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The stress response of three-spined sticklebacks is modified in proportion to effluent exposure downstream of wastewater treatment works

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Abstract

This study was conducted to investigate whether exposure to wastewater treatment works (WWTW) effluent affects the adaptive stress axis of fish resident within the receiving water. Three-spined sticklebacks (*Gasterosteus aculeatus*) were sampled from sites downstream of ten WWTWs in north-west England, selected to represent a range of human population equivalents between 1,000 and 125,000. Following capture, indices of stress (whole-body cortisol and glucose concentrations) were measured both prior to, and following, the imposition of a standardised stressor to establish both baseline and stress-induced concentrations of cortisol and glucose. There was considerable between-site variation in size, and to a lesser extent condition, of the fish. Pre- and post-stress cortisol and glucose concentrations also varied significantly between-sites. A large proportion of the variation in both the somatic data and the stress response was explained by variation in the proportion of effluent contributing to total river flow at the study sites. Mass ($r^2 = 0.35$, $P < 0.001$) and length ($r^2 = 0.37$, $P < 0.001$) of the fish, and cortisol ($r^2 = 0.26$, $P < 0.001$) and glucose ($r^2 = 0.12$, $P < 0.01$) concentrations in unstressed sticklebacks, were positively related to the concentration of effluent across the sample sites. However, in stressed fish, cortisol ($r^2 = 0.32$, $P < 0.001$) and glucose ($r^2 = 0.14$, $P < 0.001$) concentrations exhibited a negative trend in relation to the effluent concentrations across sites. Individual variation in fish size did not account for the variation in either cortisol or glucose levels. These data provide the first indication that modulation of the stress axis in fish by anthropogenic factors might be widespread and of greater significance than hitherto assumed.

Keywords: stickleback, stress, endocrine disruption, wastewater, cortisol, glucose.

1. Introduction

Fish, in common with all vertebrates, possess a suite of neuroendocrine, metabolic and behavioural responses, collectively termed the stress response, that are rapidly activated to help cope with challenging circumstances (Pankhurst, 2011). A core element of the stress response, the hypothalamic-pituitary-interrenal (HPI) stress axis, is susceptible to interference by chemicals (reviewed by Pottinger, 2003) including metals, pharmaceuticals, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and herbicides (Bisson and Hontela, 2002; Gesto et al., 2008; Hontela, 2006; Levesque et al., 2003). Chemical interference with the activity of the stress axis has been reported to result in an attenuated release of cortisol, the primary corticosteroid secreted by the interrenal tissue of stressed fish. Although clear evidence of higher-level effects arising from disruption of the stress response is limited, it is reasonable to assume that there may be adverse effects on the fitness of affected fish.

During a recent investigation of the effects of extreme weather events on the stress axis of three-spined sticklebacks (*Gasterosteus aculeatus*) in rivers in south-west England (Pottinger et al., 2011a) we detected trends in whole-body levels of cortisol which suggested that the magnitude of the stress response following capture was influenced by the proximity of the capture site to a wastewater treatment works (WWTW) effluent discharge. The present study was conducted to evaluate whether these observations constituted evidence that the complex chemical milieu present in rivers downstream of WWTWs, one of the most abundant point-sources of aquatic pollutants in UK waters, can affect the functioning of the stress axis in fish.

To address this aim, resident populations of three-spined sticklebacks were sampled at sites downstream of ten WWTWs in the north west of England serving a range of human populations (1,000 – 125,000). The freshwater three-spined stickleback offers several advantageous characteristics for a study of this nature, including small body size, a wide distribution, local abundance, a short life span often resulting in a single predominant year class, a relatively sedentary lifestyle, and no significance to recreational anglers (Katsiadaki et al., 2007; Pottinger et al., 2002). For fish captured at each site indicators of stress (whole-body concentrations of cortisol and glucose) were quantified in order to assess the status of the stress axis in individuals immediately following capture (unstressed – baseline cortisol and glucose) and after a short period of confinement (stressed – elevated cortisol and glucose). These data were examined in the context of several measures by which the environmental impact of the upstream WWTWs could be characterised: population equivalents, dry weather flow (daily discharge) and modelled effluent concentration at the sample sites.

2. Materials and methods

2.1. Site selection

The geographical distribution of sampling sites is shown in Fig. 1. Ten sites located 0.5 – 1 km downstream of WWTWs serving population equivalents of between 1,000 and 125,000 were identified (see Table 1). We had difficulty in identifying any field sites that could be categorised as uncontaminated with a high degree of confidence so in order to provide fish from an uncontaminated environment for comparative purposes, the population of three-spined sticklebacks maintained in the CEH aquarium was sampled. The CEH aquarium receives untreated water from a natural source (Blea Tarn, SD 4934 5850), which is free of

sewage, and fish are held in a flow-through system. Fish were sourced, as by-catch, from a commercial fish farm (Moore & Moore Carp, Reading, U.K.) and had been held in the aquarium for more than 6 months. The assumption was made that in the absence of any chemical challenge these fish should provide a physiologically unbiased baseline for comparative purposes.

2.2. Sampling procedure

Sampling was conducted between the 14th March and 1st April 2011. 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*. processed within 5 minutes - before the stress caused by netting had caused a detectable cortisol response – T. G. Pottinger, unpublished data) 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 N_2 dry shipper (Taylor-Wharton CryoExpress CX100 and CX500, Jencons plc). Where possible, a minimum of 10 additional fish from each site were designated as "stressed". Immediately after capture these were transferred to a 10 L bucket containing river water and held for between 30 and 60 mins. This time period was selected based on experimental data (T. G. Pottinger, unpublished) showing that maximal whole-body cortisol concentrations in sticklebacks held under these conditions was achieved within 30 minutes and did not vary significantly for at least an additional 30 minutes. Sticklebacks were then transferred to a lethal concentration of sedative and treated as

described above for the unstressed individuals. At CEH Lancaster the samples were transferred to a freezer (-70^{o}C) until they were processed for analysis within one month of capture. (Imposition of post-capture stress was conducted under the authority of a UK Home Office project and personal licence held by TGP and was approved by the local ethical committee). 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. Processing of fish

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) to provide material for subsequent analyses. 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

Cortisol was extracted from 100 µl aliquots of whole homogenate using 400 µl of ethyl acetate. After vortex-mixing, the extracts were centrifuged and a 50 µl aliquot 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) and cortisol antibody (David E. Kime, Sheffield University; anti-cortisol 161). Glucose concentrations in the homogenate supernatant were determined using a microplate assay (hexokinase reagent and standard glucose solution: Sigma-Aldrich).

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 discharged 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 are best estimates based on the population served by the works plus any base load provided by industrial discharges, most of which are delivered to the larger WWTWs. We included both DWF and PE in these analyses because of the possibility that one or other metric more accurately defined the biological impact of the discharge. In addition, to more effectively quantify the exposure of fish downstream of the WWTWs to water-borne contaminants the percentage of WWTW-derived effluent at each sampling site was estimated using the LF2000-WQX model (Keller and Young, 2004; Williams et al., 2009; Williams et al., 2012). 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 (the length of river between 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^{-1}. The modelled concentration in ng l^{-1} is the estimate of the percentage dilution thus: Percentage effluent = $[(C_{eff} \times DWF) + (F_r \times C_r)]/(F_r + DWF)$, 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 river flow data which employed a standard climate period of 30 years (1961 - 1990), and the river 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 available to estimate percent effluent in Tara Carr Gutter (receiving water for Longton WWTW).

2.6. Statistical analysis

An analysis of variance (ANOVA) approach was adopted to test for differences in variables between sample sites, sex of the fish, and stress status (unstressed / stressed). Where data did not conform to a normal distribution they were log-transformed and re-tested for normality. Selected first order interactions were included to determine whether the effect of capture and confinement stress was the same across all rivers. Within-river variation was assessed by ANOVA with a Tukey correction for multiple testing. For glucose and cortisol concentrations linear relationships with mass, length and condition from the same fish were assessed 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. 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. The comparison of the relationship between log(weight) and log(length) for fish across sampling sites, prior to calculating and comparing condition factors, was conducted using ANCOVA. These analyses were conducted using R (v. 2.10.1; R Development Core Team (2009). Regression analyses of WWTW metrics and biological data were conducted using Minitab v.16 (Minitab Inc.).

3. Results

3.1. Somatic data

There was a four-fold difference in the mean mass of fish across the WWTW sites (Fig. 2a; $\log_{10}$ body mass, ANOVA, F (10,334) = 57.4, $P < 0.001$) with the largest fish in the R. Darwen (upstream, 1894 ± 117 mg, n = 35) and the smallest in Bushburn Brook (515 ± 66 mg, n = 29; Fig. 2a). Male and female body mass differed overall (male: 1476 ± 55 mg, n = 171; female: 1315 ± 47 mg, n = 164; ANOVA, F (1,334) = 3.9, $P = 0.048$) but there was no river*sex interaction ($P = 0.17$). Fork length varied significantly (Fig. 2b; ANOVA, F (10,334) = 56.8, $P < 0.001$) among the wild-caught fish with the R. Darwen (upstream, 53.5 ± 0.9 mm, n = 35) fish longest and fish from Bushburn Brook (35.8 ± 1.4 mm, n = 29) the smallest. Length differed between male and female fish overall (male: 49.8 ± 0.6 mm, n = 171; female: 48.0 ± 0.5, n = 164; ANOVA, F (1,334) = 3.9, $P = 0.05$) but again there was no river*sex interaction ($P = 0.32$). Length frequency distributions were unimodal for all sites. There was no significant variation in the gradient of $\log_{10}$(weight) v. $\log_{10}$(length) plots across the sample sites (slope estimate 0.31 at all sites; ANCOVA) and therefore a comparison of condition factor (coefficient of condition, Fulton's condition factor K: 100*weight/length^3; Bolger and Connolly, 1989) between sites was conducted. Condition varied significantly between rivers (Fig. 2c; ANOVA, F (10,334) = 5.2, $P < 0.001$) being highest for fish from the R. Darwen (upstream, 1.18 ± 0.02, n = 35) and lowest for fish from the R. Yarrow (1.00 ± 0.02, n = 20). There was no difference in condition between males and females overall ($P = 0.46$) and no river*sex interaction ($P = 0.64$). The aquarium fish were significantly larger overall than wild-caught fish but the mean coefficient of condition for the aquarium-reared fish did not differ significantly from that of the wild-caught fish.

3.1.2. WWTW metrics and somatic data

Both body mass and fork length were highly correlated with the estimated concentration of effluent at each sampling site (Fig. 3a, b) with this relationship explaining up to 37% of variation in the somatic data (Table 2). The best fit was obtained by excluding the data for fish from Thistleton Brook and by using the effluent concentrations estimated from the long-term average flow data (1961 - 1990). Exclusion of the data for Thistleton Brook was not undertaken arbitrarily. Their position in the mass and length plots appeared suspicious, with none of the data points falling within the 95% confidence intervals derived from the remainder of the data. Their exclusion from the regression analysis substantially improved the fit of the remaining data indicating that this group of points disproportionately influenced the outcome of the regression. The results of the regression analyses conducted both with and without the Thistleton Brook data are shown (Table 2) to allow the outcomes to be compared (4% cf. 35-37% of variation explained). Body condition was also significantly related to percent effluent at site but only 8% of variation in condition was explained by this relationship. 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%).

3.2. Cortisol

3.2.1. Unstressed fish

Mean whole-body cortisol concentrations were higher in unstressed female than male sticklebacks but this difference was not statistically significant (male: 8.6 ± 0.6 ng g^{-1}, n = 63; female: 13.6 ± 2.3 ng g^{-1}, n = 67; ANOVA, F (1,129) = 1.4, P = 0.23) and there was no significant river*sex interaction among unstressed fish (P = 0.72). Cortisol concentrations in

unstressed sticklebacks varied significantly between rivers (Fig. 4a; F (10,129) = 12.3, $P < 0.001$) with a four-fold range in mean values across WWTW sites. The lowest mean cortisol concentration was in unstressed fish from Sankey Brook (4.1 ± 0.7 ng g^{-1}, n = 9) and the highest in fish from Pendle Water (17.9 ± 2.2 ng g^{-1}, n = 16). Two ostensibly unstressed fish with atypically high concentrations of cortisol captured at Sankey Brook (89 ng g^{-1}) and the R. Lostock (172 ng g^{-1}) were considered outliers and were omitted from the analysis. Mean cortisol levels in fish from the CEH aquarium were within the higher range of those from the sampled sites but did not differ significantly from fish captured at any of the field sites.

3.2.2. Stressed fish

Stress-induced cortisol concentrations varied significantly between males and females (male: 86.4 ± 4.5 ng g^{-1}, n = 108; female: 112.7 ± 5.1 ng g^{-1}, n = 96; ANOVA, F (1,203) = 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 (10,203) = 3.5, $P < 0.001$) in an unsystematic manner. Combined data have been plotted for clarity. Concentrations of cortisol in sticklebacks subjected to a period of confinement following capture were significantly elevated in fish at all sites relative to unstressed fish at the same sites ($P < 0.001$). Mean cortisol concentrations in stressed fish varied significantly between rivers (Fig. 4b; ANOVA, F (10,203) = 11.8, $P < 0.001$) with a three-fold range in mean concentrations between fish from the R. Darwen (upstream) (54.0 ± 4.2 ng g^{-1}, n = 25) and Sankey Brook (155.5 ± 30.4 ng g^{-1}, n = 8). The proportional difference in mean cortisol concentrations pre- and post-stress varied between 267% (R. Darwen us) and 3702% (Sankey Brook). No significant relationship was evident between mean pre-confinement cortisol concentrations and mean post-confinement cortisol levels ($r^2 = 0.019$, $P = 0.63$).

Mean cortisol levels in fish from the CEH aquarium exposed to a similar confinement stressor were significantly lower than mean post-stress cortisol levels in fish from Thistleton Brook, Pendle Water, R. Yarrow and Sankey Brook.

3.2.3. Cortisol in relation to 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 (P = 0.1 - 0.5) and mass and length were also unrelated to cortisol concentrations in stressed sticklebacks. However, there was a significant negative relationship between condition and stress-induced cortisol concentrations (r^2 = 0.06; P = 0.001).

3.2.4. WWTW metrics and cortisol

Regression analyses were initially conducted for male and female fish separately. However, no differences between the sexes were observed and therefore the data were combined. Cortisol levels in unstressed fish were significantly related (P = 0.001) to only the effluent concentration derived from the long-term (1961 – 1990) flow data. However, this relationship accounted for only a small proportion of variation in the data (9%; Table 2). By excluding the data for the largest WWTW (St Helens; Sankey Brook), which appeared from the plots to be atypical, the proportion of variation explained by this relationship increased to 26% and resulted in a significant positive relationship between cortisol and all the WWTW metrics, (Table 2; Fig. 5a). For stressed fish the relationship between effluent concentration and cortisol was the inverse of that for unstressed fish and was similarly improved by excluding data for St Helens WWTW (Table 2). However, in contrast to the unstressed fish the greatest portion of variation in cortisol levels in stressed fish (32%; Table

2; Fig. 5b) was explained by the variability in effluent concentration derived from the 2010/11 flow 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) but cortisol concentrations in stressed fish were significantly and inversely related to water temperature (r^2 = 0.12, *P* < 0.001).

3.3. Glucose

3.3.1. Unstressed fish

Whole-body glucose levels in unstressed sticklebacks varied significantly between rivers (Fig. 6a; ANOVA, F (10,129) = 5.2, *P* < 0.001) but no significant difference was evident between males and females overall (*P* = 0.76) and there was no significant river*sex interaction among unstressed fish (*P* = 0.6). The lowest mean concentrations of glucose in unstressed fish occurred in fish from the R. Lostock (1.30 ± 0.08 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 those in fish from Thistleton Brook, R. Lostock, R. Yarrow, and Sankey Brook.

3.3.2. Stressed fish

There was a small but significant difference in stress-induced glucose levels between males (1.86 ± 0.04 mg g^{-1}, n = 108) and females (1.64 ± 0.04 mg g^{-1}, n = 96; ANOVA, F(1,203) = 4.5, *P* = 0.03) but no river*sex interaction (*P* = 0.47). The overall mean glucose concentration in stressed fish across all sites was slightly, but significantly elevated (1.75 ± 0.03 mg g^{-1}, n = 204) compared to that in unstressed fish (1.47 ± 0.03 mg g^{-1}, n = 130) (ANOVA, F (1,333) = 33.1, *P* < 0.001). There was also significant variation between sampling sites in glucose

concentrations in stressed fish (Fig. 6b; ANOVA, F (10,203) = 8.1, $P < 0.001$) but when restricted to pairwise within-site comparisons significant elevation of whole-body glucose concentration following stress was demonstrable only for Thistleton Brook ($P < 0.001$). Mean post-stress glucose concentrations were highest in fish from Thistleton Brook (2.17 ± 0.11 mg g^{-1}, n = 24) and lowest in the R. Darwen, downstream (1.48 ± 0.06 mg g^{-1}, n = 24). Mean levels of glucose in fish from the CEH aquarium were higher than four of the sampled sites. No significant relationship between the mean whole-body cortisol and mean whole-body glucose concentrations was evident in stressed or unstressed sticklebacks ($P = 0.1 – 0.4$)

3.3.3. Mass, length and condition

Glucose concentrations in unstressed fish were unrelated to mass ($P = 0.3$) and length ($P = 0.9$) but were significantly and positively related to condition ($r^2 = 0.04$; $P = 0.015$). In stressed fish glucose concentrations were significantly and positively related to both mass ($r^2 = 0.03$; $P = 0.01$) and condition ($r^2 = 0.03$; $P = 0.01$).

3.3.4. WWTW metrics and glucose

Glucose concentrations in unstressed fish were positively related to the concentration of effluent estimated from the 2010 - 2011 flow data, after omitting data for St Helens, the largest WWTW ($r^2 = 0.12$, $P = 0.001$; Table 2; Fig. 7a). For stressed fish (Table 2; Fig. 7b) a negative relationship between glucose concentrations and WWTW metrics was seen, with population equivalent ($r^2 = 0.15$, $P = 0.0$), dry weather flow ($r^2 = 0.14$, $P = 0.0$) and the concentration of effluent estimated from the long-term flow data ($r^2 = 0.14$, $P = 0.0$) explaining the greatest proportion of variation in glucose levels in stressed fish. In this case

similar results were seen with or without inclusion of the St Helens data (Table 2). Whole-body glucose levels in unstressed fish were significantly related to water temperature at time of capture ($r^2 = 0.05$, $P < 0.01$) and this relationship approached significance for stressed fish ($r^2 = 0.02$, $P = 0.07$).

4. Discussion

This study is the first to investigate the function of the stress axis in multiple free-living populations of a fish species with lifelong exposure to WWTW effluent. The results showed that both baseline and stress-induced cortisol concentrations in three-spined sticklebacks resident downstream of rural and urban WWTWs in north-west England varied in proportion to the volume of effluent (as a percentage of total river flow) estimated to be present at the sites from which the fish were sampled. Baseline cortisol levels were directly related, whereas stress-induced concentrations of cortisol were inversely related, to effluent exposure. The activity of the HPI axis also exhibited trends in relation to other measures of WWTW impact (the population equivalent and dry weather flow) and these relationships were evident for baseline and stress-induced glucose levels also. The most plausible and parsimonious explanation for these observations is that elements of the chemical content of WWTW effluent modulate the function of the hypothalamic-pituitary-interrenal system in fish. These data also illustrate the utility of geographical information systems-based model-derived parameters (Keller and Young, 2004; Williams et al., 2009) in explaining variation in real-time physiological measurements in fish. In this context, these findings extend those of a previous study in which the incidence and severity of intersex in wild roach were significantly correlated with model-predicted concentrations of estrogens, using a forerunner of the model employed in the present study (Jobling et al., 2006).

4.1. Consistency of these findings with previous studies

Both of the primary effects observed in the present study, elevation of baseline cortisol and suppression of stress-induced cortisol in effluent-exposed sticklebacks, are consistent with data from laboratory studies and field surveys that show that the stress response of fish is altered by exposure to a range of environmental contaminants including PAHs (Gesto et al., 2008), PCBs (Quabius et al., 1997), organochlorines (e.g. DDT: Benguira et al., 2002), and metals (Gagnon et al., 2006; Gravel et al., 2005; Hontela, 1998; Laflamme et al., 2000; Norris et al., 1999). In particular, effects on the stress axis similar to those observed in the present study have recently been reported for rainbow trout, *Oncorhynchus mykiss*, exposed to municipal waste water effluent for 14 days (Ings et al., 2011a,b). Nor are these observations unique to fish. Exposure to mixtures of potential toxicants has also been associated with stress axis dysfunction in birds (Verboven et al., 2010). Considered in the context of these reports, the pattern of baseline and stress-induced cortisol concentrations observed in sticklebacks downstream of WWTWs in the present study suggests that these fish exhibit a chemically compromised stress axis. Whether this can be characterised as a form of endocrine disruption depends on whether adverse effects associated with alterations in stress axis function can be identified (WHO/IPCS, 2002). The broadly linear proportionality of the biological response to the percent effluent content in the receiving waters from which the fish were sampled suggests that the potency of the effluents as disruptors of the stress axis does not vary markedly, with the possible exception of that discharged by the largest WWTW, St Helens.

4.2. Which WWTW impact measures are associated with variation in the stress axis?

Highly significant regressions between somatic and physiological variables and WWTW metrics were obtained after excluding either the smallest WWTW (Elswick, Thistleton Brook – atypical for mass, length, condition) or the largest (St Helens, Sankey Brook – atypical for cortisol). Additionally, for somatic data and cortisol concentrations in unstressed fish a best fit was obtained with effluent concentrations derived from long-term flow data (1961 – 1990) whereas for cortisol concentrations in stressed fish the effluent concentrations based upon contemporaneous flow data (2010 – 2011) provided the best fit. We interpret these inconsistencies as being likely to arise from a combination of factors. Given the robustness of the positive relationship of mass and length with WWTW effluent concentration overall, the failure of fish from Thistleton Brook (Elswick WWTW) to conform to this relationship (they were larger than predicted by the regression) suggests that growth of fish at this site may be affected by factors additional to the nutrient input of the WWTW (see 4.4.). The site is rural and may be disproportionately affected by agricultural run-off or discharges from nearby farms, resulting in an augmentation of the enrichment effect of the WWTW effluent. The body size data for fish captured downstream of the largest WWTW (St Helens WWTW, Sankey Brook) fitted the relationship between size and effluent concentration that was evident for the sites overall, suggesting that the estimated effluent concentration at St Helens was proportional to actual nutrient input. However, the cortisol data for fish captured downstream of St Helens WWTW did not comply with overall trends across other sites. This may be due to a qualitative difference in the non-nutrient chemical content of the St Helens effluent, and thus its physiological effects, relative to the other WWTWs. In this context it is interesting to note that excluding all of the three largest WWTWs from the regression (Blackburn and Burnley in addition to St Helens) increased the proportion of variation in post-stress cortisol levels explained by effluent concentration from 32% to 43%.

This might indicate that there is a consistent qualitative difference in the chemical composition of effluent from WWTWs serving > 100,000 PEs and effluent from WWTWs serving < 100,000 PEs, possibly related to the proportion of non-domestic waste entering the WWTWs. Further investigations, particularly regarding the chemical profile of these discharges, are needed to resolve this anomaly.

Differences in the effectiveness of effluent concentrations calculated from the long- and short-term river flow data in explaining variation in fish size, and cortisol and glucose concentrations, are surprising if the contemporary flow data are assumed to best represent environmental conditions experienced by the generation of fish that was sampled. The results suggest instead that historical flow data, derived from a thirty-year period 20 years prior to the study, more accurately captured the extent to which between-site variation in flow, and consequently effluent concentration, affected body size and baseline cortisol. It is possible that the size of fish, and status of the unstimulated stress axis, are population characteristics, established by adaptation to local conditions over the long-term. In contrast, variation in stress-induced cortisol concentrations was better explained by variation in effluent concentrations derived from river flow data for the single year during which the study was conducted suggesting that the stress-induced increase in cortisol is constrained to a greater extent by factors aligned with current or recent variation in conditions. Modulation of the stress response in sticklebacks in relation to short-term variation in hydrological variables has previously been reported (Pottinger et al., 2011a). The converse was true for glucose: levels in unstressed fish showed a relationship with variation in flow only within the current year whereas glucose levels in stressed fish were related both to WWTW effluent concentrations derived from the historical river flow data but also to the

corresponding population equivalents and dry weather flows (see Table 2). Unlike cortisol, whole-body glucose concentrations in sticklebacks are in part defined by nutritional factors (Pottinger et al., 2002) and it is therefore possible that variation in glucose concentrations following stress – the incremental increase in which presumably reflects the mobilisation of stored reserves – is linked to the relative productivity of the sampling sites. Despite this, however, in both stressed and unstressed fish only a trivial component of variation in glucose was explained by variation in somatic characteristics at an individual level (0 – 4%). It is likely that a straightforward interpretation of these data is confounded by the simultaneous involvement of three factors which modify glucose concentrations – nutrition, the chemical milieu, and stress.

4.3. Effects of WWTW effluent on fish growth

Many U.K. stickleback populations are annual, with very high post-spawning and/or over-winter mortality among the 1+ adults (Pottinger et al., 2011b; Wootton and Smith, 2005). In this study, length frequency distributions for the fish captured at each site were unimodal and we therefore assumed that the majority of fish at each site represented the current year class of 0+ years old fish. A large proportion of the variation in mass and length of the fish, although not condition, was explained by variation in the concentration of effluent at the sample sites. Positive effects of WWTW effluent on the growth of fish have been reported (Pottinger et al., 2011b; Tetrault et al., 2011) and are likely to result from the effects of sewage-derived nutrients on food-web productivity (deBruyn et al., 2003) coupled with effluent-induced increases in water temperature (Pottinger et al., 2011b). The relationship between fish size and effluent concentration was most pronounced for estimates of effluent concentration based on long-term (1961 – 1990) flow data rather than

estimates of effluent concentration based on flow for the period during which the fish were resident. This presumably reflects the fact that variation in the productivity of the river ecosystems downstream of WWTWs is a function of the longer-term nutrient input and this best relates to the longer-term flow data.

4.4. The relationship between condition and cortisol concentrations

In the present study there was no relationship between fish size and activity of the stress axis at an individual level, suggesting that the variation in size in relation to effluent exposure was independent of any impact of effluent on the stress axis. There was, however, a significant inverse link between fish condition and cortisol concentration. In both the present study, and in a previous field study on the same species (Pottinger et al, 2011a), there was a strong trend among stressed sticklebacks for fish with lower condition factors to exhibit higher post-stress whole-body cortisol concentrations. This relationship was not evident in unstressed fish, suggesting that baseline cortisol concentrations were not causally involved in defining condition, or conversely that variation in baseline cortisol levels was not influenced by condition of the fish, or by factors underlying variation in condition. A relationship between condition and function of the stress axis has been widely reported among vertebrates but less specifically for fish. In a recent study, Cook et al. (2012) observed a negative relationship between condition and the degree of variability in post-stress cortisol concentrations in bluegill sunfish (*Lepomis macrochirus*), and an inverse relationship between corticosteroid levels and condition has been noted in studies encompassing reptiles (Romero & Wikelski, 2001), birds (Harms et al., 2010; Poisbleau et al., 2010; Raja-aho et al., 2010) and mammals (Cabezas et al., 2007). The interpretation of the functional significance of this relationship tends to vary. In the present study, relatively little

of the variation in condition of fish across sites was explained by variation in the proportion of WWTW effluent to which the fish were exposed suggesting that indirect rather than direct effects of effluent components are likely. Further investigation of the links between condition and stress responsiveness in fish is merited.

4.5. Mechanism(s) by which WWTW effluent may affect the stress axis

Putative effects of effluent exposure on the stress axis of the sticklebacks were context-dependent and it may prove to be challenging to identify and reconcile the mechanisms underlying superficially opposing effects. The first of these was the association of higher baseline cortisol concentrations with higher effluent concentration. Elevated baseline levels of cortisol in unstressed fish exposed to increasing amounts of effluent may reflect a contaminant-induced stress response. For the individual to cope with the chemical challenge associated with WWTW effluent requires allocation of resources to, for example, detoxification mechanisms which in turn requires diversion of resources away from other demands (allostasis; 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 elevated baseline cortisol concentrations in unstressed fish are a result of direct interactions of environmental contaminants with the stress axis via interference in signalling or biosynthetic pathways. For example, Ings et al. (2011a) attributed the elevation of baseline cortisol levels in trout exposed to 100% WWTW effluent to disruption of the cortisol clearance mechanism.

The second effect we observed was a progressive decline, in proportion to effluent exposure, in the magnitude of the stress-induced elevation of cortisol following exposure to

a stressor. Given the fact that circulating cortisol concentrations can modulate the activity of the stress axis by negative feedback it might reasonably be suggested that variation in post-confinement cortisol concentrations between populations was related to variation in resting (unstressed) cortisol concentrations. However, there was no statistically significant relationship between pre- and post-stress cortisol concentrations. Instead, attenuation of the post-stress cortisol response may have been due to interference with the function of cortisol-producing interrenal cells. For example, the organochlorine insecticide endosulfan interferes with the secretory function of teleost interrenal steroidogenic cells (Leblond et al., 2001) and similar effects have been observed in steroidogenic cells exposed to pesticides (Bisson and Hontela, 2002) and metals (Lacroix and Hontela, 2004). Chemical impairment of cortisol secretion during stress is reportedly due to modulation of the activity of 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. It has also been suggested (Arukwe, 2008) that contaminants may affect the negative feedback control of steroid hormone synthesis. While these mechanisms are in part consistent with the effects observed in the present study, given the chemical complexity of WWTW effluents it may be the case that as yet unidentified mechanisms and causal factors are involved.

4.6. Causal factors underlying disruption of stress axis function

A broad range of chemicals enters water bodies via WWTW effluent (Kolpin et al., 2002) including personal care products (Brausch and Rand, 2011; Snyder et al., 2003) and pharmaceuticals (Heberer, 2002; Kosma et al., 2010; Pedrouzo et al., 2011) whose effects singly and in combination on aquatic biota are largely unknown. With estimated effluent

concentrations at the sampling sites in the present study approaching 50% in some cases there is clearly potential for the exposure of fish to combinations of contaminants at substantial aggregated concentrations. Identifying candidate modulators of the stress axis is hampered by the lack of relevant toxicological data (Fent et al., 2006), coupled with the uncertainties introduced by concurrent exposure to a range of micropollutants, with 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. However, a surprisingly linear relationship was observed between effects on both baseline and stress-induced cortisol levels and effluent concentration (with the exception of one site) suggesting that possibly the major factor(s) responsible occur in WWTW effluents at similar concentrations. Most probably this is a reflection of the range of products entering WWTWs from domestic sources, whose relative usage is unlikely to vary within the sampling locale. Further investigation will be required to resolve this issue.

4.7. Laboratory stickleback cortisol and glucose data

Whole-body cortisol concentrations were measured in a laboratory population of sticklebacks in order to provide reference data for the stress axis in fish not exposed to any chemical contaminants. Although there was considerable variation in mean cortisol concentrations among fish from different sites (4.1 – 17.9 ng g^{-1}), mean baseline cortisol concentrations in control fish from the CEH aquarium population (11.4 ng g^{-1}) were not significantly different from those in fish from any of the sampled sites. Similar cortisol levels have been reported previously for unstressed laboratory-held sticklebacks (~ 5.0 ng g^{-1}: Pottinger et al., 2002; ~ 15 ng g^{-1}: Bell et al., 2007). The range of cortisol concentrations observed among stressed fish across the sampling sites (54.0 ng g^{-1} – 155.0 ng g^{-1}) is also

consistent with previous data for both laboratory studies (~ 35.0 ng g^{-1}: Pottinger et al., 2002; ~ 100 ng g^{-1}: Bell et al., 2007) and wild-caught sticklebacks (~ 40.0 – 120.0 ng g^{-1}: Pottinger et al., 2011a). Glucose data in the present study were similarly consistent with earlier studies (Pottinger et al., 2011a). However, although the data from captive stickleback populations are broadly consistent with those from wild-caught fish, it is perhaps unjustified to assume that these data provide a reliable surrogate for the unmodified baseline and stress-induced cortisol and glucose concentrations that would be evident in wild populations from clean environments. The research aquarium environment may impose certain constraints or modifying stimuli on the stress axis of captive fish, particularly with respect to habituation or acclimation to disturbance, and additional data from free-living stickleback populations inhabiting demonstrably pristine environments are needed to provide a more directly relevant baseline with which to assess the magnitude of any effects introduced by exposure to WWTW effluents.

4.8. Functional consequences of stress axis disruption

In possibly the only study to examine the issue of fitness and the stress axis in fish, elevated baseline plasma cortisol levels were 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) these reports suggest that the elevation of baseline cortisol concentrations in sticklebacks might result in detrimental effects on fitness. Further

investigation will be needed to resolve this possibility. With regard to the apparent effects of effluent exposure on stress-induced cortisol concentrations, the stress response is a key aid to survival that has been conserved throughout the evolution of the vertebrates. An attenuated stress response implies that the ability of the fish to implement 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. In perhaps the only experimental study to inform these issues the magnitude of the chronic stress response in rabbits, exposed to a prolonged period of captivity, was 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. Conclusions

These data strongly suggest that the function of the stress axis of three-spined sticklebacks that are resident 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. Although earlier studies have identified effects of a wide range of chemicals on the stress axis of fish we believe this to be the first study to demonstrate an effect across multiple populations of fish exposed to complex waste water effluents. This is the first indication that modulation of the stress axis in fish by

anthropogenic factors might be a widespread phenomenon and therefore of greater significance than hitherto assumed.

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REFERENCES

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

Angelier, F., Wingfield, J. C., Weimerskirch, H. Chastel, O., 2010. Hormonal correlates of individual quality in a long-lived bird: a test of the 'corticosterone–fitness hypothesis'. Biol. Lett. 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 Biol. Toxicol. 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*). Physiol. Behav. 91, 15-25.

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

Bisson, M., Hontela, A., 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol. Appl. Pharmacol. 180, 110-117.

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

Bonier, F., Martin, P. R., Moore, I. T., Wingfield, J. C., 2009. Do baseline glucocorticoids predict fitness? Trends Ecol. Evol. 24, 634-642.

Brausch, J. M., Rand, G. M., 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. Chemosphere 82, 1518-1532.

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

Cabezas, S., Blas, J., Marchant, T. A., Moreno, S., 2007. Physiological stress levels predict survival probabilities in wild rabbits. Horm. Behav. 51, 313-320.

Cook, K. V., McConnachie, S. H., Gilmour, K. M., Hinch, S. G., 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. Horm. Behav. 60, 489-497.

Cook, K. V., O'Connor, C. M., McConnachie, S. H., Gilmour, K. M., Cooke, S. J., 2012. Condition dependent intra-individual repeatability of stress-induced cortisol in a freshwater fish. Comp. Biochem. Physiol. A 161, 337–343.

de Bruyn, A. M. H., Marcogliese, D. J., Rasmussen, J. B., 2003. The role of sewage in a large river food web. Can. J. Fish. Aquat. Sci. 60, 1332–1334

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159.

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

Gesto, M., Soengas, J. L., 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. Aquat. Toxicol. 86, 341–351.

Gravel, A., Campbell, P. G. C., Hontela, A., 2005. Disruption of the hypothalamo-pituitary-interrenal axis in 1+ yellow perch (*Perca flavescens*) chronically exposed to metals in the environment. Can. J. Fish. Aquat. Sci. 62, 982-990.

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

Hontela, A., 1998. Interrenal dysfunction in fish from contaminated sites: in vivo and in vitro assessment. Environ. Toxicol. Chem. 17, 44–48.

Hontela, A., 2006. Corticosteroidogenesis and StAR protein of rainbow trout disrupted by human-use pharmaceuticals: Data for use in risk assessment. Toxicol. Sci. 93, 1-2.

Ings, J. S., Servos, M. R., Vijayan, M. M., 2011a. Exposure to municipal wastewater effluent impacts stress performance in rainbow trout. Aquat. Toxicol. 103, 85-91.

Ings, J. S., Servos, M. R., Vijayan, M. M., 2011b. Hepatic transcriptomics and protein expression in rainbow trout exposed to municipal wastewater effluent. Environ. Sci. Technol. 45, 2368-2376.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C. R., van Aerle, R., Santos, E., Brighty, G., 2006. Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environ. Health Perspect. 114, 32-39.

Katsiadaki, I., Sanders, M.B., Sebire, M., Nagae, M., Soyano K., Scott, A.P., 2007. Three-spined stickleback: an emerging model in environmental endocrine disruption. Environ. Sci. 14, 263-283.

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

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

Kosma, C. I., Lambropoulou, D. A., Albanis, T. A., 2010. Occurrence and removal of PPCPs in municipal and hospital wastewaters in Greece. J. Hazard. Mater. 179, 804–817.

Lacroix, A., 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*. Aquat. Toxicol. 67, 13-21.

Laflamme, J.-S., Couillard, Y., Campbell, P. G. C., Hontela, A., 2000. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, *Perca flavescens*, from Abitibi lakes. Can. J. Fish. Aquat. Sci. 57, 1692-1700.

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

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

Nichols, J. W., Breen, M., Denver, R. J., DiStefano III, J. J. Edwards, J. S., Hoke, R. A., Volz, D. C., Zhang, X., 2011. Predicting chemical impacts on vertebrate endocrine systems. Environ. Toxicol. Chem. 30, 39–51.

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

Pankhurst, N. W., 2011. The endocrinology of stress in fish: An environmental perspective. Gen. Comp. Endocrinol. 170, 265-275.

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

Poisbleau, M., Demongin, L., Chastel, O., Eens, M., Quillfeldt, P., 2010. Reversed hatching order, body condition and corticosterone levels in chicks of southern rockhopper penguins (*Eudyptes chrysocome chrysocome*). Gen. Comp. Endocrinol. 169, 244–249.

Pottinger, T. G., 2003. Interactions of endocrine disrupting chemicals with stress responses in wildlife. Pure Appl. Chem. 75, 2321-2333.

Pottinger, T. G., Carrick, T. R., 2001. Stress responsiveness affects dominant-subordinate relationships in rainbow trout. Horm. Behav. 40, 419-427.

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. J. Fish Biol. 61, 207-229.

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

Pottinger, T. G., Cook, A., Jürgens, M. D., Rhodes, G., Katsiadaki, I., Balaam, J. L., Smith, A. J., Matthiessen, P., 2011b. Effects of sewage effluent remediation on body size, somatic

RNA:DNA ratio, and markers of chemical exposure in three-spined sticklebacks. Environ. Int. 37, 158-169.

R Development Core Team (2009). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Raja-aho, S., Suorsa, P., Vainio, M., Nikinmaa, M., Lehikoinen, E., Eeva, T., 2010. Body condition is associated with adrenocortical response in the barn swallow (*Hirundo rustica* L.) during early stages of autumn migration. Oecologia 163, 323–332.

Romero, L. M., Wikelski, M., 2001. Corticosterone levels predict survival probabilities of Galápagos marine iguanas during El Niño events. Proc. Nat. Acad. Sci. 98, 7366–7370.

Snyder, S. A., Westerhoff, P., Yoon, Y., Sedlak, D. L., 2003. Pharmaceuticals, personal care products, and endocrine disruptors in water: implications for the water industry. Environ. Eng. Sci. 20, 449-469.

Tetreault, G. R., Bennett, C. J., Shires, K., Knight, B., Servos, M. R., McMaster, M. E., 2011. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquat. Toxicol. 104, 278-290.

Verboven, N., Verreault, J., Letcher, R. J., Gabrielsen, G. W., Evans, N. P., 2010. Adrenocortical function of Arctic-breeding glaucous gulls in relation to persistent organic pollutants. Gen. Comp. Endocrinol. 166, 25–32.

WHO/IPCS, 2002. Global assessment of the state-of-the-science of endocrine disruptors. World Health Organisation, Geneva.

Williams, R., Johnson, A., Keller, V., Young, A., Holmes, M., Wells, C., 2008. Catchment risk assessment of steroid oestrogens from sewage treatment works. Environment Agency Science Report. SCHO0308BNVO-E-P. Environment Agency, Bristol, UK.

Williams, R. J., Keller, V. D. J., Johnson, A. C., Young, A. R., Holmes, M. G. R., Wells, C., Gross-Sorokin, M., Benstead, R., 2009. A national risk assessment for intersex in fish arising from steroid estrogens. Environ. Toxicol. Chem. 28, 220-230.

Williams, R. J., Churchley, J. H., Kanda, R., Johnson, A. C., 2012. Comparing predicted against measured steroid estrogen concentrations and the associated risk in two United Kingdom river catchments. Environ. Toxicol. Chem. 31, 892-898

Wootton, R. J., Smith C., 2000. A long-term study of a short-lived fish: the demography of *Gasterosteus aculeatus*. Behaviour 137, 981–97.

Table captions

Table 1. Summary of sample sites. (us) = upstream; (ds) = downstream. SAS: secondary activated sludge; SB: secondary biological filter; TA2: activated sludge; TB2: biological filter.

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) and stress-related (cortisol, glucose, unstressed, stressed) data. Regressions with non-significant outcomes are italicised, those that explain the greatest amount of variation for each metric are in bold.

Figure captions

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.

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. Sites are ordered left to right by increasing population equivalents served. us – upstream, ds – downstream.

Figure 3. The relationship between the concentration of effluent at each site, estimated from the long-term river flow data (1961 – 1990), and (a) mass and (b) fork length of individual fish sampled downstream of each WWTW. ▌ - denotes data from Sankey Brook (St Helens WWTW); $\mathbf{V}$ - denotes data for Thistleton Brook (Elswick WWTW). Thistleton Brook data were excluded from the regression analysis (see Table 2 and Section 3.1.4. for explanation). The best-fit regression lines and 95% confidence intervals are plotted. See Table 2 for the corresponding regression parameters.

Figure 4. Whole-body cortisol concentrations in (a) unstressed and (b) stressed fish. Bars represent the mean + SEM (n for each mean is presented within the corresponding bar). Means sharing the same letter are not significantly different. Asterisks denote significant differences between unstressed and stressed mean cortisol within the same site. Note the different y-axis scales for (a) and (b). Sites are ordered left to right by increasing population equivalents served. us – upstream, ds – downstream.

Figure 5. Individual whole-body cortisol concentrations in (a) unstressed and (b) stressed fish at each site in relation to the concentration of WWTW effluent at the sample sites. For (a) the concentration of effluent at the sample sites was estimated from the long-term river flow data (1961 – 1990) and for (b) the concentration of effluent was estimated from the river flow data for March 2010 – April 2011 (see Table 2 and section 3.2.4). ▌- denotes data from Sankey Brook (St Helens WWTW) which were excluded from the regression. Note log scale for y axes.

Figure 6. Whole-body glucose concentrations in (a) unstressed and (b) stressed fish at each site. Bars represent the mean + SEM (n for each mean is presented within the corresponding bar). Means sharing a letter are not significantly different. Asterisks denote significant differences between unstressed and stressed fish within the same site. Sites are ordered left to right by increasing population equivalents served. us – upstream, ds – downstream.

Figure 7. Individual whole-body glucose concentrations in (a) unstressed and (b) stressed fish at each site in relation to the effluent concentration at the sample site. For (a) the concentration of effluent at the sample sites was estimated from the flow data for March

2010-April 2011and for (b) the concentration of effluent was estimated from the long-term river flow data (1961-1990) (see Table 2 and section 3.3.4.). O– denotes data from Sankey Brook (St Helens WWTW) which were excluded from the regressions.

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
Sankey Brook	14.3.11	SJ 5421 9576	St Helens	SJ 5390 9591	124,209	37600	SAS
R. 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
R. Lostock	16.3.11	SD 5166 2000	Leyland	SD 5216 2083	41,526	11000	TA2
R. 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

Table 2.

Metric	Stressed / unstressed	Sites excluded	Population equivalent		Dry weather flow		% effluent at site (1961-90)[1]		% effluent at site (2010-11)[2]	
			r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*
Body mass	all	None	*0.01*	*0.103*	0.02	0.002	0.04	0.001	0.05	0.000
Body mass	all	Thistleton	0.03	0.003	0.05	0.000	**0.35**	**0.000**	0.22	0.000
Fork length	all	None	0.02	0.008	0.04	0.001	0.04	0.001	0.03	0.003
Fork length	all	Thistleton	0.07	0.000	0.08	0.000	**0.37**	**0.000**	0.18	0.000
Condition[3]	all	None	*0.00*	*0.447*	*0.00*	*0.475*	0.01	0.052	0.02	0.037
Condition[3]	all	Thistleton	*0.01*	*0.205*	*0.01*	*0.249*	**0.08**	**0.000**	0.05	0.001
log_{10}Cortisol	Unstressed	none	*0.01*	*0.39*	*0.01*	*0.310*	**0.09**	**0.001**	*0.01*	*0.317*
log_{10}Cortisol	Unstressed	St Helens	0.03	0.034	0.03	0.030	**0.26**	**0.000**	0.08	0.003
log_{10}Cortisol	stressed	None	*0.01*	*0.141*	*0.02*	*0.101*	0.15	0.000	**0.18**	**0.000**
log_{10}Cortisol	stressed	St Helens	0.03	0.011	0.03	0.012	0.22	0.000	**0.32**	**0.000**
Glucose	Unstressed	None	*0.03*	*0.068*	*0.02*	*0.096*	*0.01*	*0.476*	*0.03*	*0.084*
Glucose	Unstressed	St Helens	*0.01*	*0.166*	*0.00*	*0.23*	*0.02*	*0.118*	**0.12**	**0.001**
Glucose	stressed	None	**0.15**	**0.000**	**0.14**	**0.000**	**0.14**	**0.000**	0.05	0.006
Glucose	stressed	St Helens	**0.14**	**0.000**	**0.13**	**0.000**	**0.13**	**0.000**	0.03	0.018

[1] Percent effluent estimated using long-term flow data for the period 1961 - 1990.
[2] percent effluent estimated using flow data for the period March 2010 - April 2011.
[3] Fulton's condition factor, K: 100*mass/length^3

Figure 1.

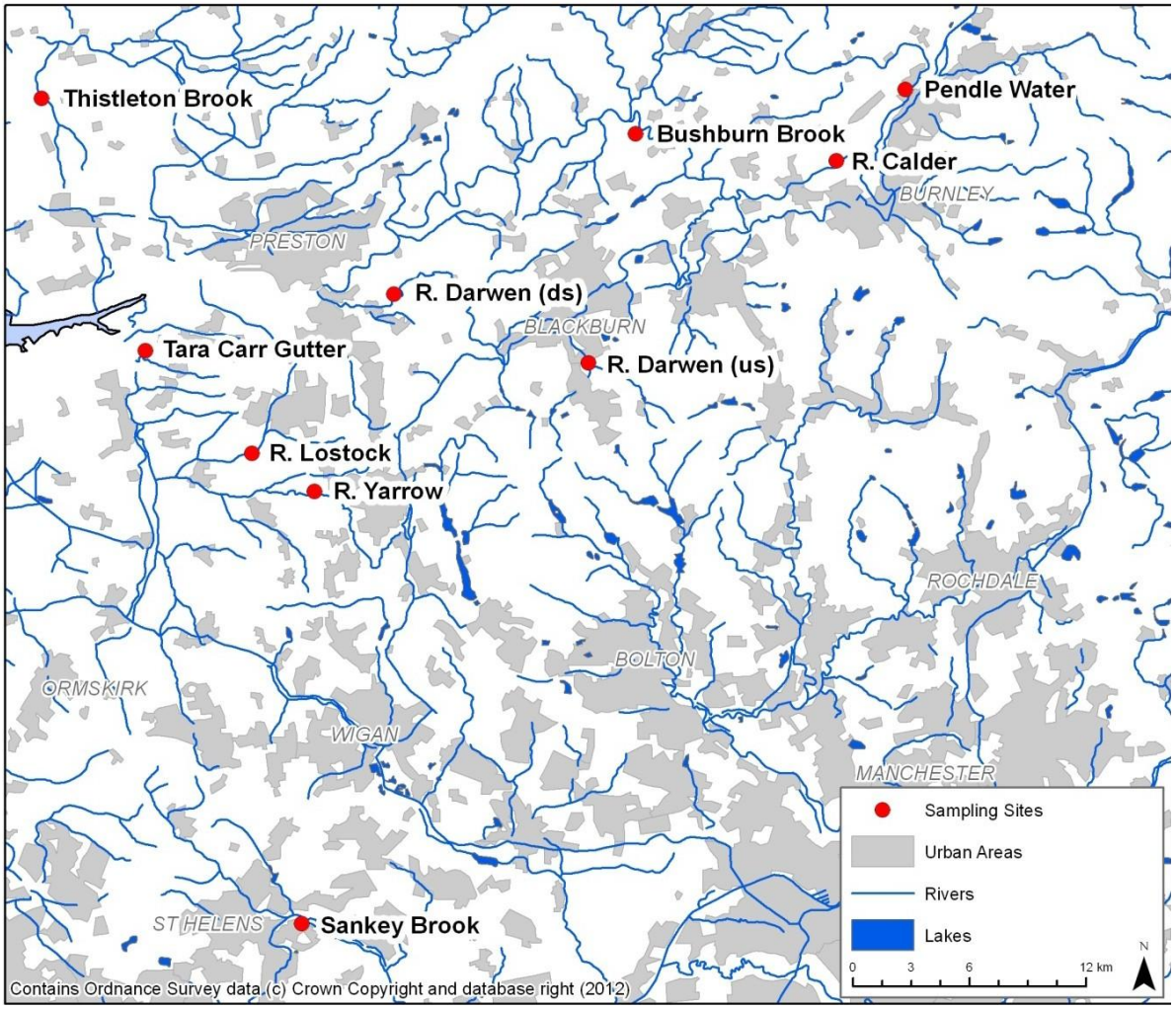

Thistleton Brook
Pendle Water
Bushburn Brook
R. Calder
BURNLEY
PRESTON
R. Darwen (ds)
BLACKBURN
Tara Carr Gutter
R. Darwen (us)
R. Lostock
R. Yarrow
ROCHDALE
BOLTON
ORMSKIRK
WIGAN
MANCHESTER
ST HELENS
Sankey Brook
Sampling Sites
Urban Areas
Rivers
Lakes
0 3 6 12 km
N
Contains Ordnance Survey data.(c) Crown Copyright and database right (2012)

Figure 2.

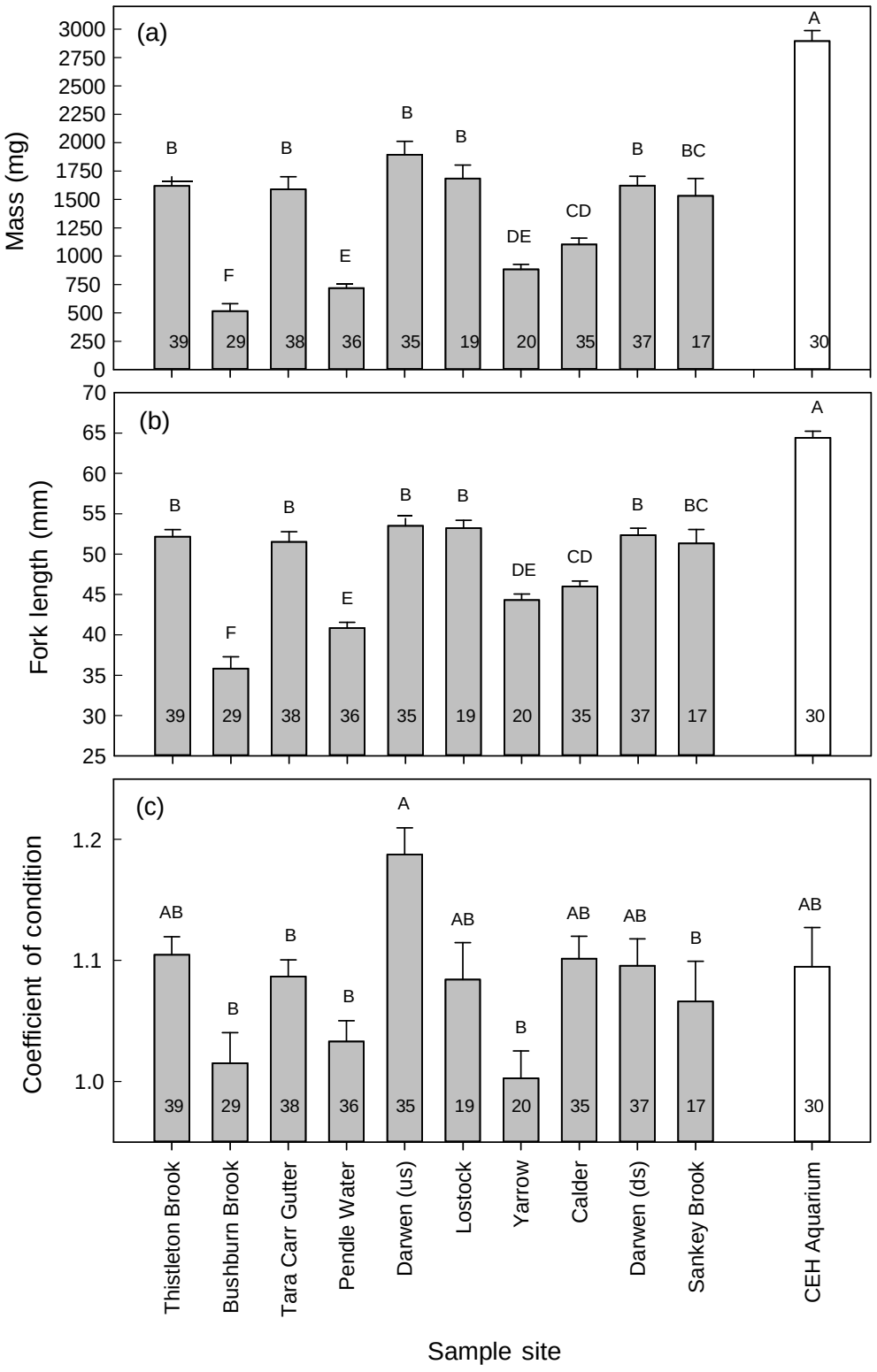

(a)
Mass (mg)
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
B
F
B
E
B
B
DE
CD
B
BC
A
39
29
38
36
35
19
20
35
37
17
30
(b)
Fork length (mm)
70
65
60
55
50
45
40
35
30
25
B
F
B
E
B
B
DE
CD
B
BC
A
39
29
38
36
35
19
20
35
37
17
30
(c)
Coefficient of condition
1.2
1.1
1.0
AB
B
B
B
A
AB
B
AB
AB
B
AB
39
29
38
36
35
19
20
35
37
17
30
Thistleton Brook
Bushburn Brook
Tara Carr Gutter
Pendle Water
Darwen (us)
Lostock
Yarrow
Calder
Darwen (ds)
Sankey Brook
CEH Aquarium
Sample site

Figure 3.

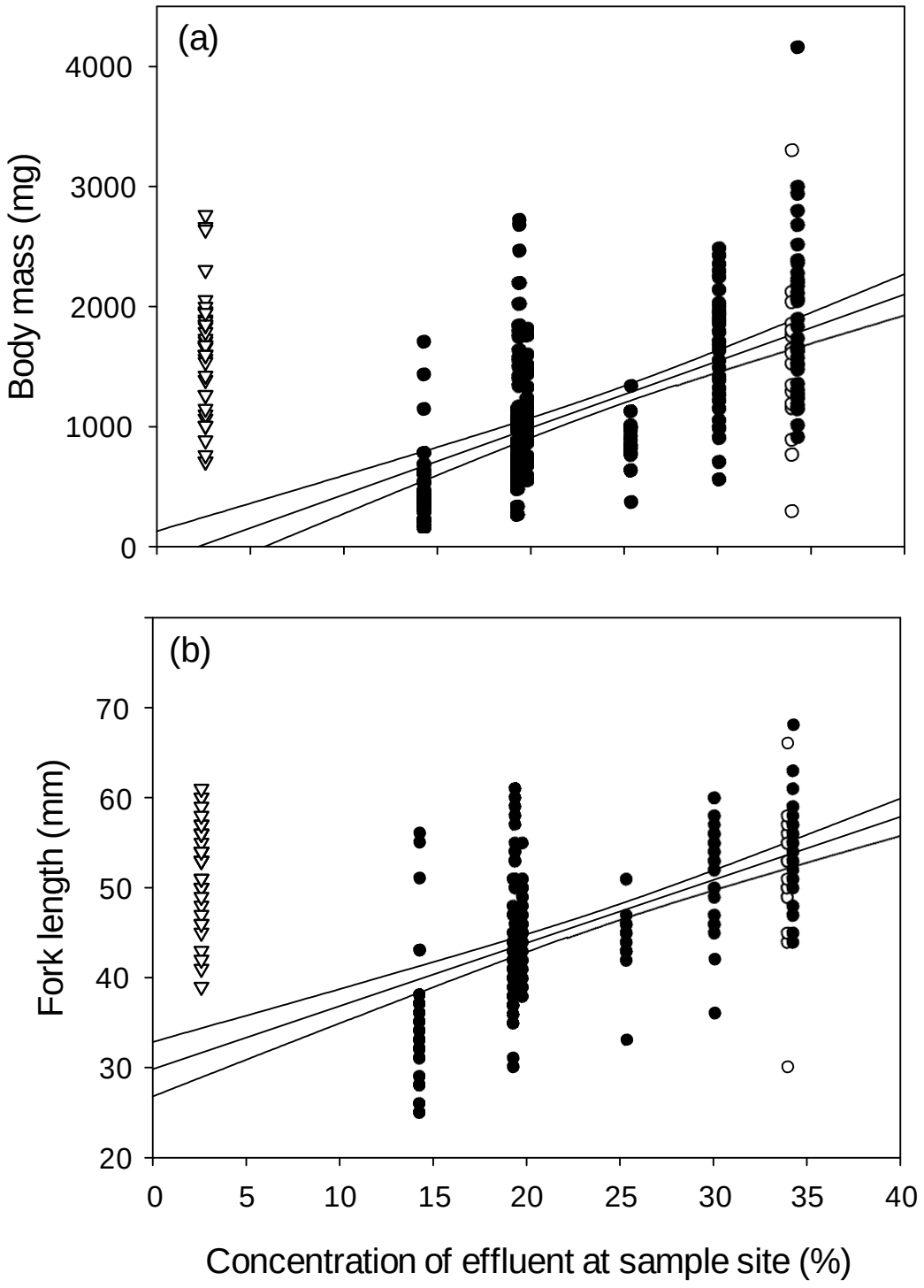

(a)
Body mass (mg)
0
1000
2000
3000
4000
(b)
Fork length (mm)
20
30
40
50
60
70
0
5
10
15
20
25
30
35
40
Concentration of effluent at sample site (%)

Figure 4.

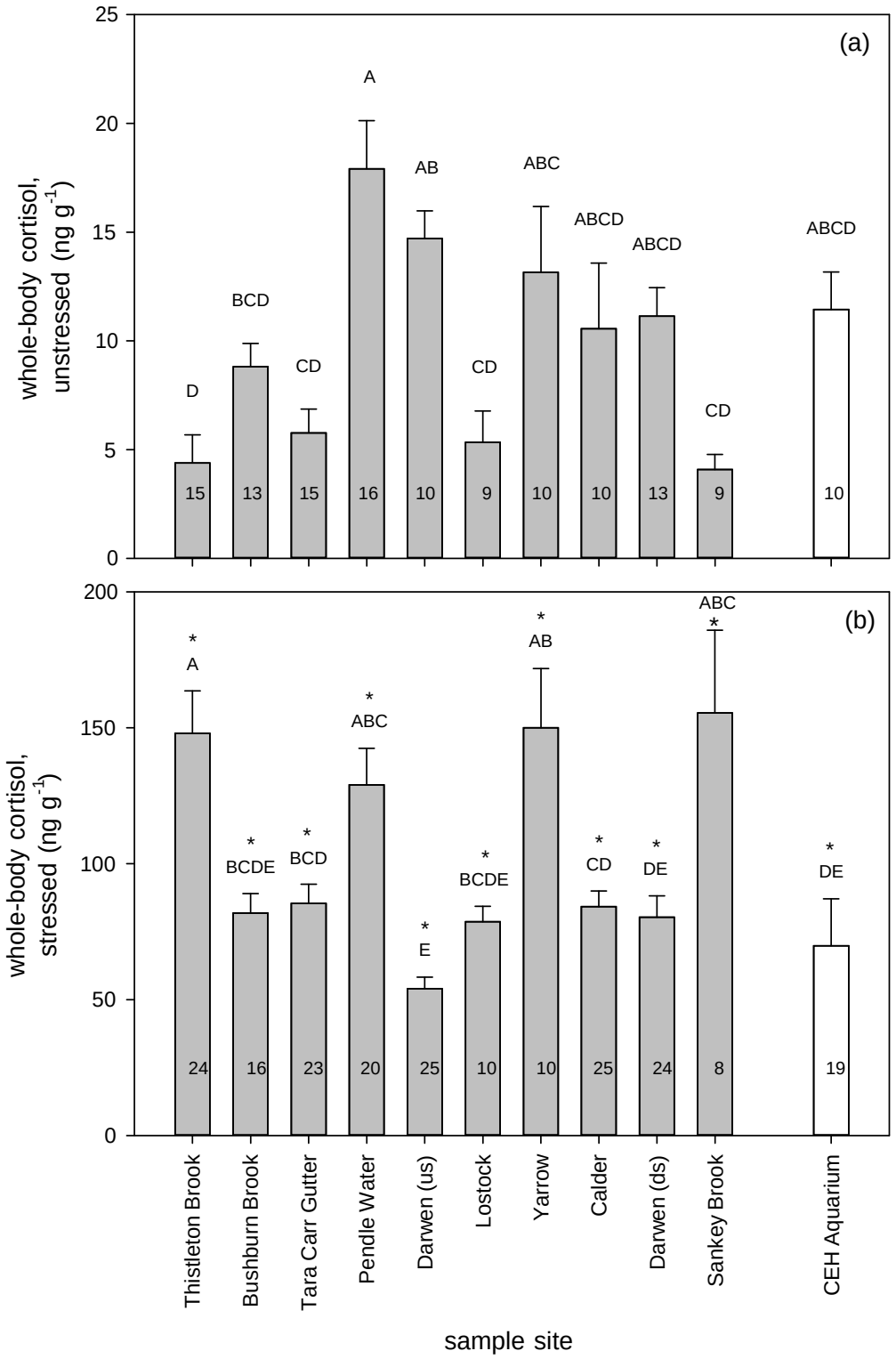

(a)
whole-body cortisol, unstressed (ng g-1)
25
20
15
10
5
0
D
BCD
CD
A
AB
CD
ABC
ABCD
ABCD
CD
ABCD
15
13
15
16
10
9
10
10
13
9
10
(b)
whole-body cortisol, stressed (ng g-1)
200
150
100
50
0
* A
* BCDE
* BCD
* ABC
* E
* BCDE
* AB
* CD
* DE
* ABC
* DE
24
16
23
20
25
10
10
25
24
8
19
Thistleton Brook
Bushburn Brook
Tara Carr Gutter
Pendle Water
Darwen (us)
Lostock
Yarrow
Calder
Darwen (ds)
Sankey Brook
CEH Aquarium
sample site

Figure 5.

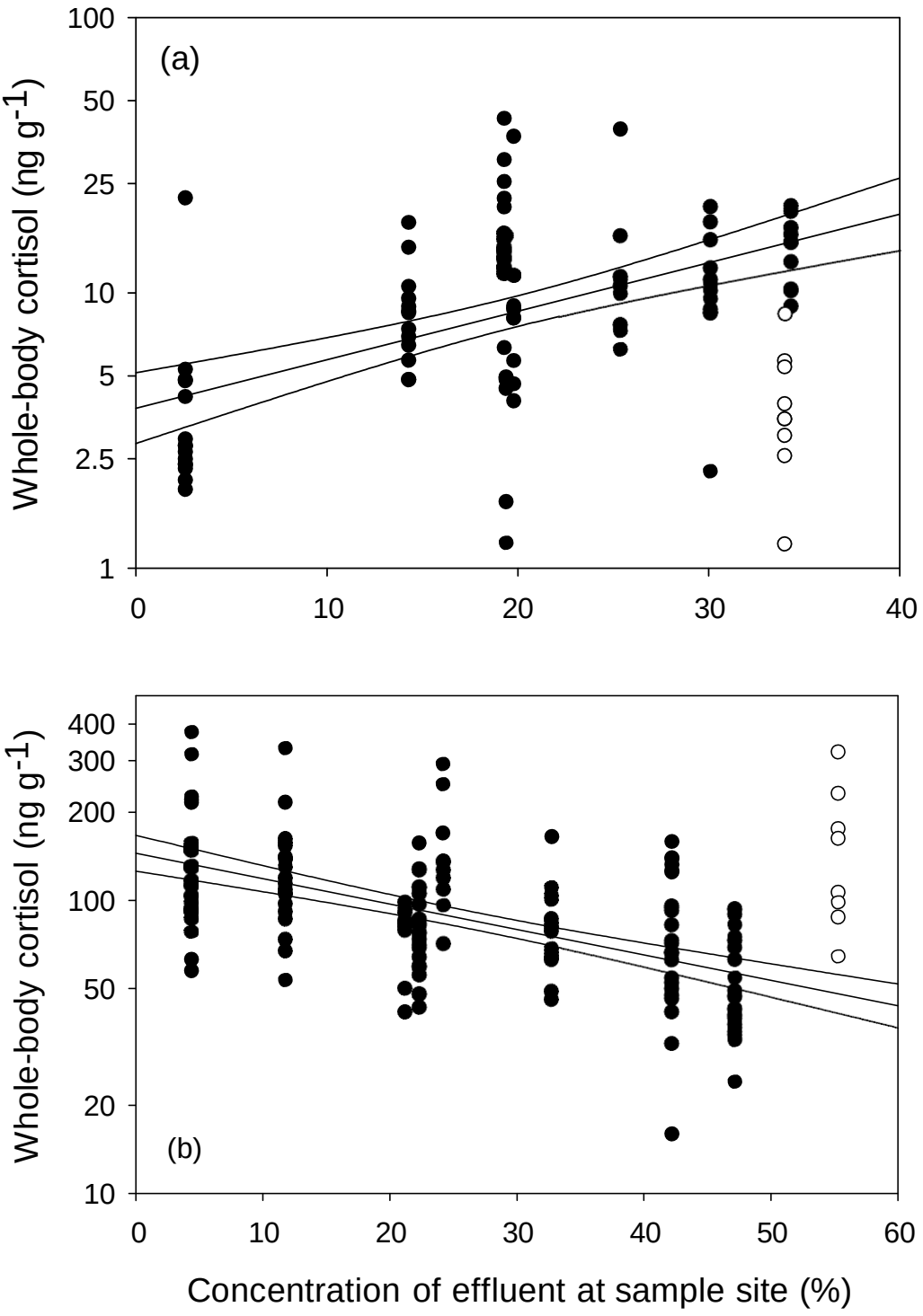

(a)
Whole-body cortisol (ng g-1)
100
50
25
10
5
2.5
1
0
10
20
30
40
(b)
Whole-body cortisol (ng g-1)
400
300
200
100
50
20
10
0
10
20
30
40
50
60
Concentration of effluent at sample site (%)

Figure 6.

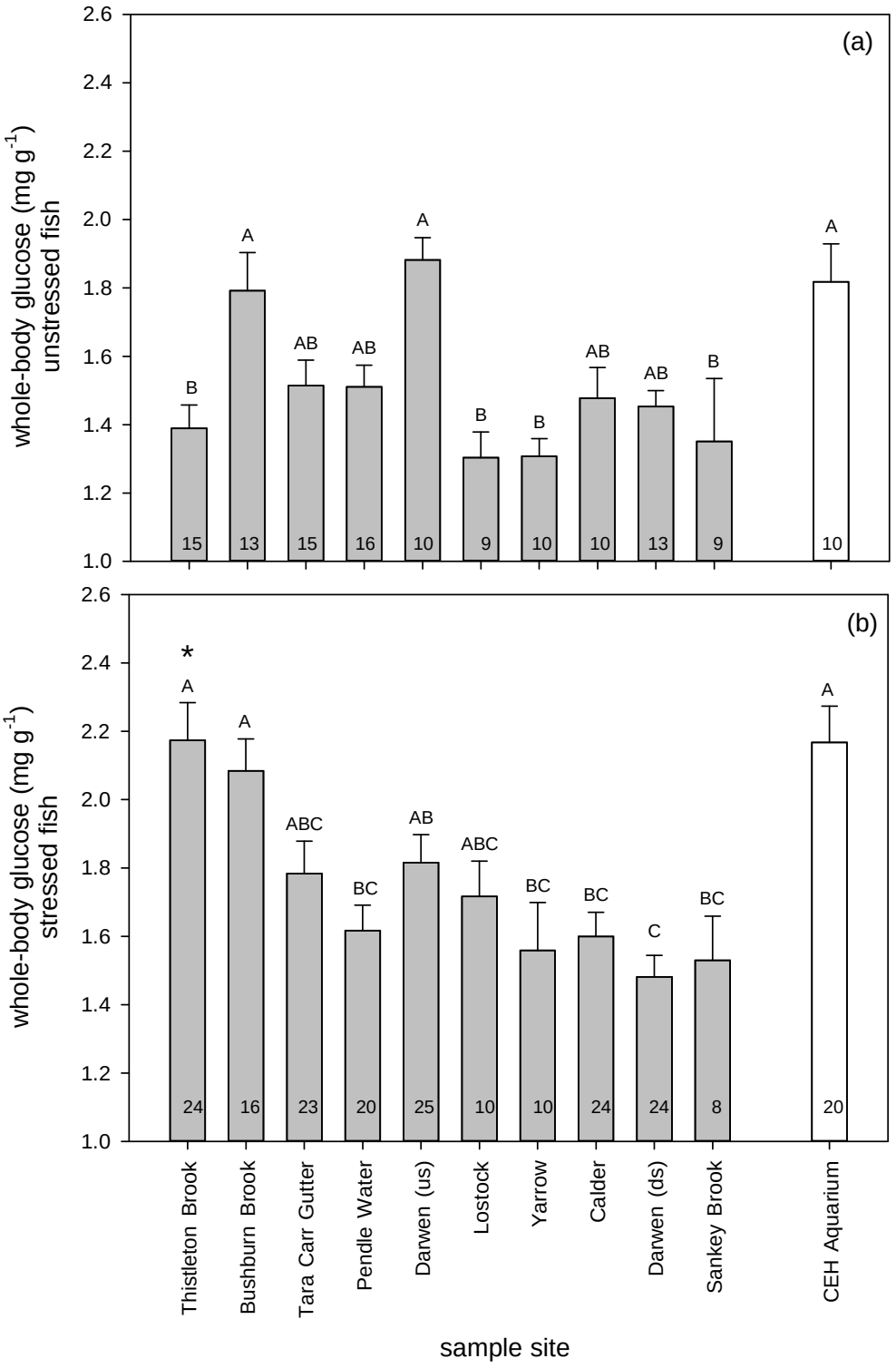
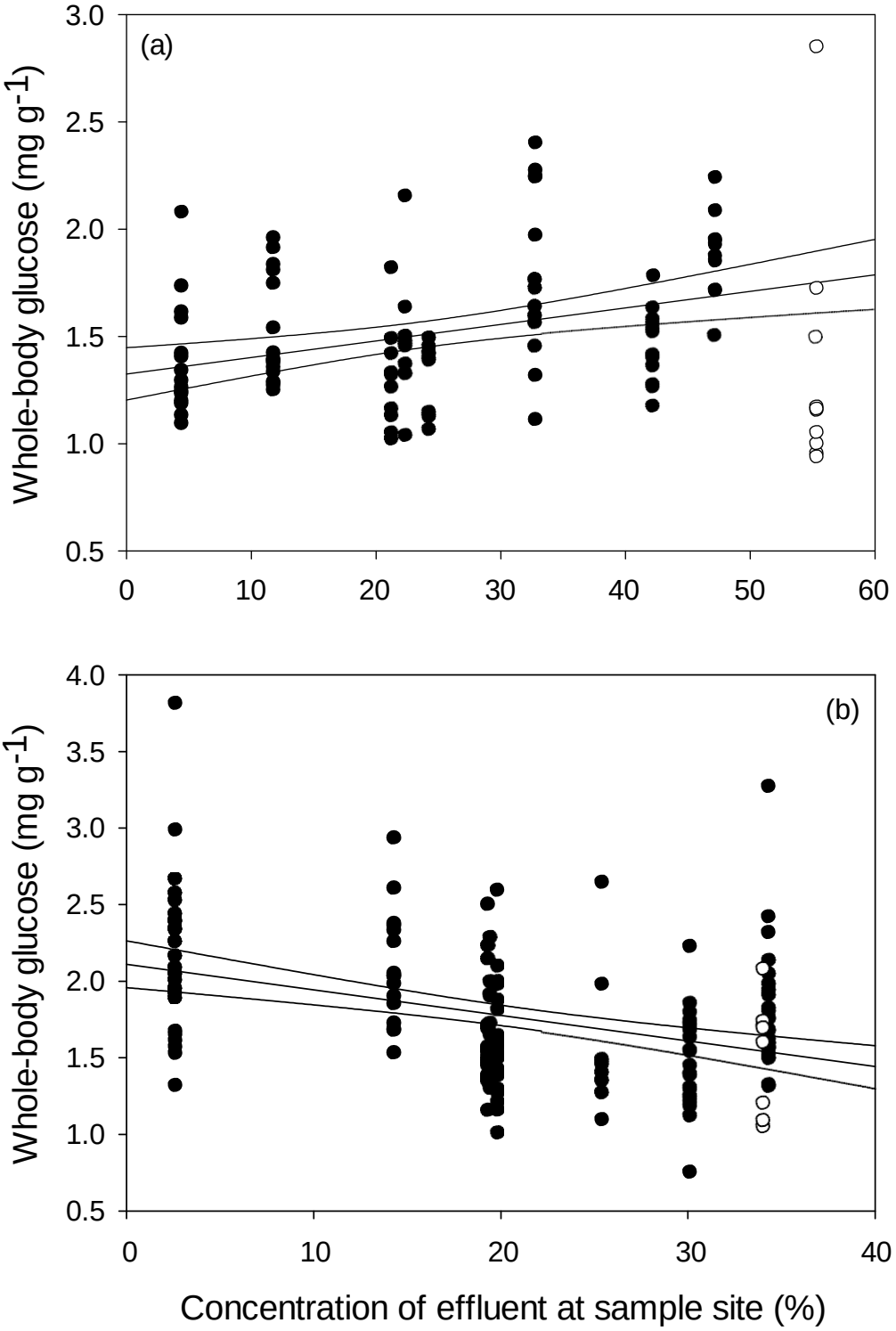

(a)
whole-body glucose (mg g-1)
unstressed fish
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
B
A
AB
AB
A
B
B
AB
AB
B
A
15
13
15
16
10
9
10
10
13
9
10
(b)
whole-body glucose (mg g-1)
stressed fish
*
A
A
ABC
BC
AB
ABC
BC
BC
C
BC
A
24
16
23
20
25
10
10
24
24
8
20
Thistleton Brook
Bushburn Brook
Tara Carr Gutter
Pendle Water
Darwen (us)
Lostock
Yarrow
Calder
Darwen (ds)
Sankey Brook
CEH Aquarium
sample site

Figure 7.


(a)
Whole-body glucose (mg g-1)
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
50
60
(b)
Whole-body glucose (mg g-1)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
Concentration of effluent at sample site (%)