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A comparison of two methods for the assessment of stress axis activity in wild fish in relation to wastewater effluent exposure

Tom G. Pottinger[a*], Richard J. Williams[b] and Peter Matthiessen[a,c]

[a] *Centre for Ecology & Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP, U.K.*

[b] *Centre for Ecology & Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, OX10 8BB, U.K.*

[c] *Present address: Dolfan Barn, Beulah, Llanwrtyd Wells, Powys LD5 4UE, U.K.*

*corresponding author at: Centre for Ecology & Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP, U.K.

E-mail address: tgp@ceh.ac.uk

ABSTRACT

Riverine fish are particularly vulnerable to chemical exposure - rivers receive chemicals of anthropogenic origin from a variety of sources, one of the most significant being the chemically complex effluents discharged by wastewater treatment works (WWTWs). The extent to which non-reproductive components of the endocrine system in fish may be vulnerable to interference by contaminants associated with WWTW effluent is not well understood, but a significant body of evidence does suggest that contaminants present in the aquatic environment may interfere with the normal function of the neuroendocrine stress axis in fish. Field investigations of stress axis function in free-living populations of fish by measurement of hormone concentrations in blood can be confounded by the remoteness of sampling locations and the size of target species. Two methods for assessing stress axis reactivity in situations where blood samples are unavailable were compared in three-spined sticklebacks in relation to their exposure to WWTWs effluent. Sticklebacks were sampled in two successive years at fifteen sites in north-west England impacted by WWTW effluent and the response of each fish to the combined stressor of capture and a brief period of confinement was evaluated using both whole-body immunoreactive cortisol concentrations (WBIC) and the rate of release of cortisol to water (CRTW). A positive relationship between the magnitude of stress-induced CRTW in sticklebacks of both sexes and WWTW effluent concentration at site of capture was observed in both years. However, the relationship between stress-induced WBIC and WWTW effluent concentration was not consistent. These results suggest that components of WWTW effluent can modulate the magnitude of the neuroendocrine stress response in sticklebacks, and by inference in other fish species, but they raise questions about the measurement and interpretation of stress

axis responses in fish via endpoints other than blood hormone concentrations. Possible factors underlying the disparity between the CRTW and WBIC results are discussed.

Key words: cortisol, stress, pollution, wastewater, three-spined stickleback.

1. Introduction

As comparative endocrinology converges with ecology and conservation the measurement of endocrine endpoints in wildlife is becoming increasingly common (Cockrem, 2005; Kersey and Dehnhard, 2014) and in this context it is important to understand the factors responsible for inter-population variation in endocrine function (Adams et al., 2013; Bourgeon et al., 2014). The possible role of environmental contaminants in modulating or disrupting endocrine systems in wildlife is of increasing concern in this regard but a comprehensive understanding of the threat is lacking, notably with respect to effects of contaminants on non-reproductive endocrine systems (Bergman et al, 2013). Riverine fish are particularly vulnerable to chemical exposure - rivers receive chemicals of anthropogenic origin from a variety of sources, one of the most significant being effluent discharged by wastewater treatment works (WWTWs). Effluent from WWTWs can comprise a substantial proportion of the total flow volume of rivers (Brooks et al., 2006): in 25% of rivers in the United Kingdom the annual median dilution factor of wastewater (total catchment river flow/total domestic wastewater effluent generated) is below 6.3 (Keller et al., 2014). The effluent discharged by WWTWs contains a complex mixture of organic and inorganic chemicals derived from domestic and industrial sources (Henze and Comeau, 2008;

Marcogliese et al., 2015) and it is well-established that some of these chemicals may modulate or interfere with the reproductive endocrine system of fish (Mills and Chichester, 2005). However, the extent to which non-reproductive components of the endocrine system in fish may also be vulnerable to interference by contaminants associated with WWTW effluent is not known.

The hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis is part of a suite of adaptive responses whose functional integrity is of great importance to the individual (Wingfield, 2013). In a previous study of stress axis function in three-spined sticklebacks (*Gasterosteus aculeatus*) inhabiting sites affected by upstream wastewater treatment works (WWTWs) an inverse relationship between the stress-induced increase in whole-body corticosteroid concentration, and the WWTW effluent concentration in the river was reported (Pottinger et al., 2013). These observations suggested that anthropogenic factors, associated with WWTW discharges, might be a driver for between-population variation in stress axis function.

Modulation of HPI axis reactivity by external factors may indicate interference with key regulatory mechanisms and it is therefore important to establish whether there are functional implications for the affected animal. In the case of the HPI axis this requires in the first instance an understanding of how target-tissue exposure to corticosteroids, key mediators of the stress response, is affected. Normally, the analysis of blood samples from appropriately selected individuals would provide the required information. However, if the targeted fish are too small to obtain a blood sample of sufficient volume, or the conditions under which the fish are collected preclude the rapid removal and processing of blood, then

whole-body immunoreactive corticosteroid concentrations (WBIC) are a viable alternative. WBIC has been widely used for assessing cortisol levels during stress, in a variety of fish species (Fuzzen et al., 2010; Pottinger and Calder, 1995; Pottinger et al., 2002). However, although measurement of WBIC is very effective at discriminating between unstressed and stressed individuals (e.g. Pavlidis et al., 2015) the precise parity between cortisol levels in the blood and WBIC may not be consistent across time (Pottinger and Mosuwe, 1994). This mismatch arises in part because measurement of WBIC by radioimmunoassay quantifies the total extractable immunoreactive steroid present in all body compartments, not only blood, and may include cortisol that is no longer in circulation and metabolites of cortisol which can cross-react with a cortisol-specific antibody (Pottinger et al., 1992; Scott et al., 2014). Therefore, although WBIC concentrations are a very effective means of distinguishing between unstressed and stressed individuals, WBIC may not be a precise surrogate for the blood-borne concentrations of cortisol to which target tissues are actually exposed. This complicates the assessment of the functional consequences of effluent-associated changes in stress axis reactivity for the individual fish, an issue that is critical in determining whether effluent-induced alterations in HPI reactivity are harmful.

An alternative to WBIC as an index of HPI axis activity is provided by the measurement of cortisol released to water (CRTW), primarily across the gills, during stress. In fish, circulating steroids, including cortisol, are transferred from the bloodstream to the surrounding water across the gill epithelium (Ellis et al., 2005) and can be quantified after extraction and concentration of water samples from holding tanks or collection vessels. Steroids retained in water samples after transfer across the gill may be a more accurate surrogate of blood steroid levels than the multi-compartment total provided by WBIC: the rate of release of

cortisol to water has been shown to be proportional to the concentration of cortisol in the blood in a number of species (Félix et al., 2013; Gabor and Contreras, 2012; Scott and Ellis, 2007; Wong et al., 2008) including three-spined sticklebacks (Sebire et al., 2007). The release of cortisol to water is an endpoint that is ideally suited to the assessment of stress responsiveness in small fish captured from field environments, in circumstances where blood sampling is not possible. As far as we are aware use of this technique has to date been restricted to controlled laboratory environments.

The present study was conducted (i) to verify earlier observations on the relationship between inter-population variation in stress axis function in sticklebacks and WWTW effluents, across a broader range of sites, and (ii) to evaluate the practicality of utilising the rate of cortisol release across the gills during a post-capture period of confinement as an endpoint for measuring stress axis reactivity in small fish in the field.

2. Methods

2.1 Experimental strategy

The three-spined stickleback is ubiquitous in U.K. lowland rivers and provides an ideal model species for comparisons between and within river systems (Pottinger et al., 2002). At each selected site sticklebacks were captured and a sub-sample of these fish used to provide an assessment of the whole-body concentration of cortisol in unstressed individuals. The remaining fish were used to determine the magnitude of the stress response to a mild stressor by (i) measuring the cortisol released to water and (ii) measuring the total cortisol accumulating in the body during a period of time following capture. Inter-population variation in baseline and stress-induced cortisol levels was then compared with the

between-site variation in WWTW effluent concentration. A simplified overview of the sampling and analytical protocols is provided in Fig. 1.

2.2 Sampling procedure

Sampling locations on rivers downstream of the effluent discharge points of fourteen wastewater treatment works (population range 22000 - 120000; Table 1; Fig. 2) in Lancashire, Cheshire, Merseyside and Greater Manchester (north-west England) were identified. Three-spined sticklebacks (*Gasterosteus aculeatus*) were sampled during April – May 2013 and March 2014. During 2013 the sampling was delayed because of exceptionally cold weather in February and March. Fish were captured using a metal-framed 45 cm D-profile hand net and were usually found in areas protected from the main flow, within emergent or overhanging vegetation adjacent to the bankside, or under floating accumulations of debris. At each site the first ten fish captured were killed immediately by immersion in sedative (2-phenoxyethanol, 1:1000) and then placed in individual labelled 12 ml capped polypropylene test tubes which were transferred to a dry shipper (Taylor-Wharton CX 100) containing liquid nitrogen. A second batch of ten fish was retained in a bucket containing 2.5 l river water for a period of approximately 30-45 minutes before being transferred to individual capped Nalgene tubs (n = 10; 150 ml, 6.5 cm diameter) containing 100 ml artificial freshwater (deionised water containing 0.33 g/l aquarium grade sea salt; Klüttgen et al., 1994) for a further 30 minutes in order to collect cortisol released across the gills during the period of confinement. Artificial freshwater, rather than the river water at each site, was used for the collection of released cortisol to minimise the inclusion of suspended solids likely to interfere with the subsequent extraction procedure, and to allow the collecting vessels to be prepared in advance. Within 30 minutes of first exposure to an

ongoing stressor levels of both plasma cortisol and whole-body cortisol in stressed three-spined sticklebacks reach a stable plateau which is sustained for at least an additional 30 – 60 minutes (Pottinger et al., 2002; T. G. Pottinger, unpublished data). After the confinement period each fish was killed by immersion in sedative as above and transferred to individual labelled 12 ml capped polypropylene test tubes which were placed in a larger capacity dry shipper (Taylor-Wharton CX500). The initial sample of unstressed fish was also transferred to this shipper for transport back to CEH Lancaster where the samples were stored at -70^{o}c prior to processing. The collection vessels containing water samples were held on ice in coolboxes until return to CEH Lancaster where they were transferred to a freezer (-20^{o}C) for storage. The confinement stress procedure was approved by the Lancaster University Animal Welfare and Ethical Review Body and was conducted under Home Office licence.

2.3 Additional stickleback samples

In April-May 2013 and March 2014 sticklebacks were sampled from a number of sites (Table S1) with no known upstream WWTW discharges in order to investigate the relationship between body mass and stress-induced cortisol release to water. The fish were treated as described in 2.2 and both WBIC and CRTW data were collected.

2.4 Fish dissection and tissue preparation

In the laboratory, each fish was weighed to the nearest mg, total length was recorded to the nearest mm and the sex of each fish was determined, after making a ventral incision, by macroscopic examination of the gonads. Partially thawed fish samples were minced on a glass petri dish using a single-edged razor blade, transferred to a 20 ml glass scintillation vial containing buffer (4:1 volume:weight; Tris-HCl, pH 8.0, 0.1 M NaCl, 0.01 M EDTA.2H_2O,

0.05% NaN_3), and homogenised (Ultra-Turrax TP 18/10; 7.5 mm dispersing element). A 250 µl aliquot of the homogenate was added to 1.0 ml of ethyl acetate in a 1.5 ml capped centrifuge tube, vortex-mixed and spun down. A 50 µl aliquot of this supernatant was taken through to the cortisol radioimmunoassay (RIA).

2.5 Extraction of water samples

Water samples within which single sticklebacks had been held post-capture for a period of 30 mins were thawed at room temperature. Each sample was pumped (Watson Marlow 202S multi-channel peristaltic pump, 10-20 ml/min, 12 active channels, 2.79 mm i.d. silicone tubing) through an inline 0.45 µm pre-filter (Pall Gellman Acrocap, Pall Life Sciences) and a Sep-Pak C18 cartridge (Waters Ltd). Sep-Pak cartridges were cleaned and conditioned by flushing with 5 ml of ethyl acetate, followed by 5 ml methanol, followed by 5 ml deionised water in a vacuum manifold. The cartridges were not allowed to dry out between conditioning and receiving the water sample. One blank (100 ml artificial freshwater only) and one recovery standard (100 ml artificial freshwater containing a 100 µl aliquot of a solution of cortisol in ethanol, 5 ng/ml) were included with each batch of ten water samples (100 ml). No interference was detected in any of the samples and recovery of added cortisol was consistently >85%. After extraction, cortisol was immediately eluted from the Sep-Pak cartridge with 2.5 ml ethyl acetate in a vacuum manifold. The eluate was dried in a heating block under a stream of air at 40^{o}C and redissolved in 350 µl ethyl acetate. A 150 µl aliquot of the reconstituted extract was taken for assay.

2.6 Cortisol radioimmunoassay

A previously validated method was employed (Pottinger and Carrick, 2001) using IgG-F-2 rabbit anti-cortisol (IgG Corp; Nashville, TN, USA) and tracer ([1,2,6,7]^{3}H-cortisol, 2.59 TBq/mmol; Perkin-Elmer, U.K.) which was added in a 25 µl aliquot of buffer at the same time as the antibody was dispensed.

2.7 Estimation of effluent concentration at WWTW-impacted sites

The percentage of WWTW-derived effluent at each sampling site was estimated using the Low Flows 2000 Water Quality eXtension model (LF2000-WQX model). The LF2000-WQX software combines hydrological models with water-quality models to make predictions on the concentration of a given chemical originating from a point source, such as WWTWs (Williams et al. 2009; Pottinger et al., 2013).

2.8 Statistical analysis

Differences in cortisol responses and in somatic data between years and between sexes were assessed on log-transformed data using one-way or two-way ANOVA with Tukey's pairwise multiple comparison test or three-way ANOVA and Holm-Sidak post-hoc tests (Sigmaplot 12; Systat Software, Inc.). The relationship between WWTW effluent concentration and the cortisol response to confinement (CRTW and WBIC) was investigated using linear regression (Sigmaplot and Minitab 16, Minitab Inc.). For female fish captured during March 2014 the total whole-body cortisol accumulated during the post-capture period was estimated by subtracting the mean whole-body cortisol concentration for all unstressed fish at each site, sampled immediately after capture, from the WBIC concentration of each individual stressed fish post-confinement, to provide a nominal

estimate of the amount of cortisol accumulated during 1h. The coefficient of condition (K, Fulton's condition index; Bolger and Connolly, 1989) was calculated as K = (100*weight)/(length^3).

3. Results

3.1 Somatic data

Comparisons between years and between sexes were conducted using only fish from sites that were sampled in both 2013 and 2014 (Altrincham, Denton, Hillhouse, Huyton and Woolton). Fish of both sexes captured in 2013 were larger and had higher coefficients of condition than fish captured from the same sites in 2014 (Table 2: mass: two-way ANOVA, $F(1,214) = 45.7$, $P < 0.001$; length: $F(1,214) = 28.5$, $P < 0.001$; condition: $F(1,214) = 67.1$, $P < 0.001$). In 2013 females were larger than males with higher coefficients of condition (mass: $P = 0.002$; length $P = 0.03$; condition: $P < 0.001$; Tukey's test). These significant between-sex differences were not evident in 2014 (mass: $P = 0.5$; length: $P = 0.7$; condition: $P = 0.13$; Tukey's Test). The sex ratio in both years (F:M; 2013 = 1.16; 2014 = 1.19) did not differ significantly from unity (Chi square test).

3.2 Accumulation of whole-body cortisol (WBIC) during stress

For fish at the five sites sampled in both 2013 and 2014 post-capture confinement significantly elevated WBIC (ng/g) concentrations by five-fold or more (three-way ANOVA, $F(1,207) = 394$, $P < 0.001$; Table 2). Overall, WBIC concentrations were higher in females than in males (ANOVA, $F(1,207) = 4.0$, $P < 0.05$; Table 2) and were more than two-fold higher in both sexes in 2014 than 2013 (ANOVA, $F(1,207) = 107$, $P < 0.001$; Table 2). Using the

complete data set (all 15 sites sampled) there were significant between-site differences in stress-induced WBIC in both years and for both sexes (Fig. 3: ANOVA, $P < 0.05$ - $P < 0.001$). In 2013, variation in stress-induced WBIC was inversely related to variation in WWTW effluent concentration at each site from which sticklebacks were sampled for both females (Fig. 3a: $r^2 = 0.37$, $P < 0.001$, n = 70) and males (Fig 3c: omitting outlying values for Woolton: $r^2 = 0.14$, $P < 0.001$, n = 45; including outlying values for Woolton: $r^2 = 0.09$, $P = 0.044$, n = 47). However, this negative trend between effluent concentration and WBIC was absent among the fish sampled in 2014 and instead a positive relationship between WBIC and effluent concentration was apparent for females, albeit with a very low coefficient of determination (Fig. 3b: $r^2 = 0.07$ $P < 0.001$, n = 64), and no relationship between WBIC and effluent concentration was present in males (Fig 3d: $r^2 = 0.05$, $P = 0.1$, n = 55). For unstressed fish in both years, there was no significant relationship between effluent concentration and WBIC ($r^2 < 0.03$, $P > 0.3$, n = 33-59) with the exception of males captured in 2014 for which a positive trend was detected ($r^2 = 0.16$, $P < 0.01$, n = 48, data not shown).

3.3 Cortisol release to water (CRTW) during stress

For fish captured at the five sites sampled in both 2013 and 2014 the mean rate of stress-induced cortisol release to water by both sexes was more than two-fold higher in 2014 than during 2013 (ANOVA $F(1,96) = 43.9$, $P < 0.001$; Table 2). Overall, there was a significant effect of sex on CRTW (ANOVA $F(1,96) = 29.8$, $P < 0.001$; Table 2) but this difference was resolved only in 2013, where mean CRTW in females was three-fold that of males, ($P = 0.07$; Table 2).

Across all the sites receiving WWTW effluent (n = 15, Table 1), there was a more than three-

fold variation in mean CRTW during post-capture confinement (Fig. 4) and significant between-site variation in CRTW was detected in both years and in both sexes (ANOVA, $P < 0.05$ - $P < 0.001$). Cortisol release rate to water during stress was proportional to the concentration of WWTW effluent at each sample site (Fig. 4) and linear regressions of CRTW against the estimated concentration of effluent were significant in all cases (females 2013: $r^2 = 0.15$; $P = 0.008$, n = 46; females 2014: $r^2 = 0.14$; $P = 0.002$, n = 64; males 2013: $r^2 = 0.27$, $P = 0.004$, n = 30; males 2014: $r^2 = 0.44$, $P < 0.001$, n = 55).

3.4 The relationship between whole-body accumulation of cortisol and cortisol release to water during stress

At an individual level, CRTW was not proportional to WBIC in fish of either sex captured at all sites in 2013 (female $r^2 = 0.0$, $P = 1.0$, n = 46; male $r^2 = 0.04$, $P = 0.3$, n = 28) but was directly proportional to WBIC (ng/g) in fish captured at all sites in 2014 (female $r^2 = 0.30$, $P < 0.001$, n = 64; male $r^2 = 0.10$, $P = 0.04$, n = 55). For female fish captured during 2014 the mean WBIC in unstressed fish at each site was subtracted from the post-confinement WBIC concentration for each fish at the same site to provide an approximation of the total corticosteroid synthesised during the post-capture period (mean = 98.7 ± 4.2 ng/g, n = 64). For the same fish during the same period the estimated total release of cortisol to water per fish (1640 ± 189 pg/g, n = 64) was approximately 1.6% of the estimated total WBIC synthesised and accumulated during this period.

3.5 The relationship between effluent concentration, fish size and normalised cortisol response to stress.

Across all the sites sampled, the body mass of sticklebacks tended to vary inversely with

effluent concentration at the site of capture (Fig. S1) in both 2013 (stressed and unstressed fish, linear regression: female r^2 = 0.04, P = 0.06, n = 103; male r^2 = 0.07, P = 0.01, n = 89) and in 2014 (female r^2 = 0.27, P < 0.01, n = 123; male r^2 = 0.33, P < 0.001, n = 103). Length of the fish was similarly related to effluent concentration although as with mass the relationship was less robust in 2013 (females r^2 = 0.01, P = 0.25; males r^2 = 0.05, P = 0.04) than in 2014 (females r^2 = 0.41, P < 0.001; males r^2 = 0.41, P < 0.001). No significant relationship between WBIC and fish mass was apparent for stressed or unstressed fish of either sex or in either year (r^2 = 0.0; P > 0.05, n = 42 - 70) at WWTW-impacted sites with the exception of a highly diffuse inverse trend between WBIC and mass for stressed males in 2014 (r^2 = 0.07, P = 0.05, n = 55). However, a negative relationship was observed between body mass and CRTW for both males and females in both years at WWTW-impacted sites (Fig. S2). For fish captured at sites with no identifiable upstream WWTW discharge no statistically significant relationship was detected between mass and CRTW for female sticklebacks in both years or for males in 2013 (r^2 < 0.16; P > 0.05, n = 10 – 58; Fig. S3). However, a significant relationship between mass and CRTW was detected for males in 2014 (r^2 = 0.28, P < 0.001, n = 38).

4. Discussion

Significant inter-population variation in two markers of stress axis function was observed in free-living three-spined sticklebacks captured at sites downstream of WWTW discharges. The magnitude of the stress response to capture and confinement, when quantified as the rate of release of cortisol to water across the gills (CRTW), showed a consistent positive

relationship with the concentration of WWTW effluent at the sites from which the fish were captured. In both years during which sampling was conducted CRTW tended to be higher among fish from sites with the greatest estimated proportion of WWTW effluent, and there was a maximum six-fold difference in CRTW among fish from sites with the lowest and highest effluent concentration (approx. 20% - 95% as a proportion of total river flow). The association between the magnitude of the CRTW stress response and WWTW effluent concentration at sampling sites suggests strongly that variation in stress axis response among these populations of sticklebacks is related to the presence of anthropogenic chemical contaminants. However, the estimated effluent concentration accounted for between 14% and 44% of the variation in CRTW suggesting that (i) factors other than WWTW effluent also contribute significantly to the variation in stress axis responsiveness and/or (ii) the modelled effluent concentration is not a consistently precise estimate of the relative concentrations of the WWTW-derived factors that may affect HPI function across all sites (Johnson et al., 2008).

The second marker of stress axis function that was employed in this study, stress-induced increase in whole-body concentrations of cortisol (WBIC), has previously been shown to vary in relation to both natural (Pottinger et al., 2011) and anthropogenic (Pottinger et al., 2013) stressors. However, in the present study WBIC was less consistently related to effluent concentrations than expected. In 2013 the magnitude of the WBIC response to capture and confinement was inversely related to effluent concentration, a finding similar to earlier observations (Pottinger et al., 2013). This trend was not repeated in 2014 when instead a positive relationship between WBIC and effluent concentration was evident in females but not in males. This lack of consistent proportionality between the WBIC response and

effluent exposure suggests that earlier conclusions that exposure to WWTW effluent is associated with a proportional attenuation of the response of the stress axis to a stressor in sticklebacks (Pottinger et al., 2013) require reconsideration and further investigation.

If CRTW is a more accurate surrogate of plasma cortisol levels than WBIC, then these data suggest that the stress axis in fish exposed to higher concentrations of WWTW effluent over-responds to stressors relative to the response seen in fish exposed to lower effluent concentrations. The rate of release of steroids, from the bloodstream to the surrounding water, across the gill epithelium of fish has been shown to be proportional to the steroid concentration in the blood during the period of collection (Félix et al., 2013; Scott and Ellis, 2007; Sebire et al., 2007) and CRTW estimates may therefore provide a more functionally relevant assessment of HPI activity than WBIC. The cortisol release rates recorded in the present study (100 – 3500 pg/g/h) are within the range reported previously for this species (Fürtbauer et al., 2015~~a~~; Sebire et al., 2007). Release rates were higher in females than males which may have a similar basis to the androgen-dependent modulation of the HPI axis observed in salmonids (Pottinger et al., 1996; Pottinger and Carrick, 2000). In the present study measurements of CRTW were restricted to stressed fish, because of the unavoidable disturbance associated with capture, handling and water-borne cortisol collection (Aubin-Horth et al., 2012; Wong et al., 2008).

Stress-induced increases in both WBIC and CRTW reflect the activation of the HPI axis and both WBIC and CRTW increase proportionally following stress (e.g. Boulton et al., 2015; Fischer et al., 2014). However, the different trends in CRTW response (consistently positive) and WBIC response (variable) in relation to WWTW effluent concentration suggest that the

two measures of stress are not strictly co-dependent or interchangeable. This view is reinforced by the absence of a significant relationship between WBIC and CRTW in sticklebacks of both sexes captured in 2013 although significant regressions between WBIC and CRTW were obtained in 2014 and in a previous study (Pottinger et al., 2011). In addition, there was a considerable disparity between the amount of cortisol estimated to have accumulated as WBIC during capture and confinement, and the amounts of cortisol released to water during the same period. For female fish captured during 2014 at sites downstream of WWTWs, the total cortisol released to water during the period (approx. 1h) between capture and sacrifice was 1.6% of the estimated total WBIC synthesised and accumulated during this period. Whole-body steroid measurements lack tissue-specificity, and there is a likelihood of detecting biologically inactive cortisol no longer in circulation together with metabolites of cortisol that are not recognised by receptors and do not play a role in the stress response. At the same time, it may be erroneous to assume that the quantity of steroid lost across the gills should be directly proportional to that accumulating in other tissues – diffusional losses across the gills are presumably a function of blood cortisol concentration, and will not necessarily reflect rates of steroid synthesis and metabolism occurring elsewhere in the fish which will, however, affect whole-body concentrations. Few data are available to help interpret these observations since different stress axis endpoints are rarely measured simultaneously in the same individual but a lack of consistent proportionality between WBIC and CRTW in the same individuals has been reported for guppies (*Poecilia reticulata*) held under laboratory conditions (Fischer et al., 2014) and differences in the relationship between plasma cortisol and CRTW has been reported between the sexes for zebrafish (*Danio rerio*; Félix et al., 2013) and in the daffodil cichlid (*Neolamprologus pulcher*; Ligocki et al., 2015). It may also be pertinent that in

zebrafish gills cortisol is converted via cortisone to 20β-hydroxycortisone (Tokarz et al., 2012) and in stressed zebrafish the concentrations of sulphated and glucuronidated 20β - hydroxycortisone release across the gills were found to be an order of magnitude greater than the corresponding conjugates of cortisol (Tokarz et al., 2013). Similar metabolism and release patterns might reasonably be suggested to occur in sticklebacks in which case cortisol itself constitutes only a proportion of the total steroid being transferred from the gills into surrounding water.

The lack of agreement between WBIC and CRTW responses observed in WWTW-exposed sticklebacks may have a mechanistic basis if a complex mixture of contaminants, such as that comprising WWTW effluent, contained chemicals that independently affect the stress axis at different loci. Effects on a range of signalling pathways have been reported in fathead minnows (*Pimephales promelas*) exposed to municipal wastewater (Arstikaitis et al., 2014) and in rainbow trout (*Oncorhynchus mykiss*) several stress-related pathways were affected by exposure to wastewater effluent (Ings et al., 2011a). Most reports of contaminant-induced effects on HPI axis function in fish report a suppressive effect on stress-induced blood cortisol concentrations by organic chemicals (e.g. Aluru and Vijayan, 2006), pharmaceuticals (e.g. de Abreau et al., 2014), metals (e.g. Gagnon et al., 2006) and whole effluents (Ings et al., 2011b) but some do report amplification of the cortisol response to stressors in fish (e.g. Gesto et al., 2008; Lerner et al., 2007; Melnyk-Lamont et al., 2014) and in other taxa (Franceschini et al., 2008; Tartu et al., 2014). Investigation of the activity of key genes responsible for coordinating HPI axis activity and for metabolising cortisol will be informative in this context.

Alternative explanations for the apparent relationship between effluent concentration and stress axis function can be considered. For example, the between-population differences in stress-induced CRTW might be a consequence of pollutant-induced alterations in gill structure and/or permeability, or of differences in respiratory/ventilatory function. Some organic and inorganic pollutants are known to cause damage to the gill epithelium resulting in changes in ion transfer processes (Evans, 1987) and alterations in these factors might affect the readiness with which steroids cross the respiratory epithelium from blood to environment. However, it seems unlikely that sticklebacks could sustain prolonged gill dysfunction without materially compromising overall fitness and gill damage has not routinely been reported as a feature of fish populations affected by WWTW discharges. Similarly, a polluted environment can increase metabolic demands in fish (Beyers et al., 1999; Peles et al., 2012) which can result in higher ventilation rates (Millidine et al., 2008) which in turn might facilitate the transfer of steroids across the gill epithelium. Given that the fish were deliberately exposed to a confinement stressor during the steroid collection procedure, and that stress is associated with an increase in ventilation rate in the stickleback (Bell et al., 2010) and other species of fish (Sanches et al., 2015), it is likely that any underlying between-population differences in resting ventilation rate were obscured. Finally, the rate of release of cortisol to water (as pg/g/h) was found to vary with body mass among sticklebacks captured at WWTW-impacted sites. However, no consistent relationship between fish size and CRTW release rates was apparent in sticklebacks sampled from populations at unimpacted sites (this study) or in aquarium-reared sticklebacks (Pottinger and Matthiessen, 2016) strongly suggesting that variation in both fish size and CRTW release are independently associated with exposure to WWTW effluent and that the relationship between fish size and CRTW is coincidental.

5. Conclusions

In three-spined sticklebacks inhabiting sites affected by upstream WWTW effluent discharges the response of the HPI axis to a standardised stressor, quantified as the rate of release of cortisol to water, was modulated in proportion to the concentration of effluent to which the fish were exposed. These results suggest that the stickleback stress response is affected by factors within, or associated with, WWTW effluent and, given that the stress axis is a key adaptive mechanism, there are likely to be functional consequences for affected fish. Making the assumption that the rate of release of cortisol to water is an accurate surrogate for circulating blood cortisol levels, these data suggest that fish exposed to WWTW effluent experience an augmented corticosteroid stress response. An over-responding stress axis is likely to be significantly detrimental to the fitness of the animal (Dickens and Romero, 2013). Recent studies on free-living birds suggest that individuals with a propensity to higher corticosteroid responses to a stressor may be disadvantaged across a range of fitness measures (Bókony et al., 2014; Bortolotti et al., 2009; Harms et al., 2015; Vitousek et al., 2014) indicating that the response of individuals to acute or transient stressors, rather than just chronic stressors, can be of significance in determining overall fitness. A number of possible mechanisms may account for these results and will require investigation before we fully understand the implications of these effects of WWTW effluent for the health and well-being of affected fish.

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Table 1. The location of sites from which sticklebacks were sampled during 2013 and 2014. The effluent concentration at each site was calculated as a percentage of the total flow using the LF2000-WQX model (Williams et al. 2009). The location of each site is show in Fig. 2.

Site no.	Upstream WWTW	Population served	Effluent concn. (%)	Receiving water	Sample location[1]	Sample date
1	Westhoughton	24162	14	Pennington Brook	SJ 656 991	2013
2	Leyland	41526	19	River Lostock	SD 517 200	2014
3	Royton	27590	25	River Irk	SD 885 062	2014
4	Tyldesley	21771	25	Moss Brook	SJ 689 985	2014
5	Westhoughton	24162	29	Westleigh Brook	SD 650 007	2014
6	Leigh	68773	30	Pennington Brook	SJ 675 979	2013
7	Glazebury	25462	30	Glaze Brook	SJ 701 922	2014
8	Blackburn	120562	30	River Darwen	SD 590 282	2014
9	Altrincham	35426	31	Sinderland Brook	SJ 738 905	2013 & 2014
10	Stretford	21904	32	River Mersey	SJ 778 934	2013
11	Darwen	30053	34	River Darwen	SD 690 246	2014
12	Huyton	63234	39	Netherley Brook	SJ 452 877	2013 & 2014
13	Denton	35050	54	River Tame	SJ 919 937	2013 & 2014
14	Woolton	46187	73	Netherley Brook	SJ 449 874	2013 & 2014
15	Hillhouse	59952	95	Maghull Hey Cop Drain	SD 353 047	2013 & 2014

[1]UK Ordnance Survey National Grid Reference.

Table 2. Summary of somatic data and cortisol data for the fish sampled downstream of the Altrincham, Denton, Hillhouse, Huyton and Woolton WWTWs in 2013 and 2014. Each value is the mean ± SEM. WBIC: whole-body immunoreactive cortisol concentration; CRTW: cortisol release to water.

	2013		**2014**	
	Female	**Male**	**Female**	**Male**
Mass (mg)	2604 ± 149	1820 ± 94	1552 ± 156	1258 ± 42
Length (mm)	57 ± 1	52 ± 1	49 ± 2	47 ± 1
Condition (K)	1.346 ± 0.03	1.192 ± 0.02	1.111 ± 0.02	1.061 ± 0.02
N (total)	63	63	50	42
WBIC (ng/g) (unstressed)	8.6 ± 1.7	5.7 ± 0.9	17.0 ± 1.8	18.8 ± 5.7
N	21	26	21	21
WBIC (ng/g) (stressed)	46 ± 4	43 ± 4	128 ± 6	115 ± 6
N	41	35	29	21
CRTW (pg/g/h) (stressed)	858 ± 103	250 ± 66	2129 ± 360	1290 ± 257
N	28	22	29	21

Figure 1. A schematic outline of the workflow employed in this study.

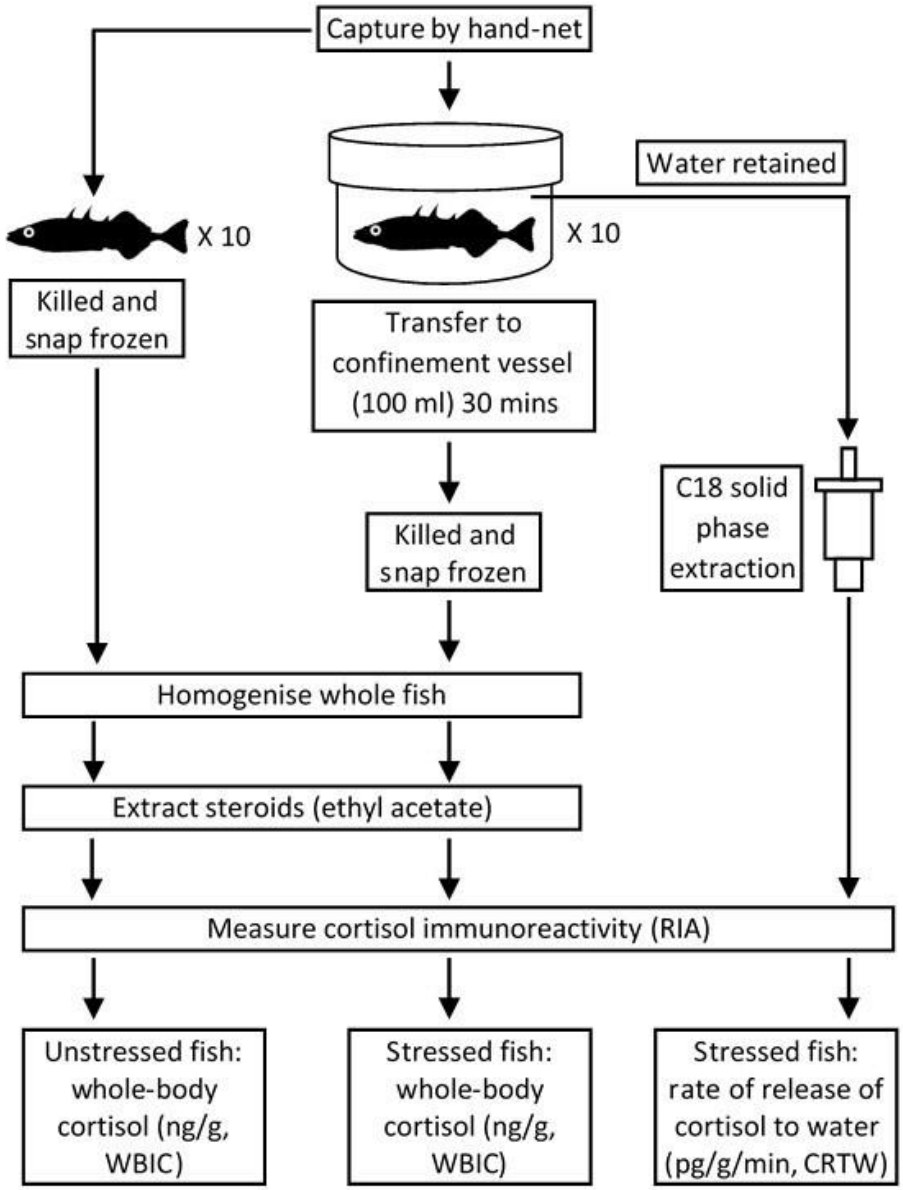


Figure 2. A map showing the location of the sites (▲) in north-west England that were sampled during this study. Numbers correspond to the site information provided in Table 1. Inset: the region of the U.K. shown in the main map.

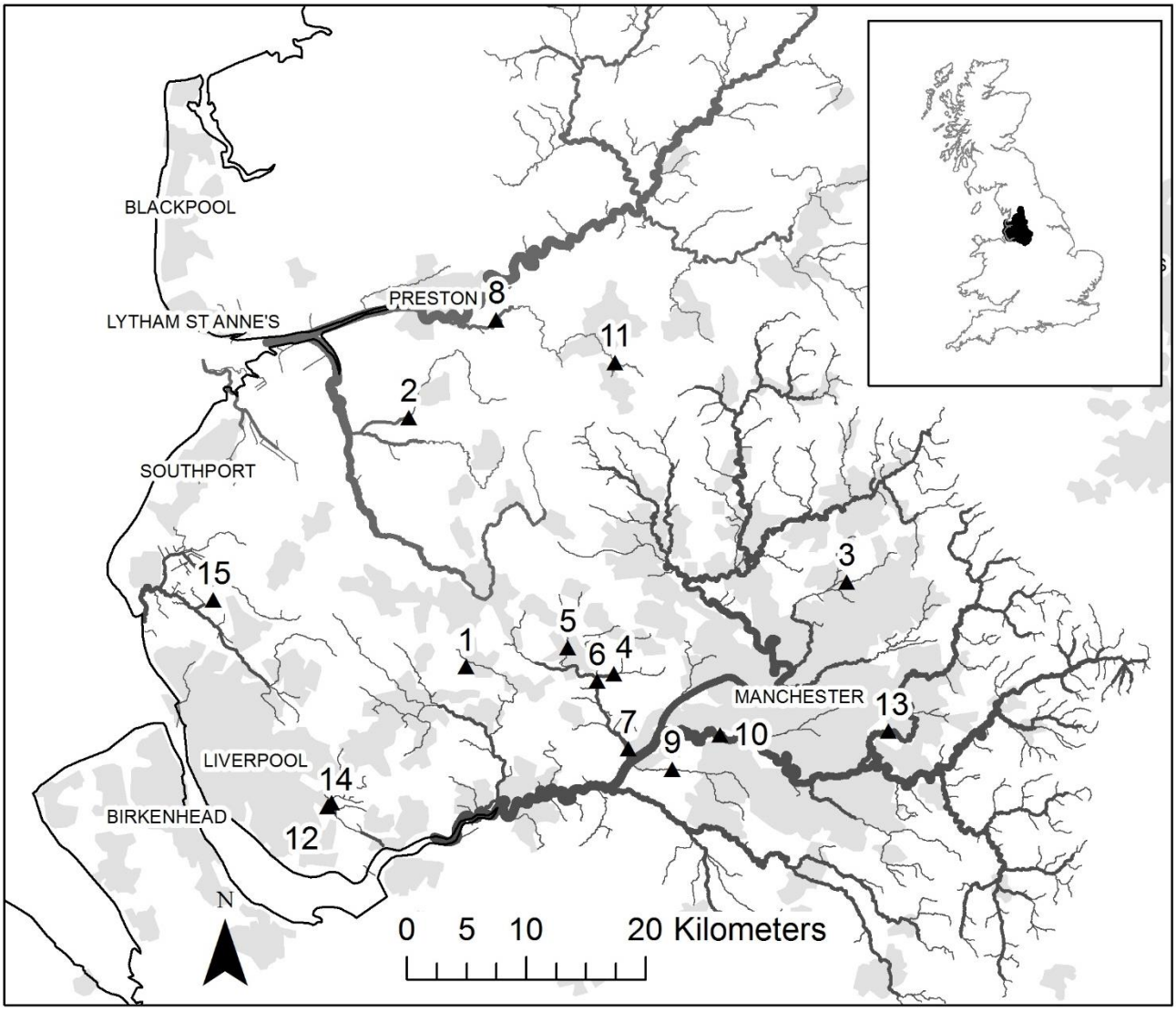


Figure 3. The stress-induced concentration of immunoreactive cortisol in whole-body homogenates (as ng/g body weight) of sticklebacks captured in 2013 (a,c) and 2014 (b,d) at all sites sampled in relation to the concentration of WWTW effluent (as a percentage of total river flow) at the corresponding sample site. Each point is the mean ± SEM. (a,b) females, n = 4-17; (c,d) males, n = 3-14. Best-fit linear regression lines and 95% confidence intervals are shown (see text for details). Regressions were conducted on raw data but the site means are depicted for clarity. Only two male fish were captured at Woolton in 2013 and these are shown (X). No males were captured at Stretford.

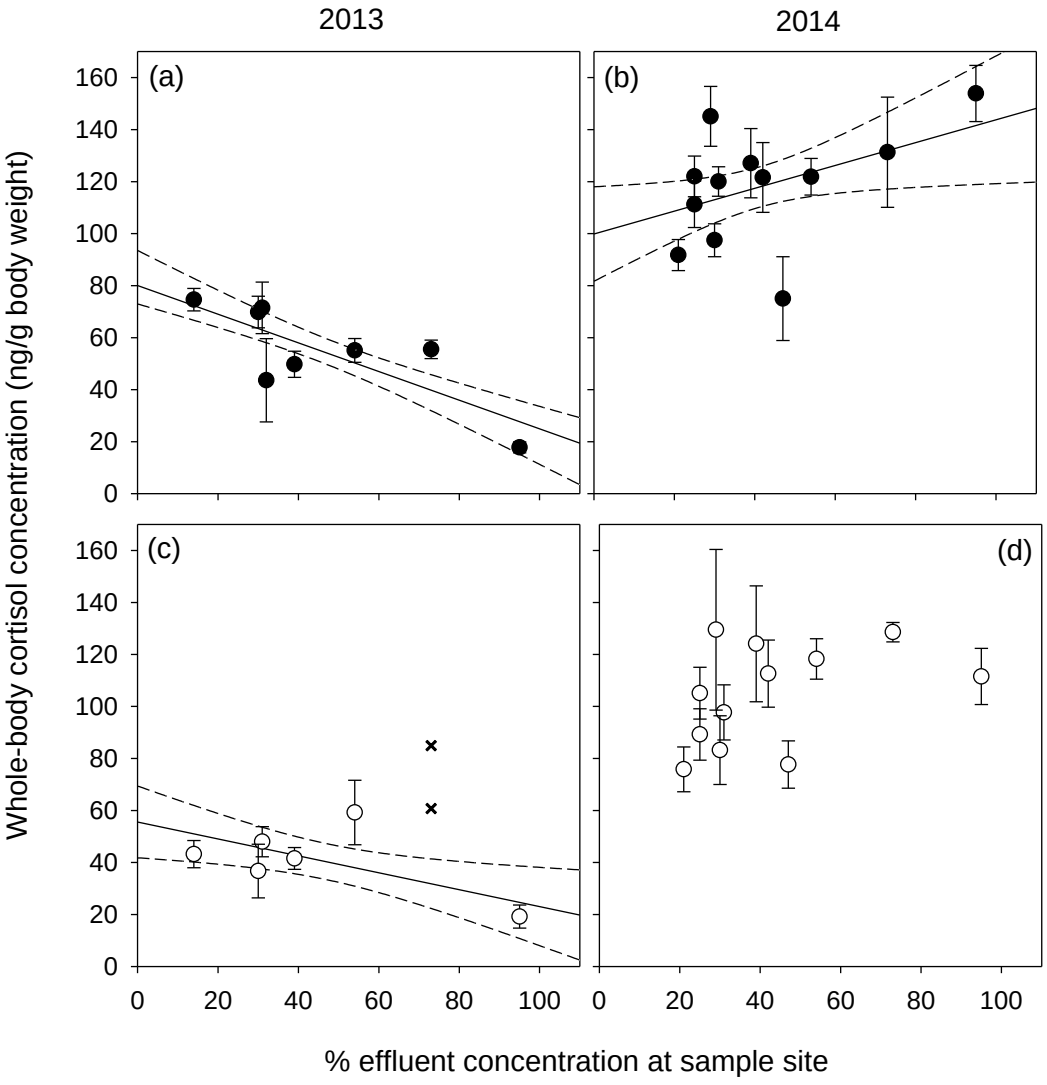
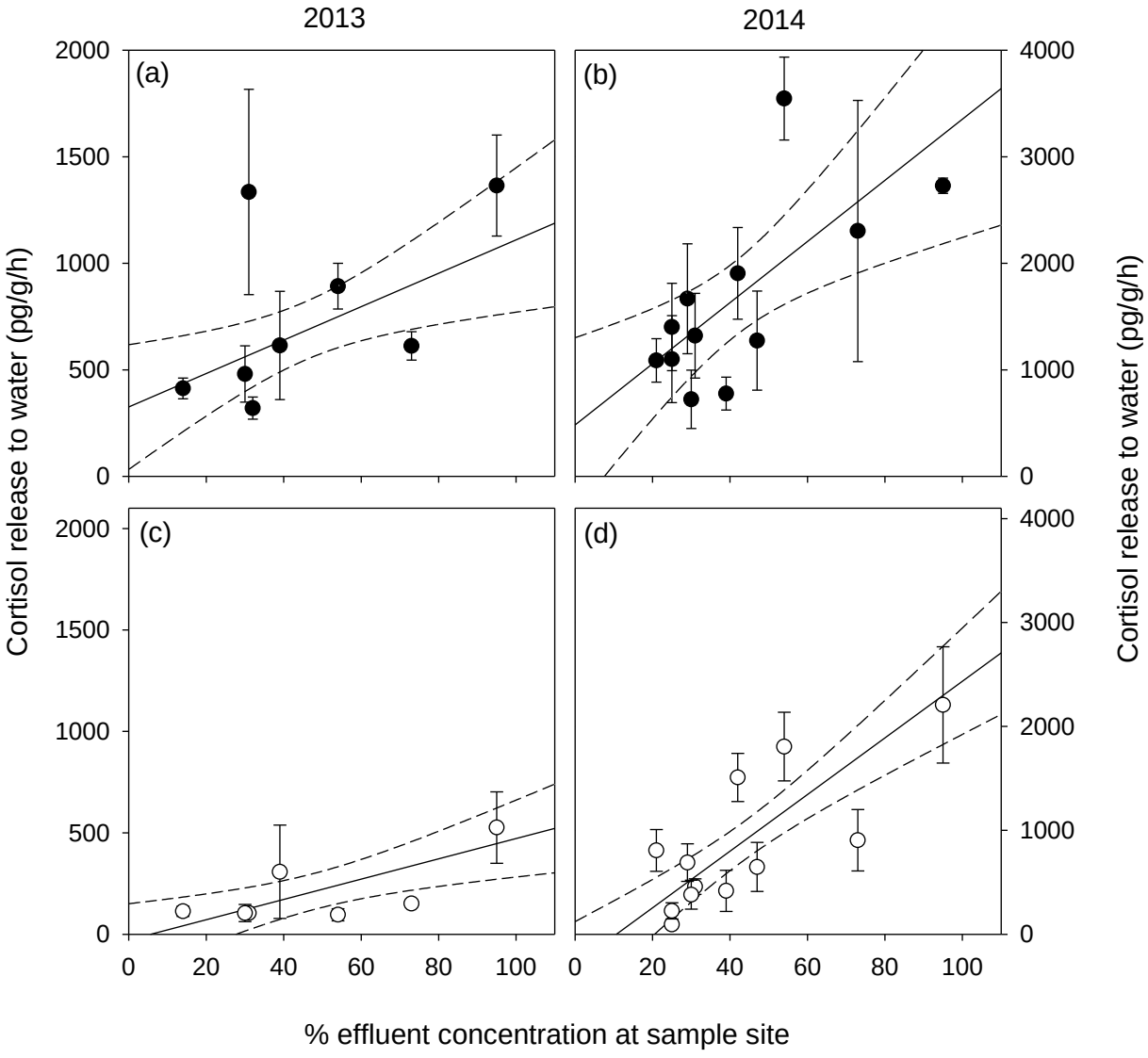


Figure 4. Mean rates of cortisol release to water by sticklebacks exposed to a post-capture confinement stressor in 2013 (a,c) and 2014 (b,d) at all sites sampled (see Table 1), in relation to the concentration of WWTW effluent (as a percentage of total river flow) at the corresponding sample site. Each point is the mean ± SEM. (a,b) females, n = 3-8; (c,d) males, n = 3-7. Best-fit linear regression lines and 95% confidence intervals are shown. Regressions were conducted on raw data but the means are depicted for clarity. Note the different y-axis scales for 2013 and 2014. In 2013 no males were captured on the R. Mersey and CRTW values for Sinderland Brook and Pennington Brook are overlaid.



SUPPLEMENTARY INFORMATION

Table S1. Sites with no known upstream WWTW inputs from which sticklebacks were sampled. DS – downstream, US – upstream.

Sample site	Location (NGR)	Sample date
Barton Brook	SD 520 364	2014
Black Brook	SD 366 137	2014
Lydiate Brook	SD 364 059	2014
Mill Brook DS	SJ 452 879	2013
Mill Brook US	SJ 453 881	2013
New Draught	SD 479 401	2014
Old Eea Brook	SJ 766 938	2014
Old River Brock	SD 480 401	2014
R. Etherow	SK 019 969	2013
Sinderland Brook	SJ 762 902	2013
Town Brook	SJ 695 991	2014
Walmer Bridge	SD 462 248	2014
Woodplumpton Brook	SD 483 356	2014
Woodplumpton Brook	SD 500 341	2014

Figure S1. The relationship between the estimated effluent concentration, as a percentage of total flow, and the body weights of sticklebacks captured at each WWTW-impacted site. Best-fit linear regression lines with 95% confidence intervals are shown where the fit was significant: (a) females, 2013, $r^2 = 0.04$, $P = 0.06$, n = 103; (b) females, 2014, $r^2 = 0.27$, $P < 0.001$, n = 123; (c) males, 2013, $r^2 = 0.07$, $P = 0.01$, n = 89; (d) males, 2014, $r^2 = 0.33$, $P < 0.001$, n = 103.

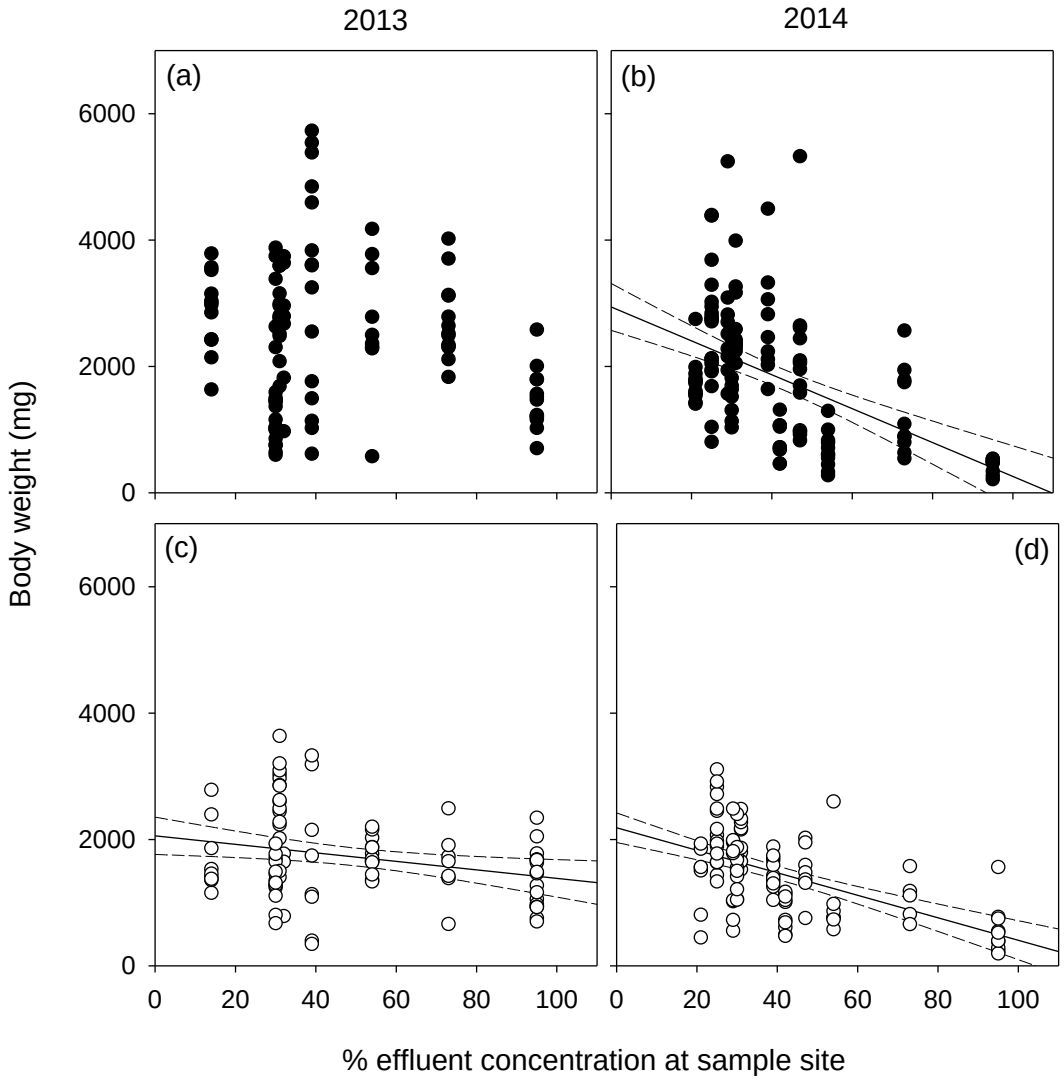


Figure S2. The relationship between the body mass of sticklebacks captured at each WWTW-impacted site and stress-induced cortisol release to water. Best-fit linear regression lines with 95% confidence intervals are shown where the fit was significant: (a) females, 2013, r^2 = 0.26, P < 0.001, n = 46; (b) females, 2014, r^2 = 0.19, P < 0.001, n = 64; (c) males, 2013, r^2 = 0.18, P = 0.02, n = 30; (d) males, 2014, r^2 = 0.52, P < 0.001, n = 55.

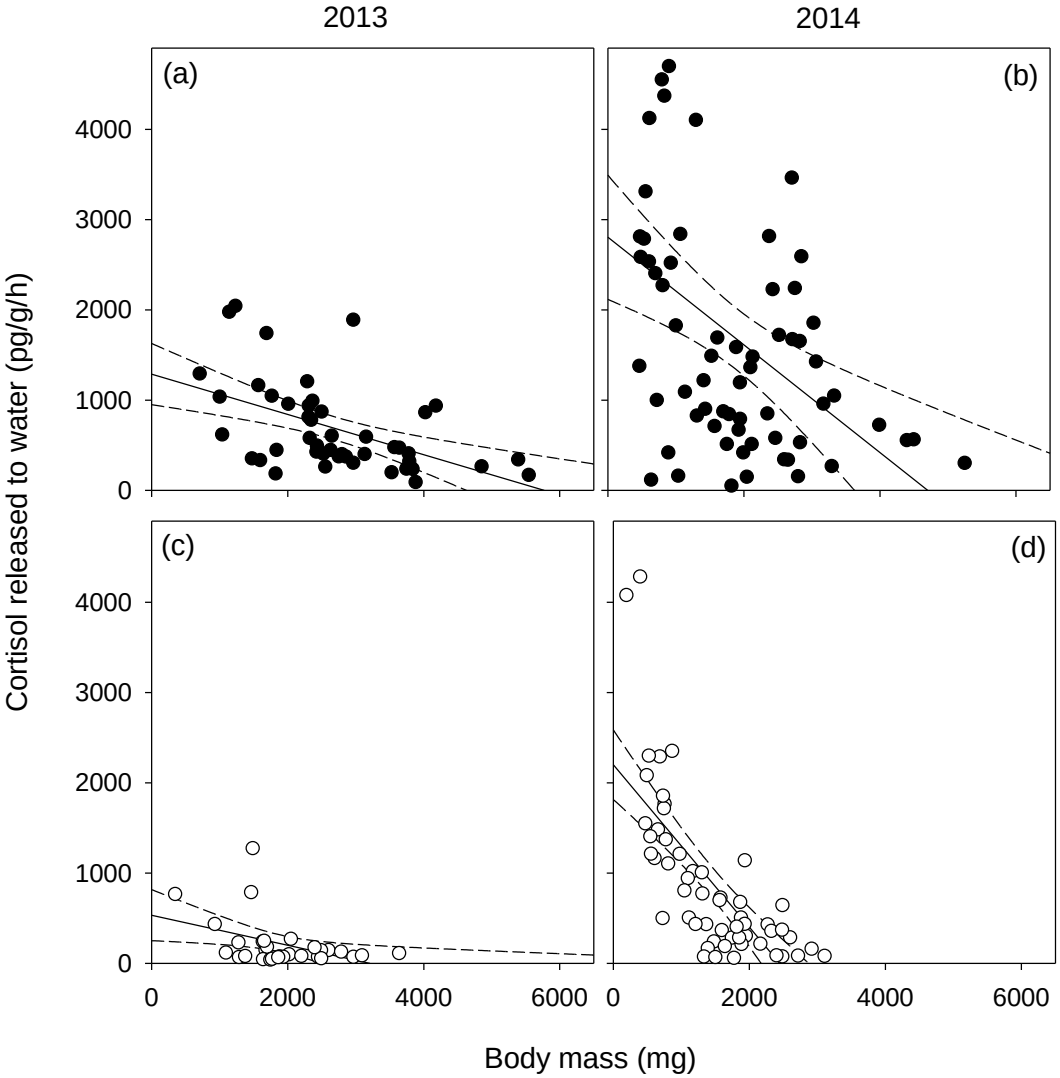


Figure S3. The rate of post-capture stress-induced cortisol release to water in relation to body mass for sticklebacks captured at sites with no known upstream WWTW discharge (Table S1). A best-fit linear regression line with 95% confidence intervals is plotted where a significant relationship between the variables was detected: (a) females, 2013, r^2 = 0.01, P = 0.6, n = 30; (b) females, 2014, r^2 = 0.0, P = 0.5, n = 58; (c) males, 2013, r^2 = 0.16, P = 0.25, n = 10; (d) males, 2014, r^2 = 0.28, P < 0.001, n = 38.