hours establishing links between various disease states and causal agents. The majority of these efforts have focused on genes and germs; that is, the genetic or pathogenic agents suspected to be the causes of cancer or major birth defects. However, studies over the last decade have clearly demonstrated that environmental – epigenetic – agents are implicated as the causal agents of many disease states and are hypothesized to be due to alterations in gene expression not gene mutations. Additionally, recent studies implicate altered gene expression as the basis for observed embryonic origins of juvenile or adult alterations in reproductive system function or disease.

The last decade has also brought a reevaluation of the role of environmental chemicals as causal agents in various alterations in the development and functioning of the reproductive system in humans and wildlife. For example, many chemicals released into the environment alter the endocrine system in detrimental ways. We will present recent examples of alterations in the development of the reproductive and endocrine systems following laboratory controlled studies. Examples will involve examining the genetic and endocrine basis for normal ovarian folliculogenesis in alligators following exposure to either contaminants naturally found in eggs of polluted lakes or after alteration of thyroid hormone signaling. We have observed that exposure *in ovo* to contaminants deposited in the egg by female alligators living on Lake Apopka, significantly alter the responsiveness of the neonatal ovary to a gonadotropin challenge. We have also observed that gene expression profiles in the neonatal ovary are significantly altered following perturbation of thyroid hormones for a short 5-day period during the period of sex determination in the alligator. The role of environmental factors in altering ovarian development and maturation will be discussed.



(S5.2) Genetic and Epigenetic Sex Reversal in Medaka, *Oryzias latipes*, with Special Reference to the Impact of Endocrine Disruptors on Wild Life

Satoshi Hamaguchi

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Medaka, *Oryzias latipes*, is a small fresh water fish endemic to East Asia. Genus *Oryzias* contains about 20 species, and medaka is the only species which can survive the cold winter of the temperate zone. From the sequence analysis of a mitochondrial gene, *cyt b*, wild medaka has been clarified to be composed of many regionally differentiated populations. This fact suggests that the responses of wild medaka to epigenetic factors might be different among local populations.

We Japanese have been kept medaka as an ornament fish for long time. In 20th century, medaka has become used as a laboratory animal in a variety of fields of biology. According to the enlargement of the laboratory use of this fish, basic devices for the laboratory animal including inbred strains and genome information, has been equipped.

In 1921, Aida first evidenced that medaka has XX-XY sex determining system, and established a $X^rX^r-X^rY^R$ strain in which male is orange-red and female white. About 40 years later, using the $X^rX^r-X^rY^R$ strain, Yamamoto (1955-69) succeeded in producing sex-reversals by administering sex steroids to juvenile fish, indicating that the plasticity of the genetic sex determination in this fish. In addition, Egami (1955-7) demonstrated that oocyte-like cells (testis-ova) can be induced by various treatments of adult fish, that is, germ cells in adult testes are sexually bipotential.

More 40 years passed, and we identified the male-determining gene on Y chromosome as *DMY* (Matsuda et al. 2002). Using *DMY* as a genetic marker, we have become able to identify the genetic sex of an individual not only of the $X^rX^r-X^rY^R$ strain but also of all medaka strains. We examined the genetic and phenotypic sex of wild medaka more than 6000 individuals, and revealed the existence of about 1% of sex-reversals (XY female and XX male), most of which result from mutations of sex-related genes. In addition, we confirmed that sex-reversal can be induced by the exposure of medaka embryos to sex steroids, and also noted that the incidence of sex-reversal was different among strains. These facts indicate a polymorphic feature of sex-related genes of this fish.

The problems of endocrine disruptors begin at the observation of sexual abnormality in wild animals. Therefore, it goes without saying that careful observations of wild animals have a crucial necessity. But the observed abnormality does not always result from epigenetic factors. We must pay attention to genetic polymorphism in the species concerned.



(S5.3) EDCAT – A UK-based research programme to investigate Endocrine Disruption in a CATchment

Peter Matthiessen

Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP

The EDCAT programme is testing the hypothesis that the degree of oestrogen contamination in some UK rivers is a risk to fish populations. EDCAT (Endocrine Disruption in Catchments) is a multi-funder programme (Defra, EA, UKWIR) which is addressing this issue by studying the River Ray downstream of an oestrogenic sewage discharge, before and after the levels of oestrogens are expected to be reduced by new treatment technology in 2008. EDCAT is studying two aspects of fish populations * breeding success of intersex *Rutilus rutilus* in experimental tanks, and population stability of wild *Gasterosteus aculeatus*. The breeding success of normal and intersex *R. rutilus* individuals will be monitored with microsatellite markers to identify their offspring. The population dynamics of *G. aculeatus* will be linked to oestrogen-specific biomarkers and to other pollutant markers. These studies will be supported by oestrogen exposure measurements and modelling so that biological responses can be explained as fully as possible.



(P1) Immunohistochemical demonstration of androgen receptor in the kidney of three-spined stickleback (*Gasterosteus aculeatus*)

Yasuhiko Ohta¹, Masaki Nagae², Taisen Iguchi³

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Male three-spined stickleback, *Gasterosteus aculeatus*, produces a glue protein in the kidney which is called spiggin and used as a cementing substance for building the nest. Since the production of spiggin is known to be regulated by androgen through its receptor (AR), spiggin is an effective biomarker of any androgenic activity caused by environmental chemicals, corresponding to the use of vitellogenin as an estrogenic biomarker. Messenger RNA of ARs (AR α and β) has been demonstrated in the stickleback kidney. To investigate the localization of AR, immunohistochemical staining using a commercial polyclonal antibody against human AR was carried out in the stickleback kidney. Various antigen retrieval methods including microwave irradiation and autoclave treatment have been introduced to the immunohistochemical field. In the present study, we examined effect of antigen retrieval solution on the signal intensity of AR immunostaining in formalin-fixed sections after autoclave pretreatment.

Kidneys were removed from the sticklebacks of both sexes in the **non-breeding season** (December, 2005) and fixed with 10% phosphate-buffered formalin. Before incubating in the anti-AR antibody (C-19, Santa Cruz), sections were autoclaved at 120 °C for 10 min in the following buffers; 0.01M citrate buffer at pH 6, 0.8 M urea and a commercial buffer (AntigenPlus™ Buffer, pH 6 and 10, Novagen). AR staining was found in nuclei of epithelial cells of renal tubules in the males, but not in the females. Intensity of the staining varied with the antigen retrieval solution. The best antigen retrieval buffer was 0.8 M urea with autoclaving for 10 min. In conclusion, antigen retrieval treatment was essential for AR immunostaining in the stickleback, and AR was expressed only in male kidneys.



(P2) Evaluation of effect of atrazine on developing *Xenopus laevis*

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This experiment was performed to investigate the effects of atrazine on metamorphosis and gonad development using *Xenopus laevis* (*X. laevis*) tadpoles. Test designs are as follows; 1) Animal: Our study was carried out using all ZZ male *X. laevis*, produced by mating wild type ZZ male with artificially feminized ZZ male. 2) Test conditions: The number of tadpole was 30 tadpoles per 10 L in a single glass tank per concentration at 22 ± 1 °C. The test medium was changed three times per week (semi-static condition). Tadpoles were exposed to atrazine from stage 49 to 66. 3) Endpoints: Metamorphosis, gonad morphology, and the hepatic VTG induction were investigated in the developing tadpoles or froglets. Furthermore, we performed the *in vitro* VTG assay using primary-cultured hepatocytes.

In the present study, atrazine had no effect on metamorphosis, developmental stage, and total length. Gonad malformation was not identified in morphological analysis. However, the effect of atrazine on gonad histology is unclear at present. Moreover, atrazine had no effect on VTG induction in the hepatic and *in vitro* study. We found atrazine had the no effect on metamorphosis and gonad development in *X. laevis*. Further study was needed to test effects of chemicals on amphibian metamorphosis based on the OECD test guideline.



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Thank you for coming