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A14–FUNCTIONAL GENOMICS IN FISH: FROM GENES TO AQUACULTURE

Organised by Patrick Prunet (INRA, Rennes, France) and Tom Pottinger (NERC, Lancaster)

A14.1 Stressgenes project: A functional genomic approach to measuring stress in fish aquaculture

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The overall aim of this study is to identify in fish candidate genes associated with resistance to stress conditions and thus provide the physiological and genetic basis for new marker-assisted selection strategies. The recent development of genomic tools, particularly microarray technology, allows systematic gene expression analysis of biological material and provides an integrated overview of the global response at the level of gene expression. Such information is of major importance for identifying genes responsible for genetic variation in response to stress and for further development of a sufficient number of molecular markers, a critical requirement for marker-assisted selection schemes. The Stressgenes project proposes to analyse several stress situations (confinement, salinity, pathogen exposure, hypoxia, temperature) and characterise stress-responsive genes as potential candidate gene markers. The scientific strategy employed in this project involved the isolation of genes that are differentially expressed in a stress specific manner in each of several functionally relevant target tissues. This has been achieved by the construction of several SSH (Suppression Subtractive Hybridisation) cDNA libraries enriched for stress-specific, differentially expressed genes and containing altogether about 30 000

clones. These SSH clones have been spotted on arrays and using a simple protocol, we have been able to select among this cDNA collection those EST which relevant to the different stress responses analysed in the project. These selected EST have been sequenced and all spotted on a Stressgenes microarray which will be further used to characterised gene expression profiles during the biological responses to stress.

A14.2 The use of cDNA microarray to profile gene expression patterns in zebrafish

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Due to some of its unique advantages for genetic studies (external development and optical clarity during embryogenesis allowing for visual analysis of early developmental processes, and high fecundity and short generation time facilitating genetic analyses), zebrafish (*Danio rerio*) has been chosen as a model system for the study of vertebrate development.

Our lab has obtained ~15 000 unique cDNA clusters via our EST sequencing project. Meanwhile, we have setup microarray facility with optimized methods and conditions and have since generated zebrafish cDNA microarrays using these unique clusters. For our own interest, we have used these microarrays for 1) profiling gene expression patterns during early embryogenesis; 2) identifying male or female/germinal enriched genes; 3) identifying liver enriched genes and some of these genes will be selected for functional study via gene knock-down methods and 4) comparing gene expression patterns between mutants versus the wild type control to identify downstream genes.

The main goal of this lab is to identify genes involved in liver initiation and development in zebrafish. We have also initiated a moderate scale of screening for liver defect mutants and 22 putative mutants have been isolated. We will clone some of these mutant genes via positional cloning method in hope to reveal how these genes are involved in controlling liver development. The combination of the established genetic and genomic

approaches will no doubt help our study to understand more about the molecular mechanism of the liver development in zebrafish. Since the fundamental molecular mechanisms of development are shared among vertebrates, the analysis of zebrafish mutants is likely to provide new information generally relevant to other classes of vertebrates.

Lo, J., Lee, SC., Xu, M., Liu, F., Ruan, H., Eun, A., He, YW., Ma, WP., Wang, WF., Wen, ZL., and Peng, J.R. (2003) 15 000 Unique Zebrafish EST clusters and their future use in microarray for profiling gene expression patterns during embryogenesis. *Genome Research* 13:455–466.

A14.3 Global definition of transcriptome regulation in the common carp during cold acclimation

A.Y. Gracey, J. Fraser, M.A. Hughes, G. Govan, W. Li, A.R. Cossins (Liverpool), Y. Fang, A. Brass (Manchester), A.P. West and J. Rogers (Sanger Institute)

Temperature fluctuations present major challenge to poikilothermic animals living in variable habitats, particularly in temperate climes. Many display powerful adaptive responses that mitigate damaging effects of temperature change and compensate for disturbances to rate processes. To identify the underpinning mechanisms we have implemented a transcript screen of genes in the common carp undergoing regulation following cooling using cDNA microarray technology. We have fabricated a 15 500 probe microarray from clones isolated from full length, directional cDNA libraries prepared from animals subjected to a range of environmental stressors. For the cooling experiment fish were cooled from 30°C down to 23, 17 and 10°C where they were held. Replicate fish were sampled, tissues excised and RNA isolated. We used ~450 arrays to compare the main tissues. Statistical procedures have determined a list of ~250 genes that are regulated in all tissues, the so-called 'common response'. This largely comprises genes involved in cellular homeostasis, turnover and catabolism, transcriptional regulation etc but includes the $\Delta 9$ -desaturase and the cold-inducible RNA binding protein. We have also defined a further 1700 genes in 23 expression clusters with tissue-specific cold-regulated expression. Gene Ontology annotations provides a functional overview of tissue-specific responses and indicates their biological significance. This identifies metabolic and remodelling transformations that contribute to the adapted phenotype with a greatly expanded list of candidate genes for further analysis. (NERC funded, cossins@liv.ac.uk).

A.14.4 The rainbow trout liver proteome: Unravelling metabolic changes

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In aquaculture, the comparatively high protein requirement of salmonid fish is met with fishmeal-based feed. The sustainability of this practice is questioned. In the present study, the feasibility of substituting fishmeal with plant-based products is investigated. Liver protein extracts were subjected to 2 dimensional electrophoresis (2DE), allowing several hundred proteins to be monitored in parallel, yielding a global picture of changes in protein profile under different metabolic states. A rainbow trout liver proteome map developed in our laboratory records proteins in terms of identity, molecular weight, isoelectric point (pI) and abundance. Protein spots that are found to be of interest, either by virtue of their response to dietary manipulation or via immunodetection methods, are subjected to peptide mass fingerprinting followed by searching of public databases, making use of the increasing availability of salmonid ESTs. We have identified a number of metabolic pathways that are sensitive to plant protein substitution in rainbow trout feed. These include pathways involved in cellular protein degradation, fatty acid breakdown, and stress response, likely linked to presence of antinutritional factors. Our results offer a more detailed and comprehensive overview of the metabolic response of rainbow trout than can be achieved by focusing on one or a few pathways. We therefore conclude that with our development of a liver proteome map, 2DE has now become a valuable diagnostic tool for assaying metabolic responses of rainbow trout to dietary or environmental stimuli.

A14.5 Multi-tissue, multi-stress transcriptional responses in the common carp

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Hypoxia is an important environmental stressor for many fish species, particularly in freshwater where it interacts with thermal stress. The cyprinid fish in general and the common carp (*Cyprinus carpio*) in particular are resilient to hypoxia and some species can withstand anoxia for long periods of time. We have explored large-scale responses to hypoxia in the common carp, and interactions between temperature and hypoxia, using transcript screening techniques. Fish were subjected at either 17°C or 30°C to progressive reduction in PO₂ down to 0.8mg O₂/l where they were held for 8 days.

Replicate fish were removed on days 0, 1, 3, 5 and 8 and tissues rapidly dissected and stored for subsequent extraction of RNA. In reference-based dual label experiments, RNA was hybridised to >600 carp cDNA microarrays comprising 15 500 separate carp probes which were obtained from a series of cDNA libraries enriched for hypoxia- and thermally-responsive genes. The resulting large-scale data set, including >5 fold biological replication, has been analysed using the *GeneSpring/GeNET* package and interpreted using the annotated gene lists provided by the *EST-Ferret*. We have defined extensive lists of genes from the 7 different tissues that respond to hypoxia, and whose hypoxia response is affected by temperature of exposure. These lists have been assessed using advanced statistical techniques to identify patterns in expression that relate to underpinning biological responses to single and combined stressors. (NERC funded, cossins@liv.ac.uk)

A14.6 Gene Expression Profiling of the Zebrafish During Development

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Vertebrate embryogenesis is a complex process controlled by a transcriptional hierarchy that coordinates the action of thousands of genes. To identify and analyze the expression patterns of these genes, we constructed a cDNA microarray containing >4500 unique genes identified from the embryonic hearts, adult hearts and skeletal muscles of zebrafish. We examined the patterns of gene expression during development in the zebrafish among five time points relative to 12h post-fertilization (hpf). Differentially expressed genes can be grouped into two categories, early genes that are expressed at 5hpf and genes expressed at 48/72/120 hpf. Furthermore, we report the utilization of cDNA microarray technology to investigate the adaptive molecular responses of zebrafish to hypoxia during development. We tested the hypotheses that hypoxia changes the gene expression profile of zebrafish embryos and that these changes can be reversed by re-exposure to a normoxic (20.8% O₂) environment. Our data were consistent with both of these hypotheses, indicating that zebrafish embryos undergo adaptive alterations in gene expression in response to hypoxia. Our study reveals dynamic changes in levels of gene expression during development and provides a striking genetic portrait of the zebrafish embryos' adaptive responses to hypoxic stress. In addition, this study demonstrates the utility of microarray technology as a tool for analyzing complex developmental processes in the zebrafish.

A.14.7 The INRA AGENAE program and the Agenes trout EST collections: first results applied to fish physiology research

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The AGENAE (Analysis of Animal Genomes) program is a vast project on animal genomics funded mainly by the French 'Institut National de la Recherche Agronomique' (INRA). One of its main objective is the understanding of physiological function regulations in different species of economic importance including one fish species, the rainbow trout, *Oncorhynchus mykiss*. To that goal we constructed specific high quality cDNA libraries and initiated a high throughput ESTs sequencing project on these libraries. Right now more than 100 000 sequences have been performed by this program (5' or 5' and 3' sequences on more than 80 000 clones) and most of this information has been already released in international databanks (EMBL, GenBank). This information currently corresponds to 70% of the overall knowledge (140 000 sequences) in this species and brings rainbow trout as one of the major fish model in term of ESTs sequence just after Zebrafish, *Danio rerio*. Using these sequenced transcript collections we initiated micro-array expression studies on different fish physiological aspects i.e., reproduction including gametogenesis, gamete quality, sex differentiation, growth and nutrition, stress and welfare. We also initiated some methodological approaches in order to validate rainbow trout micro-arrays as a heterologous tool for other species like for instance the european seabass, *Dicentrarchus labrax*. I will review some of the first results concerning these functional genomics programs. [this work has been substantially supported by an INRA-CIPA-OFIMER/EU research grant].

A14.8 Evolution of cold-responsive desaturase genes in cyprinid fish

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The Δ^9 -acyl CoA desaturase is a key environmentally-regulated gene directing the production of increased proportions of unsaturated fatty acids in the cold. It is transcriptionally up-regulated by cold in a wide range of organisms, from bacteria and plants to poikilothermic animals, including the common carp, *Cyprinus carpio*.

The carp has recently been shown to possess a second hepatic desaturase but which responds transcriptionally to dietary treatment and not by cold. This situation contrasts with the closely related grass carp, *Ctenopharyngodon idella*, which appears to possess only one desaturase homologue. We have tested the hypothesis that the two carp isoforms arose by genomic duplication and promoter divergence from an unduplicated ancestor similar to the single desaturase of grass carp. Sequence comparisons of des cDNAs from these carp and other vertebrates indicates that the two common carp genes are more closely related to each other than either are to grass carp. All three have identical synteny in the genomic sequence, as established by sequencing 30Kb fosmid clones, consistent with a proposed recent genomic duplication. Phylogenetic footprinting has indicated conserved and divergent non-coding regions that might account for the different regulatory characteristics of the two carp genes. All three carp genes shown coding sequence similarity to a fugu isoform, but quite dissimilar to a second fugu isoform. We thus suggest that desaturase evolve through an early duplication event, and in common carp with a more recent duplication that gave rise to isoforms with divergent regulatory characteristics. These characteristics are likely to be combined in the single grass carp desaturase promoter. (NERC-funded, cossins@liv.ac.uk)

A14.9 Transcriptional response of rainbow trout head kidney to confinement

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The work described here is part of the EU Stressgenes program. The aim of the study presented here was to improve our knowledge on pathways developed in trout to adapt to confined conditions. mRNA from head kidney samples from control and confined fish (3 fish per time and treatment, sampled at early (2h), middle (48h) and late stages (21days)) have been used to perform SSH-MOS libraries in both forward and reverse directions. 1248 sequenced PCR products (29% redundancy) from those SSH were spotted on macro- and microarrays. Hybridisation was performed with complexes RNA probes prepared from three stressed and three control fish for each sampling time. Cluster analysis using the 3 stages (2, 48h and 21d) revealed several clusters with differentially expressed clones. For example, one of them obtained in macro- and micro-arrays hybridisation

containing 89 and 72 clones respectively. Besides, 56 clones are common between these 2 techniques. Many of cluster sequences are not represented in available databases. To increment the Stressgenes collection we selected 309 differentially expressed clones (60% unidentified). This selection was performed using change fold method and statistical methods (Clustering, SAM). Most of the clones selected by statistical methods were already selected using change fold method. The implication in confinement of genes selected in our experiments and by other partners of the Stressgenes project would be analysed and validated in future experiments using the common Stressgenes array.

A.14.10 The Dicer gene in fish: Comparative genomics and expression studies

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Dicer is a member of the RNase III family involved in post-transcriptional gene silencing (PTGS). It hydrolyses long double stranded (ds)-RNA molecules into 21–23 bp short interfering (si)-RNA that direct the specific degradation of homologous transcripts resulting in a drastic down regulation. We present here the full amino acid sequence of the Dicer gene in zebrafish and fugu. The genomic organisation of the Dicer gene in Human, Mouse, Fugu and Zebrafish was determined and compared. The Fugu and Zebrafish Dicer amino acid sequence is about 82% and 85% similar to mammals, respectively suggesting that strong selection pressure have maintained the structure of Dicer. A partial sequence for the rainbow trout Dicer has also been isolated. The expression of this gene have been studied in vivo in zebrafish and in vitro in zebrafish (SJD.1) and rainbow trout (RTG-2) cells.

A14.11 Gene expression profiling for performance traits in Atlantic salmon

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Many physiological traits that are of commercial importance to aquaculture are under genetic control. As part of a larger project, *Salmon Traits*, we are studying two major physiological processes in salmon, immunological responses and mechanism of protein metabolism. Challenges on salmon parr have been designed to stimulate the immune system by *intra peritoneal* injection with

Aeromonas salmonicida for 24 and 48 hours. This route of infection is known to produce a broad based antibacterial response. The second challenge has been to alter rates of protein turnover by short term food withdrawal, which will change the balance between protein synthesis and protein degradation and the related mRNAs. We have used suppressive subtractive hybridizations to enrich for differentially expressed genes. Enriched cDNA libraries have been constructed from head kidney and gill for the disease challenge and from liver and muscle for protein metabolism. Characterisation of these enriched libraries showed inserts varied from 100 bp to > 1.5 Kbp. Approximately 500 clones will be sequenced from each library. Once analysed these will be used to enhance a cDNA microarray that will be constructed to include genes involved in a variety of other important physiological processes. Microarray analysis of a salmonid cDNA chip from the Genomics Research in Atlantic Salmon Program (GRASP), Canada, is also being used during this study for future comparison with the salmon traits chip.

This work is funded by BBSRC Grant EGA17675.

A.14.12 Effects of confinement stress on gene expression in selected tissues of rainbow trout: evaluation by SSH and by microarray analysis

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This study comprises one element of a larger EU project (STRESSGENES), that has investigated effects of a range of stressors on fish (temperature, salinity, hypoxia, pathogen exposure and confinement) with the aim of constructing a multi-tissue, multi-stressor trout microarray. The work described here was carried out to identify the range of genes directly altered during exposure to a prolonged confinement stressor. Control and stressed fish were sampled at intervals over a 4-week period and at each time point tissues were removed from 12 fish. Plasma ACTH, cortisol, glucose and lactate were measured and RNA was isolated from pituitary, brain and liver. In order to generate a microarray of genes associated with this stressor, an initial enrichment of differentially regulated genes was achieved by Suppression Subtractive Hybridisation (SSH). Two different time periods were considered: 'early' (2h–48h) for an acute response and 'late' (96h–356h) for a more prolonged/adaptive response. A representative number of clones from each library were PCR-amplified, printed onto slides, and screened with appropriate targets. Although SSH enriches for differentially expressed genes, a high

level of redundancy in the libraries was still to be expected. The goal at this stage therefore was simply to carry out an initial screen to allow for a truer selection of differentially expressed clones. We will present some details both on the characterisation of our SSH libraries and on our initial microarray results.

A14.13 Using transcriptomics to profile dominance-subordinate relationships in rainbow trout

L.U. Sneddon, J. Margareto & A.R. Cossins (University of Liverpool)

Dominance hierarchies are prevalent in animals living in groups and rank or position within a hierarchy influences the access to resources such as food, shelter and mates. Therefore, position within a hierarchy influences the probability of survival and overall fitness. In aquaculture situations, aggression can be problematic with one or a few dominant animals obtaining the majority of food and showing much higher growth rates than animals lower in the pecking order and, therefore, they have limited access to food, show poor growth and suffer the detrimental effects of stress. At present there is no information on the gene expression differences governing position within a dominance hierarchy, therefore, it was the purpose of the present study to investigate the molecular differences between rank members in the dominance hierarchies of rainbow trout (*Oncorhynchus mykiss*). This study aimed to understand the correlates of dominance position by using microarray technology to detect gene expression differences between dominants, subdominants and subordinates. The results for brain gene expression were assessed and approximately 300 genes were found to be differentially expressed between the ranks. Understanding the gene expression differences between animals of different status may lead to understanding the causes of this behaviour in the future and potentially identifying ways of controlling it. The trout library was made available via collaboration with the EC Stressgenes programme.

A14.14 Effects of stress and dietary supplement of L-tryptophan on gene expression in the brain of rainbow trout

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Isolated juvenile rainbow trout were fed a feed supplemented with L-tryptophan (TRP) for 7 days, after which they were either sampled directly (undisturbed controls) or subjected to a standardised 2, 6 or 10 hour confinement stress prior to sampling. Another group were treat-

ed in the same way (undisturbed controls and confinement stressed) and received the same feed but without any supplementary TRP. Stress resulted in a significant elevation of plasma [cortisol] in fish fed normal feed. However, fish fed TRP-supplemented feed for 7 days showed significant lower levels of plasma [cortisol] in response to confinement stress. Following blood sampling the brains were rapidly removed and put in Trizol for total RNA extraction. The purified total RNA were then used in microarray experiments for studies and comparison of gene expression patterns in brains of confinement stressed fish with or without TRP-supplemented feed. The experiments were conducted on a collection (within the stressgenes project) of rainbow trout brain SSH cDNA libraries from fish that had been subjected to confinement stress. This collection of cDNA clones were spotted on glass for microarray hybridisation. The experiment was designed to compare all different treatments i.e. undisturbed controls and 2, 6, 10 hour confinement stressed fish with or without TRP-supplemented feed, with a common reference. This allowed us to compare expression patterns in brains from undisturbed fish with brains from confinement stressed fish and also brains from fish with or without TRP-supplemented feed.

A14.15 Salinity stress and gene expression profiling in rainbow trout

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Total transcript expression of rainbow trout exposed to salinity stress was studied using macroarray analysis. Salinity represents a stress for organisms and generates ions imbalance and water deficit. The aim of this study is to identify differentially expressed genes at the early, middle and late stages of stress after transfer of fish from fresh water to sea water. A three time points experiment (at 8h, 72h and 21 days) was performed with four biological replicates for each time point on fish directly transferred into sea water. Using these material, SSH cDNA libraries were generated in both forward and reverse directions for gills, brain, liver and intestine. Clones from these libraries were spotted on nylon membrane macroarrays and further and hybridised with complexes RNA probes. A preliminary analysis of these data indicated that among the 6624 clones analysed, 942 could be selected for showing over-expression (ration between stress and control expression above 1.5) or under-expressed (ratio between stress and control expression bellow 0.6). Among these clones, 349 clones

selected from gills and brain were already sequenced and annotated. The analysis of sequences showed 9% of redundancy for brain and 21% for gills. Up today 70% of sequences are not represented in available databases. This analysis indicated a first list of potentially interesting candidate genes involved in the salinity stress responses in osmoregulatory organs and in brain. Cluster analysis of these data will be carried out in order to confirm the major molecular pathway mobilised after salinity stress exposure.

A14.16 Genetic and phenotypic transcriptome variation-A comparison of isogenic and outbred carp

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Environmental stress induces substantial but characteristic changes to the transcriptome, both as a direct response to altered conditions but also as part of adaptive processes. There is substantial variation between individuals, and possibly between population, in transcript responses to environmental perturbation though the origins and functional significance of these variations are not understood. We have sought to define the genetic basis both of variations transcript expression and in responses of individuals to environmental challenge by comparing the properties of an outbred carp population with an isogenic line prepared by reproductive manipulation. We compared fish liver RNA isolated from replicate animals using the Liverpool carp microarray consisting of 15 500 cDNA probes, followed by statistical analysis on the *GeneSpring/GeNET* package. The results demonstrate the powerful phenotyping capability of the microarray technique since clustering of the data enabled the exact stock and date of sampling of animals to be distinguished from the global profile. We have assessed the level of variation in transcript levels and in the effects of environmental perturbations on transcript abundance, comparing individuals from within and between the two groups. The statistical methods for global assessment of the data will be described and the genes most powerfully indicative of genetic background outlined. (NERC-funded, c.vernon@liverpool.ac.uk).

A.14.17 Expression of seabream (*Sparus aurata*) parathyroid hormone-related protein (PTHrP) and functional study

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Recombinant seabream (*Sparus aurata*) parathyroid hormone-related protein (PTHrP) was expressed using

the IMPACT™-CN system, allowing single-column purification of free recombinant peptide. The cDNA encoding seabream PTHrP was cloned into the pTYB2 vector to create an in-frame fusion at the N-terminus of the cleavable intein (454-amino-acid residues from the *Saccharomyces cerevisiae* VMA1 gene), which binds to chitin beads. The recombinant plasmid was transfected into a protease-deficient *E. coli* strain ER2566 and synthesis of PTHrP fusion protein was induced with isopropyl- β -D-thiogalactopyranoside (IPTG). After loading onto chitin bead column, in the presence of β -mercaptoethanol, the intein undergoes specific self-cleavage to release the highly purified, non-tagged PTHrP. The recombinant PTHrP, whose molecular weight is about 17KD, was identified by western blots with primary rabbit antibodies to both C-terminal and N-terminal seabream PTHrP. The final yield efficiency of recombinant seabream PTHrP was 0.6–0.8mg/liter culture, allows further study of its biological functions, as well as structure.

This work was supported by EU Project Grant (Q5RS-2001-01465). We would like to thank Dr. D. Power of CCMAR, Universidade do Algarve, for supplying the anti-PTHrP antiserum.

A14.18 Bioinformatic approaches to the functional annotation of environmentally responsive genes in fish

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Microarray technology is proving a highly effective means of investigating responses of biological systems to experimental manipulation, environmental perturbation and disease. Most studies involve a limited range of model species for which strong research communities have developed impressive genomic resources including genomic sequences. In the comparative and environmental area most work is conducted using non-model species with non-existent or limited resources. It is therefore necessary to develop methods for the large-scale production of cDNA clone sets, and for these clones to be identified by end-sequencing. We have developed an informatics package, *EST-ferret*, that deals with the cleanup, collation and database submission of large-scale EST sequencing data, and we will describe its case study of its use in producing CarpBASE, an SQL database containing the resulting data for a NERC-funded carp transcript project. This package includes an extensive annotation capability using gene ontology, KEGG and EC classification schemes and also the

means to profile the annotation categories of gene lists provided through microarray clustering procedures. The package incorporates a number of open source algorithms linked by PERL scripts. (NERC-funded, Cossins@liv.ac.uk).

A14.19 Pollutant-responsive genes identified by cDNA microarray with European flounder (*Platichthys flesus* L.)

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We initiated our microarray study of the effects of pollutants on European flounder by constructing a custom cDNA microarray of 132 genes of interest. Comparisons of fish from the polluted Tyne estuary and the relatively pristine Alde estuary resulted in statistically significant modulation of 11 genes including induction of biomarker genes such as CYP1A and UDPGT in fish from the Tyne estuary. To extend this approach, providing clones of further differentially expressed genes, we have completed three suppressive subtractive hybridisation (SSH) comparisons; Tyne vs Alde flounder, benzo(a)pyrene (BaP) treated vs untreated flounder and cadmium treated vs untreated flounder.

As part of the GENIPOL consortium we have constructed a 13 270 clone microarray. This includes the above flounder clones, but most were randomly picked from a normalised flounder liver cDNA library constructed in Lambda TriplEx 2. The library was produced by pooling RNA from fish livers 48h post-treatment with various model stressors. Preliminary experiments compared flounder treated with dibenzanthracene and ethinyl-estradiol individually and in combination. Many differences in transcript levels were seen. For example, in ethinyl-estradiol treated flounder liver, the egg proteins vitellogenin and choriogenin were found to be highly induced, whereas dibenzanthracene induced CYP1A and fatty-acid binding protein. With the co-treatment, this induction of CYP1A and fatty-acid binding protein was reduced, but expression of vitellogenin and choriogenin was maintained. Further changes in gene expression are being assessed for use as biomarkers of marine pollution.