ENDOCRINE DISRUPTION IN THE MARINE ENVIRONMENT (EDMAR)

1st Annual Seminar - 18th March 1999

Central Science Laboratory , York

Chairs: **Dr Mike Roberts (DETR)**
Dr Peter Matthiessen (CEFAS)

Rapporteur: **Mr Mark Kirby (CEFAS)**

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INTRODUCTION AND BACKGROUND

The seminar was opened by Dr. Mike Roberts of the Department of Environment, Transport and the Regions (DETR) who outlined the aims of the meeting and indicated that it should not only act as a medium for the exchange of information and progress but also as a conduit for interested parties to have a direct input into the programme and for concerns and related issues to be raised.

Dr Peter Matthiessen (EDMAR project manager) of the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) outlined the background to EDMAR. Previous work carried out by CEFAS had demonstrated that flounder populations in a number of UK estuaries were exhibiting symptoms of endocrine disruption, specifically those caused by exposure to oestrogens and their mimics. Major findings were the presence of the egg yolk protein vitellogenin (VTG) in male blood plasma and the occurrence (in the Tyne and Mersey) of a condition known as intersex where ovarian tissue was found in the testes of male flounder. This and other work triggered the need for a much larger programme to investigate the potential threat to marine fish and crustacean populations from endocrine disrupters – EDMAR. The EDMAR programme runs from 1998 – 2001, will cost £1.4 million and comprises a joint initiative between 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 Council (CEFIC), with research conducted by several major UK environmental laboratories.

The programme covers six main objectives:

- Develop biomarkers for detecting androgenic activity in marine fish and invertebrates and oestrogenic activity in invertebrates.
- Conduct surveys of oestrogenic and androgenic activity in key indicator species in the estuarine, coastal and offshore environment.
- Observe the impact of this activity on reproductive output in a fish and invertebrate species.
- Isolate the substances causing this marine endocrine disruption and identify the main sources of marine contamination.
- Conduct confirmatory experiments with suspect effluents and substances in laboratory test systems.
- Model the possible effects at the population level.

Progress towards these objectives was described by each participating laboratory through the following presentations:

A. BIOMARKER DEVELOPMENTS

Development of Biomarkers of Oestrogen and Androgen Exposure in Marine Crustaceans

Shaw Bamber and Mike Depledge, **University of Plymouth.**

Abstract

One of the major aims of the period under review was the development of an enzyme-linked immunosorbent assay (ELISA) for crustacean vitellin. There are several stages of development necessary in creating an ELISA, each of which was described briefly during the presentation. The initial stage involves obtaining tissue containing the protein of interest. Shore crabs (*Carcinus maenas*) were selected for the initial experiments because of their large size, availability and obvious sexual dimorphism. Dissection of mature ovaries, a rich source of vitellin, was straightforward and provided ample material from which to generate extracts. The second stage involved examination of the protein content of this extract, together with others obtained from testes and haemolymph samples, to determine the presence of sexually specific proteins. Polyacrylamide gel electrophoresis (PAGE) was used for this, running both native and denatured gels. Once vitellin was recognised within the extract the next step was purification. Aliquots of purified crab vitellin were sent to Zeneca where polyclonal antibodies were raised in rabbits. Test sera taken during the inoculation process were assessed for cross-reactivity using the Western blotting technique. Tests proved the antibody to be specific to crab vitellin. The final sera bleeds are now being used to optimise the ELISA. Zeneca are currently raising a polyclonal antibody to vitellin extracted from brown shrimp (*Crangon crangon*) ovaries and once this has been tested for specificity an ELISA will be developed following the same protocol as that used for the shore crab. Plans for the near future include the commencement of laboratory exposures of crabs and shrimps to known oestrogenic chemicals and the subsequent use of the ELISA to detect the induction, or otherwise, of vitellin/vitellogenin in haemolymph and tissue extracts.

Presentation

A general overview of crustacean endocrinology was presented, including detailed descriptions of the primary organs (X organ sinus gland complex, ovaries and androgenic glands) that have been targeted for tissue analysis. Early work has concentrated on the isolation and purification of vitellin, the VTG-equivalent in crustacea, from the ovaries of the shore crab (*Carcinus maenas*) and the brown shrimp (*Crangon crangon*).The raising of polyclonal antibodies against shore crab vitellin and the subsequent development of an ELISA for this protein were reported. All techniques were described and results were presented to demonstrate that the assay was specific to *Carcinus* vitellin. Ongoing work is concerned with the development of a similar ELISA technique for the detection of brown shrimp vitellin and it is anticipated that this will be available in the near future. Future objectives were outlined as below:

- Completion of ELISA development for *Crangon crangon.*
- Laboratory exposures of *Carcinus* and *Crangon* to oestrogens to determine thresholds for response and detection.
- Analysis of field sampled haemolymph using the assays.
- Development of a vitellin assay for *Chaetogammarus.*
- Search for appropriate biomarkers in molluscs.

- Eventual development of biomarkers for androgens in crustacea.

Discussion/Questions

With respect to sampling logistics, is the vitellin stable and how is it stored? – *Yes, the protein is highly stable and can be stored at -80°C for many months. In the field and at sea it is stored in liquid nitrogen.*

Has there been any evidence of gross endocrine disruption (ED) symptoms, e.g. intersex, in shore crabs? – *None that the author was aware of, although a recent Canadian study suggests that this may occur in lobsters.*

Are there any plans to use the assay in the River Tyne? – *There will eventually be a general survey deploying all biomarkers in over 10 estuaries and the Tyne will be included.*

Is the size of the organism a problem for the assay e.g. for use with *Chaetogammarus*? – *Inevitably this would cause some difficulty but could possibly be overcome by using homogenates of whole organisms.*

Would parasitism affect the assay if whole organisms e.g. *Chaetogammarus* were used? *Unknown but potentially*.

Is it known exactly in what organ the vitellin is produced in crustaceans to aid sampling and where would it be produced in induced males? – *Probably mid-gut gland but also possibly the ovarian tissue in females, however this is still unclear.*

Development of Robust Biomarker Protocols for Routine Monitoring
Tom Hutchinson, Zeneca Environmental Laboratory

Abstract

Supporting the work on crustacean biomarkers at the University of Plymouth, Zeneca has continued to help supply animals (e.g. brown shrimp) from wild populations in Tor Bay, south Devon. Purified vitellin from shore crab (scVTG) was received by Zeneca in October 1998 and anti-sera has since been sent for evaluation of cross-reactivity with purified scVTG. These antisera are now being used to optimise an ELISA to scVTG. Since it appears that the rabbit polyclonal antibodies (PAbs) to scVTG may not cross-react with brown shrimp vitellin (bsVTG), Zeneca are now raising PAbs to this antigen as an additional stage to this project milestone.

Good progress has been achieved, in parallel, with the Zeneca Ltd funded work on marine copepod bioassays. Focusing on a species used within the Direct Toxicity Assessment programme, life-cycle studies with *Tisbe battagliai* (Crustacea, Copepoda, Harpacticoida) have proved to be a practical means of measuring developmental and reproductive effects of ecdysteroids (e.g. 20-hydroxyecdysone) and xenoestrogens (e.g. diethylstilbestrol). Further research aimed at understanding the potential endocrine mechanisms behind such developmental and reproductive effects is scheduled to start in Spring 1999. This work will employ oestrogen antagonists (e.g. tamoxifen citrate) and other pharmaceutical compounds as research tools.

Presentation

The presentation outlined the Zeneca Environmental Laboratory involvement with EDMAR on two main fronts. First, in liaison with the Universities of Plymouth and Liverpool, Zeneca have provided the facilities to produce polyclonal antibodies for use in ELISAs. So far, this has involved the raising of polyclonal antibodies to shore crab and

brown shrimp vitellin by injection into rabbits and consequent isolation. Secondly, the work investigating the *in vivo* effects of oestrogens on the marine harpacticoid copepod *Tisbe battagliai,* which was described in greater detail.

The ecology and life history of *Tisbe battagliai* were described and the advantages it offers - i.e. ecological importance, field evidence of intersex in other harpacticoid copepods in the Forth, sensitivity, established test species and the fact that it offers whole life cycle testing - were highlighted. Preliminary results were presented on tests using two chemicals of interest, trans-diethylstilbestrol (DES) and 20-hydroxyecdysone (HEC). HEC is involved in the moulting cycle of crustacea, it decreases during moulting and therefore exposure causes inhibition of moulting. It was used as a positive control in 21day DES exposure experiments. No significant mortalities were observed at concentrations up to 10 μgl^{-1} DES whilst complete mortality after 10 days was observed at 100μgl^{-1}. The 10d LC_{50} and 21 d NOEC for DES to *Tisbe* is compared to other organisms in the following table:

	24hr EC_{50} DES (μgl^{-1})	10d LC_{50} DES (μgl^{-1})	21d NOEC DES (μgl^{-1})
Tisbe		31.6	10
Daphnia sp.			500
Oyster embryo	51.2		
Fathead minnow		320	

Table 1: Comparison of 24hr EC_{50} , 10d LC_{50} and 21 d NOEC concentrations for diethylstilbesterol.

Further work will be done in the 10 – 100 μgl^{-1} DES range.

It was concluded that the *Tisbe battagliai* assay was a promising system for investigating the effects of endocrine disrupters in crustacea and had proved sensitive to DES, even though the mechanism of toxicity was not yet fully understood. The ability to do assays using small volumes of test solution was especially useful.

Discussion/Questions

How much of a problem is it that there are currently no oestrogen-sensitive biomarkers developed for *Tisbe* ? – *This is a potential problem for its future use, however, immunocytochemical methods using the species are a possibility.*

At what developmental stage was exposure started during the 21 day experiments and could effects be monitored for several generations? – *Newly released (<24hr) nauplii were the stage at which experiments were started and the assay does have the potential for running over several generations though the effort would increase substantially.*

The oyster embryo results for DES were just as sensitive, could this be an alternative? – *Potentially yes, but the Tisbe assay offers important advantages, such as lifecycle testing.*

Development of a Biomarker of Androgen Exposure in a Suitable Marine Fish Species

Ioanna Katsiadaki and Sandy Scott, CEFAS Lowestoft

Abstract

The three-spined stickleback (*Gasterosteus aculeatus*) offers the potential for the assessment of reproductive disturbances caused by xenobiotic androgens. Male sticklebacks have pronounced androgen-dependent secondary sexual characteristics during their breeding season. One of these involves the kidney, which hypertrophies to produce a 'glue' protein, which is used when building a nest.

Androgens were administered to sticklebacks via the ambient water. Kidney epithelium height (KEH) measurements, which provide an objective measure of the kidney hypertrophy, indicated significant stimulation in both males and females. Furthermore, the 'glue' protein, spiggin, was localised histochemically in the tubules of the secondary proximal segment. The effect was clear despite the fact that the fish were exposed to the hormones for only two weeks. The previously published procedure involved daily injection of steroids into castrated male sticklebacks - so this work has made a considerable advance by demonstrating that the androgens can be applied via the water, and that there is no need to use operated fish.

In order to further improve the sensitivity and the speed of the bioassay for environmental androgens, the glue protein, spiggin was collected from the urinary bladders of fish in breeding condition as well as from nest threads and was purified with SDS-PAGE and injected into rabbits. An ELISA for spiggin is being developed.

Presentation

The work has so far concentrated on the use of the three-spined stickleback, *Gasterosteus aculeatus*, and the reproductive physiology of this species was briefly described. This species, in particular, is very appropriate for investigating the effects of androgens because it demonstrates strong androgen dependent secondary sexual characteristics, i.e. nuptial colouration, territorial/ nest building behaviour and kidney hypertrophy (measured by the kidney epithelium height).

Preliminary results were presented describing the effects on stickleback renal histology of exposure to the androgens methyltestosterone (MT) and 11-keto-testosterone (11-KT) and the anti-androgen flutamide. Both MT and 11-KT stimulated an increase in KEH in both males and females. Flutamide, however, failed to block this androgenic effect in MT-dosed males. The nephrosomatic index (NSI) increased in androgen dosed fish, but whilst there was a good NSI:KEH correlation in males this was not as apparent in females.

The presenter went on to describe the development of a new ELISA for the male specific protein spiggin which is an integral component of the nest building 'glue'. It was described how this protein was identified, purified and how polyclonal antibodies were raised in rabbits. Early work has detected the presence of spiggin in the urinary bladder, nest material and kidneys of male sticklebacks and it is being further evaluated as a biomarker for androgenic activity in females.

Discussion/Questions

Why did flutamide not act as an anti-androgen? – *Its anti-androgenic effect does not appear to be as potent in this species as suspected and it was thought that these results might be caused by under-dosing [of flutamide].*

Does exposure to androgenic steroids induce male behavioural patterns in females e.g. nest-building? – *Whilst female nest-building has not been observed a definite increase in territorial aggression is apparent.*

In what tissue will spiggin be routinely assayed? – *Work is still on-going but initial efforts will concentrate on blood.*

Development of Simple Histochemical Methods for Measuring Vitellogenin/Vitellin, and other Oestrogenic/Androgenic biomarkers, in Small Tissue Slices of Fish and Crustaceans

Rick Leah and Mike Simpson, CMACS – University of Liverpool

Abstract

Development work has been progressing using mainly flounder tissues from fish caught in the Mersey and Dee estuaries. Initial validation trials are underway.

There are no commercially available antibodies for oestrogen and androgen receptors in fish so mammalian receptor antibodies have been tested for cross reactivity in teleosts using standard protocols. Teleost tissues proved unresponsive in early tests but it has now been shown that there are unexpected sources of variation to be taken into account. Recently, positive staining has been obtained in some flounder (*Platichthys flesus*) tissues using antibody obtained from one commercial source. All of the mammalian antibodies tested prove positive in suitable mammalian positive control tissue such as human breast. Some have given what may be a positive result with the oestrogen receptor having a nuclear localisation in hepatocytes of flounder liver. In the gonad, oestrogen receptor appears to be localised in the cal/granulosa layer but with some staining around the subfollicular yolk.

In the testes, oestrogen receptors appear to be localised primarily in the Leydig cells. Preliminary results with the antibody (AR52) raised to a peptide conserved in human and rat androgen receptors also showed positive staining in the Leydig cells (and human prostate tissue used as positive control). However, these liver and gonadal results are not yet conclusive and further development and application is being undertaken.

It has not yet proved possible to obtain an antibody to the aromatase (CYP 19) that converts androgen into oestrogen within the brain so no results are yet available for this biomarker but the topic is still being pursued.

Bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA) have been tested as biomarkers of cell proliferation. It has not proved possible to use BrdU by simple incubation but it has been shown to work by injection *in vivo*. PCNA has now been successfully tested as a cell proliferation biomarker in flounder liver. Preliminary results have shown that livers from Mersey fish contain more active cells than livers from the comparison sites at Millport, Scotland and the estuary of the Welsh Dee. In addition, a number of probable artifacts have been identified. These will be taken into account during forthcoming quantification of PCNA in the sections.

Detailed examination of the histology of the flounder collected during the study is now fairly advanced. The majority of fish have been normal but a number of interesting features have been noted which merit further investigation. Biological variation arising from a number of natural sources will also have to be investigated before full validation of the methodology can be completed.

Presentation

The presenter explained the basis of the technique. The principle is to treat tissue slices of fish/crustacea with a primary antibody which will bind to the sites of potential vitellin/vitellogenin production. A secondary antibody, which has been conjugated with biotin-avidin complex and linked with horse radish peroxidase, is then bound to the primary. The location of the bound material is then visualised with the chromogene, diamino benzidine. (Similar immunocytochemical methodologies to this, but which differ in detail of the process chemicals etc., are also used for the other biomarkers of interest). The study has been based on species caught in the Mersey estuary and has so far concentrated on the use of flounder. Intentions are also to develop techniques for use with sticklebacks and sand gobies, but so far these have been difficult to catch in the study area.

The cycle of VTG production in flounder was reviewed and the consequent increase in hepato-somatic index (HSI) was highlighted. Whilst it is understood that HSI increases may not be solely caused by oestrogenic exposure, the consequent rise in cell-cycling was thought to be one possible starting point for developing a simple monitoring method detecting impact. A summary of the phases of the cell-cycle was shown and a marker of cell activity, proliferating cell nuclear antigen, was described. The colour reaction was based on the presence of PCNA and was first looked at in intestinal tissue where high levels of staining were apparent. The use of the technique with liver tissue was then demonstrated by the comparison of tissue from a Dee-caught flounder with a Mersey-caught flounder. Cycling (active) cells showed staining and a marked increase in positively stained nucleii was apparent in the Mersey tissue. The liver tissue showed a very patchy distribution of activity, but it was suggested that the technique was able to show differences at the qualitative level – quantitative assessment is being worked on.

Further work was briefly described on the use of similar techniques in testicular material where it has been possible to stain oestrogen receptors. Some questions as to the validity of this approach were raised as it involved the use of mammalian antibodies, but work is on-going to test their utility as a marker for endocrine disruption in male fish.

Discussion/Questions

What is the rationale for looking at oestrogen receptors in testicular material? – *Because oestrogen receptor numbers are usually upregulated during VTG synthesis so one might expect increased numbers of E_2 receptors in vitellogenic males.*

The reading of stained liver tissues in the way described can be very subjective. Are the slides read blind and how was the problem of the patchiness in liver activity addressed? – *Yes, the slides are read blind and ways of taking into account the patchiness of liver staining (activity) are being assessed. The inhomogeneity of the staining was unexpected and it is hoped that an automated method of slide reading can be developed to minimise any subjectivity.*

Are any other pathological changes, e.g. foci of cellular alteration, being assessed? – *Not yet but this will be done.*

Further clarification on the significance of the work in testicular tissue was addressed. In particular, it was noted that the cells showing staining were interstitial cells which contained sites of both oestrogen and androgen receptor antibody binding – the significance of this is under consideration.

Development of a Molecular Probe for Fish

John Craft and Fiona Robertson, Glasgow Caledonian University

This project is not formally part of the EDMAR Programme, but independently funded alongside by MAFF. It is intended that the probes developed will be utilised within EDMAR.

Abstract

Male expression of vitellogenin is a frequently used measure of the exposure of fish to environmental oestrogens. Traditionally, this has been determined in serum by ELISA or radio-immunoassay. However, it is not always possible to obtain serum from useful sentinel species and available antibodies have limited cross-reactivity between species. Therefore, this project is isolating cDNA probes for VTG determination in hepatic RNA. The strategy, previously used for the isolation of CYP1A and metallothionein probes from a number of species, is to use reverse transcriptase - polymerase chain reaction (RT-PCR) to generate DNA fragments using primers based on conserved VTG gene sequences in other genera/species which are available through sequence databases. Using this approach, a 1.57kbp fragment from plaice (*Pleuronectes platessa*) hepatic RNA was generated, which hybridised with a 5kbp mRNA in RNA from oestradiol-treated male and female plaice but not in untreated animals of either gender. The probe has also been used to assess VTG production in flatfish from various sites in the Clyde estuary and in this study none of the male fish were found to be producing VTG mRNA. Currently, an attempt is being made to isolate equivalent probes from sand goby (*Pomatoschistus minutus*) and flounder (*Platichthys flesus*) and this presentation discusses the results of this work.

Presentation

The presentation described the traditional methods of VTG determination by ELISAs in plasma and emphasized the difficulties associated with this technique in smaller species such as the sand goby. The alternative suggested was to measure the levels of the mRNA specific for VTG in liver tissue.

The principles of isolation and purification of cDNA probes specific for VTG were described and an example of its use in an environmental context was shown using plaice. In this study, positive results were gained for VTG in females and no positives were found in males from the Clyde estuary. A further advantage of this approach was highlighted in that a single probe can be used for other closely related species e.g. the plaice probe cross-reacts well with dab.

A working probe has been successfully developed for flounder (similar to plaice), but the main objectives that are now being targeted are the development of VTG mRNA probes for use in the sand goby and the viviparous blenny (*Zoarces viviparus*). Preliminary work with the goby was presented suggesting that a workable probe is near completion.

Discussion/Questions

Will the method be able to be used quantitatively? – *It is already being used in a semi-quantitative manner but will need further work.*

The role of inter-gender differences was discussed. It was stated that mature male flounder can also produce oestradiol naturally at high enough concentrations to produce some VTG and therefore the assay needs to be quantitative to be of real use. It was suggested that, bearing this in mind, the completely negative results on the Clyde were surprising.

Further discussion highlighted the possible use of cell-cycling and immunocytochemistry approaches in this area.

B. FIELD SURVEYS

Field Investigations of ED Biomarker Responses and Reproductive Success in a Fish Species and a Crustacean Species Which Breed in Estuaries

Mark Kirby and John Thain, CEFAS Burnham-on-Crouch

Abstract

The initial effort for this objective has concentrated on estuarine trawling surveys in order to establish the geographical extent of all the target species. This work has progressed well and a good information base for species distribution has been assembled. This has informed the more targeted main sampling effort which started in February 1999.

It has been found that stickleback (*Gasterosteus aculeatus*) appear to be almost completely absent from the estuarine areas sampled and that viviparous blenny (*Zoarces viviparus*), too, have been difficult to capture using trawling. The sand goby (*Pomatoschistus sp.*) has been caught in sufficient numbers in a number of the estuaries surveyed so far. However, the predominance of separate, but closely related, species in certain areas has lead to problems with the intercomparability of some samples.

Values of length, weight, hepato-somatic index, gonado-somatic index (GSI) and sex ratio will be measured for each population in order to determine the occurrence of physical signs of endocrine disruption.

Note has also been made of the presence and abundance of certain crustacean species; the shore crab (*Carcinus maenas*), the brown shrimp (*Crangon crangon*) and *Chaetogammarus* species, to inform the crustacean sampling soon to be initiated. The brown shrimp *Crangon crangon*, as expected, has been found to be abundant and ubiquitous.

Presentation

The presenter outlined the rationale behind the choice of sand gobies, viviparous blennies and sticklebacks as the target organisms for assessing potential endocrine disrupter effects on reproductive processes. It was explained that whilst ED effects (e.g. plasma VTG and intersex gonads) had been found in flounder there was some difficulty in relating the effects to contamination at the point of capture as the fish were semi-migratory. Moreover, flounder move offshore to breed and therefore the eggs and larvae, which are the most sensitive stages, are potentially not exposed to the relatively high levels of ED substances associated with some estuaries. Therefore, to gain more confidence in relating effects to discharges, and in order to take into account early life-stage exposure, appropriate fish and crustacean species that spend their entire life-cycle in estuaries were chosen.

Data was presented from trawling surveys in seven estuaries (Alde, Forth, Humber, Mersey, Tees, Thames and Tyne) conducted between October 1998 and January 1999. The use of a 2 metre beam trawl was found to be successful for the capture of benthic dwelling species. Sticklebacks were apparently absent in all lower estuary areas, viviparous blenny were recovered sporadically (only being abundant in the Forth) but sand gobies were caught in sufficient numbers from most estuaries. This initial survey supported the main choice of species (sand goby) and three estuaries; Tees

(contaminated), Thames (intermediate) and Alde (clean), for which a quarterly sampling programme was started in February 1999.

Although the sand goby was a reasonable study selection for the project, the occurrence of three very closely related species (*Pomatoschistus minutus, P. lozanoi and P. norvegicus*) has meant that 'on-site' identification proved difficult. The presenter demonstrated the best way of distinguishing between them by means of the pattern of sensory papillae on the cheeks of the fish. The situation is further complicated by the occasional occurrence of intermediate forms and the existence of *minutus/lozanoi* and *lozanoi/norvegicus* hybrids.

A number of variables will be measured in fixed specimens (length/weight, sex ratio, HSI, GSI, anal papillae size, fecundity etc.) as well as providing tissue for the goby VTG probe and histological analysis of liver and gonad tissue. The anal papilla size is potentially useful because it increases at maturity and there are clear inter-gender morphological details which are hormonally-mediated. Early data on species distribution were presented that showed *P. lozanoi* dominating in the Tees estuary whilst *P. minutus* was more common elsewhere. A suitably clean site will therefore need to be found that will provide *lozanoi* for comparison to the Tees, or another contaminated estuary will need to be found to provide *minutus.*

Finally, future research plans were outlined which included:

- Quarterly sampling of Tees(?), Thames and Alde to establish seasonality of effects.
- Wider sampling of 10-12 estuaries in late 1999 to establish extent of ED in various target species.
- Laboratory breeding experiments with wild caught populations to establish reproductive performance in different estuarine populations.
- Concurrent work on crustacea (probably *Crangon*) to begin soon.

Discussion/Questions

Are the sensory papillae used for identification retained intact after fixation? – *Yes, although they are only well observed using oblique light. If these are damaged or difficult to see, there are other helpful characteristics e.g. colouration (especially during the breeding season) and the morphology of the branchiostegal membrane.*

There were insufficient goby numbers retrieved from the Tyne, is there any chance of siting caged fish? – *Yes, it is possible. Previous studies have shown the fish to be robust enough for cage studies.*

There was a general call for information on target species distribution and abundance. The Manchester Ship Canal (MSC) was suggested as a good place to find sticklebacks in the Mersey estuary, but it was also suggested that the ability to site caged fish could help to study areas with poor target species abundance.

Field Surveys Using Biomarkers for Androgen Exposure in Fish, and for Androgen and Oestrogen Exposure in Crustaceans

Sandy Scott and Ioanna Katsiadaki, CEFAS Lowestoft

Abstract

This work, developing an androgen biomarker sticklebacks and described earlier, has so far all been carried out in the laboratory. Sticklebacks had been sought as part of the field programme, but so far had yielded insignificant numbers. The sampling method (netting) and time of year were probably unsuitable for sticklebacks in estuaries. There are two approaches which could be taken: try to collect wild sticklebacks by trapping, or deploy sticklebacks in cages. Initially, it would be preferable to sample sticklebacks from a limited number of 'representative' sites - e.g. clean river estuary; sewage-affected river; industrially-affected river; and pulp-mill affected river. Skill (and consistency) would be required to collect the fish. Kidneys would be taken and blood collected.

Presentation

The substance of this objective was not yet fully underway. The vast majority of the work had been laboratory based in developing the biomarkers (see earlier presentation on KEH measurements and the spiggin assay) and there had been difficulties in catching suitable numbers of sticklebacks in recent estuarine surveys. The presenter, therefore, took this opportunity to raise a number of issues with respect to sample collection. It was suggested that the current beam-trawling process may not be suitable for stickleback collection and that other methods, e.g. drop nets or baited traps, may need to be deployed. Although deployment of caged sticklebacks was an option, wild caught fish were preferred and a clear need to take into account seasonal influences and migration patterns in any sampling programme was also highlighted.

Once appropriate, key areas had been established, sampling would be conducted. Suitable areas for investigation would be:

- A clean river and estuary
- A sewage affected river and estuary
- An industrially affected river and estuary

The study of an area affected by a pulp-mill discharge was also suggested, because it would probably contain androgenic substances - as demonstrated by recent U.S. research in mosquitofish, where females developed some male morphology. Suggestions from the audience for appropriate sites were invited.

Discussion/Questions

Can the effects of anti-androgens be looked at in the stickleback? – *Potentially yes. The approach would be to look for a reduction in kidney hypertrophy in androgen-induced animals.*

There are various 'races' of three-spined stickleback, are they likely to respond differently? - *This is unknown but unlikely as they all have the same 'glue' making capacity for nest making. Sculpins in marine waters also produce nests and may be another area for investigation.*

Will any effort be expended on the study of effects in protected areas? - *This is definitely a possibility, but by their very nature they tend to be relatively clean and therefore these biomarkers may not show any positive results.*

Would fish used in cage studies be wild caught or laboratory bred? – *Probably wild caught from known clean areas e.g. trout farms.*

Is it not a contradiction to look at allegedly sensitive yet abundant species? If they are sensitive why are they abundant?– *Point taken, but for practical reasons an abundant species has to be used. They still show effects whilst not reaching the point of extinction. However, this does not mean that the population will not be reproductively impaired and they can still act as reasonable sentinels for the study of effects in fish in general.*

Other discussion focused around the need not to ignore the study of sensitive protected areas. The Manchester Ship Canal was again suggested as an area worth sampling for sticklebacks and the offer of histological information on fish from the MSC was made by Mike Simpson of Liverpool University. A discharge of pulp-mill effluent that enters the Mersey was also identified as an item for potential study.

C. IDENTIFICATION OF SUSPECTED SUBSTANCES

Isolation/Quantification of Oestrogenic Substances and Tracking to Sources

Kevin Thomas and Mike Waldock, CEFAS Burnham-on-Crouch

Abstract

In order to determine whether estuarine waters show oestrogenic activity, Toxicity Identification Evaluation (TIE) procedures, using the YES (yeast oestrogen screen) assay, were conducted on samples collected from six estuaries. Waters collected from the Rivers Mersey, Tyne and Tees provided positive responses in the assay at 100x the original estuarine concentrations, whilst a sample collected from downstream of Howdon sewage treatment works (STW) on the Tyne showed activity without pre-concentration. On the basis of these preliminary results, further estuarine and effluent samples were collected from the Tees and Tyne. Effluent samples were collected from the discharge at Howdon STW (Tyne) and Dabholm Gut (Tees) and estuarine samples were collected both up- and downstream of these sites. On return to the laboratory these samples were immediately tested for oestrogenic activity using the YES assay, with no response observed. Pre-concentration of these samples using a novel layered SPE approach produced a series of fractions that were also tested for activity. All the activity detected was associated with the C8 SPE extract and was highest in the effluent collected from Howdon STW (23.6 ngl^{-1} 17-β oestradiol equivalents). This compares with 6.3 ngl^{-1} for Dabholme Gut and approx. 0.5-4 ngl^{-1} in estuarine waters from the Tyne and Tees. These very preliminary assays confirm that as expected the STW effluent value falls within the range found previously for samples containing natural steroids. Interestingly, the industrial effluent has less oestrogenic "potency" and the estuarine water is only weakly oestrogenic. The implication (on the basis of this one investigation) is that animals exposed at these low water concentrations would not exhibit the marked vitellogenic response seen in wild flounder. There could be a number of reasons for this including sample variability, a more complex uptake route than a direct aqueous one, bioaccumulation of the responsible agent in the fish, or even involvement of mechanisms other than receptor binding.

Having established suitable study sites with discharges showing oestrogenic activity, caged flounder (to study VTG induction) were deployed up- and down-stream of Dabholm Gut (Tees), as well as in the effluent plume. Water and sediment samples were also collected for steroid, alkylphenol and *bis*-phenol A analysis and TIE investigations. Water samples (20 l) were extracted by C8 SPE and shown by the yeast assay to have an activity of 1.3 (0.3-2.3) ngl^{-1} 17-β oestradiol equivalents. This extract was then fractionated by reverse phase HPLC to give thirty fractions. Testing of each fraction using

the yeast assay identified 5 fractions showing oestrogenic activity which will be analysed to identify the oestrogenic compounds present. A number of steroids and other oestrogen mimicking substances were measured in Tees water. Values for steroids were lower than detection limits (<4 ngl^{-1} of 17β oestradiol, <3 ngl^{-1} of oestrone, <3 ngl^{-1} of ethynyl oestradiol). Some oestrogen mimicking substances were detected, i.e. 0.04-1.14 μgl^{-1} *bis*-phenol A, 0.1-1.37 μgl^{-1} nonylphenol, 0.3-1.9 μgl^{-1} NP1EO & NP2EO. A robust mass balance of oestrogenic substances measured by the YES assay and by direct analytical measurement is not yet possible, but early indications are that the measured substances do not account for all the oestrogenic activity. Samples collected from the caged flounder are currently being analysed for VTG induction which, when combined with the water and sediment TIE data, will meet the principal objectives of identifying and quantifying key oestrogenic contaminants.

Presentation

The presenter initially went through the principles of the approach i.e. the use of TIE in conjunction with the YES assay. Early developmental work used sewage treatment effluents and a layered SPE system in which samples were sequentially passed through a C8 column (to retain non-polar organics), then an ENV column (to retain the mid-non-polar organics) and finally a graphitic column (as a 'catch-all'). Consequent elution of these columns and screening with the YES assay proved that all oestrogenic activity resided in the C8 column, so this was used in the extractions thereafter. Further fractionation of C8 extracts revealed 3 active fractions believed to be steroidal, nonyl phenol and a third unknown component respectively. Deployment of this technique in a number of estuaries resulted in the targeting of the Tees (Dabholm Gut effluent), the Tyne (Howdon STW effluent) and the Alde (clean) for more detailed investigation including the siting of caged flounder for VTG induction analysis.

The initial results for the Dabholm Gut effluent were then presented. Targeted analysis showed steroids to be below the limit of detection but relative high levels of nonyl phenol, octyl phenol and bis-phenol A were discovered. Flounder from the clean River Alde were placed in cages near, and sites up and down stream of, the effluent. The difficulties associated with this approach were discussed - e.g. small fish were not very suitable and some stress was likely through the effects of continual water flow. These fish were left out for two weeks, at which time approximately 20% mortality had occurred at the effluent site, other sites had 100% survival. Analysis of the plasma by VTG ELISA showed very low levels in males with VTG present in only a few ripening females. It was suggested that this 'non-response' could be attributed to four possible scenarios:

- The fish were not exposed for long enough
- The concentrations of oestrogenic material in the effluent was not high enough.
- The fish were not in contact with the effluent, i.e. it was less dense than seawater and floated on the surface.
- The main route of exposure was by-passed - feeding may be a very important uptake route of oestrogenic contaminants.

Further studies have commenced on the Tyne Howdon STW effluent and it is hoped the fish can be deployed for longer.

Discussion/Questions

Is all the oestrogenic activity of the effluent accounted for by the fractions? - *Not entirely, about 66% is accounted for by the fractions from Dabholm Gut.*

Is the variation in the ELISA results worrying? – *No, the responses gained were normal. Further analysis by radio immuno assay (RIA) will be less variable.*

Do VTG levels in stressed fish decrease? – *It is possible, but most of those retrieved alive looked in good condition. Other studies, especially in trout, have shown strong VTG responses in stressed fish.*

Some discussion took place regarding the best siting of caged fish. For example, sites close to the effluent may mean that the larger ethoxylated akylphenols have not had time to degrade to their more active breakdown products. Also, the variability of the effluent quality could mean that one-off samples may give a misleading picture.

The non-response of the caged flounder was also discussed in more depth. It was hypothesised that the caged fish were, in some way, not responding in the same way as feral fish. It was suggested that the high levels of VTG observed in feral fish could be attributed to contaminated diet, a route of exposure that caged fish would not experience. A case for laboratory experiments using clean fish fed on contaminated natural food was put forward.

Finally, a great deal of interest was expressed in the outcome of the soon to be complete Tyne caged fish study.

D. LABORATORY STUDIES

Laboratory Studies of the Effects of Sewage Effluent and other Suspect Materials on Breeding Success in Sand Gobies

Ron Stagg and Craig Robinson, FRS Marine Laboratory

Abstract

Preliminary work in this laboratory has shown elevated vitellogenin levels in male plaice from contaminated sites in the Clyde which correlates with CYP1A induction at the same sites. The ecological significance of this is unknown and therefore this study is designed to investigate effects of exposure to individual and mixed suspected oestrogen mimics upon vitellogenesis (a sensitive biomarker of oestrogen exposure) in the sand goby, and the relationship between this and reproductive success. Sand gobies are being exposed to sewage effluent from the Irvine Valley Sewer (1% and 0.1% v/v) and the synthetic oestrogen ethynyl oestradiol (6ng l^{-1}) for several months during the maturation of their gonads. Irvine Valley Sewer serves a large industrial and domestic catchment, discharging untreated effluent via long sea outfall into Irvine Bay, in the Firth of Clyde. Historically the effluent has been shown to contain the oestrogen mimic nonylphenol and it's ethoxylated compounds. The study is ongoing, with the main breeding experiments due to start in April. Hepato- and gonado-somatic indices have been measured and appear to show some treatment effects. Chemical analysis of a recent effluent sample showed concentrations of nonylphenol and other suspected oestrogens to be much reduced compared with historical values and reasons for this were discussed, particularly the fact that measures have been taken to reduce the use and discharge of these chemicals. Vitellogenin induction is to be measured (as vitellogenin mRNA expression by Glasgow Caledonian University) and methods for measuring protein production are being investigated. The small size of gobies makes nondestructive sampling impractical and purification of vitellogenin from blood plasma impossible. Western blotting of sand goby liver homogenates with an antibody to plaice vitellogenin showed no protein cross reactivity and further attempts will be made to investigate whether goby gonads could be

used as a source for purifying lipo-vitellin or phosvitin in order to generate an antibody suitable for detecting goby vitellogenin in very small blood samples. During the breeding experiments (throughout which the fish will continue to be dosed), within-treatment crossings will be performed and breeding success measured by the numbers of viable and non-viable eggs and larvae produced. Liver samples continue to be taken for measurements of somatic index and RNA expression, gonad samples for somatic index, histological examination and possibly lipo-vitellin or phosvitin determinations.

Presentation

The presentation started with an outline of previous relevant work. This had focused on the measurement of CYP1A levels and VTG induction in plaice from areas in the Clyde estuary. Increased levels of VTG had been observed as compared to the study reference site off Colonsay, Isle of Mull. An effluent from a major STW, Irvine Valley, on the Clyde was found to contain quite high levels of oestrogenic chemicals (e.g. phthalates, nonyl phenol and nonyl phenol ethoxylates) and therefore was chosen for the conduct of laboratory studies with sand goby.

The objectives of the study were outlined. Briefly, pairs of breeding gobies would be exposed to STW effluent and their reproductive success would be measured (e.g. pairing success, egg viability, larvae viability etc.) and this would then be linked to biomarkers (e.g. VTG induction, VTG mRNA, gonad/liver histopathology etc.). The reproductive model is based on several measurements of batch production, egg production, viability and survival. Previous data were presented which showed that various reproductive parameters: batches per female, total batches, eggs per g, egg incubation time, larvae per batch, larval survival etc., can all be adversely affected by exposure to STW sludge. New studies are on-going in which fish will be exposed during gametogenesis and mating to Irvine Valley effluent and ethynyl oestradiol (EE2) as a positive control. These exposures are in their early stages but many reproductive parameters will be measured as well as histology, gonado somatic index and hepato somatic index. Female GSIs are yet to start increasing but early indications show an apparent reduction in GSIs in males exposed to Irvine Valley effluent and EE2.

Discussion/Questions

How is the effluent dosed and how often is it changed? - *A fresh batch of effluent is collected weekly and is constantly dosed to the tanks to maintain the appropriate concentration.*

With respect to chemical analysis, how does the quality of the effluent vary from batch to batch? - *We do not have those data yet, but this will be investigated.*

Is it possible that all the measurements required for the reproductive model could be simplified to just overall production of eggs or fry? - *At a later date this may be possible but at the moment a comprehensive set of variables needs to be measured in order to minimise the chance of missing something.*

Could you collect eggs from the field and bring them back to be hatched in the laboratory? - *Theoretically yes , but this would require the use of divers which could offer a number of logistical problems.*

Would it not be better to look at effects in fish collected from the wild? - *We need to do the laboratory work in order to recognise what the effects might be in wild populations.*

With respect to goby collection, are they sub-tidal and can they be caught from the shore? - *They can occur sub-tidally but catching techniques and abundance are uncertain.*

Laboratory studies of the effects of sewage effluent and other suspect materials on breeding success in a crustacean.
John Thain, CEFAS Burnham-on-Crouch

Abstract

Work on this objective was due to commence in June 1999 at the CEFAS Laboratory, Burnham on Crouch. An outline of the proposed experimental system was presented. Previous studies at the Burnham Laboratory had indicated that the amphipod crustacean *Chaetogammarus marinus* would be a suitable species for carrying out breeding experiments. *C. marinus* occurs widely in tidal regions and is found in most estuaries. It lives among *Fucus* fronds or under rocks, and has a salinity tolerance range of 7-30 ppt. *C. marinus* has a life cycle of three months; the females carry their eggs between their legs and embryonic development is complete in 10 days at 15°C. After emergence, the young lead a benthic existence; the newly hatched larvae are *ca*. 2mm in length. The sexes are separate and fully grown adults are 13 mm and 18 mm in length for females and males respectively. *C. marinus* can be fed on fresh or dried *Fucus* or 'Tetramin' and is ideal for laboratory studies. An experimental system has been designed for the breeding experiments and will be set up in May to establish baseline parameters.

Presentation

The presenter started by mentioning several of the species that could be appropriate for such studies (*Crangon, Carcinus etc*.) and went on to talk in more depth about the species chosen for this study, *Chaetogammarus marinus.*

Chaetogammarus was described as being widespread in most UK estuaries in the inter-tidal zone. It can tolerate a wide range of salinities (7 - 30 ppt) and has a life-cycle of about 3 months. Like most amphipods, the female carries the eggs until the juveniles (of approximately 2mm) hatch; the juveniles then live in the benthos for their early development. Like some other crustacea (e.g. *Crangon*) *Chaetogammarus* do have certain culturing problems, however the presenter went on to describe a successful culturing system that was developed some years ago for this species and this would be set-up in the coming months.

Early exposure trials, probably with pairs of animals in 2-3 litre tanks, were due to start soon with reference compounds, during which appropriate effects endpoints would be established.

Discussion/Questions

Do *Chaetogammarus* have specific advantages for this type of work over other routinely used crustacean species such as *Tisbe* ? - *Yes, firstly they are much larger and therefore easier to manipulate and secondly their size will mean that enough tissue will be available on which to measure biomarkers of exposure (e.g. vitellin production).*

E. GENERAL DISCUSSION

After the presentations there was an open forum for delegates to express their views and ask further questions. Below are a selection of the main points:-

Concern was expressed by some that more emphasis should be given to the isolation, quantification and identification of causative substances and sources and that the programme should cover wider issues of endocrine disruption, not just those associated with androgens and oestrogens. The chair noted these concerns and pointed out that many were already being addressed but that, although a large programme, funds were inevitably limited and that spreading resources too thinly must be avoided.

It was stated that temporal changes in effluent composition and quality (especially with respect to industrial effluents) meant that the characterisation of contaminant sources and the consequent implications for the route and duration of exposure to fish was a particularly complex issue that would need addressing in a thorough manner. The point was acknowledged and it was noted that the project fully recognised these problems. The studied effluents were being revisited on several occasions to get a more temporally integrated picture.

Continuing the discussion about the nature of the suspect effluents, it was suggested that many were particularly complex and that many other related biological effects would be occurring simultaneously (e.g. induction of the mixed function oxygenase system) that could have a bearing on the analysis and interpretation of endocrine disruption. It was stressed that the use of other related biomarkers (e.g. EROD) should not be overlooked. This was agreed to be a very valid point. It was again stated that not everything could be looked at, but that the complex nature of the effluents and the interaction between biological systems will not be forgotten during interpretation.

Further discussion continued to address routes of exposure. The caged fish approach which looked at the water exposure route was questioned as not perhaps the most appropriate and that routes via the sediment and food should be more thoroughly investigated. This was agreed and those present reminded that sediments from the study areas were to be taken and analysed for ED substances. The issue of food chain exposure is more problematical, but will be considered in further approaches (e.g. experimental fish could be fed with benthic invertebrates from contaminated estuaries).

The steering group was strongly urged by some participants to continue to investigate effects in the River Tyne. This, after all, was one of the estuaries pinpointed as a 'hotspot' of ED during previous studies and concern was expressed that because certain target species (i.e. sand gobies) were not readily available, the efforts in this estuary might be minimal. The chair said that unless target species were caught it was unfortunately difficult to concentrate effort there, however, if techniques such as transplanting caged fish were feasible, then the Tyne would definitely be investigated further.

The issue of how to address effects at the population level was raised. It was acknowledged by the group that it would be particularly difficult to measure population effects in the field. It was explained that a strategic decision had been made to look at

reproductive parameters from field samples and attempt to use this in a predictive model to come to some conclusions about possible population effects.

The question of quantifying the risk EDs posed to already endangered species was put forward. It was noted that all studies so far had concentrated on common species (e.g. flounder and sand goby) that did not appear to be under threat. However, protected species (e.g. lampreys, shad etc.) could potentially be under much greater threat at the population level. This was acknowledged as a good point but it was felt that, realistically, protected species could not be investigated directly and that common species were used as surrogates from which to extrapolate. It was also noted that, at present, the areas where the strongest effects had been observed were not generally important with respect to protected species anyway.

The question of complementary programmes elsewhere was raised. It was suggested that duplication of research must be avoided. Dr. Matthiessen said that there was a large programme known as COMPREHEND that focused mainly on issues related to effluents discharging to freshwater and that he also knew of smaller studies in the Netherlands, Norway, USA and Canada. He was in contact with most of these groups and was forging closer links and promoting co-operation where appropriate. Further enquiries were made concerning the links EDMAR has with OSPAR. Although no formal links exist, part of the justification for Government funding of programmes such as EDMAR was to provide information to feed into OSPAR activities and the EDMAR Steering Group will ensure that all information arising out of this programme will be made available internationally.

PUBLICATIONS

All EDMAR researchers are actively encouraged to publish their findings in the peer-reviewed scientific literature. Publications to date are listed below:

Allen Y.T., Scott A.P., Matthiessen P., Howarth S., Thain J.E. and Feist S.W. (1999). A survey of oestrogenic activity in UK estuarine and coastal waters and its effects on gonadal development of the flounder Platichthys flesus *Environ. Contam. Toxicol.* (In press).

Allen Y.T., Matthiessen P., Scott A.P., Howarth S., Feist S.W. and Thain J.E. (1999). The extent of oestrogenic contamination in the UK estuarine and marine environment - further surveys of flounder. *Science of the Total Environment* (In press).

Hutchinson T.H., Pounds, N.A., Hampel, M. and Williams, T.D. (1999). Impact of natural and synthetic steroids on the survival, development and reproduction of marine copepods (Tisbe battagliai). *Science of the Total Environment* (In press).

Matthiessen P., Allen Y.T., Allchin C.R., Feist S.W., Kirby M.F., Law R.J., Scott A.P., Thain J.E. and Thomas K.V. (1998). Oestrogenic endocrine disruption in flounder (Platichthys flesus L.) from United Kingdom estuarine and marine waters. *Science Series Technical Report* No. 107. Centre for Environment, Fisheries and Aquaculture Science, Lowestoft. 48pp.

Scott A.P. and Hylland K. (1999) Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for the measurement of marine fish vitellogenins. *ICES Techniques in Marine Environmental Sciences*. International Council for the Exploration of the Sea, Copenhagen (In press).

FURTHER INFORMATION

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

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