

ENDOCRINE DISRUPTION IN THE MARINE ENVIRONMENT (EDMAR)

Report of the 2nd Annual Seminar – 17th April 2000

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Transport and the Regions, Great Minster House,
London**

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The Endocrine Disruption in the Marine Environment (EDMAR) research programme is a joint initiative between UK Government and European Industry. It is funded by the Department of the Environment, Transport and the Regions (DETR), the Ministry of Agriculture, Fisheries and Food (MAFF), the Environment Agency, the Scotland and Northern Ireland Forum for Environmental Research (SNIFFER) and the European Chemical Industry Association (CEFIC). Research is carried out at a number of institutions around the UK, namely the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) laboratories at Weymouth, Lowestoft and Burnham-on-Crouch, the Plymouth Environmental Research Centre at the University of Plymouth, the Centre for Marine and Coastal Studies at the University of Liverpool, the FRS Marine Laboratory Aberdeen (with assistance from the Scottish Environment Protection Agency) and the AstraZeneca Environmental Laboratory Brixham. Associated research is being conducted at Glasgow Caledonian University.

WELCOME AND INTRODUCTION

Dr Mike Roberts (DETR) opened the second annual seminar and welcomed participants. The aims of the meeting were to present the results obtained to date, identify the challenges ahead and afford the opportunity for interested parties to ask questions directly to the researchers involved in the programme.

Dr Peter Matthiessen (CEFAS Burnham - EDMAR Project Manager) outlined the aims of the EDMAR programme and summarised overall progress so far. EDMAR aimed to determine whether endocrine disruption (ED) was occurring in marine organisms and, if so, identify the causal substances. This involved the development of new biomarkers for oestrogenic and androgenic exposure, identification of the sources of any observed endocrine disruption, investigation of effects in crustacea and the construction of models for predicting the impact of any observed effects at the population level of ED.

Developments since the last seminar in April 1999 were summarised as follows:

Biomarker Development:

- Stickleback spiggin and epithelial cell height assays have been developed to detect androgen exposure and were now operational;
- Crab and shrimp vitellin (VT) assays were also operational, but vitellin has been found not to be sensitive to oestrogen exposure in these groups;
- Histochemical assays for oestrogen receptors and vitellogenin (VTG) in flounder were also being developed;
- Gene probes for VTG messenger RNA (mRNA) in sand goby and viviparous blenny have been successfully developed and were now operational.

Laboratory Experiments:

- Reproduction experiments with the marine amphipod *Chaetogammarus marinus* have been suspended, since no suitable biomarker was available;
- Experiments with sand goby have shown that 6ng/l ethinyloestradiol (EE2) interfered with reproductive success in this species;
- Irvine Valley sewage effluent did not affect sand goby reproduction at the concentrations tested (0.3 and 0.03%);
- Both EE2 and octylphenol induced male sand goby VTG mRNA.

Biological Responses in Estuaries:

- Wild flounder from some industrialised estuaries still exhibited strong VTG induction, indicating that oestrogen exposure was still occurring;
- Wild caught sand gobies have shown no VTG mRNA induction or intersex so far, but male gobies in some estuaries have exhibited possible feminisation of secondary sex characteristics;
- Viviparous blennies caught in some estuaries have shown induction of VTG mRNA;
- One case of blenny intersex has been documented;
- Caged stickleback work was currently in progress for androgen assays.

Causative Substances and Sources:

- Toxicity Identification and Evaluation (TIE) procedures, deployed on the Tyne and Tees estuaries, have identified 3 natural and 2 industrial oestrogenic materials;
- High oestrogenic activity has been measured in sediments, but the causative chemicals have not yet been firmly identified;
- Caged flounder in the Tees have shown no VTG induction after 3 weeks exposure;
- Further experiments with flounder were in progress, to identify whether feeding provided an exposure route for endocrine disruption.

Although considerable progress has been made, results have not always been predictable and some entirely unexpected discoveries have occurred. This was in the nature of scientific research and to be welcomed, but it implied the need to re-deploy some of the remaining EDMAR resource into the most apparently fruitful areas. This was a matter for the EDMAR Steering Committee, but questions which needed to be addressed included:

- Should the crustacean work be put on hold in the absence of responsive biomarkers?
- Was viviparous blenny the most suitable fish species to use for measuring reproductive output, in order to feed into the population modelling work?
- Should the population effects of tributyltin (TBT) in dogwhelks be modelled?
- Would it be important to develop an understanding of the functional significance of feminised secondary sex characteristics in male gobies?
- Could gobies be used to determine the effects of defined mixtures?

One change which has already been put into effect was the use of sea trout instead of salmon in the study on migratory salmonids in the Tees.

EDMAR Contractor Reports*(Note: ^P denotes presenting author)***A. BIOMARKER DEVELOPMENT****Objective 1. Development of a biomarker of androgen exposure in a suitable marine species.***Ioanna Katsiadaki^P and Sandy Scott, CEFAS Lowestoft**Abstract*

A dose response for methyl-testosterone has been constructed, based on spiggin induction in the female stickleback kidney. This was assessed histologically through measurements of the kidney epithelium height (KEH). Flutamide, at a concentration of 500 ng/l of aquaria water, inhibited or reduced the kidney stimulation induced by methyltestosterone (MT) at concentrations between 1 and 10µg/l. The results of the effect of nonylphenol on the stimulation of the stickleback kidney were not conclusive. Further development of the Enzyme-Linked Immunosorbent Assay (ELISA) gave satisfactory results regarding the specificity of the antibody against spiggin. The antiserum obtained from the last boost immunisation of the rabbit (277/2) gave the strongest reaction with the spiggin-coated ELISA plates. A pool of spiggin from several nests was created to use as a coating material for the ELISA plates and combining hypertrophied kidneys and urinary bladders from breeding males made another spiggin standard pool. The latter was given the arbitrary value of 100,000 IK units per ml. In this way a smooth standard curve was obtained. The validation of the developed ELISA for spiggin involved the comparison of the histological method KEH with the spiggin units per whole kidney, using half of the kidney for each method. An excellent correlation was obtained. The glue protein of the 15-spined stickleback has been isolated and its molecular weight was found to be identical to spiggin and equal to 203kDa. Some cross reactivity of this protein with the spiggin antiserum was observed.

Presentation

The reasons for concentrating this particular work on the three-spined stickleback (*Gasterosteus aculeatus*) included the ubiquity of the species in the aquatic environment, ease of maintenance in the lab, short life span, well documented reproductive behaviour and biology and pronounced androgen-dependent male sexual characteristics during the breeding season.

Typical thin sections of non-breeding and breeding male kidneys exhibited the increase in kidney epithelial cell height, one of the assays used to measure androgenic effects. This increase in height was due to the production of the nest glue protein, spiggin. Exposure of males and females to the androgen methyltestosterone for three weeks stimulated increases in KEH, and the anti-androgen flutamide was shown to block this androgenic effect. Although KEH was a good assay to use, it was very time consuming and therefore unsuitable for assessing the field samples.

A specific ELISA for the nest glue protein spiggin was therefore developed, involving identification of the protein by SDS-PAGE techniques, preparation of antisera by injection into rabbits and the preparation of standard dilution curves.

Female stickleback treated with MT for three weeks showed a significant increase in spiggin production at concentrations greater than 1µg/l. Again, flutamide inhibited this kidney stimulation.

To validate the ELISA technique, it was compared with the KEH and an excellent correlation was observed. Both assays appeared to be similar in sensitivity.

Material from the nests of male 15 spined stickleback was collected and subsequently identified as the glue protein spiggin. This was shown to have significant cross reactivity with the 3 spined stickleback glue protein antiserum.

In comparison, the immunoassay had several advantages over the histological assay, including rapidity (3 days versus 4 weeks), sensitivity (100 000-fold variation versus 4-fold variation) and specificity (the KEH may be influenced by other factors).

Discussion/Questions:

David Pascoe: Were the observations androgen specific?

Ioanna Katsiadaki: *They seem to be androgen specific at the moment, but other chemicals may be found to induce the same results. Nonylphenol has been shown to stimulate epithelial height, but this may have been due to contamination from MT in nearby tanks (500mg/l). It is more likely to be a toxic effect, but additional experiments are needed.*

Tom Hutchinson: An androgen such as MT will actually cause a feminising effect, due to aromatisation to oestradiol. It would be worth doing experiments using MT and nonylphenol in combination.

David Pascoe: Previous work has shown that cadmium inhibits nesting behaviour. Have you looked at nest building behaviour at the concentrations of MT tested?

Ioanna Katsiadaki: *Treated females developed male breeding colours and attempted to build nests. In treated tanks, all males built nests, whereas in control tanks only a couple behaved in this way.*

Objective 2. Development of biomarkers to oestrogen and androgen exposure in marine crustaceans

Shaw Bamber^P, Malcolm Jones and Mike Depledge, Plymouth Environmental Research Centre (PERC), University of Plymouth.

Abstract

The first nine months of the project culminated in the development of an ELISA for shore crab (*Carcinus maenas*) vitellin. This test method has now been used to assess vitellin content from male and female crab haemolymph, sampled as part of field evaluations both locally to Plymouth and nationally as part of the widespread EDMAR sampling programme. In addition, a number of laboratory based exposure trials have been conducted, assessing the impact on crabs of contaminants that have been shown to interfere with vertebrate endocrine systems. The lack of cross-reactivity of antibodies raised against *Carcinus* vitellin to brown shrimp (*Crangon crangon*) vitellin necessitated the raising of a second antibody. Anti-sera to vitellin purified from *Crangon crangon* ovaries arrived at PERC in May 1999 and Western blotting indicated the antibody to be specific to *Crangon* vitellin. An ELISA, following similar lines to that generated for shore crabs, has been developed and used to assess vitellin content in shrimps sampled as part of the EDMAR programme.

It appears from the analyses carried out to date that vitellin is not induced in natural populations of crabs collected from estuarine areas, including sites within a few metres of a domestic sewage outfall. Haemolymph samples have been obtained

from shore crabs collected from a total of 5 estuarine sites in the Plymouth area, three of which can be considered polluted. Analysis has shown that of 70 female haemolymph samples tested, 51 contained vitellin, whereas none of the 117 male haemolymph samples tested indicated the presence of the protein. Analyses of the samples collected as part of the EDMAR national sampling effort are almost completed. Of 339 males tested, only 1 gave a positive result, while 118 of 181 females tested gave a positive response.

Results from laboratory exposures of male shore crabs to technical grade nonylphenol (supplied by AstraZeneca) and diethylstilbestrol have indicated that neither chemical, at the concentrations used, induced vitellin production in male crabs. Furthermore, physiological assessments indicated that exposure to these chemicals did not significantly interfere with the control of osmoregulation or heart rate, both processes known to be under endocrine control in crustaceans (Kleinholz and Keller, 1979; Kleinholz, 1985).

The search for potential biomarkers of androgenic disruption in crustaceans has centred on experimental procedures using male shore crabs infected with the parasitic barnacle *Sacculina carcini*. Male crabs afflicted with these parasites undergo various degrees of feminisation. It is thought that the barnacle suppresses the secretion of hormones from the androgenic gland which are responsible for the development and maintenance of the masculine state, thus effectively mimicking an anti-androgenic chemical. Both external and internal structural changes occur in affected male crabs, with the most obvious change being the presence of a female form abdomen on the male crab. The biology and life cycle of these parasitic barnacles have been reviewed by Høeg (1995). Twelve infected crabs were purchased from Millport research station, with a further seven infected males obtained from the EDMAR sampling programme. Haemolymph samples taken from infected crabs have been assessed using SDS electrophoresis and the vitellin ELISA; none of the samples provided a positive indication of the presence of vitellin.

Protein extracts from the eggs of *Chaetogammarus marinus* (live animals supplied by CEFAS Burnham-on-Crouch) have been prepared and subsequently analysed in ELISA tests to assess the extent of cross-reactivity with antibodies raised against crab and shrimp vitellin. There was no indication of inter-specific cross-reactivity in any of the tests conducted.

Investigations into potential endocrine disruption in the brown shrimp *Crangon crangon* have also been undertaken. The generally small size of shrimp precludes the efficient extraction of haemolymph possible in shore crabs, so other methods needed to be found to generate a suitable sample to add to the microtitre plate wells. In addition to problems associated with size, shrimps do not show obvious sexual dimorphism and need to be examined under a microscope to determine sexual status. *Crangon* are protandrous hermaphrodites and all stages of sexual development, male, intermediate and female, will typically appear within a single population. A sample of shrimp obtained from Brixham Environmental Laboratory was sized, sexed and assessed for vitellin content using the new protocol. There was no indication of vitellin in any of the twenty four males assessed and only one in the twenty two intermediates examined. A positive indication was given in the majority of females tested, principally those carrying eggs. Shrimps from the EDMAR sampling programme have also now been assessed. From 306 males, a single individual gave a positive indication of vitellin. From 156 individuals of intermediate sexual status, 9 gave a positive result, while 54 females tested positively out of 156.

Laboratory based studies have recently commenced which are designed to assess the influence of exogenous chemicals on the process of vitellogenesis in female crabs. The ELISA developed against crab vitellin can be used to track to progress of vitellin changes in the haemolymph of sexually mature females. In the first set of experiments, crabs were exposed to solutions containing the vertebrate hormone oestradiol and the crustacean ecdysteroid, 20-hydroxyecdysone. This crustacean hormone is involved in many of the regulatory processes in crustaceans, including moulting and reproduction (Fingerman, 1987).

The purpose of these exposures is to determine if exogenous chemicals, that could potentially mimic the action of this ecdysteroid, can influence the normal endogenous hormonal processes of the crab. Further exposure experiments using both male and female crabs are planned in which processes, including moulting and endogenously controlled rhythmic locomotor activity, will be assessed.

Presentation

The results obtained with the development of a crab and shrimp ELISA for vitellin were summarised. For the crab ELISA, this involved an evaluation of local pollution 'hotspots' near Plymouth, laboratory experiments with known oestrogens and studies of androgenic effects. Vitellin was found to be absent in male crabs, but present in females. As there was no cross reactivity of crab vitellin with shrimp vitellin, a separate ELISA was developed from purified shrimp vitellin.

For the field work, 5 sites were chosen – Bantam (clean), Yealm (intermediate), Plym (polluted from a landfill leachate lagoon), Pomphlett Creek (polluted - receives treated waste water) and Hooe Lake (polluted by treated waste water). Crabs were collected by baited drop net, which is quick and exerts minimal stress to the animals; a haemolymph sample was taken and immediately frozen. No vitellin was found in male crabs from either the clean or polluted sites, but 51 of the 70 females contained vitellin.

Male and/or female crabs from the clean location were exposed in the laboratory, via water, to a range of chemicals known to have oestrogenic properties: diethylstilbestrol, nonylphenol, ethynylöestradiol and the invertebrate hormone hydroxyecdysone. Physical processes such as heart rate and osmoregulation were also examined, as well as vitellin production. No vitellin was found in males and the pattern of effect was inconclusive for females. No effects were found with the physiological measurements.

Further work was being carried out to assess the anti-androgenic effects of the parasite *Sacculina carcini*, which suppresses crab moulting and destroys the androgenic gland. Samples of male crabs feminised through such parasitism contained no vitellin.

The field survey samples collected from estuaries around the UK also revealed no vitellin in male crabs or shrimp. In short, there is no parallelism of vitellogenesis with fish in either crabs or shrimp, which is probably due to the different chemical structure of the proteins and receptors, but other endocrine processes could be affected. Future work will include laboratory exposure of these species to anti-androgens, exogenous sources of crustacean hormones and an investigation of the potential effect of vitellogenesis in females.

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Discussion/Questions

Tom Hutchinson: Could you clarify what a positive response is in the ELISA?

Shaw Bamber: *A green coloured product is formed – it is a yes/no response and not yet quantifiable. We are looking at quantification for use with females.*

Jim Readman: Have you considered selecting another group of organisms to study, for example a sediment dweller, as sediments can contain higher amounts of contaminants?

Shaw Bamber: *Annelids could be worked on – Platynereis can be cultured in the laboratory; also, molluscs, from the TBT angle, would be interesting to look at.*

Rick Leah: Is there any evidence of endocrine disruption in fish from the sites you sampled for crab?

Shaw Bamber: *Not from the local sites, but surveys of the larger estuaries are also covered by Objective 8, which found endocrine disruption in some of them.*

Geoff Brighty: Are there any other studies that provide evidence for a positive result of ED with Carcinus? Isn't there a study from North America?

Shaw Bamber: *There is not a great deal of evidence – there has been one case of intersex documented, but this is doubtful.*

Objective 3. Develop simple histochemical methods for measuring vitellogenin/vitellin, oestrogen and androgen biomarkers in small tissue slices of fish and crustacea.

Rick Leah^P, Mike Simpson, Pete Walker and Anne Kleinkauf, CMACS, University of Liverpool.

Abstract

Tissues (liver and gonad) from a large number of flounder have been processed to test the immunocytochemical methods for vitellogenin developed during this project, using material obtained from male and female flounder which exhibited a wide range of vitellogenin concentrations in blood plasma. These include fish exposed to oestradiol in the laboratory as well as male and female fish from the estuaries of the Mersey and the Welsh Dee. Soluble-compound autoradiography, using radio-labelled oestradiol, has been successfully utilised to locate sites of oestrogen binding for comparison with sites visualised with immunohistochemistry. Positive staining using immunohistochemical methods has been achieved in stickleback tissues known to contain the sex-specific protein, spiggin. This methodology could now be fully trialled as a biomarker method for androgen exposure in female sticklebacks. Materials and reagents are now available to test for localisation of vitellin in *Carcinus*

and *Crangon* when desired. This includes whole *Carcinus*, which have been successfully decalcified and embedded.

Presentation

The immunocytochemical methods being developed offered the potential to supplement or replace the radioimmunoassay. They could be used to see what is happening at the reaction site *in situ* and to compare the concentration of VTG in blood with that of the liver. The methods developed for flounder involve colour localisation of vitellogenin and the oestrogen or androgen receptors on thin sections of tissue – a paraffin embedded method is currently being compared with fresh frozen cryostat material; this latter method may provide a better visualisation of the vitellogenin antibody reaction.

Samples of stained female and male liver hepatocytes from fish from the Dee and Mersey exhibited varying amounts of plasma vitellogenin. The hepatocytes stained positive for VTG, but the intensity of staining showed a degree of variation, which is being analysed. Particular attention is being focused on whether there are consistent differences in staining between males and females within and between the two estuaries. It is unclear as yet whether there is any relationship between plasma VTG levels and immunostaining of VTG – a confounding factor may be the systemic nature of the plasma VTG.

The methods deployed for localisation of the oestrogen receptor in Dee flounder used soluble compound autoradiography and antibody to trout oestrogen receptors. The locations of the receptor in flounder liver, testicular germ cells and secondary ovarian follicles have been identified and sections of each of these tissues silver stained. Future research will focus on determining any variation in receptor expression in the gonads during the gonadal cycle and receptor expression in the liver, where changes may occur in relation to gonadal cycling. Receptor expression might also change in flounder exposed to endocrine disrupters. The utility of this method will be explored for other teleost species.

Studies of cell cycling in fish liver and gonad tissue have been carried out using two cell cycle markers; proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU). Fish injected intramuscularly with oestradiol and subsequently with BrdU produced negative results, but this may have been due to the route of administration and further work will be carried out to find other options. Positive staining using the PCNA technique has been achieved in flounder and stickleback and this work will be developed further.

Another stain for stickleback spiggin protein has been trialed, which showed weak but positive staining in kidney hypertrophic tubules. The staining was more intense in the microvilli and lumen of the renal tubules. This immunocytochemical technique can now be fully trialed as a biomarker of androgen exposure in this species.

Discussion/Questions:

Peter Matthiessen: At a recent meeting in Japan, a technique was described which showed non-specific staining of VTG in blood plasma. Have you detected this?

Rick Leah: *All work so far has been on tissue sections, looking to see if unfixed thin sections are similar to paraffin fixed sections.*

Peter Matthiessen: The aim is to have histochemical techniques that all laboratories could handle. How practical are the histochemical techniques you have developed?

Rick Leah: *It is a reasonably good technique for laboratories with staining experience. We are unlikely to get a quantitative analysis as yet – it is subjective at the moment, but we can distinguish between females and males.*

Peter Matthiessen: It would be very interesting to find out if males exposed to oestrogens early on have an increased number of oestrogen receptors and if histochemical tools would be able to detect this.

Objective 12. Protocols for routine monitoring and marine invertebrate life-cycle tests.

Tom Hutchinson^P, NA Pounds, M Hampel and TD Williams, Brixham Environmental Laboratory (BEL) AstraZeneca UK Ltd, and LR Dinan, Exeter University.

Abstract

AstraZeneca has produced anti-sera to vitellin purified at PERC from shore crab (scVT), brown shrimp (bsVT) and from marine gammarids (mgVT) (*Chaetogammarus marinus*). These have been supplied to the Plymouth group who are now refining the ELISA methods for application in field and laboratory studies. For the ELISA method development work, BEL has provided PERC with experimental animals from wild populations in Tor Bay, south Devon. For the laboratory studies on oestrogen biomarkers in Crustacea, BEL has provided PERC with samples of technical grade nonylphenol. Work is on going opposite biomarker responses for VT and Cypris Major Protein (CMP) in shore crab and brown shrimp at PERC. Working with CMACS colleagues, a draft protocol for histological sectioning of copepods is in preparation, with a view to the future exploration of copepod immunocytochemistry at various life-stages. The *in vivo* life-cycle studies using marine copepods (*Tisbe battagliai*) have shown significant inhibition for 20-hydroxyecdysone and diethylstilbestrol (DES), however based on visual analysis of these new data, no significant effects have been observed for steroidal oestrogens - e.g. oestradiol (E2) and EE2 - anti-oestrogens (the drug ZM189,154), testosterone or flutamide. Finally, a self-funded project at Exeter University has begun to screen several environmental contaminants (eg nonylphenol, organochlorine pesticides, PAHs) for potential agonist or antagonist activity in the *in vitro* ecdysteroid cell proliferation assay. Again, results are being analysed.

Presentation

The objectives for this programme were to provide antibodies for the crustacean biomarkers being developed at Plymouth and assess the *in vivo* development and reproductive effects of natural and synthetic oestrogens and androgens in marine crustacea. The life-cycle of the harpacticoid copepod *Tisbe battagliai* was described and the advantages of using this organism discussed. Several compounds have been tested for their effects on survival and reproduction – the oestrogens trans-DES, 17 β -E2, ethinyloestradiol and oestrone, the androgen testosterone, the anti-androgen flutamide, the invertebrate moulting hormone agonist 20-hydroxyecdysone (20-HEC) and the drug ZM 189,154 (an anti-oestrogen developed by AstraZeneca for the treatment of breast cancer).

Cumulative number of offspring was significantly reduced at a concentration of 26.9 µg/l 20-HEC, whilst at 86.5 µg/l and above breeding females were absent. With DES, effects on survival and reproduction were evident at 100 µg/l and no effects were observed at 10 µg/l. These results indicate that *Tisbe* are more sensitive than daphnids. There is no evidence so far of adverse effects for E2, oestrone, EE2, flutamide, testosterone or ZM 189,154.

To screen for ecdysteroid activity, a new *in vitro* assay, the Fruitfly BII assay, is being used. This is a cell proliferation assay which can measure the agonistic and antagonistic effects of environmental contaminants. To date, ten chemicals have been tested, with positive results for Bisphenol-A (antagonist), diethylphthalate (antagonist), 20-HEC (agonist) and lindane (antagonist). More results are currently being analysed. The *Tisbe* assay may be more useful for screening general industrial chemicals rather than steroids and the BII assay could potentially be used in TIE procedures for screening chemical extracts for anti-ecdysone activity, adding to the battery of tests such as the yeast oestrogen screen (YES) assay.

Discussion/Questions:

Andrew Wither: Is the Dee estuary as clean as suggested? There is a lot of industry along this estuary. In terms of endocrine disrupting pollution, there is no effect, but what about metal pollution for example?

Rick Leah: *Mercury concentrations in tissues of flounder from the Dee and the Mersey are similar.*

Gwynne Lyons: Should the effects of aromatase inhibitors be looked at, in molluscs, annelids or crustacea?

Peter Matthiessen: *There are no plans within EDMAR as yet to do this. The endocrinology of annelids, for example, is still poorly understood.*

Tom Hutchinson: *There is no mechanistic rationale for looking at aromatase inhibitors in crustacea.*

Q: Would we expect to see endocrine disruption in annelids? They are more related to arthropods than molluscs, therefore it would be a good idea to look at them and take a top-down approach, comparing them with molluscs/crustacea.

B. FIELD STUDIES

Objective 8. Field investigations of endocrine disruption biomarker responses and reproductive success in a fish species and a crustacean species breeding in estuaries.

Mark Kirby^P, Steve Feist and Rob Dyer, CEFAS Burnham-on-Crouch and *CEFAS Weymouth.*

Abstract

The past year has seen the continuation of a very extensive field sampling programme that has contributed not only to this objective but also several others. The focus has been to obtain whole organism specimens and appropriate tissues from fish and crustacean species which breed in the target areas. Measurements and biomarkers (as developed under other EDMAR objectives) will then be applied to assess the presence and extent of endocrine disruption and make further investigations into potential effects on reproductive success. The large sampling programme has provided a very comprehensive range of specimens from several species across the UK. Whilst a great number of the samples remain in storage and some of the biomarkers require further development before they can be used, the work has already produced a wealth of data, including: continued evidence of endocrine disruption in flounder; firm evidence of endocrine disruption and intersex in the viviparous blenny which breeds in estuaries; preliminary data that suggest feminisation at a gross morphological level might be occurring in another estuarine fish species, the sand goby; and a substantial body of data that leads to the conclusion that crustaceans (specifically the brown shrimp) are not responding in an obvious way to endocrine disrupting chemicals.

Presentation

The main focus of this objective was to carry out surveys of UK estuaries in order to investigate endocrine disrupting effects in the field using species that breed in estuaries, estimate the abundance of target fish and crustacea and potential effects on somatic and morphological indices, apply the biomarkers for oestrogenic and androgenic exposure developed under other objectives and investigate potential effects on fecundity, sex ratios, recruitment etc.

A large-scale field work programme began in 1999, which involved a quarterly survey of the Alde, Crouch, Thames and Tees estuaries and also a survey of 14 estuaries between September and November. Samples of flounder, sand goby, viviparous blenny, brown shrimp and shore crab were collected, to which a variety of histological and biomarkers were applied. Gobies were also collected from power station inlets and offshore areas. In this species, somatic and morphological measurements were also made.

As Dr Bamber had already presented the results of the crustacean work, the results of the fish work were described. Specific measurements were made depending on the species, as follows: flounder – VTG, liver ethoxyresorufin-o-deethylase (EROD) and histology; sand goby – liver VTG gene probe, histology, morphology; viviparous blenny – EROD, histology, VTG gene probe. Flounder was sampled opportunistically, as it was not a target species under Objective 8; plasma VTG was found to be elevated in several estuaries, similar to the results obtained in the 1997 CEFAS survey.

Sand gobies were found to be widespread and abundant. There are three closely related species which exist as mixed populations in UK waters; they can be distinguished by the cheek papillae, but hybrids do occur. The VTG gene probe developed at Glasgow Caledonian University gave a positive response in laboratory experiments with sand gobies dosed with E2, but there was no response in over 100 samples obtained from the field surveys. It was concluded that there was a need to establish a response threshold with this species. The somatic/morphological measurements with gobies showed that length/weight increased over the season. The Gonado-somatic Index (GSI) increased overall with breeding cycle, but gobies are multiple breeders and individual specimens exhibited increased and decreased indices; there was evidence of possible testicular growth retardation. The Hepato-somatic Index (HSI) increased with known contamination and Uro-genital Papilla Length Index (U-GPLI) increased with the oncoming breeding season. A newly termed phenomenon, Morphologically Intermediate Papilla Syndrome (MIPS) was described, in which male sand gobies showed some attributes of females in terms of the morphology of the uro-genital papilla. Scanning electron micrographs were shown of normal males and females, together with intermediate individuals. The occurrence of MIPS in the surveyed estuaries was 0% in the control (Alde) and up to 70% in the Tees and Mersey.

Viviparous blenny had a limited distribution along the north and east coasts, including a good spread in contaminated and clean estuaries. It was noted that this was the best species to look at for assessing fecundity in the wild. The Glasgow gene probe showed a 70% occurrence of VTG in fish from the Clyde and a 20% occurrence in fish from the Tyne. No response was found on the Tees. Histology showed that melanomacrophage centres in gonads, which are a result of general stress, were highest in the Tyne and Clyde, and 1 intersex fish was found.

Work to be completed in 2000/2001 includes measurements of crustacean sex ratios and carapace morphology, further work on MIPS to determine if the response can be induced by laboratory exposure to known endocrine disrupters and whether sperm/egg deposition is affected, together with studies of survival, fecundity and recruitment using goby breeding tests and field collected blennies.

Objective 4. Field surveys using biomarkers for androgen exposure in fish, and for androgen and oestrogen exposure in crustaceans.

John Thain^P, Andy Smith, Mark Kirby, Steve Feist and Ioanna Katsiadaki, CEFAS Burnham-on-Crouch, CEFAS Weymouth and CEFAS Lowestoft.

Abstract

Attempts to collect significant numbers of 3-spine sticklebacks from wild estuarine populations have so far been unsuccessful. Further attempts will be made during the spring when the likelihood of success would appear to be more favourable. However, sticklebacks from a freshwater source have been acclimated to full salinity seawater and caged trials have been carried out on the Tees and Crouch estuaries. Further caged trials are now being carried out on the Tees, Tyne and Crouch.

Presentation

This objective began in June 1999, with the aim of using the three spined stickleback as the target species and measuring androgen exposure via histological and ELISA procedures. The expectation was to use wild fish caught from estuaries,

but surveys on 10 estuaries carried out under Objective 8 resulted in no specimens being caught. There were several problems with using this species as a study organism: their occurrence in estuaries was seasonal, patchy and localised and hence there was only a very small window for catching them; the various capture methods deemed suitable were not necessarily the best to use in open estuaries and the most suitable method was dependent on the habitat. Targeted field surveys of wild fish were continuing, but caged fish were being used as an alternative. Twelve hundred fish captured from a freshwater lagoon had been acclimated to seawater in the laboratory. A variety of types of cage had been trialed, the definitive design held 50 fish >25mm length and was deployed 2-3m above the seabed, suspended from a wharf or jetty. Cages had now been deployed on the Tees and Crouch estuaries, with initial survival rates after 4 weeks of 90% on the Crouch and 65% on the Tees. Survivors were fixed in formalin and examined histologically using the KEH assay, but as fixation was incomplete no conclusions could be made. Control males were found to be in breeding condition, but this was not the case in Tees males. Also, females from the Tees were vitellogenic, but control females were not.

Survival was still good in the Crouch cages after ten weeks and the fish were feeding on the fouled netting. This was originally thought to be a problem, but was now considered to be an advantage. New cages have been deployed on the Tees and Tyne, for which the spiggin assay will be deployed. The length of deployment for induction of biomarkers needed to be determined before an extensive estuarine caged survey began. The advantages of using *in situ* caged studies were that they offered site-specific, location-controlled exposure, uniformity in fish size and origin and control over sampling times; additional environmental information could be obtained by the use of loggers within the cages.

Objective 7. Field studies to assess the impact of oestrogenic compounds on juvenile and adult salmon during estuarine migration.

Andy Moore and Sandy Scott^P, CEFAS Lowestoft.

Abstract

No abstract submitted, as this work has only just begun.

Presentation

This objective aims to examine the possible effects of sewage derived oestrogens on sea trout smolt physiology and adaptation to the marine environment and also measure the levels of plasma VTG in adult salmon and sea trout from “clean” and polluted estuaries within England and Wales. Amongst the physiological parameters to be measured are gill sodium and potassium ATPase activity, plasma ion levels and plasma osmolarity. These will be related to plasma VTG levels in sea trout smolt maintained in the Tees estuary. The migration pattern of salmonids was briefly described. Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta*) have a seaward migration of smolts in the spring and a spawning migration of adults from spring to autumn. The possible effects of oestrogens on fish during these migrations through estuaries were then reviewed. For smolts, exposure could lead to inhibition of smoltification and migration, therefore resulting in poor marine survival and a decline in stocks. Adults’ reproductive status may be enhanced, viability and reproductive success may be reduced, run-timing may change, all of which could lead to declines in stocks and fisheries.

In the light of concerns that salmon are too valuable to use in this type of work another salmonid, the sea trout, will be used. It is intended to trap sea trout on the Tees estuary and cage them below the Tees barrage, at Dabholm Gut and on the Esk estuary. They will be maintained there for as long as possible, with plasma VTG concentrations being measured at the start and after 4 weeks. Tissue samples will also be taken for the measurement of ATPase.

The work will begin as soon as smolts start running down river – personnel are on standby.

C. IDENTIFICATION OF SUSPECTED SUBSTANCES

Objectives 5 and 6. Isolation/quantification of oestrogenic and androgenic substances and tracking to sources.

Kevin Thomas^P, Yvonne Allen, Mark Hurst, Andy Smith, John Thain^P and Mike Waldoock, CEFAS Burnham-on-Crouch.

Abstract

Through the application of toxicity identification and evaluation procedures, key oestrogenic compounds were identified in surface water and sediment pore water samples collected from the Tyne and Tees estuaries. The oestrogenically active surface water sample collected from Howdon sewage treatment works (STW) on the Tyne was shown to contain 17 β -oestradiol, androsterone and an unknown oestrogenic compound. Most of the activity contained in the sample collected from Dabholm Gut on the Tees was also due to 17 β -oestradiol with additional activity from nonylphenol and *bis*(2-ethylhexyl) phthalate. The only sediment pore waters to demonstrate oestrogenic activity were collected from Dabholm Gut. The cause of this activity was an unidentified steroid degradation product. Extracts of sediment particulate material collected from both estuaries were shown to be highly active (0.5–5.5 $\mu\text{g E2 equiv. g}^{-1}$), although, only nonylphenol has been identified to date as a component of this activity. An experiment is under way to establish whether feeding was the main uptake route for oestrogenic exposure in flounder.

Presentation

The initial approach of this objective was to identify estuaries that had high oestrogenic activity with the YES assay, select suitable study sites on these estuaries and then deploy caged flounder (VTG biomarker), water, sediment and biota TIE and chemical analysis at each location.

As the YES assay cannot be used on raw water samples, a system was developed for extracting the water samples and isolating the oestrogenic fractions. This involved a three-layer SPE system to separate the non-polar, mid-polar and polar organic fractions. The YES assay was then deployed on each extract. Samples giving a positive response were fractionated further using HPLC and these fractions again tested for an oestrogenic response with the YES assay; positive samples were finally analysed using GC-MS.

The Mersey, Tees and Tyne were found to produce an oestrogenic response with surface water samples. Further work concentrated on the Tees and Tyne estuaries. On each of these, three sites were chosen: on the Tees the site of particular interest was Dabholm Gut which receives STW and industrial effluents; on the Tyne, Howdon STW was selected; upstream and downstream sites were also used for comparison.

The greatest activity was found in the STW effluents themselves and the activity differed between effluents. The causative substances were identified as the natural hormone oestradiol, which accounted for 70-80% of the activity in both effluents; androsterone, a testosterone metabolite (Tyne only); nonylphenol (Tees); and a phthalate (Tees).

Techniques have also been developed for the extraction and fractionation of sediments. In sediment porewater, activity was found in the Tees but remains to be identified. Sediment particulates from both the Tyne and the Tees had greater levels of oestrogenic activity than sediment porewaters or surface waters. Some of the

activity in the Tyne particulate sample was identified as nonylphenol – this is the only oestrogenic substance identified to date in sediment.

The original aim in deploying the flounder caging and laboratory feeding studies was to pinpoint the location of oestrogenic effluents via measurement of VTG induction. To this end, deployments were made upstream, downstream and at sites of high industrial and domestic effluent inputs on the Tyne and Tees estuaries. No induction of VTG was found in male flounder exposed in this way for three weeks, which led to questions about the main exposure route(s) – water, sediment or food? As a consequence, a new line of research was initiated to determine whether male flounder could obtain significant exposure through feeding on invertebrates, using the following experimental approach. Wild fish were collected from the Alde control estuary, with the aim of feeding them in the laboratory on invertebrate food collected from the Tees estuary and measuring VTG induction at frequent intervals. However, a survey of the benthic community in the lower Tees showed that species diversity and abundance was poor. Brown shrimp was the only species that could be caught in sufficient quantities to support a feeding study, but this species is very mobile and a poor accumulator of contaminants. To overcome this problem, a separate study is being conducted, feeding the flounder caged mussels. As an initial phase in this study, mussels were caged and sited at four locations on the Tees and also at a control site on the coast at Redcar. The mussels were retrieved after six weeks and analysed for PAHs, OCs, PCBs, TBT and a suite of metals. Contamination was highest in mussels caged at Teesport and subsequently 200kg (an amount sufficient to support a feeding study) were placed there for a period of two months. Control mussels were obtained from the west coast of Scotland and caged in the Crouch estuary adjacent to the Burnham laboratory.

The experimental design for the shrimp study entailed four tanks each containing 35 fish, which were fed brown shrimp on a daily basis – 2 tanks with contaminated shrimp from the Tees, 1 tank with control shrimp from the Crouch and another with clean shrimp but dosed with 10 ng/l EE2 as a positive control. The food ration was 3+ shrimps/fish/day. On days 0, 21 and 56 the fish were measured and a sample of blood taken for plasma VTG quantification by ELISA. Fish mortality increased towards the end of the experiment and lice infestation became a problem. There was no significant effect of feeding flounder on contaminated Tees shrimp on VTG concentration after 21 or 56 days. The fish dosed with EE2 responded as expected, producing high concentrations of plasma VTG after 21 days.

Discussion/Questions (for section B and C presenters):

Shaw Bamber: Are there stickleback present naturally at the locations identified for the stickleback caging studies?

A: *It does not matter if they are there or not – the purpose of these studies is to use them as a screen for androgen exposure. We are standardising on our test organism.*

Q: Could the feeding strategy of flounder explain the results of the caged study?

A: *In the cages, flounder were exposed to water and sediment, as the cages were heavy enough to sink into the mud. However, the normal food source was not present as a possible source of oestrogens and hence the laboratory studies were set up.*

Shaw Bamber: You also have to think about the added complication of exposure through both sediment and food, which would be the real world situation.

Richard Moxon: Do flounder have particular feeding habits that are different to viviparous blenny or goby that make them more sensitive?

A: Flounder are more intimate with sediments, which have a large amount of oestrogenic activity and could be stripped off in the guts of the fish. Gobies and blennies do live in association with sediments, but not to the same extent – more studies are required.

D. LABORATORY STUDIES

Objective 9. Laboratory studies of the effects of sewage effluent and other suspect materials on breeding success in sand gobies.

Craig Robinson^P, Ron Stagg, Gerry Best, David Pirie, Ian Davies and Colin Moffat, FRS Marine Laboratory, Aberdeen and SEPA West.

Abstract

Work reported covers a long exposure and breeding test and a short exposure alkylphenol dose-ranging test. The breeding test studied the effects of long term exposure to Irvine Valley sewage effluent (IVS), ethinyloestradiol and a methanol control. The test took place during May and June 1999, utilising within-treatment pairings, and measured male reproductive behaviour, egg production at individual and population levels, egg fertility, larval production, larval survival and larval viability. In all parameters, the main response was a negative impact in the EE2 treated population. The short, dose-ranging test used 4-*tert*-octylphenol (4-*t*-OP) over the concentration range 0 - 100 µg/l, measuring hepatic mRNA induction and plasma alkali-labile phosphate production; these were dose responsive. Long term exposures to 4-*t*-OP are underway with the fish being exposed at concentrations of approximately 30 µg/l and 100 µg/l, along with appropriate water and solvent control treatments. The second breeding experiment, investigating the effect of these exposures, started at the beginning of April 2000.

Presentation

The Irvine Valley sewage treatment works serves textile, pharmaceutical and aerospace industries and alkylphenol ethoxylates are known to be present in its effluent. Two different tests have been conducted – a long-term exposure and a breeding test. The long-term experiment continued for 8 months, exposing fish to either 6 ng/l EE2, 0.3% IVS, 0.03% IVS or a methanol control. Effluent from the treatment works was sent to the laboratory on a weekly basis and fish were sampled monthly for somatic indices and hepatic VTG mRNA measurements. Each batch of effluent was chemically analysed on collection, on arrival at the laboratory and at the end of batch usage. For the breeding experiment, 12 aquaria were set up per treatment, containing clean sand and a clay pot for nest building. Each received a continuous dose of the relevant test material. Pairs of fish were added to each tank and daily examinations made for presence of nests and eggs. If eggs were present, they were removed, counted and incubated in clean water until hatching. The number and length of larvae were recorded. At the end of the experiment all fish were examined for colouration, measured and tissues sampled.

The chemical analysis of IVS showed that it was a complex mixture, with high concentrations of the pesticide permethrin, total alkylphenol ethoxylates, technical nonylphenol and 4-n-nonylphenol.

After 8 months exposure to IVS or EE2, males showed no induction of VTG or zona radiata protein (ZRP) in the effluent. Male nuptial colouration was absent after 6 months exposure to EE2 and was delayed, as well as slightly inhibited, in the 0.3% IVS treatment. Male nesting behaviour was inhibited by EE2, but not by IVS. The same results were obtained for other endpoints measured, such as number of pairs breeding, egg fertility and estimated reproductive output.

A recent short-term exposure trial was conducted with 4-tert-octylphenol; VTG and ZRP mRNA were induced at concentrations greater than 30 µg/l, plasma VTG was also produced at the same concentrations. A second long-term exposure test began in December 1999 and breeding trials from this are about to begin.

Discussion/Questions:

Jon Nash: Do you have plans, or is it possible, to raise the next generation of fish from the viable larvae?

Craig Robinson: *We do not know how to feed the larvae and knowledge of rearing this species is limited. For the next experiment, we plan to use eggs exposed to contaminated water, not clean sediment as before.*

Objective 10. Laboratory studies of the effects of sewage effluent and other suspect materials on breeding success of a crustacean.

John Thain^P and Yvonne Allen, CEFAS Burnham-on-Crouch.

Abstract

This work objective commenced in April 1999 and has so far involved the setting up of a culture system for *Chaetogammarus marinus*, developing an exposure test system for conducting chronic experiments, developing an appropriate experimental design and preparing standing operating procedures for culturing and sampling the test organism.

Presentation

This objective firstly aimed to set up a culture system for the test organism *Chaetogammarus marinus* and proceeded by developing an exposure system for chronic experiments and running some tests. In addition, CEFAS provided material to PERC for the development of crustacean biomarkers. The amphipod *C. marinus* is found widely in the intertidal zone and has a salinity tolerance range of 7 to 30, with a life-cycle of approximately three months; the female carries eggs in a brood pouch until hatching. Embryonic development is complete within 10 days at 15°C and the newly emerged larvae are approximately 2mm in length (which makes them amenable to counting) and are benthic. The sexes are separate, males reaching a length of approximately 18 mm and females 13 mm. In the laboratory, they are fed seaweed (*Fucus* spp.), either in short strands or as discs. Cannibalism can be a problem.

The culture system was modified from an earlier flow-through apparatus. Ten litre capacity trays hold breeding pairs and sub adults are sieved on a weekly basis to obtain juveniles to start the next generation. The scale of the culture was increased to 50 litres to obtain sufficient animals to start tests. Pre-copula pairs were separated into beakers periodically in order to collect freshly laid eggs for PERC.

Two types of experimental design were employed. In the first, 25 immature individuals were maintained in 10 litre containers. When pairs formed, they were placed into meshed glass beakers within the test aquaria and monitored daily until the

female laid eggs. The male was then removed and returned to the test aquarium, and the eggs hatched approximately 10 days later. In each treatment, measurements of pairing frequency, female size, and number and size of offspring were made.

The second design was a less labour intensive one, involving exposing 100-week-old individuals in test aquaria for a period of two months, with weekly sieving into fresh test solution. At the end of the exposure period, animals are sieved and sorted into size classes and the effects on growth and reproduction assessed.

The initial test substances chosen were the known oestrogen ethinyloestradiol, the oestrogen mimic nonylphenol and the ecdysone mimic 20-hydroxyecdysone. Acute sighting tests were carried out with each of these and sublethal tests planned at environmentally realistic concentrations. However, eggs sent to PERC had not shown any cross reactivity to shrimp and crab vitellin; as explained in earlier presentations, there is currently no crustacean biomarker for oestrogenic exposure available. In the light of this, plus delays encountered in developing appropriate handling and operating procedures and an inability to reduce the lifecycle time scale, it was decided to shift effort at CEFAS to the flounder feeding studies for the time being. Work with *C. marinus* currently in progress will be completed and analysed.

Discussion/Questions:

Tom Hutchinson: Would flow-through systems work equally as well with this species?

John Thain: *There is no reason why not, but they would be more expensive for chemical analyses.*

E. THE POPULATION LEVEL

Objective 11. Modelling field and laboratory data to predict population-level effects of endocrine disruption.

Mike Smith, Marinelle Basson^P and Mark Bravington, CEFAS Lowestoft.

Abstract

No abstract submitted, as this work has not begun.

Presentation

There are essentially two approaches to population-level modelling of ED contamination: empirical and mechanistic; this presentation focuses on the mechanistic approach. At a basic level, population size is governed by births and deaths. Births are governed by the number of reproducing adults and their inherent fecundity and the number of adults are governed by the survival of young through the various life-history stages up to mature adulthood. If a population is not increasing or decreasing, then births and deaths will balance and *fecundity x survival* will be close to 1; in ecological terms, the population would be in equilibrium (in reality, though, populations will fluctuate around the equilibrium level).

In a simple, model population, a decrease in fecundity (due to endocrine disruption, for example) would lead to a decline in population size and ultimately to extinction, or near extinction, since births would no longer balance deaths. The extent of the drop in fecundity would determine how rapidly the population declined and how long it took to become extinct. However, in nature, a population may decline initially due to decreased fecundity, but will generally stabilise at a lower level due to

compensating density dependent (DD) factors - through, for example, more food or more breeding sites becoming available per individual. DD is part of the basic biology/ecology of a population, not a mathematical convenience and, unless it is incorporated in models, realistic predictions about population-level responses cannot be made, only a simple assessment of initial rate of decline.

Unfortunately, there is a lack of knowledge of DD processes acting on the EDMAR study organisms and this precluded the usefulness of attempting to develop predictive population models. In sand gobies, one DD factor might be that numbers of successfully hatched eggs were limited by availability of mussel shells. Although the exact functional form of such a process was unknown, it might be used as a starting point to look at 'extremes' and determine which parameters were most important in terms of predicting population level effects. A possible way forward was therefore to consider a range of estuarine species and conduct a broad-brush modelling exercise to determine which types of life history were most likely to be vulnerable to endocrine disruption and which processes and parameters would be particularly relevant or important to study/measure in the field or laboratory.

Discussion/Questions:

Mark Kirby: Potentially, could the use of mesocosm experiments standardise "confounding" factors which may affect density dependent factors?

Marinelle Basson: *You would need to set up a matrix of experiments at different levels of densities, and think carefully about which specific question you want to address.*

Peter Matthiessen: Mesocosm experiments are expensive – would the pay-off be better focusing on viviparous blenny fieldwork?

Marinelle Basson: *I'm not convinced that more fieldwork will fill in the gaps needed for modelling work.*

John Thain: Using the TBT and dogwhelk situation as a model is good. There is a well-defined population to work with because there is no pelagic larval stage. The problem with fish is their mobility, which makes it very difficult to define a population.

F. ASSOCIATED RESEARCH

A study for English Nature of the risks posed by endocrine disruptors to marine protected areas.

Yvonne Allen^P, Jacquie Reed and Peter Matthiessen, CEFAS Burnham-on-Crouch.

This presentation described a contract which CEFAS were currently carrying out for English Nature, to review the endocrine disrupting properties of chemicals likely to be present in the marine environment and assess the implications of their presence in Special Areas of Conservation (SAC) and Special Protection Areas (SPA).

A literature review listed the main groups of chemicals for which there is strong evidence of endocrine disrupting effects and described the main uses and likely pathways to the marine/estuarine environment. Evidence for endocrine disrupting effects was briefly summarised, along with information, where available, on measured

levels in water, sediments and biota. In addition, there was a comprehensive up-to-date review of the evidence for endocrine disruption in four main groups of animals found in the marine environment: fish, invertebrates, marine mammals and birds. This evidence was drawn from laboratory based studies, controlled field experiments and field observations.

The second phase of the contract involved collation of relevant chemical data held on databases by CEFAS and the Environment Agency, in 15 designated SACs and 8 SPAs. All the information would be summarised in a series of maps and graphs. Chemical data had been retrieved for five matrices: marine sediments, seawater, fish, marine mammals, and marine biota between 1995-1999. The final report would include a summary of the features/subfeatures/species for which each of the SACs and SPAs had been selected, and, using the information gathered in the literature review and data collation exercise, a preliminary assessment of the potential risks posed by endocrine disrupters made.

GENERAL DISCUSSION

The seminar concluded with an open forum:

Geoff Brighty expressed concern over the switch from salmonids to trout under Objective 7 - salmon should not be ruled out entirely in the programme. A population on the west coast could possibly be studied. Laboratory work has been carried out looking at oestrogenic effects on smoltification and field work was about to begin.

Jim Readman asked how much oestrogenic activity was lost during the fractionation process in the TIE procedure - i.e. what was the mass balance? **Kevin Thomas** replied it was estimated that about 20% of the activity was lost during the process. **Geoff Brighty** commented that it was reassuring from these studies that no "unknown" industrial chemical is popping up as being a suspect endocrine disrupter.

Oliver Warwick commented that work should be done to investigate whether the vitellogenin response in different species of fish was the same - i.e. was VTG induced in flounder, gobies, blennies etc at the same concentrations in the laboratory? There was general agreement that this was a good point that should be borne in mind.

Peter Matthiessen also suggested that there was a need to look at levels of VTG over life-cycles, as there was little information to interpret VTG induction and effects on reproduction. **John Thain** added that the yearly cycle of VTG levels need to be assessed and **Mark Kirby** mentioned that some estuaries were being sampled at quarterly intervals - the recent samples obtained in March could complete a year's worth of surveys and help to address this issue. **Tom Hutchinson** referred delegates to the work of Peter Janssen of the Netherlands, who, as part of his PhD thesis measured VTG cycles of flounder from the Wadden Sea. **Jon Nash** pointed out that fish in different estuaries would be at different states of the breeding cycle, which would confuse the issue and data interpretation.

Sandy Scott asked if the conditions in the Mersey, in terms of pollution, were improving, since there had been a decline in the concentrations of VTG in Mersey flounder in the last survey. **Geoff Brighty** commented that he hoped this was the case,

but there is a need to continue observation on the quality of the effluents discharging to the estuary.

Oliver Warwick pointed out that although the invertebrate studies had given a negative response so far to vertebrate hormone substances, there was still much value in continuing the work to investigate the effects of invertebrate hormones and their mimics.

Peter Matthiessen highlighted the issue of measuring plasma vitellogenin vs. vitellogenin mRNA; Ankley and colleagues in the USA had measured both using fathead minnow exposed to known oestrogens and these studies showed a short-lived increase with mRNA, whereas plasma VTG increased to a peak over a longer time period. There was thus potential for missing the mRNA peak.

External assessor **Jim Readman** summed up the meeting by stating that the overall productivity of the EDMAR programme had so far been good. The main points made in each of the presentations were summarised and he concluded that EDMAR had proved to be successful to date and many results were just beginning to be produced after much development work.

Peter Matthiessen closed the meeting by concluding that the issues involved in EDMAR and the biological significance of the results were just beginning to be understood.

FURTHER INFORMATION

If you would like additional copies of this report, or to be kept informed of developments within the EDMAR programme, please contact:

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