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# Field surveys reveal the presence of anti-androgens in an effluent-receiving river using stickleback-specific biomarkers

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**Abstract**

This study was designed to assess whether the removal of endocrine disrupting chemicals (EDCs) and other substances from a Waste Water Treatment Works (WWTW) effluent (receiving water: R. Ray, Swindon, UK) by granular activated carbon (GAC) affected biomarkers of exposure to EDCs [vitellogenin (VTG) and spiggin] in male and female three-spined sticklebacks in the receiving water. A nearby river (R. Ock), with a negligible effluent loading, was used as a control. On each river fish were sampled from four sites on five occasions both before and after remediation of the WWTW effluent. The results show for the first time in a UK field study a clear seasonality of blood VTG concentrations in wild male fish, following closely the VTG profile in female fish from both rivers. VTG levels in male fish from the R. Ray were significantly reduced after the GAC installation. However, VTG levels in males from the control sites also varied significantly across the same period, reducing the significance of this finding. A laboratory exposure to oestradiol (using site-specific lower and upper levels of oestrogenic activity) failed to elevate VTG concentrations in male sticklebacks suggesting that concentrations in the effluent, even prior to remediation, may not have exceeded a critical sensitivity threshold. Most importantly, a significant increase in female kidney spiggin content (a highly specific biomarker of xeno-androgen exposure) occurred in fish in the R. Ray after the GAC installation to levels comparable with those in fish from the control river. The significance of this finding is strengthened by the fact that during the pre-remediation period in the R. Ray, female spiggin levels increased with increasing distance from the WWTW. Our results provide the first *in vivo* evidence of the presence of anti-androgens in a U.K. WWTW effluent. To our knowledge this is the first U.K.-based comprehensive field study on the effects of a WWTW upgrade on biomarkers of EDC

exposure using a sentinel fish species and our findings confirm the value of the stickleback as a model species for studying EDCs both in the laboratory and in the wild.

**Key words: endocrine disruption, wild fish survey, vitellogenin, spiggin.**

## 1. Introduction

Endocrine disrupting chemicals (EDCs) comprise a large group of natural and synthetic substances with the ability to interact with the vertebrate endocrine system. The presence of EDCs in the aquatic environment has been linked with several developmental and physiological perturbations in a variety of fish species native to the UK including trout (Purdom *et al.*, 1994) roach (Jobling *et al.*, 2002), carp (Folmar *et al.*, 1996), stickleback (Sanchez *et al*., 2008; Björkblom *et al*., 2012), flounder (Allen *et al.*, 1999) and even marine species such as cod (Scott *et al.*, 2006). The overarching concern for the past decade has been that exposure to oestrogenic contaminants may result in alterations in the reproductive output of individuals leading to effects on population structure and size (Nash *et al.*, 2004; Kidd *et al.*, 2007; Harris *et al*., 2010). The EDCs that have been studied in greatest detail to date in both wild and laboratory fish are steroidal and non steroidal oestrogens, due in part to the high promiscuity of the oestrogen receptors (ERs) coupled with an unambiguous biomarker response (vitellogenin; VTG). Wastewater treatment works (WWTWs) represent the predominant source of EDCs in rivers due to their role in domestic and industrial wastewater management and their limited effectiveness in removing natural and synthetic oestrogens from effluent during the treatment process (Johnson and Sumpter, 2001; Sole *et al.*, 2001; Zhang and Zhou, 2008). Consequently, there has been a move to develop and adopt additional treatment

procedures for managing WWTW effluent (Ze-hua *et al.*, 2009). One promising approach is the adsorption of EDCs onto granulated activated carbon (GAC) which is also effective in the removal of a variety of environmental contaminants, including plasticisers, pharmaceuticals and surfactants (Choi *et al.*, 2005; Snyder *et al.*, 2007). As part of the Endocrine Disruption in Catchments (EDCAT) research programme (Balaam *et al.*, 2010; Grover *et al.*, 2011a,b; Pottinger *et al.*, 2011a,b), the present study sought to use established biomarkers in an ecologically relevant species, the three-spined stickleback (*Gasterosteus aculeatus*), to assess the impact of EDCs in a river receiving WWTW effluent, both before and after the introduction of GAC treatment. The advantages of the stickleback as a sentinel species and as model for assessing the effects of EDCs have previously been discussed in detail (Pottinger *et al.*, 2002; Katsiadaki *et al.*, 2007).

In order to identify *in vivo* effects that might be attributed to the presence of oestrogenic/anti-oestrogenic and androgenic/anti-androgenic chemicals in either river, levels of both VTG and spiggin were measured in three-spined sticklebacks, during this study. Measurement of VTG in male fish has become the default *in vivo* biomarker for aquatic environmental contaminants with oestrogenic activity (Kimea *et al.*, 1999) and the androgen-regulated protein spiggin (Jakobsson *et al.*, 1999), unique to sticklebacks, has been shown in females to be as effective a biomarker for androgenic and anti-androgenic xenobiotics as VTG is for oestrogens (Katsiadaki *et al.*, 2000; 2002a; 2006).

Our aim was to collect sufficient data during the period before installation of the GAC plant at Rodbourne WWTW on the R. Ray, when oestrogenic content of the discharge was expected to be moderately high (>5ng $L^{-1}$ oestradiol-17β equivalents), to allow a robust comparison with the status of the same populations of fish over a similar period

after the installation of the plant, when oestrogens (and other organic chemicals) were expected to have been eliminated from the effluent or at least very much reduced in concentration. A second river (the R. Ock) in a similar geographical location as the R. Ray was selected to serve as a relatively unimpacted control river. Table 1 presents a summary of the catchment characteristics of both rivers.

Analysis of water samples collected during the EDCAT programme indicated that oestrogen content of the receiving water before remediation was equivalent to <5ng $L^{1}$ oestradiol-17β (E2; Grover *et al.*, 2011a, b). This concentration was unexpectedly low, so a subsidiary aim of this study was to generate data from a controlled laboratory experiment, in which sticklebacks were exposed over a long period to concentrations of E2 comparable with those found in the catchment area in order to provide a point of comparison with the field data.

## 2. Methods

### *2.1. Site selection*

Four sites were identified on the R. Ray, with the first of these immediately adjacent to the WWTW outfall (Rodbourne) and the final site (Seven Bridges) approximately 10 km further downstream (Figure 1). Three additional sites on the R. Ock and one on Childrey Brook (Figure 1) were identified as suitable un-impacted reference sites since they receive a minor amount of effluent from upstream sources. Water temperature was recorded at 20 minute intervals at two sites (R. Ray: downstream of Rodbourne WWTW; R. Ock: Charney Bassett) using multi-parameter sondes (YSI 6920; Yellow Springs Instruments; www.ysi.com) and a single temperature measurement was taken on the occasion of each sample at each site (Hannah

Instruments HI 93530 thermocouple thermometer). Sampling dates and fish numbers caught in each occasion are provided in Table 2.

*2.2. Frequency of sampling*

Fish were collected on 10 occasions from each of the sites on the R. Ray and the R. Ock. Sampling was conducted at 2-monthly intervals from April 2007 to November 2008 (Table 2). This between-sample interval was considered sufficiently infrequent to prevent depletion of fish at the selected sites, but frequent enough to detect seasonal trends in the biomarker responses. Sampling dates in 2008 were timed to match those collected during 2007 in order to permit comparison of year to year consistency within rivers during the periods prior to and following installation of the GAC plant on the Ray in February 2008 and to allow sufficient temporal resolution to assess seasonal changes in the reproductive status of the fish between years.

*2.3. Fish capture methods*

Fish were collected by the use of a large hand net (38 cm D-frame, 0.5 cm mesh, 1.5 m handle) while wading. On occasions when the water level was too high to safely enter the river, fish were captured by hand-netting suitable areas immediately adjacent to the river bank. This was only successful where trailing and emergent vegetation was present.

*2.4. Fish processing*

All field work was conducted with the appropriate licensing and consent of the Environment Agency of England and Wales. Fish were held in buckets for between 30 and 60 minutes before they were humanely killed with a lethal dose of anaesthetic

(2-phenoxyethanol, 1:2000). Upon confirmation of death, the fish were sealed in individually labelled, 12 ml polypropylene centrifuge tubes and transferred to a cryo-resistant bag before being frozen in a liquid N2 dry shipper (Taylor-Wharton CryoExpress CX500, Jencons plc) for transfer to CEH Lancaster. The fish were kept in frozen storage (-80°C) until processed. The total weight (mg) and fork-length (mm) of each fish was recorded whilst the fish were still frozen. Similarly, before fully thawed the heart and kidney from each fish were removed, weighed, transferred to individual 1.5 ml tubes and stored in a freezer (-20°C) prior to transport to the Cefas laboratory (on dry ice) where all VTG and spiggin analyses took place.

*2.5. Laboratory Exposure*

In order to assess the VTG responses of sticklebacks to an oestrogenic challenge of a similar magnitude to that presented by the R. Ray a controlled long-term laboratory exposure was conducted. Laboratory-reared sticklebacks were exposed to E2 at nominal concentrations of 0, 1, and 5 ng $L^{-1}$ in 30 L aquaria under flow-through conditions (100 ml $min^{-1}$) at 17ºC for 84 days (12 weeks). Concentrations of E2 of 1 ng $L^{-1}$ and 5 ng $L^{-1}$ were selected to represent the lower and higher range of predicted and measured oestrogenicity in the R. Ray (Balaam *et al*., 2010; Grover *et al*., 2011a). The exposure took place between January and April 2008, started when the fish were 8 months old and finished when the fish were 1 year old. Daylength was initially set at 12L:12D, with a 30-min dusk/dawn cycle, increasing by 0.5 h every 2 weeks throughout the study. A stock solution of E2 was prepared in acetone at 25 mg $L^{-1}$ from which daily working stocks were prepared by the addition of an appropriate amount of E2 stock to tank water (de-chlorinated tap water). Acetone concentration in all tanks was 0.001% throughout the exposure period. Exposure concentrations

were achieved by the addition of working stocks to the inflow water via a peristaltic pump (Watson Marlow). All treatments were tested in duplicate tanks. Each tank was initially stocked with 21 fish, seven of which were sampled on days 28, 56 and 84. At each sampling point, fish were humanely killed with a lethal dose of anaesthetic (MS222), weighed (nearest mg) and their fork length measured (nearest mm). Blood was collected after severing the caudal peduncle into a heparinised micro haematocrit tube (Fisher Scientific, UK). The blood samples were transferred to 0.5 ml tubes and centrifuged at 16,000 g for 10 min. The plasma was collected, volume recorded and stored at -20ºC, prior to VTG analysis.

At weekly intervals throughout the exposure period water samples were collected from each tank to verify the actual concentrations of E2 in the exposure tanks. Water extraction methods and analysis by radioimmunoassay (RIA) have been described in detail before (Katsiadaki *et al.*, 2010).

*2.6. Vitellogenin and spiggin analysis*

For the measurement of VTG in wild-caught fish, the heart tissue was used as a surrogate for blood which it is not possible to collect from sticklebacks under field conditions. Our own unpublished data show that plasma and heart VTG levels are highly comparable. For this, 100 µl of assay buffer (0.1 M sodium phosphate: 72 mM di-basic salt, 28 mM mono-basic salt, 140 mM sodium chloride, 27 mM potassium chloride, 0.05% Tween 20 [v/v], 0.1% BSA [w/v], 0.15 mM sodium azide) were added to the 1.5 ml tube containing the hearts. The heart tissue was disrupted using a pellet homogeniser (pellet pestle, Sigma-Aldrich) for 30 seconds and the homogenate was centrifuged at 1,6000g for 10 minutes. A small aliquot of the supernatant (15 µl) was used for VTG analysis. VTG from laboratory exposed animals was analysed in

plasma as described for the heart homogenate. Typically, 3-5 µl of plasma was obtained from each stickleback so a 10-fold dilution with assay buffer was necessary prior to analysis, which required a minimum volume of 15 µl. A homologous stickleback VTG ELISA was used; this has been validated previously and has a high specificity and sensitivity (Katsiadaki *et al*,. 2002b; Hahlbeck *et al*,. 2004). The detection limit of the assay is 2 ng VTG $ml^{-1}$, the quantification limit is 20 ng VTG $ml^{-1}$ and the inter-assay coefficient of variation is less than 8%. Spiggin was analysed in the kidney using a specific ELISA as previously described (Katsiadaki *et al.*, 2002a). The detection and quantification limits of the spiggin ELISA is 0.4 and 40 U $g^{-1}$ body weight respectively. The intra-assay and inter-assay coefficients of variation for the spiggin ELISA during method validation were 9%, and 13 % respectively.

*2.7. Statistical analysis*

We first examined the extent to which VTG and spiggin concentrations varied across sample sites in both the R. Ray and the R. Ock. To avoid confounding effects of seasonal variation, comparisons were carried out only where samples were available at each site for the same time points. Differences between sites were therefore investigated: (i) on the R. Ock using data collected during 2007 in April, May, July, September, and November; and (ii) on the R. Ray, using data collected during both 2007 and 2008 in July, September, and November, the only months for which data were available from every sample site. The comparisons between sites within rivers at the sampling times listed above were carried out using a one-way ANOVA design with Tukey's correction for multiple testing. Differences in the concentrations of VTG and spiggin were also examined with respect to the effect of remediation (pre and post) and location (Ray or Ock) resulting in a two by two table of levels within

factors: (i) between rivers pre-remediation, (ii) between rivers post-remediation, (iii) between pre- and post-remediation periods on the R. Ock, and (iv) between pre- and post-remediation periods on the R. Ray. Data for males and females were analysed separately for both VTG and spiggin. For each test the null hypothesis supposed that there was no difference in VTG and spiggin levels in males and females between the different levels of the grouping factor (remediation period or river). The statistical significance of each test was determined by repeatedly sampling the data under the null hypothesis in a non-parametric bootstrap approach (Efron and Tibshirani, 1993). The distinct temporal nature of the data was retained by running the bootstrap procedure separately for each time point. To produce confidence intervals based on the null hypothesis that the levels are the same in both groups, we took the 2.5th and 97.5th percentile of the estimated means from the resampled data sets. P values were obtained by observing the proportion of the 1000 bootstrap based estimates of the mean that were more extreme than the observed mean value. The results of the lab exposure study were subjected to a two-way ANOVA followed by a pairwise multiple comparison procedure (Holm-Sidak method). Data were log-transformed prior to analysis to improve homogeneity of variance. Comparisons of temperature data were conducted with a two-way ANOVA followed by a pairwise multiple comparison procedure (Holm-Sidak method).

## 3. Results

### *3.1 Water temperature*

The R. Ray, which received a large volume of treated sewage effluent, displayed consistently higher water temperatures than the R. Ock (2$^{o}$C to 5$^{o}$C difference in monthly means; Fig. 2a). This was evident for both years and at all study sites. There

were also inter-annual differences in temperature within each river. In the R. Ray, mean daily temperatures were significantly higher in 2007 in comparison to 2008 during January (10.4 cf. 9.8$^{o}$C ; $p < 0.05$), March (11.1 cf. 10.4$^{o}$C; $p < 0.01$) and April (14.9 cf. 13.2$^{o}$C; $p < 0.001$) whilst in the R. Ock temperatures were higher in February (6.7 cf 5.7 $^{o}$C; $p < 0.01$) and March (7.8 cf 7.1$^{o}$C; $p < 0.05$) 2007.

*3.2 Female VTG*

There was no evidence of significant between-site variation in female VTG in the R. Ray in either year ($p = 0.48 – 1.0$) and although in the R. Ock mean VTG levels in females tended to be higher at two sites (Garford and Venn Mill) only one significant difference was detected (2007; VM v. GAR: $p < 0.05$). The site data were therefore consolidated within rivers for investigation of pre- and post-remediation differences. There was clear seasonal variation of VTG concentrations in both rivers and for both years (Figure 2b). Comparing rivers within years, there was a difference in the timing of these changes. In R. Ray females VTG decreased earlier compared with females in the R. Ock. Females from the R. Ock had significantly higher VTG levels in both July 2007 (4,483 μg ml$^{-1}$, $p < 0.001$) and July 2008 (4,664 μg ml$^{-1}$, $p < 0.01$) compared with females from the R. Ray (8.37 and 299 μg ml$^{-1}$ respectively; Fig. 2b). Because female VTG titres increased sharply in early spring we avoided the direct comparison of the VTG data from April 2007 with March 2008 (this was the only timepoint at which the data that were not fully matched; table 2). Comparing years within rivers, there was significantly more VTG in females in the R. Ock in November 2008 than in 2007 ($p < 0.05$) but significantly less during May ($p < 0.05$) 2008 than 2007 (Fig. 2b). In the R. Ray VTG was lower in November ($p < 0.05$) 2008 but higher in May 2008 than in 2007 ($p < 0.05$; Fig. 2b).

*3.3. Male VTG*

As was the case for the female fish, no significant between-site variation in VTG was detected for fish from either river. There was no significant overall difference in site means for VTG in male fish in the R. Ray between the pre- and post-remediation periods ($p = 0.34$). Concentrations of VTG in male fish were more than two orders of magnitude lower than those in females (maximum mean VTG values: males = 135 μg $ml^{-1}$; females = 18,139 μg $ml^{-1}$). However, despite these very low concentrations in males, a clear seasonality was apparent which followed the female VTG pattern (Fig. 2c). For this reason we avoided the direct comparison of male VTG data between April 2007 and March 2008 as in the female fish.

During the 2007 pre-remediation period males in the R. Ray displayed higher mean VTG values than those in the R. Ock throughout most of the year (Figure 2c). Mean VTG levels were higher in male sticklebacks from the R. Ray in April (135 cf 10 μg $ml^{-1}$; $p < 0.001$) May (128 cf 34 μg $ml^{-1}$; $p = 0.078$) and July 2007 (2.3 cf 0.02 μg $ml^{-1}$; $p < 0.001$) compared with those from the R. Ock during the same months. There were no significant differences in VTG concentrations between males from the two rivers during the post-remediation period in 2008. This can be attributed to mean VTG levels in males from the R. Ray being lower in 2008 than 2007 during May ($p = 0.068$) and VTG levels in fish from the R. Ock being higher in July ($p < 0.001$) and September ($p < 0.01$).

*3.4. Laboratory exposure to E2*

The concentrations of E2 that were achieved, although lower than the nominal concentrations (59-61%), were maintained at a constant level (±20% of the mean

measured concentration) during the exposure period and were on average 0.59 ng $L^{-1}$ for the 1 ng $L^{-1}$ nominal concentration group and 3.07 ng $L^{-1}$ for the 5 ng $L^{-1}$ group respectively.

No induction of VTG in was evident in male sticklebacks during the course of the study (two-way ANOVA, $p = 0.85$; Fig. 3a) and no distinct trends were evident in relation to sample date ($p > 0.05$). Similarly, for females overall there was no significant difference in VTG levels between treatments ($p = 0.41$; Fig. 3b). However, there was a significant effect of sample day (28, 56 or 84) on VTG ($p < 0.001$) in females; for all treatments female VTG levels on day 84 were greater than those on day 56 and day 28.

*3.5. Female spiggin*

During the pre-remediation period there was a significant trend for spiggin concentrations in female fish from the R. Ray to increase with distance from the effluent discharge point (Figure 4, $p < 0.05$). This trend was abolished following remediation when spiggin concentrations in female fish at all sites were significantly higher than those during the pre-remediation period ($p < 0.001$; Figure 5). In the R. Ock there were no significant site-related differences in spiggin concentrations in female fish (data not shown).

During both 2007 and 2008, spiggin levels tended to be higher in females from the R. Ock, when compared with those from the R. Ray (Figure 5b) and this was most pronounced during 2007 when significant differences were evident at four out of five time points: April ($p < 0.05$), May ($p < 0.001$), September ($p < 0.05$) and November ($p < 0.001$). Post-remediation, in 2008, concentrations of spiggin in females from the R. Ray were up to two-fold higher than those in 2007 at all time points ($p < 0.05 – p$

< 0.001) and were closely comparable to spiggin levels in the R. Ock females, although levels in fish from the R. Ock remained significantly higher on two occasions, in May ($p < 0.01$) and September ($p < 0.001$). We observed higher levels of spiggin in females from the R. Ock in the post-remediation period compared with the pre-remediation period on only two occasions (July, $p < 0.05$ and September $p < 0.001$).

*3.6. Male spiggin*

During 2008 spiggin concentrations in males at Tadpole Bridge on the R. Ray were significantly higher than those at the sites upstream ($p < 0.05$) but no other significant between-site variation in spiggin concentrations in males was evident in the R. Ray or in the R. Ock. Mean spiggin levels in male sticklebacks were more than 3.5 orders of magnitude higher than levels in females (maximum mean spiggin in males = 502,943 spiggin units $g^{-1}$; females = 220 spiggin units $g^{-1}$). A marked seasonality was evident in spiggin concentrations (Figure 5c) which was analogous to the female VTG pattern (Figure 2b). In contrast to the female spiggin levels, the pattern and concentration of spiggin in males from the R. Ray were almost identical to those in the R. Ock during 2007 with only two statistically significant differences; mean male spiggin was higher in fish from the R. Ray in April ($p < 0.01$) and lower in November ($p < 0.001$) in comparison to fish from the R. Ock. In 2008 spiggin levels were significantly higher in fish from the R. Ray compared to those in the R. Ock during March ($p < 0.001$; Fig. 5c) and were lower during July ($p < 0.01$).

## 4. Discussion

The aim of this study was to investigate whether the reproductive health of three-spined sticklebacks downstream of an urban WWTW discharge (R. Ray, Rodbourne WWTW, Swindon, U.K.) was improved when works were undertaken to reduce the organic chemical load within the effluent. To accomplish this aim, two key biomarkers of endocrine disruption of the reproductive system in sticklebacks (VTG and spiggin) were assessed at intervals during a period prior to and following effluent remediation. Fish in the nearby R. Ock, which received relatively little effluent, were sampled at the same time to provide a point of comparison.

Although the inclusion of a reference or control site is common practice in field studies, some of the data reported in the present study throw some doubt on the usefulness of this approach. The WWTW effluent affected both the temperature profile in the R. Ray and the degree of enrichment (Pottinger *et al*., 2011a) and the direct and indirect effects of these factors on the growth of the resident fish populations, and timing of reproduction, meant that a direct comparison of spiggin and VTG levels in fish from the two rivers at the same time points was inappropriate. We have therefore placed greater weight on relative changes within rivers across the pre- and post-remediation periods. However, even this approach assumes that conditions remain stable in the reference river, or if changes do occur, they are as a result of factors affecting both rivers equally (for example, rainfall).

Chemical analysis of the receiving water conducted during the same period as the fish sampling took place showed that after installation of the GAC plant there was a marked decline in the downstream concentrations of endocrine active substances. Concentrations of oestrone, E2 and 17α-ethinylestradiol were reduced from 3.5, 3.1 and 0.5 ng $L^{-1}$ respectively during the pre-remediation period to undetectable levels

after remediation, and this was accompanied by an 85% reduction in anti-androgenic activity in the effluent (Grover *et al*., 2011a). The dissolved concentrations of a range of pharmaceutical compounds downstream of the WWTW were also reduced by between 17% and more than 98% after installation of the GAC plant (Grover *et al*., 2011b). Given the effectiveness of the GAC plant in reducing chemical contamination in the WWTW effluent it was expected that the remediation process would be reflected in alterations in the key biomarkers of oestrogenic and anti-androgenic exposure in the resident stickleback population.

*4.1. Vitellogenin in male sticklebacks*

During the pre-remediation period VTG concentrations were higher overall in male fish from the R. Ray than in fish from the relatively unimpacted R. Ock. There were no significant differences between VTG concentrations in male fish from the two rivers following remediation. These observations could reflect the near total elimination of oestrogens from the R.Ray effluent by the GAC treatment that should be noted even pre-remediation did not carry a heavy loading of oestrogens. However, this conclusion is challenged by two other findings: post-remediation VTG levels were also higher in males in the R. Ock at two time points relative to the pre-remediation period and there was no obvious trend between male VTG levels and distance from WWTWs within the R. Ray. During 2008 both EROD and CYP1A biomarkers indicated PAH presence in the R. Ock to a level at least as high as that in the R. Ray (Pottinger *et al*., 2011a). The sources of these contaminants were not identifiable and it is unclear whether they affected VTG levels in male fish within the R. Ock. Therefore it is possible that the between-year variation in VTG concentrations in the male sticklebacks could be a result of interferences from

chemicals or from endogenous factors that promote VTG synthesis in males, rather than the presence of oestrogenic substances alone. The lack of male VTG response in the long-term laboratory trial, where sticklebacks were exposed to E2 at concentrations corresponding to the upper and lower levels of oestrogenic activity measured at the study site (Grover *et al*., 2011a; b), agrees with the data obtained from 21-day laboratory exposures in the stickleback (Allen *et al*, 2008) and other fish (OECD, 2006).

The fish populations in both rivers were sampled at several intervals both prior to and following remediation. These time-course data clearly illustrate, for the first time in a U.K. field study, a pronounced seasonal variation in VTG concentrations in the male fish. This is not the first report of cyclical changes in plasma VTG concentrations in male fish (Hotta *et al.*, 2003; Ma *et al.*, 2005; Scott and Robinson, 2008) and it is a matter of debate as to whether variation in male VTG concentrations is due to exposure to exogenous oestrogens, cyclical changes in sensitivity to oestrogens, or to endogenous oestrogen production. Measurable levels of E2 have previously been detected in laboratory male sticklebacks (Sebire *et al.*, 2007); this supports the possibility that the seasonal variation in VTG levels detected in male fish in the present study might be due endogenous production of E2 by the males.

### *4.2. Vitellogenin in female sticklebacks*

Significant differences in VTG concentrations in females, both between rivers and between years, were evident during the study. However, it was not possible to ascribe any of these differences to the chemical contaminants present in the WWTW effluent in the R. Ray, or their removal, for several reasons. Female VTG levels are not normally considered a useful biomarker of endocrine disrupting influences in fish

because the pivotal role played by VTG in the female reproductive process means that through the life-cycle of the female fish circulating levels of VTG extend across a concentration range encompassing several orders of magnitude and exhibit considerable inter-individual variability. Both of these factors potentially mask effects of exogenous endocrine modulators. In addition, the seasonality of the reproductive cycle in sticklebacks, and thus of VTG levels, is driven in part by water temperature (Baggerman 1957, 1989) and clear differences in the annual temperature cycle in the two rivers were apparent, driven by the warming effect of the effluent in the R. Ray. The likely role of differences in the temperature regime in the two rivers on growth and the reproductive cycle are discussed elsewhere (Pottinger *et al*., 2011a) but inspection of the VTG data presented here, together with histological examination of gonadal tissues (C. Mungo, unpublished data) indicate that sticklebacks in the R. Ray spawned at least a month earlier than those in the R. Ock. This is reflected in the asynchronous nature of the VTG profiles for each river. Curiously, the lack of synchrony in variation of VTG levels between females in the R. Ock and R. Ray was not consistently evident for VTG concentrations in male fish in the two rivers, suggesting that regulation of VTG cyclicity is different between the two sexes.

*4.3. Spiggin in male sticklebacks*

The use of spiggin concentrations in male sticklebacks in a diagnostic context is subject to the same constraints as the use of VTG in female fish because spiggin concentrations in maturing male sticklebacks extend across several orders of magnitude. In the present study mean spiggin concentrations ranged between $10^2$ and $10^6$ U $g^{-1}$ body weight and therefore against this background the influence of exogenous androgens or anti-androgens was unlikely to be evident. Overall, the

seasonal pattern of spiggin concentrations in male fish in the present study was similar to that of VTG in females, with maximum values of spiggin occurring during spring and early summer, concurrent with spawning activity, and declining thereafter. The spiggin profile in males differed quite markedly between the pre-remediation (2007) and post-remediation (2008) periods. During 2007 spiggin concentrations in males from both rivers were very closely aligned, and in both rivers exhibited a precipitous drop between May and July. In contrast, during 2008 although minimum and maximum spiggin concentrations were similar to the preceding year the pattern of change was quite different for fish in both rivers and was reminiscent of the seasonal change in VTG in females. Spiggin concentrations in males during May 2008 were significantly lower than those for the same period in 2007, consistent with the assumption that sexual maturation in these fish began later in 2008 than in 2007. It is likely that these differences arose because of the cooler water temperatures at the start of 2008 in comparison to the same period during 2007.

*4.4. Spiggin in female sticklebacks*

As is the case for VTG in male sticklebacks, there is as yet no known functional relevance for the presence of spiggin in the kidney of female sticklebacks. However, the induction of spiggin production in females by the administration of exogenous androgens indicates that the female kidney is fully competent to synthesise the protein (Katsiadaki *et al*., 2002a; 2006). The presence of spiggin in wild-caught female sticklebacks, at very low concentrations compared to levels in males, is either a consequence of the presence of androgens of endogenous origin in female fish (Lokman *et al*., 2002), or reflects unstimulated constitutive expression. In contrast with VTG in males temporal variation in spiggin concentrations in female

sticklebacks was not clearly aligned with the reproductive cycle. There was, however, an up to two-fold increase in spiggin concentrations in females in the R. Ray following remediation. Furthermore the female spiggin levels in the R. Ray during the pre-remediation period increased significantly with distance from the WWTW, strongly suggesting that the causative agents were originating from the effluent. Grover *et al*. (2011a) reported that annual mean anti-androgenic activity in the Rodbourne WWTW effluent was reduced from 149 to 22 μg $L^{-1}$ flutamide equivalents following remediation. In laboratory studies, flutamide totally inhibited spiggin induction in androgenised female sticklebacks at a concentration of 250 μg $L^{-1}$ and exhibited a LOEC greater than 22 μg $L^{-1}$ (Katsiadaki *et al*., 2006; OECD, 2010). Taking all the evidence (direct and circumstantial) together we propose that the increase in spiggin concentrations in females in the R. Ray post-remediation was related to the removal of anti-androgens from the effluent. In 2003 and 2004, two nationwide surveys revealed significant anti-androgenic activity present in U.K. final sewage effluents (Johnson *et al.*, 2007). More recently, significant anti-androgenic activity was reported in water and river sediments in Italy (Urbatzka *et al.*, 2007) and France (Kinani *et al.*, 2010). However, thus far the concentration of specific contaminants with anti-androgenic activity identified in water and sediment samples accounts only for a fraction of the *in vitro* activity detected (Hill *et al.*, 2010; Kinani *et al.*, 2010). Several biocides have been reported to posses anti-androgenic activity *in vitro* (e.g. Orton *et al.*, 2011) but the occurrence of these is not directly correlated with WWTW. Nevertheless, the list of compounds with anti-androgenic activity in domestic effluents is increasing and includes several domestic and personal care products (Chen *et al.*, 2007; Rostkowski *et al*., 2011). However, it is unknown whether the anti-androgenic effect observed is only due to chemicals with androgen

receptor antagonistic activity (an area where most of the recent studies was focused) or includes other mechanisms of action (i.e. interference with steroid biosynthesis). Parallel studies on the declining human male reproductive health have implicated chemicals with mechanisms other then receptor antagonism such as phthalates (Scott *et al*., 2009) and more recently non steroidal anti-inflammatory drugs (Kristensen *et al*., 2011). Hence, it is likely that both the nature and the mechanism of action of anti-androgenic compounds in the pre-remediation effluent were multiple.

## 5. Conclusions

The current study represents the first attempt to survey the endocrine-disrupting potential of an effluent in the U.K. using the three-spined stickleback, and the first to monitor ED biomarker concentrations in a target species across multiple time-points following a WWTW upgrade in the field. Our findings on species suitability and sensitivity agree with those of other studies in which sticklebacks were used successfully to characterise effluents (Sanchez *et al.*, 2007; 2008; Björkblom *et al.*, 2012). The current study provides strong evidence that spiggin concentrations in female sticklebacks downstream of an urban WWTW discharge were increased following remediation of the effluent, most likely due to the removal of compounds with anti-androgenic activity. Although significant reductions in VTG concentrations in male fish were also observed it was difficult to tie these unequivocally with remediation of the effluent because of variation in male VTG levels in fish in the R.Ock where no remediation took place. Nevertheless, the results of a laboratory exposure study suggested that the effluent oestrogenicity was too low to evoke an effect; this may be indirect evidence that VTG responses are affected by chemicals other than oestrogens. For the first time we report that VTG concentrations in male

fish, although extremely low in comparison with those in females, presented a clear seasonality closely aligned with the pattern of change in females. It is unclear whether this is due to endogenous or exogenous oestrogen stimulation. The data also show that relying upon single-point samples during either the pre-remediation or post-remediation periods would not necessarily have captured the actual remediation outcome reliably. Where the biomarker employed varies with sex and season, as is the case for both VTG and spiggin, and where subtle effects may be anticipated, several samples may be necessary to counter natural variation and reliably identify genuine effects. Similarly, the possibility that the physicochemical characteristics of the reference site may vary from that of the study site in such a way as to affect any between-river comparisons must also be considered.

Finally, based on biomarker responses physiologically relevant to each sex (female VTG and male spiggin) we found no evidence that the reproductive health of sticklebacks in the R. Ray was adversely impacted. However, one should take into account that stickleback populations have been under substantial environmental pressures in this location and some form of genetic selection to cope with prevailing conditions in the R. Ray may have already taken place. It is well known that sticklebacks are particularly adaptive to environmental changes and have demonstrated a very quick genetic divergence both after geographic isolation (Kristjánsson, *et al.*, 2002) and after contaminant exposure (Lind and Grahn, 2011).

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**FIGURE CAPTIONS**

Figure 1. Locations of sampling sites on the Rivers Ray and Ock. ROD – Rodbourne WWTW; EB – Elborough Bridge; TB – Tadpole Bridge; 7B – Seven Bridges; R. Ock: CB – Charney Bassett; GAR – Garford; VM – Venn Mill (on Childrey Brook); MM – Marcham Mill.

Figure 2. (a) Water temperatures (smoothed daily mean) in the River Ray (at Rodbourne WWTW; solid line) and River Ock (at Charney Bassett; dotted line) during 2007 and 2008; (b) VTG concentrations in female sticklebacks from the R. Ray (●) and R. Ock (○) during 2007 (pre-remediation) and 2008 (post-remediation); (c) VTG concentrations in male sticklebacks from the R. Ray (●) and R. Ock (○) during the same periods. Each point is the mean + SEM (n detailed in table 2). Significant differences *between* rivers at the same time point within either period are denoted by *. Significant differences ($p < 0.05$) between corresponding time points *within* rivers during the pre- and post-remediation periods are denoted by + except (+) where $p = 0.057$.

Figure 3. VTG concentrations in (a) male sticklebacks and (b) female sticklebacks exposed to oestradiol (1 and 5 ng $l^{-1}$ nominal) or to vehicle only, for 84 days. Fish were sampled on days 28, 56 and 84. Each bar represents the mean ± SEM [n = males (left to right): 8, 6, 3, 8, 6, 2, 5, 9, 4; females: n = 6, 8, 11, 6, 8, 12, 9, 5, 10]

Figure 4. Spiggin concentrations in female sticklebacks captured from the R. Ray (see figure 1 for abbreviations used) during the pre-remediation and post-remediation

periods, during months for which data were available at each site for each year (July, September, November). Each bar is the mean ± SEM (n = 18 - 61). Dissimilar letters denote significant differences between sites within that year, or between years overall.

Figure 5. (a) Water temperatures (smoothed daily mean) in the River Ray (at Rodbourne WWTW; solid line) and River Ock (at Charney Bassett; dotted line) during 2007 and 2008; (b) spiggin concentrations in female sticklebacks from the R. Ray (●) and R. Ock (○) during 2007 (pre-remediation) and 2008 (post-remediation); (c) spiggin concentrations in male sticklebacks from the R. Ray (●) and R. Ock (○) during the same periods. Each point is the mean + SEM (n detailed in table 2). Significant differences *between* rivers at the same time point within either period are denoted by *. Significant differences ($p < 0.05$) between corresponding time points *within* rivers during the pre- and post-remediation periods are denoted by + except (+) where $p = 0.059$.

**TABLES**

**Table 1.** Catchment characteristics of the Rivers Ray and Ock

| River | Catchment area ($km^2$) | Rainfall (mm/year) | River discharge ($m^3$/sec) | Geology | Land use | WWTW dry weather flows ($m^3$/sec) |
|---|---|---|---|---|---|---|
| Ray | 84.1 | 698 | 1.34 | Impermeable | Arable | Rodbourne (0.51) |
| Ock | 234 | 639 | 1.55 | Mixed; pervious | Chalk land; arable | 6 small scale discharges (only 0.009 upstream of study area) |

**Table 2.** Sampling dates and number of fish caught in each occasion.

| Sampling dates | River | No of male fish | No of female fish |
|---|---|---|---|
| 18/19 April 2007 | Ray | 4 | 4 |
| 18/19 April 2007 | Ock | 35 | 31 |
| 15/16 May 2007 | Ray | 3 | 7 |
| 15/16 May 2007 | Ock | 25 | 34 |
| 17/18 July 2007 | Ray | 34 | 40 |
| 17/18 July 2007 | Ock | 4 | 30 |
| 11/12 September 2007 | Ray | 28 | 55 |
| 11/12 September 2007 | Ock | 30 | 44 |
| 13/14 November 2007 | Ray | 48 | 41 |
| 13/14 November 2007 | Ock | 34 | 51 |
| 11/12 March 2008 | Ray | 21 | 33 |
| 11/12 March 2008 | Ock | 21 | 32 |
| 20/21 May 2008 | Ray | 18 | 20 |
| 20/21 May 2008 | Ock | 26 | 77 |
| 15/16 July 2008 | Ray | 38 | 44 |
| 15/16 July 2008 | Ock | 31 | 44 |
| 9/10 September 2008 | Ray | 37 | 52 |
| 9/10 September 2008 | Ock | 19 | 43 |
| 11/12 November 2008 | Ray | 45 | 55 |
| 11/12 November 2008 | Ock | 34 | 46 |

Figure 1.

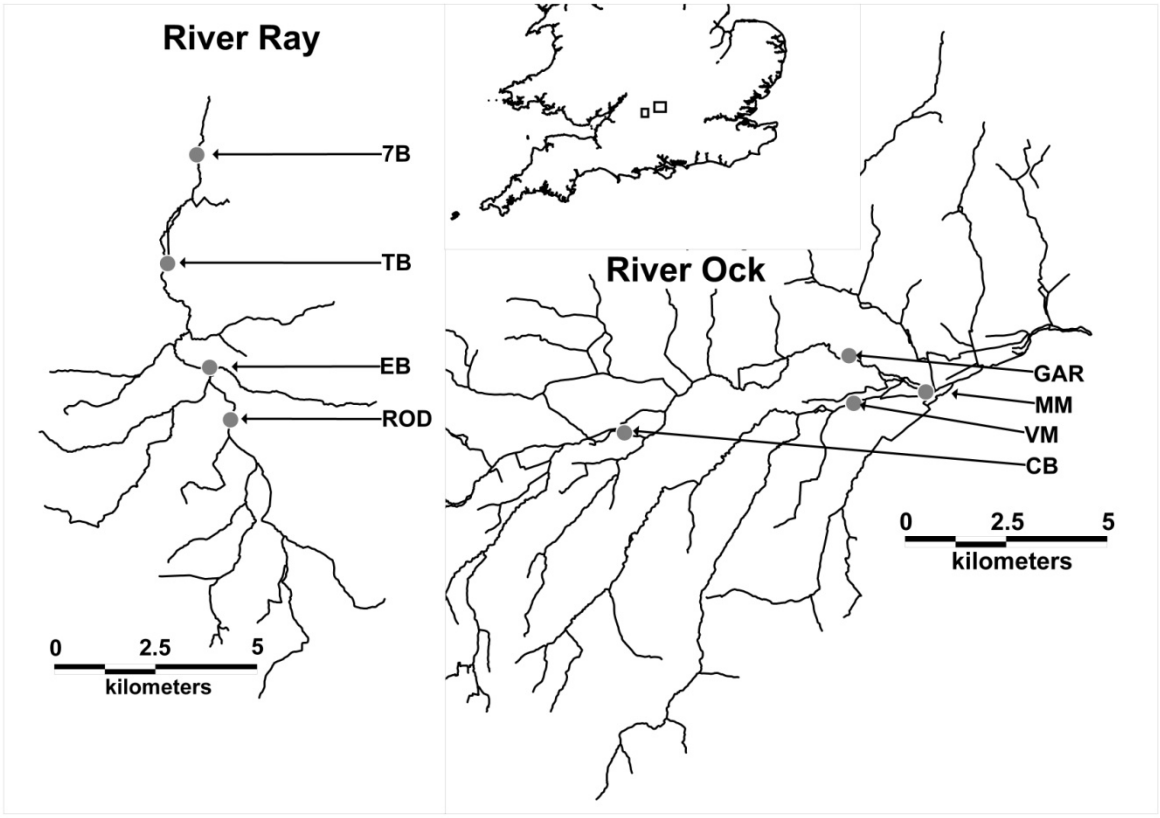

River Ray
7B
TB
EB
ROD
0
2.5
5
kilometers
River Ock
GAR
MM
VM
CB
0
2.5
5
kilometers

Figure 2.

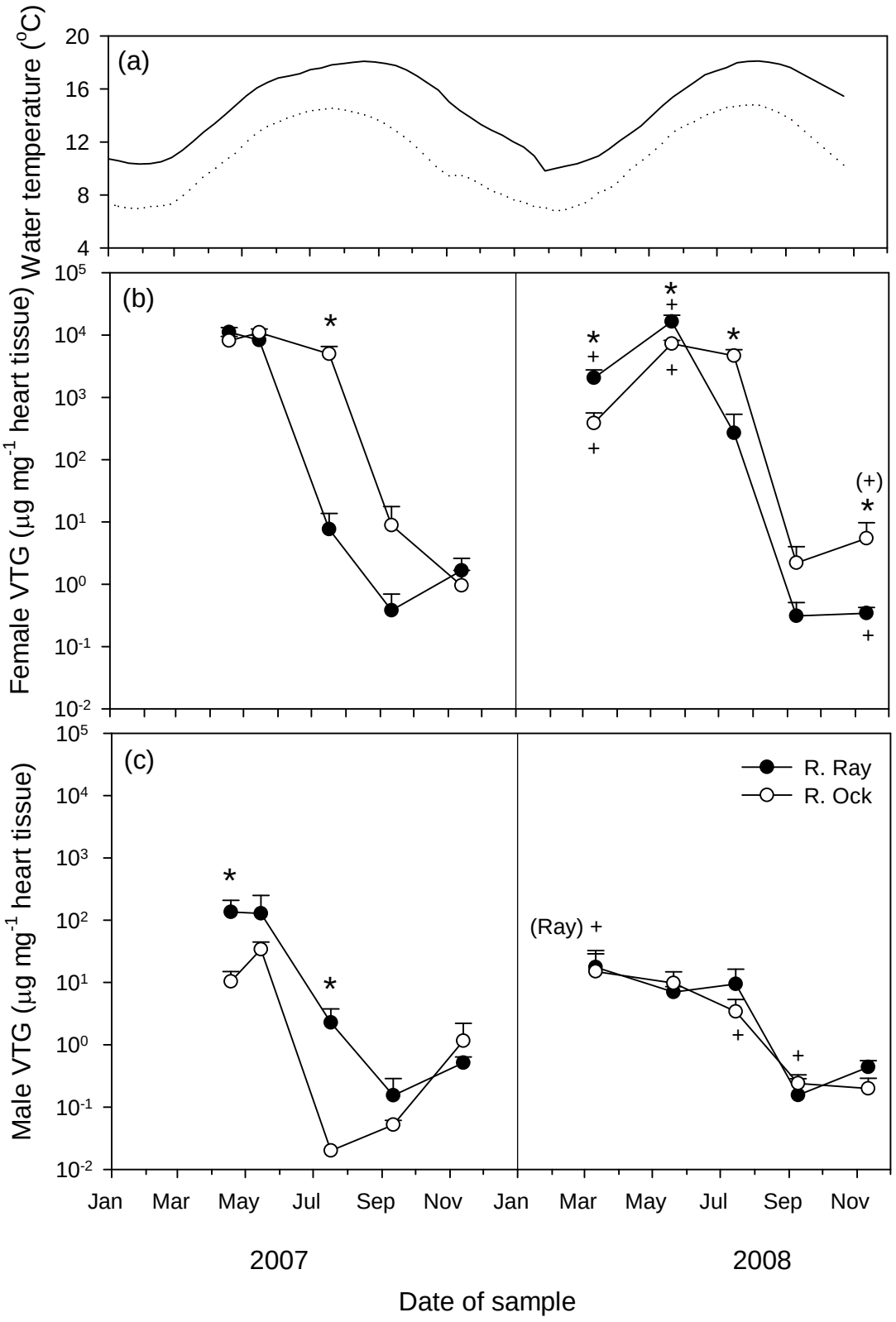

(a)
Water temperature (°C)
20
16
12
8
4
(b)
Female VTG (µg mg-1 heart tissue)
(c)
Male VTG (µg mg-1 heart tissue)
R. Ray
R. Ock
(Ray) +
(+)
Jan
Mar
May
Jul
Sep
Nov
2007
2008
Date of sample

Figure 3.

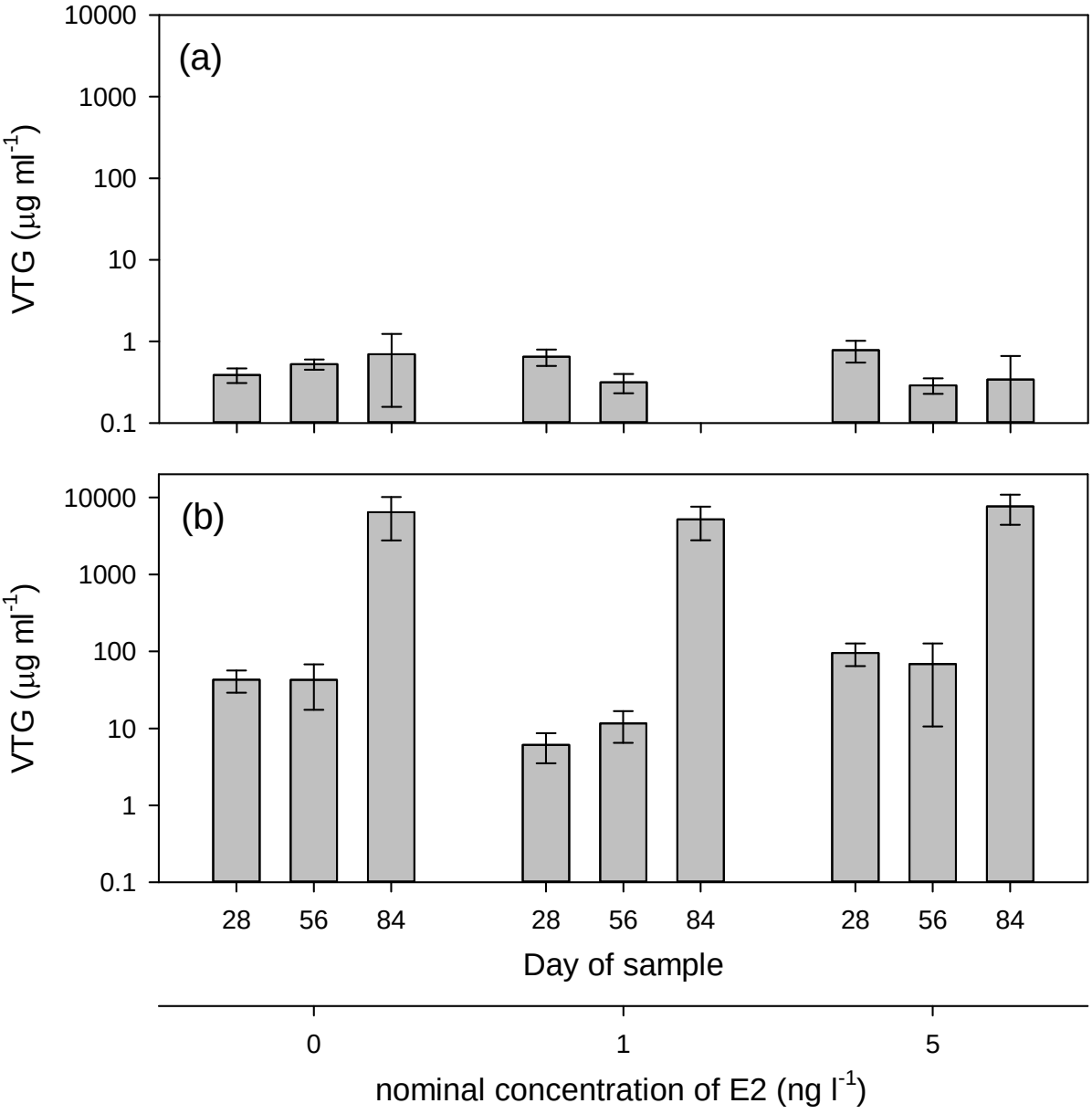

(a)
(b)
VTG (µg ml-1)
10000
1000
100
10
1
0.1
28
56
84
Day of sample
0
1
5
nominal concentration of E2 (ng l-1)

Figure 4.

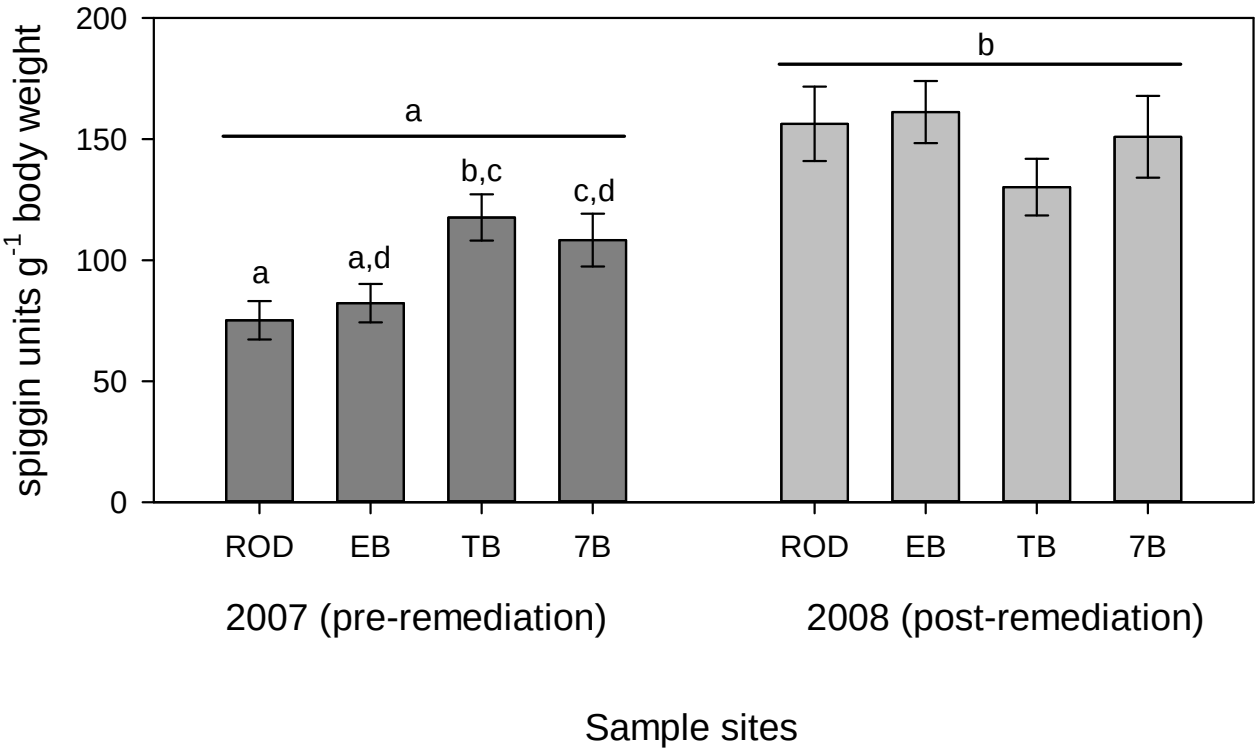
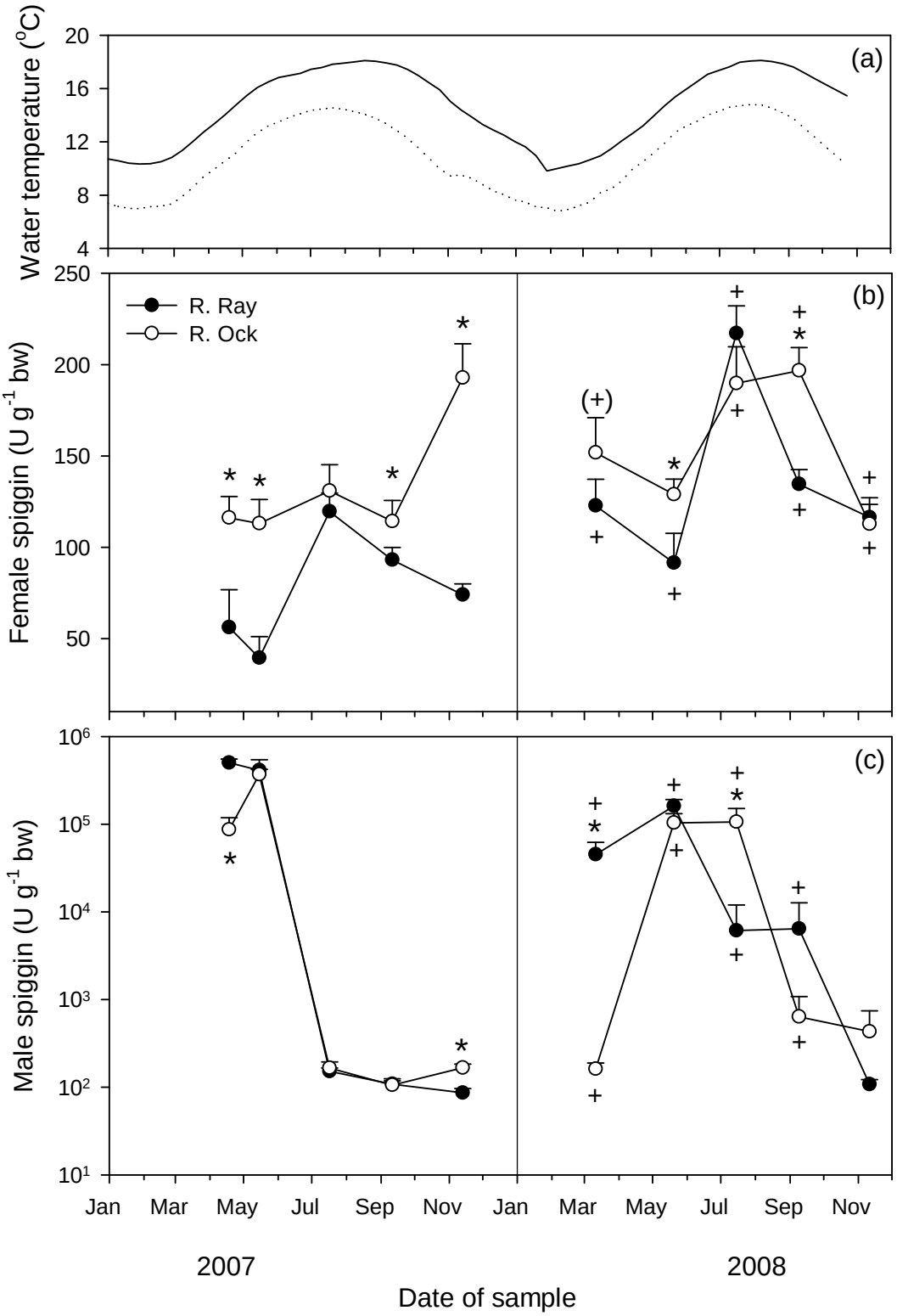

spiggin units g-1 body weight
200
150
100
50
0
a
b
a
a,d
b,c
c,d
ROD
EB
TB
7B
ROD
EB
TB
7B
2007 (pre-remediation)
2008 (post-remediation)
Sample sites

Figure 5.


Water temperature (°C)
20
16
12
8
4
(a)
Female spiggin (U g-1 bw)
250
200
150
100
50
R. Ray
R. Ock
(b)
(+)
Male spiggin (U g-1 bw)
10⁶
10⁵
10⁴
10³
10²
10¹
(c)
Jan
Mar
May
Jul
Sep
Nov
Jan
Mar
May
Jul
Sep
Nov
2007
2008
Date of sample