Endocrine disruption in the marine environment (EDMAR)

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ENDOCRINE DISRUPTION IN THE MARINE ENVIRONMENT (EDMAR)

The University of Liverpool
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1. EXECUTIVE SUMMARY

- Previous research on endocrine disruption in the freshwater and marine aquatic environments has shown that some fish species, particularly rainbow trout, roach and flounder are showing signs of exposure to exogenous oestrogens, as indicated by the presence of the female yolk protein vitellogenin (VTG) in blood plasma and intersex testes in male fish. In light of this work the Endocrine Disruption in the Marine Environment (EDMAR) research programme was initiated in 1998. The overall objectives of this three-year research programme were to investigate whether there is evidence for changes in the reproductive health of both marine fish and invertebrates due to endocrine disruption, and, if so, the possible causes.

- EDMAR was funded by a consortium of UK Government Departments and Agencies - the Department for Environment, Food and Rural Affairs (DEFRA), the Environment Agency (EA) and the Scotland and Northern Ireland Forum for Environmental Research (SNIFFER). The European Chemical Industry Association (CEFIC) also made a contribution. Six major UK laboratories conducted the research: the CEFAS (Centre for Environment, Fisheries and Aquaculture Science) Burnham, Lowestoft and Weymouth laboratories, the Plymouth Environmental Research Centre (PERC), the Centre for Marine and Coastal Studies in Liverpool (CMACS), the Fisheries Research Service (FRS) Marine Laboratory Aberdeen, the AstraZeneca Environmental Laboratory, Brixham and Glasgow Caledonian University.

- To achieve the EDMAR objectives, a total of 12 scientific workpackages were set up to conduct the research required. Broadly, these comprised development of new methods for detecting endocrine disruption in the marine environment, measurement of biological responses in the field and identification of causal substances and sources. Collectively, these would help to build a picture of biological effects in the UK marine environment and possible casual subastances. Each workpackage was the responsibility of one research organisation, although many were dependent on information inputs and some research support from one or more of the other research organisations.

- Since androgen-specific biomarkers for fish were absent, a suitable technique needed to be developed which could subsequently be applied in the field. Two biomarkers of androgenic exposure in the three spined stickleback, *Gasterosteus aculeatus,* were developed and validated: induction of the nest building glue protein, spiggin, using an ELISA (Enzyme-Linked Immunosorbent Assay) technique, and increase in kidney epithelial cell height, as measured in histological sections. Both showed similar and dose dependent sensitivity to model androgens and were further validated with the anti- androgen flutamide. Both assays were used to investigate androgenic and anti-androgenic effects in the field, using caged and wild fish. A few females caged in the Tees estuary showed low levels of spiggin induction.
  Two biomarkers of androgenic exposure were successfully developed and deployed in the field. At the sites examined there was little evidence of androgen exposure.

- To obtain an invertebrate biomarker of exposure to environmental oestrogens analogous to vitellogenin in vertebrates, ELISAs for VTG were developed and tested for two species of marine crustaceans, brown shrimp (*Crangon crangon*) and shore crab (*Carcinus maenas*). However, this protein was not induced on exposure to model oestrogens and was absent in males from a number of oestrogen-contaminated field sites. Male crabs which had been feminised through parasitism by *Sacculina carcini* also did not express VTG.
  These results indicate that VTG induction does not provide evidence of endocrine disruption in response to oestrogens and androgens. This does not necessarily mean that endocrine disruption is not occurring in crustaceans.

- Molecular probes for VTG and zona radiata protein (ZRP) mRNA were developed for the sand goby *Pomatoschistus minutus* and the viviparous blenny *Zoarces viviparus* and tested in the laboratory using a reference oestrogen (ethynyloestradiol (EE2)) and and oestrogenc mimic (octylphenol). This technique does not require large volumes of blood plasma, which are difficult to obtain from small specimens.
  Initial laboratory experiments dosing sand goby demonstrated a similar sensitivity to VTG induction in flounder.

- Protocols were developed for the immunocytochemical localisation of the oestrogen receptor and VTG in flounder liver and gonad tissue slices, and spiggin in stickleback kidney. Localisation of the oestrogen receptor, using mammalian antibodies, failed to produce reliable and reproducible data, but one antibody which gave a positive result indicated that it may have a diffuse localisation in liver hepatocytes, and be localised in the thecal/granulosa cell layer of female gonads and in the Sertoli cells and interstitium of male gonads. Immunocytochemical techniques for localising VTG in flounder liver and gonads and spiggin in stickleback kidney were partially successful. Localisation of VTG was indistinct in hepatocyte cytoplasm; in the gonad, staining was strongly positive in early VTG stage oocytes around the periphery. In the stickleback, spiggin

was localised in the renal epithelium of kidney tubules.

These techniques require further development and validation before being used as biomarkers for evaluating endocrine disruption.

- To assess the extent and effects of endocrine disruption in both fish and invertebrates in the field, an extensive field survey programme was undertaken collecting a number of sentinel species from 14 estuaries and coastal areas around the UK - flounder (*Platichthys flesus*), sand goby (*Pomatoschistus minutus*), viviparous blenny (*Zoarces viviparus*), salmon (*Salmo salar*), sea trout (*Salmo trutta*), brown shrimp (*Crangon crangon*) and shore crab (*Carcinus maenas*).

  - VTG concentrations in male flounder continue to be elevated in some estuaries, particularly in the Clyde, Tyne, Tees and Mersey, although some sites may be showing a downward trend.

  - In keeping with previous studies, intersex in male flounder (i.e. ovotestis) was found to have a low but consistent prevalence. The highest occurrences were found in the Mersey and Tyne. Lower incidences were found in the Clyde and Thames.

  - No VTG or ZRP gene induction or male intersex were observed in wild sand goby caught from 10 estuaries, but this may be due to the short half-life of mRNA which could therefore be missed if exposures to oestrogens are transient. Some wild male sand goby exhibited morphological abnormalities in the uro-genital papilla (UGP), an organ which shows secondary sexual characteristics and is known to be involved in gamete deposition. These males had UGPs with a shape intermediate between male and female. The highest incidences of this condition, termed Morphologically Intermediate Papilla Syndrome (MIPS) were found in males from the Tees, Mersey and Clyde; it was rare or absent in the Alde (the clean reference estuary), Crouch and Thames. The biological significance of this is as yet unclear.

  - More limited surveys of the viviparous blenny showed evidence for oestrogenic endocrine disruption. Up to 100% of males produced VTG in some estuaries (Tyne, Tees and Clyde) and intersex males were found in the Clyde and Tyne.

  - Potential effects of oestrogenic endocrine disruption on exoskeletal morphology of wild shore crabs were investigated using males from four sites where strong oestrogenic contamination is present - the Tyne, Tees, Clyde and Nene - and the Alde reference site.

Surveys of intersex and VTG induction in male flounder indicate that they are still being exposed to high concentrations of oestrogenic substances in some estuaries, particularly in the Clyde, Tyne, Tees and Mersey, although some sites may be showing a downward trend.

Morphological abnormalities of secondary sexual characteristics, which have not been observed previously, were found in male sand goby.

Although retarded growth in some characteristics of male shore crab was evident, it was difficult to identify any clear differences between the reference site and contaminated sites.

- The possible effects of delayed migratory passage through contaminated estuaries on smolt physiology and seawater adaptation of Atlantic salmon and sea trout were investigated. Salmon smolts caged for a realistic migration time period of 5 days within the Tees estuary below the Barrage and in an adjacent control estuary (River Esk) showed no induction of VTG, and no adverse effects on gill physiology, which indicates no effect on seawater adaptation. Adult male migratory Atlantic salmon and sea trout obtained from several estuaries showed little or no induction of VTG. Those with detectable levels were from the Tees and Tyne estuaries and coastal waters outside the River Tyne. There were no effects on gill physiology and no evidence of intersex in any of the males.

  These results suggest that endocrine disruption in salmonids within estuaries is not widespread and it is unlikely that exposure to oestrogenic contamination is a major factor regulating salmonid populations in England and Wales.

- The Toxicity Identification and Evaluation (TIE) technique with yeast oestrogen and androgen screens (YES and YAS) was used to identify and quantify oestrogenic and androgenic substances in samples from a range of estuaries. Oestrogenic activity was highest in the Tyne and Tees. In water and effluents substances identified included the natural steroids 17β-oestradiol and androsterone, and the synthetic compounds nonylphenol (a surfactant metabolite) and bis(2-ethylhexyl)-phthalate. Much higher oestrogenic activity was found in sediments and nonylphenol, cinnarizine (an anti-histamine drug) and cholesa-4,6-dien-3-one (a natural cholesterol degradation product) were positively identified as minor contributors. Androgenic activity was highest in the Clyde estuary. In the Irvine Valley Sewer effluent the natural steroids/steroid metabolites dehydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone and epi-androsterone were responsible for nearly all of the androgenic activity.

Both natural steroids and industrial chemicals are responsible for the oestrogenicity in the estuaries sampled. Androgenicity has so far been attributed to natural steroids.

- Male flounder caged on sediment for 2 weeks in the Tyne and Tees showed no VTG induction, so feeding experiments with shrimp and mussels from the Tees were initiated to investigate other exposure pathways. No VTG induction was observed in male flounder from the Alde reference site fed with wild-caught shrimp from the Tees, but those fed on mussels that had been caged for several months on the Tees showed some induction. This increase was much lower than the levels observed in wild-caught flounder.
  Although food chain exposure of wild fish to oestrogenic substances appears to be a possibility, the results indicate that several exposure factors may be interacting in the natural environment.

- The effects of long-term exposure to sewage effluent (from the River Irvine in Scotland), and known oestrogenic substances on biomarker responses (VTG mRNA), maturation indices and reproductive success were investigated in the sand goby. Exposure to known oestrogens induced VTG mRNA expression, reduced testis and seminal vesicle size and delayed/inhibited nuptial colouration. Long-term exposure to environmentally realistic concentrations (0.3 and 0.03%) of Irvine Valley Sewage (IVS) effluent produced only weak (not statistically significant) effects on markers of oestrogenic exposure (VTG mRNA, seminal vesicle size, nuptial colouration) at the higher concentration. Although exposure to known oestrogens significantly reduced population reproductive output (fertile egg production), it was unaffected by exposure to IVS, but reduced adult survival resulted in reduced overall output.
  Long term exposure to sewage effluent weakly affected primary and secondary sexual characteristics but did not affect reproductive parameters. Survival of adults was affected resulting in impacts on laboratory population size.

- As part of EDMAR associated research, a laboratory based programme was initiated using a marine copepod (*Tisbe battagliai*) life-cycle test to investigate the potential for environmental endocrine disrupters to affect population relevant parameters (survival, development, reproduction and sex ratio) in indigenous Crustacea. It was demonstrated that a range of steroidal oestrogens and androgens showed no reproductive or developmental toxicity to *T. battagliai*. The ecdysteroid (moulting hormone) 20-hydroxyecdysone and the reference oestrogen diethylstilbestrol (DES) did cause significant inhibition of development and reproduction of these copepods. A newly developed *in vitro* ecdysteroid receptor (EcR) binding assay showed that natural and synthetic oestrogens/androgens and their antagonists did not bind to the EcR, but Bisphenol-A, diethylphthalate and lindane were weak EcR antagonists.
  Natural vertebrate steroids did not affect the life-cycle of a model crustacean. Several synthetic compounds, including a pesticide, were antagonists for the invertebrate moulting hormone receptor.

- In summary, the EDMAR project has shown that oestrogenic effects at the biochemical, cellular or gross morphological levels are present in five species of estuarine fish from a range of industrialised (past and/or present) estuaries. Insufficient field data are yet available to indicate whether these changes have consequences for reproductive success, although experimental data from sand gobies suggests that this is a possibility. A range of natural and synthetic oestrogens is present in the estuaries where the most marked effects have been observed. Most of the activity is strongly adsorbed on sediment particles and much remains to be identified. However, it seems likely that flounder (and possibly other fish) obtain some oestrogenic exposure through feeding on benthic invertebrates. Whether crustaceans themselves are susceptible to oestrogenic exposure remains an open question; apparent changes of shore crab exoskeletons from oestrogen-contaminated locations are not conclusive and require further investigation. In comparison with oestrogenic effects, those caused by exposure to androgens appear to be weak or non-existent in fish (stickleback), although few data are yet available. However, weak androgenic activity is present in the vicinity of poorly treated sewage discharges and is composed exclusively of natural substances in the single sewage effluent subjected to TIE.

- Various research recommendations flowing from EDMAR and other UK research programmes on endocrine disruption in freshwaters were discussed at a DEFRA sponsored workshop at CEFAS Weymouth in September 2001. The report of this workshop "**Future directions for government funded research on endocrine disruption in the aquatic environment**", can be obtained from the EDMAR secretariat at DEFRA, 3/E6 Ashdown House, 123 Victoria Street, London SW1E 6DE.

2. BACKGROUND AND INTRODUCTION

2.1 Background to endocrine disruption

Some substances that enter the aquatic and terrestrial environments have the potential to disrupt the normal function of the hormonal (endocrine) system of humans and wildlife. These can either be naturally occurring or synthetic and are collectively termed "endocrine disrupters" (EDs). Much of the initial research on endocrine disruption focussed on vertebrate sex steroid hormone systems, but the field is broadening to include other vertebrate as well as invertebrate systems. Endocrine disrupting chemicals can be classed according to their mode(s) of action: substances that mimic or block the natural female sex hormone oestradiol are termed oestrogens and anti-oestrogens respectively; those that mimic or block the natural male sex hormone testosterone are termed androgens and anti-androgens. Those chemicals that interact directly with steroid receptors have the potential to produce effects at extremely low concentrations. Since gonadal steroid hormones control and regulate embryonic development and sex differentiation, it is during this period that the effects of EDs may be particularly severe. In addition, endocrine disrupters can interfere with sex steroid synthesis and metabolism (Kelce and Wilson, 1997; Sonnenschein and Soto, 1998), and with the pituitary, thyroid and interrenal hormone systems (Van der Kraak *et al*, 1992; Leatherland, 1993). Their effects therefore potentially reach to every significant biological process.

A large number of compounds has been reported to possess endocrine modulating activity. These include: natural products (e.g. coumestrol, genistein), and some members of the following categories of chemicals: pesticides, fungicides and insecticides (e.g. dieldrin, toxaphene, endosulfan, phenylphenol, DDT and its metabolites, methoxylchlor, vinclozolin), medical drugs (e.g. hydroxyflutamide, nilutamide, tamoxifen, diethylstilbestrol, oral contraceptives such as ethynylestradiol), commercial and/or industrial chemicals such as bisphenol-A, alkylphenols (*p*-nonylphenol), polychlorinated biphenyls (PCBs), phthalate plasticisers, PAHs and some metals.

Most endocrine disruption research has focussed on the effects of oestrogens, which can have a feminising effect. In humans, reproductive disorders and reduced sperm counts have been attributed to oestrogens (Sharpe and Skakkebaek, 1993; Henderson *et al*, 1988). Oestrogenic effects in fish, both in the laboratory and in the field, have been extremely well documented, including induction of the female yolk protein in males (vitellogenesis: e.g. Folmar *et al*, 1996, Lye *et al*, 1997; Tyler and Routledge, 1998; Kime *et al*, 1999), abnormal gonad development (e.g. Jobling *et al*, 1996, 1998; Gimeno *et al*, 1996) and alterations in sex steroid titres (Folmar *et al*, 1996).

Far less research has been carried out on compounds with androgenic (and also anti-androgenic) effects. One of the clearest observations of androgenicity in the aquatic environment has been made in female mosquitofish (*Gambusia* sp.), living downstream of kraft mill effluent discharges. These develop anal fin appendages (gonopodia) that are normally only found in males (Howell *et al*, 1980; Howell and Denton, 1989; Cody and Bortone 1997). The active compounds in the effluent have been tentatively identified as bacterial degradation products of the plant sterol, stigmastanol (Denton *et al*, 1985). Other studies have revealed a decrease in the gonad size of female fish living near coastal water receiving bleached pulp mill effluent (Andersson *et al*, 1988; Sandström *et al*, 1988) and significantly male-biased eelpout (*Zoarces viviparous*) broods near a large pulp mill (Larsson *et al*, 2000).

Compounds with anti-androgenic activity include pesticides and insecticides such as fenitrothion, vinclozolin, procymidone, linuron, iprodione, chlozolinate, ketoconazole, DDT and its metabolite, p,p',-DDE, as well as several pyrethroids. (Gray *et al*, 1999; Ostby *et al*, 1999; Ashby and Lefevre 2000; Makynen *et al*, 2000; Sohoni *et al*, 2001; Tamura *et al*, 2001). To this list can be added bisphenol A (Sohoni and Sumpter, 1998) and certain pharmaceuticals like cyproterone acetate and the well-known pure anti-androgen flutamide (Bratoeff *et al*, 2000).

Evidence for endocrine disruption in invertebrates, either terrestrial or aquatic, that is associated specifically with an established cause is very sparse. The effects of tributyltin (TBT), an ingredient of antifouling paint for boats and ships, provides one of the few clear examples of endocrine disruption in aquatic invertebrates. It is now well established and understood that imposex and intersex in gastropod molluscs is induced almost uniquely by TBT (Bryan *et al*, 1987). The adverse effects and mechanisms of action of TBT on marine organisms are comprehensively reviewed in Bryan and Gibbs (1991), Alzieu (1996) and Matthiessen and Gibbs (1998).

2.2 Endocrine disruption research in the UK aquatic environment

Concern over the effects of oestrogenic chemicals in the UK aquatic environment was first expressed by anglers and water authorities, who reported the presence of hermaphrodite fish in the settlement lagoons of some sewage treatment works (STWs). Subsequent research has indicated that many STW final effluents contain substances that are oestrogenic to male fish, as manifested by the induction of the female

yolk precursor protein vitellogenin (VTG) (Purdom *et al*, 1994; Harries *et al*, 1996, 1997) and appearance of oocytes (i.e. intersex) in the testes (Jobling *et al*, 1998; Nolan *et al*, 2001). The causal agents of oestrogenic activity in some UK STW effluents have been identified as being the natural hormones 17ß-oestradiol and oestrone and the synthetic hormone ethynylestradiol (Desbrow *et al*, 1998). In some locations the surfactant nonylphenol has been implicated (Harries *et al*, 1997).

To investigate whether endocrine disrupting effects (specifically oestrogenic effects) were occurring in the marine environment, laboratory studies and extensive field surveys of UK estuaries were carried out using the euryhaline flounder *Platichthys flesus* as the monitoring species. The results confirmed that several UK estuaries, particularly the Tyne, Tees and Mersey were severely contaminated with oestrogens (Matthiessen *et al*, 1998; Allen *et al*, 1999 a,b), again manifested as VTG induction in males and the presence of intersex testes.

With the exception of research on the impacts of TBT, research on endocrine disrupting effects in marine invertebrates in the UK is limited. In the Firth of Forth, Scotland, there have been reports of a high prevalence of intersex harpacticoid copepods in the vicinity of sewage treatment works outfalls (Moore and Stevenson, 1991, 1994), but no conclusive causal relationship has been determined.

There are therefore good indications that endocrine disrupting chemicals are entering the UK marine environment and causing biological responses. However, apart from the case of TBT, the causal substances are unknown, as is the extent of potential impacts at the population level. Although reliable biomarkers exist for measuring the presence of exogenous oestrogens in fish, there are no biomarkers for androgenic effects, other than the measurement of oestrogen-androgen ratios. In addition, there is very little information about the effects of oestrogenic and androgenic contamination on invertebrates, which is partly due to the absence of reliable specific biomarkers for detecting endocrine disruption.

2.3 The EDMAR programme

In the light of these gaps in knowledge, the **E**ndocrine **D**isruption in the **Mar**ine Environment (EDMAR) research programme was initiated. The overall aim was to investigate whether there is evidence for changes in the reproductive health of both marine fish and invertebrates associated with endocrine disruption, and, if so, the possible causes and potential impacts. EDMAR started in June 1998 and was completed in December 2001.

EDMAR was funded by a consortium of UK Government Departments and Agencies - the Department for Environment, Food and Rural Affairs (DEFRA, formerly as the Department of Environment, Transport and the Regions (DETR) and the Ministry of Agriculture, Fisheries and Food (MAFF)), the Environment Agency (EA) and the Scotland and Northern Ireland Forum for Environmental Research (SNIFFER). The European Chemical Industry Association (CEFIC) also made a contribution. Six major UK laboratories conducted the research: the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) Burnham, Lowestoft and Weymouth laboratories, the Plymouth Environmental Research Centre (PERC), the Centre for Marine and Coastal Studies in Liverpool (CMACS), the Fisheries Research Service (FRS) Marine Laboratory Aberdeen, the AstraZeneca Environmental Laboratory, Brixham and Glasgow Caledonian University. In addition, the Scottish Environment Protection Agency (SEPA) provided assistance with research carried out by FRS.

To achieve the EDMAR aim, a number of scientific objectives were identified, which broadly comprised development of new methods for detecting endocrine disruption in the marine environment, measurement of biological responses in the field and identification of causal substances and sources.

A series of workpackages were set up to conduct the research required to address these objectives. Each workpackage was the responsibility of one research organisation, although many were dependent on information inputs and some research support from one or more of the other research organisations. The workpackages were as follows:

A. Development of new methods to detect endocrine disruption in the marine environment

1. Develop a biomarker for androgen exposure in a suitable fish species.
2. Develop biomarkers for oestrogen and androgen exposure in marine crustaceans.
3. Develop simple histochemical methods for measuring vitellogenin/vitellin, oestrogen and androgen biomarkers in small tissue slices of fish and crustaceans.
4. Develop robust biomarker protocols for routine monitoring.

B. Biological responses in the field

5. Field surveys using biomarkers for androgen exposure in fish, and for oestrogen and androgen exposure in crustaceans.
6. Field studies to assess the impact of oestrogenic compounds on juvenile and adult salmon during estuarine migration.
7. Field investigation of ED biomarker responses and reproductive success in a fish species which breeds in estuaries.

C. Investigating Causal Links

8. Isolation/quantification of oestrogenic substances and tracking to sources.
9. Isolation/quantification of androgenic substances and tracking to sources.
10. Laboratory studies of the effects of sewage effluent and other suspect materials on breeding success in sand gobies.

In addition to the main EDMAR programme, there were a number of areas of Associated Research (see section 3.1.3 and Appendix 1). Briefly, Glasgow Caledonian University, with funding from DEFRA (formerly MAFF), developed molecular probes for vitellogenin (VTG) and zona radiata protein (ZRP) in a range of fish which were used for the measurement of VTG and ZRP-specific messenger RNAs. AstraZeneca funded work at their Brixham Environmental Laboratory aimed at assessing developmental and reproductive effects of endocrine disrupters using marine copepod life-cycle studies. A project with Exeter University was also initiated to screen a range of contaminants for potential endocrine disruption using an ecdysteroid-sensitive assay.

This report gives a summary of the EDMAR research programme, its results and conclusions. Further details can be found in the main contract report, available from the EDMAR Secretariat, DEFRA, 3/E6 Ashdown House, 123 Victoria Street, London SW1E 6DE.

3. APPROACHES USED

3.1 Biomarker development

3.1.1 Stickleback KEH/spiggin

To address the lack of a biomarker for androgenic exposure, EDMAR aimed to develop an *in vivo* androgen biomarker using the three-spined stickleback (*Gasterosteus aculeatus* L.). This species is widely distributed in most parts of the world and is endemic to Europe. Male sticklebacks have pronounced secondary sexual characteristics during their breeding season which are controlled by androgenic hormones. These include enlargement of the the kidney epithelium cells to produce a glue protein called spiggin, used when building a nest. EDMAR aimed to develop and validate a rapid and sensitive immunoassay for spiggin and correlate this with the kidney epithelium cell height (KEH) as measured in histological sections.

3.1.1.1 Development of ELISA for spiggin

The procedures for the spiggin immunoassay are described in detail in Katsiadaki *et al* (2002). This essentially involved the following steps:

- Isolation and purification of spiggin from hypertrophied kidneys and urinary bladder contents from breeding males, as well as nest threads
- Injection of spiggin into rabbits to produce spiggin antibodies.
- Preparation of a spiggin standard.
- Optimisation of ELISA incubation times and concentrations of reagents and antibodies.
- Validation of ELISA with KEH measurements.

3.1.1.2 Laboratory exposure to androgens and anti-androgens

Fish were maintained in glass aquaria containing either brackish water or full sea water and fed daily with a combination of dried flakes (Tetramin) and live *Daphnia*.

A range of steroids, dissolved in methanol were applied via the water at different concentrations: keto-testosterone (11-KT) at 10 and 20 $\mu g\ l^{-1}$, methyl-testosterone (17α-MT) between 0.01 and 50 $\mu g\ l^{-1}$, dihydrotestosterone (DHT) at 5 $ng\ l^{-1}$ to 50 $\mu g\ l^{-1}$ and ethynylestradiol (EE2, as a negative control) at 20 $ng\ l^{-1}$. The anti-androgen flutamide (FL) was tested at 500 $\mu g\ l^{-1}$ along with 17α-MT or DHT. The fish were exposed to the compounds for various periods from one to five weeks. At the start of the study, a semi-static system was used for the administration of the compounds to the water. In later experiments, the fish were dosed continuously in a flow-through arrangement.

At the end of each experiment the kidneys were dissected for spiggin and KEH measurements.

3.1.2 Crab and shrimp VTG ELISAs

The potential for vertebrate-type hormones to influence the hormonal control systems of marine crustaceans is unknown. The purpose of this area of work was to establish whether or not such a risk exists and whether it is possible to develop a biomarker to recognise any subsequent consequences.

Female crustaceans synthesis egg yolk protein as a nutritional store for developing embryos in much the same way as fish. The presence of vitellogenin in male crustacean haemolymph samples could provide an effective biomarker for endocrine disruption, in a manner similar to fish. An enzyme linked immunosorbent assay (ELISA) sensitive to shore crab and brown shrimp vitellogenin was developed, following a similar methodology to that employed in the development of the test for fish (e.g. Mourot and Le Bail, 1995).

The shore crab, *Carcinus maenas*, was selected as it is ubiquitous around the coast of Britain and has a particular association with estuaries, a habitat often susceptible to contamination from anthropogenic sources. This crab is relatively large in size and it is possible to obtain a series of non-destructive samples of haemolymph from each individual. They are single sexed throughout life, with males and females readily identifiable. The brown shrimp, *Crangon crangon*, is also associated with coastal waters, including estuaries.

Unlike shore crabs, brown shrimp are sequential hermaphrodites, existing in male, intermediate and female forms. Vitellogenin was measured in each of these sexual states.

Potential biomarkers of androgenic disruption in crustaceans were also investigated. Shore crabs can be parasitised by the barnacle *Sacculina carcini*, which in males can lead to feminisation of their physical form via supression of male hormone secretion. Various assessments were run to identify suitable biomarkers of such anti-androgenic activity. In addition to these studies on males, the impact of androgenic compounds on the progress of vitellogenesis in female crabs was examined, with test crabs exposed to testosterone in the laboratory.

3.1.2.1 ELISA development and methodology

Mature oocytes taken directly from the ovaries of crabs and shrimps provided the source material for the antigen (VTG). This was purified and sent to AstraZeneca, who raised the polyclonal antibodies for both shore crab and shrimp VTG in rabbits, following their inoculation with a sample of the antigen.

The specificity of the polyclonal antibody was tested using the western blotting technique before the ELISA was developed and conducted (see Appendix 3 for details).

3.1.2.2 Laboratory exposure of crabs to nonylphenol, diethylstilbestrol and testosterone

Male and female crabs collected from a clean site (Bantham, South Devon) were exposed to known oestrogens (Diethylstilbestrol (DES) and nonylphenol) and an androgen (testosterone) for twenty one days at a concentration of 100 $\mu g\ l^{-1}$. Haemolymph samples were taken from each crab at the start of the exposure period and then after 7, 14 and 21 days and immediately frozen in liquid nitrogen. All samples were subsequently analysed using the VTG ELISA.

3.1.2.3 Analyses of crabs parasitised by Sacculina carcini

Shore crabs carrying the parasitic barnacle *Sacculina carcini* were obtained from Millport Research Station (University of Glasgow) and collected as part of the EDMAR field survey programme. Samples of haemolymph were taken from each individual and proteins analysed using gel electrophoresis (see main EDMAR contract report for details). In addition, the presence or absence of vitellogenin in the haemolymph of crabs was determined using the ELISA methodology described above.

3.1.3 Blenny and goby VTG/ZRP mRNA

VTG and zona radiata protein (ZRP) (a structural component of the egg envelope) have been used as biomarkers for oestrogenic exposure in fish. Historically, these proteins have been measured in serum with immunological reagents which are obtained after purification of the egg proteins. The purified proteins are used to generate antibodies which under assay conditions react with VTG or ZRP from the serum of test animals to produce specific signals. This is a robust and convenient method for the determination of VTG and ZRP, however, significant quantities of serum are required for protein purification, which places restrictions on the size of fish that can be used. In addition, the antibodies to one species tend not to react with the egg proteins of other species so antibodies need to be prepared for each species to be investigated. Two of the species chosen for study under the EDMAR programme, sand goby (*Pomatoschistus minutus*) and viviparous blenny (*Zoarces viviparus)* were small, and hence it is difficult to obtain serum for purification or assay. Additionally there were no "off-the-shelf" antibody reagents available to measure their production of VTG or ZRP. An alternative strategy for the measurement of the production of these proteins is to look in liver rather than serum and at messenger RNA (mRNA) rather than protein. Molecular probes were therefore developed to measure VTG and ZRP mRNA in sand goby and viviparous blenny, as biomarkers of oestrogenic exposure.

Full technical descriptions of the development of the molecular probe methodologies are contained in various publications (Craft *et al*, 2002; Robinson *et al*, 2001; Kirby *et al*, in press). In addition, the sequence data has been made publicly available by submission to the global sequence database (GenBank/ EMBL - see References for accession numbers).

Briefly, the technique (Figure 1) requires production of a DNA copy of the mRNA under investigation, via conversion of RNAs to single stranded complementary DNA (cDNA) followed by the production of double stranded cDNA, a step achieved by the Polymerase Chain Reaction (PCR). The resulting fish cDNA fragment is fused with another DNA molecule that acts as a vehicle, allowing the recombinant molecule to be introduced into bacterial cells. These produce multiple replicates of the DNA which can be purified and sequenced. Finally the isolated cDNA can be tested for its ability to react with the mRNA of interest.

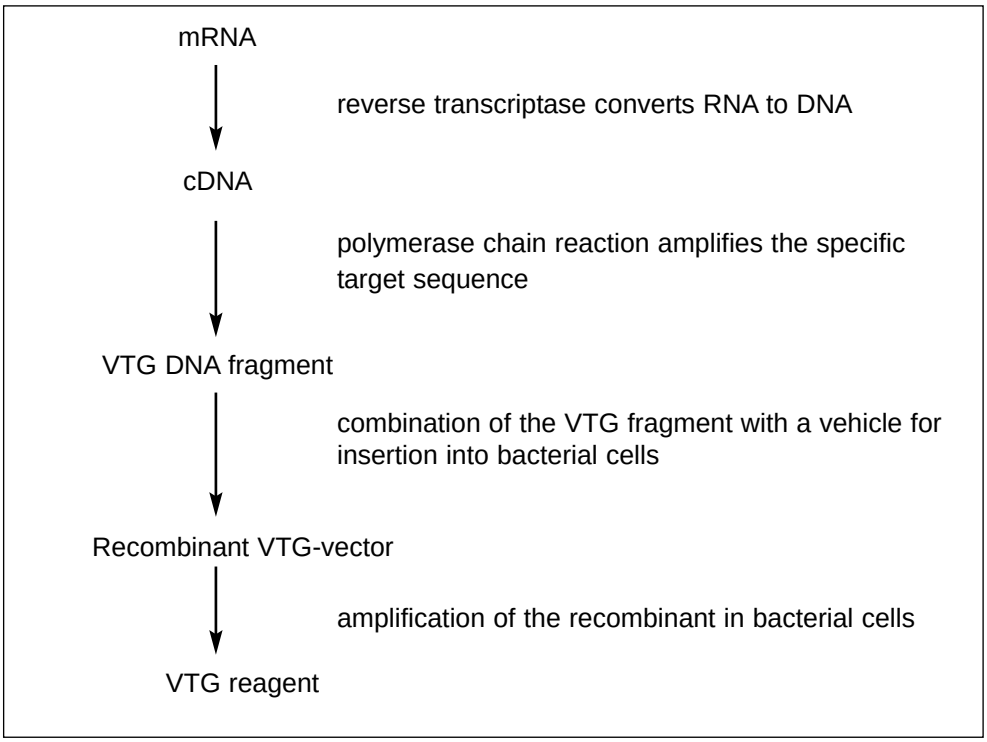


Figure 1. Schematic representation for the preparation of VTG cDNA reagents

3.1.4 Flounder RIA/ELISA

A specific radioimmunoassay (RIA) for the precise measurement of VTG concentrations in flounder blood plasma has been previously developed and validated (Matthiessen *et al*, 1998, Allen *et al*, 1999a). Briefly, the following steps are required:

- Injection of male flounders with oestradiol (E2) in order to induce VTG production
- Bleeding of fish after two weeks, to collect plasma
- Precipitation of VTG from the plasma by addition of salts and distilled water
- Purification of VTG by ion-exchange chromatography and freeze drying
- Injection of VTG into rabbits to induce antibody production
- Radiolabelling of VTG with Iodine - I25 and preparation of a standard
- Validation experiments to establish optimum concentrations of reagents and incubation times for RIA.

An Enzyme-Linked Immunosorbent Assay (ELISA) for flounder VTG was also developed for EDMAR using the same procedure as for the development of the spiggin ELISA (Katsiadaki *et al*, 2002).

3.1.5 Histochemical techniques

Immunohistochemistry is a technique which can visualise the localisation of cellular complexes, thereby allowing pathological interpretation. The EDMAR programme sought to develop histochemical techniques for detecting and quantifying oestrogen and androgen biomarkers as these can be more amenable for measuring ED in small tissue slices of fish or crustaceans compared with RIA/ELISA methods, which normally require relatively larger sample volumes. The main areas of research were to localise the oestrogen receptor in the liver and gonads of the flounder, and secondly to localise VTG in flounder liver and gonads and the glue protein spiggin in stickleback kidney.

Flounder and stickleback were collected and maintained in aquaria until dissection of tissue samples (maximum of 2 days). Once removed, all tissues were fixed in neutral buffered formalin.

Two different methods were used to localise the oestrogen receptor in flounder liver and gonads. In the first, termed immunolocalisation, antibodies of the mammalian oestrogen receptor were applied to flounder histological sections, which on successful binding to the antigen (flounder oestrogen receptor) produced a reaction product which could be visualised microscopically. The second technique used soluble compound autoradiography. Fish were injected with radiolabelled oestradiol and liver and gonad sections subsequently developed with a photographic developer.

Sites of VTG production in flounder liver and gonad and spiggin production in stickleback were localised using anti-vitellogenin and anti-spiggin antibodies respectively, which were raised in rabbit. As with the first oestrogen receptor technique described above, a dye was used to visualise the antibody-antigen binding sites.

Full details of the immunocytochemistry techniques are provided in the EDMAR contract report.

3.2 Biological responses in estuaries

3.2.1 Field surveys

To investigate the extent and severity of endocrine disruption in the estuarine environment, 6 key species were chosen with which to conduct extensive field surveys. These were the flounder (*Platichthys flesus*), two sand goby species (*Pomatoschistus minutus* and *P. lozanoi*), viviparous blenny (*Zoarces viviparus*), brown shrimp (*Crangon crangon*) and shore crab (*Carcinus maenas*). All except the flounder spend their whole lifecycle within estuaries.

Previous research using flounder clearly demonstrated that oestrogenic endocrine disruption was occurring in some UK estuaries (Allen *et al*, 1999a,b; Matthiessen *et al*, 1998). Although flounder stay in estuaries for most of the spring/summer and hence can reflect local conditions for a part of the year, they do migrate offshore in the winter to spawn (Vethaak and Jol, 1996). This presents difficulties with data interpretation for the period immediately after migration and has been suggested as the reason for high vitellogenin levels in flounder caught far afield from known sources of oestrogenic contamination (Matthiessen *et al*, 1998). In addition, offshore spawning means that the most sensitive stages of the lifecycle (e.g. eggs and larvae) which may be affected the most by endocrine disruption are not exposed to the high levels of endocrine disrupting chemicals which are found in some estuaries.

Plasma VTG in male flounder is the most widely applied biomarker of estuarine oestrogenic endocrine disruption in the UK. Early surveys established that plasma VTG levels were highest in flounder from the estuaries of the rivers Mersey, Tees and Tyne. EDMAR flounder surveys aimed to continue this work to establish trends.

The sand goby (*Pomatoschistus* sp.) is a small fish ubiquitous in UK estuarine and coastal environments and was chosen as a monitoring species for EDMAR because of its abundance, short life history and sedentary nature. Three closley related species occur in UK waters, two of which are common to estuarine and coastal habitats - *P. minutus* and *P. lozanoi*. The aim of the goby surveys was to determine whether they are responding to oestrogenic endocrine disrupters in the same way as flounder. Molecular probes to measure the induction of vitellogenin (VTG) and zona radiata protein (ZRP) mRNA were developed and applied for this purpose (see section 3.1.3). In addition, testes were examined for the determination of intersex and the external appearance of the uro-genital papilla (UGP) examined. The UGP is a secondary sexual chacteristic and demonstrates very obvious sexual dimorphism: specimens are normally sexed by examination of this organ. The potential use of the uro-genital papilla length index (UGPLI) as an effect indicator in environmental samples was assessed.

The viviparous blenny (*Zoarces viviparus*) was identified as a suitable species for use in surveys because it spends the majority of its life cycle in estuaries and therefore effects observed would reflect a cumulative exposure, potentially over several years. Furthermore, the viviparous nature of this species i.e. the young develop inside the mother, allows fecundity, sex ratios and larval viability/deformities to be measured, and hence effects on recruitment can be indirectly assessed. Viviparous blenny has a limited distribution in the UK, being absent from the west and south coasts of England (Wheeler, 1969). However, their presence in the key estuaries of the Alde, Tyne, Clyde and Forth ensured a reasonable spread of data from impacted and non-impacted areas. As with sand goby, an mRNA molecular probe for VTG was developed and applied for blenny.

Male-skewed larval sex ratios have been noted in *Z. viviparus* near to pulp mill effluent discharges in Sweden, with exposure to androgens believed to be the cause (Larsson *et al*, 2000). Since some of the sites in the EDMAR surveys are known to be contaminated with oestrogens, larval sex ratios were examined to explore the hypothesis that these would have a female skew in these areas.

Over the duration of the EDMAR programme a total of 14 estuarine or near-shore areas were surveyed, some on numerous occasions. Environment Agency research vessels or other chartered boats were used and specimens caught using a beam trawl fitted with a mesh liner. Figure 2 shows the sampling site locations.

3.2.1.1 Flounder

Four flounder surveys (September 1999, March 2000, November 2000 and February 2001) were conducted. The 'hotspot' estuaries of the Tyne, Tees and Mersey were re-targeted as well as two estuaries in Scotland, the Clyde and Forth (Figure 2). At each site the aim was to sample as many male flounder as were available, with a preferred minimum number of 10. However, during some surveys a number less than this was obtained. Blood samples were taken from each fish, centrifuged to obtain the plasma and stored frozen in liquid nitrogen until analysis for VTG using the method described in Matthiessen *et al* (1998). Samples of gonad from each fish were fixed in formalin for histological examination,

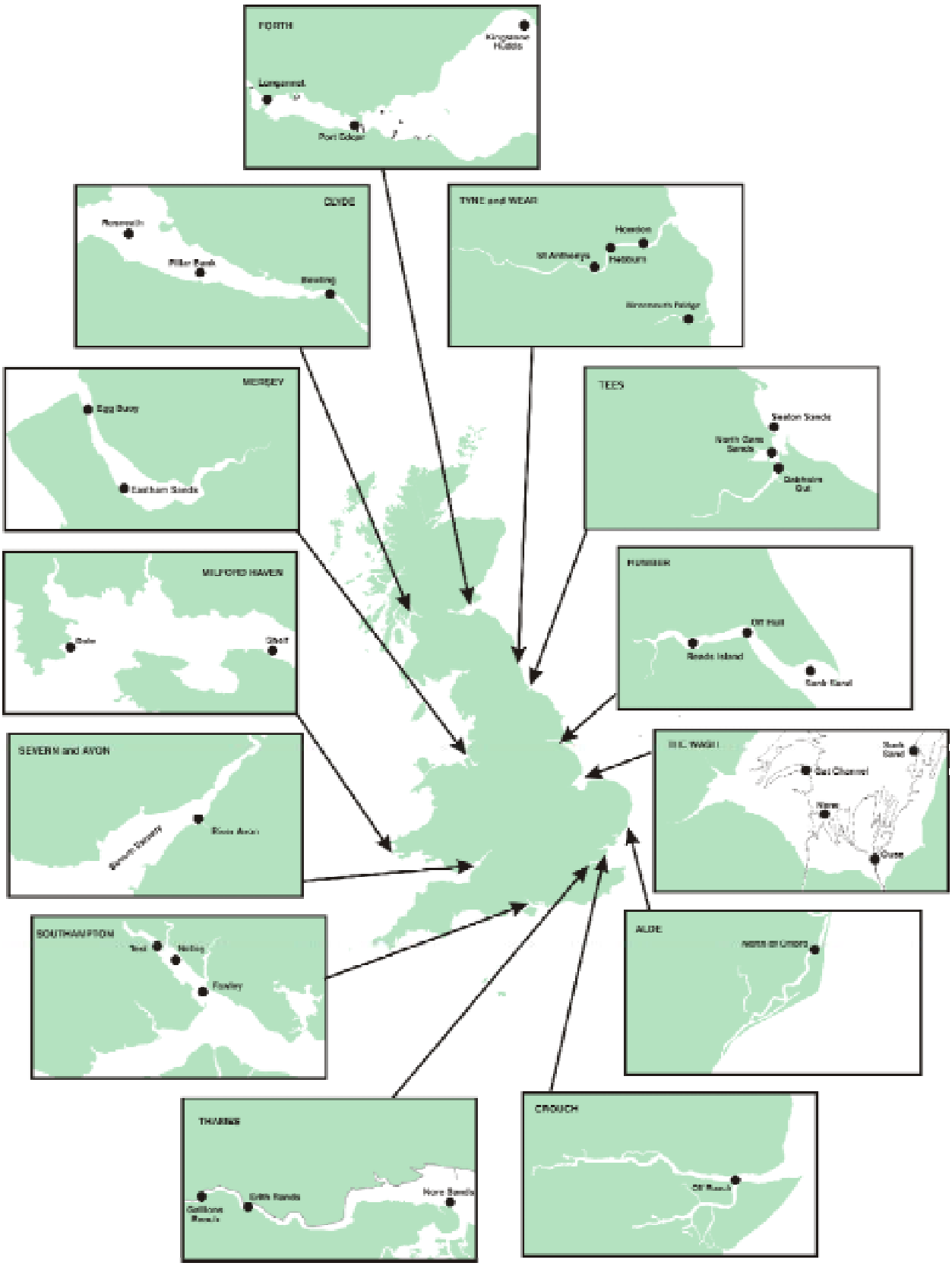


Figure 2. Locations of sampling sites for the EDMAR field surveys

including examination for the presence of intersex in male testes.

3.2.1.2 Sand goby

Pomatoschistus minutus and *P. lozanoi*, identified by markings on the cheeks, were commonly found together, although generally one species was dominant.

Fish were sorted into species and sex. Liver tissue was excised and a sub sample frozen for measurement of VTG mRNA using the newly developed molecular probe. The remaining liver and gonad tissues were fixed for histological examination and calculation of somatic indices. Fish were also preserved for further examination in the laboratory.

Examination of the UGP in early samples, to make preliminary determinations of sex and to measure the UGPLI, revealed external abnormalities in some specimens. In a number of samples male gobies with slightly feminised UGPs were discovered and sex had to be confirmed by examination of the gonads. This condition was denoted MIPS (*Morphologically Intermediate Papilla Syndrome*). Subsequent to this discovery, field collected gobies were carefully examined under a stereo-microscope and noted as either female, male or intermediate (MIPS).

Female-type UGP are relatively broad with villous processes encircling the oviduct opening at the tip (Figure 3 c and d). Male-type UGP are relatively longer and more conical in shape with a generally featureless tip (Figure 3 a and b). A male papilla exhibiting the MIPS condition is generally male in size and shape but shows some evidence of the growth of villous processes (Figure 3 e and f).

3.2.1.3 Viviparous blenny

As with the sand goby, liver tissue was removed and frozen for determination of VTG mRNA. Testes tissue was also taken and fixed for the determination of intersex. Between September 1999 and March 2001 the broods from pregnant females were removed for a series of measurements including larval size/development. Larvae were then placed in a solution which stains the gonads, allowing sex to be determined macroscopically.

3.2.1.4 Crab and Shrimp

3.2.1.4.1 Vitellogenin in local field populations of the shore crab

Haemolymph samples for evaluating the vitellogenin ELISA were taken from shore crabs collected from 5 sites local to Plymouth University (Figure 4). Three were classed as contaminated: Hooe Lake, a small enclosed bay with a sewage treatment works on its banks, a site on the R. Plym downstream from a waste pipe serving a leachate lagoon associated with a large landfill site, and Pomphlett creek, a narrow finger of water leading off from the main Plym estuary which receives treated waste water. Two cleaner sites were Yealm and Bantham, on the Yealm and Avon estuaries respectively.

3.2.1.4.2 EDMAR national sampling programme

Crab haemolymph and whole shrimp were collected from several estuaries during the EDMAR surveys for determination of VTG in natural populations, using the ELISA. Notes on each crab were made at the time of collection providing information on sex, size and colouration, together with any other unusual features, such as the presence of epibionts or the parasitic barnacle *Sacculina carcini*.

Whole crabs were collected from 11 estuaries and fixed in formalin for laboratory measurements of exoskeletal variables. Using vernier calipers, measurements were taken of the carapace width, claw depths, pleopod heights and abdomen dimensions (see Figure 5).

3.2.2 Stickleback field studies

To investigate androgenic effects in the field, two separate studies took place using the stickleback. In April 1999 wild sticklebacks were collected from the River Weaver near its confluence with the Manchester Ship Canal, and from two clean sites, one in Kent and the other in Lowestoft, Suffolk. These were fixed in formalin and processed for measurement of KEH.

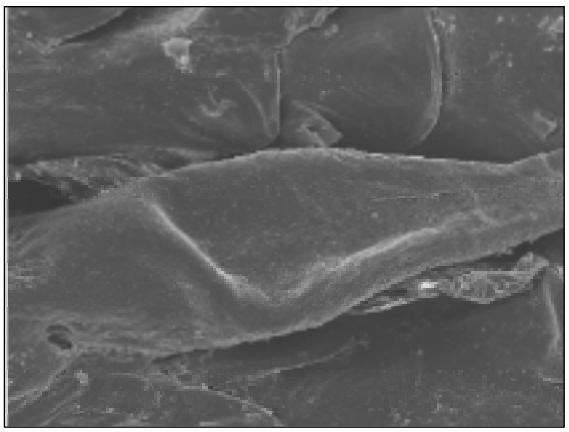
In the spring of 2000, two hundred seawater-adapted fish were caged in the Tyne estuary for 3 weeks (downstream and upstream of Howdon) and in the Tees estuary (at Dabholm Gut) for either 3 or 7 weeks. Thirty sticklebacks were kept in laboratory aquaria as controls. A total of 120 fish were recovered from the river deployments. The kidneys of all fish were removed for the measurement of spiggin by ELISA.

3.2.3 Trout/salmon field studies

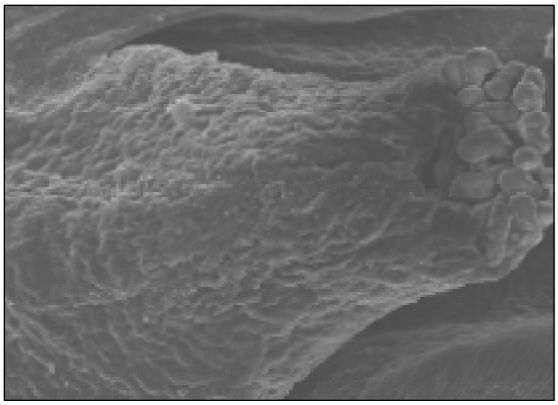
The purpose of these EDMAR studies was to determine the effects of oestrogenic contamination within estuaries on adults and smolts of two salmonid species. Atlantic salmon (*Salmo salar* L.) and sea trout (*Salmo trutta* L.) migrate through river estuaries at least twice during their life cycle; firstly when smolts emigrate from the river to the marine environment to feed and later as mature adults returning from the feeding grounds into the river to spawn.

At the smolt stage, a number of physiological and behavioural changes occur which pre-adapt the young fish, while still in freshwater, for life in the sea. One of the changes is an increase in the gill Na^+K^+ATPase activity, an enzyme involved in osmoregulation. The level of gill Na^+K^+ATPase activity is a good indicator of how well the smolts will survive in the marine environment. Previous work has shown that oestrogenic compounds can significantly inhibit the development of smolt physiology (Madsen *et al*, 1997).

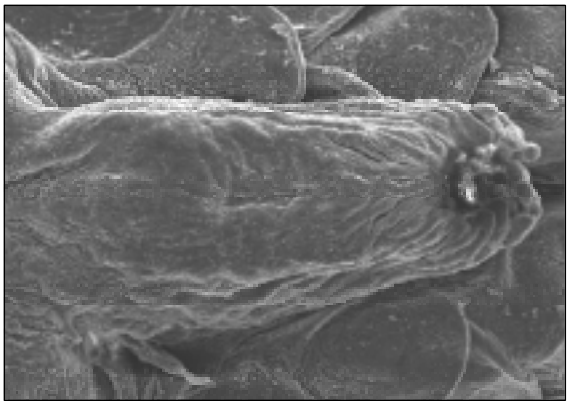
(a) P. minutus, male, Clyde



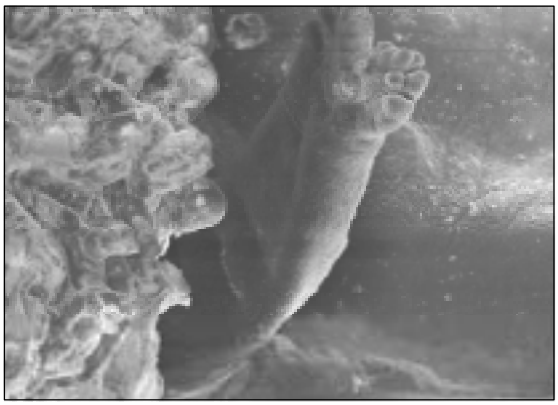
(b) P. minutus, male, Alde



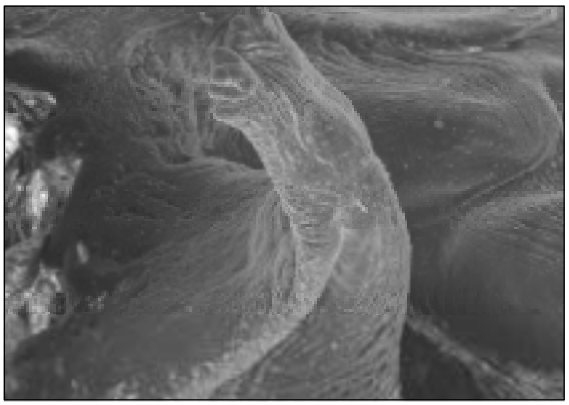
(c) P. lozanoi, female, Severn



(d) P. minutus, female, Alde



(e) P. minutus, male (MIPS), Tees



(f) P. minutus, male (MIPS), Tees

Figure 3. Electron micrographs showing normal male and female sand goby uro-genital papillae external morphology, and males with the MIPS condition

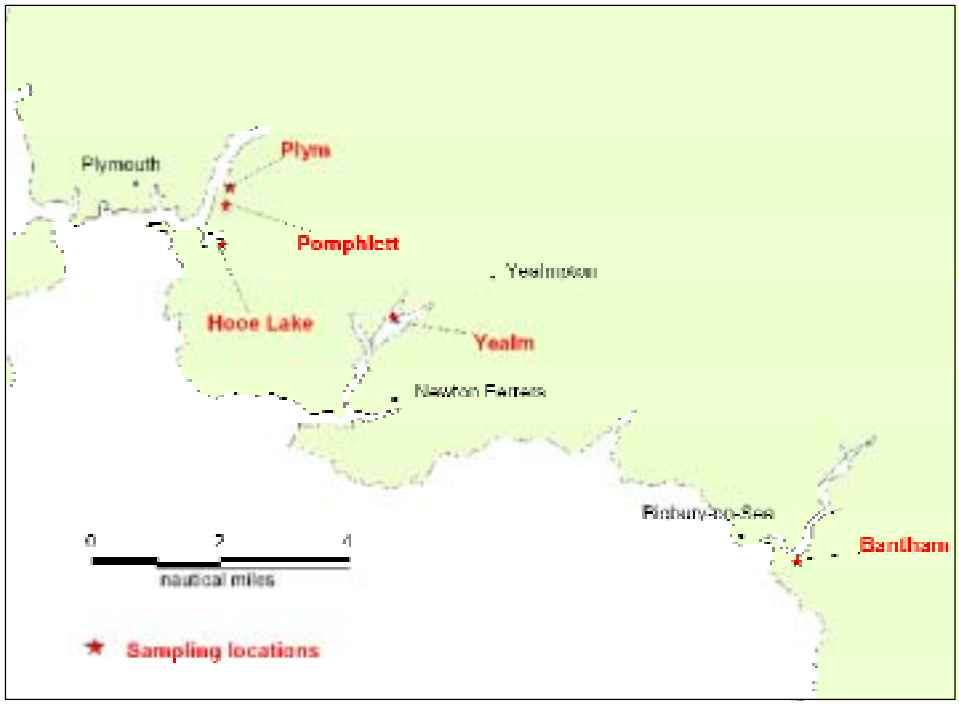


Figure 4. Map to show the locations of shore crab sampling sites in the Plymouth area

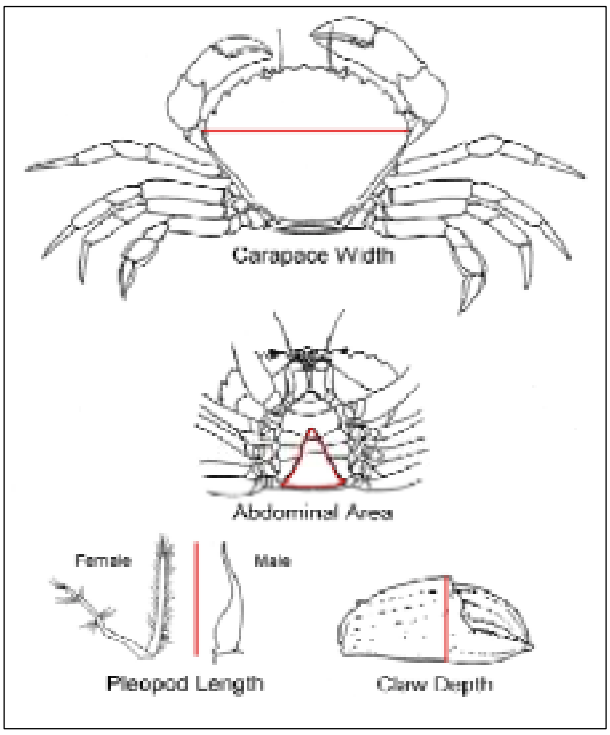


Figure 5. Diagram showing the shore crab exoskeletal measurements made in order to investigate cuticular abnormalities

The estuaries of the Rivers Tees and Tyne were chosen for study since they both support spawning populations of salmonids and have been shown to be contaminated with oestrogens (Matthiessen *et al*, 1998; Allen *et al*, 1999a). It was hypothesised that exposure of young fish to oestrogenic contamination may inhibit the physiological changes associated with smoltification and exposure of adult male salmon and sea trout may affect their ability to successfully reproduce.

3.2.3.1 Smolt exposure in estuaries

In the spring of 2000, salmon smolts were captured at the River Tees Barrage in the North-east of England. Blood and gill tissue were sampled immediately from one group (freshwater control group). Two further groups were placed in floating cages for 5 days, either in the upper Tees estuary or the upper estuary of the adjacent River Esk, which acted as the clean control site. Blood and gill tissue were then sampled for analysis.

3.2.3.2 Adult sampling

Male salmon and sea trout were sampled by various methods in collaboration with the Environment Agency and commercial fishermen. Between May and December 2000, sea trout were sampled from the Rivers Avon, Dee, Itchen and Tees, together with a number of salmon from the coastal fishery adjacent to the River Tyne.

Additional samples of salmon and sea trout were obtained during December 1999 and early 2000 from brood-stock held at various Environment Agency hatcheries. Further samples were obtained from the Tyne and Tees between July and October 2001. Blood and gill tissue samples were taken from all fish for analysis.

3.2.3.3 Biomarker measurements

Plasma VTG levels were measured using an ELISA technique, the procedures for which were identical to those developed for the spiggin ELISA (Katsiadaki *et al*, 2002). Gill Na^+,K^+-ATPase activity was measured in gill homogenates using the method of Zaugg (1982), but using isobutanol extraction.

3.3 Investigating causal links - substances and sources

3.3.1 Toxicity Identification and Evaluation

One technique for identifying toxic compounds in complex environmental matrices is toxicity identification evaluation (TIE), which uses bioassays to identify toxic fractions of samples prior to chemical analysis. Through the application of TIE techniques, Desbrow *et al* (1998) identified the steroid hormones 17β–oestradiol, estrone, and ethynylestradiol as the cause of oestrogenic activity in several UK sewage treatment works (STW) effluents, and it is likely that these steroid hormones are also partly responsible for the oestrogenic activity observed in UK estuaries. There are very few studies which have used TIE for the identification of androgens in the aquatic environment: the only environmental androgen so far reported is androstenedione (Jenkins *et al*, 2001), identified through the use of an *in vitro* androgenic assay, and is thought to be a biotransformation product of natural plant sterols.

The purpose of this EDMAR objective was to identify oestrogenic and androgenic compounds in surface waters, sediment pore waters, and sediments from a range of estuaries in England and Scotland through application of TIE modified for marine samples using the yeast oestrogen and androgen screens (YES and YAS).

3.3.1.1 The Yes and YAS Assays

Samples were tested for oestrogenic activity using a yeast-based oestrogen screen (YES) based on the method of Routledge and Sumpter (1996). The yeast cell has the human oestrogen receptor implanted into the genome. In the presence of oestrogens or chemicals with oestrogenic activity, which bind to, and activate the receptor, a chemical is synthesised by the yeast, secreted into the assay medium which breaks down a substance to change the colour of the test from yellow to red. The concentration of this red product can be measured by absorbance and the oestrogenic activity for each sample determined by comparing responses to a standard (the natural hormone oestradiol (E2)). Results are expressed as equivalent E2 values (ng E2 equivalents l^{-1}) for the initial sample, taking into account relevant concentration factors.

A yeast-based androgen screen (YAS) was used to test the samples for androgenic activity (Sohoni and Sumpter, 1998). This bioassay is identical to the YES assay, except that the human androgen receptor has been integrated into the yeast genome. Androgenic activity for each sample was determined by comparing responses to a dihydrotestosterone (DHT) standard and reported as DHT equivalent concentrations (ng DHT equivalents l^{-1}).

3.3.1.2 Sample collection

Surface water samples were collected using a stainless steel bucket and alloy churn (oestrogens) or in clean glass bottles (androgens). Sediment samples were collected by van Veen grab and stored either in 10 l

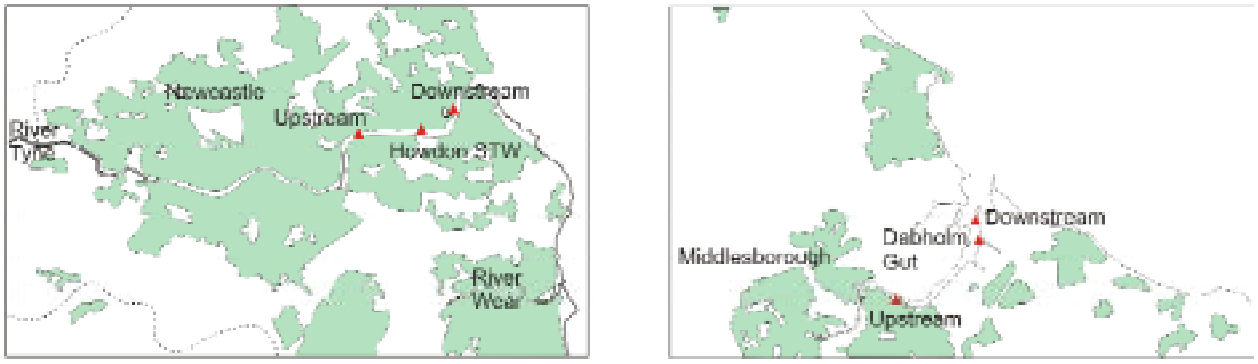


Figure 6. Location of sampling sites on the Tyne and Tees estuaries for TIE studies. See Figure 2 for location of the Alde reference site

stainless steel containers (oestrogens) or in hexane-rinsed glass jars (androgens). Effluent samples were either collected from the end of the discharge pipe or from within the works at a point downstream of the last conventional water treating system or other designated sampling location. Samples from the Tees and Tyne estuaries (Figure 6) were evaluated for oestrogens. Samples from a total of seven estuaries - the Tees, Tyne, Mersey, Thames, Southampton Water, Clyde and Forth were evaluated for androgens.

3.3.1.3 TIE procedure
TIE basically involved 4 stages, which are shown in Figure 7:

1. Bioassay of initial sample: the YES/YAS screen was conducted on raw water samples to identify any oestrogenic/androgenic activity
2. Sample extraction and bioassay: water samples were passed through a solid phase extraction (SPE) column; sediments were placed in Teflon tubes and centrifuged to separate the pore water from the particulate material. Pore water was passed through Teflon SPE columns. The extracted compounds were recovered from the SPE columns using methanol, while compounds on sediment particulates were extracted using dichloromethane. Fractions were tested for oestrogenic/androgenic activity using YES/YAS
3. Fractionation and bioassay: the SPE fractions exhibiting oestrogenic or androgenic activity were further separated into fine fractions by High Powered Liquid Chromatography (HPLC), and tested once more using YES/YAS
4. Fraction analysis: all HPLC fractions demonstrating oestrogenic or androgenic activity were chemically analysed using gas chromatography-mass spectrometry (GC-MS).

3.3.2 Flounder caging trials

The aim of this work was to locate oestrogenic effluents by deploying caged flounder and measuring VTG induction. In early 1999, flounder from the Alde estuary, a clean control site, were placed in galvanised steel cages on the Tees and Tyne estuaries. Three sites were selected on each - one directly at the point of an effluent discharge, and one upstream and downstream of the discharge. Effluent discharge sites were at Dabholm Gut on the Tees and the sewage outfall at Howdon on the Tyne. Cages were placed below the mean low water mark at each site, secured to a jetty. The cages, because of their weight, sank a few centimetres into the sediment, which provided the flounder with its natural habitat while confined. Twenty mixed-sex flounder were added to each cage. In addition, a 'control' batch of 20 fish was retained at the CEFAS Burnham laboratory over the same period. After two weeks exposure surviving fish (>80% at all sites) were removed and a blood sample taken from each for VTG analysis using a radioimmunoassay technique (for details of methods see Allen *et al*, 1999a).

3.4 Investigating causal links - laboratory experiments

3.4.1 Flounder feeding experiments

Flounder trawled from the Alde estuary (the clean reference site) and acclimated to laboratory conditions were used in two experiments to assess the role of food as a route of exposure to oestrogens in the environment.

In the first experiment, flounder were fed to satiation with live brown shrimp which had been trawled either from the Tees estuary or the Crouch estuary, the latter acting as the control. In addition, some flounder were fed control shrimp and continuously dosed with EE2 (10

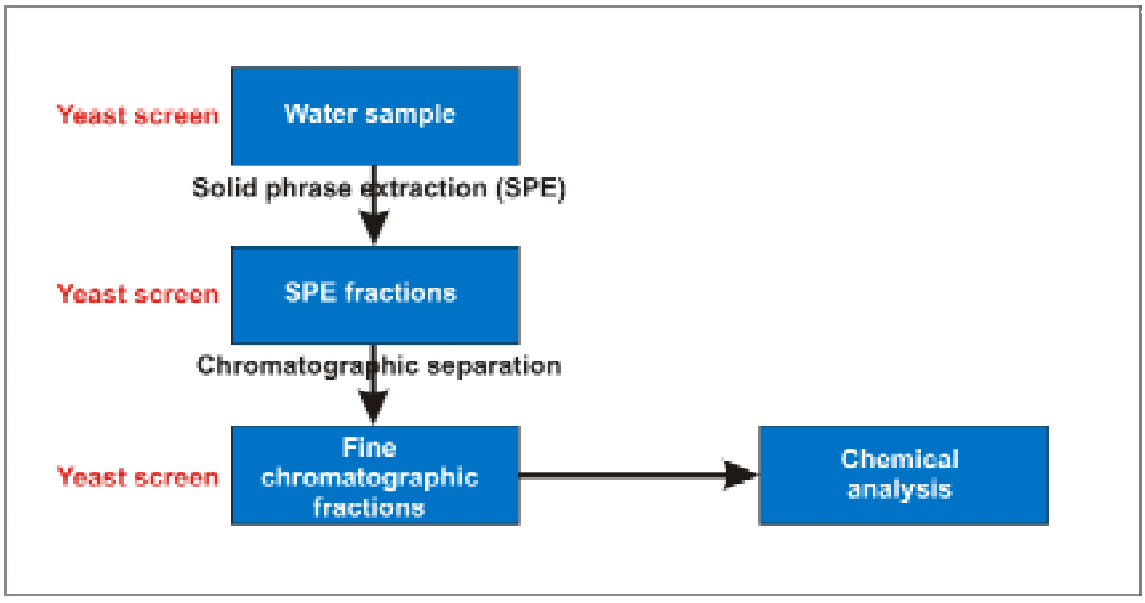


Figure 7. Diagramatic representation of the TIE scheme for isolating oestrogenic and androgenic compounds

ng l^{-1}), which acted as a positive control to check that the flounder were still able to produce the VTG response. Starting at the beginning of February 2000, brown shrimp were trawled from the Tees and Crouch on a weekly basis. On the Tees, the shrimp were sampled from an area between Dabholm Gut and Teesport, a site that receives a large amount of effluent from a sewage treatment works and an industrial plant. Samples of shrimp from both locations were frozen for chemical analysis. Blood samples were taken from each fish for analysis of plasma VTG by ELISA at the beginning of the experiment, after 21 days and at the end of the experiment (56 days).

In the second experiment, flounder from the Alde were fed mussels (*Mytilus edulis*) originally obtained from a clean site and caged for three months either on the Tees estuary (at Teesport) or on the River Crouch (control). Cages were suspended from a jetty approximately 1.5 m above low tide, so that the mussels experienced a normal tidal cycle with air and water exposure. Although conditions in the Crouch are not completely pristine, holding the control animals there was preferable to maintaining them in filtered seawater in the laboratory, since the latter would probably have induced spawning, which has undesirable effects on the nutritional quality of the mussels. Flounder were fed with ~ 5% body weight of mussel flesh per fish per day. A sufficient quantity of mussels was collected from the Tees and Crouch on a weekly basis. Mussel tissue from both locations were frozen for chemical analysis. Blood samples for analysis of plasma VTG by ELISA were taken at test initiation, after approximately three weeks exposure, and at test termination (43 days).

3.4.2 Sand goby Experiments

The aims of these studies were to investigate whether exposure to substances which are known to have or suspected of having oestrogenic activity could also affect the reproductive success of a marine fish, and to relate reproductive success to biomarkers of oestrogenic exposure.

The breeding strategy and small size of the sand goby makes it a suitable fish for use in breeding experiments. Irvine Valley sewage effluent (IVS) was chosen for study since it is known to contain significant concentrations of alkylphenolic compounds (Pirie *et al*, 1996), some of which are oestrogen mimics. EE2 and E2 were used as positive controls, and octylphenol (OP) was used as a model alkylphenol, being the most potent isomer (Pedersen *et al*, 1999).

3.4.2.1 Sand Goby breeding experiments

The methodologies employed during these studies are detailed in Robinson *et al* (2001b).
In total, four experiments were conducted:

1. Seven-month exposures to IVS and EE2 including a within-treatment breeding trial
2. Four-week exposure to octylphenol (OP)
3. Six-month exposure to OP
4. Eight-month exposure to E2 including within and between treatment breeding trials

In the first experiment, juvenile sand goby collected from the Ythan estuary, NE Scotland, were exposed to IVS effluent (nominal 0.3% or 0.03% v/v), EE2 (nominal 6 ng l^{-1}) or solvent carrier control (methanol) between November 1998 and the end of June 1999. Breeding trials using within-treatment crosses took place during the last two months.

Fish were sampled periodically to make measurements for various maturation indices (e.g. UGP length, seminal vesicle weight, nuptial colouration) and to obtain liver tissue for measurement of VTG/ZRP mRNA.

The breeding trials broadly followed the method of Waring *et al* (1996). Goby pairs continued to be exposed to the test substance. They were provided with nesting material (a sandy substrate and broken clay pot) and the aquaria were examined daily for nest building activity and the presence of eggs. Figure 8 shows a typical nest in one of the breeding aquaria. Once eggs appeared on a nest, it was removed and the adults

Figure 8. Male sand goby (Pomatoschistus minutus) and nest. A sand substrate and broken clay pot were provided in each tank as nesting material. The male sand goby covers the pot and restricts its entrance with sand to make a nest. He then guards eggs laid by the female

replaced with a new pair. Nests with eggs were incubated in aerated, untreated seawater until hatch. After incubation for 9-11days the percentage of fertile eggs in each brood was determined by the presence of eye spots. The numbers of fertile eggs were counted in approximately half the broods, and individual fecundity determined as the mean number of fertile eggs per brood. Population reproductive success was estimated as mean individual fecundity x total number of broods produced, normalised to the number of females available to breed at the start of the breeding period (i.e. fertile eggs per available female).

In the second experiment, sand goby were exposed for 4 weeks to OP concentrations of 3, 20, 31 and 101µg l^{-1},a solvent control and a water control in glass aquaria with a flow-through system. At the end of the experiment the fish were sampled as above and livers analysed for expression of VTG mRNA.

The third and forth experiments were long-term exposures to OP and E2 respectively. Fish were sampled periodically as above and analysed for changes in maturation indices and VTG mRNA expression. The OP exposure was conducted between January and June 2000. The E2 exposure took place between October 2000 and June 2001, with the breeding trials conducted in April, May and June.

In the E2 breeding trials fish were paired for seven days, with nesting and egg-laying behaviour noted daily. On the seventh day, any eggs were removed and all fish replaced with new pairs. Pots with eggs attached were transferred to cages of fine mesh netting and suspended in the tanks within which fish were being exposed to the test substance. Egg fertility and individual fecundity were determined as before. Population reproductive success was defined as the number of eggs produced per female available to breed at the beginning of the breeding trial. Following the within-treatment trials, a short between-treatment trial was conducted using water control fish and those exposed to the highest E2 concentration.

3.4.2.2 MIPS Response in Gobies

In order to investigate the possible link between exposure to oestrogens and development of the MIPS condition, a laboratory experiment was conducted.

'Young of the year' gobies (4-6 months old) were continuously exposed to 17β-oestradiol at nominal concentrations of 10, 100 and 1000 ng l^{-1}, together with solvent (propan-2-ol, less than 0.003% v/v) and water controls. Exposures were conducted in duplicate tanks between mid-October 2000 and March 2001, when tanks from each treatment were combined. Thereafter the exposures took place in single tanks, until they were terminated. Following high male mortality rates in the highest treatment, the E2 concentration was reduced to a nominal 600 ng l^{-1} in mid-March 2001. Actual E2 exposure concentrations were determined at intervals of approximately three weeks, by GC-MSD. Fish were sampled after 13, 22 and 32 weeks exposure and examined for the presence of MIPS.

4. RESULTS

4.1 Biomarker development

4.1.1 Stickleback KEH/spiggin

Results of the effect of waterborne androgens are highlighted for female fish only, since laboratory conditions often induce breeding in intact males. Exposure to four concentrations of 17α-MT, ranging from 10 ng l^{-1} to 10 µg l^{-1}, for three weeks produced a concentration dependent increase in female mean KEH, with the highest concentration resulting in a KEH similar to that found in reproductively mature males (Figure 9). In the experiment using 11-KT, the two concentrations tested (10 and 20 µg l^{-1}) produced an average KEH greater than 17 µm, which provides evidence of kidney stimulation (Borg, 1981).

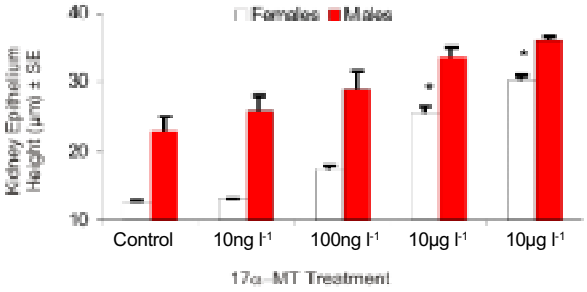


Figure 9. Kidney epithelium height (KEH) of sticklebacks treated with 17α–MT. Asterisks indicate significantly different from the control group (p < 0.01)

Stained kidney sections from females dosed with 17α-MT demonstrated the increasing presence of a carbohydrate-containing protein with increasing exposure concentrations (Figure 10, plates a to f). The changes involved in the transformation of an excretory organ to a secretory gland are clearly illustrated.

The validation of the spiggin immunoassay was accomplished by comparing the KEH of 160 fish (that received different concentrations of 17α-MT and/or flutamide), as measured on histological sections of half of the kidney with the amount of spiggin units as

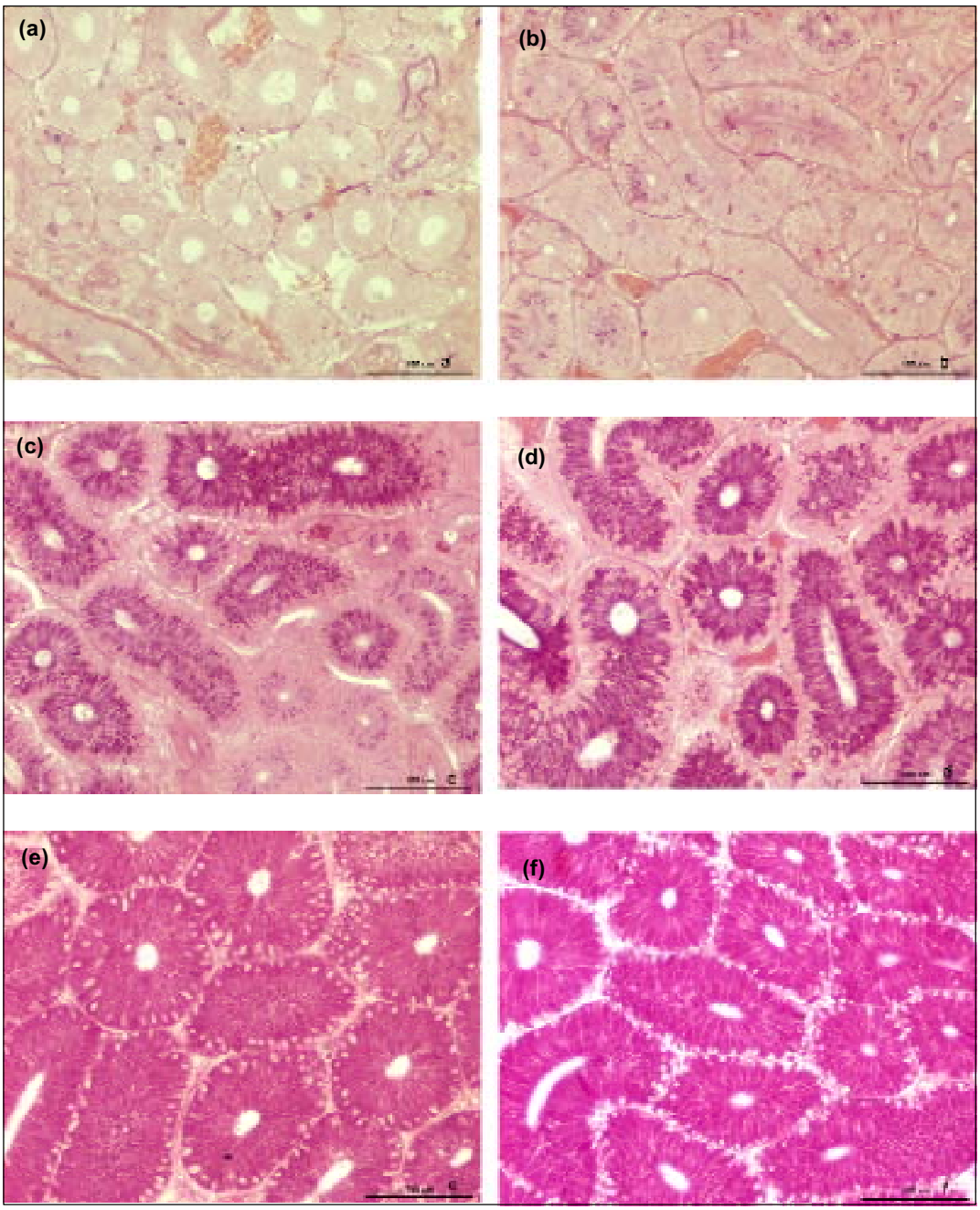


Figure 10. Kidney sections of female sticklebacks treated with increasing concentrations of 17α-Methyltestosterone (a-e) and of a naturally breeding male (f). Sections were stained with PAS, all photomicrographs were taken under the same magnification.
a: Control, KEH=13.5 µm; b: 100 ng l^{-1}, KEH=18 µm; c: 1 µg l^{-1}, KEH=24.2 µm;
d: 10 µg l^{-1}, KEH=29.7 µm; e: 500 µg l^{-1}, KEH=36.2 µm; f: breeding male, KEH=34.5 µm

measured in the other half of the kidney. This process resulted in an excellent correlation (r^2=0.93, Figure 11). The spiggin assay was completed in three days, while the histological method took approximately four weeks. In addition, spiggin concentrations were more sensitive showing a 100,000-fold variation, as opposed to a 4-fold difference in KEHs.

Subsequent studies focussed upon development of dihydrotestosterone (DHT) as an androgenic standard, with the aim of applying the assay in the field of ecotoxicology.

Female stickleback exposed to DHT (between 1 and 5 µg l^{-1}) for either three or five weeks produced increasing amounts of spiggin with increasing DHT (Figure 12). Statistical analysis of the results showed that the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) were 3 µg l^{-1} and 4 µg l^{-1} and 1 µg l^{-1} and 2 µg l^{-1} for a three-week and five-week exposure respectively.

The anti-androgenic effect of flutamide (FL) was confirmed in experiments using a single concentration of 500 µg l^{-1} FL in combination with several doses of

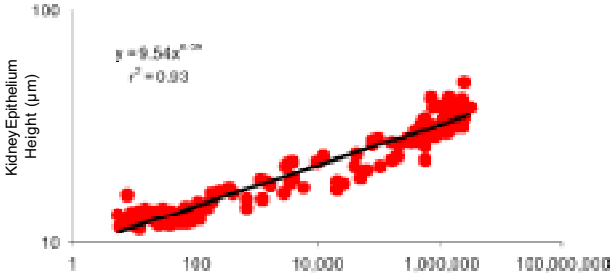


Figure 11. Comparison of the ELISA for spiggin with the KEHs obtained from histological examination

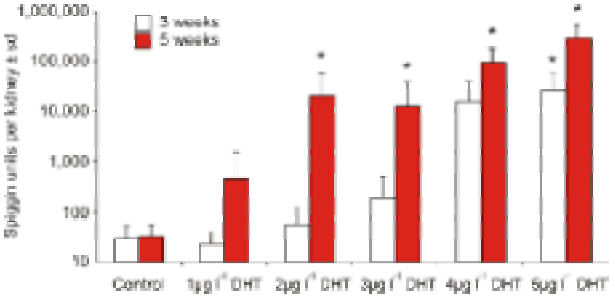


Figure 12. Dose response curve for DHT after 3 and 5 weeks exposure. Asterisks denote significantly different from control group (p<0.05)

either 17α-MT or DHT, demonstrating the potential of this *in vivo* method to screen and evaluate the anti-androgenic activity of various chemicals and pharmaceuticals. The results of the 17α-MT experiment are shown in Figure 13. Spiggin concentrations of flutamide-treated males were statistically significantly lower than those of males treated with 100 ng l^{-1} 17α-MT, while the same significance was found in the females treated at 1 µg l^{-1} 17α-MT. Identical results were obtained using KEH measurements.

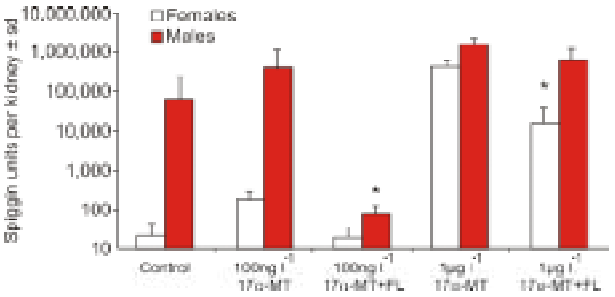


Figure 13. Anti-androgenic effects of flutamide on spiggin production in 17α-MT treated male and female stickleback. Asterisks denote 17α-MT and flutamide treatments that were significantly different from 17α-MT only group (p<0.05)

4.1.2 Crab and shrimp VTG ELISAs

The optimised ELISAs successfully indicated vitellogenin (VTG) in female crab haemolymph samples, allowing the screening of many hundreds of crustacean test samples (see section 4.2.1.4).

4.1.2.1 Exposure of crabs to nonylphenol, diethylstilbestrol and testosterone

An ELISA of the groups of males exposed to nonylphenol and diethylstilbestrol indicated that VTG was not induced in any of the animals. A further ELISA examining the impact of nonylphenol on the haemolymph concentration of VTG in females over 21 days exposure also showed no obvious pattern of effect.

Similarly, there was no significant effect of testosterone on the levels of VTG in female crabs, suggesting that testosterone does not interfere with the process of vitellogenesis in shore crabs.

4.1.2.2 Analyses of crabs parasitised by Sacculina carcini

Analysis of proteins in haemolymph of parasitised and non-parasitised males showed no differences in the profiles between the two groups. In addition, an ELISA run to detect *Carcinus* VTG gave negative results. Negative results were also obtained for haemolymph from males with parasites collected as part of the EDMAR national field sampling programme.

4.1.3 Blenny/goby VTG/ZRP mRNA

The preparation and characterisation of cDNAs for VTG and ZRP for sand goby and blenny were completed successfully. Subsequently, the specificity of reaction of the VTG and ZRP cDNA reagents were assessed, which involved showing that the cDNAs reacted with their own mRNAs, determining the size of the mRNAs they detected and demonstrating detection of differential expression of the mRNAs based on gender and after exposure of animals to oestrogenic substances.

Once the specificity of reaction of the cDNA reagents had been established it was possible to develop a method suitable for the high throughput of samples from laboratory studies and environmental monitoring. The methodology was subsequently successfully deployed for analysis of VTG/ZRP mRNA in fish from the field (see section 4.2.1) and laboratory exposed fish (see section 4.4.2).

4.1.4 Histochemical techniques

4.1.4.1 Localisation of the oestrogen receptor

Although several different mammalian antibodies successfully located the oestrogen receptor in human breast tissue histological sections, used as the positive control (Figure 14), the majority did not work well in localising the flounder oestrogen receptor. However, one did appear to cross react in flounder, showing the receptor to be associated with hepatocyte nuclei in the liver, with the granulosa/thecal cell layer in the ovary and in interstitial (Leydig) and possibly sertoli cells in the testes (Figure 15). However, these results were not reproducible. An oestrogen receptor antibody for rainbow trout worked in histological sections of rainbow trout liver and gonad but did not cross-react in sections of flounder liver and gonad.

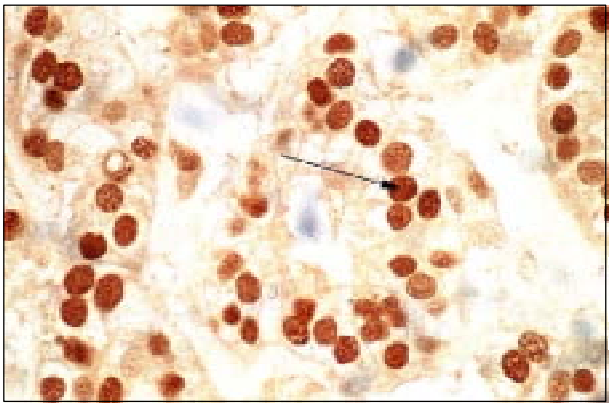


Figure 14. Immunocytochemical localisation of the oestrogen receptor, using mammalian antibodies, in human breast tissue. This was used as the postitive control for flounder liver/gonad samples

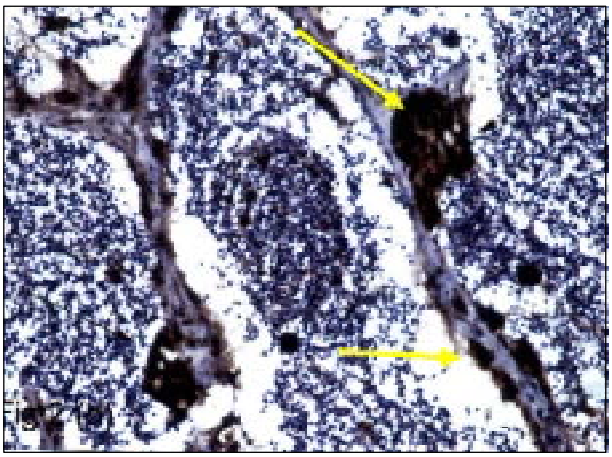


Figure 15. Oestrogen receptor immuno-cytochemistry in flounder testis. The label is found in the interstitial (Leydig) cells and possibly in the sertoli cells around unlabelled sperm

Autoradiography using radiolabelled oestradiol showed that the hepatocytes/hepatopancreatic cells of both sexes were labelled with silver grains. The testes contained radiolabelled oestradiol in the interstitium (Figure 16). In females, oestradiol was present in the yolk of vitellogenic ovaries (Figure 17). Spermatogonia, oogonia and control tissues were unlabelled.

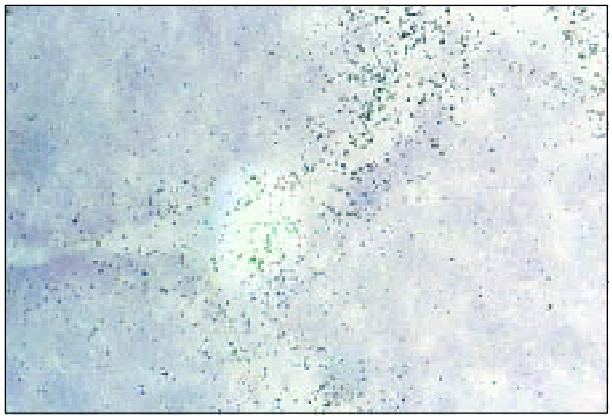


Figure 16. Localisation of the oestrogen receptor in flounder testis with ripe sperm. Radiolabelled oestradiol is present in interstitial tissue. Unfixed cryostat section

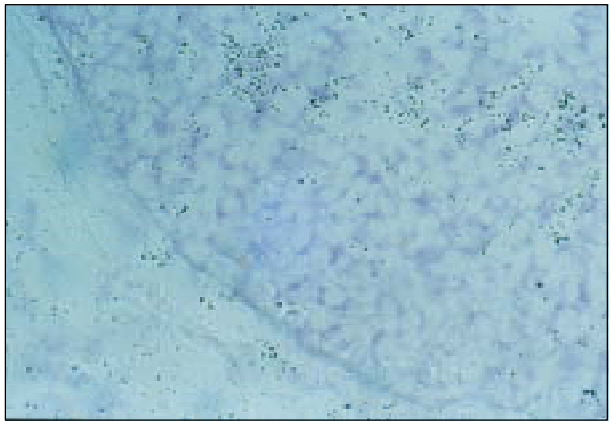


Figure 17. Localisation of oestrogen receptor in flounder ovary in vitellogenesis. Oestrogen receptor, labelled with 17β–oestradiol, is found in ripe yolk but not in egg membranes

4.1.4.2 Localisation of stickleback spiggin

The kidney tubular epithelia cells express and secrete spiggin into the tubule lumen, and immunohistochemical staining visualised it within the cytoplasm as discrete spherical or ovoid vesicles. Determining whether these were discretely packaged vesicles of spiggin, or part of the extensive endoplasmic reticulum which produces spiggin was constrained by the resolution limits of the microscope, but they may indeed be both.

The luminal surface of the tubular epithelial cell also stained positive and was more intense than the cytoplasmic staining. The spiggin was visualised on the exterior of the cell membrane which extended into the lumen of the nephron, corresponding with the membrane across which spiggin would be transported (Figure 18).

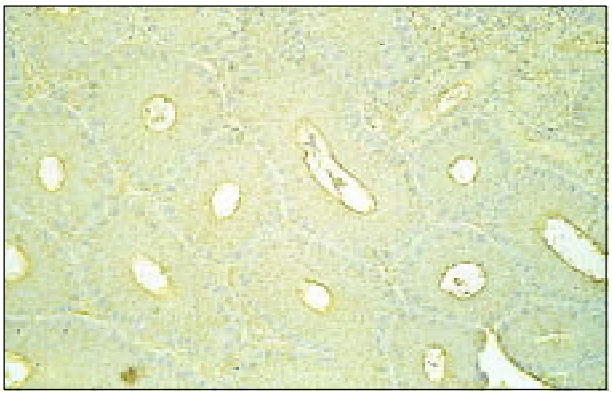


Figure 18. Immunocytochemical labelling of spiggin in male stickleback

4.1.4.3 Localisation of VTG

Mild visualisation of VTG was observed in flounder liver hepatocytes, with no particular focal areas of cellular expression (Figure 19); the absence of intense staining indicated that VTG is not stored in these cells. There was a stronger staining of VTG around blood vessels within the liver.

Staining for VTG was positive and particularly distinct during the early stages of vitellogenesis. In ovaries which had both developing oocytes and oocytes in early vitellogenesis (i.e accumulating yolk), staining for VTG was negative in the former and positive for the latter. The early VTG stage oocytes stained lightly across the ooplasm and strongly around the periphery where the yolk vesicles were located (Figure 20). These positively stained vitellogenic oocytes contrasted sharply against the negatively stained oogonia (pre-oocyte cell) and non-vitellogenic early oocytes. There was also positive staining associated with the surrounding follicle cells.

Figure 19. Immunocytochemial staining of VTG in the liver of Mersey flounder

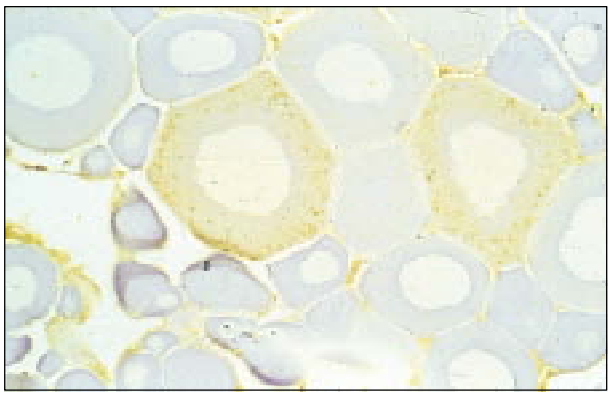


Figure 20. Immunocytochemical staining of vitellogenic oocytes of flounder

4.2 Biological responses in estuaries

4.2.1 Field surveys

4.2.1.1 Flounder

Figure 21 summarises the male plasma VTG data that have been collected since 1996 (which includes data from Allen *et al*, 1999a,b).

The data suggest that in some areas, plasma VTG concentrations in male flounder are falling. Plasma VTG concentrations in Mersey males declined by two orders of magnitude between 1996 and 2000, although the 2001 dataset suggested a small rise. The greatest reductions were observed at Hebburn and Howdon on the Tyne: males routinely had high concentrations which have declined to near baseline levels since late 2000. Conversely, plasma VTG in males from St Anthonys on the Tyne and at Dabholm Gut on the Tees have shown little reduction over the study period.

Flounder intersex incidence is presented in Table 1, Appendix 2. The condition is not highly prevalent in UK populations, reflecting the pattern observed in previous years. As with pre-1999 data, most of the intersex cases occurred in fish from the Mersey and Tyne, but isolated examples in fish from the Clyde and Thames suggest that it may occur in all fish populations from contaminated locations.

4.2.1.2 Sand goby

The somatic index data generated for male and female sand goby are shown in Tables 2 and 3 in Appendix 2. These showed no obvious non-seasonal trends.

Since analysis for MIPS could be suceptible to subjectivity, random samples were double-checked 'blind' by other analysts to ensure agreement. Sex was confirmed by examination of the gonads.

The percent occurrence of MIPS at each surveyed site is shown in Figure 22. Absolute numbers caught are given in Table 4, Appendix 2. A background level of <10% occurrence in the male population was found at

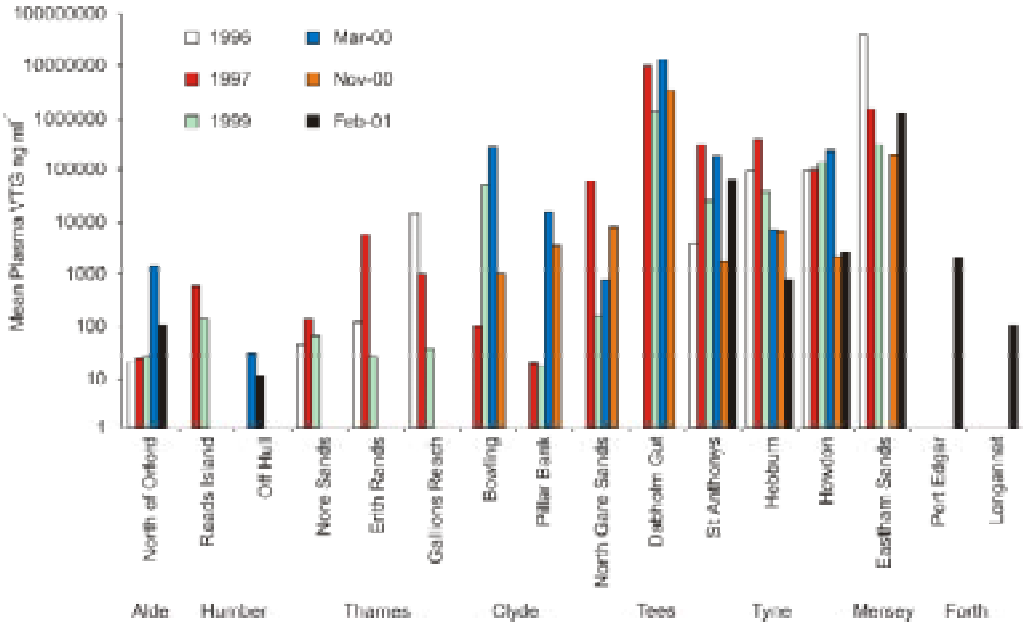


Figure 21. Mean plasma VTG (ng/ml) in male flounder from surveys carried out between 1996 and 2001

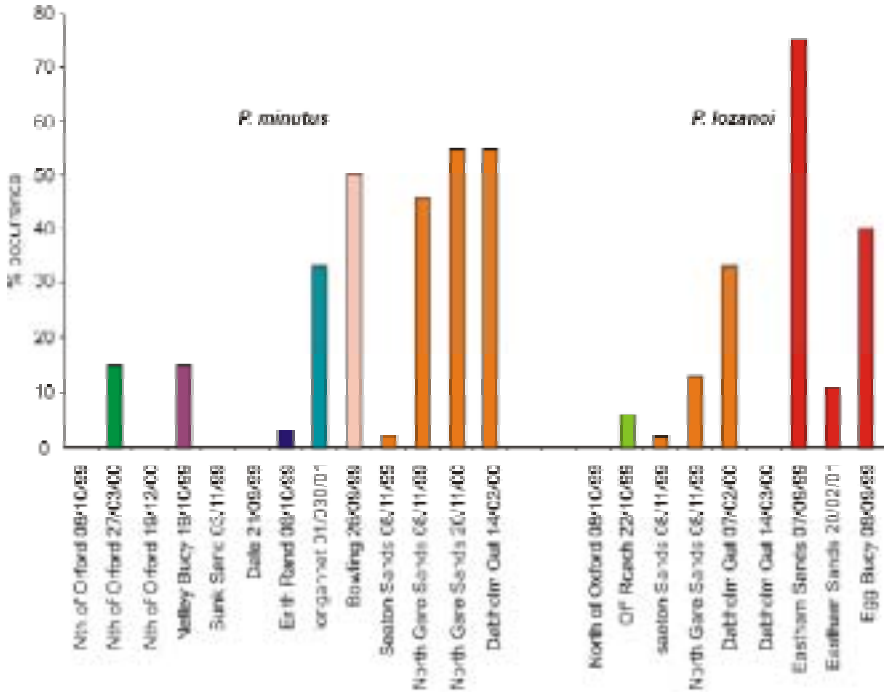


Figure 22. Percentage occurrence of the MIPS condition in wild-caught male sand goby from clean and contaminated estuaries

the majority of sites, but this was elevated to >20% at some sites on the Tees, Clyde and Forth. Dabholm Gut and North Gare Sands on the Tees, Bowling on the Clyde and Eastham Sands on the Mersey had the highest levels with >50% occurrence.

There was no evidence of VTG gene expression in male gobies from any of the sample sites. These results were slightly surprising since other fish species (specifically flounder and viviparous blenny) caught in some of the same areas expressed markers of oestrogenic endocrine disruption. Male gobies exposed to the synthetic oestrogen EE2 in the laboratory responded by producing ZRP mRNA, which is another biomarker for oestrogenic exposure which provides a parallel measurement to VTG mRNA, and the effect threshold is between 1-10 ng l^{-1}, similar to that noted for VTG induction in other fish species.

No wild male gobies showed any evidence of intersex.

Due to the labour- and time-consuming nature of UGP characterisation only a limited number of papillae could be processed histologically. A transverse section of the male UGP (Figure 23) clearly shows the positioning of the sperm duct (labelled with a single arrow) and urinary duct (labelled with a double arrow). Serial sections of UGPs from normal male and female sand goby were made, thus obtaining a complete morphological sequence from the external villi to the gonad (Figure 24 a-d). These served as control material for comparison with fish that exhibited MIPS.

The gonadal duct of females was surrounded by spongiform-like tissue (Figure 24 b and d) which was not observed in normal males or male *P. minutus* with MIPS, but was noted in one specimen of *P. lozanoi* with MIPS (Figure 24 f). In males with MIPS (*P. minutus* only) the normal dorso-ventral alignment of the urinary and gonadal ducts had altered towards the tip of the papillae and were positioned approximately laterally to one another (Figure 24 e). The ducts returned to their normal position towards the base of the papillae.

4.2.1.3 Viviparous blenny

Viviparous blenny were caught in the Forth, Clyde, Tyne and the Alde (reference) estuaries, which although a limited distribution, included both clean and contaminated areas.

Figure 25 shows the total percentage of males from the whole survey period at each of the sites which were positive for the VTG mRNA probe. No VTG mRNA was detected in fish from the Alde, but a significant proportion of males from the estuaries of the Tyne, Tees, Forth and Clyde were positive. The geographic distribution of the responses correlates well with plasma VTG results in flounder.

Tables 5 and 6 in Appendix 2 show the brood measurements and larval sex ratios from these broods from pregnant females collected from September 1999 to March 2001. Unfortunately, no pregnant females were caught from the Alde reference site for comparison with contaminated sites. The sex ratios for all sites showed a female bias, but there was no significant difference between sites, largely due to the variation in sex ratio between broods and low sample numbers.

The percent occurrence of intersex in male blennies is listed in Table 7, Appendix 2. As with flounder, this condition is not highly prevalent, with only 10 examples being found during the EDMAR programme. The highest incidence was in males from the Tyne, followed by the Clyde, which corresponds to the VTG mRNA data. Figure 26 gives an example of an intersex testis from a Tyne male blenny. This is the first time the condition has been noted in an estuarine species other than flounder.

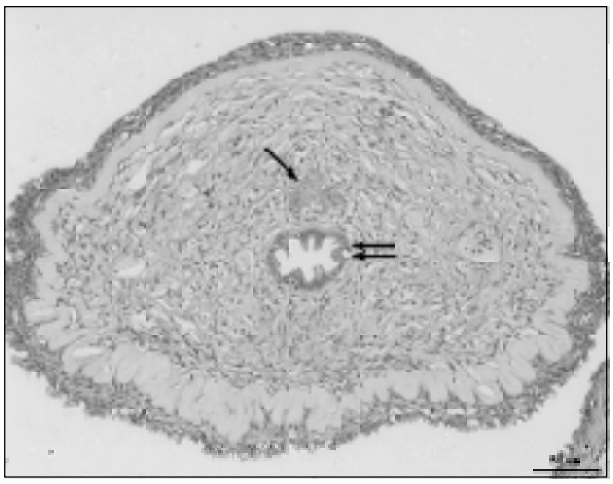


Figure 23. Transverse section of sand goby male uro-genital papilla. Gonadal duct is indicated by the single arrow, the urinary duct by the double arrow

4.2.1.4 Crab and shrimp

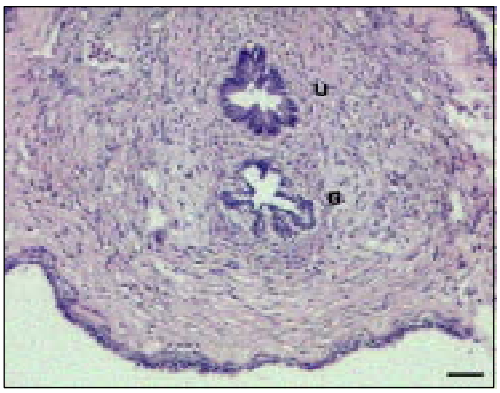
4.2.1.4.1 Vitellogenin in local field populations of the shore crab

A total of 187 crabs from a range of clean and contaminated sites were assessed for the presence of VTG using the ELISA. A high percentage of the females gave a positive result, but none of the males tested positive.

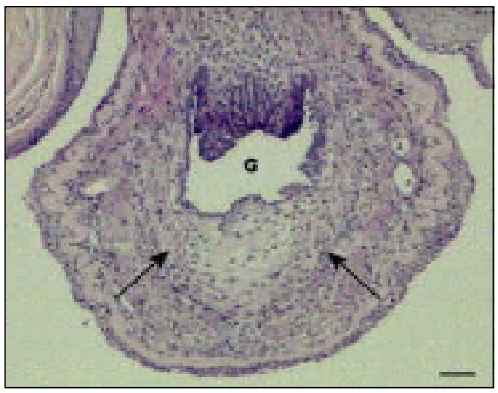
4.2.1.4.2 EDMAR national sampling programme

Of 339 male samples analysed just one gave a positive indication of VTG, which was probably due to sampling error. *Crangon* samples were sexed and relative proportions of male, female and intermediate obtained. Sub-samples of each group were analysed using the *Crangon* ELISA. Of 306 males assessed just one gave a positive indication of vitellogenin; of 156 intermediates tested 9 gave a positive result and of 156 females 54 were positive for VTG.

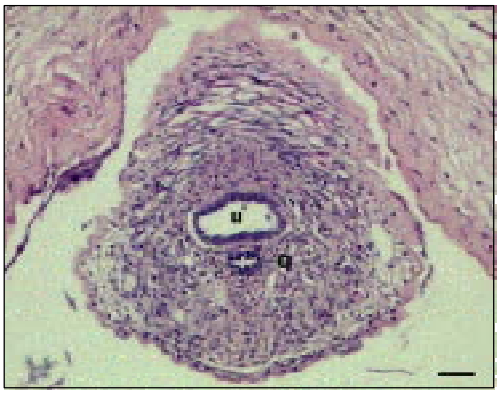
Since it was difficult to obtain crabs of a standard size from each site, morphological data were compared after generating a ratio for each measurement to carapace width. The mean data for the shore crab morphometric measurements are shown in Table 8 of Appendix 2. No obvious patterns are discernible. Figure 27 compares the morphometric ratios of crabs from the reference site (the Alde) to samples taken at four other sites where mean carapace width was comparable to the Alde. The sites represent three estuaries in which oestrogenic effects have been noted in fish (Tees, Tyne and Clyde) and one coastal site that was expected to be relatively free of contamination (Wash - Off Nene). All four sites had significantly smaller right claw depth and left pleopod length ratios compared to the Alde.



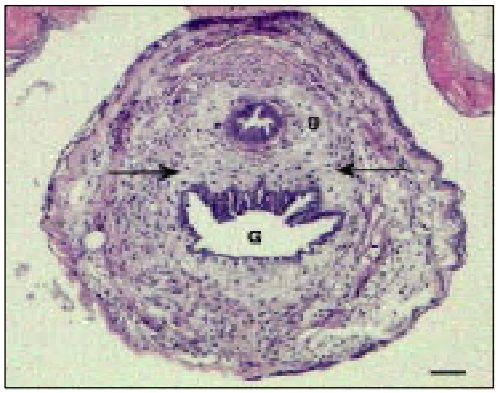
(a) Transverse section through the uro-genital papilla of a male P. minutus. Urinary duct (U), gonadal duct (G)



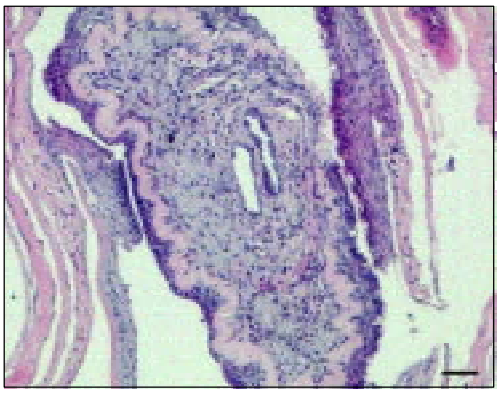
(b) Transverse section through the uro-genital papilla of a female P. minutus. Gonadal duct (G). The urinary duct is located above the upper margin of this figure. Note the presence of pale stained "spongiform" tissue (arrows) surrounding the gonadal duct



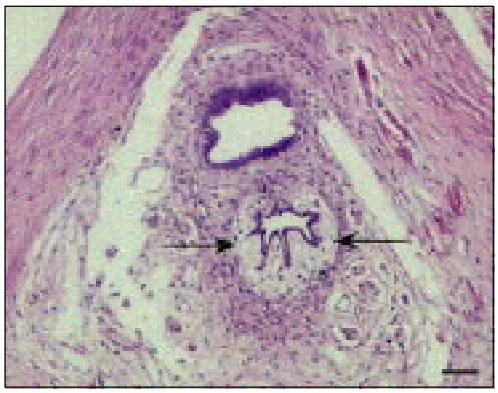
(c) Transverse section through the uro-genital papilla of a male P. lozanoi. Urinary duct (U), gonadal duct (G)



(d) Transverse section through the uro-genital papilla of a female P. lozanoi. Note pale stained "spongiform" tissue surrounding both the urinary (U) and gonadal (G) ducts



(e) Transverse section of P. lozanoi exhibiting MIPS. Note the lateral displacement (torsion) of the ducts



(f) Transverse section of P. lozanoi exhibiting MIPS. Note the pale stained "spongiform" tissue surrounding the gonadal duct

Figure 24. a-f Internal structure of male and female sand goby uro-genital papillae (scale bar = 100 µm)

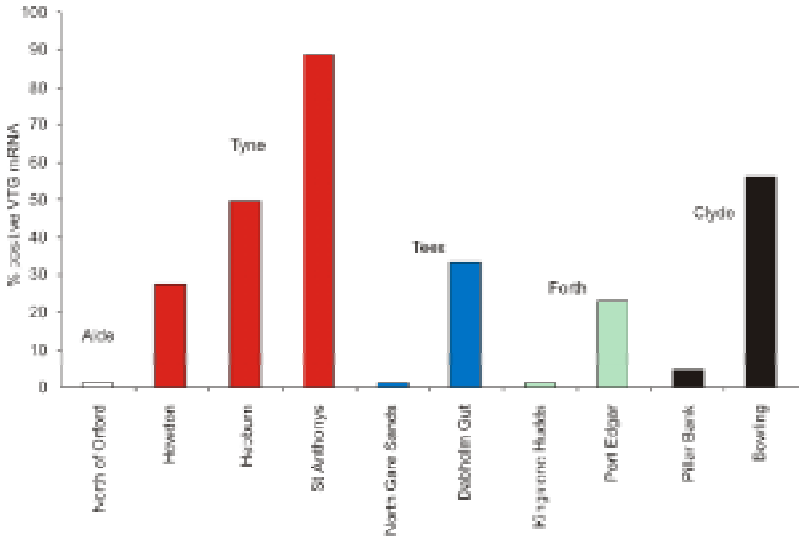


Figure 25. Percent VTG mRNA positive results over all sampling occasions (September 1999- February 2001) in male viviparous blenny

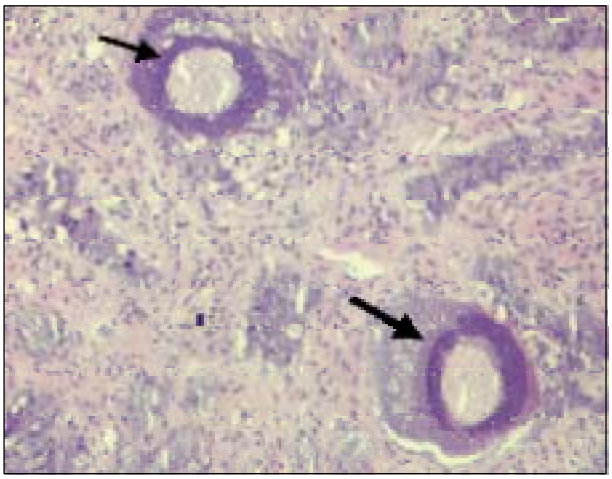


Figure 26. Ovotestis in viviparous blenny from the Tyne estuary, showing two primary oocytes embedded in testicular tissue (arrows)

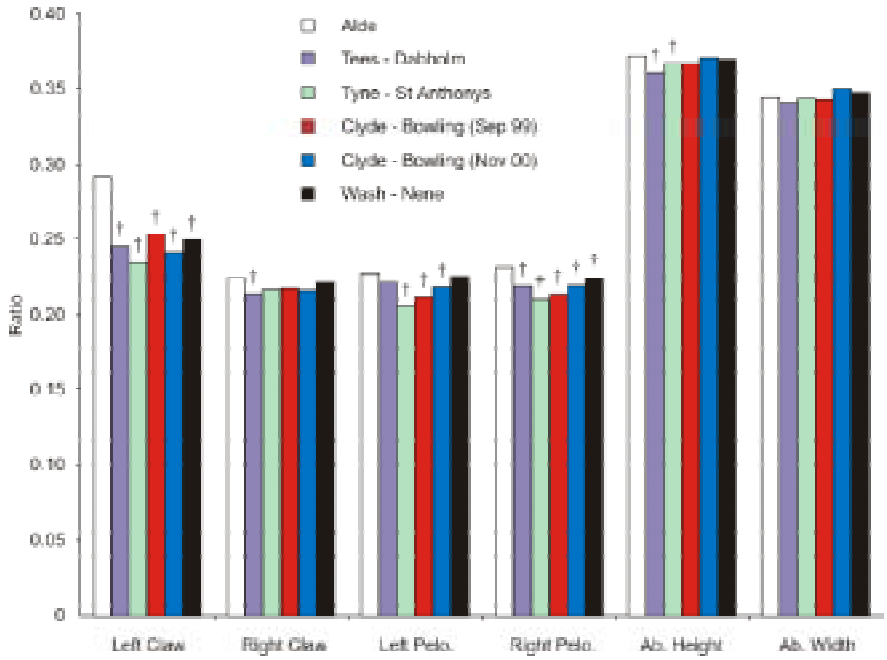


Figure 27. Comparison of morphometric data in male shore crabs sampled from 4 contaminated estuaries and the reference estuary. Columns marked † denote significant difference from the reference (Alde) estuary ($p<0.05$)

4.2.2 Stickleback field studies

The KEH of wild sticklebacks collected from the River Weaver (heavily polluted), Lowestoft and Kent (clean) sites are displayed in Table 1. Females from the Weaver had statistically significant higher KEHs than the reference sites. However, because of several possible confounding factors (including parasitism, presence of rodlet cells in the kidney and atresia of oocytes in the ovaries) this greater KEH could not be confidently attributed to androgen exposure. Male fish from Kent had all entered breeding, displaying full kidney hypertrophy and a significantly higher KEH than all other male groups. This may be because Kent is the most southern site and the survey took place slightly later than the other sites.

Table 1. Kidney epithelium height of wild sticklebacks

Location	Grid reference	Date	Female KEH (µm)	Male KEH (µm)
Inner Weaver	SJ 535772	04/04/99	15.9 (n=26)	26.6 (n=19)
Weaver mouth	SJ 530784	04/04/99	15.8 (n=8)	25.6 (n=5)
Kent	TQ 861529	24/04/99	13.5 (n=23)	36.5 (n=6)
Suffolk	TM 536919	20/04/99	14.3 (n=12)	25.8(n=6)

Results from the Tyne and Tees caging study are presented in Figure 28. Several males were into breeding condition and therefore had several thousand spiggin units per kidney. Females caged on the Tyne, and in the Tees for three weeks were negative. However, three out of the fifteen female sticklebacks which were deployed in the Tees for 7 weeks were weakly positive, showing values of 369, 452 and 540 spiggin units per kidney.

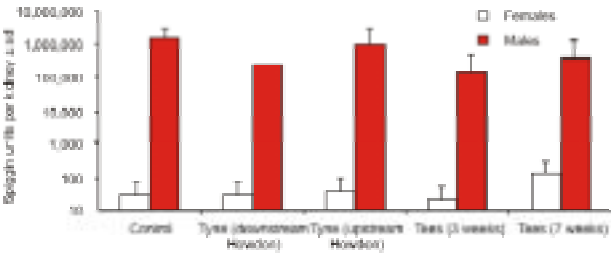


Figure 28. Spiggin units of sticklebacks caged for three or seven weeks in the Tyne and Tees estuaries

4.2.3 Trout/salmon field studies

4.2.3.1 Smolts

Salmon smolts retained for a period of 5 days within the Tees estuary downstream of the barrage had plasma levels of VTG below the level of detection (< 0.1 µg ml^{-1}). There was no significant difference in the levels of

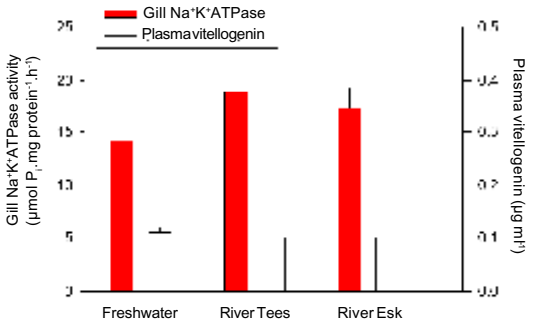


Figure 29. Gill Na^+K^+ATPase activity and plasma VTG in Atlantic salmon smolts sampled in freshwater or retained in the River Tees (ostrogen contaminated) and the River Esk (clean estuary) for a period of 5 days. The levels of plasma VTG are below the detectable level of the assay (<0.1 µg ml^{-1}). The data represent the mean and standard error (SE) of 10 smolts

plasma VTG between these fish and the control group retained for a similar period at the "clean site" in the adjacent River Esk estuary. The mean gill Na^+K^+ATPase activity in the fish retained within the River Tees was also not significantly different to the control group (Figure 29).

4.2.3.2 Adults

Plasma VTG levels in adult female sea trout were between 192 and 4300 µg ml^{-1} (n = 4). In male adult sea trout plasma VTG levels were predominantly below the level of detection (< 0.1µg ml^{-1}) (n = 41), although three males had slightly elevated levels in the range 0.9 to 1.8 µg ml^{-1}. Two of these fish were from the River Tees and one from the River Tyne. Gill Na^+K^+ATPase activity in males with elevated VTG was not significantly different to males with undetectable VTG levels. Similarly there was no evidence of intersex or significant gonadal pathology in these two groups of fish.

In adult female Atlantic salmon the levels of plasma VTG were between 410 and 470 µg ml^{-1} (n = 3). In male Atlantic salmon the levels were below the level of detection (< 0.1 µg ml^{-1}) (n = 48).

4.3 Investigating causal links - substances and sources

4.3.1 Toxicity Identification and Evaluation

4.3.1.1 Oestrogens

The oestrogenic activity of estuarine water samples without extraction was < 15 ng E2 equivalents l^{-1}. After solid phase extraction of water samples, the YES assay showed that the highest amount of activity was in the Howdon STW sample (24 ng E2 equivalents l^{-1}) with lesser activity detected up- and downstream of the discharge point. Lower activity was detected in the Dabholm Gut sample, with very little activity in the samples collected up- and down stream of the point of discharge.

Fractionation of the Dabholm Gut and Howdon STW extracts by HPLC isolated a number of oestrogenic fractions (Figure 30; Fractions 21-23; Howdon STW 90 % of total activity; Dabholm Gut 84 % of total activity). GC-MS analysis identified 17β-oestradiol, *bis*(2-ethylhexyl)phthalate, nonylphenol, androsterone, and an unknown agent to be responsible for the observed activity (Table 2). The majority of the activity was associated with the natural steroid hormone 17β-oestradiol derived from domestic STW effluent.

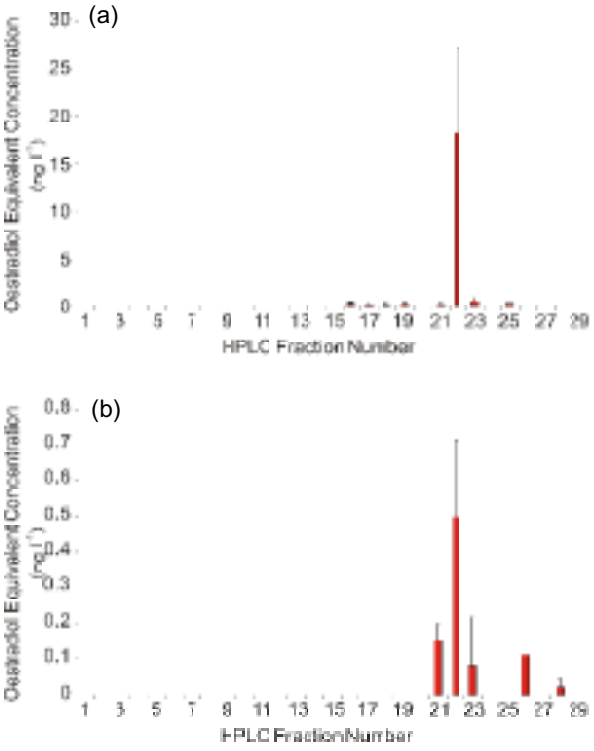


Figure 30. Oestrogenic activity of HPLC fractions from, (a) Howdon STW effluent (Tyne) and (b) Dabholm Gut effluent (Tees) represented as 17β–oestradiol equivalents

The sediment pore water extract obtained from Dabholm Gut (Tees) had an oestrogenic effect equivalent to 7 ng E2 l^{-1}. No activity was detected in the pore waters collected from the Tyne. Fractionation of the active extract by HPLC produced five consecutive oestrogenic fractions (Fractions 19-23). GC-MS analysis of these fractions failed to identify any candidate oestrogens.

Higher oestrogenic activity (0.5 to 5.5 µg E2 equivalents kg^{-1}) was detected in the extracts of the sediment particulate material obtained from both Howdon and Dabholm Gut. Fractionation and GC-MS analysis identified nonylphenol, cinnarizine and cholesta-4,6-dien-3-one in the Howdon STW sediment extract (Table 3). However, these three compounds account for a very small amount of the observed activity (<1 %). The compounds responsible for a significant amount of the activity (and the total activity of the Dabholm Gut sample) remain unidentified.

4.3.1.2 Androgens

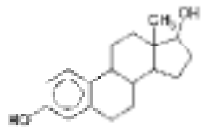
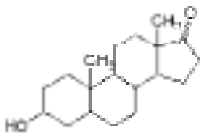
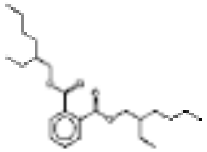
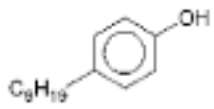
The level of *in vitro* androgenic activity determined in surface waters, sediment pore waters and sediment extracts of samples collected from seven UK estuaries are presented in Figure 31(and Table 9, Appendix 2).

The *in vitro* androgenic activity of estuarine surface waters was shown to be between <2 and 9 ng DHT equivalents l^{-1}. The highest levels of activity were determined in a sample collected on the Clyde estuary (9 ng DHT equivalents l^{-1}) and downstream of Dabholm Gut on the Tees estuary (8 ng DHT equivalents l^{-1}). The majority of samples showed levels of androgenic activity below the detection limit of the YAS assay (<2 ng DHT equivalents l^{-1}). These data indicate that *in vitro* androgenic activity in the surface waters of the surveyed UK estuaries is generally low.

Samples of sediment pore water showed much higher *in vitro* androgenic activity (Figure 31; Table 9, Appendix 2), being between <45 and 187 ng DHT equiv. l^{-1}. However, only eight of the forty-one samples collected were above the limit of detection for the YAS assay system. The sediment pore water samples with measurable *in vitro* androgenic activity were from the estuaries of the rivers Clyde, Forth, Tees, Tyne and Thames.

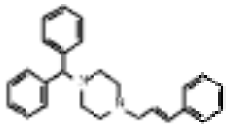
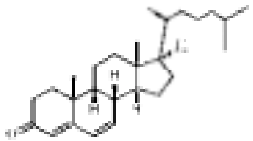
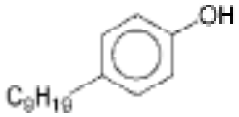
Sediment extracts collected from ten sites (Figure 31) showed elevated levels of androgenic activity (1,020 - 15,300 ng DHT equivalents kg^{-1} wet weight). These data should be viewed with caution, since the extracts tested are complex mixtures of very diverse compounds. The influence of non-androgenic compounds (e.g. anti-oestrogenic compounds) on the mechanism of the YAS assay is unclear and the values obtained may be an overestimate of the true level of *in vitro* androgenic activity present in the sample.

Table 2. Summary of oestrogens identified in Howdon STW and Dabholm Gut effluent and associated chemical data

Effluent/Fraction	Compound	Chemical structure	Chemical Abstract Service (CAS)	Log K_{OW}	Activity (ng E2 equiv. l^{-1})	Oestrogenic potency†	Estimated conc. in effluent ($\mu g\, l^{-1}$)	Source
Howdon STW: 16-19	Unknown	-	-	-	1.0	-	-	-
Howdon STW: 21-23	17β-oestradiol		50-28-2	3.1	19.1	x 1	0.0191	Natural steroid hormone
Howdon STW: 25	androsterone		53-41-8	-	0.4	x $5x10^{-4}$	0.8	Testosterone metabolite
Dabholm Gut: 21-23	17β-oestradiol	As above	50-28-2	3.1	0.73	x 1	0.00073	Natural steroid hormone
Dabholm Gut: 26	bis(2-ethylhexyl)phthalate		117-81-7	4.9	0.11	x $2.8x10^{-7}$*	393	Plasticizer
Dabholm Gut: 28	nonylphenol		60-57-1	4.5	0.02	x $2.6x10^{-5}$	0.77	Surfactant metabolite

† *As compared to 17β-oestradiol using the YES assay.*
* *Tentative quantification since plasticware is used in the extraction procedure, however blanks do not show a phthalate induced response.*

Table 3. Summary of oestrogens identified in Howdon STW sediment and their associated chemical data

Effluent/Fraction	Compound	Chemical structure	Observed Activity in fraction (ng E2 kg^{-1})	Oestrogenic potency[1]	Estimated conc. in sample (ng kg^{-1})[2]	Activity due to identified compound (ng E2 kg^{-1})[3]	Source
Howdon STW sediment extract: DCM 20-21	Nonylphenol		5000	x 2.6 x 10^{-5}	-	-	Surfactant metabolite
Howdon STW sediment extract: DCM 20-21, 5	Cinnarizine		100	x 3.37x 10^{-7}	5600	1.90x10^{-3}	Anti-histamine
Howdon STW sediment extract: DCM 20-21, 5	Cholesta-4,6-dien-3-one		100	x 1.57 x 10^{-6}	800	1.31x10^{-3}	Cholesterol oxidation product
Howdon STW sediment extract: AcOH 30	Nonylphenol		-	x 2.6 x 10^{-5}	-	-	Surfactant metabolite

[1] *As compared to 17β-oestradiol using the YES assay (E2=1),*
[2] *From GC-MS data,*
[3] *Oestrogenic Potency x Concentration, - data not available.*

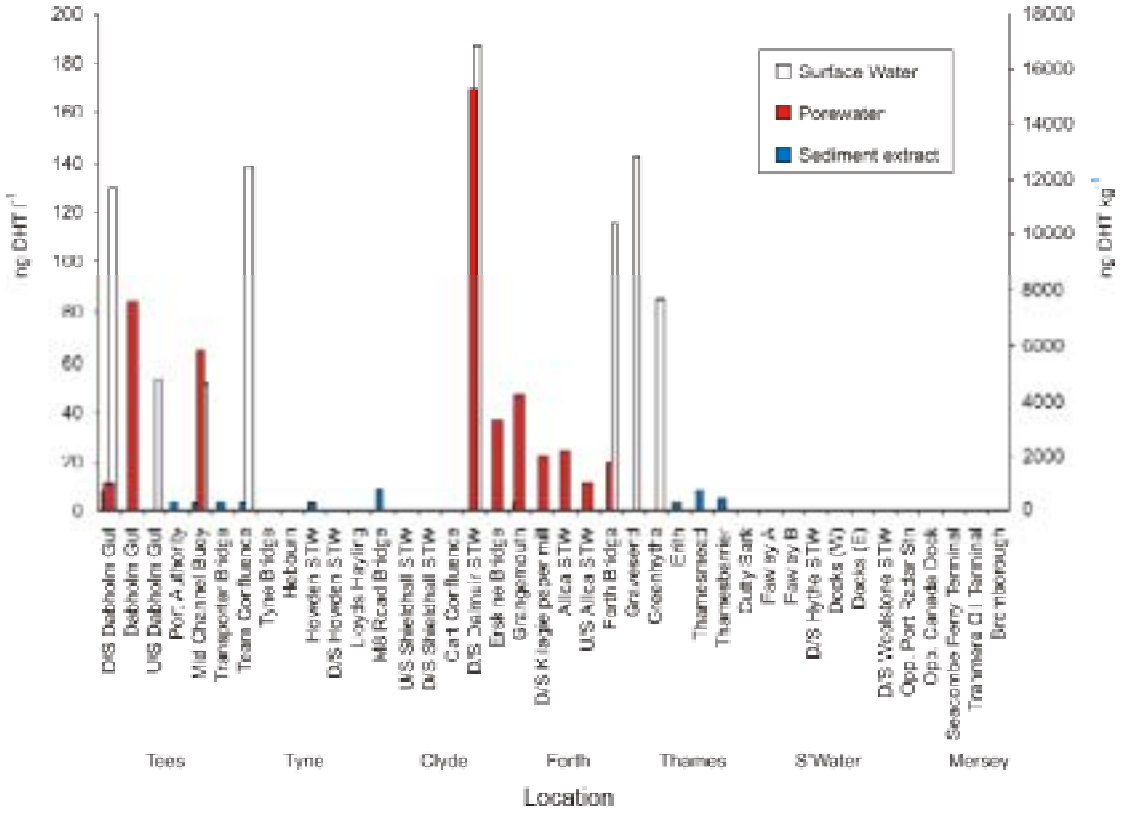


Figure 31. In vitro androgenic activity of estuarine surface waters, sediment pore waters and sediment extracts

The *in vitro* androgenic activity of a selection of effluents discharging into estuaries was also determined (Table 10, Appendix 2). The level of androgenic activity ranged from below the limit of detection (< 23 ng DHT equiv. l^{-1}) to 598 ng DHT equiv. l^{-1}. Only two effluents had measurable androgenic activity: the Irvine Valley Sewer on the Forth of Clyde (34 - 598 ng DHT equiv. l^{-1}) and Howdon STW on the Tyne (191 - 635 ng DHT equiv. l^{-1}). The main difference between the five effluents sampled is the level of treatment that each receives. Where the level of androgenic activity is high the effluent had only received primary treatment prior to discharge. However, where the level of androgenic activity is below the detection limit the effluent had received a more advanced level of treatment. From these few data, it therefore appears that the compounds responsible for STW effluent androgenic activity are removed by percolating filter bed and activated sludge systems, but not completely removed by primary treatment processes.

Effluent from Irvine Valley Sewer was chosen for the TIE study since it represented the greatest input of *in vitro* androgenic activity detected in the survey. Solid phase extraction of the effluent produced an androgenic extract that when fractionated by HPLC isolated the androgenic activity to a cluster of nine fractions with a total activity of 58 ng DHT equiv. l^{-1} (Figure 32).

GC-MS characterisation of all androgenic fractions identified dehydrotestosterone, androstenedione,

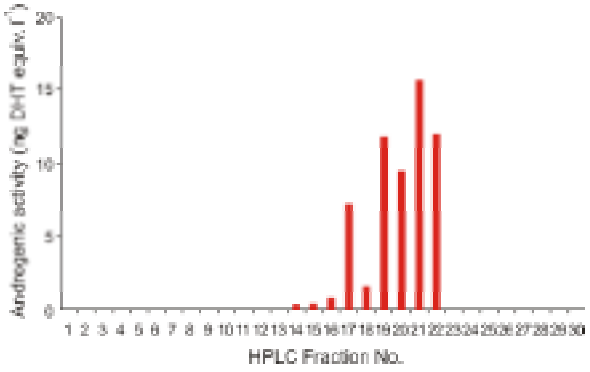


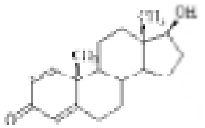
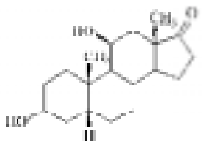
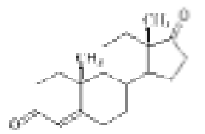
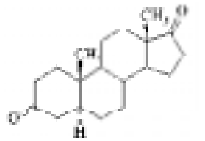
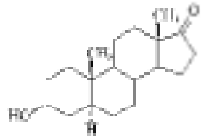
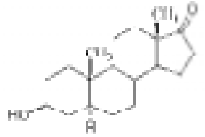
Figure 32. Androgenic activity of Irvine Valley Sewer reverse phase HPLC fractions

androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone, and epi-androsterone as responsible for 99% of the *in vitro* activity determined in the effluent (Table 4).

4.3.2 Flounder caging trials

Very low plasma VTG levels (20-30 ng ml^{-1}) were measured in the laboratory control fish at the beginning of the study. No significant increase in VTG levels was measured in male or female flounder after 14 days exposure to estuarine waters at any of the sites in the Tyne and Tees (Table 11, Appendix 2).

Table 4. Summary of the androgens identified in Irvine Valley Sewer effluent and associated chemical data

Fraction No.	Compound	Chemical structure	Chemical Abstract Service (CAS)	Kovats Retention Index	Diagnostic ions (m/z)	Total activity	Androgenic potency† (ng DHT equiv. l^{-1})	Estimated conc. in effluent (ng l^{-1})
14, 15	Unknown	-	-	-	-	0.6	-	-
16, 17, 18	1-Dehydrotestsosterone		846-48-0	2664	122 (100), 286 (5) $[M]^+$	8.6	x 0.05	172
18	5β-Androstane-3α,11β-diol-17-one		739-26-4	2695	232 (100) $[M]^+$, 191 (90), 150 (80)	0.8	x 0.0002	4000
19, 20	4-Androstenedione		63-05-8	2615	286 (100) $[M]^+$, 244 (80)	21.0	x 0.2	105
21, 22	5α -Androstanedione		846-46-8	2533	288 (100) $[M]^+$, 244 (80)	19.1	x 0.2	96
22	Androsterone		53-41-8	2579	290 (100) $[M]^+$, 246 (95)	8.3	x 0.1	83
23	Epi-androsterone		1239-31-2	2508	290 (100) $[M]^+$, 246 (90)	< 0.1	x 0.03	33

† *As compared to 5α- dihydrotestosterone using the YAS assay.*

4.4 Investigating causal links - laboratory studies

4.4.1 Flounder feeding experiments

Plasma VTG concentrations in male flounder in the control and shrimp fed tanks at the three time intervals are presented in Figure 33. Initial mean concentrations of VTG in all tanks were low, approximately 100 ng ml^{-1}. Feeding the fish either contaminated shrimp from the Tees or clean shrimp from the Crouch had no significant effect on VTG concentration. Fish exposed to EE2 (the positive control) for three weeks responded well, showing 5000-fold increases in plasma VTG concentrations.

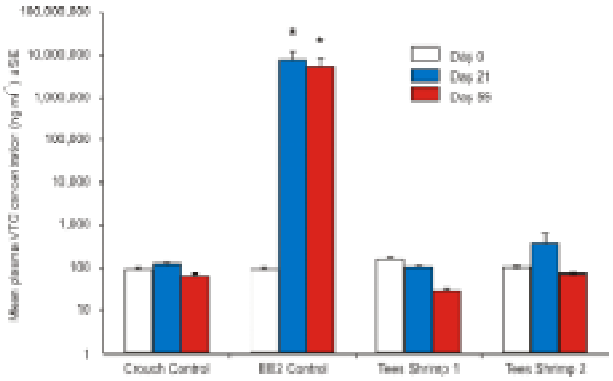


Figure 33. Plasma VTG concentrations in male Alde flounder fed in the laboratory with brown shrimp caught in the Tees estuary. * denotes statistically significant from control (p<0.01)

Plasma VTG concentrations in male flounder from the control and mussel fed tanks at each of the three time intervals are presented in Figure 34. For the treatment tanks, fish are separated into those that gained weight during the experiment (i.e. they were feeding) and those that maintained or lost weight (i.e. were not feeding). It is evident that male flounder which fed on contaminated mussels had increased mean plasma VTG concentrations after 21 and 43 days, whereas those that did not feed showed either no increase or a reduction in VTG. Statistical analysis of data from male fish that fed and gained weight during the experiment showed that there was no significant effect of the source of the mussels. The elevated mean VTG in males fed Tees mussels was due to very high concentrations in one male fish in each tank.

Chemical analysis of the shrimp samples was not performed due to lack of response of male flounder. However, in view of other information that crustacea are not significant bioaccumulators, it is likely that shrimp from the Tees did not contain significantly higher amounts of contaminants compared to Crouch shrimp.

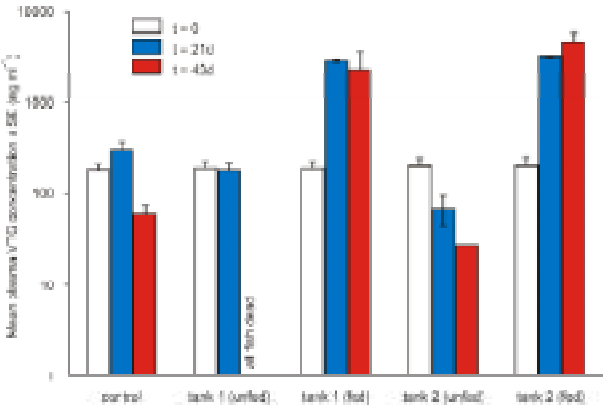


Figure 34. Plasma VTG concentrations in male Alde flounder fed in the laboratory with mussels caged in the Tees estuary

Concentrations of measured chemicals in mussel flesh are presented in Table 12 of Appendix 2. In general, mussels from the Tees had higher concentrations of contaminants in their tissue than those which had been held in the clean estuary. In particular, hydrocarbons and tributyltin (TBT) were 20 and 7 times higher, respectively, in Tees mussels compared to those caged on the Crouch. It was also anticipated that the levels of PCBs would differ substantially between the contaminated and clean site, but this was not the case. Bisphenol-A concentrations were generally below the limit of detection, although they were elevated in some samples of Tees mussels.

4.4.2 Sand goby experiments

4.4.2.1 Sand goby breeding experiments

A full account of the results from this series of experiments can be found in the EDMAR contract report and in Robinson *et al* (2001a). Additionally, several papers are currently being prepared for peer-reviewed publication.

4.4.2.1.1 IVS/EE2 experiment

IVS effluent was analysed for alkylphenols and only nonylphenol (NP) was detected. Its concentration was highly variable between effluent batches (99 ± 102 µg l^{-1}; all isomers) but appeared to be higher than previously reported (26 µg l^{-1}; Pirie *et al*, 1996).

Exposure to 6 ng l^{-1} EE2 (nominal) reduced male growth and delayed and inhibited male maturation by reducing gonad size, seminal vesicle size and nuptial colouration (Figure 35). VTG and ZRP mRNA expression were induced in both sexes (Figure 36). Male nesting and breeding behaviour were reduced, and individual fecundity, egg fertility and population output were also significantly lower than in controls (Figure 37). Exposure to IVS did not induce male VTG/ZRP mRNA expression (Figure 36) or affect maturation indices, although at the higher concentration male fish had

Figure 35. Photographs of a mature male sand goby (Pomatoschistus minutus; left), showing the normal nuptial colouration (blue anal fin, dark ventral fins and dark vertical bars), and of a male exposed for 6 months to nominal 6 ng l^{-1} 17α-ethynyl oestradiol (EE2; right). Note reduced size and colouration of male exposed to EE2 compared to control

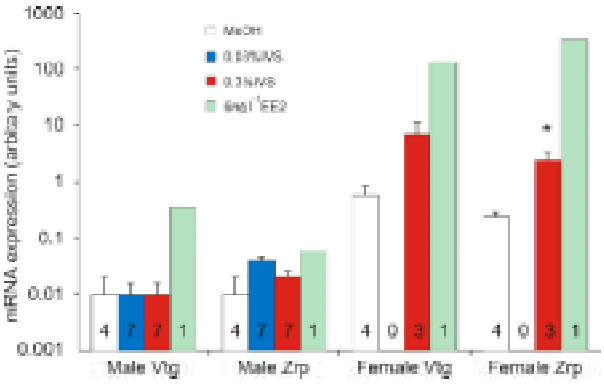


*Figure 36. Effects on hepatic vitellogenin (VTG) and zona radiata protein (ZRP) mRNA expression in post-spawning sand goby after seven months exposure to solvent control (MeOH; <0.002% methanol), Irvine Valley Sewage effluent (IVS; 0.03 or 0.3% v/v), or nominal 6 ng l^{-1} ethynyl oestradiol (EE2). Significant differences between treatment and solvent control (*p<0.05), and the number of observations are indicated*

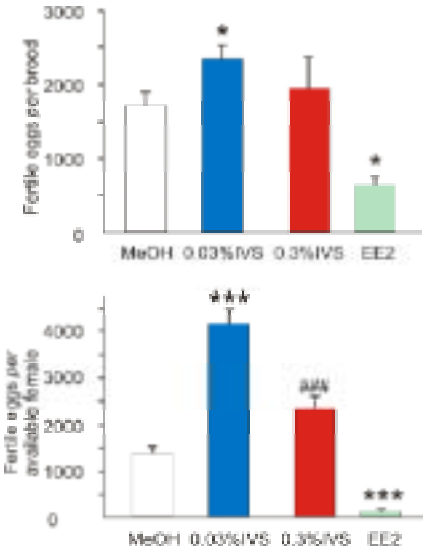


*Figure 37. Effects on individual fecundity and population egg production of sand goby after a total of seven months exposure to solvent control (MeOH; <0.002% methanol), Irvine Valley Sewage effluent (IVS; 0.3% or 0.03% v/v), or nominal 6 ng l^{-1} ethynyl oestradiol (EE2). Significant differences between treatment and solvent control (*p<0.05, **p<0.01, ***p<0.001), and between 0.03 and 0.3 % v/v IVS (###p<0.001) are indicated*

smaller urogenital papillae (as did EE2-exposed fish) and a higher mortality rate than controls. Exposure to IVS caused an inverted U-shape dose response curve on population egg production, with higher egg production in fish exposed to 0.03% IVS than either methanol controls or 0.3%-exposed fish (Figure 37).

4.4.2.1.2 OP experiments

At the end of the four-week OP exposure, male sand goby were expressing VTG mRNA at exposure concentrations greater than 30 µg l^{-1} (Figure 38).

In the long-term OP experiment, all fish exposed to the higher concentration (119 µg l^{-1} median measured concentration) died after 7 weeks. Fish exposed to the lower concentration (29 µg l^{-1} OP median measured concentration) had a higher mortality rate than controls and maturation of males was inhibited (smaller seminal vesicles and reduced nuptial colouration). Males did not show any feminisation of the urogenital papilla.

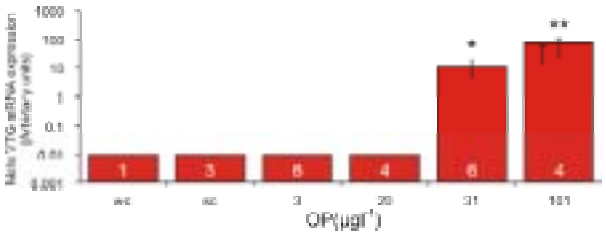


Figure 38. Effects of exposure to OP for four weeks on hepatic vitellogenin (VTG mean and standard error) mRNA expression. The number of observations and significant differences between treatment and water control (wc), (*p<0.05, **p<0.01), are indicated

VTG mRNA expression (of both sexes) tended to increase with increasing OP concentration and duration of exposure, for the first three months. Post-150 days exposure however, mRNA expression had decreased in both sexes at the single remaining treatment (29 µg l^{-1}), although it was still higher than control fish levels.

4.4.2.1.3 E2 experiment

In the final experiment, median measured E2 concentrations for the water/solvent controls and the three test concentrations were <5, 17, 70 and 530 ng l^{-1} respectively. There were no significant differences between the measured biological parameters of the two control groups, and data were therefore combined.

Exposure to high concentrations of E2 affected fish health, particularly males, which became anaemic and had reduced growth compared to controls. Additionally, males exposed to the two highest concentrations showed feminisation of the urogenital papilla: it was shorter than controls, and there was a dose related increase in occurrence of the MIPS condition.

There were both temporal and treatment effects upon maturation indices of fish exposed to E2 (Figure 39). Females showed the expected seasonal increase in gonad weight (gonadosomatic index; GSI) between October and March, however ovarian growth was initially slower in the treated fish, such that the GSI of all E2 treated females was smaller in January than for controls. All male maturation markers (GSI, seminal vesicle somatic index (SVSI), nuptial colouration) showed a seasonal increase to March, with GSI and SVSI then being smaller post-spawning. However, there were no seasonal changes in these indices for 530 ng l^{-1}-exposed males. In March, maturation of males exposed to 70 ng l^{-1} E2 was also inhibited compared to controls.

Male VTG mRNA expression showed a dose related increase, but no significant temporal effects. Although also present in females, the dose related increase in VTG mRNA expression was complicated by seasonal effects (Figure 40).

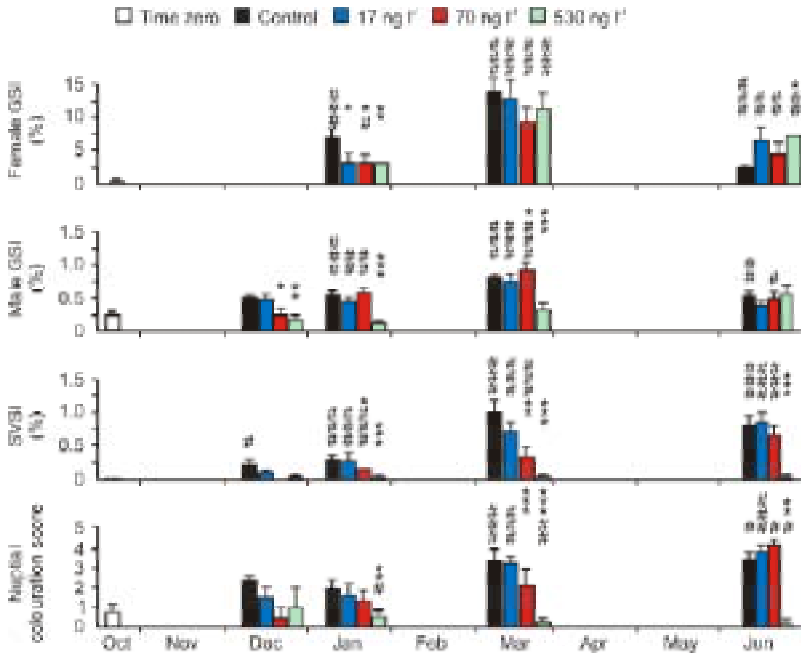


Figure 39. Effects of treatment and length of exposure on maturation indices of sand goby exposed to 17β-oestradiol (E2; 17, 70, or 530 ng l^{-1}) or controls (combined water control and solvent control: propan-2-ol, <0.002%). Mean and standard error of 7-8 observations per treatment and time point (14-15 for controls), except December (2 observations per treatment, 4 for controls) and June (for only 1 female treated with 530 ng l^{-1}). GSI = gonadosomatic index (tissue weight as percentage of total weight); SVSI = seminal vesicle somatic index (tissue weight as percentage of total weight); nuptial colouration scored according to presence or absence of dark anal fin, blue anal fin, dark ventral fins plus 0.5 x no. of vertical bars. Differences from control at each time point (*p<0.05, **p<0.01, ***p<0.001), and from time zero for each treatment (#p<0.05, ##p<0.01, ###p<0.001) are indicated

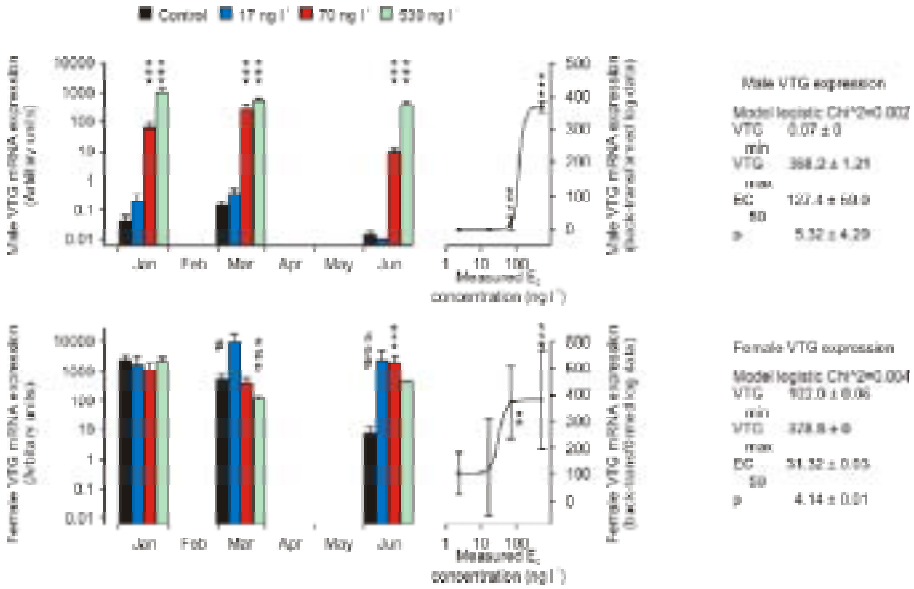


Figure 40. Effects of treatment and length of exposure on hepatic expression of vitellogenin (VTG) mRNA in sand goby exposed to 17β-oestradiol (E2; 17, 70, or 530 ng l^{-1}) or controls (combined water control and solvent control: propan-2-ol, <0.002%). Mean and standard error of 7 observations per treatment and time point (14-15 for controls), except in June, when 9 control females and 1 female treated with 530 ng l^{-1} were analysed. Differences from control at each time point (*p<0.05, **p<0.01, ***p<0.001) and from time zero for each treatment (#p<0.05, ##p<0.01, ###p<0.001) are indicated. EC50 graphs are of data from all time points

During the breeding trials 530 ng l^{-1}-exposed fish produced no fertile eggs. Even when crossed with water control fish, 530 ng l^{-1}-exposed females did not spawn and 530 ng l^{-1}-exposed males did not nest, although within-control crosses bred successfully. At the population level, fish exposed to 70 ng l^{-1} E2 produced 22% fewer fertile eggs than control fish (Figure 41), although there were no effects on individual fecundity. No effects were seen on egg production by 17 ng l^{-1}-exposed fish.

The EC50s (the concentration of a chemical/effluent that reduces the measured parameter to 50% relative to the control population) for male VTG mRNA expression and population fertile egg production are very similar (127±59 and 120±73 ng l^{-1}, respectively). When plotted together there is a strong decrease in population egg production with increasing male VTG mRNA expression (Figure 42). This model suggests that an approximately 1700-fold induction in male mRNA expression was associated with a 50% reduction in egg production.

4.4.2.2 MIPS Response in gobies

MIPS was shown to be inducible *in P. minutus* by exposure to the oestrogen E2. Early signs of this condition became apparent after 11 weeks exposure (Figure 43). The percent prevalence of MIPS at each time interval and E2 concentration is presented in Figure 44. Both a concentration- and time-dependent increase in the incidence of MIPS was observed, with the highest occurrence being 75% at 1000 ng l^{-1} after 32 weeks exposure.

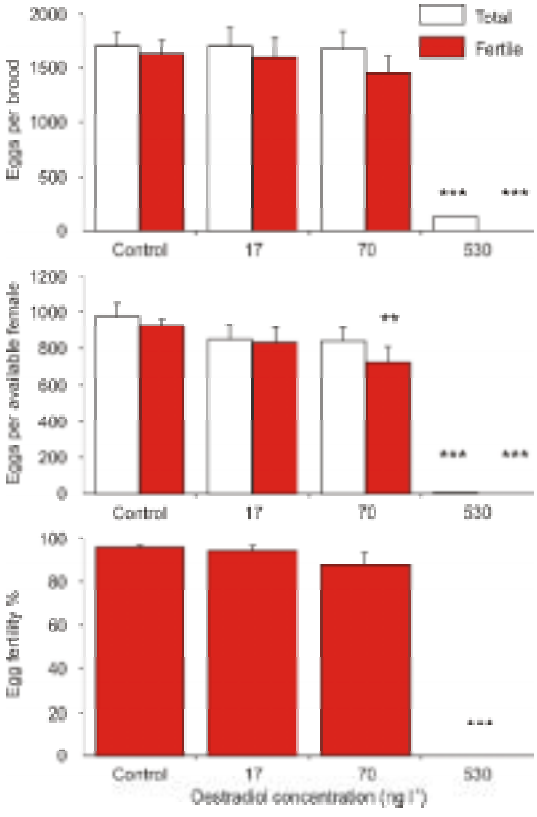


Figure 41. Effects on individual fecundity (total or fertile eggs per brood), population egg production (total or fertile eggs produced per female available to breed) and egg fertility of sand goby exposed to 17β-oestradiol (E2) for a total of 8 months. Significant differences between treatments and control (**p<0.01, ***p<0.001) are indicated

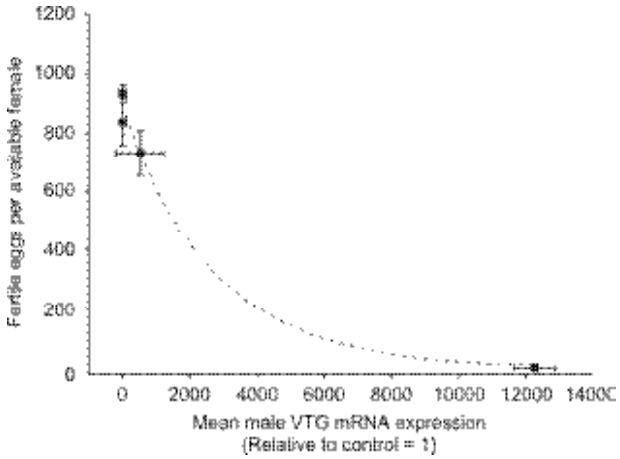


Figure 42. Effects of eight months exposure to 17β-oestradiol (E2) upon sand goby population egg production (number of fertile eggs produced per female available to breed) in relation to male hepatic vitellogenin (VTG) mRNA expression (relative to control = 1). The fitted curve is $y = y_{max}.e^{-(xla)}$, where $y_{max} = 885$ and $a = 2683$

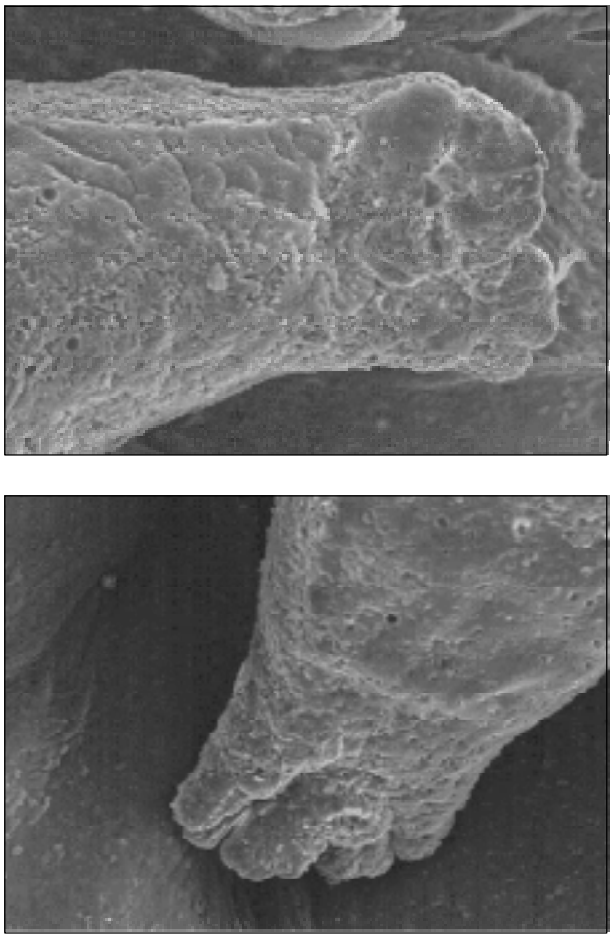


Figure 43. Examples of male P. minutus uro-genital papillae showing the MIPS condition after 13 weeks exposure to 1000 ng l^{-1} oestradiol

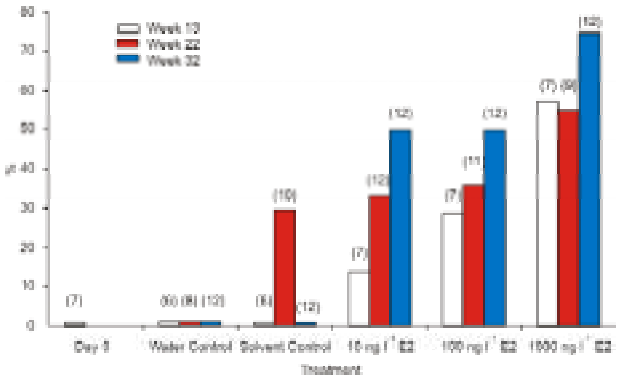


Figure 44. Percent MIPS induced in male P. minutus exposed to oestradiol (total number of specimens in brackets) for periods of 13, 22 or 32 weeks

5. DISCUSSION

5.1 Biomarker development

5.1.1 Biomarkers for androgenic effects in fish

In vivo androgenic effects in fish, both in the laboratory and in the field, have historically been assessed using responses such as development of external male secondary sexual characteristics (e.g. in fish caught near pulp mill effluents (Cody and Bortone, 1997)). The stickleback studies here succeeded in developing a robust and sensitive *in vivo* biomarker for measuring exposure to androgens, their mimics and antagonists in the aquatic environment, based on the induction of spiggin in the kidneys of female sticklebacks (which do not normally synthesise this protein themselves). These will be invaluable for screening chemicals in the laboratory and for monitoring studies, not just in the UK, but on a global scale.

Borg *et al* (1993) established that stickleback kidney epithelial cell height shows a highly specific response to androgens and laid the foundation for the present study. However, from an ecotoxicological perspective the bioassay used in Borg's study has a number of drawbacks: it requires male sticklebacks to be castrated prior to carrying out the bioassay, the fish have to be injected with the test compound on a daily basis for three weeks and the kidneys of males then have to be processed histologically and KEH measurements made on each section. Clearly, all three procedures are time consuming. Also, the first and the second procedures rule out the possibility of using the bioassay for testing androgen exposure in the field. The results of the EDMAR work demonstrate that females are a practical substitute for castrated males and that androgen exposure can be via the water rather than by injection and thus more environmentally relevant.

Although the histological measurement of kidney cell height proved to be a valid bioassay, the processing of a large number of samples for microscopy was time-consuming. The development of an immunoassay based on the androgen-induced protein, spiggin, was expected to provide a much faster and specific bioassay. The spiggin ELISA showed a strong correlation with the histological method, enabling KEH measurement to be replaced, with subsequent large gains in resolution and processing speed.

Female stickleback treated with the androgens 17α-MT, DHT and 11-KT showed dose-dependent increases in spiggin, and the results suggest that 17α-MT may be slightly more potent than DHT. The spiggin assay was further validated by studies using the anti-androgenic drug flutamide, which inhibited spiggin induction by 17α-MT and DHT, suggesting that these steroids are acting directly on a receptor (or receptors). The kidneys of male sticklebacks also show an increase in spiggin production in response to water-borne androgens. However, the fact that for much of the year they are able to come into maturity means that control male fish tend to have high background levels of spiggin. Males are likely to prove more useful for pinpointing anti-androgenic activity.

It has been proposed that the stickleback be included as one of the test species in an Organisation for Economic Co-operation and Development (OECD) short-term fish screening protocol, which is currently being developed and validated. This would initially be used to screen for androgens and anti-androgens, but it is envisaged that with further development and validation of a stickleback VTG assay (currently underway at CEFAS), the same species can also be used for screening oestrogens and anti-oestrogens. Furthermore, in the future, it may be possible to include the stickleback in long-term testing protocols, currently being developed using the fathead minnow, to evaluate the effects of oestrogens, androgens and their antagonists on apical endpoints such as growth and reproduction. The possibility of measuring all these effects in a single species carries implications for reduced testing costs and reduced use of experimental animals.

5.1.2 Biomarkers for oestrogenic effects in invertebrates

The development of a biomarker based on the detection of vitellogenin in male crustaceans followed the assumption that as this measure works well in some fish species it might also work with crustaceans. However, it became clear during the course of the EDMAR research that endocrine processes associated with vitellogenesis in crustaceans are not influenced by environmental contaminants in the same way as in vertebrates, particularly fish. Vitellogenin was not detected in the haemolymph of male crab or shrimp collected from local populations or as part of the wider EDMAR survey programme.

Even though the process of vitellogenesis in crustaceans is broadly similar to that seen in vertebrates the hormones controlling this process are different. Thus, xenobiotics that can mimic, block or otherwise disrupt the hormone-receptor interaction in vertebrates do not necessarily have the same effect on the different set of receptors found in crustaceans. The possibility remains, however, that other, as yet unidentified chemicals, could disrupt crustacean endocrine systems in much the same way. The newly developed assays, nevertheless will be used in many other areas of work to assess the biological significance of contaminant effects on the process of vitellogenesis in crustaceans.

Experimental evidence has indicated that vitellogenin is not suitable as an indicator of anti-androgenic activity, since it could not be detected in the haemolymph of crabs known to have lost the secretions from their androgenic glands following infection with the parasitic barnacle *Sacculina carcini.* Further investigations will need to be undertaken to determine other suitable biomarker candidates for potential androgenic and anti-androgenic disturbance in crustaceans.

There appears to be no obvious detriment to vitellogenesis when female crabs are exposed to testosterone under the experimental conditions used in this study. These results indicate that the potential for vertebrate type androgens to disrupt overt reproductive processes in crabs concurs with the oestrogens situation.

Vitellogenesis is only one component of the reproductive process in animals and its easily measurable nature has proved useful for examining endocrine disruption in fish. The lack of a measurable response in this process in crustaceans, exposed to chemicals known to disrupt the same process in vertebrates, should not of course lead to the conclusion that endocrine systems of crustaceans are not susceptible to disruption by vertebrate steroids and their mimics. It may be that reproductive and other physiological processes of crustaceans under endocrine control are indeed susceptible to the threat of exogenous chemicals and it is merely that detriment leading from such exposures has yet to be recognised. A review of crustacean (invertebrate) physiology under endocrine control, coupled with the development of suitable endpoints to measure the effectiveness of these systems in the face of exposure to test contaminants, could help to identify areas of potential concern.

5.1.3 Biomarkers for oestrogenic effects in small fish species

The traditional method of detecting egg-protein production in the serum of fish with antibody-reagents can now be augmented with an alternative molecular methodology of measuring egg-protein mRNA in liver. Isolation of the cDNA reagents can be readily achieved and were produced for four teleost species. Each of the sequences generated during this project have been submitted to the appropriate GenBank database. The isolated reagents reacted specifically with their matching mRNA but not with the mRNA of other species. Specificity of reaction was also apparent from observations that the cDNA reagents reacted with RNA from breeding females but not males. VTG and ZRP genes were only expressed in males when pre-treated with oestrogenic chemicals.

Having established the specificity of the reagents a modified high throughput assay was devised enabling the measurement of egg-protein mRNAs in livers of individual fish.

5.1.4 Histochemical techniques

5.1.4.1 Oestrogen Receptor

Positive results were obtained for the human breast cancer tissue (the positive control) using several different mammalian antibodies. Although binding was achieved in the flounder sections, the expected staining pattern, as seen in the positive control, was not obtained. Since there is no antibody specific for the flounder oestrogen receptor, the immunocytochemistry technique is not yet viable. Due to the poor quality of the cryostat sections, the results from the *in vivo* autoradiography work were invalid. A useful next step may be to examine the various fixed tissues that are archived and use conventional methods of processing to look for evidence of retention and possibly better localisation of the labelled isotope. Also, there is a need to investigate the use of tritium-labelled androgens.

5.1.4.2 Localisation of flounder VTG and stickleback spiggin

The immunocytochemical methods for localisation of the male stickleback glue protein spiggin and flounder VTG were partially successful. Spiggin was present in the epithelium of the proximal tubules and VTG was weakly present in the hepatocytes. However, because the staining was weak the practical use of these techniques as immunocytochemical biomarkers is unlikely at present.

5.2 Biological responses in estuaries

5.2.1 Field surveys

5.2.1.1 Flounder

The VTG data indicate that levels in male flounder are decreasing in certain areas. The clearest example is the Mersey, with a two orders of magnitude reduction over a four year period, but other sites, such as the Tyne and Clyde also showed large reductions. These declines may reflect improved clean-up techniques in certain places, but some caution is still required when interpreting the results, since they are based on limited sampling and could be due to other factors such as significant migration into the area or changed feeding habits. Further trend data needs to be gathered in order to address the issue properly. Future VTG measurements should be made in conjunction with examination of effluent quality data and interpreted with reference to local effluent clean up technology.

Early studies of plasma VTG and the occurrence of intersex revealed no close relationship between the two. Studies from 1996 demonstrated that the intersex condition occurred solely in flounder from the Mersey; surveys from 1997 revealed the condition in both the Mersey and Tyne (Matthiessen *et al*, 1998; Allen *et al*, 1999a,b). The results from the EDMAR surveys show that these estuaries continue to be the 'hotspots' although there is good evidence that, in some areas including these, the VTG response in male fish appears to be lower. It remains difficult to draw any firm conclusions with respect to the presence of intersex and contamination by EDs. The Mersey and Tyne intersex 'hotspots' coincide with areas having high male plasma VTG levels. The unexpected absence of intersex in Tees fish could simply be due to the small sample sizes, or may indicate that the condition is caused by specific oestrogenic chemicals that are more prevalent in the Tyne and Mersey.

5.2.1.2 Sand goby

The occurrence of the MIPS condition correlated with areas of known oestrogenic contamination, with >50% occurrence at sites on the Tees, Mersey and Clyde. Insufficient numbers of gobies were caught in the Tyne to make a judgement for this estuary.

The significance of the MIPS condition in terms of repercussions for reproductive output can only be speculated upon since the precise function of the uro-genital papilla (UGP) is not entirely understood. However, it is known that the UGP has a role during reproduction and courtship, primarily in sperm

deposition and attachment in males, and oviposition in females. It is therefore entirely plausible that malformations in the UGP could interfere with mating behaviour and compromise reproductive performance.

A link between oestrogen exposure and the development of the MIPS condition was established in the laboratory (section 4.4.2.2). However, not all fish showed this change and the severity of MIPS was very mild. Nevertheless, this has provided good evidence that the MIPS condition observed in oestrogen contaminated areas is probably caused by exposure to environmental endocrine disrupters.

Whilst investigating MIPS, two possible accompanying observations to this condition were noted - the appearance of spongiform type tissue as found in females and twisting of the orientation of the urinary and gonadal ducts. However, it cannot be confirmed that these are related to oestrogen exposure since very few specimens have been processed.

The absence of VTG mRNA in wild caught male gobies suggests that they either do not respond to xeno-oestrogens in the same manner as other fish, or their sensitivity is much lower. Laboratory experiments have disproved both theories, having shown that males do indeed express VTG mRNA, and are as sensitive to water-borne oestrogen exposure as flounder. It remains unknown why VTG or ZRP mRNA are not detectable in wild fish; however, a possible explanation is that exposure to oestrogens is intermittent and the induced mRNA, which has a short half-life compared to plasma VTG, rapidly disappears from the body.

5.2.1.3 Viviparous blenny

It is clear from the VTG mRNA data that viviparous blenny are being exposed to environmental oestrogens in some areas, notably the estuaries of the Tyne, Tees, Clyde and Forth. Intersex was observed in male fish from the Clyde and Tyne, sites where intersex has been observed in male flounder. This dataset is too small to draw any firm conclusions about causality, but adds to the growing body of circumstantial evidence that the intersex condition is linked to oestrogen exposure in certain areas. It is significant that this is the first time that evidence of oestrogenic endocrine disruption has been demonstrated in wild fish of an estuarine species other than flounder.

No firm conclusions about the effects of ED on reproduction and sex ratios could be made, since the brood variables dataset was too limited and comparisons with a clean reference site were not possible. However, it is worth noting that all the ratios were slightly skewed towards females. This could be a result of oestrogenic exposure but clearly more research is required. The information has initiated a database which, it is hoped, will be built upon in future years.

5.2.1.4 Crab and shrimp

Brown shrimp and shore crab clearly do not respond to oestrogenic exposure in a similar way to fish. There has been no evidence that male field-collected samples of either species are producing female-specific yolk protein. The implications of this lack of response are discussed in more detail in section 5.1.2.

The crab morphometric approach may have some potential as a measure of endocrine disruption, but as yet no firm evidence exists to support a conclusion that exoskeletal abnormalities are caused by oestrogens, and possible mechanisms are unknown.

In normal male shore crabs, right claws are comparatively enlarged compared to the left, and this is clear from the EDMAR data. This secondary sexual characteristic could be influenced by oestrogenic exposure and indeed right claws from almost all sites were smaller than those from the Alde. However, while known oestrogen-contaminated sites, such as Dabholm Gut and Clyde Bowling, show evidence of retarded growth in some male characteristics, similar effects are also seen at a number of possibly cleaner sites (e.g. The Wash). It should, however, be noted that even the samples from the Wash may have been affected by water from the Nene, a river known to contain high concentrations of oestrogenic substances (Jobling *et al*, 1998). These data are clearly not to be simplistically interpreted and should form the basis of further investigation.

5.2.2 Stickleback field studies

The results of the caging study suggested the presence of low concentrations of androgenic compounds in the Tees estuary, and this has indeed been confirmed by analytical work (see section 4.3.1.2). However, further investigations are clearly required to evaluate the full biological implications of these findings. The complexity of a site like the Tees estuary should be taken into account when evaluating such a weak signal. The low percentage of breeding males in the Tees deployments in comparison to the Tyne and the control group could indicate the presence of anti-androgens which may be antagonising the androgen-controlled development of male reproductive charcteristics. The fact that the deployment took place during May (well into the breeding season of the stickleback in the UK) and three weeks later than the control sampling supports this possibility. Further cage deployments are currently taking place in the Irvine valley, where primary sewage effluent is discharged and is a site of suspected androgenicity.

5.2.3 Field studies on salmonids

Retention of salmon smolts within the Tees estuary resulted in no measurable increase in plasma VTG levels and no effect on seawater adaptation as measured by changes in gill Na^+ K^+ATPase activity. These results

suggest that there was no observable endocrine disruption in the salmon smolts as a result of exposure within the Tees estuary. Retention of the smolts within the estuary for 5 days was an environmentally realistic exposure period. Previous studies on the migratory behaviour of salmon smolts within estuaries have indicated that movement is rapid and fish only reside within the estuary for a maximum of 2-3 days (Moore *et al*, 1995, 1998). In general, smolts are considered to migrate too quickly through estuaries to be affected by the presence of oestrogenic chemicals.

Although there was a slight elevation in plasma VTG in three (out of 18) adult male sea trout from the Rivers Tees and Tyne, the results suggest that the majority of the salmon and sea trout were unaffected. In addition, histology of the gonads showed no evidence of intersex or significant gonadal pathology. Therefore, it appears from the present study that endocrine disruption in salmonids resulting from oestrogenic contamination within estuaries is not widespread and is unlikely to have a significant impact on wild populations.

5.3 Investigating causal links - substances and sources

5.3.1 Identifying estuarine oestrogens and androgens

The Toxicity Identification and Evaluation approach was successful at identifying a number of oestrogenically active compounds in surface water samples collected from the rivers Tyne and Tees. A range of compounds was identified that may be contributing to the physiological effects (elevated VTG and intersex) reported in flounder collected from these estuaries. The data suggest that the natural steroid hormone 17β-oestradiol, derived from domestic STW effluent, plays a significant role in the oestrogenic activity of estuarine surface waters. Androsterone, a second natural steroid detected, is produced by the metabolism of testosterone in the prostate. Although classified as an androgen, it is also an oestrogen agonist. The nonylphenol and bis(2-ethylhexyl)phthalate identified are both ubiquitous contaminants derived from surfactants and plastics respectively and have been previously reported as oestrogenically active (Jobling *et al*, 1996; Keith, 1997). This suggests that in domestically derived STW effluent natural steroid hormones are the main cause of oestrogenic activity.

Nonylphenol, cinnarizine, and cholesta-4,6-dien-3-one were the only oestrogenic compounds successfully identified in estuarine sediments: unfortunately their contribution to the overall activity determined was small. The high level of activity in the particulate phase of the sediment suggests that the majority of the activity is associated with compounds that have a high affinity for particulate matter (i.e. non-polar compounds), however the fact that activity elutes from the SPE system using mid-polar solvents (rather than hexane) suggests a more polar structure. Further work will be necessary to both identify the compound(s) and address the issue of bioavailability to benthic organisms.

The *in vitro* androgenic activity of surface waters collected from UK estuaries was generally low. Higher levels were measured in sediment pore waters and bound to particulate material. Detectable levels of *in vitro* androgenic activity were only measured in two discharges receiving primary treatment. It would appear that the compounds responsible for this activity are removed by more advanced treatment. The application of TIE procedures was successful in identifying six natural steroids as the cause of androgenic activity in primary treated effluent.

5.3.2 Flounder caging trials

The lack of plasma VTG induction in flounder caged in the Tees and Tyne estuaries could be due to one or a combination of several reasons. Firstly, the 14 day exposure period may have been too short to induce plasma VTG. Secondly, the concentration of VTG-inducing compounds in the Howdon and Dabholm Gut effluents may have been too low and, thirdly, the main route of exposure may not be through the water column but through the sediment and/or food or a combination of all three.

Male flounder have previously been shown in laboratory experiments with dissolved oestrogen to produce VTG within a three week exposure period, although this was using the potent synthetic steroid EE2 (Allen *et al*, 1999a). A concentration of 10 ng l^{-1} EE2 is sufficient to induce VTG in males in this time period and the response time is likely to be shorter than three weeks. The less potent natural oestrogen E2 was identified in both effluents in which the cages were placed (see section 4.3.1.1). Although there are no data for VTG induction thresholds of E2 in male flounder, if they are similar to those observed in roach and trout (Lowest Observed Effect Concentration (LOEC) = 100 ng l^{-1}, No Observed Effect Concentration (NOEC) = 10 ng l^{-1}; Routledge *et al*, 1998), the estimated E2 concentration in Howdon effluent (19 ng l^{-1}) would be unlikely to stimulate VTG to any great extent. However, in view of the fact that other oestrogens were present which could have exerted potential additive action, it is perhaps surprising that VTG was not even slightly induced. The highest *in vitro* oestrogenic activity was found to be associated with the sediments and it may have been the case that the flounder were not sufficiently exposed to this source of oestrogens, although the cages did settle a few centimetres into the estuary bed. It is not possible to pinpoint the specific cause for lack of response, but it is likely to be a combination of the three main factors stated above.

The laboratory feeding experiments were subsequently set up to investigate the role of food as an exposure route for oestrogens.

5.4 Investigating causal links - laboratory experiments

5.4.1 Flounder feeding study

Male flounder feeding on shrimp caught in a contaminated (not necessarily with oestrogens) area of the Tees showed no induction of VTG, but it is likely that the shrimp were poor bioaccumulators of contaminants. A second experiment feeding male flounder on mussels (which are efficient bioaccumulators of contaminants) caged for several months on the Tees, showed that there was a clear VTG response in some male flounder that fed on contaminated mussel meat, and were therefore exposed to bioaccumulated contaminants. Fish that did not respond were, generally, those that had lost weight during the experiment, implying that they did not feed.

However, the absolute levels of the response were not very big. The maximum individual concentration of plasma VTG in the experimental group was only 20 times greater than the maximum in the control group, compared with the 10^5 fold induction seen in wild flounder. Although there may be wild invertebrates with higher levels of oestrogenic contamination, it is unlikely that they will be orders of magnitude higher than in mussels kept in the estuary for several months. The high levels of VTG seen in some wild flounder therefore still need further explanation, since neither caged flounder exposed to sediment and water, or laboratory fish fed invertebrates from the Tees, produced large responses. One possible explanation for this discrepancy is that wild fish in oestrogen-contaminated estuaries are exposed to oestrogens from the egg/larval stage, either via transfer from the female or via the external environment, which could make them more susceptible than 'clean' fish. Studies by Metcalfe *et al* (2000) have shown that male Japanese medaka exposed as fry to an oestrogen mimic (o,p'-DDT) and subsequently dosed with E2 as adults gave a 5-fold larger VTG response than control males. To investigate this, flounder from the Alde control estuary were tagged and released into the Tyne estuary, with the aim of comparing VTG levels in recaptured fish with native wild populations. To date, no tagged flounder have been recaptured in subsequent estuarine surveys. However, if the sensitisation hypothesis is correct, any re-captured fish should have lower VTG titres than indigenous Tyne fish.

5.4.2 Sand goby breeding experiments

The oestrogens (E2 and EE2) and oestrogen mimic (OP) used in these studies induced vitellogenesis in male sand goby at concentrations similar to those seen in other non-salmonid species (i.e. approximately 10 ng l^{-1} EE2 (Matthiessen *et al*, 1998; Folmar *et al*, 2000), 30 µg l^{-1} OP (Routledge *et al*, 1998) and between 10 and 100 ng l^{-1} E2 (Kramer *et al*, 1998; Panter *et al*, 1998; Folmar *et al*, 2000)).

As with carp (Gimeno *et al*, 1998a,b), sand goby growth was reduced following exposure to steroidal oestrogens (EE2 and E2), but not with exposure to alkylphenols. Exposure of sand goby to EE2 and E2 altered male testicular development at concentrations similar to those which demasculinise mature male carp (Gimeno *et al*, 1998b), feminise young male carp (Gimeno *et al*, 1998a), or induce intersex in medaka (Gray and Metcalfe, 1997; Gray *et al*, 1999). In addition, male sand goby exposed to all three test compounds had delayed or inhibited nuptial colouration development which has been shown to be under hormonal control (Borg and Mayer, 1995).

Sand goby also showed two novel markers of oestrogenic exposure: a reduction in the size of the seminal vesicle (sperm duct gland), and the feminisation of the UGP. Seminal vesicle development was reduced by exposure to all three test substances and appears to be a very sensitive marker for oestrogenic exposure in this species. The functions of these glands are not known, although they are thought to play an important role in reproduction (Webb, 1980; Miller, 1984), possibly through the secretion of pheromones (Colombo *et al*, 1980).

In the IVS effluent breeding experiments, population production of fertile eggs showed an inverted U-shaped dose response curve. A similar result was reported for fathead minnow exposed to low concentrations of nonylphenol (Giesy *et al*, 2000), where egg production was elevated relative to controls at a nominal concentration of 0.1 µg l^{-1} NP. In the IVS study, fish in the nominal 0.03% IVS exposure would have experienced a comparable NP concentration (the mean effluent NP concentration was approximately 100 µg l^{-1}).

Exposure to EE2 and E2 led to inhibited maturation and subsequent inhibition of reproductive behaviour, with fewer males nesting and fewer pairs producing eggs. Exposure to 6 ng l^{-1} EE2 and 530 ng l^{-1} E2 also reduced individual fecundity, egg fertility and population reproductive success. The latter parameter was also reduced by exposure to 70 ng l^{-1} E2. Reduced reproductive output due to failure to spawn has recently been seen in medaka exposed to 10 ng l^{-1} EE2 (Scholz and Gutzeit, 2000). Reduced fertility has been shown in zebra fish exposed to 5 ng l^{-1} EE2 (Kime and Nash, 1999) and reduced fecundity following exposure to E2 or EE2 has been seen in medaka (1.7 µg l^{-1} E2, Nimrod and Benson, 1998; 10 ng l^{-1} EE2, Scholz and Gutzeit, 2000) and in fathead minnow (EC50 = 20 ng l^{-1} E2, Kramer *et al*, 1998).

The similarity between concentrations of E2 that induced male vitellogenesis and reduced population egg production in sand goby suggests that wild populations exposed to oestrogens (such that males are expressing VTG mRNA) are likely to have reduced reproductive success. However, there is no evidence of males in UK estuaries expressing VTG mRNA, hence it

remains in doubt whether there are population-level effects in wild sand goby.

The ecological significance of any reduction in reproductive output in goby would depend upon three main factors: a) the degree of exposure, and therefore its impact upon reproduction; b) the mortality rate of fish within the impacted area (both natural, and any due to exposure to chronic toxicants, as occurred during these experiments); and c) the rate of recruitment of young fish from outside the impacted area. The sand goby life cycle involves paternal care of the eggs, reducing the time available for dispersion of larvae compared to animals that release eggs into the water column. Where fish are impacted by oestrogenic contamination, goby populations may therefore be more at risk than fish without parental care. Taken a stage further, if fish that also give parental care to their larvae and young have reduced reproductive success, then the potential impact on their populations would be greater, because dispersal of the young is minimal hence recuitment from elsewhere would be very limited. The viviparous blenny (*Zoarces viviparus*) is one such fish, and in some UK estuaries males have been shown to be producing VTG and occasionally displaying intersex. However, unlike sand goby, the data to correlate VTG production and intersex with reproductive impacts are currently lacking. With its specialised reproductive strategy and limited geographical range in the UK, populations of viviparous blenny may be at greater risk than other fish from endocrine disrupting chemicals.

6. CONCLUSIONS

The EDMAR programme has, overall, achieved its main objectives and enhanced our understanding of the extent and severity of endocrine disruption, in terms of oestrogenic and androgenic effects, in the UK marine environment.

The main achievements and conclusions can be summarised as follows:

Toolkit development

- **Two biomarkers for androgenic exposure, kidney epithelial cell height and spiggin (glue protein) induction in female stickleback, were successfully developed and validated.**
  - Spiggin responds to synthetic androgens in a dose and time-dependent manner and induction is antagonised by the pure anti-androgen, flutamide.
  - The androgen biomarker can be also be adapted to detect anti-androgens.
- **ELISA methodologies for shore crab and brown shrimp vitellogenin were successfully developed and used to screen for its presence in individuals from the field.**
  - VTG was absent from wild male crabs and shrimp.
  - Laboratory exposures to known oestrogens did not produce a response.
  - Other unidentified xenobiotics may still be able to interact with invertebrate endocrine systems.
- **Histochemical biomarkers for ED in fish require substantial further development and validation before they can be considered as useful tools for evaluating endocrine disruption.**
- **Molecular probes for VTG and ZRP mRNA were successfully developed for sand goby and viviparous blenny.**
  - These were effectively used to measure VTG induction in fish sampled from the field and exposed to oestrogens in the laboratory.

Biological responses in estuaries

- **A total of 14 UK estuaries were surveyed for 5 sentinel species and a range of biomarkers for oestrogenic exposure applied.**
- **Flounder plasma VTG concentrations were determined over a three year period to establish trends. There was some evidence of a decline at selected sites, while others remained stable. Intersex flounder continues to be observed at a relatively low but consistent level in some estuaries .**
- **Some wild male sand goby exhibited morphological abnormalities of the uro-genital papilla, with a shape intermediate between male and female. This condition was termed Morphologically Intermediate Papilla Syndrome (MIPS)**
  - The highest incidences of MIPS were in the Tees, Mersey and Clyde; it was rare or absent in the reference estuary (Alde). The biological significance of this is as yet unclear.
  - No VTG or ZRP gene induction or male intersex were observed in wild caught sand goby. The reason for this is not yet fully understood.

❖ **Viviparous blenny showed signs of oestrogenic exposure in some estuaries**

- High levels of VTG mRNA were found in males from the estuaries of the Tyne, Tees, Forth and Clyde.
- Intersex males were found, particularly in the Tyne, although overall prevalence was low.
- Larval sex ratios in all estuaries surveyed showed a slight female skew, but reference samples could not be obtained for comparison.

❖ **Fieldwork to investigate endocrine disrupting effects on migratory salmonids (Atlantic salmon and sea trout) in estuaries revealed no major evidence of effects on the smoltification process or on male vitellogenesis. These results suggest that endocrine disruption in salmonids within estuaries is not widespread and it is unlikely that exposure to oestrogenic contamination is a major factor regulating salmonid populations in England and Wales.**

Causal links - substances and sources

❖ **A number of endocrine disrupting substances in water, effluent and sediments from several UK estuaries were identified using a Toxicity Identification Evaluation technique, specifically tailored to identify oestrogenic and androgenic compounds**

- Oestrogenic and androgenic activity in surface waters was generally low. Much higher levels of activity were determined in sediment pore waters (androgens) and extracts of particle bound material (oestrogens and androgens).
- Several natural and synthetic compounds were identified as the cause of oestrogenic activity in estuarine surface waters. The cause of oestrogenic activity in sediments is largely unknown.
- Several natural steroids were identified as the cause of androgenic activity in a primary treated effluent.

Causal links - laboratory experiments

❖ **Flounder feeding studies with mussels caged on the Tees for several months demonstrated that food probably has a role in exposure of these fish to exogenous oestrogens and that food-chain transfer can occur.**

- The magnitude of the response in the laboratory was much lower than that measured in wild flounder, which may be due to longer exposure during a critical time period or alternatively to "hypersensitisation" of larval/juvenile feral fish to exogenous oestrogens in contaminated estuaries.

❖ **Several long-term studies with sand goby were conducted exposing fish to Irvine Valley sewage effluent (IVS) and known oestrogenic substances, measuring biomarker responses and effects on maturation and reproduction**

- Reproductive output was unaffected by exposure to 0.3 and 0.03% IVS. Weak effects on biomarkers observed at the higher concentration.
- VTG was induced in male sand goby by E2, EE2 and OP.
- Exposure to 6 ng l^{-1} EE2 reduced breeding success by over 90% without producing a large VTG response in males. Exposure to 70 ng l^{-1} E2 reduced population egg production by approximately 25% and a large VTG response.
- A novel marker (seminal vesicle size) appears to be particularly sensitive to oestrogenic exposure.

❖ **Exposing juvenile sand gobies to E2 for greater than three months induced a mild MIPS condition. MIPS observed in wild fish is therefore probably due to oestrogen exposure and the more severe cases could be due to prolonged exposure and/or exposure during early life stages.**

❖ **Various research recommendations flowing from EDMAR and other UK research programmes on endocrine disruption in freshwaters were discussed at a DEFRA sponsored workshop at CEFAS Weymouth in September 2001. The report of this workshop " Future directions for government funded research on endocrine disruption in the aquatic environment", can be obtained from the EDMAR secretariat at DEFRA, 3/E6 Ashdown House, 123 Victoria Street, London SW1E 6DE.**

7. REFERENCES

Allen YT, Scott AP, Matthiessen P, Haworth S, Thain JE and Feist SW. (1999a) Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. *Environ. Toxicol. Chem.* **18**: 1791-1800.

Allen Y, Matthiessen P, Scott AP, Haworth S, Feist S and Thain JE (1999b). The extent of oestrogenic contamination in the UK estuarine and marine environments-further surveys of flounder. *Sci. Total Environ*. **233**: 5-20

Alzieu C. (1996) Biological effects of tributyltin on marine organisms. *In*: *Tributyltin: Case study of an environmental contaminant* (de Maora SJ, Ed), Cambridge University Press, Cambridge, UK, pp167-211.

Andersson T, Forlin L, Hardig J and Larsson A (1988). Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. *Can. J. Fish Aquat. Sci*. **45**: 1525-1536.

Ashby J and Lefevre PA (2000). The peripubertial male rat assay as a replacement for the Hershberger castrated male rat assay. *J. Appl. Tox.* **20**: 35-47.

Baldwin WS, Milan DL and LeBlanc JA. (1995) Physiological and biochemical perturbations in *Daphnia magna* following exposure to the model environmental estrogen diethylstilbestrol. *Environ. Toxicol. Chem*. **14**: 945 - 952.

Bodar CWM, Voogt PA and Zandee DI. (1990) Ecdysteriods in *Daphnia magna*: their role in moulting and reproduction and their levels upon exposure to cadmium. *Aquat. Toxicol*. **17**: 339-350.

Borg B. (1981) Effects of methyltestosterone on spermatogenesis and secondary sexual characters in the three-spined sticklebacks, *Gasterosteus aculeatus* L. *Gen. Comp. Endocr*. **44**: 177-180.

Borg B and Mayer I. (1995) Androgens and behaviour in the three-spined stickleback. *Behaviour* **132**: 1025-1035.

Borg B, Antonopoulou E, Andersson E, Carlberg T and Mayer I. (1993) Effectiveness of several androgens in stimulating kidney hypertrophy, a secondary sexual character, in castrated male three-spined sticklebacks, *Gasterosteus aculeatus. Can. J. Zool*. **71**: 2327-2329.

Bratoeff EA, Herrera H, Ramirez E, Solorzano K, Murillo E, Quiroz A and Cabeza M. (2000) Anti-androgenic effect of 16-substituted, non-substituted and D-homopregnane derivatives. *Chem. Pharm. Bull*. **48**: 1249-1255.

Bryan GW and Gibbs PE. (1991) Impact of low concentrations of tributyltin (TBT) on marine organisms: a review. In: *Metal Ecotoxicology: Concepts and Applications.* (Newman MC, McIntosh AW, Eds), CRC, Boca Raton, FL, USA, pp 323-361.

Bryan GW, Gibbs PE, Burt GR and Hummerstone LG. (1987) The effects of tributyltin (TBT) accumulation on adult dog whelks, *Nucella lapillus* : Long term field and laboratory experiments. *J. Mar. Biol. Assoc. UK* **67**: 525 544.

Cody RP and Bortone SA. (1997) Masculinisation of mosquitofish as an indicator of exposure to kraft mill effluent. *Bull. Environ. Contam. Toxicol*. **58**: 429-436.

Colombo L, Marconato A, Colombo Belvedere P and Frisco C. (1980) Endocrinology of teleost reproduction: a testicular pheromone in the black goby *Gobius jozo* L. *Boll. Zool.* **47**: 355-364.

Craft JA, Robertson FE and Brown E. (2002) Endocrine disruption in teleosts: cloning and characterization of cDNA probes for vitellogenins and zona radiata proteins. EDMAR Final Report to DEFRA, 234pp.

Denton TE, Howell WM, McCollum CJ, Marks EB and Allison JJ. (1985) Masculinization of female mosquitofish by exposure to plant sterols and *Mycobacterium smegmatis*. *Bull. Environ. Contam. Toxicol*. **35**: 627-632.

Desbrow C, Routledge EJ, Brighty G, Sumpter JP and Waldock M. (1998) Identification of oestrogenic chemicals in STW effluent: Chemical fractionation and screening. *Environ. Sci. Technol*. **32**: 1549-1558.

Dinan L, Bourne P, Whiting P, Dhadialla TS and Hutchinson TH. (in press) Screening of environmental contaminants for ecdysteroid agonist and antagonist activity using the *Drosophila melanogaster* B_{II} cell *in vitro* assay. *Environ. Toxicol. Chem*.

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J and Guillette LJ Jr. (1996) Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinous carpio*) captured near major metropolitan sewage treatment plant. *Environ. Health Perspect.* **104**: 1096-1101.

Folmar LC, Hemmer M, Hemmer R, Bowman C, Kroll K and Denslow ND. (2000) Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. *Aquat. Toxicol.* **49**: 77-88.

GenBank/EMBL accession numbers: vitellogenins AJ416326 (*Zoarces viviparus*); AJ416327 (*Platichthys flesus*); AJ416328 (*Pleuronectes platessa*); AJ416329 (*Pomatoschistus minutus*). ZRP AJ416330 (*Pomatoschistus minutus*).

Giesy JP, Pierens SL, Snyder EM, Miles-Richardson S, Kramer VJ, Snyder SA, Nichols KM and Villeneuve DA. (2000) Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* **19**: 1368-1377.

Gimeno S, Gerritsen A, Bowmer T and Komen H. (1996) Feminization of male carp. *Nature* **384**: 221-222.

Gimeno S, Komen H, Gerritsen AGM and Bowmer T. (1998a) Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. *Aquat. Toxicol.* **43**: 77-92.

Gimeno S, Komen H, Jobling S, Sumpter J and Bowmer T. (1998b) Demasculinisation of sexually mature male common carp, *Cyprinus carpio,* exposed to 4-*tert*-pentylphenol during spermatogenesis. *Aquat. Toxicol.* **43**: 93-109.

Gray MA and Metcalfe CD. (1997) Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to *p*-nonylphenol. *Environ. Toxicol. Chem.* **16**: 1082-1086.

Gray LE Jr, Wolf C, Lambright C, Mann P, Price M, Cooper RL and Ostby J. (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health* **15**: 94-118.

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, Rycroft R, Sumpter JP, Tylor T. (1996) A survey of estrogenic activity in United Kingdom inland waters. *Environ. Toxicol. Chem.* **15**: 1993-2002.

Harries JE, Sheahan DA, Jobling S, Mathhiessen P, Neall P, Sumpter JP, Tylor T and Zaman N. (1997) Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Toxicol. Chem.* **16**: 534-542.

Henderson BE, Ross R and Bernstein L. (1988) Estrogens as a cause of human cancer. The Richard and Hilda Rosenthal Foundation Award Lecture. *Cancer Res*. **48**: 246-253.

Howell MW, Black DA and Bortone SA. (1980) Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally induced masculinisation. *Copeia* **4**: 676-681.

Howell MW and Denton TE. (1989) Gonopodial morphogenesis in female mosquitofish, *Gambusia affinis affinis*, masculinised by exposure to degradation products from plant sterols. *Environ. Biol. Fish.* **24**: 43-51.

Hutchinson TH, Pounds NA, Hampel M and Williams TD. (1999a) Life-cycle studies with marine copepods (*Tisbe battagliai*) exposed to 20-hydroxyecdysone and diethylstilbestrol. *Environ. Toxicol. Chem*. **18**: 2914-2920.

Hutchinson TH, Pounds NA, Hampel M and Williams TD (1999b) Impact of natural and synthetic steroids on the survival, developmental and reproduction of marine copepods *Tisbe battagliai*. *Sci. Total Environ*. **233**: 167-179.

Jenkins R, Angus RA, McNatt H, Howell WM, Kemppainen JA, Kirk M and Wilson EM. (2001) Identification of androstenedione in a river containing paper mill effluent. *Environ. Toxicol. Chem*. **20**: 1325-1331.

Jobling S, Sheahan D, Osborne JA, Matthiessen P and Sumpter JP. (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkyl-phenolic chemicals. *Environ. Toxicol. Chem*. **15**: 194-202.

Jobling S, Nolan M, Tyler CR, Brighty G and Sumpter JP. (1998) Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **32**: 2498-2506.

Katsiadaki I, Scott AP, Hurst MR, Matthiessen P and Mayer I. (2002) Detection of environmental androgens: a novel method based on Enzyme-Linked Immunosorbent Assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environ. Toxicol. Chem.* **21**:1946-1954.

Keith LH. (1997) Environmental Endocrine Disruptors: a handbook of property data. John Wiley and Sons, New York, USA.

Kelce WR and Wilson EM. (1997) Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. *J. Mol. Med*. **75**: 198-207.

Kelce WR, Gray LE and Wilson EM. (1998) Antiandrogens as environmental endocrine disruptors. *Reprod. Fertil. Dev*. **10**: 105-111.

Kime DE and Nash JP. (1999) Gamete viability as an indicator of reproductive endocrine disruption in fish. *Sci. Total Environ.* **233**: 123-129.

Kime DE, Nash JP and Scott AP. (1999) Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. *Aquaculture* **177**: 345-352.

Kirby MF, Bignell J, Brown E, Craft J, Davies I, Dyer RA, Feist SW, Jones G, Matthiessen P, Robertson FE and Robinson C. (in press) The presence of morphologically intermediate papilla syndrome (MIPS) in UK populations of sand goby (*Pomatoschistus* spp.): Endocrine Disruption? *Environ. Toxicol. Chem*.

Kirby MF, Dyer RA, Craft J, Robertson FE, Brown E, Feist SW, Jones G, Bignell J, Scott S, Katsiadaki I, Tolhurst L and Matthiessen P. (2002) Field investigations of ED biomarker responses and reproductive success in estuarine fish species. EDMAR final report to DEFRA, 234 pp.

Kramer VJ, Miles-Richardson S, Pierens SL and Giesy JP. (1998) Reproductive impairment and induction of alkali-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to water-borne 17â-estradiol. *Aquat. Toxicol.* **40**: 335-360.

Larsson DGJ, Hällman H and Förlin L. (2000) More male fish embryos near a pulpmill. *Environ. Toxicol. Chem*. **19**: 2911-2917.

Leatherland JF. (1993) Field observations on reproductive and developmental dysfunction in introduced and native salmonids from the Great Lakes. *J. Great Lakes Res*. **19**: 737-751.

Lye CM, Frid CLJ, Gill ME and McCormick D. (1997) Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from a sewage treatment works. *Mar. Poll. Bull.* **34**: 34-41.

Madsen SS, Mathiesen AB and Korsgaard B. (1997) Effects of 17β-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon Salmo salar. *Fish Physiol. Biochem*. **17**: 303-312.

Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van Der Kraak G and Ankley GT. (2000) Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). *Aquat.Toxicol*. **48**: 461-475.

Matthiessen P and Gibbs PE. (1998) Critical appraisal of the evidence for tributyltin-mediated endocrine dis-ruption in mollusks. *Environ. Toxicol. Chem.* **17**: 37-43.

Matthiessen P, Allen YT, Allchin CR, Feist SW, Kirby MF, Law RJ, Scott AP, Thain JE and Thomas KV. (1998) Oestrogenic endocrine disruption in flounder (*Platichthys flesus*) from United Kingdom estuarine and marine waters. CEFAS Science Series Technical Report No. **107**, 48pp.

Metcalfe TL, Metcalfe CD, Kiparissis Y, Nimi,Aj, Foran CM, Bensson WH. (2000) Gonadal development and endocrine responses in Japanese medaka *(Oryzias latipes*) exposed to o,p'-DDT in water or through maternal transfer. *Environ. Toxicol. Chem*. **19**: 1893-1900.

Miller PJ. (1984) The tokology of gobioid fishes. In: *Fish Reproduction*: *strategies and tactics* (Potts GW, Wooton RJ, eds), Academic Press, London, pp 119-153.

Moore A, Potter ECE, Milner NJ and Bamber S. (1995) The migratory behaviour of wild Atlantic salmon smolts in the estuary of the River Conwy, North Wales. *Can. J. Fish. Aquat. Sci*. **52**: 1923-1935.

Moore A, Ives S, Mead TA and Talks L. (1998) The migratory behaviour of wild Atlantic salmon (*Salmo salar* L.) smolts in the River Test and Southampton Water. *Hydrobiol*. **371/372**: 295-304.

Moore CG and Stevenson JN. (1991) The occurrence of intersexuality in harpacticoid copepods and its relationship with pollution. *Mar. Poll. Bull*. **22**: 72-74.

Moore CG and Stevenson JN. (1994) Intersexuality in benthic harpacticoid copepods in the Firth of Forth, Scotland. *J. Nat. Hist.* **28**: 1213 - 1230.

Mourot B and Le Bail PY. (1995) Enzyme-linked immunosorbent assay (ELISA) for rainbow trout (*Oncorhynchus mykiss*) vitellogenin. *J. Immunoassay* **16**: 365-377.

Nimrod AC and Benson WH. (1998) Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquat. Toxicol.* **44**: 141-156.

Nolan M, Jobling S, Brighty G, Sumpter JP and Tyler CR. (2001) A histological description of intersexuality in the roach. *J. Fish Biol*. **58**: 160-176.

Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P and Gray LE. (1999) The fungicide procymidone alters sexual differentiation in the male rat by acting as androgen-receptor antagonist *in vivo* and *in vitro*. *Toxicol. Ind. Health* **15**: 80-93.

Panter GH, Thompson RS and Sumpter JP. (1998) Adverse reproductive effects in male fathead minnows (*Pimphales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat. Toxicol.* **42**: 243-253.

Pedersen SN, Christiansen LB, Pedersen KL, Korsgaard B and Bjerregaard P. (1999) *In vivo* estrogenic activity of branched and linear alkylphenols in rainbow trout (*Onchorynchus mykiss*). *Sci. Total. Environ.* **233**: 89-96.

Pirie D, Steven L, McGrory S and Best G. (1996) Survey of hormone disrupting chemicals. Scottish Environment Protection Agency, 13 pp.

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR and Sumpter JP. (1994) Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **8**: 275-285.

Robinson CD, Craft JA, Davies IM, Moffat CF, Pirie D, Robertson F, Brown E, Stagg RM and Struthers S. (2001) Effects of ethynyl oestradiol and sewage effluent upon maturation, molecular markers of oestrogenic exposure, and reproductive success in a marine teleost (*Pomatoschistus minutus*, Pallas). Fisheries Research Services Technical Report 03/01.

Robinson CD, Craft JA, Davies IM, Moffat CF, Pirie D, Robertson F, Brown E, Struthers S, Stagg RM, Best G and Megginson C. (2002) Laboratory studies on the effects of sewage effluent and other suspect materials on breeding success in sand goby. EDMAR Final Report to DEFRA, 234pp.

Routledge EJ and Sumpter JP. (1996) Oestrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem*. **15**: 241-248.

Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M and Sumpter JP. (1998) Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environ. Sci. Technol.* **32**: 1559-1565.

Sandström O, Newman E and Karås P. (1988) Effects of a bleached pulp mill effluent on growth and gonadal function in Baltic coastal fish. *Water Sci. Technol*. **20**: 107-118.

Scholz S and Gutzeit HO. (2000) 17á-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias laipes*). *Aquat. Toxicol.* **50**: 363-373.

Sharpe RM and Skakkebaek NE. (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* **B41**: 1392-1395.

Sohoni P and Sumpter JP. (1998) Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**: 327-339.

Sohoni P, Lefevre PA, Ashby J and Sumpter JP. (2001) Possible androgenic/anti-androgenic activity of the fungicide fenitrothione. *J. Appl. Tox.* **21**: 173-178.

Sonnenschein C and Soto A. (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J. Steroid Biochem. Molec. Biol*. **65**: 143-150.

Tamura H, Maness SC, Reischmann K, Dorman DC, Gray LE and Gaido KW. (2001) Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol. Sci*. **60**: 56-62

Tyler CR and Routledge EJ. (1998) Oestrogenic effects in fish in English rivers with evidence of their causation. *Pure Appl. Chem*. **70**: 1795-1804.

Van Der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB and Chang JP. (1992) Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of White Sucker at multiple sites. *Toxicol. Appl. Pharmacol.* **115**: 224-233

Vethaak AD and Jol J. (1996) Diseases of flounder *Platichthys flesus* in Dutch coastal and estuarine waters, with particular reference to environmental stress factors. I. Epizootiology of gross lesions. *Dis. Aquat .Org.* **26**: 81-97.

Waring CP, Stagg RM, Fretwell K, McLay HA and Costello MJ. (1996) The impact of sewage sludge exposure on the reproduction of the sand goby (*Pomatoschistus minutus*). *Environ. Poll.* **93**:17-25.

Webb CJ. (1980) Systematics of the *Pomatoschistus minutus* complex (Teleostei: Gobioidei). *Philos. Trans. R.. Soc. Lond. B* **291**: 201-241.

Wheeler A. (1969) *The fishes of the British Isles and North West Europe*. Macmillan and Co. Ltd., London.

Zaugg WS. (1982) A simplified preparation for adenosine triphosphatase determination in gill tissue. *Can. J. Fish. Aquat. Sci*. **39**: 215-217.

APPENDIX 1 - SUMMARY OF ASSOCIATED RESEARCH

AstraZeneca's Brixham Environmental Laboratory (BEL) successfully delivered a laboratory based research programme using a marine copepod (*Tisbe battagliai*) life-cycle test to investigate potential developmental and reproductive toxicity. This work was prompted by observations on intersex copepods near the Edinburgh sewage outfall in the Firth of Forth (Moore and Stevenson, 1991,1994) and also as a link to related UK programmes on hazardous substances (e.g. Direct Toxicity Assessment). Finally, this EDMAR associated work has already led to a productive on-going research project with Dr. Laurie Dinan at Exeter University, to investigate mechanisms of action using an *in vitro* ecdysteroid receptor-based screening assay derived from the B_{II} blood cell line of *Drosophila melanogaster*.

Copepod life-cycle test protocol

The 21-day life-cycle test monitors naupliar (larval) and copepodid (juvenile) survival and development. Adults are then sexed, typically 10 days at 20 ± 1°C. Mature males and females are then paired and exposures continued to address reproductive output (Hutchinson *et al*, 1999a, b).

To start the bioassay, individual copepod nauplii (< 24 hr post release) were added to 2 ml of test solution in microplate cell wells and checked at 24 hourly intervals using a stereomicroscope with darkfield illumination. Mortalities were recorded when there was an absence of any movement by the organism for a period of 15 seconds. Test solutions were renewed (80% of volume) three times per week and animals fed with microalgae (*Isochrysis galbana* and *Rhodomonas reticulata*).

Under these conditions, healthy *T. battagliai* nauplii typically reach the first juvenile (copepodid) stage after four days at 20 ± 1°C, attaining the adult stage after 10 days. The sexually dimorphic adults normally have mated by this stage, the inseminated female being capable of utilising stored sperm to subsequently produce several broods of nauplii from a single copulation. Adult females were then randomly selected from the surviving test population and transferred individually into clean cell wells and static-renewal exposures continued until day 21. Under the conditions described, healthy female copepods normally produced a first brood of nauplii by day 12 and a further two broods by day 21.

In summary, this experimental design allows the observation of effects in terms of naupliar, copepodid and adult survival, delays in attainment of sexual maturity and sex ratios in the F0 generation and quantification of the reproductive output in adult females (total number of nauplii from three broods).

Observations of percent mortality of copepods after 10 days exposure to the test substance were used to calculate the median effect concentration (EC50) and 95% confidence intervals. Fisher's Exact Test was used to calculate the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values based on survival after 10 and 21 days. Data on reproductive output (total number of nauplii per female) were analysed by ANOVA techniques.

The key features of the copepod life-cycle assay are summarised in Table 1.

Table 1. Experimental parameters for the life-cycle study using the marine copepod Tisbe battagliai

Experimental parameter	Comments
Dilution water	Natural seawater (salinity approx. 35‰)
Duration	Total period of 21 days.
Feeding	Mixed diet of microalgae, *Isochrysis galbana* & *Rhodomonas reticulata.*
Holding conditions	Static-renewal (3x per week), total test volume approx. 1.5 litres
Photoperiod	16 h light followed by 8 h dark
Test organisms	start with newly released nauplii (<24 hrs old); 40 per treatment continue with F0 adults; 15 per treatment
Temperature	20 ± 1°C
Test vessels	Tissue culture vessels (Cellwells™) with copepods held in individual wells (working volume 2.0 or 5.0 ml per well)
Toxicity endpoints	Survival (days 0 - 21) Development (days 0 - 11) Sex ratio (at day 11) Reproduction over three broods (days 11 - 21).

Selection of reference compounds

Based on the work of Bodar *et al* (1990), the arthropod moulting hormone, 20-hydroxyecdysone, was used as a positive control. According to the parameters outlined in Table 1, a full life-cycle study was completed for 20-hydroxyecdysone prior to further studies. Subsequently, diethylstilbestrol (DES), 17β-oestradiol, oestrone and 17α-ethynylestradiol were selected as reference oestrogens, based on either their environmental relevance or the availability of published data on the impact of this compound on other crustacean species (Baldwin *et al*, 1995). This same approach was taken for anti-oestrogen (ZM189,154) or (anti-) androgenic chemicals (namely, testosterone and flutamide).

Based on the published literature, the nominal concentrations of reference compounds used for the copepod studies were for 20-hydroxyecdysone - 8.7 to 269 µg per litre, and for all other chemicals were 0.1 - 100 µg per litre.

Copepod life-cycle test results

After 21 days exposure of to 20-hydroxyecdysone, 87 µg l^{-1} caused 100% mortality, with 8.7 - 26.9 µg l^{-1} having no impact on copepod survival. Examination of the animals at 10 days (adult stage in controls) indicated no effect on the F0 sex ratio. Reproductive output (measured as the total number of nauplii per female) was further affected by 20-hydroxyecdysone, with 21-day LOEC and NOEC values of 26.9 and 8.7 µg l^{-1}, respectively.

For diethylstilbestrol, significant inhibition of naupliar survival was observed after four days exposure at 100 µg l^{-1}. Adult males and females were paired after day 10 and exposures continued to investigate effects on reproductive output (21 days total exposure). In summary, the 21 day LC50 value (with 95% confidence intervals) for DES was 31.6 µg l^{-1} (10 - 100). Survival and reproduction were equally sensitive and both gave a NOEC value of 10 µg l^{-1} (all based on nominal concentrations). None of the other compounds showed any significant toxicity over the copepod life-cycle (Table 2).

Ecdysteroid screening assay

Briefly, the B_{II} cell line is grown in microtitre plates and cells respond to ecdysteroid agonists with reductions in cell density over 6 days at 25°C. The *in vitro* response measured is based on turbidity and the B_{II} assay can identify agonist, antagonist or cytotoxic effects of the test compound.

The results showed that neither the natural vertebrate oestrogens nor the synthetic oestrogens bind to the ecdysteroid (EcR). The same absence of EcR binding was demonstrated for the androgen agonists and antagonists, although bisphenol A, diethylhthalate and lindane were weak EcR antagonists (Dinan *et al*, in press). On-going work co-funded by NERC and AstraZeneca is extending this search for (anti-) ecdysteroidal activity for other classes of natural and synthetic chemicals.

Table 2. Summary of effects of reference chemicals on Tisbe battagliai at 20 ± 1°C

Endpoint	Nominal test concentration (micrograms per litre)							
	20-HEC	DES	E2	E3	EE2	ZM189,154	Testosterone	Flutamide
Effects on survival								
10 d LC50	≥ 269	31.6[d]	>100	>100	>100	>100	>100	>100
95% confidence intervals	(-)	(10 -100)	-	-	-	-	-	-
LOEC survival at 10 d	269[a]	100	>100	>100	>100	>100	>100	>100
NOEC survival at 10 d	86.5	10	≥ 100	≥ 100	≥ 100	≥ 100	≥ 100	≥ 100
21 d LC50	53.4[b]	31.6[d]	> 100	> 100	> 100	> 100	> 100	> 100
95% confidence intervals	(36.5 - 78.7)	(10 -100)	-	-	-	-	-	-
LOEC survival at 21 d	86.5[a]	100	>100	>100	>100	>100	>100	>100
NOEC survival at 21 d	26.9	10	≥ 100	≥ 100	≥ 100	≥ 100	≥ 100	≥ 100
Effects on reproduction								
LOEC at 21 d	26.9c	100	> 100	> 100	> 100	> 100	> 100	> 100
NOEC at 21 d	8.7	10	≥ 100	≥ 100	≥ 100	≥ 100	≥ 100	≥ 100

[a] *- Significantly different from dilution water controls (Fisher's Exact Test, p<0.05).*
[b] *- Calculated using moving average angle method.*
[c] *- Significantly different from dilution water controls (ANOVA, p<0.01).*
[d] *- Calculated using the binomial method.*

APPENDIX 2 - TABLES

Table 1. Occurrence of ovotestis in male flounder (1999-2001)

Estuary	Location	Date	Total No.	No. with ovotestis	%
Alde	N. of Orford	08/10/99	10	0	0
	N. of Orford	27/03/00	12	0	0
	N. of Orford	19/12/00	26	0	0
Crouch	Off Roach	22/10/99	4	0	0
Humber	Reads Isl.	02/11/99	11	0	0
	Off Hull	02/11/99	5	0	0
Thames	Nore Sands	05/10/99	4	0	0
	Erith Rands	06/10/99	14	1	7
Mersey	Eastham	07/09/99	6	0	0
	Eastham	01/11/00	20	2	10
	Eastham	20/02/01	7	1	14
Forth	Longannet	15/09/99	10	0	0
	Longannet	01/03/01	3	0	0
	Port Edgar	16/09/99	5	0	0
	Port Edgar	16/03/00	7	0	0
	Port Edgar	01/12/00	15	0	0
	Port Edgar	28/02/01	10	0	0
Clyde	Roseneath	28/11/00	25	0	0
	Pillar Bank	30/09/99	17	1	6
	Pillar Bank	14/03/00	11	1	9
	Bowling	29/09/99	17	0	0
	Bowling	14/03/00	5	0	0
	Bowling	27/11/00	29	0	0
Tees	North Gare	08/11/99	8	0	0
	North Gare	21/03/00	2	0	0
	North Gare	21/11/00	2	0	0
	Dabholm Gut	09/11/99	2	0	0
	Dabholm Gut	20/03/00	2	0	0
	Dabholm Gut	20/11/00	25	0	0
Tyne	St Anthonys	10/11/99	13	0	0
	St Anthonys	23/03/00	15	0	0
	St Anthonys	23/11/00	14	0	0
	St Anthonys	13/02/01	15	2	13
	Hebburn	10/11/99	14	0	0
	Hebburn	22/03/00	12	0	0
	Hebburn	23/11/00	18	1	6
	Hebburn	13/02/01	21	0	0
	Howdon	11/11/99	18	1	6
	Howdon	22/03/00	20	0	0
	Howdon	22/11/00	23	0	0
	Howdon	12/02/01	30	1	3

Table 2. Mean measurements from Pomatoschistus minutus populations - MALES. Standard deviations in brackets

Estuary/Site	Date	No. (n)	Length (mm)	Weight (g)	GSI	HSI	UGPLI
Alde							
N. of Orford	11/01/99	21	71.2 {9.4}	2.3 {0.98}	1 {0.66}	3.7 {1.81}	2.9 {0.61}
	02/03/99	53	72 {9.4}	2.4 {0.95}	1.53 {0.40}	3.3 {1.25}	2.6 {0.40}
	25/05/99	0	-	-	-	-	-
Crouch							
Off Roach	29/03/99	33	67.1 {7.8}	1.8 {0.66}	1.79 {0.53}	2.9 {1.4}	2.8 {0.33}
	26/05/99	7	55.1 {1.7}	1.1 {0.39}	1.57 {0.22}	1.97 {0.49}	3.3 {0.53}
Thames							
Nore Sands	12/10/98	20	60.2 {8.3}	1.4 {0.62}	0.09 {0.12}	2.3 {1.37}	1.8 {0.81}
	16/02/99	6	62.8 {6.4}	1.57 {0.48}	1.48 {0.36}	4.34 {2.58}	2.32 {0.21}
	01/06/99	0	-	-	-	-	-
Erith Rands	12/10/98	50	59.5 {8.2}	1.35 {0.58}	0.16 {0.16}	2.74 {1.22}	1.98 {0.44}
	16/02/99	12	70.5 {3.9}	2.2 {0.36}	0.58 {0.29}	6.1 {2.16}	3 {0.34}
	01/06/99	0	-	-	-	-	-
Gallions Reach	14/10/98	19	58.1 {6.6}	1.2 {0.39}	0.14 {0.12}	3.7 {1.23}	1.7 {0.43}
	16/02/99	0	-	-	-	-	-
	01/06/99	0	-	-	-	-	-
Tees							
Seaton Sands	11/12/98	9	52 {4.72}	0.7 {0.01}	0.34 {0.10}	3.5 {0.94}	2.5 {0.44}
	11/03/99	5	58.8 {9.63}	1.5 {0.8}	1.79 {0.40}	2.06 {0.70}	2.29 {0.78}
	15/06/99	0	-	-	-	-	-
North Gare Sands	11/12/98	0	-	-	-	-	-
	11/03/99	2	68 {4.24}	2.1 {0.84}	0.34 {0.34}	4.13 {3.15}	2.02 {0.13}
	15/06/99	0	-	-	-	-	-

* *gonadosomatic index*
** *hepatosomatic index*
*** *uro-genital papilla length index*

Table 3. Mean measurements from Pomatoschistus minutus populations - FEMALES. Standard deviations in brackets

Estuary/Site	Date	No. (n)	Length (mm)	Weight (g)	GSI*	HSI**	UGPLI***
Alde							
N. of Orford	11/01/99	12	62.7 {7.4}	1.5 {0.58}	2.53 {2.14}	3.2 {1.72}	2 {1.12}
	02/03/99	15	58.1 {9.9}	1.34 {0.69}	10.9 {6.1}	3.34 {1.39}	1.81 {0.47}
	25/05/99	0	-	-	-	-	-
Crouch							
Off Roach	29/03/99	33	60.9 {6.95}	1.51 {0.57}	11.13 {5.3}	3.45 {1.3}	1.94 {0.55}
	26/05/99	2	62 {4.24}	1.53 {0.37}	3.55 {0.41}	3.47 {0.81}	1.42 {0.38}
Thames							
Nore Sands	12/10/98	23	52.2 {8.33}	0.84 {0.41}	0.59 {0.87}	2 {1.5}	1.7 {0.77}
	16/02/99	9	58.2 {9.95}	1.47 {1.0}	11.28 {7.95}	3.87 {1.46}	1.26 {0.44}
	01/06/99	0	-	-	-	-	-
Erith Rands	12/10/98	61	57.3 {7.29}	1.12 {0.39}	0.36 {0.15}	3.44 {1.47}	1.27 {0.41}
	16/02/99	12	64.3 {7.2}	1.7 {0.50}	4.59 {2.68}	5.9 {2.51}	2.56 {0.55}
	01/06/99	0	-	-	-	-	-
Gallions Reach	14/10/98	23	54.5 {8.91}	0.93 {0.47}	0.48 {0.28}	3.4 {1.42}	1.2 {0.51}
	16/02/99	0	-	-	-	-	-
	01/06/99	0	-	-	-	-	-
Tees							
Seaton Sands	11/12/98	8	52 {9.45}	0.75 {0.43}	0.64 {0.23}	3.6 {1.06}	1.5 {0.51}
	11/03/99	4	58.8 {3.4}	1.52 {0.33}	9.79 {4.39}	3.73 {0.28}	1.92 {0.43}
	15/06/99	0	-	-	-	-	-
North Gare Sands	11/12/98	0	-	-	-	-	-
	11/03/99	5	64.6 {7.67}	2.05 {0.94}	10.95 {6.8}	4.29 {2.01}	2.24 {0.10}
	15/06/99	0	-	-	-	-	-

* *gonadosomatic index*
** *hepatosomatic index*
*** *uro-genital papilla length index*

Table 4(a). The % occurrence of MIPS in estuarine P. minutus

Estuary	Location	Date	No.	% MIPS
Alde	Nth of Orford	08/10/99	40	0
	Nth of Orford	27/03/00	27	15
	Nth of Orford	19/12/00	10	0
Crouch	Off Roach	22/10/99	24	8
S'oton Water	Netley Buoy	19/10/99	27	15
Humber	Reads Island	02/11/99	33	10
	Sunk Sand	03/11/99	28	0
The Wash	Gat Channel	13/10/99	24	4
Milford Haven	Dale	21/09/99	18	0
Thames	Erith Rands	06/10/99	40	3
	Gallions Reach	06/10/99	40	5
Forth	Longannet	01/03/01	6	33
Clyde	Roseneath	28/09/99	44	18
		28/11/00	96	5
	Bowling	29/09/99	8	50
		27/11/00	22	55
Tees	Seaton Sands	08/11/99	42	2
	North Gare Sands	08/11/99	26	46
	North Gare Sands	21/03/00	18	50
	North Gare Sands	20/11/00	22	55
	North Gare Sands	15/02/01	5	20
	Dabholm Gut	14/02/00	11	55

Table 4(b). The % occurrence of MIPS in estuarine P. lozanoi

Estuary	Location	Date	No.	% MIPS
Alde	North of Orford	08/10/99	2	0
Crouch	Off Roach	22/10/99	17	6
Tees	Seaton Sands	08/11/99	42	2
	North Gare Sands	08/11/99	16	13
	Dabholm Gut	07/02/00	3	33
	Dabholm Gut	14/03/00	33	0
Mersey	Eastham Sands	07/09/99	16	75
	Eastham Sands	20/02/01	9	11
	Egg Buoy	08/09/99	5	40

Table 5. Measurements (means) in pregnant blennies and their broods

Estuary	Location	Date	Mother					Fry				
			No.	Weight (g)	Length (cm)	CF*	Brood weight (g)	No.	Length (mm)	Weight (g)	Relative fecundity#	ESI&
Clyde	Bowling	28/11/00	4	19.9	17.8	0.34	3.3	22.5	33	0.15	1.1	15.6
	Pillar Bank	28/11/00	3	21.0	18.5	0.33	3.8	21.0	35.8	0.18	1.0	18.1
Forth	K. Hudds	11/02/99	4	31.3	23.0	0.25	8.4	22.8	47.4	0.37	0.7	27.2
	Longannet	02/11/00	1	28.3	20.7	0.32	3.7	25.0	36.5	0.15	0.9	12.9
	Port Edgar	01/12/00	19	22.9	19.1	0.29	3.3	15.5	37.9	0.20	0.7	13.7
	Port Edgar	28/02/01	8	26.3	20.8	0.29	7.3	20.5	46.5	0.36	0.8	27.8
Tyne	Howdon	23/11/00	4	87.4	29.2	0.34	20.4	62.0	43	0.33	0.7	22.9
	Howdon	10/01/01	6	54.2	25.8	0.31	18.0	31.2	50.1	0.64	0.6	29.7
	Howdon	12/02/01	1	36.2	22.9	0.30	17.4	26.0	51	0.67	0.7	48.2
Wear	Qu Alex Br	22/11/00	2	23.3	18.5	0.37	2.7	12.5	38.6	0.22	0.5	11.5

**= Condition Factor (100*weight/(length)^3); # = Relative Fecundity (fry no./somatic weight) *100; & = Embryo somatic index (weight of brood/somatic weight)*100*

Table 6. Larval sex ratios in individual broods of viviparous blenny

Estuary	Site	Date	M	F	F:M Ratio	% F	% F for site
Clyde	Bowling	27/11/00	9	10	1.1	53	58
		28/11/00	11	15	1.4	58	
		29/11/00	13	24	1.8	65	
		30/11/00	4	3	0.8	43	
	P Bank	28/11/00	6	10	1.7	63	59
		29/11/00	8	14	1.8	64	
		30/11/00	11	12	1.1	52	
Forth	K. Hudds	12/02/99	5	14	2.8	74	52
		13/02/99	8	12	1.5	60	
		14/02/99	14	9	0.6	39	
		15/02/99	18	14	0.8	44	
	Longannet	30/11/00	11	14	1.3	56	56
	P.Edgar	30/11/00	4	6	1.5	60	54
		30/11/00	12	7	0.6	37	
		30/11/00	10	7	0.7	41	
		30/11/00	9	11	1.2	55	
		30/11/00	7	9	1.3	56	
		30/11/00	9	9	1.0	50	
		30/11/00	5	8	1.6	62	
		30/11/00	12	6	0.5	33	
		30/11/00	2	5	2.5	71	
		30/11/00	4	6	1.5	60	
		30/11/00	5	11	2.2	69	
		30/11/00	15	17	1.1	53	
		30/11/00	3	5	1.7	63	
		30/11/00	4	5	1.3	56	
		30/11/00	4	6	1.5	60	
		30/11/00	24	16	0.7	40	
		30/11/00	7	9	1.3	56	
		30/11/00	1	6	6.0	86	
		30/11/00	7	11	1.6	61	
		28/02/01	21	29	1.4	58	
		28/02/01	8	9	1.1	53	
		28/02/01	3	5	1.7	63	
		28/02/01	10	7	0.7	41	
		28/02/01	10	9	0.9	47	
		28/02/01	4	10	2.5	71	
		28/02/01	7	8	1.1	53	
		28/02/01	9	15	1.7	63	
Tyne	Howdon	22/11/00	34	29	0.9	46	56
		22/11/00	28	36	1.3	56	
		22/11/00	43	48	1.1	53	
		22/11/00	11	19	1.7	63	
		09/01/01	23	26	1.1	53	
		09/01/01	17	16	0.9	48	
		09/01/01	24	40	1.7	63	
		09/01/01	14	20	1.4	59	
		09/01/01	2	4	2.0	67	
		09/01/01	10	15	1.5	60	
		12/02/01	7	19	2.7	73	
Wear	Q.A. Bridge	22/11/00	6	9	1.5	60	60
		22/11/00	4	6	1.5	60	

Table 7. Occurrence of intersex condition in male viviparous blenny

Estuary	Location	Date	Total	Intersex No.	% No.
Alde	N. of Orford	08/10/99	4	0	0
	N. of Orford	27/03/00	6	0	0
Forth	Kingstone H.	14/09/99	10	0	0
	Port Edgar	16/09/99	14	0	0
	Port Edgar	16/03/00	5	0	0
	Port Edgar	30/11/00	19	0	0
	Port Edgar	28/02/01	15	1	7
Clyde	Pillar Bank	30/09/99	12	0	0
	Pillar Bank	14/03/00	9	0	0
	Pillar Bank	28/11/00	6	0	0
	Bowling	29/09/99	16	0	0
	Bowling	14/03/00	20	1	5
	Bowling	27/11/00	13	2	15
	Bowling	27/02/01	3	0	0
Tees	North Gare	21/03/00	2	0	0
Tyne	St Anthonys	23/03/00	12	0	0
	Hebburn	10/11/99	5	0	0
	Hebburn	22/03/00	5	1	20
	Hebburn	13/02/01	2	1	50
	Howdon	11/11/99	10	1	10
	Howdon	22/03/00	13	0	0
	Howdon	23/11/00	8	1	13
	Howdon	09/01/01	11	2	18
Wear	Q. A. Bridge	22/11/00	2	0	0

Table 8. Shore crab morphometric data - Males only (All measurements in mm)

Estuary	Site	Date	No.	Carapace	Right Claw		Left Claw		Right Pleopod		Left Pleopod		Abdomen Height		Abdomen Width	
			Sampled	(mm)	Value	Ratio	Value	Ratio	Value	Ratio	Value	Ratio	Value	Ratio	Value	Ratio
Alde		08/10/99	41	55.2	16.4	0.29	12.4	0.22	12.6	0.23	12.8	0.23	20.7	0.37	19	0.34
Tees	Dabholm Gut	09/11/99	39	68.7	18.2	0.27	16.1	0.24	14	0.2	14.1	0.21	24.5	0.36	23.2	0.34
	Dabholm Gut	20/11/00	28	67	18.4	0.27	16.3	0.24	13.6	0.2	13.6	0.2	24.6	0.36	22.8	0.34
	Dabholm Gut	14/02/01	18	56	13.7	0.25	12.1	0.21	12.6	0.22	12.3	0.22	20.3	0.36	19.1	0.34
	North Gare Sands	08/11/99	40	69.8	20.3	0.29	17.1	0.24	14.5	0.21	15	0.21	25	0.36	23.5	0.34
Tyne	Howdon	11/11/99	33	66.7	18.4	0.27	16.1	0.24	14.1	0.21	14.3	0.21	24	0.36	23.3	0.35
	Howdon	22/11/00	23	65.2	17.3	0.26	15.6	0.24	13.3	0.21	13.8	0.21	23.7	0.36	22.3	0.34
	St Anthonys	10/11/99	36	57.1	13.5	0.24	12.3	0.22	11.7	0.21	12	0.21	20.9	0.37	19.6	0.34
Forth	Longannet	15/09/99	35	68.6	20.2	0.31	17.4	0.27	14.8	0.23	14.9	0.23	25.6	0.4	23.9	0.37
	Port Edgar	16/09/99	22	65.8	18.6	0.28	16	0.24	14.2	0.21	13.7	0.21	25	0.38	23.2	0.35
Clyde	Bowling	29/09/99	57	56.7	14.5	0.25	12.6	0.22	11.9	0.21	12	0.21	20.8	0.37	19.5	0.34
	Bowling	27/11/00	55	55.6	13.5	0.24	12	0.22	12.1	0.22	12.2	0.22	20.6	0.37	26.6	0.35
	Pillar Bank	30/09/99	37	58.7	16.7	0.28	13.3	0.22	12.6	0.21	12.6	0.21	22.1	0.38	20.4	0.35
	Roseneath	28/09/99	15	51.5	14.3	0.27	10.6	0.21	12.8	0.25	13	0.26	20.4	0.4	18.3	0.36
Mersey	Eastham	07/09/99	34	50.4	12.8	0.26	11.5	0.23	11.5	0.23	11.4	0.23	18.5	0.37	17.5	0.35
	Egg Buoy	08/09/99	4	45.5	12.7	0.27	10	0.22	10.4	0.23	10.9	0.24	17.3	0.38	15.8	0.35
Humber	Sunk Sand	03/11/99	25	59.8	16.1	0.27	13.7	0.23	13.1	0.22	13.1	0.22	22	0.37	20.4	0.34
Milford Haven	Shelf	22/09/99	10	44.7	10.4	0.23	9.1	0.2	10	0.22	9.9	0.22	17.2	0.38	15.7	0.35
	Dale	21/09/99	14	49	11.1	0.23	9.9	0.2	11.7	0.24	12	0.25	19.5	0.4	17.4	0.35
S'oton Water	Test	20/10/99	3	48.6	13.9	0.29	10.5	0.22	11.2	0.23	11.3	0.24	18.2	0.38	17.3	0.36
	Netley	19/10/99	13	49	11.4	0.23	11.5	0.23	10.6	0.22	11.2	0.23	17.9	0.36	17.3	0.35
	Fawley	20/10/99	38	48.4	14.6	0.3	11.7	0.24	10.6	0.22	10.4	0.22	17.8	0.37	16.7	0.34
Thames	Nore Sands	05/10/99	24	52.8	13.8	0.26	12	0.23	11.6	0.22	11.7	0.22	19.2	0.36	18.2	0.34
Wash	Nene	13/10/99	47	55.2	13.7	0.25	12.3	0.22	12.4	0.23	12	0.22	20.4	0.37	19.2	0.35
	Gat Channel	13/10/99	26	58.6	14.4	0.25	13.3	0.23	13.3	0.23	13.2	0.22	21.6	0.37	20.3	0.35
	Sunk Sand	12/10/99	32	49.3	11.5	0.23	11.4	0.22	11.3	0.23	11.3	0.23	18.3	0.37	17.3	0.35

Table 9. In vitro androgenic activity of estuarine surface waters, sediment pore waters and sediment extracts

Estuary	Location	Date Collected	Latitude	Longitude	*In vitro* androgenic activity		
					Surface waters (ng DHT kg^{-1})	Sediment porewater (ng DHT l^{-1})	Sediment particulates (ng DHT kg^{-1})
Tees	D/S Dabholm Gut	9 July 2000	54 36.999 N	01 09.072 W	8	129	1020
	Dabholm Gut	9 July 2000	54 36.270 N	01 09.401 W	<2	Toxic	7590
	U/S Dabholm Gut	9 July 2000	54 36.069 N	01 09.955 W	<2	53	<454
	Port Authority	9 July 2000	54 35.550 N	01 10.869 W	4	<45	<454
	Mid Channel Buoy	9 July 2000	54 34.945 N	01 12.571 W	3	51	5850
	Transporter Br.	9 July 2000	54 35.136 N	01 13.864 W	4	<45	<454
Tyne	Team Confluence	10 July 2000	54 57.481 N	01 38.216 W	3	138	<454
	Tyne Bridge	10 July 2000	54 58.248 N	01 35.632 W	<2	<45	<454
	Hebburn	10 July 2000	54 58.986 N	01 31.935 W	<2	<45	<454
	Howdon STW	10 July 2000	54 59.263 N	01 27.726 W	3	<45	<454
	D/S Howdon STW	10 July 2000	54 59.782 N	01 26.568 W	<2	<45	<454
	Lloyds Hayling	10 July 2000	55 00.577 N	01 25.763 W	<2	<45	<454
Clyde	M8 Road Bridge	11 July 2000	55 51.344 N	04 16.283 W	9	<45	<454
	U/S Shieldhall STW	11 July 2000	55.51610 N	04 17.923 W	<2	<45	<454
	D/S Shieldhall STW	11 July 2000	55 52.172 N	04 20.634 W	<2	<45	<454
	Cart Confluence	11 July 2000	55 52.762 N	04 24.574 W	<2	<45	<454
	D/S Dalmuir STW	11 July 2000	55 53.785 N	04 24.799 W	<2	187	15300
	Erskine Bridge	11 July 2000	55 55.121 N	04 27.688 W	<2	<45	3360
Forth	Grangemouth	12 July 2000	56 02.179 N	03 40.928 W	3	<45	4180
	D/S Kibagie paper mill	12 July 2000	56 04.238 N	03 43.912 W	<2	<45	1990
	Alloa STW	12 July 2000	56 06.240 N	03 47.634 W	<2	<45	2190
	U/S Alloa STW	12 July 2000	56 06.592 N	03 48.590 W	<2	<45	1024
	Forth Bridge	12 July 2000	55 59.874 N	03 24.335 W	<2	115	1748
Thames	Gravesend	11 August 2000	51 26.584 N	00 23.737 W	<2	143	<454
	Greenhythe	11 August 2000	51 27.340 N	00 16.463 W	<2	86	<454
	Erith	11 August 2000	51 28.940 N	00 11.034 W	3	<45	<454
	Thamesmead	11 August 2000	51 30.025 N	00 09.865 W	8	NS	NS
	Thamesbarrier	11 August 2000	51 30.028 N	00 04.703 W	5	<45	NS
	Cutty Sark	11 August 2000	51 28.960 N	00 00.873 W	<2	NS	<454
South-ampton Water	Fawley A	12 August 2000	50 49.872 N	01 19.555 W	<2	<45	<454
	Fawley B	12 August 2000	50 50.420 N	01 19.619 W	<2	<45	<454
	D/S Hythe STW	12 August 2000	50 52.061 N	01 23.056 W	<2	<45	<454
	Docks (W)	12 August 2000	50 54.318 N	01 26.706 W	<2	<45	<454
	Docks (E)	12 August 2000	50 53.783 N	01 24.728 W	<2	<45	<454
	D/S Woolstone STW	12 August 2000	50 53.524 N	01 23.252 W	<2	<45	<454
Mersey	Opp. Port Radar Stn.	25 July 2000	53 27.800 N	03 02.800 W	<2	<45	<454
	Opp. Canada Dock	25 July 2000	53 26.180N	03 00.700 W	<2	<45	<454
	Seacombe Ferry Terminal	25 July 2000	53 24.930 N	03 00.800 W	<2	<45	<454
	Tranmere Oil Terminal	25 July 2000	53 22.720 N	02 59.350 W	<2	<45	<454
	Bromborough	25 July 2000	53 20.100 N	02 57.200 W	<2	<45	<454

Table 10. In vitro androgenic activity of effluents

Effluent	Date collected	Consented discharge volume (m^3 day^{-1})	Level of treatment at time of collection	*In vitro* androgenic activity (ng DHT l^{-1})	*In vitro* androgenic activity (g DHT day^{-1})
Irvine Valley Sewer	18 July 2000	570,240	Primary	66	37.6
Irvine Valley Sewer	19 July 2000	570,240	Primary	598	341
Irvine Valley Sewer	25 July 2000	570,240	Primary	219	125
Irvine Valley Sewer	26 July 2000	570,240	Primary	283	161
Irvine Valley Sewer	02 August 2000	570,240	Primary	34	19.4
Paisley STW	20 July 2000	79,998	Trickling filter	<23	<2
Sheildhall STW	18 July 2000	273,024	Activated sludge	<23	<5
Sheildhall STW	19 July 2000	273,024	Activated sludge	<23	<5
Sheildhall STW	25 July 2000	273,024	Activated sludge	<23	<5
Sheildhall STW	26 July 2000	273,024	Activated sludge	<23	<5
Sheildhall STW	02 August 2000	273,024	Activated sludge	<23	<5
Dalmuir STW	20 July 2000	213,940	Trickling filter	305	65.3
Howdon STW	05 September 2000	230,000	Primary†	330	75.9
Howdon STW	12 September 2000	230,000	Primary†	369	84.9
Howdon STW	19 September 2000	230,000	Primary†	191	43.9
Howdon STW	26 September 2000	230,000	Primary†	198	45.5
Howdon STW	02 October 2000	230,000	Primary†	570	131
Howdon STW	12 October 2000	230,000	Primary†	635	146

† *Secondary treatment now installed.*

Table 11. Plasma VTG concentrations in male and female flounder caged for two weeks at several sites on the Tyne and Tees estuaries

Site	VTG levels µg ml^{-1} Male	VTG levels µg ml^{-1} Female
Tyne Estuary		
Control Day 0	0.037	0.034
Control Day 14	0.036	0.040
Downstream Howdon	0.120	0.030
Howdon	0.020	0.026
Upstream Howdon	0.027	0.020
Tees Estuary		
Control Day 0	0.015	0.010
Control day 14	0.019	0.024
Downstream Dabholm Gut	0.022	0.054
Dabholm Gut	0.016	0.016
Upstream Dabholm Gut	0.042	0.016

Table 12. Concentrations of contaminants in mussel tissue from animals caged on the Tees and Crouch estuaries

Sampling Location/Time	Total PAH µg/kg wet wt.	Sum 25 PCBs ppm wet wt.	Bisphenol -A	DBT ppm	TBT ppm	NP ng g^{-1} wet wt.
Tees week 1	151.6	0.0011	<0.00002	0.023	0.098	ND
Tees week 2	735.9	0.0032	<0.00002	0.028	0.236	ND
Tees week 3	557.4	0.0052	0.11	0.03	0.314	ND
Tees week 4	979.1	0.001	<0.00002	0.029	0.21	ND
Tees week 5	878.5	0.0011	0.12	0.029	0.211	ND
Crouch week 5	42.1	0.0015	<0.00002	0.012	0.028	ND
Crouch 1 year	74.5					<MDL
Farmed mussels	156.25					16.9

APPENDIX 3 - CRAB/SHRIMP ELISA DEVELOPMENT

A similar approach to that used in the fish studies was adopted to generate an ELISA sensitive to shore crab and brown shrimp vitellogenin.

The source material for the antigen (VTG) was mature oocytes taken directly from the ovaries of crabs and shrimps. Following homogenisation of the oocytes in buffer solution and subsequent centrifugation, vitellin was separated from the remaining proteins in the supernatant using a combination of column chromatography and centrifugation, thus producing a pure antigen. The antigen was dispatched to AstraZeneca who raised the polyclonal antibodies for both shore crab and shrimp VTG in rabbits, following their inoculation with a sample of the antigen.

The specificity of the polyclonal antibody was tested using the western blotting technique. Briefly, this involves electrophoresis of crustacean tissue samples to generate an array of polypeptide units within a gel, which are associated with a variety of proteins. Which of these polypeptide units cross-react with the antibody is then determined. If cross-reactivity is observed between polypeptides not known to be associated with VTG then the antibody will be non-specific and therefore unsuitable. If, however, the antibody is seen to cross-react only with those regions known to originate from VTG then it can be used successfully within an ELISA. The major steps are illustrated in Figure 1.

Typically, two identical gels are run (see top left gel). The first is stained with coomassie blue to visualise the protein bands and the invisible but identical bands of the second gel are transferred by electrophoresis onto the surface of a nitrocellulose membrane. This transfer takes the proteins out of the matrix of the gel and sits them on the surface of the membrane where they offer a much larger surface area for the antibody to potentially interact with. The membrane is then incubated with a dilute solution of the antibody. If suitable antigens are on the surface of the membrane the antibodies will bind to them. Since there is no obvious visual indication that cross-reactivity has taken place, a second more general-acting antibody is used which recognises the first. This second antibody has an enzyme conjugated to it which reacts with a reagent to produce a coloured product visible to the eye. In the absence of an initial cross-reaction between the protein and the primary antibody the secondary antibody will not appear, thus only those regions where cross-reactivity with the primary antibody has taken place will produce a coloured product. The stained gel is then compared with the Western blot to determine which proteins or polypeptides have cross-reacted and, just as importantly, those that have not. Figure 1 shows that on the final gel only the polypeptides associated with vitellin have cross-reacted with the primary antibody thus demonstrating the specificity of the primary antibody and its suitability for use in the ELISA.

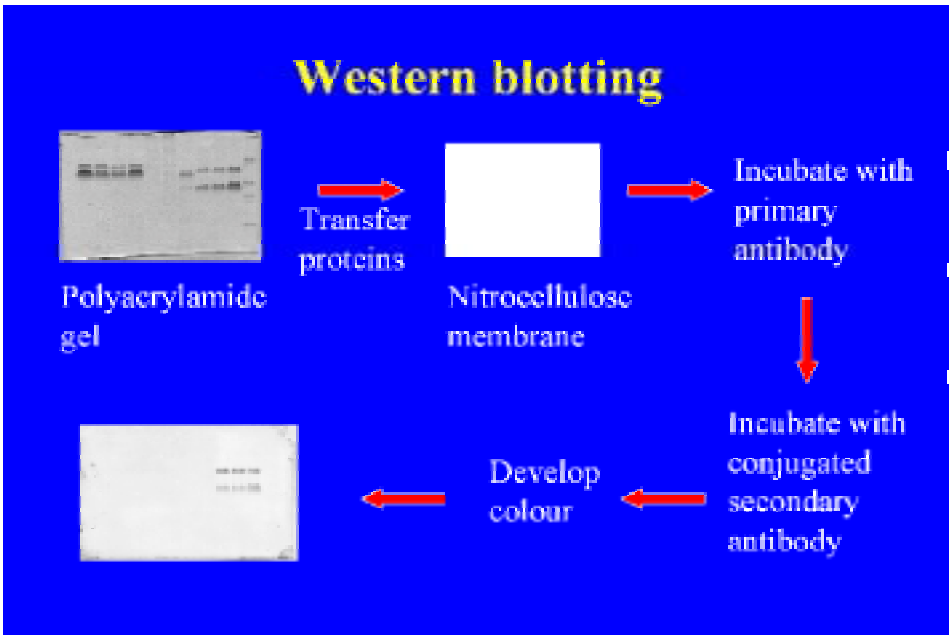


Figure 1. Procedural steps in the Western Blotting technique for assessing the specificity of invertebrate polyclonal antibodies

The basic principles of the ELISA are similar to that described for Western blotting with a few important differences:

(1) ELISAs offer much greater sensitivity
(2) Tests are carried out in 96 well plates allowing large numbers of samples to be tested simultaneously.
(3) Test plates are read accurately and rapidly in an automated plate reader.
(4) Dedicated software is used to analyse the data.

The major difference between Western blotting and ELISAs is in the way the protein sample is presented. In Western blotting the various components of a sample are separated on a gel prior to exposure to antibody. Using ELISAs the entire sample is placed into the well in a heterogeneous solution. In order to have confidence that any positive response shown in an ELISA test is due entirely to the protein or antigen of interest there must be confidence that the antibody is specific to this particular protein. This is why Western blotting is an essential preliminary step in the development of ELISAs, providing information on antibody specificity.

The basic methodology used for the ELISA is illustrated in Figure 2. Haemolymph was the source material for all vitellogenin ELISA assessments undertaken on shore crabs. The small size of shrimps meant that obtaining non-destructive (or even destructive) haemolymph samples was difficult. To overcome this a standardised protocol was developed which allowed body fluids, chiefly extra-cellular fluids including haemolymph, to passively exude from individual animals into a medium of coating buffer, prior to further dilution and loading into the plate well. Preliminary tests showed that there was little cross reactivity between the polyclonal antibody raised against crab VTG and shrimp VTG and vice versa. This necessitated the production of separate antibodies for each of the two species.

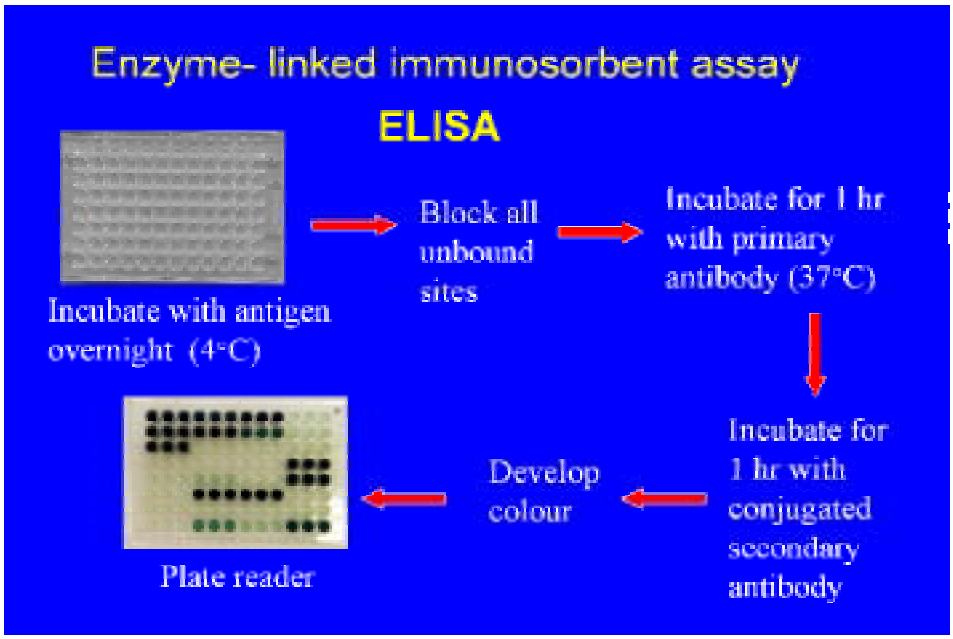


Figure 2. Procedural steps for an enzyme linked immunosorbent assay (ELISA), as used for detection of shore crab and shrimp vitellogenin

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