General Enquiries on the form should be made to:
Defra, Procurements and Commercial Function (Evidence Procurement Team)
E-mail: research.competitions@defra.gsi.gov.uk

Evidence Project Final Report

- **Note**
  In line with the Freedom of Information Act 2000, Defra aims to place the results of its completed research projects in the public domain wherever possible.
  The Evidence Project Final Report is designed to capture the information on the results and outputs of Defra-funded research in a format that is easily publishable through the Defra website
  An Evidence Project Final Report must be completed for all projects.
- This form is in Word format and the boxes may be expanded, as appropriate.
- **ACCESS TO INFORMATION**
  The information collected on this form will be stored electronically and may be sent to any part of Defra, or to individual researchers or organisations outside Defra for the purposes of reviewing the project. Defra may also disclose the information to any outside organisation acting as an agent authorised by Defra to process final research reports on its behalf. Defra intends to publish this form on its website, unless there are strong reasons not to, which fully comply with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.
  Defra may be required to release information, including personal data and commercial information, on request under the Environmental Information Regulations or the Freedom of Information Act 2000. However, Defra will not permit any unwarranted breach of confidentiality or act in contravention of its obligations under the Data Protection Act 1998. Defra or its appointed agents may use the name, address or other details on your form to contact you in connection with occasional customer research aimed at improving the processes through which Defra works with its contractors.

Project identification

1. Defra Project code: CB0446
2. Project title: Chemical Exposure and Stress Responses in UK Fish
3. Contractor organisation(s):
   Centre for Ecology & Hydrology
   Lancaster Environment Centre
   Library Avenue
   Bailrigg
   Lancaster LA1 4AP
4. Total Defra project costs (agreed fixed price): £ 76,778
5. Project: start date 01/11/2010
   end date 31/03/2012

6. It is Defra's intention to publish this form.

   Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

   (a) When preparing Evidence Project Final Reports contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.

   Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the Evidence Project Final Report can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.

   In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

   (b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

1. This scoping study was conducted to assess whether evidence exists for effects of wastewater treatment works (WWTW) effluent on the adaptive stress axis of fish.

2. A total of 427 three-spined sticklebacks were sampled during March and April 2011 from sites downstream of ten WWTWs in north-west England, serving rural and urban areas with population equivalents of between 1,000 and 120,000. Sticklebacks were also sampled from three sites with known heavy metal contamination and from a laboratory population maintained in uncontaminated water.

3. Somatic (mass, length, sex) data were collected from the sticklebacks to provide information on the relative growth and condition of the sampled populations. Indices of stress (whole-body cortisol and glucose) were measured both prior to and following a standardised stressor to establish both the baseline activity and stress-induced responsiveness of the stress axis in fish at each site. Biomarkers of chemical exposure (metallothionein, cytochrome P4501A, choriogenin gene expression) were measured to provide information on the relative exposure of fish at each site to polycyclic aromatic hydrocarbons, heavy metals and estrogenic compounds, all of which are known to modify the activity of the stress axis in fish.

4. The relative impact of the selected WWTWs on the receiving waters was characterised by three indirect measures: (i) population equivalents, (ii) dry weather flow and (iii) the percentage of effluent calculated to be present at each sampling site.

5. There was considerable between-site variation in all the parameters measured in sticklebacks. For the fish sampled downstream of the WWTWs much of this was found to be related to the impact metrics of the upstream WWTWs, particularly the percent effluent present at the study site.

6. For the sticklebacks sampled downstream of WWTWs mass, length, and to a much lesser extent condition, were positively related to the concentration of effluent present at the sample site. This is

consistent with previous findings that indicate the positive effects of effluent-derived enrichment and elevated temperature on fish growth. Somatic measurements for fish sampled from metal-contaminated sites were within the range of those for fish at WWTW sites.

7. Cortisol and glucose concentrations in unstressed sticklebacks downstream of WWTWs were positively related to the percent effluent concentration at each site. Variation in somatic data did not account for the variation in cortisol levels in unstressed fish. Cortisol and glucose concentrations in unstressed fish sampled from metal-contaminated sites were within the range of those of fish at WWTW sites.

8. After the exposure of sticklebacks to a standardised stressor (confinement) following capture, both cortisol and glucose concentrations in whole-body extracts of the stressed fish were elevated. In fish downstream of WWTWs the magnitude to which cortisol and glucose concentrations were elevated was reduced with increasing effluent concentration at each site. Cortisol and glucose concentrations in fish sampled from metal-contaminated sites and also subjected to a period of confinement were within the range of those for fish at WWTW sites

9. For sticklebacks downstream of the WWTWs little of the variation in biomarker gene expression between sites was explained by the variation in impact measures between the WWTWs. Overall there was a small but significant negative trend evident between percent effluent at each sample site and biomarker activity in the resident fish. The range in the magnitude of biomarker gene expression was similar among fish downstream of the WWTWs and fish sampled from metal-contaminated sites.

10. These data suggest that the function of the stress axis in sticklebacks downstream of these WWTWs was modified by exposure to the discharged effluent. In unstressed fish the stress axis was more active at sites receiving a higher proportion of effluent, which might be interpreted to indicate that exposure to the effluent constitutes a chronic stressor. The stress axis became less responsive to an additional stressor in fish exposed to higher effluent loads, suggesting that elements within the effluent impeded the normal functioning of the stress axis.

11. A lack of pronounced variation in biomarker gene expression across the range of WWTWs suggests that these alterations in the function of the stress axis are not solely the result of exposure to high concentrations of polycyclic aromatic hydrocarbons, heavy metals or estrogenic compounds. The effects may instead be mediated by a combination of components within the complex WWTW effluents, including possibly pharmaceuticals and personal care products (PPCPs).

12. The results of this scoping study suggest that exposure to WWTW effluents can perturb the function of the stress axis in sticklebacks. Further work is needed to (i) demonstrate that the effect is consistent across a wider range of sites, (ii) investigate the causality of the effect, and (iii) investigate the implications for the fitness of fish populations downstream of WWTWs.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and

- any action resulting from the research (e.g. IP, Knowledge Exchange).

1. Background to the project

1.1 Chemicals in the aquatic environment

The health and well-being of the aquatic environment is threatened by a range of factors, among the most important of which are chemical contaminants from both discrete and diffuse sources. While the regulatory framework in the UK is effective in ensuring that concentrations of individual chemicals are present in discharged effluents or field run-off/drainage at concentrations well below those likely to cause immediate adverse effects in the receiving water, concern has focused on two issues.

The first is the prospect that the mixture of chemicals present in the aquatic environment may act in concert, and evoke effects on exposed animals even when present individually at "safe" concentrations (Schwarzenbach *et al*., 2006). The second is the now well-established fact that some chemicals, at concentrations below those associated with “conventional” toxicity, can interfere with the normal functioning of the endocrine system in aquatic animals.

In the aquatic environment this has been best studied with regard to effects on the reproductive endocrine system in fish and has been termed endocrine disruption (Caliman and Gavrilescu, 2009; Burkhardt-Holm, 2010). This scoping study was designed to investigate whether the complex chemical milieu in rivers downstream of wastewater treatment works (WWTW) can affect the functioning of another endocrine-based system in fish, the stress axis.

1.2 The stress response in fish

Optimising reproductive success is critically important in sustaining populations, but animals must survive long enough to reach reproductive maturity. To do this they have to cope with many challenges, ranging from intra-specific competition and predation, to effects of extreme weather events.

Fish, in common with all vertebrates, possess a suite of adaptive responses that are rapidly invoked to assist the animal in coping with challenging circumstances (Pankhurst, 2011). Collectively, this array of neuroendocrine, metabolic and behavioural alterations is termed the stress response and is activated when the fish is faced with actual or perceived threats, of biotic or abiotic origin. Distinct response types have been identified within populations of fish and these are linked with specific behavioural traits (Øverli et al., 2005). Thus, for example, individuals can be categorized as high-responding and reactive/risk-averse or low-responding and pro-active/risk-taking, categories that have been allied to the notion of coping strategies (Øverli et al., 2007). Variation in the magnitude of the stress response is to a large extent under genetic control (Pottinger and Carrick, 1999).

In recent years there has been an increasing awareness of the significance of the stress response in ecological and environmental contexts (Romero, 2004; Wikelski and Cooke, 2006; Young et al., 2006; Bradshaw, 2007; Wingfield, 2008).

The stress response is an adaptive mechanism that is not allied with any specific life-history event, such as reproduction. Instead it provides an ongoing protective mechanism that increases the likelihood of survival under adverse conditions or unpredictable challenges.

The core of the stress response is neuroendocrine – both metabolic and behavioural elements of the response can be linked with changes in the neuroendocrine system. Key indicators of stress include the release of corticosteroids from the interrenal tissues (homologous with the adrenal cortex in mammals) resulting in increased blood cortisol levels, and mobilisation of energy leading to an elevation of blood glucose concentrations. In recent years, a considerable body of work, mostly originating from North America, has demonstrated that the stress axis in fish, like the reproductive axis, is susceptible to interference by chemicals (see Pottinger, 2003).

1.3 Chemical effects on the stress axis of fish

The primary manifestation of interference by chemicals with the stress axis, as reported to date, is the inability of the fish to mount an appropriate response to a challenge, as indicated by an attenuated release of corticosteroids into the bloodstream. This, it is assumed, has adverse implications for the ability of the fish to deal with threats to its well-being. Clear evidence of higher-level effects is limited, as is the case for reproductive endocrine disruption. However, at its extreme, adrenal insufficiency has been demonstrated to have lethal consequences among post-operative humans (Hinson and Raven, 2006) and cortisol deficiency is a well-identified issue in veterinary practice, requiring replacement therapy (Plechner, 2004).

It is therefore reasonable to assume that the fitness and survival of fish exposed to contaminants that interfere with the normal function of the stress response will be adversely affected.

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; Levesque et al., 2003; Hontela, 2006; Gesto et al., 2008). Currently it is not known whether the functioning of the stress axis of fish in British waters is affected by exposure to chemical contaminants.

1.4 Aim of this scoping study

The aim of this scoping study was twofold:

1. To determine whether the stress response in three-spined sticklebacks varies significantly between sites affected or unaffected by chemical contaminants.

2. To evaluate the extent to which such variation in the stress axis can be attributed to differences in chemical contamination.

To address these goals three-spined sticklebacks (*Gasterosteus aculeatus*) were sampled from sites downstream of ten WWTWs in the north west of England serving a range of populations (1000 – 125,000) and from three sites affected by elevated dissolved metal concentrations but not WWTW effluent. Indicators of stress (cortisol and glucose) were quantified in these fish in order to assess the status of the stress axis, both in individuals immediately following capture (unstressed – baseline cortisol and glucose) and after a short period of confinement (stressed – elevated cortisol and glucose). The extent to which the sampled fish had been exposed to certain types of key contaminants was also measured using the expression of selected biomarker genes (*i.e*. cytochrome P450 1A as a marker for exposure to planar organic molecules such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls; metallothionein as a marker for exposure to heavy metals such as copper and zinc; choriogenin as a marker for exposure to estrogens). Estradiol-17β was also measured as an indicator of reproductive stage and endogenous estrogen exposure.

2. Materials and methods

2.1 Site selection

2.1.1. Polluted sites. The geographical distribution of sampling sites is shown in Fig 1. Ten sites downstream of WWTWs serving population equivalents of between 1k and 125k, and within a 50 km radius of CEH Lancaster, were identified as being likely to support sticklebacks and as being accessible for sampling (see Table 1). In addition, three sites not immediately downstream of WWTWs but at which water-borne metal concentrations were high, were selected. These latter sites were identified using data provided by the CEH Ribble & Wyre catchment observatory study, which has recently investigated the water quality in two major river basins in north-west England (Rowland et al., 2010; Neal et al., 2011). Mean total concentrations of Zn, Hg, Al and V were used to rank the 25 sites for which data were available and of these, three (see Table 1) high-scoring sites were selected, on the basis that they were accessible for sampling and were likely to support populations of sticklebacks.

2.1.2. Control sites. It was intended to collect fish both from contaminated sites and from sites with no identifiable contaminant input. However, during the site selection process it became evident that it was difficult to identify sites that could be categorised as uncontaminated with a high degree of confidence. To provide a comparison with fish from a demonstrably pristine environment, the population of three-spined sticklebacks maintained in the CEH aquarium was selected. The CEH aquarium receives water from a tarn (Blea Tarn, SD 4934 5850) used as a drinking water reservoir (United Utilities) and fish are held in a constant flow-through system. The assumption was made that in the absence of any chemical challenge these fish should provide a physiologically neutral baseline for comparative purposes.

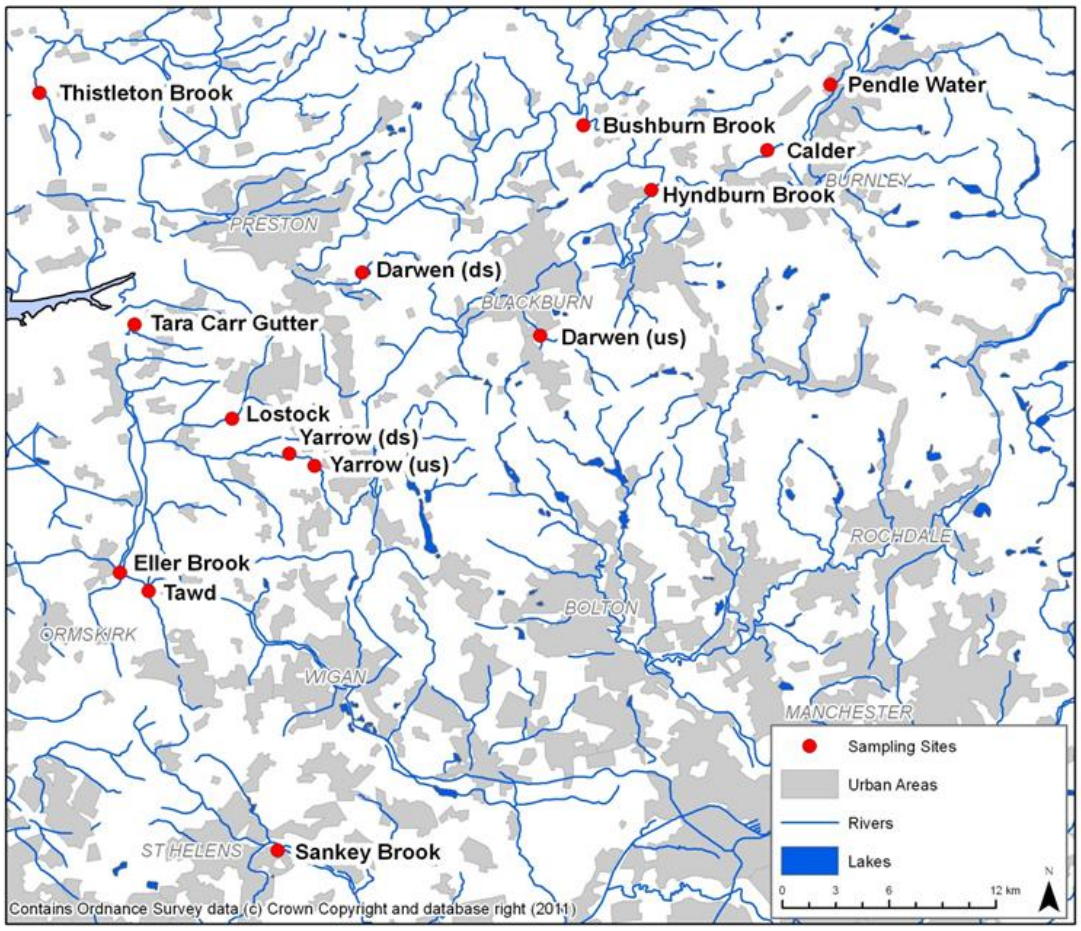


Figure 1. The location of sampling sites in north-west England (Lancashire and Merseyside) in relation to major conurbations and river systems; us = upstream, ds = downstream. Note – the R. Yarrow, upstream site, was sampled mistakenly. Because the data from these fish are presented in the report, its position is given here and in Table 1.

Sample site	Date of sample	Sample site grid reference	Associated WWTW	WWTW discharge grid reference	Population served by WWTW	Daily dry weather flow (m^3)	Treatment type
Sites downstream of WWTWs - affected by effluent							
Sankey Brook	14.3.11	SJ 5421 9576	St Helens	SJ 5390 9591	124,209	37600	SAS
Darwen (ds)	25.3.11	SD 5897 2819	Blackburn	SD 6047 2941	120,562	49700	SB
R. Calder	31.3.11	SD 8171 3506	Burnley	SD 8255 3527	113,332	28600	TA2
R. Yarrow	24.3.11	SD 5488 1804	Chorley	SD 5627 1740	45,211	13400	TA2
Lostock	16.3.11	SD 5166 2000	Leyland	SD 5216 2083	41,526	11000	TA2
Darwen (us)	1.4.11	SD 6896 2464	Darwen	SD 6899 2434	30,053	8800	SB
Pendle Water	25.3.11	SD 8526 3875	Colne	SD 8702 3947	21,073	6400	TB2
Tara Carr Gutter	17.3.11	SD 4618 2528	Longton	SD 4687 2528	13,606	3600	TB2
Bushburn Brook	1.4.11	SD 7139 3644	Billington	SD 7134 3612	5,889	1500	SB
Thistleton Brook	17.3.11	SD 4084 3828	Elswick	SD 4105 3817	1,013	300	SB
Sites affected by high dissolved metal concentrations							
Eller Brook	15.3.11	SD 4537 1135	-	-	-	-	-
Hyndburn Brook	31.3.11	SD 7519 3284	-	-	-	-	-
R. Tawd	24.3.11	SD 4698 1031	-	-	-	-	-

Table 1. Summary of sample sites. (us) = upstream; (ds) = downstream. SAS: secondary activated sludge; SB: secondary biological filter; TA2: activated sludge; TB2: biological filter. Details are not provided in the Table for the R. Yarrow (upstream) site (SD 5630 1735), which is shown in Fig. 1, and was sampled mistakenly (24.3.11).

2.2. Sampling procedure

Sampling was conducted between the 14th March and 1st April 2011. The total number of fish caught was 427 with numbers captured at each site varying between 18 and 39 (with the exception of the R. Tawd at which only seven fish were captured). Fish were captured using a large hand net (38 cm D-frame, 0.5 cm mesh, 1.5 m handle) dragged through areas adjacent to and under the river bank where trailing and emergent vegetation was evident.

Immediately after capture, fish designated "unstressed" (*i.e*. before the stress caused by netting had caused a detectable neuroendocrine response) were transferred to a 10 l bucket containing a lethal concentration of the sedative 2-phenoxyethanol (1:1000) in river water. When opercular movement had ceased and the fish were unresponsive to a tail pinch using forceps they were placed individually in labelled, capped, 12 ml polypropylene centrifuge tubes which were transferred to a liquid N2 dry shipper (Taylor-Wharton CryoExpress CX500, Jencons plc).

Where possible, a minimum of 10 fish were designated as "stressed". Immediately on capture these were transferred to a 10 L bucket containing river water and held for between 30 and 60 mins (in order to allow a maximal activation of the stress axis) before being transferred to a lethal concentration of sedative and treated as described above. At CEH Lancaster the samples were transferred to a freezer in racks (-70^{o}C) for storage until they were processed for analysis within one month of capture. (Imposition of post-capture stress was conducted under the authority of a Home Office project and personal licence held by TGP).

For the control aquarium population, a similar procedure was adopted. Fish were netted from a previously undisturbed aquarium and transferred to a bucket containing tank water. After 45 minutes they were killed and processed as above. Fish from a second aquarium were netted directly into sedative and killed immediately to provide unstressed individuals.

2.3. Tissue processing

Tubes containing fish were removed from the freezer in groups of six and placed on ice. While still frozen, each fish was removed from its tube and body mass (mg) and fork length (mm) were recorded. A ventral incision was made using dissecting scissors and the liver was removed and transferred to RNA extraction buffer (RNeasy mini kit, Qiagen). The sex of each fish was recorded. While still frozen 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), with cooling on ice between bursts. The homogenate was stored frozen (-70^{o}C) until required for assay.

2.4. Analytical procedures

***2.4.1. Cortisol and estradiol-17β (E2)*:** Steroids were extracted from a 100 µl aliquot of whole homogenate with 400 µl ethyl acetate. Extracts were centrifuged and a 50 µl aliquot (cortisol) or 100 µl aliquot (E2) of each supernatant was analysed by radioimmunoassay (RIA; Pottinger & Carrick, 2001; Bell et al., 2007). The only deviation from previously published methods was the formulation of dextran coated charcoal (1% dextran and 5% activated charcoal in assay buffer). Cortisol antibody was supplied by David Kime (Sheffield University), estradiol antibody from AbCam (Abcam anti-E2 3).The lowest concentration of E2 that was consistently detected by the assay was 0.3 ng g^{-1}. For those samples in which E2 was undetectable a nominal value of 0.15 ng g^{-1} was attributed. Cortisol was detected in all samples, with a limit of detection of 0.5 ng g^{-1}.

***2.4.2. Glucose*:** Glucose concentrations in the homogenates were determined using a microplate assay (hexokinase reagent and standard glucose solution: Sigma-Aldrich).

***2.4.3. Real Time-PCR for quantification of gene expression*:** Details of the procedures adopted for quantifying gene expression in stickleback liver tissue are provided in Pottinger et al. (2011a). The sequences for amplification primers and minor groove binding (MGB) Taqman fluorogenic probes were derived from previous work (CYP1A: Geoghegan et al., 2008; T. Williams, University of Birmingham, pers comm..). Because sufficient RNA was not obtained from every fish, a sub-set of each sample was analysed.

Cytochrome P4501A: CYP1AFP (5'-GGAATTGTCAATGACCTGTTTGG–3'); CYP1ARP (5'-CGGATGAGCCACCATGTACA-3'); MGB Taqman probe CYP1ATP (5'-6FAM-ACACCGTCAGCACGACATTGTCATGG-3').

Metallothionein: MTFP (CCTGCAACTGCGGAGGAT); MTRP (GCCAGAGGCGCATTTTGT); Taqman Probe (6FAM-TGCACAAACTGCTCCTGCACCACC-TAMRA).

Choriogenin H: ChgHFP (5'- GATGCCACTCTGCCAAGCA); ChgHRP (5'-TGGCCCATCTCCCAAAAG); Taqman probe ChgHTP (6FAM-CGACCTCGAATCAA).

Primers and probes were checked for specificity by using BLASTn (Altschul et al., 1997) within the NCBI suite of facilities (www.ncbi.nlm.nih.gov). Expression of biomarker genes was normalised to expression of 18s rRNA using the equation R=1000 [(2Ct18s)/(2CtCYP1A)] where R is relative expression level, and Ct is the cycle threshold for target and control genes. Amplification efficiencies were not adjusted for each sample. Recent studies indicate that the expression of 18s remains constant in sticklebacks during exposure to chemical stressors (Williams et al., 2009).

2.5. Characterisation of WWTW impact

Two quantitative descriptors were available with which to characterise each WWTW: the dry weather flow per day (DWF) and the size of population served, *i.e*. the population equivalent value (PE). The DWF is the flow from the works after a period of no rainfall and can be comprised of a mixture of domestic and industrial waste. DWF values used in this study were provided to CEH in 2007 by the water companies. They are not measured values since no requirement existed to report flows to the Environment Agency. Hence the DWF is a best estimate based on the population served by the works plus any base load provided by industrial discharges, most of which are to the larger WWTWs. We have included both measures in these analyses because of the possibility that one or other metric defines more accurately the biological impact of the discharge.

In addition, to quantify more effectively the exposure of fish downstream of the WWTWs to contaminants it was possible to estimate the percentage of WWTW-derived effluent at each sampling site using the LF2000-WQX model (Keller and Young, 2004; Williams et al., 2009). Briefly, the model is a geographical information-based system that combines hydrological models with a range of water-quality models, including a catchment-scale water-quality model. This model generates spatially explicit statistical distributions of down-the-drain chemicals for both conservative and degradable compounds. It uses a Monte Carlo mixing-model approach to combine statistical estimates of chemical loads at specific emission points with estimated river flow- duration curves for the whole river network of interconnected model reaches (a reach is the river stretch between model features e.g. major tributaries, WWTWs). Thus, working from the low order streams at the head of the river network to the outlet from the river basin, the model accounts for the accumulation of point loads and the accumulation of water in which these loads are diluted. Degradable chemicals are removed from the river water by a non-specific dissipation process, assuming first-order kinetics.

A database within the model provided information on WWTWs within England and Wales including the population served, the DWF and the type of treatment used (Williams et al., 2008). The percentage effluent was estimated as the concentration modelled for a conservative chemical discharged from all WWTWs in the river system at a fixed concentration of 100 ng/L. The modelled concentration in ng/L is the estimate of the percentage dilution thus:

$$\text{Percentage effluent} = ((C_{eff} \times DWF) + (Fr \times C_r))/(F_r + DWF)$$

Where C_{eff} is the effluent concentration (= 100 ng L^{-1}), DWF is the effluent dry weather flow ($m^3\,day^{-1}$), F_r is the river flow at the discharge point ($m^3\ day^{-1}$) and C_r is the river concentration of the conservative chemical already in the river that has been discharged from any STWs upstream (ng L^{-1}).

Since both F_r and DWF are expressed as distributions, this calculation is carried out 2000 times, each time selecting a value randomly from these distributions to produce a distribution of estimated percentage effluents, from which the mean value has been selected for use in this analysis. Estimates of the percent effluent at each sampling site were calculated using both the long-term average flow data (1961-1990), and the flow data for the period during which the fish were resident in the river (March 2010 – April 2011). The model excludes WWTWs within 1 km of the coast that might discharge to the sea or to rivers that are tidally influenced, therefore no supporting data are currently available to estimate percent effluent in Tara Carr Gutter (Longton WWTW).

2.6. Statistical analysis

To test for differences in a selection of variables between rivers, sexes and stress levels, we employed an analysis of variance (ANOVA) approach. The first stage of the analysis was to ensure that the fundamental assumption of normality made in the ANOVA procedure was met and that any inference drawn from the results was as accurate as possible. Where data did not conform to a normal distribution they were log transformed and re-tested for normality. In all but one case (E2), the raw data or the log transformed data conformed to a normal distribution and hence a standard ANOVA was carried out for

these variables. For all metrics the effects of river, sex and stress (unstressed, stressed) were tested using a straightforward ANOVA approach. Selected first order interactions were also included. This enabled us to determine, for example, whether the effect of capture and confinement stress was the same across all rivers. To test variables for fish within individual rivers against one another we used an ANOVA design with a Tukey correction for multiple testing. This ensured that we accounted for the fact that we were more likely to see a significant effect by performing more tests (Type I error). This post-hoc Tukey test was carried out only once a significant effect of river was found in the initial ANOVA analysis.

For the E2 analysis, the data could not be sufficiently transformed to coincide with a normal distribution because of the highly inflated count of not-detectable (set at 0.15 ng g^{-1}) values. Rather than using more complicated zero inflated based models, we converted the data to binary (1,0) data representing the detection and non-detection of E2 respectively. This allowed us to model the binary response and hence infer whether certain groups (e.g. river, sex or stress condition) had significantly more instances of measurable E2 than others. To model this binary data a generalised linear model based approach with binomial distribution and logit link function was used. The link function transformed the binary data to a continuous scale and using the binomial distribution ensured that the associated errors were correct. Results from this analysis are similar to the standard ANOVA output providing significance effects of variables together with their impact on the proportion of detectable levels of E2.

For glucose and cortisol concentrations we investigated linear relationships to other measured variables of mass, length and condition from the same fish using data pooled from across all rivers. Because of pooling the data across rivers and the expectation that data from within the same river is likely to be less variable than data from different rivers, a mixed model based approach was adopted in which river was included as a random effect. Essentially this allowed for the differing variation we expected between-rivers compared to that within-river. Results from this analysis are analogous to those obtained from standard regression, returning the significance of variables along with the linear relationship between the response and the predictor variables.

Additional regression analyses were conducted using Minitab v.16 (Minitab Inc.).

3. Results

3.1. Somatic data

***3.1.1. Body mass*:** There was a highly significant four-fold difference in the mean mass of fish across the sample sites (Fig 2a; $\log_{10}$ body mass, ANOVA, F (14,412) = 50.5, $P < 0.001$). The largest fish were caught in the R. Tawd (2157 ± 93 mg, mean ± SEM, n = 7) and R. Darwen (upstream, 1894 ± 117 mg, n = 35) and the smallest in Bushburn Brook (515 ± 66 mg, n = 29). Statistically significant differences between rivers are indicated on Fig. 2a. Fish sampled from the CEH aquarium were two-years old (transferred to the aquarium at one year old and held for one year) and therefore older than all the wild-caught samples (which were assumed to be no more than 1 year old – but see Discussion) and therefore larger also.

There was a small but significant difference between male and female body mass overall (male: 1476 ± 55 mg, n = 210; female: 1315 ± 47 mg, n = 217; ANOVA, F (1,397) = 5.7, $P = 0.018$). There was no river*sex interaction ($P = 0.37$) – the relationship between the mass of male and female fish was similar at each site. Overall the range of values for fish sampled downstream of WWTWs was similar to that for fish collected from Ribble & Wyre observatory sites (Eller Brook, Hyndburn Brook, R. Tawd).

3.1.2. Fork length: Similar to mass, fork length varied across rivers (Fig. 2b; ANOVA, F (14,412) = 48.9, $P < 0.001$) with fish from the R. Tawd (56.9 ± 0.6 mm, n = 7) and R. Darwen (53.5 ± 0.9, n = 35) exhibiting the greatest mean length and fish from Bushburn Brook (35.8 ± 1.4, n = 29) the smallest. As was the case for mass there was a small but significant difference in length between male and female fish overall (male: 49.8 ± 0.6 mm, n = 210; female: 48.0 ± 0.5, n = 217; ANOVA, F (1,397) = 5.5, $P = 0.02$) with no river*sex interaction ($P = 0.66$). As for mass, mean length was within similar ranges for fish from both the WWTW sites and Ribble & Wyre observatory sites.

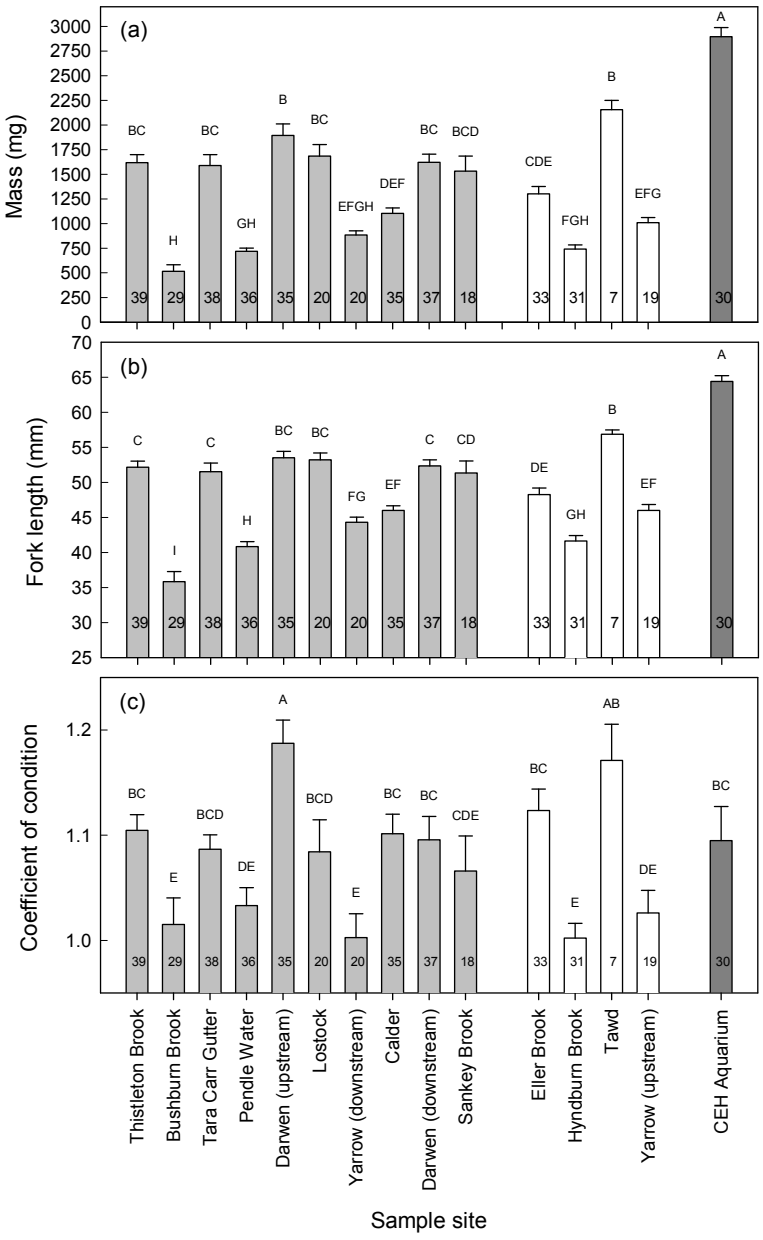


Figure 2. Somatic data. Each bar represents the mean + SEM (n for each mean is presented within the corresponding bar) for (a) mass, (b) fork length and (c) coefficient of condition (100*mass/length^3). Means sharing the same letters are not significantly different.

***3.1.3. Condition*:** The condition factor (coefficient of condition, Fulton's condition factor K: 100*weight/length^3; Bolger and Connolly, 1989) also varied significantly between rivers (Fig. 2c; ANOVA, F (14,412) = 5.9, $P < 0.001$) with highest condition factors recorded for fish from the R. Darwen (upstream) (1.18 ± 0.02, n = 35) and lowest condition factors for fish from Hyndburn Brook (1.00 ± 0.01, n = 31). There was no difference in condition between males and females overall ($P = 0.37$) and no river*sex interaction ($P = 0.47$). In this context it should be noted that in order to compare the condition factor of different populations it must be assumed that growth is isometric, that is, the relationship between mass and length remains the same independent of fish size (Cone, 1989). When growth is isometric the gradient "*b*" in the equation $\log_{10}(\text{mass}) = \log_{10}(a) + b.\log_{10}(\text{length})$ is equal to 3. However, when regressions were carried out on log-transformed mass and length data for fish at each site some variation between sites was observed with *b* ranging from 2.6 (R. Yarrow upstream) to 3.62 (R. Lostock). Whether this has any bearing on the interpretation of these condition data is not clear.

3.2. Cortisol

***3.2.1. Cortisol in unstressed fish*:** Overall, there was a significant difference in whole-body (WB) cortisol concentrations between male and female sticklebacks processed immediately after capture (unstressed) (male: 8.6 ± 0.6 ng ml^{-1}, n = 76; female: 13.6 ± 2.3 ng g^{-1}, n = 87; ANOVA, F (1,135) = 7.3, *P* = 0.008). There was no significant river*sex interaction among unstressed fish (*P* = 0.35) – the relationship between cortisol levels in male and female fish was similar at each site (data not shown).

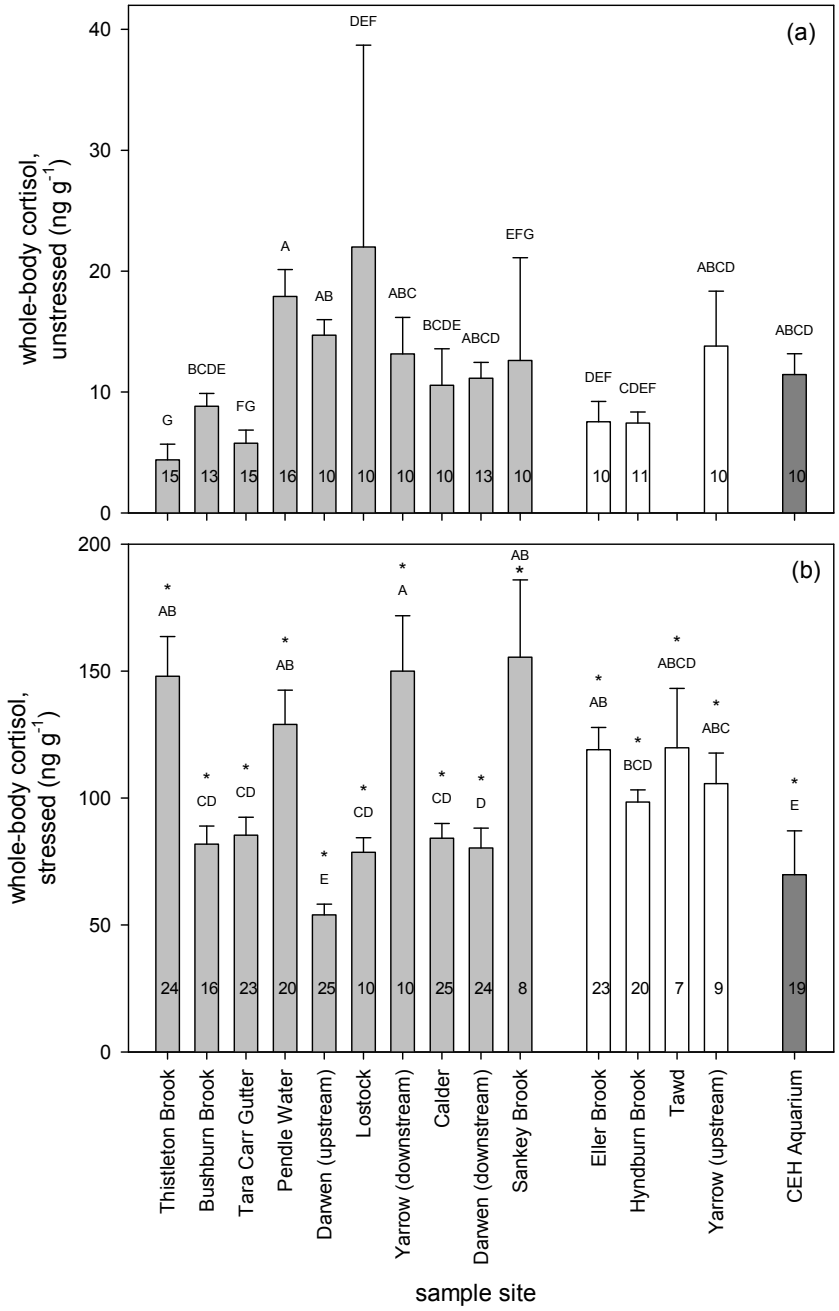


Figure 3. Whole-body cortisol concentrations in (a) unstressed and (b) stressed fish. Each bar represents the mean + SEM (n for each mean is presented within the corresponding bar). Means sharing the same letter are not significantly different. Note the different y-axis scales for (a) and (b).

Whole-body cortisol levels in unstressed sticklebacks varied significantly between rivers (Fig. 3a; arithmetic means presented but ANOVA carried out on log_{10} transformed data, F (13,149) = 5.8, *P* < 0.001) with a four-fold range in mean values across sites. The lowest concentrations of cortisol in unstressed fish were seen in fish from Thistleton Brook (4.4 ± 1.3 ng g^{-1}, n = 15) and the highest in fish from the R. Lostock (22.0 ± 16.7 ng g^{-1}, n = 10). There were two ostensibly unstressed fish with very high

concentrations of cortisol within the samples for Sankey Brook (89 ng g^{-1}) and the R. Lostock (172 ng g^{-1}) leading to the high intra-sample variability seen for these two sites. Overall, mean cortisol levels in fish from WWTW sites were of a similar range to those for fish from the Ribble & Wyre observatory sites. Mean cortisol levels in fish from the CEH aquarium were within the mid-range of those from the sampled sites.

***3.2.2. Cortisol in stressed fish*:** Concentrations of cortisol in sticklebacks exposed to a period of confinement following capture were significantly elevated at all sites compared to concentrations of cortisol in unstressed fish at the same sites (ANOVA, F (13, 397) = 10.4, $P < 0.001$).

Mean cortisol concentrations in stressed fish varied significantly between rivers (Fig. 3b; $\log_{10}$ transformed, ANOVA, F (14,248) = 10.0, $P < 0.001$) with a three-fold variation in mean concentrations between fish from the R. Darwen (upstream) (54.0 ± 4.2 ng g^{-1}, n = 25) and fish from Sankey Brook (155.5 ± 30.4 ng g^{-1}, n = 8). Mean cortisol levels in fish from the CEH aquarium exposed to a similar stressor were significantly lower than all except one sampled site (Darwen upstream; comparison conducted on log-transformed data).

There was a significant difference between males and females in stress-induced cortisol concentrations (male: 86.4 ± 4.5 ng g^{-1}, n = 133; female: 112.7 ± 5.1 ng g^{-1}, n = 130; ANOVA, F (1,233) = 12.9, $P < 0.001$). In contrast to cortisol concentrations in unstressed fish, differences between male and female fish following stress varied between rivers (river*sex interaction, ANOVA, F (14,233) = 3.5, $P < 0.001$). Mean cortisol concentrations in fish from the Ribble and Wyre observatory sites were midway between the highest and lowest values for fish downstream of WWTW sites.

The increment between mean cortisol concentrations in unstressed fish and mean cortisol concentrations in stressed fish at each sample site varied between 54 ng g^{-1} (Darwen upstream) and 148 ng g^{-1} (Thistleton Brook) but there was no relationship between mean cortisol concentrations in unstressed and stressed fish from each site ($y = -0.96x + 113.9$, $r^2 = 0.02$, $P = 0.63$) or between mean cortisol concentrations in unstressed fish and the stress-induced incremental change in cortisol (linear regression, $y = -1.96x + 113.9$, $r^2 = 0.08$, $P = 0.33$).

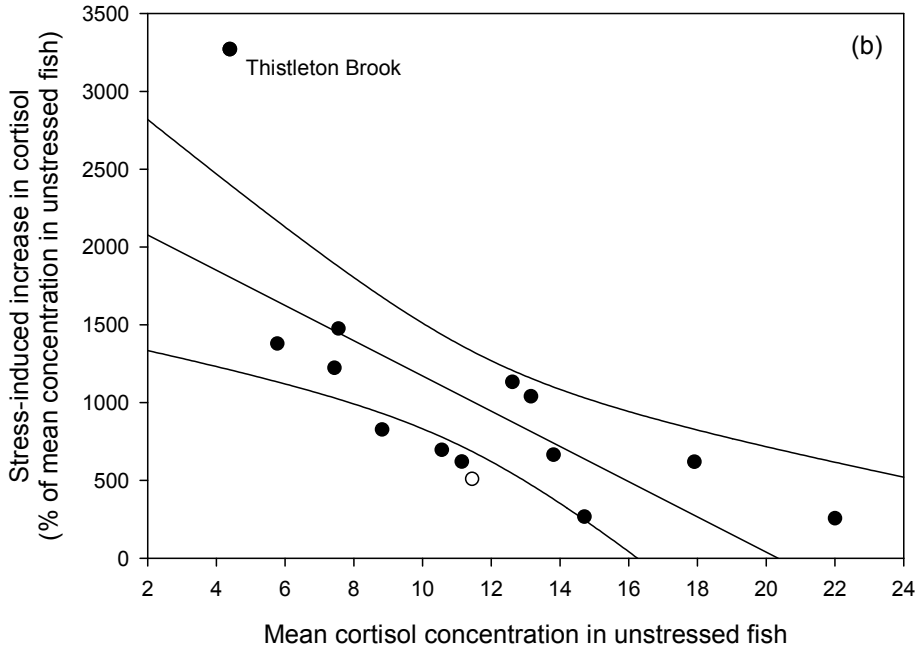


Figure 4. The relationship between mean cortisol levels in unstressed fish and the magnitude of the stress-induced change in cortisol as a percentage of the mean of the corresponding unstressed group (linear regression, $y = -113.2x + 2303$, $P < 0.01$, $r^2 = 0.51$). The best-fit line and 95% confidence intervals are shown. The data for Thistleton Brook were excluded from the analysis. The mean for the aquarium sample is indicated by an unfilled circle.

The increase in mean cortisol concentration following confinement, as a percent of the mean concentration in unstressed fish at the same site, varied between 257% (R. Lostock) and 1476 % (Eller Brook), with the exception of Thistleton Brook where the percent increase following stress was >3000 %. There was a highly significant negative relationship between WB cortisol levels in unstressed fish and the subsequent increase following imposition of the confinement stressor (Fig. 4: linear regression, y = -113.2x + 2303, $P < 0.01$, $r^2 = 0.51$).

***3.2.3 Cortisol: mass, length and condition*:** Overall, body mass, fork length and condition did not account for any of the variation in $\log_{10}$ cortisol concentrations in the unstressed fish (e.g. for condition: linear regression, $r^2 = 0.0$, $P = 0.8$). However, there was a significant effect of condition on stress-induced cortisol concentrations ($r^2 = 0.03$, $P < 0.01$) with higher condition being associated with a trend for a lower stress-induced WB cortisol concentration.

3.3 Glucose

***3.3.1 Glucose in unstressed fish*:** Whole-body glucose levels in unstressed sticklebacks varied significantly between rivers (Fig. 5a; ANOVA, F (13,149) = 7.4, $P < 0.001$) but no significant difference was evident between males and females overall ($P = 0.58$). There was no significant river*sex interaction among unstressed fish ($P = 0.62$) – the relationship between glucose levels in male and female fish was similar at each site.

The lowest mean concentrations of glucose in unstressed fish were measured in fish from the R. Yarrow (upstream) (1.08 ± 0.06 mg g^{-1}, n = 10) and the highest in fish from the R. Darwen (upstream) (1.88 ± 0.07 mg g^{-1}, n = 10). Mean glucose levels in fish from the CEH aquarium were significantly higher than mean levels for fish from several sampled sites, including Thistleton Brook, R. Lostock, R. Yarrow, Sankey Brook and Eller Brook.

***3.3.2 Glucose in stressed fish*:** The overall mean WB glucose concentration in stressed fish was slightly, but significantly elevated (1.75 ± 0.03 mg g^{-1}, n = 263) compared to that in unstressed fish (1.47 ± 0.03 mg g^{-1}, n = 163) (ANOVA, F (1,397) = 46.9, $P < 0.001$). There was also significant variation in glucose concentrations between sampling sites (Fig. 5b; ANOVA, F (14,248) = 9.0, $P < 0.001$). However, when restricted to within-site comparisons significant elevation of whole-body glucose concentration following stress was demonstrable only for Thistleton Brook. Mean post-stress glucose concentrations were highest in fish from the R. Tawd (2.25 ± 0.2 mg g^{-1}, n = 7) and were lowest in fish from Eller Brook (1.31 ± 0.04 mg g^{-1}, n = 23). A similar range of mean values was evident for fish from the Ribble and Wyre observatory sites and fish from sites downstream of WWTWs. Mean levels of glucose in fish from the CEH aquarium were higher than all but three of the sampled sites (Thistleton Brook, Bushburn Brook and R. Tawd).

There was a small but significant difference in stress-induced glucose levels between males (1.86 ± 0.04 mg g^{-1}, n = 133) and females (1.64 ± 0.04 mg g^{-1}, n = 130; ANOVA, F(1,233) = 4.3, $P < 0.05$) but no river*sex interaction ($P = 0.33$).

In common with the results for cortisol, fish from Thistleton Brook exhibited atypical responses in terms of the increment between mean glucose concentrations in unstressed fish and mean glucose concentrations in stressed fish when expressed in absolute terms (mg g^{-1}) or as a percentage of concentrations in unstressed fish. No significant relationship was evident between the mean concentration of glucose in unstressed fish and the absolute mean increment in stressed fish (linear regression, y = -0.0.1x + 0.4, $r^2 = 0.05$, $P = 0.43$), ignoring Thistleton Brook as an obvious outlier. There was, however, evidence of a (near-significant) negative trend in the relationship between mean glucose concentration in unstressed fish and the percent change in mean glucose after capture, linear regression, y = -18.9x + 42.9, $r^2 = 0.18$, $P = 0.08$).

In contrast with the results for WB cortisol, there was a strong positive relationship between the mean glucose concentration in unstressed and stressed fish from the same site (Fig. 6, linear regression, y = 0.82x + 0.51, $r^2 = 0.51$, $P < 0.01$) reflecting the small but consistent change in WB glucose concentrations between unstressed and stressed fish.

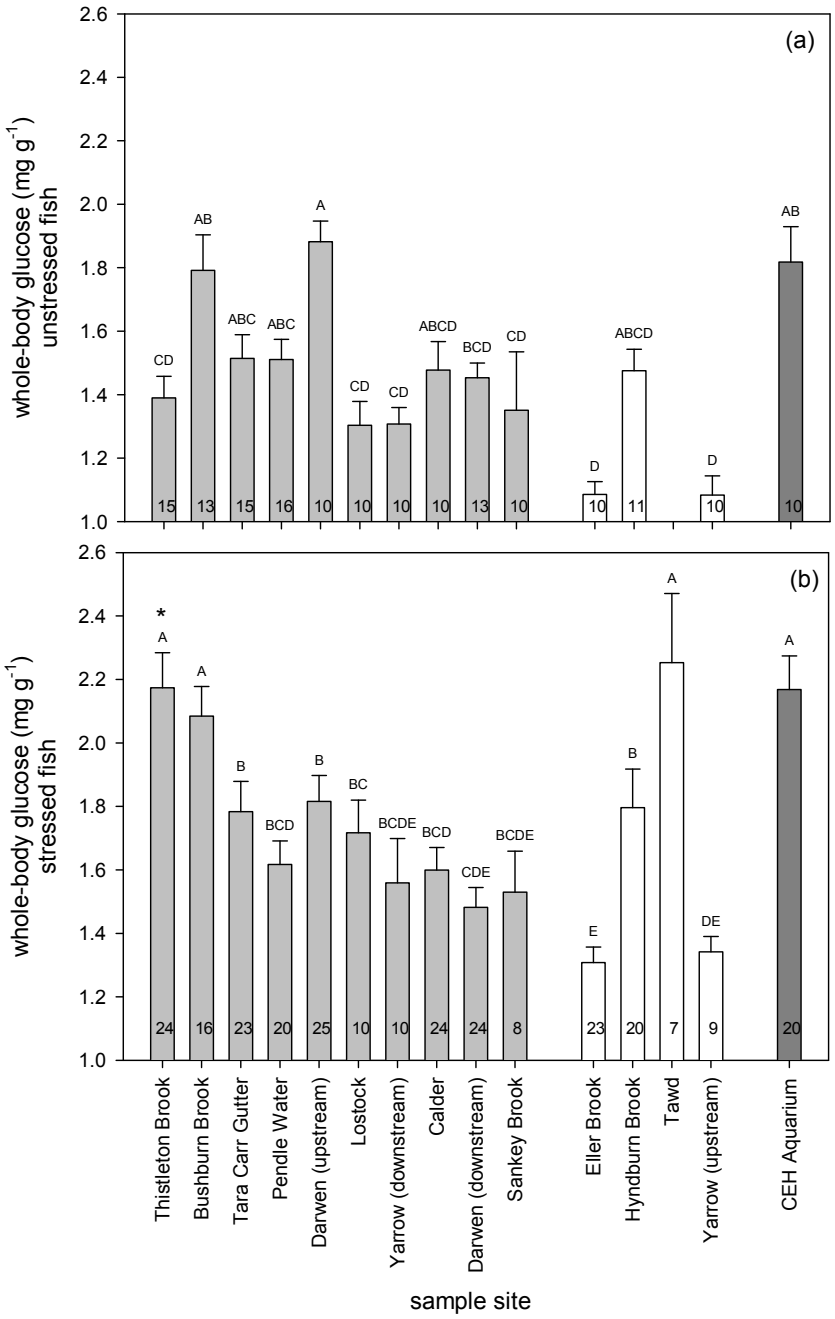


Figure 5. Whole-body glucose concentrations in (a) unstressed and (b) stressed fish. Each bar represents the mean + SEM (n for each mean is presented within the corresponding bar). Means sharing a letter are not significantly different.

***3.3.3 Glucose: mass, length and condition*:** Glucose concentrations in unstressed fish exhibited a significant relationship with mass (r^2 = 0.08, P < 0.001), length (r^2 = 0.02, P < 0.05) and condition (r^2 = 0.06, P < 0.01) and these relationships were also evident in stressed fish (mass: r^2 = 0.09, P < 0.001; length: r^2 = 0.06, P < 0.001; condition: r^2 = 0.02, P < 0.05).

3.3.4 Glucose and cortisol – interactions: For the purposes of these comparisons the data point for Thistleton Brook (identified as an outlier, see above) was excluded. Using all the available data there was no significant relationship between the mean whole-body cortisol and mean whole-body glucose concentrations in unstressed sticklebacks (linear regression, r^2 = 0.001, P = 0.7). Nor was any relationship evident between cortisol and glucose in stressed sticklebacks (r^2 = 0.0, P = 0.9).

No relationship was evident between cortisol and glucose for differences between unstressed and stressed means (linear regression, $r^2 = 0.16$, $P = 0.16$) or between the unstressed/stressed differences in cortisol and glucose when these were expressed as a percent of unstressed levels (linear regression, $r^2 = 0.06$, $P = 0.4$).

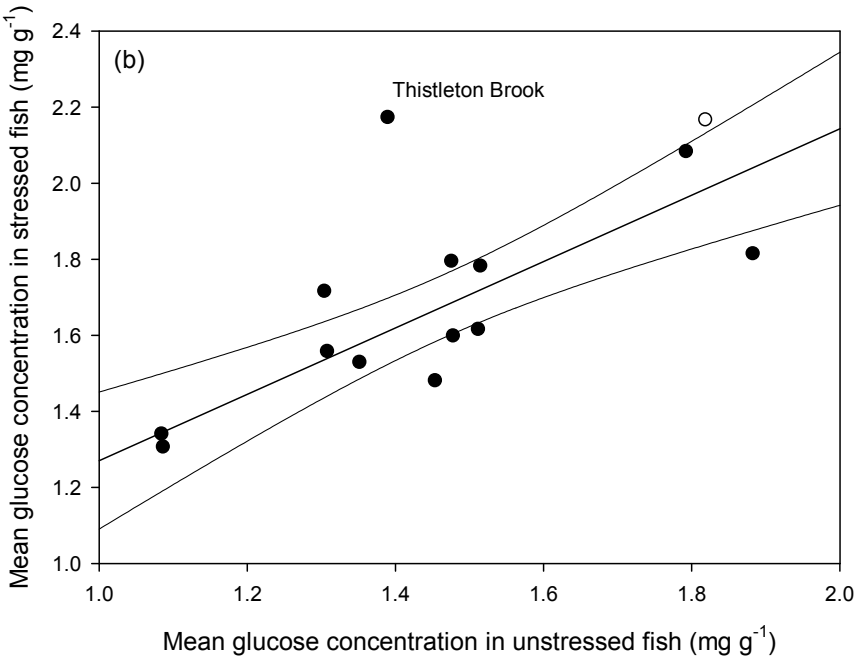


Figure 6. The relationship between mean glucose concentrations in unstressed fish and mean glucose concentrations in stressed fish from the same sampling sites (linear regression, $r^2 = 0.51$, $P < 0.01$). The data for Thistleton Brook are plotted but did not contribute to the regression analysis. Data for the aquarium sample are indicated by an unfilled circle.

3.4 Estradiol-17β

Concentrations of estradiol-17β (E2) were determined in all fish, primarily to provide a point of reference with choriogenin (ChgH) gene expression data (see below). This was in order to establish whether variation in ChgH could be attributed to variation in endogenous E2 concentrations, or might instead be indicative of exposure to estrogens of environmental origin. Concentrations of E2 also provide information on the relative stage of reproductive advancement at each sample site. Reproductive status and in particular levels of gonadal steroids are known to modulate the stress axis in some species of fish (Pottinger *et al*., 1995, 1996) The E2 data were not normally distributed due to the high frequency of fish exhibiting non-detectable concentrations of E2.

There was little difference between the mean E2 concentration in unstressed fish (3.7 ± 0.9, n = 163) and stressed fish (4.0 ± 0.8, n = 264) and using the binary response model to investigate the E2 data no effect of capture and confinement stress on the proportion of fish with measurable E2 concentrations was evident ($P = 0.08$). Therefore, data from unstressed and stressed fish were consolidated for analysis. The relative proportions of fish of both sexes at each site exhibiting detectable E2 levels are shown in Fig 7a.

There was no overall difference in the frequency of individuals with measurable E2 concentrations between the sexes ($P = 0.08$) and more surprisingly mean concentrations were very similar for the sexes (female = 4.4 ± 0.9, n = 217, Fig. 7b; male = 3.4 ± 0.8, n = 210, Fig. 7c). Concentrations of E2 were highest in both males and females at three sites: R. Darwen (upstream), R. Calder and R. Darwen downstream. All three of these sites were downstream of WWTWs.

There was no relationship between the proportion of fish with measurable E2 concentrations at any site and fish mass (P = 0.339), fork length (P = 0.503) or condition (P = 0.94). However, significant regressions were obtained between the proportion of fish with measurable E2 concentrations and indices of WWTW function and with biomarker expression levels (see below).

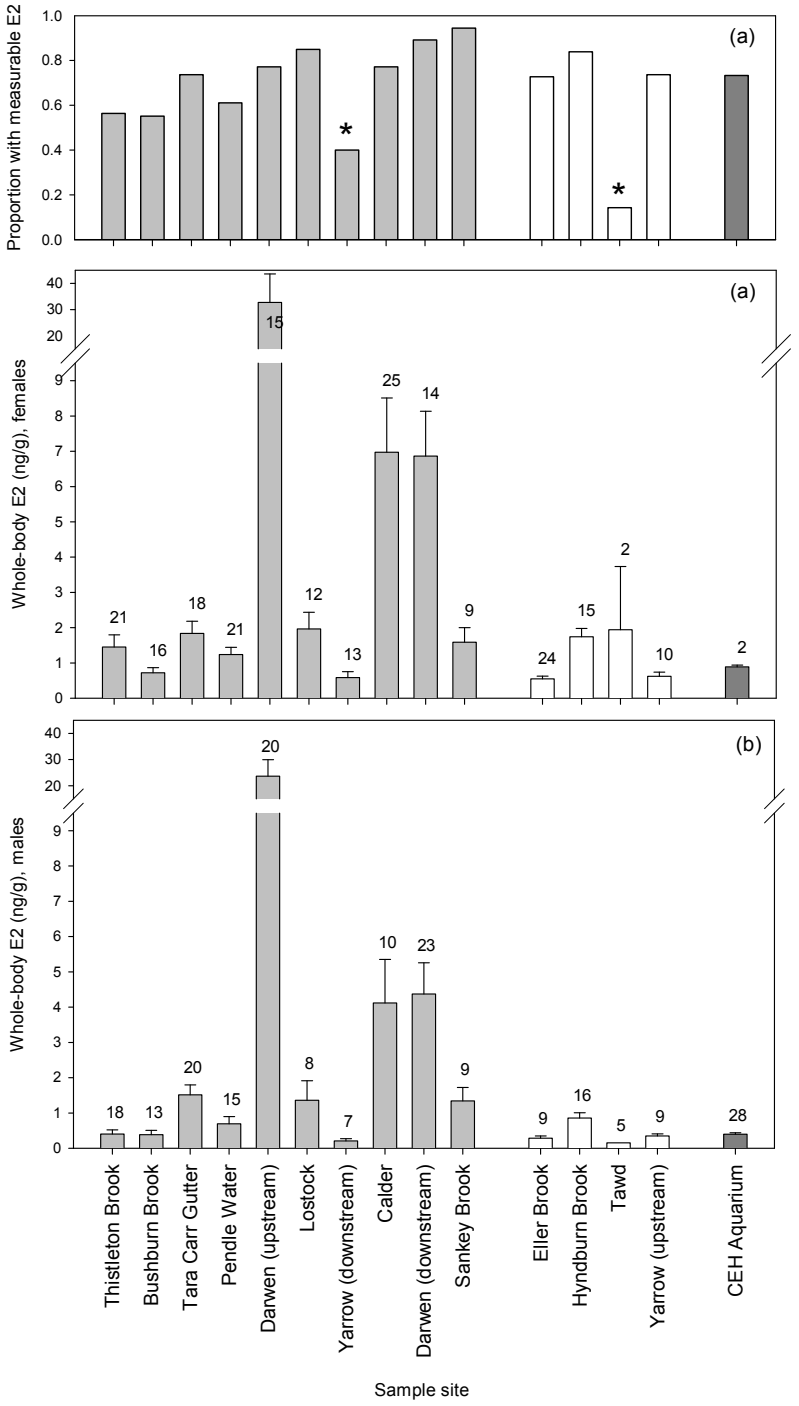


Figure 7. (a) The proportion of fish at each sample site with detectable E2 concentrations; and whole-body concentrations of estradiol-17β in (b) female and (c) male sticklebacks from each sampling site. Each bar represents the mean + SEM. For each sample, n is depicted above the bar. Asterisks indicate sites at which the proportion of fish with measurable E2 was significantly lower than the overall mean.

3.5 Biomarker gene expression

3.5.1 Cytochrome P4501A: CYP1A was expressed to a different degree by males and females (females: 7.3 ± 2.3, n = 77, Fig. 8a; males: 9.32 ± 3.33, n = 56, Fig. 8b; ANOVA, F (1,190) = 3.9, $P < 0.05$). Within sexes there were significant differences in CYP1A expression between the sample sites (females: ANOVA, F (14,82) = 4.46, $P < 0.001$; males: ANOVA, F (14,80) = 3.4, $P < 0.001$). Among females highest expression of CYP1A was detected in fish from the R. Yarrow (downstream), Hyndburn Brook, and Pendle Water. Similar trends were evident among the male fish. Expression levels of CYP1A in fish from the CEH aquarium were statistically indistinguishable from almost all the sampled sites.

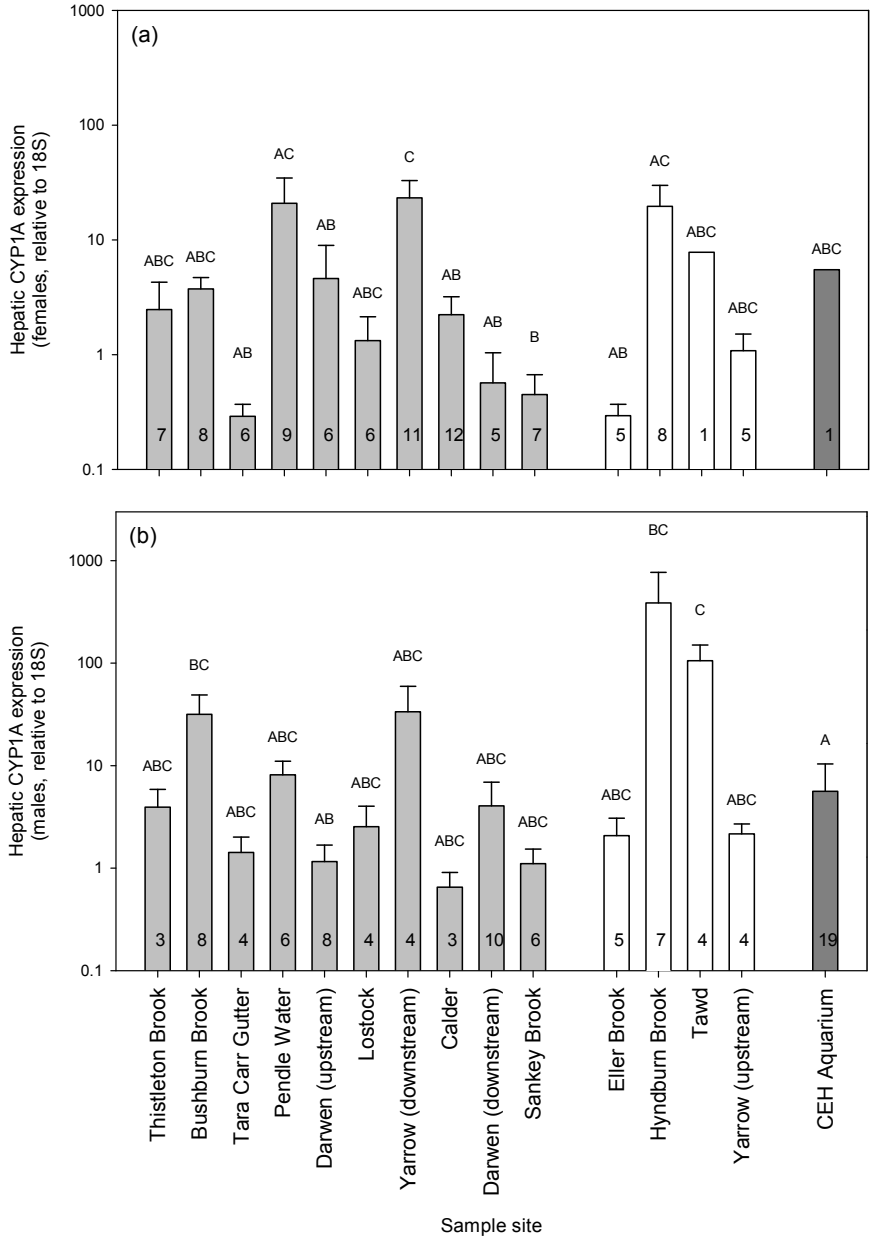


Figure 8. Expression of cytochrome P450 1A1 (CYP1A) RNA relative to 18s ribosomal RNA in liver tissue of (a) female and (b) male sticklebacks from each sampling site. Each bar is the mean + SEM. For each sample, n is depicted within the bar. Means sharing the same letter are not significantly different.

3.5.2 Metallothionein: MT was expressed to a different degree by males and females (females: 1.82 ± 0.6, n = 80; males: 1.34 ± 0.6, n = 63; ANOVA, F (1,220) = 10.3, $P < 0.01$). Within sexes there were significant differences in MT expression between the sample sites (females: ANOVA, F (14,97) = 6.15, $P < 0.001$, Fig. 9a; males: ANOVA, F (14,95) = 16.7, $P < 0.001$, Fig. 9b) with fish from Hyndburn Brook exhibiting highest levels in both males and females. Expression of MT among females showed greater variation between sites than for males. Highest overall expression levels among male fish were detected in fish from the CEH Aquarium (see Discussion).

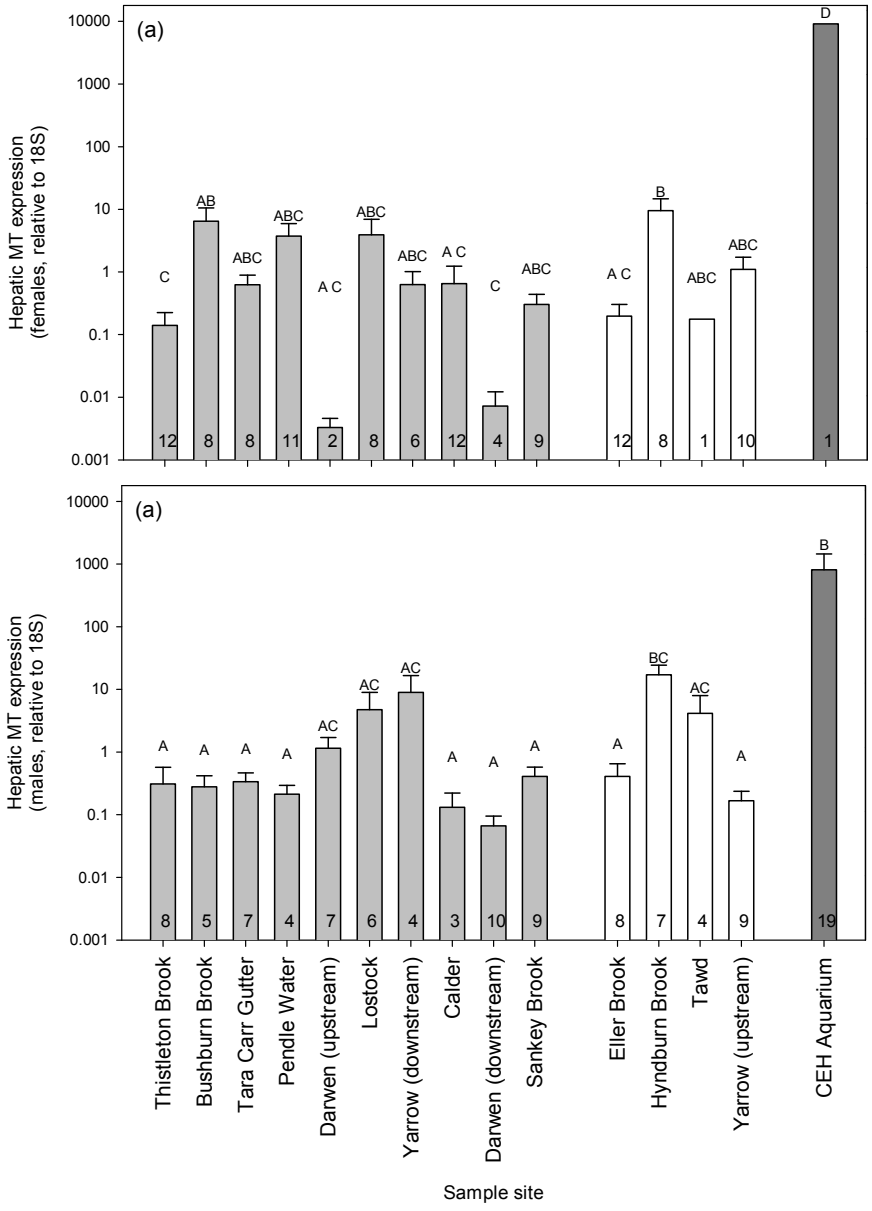


Figure 9. Expression of the metallothionein (MT) gene RNA relative to 18s ribosomal RNA in liver tissue of (a) female and (b) male sticklebacks from each sampling site. Each bar is the mean + SEM. For each sample, n is depicted above/within the bar. Means sharing the same letter are not significantly different.

3.5.3 Choriogenin: ChgH was expressed to a very different degree by males and females (females: 35.8 ± 11.8, n = 93; males: 2.97 ± 0.9, n = 68; ANOVA, F (1,236) = 84.2, *P* < 0.001). Within sexes there were significant differences in ChgH expression between the sample sites (females: ANOVA, F (14,110) = 3.3, *P* < 0.001, Fig. 10a; males: ANOVA, F (14,98) = 2.01, *P* < 0.05, Fig. 10b). Among females this manifested as highest expression in fish from Pendle Water and lowest in fish from the R. Lostock, Sankey Brook and Eller Brook. Among the males, fish from Pendle Water also exhibited relatively high levels of expression, but the most significant contrast was between levels in fish from Thistleton Brook and levels in fish from Bushburn Brook and Hyndburn Brook. ChgH expression in female fish from the CEH aquarium was statistically indistinguishable from females at the sampled sites and for males levels of expression in CEH aquarium fish were similar to, or substantially lower, than those in fish from the sampled sites.

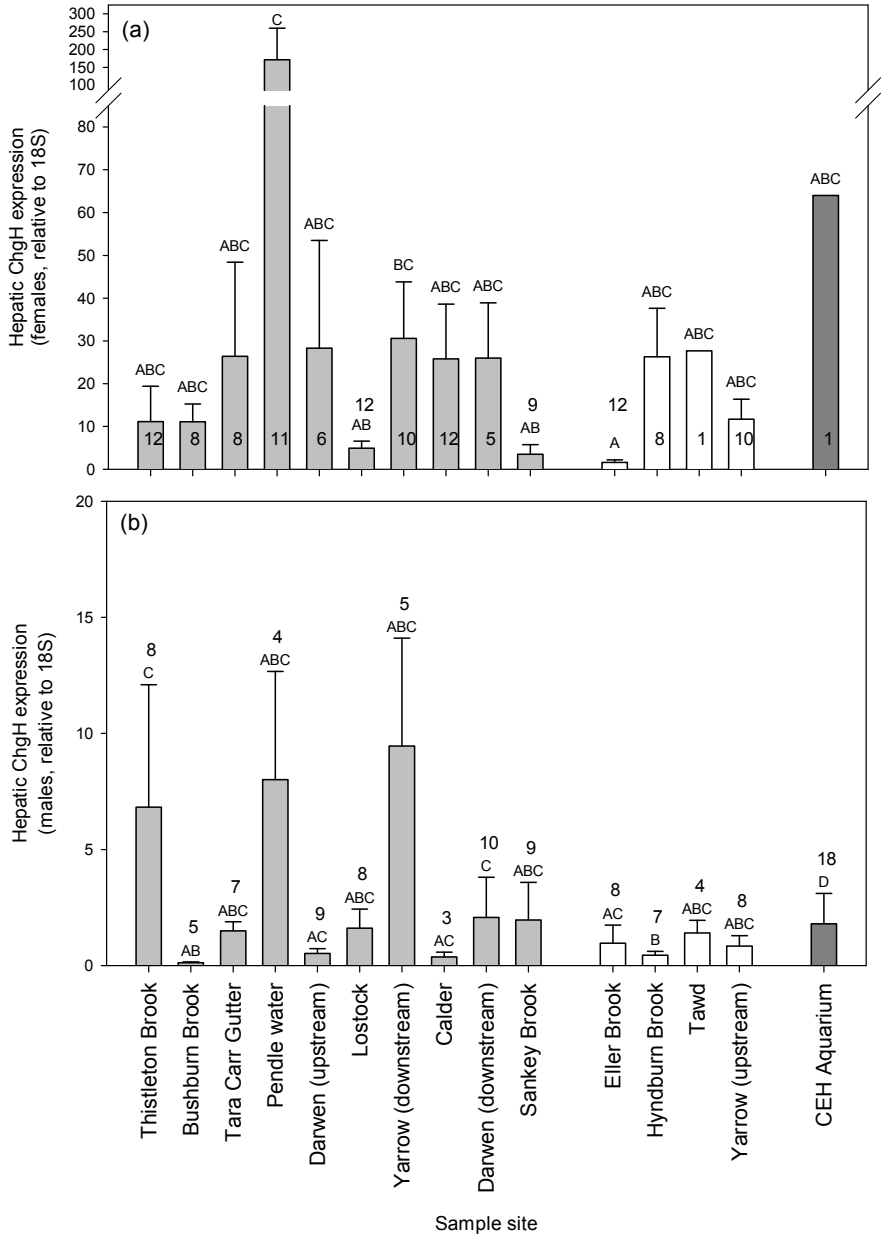


Figure 10. Expression of choriogenin (ChgH) RNA relative to 18s ribosomal RNA in liver tissue of (a) female and (b) male sticklebacks from each sampling site. Each bar is the mean + SEM. For each sample, n is depicted above/within the bar. Means sharing the same letter are not significantly different.

3.6 Relationship of measured variables in fish downstream of WWTWs with population equivalents, dry weather flow, effluent concentration and water temperature

Three metrics were available with which to characterise the relative impact of each WWTW on the receiving water; population equivalents (PE); dry weather flow (DWF); percentage of effluent at the sample site. Given their interdependencies it was initially assumed that dry weather flow would be correlated with population size to a similar extent for each WWTW, even allowing for the additional loading resulting from industrial inputs to the WWTWs. However, when the two measures for each WWTW were plotted against each other it was evident that while the relationship was directly linear for the seven WWTWs serving populations of between 1,000 and 50,000 the relationship was not as consistent for the sites serving populations > 100,000 (Burnley, Blackburn, St Helens; Fig. 11a). This misalignment was emphasised when the calculated value for percent effluent at the sample site was plotted against population (Fig. 11b), perhaps unsurprisingly since DWF is a component of the equation determining percent effluent concentration. These plots highlighted the possibility that for the three largest WWTWs the proportionality of the biological response downstream of the discharges to metrics describing the WWTW function (DWF, PE, effluent concentration) might be different from that at sites serving smaller populations. Therefore, regressions of WWTW metrics against biological data were carried out both for the full range of treatment plants and for the reduced set, excluding the largest three sites. The outcomes of these regressions are reported below with the regression parameters summarised in Table 2.

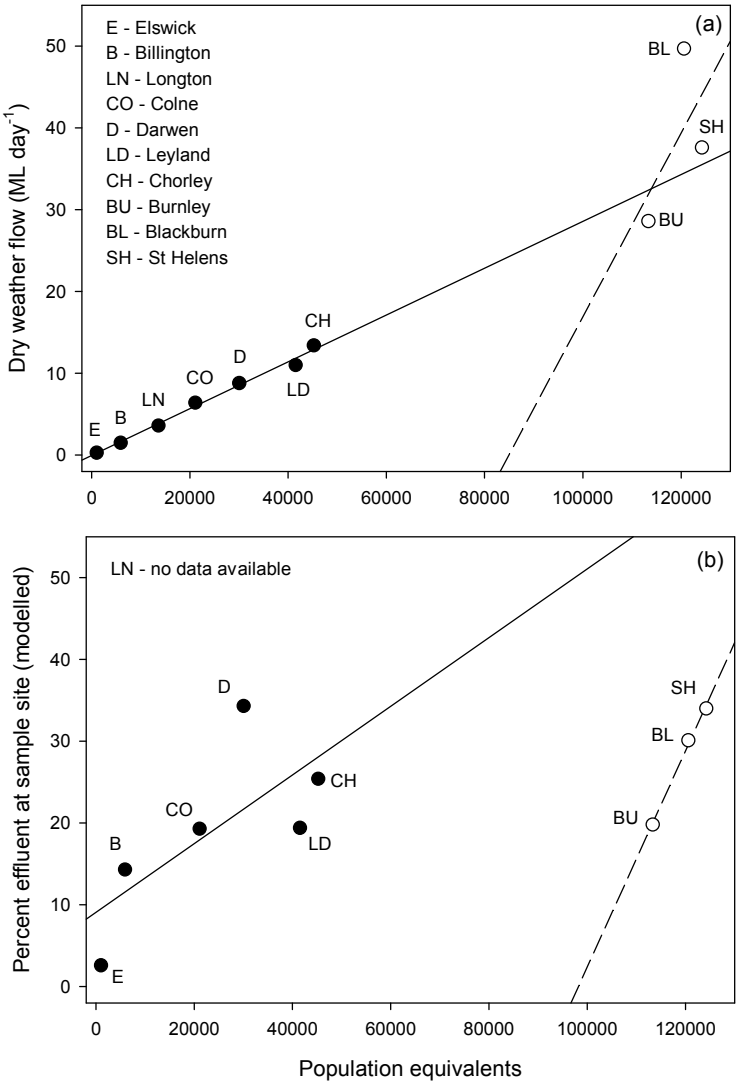


Figure 11. WWTW population equivalents plotted against (a) the dry weather flow and (b) the percent effluent at the sample site. Linear regression lines are plotted separately for sites with PE < 100,000 and sites with PE > 100,000.

Water temperature on the day of sampling was included as a variable of possible relevance to the activity of the stress axis in this context, given the dependence of many physiological processes in fish on temperature and the likelihood that the WWTW discharges resulted in elevation of downstream temperatures. However, no significant relationships between water temperature on the day of sampling and PE, DWF or percent effluent were observed, although in every case a positive trend line was apparent.

3.6.1 Somatic data: Both body mass and fork length were highly correlated with the calculated concentration of effluent at each sampling sites (Fig. 12a, b) with this relationship explaining up to 37% of variation in the data (Table 2). The best fit for these data was obtained using the effluent concentrations estimated from the long-term average flow data, rather than the estimate obtained using the flow data for the period in which the sampled fish were resident within the rivers (March 2010 – April 2011; see Table 2). Although condition was significantly related to percent effluent at site, the proportion of variation (8%) explained by this relationship was very much smaller than for mass and length. Both DWF and PE also provided significant regressions with mass and length (although not condition) but these relationships explained much less of the overall variation (<8%). These regressions were carried out on the data set minus the data for Thistleton Brook, the inclusion of which markedly reduced the proportion of variance explained by the regression. As noted elsewhere, the Thistleton Brook data were in some instances anomalous. No significant relationship between water temperature on the day of sampling and body mass (r^2 = 0.002, P = 0.4), length (r^2 = 0.0, P = 0.99), or condition (r^2 = 0.007, P = 0.14) were detected for any site.

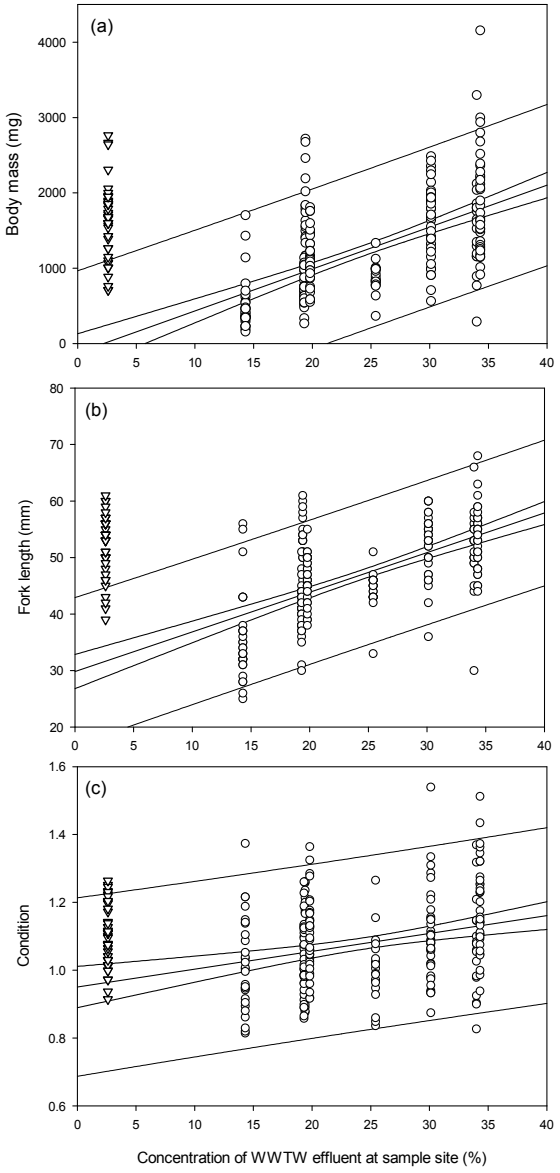


Figure 12. The relationship between the concentration of effluent at each site and (a) mass, (b) fork length, and (c) condition of individual fish sampled downstream of each WWTW. Thistleton Brook data (triangles) were excluded from the regression analysis (see text for explanation). The regression lines, 95% confidence intervals and prediction intervals are plotted. See Table 2 for the corresponding regression parameters.

3.6.2 Cortisol: Regression of cortisol levels in unstressed fish against WWTW metrics for all WWTWs provided a significant regression (P = 0.001) for the effluent concentration derived from the long-term flow data. However, this relationship accounted for only a small proportion of variation in the data (9%; Table 2). When the regression was carried out on the data set for only WWTWs with PEs < 100,000 a clear positive relationship was obtained between cortisol and all WWTW parameters, with 27% of variation in cortisol levels explained by variability in effluent concentration at the sample site (P<0.001; Fig. 13a,b,c).

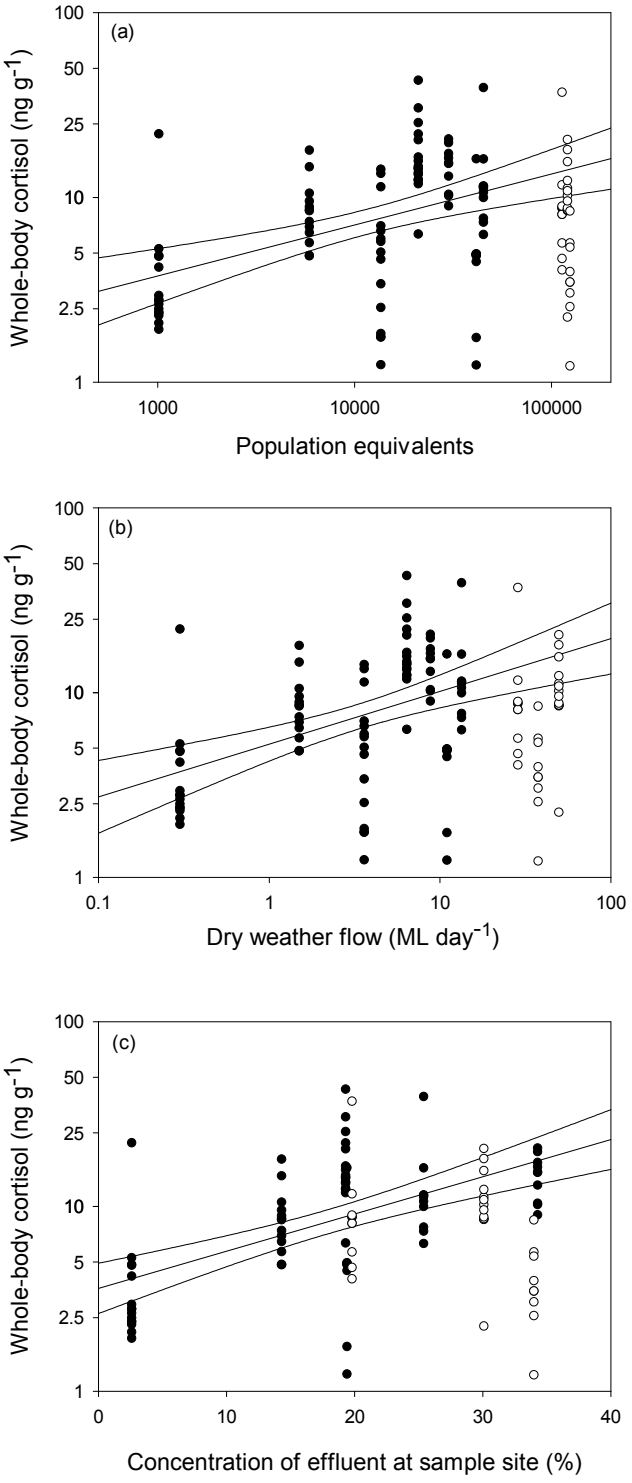


Figure 13. Regressions of whole-body cortisol concentrations in individual unstressed fish at each site against (a) population equivalents, (b) dry weather flow and (c) effluent concentration for the corresponding WWTW. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text and Table 2 for further detail). Note log scale for y axes.

For stressed fish, an even greater proportion of the variation in cortisol levels was explained by regression with effluent concentration. Here, the greatest amount of variation in cortisol data was explained by the variability in effluent concentration derived from the 2010/11 flow data (43%; Table 2; Fig. 14c) excluding WWTWs with PEs > 100,000. The % change in cortisol between unstressed and stressed fish was significantly related to the effluent concentration at the sample sites (Table 2) but this trend was

dependent upon the presence of the Thistleton Brook data.

Whole-body cortisol concentrations in unstressed fish captured downstream of WWTW sites exhibited no significant relationship with water temperature on the day of sampling (r^2 = 0.001, *P* = 0.76; Fig. 19a) but cortisol concentrations in stressed fish were significantly and inversely related to water temperature (r^2 = 0.12, *P* < 0.001, Fig. 19b).

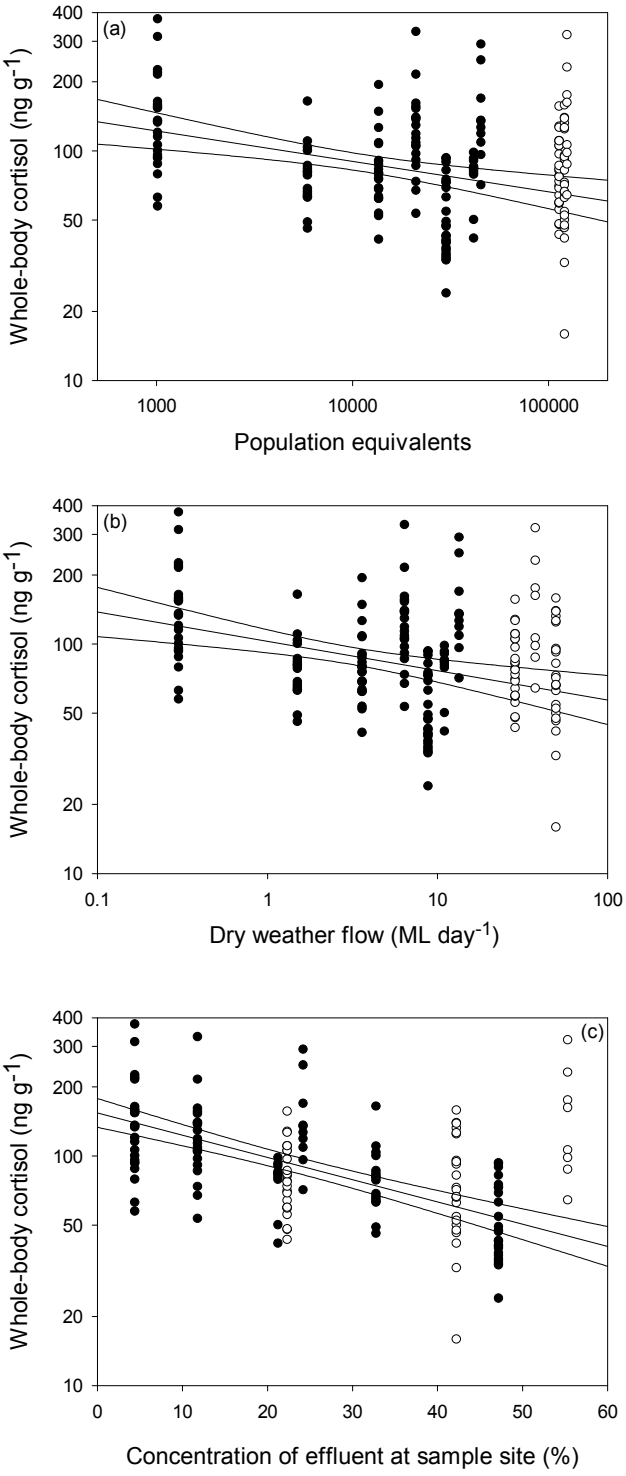


Figure 14. Regressions of whole-body cortisol concentrations in individual stressed fish at each site against (a) population equivalents, (b) dry weather flow and (c) effluent concentration for the corresponding WWTW. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text and Table 2 for further detail). Note log scale for y axes.

3.6.3 Glucose: For glucose concentrations in unstressed fish a highly significant regression was obtained with the concentration of effluent derived from the 2010/11 flow data which explained 22% of variability (Table 2; Fig. 15c). As was the case for cortisol, glucose concentrations in unstressed fish tended to increase with increasing effluent concentration.

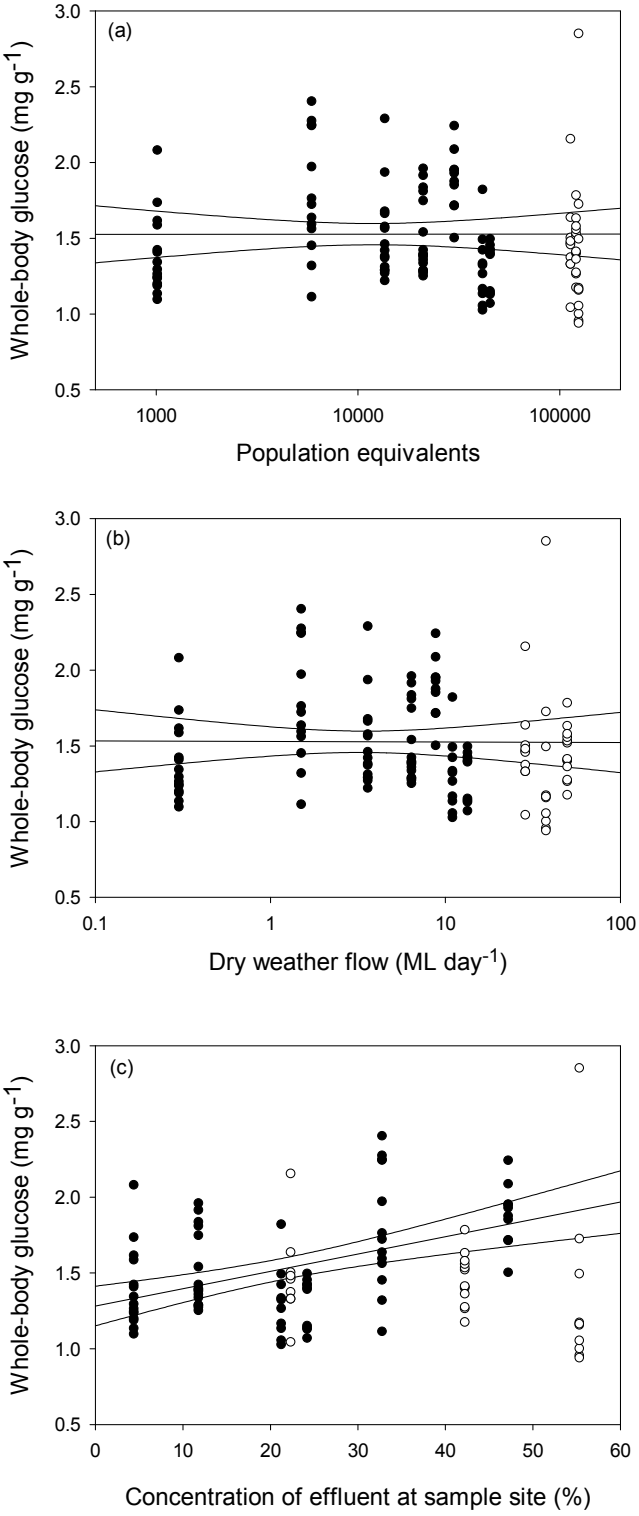


Figure 15. Regressions of whole-body glucose concentrations in individual unstressed fish at each site against (a) population equivalents, (b) dry weather flow and (c) effluent concentration for the corresponding WWTW. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text and Table 2 for further detail).

For stressed fish, significant regressions were obtained for all combinations with one exception (Table 2; Fig. 16a,b,c). Dry weather flow at all sites explained the greatest proportion of variation in glucose levels in stressed fish (14%).

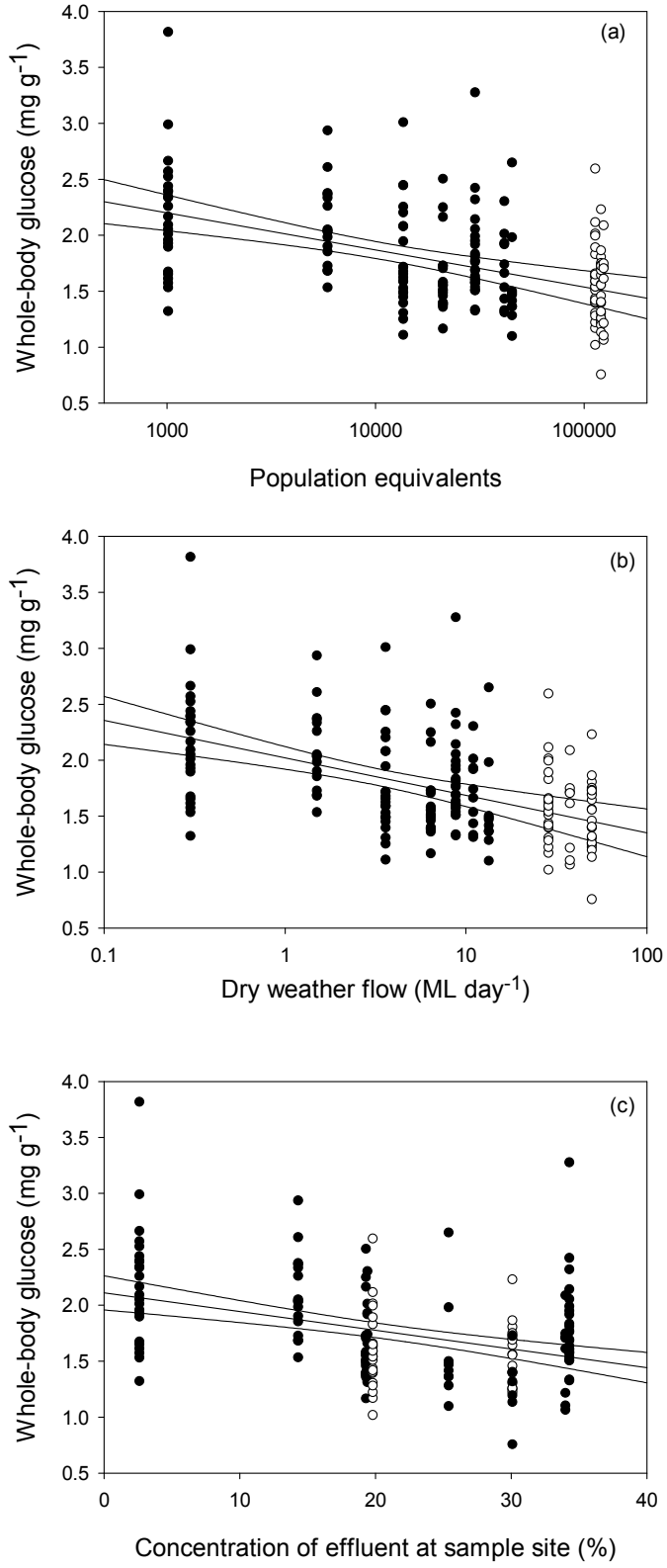


Figure 16. Regressions of whole-body glucose concentrations in individual stressed fish at each site against (a) population equivalents, (b) dry weather flow and (c) effluent concentration for the corresponding WWTW. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text and Table 2 for further detail).

The % change in glucose between unstressed and stressed fish was inversely related to the effluent concentration at the sample sites (Table 2; Fig. 17c) and unlike the cortisol data this relationship was not wholly dependent upon the Thistleton Brook data point.

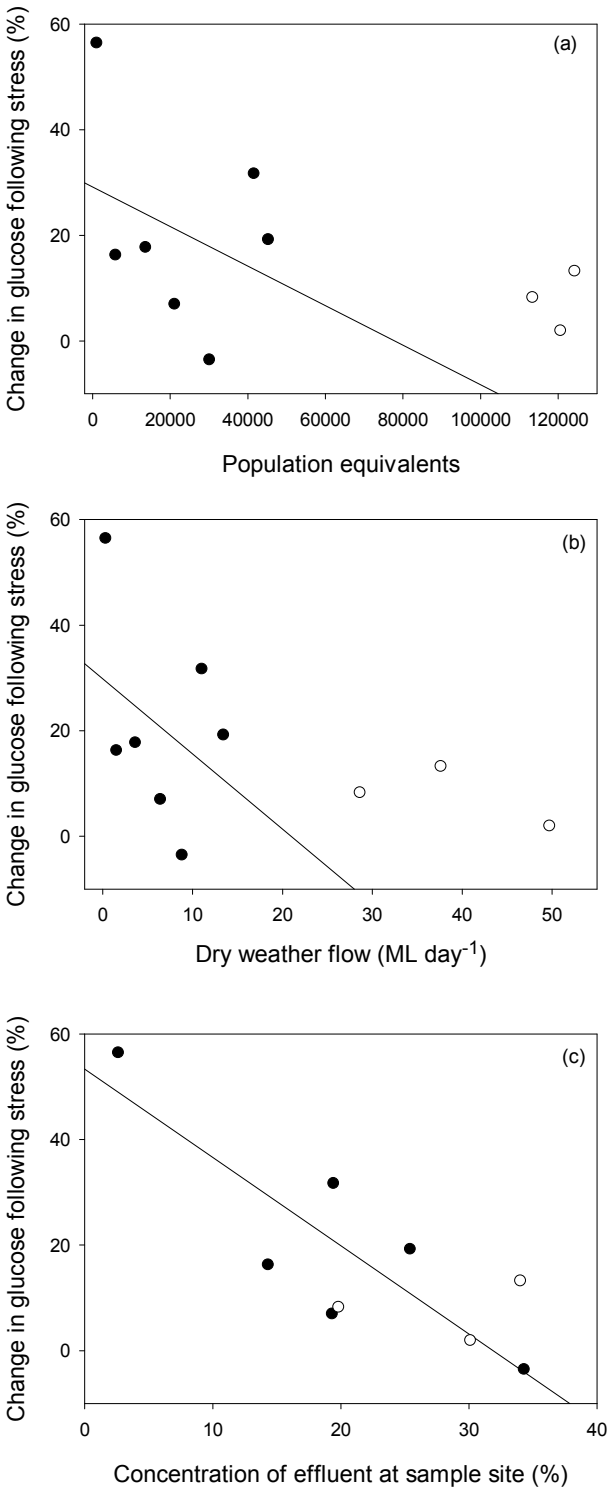
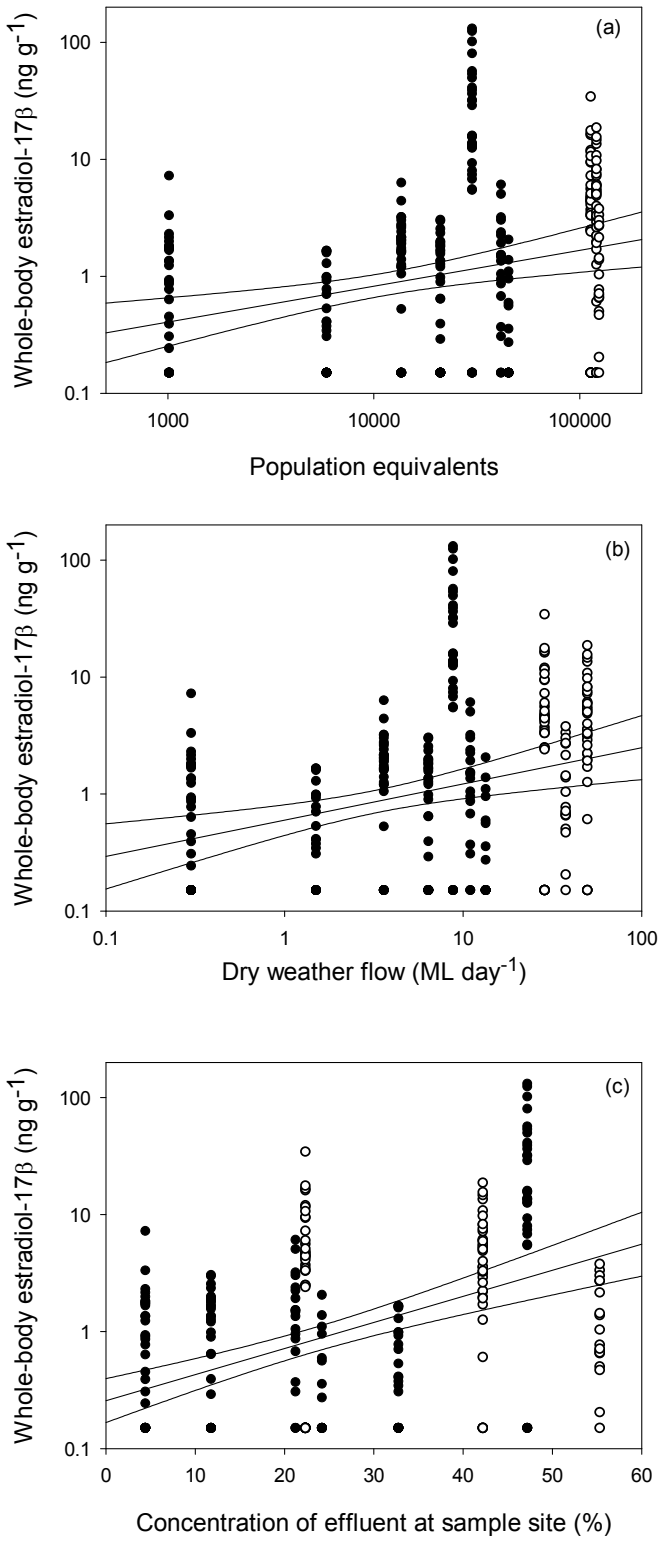


Figure 17. Regressions of the percent change in mean whole-body glucose concentration following stress for fish at each site against (a) population equivalents, (b) dry weather flow and (c) effluent concentration for the corresponding WWTW. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text and Table 2 for further detail).

Whole-body glucose levels in unstressed fish (immediately post-capture) at sites downstream of the WWTWs were significantly related to water temperature (linear regression, $r^2 = 0.05$, $P < 0.01$, Fig. 19c) but this relationship was not significant for stressed fish ($r^2 = 0.02$, $P = 0.07$; Fig. 19d).

3.6.4 Estradiol-17β: No relationship was evident between E2 concentrations and WWTW PE or DWF. However, whole-body E2 concentrations were significantly (P = 0.000) and positively related to the effluent concentration at sample sites using both the long-term and 2010/11 flow data (Table 2; Fig. 18c). Effluent concentration explained up to 20% of variation in E2 concentrations.

Whole-body E2 concentrations were also significantly related to water temperature at sites downstream of WWTWs (r^2 = 0.09, P < 0.001, Fig. 19e).



The proportion of fish at each site with detectable E2 concentrations was found to show some distinct trends. Both fish mass and fork length were significantly related to the proportion of fish with measurable E2 concentrations (mass: r^2 = 0.42, P = 0.05; length r^2 = 0.37, P = 0.06). In addition, the proportion of fish with detectable E2 concentrations was significantly and positively related to both WWTW PE (r^2 = 0.42, P = 0.04) and DWF (r^2 = 0.42, P = 0.042). The relationship between the proportion of fish with measurable E2 and the percentage of effluent present at the sample site approached significance (r^2 = 0.35, P = 0.096).

In addition, the proportion of fish with measurable E2 levels showed a distinct decline with increasing expression of CYP1A in both male (r^2 = 0.59, P = 0.009) and female (r^2 = 0.49, P = 0.024) fish. A similar, though less pronounced relationship was apparent between the proportion of fish with detectable E2 concentrations and ChgH expression in males (r^2 = 0.44, P = 0.04). No such relationship was evident with ChgH expression in females, or with MT expression levels.

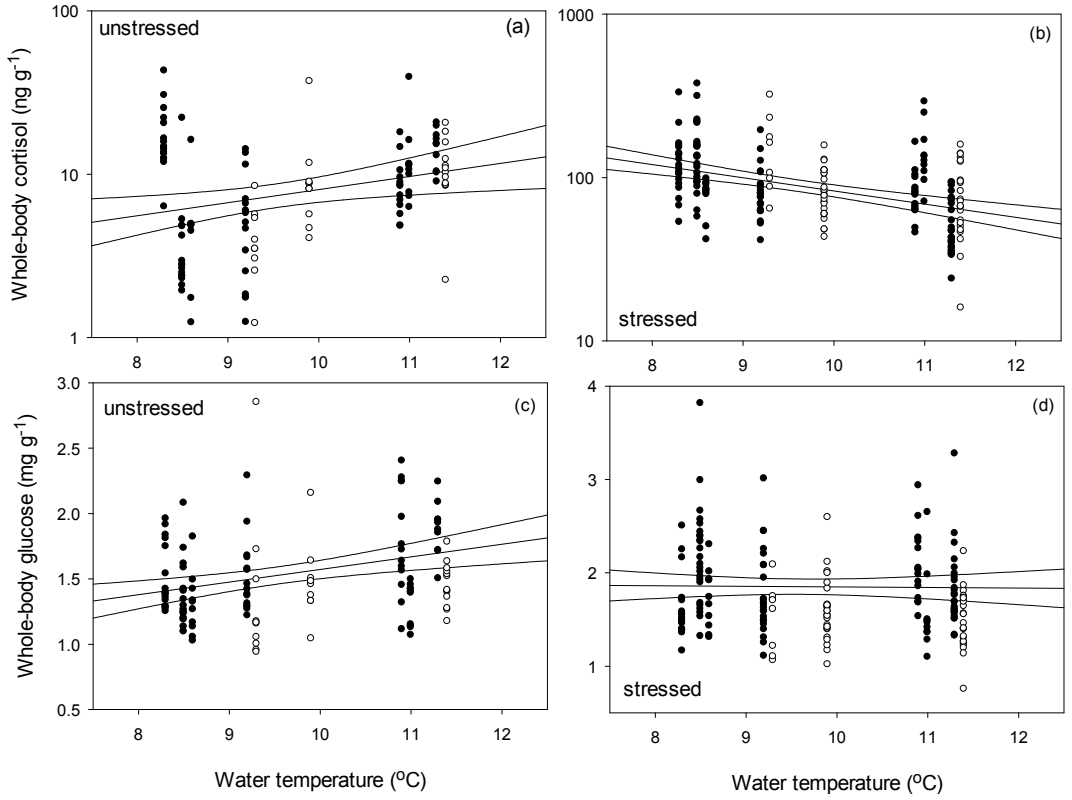
Figure 18. Regressions of whole-body E2 concentrations for fish at each site against (a) population equivalents, (b) dry weather flow, and (c) effluent concentration for the corresponding WWTW. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text and Table 2 for further detail). Note log scale for y axes.

***3.6.5 Biomarker expression**:* There was a significant negative relationship between CYP1A and all measures of WWTW function although these explained relatively little of the variation within the data (Table 2). Removal of the three largest WWTW sites eliminated this significant trend.

There were significant negative regressions evident for MT with PE and DWF (Table 2) but again relatively little of the variation was explained by these trends. MT exhibited no relationship with the concentration of effluent at the sample site.

For female fish ChgH exhibited no significant relationship with any measure of WWTW function (Table 2). However, for males significant regressions were obtained with the concentration of effluent at sample sites derived from the 2010/11 flow data indicating a negative trend in expression with increasing percentage of effluent.

No relationship with water temperature was evident for any of the biomarkers.



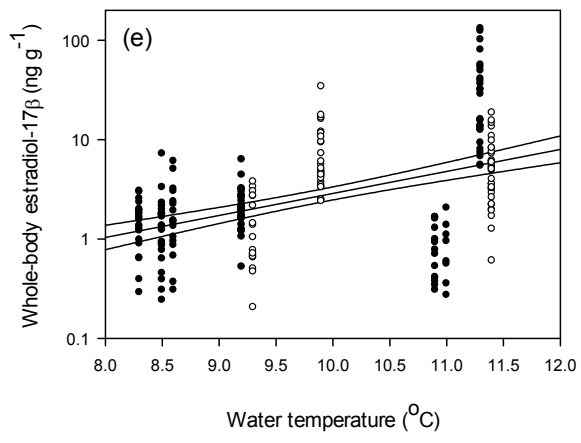


Figure 19. Regressions of water temperature against: (a) whole-body cortisol concentrations in unstressed fish and (b) stressed fish; (c) whole-body glucose concentrations in unstressed and (d) stressed fish and (e) whole-body E2 concentrations in all fish. ● - fish collected at sites with PE < 100,000; O - fish collected at sites with PE > 100,000. Regressions are plotted for the former only (see text for further detail).

Table 2. Summary of regression outcomes for relationships between indices of WWTW function [population equivalents, dry weather flow, % effluent at site based on long-term flow data (1961-1990), % effluent at site based on 2010/11 flow data only] and somatic (mass, length, condition), stress-related (cortisol, glucose, unstressed, stressed) and biomarker (CYP1A, MT, ChgH) data.

Metric	Condition	Excluding	Population equivalent		Dry weather flow		% effluent at site (1960-90)		% effluent at site (2010/11)	
			r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*
Body mass	all	Thistleton	**0.03**	**0.003**	**0.046**	**0.000**	**0.35**	**0.000**	**0.22**	**0.000**
Fork length	all	Thistleton	**0.065**	**0.000**	**0.08**	**0.000**	**0.37**	**0.000**	**0.18**	**0.000**
Condition	all	Thistleton	*0.006*	*0.205*	*0.005*	*0.249*	**0.08**	**0.000**	**0.049**	**0.001**
log_{10}Cortisol	Unstressed	none	*0.006*	*0.388*	*0.009*	*0.309*	**0.09**	**0.001**	*0.01*	*0.317*
log_{10}Cortisol	Unstressed	>100,000	**0.116**	**0.001**	**0.138**	**0.000**	**0.27**	**0.000**	**0.08**	**0.012**
log_{10}Cortisol	stressed	none	*0.012*	*0.141*	*0.015*	*0.101*	**0.149**	**0.000**	**0.179**	**0.000**
log_{10}Cortisol	stressed	>100,000	**0.019**	**0.040**	**0.036**	**0.031**	**0.27**	**0.000**	**0.427**	**0.000**
% change in cortisol		>100,000	*0.4*	*0.127*	*0.38*	*0.139*	**0.63**	**0.059**	0.42	0.16
Glucose	Unstressed	none	*0.028*	*0.068*	*0.023*	*0.096*	*0.005*	*0.476*	*0.028*	*0.084*
Glucose	Unstressed	>100,000	*0.029*	*0.110*	*0.026*	*0.129*	**0.055**	**0.044**	**0.219**	**0.000**
Glucose	stressed	none	**0.15**	**0.000**	**0.144**	**0.000**	**0.142**	**0.000**	**0.047**	**0.006**
Glucose	stressed	>100,000	**0.128**	**0.000**	**0.132**	**0.000**	**0.105**	**0.001**	*0.01*	*0.310*
% change in glucose		none	*0.189*	*0.210*	*0.19*	*0.196*	**0.626**	**0.011**	**0.404**	**0.066**
E2	all	none	*0.001*	*0.600*	*0.001*	*0.678*	**0.102**	**0.000**	**0.089**	**0.000**
E2	all	>100,000	**0.03**	**0.008**	**0.036**	**0.005**	**0.176**	**0.000**	**0.199**	**0.000**
Log_{10}CYP1A	all	none	**0.068**	**0.002**	**0.059**	**0.005**	**0.03**	**0.045**	**0.068**	**0.004**
Log_{10}CYP1A	all	>100,000	*0.013*	*0.285*	*0.02*	*0.187*	*0.001*	*0.759*	*0.016*	*0.270*
Log_{10} MT	all	none	**0.03**	**0.038**	**0.037**	**0.022**	*0*	*0.940*	*0.002*	*0.595*
Log_{10} MT	all	>100,000	**0.049**	**0.030**	**0.046**	**0.036**	*0.034*	*0.099*	*0.026*	*0.150*
Log_{10} ChgH	females	none	*0.002*	*0.677*	*0.001*	*0.830*	*0.001*	*0.766*	*0.002*	*0.185*
Log_{10} ChgH	females	>100,000	*0.008*	*0.459*	*0.016*	*0.315*	*0.032*	*0.174*	*0.001*	*0.862*
Log_{10} ChgH	males	none	*0.02*	*0.231*	*0.02*	*0.255*	*0.023*	*0.242*	**0.08**	**0.023**
Log_{10} ChgH	males	>100,000	*0.008*	*0.560*	*0.012*	*0.467*	*0.013*	*0.497*	**0.114**	**0.035**

Non-significant regressions are italicised, significant regressions are in bold and regressions explaining the greatest amount of variation in the data are shaded.

4. Discussion

The data set generated by this scoping study is extensive; consequently this Discussion will focus on the key aspects of the results that are directly relevant to the aims of the study, primarily between-site variability in the stress axis as determined by measurement of whole-body cortisol concentrations.

After evidence was obtained during the EDCAT programme that glucose concentrations, as well as cortisol, in free-living sticklebacks were modified by stress (Pottinger et al., 2011b) both cortisol and glucose concentrations were assessed in fish during the present study. Glucose concentrations showed distinct trends associated with exposure to WWTW effluent. However, because these tended to match the trends exhibited by cortisol and were relatively modest in magnitude, and given the pivotal role played by cortisol in the stress response, the cortisol data are given prominence in this Discussion. Similarly, whole-body concentrations of E2 were determined primarily to provide a point of comparison with choriogenin (Chg) expression. While Chg expression was strongly related to sex of the fish the E2 concentrations were not. This observation, while of interest, is not of relevance to the primary purpose of the study. These and related issues will be addressed more fully in a paper to be prepared for publication.

4.1 Variation in the activity of the stress axis in fish captured downstream of WWTWs

The findings of this scoping study suggest that the function of the stress axis in sticklebacks occupying habitats downstream of WWTWs is affected by exposure to chemical contamination.

This conclusion is based on the degree to which variation in indices of stress in stickleback populations downstream of WWTWs (cortisol, glucose) is explained by concurrent variation in key measures of WWTW impact.

The data show that the stress axis varied with contaminant exposure in two respects:

(i) baseline levels of markers of stress axis activity (cortisol and glucose) were elevated, and

(ii) the magnitude of the response to a standardised stressor was reduced.

Both effects are consistent with data that show in both laboratory studies and field surveys that the response of the stress axis of fish to a stressor is altered by exposure to environmental contaminants including polycyclic aromatic hydrocarbons (PAHs: Gesto et al., 2008), polychlorinated biphenyls (PCBs: Quabius et al., 1997), organochlorines (e.g. DDT: Benguira et al., 2002), and metals (Hontela, 1998; Norris et al., 1999; Laflamme et al., 2000; Gravel et al., 2005; Gagnon et al., 2006).

In particular, effects on the stress axis similar to those observed in the present study have recently been reported for rainbow trout exposed to municipal waste water effluent (Guelph, Ontario, Canada). Cortisol concentrations in the blood of otherwise unstressed rainbow trout exposed to effluent were elevated and when exposed to a stressor the post-stress increase in cortisol in these fish was reduced (Ings et al., 2011). A similar pattern of effects has been reported for trout exposed to agrochemicals at environmentally relevant concentrations with a stimulation of the stress axis seen in otherwise unstressed fish accompanied by an attenuation of the ability of the fish to mount an appropriate cortisol response to an additional stressor (Tierney et al., 2011). Finally, administration of selenium to rainbow trout has also been shown to result in elevated “resting” cortisol concentrations accompanied by an inability to respond appropriately to an additional stressor (Wiseman et al., 2011). These effects are not unique to fish. Exposure to mixtures of potential toxicants has also been associated with stress axis dysfunction in birds. In the glaucous gull (*Larus hyperboreus*), a top predator in the Arctic marine environment, elevated concentrations of organochlorines, brominated flame retardants and metabolically-derived products in the blood of gulls were associated with high baseline corticosterone concentrations in both sexes and a reduced stress response in males (Verboven et al., 2010).

In the present study measures of the activity of the stress axis (cortisol, glucose) were correlated with measures of WWTW impact at all sites. However, to obtain “best fits” for some of the data it was

necessary to exclude either the smallest WWTW (Elswick – for mass, length, condition) or the three largest (St Helens, Blackburn, Burnley – for cortisol and glucose). See the discussion in section 3.6 for more detail supporting this approach. In some cases a better fit for the data was obtained with WWTW metrics based on long-term data (mass and length, percent change in cortisol and glucose following stress) and in some cases data derived from the 2010/2011 period provided the best fit (post-stress cortisol concentrations, male Chg expression). We believe that this is a legitimate approach to interpreting the results and is not arbitrarily selective. The nature and concentration of chemical contaminants at each of the sites is unknown and it is quite likely that there are differences in both the abundance and combination of chemicals present, particularly between the largest WWTWs and the smaller sites. We also do not yet understand how environmental factors interact with the stress axis and the time-course over which this occurs. We are therefore reluctant to make an unfounded assumption that all the discharges were identical in nature, and that only characteristics pertaining to the period 2010-2011 are relevant. The regression data for all the combinations have been reported so that the presence and absence of potentially causal relationships is clearly evident.

Overall, and given how far-removed our physiological measures (derived from analysis of a single sample of fish from each site, collected on a single random day) are from the WWTW metrics (none of which were based entirely on measured contemporaneous data) the existence of any relationships between the two must be strongly indicative of a robust underlying interrelationship between WWTW function and physiological status of fish downstream of the discharge.

Taking all these considerations into account, the pattern of baseline and stress-induced cortisol concentrations observed in sticklebacks downstream of WWTWs is consistent with a chemically compromised stress axis.

4.2 Possible causes of the observed variation in the activity of the stress axis in fish exposed to WWTW effluent

Two observations need to be considered: the trend for cortisol concentrations in unstressed fish to increase with exposure to increasing concentrations of WWTW effluent, and the progressive reduction in responsiveness of the stress axis to a post-capture stressor with exposure to increasing concentrations of WWTW effluent.

4.2.1 Elevated baseline cortisol in unstressed fish: Although there have been numerous studies concerning chemical effects on the activity of the stress axis, few provide details of pre-stress cortisol concentrations (but see recent papers by Ings et al., 2011; Tierney et al., 2011; Wiseman et al., 2011). The most obvious hypothesis to explain the rise in baseline cortisol concentrations with increasing WWTW is that the elevation reflects the stress associated with an ongoing chemical challenge. That is, for the individual to cope with the chemical challenge associated with WWTW effluent requires investment in, for example, detoxification mechanisms which in turn requires diversion of resources away from other demands. This process is termed allostasis and is characterised as changes to one or more components of a system as an organism strives to cope with an external stressor (Nichols et al., 2011). In the longer term, if the allostatic load is not fully compensated for, a state of chronic stress may arise, resulting in elevated cortisol concentrations. It is also possible that the potential interactions of environmental contaminants with the stress axis, as described below, is complex and results in a net elevation (or possibly reduction, see below) of cortisol levels in unstressed fish via interference in signalling or biosynthetic pathways.

4.2.2 Attenuation of the cortisol response in stressed fish: The reduction in the cortisol response to stressors that is reported to occur in fish exposed to contaminants has so far been shown to be primarily due to interference with the function of cortisol-producing interrenal cells (homologous with the adrenal cortex in mammals) although more complex interactions are likely (Hinson and Raven, 2006). The organochlorine insecticide endosulfan interferes with the secretory function of teleost interrenal steroidogenic cells (Leblond et al., 2001) and similar results were observed in steroidogenic cells exposed to pesticides (atrazine, mancozeb, diazinon, endosulfan; Bisson and Hontela, 2002) and metals (Lacroix and Hontela, 2004). Impairment of cortisol secretion during stress appears to be due to modulation of the

activity of two key steroid biosynthetic enzymes in the interrenal: steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (P450scc; Aluru et al., 2005) which are involved with the first step in the synthesis of cortisol, the uptake and conversion of cholesterol to pregnenolone. This occurs because of interference in receptor-mediated signalling upstream of pregnenolone synthesis (Sandhu and Vijayan, 2011). It has also been suggested (Arukwe, 2008) that contaminants may affect the negative feedback control of steroid hormone synthesis. Additional considerations include the fact that species differences have been reported for sensitivity to toxicants and for mechanisms of interrenal toxicity (Miller and Hontela, 2011) and that given the complexity of WWTW effluents (see below) it cannot be assumed that the mechanisms described above are the only processes involved in modifying the function of the stress axis.

4.2.3 Contaminants with potential interrenal-disrupting effects: The identity of the chemical(s) that may be responsible for modifying the activity of the stress axis of sticklebacks in the current study is unknown. High metal concentrations have repeatedly been associated with dysfunction of the stress axis in fish (e.g. Laflamme et al., 2000; Gravel et al., 2005) and three sample sites in the current study were selected on the basis of having reportedly high metal concentrations but no association with upstream WWTWs (Eller Brook, Hyndburn Brook, R. Tawd). In fish sampled from Hyndburn Brook, which experiences particularly high zinc (> 100 µg l^{-1}), arsenic (> 8 µg l^{-1}) and cadmium (> 0.1 µg l^{-1}) concentrations relative to other nearby rivers (see supplementary data for Neal et al., 2011,) the expression of MT, a biomarker for metal exposure (Santos et al., 2010), was greater than MT expression levels in fish from Eller Brook, which periodically experiences exceptionally high mercury concentrations (> 200 ng l^{-1}; Rowland et al., 2010). These concentrations of dissolved metals are within a similar range to those reported by Laflamme et al. (2000) to have effects on the stress axis in yellow perch.

MT expression in fish sampled at most of the WWTW-impacted sites was similar to that observed at the "high metal" sites and the functionality of the stress axis in fish from the three "high metal" sites was within the mid-range of that for fish from WWTW-affected sites with mean post-stress cortisol levels exceeding levels in fish at six of the WWTW sites. Discharge data for WWTWs that are posted on the EA website (http://www.environment-agency.gov.uk/homeandleisure/37793.aspx) confirm that a range of metals are present in most WWTW effluents. However, there was a distinct lack of positive correlation between the percentage of effluent calculated as being present at each site and MT expression in sticklebacks captured at these sites. This suggests that overall the concentrations of metals at the sample sites were insufficient to induce differential expression of MT across sites. This does not preclude metals from being involved in the effects observed on the stress axis – the threshold for effects on the stress axis may be lower than the threshold concentrations required to induce MT expression.

To complicate interpretation of the data further it might also be argued that the critical exposure route to metals for fish is not via those dissolved in the water column, for which measurements are available, but instead via the metal content of invertebrate food items ingested by the fish and that therefore the relevant concentrations of metals are those in sediments and related biota, for which we have no data.

As well as an absence of clear evidence implicating metals in the effects observed on the stress axis of the sampled fish the biomarker data do not provide support for effects due to PAHs. There was no positive trend in CYP1A expression, a biomarker of PAH exposure (Gao et al., 2011) with increasing concentration of effluent at the study sites. Furthermore, judged by the lack of trends in Chg expression, a biomarker for estrogen exposure (Chen et al., 2008), there was no evidence that estrogenic components of the WWTW effluent played a dominant role in the effects observed. In fact the most significant association between any of the biomarkers and a measure of WWTW impact was observed for Chg expression in male fish only. Here the relationship was a negative one, with expression declining with increasing concentrations of effluent.

The absence of a strong positive association between effluent concentration and biomarker may indicate that there were relatively low concentrations of planar organics, metals and estrogens present in the effluents, at least in terms of the concentrations necessary to induce substantial expression of these enes in sticklebacks – not necessarily in terms of the concentrations needed to exert effects on the stress axis. However, it is also possible that elements within the complex cocktail of chemicals present in the WWTW

effluents had a suppressive effect on the expression of one or more biomarker genes, which might account for the otherwise puzzling downward trend in biomarker expression with increasing effluent concentration (assuming that contaminant concentration within effluents remains similar across WWTWs dealing with different PEs and it is volume of effluent produced that is the main variable between sites). This possibility has recently been discussed at length by Celander (2011) who noted that biomarker responses can be extensively altered by concurrent exposure to chemicals that are capable of affecting relevant pathways and that new approaches to assessing exposure may need to be adopted in cases where complex mixtures are involved.

These considerations highlight the complications inherent in attributing causality for the effects observed on the stress axis of fish downstream of WWTWs because these effluents are complex mixtures of contaminants (Schwarzenbach et al., 2006).

***4.2.4 The complexity of WWTW effluents*:** The wide range of chemicals (micropollutants) prevalent at sites downstream of WWTWs is illustrated by the results of a survey carried out in the US in which the concentrations of 95 selected organic contaminants were determined in samples from 139 streams receiving waste water (Kolpin et al., 2002). Among the most frequently detected compounds were cholesterol, N,N-diethyltoluamide (an insect repellent), caffeine, triclosan (an antimicrobial agent), tri(2-chloroethyl)phosphate (a fire retardant) and 4-nonylphenol (non-ionic detergent metabolite). While the specific chemicals present in UK WWTW effluents are likely to differ from those in the US, there is no reason to believe that the diversity of contaminants will not be as great.

Of particular interest is the high number of personal care products (Snyder et al., 2003) and in particular pharmaceuticals (Heberer, 2002; Fent et al., 2006) present within WWTW effluents. For example, among 23 pharmaceuticals determined in WWTW effluent in Spain those occurring at highest concentrations (up to 1.0 μg l^{-1}) in the effluent included caffeine, diclofenac and ibuprofen (Pedrouzo et al., 2011). In a similar survey in Greece, caffeine, ibuprofen, and salicylic acid were present at up to 14 μg l^{-1} in WWTW effluent (Kosma et al., 2010). With effluent concentrations at the sampling sites in the present study approaching 50% in some cases, if contaminant concentrations in UK effluents approach those in Spain and Greece there is clearly potential for the exposure of fish to combinations of contaminants at substantial aggregated concentrations. Predicting the likely effect in fish of exposure to the most abundant pharmaceutical contaminants is difficult, with the lack of relevant toxicological data being a major obstacle (Fent et al., 2006).

In the case of pharmaceuticals some degree of interaction with exposed biota is almost certain given that these compounds are targeted to be reactive with biological systems. Even with adequate coverage of ecotoxicological data, predicting and understanding the nature of the effects likely to be imposed upon exposed biota by this assemblage of micropollutants is confounded by the potential for a complex array of additive, synergistic, inhibitory, stimulatory and competitive interactions between the constituents of the effluent and target tissues within the exposed organisms.

The stress axis in vertebrates comprises multiple neuroendocrine pathways together with a cognitive component. The response of the stress axis to a stressor may integrate multiple influences, chemical and non-chemical (e.g. Pottinger et al., 2011b). There is considerable scope for interference with, or modulation of, the stress axis at a range of loci. In addition, disruption can be envisaged as arising from single reactive contaminants or from a combination of agents. The two-phase nature of the stress axis (stimulated/unstimulated) also adds complexity to the interpretation and prediction of effects arising from contaminant exposure.

4.3 The stress axis in laboratory-held control fish – an appropriate point of comparison?

Whole-body cortisol concentrations were measured in a laboratory population of sticklebacks in order to provide “control” data for activity of the stress axis in fish not exposed to any chemical contaminants. The results of these measurements were not as initially predicted and so are discussed in more detail below.

4.3.1 Cortisol concentrations in unstressed fish: Mean baseline cortisol concentrations in control fish from the CEH aquarium population were within the mid-range of values measured in fish from the field sites. This result might at first appear to be unexpected – if exposure to WWTW effluent is associated with increased baseline levels of cortisol then the aquarium fish, which were held in clean water, should exhibit baseline cortisol levels lower than the fish from all the field sites. However, if the effects of exposure to WWTW effluent are more complex than we assume, and the nature of their influence alters with increasing concentration of effluent (and/or size of WWTW) it is possible that at lower concentrations of effluent there is a suppression of activity in the stress axis of unstressed fish and at higher concentrations a positive influence on baseline cortisol concentrations. In effect this would constitute a relationship between WWTW effluent concentration and the stress axis in which low and high levels of exposure to effluent elicit different responses, similar to the concept of hormesis (Calabrese, 2004). A further possibility is that baseline levels of cortisol in wild fish are lower than those in laboratory held fish. As far as the authors are aware there have been no systematic comparisons of the stress axis in the same species of fish under both laboratory and natural conditions. The "set point" for resting levels of cortisol in fish held under laboratory conditions, and consequently subject to a certain amount of regular disturbance, may differ from that of free-living fish.

4.3.2 Cortisol concentrations in stressed fish: The mean maximum elevation of cortisol in the aquarium fish following a confinement stressor was significantly lower than that observed in fish from most of the field sites. If, as the results of the study suggest, exposure to WWTW effluent is associated with reduced post-stress cortisol levels then the aquarium fish, which were held in uncontaminated water, should have exhibited post-stress cortisol levels higher than fish from all the field sites, which were all contaminated to some degree.

Two issues may be relevant to these observations. The first is the manner in which the stress axis is regulated. There is a cognitive element within the axis which allows the perception of a stressor by the fish to modify the subsequent response and here novelty and unfamiliarity are important factors (e.g. Pickering and Pottinger, 1985). The aquarium fish were unavoidably more familiar with human disturbance and handling than the wild-caught fish and it is possible that this accounts for the lower maximum response of the aquarium fish to an identical stressor. In support of this interpretation, the full time-course response of the aquarium sticklebacks used in the present study to confinement was very similar in dynamic and magnitude to an earlier study with lab-maintained sticklebacks sourced from a different supplier (Pottinger et al., 2002).

The second issue arises from the unexpectedly high expression levels for MT seen in the aquarium fish. Whereas CYP1A expression in the aquarium fish was not significantly different from that in wild-caught fish, MT expression in aquarium fish was significantly higher than that observed at most of the field sites. If the expression of MT in the aquarium fish does relate to contaminant exposure it is possible that this would account for the lower than predicted post-stress cortisol response of these fish. This interpretation would require that the aquarium fish were exposed to concentrations of metals high enough to elicit expression of the biomarker, and higher levels than were apparently present at any of the field sites, which seems unlikely. Analysis of the incoming aquarium water supply for more than 30 potential metal contaminants by the CEH analytical chemistry department revealed the aquarium (Blea Tarn) water to be indistinguishable from a good quality upland stream in terms of metal content. The only other feasible route of exposure for these fish is via their food, a commercial pellet formulation. Analysis of feed samples for metals is underway at the time of writing.

4.4 What are the functional implications for fish of variation in the responsiveness of the stress axis?

Two issues are raised by these data. The first is the elevation of "resting" cortisol concentrations that were evident in fish caught and processed without the imposition of any additional stressor. The second is the decline in responsiveness of the stress axis that was evident as the exposure to WWTW effluent increased in fish that were subject to a period of confinement following capture. Relatively few studies have addressed these questions and of those that have most focus on birds.

4.4.1 Elevated cortisol in unstressed fish: In possibly the only study to examine the issue of fitness and the stress axis in fish, elevated baseline plasma cortisol levels were observed to be associated with reduction in reproductive behavioural activity in pink salmon (*Oncorhynchus gorbuscha*), with a complete failure to spawn in some cases, and with greater mortality (Cook et al., 2011).

Elevated baseline corticosteroid concentrations (corticosteroid concentrations in ostensibly unstressed individuals) have also been shown to be associated with poor fitness in birds (survival, breeding frequency and breeding success; Angelier et al., 2010). Although this relationship is not consistent across all studies (reviewed by Bonier et al., 2009) there are sufficient data supporting a relationship to allow formulation of the Cort-Fitness Hypothesis which posits that baseline corticosteroid concentrations can be used as a proxy for environmental challenges facing individuals or populations (Bonier et al., 2010).

White-crowned sparrows living in urban environments had higher baseline corticosteroid concentrations than individuals in a rural habitat (Bonier et al., 2007) and higher baseline corticosterone (= cortisol in teleost fish) levels was inversely associated with reproductive success. A very similar link between urbanization and baseline corticosterone concentrations has been reported for tree sparrows in China and interpreted to reflect the greater environmental challenges facing birds in an urban environment (Zhang et al., 2011). In house sparrows, pre-breeding baseline corticosterone was found to be negatively correlated with the reproductive success of females (Ouyang et al., 2011).

Overall, these reports suggest that the elevation of baseline cortisol concentrations in sticklebacks might be associated with detrimental effects on fitness. Further investigation will be needed to resolve this possibility.

4.4.2 Reduction in the magnitude of the cortisol response in stressed fish: Most research into the effects of elevated cortisol levels in fish concerns the longer-term adverse effects associated with chronic stress. On the basis of the well-established negative association between elevated corticosteroid levels and immunocompetence, reproduction and growth, it might be supposed that a reduction in the magnitude of the stress response would actually be beneficial to the individual. However, this is to fail to discriminate between the response to an acute stressor (which typify the type of challenge the stress response has evolved to cope with) and to a chronic stressor (more likely to be anthropogenic in origin) and to overlook the fact that the stress response is a key aid to survival that has been conserved throughout the evolution of the vertebrates. An attenuated response to a stressor implies that the ability of the fish to mount an appropriate adaptive response to a challenge is impeded. This, it may be assumed, has adverse implications for the ability of the fish to deal with threats to its well-being and will ultimately modify fitness. Clear evidence of higher-level effects of a dysfunctional stress axis is limited, as is the case for most examples of reproductive endocrine disruption. However, at its extreme, adrenal insufficiency has been demonstrated to have lethal consequences among post-operative humans (Hinson and Raven, 2006) and cortisol deficiency is a well-identified issue in veterinary practice, requiring replacement therapy (Plechner, 2004). It is reasonable to assume that the fitness of fish exposed to chemicals that interfere with the normal function of the stress response will be adversely affected.

An additional complication in the interpretation of possible effects on fitness associated with the attenuation of the cortisol response to a stressor arises due to the fact that populations are believed to comprise of sub-sets of individuals with a propensity for a high or low response to a stressor, relative to the population mean response (Øverli et al., 2007). The high- and low-responding phenotypes are characterised by distinct combinations of behavioural traits that have been termed reactive and proactive coping strategies. It is unclear to what extent the stress axis and behavioural repertoire are functionally inter-dependent and therefore how interference with the normal function of the stress axis would impact upon the behaviour of the animal. For example, it could be construed that attenuation of the stress response could alter behavioural types in affected individuals to more closely align with those of low-responding pro-active individuals. The consequences for fitness of the individual so affected, or for performance of the population as a whole, are impossible to predict with the limited data available to us.

Very few relevant experimental data are available. In perhaps the only experimental study to inform consideration of these issues corticosteroid concentrations in rabbits, exposed to a prolonged period of

captivity, were negatively associated with condition but positively associated with survival, suggesting that a fully functional stress response was a critical element in assuring survival of the individual on return to the wild (Cabezas et al., 2007). The complexities of interpreting the available data in terms of conservation-relevant variables such as fitness are discussed by Busch and Hayward (2009) who conclude that the relationship between corticosteroids and fitness parameters is complex and does not always fit predictions.

5. Requirements for further research

The data collected during this scoping study strongly suggest that the stress axis of sticklebacks downstream of WWTWs is affected by exposure to effluent and that the magnitude of effects on both the unstimulated and stimulated stress axis is proportional to the concentration of effluent present.
These findings lead to several important follow-up questions:

Q1. Are the trends detected in this scoping study evident at other WWTWs?

The most informative data in this study were collected from fish downstream of ten WWTWs. However, while the somatic characteristics of fish collected downstream of the three largest of these appear to be consistent with fish from the other sites (with one exception) the characteristics of the stress axis were not consistent for fish downstream of the three largest WWTWs. The measurements made during this scoping study should be replicated at a wider range of WWTWs to establish that the trends detected are a reflection of a widespread phenomenon. The reasons for the discontinuity between the characteristics of the stress axis in fish downstream of WWTWs serving < 100,000 PE compared with fish downstream of WWTWs serving populations > 100,000 should be investigated.

Q2. Is exposure to effluent the causal factor that accounts for variation in the stress axis between sites?

If samples of fish from affected sites are transferred to the laboratory environment and held in clean water, do baseline cortisol concentrations and the reactivity of the stress axis return to a species “norm”? Conversely, does exposure of laboratory-reared fish to effluent evoke changes in the function of the stress axis that resemble those seen in wild fish downstream of those WWTWs?

Q3. Do the changes evident in the stress axis of fish downstream of WWTWs have any adverse effect on the fitness of those fish?

Straightforward measures of size, growth and reproductive performance are confounded by the enriching effect of the WWTW effluent on the environment downstream of WWTWs. Alternative measures of fitness will need to be identified and employed to investigate whether interference in the function of the stress axis has adverse consequences for the individual and by inference the population.

Acknowledgements
The authors thank Martin Rossall for assistance with field work, Claire Wood for preparing the site maps, Alan Lawlor for conducting metal analysis, and Glenn Rhodes for help with RT-PCR assay design.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., 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.

Aluru, N., Renaud, R., Leatherland, J. F. and Vijayan, M. M. (2005). Ah Receptor-mediated impairment of interrenal steroidogenesis involves StAR protein and P450scc gene attenuation in rainbow trout. Toxicological Sciences 84, 260–269.

Angelier, F., Wingfield, J. C., Weimerskirch, H. and Chastel, O. (2010). Hormonal correlates of individual quality in a long-lived bird: a test of the 'corticosterone–fitness hypothesis'. Biology Letters 6, 846-849.

Arukwe, A., (2008). Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish. Cell Biology and Toxicology 24, 527-540.

Bell, A. M., Backström, T., Huntingford, F. A., Pottinger, T. G., Winberg, S. (2007). Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks (Gasterosteus aculeatus). Physiology & Behavior 91, 15-25.

Benguira, S., Leblond, V. S., Weber, J.-P. and Hontela, A. (2002). Loss of capacity to elevate plasma cortisol in rainbow trout (*Oncorhynchus mykiss*) treated with a single injection of o,p'-dichlorodiphenyldichloroethane. Environmental Toxicology and Chemistry 21, 1753-1756.

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.

Bolger, T. and Connolly, P. L. (1989). The selection of suitable indices for the measurement and analysis of fish condition. Journal of Fish Biology 34, 171-182.

Bonier, F., Martin, P. R., Sheldon, K. S., Jensen, J. P., Foltz, S. L. and Wingfield, J. C. (2007). Sex-specific consequences of life in the city. Behavioural Ecology 18, 121-129.

Bonier, F., Martin, P. R., Moore, I. T. and Wingfield, J. C. (2009). Do baseline glucocorticoids predict fitness? Trends in Ecology & Evolution 24, 634-642.

Bonier, F., Martin, P. R., Moore, I. T. and Wingfield, J. C. (2010). Clarifying the Cort-Fitness Hypothesis: A response to Dingemanse et al. Trends in Ecology & Evolution 25, 262-263.

Bradshaw, D. (2007). Environmental endocrinology. General and Comparative Endocrinology 152, 125-141.

Burkhardt-Holm, P. (2010). Endocrine disruptors and water quality: a state of the art review. International Journal of Water Resources Development 26, 477-493.

Busch, D. S. and Hayward, L. S. (2009). Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation 142, 2844-2853.

Cabezas, S., Blas, J., Marchant, T. A. and Moreno, S. (2007). Physiological stress levels predict survival

probabilities in wild rabbits. Hormones and Behavior 51, 313-320.

Calabrese, E. J. (2004). Hormesis: a revolution in toxicology, risk assessment and medicine. EMBO Reports 5 (Suppl 1) S37-S40.

Caliman, F. A and Gavrilescu, M. (2009). Pharmaceuticals, personal care products and endocrine disrupting agents in the environment – A review. Clean 37, 277-303.

Celander, M. C. (2011). Cocktail effects on biomarker responses in fish. Aquatic Toxicology 105S, 72-77.

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.

Cone, R. S. (1989). The need to reconsider the use of condition indices in fishery science. Transactions of the American Fisheries Society 118, 510-514.

Cook, K. V., McConnachie, S. H., Gilmour, K. M., Hinch, S. G. and Cooke, S. J. (2011). Fitness and behavioral correlates of pre-stress and stress-induced plasma cortisol titers in pink salmon (*Oncorhynchus gorbuscha*) upon arrival at spawning grounds. Hormones and Behavior 60, 489-497.

Fent, K., Weston, A.A. and Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. Aquatic Toxicology 76, 122–159.

Gagnon, A., Jumarie, C. and Hontela, A. (2006). Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 78, 59-65.

Gao , K., Brandt, I., Goldstone, J. V. and Jönsson, M. E. (2011). Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. Comparative Biochemistry and Physiology, Part C 154, 42–55.

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, -naphthoflavone and benzo(a)pyrene) treatment. Aquatic Toxicology 86, 341–351.

Gravel, A., Campbell, P. G. C. and Hontela, A. (2005). Disruption of the hypothalamo-pituitary-interrenal axis in 1+ yellow perch (*Perca flavescens*) chronically exposed to metals in the environment. Canadian Journal of Fisheries and Aquatic Science 62, 982-990.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters 131, 5-17.

Hinson, J. P. and Raven, P. W. (2006). Effects of endocrine-disrupting chemicals on adrenal function. Best Practice & Research Clinical Endocrinology & Metabolism 20, 111-120.

Hontela, A. (1998). Interrenal dysfunction in fish from contaminated sites: *in vivo* and *in vitro* assessment. Environmental Toxicology and Chemistry 17, 44–48.

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.

Ings, J. S., Servos, M. R. and Vijayan, M. M. (2011). Exposure to municipal wastewater effluent impacts

stress performance in rainbow trout. Aquatic Toxicology 103, 85-91.

Keller, V. and Young, A. R. (2004). Development of the integrated water resources and water quality modelling system. (Science Report). Science Report P2-248/SR. Bristol, UK.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B. and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. Streams, 1999-2000: A national reconnaissance. Environmental Science and Technology 36, 1202-1211.

Kosma, C. I., Lambropoulou, D. A. and Albanis, T. A. (2010). Occurrence and removal of PPCPs in municipal and hospital wastewaters in Greece. Journal of Hazardous Materials 179, 804–817.

Lacroix, A. and Hontela, A. (2004). A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch, *Perca flavescens*. Aquatic Toxicology 67, 13-21.

Laflamme, J.-S., Couillard, Y., Campbell, P. G. C. and Hontela, A. (2000). Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. Canadian Journal of Fisheries and Aquatic Science 57, 1692-1700.

Leblond, V. S., Bisson, M. and Hontela, A. (2001). Inhibition of cortisol secretion in dispersed head kidney cells of rainbow trout (*Oncorhynchus mykiss*) by endosulfan, an organochlorine pesticide. General and Comparative Endocrinology 121, 48–56.

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.

Miller, L. L. and Hontela, A. (2011). Species-specific sensitivity to selenium-induced impairment of cortisol secretion in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). Toxicology and Applied Pharmacology 253, 137-144.

Neal, C., Rowland, P., Scholefield, P., Vincent, C., Woods, C. and Sleep, D. (2011). The Ribble/Wyre observatory: Major, minor and trace elements in rivers draining from rural headwaters to the heartlands of the NW England historic industrial base. Science of the Total Environment 409, 1516-1529.

Nichols, J. W., Breen, M., Denver, R. J., DiStefano III, J. J. Edwards, J. S., Hoke, R. A., Volz, D. C. and Zhang, X. (2011). Predicting chemical impacts on vertebrate endocrine systems. Environmental Toxicology and Chemistry 30, 39–51.

Norris, D. O., Donahue, S., Dores, R. M., Lee, J. K., Maldonado, T. A., Ruth, T. and Woodling, J. D. (1999). Impaired adrenocortical response to stress by brown trout, *Salmo trutta*, living in metal-contaminated waters of the Eagle River, Colorado. General and Comparative Endocrinology 113, 1–8.

Ouyang, J. Q., Sharp, P. J., Dawson, A., Quetting, M. and Hau, M. (2011). Hormone levels predict individual differences in reproductive success in a passerine bird. Proceedings of the Royal Society B 278, 2537-2545.

Øverli, Ø., Winberg, S. and Pottinger, T. G. (2005). Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout - a review. Integrative and Comparative Biology 45, 463-474.

Øverli, Ø., Sørensen, C., Pulman, K. G. T., Pottinger, T. G., Korzan, W., Summers, C. H. and Nilsson, G. E. (2007). Evolutionary background for stress coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. Neuroscience and Biobehavioral

Reviews 31, 396-412.

Pankhurst, N. W. (2011). The endocrinology of stress in fish: An environmental perspective. General and Comparative Endocrinology 170, 265-275.

Pedrouzo, M., Borrull, F., Pocurull, E. and Marcé, R. M. (2011). Presence of pharmaceuticals and hormones in waters from sewage treatment plants. Water, Air, and Soil Pollution 217, 267-281.

Pickering, A.D. and Pottinger, T.G. (1985) Acclimation of the brown trout, *Salmo trutta* L., to the stress of daily malachite green treatment. Aquaculture 44, 145-152.

Plechner, A. J. (2004). Adrenal toxicity in dogs and cats as a contributing cause of hormonal and immune destabilisation. Journal of Applied Toxicology 24, 53–58.

Pottinger, T. G. (2003). Interactions of endocrine disrupting chemicals with stress responses in wildlife. Pure and Applied Chemistry 75, 2321-2333.

Pottinger, T. G. and Carrick, T. R. (1999). Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. General and Comparative Endocrinology 116, 122-132.

Pottinger, T. G. & Carrick, T. R. (2001). Stress responsiveness affects dominant-subordinate relationships in rainbow trout. Hormones and Behavior 40, 419-427.

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., Carrick, T. R., Hughes, S. E. and Balm, P. H. M. (1996). Testosterone, 11-ketotestosterone and estradiol-17□ modify baseline and stress-induced interrenal and corticotropic activity in trout. General and Comparative Endocrinology 104, 284-295.

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., Cook, A., Jürgens, M. D., Rhodes, G., Katsiadaki, I., Balaam, J. L., Smith, A. J. and Matthiessen, P. (2011a). Effects of sewage effluent remediation on body size, somatic RNA:DNA ratio, and markers of chemical exposure in three-spined sticklebacks. Environment International 37, 158-169.

Pottinger, T. G., Cook, A., Jürgens, M. D., Sebire, M., Henrys, P. A., Katsiadaki, I., Balaam, J. L., Smith, A. J. and Matthiessen, P. (2011b). Indices of stress in two populations of three-spined sticklebacks are altered by extreme weather events and exposure to waste-water effluent. Journal of Fish Biology 79, 256-279.

Plechner, A. J. (2004). Adrenal toxicity in dogs and cats as a contributing cause of hormonal and immune destabilisation. Journal of Applied Toxicology 24, 53–58.

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. TRENDS in Ecology and Evolution 19, 249-255.

Rowland, A. P., Neal, C., Scholefield, P., Halford, A. P., Vincent, C. D. and Hockenhull, K. (2010). Mercury in rivers in NW England: from rural headwaters to the heartlands of the historic industrial base. Journal of Environmental Monitoring 12, 2299-2306.

Sandhu, N. and Vijayan, M. M. (2011). Cadmium-mediated disruption of cortisol biosynthesis involves suppression of corticosteroidogenic genes in rainbow trout. Aquatic Toxicology 103, 92–100.

Santos, E. M., Ball, J. S., Williams, T. D., Wu, H., Ortega, F., Van Aerle, R., Katsiadaki, I., Falciani, F., Viant, M. R., Chipman, J. K. and Tyler, C. R. (2010). Identifying health impacts of exposure to copper using transcriptomics and metabolomics ina fish model. Environmental Science and Technology 44, 820-826.

Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U. and Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. Science 313, 1072-1077.

Snyder, S. A., Westerhoff, P., Yoon, Y. and Sedlak, D. L. (2003). Pharmaceuticals, personal care products, and endocrine disruptors in water: implications for the water industry. Environmental Engineering Science 20, 449-469.

Tierney, K. B., Williams, J. L., Gledhill, M., Sekela, M. A., and Kennedy, C. J. (2011). Environmental concentrations of agricultural-use pesticide mixtures evoke primary and secondary stress responses in rainbow trout. Environmental Toxicology and Chemistry 30, 2602–2607.

Verboven, N., Verreault, J., Letcher, R. J., Gabrielsen, G. W. and Evans, N. P. (2010). Adrenocortical function of Arctic-breeding glaucous gulls in relation to persistent organic pollutants. General and Comparative Endocrinology 166, 25–32.

Wikelski, M. and Cooke, S. J. (2006). Conservation physiology. Trends in Ecology and Evolution 21, 38-46.

Williams, R. J., Keller, V. D. J., Johnson, A. C., Young, A. R., Holmes, M. G. R., Wells, C., Gross-Sorokin, M., and Benstead, R. (2009). A national risk assessment for intersex in fish arising from steroid estrogens. Environmental Toxicology and Chemistry 28, 220-230.

Williams, R., Johnson, A., Keller, V., Young, A., Holmes, M. and Wells, C. (2008). Catchment Risk Assessment of Steroid Oestrogens from Sewage Treatment Works. (Environment Agency Science Report). SCHO0308BNVO-E-P. Bristol, UK.

Williams, T .D., Wu, H., Santos, E. M., Ball, J., Katsiadaki, I., Brown, M. M., Baker, P., Ortega, F., Falciani, F., Craft, J. A., Tyler, C. R., Chipman, J. K. and Viant, M. R. (2009). Hepatic transcriptomic and metabolomic responses in the stickleback (Gasterosteus aculeatus) exposed to environmentally relevant concentrations of dibenzanthracene. Environmental Science and Technology 43, 6341–6348.

Wingfield, J. C. (2008). Comparative endocrinology, environment and global change. General and Comparative Endocrinology 157, 207-216.

Wiseman, S., Thomas, J. K., McPhee, L., Hursky, O., Raine, J. C., Pietrock, M., Giesy, J. P., Hecker, M. and Janz, D. M. (2011). Attenuation of the cortisol response to stress in female rainbow trout chronically exposed to dietary selenomethionine. Aquatic Toxicology 105, 643– 651.

Young, J. L., Bornik, Z. B., Marcotte, M. L., Charlie, K. N., Wagner, G. N., Hinch, S. G. and Cooke, S. J. (2006). Integrating physiology and life history to improve fisheries management and conservation. Fish and Fisheries 7, 262-283.

Zhang, S., Lei, F., Liu, S., Li, D., Chen, C. and Wang, P. (2011). Variation in baseline corticosterone levels of tree sparrow (*Passer montanus*) populations along an urban gradient in Beijing, China. Journal of Ornithology 152, 801-806.