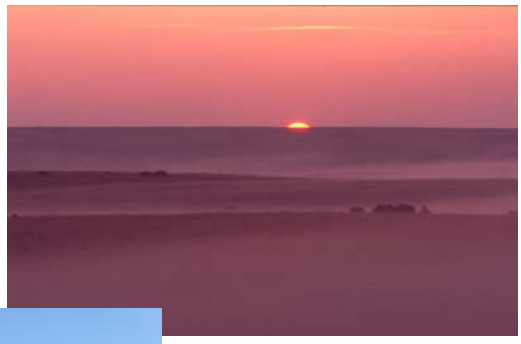
10th Annual Scientific Workshop

UK-Japan Co-operation for Research on Endocrine Disrupters in the Aquatic Environment

Bovey Castle, Dartmoor National Park, Devon, UK

5-8th October 2008




defra
Department for Environment Food and Rural Affairs

NATURAL ENVIRONMENT RESEARCH COUNCIL

環境省
Ministry of the Environment

10th Annual Scientific Workshop

UK-Japan Co-operation for Research on Endocrine Disrupters in the Aquatic Environment

Bovey Castle

North Bovey, Dartmoor National Park, Devon, UK

5th – 8th October 2008

Abstracts and Programme Information

Contents

The 10th Annual Scientific Workshop on Research into Environmental Endocrine Disrupting Chemicals

Bovey Castle, North Bovey, Dartmoor National Park, Devon

5-8th October 2008

Programme

Sunday, 5th October

Arrival of Participants at Bovey Castle

19:00 - 21:00 **Welcome reception, Dartmoor Suite** (Buffet and Drinks)

Opening Address/Welcome by

Dr Mike Roberts (Defra, UK)
Dr Hirotsugu Kimura (Ministry of the Environment, Japan)

Monday, 6th October

Dartmoor Suite

WELCOME and INTRODUCTION

8:30 - 9:15 **Welcome to the Science**

Logistics and meeting events

Prof Charles Tyler

Achievements under the UK-Japan partnership 2005-08

Prof Taisen Iguchi

SESSION - CORE PROJECTS

Chairs: Dr Christina Lye and Prof Yasuhiko Ohta

9:15 - 9:55 **CORE PROJECT 1**

Evaluation on Effects of Reducing Estrogenic Activity in Sewage

Prof. Hiroaki Tanaka, Dr Yutaka Suzuki and Dr Andrew Johnson

9:55 - 10:35 **CORE PROJECT 2**

Basic Understandings of Spiggin Synthesis in Kidney for the Effective Evaluation of Endocrine Disruptors

Dr Masaki Nagae

Summary of the *in vivo* Stickleback Tests Aimed to be Part of the Official OECD Guidelines for Endocrine Disruptor Testing

Dr Ioanna Katsiadaki

10:35 -11:00 Coffee Break

Chairs: Prof Alan Pickering and Prof Yasuhiko Ohta

11:00 - 11:40 **CORE PROJECT 3**

Comparative Responsiveness and Sensitivity of Fish Oestrogen Receptors to Steroid Oestrogens and other Environmental Oestrogens

Dr Anke Lange and Dr Yoshinao Katsu

11:40 - 12:20 **CORE PROJECT 4**

Development of Methods for Detecting and Assessing Impacts of Chemicals on Amphibians

Dr Minoru Takase

Progress and the need for integration of laboratory and field studies concerning ED in amphibians

Dr Daniel Pickford

12:20 - 13:45 BUFFET LUNCH and POSTER PRESENTATIONS

SESSION – INVITED PRESENTATIONS

Chairs: Prof. Charles Tyler, Prof. Taisen Iguchi

13:45 - 14:05 **Initial investigations into the ecotoxicology of nanoparticles in the aquatic environment**

Rhys Goodhead (DEFRA-funded PhDstudent, University of Exeter, UK)

14:05 - 14:40 **Ecosystem level impacts of ethinyloestradiol – Lake 260, Canada**

Dr Karen Kidd (University of New Brunswick, Canada)

14:40 - 15:15 **Testing methods for invertebrate's endocrine disrupting chemicals using cladoceran crustaceans, *Daphnia magna***

Dr Norihisa Tatarazako (NIES, Japan)

15:15 -15:35 Coffee Break

15:35 - 15:55 **Putting science into policy**

Dr Mike Roberts (Defra, UK)

15:55 - 16:20 **Discussion / QA on core projects**

16:25 – as required Break-away discussions on 'What are the major issues in ecotoxicoogy that the UK-J partnership should be addressing?'

19:30 – CONFERENCE DINNER (Adam Room)

Tuesday, 7th October

Dartmoor Suite

SESSION

Chairs: Prof. Taisen Iguchi and Prof. Charles Tyler

9:00 - 11:00 Taking UK-J into next generation of ecotoxicological science (Individuals from core themes and governmental bodies to provide their thinking on the critical issues in ecotoxicology and how the UK-J partnership can help to take them forward

11:00 -11:20 Coffee Break

11:20 - 12:00 Summing up, and closing

Prof. Charles Tyler

Taisen Iguchi

Dr Mike Roberts

Dr Hirotsugu Kimura

12:00 Collection of Packed Lunch

12:45 Depart for Excursion – Historic and Beauty Sites on Dartmoor

~ 18:30 Pub Dinner – The Old Inn, Widecombe-In-The_Moor

Wednesday, 8th October

Departure - room checkout by 11 am

POSTERS

P1 **Estrogen receptor Expression in American Alligator Oviduct**

Ohta, Y., Katsu, Y., Iguchi, T., Kohno, S., Brandon, M. and Guillette, L.J.

P2 **Embryo toxicity in Japanese medaka and zebrafish exposed to endocrine disrupters, pesticides, and PPCPs**

Norihisa Tatarazako, Narisato Hirai and Taisen Iguchi

P3 **Mentoring the Next Generation of Scientists**

Thea M. Edwards and Louis J. Guillette, Jr.

P4 **Sexual Re-programming and Oestrogenic Sensitization in Wild Fish Exposed to Ethinyloestradiol**

Anke Lange, Gregory C. Paull, Yoshinao Katsu, Hiroshi Urushitani, Rie Ichikawa, Tobias S. Coe, Taisen Iguchi, Charles R. Tyler

Abstracts

(C1) Evaluation on Effects of Reducing Estrogenic Activity in Sewage

Hiroaki Tanaka[1], Yutaka Suzuki[2] and Andrew Johnson[3]

[1] Kyoto University
[2] Public Works Research Institute
[3] Centre for Ecology and Hydrology

The main objective of this study was to assess the overall impact of steroid estrogens on endocrine disruption in British and Japanese rivers. To this end, the fate and behaviour of estrogens in Japanese and British sewage treatment plants and river catchments was examined. This included understanding their removal in sewage treatment and developing analytical and modelling methods for estrogens in rivers. It is intended that these studies assist in the management of endocrine disruption in the two countries.

(1) Improvement of analytical method for estrogens including conjugates

We attempted to improve conventional analysis for conjugated estrogens in addition to free estrogens. The developed method showed high recoveries of conjugated estrogens by skipping cleanup of florisil and aminopropyl cartridges and by changing elusion solvents. We confirmed that the method gives high recovery not only for standard solutions but also for environmental samples. We also examined deconjugation method for estimating total conjugated estrogens by enzymes, but found that the amount of estrogens after deconjugation treatment did not coincide with that before the treatment even for standard solution. Thus, further work is needed for development of estimation method for total conjugates.

We compared the occurrence of estrogens in sewage, treated sewage and receiving river water between UK and Japan based on the same analytical method described above. The major difference between the countries was that ethynylestradiol was prevalent in sewage, treated sewage and receiving water in UK while it was below detection limit in all samples from Japan. In both the countries glucuronide conjugates of estrogens exist in sewage, but rarely in treated sewage, while sulfate conjugates of estrogens exist in both sewage and treated sewage.

(2) Developing improvements to the removal of estrogens in sewage treatment

From our research last year, dissolved oxygen (DO) concentration was found to be a very important operation factor for the estrogen removal in a sewage treatment plant (STP) with relatively short solid retention time (SRT). We attempted to provide a reliable and efficient tertiary treatment process for the STP which can not afford high DO concentration in the aeration tank because of limited treatment capacity. The tertiary process contained plastic media for the attachment of microorganisms which can degrade estrogens, and the media were fluidized by aeration. Hydraulic retention time was set at 2 hours. The process could effectively remove estrogens of around 20ng/L in the STP effluent to below the detection limit. Relationship was observed between the estrogen

removal rate and the TOC concentration in the STP effluent, which suggests the co-metabolism of estrogens with organic matter contained in the STP effluent.

The performance of the tertiary process to prevent fish feminization was confirmed using Medaka, and it was revealed that the effluent of the tertiary process did not cause any feminization effect on Medaka just like de-chlorinated tap water.

(3) Fate of estrogens in receiving waters

Four field surveys were conducted in Yodo River systems to understand the fate of estrogens in the waters receiving STP discharge. The results indicated that most of estrogen load is derived from STP discharge in this river basin and consists of estrone. The following findings were derived from the mass balance of estrogens in the river systems:

- Discharged and transferred loads of estrogens is related to the time of year (seasons)
- Estrogen discharge from STPs significantly increased during periods of lower temperature
- Estrogen load in receiving waters decreased according to travel time;
- Estrogen load in the river was less reduced during its journey downstream during periods of lower temperature.

Biodegradation tests of estrogens have recently started using batch experiments. At a room temperature (20 degree C) glucuronide conjugates in sewage tended to be easily degraded under aerobic condition to release free estrogens, which themselves were quickly degraded. However, sulfate conjugate estrogens were degraded at least one-order magnitude slower than glucuronide conjugates. This suggests that free and glucuronide conjugate estrogens are easily degraded, but sulfate conjugates are slowly degraded at room temperature. Deconjugation and degradation of conjugates and free estrogens at lower temperature should be further conducted to understand the fate of estrogens in receiving water as well as sewage treatment.

The results of field surveys also demonstrated that 2 to 6 mg/L of ozone dose after biological treatment process can significantly decrease free estrogens to below detection limit. This indicates ozonation is a prospective and stable method for reducing estrogen concentrations in addition to improvement of biological treatment.

(4) Future perspectives

At the present, prediction model for hotspots of estrogens in rivers is under construction based on the results of field survey and the degradation tests. Understanding the difference of pollution and sewage treatment system in both the countries is helpful to understanding and managing estrogenic pollution. The next challenge is how to expand the shared knowledge and expertise gained from this estrogen study to other emerging pollutants such as pharmaceuticals and personal care products (PPCP). As with estrogens, these PPCPs share a sewage origin and have the potential to cause adverse effects on aquatic wild life as well as human health.

(C2.1) Basic understandings of spiggin synthesis in kidney for the effective evaluation of endocrine disruptors

Masaki Nagae[1], Masanobu Sakai[1], Subaru Matsuo[1], Kiyoshi Soyano[1], Yoshinao Katsu[2], Yasuhiko Ohta[3], Akihiko Hara[4], Ioanna Katsiadaki[5] and Alexander P. Scott[5]

[1] Nagasaki University, Japan
[2] Okazaki Institute for Integrative Bioscience, Japan
[3] Tottori University, Japan
[4] Hokkaido University, Japan
[5] Cefas, 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 spawning. The synthesis of spiggin is strongly controlled by androgen. However, there is room for basic and comprehensive understandings of spiggin synthesis in kidney. In this presentation, with previous progress in our studies about spiggin, the effect of water temperature on spiggin synthesis will be talked. In addition, we also will be shown the changes in androgen receptor (ARα and ARβ) mRNA levels during androgen exposure.

Immature male and female sticklebacks were treated for 1 week with 17α-methyltestosterone (MT) at 10, 1 and 10^{-1} μg/L (nominal concentration) at 5, 10 and 15 degrees centigrade. Kidney RNA samples were extracted and applied to spiggin and ARα and ARβ mRNA quantification.

Water temperature strongly influenced spiggin synthesis in MT treated stickleback. At 15 degrees centigrade, spiggin mRNA levels in kidney were extremely high, there was closely positive correlation between MT concentration and spiggin synthesis in kidney. On the other hand, at lower temperature, particular in 5 degrees centigrade, spiggin mRNA levels markedly decreased. These findings indicate that water temperature should be controlled intensively when we do in vivo exposure test for the analysis of androgenic potency of chemicals using spiggin as a biomarker.

ARα mRNA levels were almost constant during MT exposure both male and female kidneys. However, ARβ mRNA levels drastically decreased by MT exposure. These results suggest that ARβ is more potent than ARα to regulate the transmission of androgenic stimulation by self-controlling of its number of molecules.

(C.2.2) Summary of the in vivo stickleback tests aimed to be part of the official OECD guidelines for endocrine disruptor testing

Ioanna Katsiadaki, Marion Sebire and Yvonne Allen

Cefas Weymouth

The internationally standardised chemical testing regimes were not specifically designed for identifying EDCs and are certainly inadequate for estimating their likely ecological or medical effects. This problem has the Organization for Economic Cooperation and Development (OECD) to expand its test guideline development programme and include procedures that can identify EDCs. Since then, several comprehensive test validation projects have been initiated and some of these are currently well underway. In the case of fish, the OECD developed (and is close to validating) a 21-day screening test guideline, which is sensitive to certain EDCs (oestrogens/androgens and their antagonists, plus aromatase inhibitors). In this screen the proposed species are the Japanese medaka, the fathead minnow and the zebrafish.

The adoption of the guideline as the main screen for the detection of EDCs recommended by the OECD presents two major limitations. The first is specific to European countries and is related to the fact that none of the proposed species is endemic in Europe, rendering data extrapolation obtained from model species to environmental risk assessment problematic. The second issue is universal and related to the lack of robust endpoints in the three core species that have a diagnostic value for androgenic xenobiotics (in particular androgen antagonists).

The potential of developing a biomarker for androgens in the three-spined stickleback (via the kidney glue protein that breeding males use to construct a nest) was recognised 10 years ago. As a consequence, under the EDMAR programme an ELISA for the only known so far androgen-regulated protein in fish, the stickleback glue spiggin, was developed and validated. More recently the stickleback assay was modified in two different ways to detect anti-androgens, a group of EDCs that poses an increasing risk to the aquatic environment as the in vitro data obtained from two nationwide surveys of final sewage effluents suggest. Importantly, the stickleback is present in Europe (and across all of the North hemisphere) and can bring together lab and field studies providing a sound basis for environmental risk assessment.

To this end, the UK (Defra CN) has financed three research programmes that underpinned the background and validation data requirements that could lead to the inclusion of the stickleback as a recommended species for EDCs screening. Here we present the outcomes of this research and provide an update of the current state of fish test guidelines for EDCs.

In addition we will be referring to a new project, funded by the National Centre of 3Rs, UK and is aiming to validate the fish sexual development test (another OECD guideline line under development) using the stickleback. The presence of a genetic sex marker in the stickleback (and the medaka) has the potential of reducing the number of fish needed for this test because genetic sex (as opposed to phenotypic) can be assigned.

(C3) Comparative Responsiveness and Sensitivity of Fish Oestrogen Receptors to Steroid Oestrogens and other Environmental Oestrogens

Yoshinao Katsu[1], Anke Lange[2], Charles R. Tyler[2] and Taisen Iguchi[1]

[1] Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Japan
[2] School of Biosciences, University of Exeter, United Kingdom

A variety of natural and man-made chemicals can mimic hormonal activities and bind to hormone receptors, resulting in the modulation of biological processes. Aquatic species are especially vulnerable to exposure to environmental oestrogens and these exposures can be both considerable and continuous. Hormonally active chemicals have been shown to not only cause disruption of sexual differentiation and development and to impact on reproduction in various fish species, but to also have wider health effects. In UK rivers, exposure of roach (*Rutilus rutilus* – a common cyprinid fish) to effluents from sewage treatment works effluents that contain complex mixtures of endocrine disrupting chemicals (EDCs) has been shown to alter sexual development and impact negatively on their reproductive capabilities. Oestrogens and oestrogen-mimicking compounds that act via oestrogen receptors, have been shown to play a major role in the disruption of sexual differentiation and development of wild roach. Some of the most potent environmental oestrogens include natural sex steroids and pharmaceutical oestrogens used in contraceptives and hormone replacement therapies.

In our collaborative research this year under the UK-Japan partnership we have applied molecular tools developed in the roach and medaka (and other fish species) to advance our understanding on the health effects of exposure to environmental oestrogens. A key aspect of this work has been to undertake studies to investigate the comparative sensitivities of a range of different fish species to oestrogens, conducted through both *in vivo* and *in vitro* studies. Additionally, we have conducted a desk top study to produce a critical analysis on endocrine disruption and the lessons learned for (eco)toxicology from these studies (Goodhead & Tyler, 2008; Tyler & Goodhead 2008), and initiated studies into the effects of engineered nanoparticles in fish (see abstract by Goodhead et al.).

Genes cloned from the roach and involved with sexual development and function have been used to study the effects of exposure to oestrogen during early life on the subsequent responsiveness of adults to oestrogen re-challenge in later life. Using qRT-PCR assays we have shown that early life exposure to environmentally relevant concentrations of ethinyloestradiol (EE_2) sensitized females to oestrogen, as determined by the measurement of the responses of oestrogen-sensitive genes in a further EE_2 challenge 398 days after the original exposure. Oestrogen receptors cloned from the roach, medaka and a series of other fish species, have been applied in the development of reporter gene assays to investigate their interactions with steroid oestrogens and oestrogen mimics *in vitro* (see later). Further molecular studies have included the isolation of a gene that might be a potential biomarker of testis-ova induction (in the medaka) and advancement of transcriptomics in the roach to better understand the evolution of intersex and the wider health effects of oestrogens.

Studies into the comparative sensitivities of fish to oestrogen have included both *in vivo* and *in vitro* studies. *In vivo* exposures of carp and rainbow trout to dihydroequilenin (a horse oestrogen used in hormone replacement therapy) and measuring the induction of vitellogenin (after a 21 day exposure via the water) has shown that trout are considerably more sensitive to this oestrogen than carp (LOEC of 0.6 ng/L versus 420 ng/L, respectively). Similarly, for a study exposing rainbow trout and sticklebacks in the same tanks for 7 days to EE_2, VTG induction occurred at the lowest concentration tested in trout (4 ng/L), but no induction was evident in sticklebacks even at 8 ng EE_2/L. These *in vivo* findings strongly suggest that there can be considerable species differences in sensitivity to oestrogen in fish. We are now undertaking exposures to oestrogen via the water of carp, trout, stickleback, and roach kept together in the same tanks and zebrafish, fathead minnow and medaka together in another tank series to define more precisely their comparative sensitivities to oestrogen *in vivo*. Our *in vivo* work has been complimented with an extensive series of studies analysing the ligand- and species-specificity of fish (medaka, roach, carp, zebrafish, fathead minnow and stickleback) ERs using reporter gene assays that we established previously. We have already shown the species-specificity of fish ERα transcriptional activity to steroid oestrogens (UK-J meeting, 2007), showing that the medaka ERα was more sensitive to diethylstilbestrol (DES) than other fish ERαs. During this years studies, we examined the effects of the environmental oestrogenic chemicals, DDD, DDE, DDT, bisphenol A and nonylphenol and found that DDT had a stronger oestrogenicity compared with DDD and DDE. Further, using these reporter assays the medaka and stickleback ERαs were found to be more sensitive to DDT and its metabolites compared with other fish ERαs. DDD and DDE had very little stimulatory transactivity for the carp ERα, even at 10-5M. Moreover, both bisphenol A and nonylphenol stimulated the transactivity of the fish ERαs examined. As for effects of DDT and its metabolites, medaka and stickleback ERαs were more sensitive to bisphenol A and nonylphenol.

Teleost fish express at least three ERs, ERα ERβ1 and ERβ2 (a fourth form, ERα2 has recently been identified in fish). We compared the ligand-specificity of 3 zebrafish ERs and found that each ER has its own oestrogen-specificity; ERα was more responsive to DES than to the other oestrogens tested, ERβ1 was more sensitive to E_2 and EE_2, and ERβ2 was more sensitive to EE_2. Further, we also compared the ligand-dependent induction of zebrafish ERs using the environmental oestrogen mimics, DDD, DDE, DDT, bisphenol A and nonylphenol. Intriguingly, zebrafish ERβ1 was more sensitive to all environmental chemicals examined compared to ERα and ERβ2.

These *in vitro* findings further indicate that the medaka, roach, carp, zebrafish, fathead minnow and stickleback ERα differ in their relative sensitivities to different oestrogens and environmental chemicals. Further, zebrafish ERβ1 is more sensitive to environmental oestrogens, suggesting it may be a main target receptor for oestrogens and other environmental oestrogenic chemicals. We now propose to examine the expression level and tissue distribution of the three ERs in detail to help unravel the functional significance of these findings *in vitro*.

(C4.1) Development of methods for detecting and assessing impacts of chemicals on amphibians

Minoru Takase[1], Tomohiro Oka[2], Maki Miyahara[2] and Osamu Tooi[2]

[1] Institute for Amphibian Biology, Graduate School of Science, Hiroshima University
[2] Institute of Environmental Ecology, IDEA Consultants, Inc., Japan

The West African clawed frog, *Xenopus* (*Silurana*) *tropicalis* is more useful for environment-related studies as well as development and genetics studies than the South African clawed frog (*X. laevis*), because of a diploid genome, shorter generation time, and available genomic information on web. To use *X.* (*S.*) *tropicalis* for evaluating and assessing influences of endocrine disrupters, it is needed that basic information on the field of development biology and endocrinology of the frog are gathered. In 2006, we created standard database on development of the *X.* (*S.*) tadpole exposed to 1 nM ethinylestradiol-17α (EE2) as test chemical in breeding water from day 3 of postfertilization (PF) through completion of metamorphosis. All tadpoles were reared according to the phase 2 method used in metamorphosis assay established in OECD. In the present study, we collected once more data on development of the tadpole to confirm the previous data. The present data were almost similar to the previous data. Additionally, half of the gonad of the metamorphosed frog day 49 PF was analyzed histologically for sexing. The histological analysis showed that sex ratio in the EE2 group was a significant excess of females. We are analyzing gene expressions in the gonad of metamorphosed frog to complete the standard database.

We have isolated *Vtg A* and *Vtg B* as estrogen-responsive genes. In the present study, *Vtg* gene expressions were investigated in the froglets exposed to estrogen by RT-PCR. The RT-PCR analysis showed that both of *Vtg A* and *Vtg B* were highly transcribed in the froglets exposed to 1 nM E2 for one week. When the froglets exposed to 1 nM E2 for one week were reared in breeding water without E2, the transcripts of the two *Vtg* genes became lower level after two weeks.

All male tadpoles are very useful for development of sensitive bioassay system for assessing and evaluating estrogenic chemicals. Sex-reversed genetic male is needed to produce all male tadpoles by crossing with genetic male. In the last year, sex reversal of the male *X.* (*S.*) tadpoles were induced by exposed to E2 from stage 50 through completion of metamorphosis. The froglets are growing to maturation.

Thus far, we have isolated cDNAs encoding estrogen receptors (ER) α and β, thyroid hormone receptor α and β, and aromatase of *X.* (*S.*) *tropicalis*, investigated their gene expressions in the tadpole during development, and developed reporter gene assay systems of ERs with great help of Dr. Katsu.

(C4.1) Progress and the need for integration of laboratory and field studies concerning ED in amphibians

Daniel Pickford[1], Severine Larroze[1], Minoru Takase[2], Tomohiro Oka[3], Naoko Mitsui[3] and Osamu Tooi[3]

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[2] Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Japan
[3] Institute of Environmental Ecology, IDEA Consultants, Inc., Shizuoka, Japan

Endocrine disrupter research in amphibians is currently taking two paths that need integration. Chemical safety assessment programs require a suit of ecologically representative apical tests for hazard characterization of potential endocrine disrupters, and OECD-coordinated efforts on amphibian testing are currently focused on the West African clawed frog, *Silurana* (*Xenopus*) *tropicalis*. This species has considerable potential as an alternative to the South African clawed frog (*Xenopus laevis*) for ecotoxicological studies, including those on endocrine disruption. Development of (partial) life cycle methods using S. tropicalis anticipates benefits from the shorter generation time and growing availability of molecular markers for this emerging model.

To this end, creating a standard database on development of the *S. tropicalis* tadpole has been identified as a key objective of Project 4 of the UK-Japan Research Liason on Endocrine Disrupter Research. Studies are being undertaken in Japan and UK to document comprehensively the effects of the model estrogen 17α-ethynylestradiol (EE2) on larval development and sexual differentiation of *S. tropicalis*, and the UK-study reported at the 2007 UK-J meeting is now completing its second phase. Histological analysis of gonads sampled at completion of metamorphosis was consistent with observations of gross gonadal morphology reported previously, indicating that gonadal differentiation was affected in all EE2-exposed groups. However, while clearly discernible ovarian structures were evident in all groups, it is possible that male gonadal differentiation was incomplete at the time of sampling. This may demand re-evaluation of the stage 66/completion of metamorphosis intermediate sampling point in any (partial) life cycle study. Assessment of reproductive potential in adult frogs grown out in unexposed conditions for the last year is currently underway, and for males will combine *in vitro* fertilization of control female eggs, sperm count and sperm motility and further histological analysis. Sperm motility optimization studies indicate significant left-right asymmetry in sperm count, while boosting males with gonadotropins seems to have little or negative effect on sperm count, potentially due to evacuation of testes subsequent to boosting. Sperm dilution studies to establish appropriate conditions for in vitro fertilization studies are underway. Availability of molecular markers of gonadal function (in particular ovarian signaling pathways) for *S. tropicalis* would be beneficial in elucidating any detectable impacts on adult reproductive function, and this need could be met through cooperation on the UK-J amphibian project.

Research on test method development in amphibians requires greater coordination with the second area of amphibian ED research, into real amphibian populations as part of

monitoring and evaluation of known or suspected environmental impacts. In assessing the potential for impacts of endocrine disrupters in the aquatic environment on amphibians (namely the Common toad, *Bufo bufo*) in the UK, we have combined environmental sampling of toad breeding sites using passive accumulation devices (SPMD and POCIS), with field-to-lab studies of survival growth and development in larval toads. Endocrine activity has been detected in a number of sites nearby agricultural activity (arable or pasture) using recombinant yeast screen and these findings are corroborated with screening of the same extracts in the *Xenopus* hepatocyte monolayer-VTG induction assay, conducted by our Japanese colleagues at IDEA consultants. Preliminary histological analysis indicates elevated incidence of testicular oocytes in male toad metamorphs from a site characterized by intense arable farming and estrogenic activity in POCIS extracts from the breeding waters. Further analysis of comparable sites is ongoing. Elucidating any robust associations between aberrant gonadal differentiation in sites with detectable ED activity and potential for exposure to ED-active pesticides or other environmental chemicals (eg effluents or animal wastes) will require in vivo studies, using partial life cycle tests. Integrating developments in promising amphibian laboratory models with the need for ecologically relevant methods is a challenge that the UK-J research liason on Endocrine Disruptors should aim to meet though its Core 4 project.

(S1) Initial investigations into the ecotoxicology of nanoparticles in the aquatic environment

Rhys Goodhead[1], Julian Moger[2], Tessa M. Scown[1], Blair D. Johnston[1], Taisen Iguchi[3] and Charles R. Tyler[1]

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[2] School of Physics, University of Exeter, Stocker Road, Exeter, EX4 4QL, United Kingdom
[3] Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Japan

This UK-J (defra)-funded studentship is focused on investigating contaminant effects on fish, including endocrine disrupting chemicals (EDC), novel pollutants such as engineered nanoparticles (ENPs) and their mixtures. The project will assess impacts on endocrine function but also address the wider health concerns of exposure, including effects on immune function and oxidative stress. The project will thus contribute knowledge towards obtaining a greater understanding of EDC effects, develop fate and biological effects information on ENPs in the aquatic environment and help to better understand mixture effects of contaminants that are of divergent natures. The huge growth in the development and commercial application of ENPs has stimulated significant interest worldwide and, in parallel, research activity into their (eco)toxicology is expanding rapidly. Taking into to account some of the lessons learnt from the research into endocrine disruption, we are trying to develop a pragmatic approach to testing and screening ENPs for possible biological effects. Given their very diverse nature (i.e. the very large number of types, shapes, forms and different functionalisations of ENPs) this initially requires a high throughput screening method and in vitro approaches potentially offer this capability.

We have conducted preliminary studies using primary hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*) under standard culture conditions to investigate the toxicity of various metal oxide NPs including, cerium dioxide (CeO_2), titanium dioxide (TiO_2) and zinc oxide (ZnO), as well as copper (Cu) and silver (Ag) NPs. Cytotoxicity (assessed via the measurement of lactate dehydrogenase, (LDH) released into the culture medium) was not evident for any of the NPs studied even at extremely high doses (500 µg/l for 24 hours). Sub-lethal toxicity (assessed via the measurement of malondialdehyde (MDA) to determine lipid peroxidation and consequently oxidative stress) was also measured.

Given the apparent very low toxicity for all of the metal oxide ENPs tested, we then considered the availability of the ENPs to the cultured hepatocytes, and indeed whether the particles were taken up into the cultured cells at all. This was performed using imaging with Coherent Anti-Raman Stokes (CARS) microscopy, a nonlinear optical technique that derives image contrast from intrinsic molecular vibrations within a sample. By exploiting the large nonlinear optical susceptibilities of metal oxides (enhanced by two-photon electronic band-gap resonance), we used CARS to provide localisation of metal oxide nanoparticles with sub-cellular resolution. 3D imaging has provided very strong evidence that, whatever the form of the nanoparticles in the culture medium (we found that much of the ENPs clumped together and precipitated out of the medium), some remained bioavailable and became internalised in the hepatocytes. Research planned in this project on ENPs in the coming year will investigate possible uptake of ENPs from the water into gills (using an in vitro gill cell culture system). In the natural environment the gill (uptake from the water) and the gut (uptake via drinking/feeding) are the most likely routes of uptake of ENPs into fish.

(S2) Ecosystem level impacts of ethinyloestradiol – Lake 260, Canada

Dr. Karen Kidd

Canada Research Chair and Professor of Biology, Canadian Rivers Institute, University of New Brunswick, Saint John New Brunswick

Women taking the birth control pill excrete the synthetic estrogen in their urine; this estrogen is not always completely broken down during municipal wastewater treatment or it is released directly into rivers in untreated sewage. Male fishes downstream of these discharges are being exposed to estrogen and, as a result, are vitellogenin (VTG) or eggs in the more severe cases. Despite widespread evidence for the feminization of male fishes in rivers receiving sewage discharges, it was not known though whether these estrogens were affecting the ability of the males to reproduce and the sustainability of the populations. To address this question, a whole-lake study was done for 7 years at the Experimental Lakes Area in northwestern Ontario, Canada to determine whether the estrogen used in birth control pills (17α-ethinylestradiol; EE2) could affect reproduction of the fish and the organisms that these fish eat. EE2 was added continuously over 3 summers (2001-2003) to achieve concentrations that have been measured in municipal wastewaters (5-6 ng/L); shortly after the additions began, male fish in this lake started producing VTG. In addition, sexual development for both the male and female fish was delayed, and males of one species (pearl dace, *Semotilus margarita*) developed intersex. In the second summer (2002), the shortest-lived fish species in the lake, the fathead minnow (*Pimphales promelas*), stopped reproducing and this lead to a near extinction of the minnow from the lake. However in 2006 this population recovered, 3 years after the EE2 additions stopped. The longer-lived species also declined in numbers in 2003 and 2004 either through direct effects of the estrogen (for pearl dace) or because of a loss of their main prey the fathead minnow (for lake trout, *Salvelinus namaycush*). In contrast to the fathead minnow, their numbers did not decrease as dramatically. Results from our whole lake experiment show that the estrogen in birth control pills can control both human and fish reproduction, and that fish at greatest risk from these compounds in waters are the ones that have a short lifespan.

(S3) Testing Methods for Invertebrate's Endocrine Disrupting Chemicals using Cladoceran Crustaceans, *Daphnia magna*.

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The invertebrate occupies 95% of all the animal species inhabiting on the earth, among which 90% are "the Arthropods such insects or Crustaceans" bearing crust as the external skeleton, growing up while undergoing repeated ecdysis. These insects require metamorphosis with ecdysis and accomplish their life.

The mechanism of the hormone of invertebrates differs from that of vertebrates, and is thus considered to have divergent hormone disrupting mechanism by endocrine disrupting chemicals. It is known that ecdysis and the metamorphosis are controlled by molting hormone (ecdysteroid) and the juvenile hormone, which are periphery hormones secreted by an epithelial endocrine organ. The ecdysteroid induces ecdysis, and juvenile hormone contributes to the continuance of the growth stage. These hormones are peculiar and universal to invertebrates and participate in reproduction, embryogenesis, control of the dormancy, as well as ecdysis, a metamorphosis.

The mechanism such as the ecdysis, the metamorphosis, the reproduction, or the breeding is unique for every invertebrate species, and these varieties are attributed to two kinds of erasion hormones mentioned above. The ecdysteroid is a type of the steroid hormones, but its chemical property is different from the steroid hormone of the vertebrates. The juvenile hormone is called sesquiterpenoid, and the detailed function is hardly elucidated.

Daphnia is a kind of the Crustacean and is also known to have ecdysterone and juvenile hormone. Currently,,Daphnia is approved as test organism of the endocrine disruptor screening method of the invertebrates by OECD.

Here, we would like to introduce in detail, the process of the development of this test method. The ring tests were conducted twice, and various background data were prepared for this process. However, a great proportion of this process, such as the mechanism of juvenile hormone and the peptide hormone of Daphnia and other invertebrates, which are not yet scientifically elucidated, must wait for a future study.

(S4) Putting Science into Policy

Dr Mike Roberts

Defra, UK

The process by which Governments translate their political vision into programmes and actions to deliver the changes they desire in the real world is known as policy development. To be effective, this process should have a sound evidence base. Science therefore underpins policy development, for which there are several examples regarding endocrine disruption research, such as the environmental impacts of tributyltin and oestrogenic sewage effluents, as well as the development of methods for chemical assessment. How should we continue to ensure that the maturing endocrine disruption science base in general, and the UK-Japan partnership in particular, continue to underpin the development of chemicals policy?

(P1) Estrogen receptor Expression in American Alligator Oviduct

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Estrogens are principally important for reproductive function in female vertebrates including reptiles. The majority of actions of estrogens are mediated by specific receptors in the target cells. In American alligators, cDNAs encoding estrogen receptors (ERs) have been cloned, and messenger RNA of ERalpha has been demonstrated in the liver, gonads and other organs. We have recently made an antibody against American alligator ERalpha. The antibody, together with a commercial antibody (MC20, Sant Cruz Biotechnology, Inc), was applied to immunohistochemistry in the oviduct of juvenile American alligators (2-year-old). Positive staining of ERalpha was evident in nuclei of epithelial, stromal and muscular cells in all parts of the oviduct, regardless of the type of antibodies. Intensity of staining was approximately the same between two antibodies. Two anti-ERalpha antibodies are useful for immunohistochemical demonstration of ERalpha in the alligator tissues.

(P2) Embryo toxicity in Japanese medaka and zebrafish exposed to endocrine disrupters, pesticides, and PPCPs

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Fish embryo toxicity is one of the most important issues in ecological risk assessment. Especially in European Union, its testing is remarkable as the alternatives of previous test method such as adult acute toxicity test (OECD TG203) from the stand of animal welfare proposed in REACH. Thus, the fish embryo toxicity testing will be an important test method in world wide chemical risk management, but its information of FET is less than those of adult acute toxicity test in recommended fish species in OECD test guideline such as Japanese medaka *Oryzias latipes* and zebrafish *Danio rerio*. Therefore, we examined the developmental toxicity in embryo and larva in Japanese medaka and zebrafish exposed to 8 chemicals including endocrine disrupters, pesticides and its related chemicals, and pharmaceuticals and personal care products. To compare the sensitivity to chemicals during early developmental period, fish embryo toxicity test (FET) was conducted with exposure during embryo and larval period (10days for embryo and 7days for larva), and fish eleuthero-embryo toxicity test (FEET) was conducted with exposure of newly hatched larva (fish within 24 hours after hatching) for 4days. LC50 and EC50 in FET were changed in each species, chemical, and test period. Therefore, to find the more sensitive phase, we compared the LC50 values in medaka for TG203 (Adult 96h), FEET (eleuthero-embryo 96h), and FET (96h after fertilization, hatching, 7days post hatch). At 96hours, LC50s in FEET indicated was within 50% range of those in adult TG203, but those in FET, most chemicals performed that LC50 at 96hours in embryo was higher than those in adult. In most chemicals, LC50 values at hatching was still low sensitive than those in TG203 (Adult 96h), but the LC50 values at the end of testing (7days post hatch) was higher sensitive than those in adult or eleuthero-embryo at 96hours. On the other hand, the result in FEET indicated the quite similar sensitivity with LC50 values of TG203, and FEET is the suitable testing in Japanese medaka as the alternatives of adult acute toxicity test such as OECD TG203. Using fish eleuthero-embryo can reduce the cost to manage the number of test fish rather than adult fish, and it can contribute to reduce the waste water in each testing, this advantage may be also better on the point of animal welfare.

(P3) Mentoring the Next Generation of Scientists

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In the United States, there is current emphasis on undergraduate recruitment and education in the sciences. This movement is being promoted by major funding organizations, including Howard Hughes Medical Institute (HHMI) and the National Science Foundation (NSF). We are currently in our second year of an HHMI funded program called GATOR, or Group Advantaged Training of Research. Our program pairs undergraduate researchers with graduate student mentors in a two-pronged approach to mentoring that benefits both undergraduates who plan to go to graduate school, and graduate students who will pursue academic careers. Quantitative evaluations from our first year indicate that, compared to the national average, our undergraduates show significantly improved gains in critical scholarly areas, such as ability to read primary literature, give oral presentations, integrate theory into practice, and self confidence (based on the HHMI-supported national SURE survey). The majority of our undergraduates have pursued a second year of research experience. Graduate students in the GATOR program report improved confidence in mentoring and appreciation of the importance of a good match between mentor and mentee. Goodness of match is affected by expectations, goals, and personalities of both members of the mentoring relationship. The details and accomplishments of the GATOR Mentoring Program will be presented.

(P4) Sexual Re-programming and Oestrogenic Sensitization in Wild Fish Exposed to Ethinyloestradiol

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It is now well established that a variety of natural and man-made chemicals mimic hormones thus modulating endocrine systems. As result they cause disruption in sexual development and impact on reproduction in wildlife species. In fish, sex determination and sexual differentiation are controlled by a delicate balance of genetic and environmental factors, and any alteration by exogenous influences, including endocrine disrupting compounds, can ultimately impact on sex assignment, even in gonochoristic species. In this study we conducted exposures of roach (*Rutilus rutilus*) to environmentally relevant concentrations of the contraceptive oestrogen 17α-ethinylestradiol (EE2) for up to two years, to investigate the impacts of long-term exposure on reproductive development. After sexual differentiation was completed, a sub-population of these fish was depurated for 400 days in clean water before being re-challenged to EE2 in order to assess how this exposure during early life subsequently impacted their responsiveness to oestrogen in later life. Long-term exposure of roach induced of a range of feminizing effects on gonad histomorphology, VTG induction, and transcription of key genes involved in oestrogen signalling. Exposure to 4 ng/L EE2 resulted in sex reversal of males leading to an all-female population. Exposure to environmental concentrations of EE2 during early life induced more subtle disruptions on normal sexual programming, but also altered the subsequent responsiveness to estrogens in later life, shown by a clear sensitization effect on vitellogenic responses and transcription of key genes involved in oestrogen signalling.

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The History of Bovey Castle

We shall perhaps never know what it was that first interested William Henry Smith to buy 5,000 acres of Devon land and property in 1880. It could have been something to do with Isambard K Brunel and his magnificent plans for the Great Western Railway. W H Smith had built up his successful chain of bookshops and, astute businessman that he was, he may have foreseen the impending rise in land prices that would follow the opening up of the Westcountry by the Railways in general and Great Western Railway in particular. It is certain that he did not buy the land to obtain a country seat as the land he bought was not graced by any Manor House but consisted of farms and dwellings in the parishes of North Bovey, Moretonhampstead and Lustleigh, and included the village of North Bovey.

If he had plans to build on his Devon estate they may have been shelved in the hurley burley of a busy life. Apart from being a very successful businessman the Rt. Hon. William Henry Smith was an able and respected politician who obtained Cabinet rank and became a close friend of Lord Randolph Churchill. He was MP for Westminster 1868-1885 and for The Strand 1885-1891 and became First Lord of the Admiralty (lampooned as Sir Joseph Porter in *HMS Pinafore*), First Lord of the Treasury, Lord Warden of the Cinq Ports and Leader of the House of Commons.

In 1891 W H Smith was to have been created the first Viscount Hambleden by Queen Victoria, who had developed great respect for him. However he died before the honour could be bestowed upon him and the title of Viscountess went to his widow and his son, William Frederick Danvers Smith born in 1868, became the second Viscount. It was Frederick, as he was know to his family, that took the decision to build the Manor House. He commissioned Walter E Mills, a well-known architect and Lewis Bearne, a master builder in Newton Abbot. The Architect was given a free hand and the splendid mansion

was built with a complete disregard for expense in the style of a Tudor castle, using fine quality stone, which blended in with its wonderful setting overlooking the valley of the River Bovey. Mr Mills was also the architect responsible for the Rectory and the restoration of Moretonhampstead Parish Church 1904-1905. It is possible that the masterly way in which the restoration of the Parish Church was carried out attracted Viscount Hambleden to award him the commission for the Manor House. The Hambleden Coat of Arms is executed in stained glass on a screen inside the main entrance, emblazoned with the family motto 'Deo Non Fortuna Fretus' – 'Relying on God, not on Fortune'. It can also be seen on the Jacobean carved stone chimney piece in the oak-panelled banquet hall, now the Cathedral Room.

The Cathedral Room is of particular interest; measuring 43 feet x 33 feet, it had a lofty roof span with oak beams, open fireplace and carved stone chimney piece. An oak screen partly shut off one end and from here an oak staircase rose to a Minstrels' Gallery. The Adam Drawing Room was a beautiful replica of the period as was the fine Dining Room, entirely panelled in Jacobean patterned oak with open stone fireplace, carved chimney piece and enriched ceiling. When completed in 1907 this was certainly one of the most beautiful of the smaller manor houses in the Westcountry.

The association of the Hambleden family with the area lasted for nearly fifty years and they were great benefactors to North Bovey.

In 1920 the Parish Church was the subject of considerable restoration work at the expense of Lord Hambleden who was patron of the 'living' and the Parish Hall was built by him and presented to the village in 1925. However, the family were only in residence for a few weeks in any year, spending most of their time at their estate of Greenlands near Henley-on-Thames. There was a small permanent staff kept at the Manor, including four gardeners, but when the family visited the Manor they would bring their own domestic staff with them.

In those days the motor car was in its infancy and it was the heyday of the Railways with branch lines into the countryside. Such a line was the branch from Newton Abbot to Moretonhampstead – a beautiful run up the valley onto the moor. Family members would arrive to be met by the Manor coach and prepare to take them to North Bovey Manor.

On 16th June 1928, at the age of sixty, Lord Hambleden died and it was decided to sell the whole estate by auction. This took place at the Globe Hotel, Newton Abbot on November 8th later that year. This proved to be the end of the Hambleden family association with Devon as the estate was parcelled into 132 lots and sold, for the most part, to the tenants who attended the sale. At the sale the most important lot was, of course, No.67 the Manor House, together with its two lodges, stables and 193 acres. However the highest bid reached only £16,750 and the property was withdrawn. It was subsequently sold by private treaty to a Mr Bartlett of Bideford who re-sold it in 1929 to the Great Western Railway Company. It was this company that developed the property as a hotel and constructed the

eighteen-hole golf course, designed by J W Abercrombie. The course was ready for play in June 1930 with a yardage of 5,600. It was not until 1956 that work, was started in remodelling and extending the course to a playing length of 6,260 yards.

It could be said that the Great Western Railway Company had acquired the Hotel towards the end of the great railway era for now the motor car was becoming increasingly popular, with the mobility to penetrate into the very heart of the countryside. It is interesting to reflect what realists the Railway directors must have been to recognise this fact and to purchase the hotel, which was not served by the Railway. It is true the branch line to Moretonhampstead was still active at this time and some guests would arrive to amble along the Devon lanes in the hotel taxi.

In 1935-1936 the building was extended but happily maintained the architectural style using matching granite stone. The picture that follows shows the hotel as it was planned. In fact although seventeen bedrooms, a new cocktail bar and dining room, together with squash and badminton courts, were added at this time, the end gable shown on the left was not completed. Perhaps if the war had not intervened in 1939 this final phase would have been added, but it was not to be …… yet.

An interesting point is that when the extension was put up it was necessary to move the massive entrance gate piers and archway with their heavy oak studded gates. Such is the degree of permanence engendered by the architectural style that if you look at these today it is hard to imagine that they have not always been in their present position albeit with the new wrought iron gates as opposed to the original oak gates.

The hotel has had a quiet history. There have been no serious fires, robberies or other catastrophic events to spoil the peace and quiet of the lovely setting which remains very much the same as when the house was completed more than 90 years ago. Since it became a hotel Bovey Castle has welcomed many interesting guests from all over the world. In the 1930's Herr Von Ribbentrop would visit the hotel with his retinue of staff and bodyguards. It used to be good business when the massive Mercedes cars arrived to fill up and change tyres at the local garage and it is said that there was a bodyguard on duty outside the hotel as well as outside his bedroom. Many golf personalities have visited the hotel and played golf on the course, including the famous Henry Cotton.

It is surprising that more film crews have not sought out the Bovey Castle as a setting for medieval drama for it rises like some Tudor castle from a Moorland scene. It did feature in the memorable English version of the film 'Hound of the Baskervilles' and was the setting for the historical film 'Knights of the Round Table'. In recent years it has been used as a location for the series 'Down to Earth' starring Pauline Quirke and most recently has played host to the cast of the 2004 Hollywood version of King Arthur starring Kiera Knightley and Clive Owen.

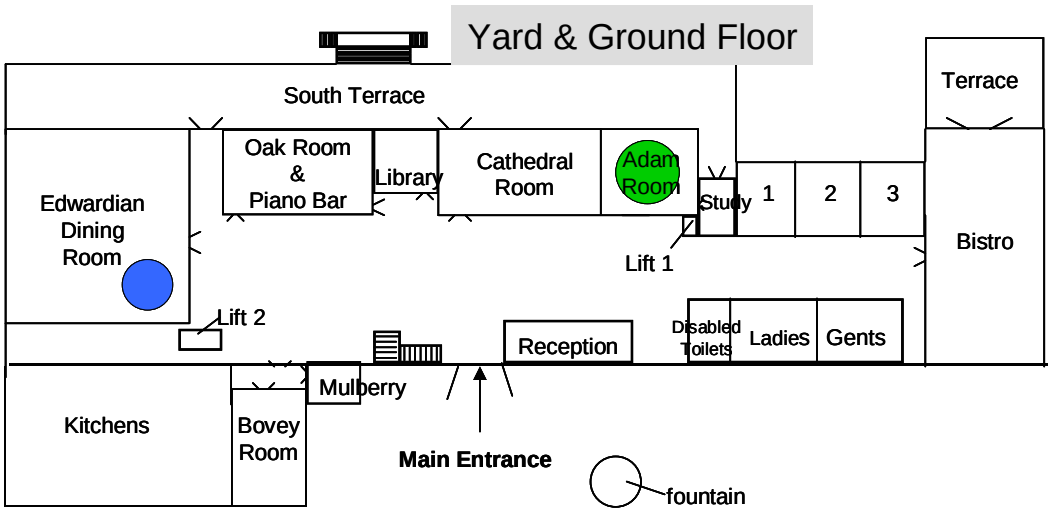
In the First World War, North Bovey Manor as it was then, became a convalescent home and when war came again in 1939 the then hotel was requisitioned by the army to become a military hospital for American, Canadian and British Officers. These wartime visitors must remember the house in a very different guise from that enjoyed by the family who built it and the guests who have subsequently stayed in it.

1946 saw the return of the property to its owners, Great Western Railways, and its reopening as a hotel again. With nationalisation of the railways in 1948 the hotel came under the control of the British Transport Hotels Ltd, inevitably fondly known as 'BTH'. It was under their direction that the alteration of the Cathedral Room took place in order to provide a modern conference facility in the form of the Bowden Room.

Change of control came in 1983 when Government policy saw the sale of the British Transport Hotels into private ownership once again and the opening of a new chapter in the history of the Castle. Principal Hotels, a private company based in Harrogate, acquired the hotel in 1991 and since then considerable expansion has taken place with the addition of 18 bedrooms in the Mews, the conversion of the North Lodge into a guest cottage and renovations to the roof and interior rooms. The golf course having slipped into disrepair, received an injection of investment and expertise and was proudly referred to as one of England's finest inland courses, and a venue for the West of England PGA Championships.

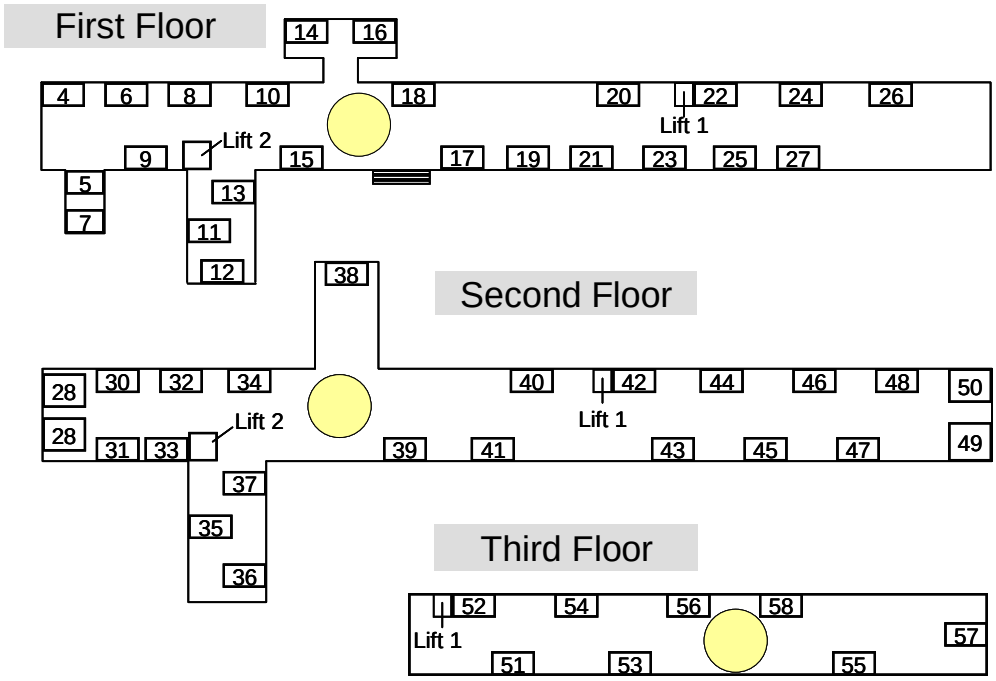
The most recent development of Bovey Castle was when the "Manor House Hotel and Golf Course" as it was then known was purchased by the entrepreneur Peter de Savary in 2003. Building on his success as the founder of the Carnegie Club at Skibo Castle Mr de Savary, originally from the West Country himself, saw the potential of the existing building and Estate and set about transforming the hotel into the five star luxury you see today. Over £30 million has been spent in the restoration of the Castle, the Old Course and the Estate with the addition of a new wing to incorporate a Clubhouse, indoor swimming pool and luxury Spa facility. Bovey Castle opened its doors under this new owner in April 2004 and was awarded a 5 star rating by both the AA and the RAC in March 2005, the first hotel in the West Country in over 50 years to receive such recommendations.

Bovey Castle Internal Map



Key

- Dartmoor Suite – Conference Room, Reception (Sun), Coffee breaks
- Adam Room – Dinner (Mon)
- Bedrooms
- Edwardian Dining Room – Breakfast & Lunch (Mon)

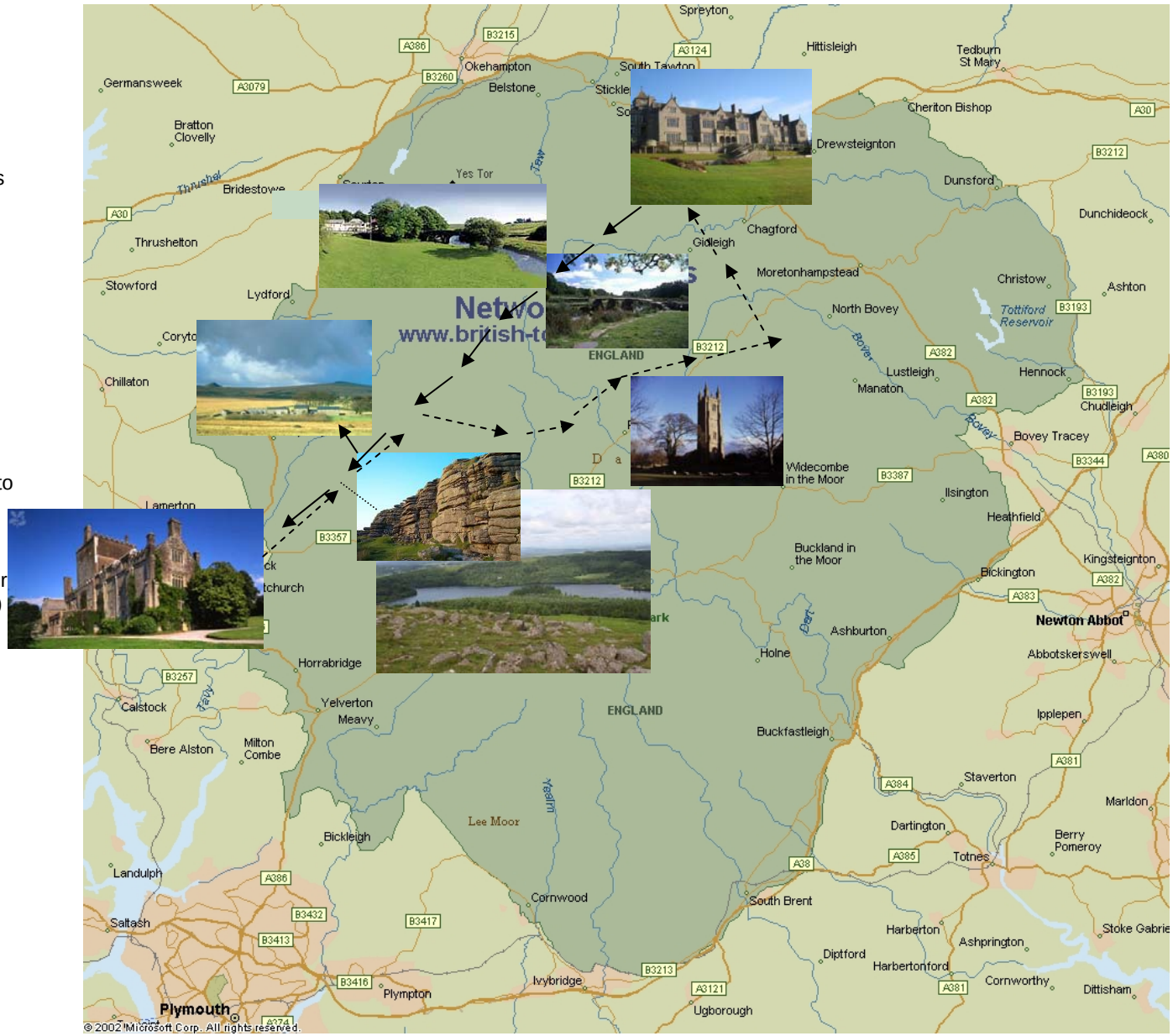


This map is not to scale

Dartmoor

Rout

- ❖ Bovey Castle
- ❖ Post Bridge
  Clapper bridge and visitors centre)
- ❖ Two Bridges
- ❖ Princetown
- ❖ Dartmoor Pottery
- ❖ Hike above Burrator Reservoir (optional)
- ❖ Buckfast Abbey home to Elizabethan seafarers Drake and Grenville
- ❖ Widecombe in the Moor (Dinner and scenic village)



Outward

Return

Wal