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Genetic markers for signalling and diagnosis of sexual disruption in roach, *Rutilus rutilus*

Science Report – SC030299/SR3

SCHO0408BNZF-E-P

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Steve Killeen

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Executive summary

The work described in this report is part of ongoing research at the University of Exeter focused on the development and application of molecular tools to advance our understanding of the effects of environmental oestrogens in the evolution of intersex in fish. This programme of work described was principally funded to Professor Charles Tyler via the NERC Environmental Genomics Thematic, together with collaborative inputs from Professor Andrew Cossins at the University of Liverpool, Professor Taisen Iguchi, Dr Yoshinao Katsu and Rie Ichikawa at the Okazaki Institute for Integrative Bioscience (Japan), and Dr Yoshihiro Kagami at Ecogenomics, Inc. (Japan).

The work described supports an Environment Agency project funded to Exeter that has been investigating the ontogeny of sexual development and effects of long-term exposure to oestrogenic chemicals on sexual development and function in the roach, *Rutilus rutilus*. The report provides a brief overview of the extensive genetic and genomic resources that have been developed for studies into sexual function in the roach. These include the cloning of 42 genes in the roach, quantitative assays to measure expression of individual genes (qRT-PCR assays) and the establishment of gene arrays (allowing for studies into the expression of thousands of roach genes simultaneously).

The report also describes how the molecular tool box developed has been applied to investigate the molecular mechanisms controlling sexual development in male and female roach and used to investigate the mechanisms of action of environmental oestrogens. In the results some of the findings are presented on the normal ontogeny of expression of oestrogen receptors and aromatase genes that are known to play central roles in reproduction. In the oestrogen exposure studies, data are presented that illustrate altered patterns in the expression of specific genes can signal (and potentially provide early warnings) for sexual disruption by environmental oestrogens. The gene array work illustrates how this approach can provide deeper insights into the modes of action of environmental oestrogens and biological processes that are subsequently disrupted.

Acknowledgements

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1 Development of a molecular toolbox for studies on roach

In collaboration with colleagues at the National Institutes of Natural Sciences (Okazaki Institute for Integrative Bioscience) in Japan, the University of Liverpool (Laboratory of Environmental Gene Regulation) and Ecogenomics, Inc., Japan, we have established a molecular toolbox allowing for investigations into both the normal molecular mechanisms controlling sexual development and function in the roach and in understanding how oestrogenic chemicals disrupt these processes. This toolbox consists of:

i. A suite of 42 genes (identified in Table 1.1) involved with the control of reproduction, including for proteins effecting sexual differentiation, -maturation, steroidogenesis and development, cloned from roach (in collaboration with Prof. Taisen Iguchi and Dr Yoshinao Katsu, Okazaki Institute for Integrative Bioscience, Japan).

ii. qRT-PCR assays (that allow for quantifying levels of expression of target genes) for ten of these genes, including for oestrogens receptors (ERα, ERβ), aromatases (A and B, enzymes that covert the male hormone testosterone into oestrogen), and genes involved with sexual differentiation, including sox9, anti-Mullerian hormone (amh) and DMRT1 (developed in collaboration with Prof. Taisen Iguchi and Dr Yoshinao Katsu, Okazaki Institute for Integrative Bioscience, Japan).

iii. A macroarray that enables quantification of expression of the 42 cloned roach cDNAs, simultaneously (developed in collaboration with Dr Yoshihiro Kagami, Ecogenomics, Inc, Japan).

iv. A custom-built roach cDNA microarray displaying 18,477 gonadal ESTs (expression sequence tags, 9676 assembled sequences of which 4959 were identified) developed from six gonadal cDNA libraries from three different fish life stages. Of these identified sequences, 2800 have gene ontology (GO) annotations (functional descriptions) and a further 556 encode for enzymes (Figure 1.1).

All the gene sequences we have produced have been submitted to GeneBank, such that they are freely available to the research community (accession nos EG530818 – EG54928). Furthermore, a database for the roach sequences has been established at the University of Liverpool (RoachBase) (in collaboration with Prof. Andrew Cossins, University of Liverpool).

Table 1.1 List of 42 genes cloned from the roach.

	Gene
Gonad development	anti-Mullerian hormone (amh)
	vasa homolog (vasa)
	Pumilio homolog
Steroidogenesis	3 beta-hydroxysteroid dehydrogenase (3b-HSD)
	17 beta-hydroxysteroid dehydrogenase (17b-HSD)
	Side-chain cleavage enzyme (cyp11a1)
	Aromatase A (cyp19a)
	Aromatase B (cyp19b)
	17 alpha-hydroxylase/17,20 lyase (cyp17)
	Steroidogenic acute regulatory protein (StAR)
(Steroid) Hormone receptor	Oestrogen receptor alpha (ERα)
	Oestrogen receptor alpha (ERβ)
	Androgen receptor (AR)
	Glucocorticoid receptor (GR)
	Mineralocorticoid receptor (MR)
	Follicle stimulating hormone receptor (FSHr)
	Gonadotropin-releasing hormone receptor
	Growth hormone receptor
	Insulin-like growth factor 1 receptor (IGF1r)
	Thyrotropin-releasing hormone receptor (THRH)
Hormone activity	Thyroid stimulating hormone, beta subunit (TSHb)
	Follicle stimulating hormone, beta polypeptide (FSHb)
	Gonadotropin-releasing hormone 2 (GnRH2)
	Gonadotropin-releasing hormone 3 (GnRH3)
	Growth hormone
	Thyroid hormone receptor alpha (Thrα)
	Thyroid hormone receptor beta (Thrβ)
Gametogenesis	Daz-like gene (Dazl)
	Follistatin
	Inhibin beta-A
	Inhibin beta-B
Transcription factors	SRY-box containing gene 21 (sox21)
	SRY-box containing gene 9 (sox9)
	Wilms tumor 1 (WT-1)
	SF-1
	Doublesex and mab-3 related transcription factor 1 (DMRT1)
Development	Wingless-type MMTV integration site family, member 4 (Wnt4)
Physiological process	Insulin-like growth factor 1 (Igf1)
	Cathepsin D
Control genes	Ribosomal protein L8 (rpl8)
	Beta-actin (bactin)
	Elongation factor 1-alpha (EF-1a)

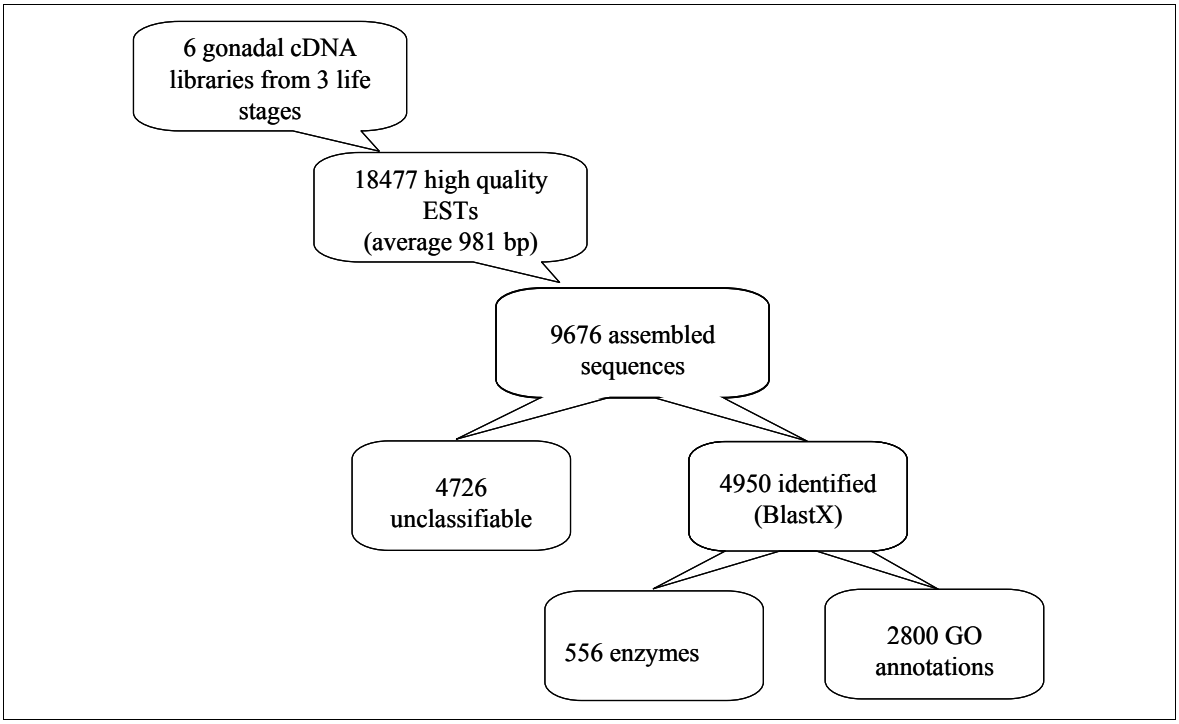

Figure 1.1 Diagrammatic representation of the establishment of the roach cDNA microarray.

2 Studies on single gene responses in roach following developmental exposure to oestrogen

Using the qRT-PCR assays we have established the effects of environmental concentrations of 17α-ethinyloestradiol (EE2) on the expression of genes that are key in controlling sexual function (ERα, ERβ, aromatase A and aromatase B) after exposure during sex differentiation and gonadogenesis and analysed this against the associated effects on the sexual phenotype.

2.1 Aromatases and oestrogen receptors

2.1.1 Aromatases

The biosynthesis of oestrogens occurs throughout the entire vertebrate phylum (Simpson et al. 2002) and is catalysed by the cytochrome P450 aromatase (Simpson et al. 1994) encoded by the *cyp19* gene which is a member of the cytochrome P450 superfamily. This enzyme catalyses the conversion of C19 androgens into C18 oestrogens, and is thus the final step during the steroidogenic pathway. It is also the rate-limiting step of oestrogen biosynthesis. Regulation of its gene dictates the ratio of androgens to oestrogens and therefore, appropriate expression of the enzyme is critical for reproduction as well as for its pivotal role in sex differentiation (Trant et al. 2001).

In vertebrates, including fish, aromatases are mainly expressed in gonads and brain, but expression also occurs in non-steroidogenic tissues such as skin fibroblasts, intestine or foetal liver (Simpson et al. 2002). 17β-oestradiol, the major product of aromatase, is essential for normal ovarian development in non-mammalian vertebrates (Wallace 1985). In fish, 17β-oestradiol is the main oestrogen and, to date, steroids have also been shown to play an important role in sexual differentiation of fish (Hunter and Donaldson, 1983, Baroiller et al. 1999, Guiguen et al. 1999). The exposure of embryos and fry to sex steroid hormones can result in functional sex changes against genetic sex (Yamamoto 1969). Oestrogen treatment, for example, leads to feminised gonads in fish (e.g. van Aerle et al. 2002, Environment Agency 2007). The disruption of aromatase activity during early life and sexual differentiation has also been shown to impair with fish development and reproduction (reviewed in Cheshenko et al. 2007).

In most vertebrates, aromatase is encoded by one single *cyp19* gene and its tissue-specific expression is controlled by tissue-specific promoters. In teleost fish, however, the presence of two different aromatase isoforms, differentially expressed in the ovary (aromatase A / Cyp19a) and brain (aromatase B / Cyp19b), respectively, is now well established. These two isoforms have their own regulatory mechanisms and are differentially programmed and regulated during early development. Both isoforms have been shown to be encoded by two distinct *cyp19* genes which show higher homologies between the same isoforms across species than for the other isoform within the same species.

2.1.2 Oestrogen receptors

Biological effects of oestrogens are principally mediated through specific nuclear receptor proteins, the oestrogen receptors (ERs), which function as ligand-activated transcription factors. It is established that there are at least two subtypes of ERs (ERα and ERβ) mediating the diverse functions of oestrogens, and these have been cloned in a wide variety of vertebrate species including fish.

ERs exhibit broad tissue expression, consistent with the diverse roles of oestrogens. In fish, tissue expression of ERα and ERβ have been shown to differ between species, but generally they appear to be concentrated in the gonad and liver (Tchoudakova et al. 1999, Ma et al. 2000, Socorro et al. 2000, Menuet et al. 2002, Filby and Tyler 2005). These tissue localisations are consistent with the pivotal role of oestrogens in gonadal sex differentiation and development (Devlin and Nagahama 2002), and in the hepatic production of the egg yolk precursor, vitellogenin (VTG), and vitelline envelope proteins (VEPs) required for oocyte synthesis. The oestrogen receptors, together with the aromatases play central roles in the sexual differentiation and gonadogenesis of the female phenotype, but they are also required for fertility in males (Eddy et al. 1996, Robertson et al. 1999).

While data available on the aromatase expression have contributed to the understanding of the processes of sex differentiation, sexual development and reproduction in fish, there is little additional information on the roles of the different ER subtypes in reproduction in fish. In addition, the occurrence of sexual disruption caused by environmental pollutants mimicking sexual hormones and/or disrupting steroidogenesis and thus altering normal reproductive function in wildlife (Tyler et al. 1998) further complicates the understanding of processes underlying reproduction in fish. Despite this widespread phenomenon, the molecular modes of action of environmental oestrogens and their effects on the steroidogenic pathway, especially the different ER subtypes, are not fully understood.

2.2 Aim

Extensive studies on wild populations of roach (*Rutilus rutilus*) inhabiting UK rivers have shown that exposure to oestrogenic effluents emanating from wastewater treatment works (WwTWs) causes altered sexual development resulting in fish with reduced fertility (Jobling et al. 1998, 2002a, 2002b). Controlled effluent exposures have demonstrated that early life stages are especially sensitive for feminised responses (Rodgers-Gray et al. 2001, Liney et al. 2005), but the underlying molecular mechanisms of oestrogenic disruption of sexual development in wild roach are not understood. Given that aromatisation ultimately leads to the formation of oestrogens and that most oestrogenic effects are mediated through ERs (Pellegrini et al. 2005), the aromatases and the ERs are likely to be pathways through which exogenous oestrogens mediate disruptions in endogenous oestrogen signalling pathways and affect sexual differentiation.

To further the understanding of the molecular mechanisms underlying sexual disruption in wild fish, roach were exposed to a series of environmental concentrations of the steroid oestrogen 17α-ethinyloestradiol (EE2) for exposure periods starting from fertilisation. The fish were subsequently sampled at various time points during sex differentiation to determine the effects of EE2 on the dynamics of expression of both the ER subtypes and aromatase isoforms in gonads and brain. At early time points (28, 56, 84 and 112 days post hatch – dph) when gonads were too small to be dissected out, gene expression was measured in body trunks and heads. 518 dph was chosen as a later time point during gametogenesis when gene expression was determined in

dissected gonads. The details of this exposure are given in a previous report to the Environment Agency (Environment Agency 2007).

2.3 Results

2.3.1 Normal developmental expression of ERs and aromatases

For roach the patterns of expression of aromatase and oestrogen receptor mRNAs were first established in body trunks and heads during sexual differentiation using the corresponding qRT-PCR assays (control fish in Figure 2.1). These studies provided the basic information on the normal patterns of expression across these life stages.

Three of these assays (ERα, ERβ and aromatase A) were also used to investigate differences in their gonadal expression between the sexes. At 518 dph, all three genes were more highly expressed in testes compared with ovaries (Figure 2.2).

2.3.2 Effects of EE2 on ER and aromatase expression during gonad development

Exposure to 4 ng L^{-1} EE2 (a concentration found in some of the more polluted WwTWs effluents) resulted in a marked impact on the expression of some of the four target genes. The most pronounced changes in expression were observed for aromatase B in both body trunks and heads (Figure 2.1G and H), but they occurred also for ERα in bodies (Figure 2.1A). In heads, a trend of increasing ERα expression with increasing exposure concentration could be observed at 28 and 56 dph, whereas at 112 dph the expression seems to decrease with increasing concentrations (Figure 2.1B). Expression of aromatase A did not appear to be affected by oestrogen exposure in body trunks or heads (Figure 2.1E and F).

At 518 dph, the expression of ERα, ERβ and aromatase A was also determined in ovaries of fish that had been continuously exposed from fertilisation to 4 ng L^{-1} EE2. At this time point it was not possible to determine the expression in testis of exposed males since this exposure regime had resulted in an all-female population (see Environment Agency 2007). In ovaries of these exposed females, the expression of ERα and aromatase A did not appear to differ from the expression in control females of the same age, whereas ERβ expression seemed to be decreased in the ovaries of exposed females.

These findings on the expression of the ERs and aromatases provide strong support that changes in the expression of key genes controlling sexual development and function can signal for subsequent adverse effects on the sexual phenotype. These findings are further supported by our ongoing studies in zebrafish (*Danio rerio*) where we show that alterations in expression of DMRT-1 and amh during early life occur as a consequence of exposure to EE2 signal for subsequent adverse effects on testis development (Schulz et al. 2007). Further details on the findings for our work on roach ERs can be found in Katsu et al. (2007).

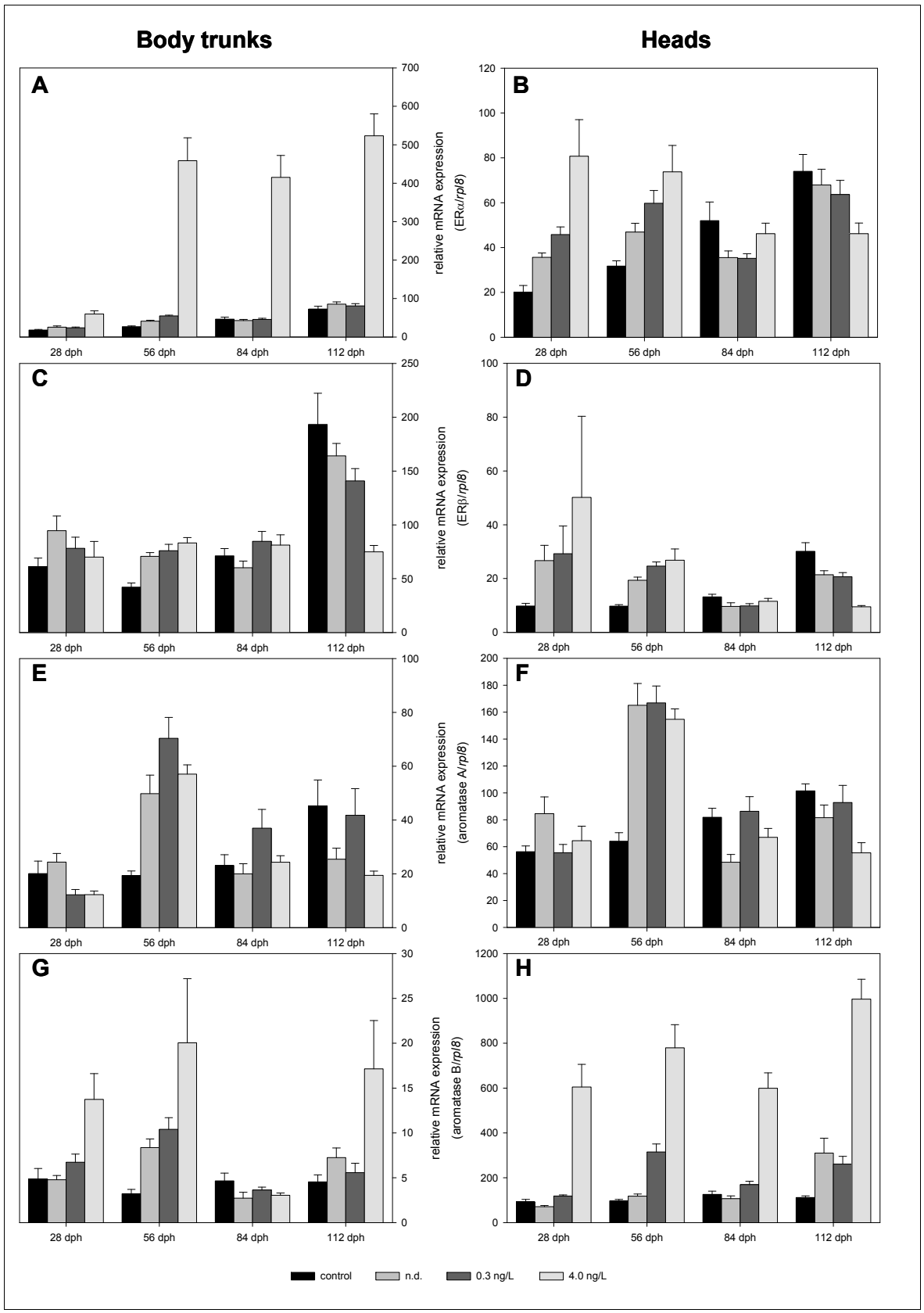

Figure 2.1 Effects of exposure to environmental concentrations of EE2 during early life on the expression of genes key in sexual function (ERα (A and B), ERβ (C and D), aromatase A (E and F) and aromatase B (G and H) in body trunks and heads of roach. Each column represents mean ± SEM. n.d. – non-detectable.

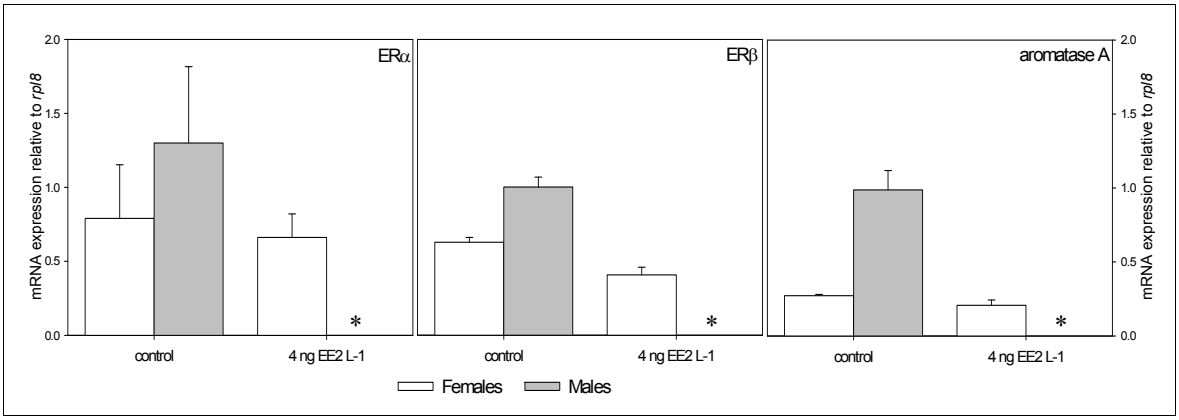

Figure 2.2 Effects of continuous exposure to 4 ng EE2 L^{-1} until 518 dph on the gonadal expression of genes key in sexual function in roach; ERα (A), ERβ (B) and aromatase A (C). * – no phenotypic males at 518 dph after exposure to 4 ng EE2 L^{-1}.

2.3.3 Studies on the expression of ERs and aromatase A mRNAs reveal sensitisation to oestrogen after exposure during early life

In a previous report to the Environment Agency (Environment Agency 2007), we showed that exposure to exogenous oestrogens during early life resulted in an enhanced vitellogenic response to oestrogen on re-exposure in later life. We investigated this phenomenon further with an investigation into the responses of the ERs and aromatase A in the ovaries of females exposed to EE2, using the qRT-PCR assays. Females only were studied as the trend in the vitellogenic response was more pronounced in females compared to males (see previous report). In this work, briefly fish were exposed to different concentrations of EE2 up to 120 dph and then maintained in clean water to 518 dph, when they were re-challenged to EE2 at a single measured concentration of 2.3 ng L^{-1}. The results are displayed in Figure 2.3 and show the same trend in the expression for all three genes as for the vitellogenic response, at least for fish originally exposed to 4.0 ng L^{-1} EE2 during early life. In these fish, the highest induction was observed for all genes after 10 days. Interestingly, fish not receiving EE2 during early life showed an inhibition of aromatase A expression after 4 and 10 days of re-exposure compared to control fish.

These data presented show that exposure to oestrogen during early life affects the response of gonadal genes key to normal sexual functioning (ERs and aromatase A) subsequently in later life, even after a very prolonged period in clean water. This finding has potential implications for risk assessment, as applied to our understanding of the impacts of oestrogens on fish and fisheries.

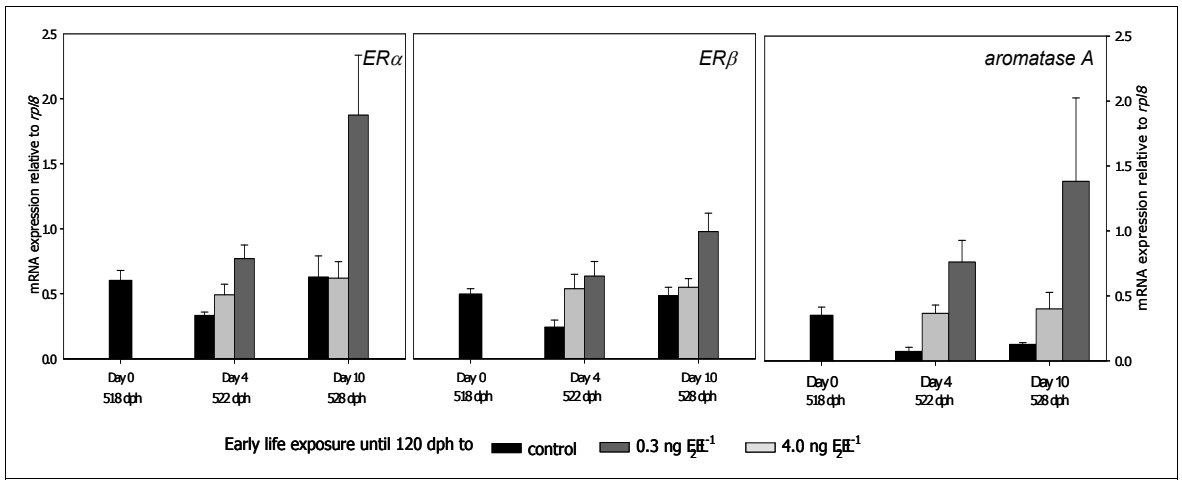

Figure 2.3 Relative expression of ERα (A), ERβ (B) and aromatase A (C) in ovaries of roach exposed to 2.3 ng L^{-1} EE2 for 10 days following early life exposure to different concentrations of EE2 until 120 dph and a subsequent depuration period for 400 days. Each column represents mean ± SEM.

3 A custom-made cDNA macroarray for the simultaneous analysis of 42 genes involved with controlling reproduction

3.1 Aim

Extensive resources have been applied to the cloning of 42 roach genes involved with controlling reproduction in fish. In this work, with colleagues in Japan, we have developed a custom-made cDNA macroarray containing all of these 42 genes and applied this array to investigate some of the differences in gonadal gene expression patterns (for fish at 518 dph) between normal (control) males and females, between control males and intersex males, and between control females and intersex males. The rationale of this work was to further investigate possible genetic markers that signal for disruptions in sexual development as a consequence of exposure to environmental oestrogens.

The samples applied to the array were obtained from an experiment described previously (Environment Agency 2007). Briefly, in that experiment roach were exposed to environmental concentrations of EE2 from fertilisation until 120 dph when fish from each exposure regime were removed from the tanks and transferred into tanks containing clean water. These fish were allowed to depurate for 400 days after which they were analysed histologically prior to biochemical and molecular analyses. Representative examples of the status of gonadal development are shown in Figure 3.1.

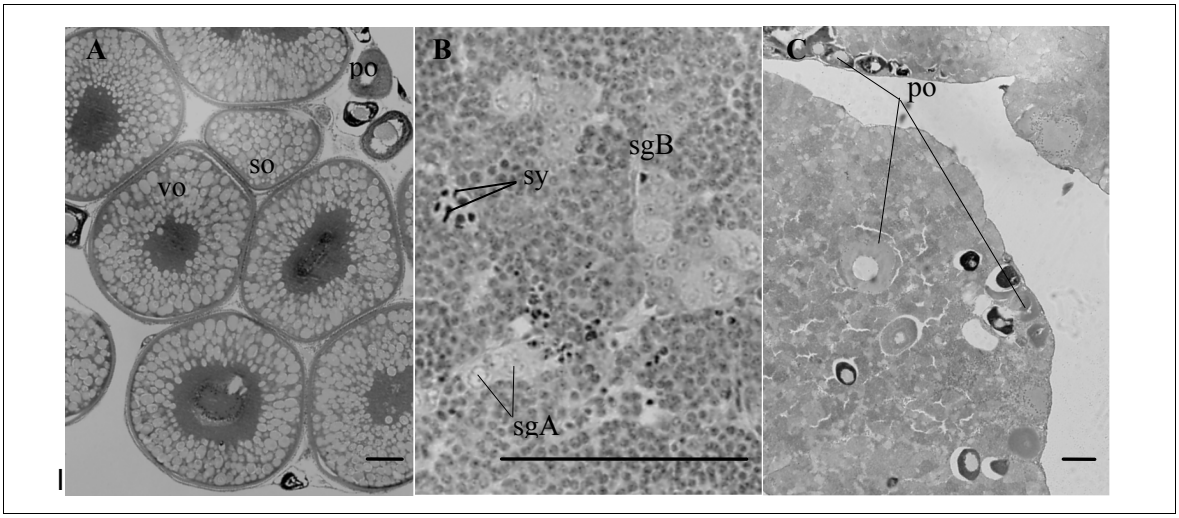

Figure 3.1 Histological sections through ovary (A), testis (B) and an intersex testis (C) from roach at 518 dph, exposed to 4 ng L^{-1} EE2 during early life (until 120 dph) and then kept in clean water for 400 days. po, primary oocyte; so, secondary oocyte; vo, vitellogenic oocyte; sgA, spermatogonia A; sgB, spermatogonia B; sy, spermatocytes; Bars 50 µm. Figure taken from Environment Agency (2007).

3.2 Results

Genes that were found to be differentially expressed between control males and females included vasa, rpl8, gapdh and amh (all down-regulated in ovaries) and pumilio, 17b-HSD, WT-1 and Daz (all down-regulated in testes), providing some insights into sex-related molecular biomarkers of gonadal development (Table 3.1).

Table 3.1 List of genes differentially expressed between ovaries and testes of control roach at 518 dph.

Gene	Expression in ovaries compared with testes[1, 2]
vasa	↓
rpl8	↓
gapdh	↓
pumilio	↑
Daz	↑
17b-HSD	↑
WT-1	↑
amh	↓

Notes: [1] ↓ – genes down-regulated in ovaries compared to testes
[2] ↑ – genes up-regulated in ovaries compared to testes

In these initial investigations only a few genes were found to be differentially expressed when comparing the patterns of gonadal gene expression in control and intersex males (vasa, rpl8 and gapdh; Figure 3.2A) and control females and intersex males (vasa, wt1, gapdh and amh; Figure 3.2B). A list of genes differentially expressed between gonads of intersex males and control males or control females with the associated fold-changes (normalised to bactin) is shown in Table 3.2. vasa, gapdh and amh were highly up-regulated in intersex testis compared with ovaries of control females, whereas wt1 was down-regulated. Comparing the gonadal gene expression of intersex males with control males, only the expression of one vasa clone was down-regulated in intersex testis.

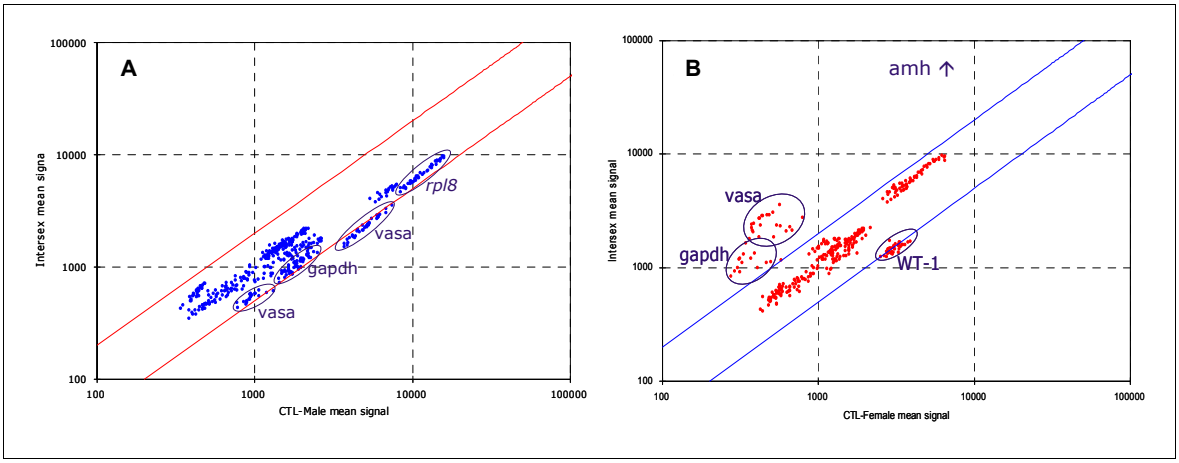

Figure 3.2 Scatter plots of differences in gonadal gene expression between intersex males and control males (A) or control females (B). The two diagonal lines indicate two-fold differences in expression between the compared conditions; genes outside the diagonal lines are considered as differentially expressed (normalised to bactin).

Table 3.2 List of genes differentially expressed between intersex testis and control testis or ovaries and associated fold-changes normalised to *bactin* (*).

Gene	Clone identifier	Fold difference[1] (normalised to *bactin*)	
		Intersex testis versus control testis	Intersex testis versus control ovary
vasa	04c	0.56	↑[2]
vasa	04d	0.55	↑
bactin *	11c	1.00	1.00
bactin *	11d	0.93	0.98
rpl8	12c	0.67	1.47
rpl8	12d	0.68	1.44
vasa	cDNA-04	0.45	5.22
bactin	cDNA-11	0.77	0.90
rpl8	cDNA-12	0.59	1.55
gapdh	cDNA-15	0.58	3.08
WT-1	cDNA-21	1.07	0.48
amh	cDNA-41	0.91	↑
cathepsin D	cDNA-47	1.35	1.10

Notes: [1] Values > 2.0 are considered as up-regulated, values < 0.5 are considered as down-regulated.
[2] ↑ – genes are up-regulated in intersex testis compared to control ovaries, but no gene expression signal was detected in control ovaries.

The expression of two *vasa* isoforms has been reported for other fish species such as tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*) or zebrafish (*Danio rerio*). Since *vasa* has been associated with early events during sex determination it is also considered as a primary germ cell marker. A sexual dimorphism in the expression of both isoforms is described after sexual differentiation of tilapia with one isoform predominantly expressed in oogenesis and the second isoform in spermatogenesis (Kobayashi et al. 2002). Currently, it is not known which isoform the cloned roach *vasa* sequence corresponds to (further sequence analysis will be necessary to identify definitively), but the sexual dimorphism of its expression in roach gonads (up-regulation of *vasa* in roach testis compared with ovaries) suggests it is the isoform predominantly expressed in spermatogenesis.

The sexual dimorphism in the expression of *amh* is in accordance with previous findings in zebrafish where *amh* was shown to be significantly more highly expressed in male fish compared with females (Schulz et al. 2007). In various fish species, a role for *amh* in sexual differentiation has been identified, but its function in adult gonads is not known.

A pertinent observation from the macroarray experiments was that some housekeeping genes (*rpl8* and *gapdh*) were found to be differentially expressed between the sexes, highlighting the importance of choosing carefully reference genes for normalisation of data sets for gene expression studies.

4 Establishing gonadal transcriptome responses to oestrogen exposure using the custom-made cDNA microarray

Gene microarray technologies allow the responses of thousands of genes to be analysed simultaneously potentially offering extensive insights into the molecular mechanisms underpinning biological function and their disruption by environmental pollutants. Most gene microarray work has been applied to organisms with sequenced genomes (e.g. mice, zebrafish etc.) and thus for non-model organisms extensive work has to be applied to develop the required genetic and genomic resources. In our work with colleagues at the University of Liverpool we have developed extensive genetic resources for DNA microarray work in the roach. The array developed is briefly described above. Here we describe the application of the roach DNA microarray to investigate sex-related differences in the gonadal transcriptome and investigate oestrogenic disruption in gonadal development in roach.

4.1 Aim

The gonadal cDNA microarray was applied to:

- establish sex-related differences in the gonadal transcriptome between mature control males and females;
- investigate mechanisms underlying oestrogenic disruption of gonadal sexual development in roach.

4.2 Methods

Gonadal RNA was extracted from male and female fish exposed for 720 dph to two environmental concentrations of EE2 (control and 4 ng L^{-1}) and applied to the custom-built roach cDNA microarray displaying 18,477 gonadal ESTs. Subsequently, the arrays were hybridised (the principle of a microarray experiment is shown in Figure 4.1) and analysed using a balanced ANOVA loop design (Figure 4.2A).

Principal Component Analysis (PCA) was used to identify the main trends in gene expression for the different treatments. Differentially expressed genes were identified by plotting a histogram of *P*-values and determining the false discovery rate (allowing estimation of the rate of features that are truly null (false), Storey and Tibshirani (2003)) which was used to filter the $P < 0.05$ cohort (Figure 4.2B). Differentially expressed genes were clustered and their biological significance investigated by analysis of bias of the distribution of GO terms in the obtained gene lists.

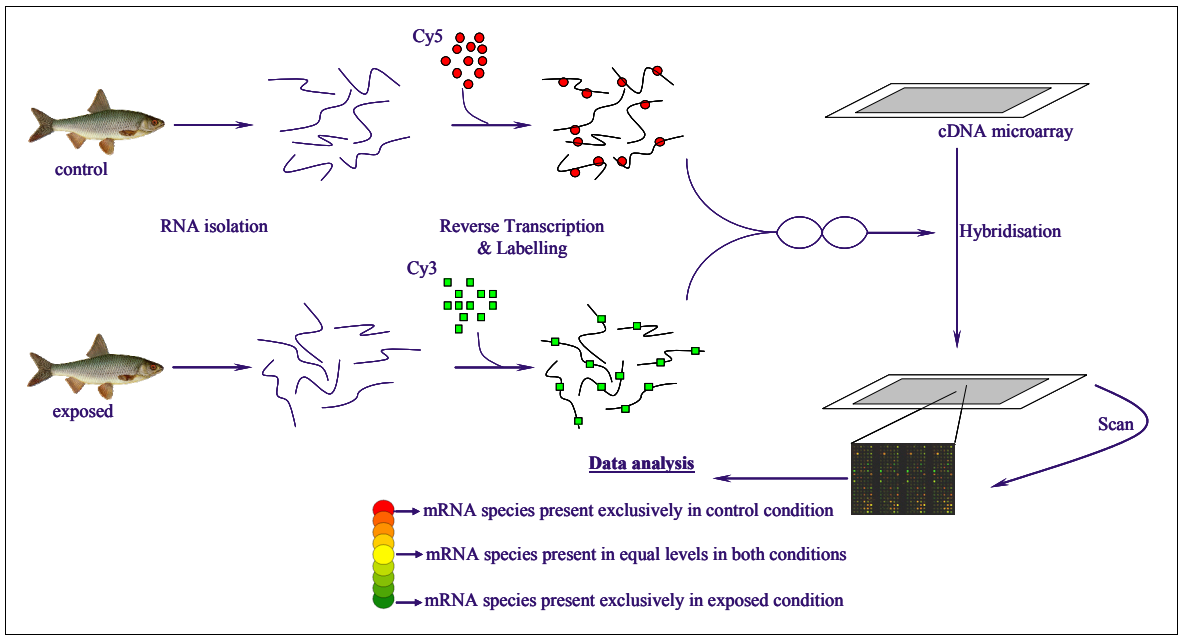

Figure 4.1 Simplified principle of a cDNA microarray experiment.

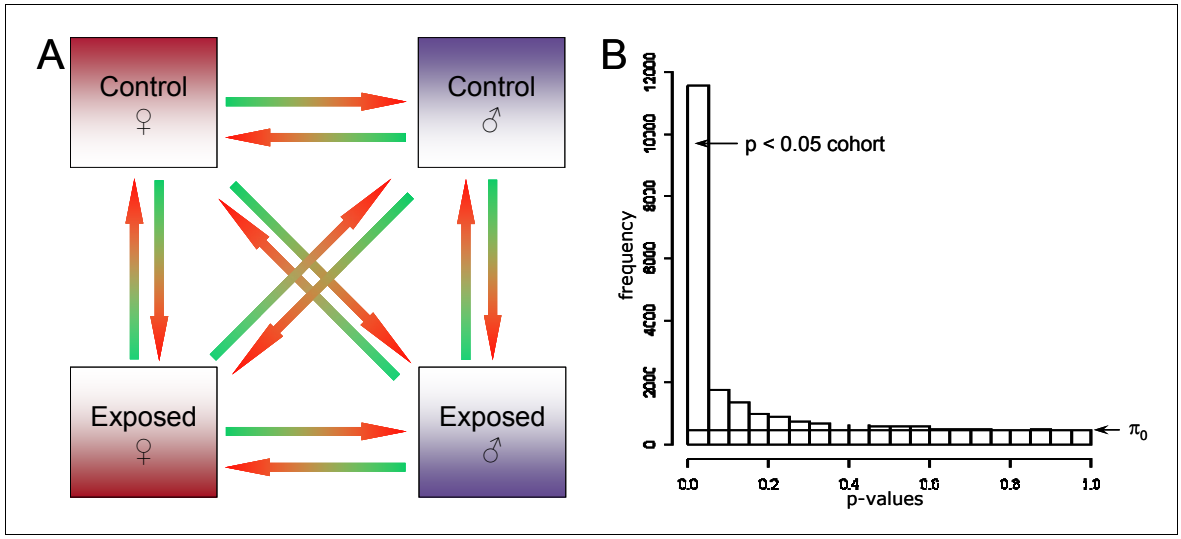

Figure 4.2 A. The balanced loop design applied for the analysis of ovaries and testis of roach exposed to EE2 (control and 4 ng L^{-1}). Six biological replicates were analysed from each treatment group. Each arrow represents two biological samples hybridised to one microarray, green arrow bases indicate Cy3-labelled samples, red arrow heads Cy5-labelled samples. B. Density histogram of the *P*-values after normalisation of raw data obtained for one treatment comparison within the loop. Cohorts of *P*-values are plotted in steps of 0.05 against the frequency of their appearance within one treatment comparison and are used to estimate the proportion of features that are truly null according to Storey and Tibshirani (2003). The determined π0 value, indicated by a solid line, allows estimation of this proportion.

4.3 Results

As previously reported (Environment Agency 2007), continuous exposure of roach from fertilisation until 720 dph to 4 ng EE2 L^{-1} was shown definitively to result in an all-female population (as assessed by gonad histology), thus causing complete gonadal sex reversal in males. At 720 dph, stages of ovarian development varied more widely in 4 ng EE2 L^{-1}-treated fish compared with the control females (that were tightly synchronised in their development), which probably reflected the presence of both females and feminised (sex-reversed) males in this treatment group. Based on the histological results, fish exposed to 4 ng EE2 L^{-1} were classified into two groups (Figure 4.3A): fish whose gonads were comparable to those of control females and which contained all stages of sex cells up to vitellogenic oocytes were considered as 'putative genetic or mature females', whereas gonads of the second group contained predominantly primary oocytes with just a few more advanced female sex cells. Due to the lack of advanced ovarian development, this group was considered as 'immature females/putative exposed males'.

PCA of the gene expression profiles in gonads of control roach clearly separated males from females for both studies, indicating that sex was the main determinant of differential gene expression in gonads. An analysis of the gene profiles for the EE2-exposed fish showed clear and major effects of treatment on the overall transcriptome of individuals. PCA on the EE2-exposed, 'female-like' fish (that would have included genetic females and males) separated them into two groups, one showing a higher similarity to the control females, and the other (immature females/presumed feminised males) to the control males. Furthermore, it was observed that oestrogenic exposure impacted the transcriptomes of males to a greater extent compared with females (Figure 4.3B).

These results give a first indication into the power of the microarray to distinguish between genetic females and sex-reversed males on the level of the transcriptome. Further analyses (of this and other data sets) are continuing.

Determination of the numbers of differentially expressed genes between the various treatment comparisons not surprisingly revealed most differences between control males and all groups of phenotypic females (control as well as both groups of exposed phenotypic females – all over 9200 differentially expressed genes), whereas the smallest difference was found between ovaries of 'exposed mature females' and control females (2307 differentially expressed genes). About 4800 genes were differentially expressed between the two groups of ovaries from exposed females (Figure 4.4). Among the genes differentially expressed between ovaries and testes were genes encoding for vitelline proteins and zona pellucida proteins, well-established oestrogen dependent genes. The results presented are preliminary and the lists of differentially expressed genes are currently under further analysis for new/unknown genes differentially expressed between ovaries and testes of control and exposed fish.

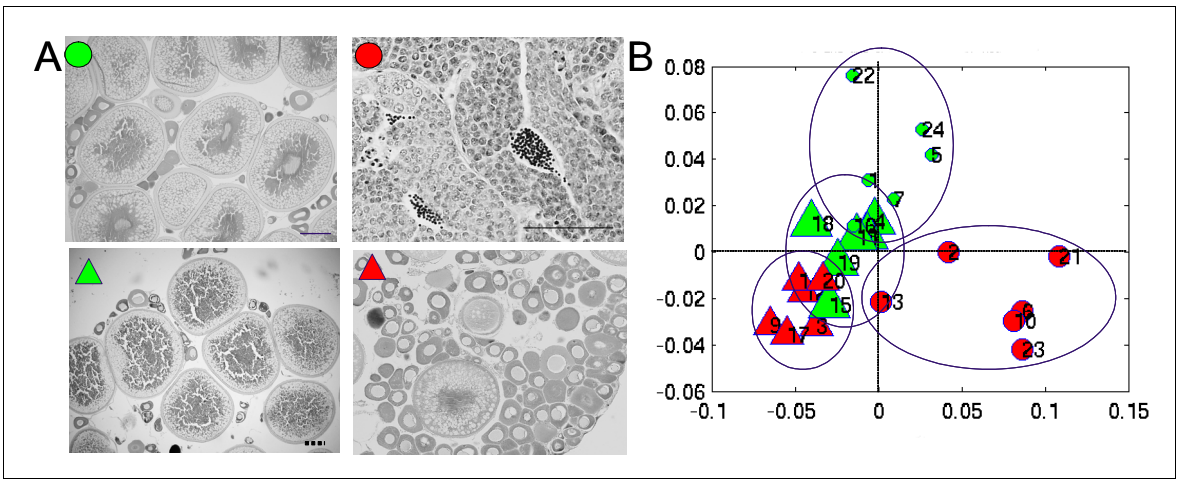

Figure 4.3 Phenotypic classification of samples used for transcriptomic analysis after exposure to EE2 for 720 dph (A) and Principal Component Analysis (PCA) of genes expressed in the gonads of control and EE2-exposed roach at 720 dph (B). The colours and symbols represent the following: green circles – control females, red circles – control males, green triangles – exposed mature females, red triangles – exposed immature females. All exposed fish showed an ovarian morphology as a result of EE2 treatment. Due to the lack of advanced ovarian development, exposed immature females (red triangles) were considered as 'putative exposed males', whereas exposed mature females (green triangles) were considered as 'putative genetic females' at 720 dph.

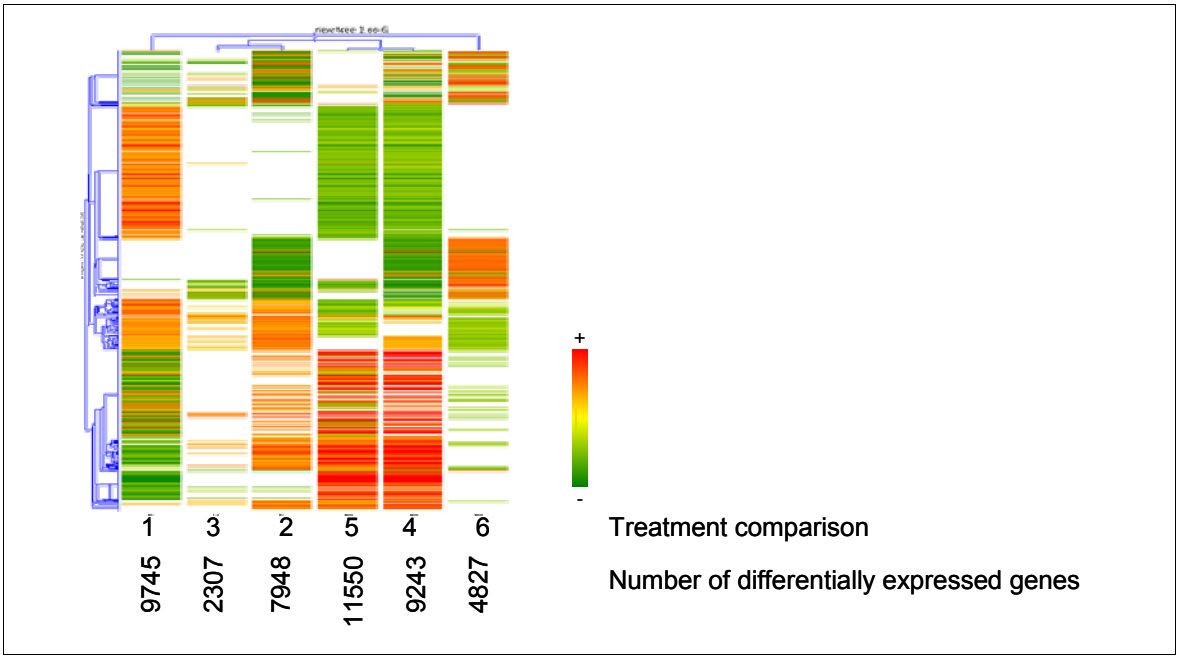

Figure 4.4 Cluster diagram of gonadal genes differentially expressed between different treatment comparisons after exposure of roach to EE2 for 720 dph. Clustering for both genes and conditions was performed using Pearson correlation as a similarity measure. The gene tree is displayed horizontally and the condition tree is displayed vertically. The treatment comparisons were: 1 – control females versus control males; 2 – exposed immature females versus control females; 3 – exposed mature females versus control females; 4 – exposed immature females versus control males; 5 – exposed mature females versus control males; 6 – exposed mature versus exposed immature females. Numbers in the lowest row indicate the numbers of differentially expressed genes for each treatment comparison.

The over-representation of biological processes within the individual gene lists was estimated by one-tailed Fisher's exact test and the processes over-represented included metabolism, biosynthesis and translation (Figure 4.5).

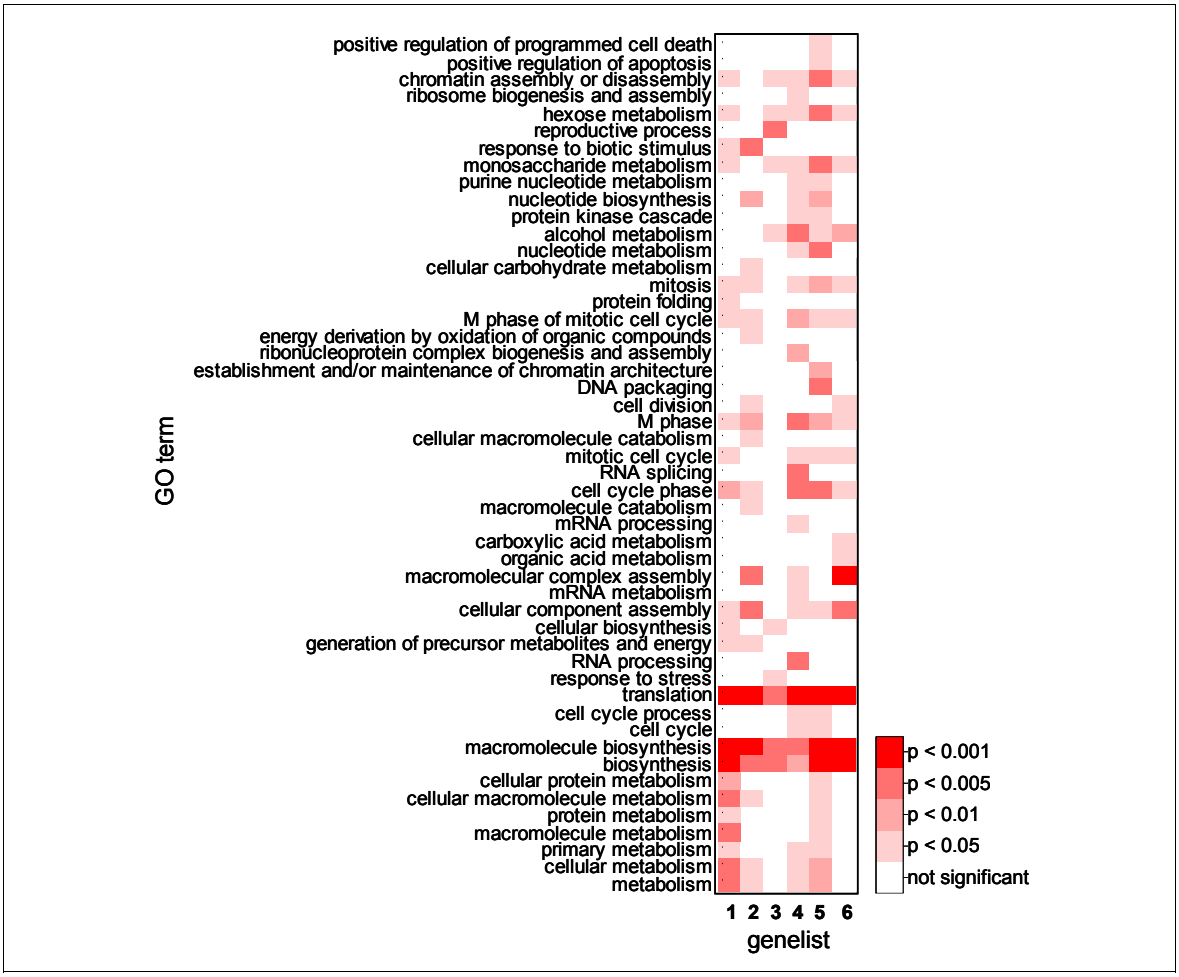

Figure 4.5 GO-Matrix (a pseudocolour map) of significantly over-represented biological processes determined by one-tailed Fisher's exact test. The gene lists 1–6 represent the different treatment comparisons described in Figure 4.4.

Further microarray studies on roach are continuing. They include investigations of:

- Effects of exposure to three environmental concentrations of EE2 (non-detectable, 0.3 and 4 ng L^{-1} EE2) on the gonadal transriptome in male and female roach at 250 dph.
- Effects of exposure to an oestrogenic WwTW effluent on the gonadal transcriptome in exposed fish. In this project, transcriptomic analyses have been run for fish exposed to a treated sewage effluent that is known to be oestrogenic. Mild effects on the sexual phenotype were induced by the WwTW effluent exposure (feminisation of the reproductive duct and in some cases the presence of a few oocytes in the testis of males) compared with that for the EE2 treatment described above, and the treatment had less effects on the overall gonadal transcriptome (Figure 4.6).

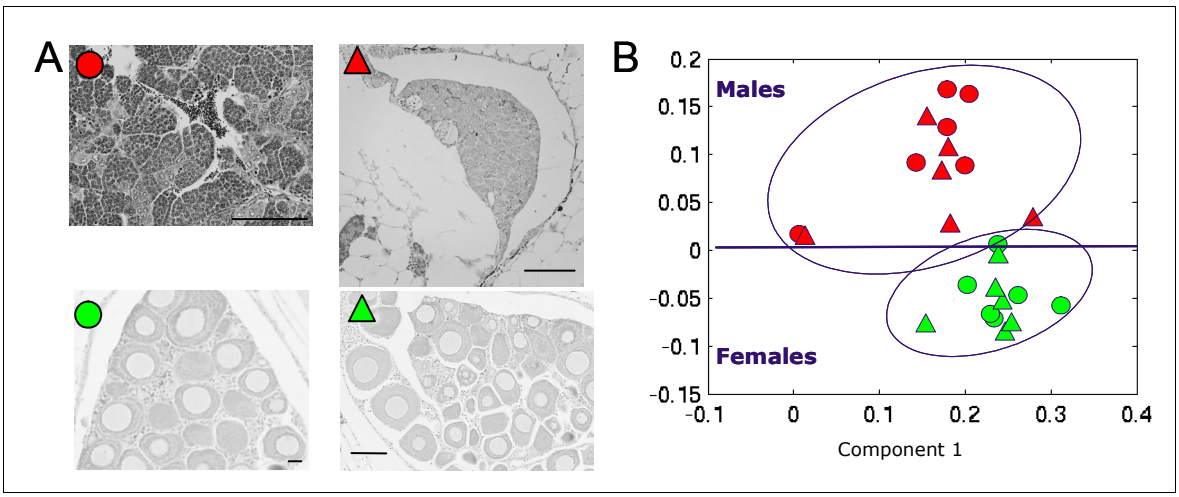

Figure 4.6 Phenotypic classification of samples used for transcriptomic analysis after exposure to 100% effluent for 390 dph (A) and Principal Component Analysis (PCA) of genes expressed in the gonads of control and effluent-exposed roach at 390 dph (B). The colours and symbols represent the following: green circles – control females, red circles – control males, green triangles – exposed mature females, red triangles – exposed males.

5 Summary

In summary this report describes the development of extensive genetic resources for studies into the molecular mechanisms controlling sexual development and function in the roach, a key environmental sentinel for studies on the effects of endocrine disruption in UK rivers. These molecular tools have been applied to develop an understanding of the normal patterns of expression of key genes involved in sexual development in the gonad and illustrate that altered expression of specific genes can signal for disruption in sexual development induced by exposure to environmental oestrogens. The gene arrays developed have started to provide extensive and comprehensive insights into the mechanisms controlling sex in fish and the pathways of effect for environmental chemicals such as environmental oestrogens.

6 Recent publications resulting from this and allied work

This list contains recent publications from the research team at the University of Exeter resulting from this and allied work on the development and application of molecular tools (including gene arrays) to understand the mechanisms of sexual disruptions in fish.

Ankley, G.T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillitt, D.E., Tyler, C.R. and Versteeg, D., 2006. Toxicogenomics in regulatory ecotoxicology. *Environmental Science & Technology* 40, 4055–4065.

Filby, A.L. and Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biology of Reproduction* 73, 648–662.

Filby, A.L. and Tyler, C.R., 2007. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. *BMC Molecular Biology* 8.

Filby, A.L. and Tyler, C.R., 2007. Cloning and characterization of cDNAs for hormones and/or receptors of growth hormone, insulin-like growth factor-I, thyroid hormone, and corticosteroid and the gender-, tissue-, and developmental-specific expression of their mRNA transcripts in fathead minnow (*Pimephales promelas*). *General and Comparative Endocrinology* 150, 151–163.

Filby, A.L., Thorpe, K.L. and Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental oestrogen in fish. *Journal of Molecular Endocrinology* 37, 121–134.

Filby, A.L., Thorpe, K.L., Maack, G. and Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquatic Toxicology* 81, 219–231.

Filby, A.L., Neuparth, T., Thorpe, K.L., Owen, R., Galloway, T.S. and Tyler, C.R., Health impacts of estrogens in the environment, considering complex mixture effects. *Environmental Health Perspectives*. doi:10.1289/ehp.10443

Filby, A.L., Santos, E.M., Thorpe, K.L., Maack, G. and Tyler, C.R. Gene expression profiling for understanding chemical causation of biological effects of complex mixtures: A Case Study for Estrogens. *Environmental Science and Technology*. Accepted, in press.

Hamilton, P.B. and Tyler, C.R., Identification of microsatellite loci for parentage analysis in roach *Rutilus rutilus* and 8 other cyprinids by cross-species amplification, and a novel test for detecting hybrids between roach and other cyprinids. *Molecular Ecology Notes*. doi:10.1111/j.1471-8286.2007.01994.

Katsu, Y., Lange, A., Ichikawa, R., Urushitani, H., Paull, G.C., Cahill, L.L., Jobling, S., Tyler, C.R. and Iguchi, T., 2007. Functional associations between two estrogen receptors, environmental estrogens and sexual disruption in the roach (*Rutilus rutilus*). *Environmental Science & Technology* 41, 3368–3374.

Santos, E.M., Workman, V.L., Paull, G.C., Filby, A.L., Van Look, K.J.W., Kille, P. and Tyler, C.R., 2007. Molecular basis of sex and reproductive status in breeding zebrafish. *Physiological Genomics* 30, 111–122.

Santos, E.M., Paull, G.C., Van Look, K.J.W., Workman, V.L., Holt, W.V., van Aerle, R., Kille, P. and Tyler, C.R., 2007. Gonadal transcriptome responses and physiological consequences of exposure to oestrogen in breeding zebrafish (*Danio rerio*). *Aquatic Toxicology* 83, 134–142.

Schulz, R.W., Bogerd, J., Male, R., Ball, J., Fenske, M., Olsen, L.C. and Tyler, C.R., 2007. Estrogen-induced alterations in amh and dmrt1 expression signal for disruption in male sexual development in the zebrafish. *Environmental Science & Technology* 41, 6305-6310.

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Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., McMeel, O.M., Martin, S.A.M. and Fostier, A., 1999. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). *Molecular Reproduction and Development* 54, 154–162.

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List of abbreviations

cDNA	Complementary DNA
cy	Brand name for fluorescent dyes
cyp19	Cytochrome P450 aromatase
DNA	Deoxyribonucleic acid
dph	Days post hatch
EE2	17α-ethinyloestradiol
ER	Oestrogen receptor
EST	Expression sequence tags
GO	Gene ontology
mRNA	Messenger ribonucleic acid
PCA	Principal Component Analysis
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RNA	Ribonucleic acid
WwTW	Wastewater treatment works

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