CORE
Metadata, citation and similar papers at core.ac.uk
Provided by NERC Open Research Archive

Report

Pottinger, Tom G.; Abel, David; Juergens, Monika D.; Rhodes, Glenn; Katsiadaki, Ioanna; Cook, Alastair; Sanders, Matthew. 2009 *A catchment-based study of endocrine disruption in surface waters: multivariate evaluation of the health of a sentinel fish species exposed to sewage treatment works effluent.* EDCAT consortium, 64pp. (CEH Project Number: C03052)

Copyright © 2009, NERC/Centre for Ecology & Hydrology

This version available at http://nora.nerc.ac.uk/7170/

NERC has developed NORA to enable users to access research outputs wholly or partially funded by NERC. Copyright and other rights for material on this site are retained by the authors and/or other rights owners. Users should read the terms and conditions of use of this material at http://nora.nerc.ac.uk/policies.html#access

This report is an official document prepared under contract between the customer and the Natural Environment Research Council. It should not be quoted without the permission of both the Centre for Ecology and Hydrology and the customer.

Contact CEH NORA team at
nora@ceh.ac.uk

A catchment-based study of endocrine disruption in surface waters: multivariate evaluation of the health of a sentinel fish species exposed to sewage treatment works effluent.

Tom G. Pottinger[1], David Abel[1], Monika D. Jürgens[2], Glenn Rhodes[1], Ioanna Katsiadaki[3], Alastair Cook[3], Matthew Sanders[3]

[1] CEH Lancaster, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP

[2] CEH Wallingford, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire, OX10 8BB

[3] CEFAS, The Nothe, Barrack Road, Weymouth, Dorset DT4 8UB

Comprising part of the Final Report of the EDCAT project to Defra.

CEH project C03052.

May 2009

Section 5.
EDCAT 5: A catchment-based study of endocrine disruption in surface waters: multivariate evaluation of the health of a sentinel fish species exposed to sewage treatment works effluent.

Project Personnel and Affiliations:

Tom G. Pottinger (CEH Lancaster)
David Abel (CEH Lancaster)
Monika D. Jürgens (CEH Wallingford)
Glenn Rhodes (CEH Lancaster)

Ioanna Katsiadaki (Cefas Weymouth)
Alastair Cook (Cefas Weymouth)
Matthew Sanders (Cefas Weymouth)

5.1 Introduction

The overall aims of the EDCAT programme are outlined elsewhere (see Section 2). The specific aims of EDCAT 5 were based on the assumption that incorporation of the Swindon STW into the EA/Defra Demonstration Programme indicated that the discharge contained estrogenic chemicals at a concentration that was considered a potential threat to aquatic wildlife.

On this basis, EDCAT 5 was designed to provide data on the status of an indicator fish species (the three-spined stickleback, *Gasterosteus aculeatus*) in the receiving water (River Ray) prior to remediation. It was intended to collect sufficient data during the period before installation of the GAC plant, when estrogenic content of the discharge was expected to be moderately high, to allow a robust comparison with the status of the same populations of fish over a similar period after the installation of the plant, when estrogens (and other organic chemicals) were expected to have been eliminated from the effluent or at least very much reduced in concentration.

Two unanticipated issues required accommodation within the project. The first of these was the surprisingly low level of estrogenic chemicals present in the discharge prior to the onset of remediation. This resulted in the absence of clear response in the selected primary biomarker of estrogen exposure, vitellogenin elevation in male sticklebacks. At the request of the EDCAT Steering Group, an additional sensitive index of estrogen exposure was incorporated into the suite of endpoints measured. The biomarker chosen was induction of the inner egg envelope protein precursor choriogenin (ChgH) which is also under estrogenic control. This was selected because preliminary microarray data from Cefas (Katsiadaki, unpublished) indicated that this gene was expressed to a greater extent in fish exposed to the Ray STW effluent than in a control group. The second issue was the installation and commissioning of the GAC plant before the end of what was intended to be a monitoring period. While this reduced the number of pre-remediation samples it did provide an opportunity to compare the pre- and post-remediation periods within the lifetime of this single project. Thus, monitoring was extended through 2008 to provide a set of samples that could be matched to corresponding pre-remediation samples.

Summary of EDCAT 5 project aims:

5.1.1. By comparing appropriate biomarkers in fish sampled from STW-impacted sites and control sites during the pre-remediation period, to determine whether there was evidence for any effects

that might be attributed to the presence of estrogenic/anti-estrogenic (or androgenic/anti-androgenic) chemicals in the former. This aim was addressed by measuring concentrations of the estrogen-dependent yolk protein precursor vitellogenin, and the androgen-dependent nest glue spiggin in male and female sticklebacks. In addition histological examination of the gonadal structure of fish captured at the impacted and non-impacted sites was employed to seek evidence of overt alterations in reproductive physiology of the fish. For a subset of matched samples from the two rivers, the relative induction of hepatic choriogenin mRNA, a biomarker of estrogen exposure, was measured.

5.1.2. By comparing appropriate biomarkers in fish sampled from STW-impacted sites and control sites during the pre-remediation period, to determine whether there was evidence for any effects that might be attributed to the presence of "conventionally" toxic chemicals. This was addressed by measurement of the activity of a key Phase I transforming enzyme in the liver of fish, either using direct enzymatic assay (EROD) or by quantifying the levels of expression of the corresponding gene (CYP1A).

5.1.3. To determine whether the adaptive capacity and energetic status of fish varied between the STW-impacted and non-impacted sites. This was addressed by measurement of indicators of stress (whole-body corticosteroid levels), metabolic status (whole-body glucose levels) and anabolic activity (RNA:DNA ratios).

5.1.4. To assess whether there were differences in population size and structure between STW-impacted and non-impacted sites. This was addressed by comparison of key somatic measures, in particular frequency distributions for fork length.

5.2 Methods

5.2.1 Site selection: Site selection on the R. Ray was constrained by the identification of vehicle access points and downstream proximity to the Rodbourne STW. Four sites were identified, with the first of these immediately adjacent to the STW outfall and the final site approximately 10 km further downstream. Matching reference sites were initially selected in the River Cole catchment which is immediately east of the Ray, and is very similar in terms of flow, geology and land-use, but with limited STW input. However, field assessment of the suitability of the Cole indicated that it was not an appropriate choice. Instead, three accessible sites on the R. Ock and one on the Childrey Brook were identified as suitable unimpacted reference sites. Locations of the sample sites are provided in Table 5.1 and Figures 5.1 and 5.2.

Table 5.1. Location and name (abbreviation) of EDCAT 5 sample sites.

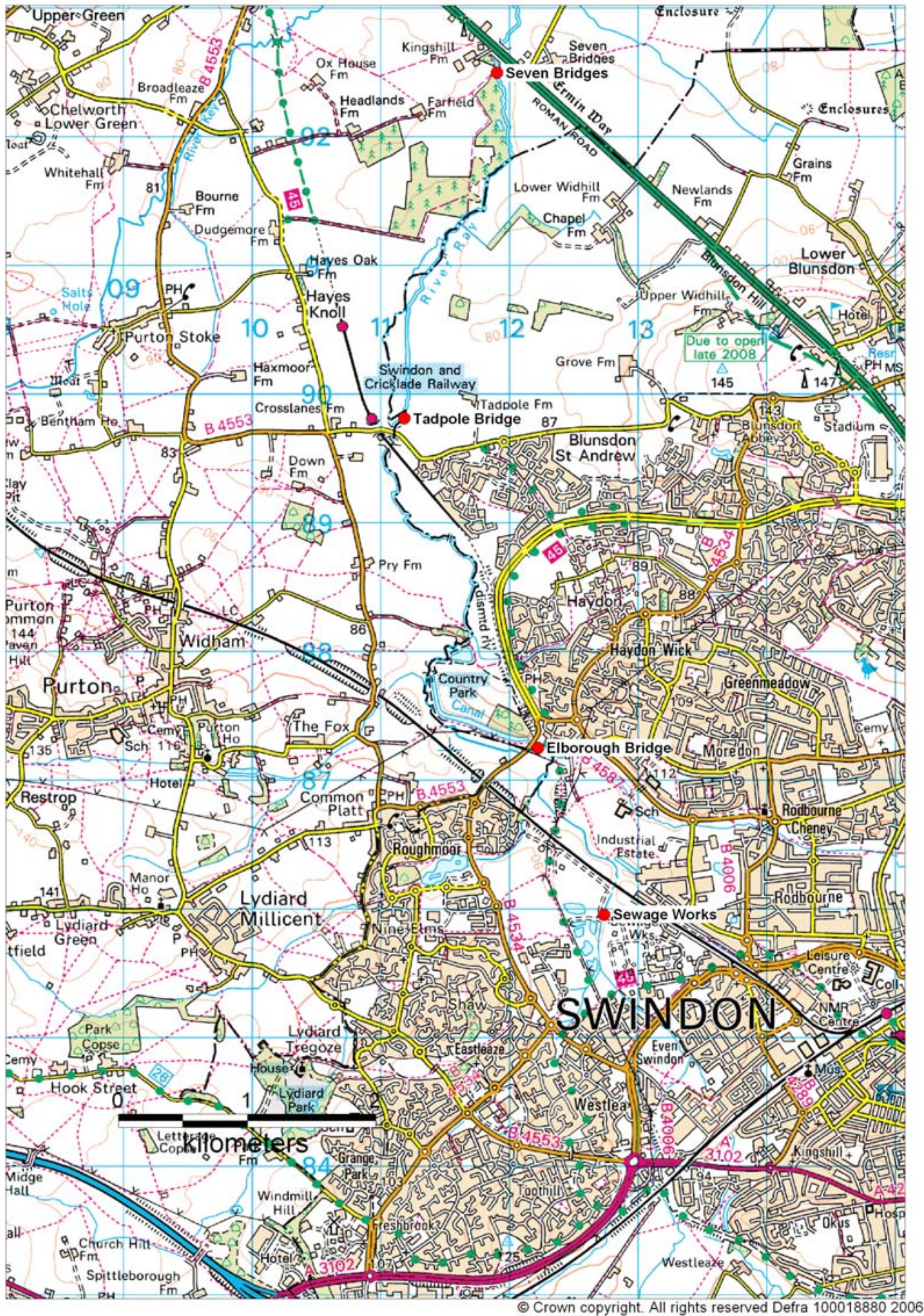
River	Site	Site No.	Grid reference
Ray	Rodbourne STW (ROD)	3	128865
Ray	Elborough Bridge (EB)	4	121872
Ray	Tadpole Bridge (TB)	6	111896
Ray	Seven Bridges (7B)	7	119925
Ock	Charney Basset (CB)	10	381944
Ock	Garford (A338 bridge) (GAR)	11	438962
Childrey Brk	Venn Mill (VM)	12	434948
Ock	Marcham Mill (MM)	13	448954

5.2.2 Frequency of sampling: A total of fifteen sample trips were made (Table 5.2). Relatively few trips were conducted during 2006 because of uncertainties that arose with regard to the absence of a vitellogenic signal in fish from the R. Ray and a perceived need to confirm that the absence of apparent estrogenic effects in the fish could be supported by the chemical analysis of water from the STW and sites downstream of it. When the decision was made to continue with the project, despite the very much lower than expected levels of estrogens in the pre-GAC Rodbourne effluent, sampling was scheduled for March 2007 (changed to April) and at 2-monthly intervals thereafter. This between-sample interval was selected based on a wish to avoid depletion of fish at the selected sites, a need to allow processing time for the collected samples, and a requirement that sufficient temporal resolution be provided to capture seasonal changes in the reproductive status of the fish.

Fortunately, the sample timing also meant that between the early commissioning of the GAC plant in February 2008 and the scheduled end of the project it was possible to complete five samples, timed to match those collected during 2007. This required an extension of the project end date in order to accommodate the additional samples and the associated analyses. The additional samples allowed a comparison of year to year consistency within both rivers during the periods prior to and following installation of the GAC plant on the Ray, providing a means to assess whether remediation caused detectable alterations in defining characteristics of the stickleback population in the Ray.

Table 5.2. Dates of EDCAT 5 sample trips.

<table>
<tr><th>Sample</th><th>Date</th><th>Sites visited</th></tr>
<tr><td>1</td><td>12 December 2005</td><td>R.Ray (4); R. Cole (1)</td></tr>
<tr><td>2</td><td>29/30 March 2006</td><td rowspan="14">R. Ray (4); R. Ock (3); Child. Brk (1)</td></tr>
<tr><td>3</td><td>3/4 May 2006</td></tr>
<tr><td>4</td><td>20/21 September 2006</td></tr>
<tr><td>5</td><td>18/19 April 2007</td></tr>
<tr><td>6</td><td>15/16 May 2007</td></tr>
<tr><td>7</td><td>17/18 July 2007</td></tr>
<tr><td>8</td><td>11/12 September 2007</td></tr>
<tr><td>9</td><td>13/14 November 2007</td></tr>
<tr><td>10</td><td>29/30 January 2008</td></tr>
<tr><td>11</td><td>11/12 March 2008</td></tr>
<tr><td>12</td><td>20/21 May 2008</td></tr>
<tr><td>13</td><td>15/16 July 2008</td></tr>
<tr><td>14</td><td>9/10 September 2008</td></tr>
<tr><td>15</td><td>11/12 November 2008</td></tr>
</table>



© Crown copyright. All rights reserved Defra 100018880 2006.

Figure 5.1. Location of EDCAT 5 sample sites on the River Ray (STW: Site 3; Elborough Bridge: Site 4;, Tadpole Bridge: Site 6; Seven Bridges: Site 7).

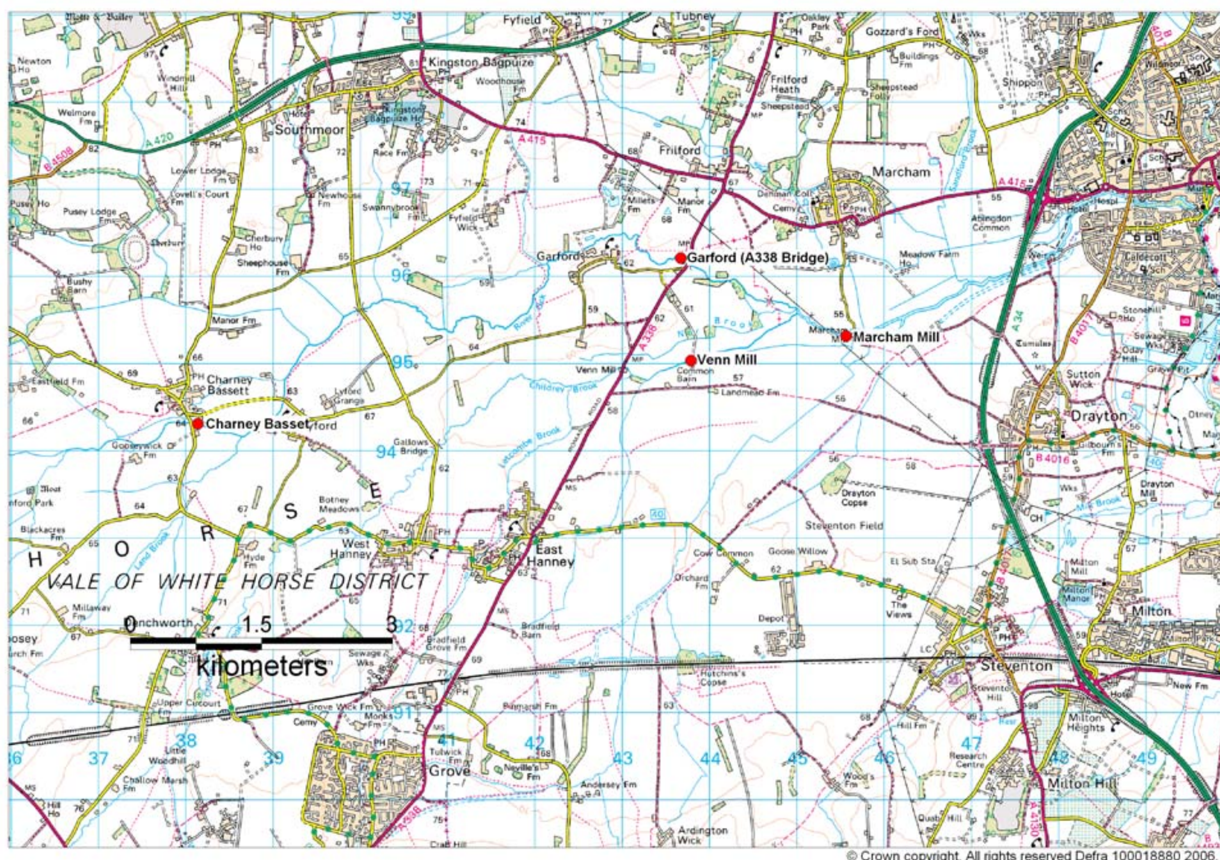


Figure 5.2. Location of EDCAT 5 sample sites on the River Ock (Charney Bassett: Site 10; Garford: Site 11; Marcham Mill: Site 13) and Childrey Brook (Venn Mill: Site 12)

5.2.3 Fish capture methods**:** Three options were considered for fish capture: electric fishing, hand nets, and traps. The use of traps presented practical difficulties associated with their deployment and retrieval, consequently their usefulness was judged to be too limited for the present situation. The application of electric fishing (InteliSYS Fish Magnet Mobile II; 200w pulsed DC; 10cm diameter anode; Copp, 1989) was not successful in removing sticklebacks at many of the study sites, to a large extent because the tendency of the fish to seek cover in dense submerged vegetation combined with the depth of the water made retrieval of stunned fish difficult, but also in part because of the reduced efficiency of electric fishing for small fish (Beaumont *et al*., 2002). The most consistently successful method of fish capture proved to be the use of a large hand net (38 cm D-frame, 0.5 cm mesh, 1.5 m handle), combined with wading. On occasions when the water level was too high to safely enter the river, fish were captured by hand-netting suitable areas immediately adjacent to the bankside. This was only successful where trailing and emergent vegetation was present.

5.2.4 Fish processing: Fish were held temporarily in buckets following capture. After each sample period ended the fish were killed with a lethal dose of anaesthetic (2-phenoxyethanol, 1:2000) and placed in individually labelled, capped, 12 ml polypropylene centrifuge tubes and transferred to a cryo-resistant bag before being frozen in a liquid N_2 dry shipper (Taylor-Wharton CryoExpress CX500, Jencons plc) for transfer to CEH Lancaster. A single dry shipper provided sufficient capacity for all the fish collected during a 2-day sampling trip. On arrival at CEH Lancaster the samples were transferred to a freezer (-20°C) for storage until required for analysis. Each fish was

coded with a unique identifier at the time of capture and this was retained throughout processing and subsequent analysis.

Shortly after return to the laboratory, the fish were processed for analysis. Tubes containing fish were removed from the freezer in groups of six and placed on ice. In turn, and while still frozen, each fish was removed from its tube and weight (mg) and fork-length (mm) were recorded. A ventral incision was made using dissecting scissors and the liver was removed and placed into 1.0 ml RNAlater (Sigma-Aldrich) in a 1.5 ml capped polypropylene centrifuge tube. The heart was removed, transferred to a pre-tared 1.5 ml capped polypropylene centrifuge tube and weighed. The kidney was removed and placed into a screw-top 1.5 ml cryo-tube. The remainder of the fish was replaced in the sample tube and returned to the freezer. The heart and kidney samples were stored frozen until transferred by courier, on dry ice, to Cefas Weymouth where VTG and spiggin analyses were completed. Liver samples in RNAlater were held at $4^{o}C$ for 24h then stored frozen ($-20^{o}C$) until required for RNA extraction.

After dissections were completed, the remaining carcass was homogenised to provide a substrate for the measurement of whole-body determinands (cortisol, glucose, RNA:DNA ratio). Each fish was minced on a glass Petri dish with a single-edged razor blade. The minced tissue was returned to the sample tube and chilled homogenisation buffer was added (4:1; volume:weight; Tris-HCl buffer, pH 8.0 containing 0.1M NaCl, 0.01 M EDTA). The mixture was homogenised using an IKA Ultra-Turrax TP18/10 with an 8 mm dispersing tool (S25 N–8 G). Homogenate was cooled on ice between bursts. The homogenate was stored frozen until required for assay.

Note concerning VTG measurements. VTG levels for fish captured in December 2005 and March 2006 were determined in plasma, removed after taking live fish from the sample sites to Cefas Weymouth. Plasma is the preferred substrate for VTG determination, providing an estimate of circulating levels of VTG in the blood at time of capture, and permitting the retention of the carcass for additional measurements or procedures. However, blood samples can successfully be collected only from larger individuals and very few fish large enough to provide blood samples were collected during the first samples in December and March. Because the quantitative assessment of VTG levels in this study was primarily being employed to facilitate between-river and between-time comparisons it was agreed that, to maximise the information content of each sample, VTG levels would subsequently be determined in heart tissue. This approach permits the quantification of VTG in even the smallest individuals, but does not require that live fish be transported back to the laboratory. The measurement of VTG in heart tissue has routinely been used in a number of projects in which both Cefas and CEH has participated and no loss of sensitivity is evident.

5.2.5 Histology: Sufficient fish were available for histological processing during April 2007, May 2007, July 2007, September 2007, November 2007 and July 2008. The fish were killed by a lethal dose of anaesthetic and transferred to 10% neutral buffered formalin. Depending on size, the fish were either processed for embedding and sectioning whole, or were further dissected. The samples were embedded in wax, sectioned at 3µm, and stained with haematoxylin and eosin. In total, 695 fish were examined and a separate report on the outcome of the histological examination is available ("*Does Sewage Treatment Plant Remediation Improve Effluent Quality in Terms of Endocrine Disrupting Compounds? The Stickleback Perspective*." By C. Mungo, Kings College London).

***5.2.6 Vitellogenin and spiggin analysis*:** Samples were assayed for VTG (heart) and spiggin (kidney) at Cefas Weymouth, using stickleback-specific ELISAs (Hahlbeck *et al*., 2004; Katsiadaki *et al*., 2002a,b). Details of the methods are appended.

5.2.7 EROD: Activity of the cytochrome P450 1A monooxygenase, ethoxyresorufin–O-deethylase (EROD) was measured in liver tissue from selected fish, before the decision was made to use molecular techniques to quantify both choriogenin and CYP1A as relative transcript abundances. EROD is widely employed as a reliable biomarker of exposure to polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). A microplate kinetic assay requiring small amounts of tissue was adopted, based on that described by Hodson *et al*. (1996). The assay method fluorimetrically detect the conversion of 7-ethoxyresorufin to resorufin, the rate of conversion being proportional to the activity of the enzyme.

Livers were dissected from frozen sticklebacks, weighed, and homogenized in a constant volume of 400 µl phosphate buffer (pH 7.4; 80.2 ml 1.0 M K_2HPO_4; 19.8 ml 1.0 M KH_2PO_4; 700 ml distilled water; 200 ml glycerol = 252.4 g) containing 1 mM EDTA and 1 mM dithiothreitol in a 1.5 ml capped centrifuge tube, using a motor-driven pestle. Homogenates were centrifuged at 4^oC for 15 minutes.

The assay was carried out in 96-well plates in a Fluoroskan Ascent plate reader with excitation at 530 nm and emission at 590 nm. For the unknowns, each well of the plate contained 50 µl liver homogenate supernatant and 50 µl of 10.0 µM 7-ethoxyresorufin in homogenisation buffer. Each sample was measured in triplicate. In addition, an eight point standard curve was included on each plate, comprising serial dilutions from 100 µl of a 50.0 µM solution of resorufin in methanol, in duplicate (50 µl resorufin + 50 µl buffer in successive wells). The plates were incubated at room temperature for 10 mins. The reaction was initiated by the addition of 10 µl of a 24 mM solution of NADPH in buffer. Each plate was scanned immediately and at 2 minute intervals for 30 minutes. Protein concentrations in the liver homogenates were determined using the method of Lorenzen (1993). A series of seven dilutions of bovine serum albumin (BSA) ranging from 0 to 1000 µg/ml using phosphate buffered saline (PBS) pH 7.4 as the diluent was carried out in duplicate in the first two rows of the plate (75.0 µg – 1.2 µg/well). To the first and 7th well in rows D-H 140 µl dw was pipetted, giving a total volume in these wells of 290 µl. A 10 µl aliquot of sample was added to the first and 7th wells in these rows (total 10 unknowns per plate). A serial dilution of each sample was accomplished by transferring 150 µl from the first well to the second and so on, to the 6th. The 150 µl remaining in the pipette was discarded. A 50 µl aliquot of 1.08 mM (3 mg/ml) fluorescamine dissolved in acetone was added to each well using a repeater pipette. Following the addition of fluorescamine the plate was shaken for one minute. Fluorescence was determined using a plate reader (Thermo Fluoroskan Ascent FL) with a 355 nm excitation filter and a 460 nm emission filter.

Enzyme activity in each sample was calculated from the gradient of the fluorescence curves (change in FU/min). FU/min was converted to molarity by reference to the resorufin standard curve and normalised for protein concentration in each liver sample to give specific activity as picomoles resorufin produced per mg protein per minute.

5.2.8 Whole-body corticosteroids: Corticosteroids were quantified by measurement of solvent-extractable immunoreactivity in whole-body homogenates after direct homogenization of minced stickleback tissue in ethyl acetate (5:1 volume:body weight (Pottinger and Carrick, 2001; Pottinger *et al*, 2002). Homogenates were centrifuged and an aliquot of each extract (10 µl) was transferred to labelled 3.5 ml polypropylene assay tubes. A standard curve was constructed by adding to a series of assay tubes, in duplicate, aliquots of ethyl acetate containing between 12.5 and 800 pg of inert cortisol (Sigma Aldrich). Blank tubes received ethyl acetate alone. A 50 µl aliquot of ethyl acetate containing 20,000 dpm of [1,2,6,7-^{3}H]cortisol (Amersham Pharmacia Biotech; 60 Ci mmol-1) was added to all the tubes and the solvent was evaporated under a vacuum. A 200 µl aliquot of anti-cortisol antibody (AbCam; 1:1500; rabbit, polyclonal) in phosphate buffered saline (PBS; Sigma) containing bovine serum albumin (RIA grade; Sigma; 0.1 %) was added to each tube (except two non-specific binding tubes which contained ^{3}H-cortisol only) and the tubes were incubated

overnight at 4°C. After incubation, racks containing the assay tubes were placed on ice and a 100 µl aliquot of chilled, stirred, dextran-coated charcoal in PBS (2.5 % activated charcoal; 0.2 % dextran) was added to each tube. After vortex mixing, the tubes were incubated on ice for 5 mins before being spun (3000g at 4°C for 10 mins). A 200 µl aliquot of the supernatant was added to 4.5 ml of scintillant (Ecoscint A; National Diagnostics) in a vial, mixed by inversion, and counted under standard ^{3}H conditions. Cortisol concentrations in the unknown samples were calculated from the equation of a 3-parameter hyperbolic function fitted to a plot of the percentage of ^{3}H-cortisol bound against pg of inert cortisol (Sigmaplot; SPSS Science).

5.2.9 Whole-body glucose: Glucose concentrations in the homogenate were measured using a hexokinase microplate method. In brief, the homogenates were thawed on ice and centrifuged at 4°C for 5 minutes. A 15 µl aliquot was taken for assay. 130 µl distilled water and 50 µl glucose standard solution were pipetted into wells A, B row 1. In the remaining wells (A2-A12, B2-B12) 90µl distilled water were pipetted. A serial dilution of the standards was performed by transferring 100 µl from wells A1, B1 into adjacent wells. This was repeated to provide the series: 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.19, 0.0 µg / well. The final wells were retained as blanks (distilled water only). A 15 µl aliquot of each sample was pipetted into the wells as required and 75 µl distilled water was added to each. A 100 µl aliquot of diluted glucose assay reagent was added to all wells and plates were incubated for 15 minutes at room temperature (18-35 °C) on a shaker. The absorbance at 340 nm versus deionized water was measured on an Ascent Multiskan reader and a standard curve was constructed. The Sigmaplot pharmacology function (4-parameter logistic curve) was used to solve for x. Results were adjusted for dilution to give glucose concentration as mg/g tissue.

5.2.10 RNA:DNA ratios: Nucleic acids were extracted from homogenised stickleback tissue and quantified using a fluorescent dye-binding method. Total RNA + DNA was first estimated and then RNA was eliminated by treatment with RNAse, the difference in fluorescence was attributed to the RNA content. The method employed was adapted from Gorokhova and Kyle (2002) and is appended in full.

5.2.11 Choriogenin gene expression: RNA was extracted from livers using the Qiagen RNeasy mini kit (Qiagen, Germany) and was converted to cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). The yield and purity of RNA extracts was assessed at 260 nm using the Nanodrop ND-1000 spectrophotometer (Labtech, UK). Relative expression of choriogenin was determined using a StepOne real-time PCR machine (Applied Biosystems) Amplification primers and a minor groove binding (MGB) Taqman fluorogenic probe were designed using Primer Express software (v2.0; Applied Biosystems) based upon a cDNA sequence (Accession number CD492922) identified as mRNA of the choriogenin H gene from *Gasterosteus aculeatus* (EST forwarded by T. Williams, Birmingham University). Primers ChgHFP (5'-GATGCCACTCTGCCAAGCA) and ChgHRP (5'-TGGCCCATCTCCCAAAAG-3') and the MGB Taqman probe ChgHTP (5-6FAM-CGACCTCGAATCAA- 3') were checked for specificity by using BLASTn (Altschul *et al*., 1997) within the NCBI suite of facilities (www.ncbi.nlm.nih.gov). All reagents and kits used in amplifications were obtained from Applied Biosystems. Optimum primer (300 nM) and MGB probe concentration (250 nM) were determined empirically using cDNA pooled from fish liver RNA extractions as template. Duplex relative gene expression real-time PCR assays were performed in 20 µl reaction volumes. All reactions received the following: 3 µl cDNA; 10 µl Taqman Gene Expression Master Mix (2x concentration); 2 µl of each primer (300 nM); 2 µl (250 nM) MGB and 1 µl Human 18S rRNA endogenous control mixture (containing limited concentration primers and VIC-labelled MGB Taqman probe specific to 18S rRNA). The cycling parameters of 1 cycle of 50°C for 2 min (activation of uracyl glycosylase) followed by 1 cycle of 95°C for 10 min (activation of Amplitaq gold) and 45 cycles of 95°C for 15 secs and 60oC for 1 min were maintained in all cases. In accordance with accepted guidelines for carrying out the

comparative cycle threshold (CT) method the relative amplification efficiencies of template and endogenous control was tested and confirmed.

5.2.12 CYP1A gene expression: Due to inconsistencies in the results obtained with the probe and primers originally designed for this assay, we have designed alternates and will repeat the analysis. Data will be made available as a supplementary file in due course.

***5.2.13 Statistical analysis*:** Results were subjected to analysis of variance (ANOVA; Genstat for Windows V. 8, Lawes Agricultural Trust; SigmaStat 3.5, Systat Software Inc.). Where appropriate, data were log-transformed prior to analysis to improve homogeneity of variance. Multiple comparison post tests to assess significant differences between times or rivers were carried out using the estimated standard error of the differences between means provided by the Genstat output or, in the case of data analysed using SigmaStat, the Holm-Sidak test was used for post-hoc pairwise multiple comparisons.

5.3 Results and Discussion

5.3.1 Somatic data

A total of 2284 individual fish contributed data to the summary of somatic data that are presented here. The data are presented in several formats to provide a comprehensive picture of the characteristics of the populations in the two river systems. For the purposes of the analysis the data from Childrey Brook (Venn Mill; VM) have been combined with sites on the R. Ock proper and the aggregate data are referred to as R. Ock throughout, except where individual sites are considered.

Summaries of the length frequency data for the River Ray and River Ock are presented in Figures 5.3 and 5.4 respectively. These data provide some insight into the population structure on the two rivers. We lack accurate abundance data because of the difficulties in matching sampling effort across sites and rivers. Distribution of the sticklebacks was found to be patchy in the extreme and catches tended to be focused upon “hotspots” characterised by a low flow and the presence of cover (submerged and emergent macrophytes) interspersed by long stretches where no sticklebacks could be found. This problem was compounded by the flooding during July 2007 following which, at Charney Bassett in particular, stretches of the river were scoured of substrate and vegetation. Interpretation of the data is simplified because the stickleback populations on both rivers appeared to be annual, with few fish surviving beyond spawning into a second year. This is most evident for the River Ray where for example, the year-class of 2007 can be followed from May, where both the smaller 0+ 2007 fish and the larger 1+ 2006 fish were caught, through the remainder of 2007 and into 2008 as the modal value progressively increases, until July where little or no evidence of the 2007 year class remains and the bulk of the catch consisted of 2008 fish (Fig. 5.3). A similar situation is apparent for the R. Ock (Fig 5.4.). However, for the Ock, progression of the modal value along the x-axis with time is not so clearly defined and divergence of successive year classes is not so evident. For the Ock, the length frequency plots for May 2007 and 2008 and corresponding modal values indicate that the bulk of the population tends to be smaller than for the corresponding periods on the Ray. This apparent difference in overall length distribution between the populations is clearly seen in a plot of the combined frequency distributions for both rivers (Fig 5.5). The comparison of length frequency data for the two rivers suggests strongly that fish on the R. Ray spawn earlier and/or grow more quickly than fish on the R. Ock. This supposition is supported by observations at the time of sampling. For example, during May 2008 only 39 fish were caught on the Ray in total but most of these were at one of the critical sites, Elborough Bridge. In contrast, at least 20 fish were caught at each of the Ock/Childrey Brook sites. During May, fish on the Ray (where caught) were larger and clearly in spawning condition whereas those on the Ock were less advanced.

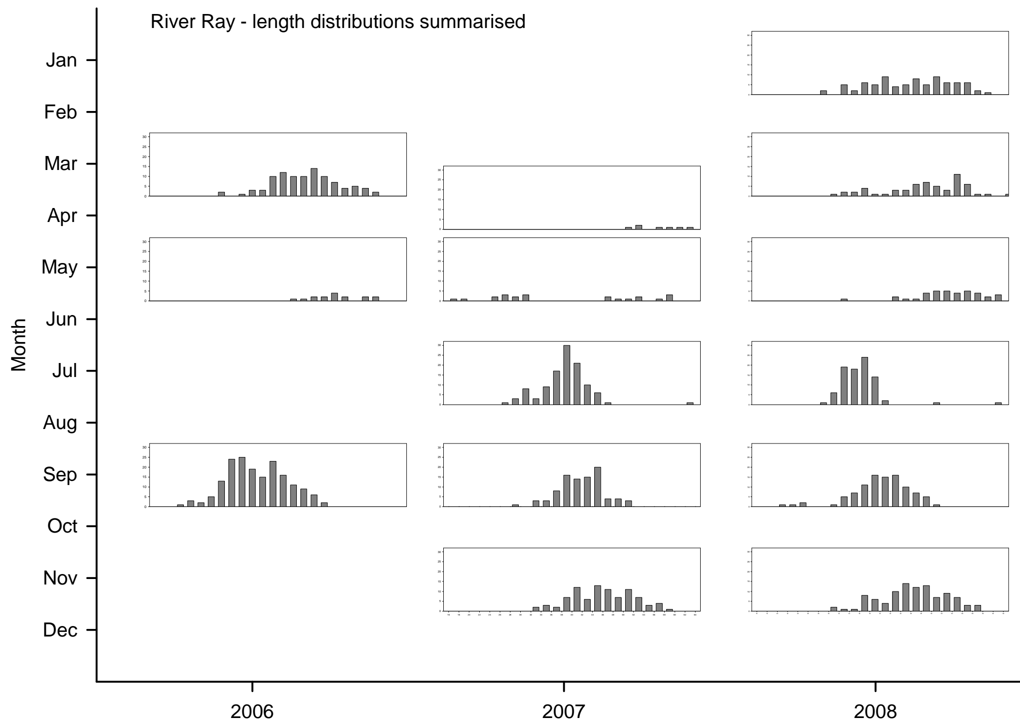


Figure 5.3. River Ray: Length frequency plots for samples 2 – 15. The x-axes (length interval) for each plot span 16-64 mm. The y-axis (frequency) for each plot spans 0-30.

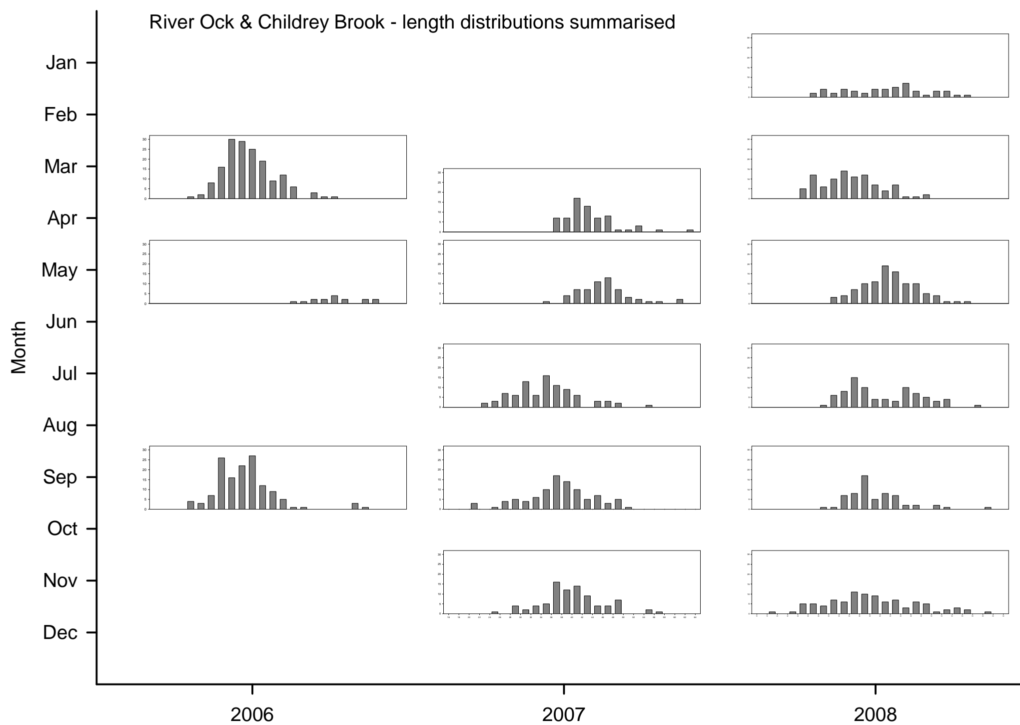


Figure 5.4. River Ock: Length frequency plots for samples 2 – 15. The x-axes (length interval) for each plot span 16-64 mm. The y-axis (frequency) for each plot spans 0-30.

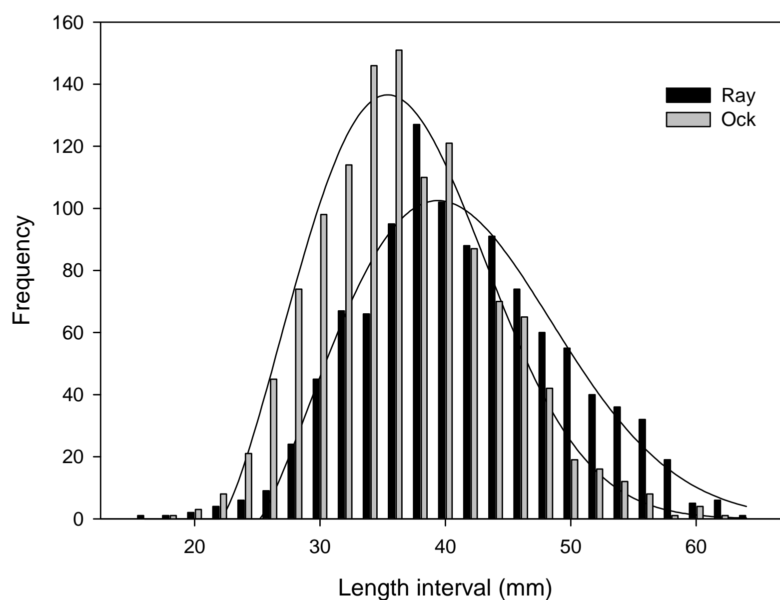


Figure 5.5. Length frequency plots for all sticklebacks at all times for both R. Ray (n = 1056) and R. Ock (n = 1217).

It is possible that the lack of fish caught in the Ray during May was due to their being dispersed on spawning beds, and/or to post-spawning mortality of adults, combined with the newly-hatched fish being too small to catch. The July sample resulted in a full complement of fish from all sites on both rivers. In the Ray most of these were clearly 0+ with very few 1+ individuals being captured. On the Ock/Childrey Brook while the majority of fish were juveniles a greater number of 1+ adults in spawning condition were caught. These observations are consistent with the hypothesis that a combination of factors results in earlier spawning of sticklebacks in the Ray.

It's likely that the underlying cause of the difference in growth/timing of reproduction is related to two aspects of the STW effluent – additional nutrient input providing a general enriching effect in the Ray (see Table 5.3, dissolved organic carbon concentrations), and the fact that the STW effluent raises the water temperature in the Ray by several degrees above that in the Ock throughout the year (see Table 5.4, and temperature logger data provided by EDCAT 3&4 in Figures 5.6 onwards). Water temperature and food availability are the two most important factors determining growth rate in fish (see Graham and Harrod, 2009, for references). Increases in temperature and food availability, either in combination or in isolation, are likely to have a growth-promoting effect on the stickleback population in the Ray, providing a longer growing season, and ultimately leading to differences in size of the fish in the two rivers.

The difference in temperature regime/nutrient loading between the two rivers, and the likelihood that this affects a number of aspects of the biology of the resident sticklebacks, in particular leading to asynchrony in the timing of reproduction, meant that the fish population in the Ock did not provide a suitable direct comparison for that in the Ray as was originally intended. This issue was further complicated by the early commissioning of the GAC plant which came on line at the end of February 2008. As noted above (5.2.2) five samples were completed after commissioning of the GAC plant, timed to match those collected during 2007 and allowing a comparison of year to year consistency within both rivers during the periods prior to and following installation of the GAC plant on the Ray.

Table 5.3. Dissolved organic carbon (DOC; mg/l) concentrations at each sample site on two occasions.

	Date of sample			
Site	November 2007		May 2008	
	DOC mg/l	mean	DOC mg/l	mean
ROD	12.00		4.96	
EB	6.32		4.40	
TB	6.39		4.82	
7B	5.93	**7.66**	5.83	**5.00**
CB	3.59		3.82	
GAR	4.08		4.22	
VM	3.69		3.86	
MM	3.11	**3.62**	6.05	**4.49**

Table 5.4. Water temperature recorded at the time of sampling on five occasions during 2008.

Site	Date of sample				
	March 08	May 08	July 08	Sep 08	Nov 08
ROD	9.8	16.5	18.5	17.2	10.8
EB	8.8	14.6	17.3	16.4	9.8
TB	9.5	15.6	17.8	17.2	10.1
7B	9.3	14.9	17.8	15.9	9.7
CB	6.2	10.1	16.8	14.4	-
GAR	6.3	10.6	16.0	14.3	6.4
MM	6.7	12.7	16.6	14.5	8.2
VM	7.1	12.1	16.9	14.7	8.1

Sample means for body mass, length and coefficient of condition for sticklebacks across the entire sampling period are presented in Figures 5.6 (males) and 5.7 (females), in which the overall larger size of the Ray fish is clear.

The fish were significantly heavier overall on the Ray (802 ± 14 mg) compared to the Ock (578 ± 13 mg; $P<0.001$) and while there was no difference overall between males and females on the Ock (561 v. 594 mg; NSD), in the Ray males were significantly larger overall than females (838 cf 765 mg; $P<0.05$) although this appears counter-intuitive because of the distribution of size across sampling times. This difference was evident for weight only, not length. Fork length of fish from

the Ray (41.6 ± 0.2 mm) was significantly greater overall than fish from the Ock (37.2 ± 0.2 mm) and on both rivers males were overall fractionally but significantly longer than females (39.7 cf 39.1 mm; $P<0.05$). There was no significant difference overall in coefficient of condition (K-factor) between rivers although in the Ock, the K-factor of female fish (1.024 ± 0.008) was slightly but significantly greater than that of male fish (0.993 ± 0.09; $P<0.05$))

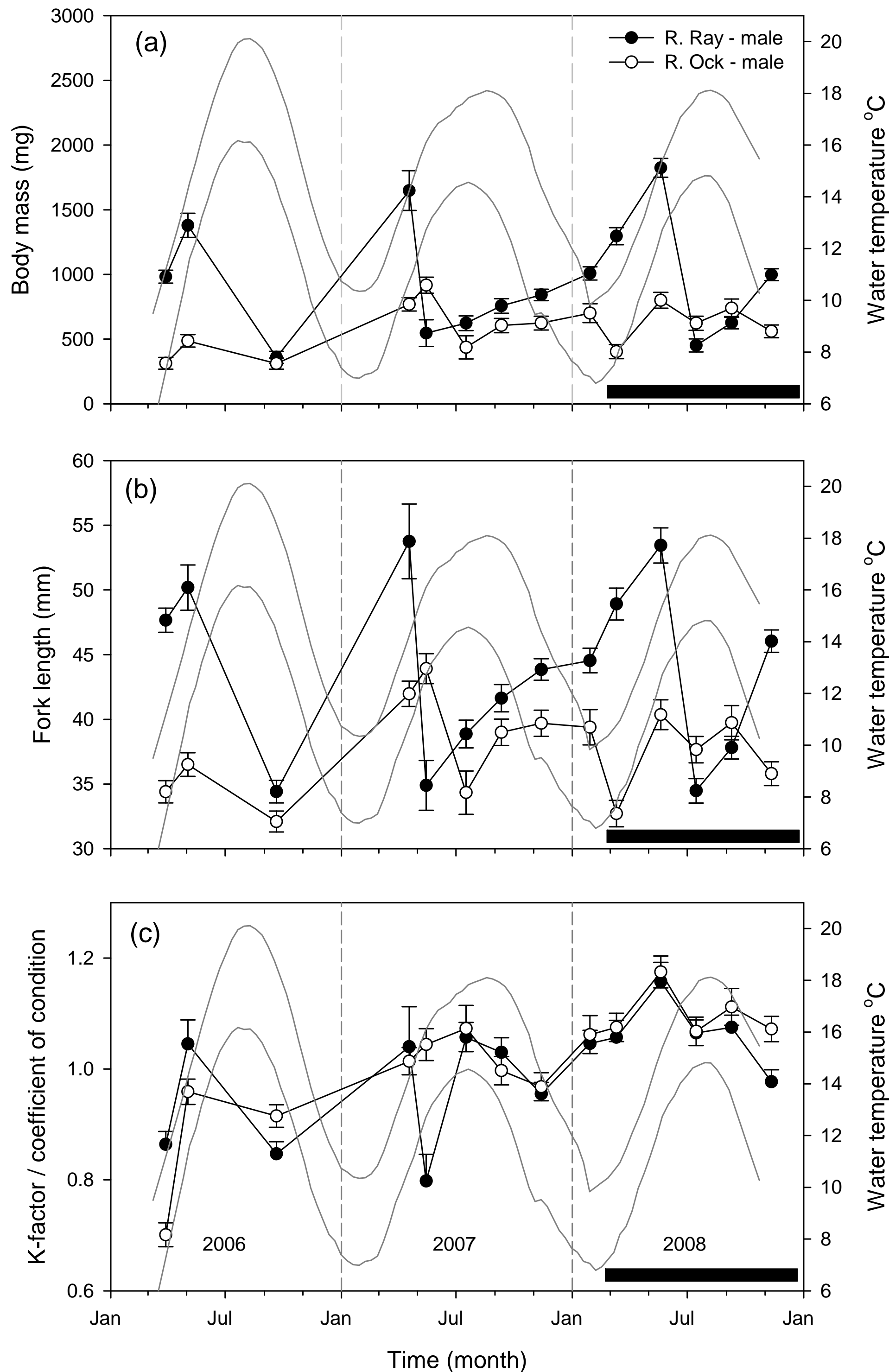


Figure 5.6. Mean values (± SEM) for (a) body mass, (b) fork length and (c) coefficient of condition for male sticklebacks in each river at each sampling time. The black bars indicate the period during which the GAC plant was operational at Rodbourne STW. The grey lines indicate water temperature on the R. Ray (upper line) and R. Ock (lower line)

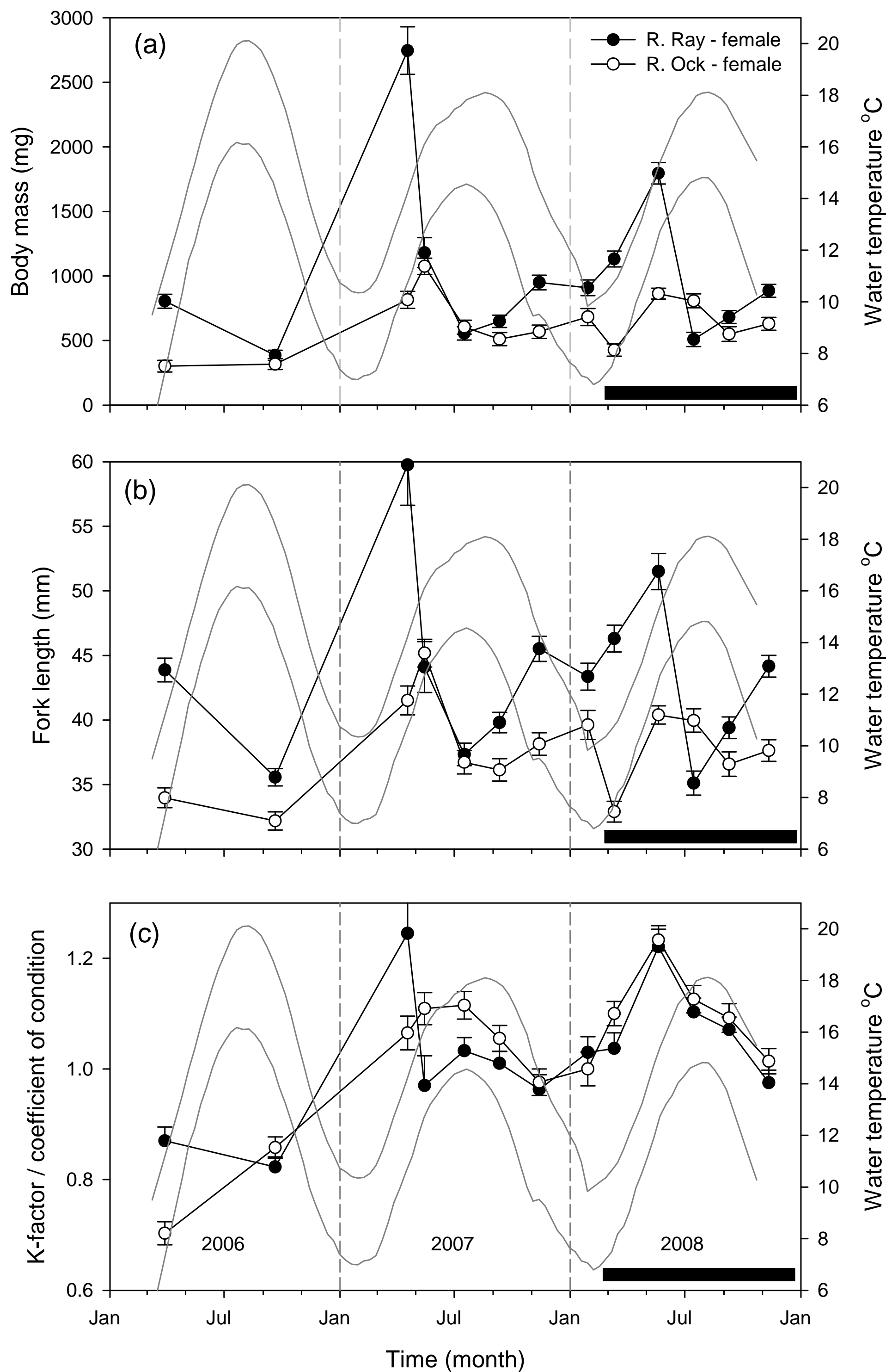


Figure 5.7. Mean values (± SEM) for (a) body mass, (b) fork length and (c) coefficient of condition for female sticklebacks in each river at each sampling time. The black bars indicate the period during which the GAC plant was operational at Rodbourne STW. The grey lines indicate water temperature on the R. Ray (upper line) and R. Ock (lower line)

Figure 5.8 presents the mean values for the three main somatic measures for each river (sexes combined) during the periods of interest (March-Nov 2007; March-Nov 2008). Overall and predictably so when considering the data shown in Figs 5.6 and 5.7, body mass of fish in the Ray was significantly greater than that of fish in the Ock (Fig. 5.8a; ANOVA, $P<0.001$). Less predictably, in the Ray there was a significant increase in body mass during the period following installation of the GAC plant (ANOVA, $P<0.001$) compared to the period prior to its installation whereas body mass of fish in the Ock remained similar across the two periods.

In addition, as evident in Figs 5.6 and 5.7 fish in the Ray were significantly longer overall than those from the Ock (Fig. 5.8b; ANOVA, $P<0.001$) and, mirroring the change in mean body mass, there was a significant increase in mean length between 2007 and 2008 in the Ray ($P<0.05$), whereas in the Ock there was a small but significant decline in mean length across the two periods ($P<0.05$). In contrast to the results of the analysis of the full data set, in this sub-set here there was a highly significant difference in coefficient of condition between rivers. Overall, the mean coefficient of condition for fish from the Ock was significantly greater than that of fish from the Ray (P<0.001). In both rivers there was a small but significant increase in condition between 2007 and 2008 ((P<0.001; Fig. 5.8c).

The coefficient of condition (K-factor, Fulton condition factor) is a means of describing body condition (Pope and Kruse, 2007) and has been shown to reliably predict the chemical composition (relative energy content) of body tissues for a number of fish species (Pangle and Sutton, 2005). As noted by these authors, the use of the coefficient of condition for comparative purposes is based on the assumption that fishes exhibit isometric growth (i.e. the slope of the mass and length relationship has an exponent = 3, body shape does not change with age) where this assumption cannot be fulfilled only comparisons between fish of similar length should be undertaken. In the present study a regression of log[body mass] against log[fork length] for the Ray and Ock populations provided very similar best-fit lines with exponents close to 3 (Ray: $y = 0.0083x^{3.0246}$; Ock: $y = 0.0057x^{3.1319}$) suggesting that comparison of the coefficient of condition provided an unbiased estimate of relative condition for fish in the two rivers, across the full range of lengths. If this is the case, the higher condition coefficients of the fish in the Ock suggest that, despite the overall larger size of the fish in the Ray, the fish in the Ock were in better condition (heavier at a given length) than fish in the Ray. This seems counter-intuitive but may be related to the assumed earlier spawning of fish in the Ray, and interplay of water temperature and food availability. Although the Ray population hatches and begins growing earlier than fish on the Ock, early growth may occur during a period when food items are more scarce, or when the energetic demands of obtaining food are greater. In contrast, the later spawning and hatching of the Ock population may coincide with greater availability of food and a better match between temperature as a driver of metabolic demand, and food availability, resulting in different patterns of length and weight gain across time between the two populations.

Over and above the difference in condition between rivers, for both the Ray and Ock there was a significant increase in coefficient of condition between 2007 and 2008 (Fig. 5.8). A more pronounced trend is evident in Figures 5.6 and 5.7 when the coefficient of condition during 2006 is compared with that in 2008. These trends may be related to trends in whole-body concentrations of corticosteroids and glucose that show similar but inverse patterns of change (Figs 5.22 and 5.24). It is suggested (see (5.3.11 and 5.3.12) that these reflect the impact of severe environmental conditions and provide indication that the resident fish were exposed to intermittently stressful changes in flow regime. It is possible that the year on year changes in condition are also related to these events.

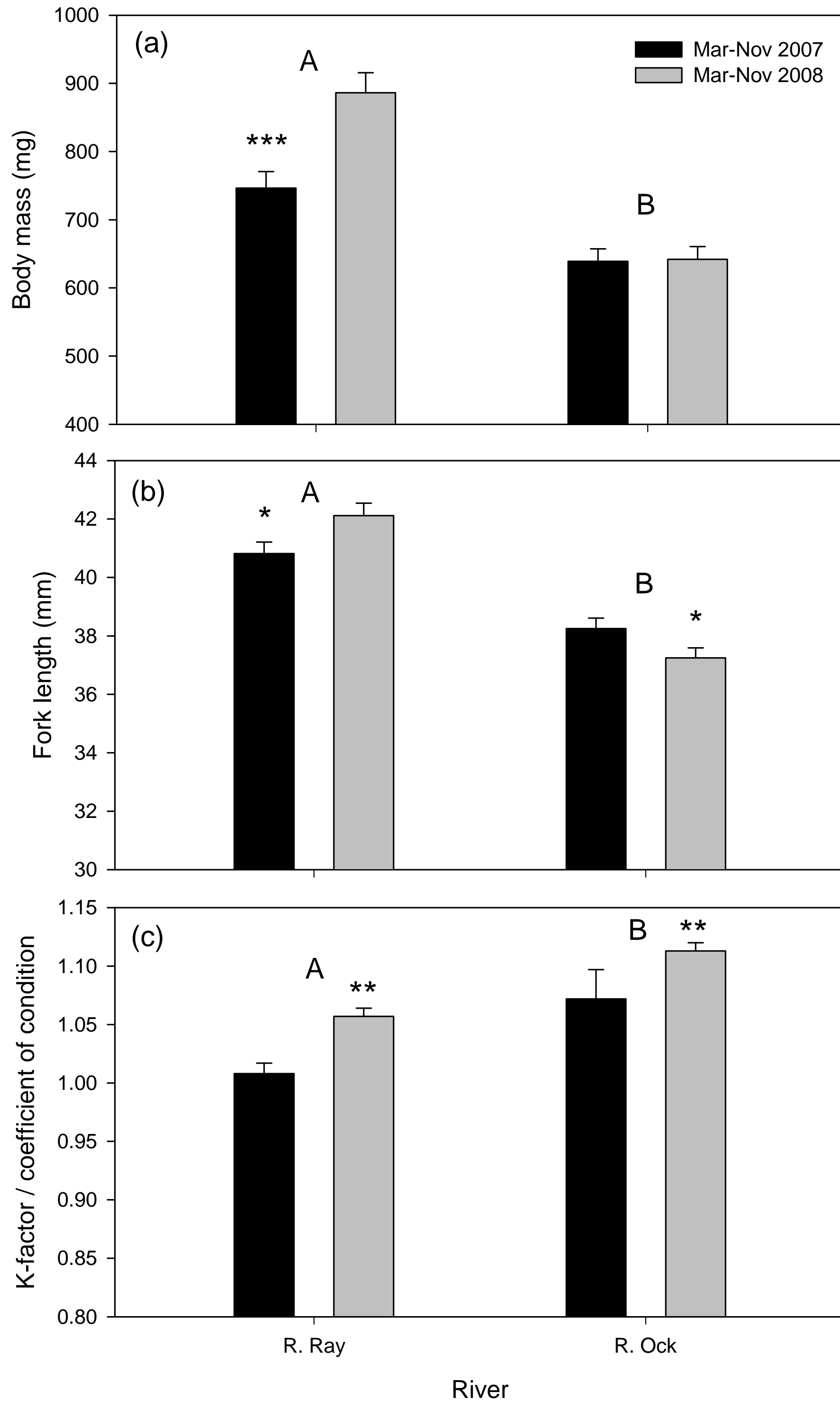


Figure 5.8.(a) body mass, (b) fork length and (c) coefficient of condition for sticklebacks from the R. Ray and R. Ock during March – November, 2007 and 2008. Each bar represents the mean (+ SEM) values of between 317 and 436 individuals. Significant differences between the two monitoring periods within a river are denoted by asterisks: * *P*<0.001; ** *P*<0.01; * *P*<0.05. Significant differences overall between rivers are indicated by different letters.**

Sex ratio: The male:female ratio deviated markedly from unity among both the fish caught from the R. Ray and those caught from the R. Ock when all samples were considered together (Table 5.5). This bias was also reflected among the fish processed for histology (see separate histology report). A similar situation was apparent for the sample period March-November 2007. However, during the period March-November 2008, the deviation from unity for the R. Ray fish was not statistically significant (Chi square test) whereas during the same period the sex ratio for the R. Ock population remained significantly biased in favour of females.

Table 5.5. Male:female sex ratios for the overall sampling period, and for the periods prior to and following installation of the GAC plant on the R. Ray. The significance with which the observed ratios deviate from the expected (1:1) is given.

	Sex ratio (M/F)			
Period	**R. Ray**	**Chi square**	**R. Ock**	**Chi square**
Overall (Mar 2006 – Nov 2007)	0.732	$P<0.001$	0.591	$P<0.001$
May-Nov 2007	0.687	$P<0.001$	0.629	$P<0.001$
May-Nov 2008	0.794	$P = 0.20$	0.528	$P<0.001$

There are few published data with which to compare these results and on first inspection they would appear surprising – while it might be hypothesised that chemical elements of the STW effluent entering the Ray may exert effects on phenotypic sex, here the ratio is biased in favour of females on both rivers and perhaps to a greater extent on the Ock than Ray. However Wootton (1984) cites several studies of stickleback populations, in all of which females outnumbered males to a significant extent. He suggests that such a situation may arise due to a higher mortality rate among males than females, assuming that no bias exists in the sex of fertilised ova. If this is the case, such mortality does not seem to be associated specifically with the spawning period, as might be expected. When the ratios for the July, September and November samples alone are considered, where the bulk of the population will be 0+ fish with no prior reproductive experience, the ratios still reflect those that are obtained from the whole sample period. This observation also makes it unlikely that the disparity in sex ratio arises from the relative "catchability" of male and female fish at spawning time either due to evasive capabilities or to unequal patterns of dispersion.

5.3.2 RNA:DNA ratios

The concentration of DNA in somatic tissues is relatively constant whereas the concentration of RNA is proportional to the amount of protein synthesis that is occurring (Clemmesen, 1994; Chícharo and Chícharo, 2008). Consequently, whole-body RNA:DNA ratios have been widely used to assess the condition and growth of larval fishes (Richard *et al*., 1991; Buckley *et al*., 1999). In three-spined sticklebacks the whole-body RNA:DNA ratio has been shown to be positively correlated with growth rate (Ali and Wootton, 1998). The RNA:DNA ratio provides a useful adjunct to other somatic data by providing information on growth status at the time of capture, rather than an integrated measure of growth prior to the time of the sample as provided by direct measures of length or body mass. The RNA:DNA ratio can therefore be more confidently related to conditions pertaining at the time of capture of the individual.

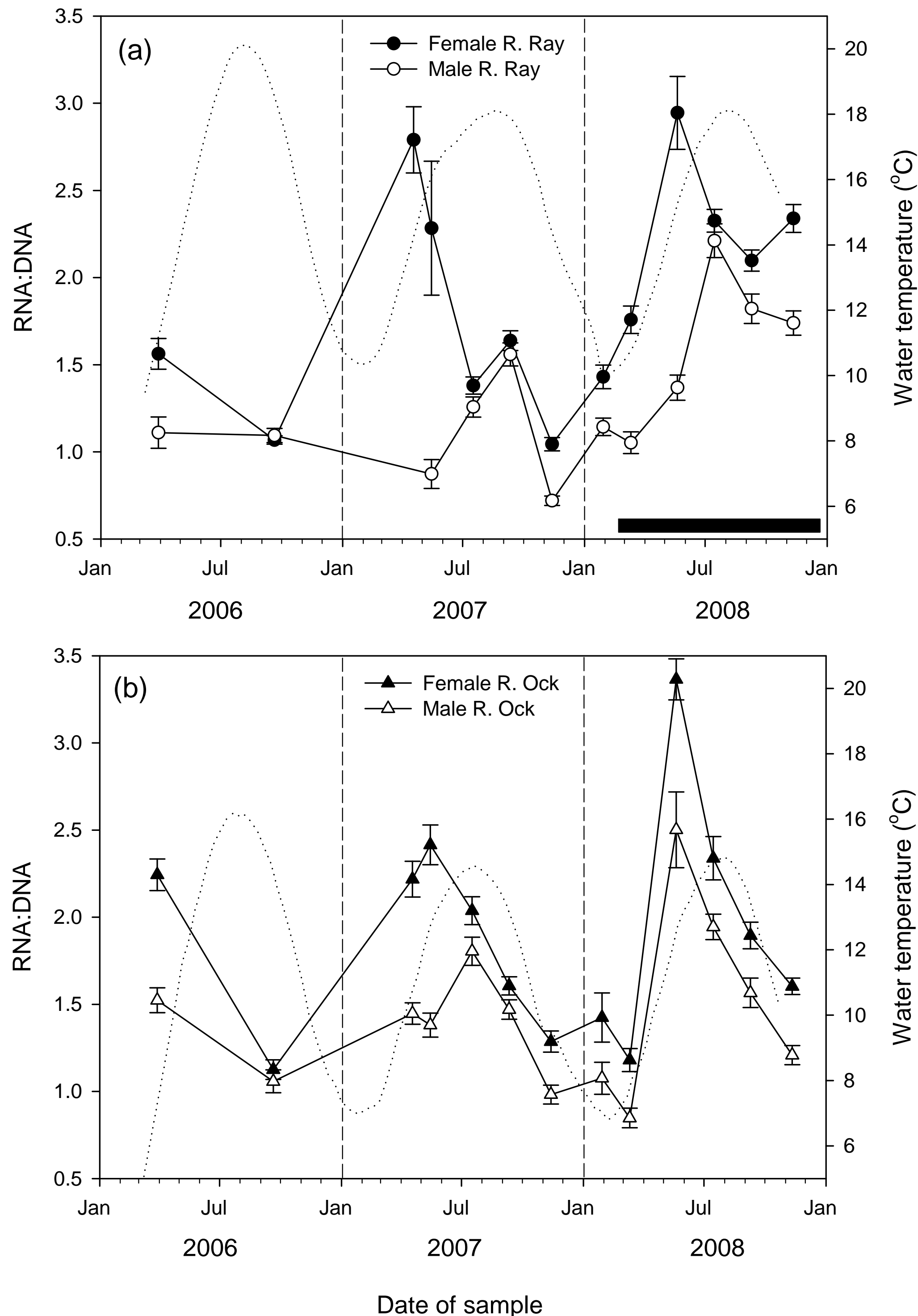


Figure 5.9. RNA:DNA ratios in male (open symbols) and female (solid symbols) sticklebacks from (a) the R. Ray and (b) the R. Ock. Each point is the mean ± SEM. The black bar denotes the period during which the GAC plant was operational on the R. Ray. Water temperature for each river is depicted by the dotted line.

Mean RNA:DNA ratios for all sample times and both rivers are shown in Fig 5.9. As is clear from the graphs, there was a difference between male and female fish with the latter having a higher RNA:DNA ratio overall ($P<0.001$; Female: 1.862 ± 0.02; Male: 1.415 ± 0.03). There was also an overall difference in RNA:DNA ratios between rivers with ratios in the Ock being higher overall

($P<0.05$; Ray: 1.593 ± 0.03; Ock: 1.685 ± 0.03). Changes in RNA:DNA ratio showed a clear seasonal periodicity and appeared to be closely entrained by temperature, as would be expected. There was a tendency for the RNA:DNA ratio in females to reach a maximum in advance of the temperature maxima whereas in the males there was closer alignment between RNA:DNA ratio and temperature although in the Ock in 2008 the changes in RNA:DNA ratio in both males and females were closely correlated. It is not immediately evident why RNA:DNA ratios for fish from the Ock, which experienced a shorter growing season and attained a smaller maximum size, should overall be higher than those in fish from the Ray – however, see data for the matched time periods in Fig 5.10 below

The RNA:DNA data do seem to clearly confirm the longer growing season for fish in the Ray. In Fig 5.9a the RNA:DNA ratio increases between November 2007 and January 2008 and continues to increase thereafter until May (females) or July (males) In marked contrast, for fish in the Ock (Fig. 5.9b) the RNA:DNA ratio only shows sustained increases between March and May 2008.

There was a significant relationship between mean RNA:DNA ratio and mean body mass, length and coefficient of condition in females ($P<0.05$ – $P<0.01$; r^2 = 0.3, 0.2, 0.3 respectively) by time but in males the regression was significant only for coefficient of condition ($P<0.05$; r^2 = 0.16). No significant relationships between somatic measures and RNA:DNA ratio could be demonstrated for either sex at an individual level.

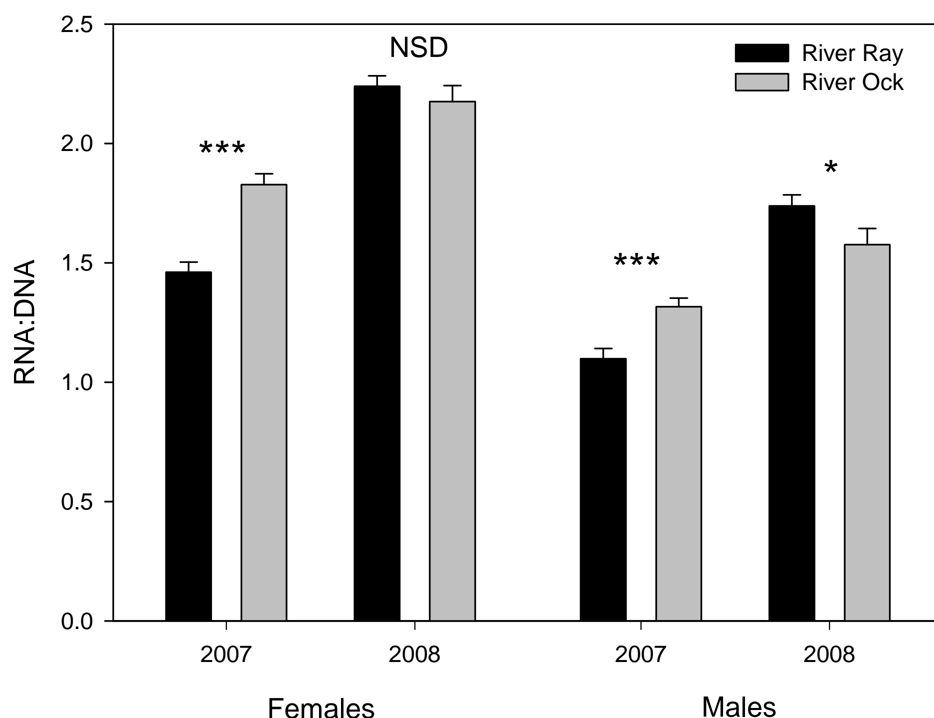


Figure 5.10. RNA:DNA ratios in female and male sticklebacks during the periods Mar-Nov 2007 and Mar-Nov 2008. Mean values for the R. Ray population are denoted by black bars, those for the R. Ock population by grey bars. Each bar is the mean + SEM. Significant differences between the two rivers within time points are denoted by asterisks: * *P*<0.001; * *P*<0.05; NSD – no significant difference.**

Analysis of the period November-March in 2007 and 2008 revealed that while during 2007, for both males and females, the RNA:DNA ratio was higher in fish from the Ock than the Ray ($P<0.001$; Fig. 5.10), in 2008 this difference had been eliminated and in fact reversed for males ($P<0.05$).

5.3.3 Summary – somatic results

1. The stickleback populations in both the Rivers Ray and Ock were found to be annual with few adults surviving into a second year. There were pronounced differences in the somatic characteristics of the populations in the two rivers with sticklebacks in the Ray being overall larger, and spawning earlier, than those in the Ock. The Rodbourne STW effluent is likely to have been the primary determinant of these differences.

2. The effluent exerts two effects that may influence the resident fish; the water temperature in the Ray was consistently 2-3°C above that in the Ock which is likely to have both direct and indirect effects on growth, and the effluent introduces additional nutrients which while not affecting the fish directly are likely to affect the abundance of food items.

3. One or both of these factors is probably responsible for the earlier spawning of sticklebacks on the Ray, and their overall larger size compared with fish in the Ock.

4. There are two key observations relating to the installation of the GAC plant at Rodbourne:

   (i) During the period following installation of the GAC plant (March-November 2008) there was a significant increase in mean body mass and length of sticklebacks in the Ray compared to the matching period in the previous year. In contrast the mean body mass of fish in the Ock remained similar across the two periods and mean length of fish in the Ock actually declined.

   (ii) During March-November 2007 the mean RNA:DNA ratio was higher in fish from the Ock than the Ray. However, in 2008 this difference had been eliminated and in fact reversed for male fish. In both rivers the RNA:DNA ratio was higher in 2008 than 2007. This may be related to negative effects on growth of the extreme weather conditions experienced during 2007.

5. Although we are constrained by having only a single year of pre- and post-remediation data, these observations suggest that performance of the stickleback population in the R. Ray improved following installation of the GAC plant.

6. It would not be unreasonable to suggest a direct causal link between remediation and improved performance – the data provided by EDCAT 3&4 clearly show that prior to 2008 the Rodbourne effluent contained a mixture of potentially harmful/disruptive organic chemicals including steroidal estrogens (E1, E2, EE2), anti-androgens (including flutamide), personal care products (triclosan) and pharmaceuticals (ibuprofen, naproxen, ketoprofen), phenols (octylphenol, bisphenol-A) and PAHs.

7. There was a broad improvement in condition of the fish between 2006 and 2008, in both rivers. This may be related to the extreme flow events during 2007 that are discussed more fully in 5.3.11 and 5.3.12.

Following installation of the GAC plant estrogen concentrations in the effluent declined markedly and its likely that a wide range of organics were removed by the same mechanism: granular activated carbon treatment is reported to remove up to 99% of dissolved organic contaminants from waste water (Kim *et al*., 2007; Ternes *et al*., 2002). Although not present individually at levels presumed to exert acute effects the consequences of long-term exposure to the mixture of potential toxicants present in the Ray, both through direct water-born exposure and via ingestion of contaminated food items, cannot easily be predicted. It may reasonably be supposed that the mixture of contaminants present, which will inevitably have been more extensive than the range

selected for quantification, represented a challenge to the resident fish, either as a consequence of direct adverse effects or by requiring a diversion of resources to cope (e.g. by detoxification or clearance). Alleviation of an ongoing chemical challenge of this nature might be expected to be reflected in improved performance.

It is also reported by EDCAT3&4 that dissolved oxygen (DO) concentrations were higher during 2006 and 2007 in the Ock (Charney Basset; site 10; 100% saturation) than in the Ray at Rodbourne (site 3) where they rarely exceeded 75% saturation. However, during 2008 in the Ray at Rodbourne DO concentrations exceeded 85% saturation. The extent to which the DO concentrations at Rodbourne are representative of those further downstream of the STW is not documented, although certainly the temperature differential between the Ock and Ray was sustained throughout the full range of sample sites. Whether the increase in DO in 2008 can be attributed to the operation of the GAC plant or not, the net effect on fish populations in the Ray is likely to be beneficial. Hypoxia has been reported to cause disruption of reproductive processes in fish (Wu *et al*., 2003) and although a DO concentration of 70% saturation is unlikely in itself to be harmful to sticklebacks the interaction between factors such as exposure to chemical contaminants, water temperature and oxygen availability is likely to be of functional significance. All these factors represent potential constraints on performance and in combination such effects are likely to be exacerbated.

With regard to specific somatic effects that may be attributed to potential endocrine-disrupting chemicals present in the STW effluent, there was a marked sex ratio bias evident in both rivers, in favour of females. However, this was more pronounced in the Ock than the Ray and was most likely related to life-history factors. Furthermore, alterations in sex ratio would be expected to be accompanied by an increased incidence of intersex individuals. However, histological examination of fish from both rivers provided no evidence of ovotestis in male fish. These findings are consistent with the chemistry data reported in Section 4. Jobling *et al*. (2006) provided estimates for the no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) for the induction of intersex in (non-native) cyprinid fish. For the three steroids measured directly by EDCAT3&4 these are respectively 1.0, 10.0 (E1); 1.0, 10.0 (E2); and 0.2, 2.0 (EE2) ng l^{-1}. For all three steroids in the Ray, mean annual concentrations failed to exceed these LOECs and the absence of intersex is unsurprising.

5.3.4 Vitellogenin - females

Vitellogenin (VTG) is a glycolipoprotein egg yolk precursor synthesised in the liver of oviparous animals such as fish. The primary endogenous regulators of VTG gene expression are the gonadal steroids 17β-estradiol (E2) and estrone (E1) but a wide range of chemicals with estrogenic activity elicit VTG production both *in vivo* and *in vitro*. In males, the VTG gene is present and inducible by estrogens but because estrogen levels in males are normally low there is little circulating VTG present. Exposure of male fish to exogenous estrogenic substances results in the synthesis of VTG. Thus elevated circulating VTG levels in male fish has become the default biomarker for aquatic environmental contaminants with estrogenic activity (Hansen *et al.*, 1998). Female VTG levels are not normally considered a useful biomarker of endocrine disrupting influences because the pivotal role played by VTG in the female reproductive process means that through the life-cycle of the female fish, circulating levels of VTG extend across an extreme concentration range, and exhibit considerable inter-individual variability, potentially masking effects of exogenous endocrine modulators. However, in the current investigation, where bi-monthly time-course sampling was deployed rather than single-point sampling, the continuity across time offered an opportunity to re-evaluate the diagnostic usefulness of VTG in female fish.

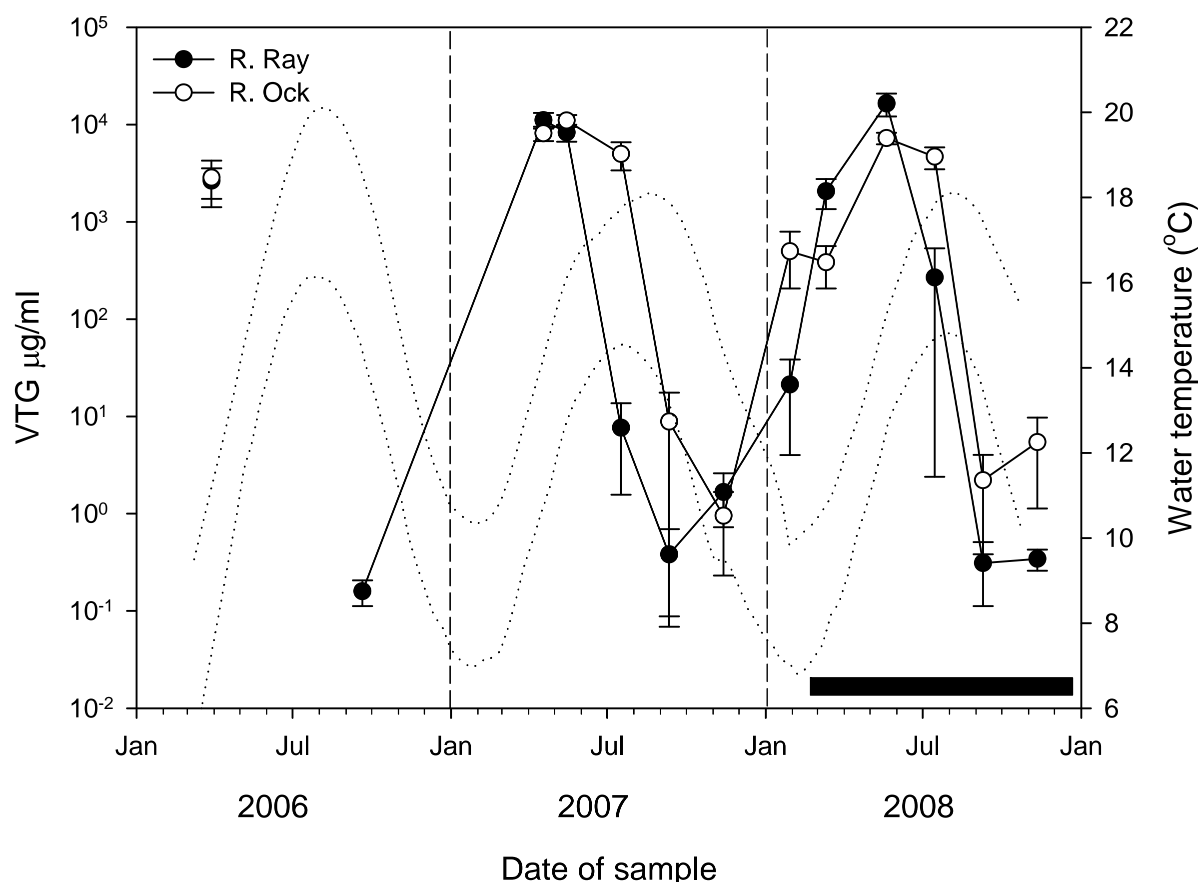


Figure 5.11. Log VTG concentrations (plasma/heart) in female sticklebacks from the R. Ray (●) and R. Ock (○) at all time points. Each value is the mean ± SEM. The upper dotted line depicts average daily water temperature at site 3 (Rodbourne STW) on the R. Ray. The lower dotted line depicts average daily water temperature at site 10 (Charney Basset) on the R. Ock. The black bar indicates the period during which the GAC plant was operational at Rodbourne STW. – Note: VTG was determined in plasma for the March and September 2006 samples, and in heart tissue for all remaining time points.

Mean vitellogenin (VTG) concentrations in female sticklebacks for each river, all sites combined, at every sample time are presented in Figure 5.11. The plots clearly show the seasonal cycle of VTG with an annual peak around May and lowest levels observed during September. The two plots depicting VTG concentrations for fish in the R. Ray and the R. Ock (Fig. 5.11) are offset suggesting that for at least two of the years studied, VTG levels in fish in the Ray were not changing in tandem with those in fish from the R. Ock. This observation is consistent with the picture presented by the somatic data, suggesting that growth and reproduction of the populations from the two rivers does not proceed synchronously.

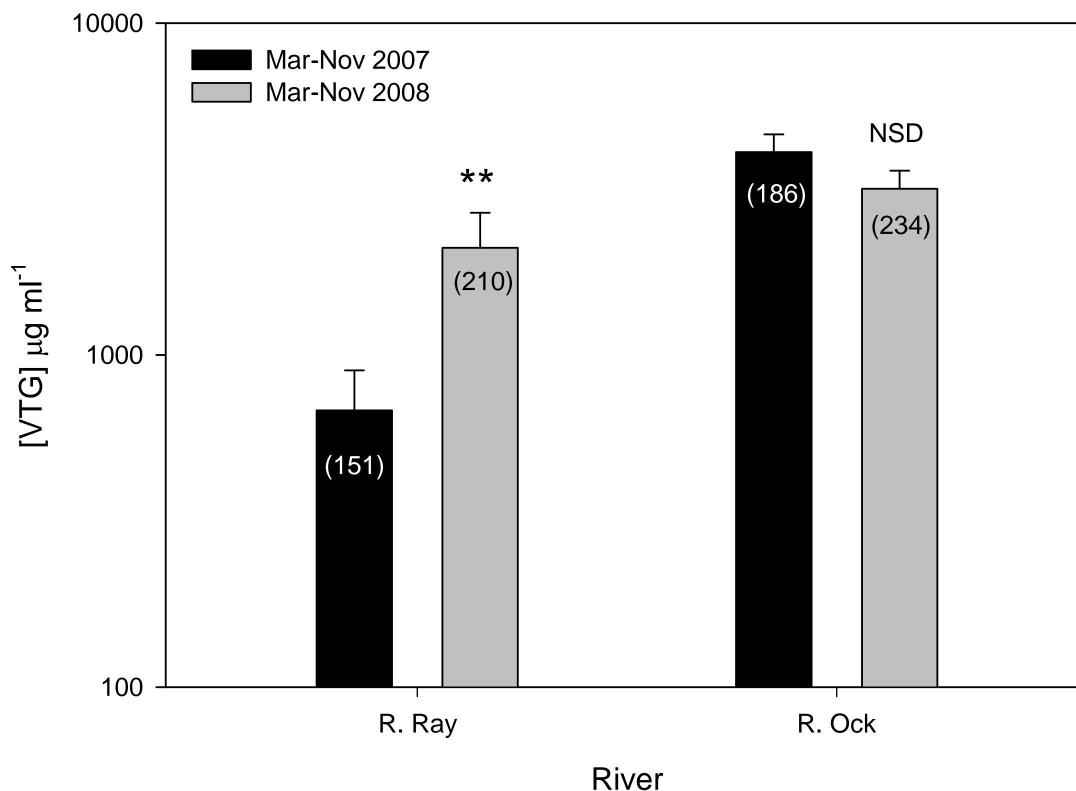


Figure 5.12. Log VTG concentrations (heart) in female sticklebacks from the R. Ray and R. Ock restricted to the periods March-November, 2007 (black bars) and 2008 (grey bars). The GAC plant came online during 2008 on the Ray. Each bar is the mean + SEM, with n indicated in brackets. Significant differences between the two time periods denoted by: ** *P*<0.01. NSD – no significant difference.

The restricted data set for the periods March-Nov 2007 and March-Nov 2008 are presented in Figure 5.12. Overall, VTG levels during these periods were significantly higher in fish from the R. Ock (3574 ± 337 µg/ml) than the R. Ray (1509 ± 349 µg/ml; ANOVA, *P*<0.001) and whereas no significant changes in VTG concentrations were detected between 2007 and 2008 for the R. Ock, in contrast, there was a significant increase (ANOVA, *P*<0.01) in mean VTG levels in fish from the R. Ray during the period in which the GAC plant was operating (Mar-Nov 2008) compared to the corresponding period a year earlier.

The extent to which VTG concentrations varied across sites is illustrated in Figure 5.13 and even here the overall change in mean VTG concentrations in female fish on the R. Ray between the periods prior to and following the commissioning of the GAC plant is clearly evident. The mean VTG concentrations at the STW site are markedly lower in both periods because few female fish were caught during the March and May samples when VTG levels were at their highest. The key

observation is that while there was no evidence of significant change in mean VTG levels for female sticklebacks from the R. Ock between the two periods, there was a clear change on the Ray, with levels overall being higher post-remediation. Whether this represents an alteration in the reproductive endocrinology of female sticklebacks on the Ray, and/or a change in timing of reproduction, is unclear. It would not be unreasonable to suggest that the remediation process may have eliminated chemicals from the effluent that were exerting an influence on one or more of these parameters, for example anti-estrogens, aromatase inhibitors or androgens.

It is also notable that the relative concentrations of VTG between sites remains similar in the pre- and post-remediation periods. On the R. Ray, fish from the Seven Bridges site exhibited the lowest mean values during both periods and on the Ock lowest mean VTG levels are evident in fish caught at Venn Mill. Some of these differences are statistically significant (e.g. GAR > VM, $P<0.001$).

The reasons for these apparently robust site-to-site differences in mean VTG levels are currently obscure but suggest local influences on the reproductive system that may be either population related (genetic) or environmental.

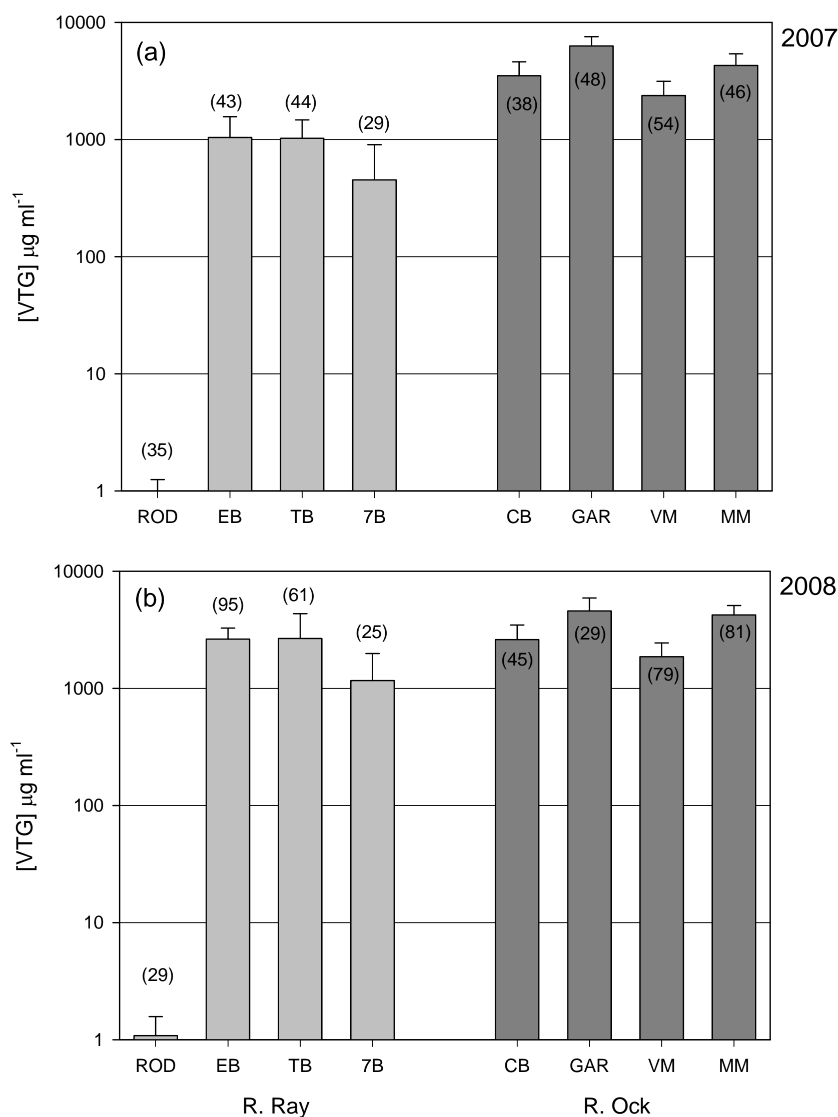


Figure 5.13. Log VTG concentrations (heart) in female sticklebacks from sites on the R. Ray (light grey bars) and R. Ock (dark grey bars) restricted to the periods (a) March-November 2007 and (b) March-November 2008. Each bar is the mean + SEM, with n indicated in brackets.

5.3.5 Vitellogenin - males

The measurement of VTG in male fish of many different species has been widely deployed as a sensitive biomarker of estrogen exposure. The male teleost liver is fully competent to synthesise VTG under estrogenic influence but normally circulating levels of VTG are very low because of the negligible levels of endogenous estrogen present in the male. Elevation of VTG in males arising from exposure to chemicals of exogenous origin is readily detected against this low baseline.

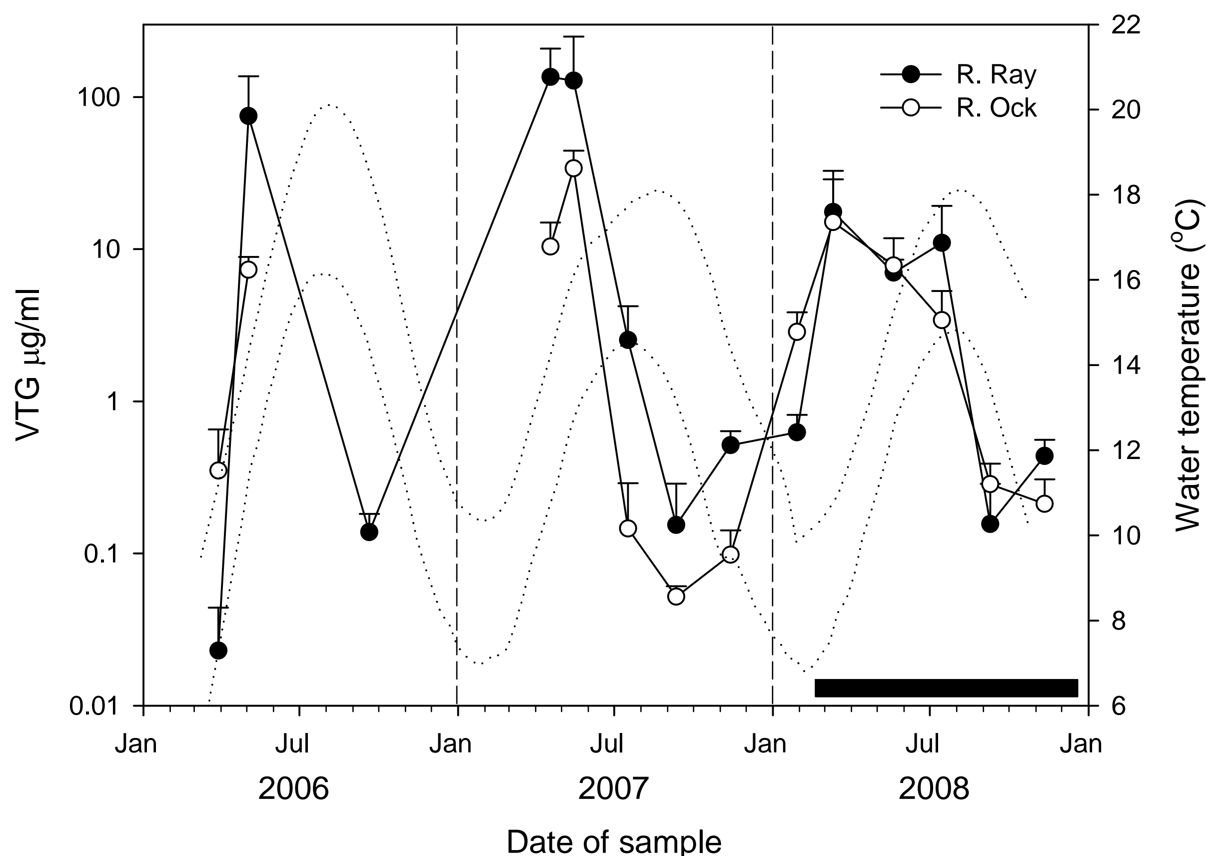


Figure 5.14. Log VTG concentrations (plasma/heart) in male sticklebacks from the R. Ray (●) and R. Ock (○) at all time points. Each value is the mean ± SEM. The upper dotted line depicts average daily water temperature at site 3 (Rodbourne STW) on the R. Ray. The lower dotted line depicts average daily water temperature at site 10 (Charney Basset) on the R. Ock. The black bar indicates the period during which the GAC plant was operational at Rodbourne STW. – Note: VTG was determined in plasma for the March and September 2006 samples, in whole-body homogenates for the May 2006 sample, and in heart tissue for all remaining time points.

The data presented in Figure 5.14 show that on both rivers VTG concentrations in male fish exhibited a seasonal cycle that closely paralleled that of VTG in the females, albeit at a very much lower concentration (maximum mean value in males ~ 100 µg/ml compared with ~ 10,000 µg/ml for the females). Detectable levels of VTG in male fish of various species not subject to a contaminant challenge have been reported previously (e.g. brown trout: Pottinger *et al*., 2005; stickleback: Allen *et al* 2008 - although Sanchez *et al* (2008) failed to detect VTG in wild-caught male three-spined sticklebacks, employing a VTG assay with a limit of detection of 256 ng ml^{-1}). It might be suggested that annual periodicity in levels of VTG in males is not unexpected given that E2 can be detected in the blood of male fish of many species (including sticklebacks: Maunder *et*

al., 2007), and the male liver is capable of producing VTG in response to E2. However, in trout at least (Pottinger and Carrick, 2000; Pottinger *et al*, 2005) although both E2 and VTG are detected in plasma, they don't show the distinct seasonality that is evident in VTG levels in male sticklebacks in the present study (Fig 5.14). An explanation for this disparity is not immediately evident.

The mean VTG concentrations for male fish during the periods Mar-Nov 2007 and 2008 are presented in Figures 5.15. There was no significant difference between mean VTG levels in male sticklebacks from the two rivers (*P* = 0.267), nor was there any significant difference between levels in fish from the two rivers after the GAC plant was brought online in 2008 (ANOVA, *P* = 0.549). Fish from both rivers displayed a similar downward trend in mean VTG concentrations between 2007 and 2008. These data provide no evidence that the STW effluent was significantly estrogenic to fish in the Ray prior to remediation.

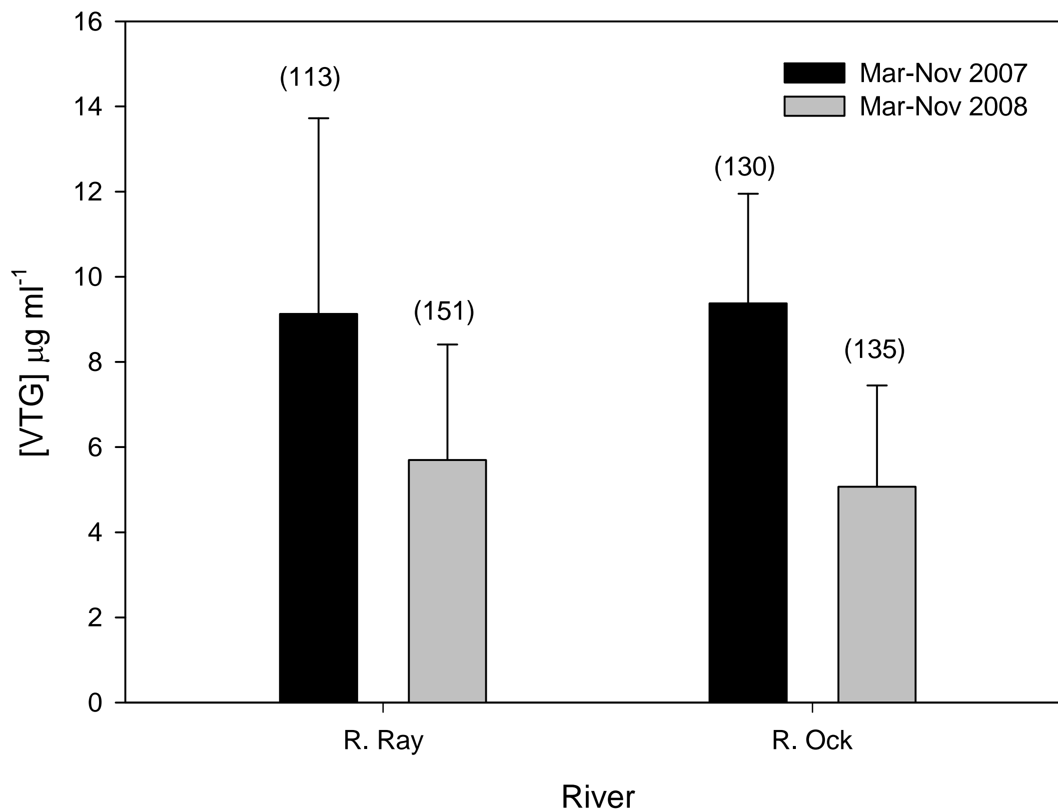


Figure 5.15. VTG concentrations (heart) in male sticklebacks from the R. Ray and R. Ock restricted to the periods March-November, 2007 (black bars) and 2008 (grey bars). The Ray GAC plant came online during 2008. Each bar is the mean + SEM, with n indicated in brackets.

The data are further broken down by site across 2007 and 2008 in Fig 5.16. .The site means broadly reflect the overall picture, with no systematic difference between rivers and lower means during 2008 than 2007. The exception appears to be the Rodbourne site. However, here the mean values are skewed by an absence of males during the periods when VTG levels were at their highest (March-May). The consistent site-to-site variation in overall mean VTG concentrations evident for the female sticklebacks (Fig 5.13) is not apparent here.

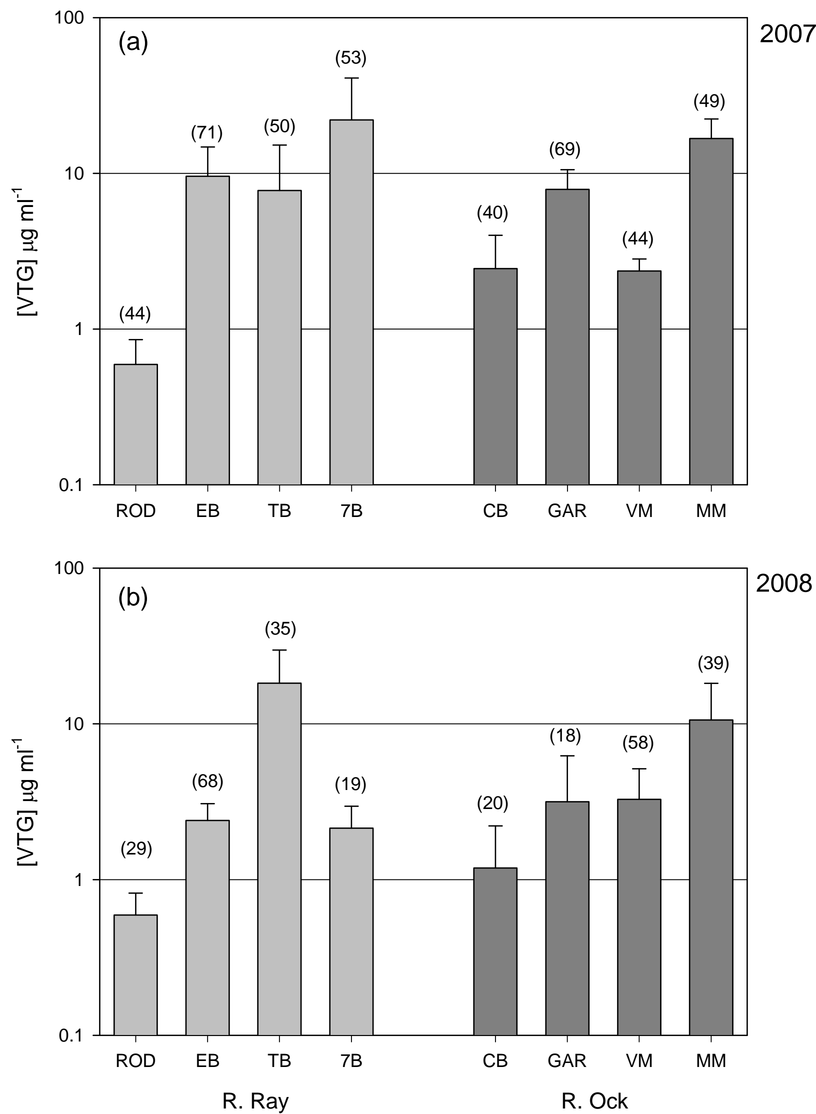


Figure 5.16. Log VTG concentrations (heart) in male sticklebacks from sites on the R. Ray (light grey bars) and R. Ock (dark grey bars) restricted to the periods (a) March-November 2007 and (b) March-November 2008. Each bar is the mean + SEM, with n indicated in brackets.

5.3.6 Summary - VTG data

Females

Taken overall, the VTG data for female sticklebacks provide no evidence that females resident in the Ray were subject to what might be described as "conventional" estrogenic endocrine disruption – augmentation of the endogenous estrogenic signal by estrogens of external origin. Instead there is strong evidence that the reproductive endocrine system in female sticklebacks in the Ray was to some extent depressed prior to the installation of the GAC plant – in female sticklebacks in the Ock VTG concentrations showed no significant alteration across 2007 and 2008, whereas in the Ray there was a significant increase in VTG concentrations in the females during 2008 relative to 2007.

As already noted (see citations in section 5.3.3) operation of the GAC plant is likely to have very significantly reduced the levels of most if not all the organics present in the effluent. It may reasonably be hypothesized that the pattern of VTG data across 2007 and 2008 in female sticklebacks in the Ray arose because the cocktail of chemicals present in the pre-remediation effluent contained some that (either alone or in combination) exerted a suppressive (anti-estrogenic) effect on the female stickleback reproductive system such that a net outcome was a reduction in the concentration of circulating VTG. The female reproductive axis is vulnerable to interference at many loci and mechanisms of anti-estrogenicity include direct interference via the estrogen receptor (ER antagonists or agonists), or via modulation of steroid synthesis or interconversion, or indirect interference via interaction with the aryl hydrocarbon receptor (AhR) (Nicolas, 1999; Navas and Segner, 2000; Aubry *et al*., 2005; Vaccaro *et al*., 2005; Kawahar *et al*., 2008; Chesenko *et al*., 2008; Palumbo *et al*., 2009) or via some other undefined mechanism.

Attention in the UK has focused primarily on male reproductive function in fish populations exposed to STW effluent largely because of the very pronounced effects apparent on VTG levels and gonadal structure evident in male fish exposed to estrogenic chemicals, and the reasonable assumption that this has significant adverse implications for population level performance. Single point measures of female reproductive status can be clouded by a high degree of inter-individual variability, particularly where multiple year-classes are present. In this case, with good replication, a single year-class population structure, and several matched samples across successive years we were able to detect differences that may not have been evident with a less comprehensive sampling strategy. It can be argued that the observed reduction in VTG concentrations in female sticklebacks in the current study is a more directly informative biomarker of adverse effect than elevation of VTG in males would be (had any been detected). In a recent paper, data from fathead minnow exposure studies with five contaminants with differing modes of action but similar effects (a reduction in plasma VTG concentrations) were consolidated to show that reduced VTG concentration provided a reliable predictor of reductions in fecundity (Miller *et al*., 2007).

Males

The concentrations of VTG in male sticklebacks reported here provide no evidence that the Rodbourne STW contained sufficient estrogenic activity to elicit VTG synthesis in male sticklebacks in the Ray. From data provided by EDCAT3&4 it is clear that even prior to the installation of the GAC plant levels of estrogenic steroids (E1, E2, EE2) in the effluent were very low. The annual average steroid concentrations presented in Fig. 4.1 show that during 2007 mean E1 concentrations did not exceed 3 ng l^{-1}; mean E2 concentrations did not exceed 6 ng l^{-1}; mean EE2 concentrations did not exceed 3 ng l^{-1}. In a series of composite samples collected directly from the effluent in February 2008, levels were even lower. The effluent also contained a number of other chemicals including pharmaceuticals, personal care products and phenols (see section 4.3.4 for details), some of which have known endocrine-disrupting potential. However, the concentrations at which they occur in the river downstream of the Rodbourne discharge (Table 4.6), combined with potencies very much lower than steroidal estrogens (e.g. ~0.01% in the case of bisphenol A), suggests that they do not represent a significant contribution to the total estrogenicity of the effluent.

Considering estrogens alone, the exposure level experienced by resident fish is an aggregate of the total for each steroid present but this assessment can be further complicated if the relative potencies of each steroid, in terms of their ability to induce VTG synthesis, are factored in. Inspection of the steroid concentration data presented in Fig 4.1 and Table 4.4 indicates that there is considerable variation in the relative concentration of each steroid between sites between years, within years, and even within a 24h period. In this case at least, attempting to assess the combined effect of the steroid complement in the Ray would be fraught with uncertainty. However, EDCAT3&4 also evaluated the estrogenic activity within the Ray using the yeast estrogen screen (YES) which

effectively integrates all estrogenic effects within the sample into a single estimate of total estrogenicity. Highest levels of total estrogenicity were detected during 2006 (Fig. 4.2) with a mean (as E2 equivalents) of less than 3.0 ng l^{-1} in the Ray at Rodbourne and an individual maximum of just over 10 ng l^{-1}. Levels in 2007 were lower still at all sites. [*Information made available to the authors during the course of preparing this report suggests that during 2004 the concentrations of steroids in the Rodbourne effluent as EE2 equivalents were measured as < 0.7 ng l^{-1} confirming the year to year variability in dissolved estrogens and raising questions about the selection of Rodbourne as the most appropriate site for a GAC pilot plant*.]

The VTG data clearly and unequivocally show that absolutely no estrogenic stimulation of VTG production in male sticklebacks in the Ray could be detected prior to remediation. In terms of its total estrogenicity alone, the effluent can be considered to have posed no threat to the reproductive health of the stickleback population in the Ray (see section 5.3.3 for comments on intersex). However, can this finding safely be extrapolated to other fish species?

The sensitivity of male sticklebacks to estrogens

The question of the sensitivity of the male stickleback to estrogenic stimulation, relative to other receptor organisms such as roach, has been raised during discussions within the course of this project. Very few relevant published dose-response data are available for sticklebacks and there are insufficient data available from which to draw a firm conclusion regarding the sensitivity of the stickleback to estrogenic chemicals. Scholz and Mayer (2008) provide a summary of the existing data indicating that currently the lowest observable effect concentration (LOEC) for E2 in sticklebacks lies between 15 and 100 ng l^{-1} and for EE2 around 50 ng l^{-1}.

A recent as yet unpublished study has indicated that rainbow trout were more sensitive (in terms of the threshold for VTG induction) to estrogenic treatment compared with the three-spined stickleback exposed simultaneously in the same tanks (Katsiadaki, pers. comm.). This is in accordance with previous studies on these two species where VTG induction has been reported in rainbow trout exposed to concentrations of EE2 as low as 0.1 ng l^{-1} (Purdom *et al*., 1994), whereas for sticklebacks, threshold concentrations are significantly higher (53.7 ng l^{-1}, in males; Andersson *et al*., 2007), both for 21 day exposures. However, Andersson *et al*. did not test any EE2 concentrations between 5 and 50 ng l^{-1} (nominals) hence 53.7 ng l^{-1} could be an underestimate of the level at which EE2 elicits positive VTG responses in the male stickleback. The NOEC nominal concentration for EE2 effects in male sticklebacks after 21 days of exposure is 6 ng l^{-1} whilst the LOEC is 10 ng l^{-1} (Katsiadaki and co-workers, pers. comm.). Nevertheless, this still appears to be at least 10-fold higher than the threshold concencentration of EE2 at which trout respond positively. Previous studies have shown rainbow trout to be particularly sensitive to environmental oestrogens compared with other fish species including roach (*Rutilus rutilus*; estradiol-17β; Routledge *et al*., 1998) and even zebrafish (*Danio rerio*; 4-tert-octyphenol; Van den Belt *et al*., 2003). It is difficult to make direct comparisons of the sensitivity of different species to estrogen exposure, for a number of reasons. Firstly not all published laboratory data make a distinction between nominal and measured concentrations of test chemicals, a fact that is very important particularly when working with compounds that display high affinity for glass surfaces such as EE2. Secondly, laboratories have not employed standardized exposure systems, thus flow and water exchange rates may vary to a substantial degree affecting factors such as the rate of chemical uptake by the fish. Thirdly but most importantly, different methods of measuring VTG exhibit a range of detection limits. The ELISA for stickleback VTG that was employed here has a detection limit of 0.02 μg ml^{-1} and a quantification limit of 0.08 μg ml^{-1}. The need to dilute the small plasma or tissue volumes that can be obtained from a small-sized fish such as the stickleback before assaying however moves the VTG detection level to almost 1 μg ml^{-1} of plasma.

Over and above these caveats, the apparently lesser sensitivity of the three-spined stickleback to estrogens compared to other species may have a functional basis. The hepatic estradiol receptor (ERbeta) in sticklebacks appears not to be up-regulated by esposure to estrogens (Geoghegan *et al*, 2008) in contrast to other species in which exposure to estrogens increases the abundance of the receptor protein and levels of transcripts. The ERalpha is reportedly up-regulated in sticklebacks after short-term exposure to 50 ng l^{-1} estradiol (Geoghegan *et al*, 2008) but not significantly so by 32 ng l^{-1} EE2 exposure. This observation could reflect genuine differences between E2 and EE2 in their ability to auto-regulate expression of ERalpha in the stickleback. The lack of significant induction of ERalpha by EE2 could also account, at least partially, for the modest sensitivity of the stickleback to EE2 exposure. In fish species that are perceived as particularly sensitive in responding to an estrogen challenge, a significant up-regulation of ERalpha is commonly observed and might be expected to provide a signal amplification effect. For example, rainbow trout (*Oncorhynchus mykiss*) exposed to 100 ng l^{-1} EE2 showed a 30 to 40-fold upregulation of ERalpha within the first 100 hours of exposure (Hook *et al*, 2007). Similarly, hepatic ERalpha mRNA was induced by more than 20-fold in zebrafish (Martyniuk *et al*, 2007) and in fathead minnow (*Pimephales promelas*; Filby *et al*, 2007) after 21 days of exposure to 10 ng/l EE2. Although the exposure period for the latter studies was much longer than that of Geoghegan *et al*. there is evidence to suggest that at least in zebrafish, ERalpha after 3 days of increased transcription level reaches a high and stable level of expression that are maintained unchanged until day 21 of exposure (Islinger *et al*, 2003). Interestingly, rainbow trout, fathead minnow and zebrafish are amongst the most sensitive species to estrogen treatment as assessed by plasma VTG levels. Taking these factors into account, it is unsurprising that no detectable effect on VTG concentrations in sticklebacks in the Ray was evident.

The status of other fish species in the Ray

Given the absence of effects in sticklebacks, and the low levels of estrogens in the effluent, what can be inferred with regard to effects on other fish species resident in the Ray, the most abundant of which are cyprinids? In a study in which the extent of endocrine disruption in roach in British rivers was correlated with predicted steroid estrogen concentrations, Jobling *et al* (2006) provided NOECs (no observable effect concentration, in ng l^{-1}) and LOECs for the induction of VTG in (non-native to UK) male cyprinid fish of 9.9, 31.8 (E1); 5.0, 25.0 (E2); and 0.07, 0.7 (EE2) respectively. Measured E1 and E2 concentrations in the Ray (Fig 4.1; Table 4.4) are lower than or barely exceed these NOECs and only mean EE2 concentrations exceeded the LOEC, during 2006 at Rodbourne, and during 2007 in the Ray upstream of the effluent discharge point. In a more recent study (Environment Agency, 2008; see also Katsu *et al*., 2007, and Lange *et al*., 2008) in which roach were exposed from hatch to EE2, induction of VTG (accompanied by presumed sex reversal) was detected in fish exposed for up to 518 days to 4.0 ng l^{-1} EE2 but VTG was not elevated in fish exposed to lower concentrations of <40 pg l^{-1} or 0.3 ng l^{-1}. In a second phase of this study a shorter-term exposure to 2.3 ng l^{-1} EE2 elevated plasma VTG significantly in both male and female roach.

Even taking into account the variability evident within these means the balance of probability suggests that the Ray effluent prior to remediation was unlikely to be capable of causing sustained VTG production in resident male cyprinids, such as roach, although the temporal variability in the estrogen content of the effluent means that the occurrence of transient spikes of estrogenicity that exceeded the LOEC cannot be excluded.

5.3.7 Spiggin – females

The stickleback kidney glue protein, spiggin, is synthesised and secreted under androgenic control and used by males when constructing nests. As is the case for VTG in males, female sticklebacks can synthesise spiggin when the appropriate androgens are provided. Concentrations of spiggin in the kidneys of female sticklebacks can therefore be used to detect exposure to water-borne androgens (Katsiadaki *et al.*, 2002b) and measurement of kidney spiggin concentrations in androgen-treated female sticklebacks provides a method for the quantitative evaluation of anti-androgens (Katsiadaki *et al.*, 2006). The production of spiggin is suppressed by anti-androgens but also by relatively high concentrations of estrogens (Katsiadaki, Mayer & Pottinger, unpublished). Spiggin concentrations were measured in sticklebacks from the R. Ray and Ock primarily to assess whether the resident fish were exposed to anti-androgenic chemicals originating from the STW effluent. Spiggin levels were determined in both male and female fish.

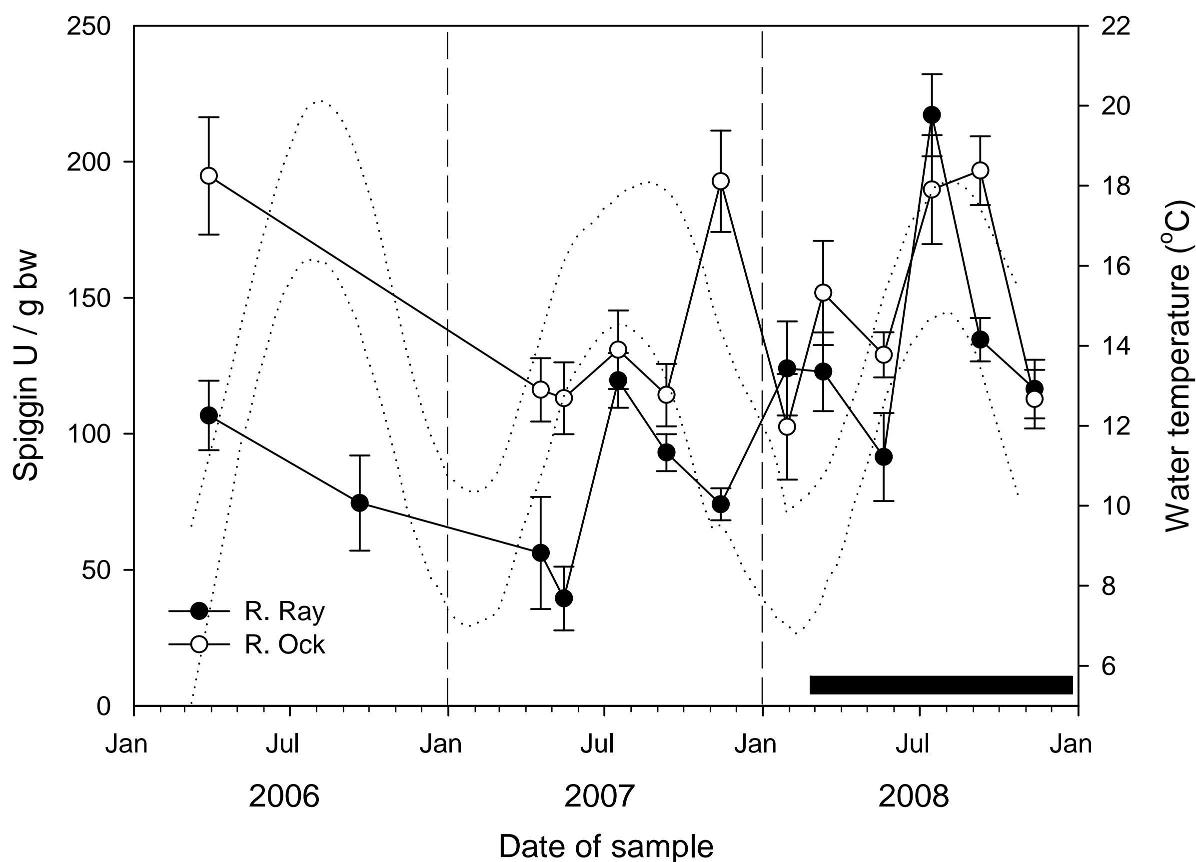


Figure 5.17. Spiggin concentrations (kidney) in female sticklebacks from the R. Ray (●) and R. Ock (○) at all time points. Each value is the mean ± SEM. The upper dotted line depicts average daily water temperature at site 3 (Rodbourne STW) on the R. Ray. The lower dotted line depicts average daily water temperature at site 10 (Charney Basset) on the R. Ock.

The mean kidney spiggin concentrations for female fish from both rivers at each sample time are presented in Figure 5.17. They are overall very low compared to spiggin concentrations in male fish (see Fig. 5.19) but nonetheless systematic variation in concentrations was evident and during 2007 and 2008 there was a broad trend for spiggin concentrations in female fish from the R. Ray to increase while spiggin concentrations in female fish from the Ock ended this period at a similar level to that at which they started. Superimposed upon this trend are at least one annual maxima for fish from both rivers. These peak levels did not coincide with the maximum VTG concentrations,

which for both male and female fish were evident around May. Peak spiggin concentrations occurred instead during the second half of the year, from July onwards.

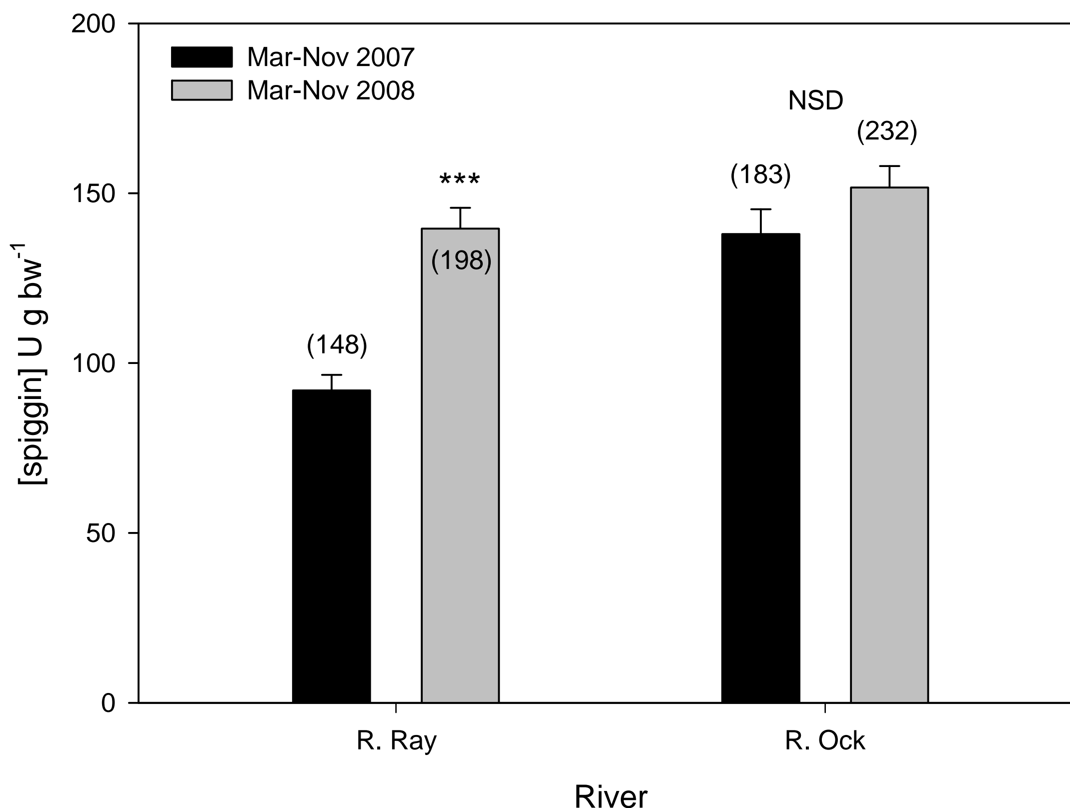


Figure 5.18. Kidney spiggin concentrations in female sticklebacks from the R. Ray and R. Ock restricted to the periods March-November, 2007 (black bars) and 2008 (grey bars). The GAC plant came online during 2008 on the Ray. Each bar is the mean + SEM, with n indicated in brackets. * - significant difference between the two time periods, $P<0.001$. NSD – no significant difference.**

The mean spiggin concentrations for the periods Mar-Nov, 2007 and 2008 (Figures 5.18) confirm these impressions. In particular there is a significant increase in spiggin concentrations in female sticklebacks in the Ray between 2007 and 2008, whereas no significant change in mean spiggin levels occurred in fish from the Ock during the same period. This interpretation is further supported by the data in Figure 5.19 which shows mean spiggin concentrations aggregated by site across all time points pre-remediation and post-remediation. Overall, mean spiggin concentrations at sites on the Ray are lower than those on the Ock in 2007. This is most marked for the Rodbourne and Elborough Bridge sites (Fig. 5.19a). This disparity is eliminated during the period in 2008 in which the GAC plant was operational (Fig 5.19b)

This observation, that a significant increase in spiggin concentration in females on the R. Ray occurred during the period when the GAC plant was operational whereas no change occurred during the corresponding period for fish in the Ock, is similar to the pattern observed for VTG levels in females. This suggests that the magnitude of spiggin concentrations in female sticklebacks is dependent upon the activity of one or more elements of the reproductive system that also influence

VTG concentrations, and/or chemical(s) affecting the production of spiggin in females were eliminated or reduced in concentration by the remediation process at Rodbourne.

Spiggin concentrations are reported as U g^{-1} body mass. A regression of all data revealed that overall, smaller females tended to have higher spiggin concentrations per g of body mass than larger females. We don't believe that this relationship alters the way in which the data should be interpreted, particularly since both spiggin concentrations and mean body mass increased in females in the Ray following remediation. If changes in spiggin between the pre- and post-remediation periods were a function only of the increased size of the fish in the Ray, a decline in spiggin concentration would be expected.

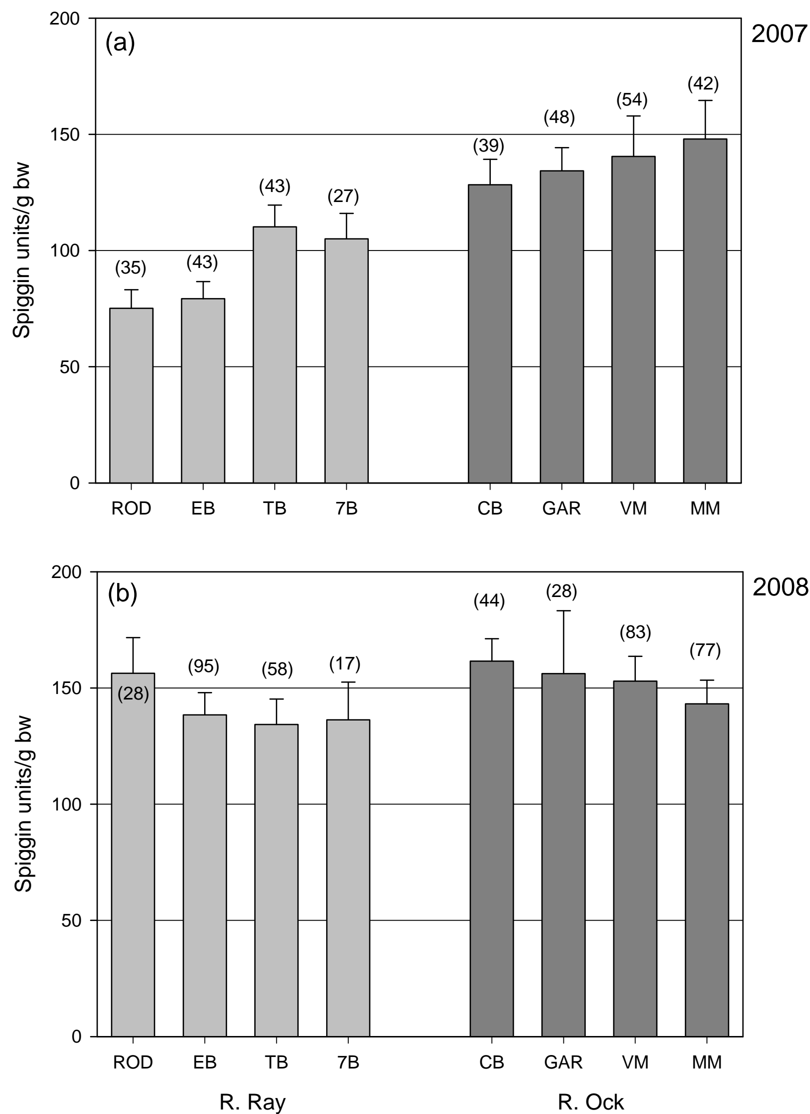


Figure 5.19. Spiggin concentrations (kidney) in female sticklebacks from sites on the R. Ray (light grey bars) and R. Ock (dark grey bars) restricted to the periods (a) March-November 2007 and (b) March-November 2008. Each bar is the mean + SEM, with n indicated in brackets.

5.3.8 Spiggin – males

The usefulness of measuring spiggin concentrations in male sticklebacks in the context of environmental monitoring is compromised by the same constraint that applies to the measurement of VTG in females – spiggin plays a major role in male reproductive processes and thus exhibits an extended concentration range and very marked inter-individual variation. However, in a study such as this where some temporal continuity is available spiggin becomes a potentially informative biomarker of interference with normal reproductive processes.

Changes over time in spiggin concentrations in male sticklebacks from the two rivers are presented in Figure 5.20. The mean values for the Ray in September 2007 and for the Ock in January 2008 were both distorted by the presence of single very high outliers. These have therefore been omitted from the plotted means (see legend for Fig. 5.20). The general pattern then evident is of a single annual peak in spiggin concentrations occurring around May-July which is consistent with the timing of the peak in VTG concentrations in both male and female fish and our assumption that spawning of fish in both rivers occurs during the period March – July. As is the case for the VTG data in females, the spiggin peak in males appears to be earlier on the Ray than the Ock again suggesting that reproduction is asynchronous on the two rivers,

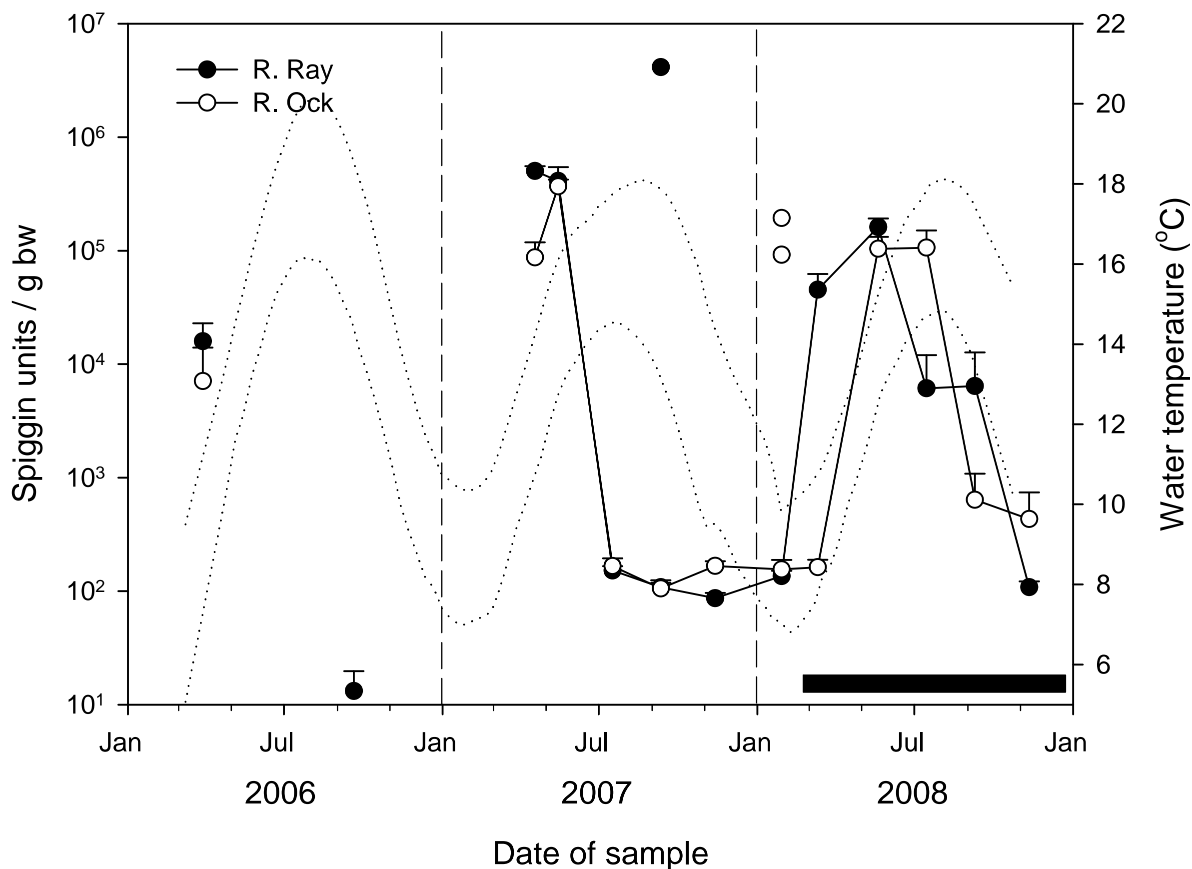


Figure 5.20. Log spiggin concentrations (kidney) in male sticklebacks from the R. Ray (●) and R. Ock (○) at all time points. Each value is the mean ± SEM. The upper dotted line depicts average daily water temperature at site 3 (Rodbourne STW) on the R. Ray. The lower dotted line depicts average daily water temperature at site 10 (Charney Basset) on the R. Ock. The black bar indicates the period during which the GAC plant was operational at Rodbourne STW. Note: A single outlier in the Sep07 Ray sample and two outliers in the Jan08 Ock samples are plotted separately because of the significant distortion introduced into the overall means by including these values

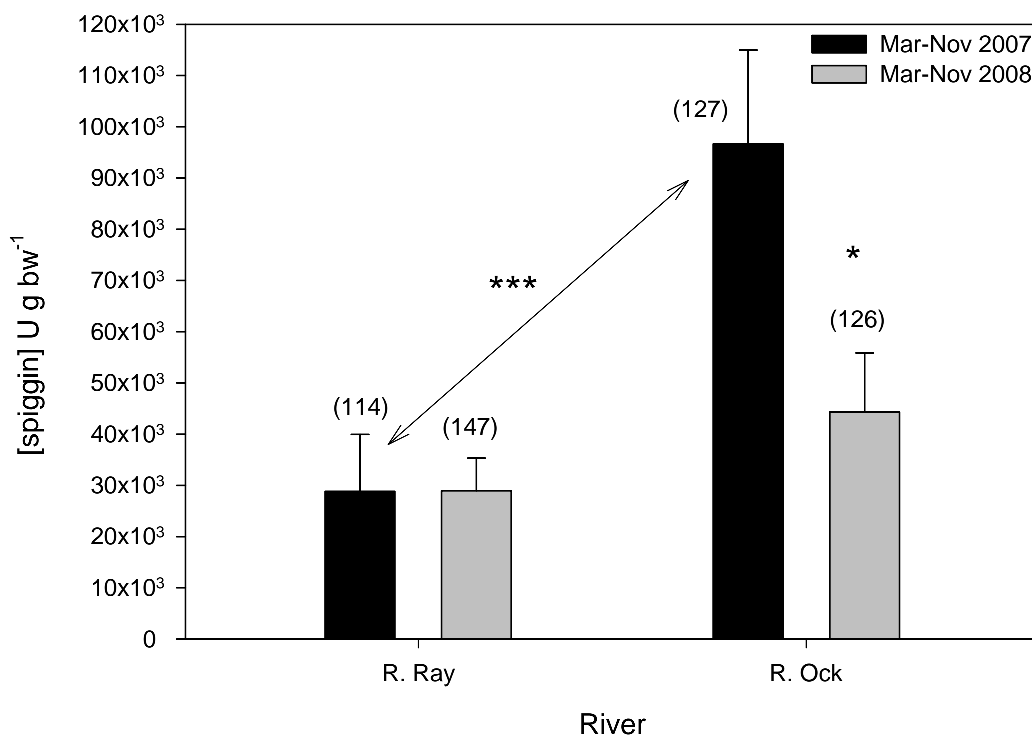


Figure 5.21. Spiggin concentrations (kidney) in male sticklebacks from the R. Ray and R. Ock restricted to the periods March-November, 2007 (black bars) and 2008 (grey bars). The GAC plant was operational during 2008 on the Ray. Each bar is the mean + SEM, with n indicated in brackets. * - significant difference between the two rivers, P<0.001. * - significant difference between 2007 and 2008 within rivers, P<0.05. Note: One outlying value (R. Ray, September 2007) was removed before statistical analysis. The remaining two outliers were present in the Jan08 sample and therefore not included in the analysis.**

When the data plot and ANOVA are restricted to the matched Mar-Nov, 2007 and 2008 samples (Fig. 5.21) it is evident that spiggin concentrations in male fish from the Ock were significantly higher than those from the Ray during Mar-Nov 2007 (P<0.001) whereas for the period Mar-Nov 2008 there was no significant difference in spiggin concentrations in male fish between the rivers. Spiggin concentrations in fish on the Ock, but not the Ray, declined significantly between 2007 and 2008 (P<0.05). These findings are reflected in the spiggin data apportioned by site within the two time periods (Fig. 5.22) where it is apparent that the changes in the overall Ock mean values are due mostly to a massive year-on-year reduction in male kidney spiggin content at two sites, Venn Mill and Marcham Mill.

Spiggin concentrations in male fish were found to be related to body mass in the same manner as for female fish, with smaller fish tending to have higher spiggin concentrations per g body mass. This relationship did not apply to mature males. We don't believe this relationship has any confounding effect on the data as they are interpreted here.

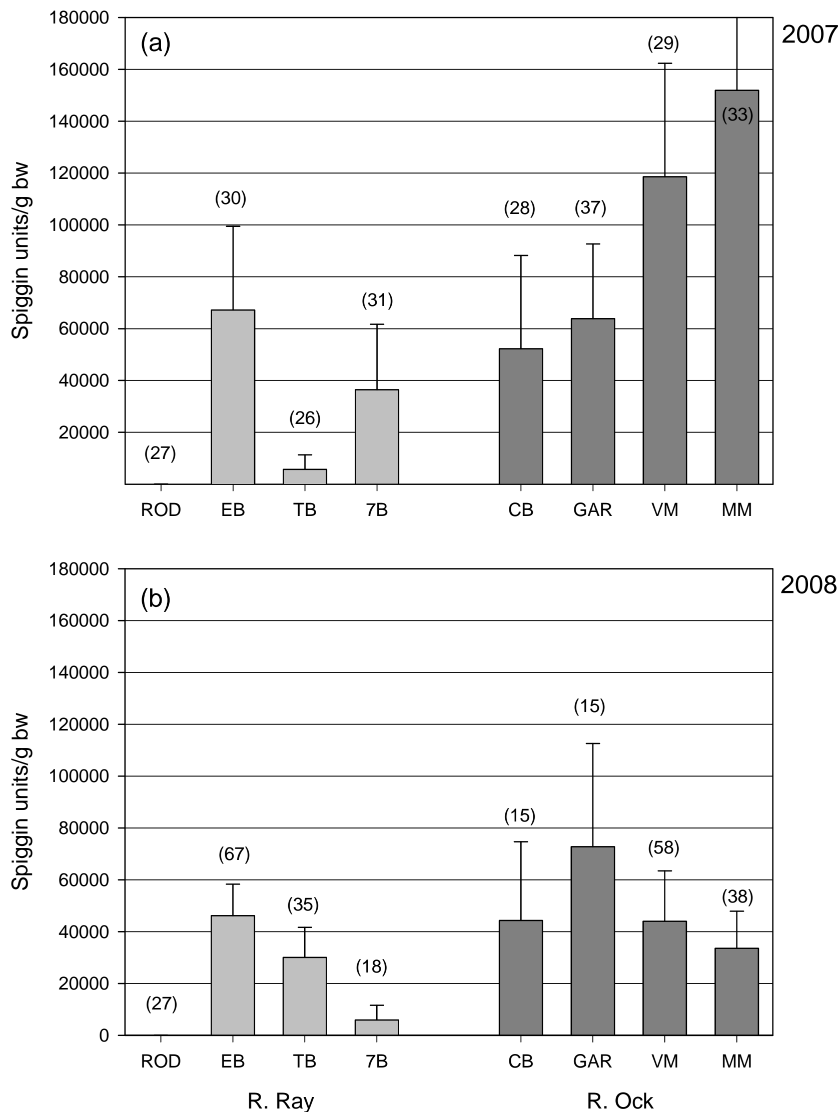


Figure 5.22. Spiggin concentrations (kidney) in male sticklebacks from sites on the R. Ray (light grey bars) and R. Ock (dark grey bars) restricted to the periods (a) March-November 2007 and (b) March-November 2008. Each bar is the mean + SEM, with n indicated in brackets.

5.3.9 Summary - Spiggin data

Alterations in stickleback kidney spiggin concentrations may be indicative of the exposure of the fish to androgenic (spiggin increased; Katsiadaki *et al*., 2002b), anti-androgenic (spiggin decreased; Katsiadaki *et al*., 2006) or estrogenic (spiggin decreased; Katsiadaki, Mayer and Pottinger, unpublished) factors. In female sticklebacks from the Ray and Ock, spiggin concentrations were very low (<200 U/g) and similar to spiggin concentrations reported previously for control females in laboratory studies (Katsiadaki *et al*., 2002b). Nonetheless, significant variation with clear seasonal trends, could be detected across time and between rivers. In the only other published study we are aware of to have measured spiggin in free-living sticklebacks at several time points, levels in female fish fell below the limit of detection for the assay employed at all times (Sanchez *et al*., 2008) possibly reflecting between-population differences but more likely a shortcoming in the sensitivity of the assay method. It is unlikely that the spiggin concentrations detected in female fish

in the present study have a physiological significance - it is more probable that spiggin in females is an incidental consequence of the presence of circulating androgens, in much the same way that the low levels of VTG detected in male sticklebacks (see 5.3.5) are a likely consequence of circulating estrogen.

In the present investigation spiggin concentrations in female sticklebacks in the Ray showed a significant increase between 2007 and 2008 whereas no change was observed between these periods in fish from the Ock. It again seems reasonable to propose that the change in the Ray is associated with the operation of the GAC plant and it might immediately be suspected that an increase in an androgen-dependent biomarker in the females is indicative of the removal of anti-androgenic substances from the effluent. In Section 4.3.3.2, EDCAT 3&4 reports levels of anti-androgenic activity in water samples (as flutamide equivalents) approaching 400 µg l^{-1} during 2007 (although mean levels were < 200 µg l^{-1}) and <50 µg l^{-1} in 2008. Estimates of annual average anti-androgenic activity derived from the analysis of POCIS extracts were higher still for 2007 and also declined dramatically during 2008. Under laboratory conditions, spiggin content of the kidney of mature male sticklebacks is significantly reduced by exposure to flutamide at 500 µg l^{-1} (Sebire *et al*., 2008) and in androgen-primed female sticklebacks kidney spiggin content is significantly reduced by the anti-androgen fenitrothion at concentrations as low as 15 µg l^{-1} (Katsiadaki *et al*., 2006). We don't know the identity of the chemicals responsible for the anti-androgenic effects detected in the Ray by the yeast androgen screen, and it is clear that there was considerable variation in levels of anti-androgenicity (with surprisingly high concentrations detected on the Ock at Charney Bassett). Nonetheless, it is possible that a sufficient concentration of anti-androgens was present in 2007 to account for the lower spiggin content observed in the female sticklebacks compared to 2008.

However, despite the presence of anti-androgenic activity in the Ray STW effluent, no change in spiggin concentrations was detected in male fish from the Ray between 2007 and 2008 during which period anti-androgenic activity in the Ray reduced markedly. On the one hand, this may be evidence that it is not anti-androgens that were responsible for the alterations in spiggin observed in the females. While this may be the case, there is an alternative explanation. Laboratory studies have shown that high androgen levels can completely mask the opposing effects of anti-androgens. Katsiadaki *et al*. (2006) reported that whereas the effects of a range of anti-androgens could be detected in female fish exposed to 17α-methyltestosterone at a concentration of 0.5 µg l^{-1}, if the fish were exposed to androgen at 5 µg l^{-1} the antiandrogenic activity of the test chemicals was completely masked. It is possible that in male sticklebacks in the Ray, endogenous levels of androgens were sufficiently high to counter the effects of the exogenous anti-androgens within the concentration range presented by the Rodbourne effluent. Sebire *et al* (2008) did observe the reduction of spiggin in naturally maturing male sticklebacks continuously exposed to flutamide at 500 µg l^{-1}, but in the Ray there was considerable variation in concentration within and between sites (Figs. 4.4 & 4.5) and annual mean values were closer to 200 µg l^{-1}.

It must also be considered possible that the alterations in spiggin in the female sticklebacks have the same cause as underlies the changes in VTG between 2007 and 2008 and may reflect the influence of factor(s) that interfere with the endocrine reproductive system at a locus that lies upstream of both estrogen and androgen action, possibly by affecting a key step in the synthesis of both groups of steroids. Assuming that both spiggin and VTG are products of the same steroidogenic pathways (androgens induce spiggin, and androgens are quickly converted to estrogens by aromatisation and induce VTG) one could speculate that these chemicals/agents affect this process either directly (i.e. via inhibition of an enzyme) or indirectly (via a negative feedback mechanism in the brain which switches off the production of gonadotropins and hence the initiation of gonadal steroidogenesis).

Finally, spiggin concentrations in male fish at two sites on the R. Ock / Childrey Brook (Marcham Mill and Venn Mill) showed three- to five-fold reductions in concentration between 2007 and 2008.

We don't have any water chemistry data that might throw light on the causes of these alterations. It is interesting to note, however, that the Venn Mill site on Childrey Brook is effectively upstream of the Marcham Mill site, with the Childrey Brook entering the R. Ock approximately 200m upstream of the Marcham Mill sample site. The remaining two Ock sites, Garford and Charney Bassett are upstream of both Venn and Marcham. It is possible that activities in or around the Venn Mill site resulted in the changes in spiggin concentration observed. It should be acknowledged that there was no evidence of similar differences at these sites in VTG concentrations in males or females, or in spiggin concentrations in females.

5.3.10 Choriogenin gene expression

A number of genes associated with gametogenesis in female fish are known to be modulated by estrogens. Among these are the gene coding for the egg envelope protein precursor choriogenin (also known as zona pellucida, vitelline envelope protein, zona radiate structural protein) which occurs in a heavy (ChgH) and light (ChgL) variant in most species so far studied and like VTG is synthesised in the liver. A number of studies have suggested that choriogenin expression may be a more sensitive biomarker of estrogen exposure than VTG (Celius and Walther, 1998; Arukwe *et al*., 1998, 2002; Shimizu *et al*., 2000; Fujita *et al*., 2004) and that ChgH may be more responsive to estrogen than ChgL (Chen *et al*., 2008). As a consequence of failure early within the life of this project to detect any evidence that VTG levels in male sticklebacks in the Ray were inappropriately elevated, and the assumption at that time (prior to chemistry being available) that pre-remediation effluents must contain appreciable concentrations of estrogenic EDCs, we undertook to assess Chg expression levels as an additional biomarker of estrogen exposure. Because of the extensive preparatory work necessary and the costly and involved nature of the analyses it was decided to restrict analysis of Chg to two samples prior to installation of the GAC plant at Rodbourne (September and November 2007) and the corresponding two samples following installation (September and November 2008).

ChgH expression was normalised to the expression of 18S rRNA a gene whose expression levels have been shown to provide an adequate degree of constancy for its application as an internal reference gene in fish (Filby and Tyler, 2007; McCurley and Callard, 2008). Overall, ChgH expression was greatest in females (F: 9.16 ± 0.6; M: 2.9 ± 0.7; P<0.001). Data for males and females were therefore analysed separately.

Expression of ChgH in female sticklebacks was overall higher in fish from the Ock than in fish from the Ray (*P*<0.001; Ray: 5.7 ± 1.0; Ock: 12.6 ± 1.1) and higher in November than in September on both rivers (Fig. 5.23). Among fish from the Ock, there was no significant change in expression levels between September 2007 and September 2008 or between November 2007 and November 2008 despite an upward trend in both cases. However, expression levels in female fish from the Ray were significantly higher in November 2008 than in November 2007 (*P*<0.001; Fig 5.23).

Levels of ChgH expression were also higher in male sticklebacks from the Ock than the Ray (*P*<0.01; Ray: 2.1 ± 0.6; Ock: 4.6 ± 0.7). In male fish from the Ray there was no significant increase in ChgH expression between 2007 and 2008 when September and November were considered separately (Fig. 5.24) although the trend was upwards for both. In the Ock, however, mean expression of ChgH among male sticklebacks was significantly higher in both September and November 2008 than the corresponding months a year earlier (*P*<0.01; Fig. 5.24).

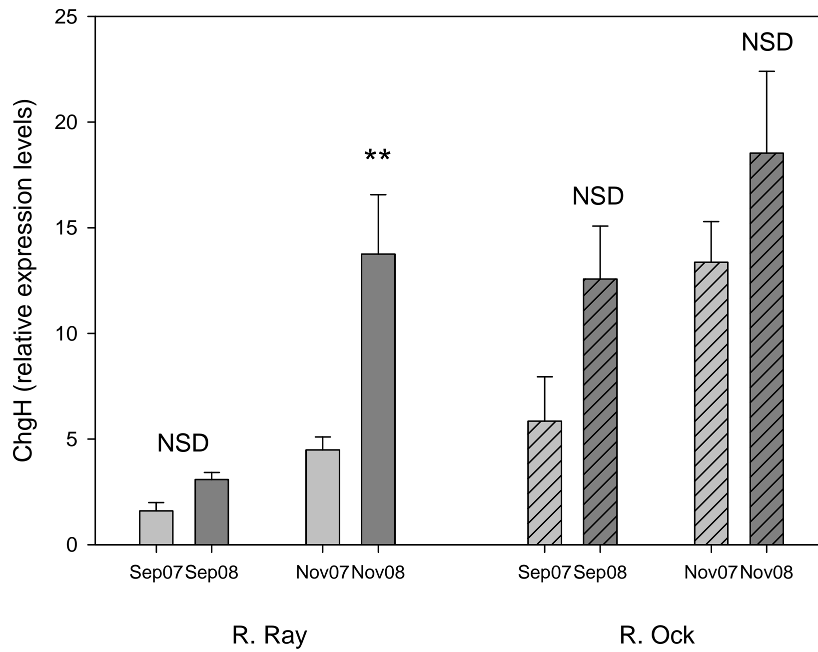


Figure 5.23. Relative expression levels for ChgH in female sticklebacks during September and November 2007 and 2008. Each bar is the mean + SEM. NSD denotes no significant difference between ChgH expression level across the two years. Significant difference denoted by asterisks: ** P<0.01.

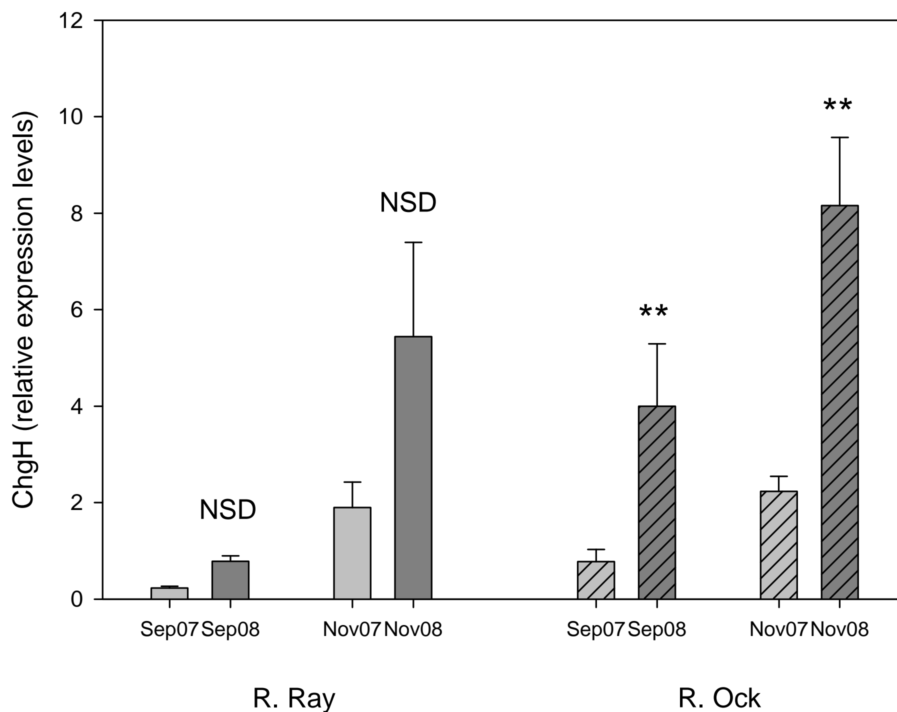


Figure 5.24. Relative expression levels for ChgH in male sticklebacks during September and November 2007 and 2008. Each bar is the mean + SEM. NSD denotes no significant difference between ChgH expression level across the two years. Significant differences denoted by asterisks: ** P<0.01.

These data provide some collateral validation for the results of other reproductive biomarker assay in the current investigation. In female sticklebacks ChgH expression was higher overall in fish from the Ock than those from the Ray, and in fish from the Ray ChgH expression was significantly elevated in 2008 relative to 2007, at least for November, whereas in fish from the Ock, there was no (significant) change across this period. This mirrors the relative levels and changes in both VTG and spiggin observed in females from the Ray which both increased in 2008 relative to 2007 (Figs 5.12 and 5.18) whereas no change was observed in fish from the Ock. Given that ChgH expression is estrogen-dependent, it is likely that whatever factor(s) is/are responsible for modulation of VTG in females from the Ray the same factors can be considered to be responsible for changes in ChgH expression. While modest in magnitude, the consistency of these effects across three very distinct elements of the female stickleback reproductive system lend weight to the conclusion that the reproductive status of sticklebacks in the Ray pre-remediation was sub-optimal.

In male sticklebacks, the ChgH results show less consistency with the other reproductive endpoints. Firstly, the very pronounced sexual dimorphism that is evident for VTG and spiggin is not apparent for ChgH - given the role played by ChgH in ovarian development the relative expression levels of the gene are surprisingly high in males, slightly less than half those in the female fish. Second, there is a very clear increase in ChgH expression in males from the Ock between 2007 and 2008. It is possible that this change is linked to the very pronounced decline in spiggin seen in fish from the Marcham Mill and Venn Mill sites between 2007 and 2008 (Fig. 5.22). In the 2007 sample, fish from Venn Mill and Marcham Mill were over-represented because of low catches at these times at other sites on the Ock .

5.3.11 Summary – biomarkers of endocrine disruption – VTG, spiggin, choriogenin.

1. There is strong evidence that the reproductive endocrine system in female sticklebacks in the Ray was to some extent depressed prior to the installation of the GAC plant – in female sticklebacks from the Ock VTG concentrations showed no significant alteration between 2007 and 2008, whereas in the Ray there was a significant increase in VTG concentrations in the females during 2008 relative to 2007.

2. It may be hypothesized that the pattern of VTG data across 2007 and 2008 in female sticklebacks in the Ray arose because the cocktail of chemicals present in the pre-remediation effluent contained some that (either alone or in combination) exerted a suppressive (anti-estrogenic) effect on the female stickleback reproductive system such that a net outcome was a reduction in the concentration of circulating VTG.

3. The lower VTG concentrations in female sticklebacks from the Ray prior to remediation may be directly indicative of adverse effects on reproductive performance – declining VTG concentrations provide a reliable predictor of declines in fecundity.

4. There was no evidence that the Rodbourne STW contained sufficient estrogenic activity to elicit VTG synthesis in male sticklebacks in the Ray.

5. Chemistry data supplied by EDCAT3&4 support this - it is clear that even prior to the installation of the GAC plant levels of estrogenic steroids (E1, E2, EE2) in the effluent were very low. In terms of its total estrogenicity alone, the effluent can be considered to have posed no threat to the reproductive health of the stickleback population in the Ray

6. With regard to possible effects on other fish populations resident within the Ray - even taking into account the variability evident within the annual mean steroid concentrations - the balance of probability suggests that the Ray effluent prior to remediation was unlikely to be capable of causing sustained VTG production in resident male cyprinids, such as roach, although the temporal variability in the estrogen content of the effluent means that the possible occurrence of transient spikes of estrogenicity that exceeded the LOEC cannot be excluded.

7. In female sticklebacks from the Ray and Ock, spiggin concentrations were very low and similar to spiggin concentrations reported previously for control females in laboratory studies. Nonetheless, significant variation in spiggin content of female kidneys with clear seasonal trends, could be detected across time and between rivers.

8. Spiggin concentrations in female sticklebacks in the Ray showed a significant increase between 2007 and 2008 whereas no change was observed between these periods in fish from the Ock. It again seems reasonable to propose that the change in the Ray is associated with the operation of the GAC plant and it might immediately be suspected that an increase in an androgen-dependent biomarker in the females is indicative of the removal of anti-androgenic substances from the effluent.

9. Data from EDCAT 3&4 show levels of anti-androgenic activity in water samples (as flutamide equivalents) approaching 400 μg l^{-1} during 2007 (although mean levels were < 200 μg l^{-1}) and <50 μg l^{-1} in 2008. Estimates of annual average anti-androgenic activity derived from the analysis of POCIS extracts were higher still for 2007 and also declined dramatically during 2008. We don't know the identity of the chemicals responsible for the anti-androgenic effects detected in the Ray by the yeast androgen screen, and it is clear that there was considerable variation in levels of anti-androgenicity. Nonetheless, it is possible that a sufficient concentration of anti-androgens was present in 2007 prior to operation of the GAC plant to account for the lower spiggin content observed in the female sticklebacks compared to 2008.

10. No change in spiggin concentrations was detected in the kidneys of male sticklebacks from the Ray between 2007 and 2008, during which period anti-androgenic activity in the Ray reduced markedly. This may be evidence that it is not anti-androgens that were responsible for the alterations in spiggin observed in the females. However, laboratory studies have shown that high endogenous androgen levels can completely mask the opposing effects of anti-androgens. It is possible that in male sticklebacks in the Ray, endogenous levels of androgens were sufficiently high to counter the effects of exogenous anti-androgens within the concentration range presented by the Rodbourne effluent.

11. It is a realistic possibility that the alterations in spiggin concentration in female sticklebacks have the same cause as underlies the changes in VTG between 2007 and 2008. Both may reflect the influence of factor(s) that interfere with the endocrine reproductive system at a locus that lies upstream of both estrogen and androgen action, possibly by affecting a key step in the synthesis, or interconversion, of either or both groups of steroids.

12. Expression of ChgH in female sticklebacks from the Ray increased significantly between 2007 and 2008 whereas no significant change took place in females from the Ock across this period. These data exhibit a similar pattern to, and provide some collateral validation for, the results of other reproductive biomarker assays (VTG, spiggin). While modest in magnitude, the consistency of these changes across three distinct elements of the female stickleback reproductive system lend weight to the conclusion that the reproductive status of sticklebacks in the Ray pre-remediation was sub-optimal.

13. ChgH expression levels in male fish from the Ray provided no evidence that males were exposed to an estrogenic signal.

An overwhelmingly dominant estrogenic contaminant fraction was absent from the Rodbourne STW effluent, even prior to remediation. The effects observed on the reproductive system of male and female sticklebacks seem consistent with exposure to a complex mixture of organic chemicals whose combined effect is difficult to predict but may be anti-estrogenic in nature.

5.3.11 Whole-body corticosteroid concentrations

Concentrations of corticosteroids in whole-body homogenates of fish (Pottinger and Mosuwe, 1995; Pottinger and Calder, 1995; Ramsay *et al.*, 2006) provide a reliable index of the activation of the neuroendocrine stress response, in which the elevation of blood cortisol levels is a key element. The stress axis is rapidly activated in fish by exposure to almost any form of disturbance that is perceived by the fish as a threat and the stress response itself comprises a critical element of the coping strategies adopted by animals to survive adverse changes in their environment. Whole-body corticosteroid levels rise during stress to reflect the elevation of blood levels, but the dynamics of change in whole-body levels are more complex than those in the blood. The degree of physical disturbance caused by the sampling procedure adopted in the current study (capture by hand net and retention in buckets) is akin to the confinement stressor previously shown to elevate whole-body corticosteroid levels in sticklebacks (Pottinger *et al.*, 2002) being characterised by physical disturbance and enforced exercise, emersion, and transfer to an unfamiliar environment combined with spatial restriction.

A considerable body of data has emerged from North America in recent years to show that the presence of organic and inorganic contaminants in the aquatic environment can modify the responsiveness of fish to a stressor, normally manifested as an inability to mount an adequate endocrine response to challenge. A wide range of chemicals, present in water bodies, have been identified as having the ability to disrupt adrenal/interrenal function. These include metals, pharmaceuticals, PCBs, PAHs, and herbicides (Bisson and Hontela, 2002; Gesto *et al.*, 2008; Hontela, 2006; Levesque *et al.*, 2003). Whole-body corticosteroid levels were measured in the present investigation in order to evaluate whether the ability of the fish to respond to a stressor (i.e. sampling) was affected by elimination of much of the organic contaminant burden in the Ray following installation of the GAC plant.

Mean whole-body corticosteroid concentrations for sticklebacks on each river are shown in Fig. 5.21. The concentration range observed is higher than that reported previously for sticklebacks exposed to a confinement stressor (40-60 ng g^{-1}; Pottinger *et al.*, 2002). Corticosteroid concentrations in undisturbed sticklebacks in the same study were found to be ~5.0 ng g^{-1} so it can safely be assumed that the sticklebacks captured in the present study were stressed at time of sacrifice.

Overall whole body corticosteroid levels were higher in fish from the Ray than in fish from the Ock ($P<0.05$; Ray: 67.7 ± 1.4 ng g^{-1}; Ock: 58.5 ± 0.9 ng g^{-1}). There was also a small but significant difference in corticosteroid concentrations between sexes on both rivers with higher corticosteroid levels in females than males ($P<0.001$, data not shown). This may be related to the tendency for stress-induced levels of cortisol to be greater in females than males, particularly in reproductively active fish (Pottinger *et al.*, 1995).

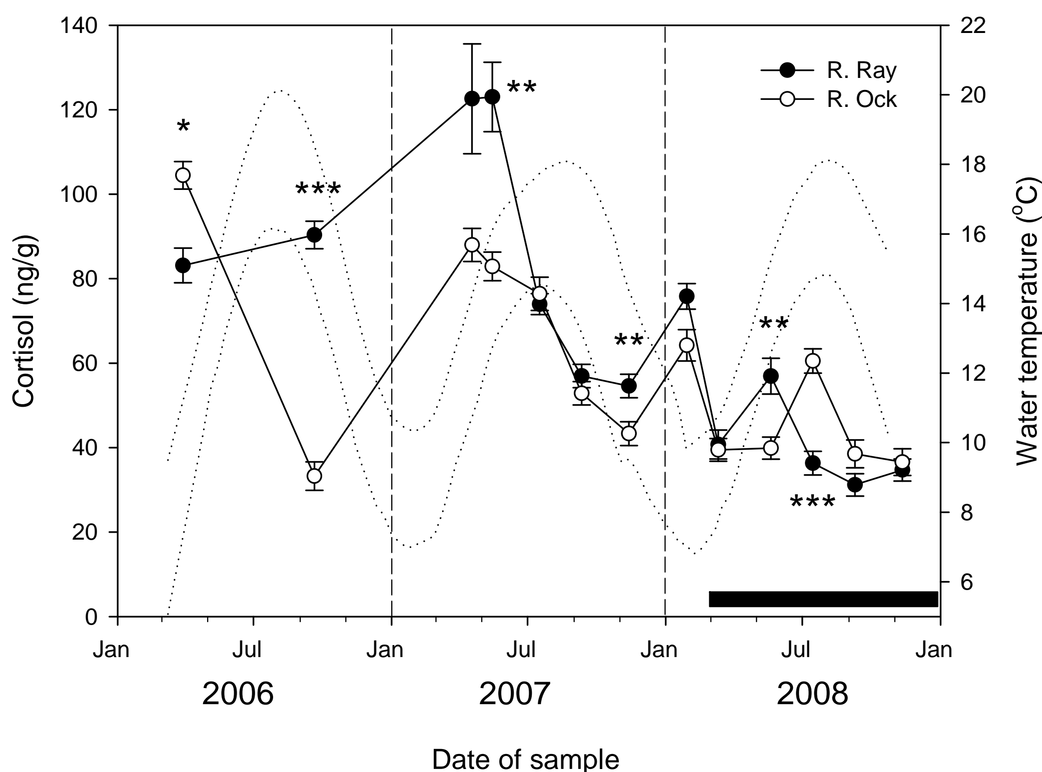


Figure 5.21. Whole-body corticosteroid concentrations in sticklebacks from the R. Ray (solid symbols) and R. Ock (open symbols). Each point is the mean ± SEM. Significant differences between rivers within times are denoted by: * - *P*<0.05; ** - *P*<0.01; * - *P*<0.001. The upper dotted line depicts average daily water temperature at site 3 (Rodbourne STW) on the R. Ray. The lower dotted line depicts average daily water temperature at site 10 (Charney Basset) on the R. Ock. The black bar indicates the period during which the GAC plant was operational at Rodbourne STW.**

There was considerable variation in mean corticosteroid levels between sampling times within rivers (*P*<0.001) and between rivers at some sample times (see Fig 5.21). In particular, during mid-2006 to mid-2007 cortisol levels were significantly higher in fish from the Ray than fish from the Ock. From July 2007 onwards, although differences were evident they were much less pronounced than was the case previous to this time. There was also a clear overall downward trend in corticosteroid concentrations across the duration of the study with lowest levels in both rivers being recorded during late 2008, those in fish from the Ray at this time being approximately half the concentrations recorded during 2006, the decline in both rivers from May 2007 to November 2008 being significant (*P*<0.001). No obvious seasonal trends were evident in either river, but overall the timing of peaks and troughs in cortisol concentration were similar across both rivers.

In sticklebacks from both rivers, but particularly the Ray, higher concentrations of cortisol were detected during May-July 2007 than during subsequent months. These high concentrations may be indicative of (i) the sensitivity/responsiveness of the fish to the capture procedure on both rivers being modulated by external factors, or (ii) the stress imposed by capture being additive with an existing state of stress, or (iii) the fish were sensitised to additional stressors by an ongoing or intermittent chronic stressor. A number of factors are known to influence the response of fish to stressors and these include temperature (Pottinger *et al.*, 1999) and water quality (Pickering and Pottinger, 1987).

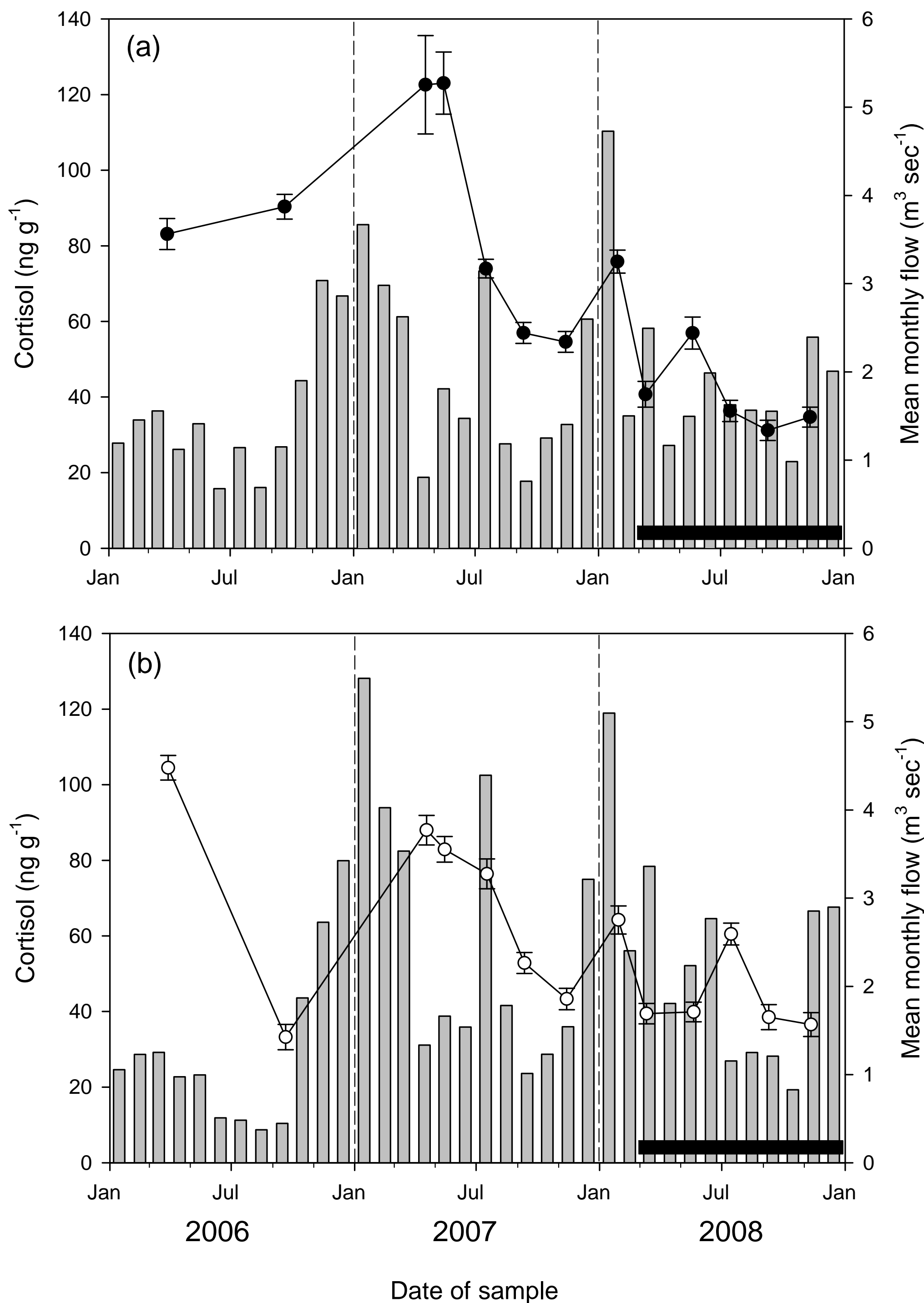


Figure 5.22. Whole-body corticosteroid concentrations in sticklebacks from (a) the R. Ray and (b) the R. Ock. Each point is the mean ± SEM. The grey bars represent the mean monthly water flow at gauging stations on the Ray (Water Eaton) and the Ock (Abingdon) derived from daily measurements (data courtesy of the National River Flow Archive, CEH Wallingford). The black bar indicates the period during which the GAC plant was operational at Rodbourne STW.

It would seem initially that temperature can be excluded as a possible cause given the well-defined and consistent annual temperature cycle observed in both rivers (but see below). However, we have only limited and incomplete data on water quality. While no clear trends are evident in the water

chemistry data provided by EDCAT 3&4 that might be associated with the pattern of corticosteroid levels the possibility that short-term fluctuations in chemical factors, or the interaction of different aspects of the water chemistry might be responsible for the long-term trends in corticosteroid status of these fish cannot be excluded.

A more obvious correlate of the corticosteroid profile is provided by water flow data (Fig 5.22) collected at gauging stations which for both rivers were downstream of all sample sites (data courtesy of the National River Flow Archive, Wallingford). For sticklebacks in both rivers, the highest whole-body corticosteroid levels were recorded in March/May 2007, following a period of sustained high river flows between October 2006 and March 2007. The extreme rainfall events of July 2007 occurred immediately following the July sample trip so it is unsurprising that there does not appear to be an association with particularly high corticosteroid levels at this time although in both rivers there is a spike in corticosteroid concentrations following higher flows recorded during December 2007. Of course, it should be noted that the spacing of samples does not necessarily allow for detection of unpredictable events within a dynamic system – that is, short-term elevation of corticosteroid levels may have occurred between sample times (e.g. between Oct 06 and Mar 07). The corticosteroid data for fish from the Ray are higher than those from the Ock during 2007, yet the monthly mean flow data suggest flows were higher in the Ock. This may be related to "missing" samples but inspection of the daily flow data indicates that although monthly mean flows were higher in the Ock the magnitude of daily change in flow during this period was greater in the Ray ranging to 15 $m^3 sec^{-1}$ whereas in the Ock, maximum daily flows did not exceed 9.4 $m^3 sec^{-1}$. There are no previous data on which to draw for comparison but it is possible that extreme hydrological events, such as flooding and resultant alterations in flow and temperature, might modify the corticosteroid status of resident fish populations, resulting in a magnified response to capture, or in an additive response.

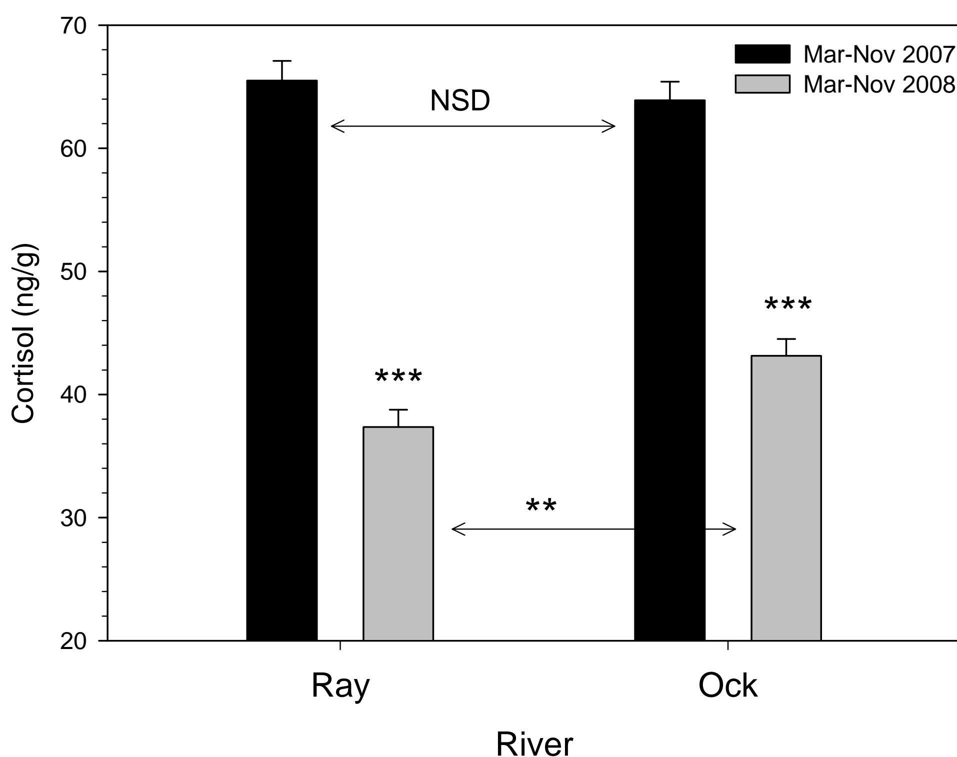


Figure 5.23. Whole-body corticosteroid concentrations in female and male sticklebacks from the R. Ray and the R. Ock during the periods Mar-Nov 2007 and Mar-Nov 2008. Each bar is the mean + SEM. Significant differences between corticosteroid concentrations during the two periods are denoted by asterisks: * *P*<0.001.**

These effects might be more pronounced during periods when food supply is limited, such as the winter months. Examination of the flow data in conjunction with the water temperature data collected by EDCAT3&4 indicates that periods of high flow caused a marked drop in temperature (data not shown). A previous study in birds suggested that a proportion of the variability in corticosteroid levels in wild-caught passerines can be explained by the proximity in time of extreme weather events (Romero *et al*., 2000) although the extent of this relationship depended upon species and life-history stage. The authors are not aware of any existing data that demonstrate the physiological or endocrine responses of fish in the natural environment to extreme events and this may be the first occasion on which such a link has been identified, albeit tentatively.

The year on year change in corticosteroid concentrations are shown clearly in Figure 5.23 in which the mean whole-body cortisol concentrations for fish from each river during the matched pre- and post-remediation periods (Mar-Nov) in 2007 and 2008 are plotted. There was no overall difference evident between means for cortisol concentrations in fish from both rivers in 2007, despite the higher peak levels observed in fish from the Ray during March and May 2007. The means for both rivers were significantly lower in 2008 than 2007 (P<0.001) and significantly lower in fish from the Ray compared to those from the Ock (P<0.01).

The difference in the mean post-capture corticosteroid concentrations between rivers, while significant, is numerically quite small and while the possibility that it arises as a result of alterations in water quality due to the operation of the GAC plant cannot be excluded it seems unlikely. The drivers of change in corticosteroid levels in these fish seem more likely to be natural environmental stressors.

In summary, with regard to the original rationale for examining corticosteroid concentrations in the resident stickleback populations, the data provide no unequivocal evidence that the stress response of fish capture in the Ray prior to installation of the GAC plant was modified by exposure to the effluent. Large variations in corticosteroid concentrations in fish from both rivers, with clear trends over time, were closely linked to perturbations in the river flow environment. Higher peak corticosteroid levels in fish in the Ray may be due to the interaction between environmental and chemicals stressors (i.e. the effluent) or may just reflect differences in the flow regime between the two rivers.

5.3.12 Whole-body glucose concentrations

Blood glucose concentrations are widely employed as an index of stress in fish – stressors evoke an immediate elevation of blood glucose as a consequence of the actions of corticosteroids and catecholamines released by the neuroendocrine pathways activated during stress. However, the limited data available (a single laboratory-based study) suggest that unlike whole-body corticosteroid levels, whole-body glucose levels do not change immediately when fish are exposed to a stressor and when changes do occur, they are manifested as a decline in whole-body concentrations, rather than an increase. – presumably as stored resources are depleted by the demands of coping with the stressor(s). The concentrations of glucose observed here in whole-body homogenates are similar to those reported for sticklebacks in a previous study (Pottinger *et al*., 2002) where mean glucose concentrations were 1.5 – 2.5 mg g^{-1} in fed/unstressed sticklebacks and approached 0.5 mg g^{-1} in fasted/stressed fish.

Whole-body glucose levels in fish from the Ray and Ock were similar for both sexes and overall there was no significant difference in glucose levels between rivers. There was however a highly significant decline in glucose concentrations across the sampling period (P<0.001; Fig. 5.24). When glucose concentrations for fish in both rivers are analysed together, the overall downward trend in concentrations is interrupted by a significant (P<0.001) increase in mean glucose concentrations during the summer of 2007.

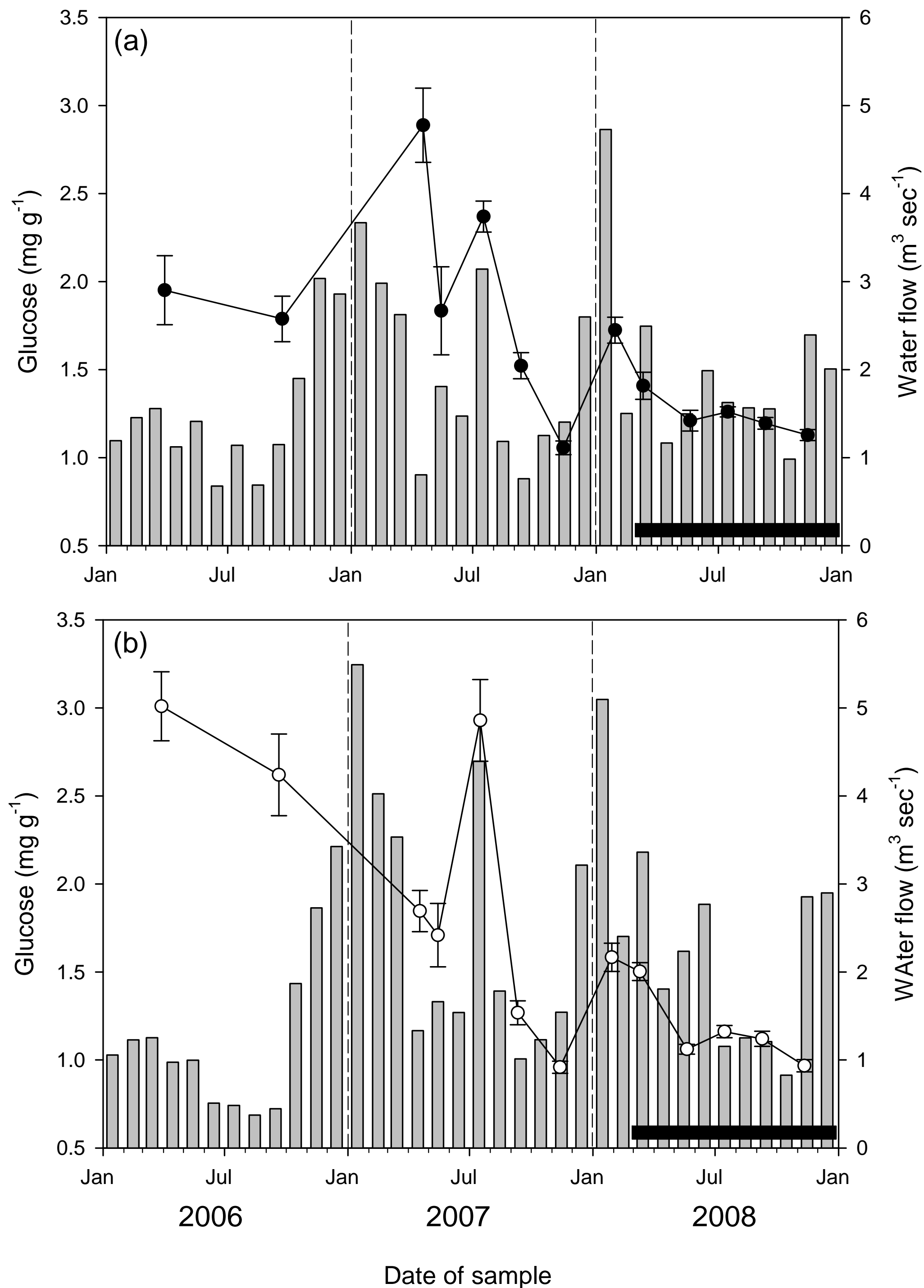


Figure 5.24. Whole-body glucose concentrations in sticklebacks from (a) the R. Ray and (b) the R. Ock. Each point is the mean ± SEM. The grey bars represent the mean monthly water flow at gauging stations on the Ray (Water Eaton) and the Ock (Abingdon) derived from daily measurements (data courtesy of the National River Flow Archive, CEH Wallingford). The black bar indicates the period during which the GAC plant was operational at Rodbourne STW.

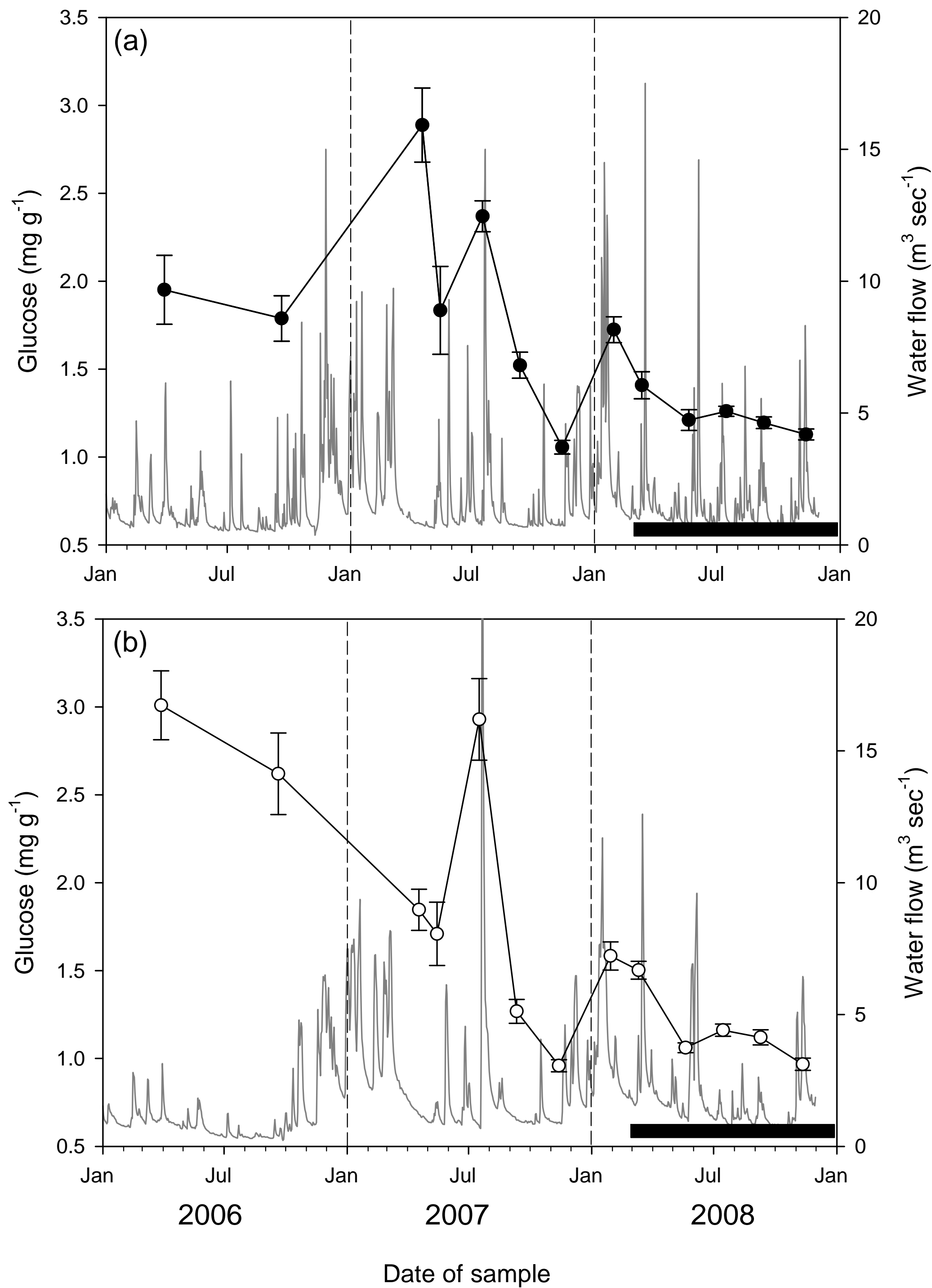


Figure 5.25. Whole-body glucose concentrations in sticklebacks from (a) the R. Ray and (b) the R. Ock. Each point is the mean ± SEM. The uninterrupted lines represent the daily water flow at gauging stations on the Ray (Water Eaton) and the Ock (Abingdon) (data courtesy of the National River Flow Archive, CEH Wallingford). The black bar indicates the period during which the GAC plant was operational at Rodbourne STW.

If it is presumed that whole-body glucose levels provide some information as to the energetic status of the fish then the data, encompassing a three-fold decline in whole-body glucose concentrations in the Ock, suggest that either the fish were exposed to an ongoing source of stress that resulted in a cumulative reduction in levels of stored energy across three successive years, or very large alterations in the energy intake of the fish in both rivers occurred during the monitoring period. Both explanations seem unlikely and there is no downward trend evident in any somatic measure (mass, length, coefficient of condition, RNA:DNA ratio) to suggest that the change in glucose concentration is symptomatic of a decline in growth performance of the stickleback populations in either river. In fact, there was a broad upward trend in coefficient of condition across the monitoring period in both rivers. It may therefore be the case that laboratory studies (of which there has been only one directly relevant to the present data) do not provide an appropriate point of reference for interpretation of the whole-body glucose data in wild-caught fish.

Inspection of these glucose data suggests that they tie more closely to variation in the status of the stress axis of the fish and the changes with time observed across the monitoring period may reflect the involvement of the same factors responsible for the alterations in corticosteroid status during this period.

This hypothesis tends to receive support from the data presented in Fig. 5.24. Among fish from the Ray, peak glucose concentrations are associated with periods of high water flow (October 2006 – March 2007; May-July 2007; December 2007; January 2008). For fish in the Ock prominent peaks are associated with high flows preceding July 2007 and January 2008. In both rivers glucose levels are high during 2006, possibly indicating events not captured by the flow gauges, or not linked with flow rate change at all. The same observation is true for the cortisol data also (Fig. 5.22).

The data in Figure 5.24 indicate high flows were present in the Ock between October 2006 and March 2007 but the glucose data for fish captured in the Ock do not indicate any increase comparable to that seen in fish from the Ray immediately following the same period. This may be related to the flow data as shown in Figure 5.25. Here, the daily flows are plotted and it can be seen that although the mean flows in the Ock were overall higher than those in the Ray during this period (Fig. 5.25b), the magnitude of change in flow was greater in the Ray (Fig. 5.25a). During October 2006 to March 2007 the daily flow rates in the Ray were 0.37 – 15.0 $m^3 sec^{-1}$ compared to 0.6 – 9.4 $m^3 sec^{-1}$ in the Ock. It may be the case that certain critical flow levels must be exceeded before conditions within the river evoke the changes in the physiological status of the fish exemplified by the whole-body cortisol and glucose data. Or associated changes such as those in temperature and turbidity, and their interactions, may play a role and the frequency of such events may also be a factor. Of course, a simpler explanation may be that the sampling periodicity was such that a peak in glucose levels during this interval was missed, recovery having occurred before the March 2008 sample was collected.

In summary, it seems safe to conclude that the Rodbourne effluent was not a factor in modulating the glucose content of the resident stickleback population - glucose concentrations in fish from both rivers during the matching periods prior to and following the installation of the GAC plant were almost identical. However, there was a small but significant overall difference in glucose concentrations in fish from the Ray and Ock during the period March-November 2007. While it seems likely that this arose because of differences in environmental factors such as the extent of perturbations in water flow, as is the case for the corticosteroid data it remains a (remote) possibility that factors in the effluent modified the response of the fish to the events driving the changes in glucose. (Fig. 5.26).

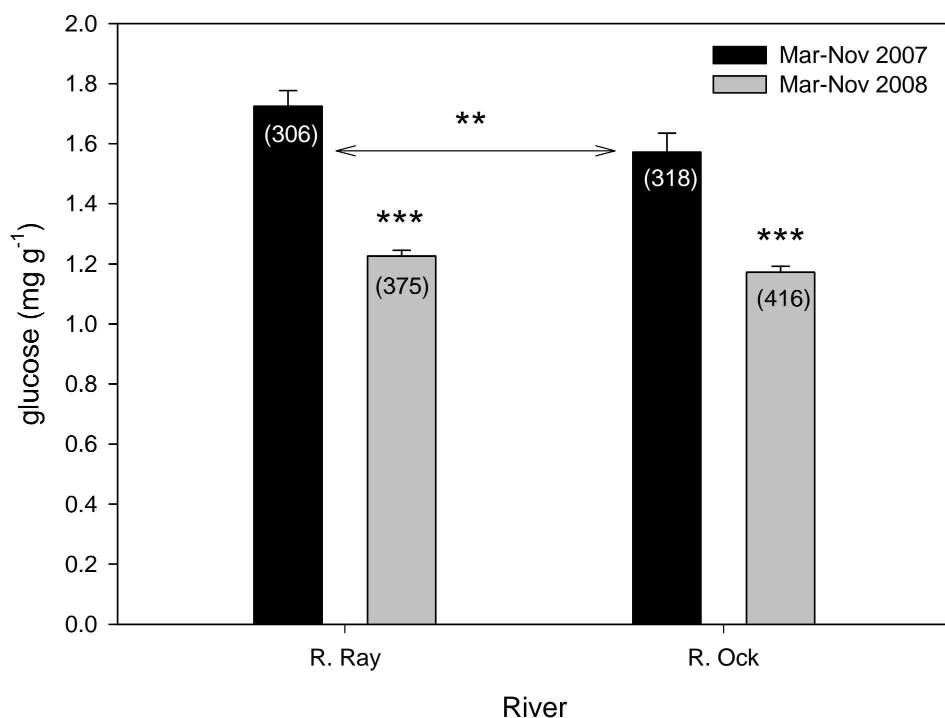


Figure 5.26. Whole-body glucose concentrations in sticklebacks from the R. Ray and the R. Ock during the periods Mar-Nov 2007 (black bars) and Mar-Nov 2008 (grey bars). Each bar is the mean + SEM. Significant differences between glucose concentrations during the two periods and between rivers are denoted by asterisks: ** $P<0.01$; *** $P<0.001$.

5.3.13 EROD

Measurement of the activity of cytochrome P450 isoform CYP1A is widely employed to evaluate the exposure of fish to organic contaminants (Whyte *et al*, 2000). This isoform is induced by dioxins, dibenzofurans, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and other compounds that bind to the aryl hydrocarbon receptor (AhR). The measurement of the activity of the monooxygenase 7-ethoxyresorufin-O-deethylase (EROD) provides a surrogate for direct quantification of CYP1A expression. In the present investigation, the EROD assay was deployed to determine whether sticklebacks resident in the Ray were subject to chemical exposures high enough to evoke an EROD biomarker response, and if so, whether this response declined following installation of the GAC. It was originally intended to use the assay throughout the study. However, with the decision to augment the suite of ED biomarkers by looking at Chg expression as well as VTG and spiggin it became necessary to retain livers for RNA extraction, meaning that they would be unavailable for direct EROD quantification. Instead, it was decided to measure CYP1A expression levels directly to best utilise the limited quantity of liver tissue available from these small fish. A limited number of EROD assays were carried out to support the CYP1A data. As noted earlier in this report, technical issues mean that the results obtained with the CYP1A RT-PCR assay will not be available until after the completion of the report.

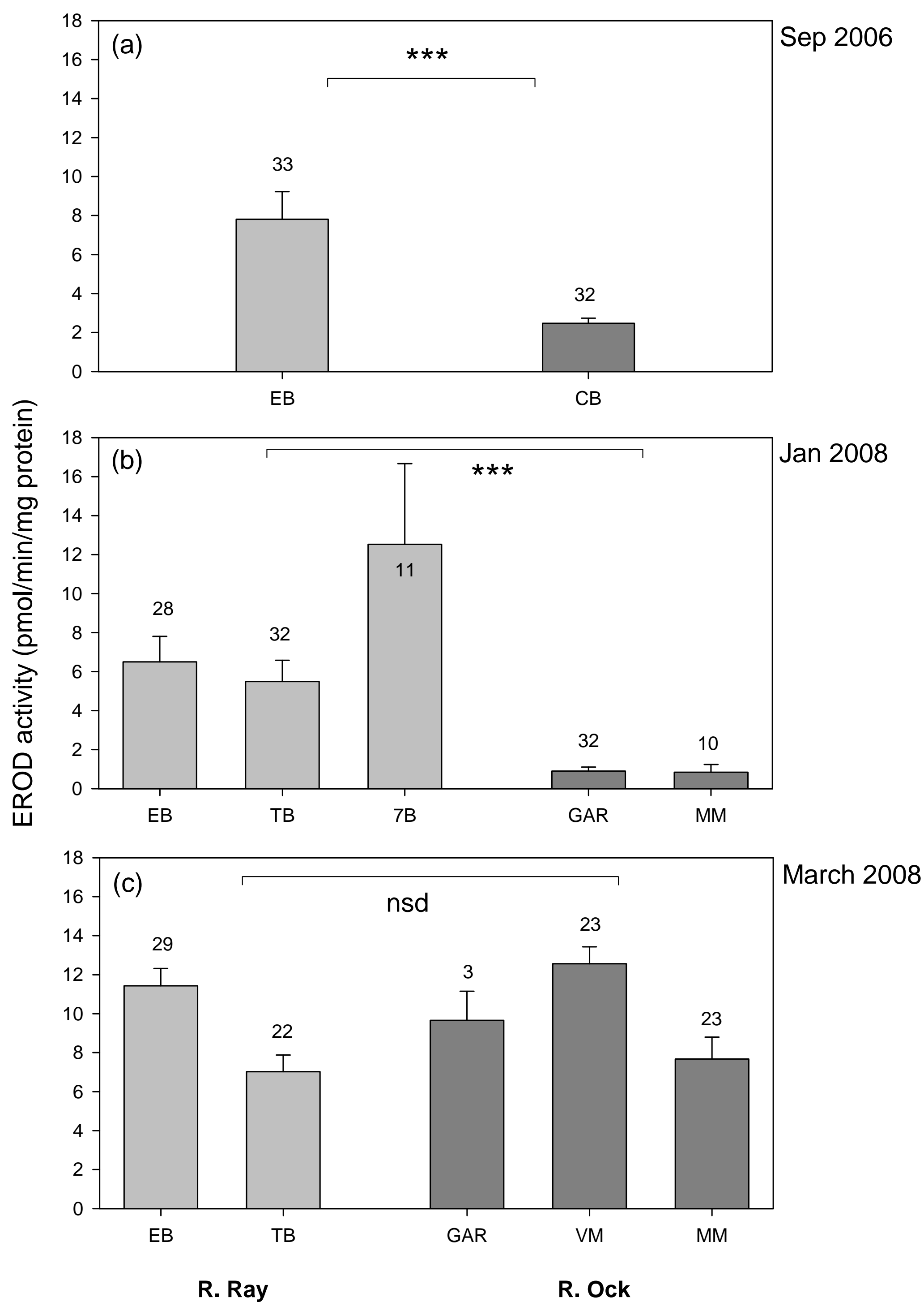


Figure 5.27. EROD activity in sticklebacks collected during (a) September 2006, (b) January 2008, (c) March 2008. Each bar is the mean + SEM. Sample sites are indicated along the x-axes: R. Ray (light grey bars): EB - Elborough Bridge; TB - Tadpole Bridge; 7B - Seven Bridges; R. Ock (dark grey bars): CB - Charney Basset; GAR - Garford; VM - Venn Mill; MM - Marcham Mill. Significant differences where they exist between rivers overall are indicated by *** P<0.001; nsd: no significant difference.

In the samples collected during September 2006 (Fig. 5.27a) and January 2008 (Fig. 5.27b) significant differences were evident between the EROD activity detected in fish collected from sites in the Ray compared to the activity in fish collected from sites on the Ock (Fig 5.27). However, in the single post-remediation sample collected in March 2008 (Fig. 5.27c) there is no difference evident between rivers; EROD activity within samples from the Ray is similar to that observed in the previous two samples (range 6 – 13 pmol min^{-1} mg $protein^{-1}$). However, EROD activity in fish from the Ock is greatly increased over previous samples (< 3 pmol min^{-1} mg $protein^{-1}$) to levels statistically indistinguishable from those in the Ray (7 – 13 pmol min^{-1} mg $protein^{-1}$). A procedural error can be eliminated from consideration as a rationale for the change in activity in fish from the Ock - the assays performed similarly on each occasion and the TB samples from January 2008 were assayed together with the March 2008 samples providing no reason to suppose that the increased activity levels observed at all three sites on the Ock in March 2008 are not real.

The EROD activity levels reported here are consistent with the limited stickleback data available elsewhere and seem indicative of a low level of chemical exposure in the Ray fish, at least during September 2006 and January 2008. Sanchez *et al*. (2007) report mean EROD activities in sticklebacks of between 2.6 and 43.3 pmol min^{-1} mg $protein^{-1}$ from a range of sites with varying degrees of chemical contamination, and values of around 0.5 - 10 pmol min^{-1} mg $protein^{-1}$ have been reported for laboratory controls and sticklebacks from ostensibly unpolluted sites (Geoghegan *et al*., 2008; Sanchez *et al*., 2008; Wartman *et al*., 2009). Under laboratory conditions Geoghegan *et al*. (2008) found a pronounced sexual dimorphism in the EROD response to dibenzanthracene with both male and female fish exhibiting significant increases over controls (~ 3-5 pmol min^{-1} mg $protein^{-1}$) but the response of males (~ 45 pmol min^{-1} mg $protein^{-1}$) being some four-fold greater than that in females (~ 12 pmol min^{-1} mg $protein^{-1}$). Sanchez *et al*. have reported similar differences in EROD activity between males and females from wild populations of sticklebacks (Sanchez *et al*., 2008). In the present study, no significant differences between sexes were evident during September 2006 (male: 5.0 ± 1.7, n = 43; female = 5.3 ± 0.9, n = 43) or January 2008 (male: 4.2 ± 0.8, n = 52; female = 5.2 ± 1.1, n = 61) but in March 2008 EROD activity in males overall was slightly but significantly greater than that in females (male: 11.0 ± 0.7, n = 41; female = 9.0 ± 0.7, n = 59; $P<0.05$). The male/female difference has been suggested to arise due to a protein dilution effect because of the large amounts of VTG being synthesised in the liver of female fish during the reproductive period and the normalisation of EROD activity to cytosolic protein concentration.

Of the chemical analyses carried out by EDCAT3&4 the most relevant to the interpretation of the EROD data are probably the PAH data which were obtained from sediments collected at Rodbourne STW, Elborough Bridge and Seven Bridges on the Ray and at Charney Bassett on the Ock. Total PAHs in the sediment samples were highest at EB, lower at 7B and were lower than both these at CB (Table 4.7). The exposure of fish to PAHs at these sites may be either direct via dissolved chemicals or chemicals adsorbed to particles in the water column, or indirect via the consumption of contaminated prey items.

To date, with only the EROD data to consider, the issue of whether the installation of the GAC plant at Rodbourne removed sufficiently high proportion of organic contaminants effluent to affect exposure of the fish cannot be resolved. Certainly, EROD activity was significantly higher in fish from the Ray than the Ock during the September 2006 and January 2007 samples suggesting levels of exposure in the Ray sufficient to trigger detoxification measures, but the elevation of EROD activity in fish from the Ock in March 2008 clouds the issue. EROD activity in fish from the Ock in March was as high as that in fish from the Ray at that time and also on previous occasions.

There are two possible explanations: (i) there are seasonal changes in baseline (constitutive) expression of EROD activity and in March baseline levels increase leading to apparently similar levels in fish from both the Ray and Ock. For this hypothesis to be valid it must be assumed that

exposure of fish in the Ray to contaminants was reduced post-remediation at the same time as a seasonal increase occurred. Countering this possibility is the failure of Sanchez *et al*. (2008) to observe any seasonal variation in EROD activity in sticklebacks across samples taken between April and October; (ii) the second possibility is that fish in the Ock were exposed to contaminants prior to the March 2008 sample, at sufficiently high concentrations to induce EROD activity. A period of gales and high rainfall preceded the March sample so it is possible that materials entered the river that would not otherwise have done so. However, although both the Garford and Marcham Mill sites are on the Ock, the Venn Mill site is on Childrey Brook and it is difficult to envisage contamination of both rivers occurring simultaneously unless it is suggested that sufficient road run-off enters both rivers during periods of high rainfall to raise the levels of EROD-inducing chemicals. Both explanations seem unlikely. Resolution of the factors underlying these anomalous observations will probably not be possible until the CYP1A expression level data set becomes available.

In summary, the EROD data confirm that levels of organic contaminants in the Ray prior to the STW upgrade were sufficient to induce hepatic Phase 1 detoxification enzyme activity in the livers of exposed fish. The single sample currently available following the upgrade on the Ray indicates no decline in EROD activity. This might be expected if the exposure route of the fish is not primarily via water-borne chemicals and is instead via ingested food items, for example. However, we don’t have direct evidence that installation of the GAC plant definitely resulted in a decline in EROD-inducing organic chemicals within the effluent, this is surmise based on the results of previous studies. The interpretation of the March 2008 EROD data set is complicated by relatively high levels of activity observed in fish from the Ock and Childrey Brook. Completion of the CYP1A gene expression studies will hopefully provide some explanation for this anomaly.

5.3.14 Summary – biomarkers of exposure to non-ED chemical and environmental stressors – corticosteroids, glucose, EROD.

1. Overall whole body corticosteroid levels were higher in fish from the Ray than in fish from the Ock. There was also a small but significant difference in corticosteroid concentrations between sexes on both rivers with higher corticosteroid levels in females than males

2. There was considerable variation in mean corticosteroid levels between sampling times within rivers and between rivers at some sample times. In particular, during mid-2006 to mid-2007 cortisol levels were significantly higher in fish from the Ray than fish from the Ock. From July 2007 onwards, although differences were evident they were much less pronounced than was the case previous to this time.

3. There was a clear overall downward trend in corticosteroid concentrations across the duration of the study with lowest levels in both rivers being recorded during late 2008, those in fish from the Ray at this time being approximately half the concentrations recorded during 2006. No obvious seasonal trends were evident in either river, but overall the timing of peaks and troughs in cortisol concentration were similar across both rivers and seemed to be linked with rainfall/flow events.

4. For sticklebacks in both rivers, the highest whole-body corticosteroid levels were recorded in March/May 2007, following a sustained period of high river flows between October 2006 and March 2007. The high rainfall of July 2007 did not seem to be associated with particularly high corticosteroid levels but in both rivers there was a spike in corticosteroid concentrations following higher flows recorded during December 2007.

5. The corticosteroid data for fish from the Ray are higher than those from the Ock during 2007, yet the monthly mean flow data suggest flows were higher in the Ock. This disparity may be related to changes that occurred between samples and were therefore not detected, but inspection of the daily flow data indicates that although monthly mean flows were higher in the Ock the magnitude of daily change in flow during this period was greater in the Ray rising to 15 $m^3 sec^{-1}$ whereas in the Ock, maximum daily flows did not exceed 9.4 $m^3 sec^{-1}$.

6. There are no previous data on which to draw for comparison but it is likely that extreme hydrological events, such as flooding and resultant alterations in flow and temperature, modifies the corticosteroid (stress) status of resident fish populations, resulting in a magnified or additive response to capture.

7. The corticosteroid data provide no evidence that the stress response of fish captured in the Ray prior to installation of the GAC plant was modified by exposure to the effluent. Higher peak corticosteroid levels in fish in the Ray may be due to the interaction between environmental and chemicals stressors (i.e. the effluent) or may just reflect differences in the flow regime between the two rivers.

8. Whole-body glucose levels in fish from the Ray and Ock were similar for both sexes and overall there was no significant difference in glucose levels between rivers. There was however a highly significant decline in glucose concentrations across the sampling period. The overall downward trend in concentrations was interrupted by a significant increase in mean glucose concentrations during the summer of 2007.

9. Inspection of the glucose data suggested that they tied more closely to variation in the status of the stress axis of the fish (corticosteroids) and the changes with time observed across the monitoring period may have reflected the involvement of the same factors responsible for the alterations in corticosteroid status during this period.

10. Among fish from the Ray, peak glucose concentrations were associated with periods of high water flow (October 2006 – March 2007; July 2007; December 2007). For fish in the Ock a single prominent peak is associated with high flows during July 2007. In both rivers glucose levels were high during 2006, possibly indicating events not captured by the flow gauges, or not linked with flow rate change at all. The same observation is true for the corticosteroid data.

11. It seems safe to conclude that the Rodbourne effluent was not a factor in modulating the glucose content of the resident stickleback population - glucose concentrations in fish from both rivers during the matching periods prior to and following the installation of the GAC plant were almost identical. However, there was a small but significant overall difference in glucose concentrations in fish from the Ray and Ock during the period March-November 2007. While it seems likely that this arose because of differences in environmental factors such as the extent of perturbations in water flow, as is the case for the corticosteroid data it remains a (remote) possibility that factors in the effluent modified the response of the fish to the events driving the changes in glucose.

12. In the samples collected during September 2006 and January 2008 EROD activity was significantly higher in fish collected from sites in the Ray compared to the activity in fish collected from sites on the Ock. The EROD activity levels reported here are consistent with the limited stickleback data available elsewhere and seem indicative of a low level of exposure to organics in the Ray fish.

13. Of the chemical analyses carried out by EDCAT3&4 the most relevant to the interpretation of the EROD data are probably the PAH data which were obtained from sediments. Total PAHs in the sediment samples were highest at EB, lower at 7B and were lower than both these at CB. The exposure of fish to PAHs at these sites may be either direct via dissolved chemicals, or chemicals adsorbed to particles in the water column, or indirect via the consumption of contaminated prey items.

14. In the single post-remediation sample collected in March 2008 EROD activity within samples from the Ray was similar to that observed in the previous two pre-remediation samples. This might be expected if the exposure route of the fish is not primarily via water-borne chemicals and is instead via ingested food items, for example.

15. However, with only the EROD data to consider, the issue of whether the installation of the GAC plant at Rodbourne removed sufficiently high proportion of organic contaminants effluent to reduce the exposure of the fish cannot be resolved. Residual contamination of the sediments would be expected for some time following remediation of the effluent (assuming that EROD-inducing chemicals are effectively removed by the GAC plant) and may be expected to be sustained in invertebrates closely exposed to the sediments.

16. In March 2008 EROD activity in fish from the Ock was greatly increased over previous samples from that river to levels indistinguishable from those in the Ray. Possible explanation for this anomaly are discussed in the report. Resolution of the factors underlying these anomalous observations will probably not be possible until the CYP1A expression level data set becomes available.

5.4 Summary of the results in the context of EDCAT 5 project aims:

1. *By comparing appropriate biomarkers in fish sampled from STW-impacted sites and control sites during the pre-remediation period, to determine whether there was evidence for any effects that might be attributed to the presence of estrogenic (or androgenic, or anti-androgenic/-estrogenic) endocrine disrupting chemicals in the former. This aim was addressed by measuring concentrations of the estrogen-dependent yolk protein precursor vitellogenin, and the androgen-dependent nest glue spiggin in male and female sticklebacks. In addition histological examination of the gonadal structure of fish captured at the impacted and non-impacted sites was employed to seek evidence of overt alterations in reproductive physiology of the fish. For a subset of matched samples from the two rivers, the relative induction of hepatic choriogenin mRNA, a biomarker of estrogen exposure, was measured.*

Conclusions: Chemistry data provided by EDCAT3&4 showed that estrogenicity of the effluent was low prior to remediation and lower still following installation of the GAC plant. No evidence of overt estrogenic effects was detected in male sticklebacks in the Ray, VTG and ChG levels were similar in males from both rivers. Nor was there any evidence of alterations in spiggin concentrations in the kidneys of males from the Ray compared to the Ock. However, VTG concentrations in female sticklebacks from the Ray were increased following the STW upgrade as were hepatic ChG transcript levels, and kidney spiggin concentrations. No changes in these elements of the reproductive system were observed in females from the Ock across the same time periods. Chemical analysis of the effluent indicated that prior to installation of the GAC plant substantial concentrations of anti-androgenic chemicals were present, together with a wide range of other organics. Concentrations of these were much reduced following the plant upgrade. It is reasonable to suppose that the changes observed in the female reproductive endocrine system following the upgrade were related to the removal of some or all of this complex mixture of

chemicals. The absence of effects in males may be related to the balance between exogenous and endogenous signals, or to the specificity of effects exerted by the chemicals present. No intersex fish were detected from either river. A significant bias in favour of females was detected in the stickleback populations in both rivers suggesting a factor associated with life-history of the fish, rather than contaminant burden, was responsible.

2. *By comparing appropriate biomarkers in fish sampled from STW-impacted sites and control sites during the pre-remediation period, to determine whether there was evidence for any effects that might be attributed to the presence of "conventionally" toxic chemicals. This was addressed by measurement of the activity of a key Phase I transforming enzyme in the liver of fish, either using direct enzymatic assay (EROD) or by quantifying the levels of expression of the corresponding gene (CYP1A).*

Conclusions: EROD activity was significantly greater in fish from the Ray than the Ock in two samples collected prior to the installation of the GAC plant (2006, 2007) and this likely reflects the differential contaminant loading in the two rivers. A single sample following the commissioning of the GAC plant (2008) indicated that EROD activity had increased among fish from the Ock while that in fish from the Ray remained unchanged. While a delayed recovery of this biomarker in fish from the Ray may be expected depending on the route of exposure (direct via water or indirect via contaminated food) the reasons for elevated EROD activity in fish from the Ock/Childrey Brook are not immediately evident. Provision of a full data set for Cyp1A expression awaits the repeat of the assay. When this is complete the factors underlying the EROD findings may become clear.

3. *To determine whether the adaptive capacity and energetic status of fish varied between the STW-impacted and non-impacted sites. This was addressed by measurement of indicators of stress (whole-body corticosteroid levels), metabolic status (whole-body glucose levels) and anabolic activity (RNA:DNA ratios).*

Conclusions: The data provide no evidence that the stress response of fish captured in the Ray prior to installation of the GAC plant was modified by exposure to the effluent. However, large variations in whole-body corticosteroid and glucose concentrations in fish from both rivers, with clear trends over time, were closely linked to perturbations in the river flow regime. Whether there was interaction between environmental and chemical factors in determining corticosteroid and glucose status is difficult to discern but it seems likely that variation in these indicators of the stress axis was driven primarily by environmental factors. The RNA:DNA ratios were closely linked with seasonal change in temperature and closely matched observed patterns of weight and length gain in stickleback populations in the two rivers. The longer growth period enjoyed by fish in the Ray was clearly evident. For both rivers, mean anabolic activity was greater during 2008 than 2007 and it seems likely that this is related to adverse effects associated with the periods of extreme flow change observed on both rivers in 2007.

4. *To assess whether there were differences in population size and structure between STW-impacted and non-impacted sites. This was addressed by comparison of key somatic measures, in particular frequency distributions for fork length.*

Conclusions: Because of the extreme patchiness of the distribution of stickleback populations in both rivers accurate abundance estimates were not obtained. However, the catch per unit effort across the life of the project was similar for both rivers. While population size, and age structure (both rivers hosted annual populations), appeared to be similar fish in the Ray were overall larger than those from the Ock, and spawned earlier. The differences in growth and timing of spawning between the rivers were likely to have been associated with the Rodbourne STW effluent. Downstream of the discharge on the Ray water temperatures were consistently 2 – 3°C above those

of the Ock. This temperature difference, in combination with the introduction of additional nutrients into the river which is likely to have affected the availaibility of food, probably accounts for the different growth profile among the sticklebacks in the two rivers. However, over and above this difference, there was a significant increase in size of sticklebacks in the Ray between the matched pre- and post-remediation periods in the Ray while no change in size of the fish in the Ock occurred during the same period. Similarly, the RNA:DNA ratio was higher in fish from the Ock during 2007 but greater in fish from the Ray during 2008. Taken together, these observations suggest that there was an improvement in the status of the fish in the Ray following the commissioning of the GAC plant, while the population in the Ock remained relatively stable. It is reasonable to suppose that this may be linked to the reduction of the chemical load entering the Ray at Rodbourne following the installation of the GAC plant. The Ray is "cleaner" now than was the case prior to remediation but remains nutrient rich and several degrees warmer than the Ock, this combination of factors providing fish in the Ray with greater scope for growth relative to populations in the Ock.

5.5 Final comments

The changes and differences in various biomarkers that were detected among the stickleback populations in the Ray and Ock during 2006 to 2008 are likely to have had several causes.

Precise attribution of cause is complicated by the fact that several potentially interacting factors can be identified: (i) changes in the chemical content of the Rodbourne effluent post-remediation; (ii) the nutrient content of the effluent; (iii) the temperature differential introduced by the effluent; and (iv) the occurrence of severe perturbations in flow rate due to rainfall events.

All of these factors have the potential to affect growth processes and may have done so. The stress axis appears to have been affected primarily by extreme changes in flow rate and the consequences of those alterations but the response of the stress axis to change may also reflect the interaction of environmental perturbations with other factors. The timing of reproduction is likely to have been affected by temperature and possibly nutritional factors.

The relatively trivial estrogen content of the pre-remediation effluent was insufficient to evoke direct estrogenic effects in the resident male sticklebacks. However, alterations in female reproductive biomarkers in the Ray appear to be linked directly with the remediation process suggesting that components of the effluent were disruptive of female reproduction and the reduction in the chemical content of the effluent brought about by remediation had an ameliorative effect on the reproductive status of the female sticklebacks. While it is impossible to predict to what extent the functional reproductive performance of female sticklebacks in the Ray was affected by exposure to the pre-remediation effluent the potential for effects should not be dismissed. Reductions in VTG have demonstrable effects on fecundity in other species and combined with possible effects on growth may have had a significant influence on recruitment.

The data suggest that STW effluent may be intrinsically harmful to the reproductive status of female fish, an issue perhaps obscured by recent focus on the reproductive health of male fish.

5.6 References

Allen, Y. T., Katsiadaki, I., Pottinger, T. G., Jolly, C., Matthiessen, P., Mayer, I., Scott, A. P., Eccles, P. and Feist, S. (2008). Intercalibration exercise using stickleback endocrine disrupter screening assay. *Environmental Toxicology and Chemistry* 27, 414-412.

Ali, M. & Wootton, R. J. (1998). Do random fluctuations in the intervals between feeding affect growth rate in juvenile three-spined sticklebacks? *Journal of Fish Biology* 53, 1006–1014.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389-3402.

Andersson, C., Katsiadaki, I., Lundstedt-Enkel, K., Orberg, J. (2007). Effects of 17α-ethynylestradiol on EROD activity, spiggin and vitellogenin in three-spined stickleback (*Gasterosteus aculeatus*). *Aquatic Toxicology* 83, 33-42.

Arukwe, A., Celius, T., Walther, B.T. and Goksøyr, A. (1998). Plasma levels of vitellogenin and eggshell zona radiata proteins in 4-nonylphenol and o,p'-DDT treated juvenile Atlantic salmon (*Salmo salar*). *Marine Environmental Research* 46, 133–136.

Arukwe, A., Kullman, S.W., Berg, K., Goksøyr, A. and Hinton, D.E. (2002). Molecular cloning of rainbow trout (*Oncorhynchus mykiss*) eggshell zona radiata protein complementary DNA: mRNA expression in 17β-estradiol- and nonylphenol-treated fish. *Comparative Biochemistry and Physiology* B 132, 315–326.

Aubry, É., Rime, H. and Monod, G.(2005).Beta-naphthoflavone inhibits the induction of hepatic oestrogen-dependent proteins by 17alpha-ethynylestradiol in mosquitofish (*Gambusia holbrooki*). *Biomarkers* 10, 439-455.

Beaumont, W. R. C., Taylor, A. A. L., Lee, M. J. and Welton, J. S. (2002). Guidelines for Electric Fishing Best Practice. Environment Agency R&D Technical Report W2-054/TR

Bisson, M. and Hontela, A. (2002). Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. *Toxicology and Applied Pharmacology* 180, 110-117.

Buckley, L., Caldarone, E. & Ong, T.-L. (1999). RNA-DNA ratio and other nucleic acid-based indicators for growth and condition of marine fishes. *Hydrobiologia* 401, 265–277.

Celius, T. and Walther, B.T. (1998). Oogenesis in Atlantic salmon (*Salmo salar* L.) occurs by zonagenesis preceding vitellogenesis *in vivo* and *in vitro*. *Journal of Endocrinology* 158, 259–266.

Chen, X., Li, V. W. T., Yu, R. M. K. and Cheng, S. H. (2008). Choriogenin mRNA as a sensitive molecular biomarker for estrogenic chemicals in developing brackish medaka (*Oryzias melastigma*). *Ecotoxicology and Environmental Safety* 71, 200–208.

Chesenko, K., Pakdel, F., Segner, H., Kah, O. and Eggen, R. I. L. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and Comparative Endocrinology* 155, 31-62.

Chícharo, M. A. and Chícharo, L. (2008). RNA:DNA ratio and other nucleic acid derived indices in marine ecology. *International Journal of Molecular Sciences* 9, 1453-1471.

Clemmesen, C. (1994). The effect of food availability, age or size on the RNA/DNA ratio of individually measured herring larvae: laboratory calibration. *Marine Biology* 118, 377–382.

Copp, G. H. (1989) Electrofishing for fish larvae and 0+ juveniles: equipment modifications for increased efficiency with short fishes. *Aquaculture and Fisheries Management* 20, 453-462.

Environment Agency (2008). Long-term exposure to environmentally relevant concentrations of ethinyloestradiol affects sexual differentiation and development in roach, *Rutilus rutilus*. Environment Agency Science Report SC030299/SR2. 27 pp.

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, article 10.

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.

Graham, C. T. and Harrod, C. (2009). Implications of climate change for the fishes of the British Isles. *Journal of Fish Biology* 74, 1143-1205.

Fujita, T., Fukada, H., Shimizu, M., Hiramatsu, N. and Hara, A. (2004). Quantification of serum levels of precursors to vitelline envelope proteins (choriogenins) and vitellogenin in estrogen treated masu salmon, *Oncorhynchus masou*. *General and Comparative Endocrinology* 136, 49–57.

Geoghegan, F., Katsiadaki, I., Williams, T. D. and Chipman, J. K. (2008). A cDNA microarray for the three-spined stickleback, *Gasterosteus aculeatus* L., and analysis of the interactive effects of oestradiol and dibenzanthracene exposures. *Journal of Fish Biology* 72, 2133-2153.

Gesto, M., Soengas, J. L. and Míguez, J. M. (2008). Acute and prolonged stress responses of brain monoaminergic activity and plasma cortisol levels in rainbow trout are modified by PAHs (naphthalene,α-naphthoflavone and benzo(a)pyrene) treatment. *Aquatic Toxicology* 86, 341–351.

Gorokhova, E. and Kyle, M. (2002). Analysis of nucleic acids in Daphnia: development of methods and ontogenetic variations in RNA-DNA content. *Journal Of Plankton Research* 24 (5), 511-522.

Hahlbeck, E., Katsiadaki, I., Mayer, I., Adolfsson-Erici, M., James, J., and Bengtsson, B. E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption - II - kidney hypertrophy, vitellogenin and spiggin induction. *Aquatic Toxicology* 70, 311-326.

Hansen, P. D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M. and Blaise, C. (1998). Vitellogenin – a biomarker for endocrine disruptors. *Trends in Analytical Chemistry* 17, 448-451.

Hontela, A. (2006). Corticosteroidogenesis and StAR protein of rainbow trout disrupted by human-use pharmaceuticals: Data for use in risk assessment. *Toxicological Sciences* 93, 1-2.

Hook, S. E., Skillman, A. D., Small, J. A. and Schultz, I. R. (2007). Temporal changes in gene expression in rainbow trout exposed to ethynyl estradiol. *Comparative Biochemistry and Physiology* C 145, 73–85.

Islinger, M., Willimski, D., Völkl, A. and Braunbeck, T. (2003). Effects of 17a-ethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish. *Aquatic Toxicology* 62, 85-103.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C. R., van Aerle, R., Santos, E. and Brighty, G. (2006). Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives* 114 (suppl 1), 32–39.

Katsiadaki, I., Scott, A. P., and Mayer, I. (2002a). The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. *Marine Environmental Research* 54 SI, 725-728.

Katsiadaki, I., Scott, A.P., Hurst, M.R., Matthiessen, P., and Mayer, I. (2002b). Detection of environmental androgens: a novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environmental Toxicocology and Chemistry* 21, 1946–1954.

Katsiadaki, I., Morris, S., Squires, C., Hurst, M. R., James, J. D.and Scott, A. P. (2006). Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive in vivo test for detection of environmental antiandrogens. *Environmental Health Perspectives* 114 (suppl 1) 115–121.

Katsu, Y., Lange, A., Urushitani, H., Ichikawa, R., 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 and Technology* 41, 3368-3374.

Kawahara, S., Hrai, N., Arai, M., and Tatarazako, N. (2008). The effect of in vivo co-exposure to estrone and AhR-ligands on estrogenic effect to vitellogenic production and EROD activity. *Environmental Toxicology and Pharmacology* 27, 139-143.

Kim, S. D., Cho, J., Kim, I. S., Vanderford, B. J. and Snyder, S. A. (2007). Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Research* 41, 1013 – 1021.

Lange,A., Katsu, Y., Ichikawa, R., Paull, G. C., Chidgey, L. L., Coe, T. S., Iguchi, T. and Tyler, C. R. (2008). Altered sexual development in roach (*Rutilus rutilus*) exposed to environmental concentrations of the pharmaceutical 17a-ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. *Toxicological Sciences* 106, 113–123.

Levesque, H. M., Dorval, J., Hontela, A., Van Der Kraak, G. J. and Campbell, P. G. C. (2003). Hormonal, morphological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures. *Journal of Toxicology and Environmental Health* 66 A, 657-676.

Marsh, T. J. and Hannaford, J. (2007) The summer 2007 floods in England and Wales – a hydrological appraisal. Centre for Ecology & Hydrology. 32pp.

Martyniuk, C. J., Gerrie, E. R., Popesku, J. T., Ekker, M. and Trudeau, V. L. (2007). Microarray analysis in the zebrafish (*Danio rerio*) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol. *Aquatic Toxicology* 84, 38-49.

Maunder, R. J., Matthiessen, P., Sumpter, J. P. and Pottinger, T. G. (2007). Rapid bioconcentration of steroids in the plasma of sticklebacks (*Gasterosteus aculeatus*) exposed to water-borne testosterone and 17β-estradiol. *Journal of Fish Biology* 70, 678-690.

McCurley, A. T. and Callard, G. V. (2008). Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Molecular Biology* 9, article 102.

Miller, D. H., Jensen, K. M., Villeneuve, D. L., Kahl, M. D., Makynen, E. A., Durhan, E. J. and Ankley, G. T. (2007). Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, 26, 521–527.

Navas, J. M. and Segner, H. (2000). Antiestrogenicity of β-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. *Aquatic Toxicology* 51, 79-92.

Nicolas, J.-M. (1999). Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. *Aquatic Toxicology* 45, 77-90.

Palumbo, A. J., Denison, M. S., Doroshov, S. I. and Tjeerdema, R. S. (2009). Reduction of Vitellogenin Synthesis by an Aryl Hydrocarbon Receptor Agonist in the White Sturgeon (*Acipenser transmontamus*). *Environmental Toxicology and Chemistry* (in press)

Pangle, K. L. and Sutton, T. M. (2005). Temporal changes in the relationship between condition indices and proximate composition of juvenile *Coregonus artedi*, *Journal of Fish Biology* 66, 1060–1072.

Pickering, A.D. & Pottinger, T.G. (1987) Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology* 30, 363-374.

Pope, K. L. and Kruse, C. G. (2007). Condition. In "*Analysis and Interpretation of Freshwater Fisheries Data*" C. S. Guy & M. L. Brown, eds. American Fisheries Society, Bethesda, pp. 423-471.

Pottinger, T. G. and Calder, G. M. (1995). Physiological stress in fish during toxicological procedures: a potentially confounding factor. *Environmental Toxicology and Water Quality* 10, 135-146.

Pottinger, T. G. & Carrick, T. R. (2000). Contrasting seasonal modulation of the stress response in male and female rainbow trout. *Journal of Fish Biology* 56, 667-675.

Pottinger, T. G. and Musowe, E. M. (1994). The corticosteroidogenic response of brown and rainbow trout alevins and fry during a 'critical' period. *General and Comparative Endocrinology* 95, 350-362.

Pottinger, T. G., Balm, P. H. M. and Pickering, A. D. (1995). Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. *General and Comparative Endocrinology* 98, 311-320.

Pottinger, T. G., Yeomans, W. E. & Carrick, T. R. (1999). Plasma cortisol and 17b-estradiol levels in roach, *Rutilus rutilus* (L.), exposed to acute and chronic stress. *Journal of Fish Biology* 54, 525-532.

Pottinger, T. G., Carrick, T. R. & Yeomans, W. E. (2002). The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *Journal of Fish Biology* 61, 207-229.

Pottinger, T. G., Pulman, K. G. T., Carrick, T. R. and Scott, A. P. (2005). Evaluation of biochemical methods for the non-destructive identification of sex in upstream migrating salmon and sea trout. *Journal of Fish Biology* 67, 1514-1533.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chemical Ecology* 8, 275–285.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfiedl, M., Kent, M. L. and Schreck, C. B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* 258, 565-574.

Richard, P., Bergeron, J.-P., Boulhic, M., Galois, R. & Person-Le Ruyet, J. (1991). Effect of starvation on RNA, DNA and protein content of laboratory-reared larvae and juveniles of *Solea solea*. *Marine Ecology Progress Series* 72, 69–77.

Romero, L. M., Reed, J. M. and Wingfield, J. C. (2000). Effects of weather on corticosterone responses in wild free-living passerine birds. *General and Comparative Endocrinology* 118, 113-122.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., Sumpter, J. P. (1998). Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environmental Science and Technology* 32, 1559-1565.

Sanchez, W., Aït-Aïssa, S., Palluel, O., Ditche, J.-M. and Porcher, J.-M. (2007). Preliminary investigation of multi-biomarker responses in three-spined stickleback (*Gasterosteus aculeatus* L.) sampled in contaminated streams. *Ecotoxicology* 16, 279-287.

Sanchez , W., Piccini, B., Ditche, J.-M. and Porcher, J.-M. (2008). Assessment of seasonal variability of biomarkers in three-spined stickleback (*Gasterosteus aculeatus* L.) from a low contaminated stream: Implication for environmental biomonitoring. *Environment International* 34, 791–798.

Scholz, S. and Mayer, I. (2008). Molecular biomarkers of endocrine disruption in small model fish. *Molecular and Cellular Endocrinology* 293, 57–70.

Sebire, M., Allen, Y., Bersuder, P. and Katsiadaki, I. (2008). The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. *Aquatic Toxicology* 90, 37-47.

Shimizu, M., Fukada, H., Fujita, T., Hiramatsu, N. and Hara, A. (2000). Serum levels of precursors to vitelline envelope proteins (choriogenins) in *Sakhalin taimen* after treatment with oestrogen and during oocyte growth.. *Journal of Fish Biology* 57, 170–181.

Ternes, T.A., Meisenheimer, M., Mcdowell, D., Brauch, H.J., Brigitte, H.G., Preuss, G., William, U., Zulei-Seibert, N. (2002). Removal of pharmaceuticals during drinking water treatment. *Environmental Science and Technology* 36, 3855–3863.

Vaccaro, E., Meucci, V., Intorre, L., Soldani, G., Di Bello, D., Longoa, V., Gervasi, P. G., and Pretti, C. (2005). Effects of 17β-estradiol, 4-nonylphenol and PCB 126 on the estrogenic activity and phase 1 and 2 biotransformation enzymes in male sea bass (*Dicentrarchus labrax*). *Aquatic Toxicology* 75, 293-305.

Van den Belt, K., Verheyen, R., Witters, H. (2003). Comparison of vitellogenin responses in zebrafish and rainbow trout following exposure to environmental estrogens. *Ecotoxicology and Environmental Safety*, 56, 271-281.

Wartman, C. A., Hogan, N. S., Hewitt, L. M., McMaster, M. E., Landman, M. J., Taylor, S.,
Kovacs, T. G. and van den Heuvel, M. R. (2009). Androgenic effects of a Canadian bleached kraft pulp and paper effluent as assessed using threespine stickleback (*Gasterosteus aculeatus*). *Aquatic Toxicology* 92, 131–139.

Whyte, J. J., Jung, R. E., Schmitt, C. J. and Tillitt, D. E. (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology* 30, 347 – 570.

Wootton, R. J. (1984). A Functional Biology of Sticklebacks. London: Croom Helm.

Wu, R. S. S., Zhou, B. S., Randall, D. J., Woo, N. Y. S. and Lam, P. K. S. (2003). Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. *Environmental Science and Technology* 37, 1137-1141.