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Effects of sewage effluent remediation on body size, somatic RNA:DNA ratio, and markers of chemical exposure in three-spined sticklebacks

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ABSTRACT

Body mass, fork length, RNA:DNA ratio, specific growth rate, and hepatic EROD activity and CYP1A expression, were measured in three-spined sticklebacks in the River Ray (south west England) at sites downstream of an urban waste water treatment works (WWTW) prior to, and following, remediation of the effluent with granular activated carbon (GAC) tertiary treatment. During the same two-year period fish were also sampled from a neighbouring reference river (R. Ock). The WWTW effluent elevated water temperatures and nutrient content in the R. Ray and rendered a direct comparison of fish populations in the two rivers untenable. Instead, the stability of population parameters within each river during matched pre- and post-remediation periods was compared. Stickleback populations in both rivers were annual but fish in the R. Ray spawned earlier and were larger than those in the R. Ock. In the R. Ray fish gained mass throughout the winter months whereas in the R. Ock growth was much reduced during this period. In fish from the R. Ray the somatic RNA:DNA ratio remained elevated during May-November after remediation, rather than declining as in the same period pre-remediation and as was the case for fish in the R. Ock during both periods. The specific growth rate of the first post-remediation generation of sticklebacks in the R. Ray was higher than that of the previous pre-remediation generation. Following remediation there was no decline in hepatic EROD activity or in the abundance of hepatic CYP1A transcripts in fish in the R. Ray suggesting that the primary route of exposure to contaminants for these fish was not via the water column, and that the change in performance of the fish post-remediation was not impeded by continued exposure to contaminants. Both EROD activity and CYP1A expression increased in fish in the R. Ock during the later stages of the study suggesting that the fish in this river were exposed to an unidentified contaminant episode. This may have been linked with the poorer performance of fish in the R. Ock during the post-remediation period. The improved performance of fish in the R. Ray suggest that there may be factors in good quality secondary treated sewage effluent which can adversely influence the performance of fish populations, directly or indirectly, and which can be removed by tertiary treatment.

Key words: Stickleback, EROD, CYP1A, RNA:DNA ratio, Pollution, Waste-water treatment, Sewage, Remediation.

1. Introduction

In 2006 the Environment Agency of England and Wales and the Department for Environment Food and Rural Affairs established a National Demonstration Programme in the United Kingdom to evaluate the effectiveness of a range of water treatment options for the removal of endocrine disrupting chemicals (EDCs) from waste water effluent (Huo and Hickey, 2007). The demonstration programme involved the collaborative participation of ten water companies in England and Wales and was coordinated by UK Water Industry Research (UKWIR, 2009). As part of this programme, a granular activated carbon (GAC) system for the adsorption of EDCs was installed at Swindon waste water treatment works (WWTW; Rodbourne, Swindon, UK). The primary purpose of the upgrade was to eliminate from the effluent, or significantly reduce in concentration, steroid estrogens and other potential endocrine disrupters. The study described here was part of a project designed to evaluate some of the effects of the GAC upgrade on the fish populations downstream of the WWTW.

The three-spined stickleback (*Gasterosteus aculeatus* L.) was selected as the target species for the study because of its local abundance, ease of capture, and status as a model species for ecological, behavioural and ecotoxicological studies (Katsiadaki et al., 2002; Pottinger et al., 2002; Sanchez et al., 2008a; Huntingford and Ruiz-Gomez, 2009). The stickleback populations in the R. Ray and in a nearby reference river unaffected by WWTW discharges, the R. Ock, were sampled at intervals during the study period. No evidence for disruption of the endocrine reproductive system or of gonadal structure, was detected in the stickleback population in the R. Ray prior to remediation (Katsiadaki et al., unpublished). However, because the GAC adsorption process is effective in

removing a high proportion of organic molecules from solution (Kim et al., 2007; Snyder et al., 2007) the possibility that the remediation of the effluent affected targets other than the reproductive endocrine system was investigated. Indices of somatic status (weight, length, RNA:DNA ratio) were assessed for fish in both rivers, making the assumption that these high-level endpoints would collectively integrate the effects of any change in conditions in the R. Ray arising from effluent remediation and the R. Ock would in addition control for any influences on the fish populations of catchment-wide events. The activity of the monooxygenase cytochrome P4501A, as both ethoxyresorufin–O-deethylase (EROD) activity and as the relative abundance of cytochrome P450 1A (CYP1A) transcription, was measured in order to determine whether exposure of the fish to one subset of organic contaminants (polyaromatic hydrocarbons) typical of urban WWTW effluent was reduced following installation of the GAC plant.

2. Materials and methods

2.1. Sampling sites

Fish were sampled from four sites on each river (Fig. 1, Table 1). On the R. Ray the first of these was immediately adjacent to the WWTW outfall (mean dry weather WWTW discharge = 0.51 m^3/sec, data courtesy Thames Water; mean annual river discharge 1.34 m^3/sec, Water Eaton Gauging Station; Marsh and Hannaford, 2008) with additional sites 1.8 km, 5.9 km and 9.5 km downstream. Three reference sites were located on the R. Ock and one on the Childrey Brook, a major tributary of the R. Ock. These sites received very little waste water (maximum dry weather WWTW flow upstream of the sampling sites = 0.009 m^3/sec, data courtesy Thames Water; mean annual river discharge 1.55 m^3/sec, Abingdon Gauging Station; Marsh and Hannaford, 2008). Samples of fish were collected from both rivers on six occasions prior to the remediation of the effluent (April, May, July, September and November 2007 and January 2008) and on five occasions

following the commissioning of the GAC plant at the Rodbourne WWTW (in March, May, July, September and November 2008).

2.2. Water chemistry

Water temperature, dissolved oxygen (DO), pH, ammonium-N, conductivity and turbidity were recorded at 20 minute intervals using sondes (YSI 6920; Yellow Springs Instruments) sited 100m downstream of the Rodbourne WWTW effluent discharge point on the R. Ray (ROD), and at Charney Bassett (CB) on the R. Ock.

2.3. Fish capture and processing

Three-spined sticklebacks were captured with a large hand net (38 cm D-frame, 0.5 cm mesh, 1.5 m glassfibre handle). After capture, fish were held temporarily in buckets containing river water and then killed with a lethal dose of anaesthetic (2-phenoxyethanol, 1:1000). Fish were placed in individually labelled 12 ml polypropylene centrifuge tubes and frozen in a liquid N_2 dry shipper (Taylor-Wharton CryoExpress CX500, Jencons plc) for transfer to the laboratory. On arrival the samples were transferred to a freezer (-80°C) for storage until required for analysis. Tubes containing fish were removed from the freezer in groups of six and placed on ice. While still frozen, each fish in turn was removed from its tube and weight (mg) and fork-length (mm) were recorded.

A ventral incision was made using dissecting scissors and the heart and kidney were removed and stored frozen for subsequent VTG and spiggin analysis (Katsiadaki et al., unpublished). For a sub-set of fish in January and March 2008 livers were processed as described in 2.5. For an additional sub-set of fish in September and November 2007 and 2008 the liver was removed and placed into 1.0 ml RNAlater (Sigma-Aldrich) in a 1.5 ml capped polypropylene centrifuge tube on ice. The remainder of each fish was returned to the sample tube and placed in the freezer. Liver samples in

RNAlater were held at 4°C for 24h and then stored (-20°C) until required for RNA extraction. After dissections were completed, the remainder of the fish was homogenised to provide a substrate for the measurement of total RNA and DNA. 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 (-20^{o}C) until required for assay.

2.4. RNA:DNA ratio

Nucleic acids were extracted from homogenised stickleback tissue using 1% sarcosyl (N-lauroylsarcosine sodium salt) in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and quantified using a fluorescent dye-binding method (Quant-It RiboGreen reagent, RNA assay kit; Invitrogen) as described by Gorokhova and Kyle (2002). RiboGreen is a non-specific nucleic acid dye allowing estimation of total RNA + DNA fluorescence (excitation 485 nm, emission 535 nm; Fluoroskan Ascent FL; Thermo Scientific) and then, following RNAse treatment of the sample, quantification of fluorescence attributable to DNA alone and by difference between the two readings the RNA content.

2.5. EROD

Activity of the cytochrome P4501A monooxygenase, ethoxyresorufin–O-deethylase (EROD) was measured in liver tissue from fish collected in 2008 from the two rivers immediately prior to (January) and following (March) commissioning of the GAC plant. A microplate kinetic assay requiring small amounts of tissue was adopted, based on that described by Hodson et al. (1996).

Livers were homogenized in a constant volume of 400 µl phosphate buffer (pH 7.4, containing 20% glycerol; 1 mM EDTA; 1 mM dithiothreitol) in a 1.5 ml capped centrifuge tube, using a motor-driven pestle, then centrifuged at 4°C for 15 minutes. The assay was carried out in 96-well plates in a plate reader (Fluoroskan Ascent FL, Thermo Scientific) with excitation at 530 nm and emission at 590 nm. For the unknowns, each well of the plate contained 50 µl liver homogenate supernatant and 50 µl of 10.0 µM 7-ethoxyresorufin in homogenisation buffer. Protein concentrations in the liver homogenates were determined using the fluorescamine-based microplate method of Lorenzen and Kennedy (1993).

2.6. CYP1A gene expression

RNA was extracted from stickleback livers (RNeasy mini kit, Qiagen) and converted to cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). The yield and purity of RNA extracts was assessed at 260 nm using the Nanodrop ND-1000 spectrophotometer (Labtech). Relative expression of CYP1A was determined using a StepOne real-time PCR machine (Applied Biosystems). The sequences for amplification primers and a minor groove binding (MGB) Taqman fluorogenic probe were derived from previous work (Geoghegan et al., 2008). Primers CYP1AFP (5'-GGAATTGTCAATGACCTGTTTGG–3') and CYP1ARP (5'-CGGATGAGCCACCATGTACA-3') and the MGB Taqman probe CYP1ATP (5'-6FAM-ACACCGTCAGCACGACATTGTCATGG-3') were checked for specificity by using BLASTn (Altschul et al., 1997) within the NCBI suite of facilities (www.ncbi.nlm.nih.gov). All reagents and kits used in amplifications were obtained from Applied Biosystems. Optimum primer (300 nM) and MGB probe concentration (250 nM) were determined empirically using cDNA pooled from fish liver RNA extractions as template. Duplex relative gene expression real-time PCR assays were performed in 20 µl reaction volumes. All reactions received the following: 3 µl cDNA; 10 µl Taqman Gene Expression Master Mix (2x concentration); 2 µl of each primer (300 nM); 2 µl (250

nM) MGB and 1 µl Human 18S rRNA endogenous control mixture (containing limited concentration primers and VIC-labelled MGB Taqman probe specific to 18S rRNA). The cycling parameters of 1 cycle of 50°C for 2 min (activation of uracyl glycosylase) followed by 1 cycle of 95°C for 10 min (activation of Amplitaq gold) and 45 cycles of 95°C for 15 secs and 60°C for 1 min were maintained in all cases. In accordance with accepted guidelines for carrying out the comparative cycle threshold (C_t) method, the relative amplification efficiencies of template and endogenous control were tested and confirmed. Baselines and cycle threshold values were automatically calculated by the StepOne software. Expression of CYP1A in each sample was normalised to that of 18s rRNA using the equation $R = 1000*[(2^{Ct18s}) / (2^{CtCYP1A})]$ where R is relative expression level, and Ct is the cycle threshold for target and control genes. Amplification efficiencies were not adjusted for each sample. The overall mean C_t value of 9.0 obtained for the internal control gene (18s rRNA) was similar to the value previously reported for 18s in zebra fish liver (11.7; Filby and Tyler, 2007) and recent studies indicate that the expression of 18s remains constant in sticklebacks during exposure to chemical stressors (Williams et al., 2009). The overall mean C_t value for CYP1A was approximately 22.5.

2.7. Statistical analysis

Body mass, fork length, RNA:DNA ratios, EROD and CYP1A expression data were transformed ($\log_{10}$(data + 1)) where necessary and differences between means were evaluated with analysis of variance (ANOVA; Genstat for Windows V. 8, Lawes Agricultural Trust; Kruskal-Wallis ANOVA, SigmaStat 3.5, Systat Software Inc.). Multiple comparison post tests to assess significant differences between times or rivers were carried out using the estimated standard error of the differences between means (Genstat) or with pairwise multiple comparisons using Dunn's method (SigmaStat). Water chemistry data were analysed with a two-way ANOVA followed by Tukey's pairwise multiple comparison test (SigmaStat). For all analyses alpha was set at 0.05. The

Kolmogorov-Smirnov two-sample test (SYSTAT 11; Systat Software Inc.) was used to determine whether the length frequency distributions differed significantly between the two rivers. Specific growth rate (SGR) for fish in the R. Ray was calculated as percent day^{-1} for either mass or length: $SGR = [100*(\ln(Wt_2/Wt_1)/(t_2-t_1)]$ where Wt_2 and Wt_1 were the mean body mass or length for fish in November and July respectively and (t_2-t_1) was the number of days within this period. A paired t-test (SigmaStat) was used to compare SGR during the pre- and post-remediation periods.

3. Results

3.1. Water chemistry and physicochemical measurements

Seasonal variation in water temperature for both rivers was evident from data collected at the continuous monitoring sites (R. Ray: Rodbourne; R. Ock: Charney Bassett; Fig 2a; Table 2) and from that collected at time of sampling (Table 3). The two rivers exhibited a markedly different temperature regime during the two-year monitoring period with the mean temperature in the R. Ray downstream of the Rodbourne WWTW in both years significantly higher than that in the R. Ock ($P<0.001$). There was a negligible decline in water temperature at sites downstream of the WWTW (Table 3) and a temperature difference between the two rivers of at least $2 – 3^{o}C$ was sustained at all sites and in both years (Table 3). Mean DO concentration (as percent saturation) was higher in the R. Ock than in the R. Ray ($P<0.01$; Fig. 2b, Table 2) during 2007. Following the installation of the GAC plant in February 2008, DO concentrations in the R. Ray were significantly elevated compared to pre-remediation levels ($P<0.01$) but remained lower overall than those in the R. Ock ($P<0.01$). The pH was significantly lower in the R. Ray than in the R. Ock ($P<0.001$) and no changes in pH were evident in the R. Ray following the installation of the GAC plant (Table 2). Ammonium–N concentrations were significantly higher in the R. Ray than in the R. Ock in both 2007 and 2008 and higher in 2008 than 2007 in both rivers (Table 2). Higher conductivity and

turbidity levels in the R. Ray indicated that the R. Ray contained higher concentrations of suspended and total dissolved solids than the R. Ock (Table 2).

3.2. Body mass, fork length, sex ratio

The data from Childrey Brook (Venn Mill; VM) were combined with those from sites on the R. Ock and the aggregate data are referred to as R. Ock throughout. Fish abundance data were unavailable because of practical constraints. Mark-recapture methods for abundance estimation were not adopted because of the unrestricted movement enjoyed by the resident fish and quantitative estimation of abundance by catch depletion and timed electric fishing was also unsuccessful because of the preferred habitat of the fish. The size selectivity of the mesh in the dip nets dictated that no fish smaller than 15 mm in length were caught. The distribution of three-spined sticklebacks at each sample site (comprising approx. 200m of river) was patchy with fish captured in areas characterised by low flow and the presence of submerged and emergent macrophytes. The stickleback populations in both rivers were annual, with few adult (1+) fish surviving beyond the spawning period into a second year, although the loss of adults post-spawning was most pronounced in the R. Ray. The shape and location of the length frequency distributions for the populations in the two rivers differed significantly ($P<0.001$). Fish in the R. Ock were smaller overall (mean ± SEM: mass, 663 ± 13 mg; length, 38.3 ± 0.2 mm; n = 782) than those in the R. Ray (mass, 841 ± 20 mg; length, 41.9 ± 0.3 mm; n = 663). No significant overall difference in body mass was evident between male and female fish during 2007 and 2008 (M: 749 ± 18 mg, n = 570; F: 742 ± 16 mg, n = 875; $P > 0.05$) although there was a small but significant difference in length (M: 40.3 ± 0.3 mm; F = 39.7 ± 0.3 mm; $P<0.05$). For clarity, data for both sexes were combined in order to compare the pre-remediation (2007) and post-remediation (2008) periods. During 2007 the sex ratio deviated from unity among both the fish caught from the R. Ray (M/F = 0.69) and those caught from the R. Ock (M/F = 0.63; $P<0.001$; Chi square test). However, during 2008, the sex

ratio in the R. Ray did not deviate significantly from unity (M/F = 0.79) whereas the R. Ock population remained biased in favour of females (M/F = 0.53). Direct observation of fish in spawning condition coupled with the results of the histological examination of gonadal tissue carried out during 2007 (C. Mungo, unpublished.) confirmed that fish in the R. Ray spawned earlier than those in the R. Ock (April/May and May onwards respectively).

Length frequency plots for the bi-monthly matched samples collected from the R. Ray in May, July, September and November of 2007 and 2008 (Fig. 3a-d) show that during May 2007 (Fig. 3a) both adults (1+ years old) and juveniles (0+ years old) were present whereas the distribution was unimodal from July onwards (Fig. 4a,b). Clearly separated year classes were not present in the length frequency distributions for the R. Ock (Fig. 3e-h), with an overlap in size from July onwards between the juvenile fish and surviving adults evident in the extended upper tail of the distributions. For fish in the R. Ray, mean body mass (Fig. 4a) and mean fork length (Fig. 4b) increased significantly ($P<0.001$) from July 2007 to a maximum in May 2008, a period during which the majority of fish captured could confidently be assumed to be of the 07/08 year class. During this period mean body mass for fish in the R. Ray increased more than 200% and fork length increased by 40%. For fish in the R. Ock, the increase in mean body mass (Fig. 4a) and fork length (Fig. 4b) during the same period was significant ($P<0.001$) but proportionally less than that observed for fish in the R. Ray with mass increasing by 84% and length by 20%. The increase in both mean length and mass among fish in the R. Ock was interrupted by a significant ($P<0.001$) decline in both parameters between January and May 2008. A direct comparison of the matched pre- and post- remediation periods show that the pattern of growth for fish in the R. Ray was similar during both periods (Fig. 4a,b). The mean mass and length of the first post-remediation generation of fish in the R. Ray sampled in July and November 2008 were not significantly different from those of fish sampled at the corresponding times in 2007. For fish in the R. Ock a different pattern was evident. Whereas during 2007 a clear and significant increase in both mean mass ($P<0.05$; Fig. 4a) and

length (P<0.001; Fig. 4b) occurred between July and November, during 2008 mean mass and length of fish in the R. Ock both declined significantly during this period (P<0.05). When mass and length of fish at different sites in the R. Ray during the period July-November was examined (data not shown) no significant consistent difference was evident between pre- and post-remediation periods, with the exception of fish at the site immediately adjacent to the WWTW discharge which were significantly smaller in the post-remediation period (P<0.01). However, both length and mass showed a significant downward trend with distance downstream from the WWTW. Fish captured at the site farthest downstream on the R. Ray (7B) were significantly (P<0.001) smaller than those captured closer to the WWTW discharge (ROD, EB). No significant between-site differences were evident for either mass or length for fish in the R. Ock during the pre-remediation period (2007). However, during the post-remediation period in 2008 fish captured at Venn Mill (VM) and Marcham Mill (MM) were significantly larger than those at the other two sites (P<0.001). For fish in both the R. Ray and R. Ock mass varied significantly across monitoring periods when length was included as covariate indicating variation in condition (ANCOVA, P<0.001). Overall, coefficients of condition were higher in both rivers in 2008 (R. Ray 1.06 ± 0.007; R. Ock 1.113 ± 0.007) than 2007 (R. Ray 1.001 ± 0.01; R. Ock 1.04 ± 0.01).

3.3 RNA:DNA ratios and specific growth rates

There was a significant overall difference in mean RNA:DNA ratios between male and female fish in both rivers (P<0.001; female: 1.862 ± 0.02; male: 1.415 ± 0.03) but both sexes exhibited the same pattern of change with time and so the data have been combined for clarity (Fig. 5). The RNA:DNA ratio showed a seasonal periodicity for fish in both rivers with higher ratios coinciding with higher water temperatures but the pattern of change within each year was different. In both rivers, the RNA:DNA ratio declined significantly between May and November 2007 (P<0.001; Fig 6) across a broadly similar range (~2.0 - ~ 1.0). Between November 2007 and March 2008, there

was a significant increase in RNA:DNA ratio in fish from the R. Ray (P<0.001; Fig. 5a) whereas in fish from the R. Ock no significant change in RNA:DNA ratio occurred during this period (Fig. 5b). In both rivers the RNA:DNA ratio showed significant increases between March and May 2008 with fish in the R. Ock increasing by 200%. Thereafter ratios in fish from the R. Ray remained at the levels achieved during May whereas in fish from the R. Ock the ratio declined significantly by more than half between May and November 2008 (P<0.001; Fig. 5b). In order to assess whether the difference in the RNA:DNA ratios in fish in the R. Ray between the pre-and post-remediation periods was reflected in performance of the fish, the specific growth rates (SGR) for fish in the R. Ray were examined. SGRs were calculated for the period when the population was composed of juvenile fish only (July-November). In the R. Ock, the length frequency distributions (Fig. 3e-h) and declining mean mass and length for this period (Fig. 4a,b) strongly suggested that the previous years adult fish survived longer than was the case in the R. Ray, resulting in a mixed year class population and rendering the calculation of SGR via population means inappropriate. To compare the SGR of fish in the R. Ray between 2007 and 2008, values were calculated for fish at each of the sample sites on the R. Ray. At three sites the SGRs for both length (Fig. 6a) and mass (Fig. 6b) during the period July-November in 2008 were greater than those in the corresponding period in 2007 and collectively this difference was significant (P<0.05). The clear exception to this pattern was Tadpole Bridge (TB) at which there was a marked decline in SGR for both length and mass of the fish between 2007 and 2008.

3.4 EROD

In the samples collected during January 2008 (pre-remediation; Fig. 7a) EROD activity was significantly greater in fish collected from sites in the R. Ray compared to those from sites on the R. Ock. However, in March 2008 (post-remediation; Fig. 7b) there was no difference in EROD activity evident between fish from the two rivers. In March 2008 EROD activity within samples from the R.

Ray was similar to that observed in January (range 6 – 13 pmol min^{-1} mg $protein^{-1}$) whereas EROD activity in fish from the Ock was increased in March compared to that in January (< 3 pmol min^{-1} mg $protein^{-1}$) to levels statistically indistinguishable from those in the Ray (7 – 13 pmol min^{-1} mg $protein^{-1}$). No significant differences between the sexes were evident during January 2008 (male: 4.2 ± 0.8, n = 52; female = 5.2 ± 1.1, n = 61) but in March 2008 EROD activity in males was slightly but significantly greater than that in females (male: 11.0 ± 0.7, n = 41; female = 9.0 ± 0.7, n = 59; $P<0.05$).

3.5 CYP1A gene expression levels

In 2007, there was no significant difference between rivers overall in relative expression levels of CYP1A (Fig. 8a) although significant differences between sites both within and between rivers were evident. For fish from the R. Ray there was a significant increase in CYP1A expression between Rodbourne WWTW and Tadpole Bridge ($P<0.05$). On the R. Ock, CYP1A expression was lowest in fish from Charney Bassett and significantly higher in fish from Venn Mill and Marcham Mill ($P<0.05$). There was a significant increase in expression of CYP1A in fish from the R. Ock between 2007 and 2008 ($P<0.05$), but not in fish from the R. Ray and in 2008 (Fig. 8b) CYP1A expression was overall significantly greater in fish from the R. Ock than in fish from the R. Ray. For fish from the R. Ray in 2008 a similar spatial trend to that observed in 2007 was evident with CYP1A expression levels significantly higher in fish from Tadpole Bridge compared to those captured at Rodbourne ($P<0.05$; Fig. 8b). No significant difference in CYP1A expression was evident between the sexes.

4. Discussion

The Rodbourne WWTW, which serves a population of approximately 135,000 in the south west of

England, is a modern activated sludge plant with biological nutrient removal. The effluent discharged is consequently of a relatively high standard. However, the R. Ray, into which the WWTW discharges, is a small river with limited dilution capacity. Water temperatures upstream of the effluent discharge point on the Ray were similar to those in the R. Ock (D. Grover, pers comm.) whereas downstream of the discharge the water temperature in the R. Ray was consistently 2-3°C higher than that in the R. Ock. The mean DO concentration in the R. Ray was lower than that in the R. Ock and dissolved nutrients and suspended and dissolved solids (indicated by higher NH_3-N concentrations, greater turbidity and higher conductance values) were higher in the R. Ray. The physicochemical conditions in the R. Ray attributable to the WWTW discharge rendered direct comparisons of the fish population in the R. Ray with that in the R. Ock inappropriate. Therefore, in order to detect effects that might be linked with effluent remediation following installation of the GAC plant, four samples of fish collected during each of two matched periods (May, July, September, November) before (2007) and after (2008) the upgrade were compared between years within rivers. It was assumed that variation in local environmental conditions that might affect the fish populations (e.g. extreme weather events) would affect both rivers similarly.

A modelling exercise and chemical analysis of the receiving water (Balaam et al., 2010) indicated that prior to remediation the mean annual total estradiol-equivalent concentrations downstream of the discharge were in excess of 1 ng l^{-1} which is the predicted no-effect concentration for endocrine disruptive effects in fish (Young et al., 2004). However, no evidence of such effects was detected in measurements of vitellogenin or spiggin, or in gonadal structure, in sticklebacks sampled from sites downstream of the WWTW discharge (Katsiadaki et al., unpublished; Mungo, unpublished). Operation of the GAC plant was found to significantly reduce or completely eliminate steroid estrogens (estrone, 17β-estradiol, 17α-ethinylestradiol) and total estrogenic activity in the effluent (Balaam et al., 2010). Granular activated carbon treatment is reported to remove up to 99% of dissolved organic contaminants from waste water (Ternes et al., 2002; Kim et al., 2007; Snyder et

al., 2007) and it can therefore be assumed that the decline in steroid estrogens was matched by a decline in the total organic chemical load delivered by the pre-remediation effluent.

4.1. Population structure

Interpretation of the somatic data for sticklebacks in the R. Ray and R. Ock was constrained by having only a single year of matched pre- and post-remediation observations. The abundance and mean size of sticklebacks in a single population can vary markedly across years (Wootton and Smith, 2000) and the original study design allowed for a 2-year monitoring period both before and after effluent remediation but the GAC plant was installed and commissioned ahead of schedule. Taken collectively, the somatic data indicate that the pre-remediation Ray, despite receiving a significant volume of WWTW effluent, was not a hostile environment for the resident stickleback population. The stickleback populations in both the R. Ray and R. Ock were found to be annual, with few adults surviving beyond the spawning period and into a second year, and with the sex ratio in both rivers biased towards females. These observations are consistent with previous reports of the age structure (Mann, 1971; Allen and Wootton, 1982; O'Hara and Penczak, 1987; Wootton and Smith, 2000; Poizat et al., 2002; Wootton, 2007) and sex ratio (Kynard, 1978; Wootton, 1984; Mori, 1993; Arnold et al., 2003) of three-spined stickleback populations. Disproportionate mortality among males due to the energetic demands and risks associated with male parental investment has been suggested to account for the female bias (Chellappa et al., 1989; Arnold et al., 2003). However, in the present data set, the bias in sex ratio was not associated specifically with the post-spawning period. The ratios for the July, September and November samples, during which the bulk of the population would have been 0+ fish with no prior reproductive experience, were similar to those that were obtained from the sample period in its entirety. The observed sex ratio may have arisen in part from sustained differences in the relative "catchability" of male and female fish due either to differences in net evasion or to different patterns of dispersion. No histological evidence of

gonadal abnormalities, in particular intersexuality, was detected among fish from the R. Ray (Mungo, unpublished) but the possibility that the sex ratio of fish in both rivers was influenced by exogenous factors cannot be excluded. The phenotypic sex of fish can be modified by exogenous steroids and other contaminants (Devlin and Nagahama, 2002) and the EROD and CYP1A data suggest that the R. Ock, contrary to expectations, was not a chemically pristine environment. Fish in the R. Ock were subject to intermittent exposure to inducers of this enzyme system, and therefore possibly other classes of chemicals as well, although the source, identity and concentration of contaminants is unknown. It therefore remains a possibility that the imbalance in sex ratio that was evident among fish from both rivers arose from exposure to chemicals during a critical developmental window of sensitivity (Hahlbeck et al., 2004). In this respect it is interesting that the sex ratio among fish in the R. Ock remained significantly biased in favour of females in 2008 whereas following remediation in the R. Ray, the ratio was not significantly different from unity.

4.2. Body mass, fork length and specific growth rate

Sticklebacks in the R. Ray were larger overall than those from the R. Ock. Inter-population differences in the growth of sticklebacks may be in part attributable to heritable traits (Wright et al., 2004) but in this case effects of the WWTW effluent on physicochemical characteristics of the R. Ray are likely to have been the primary cause of overall differences in size of the fish in the two rivers. Both abiotic (temperature, oxygen availability) and biotic (food abundance, predation) factors are likely drivers of variation in individuals size between stickleback populations (Poulin and Fitzgerald, 1989; Wootton and Smith, 2000) with water temperature and food availability the two most important factors determining growth rate in fish (see Graham and Harrod, 2009, for references). Higher temperatures in themselves promote metabolic activity in poikilotherms and the warmer water coupled with the nutrient content of the effluent (exemplified by higher ammonium- N concentrations in the R. Ray) is likely to have resulted in a more abundant food supply in the R.

Ray (Friberg et al., 2009), typical of rivers downstream of modern WWTW discharges (e.g. Gücker et al., 2006). There was a consistent trend for fish to decline in size with increasing distance downstream from the WWTW but the water temperature changed little downstream of the WWTW discharge, suggesting that a direct effect of temperature on the fish themselves was not the primary driver for the size difference between the fish from the R. Ray and R. Ock..

In the R. Ray, fish exhibited a pattern of growth similar to that reported for stickleback populations in Mediterranean streams (Clavero et al., 2009) with growth continuing through the winter months This resulted in fish with mean lengths in May in excess of those reported for one-year old sticklebacks in other UK populations (Mann, 1971; Allen and Wootton, 1982; O'Hara and Penczak, 1987) and with clear separation between the current (0+, 2008) and previous (1+, 2007) year classes. In July very few fish that could be apportioned to the preceding year class were caught in the R. Ray. The disappearance of post-spawning adult sticklebacks in early summer has been reported by others (Mann, 1971; Clavero et al., 2009) and it is only possible to speculate that the larger adults evident in the May sample from the R. Ray in both years either evaded capture in subsequent months, or succumbed to disease or size-selective predation. There was no statistically significant difference in the mean body mass and fork length of juvenile (0+) sticklebacks in the R. Ray during July-November 2008 (post-remediation) and 2007 (pre-remediation). However, differences in growth rate were evident between these periods, with specific growth rates of fish at three out of four sites on the R. Ray significantly greater following remediation than during the pre-remediation period. Interestingly, growth rate did not increase but actually declined between 2007 and 2008 at Tadpole Bridge, the site at which the highest CYP1A expression levels for fish in the R. Ray were recorded.

In the R. Ock, the annual growth profile for sticklebacks resembled that reported for other populations in the UK (Allen and Wootton, 1982) with little growth evident between November and

March, contrasting with the continued growth of fish in the R. Ray during this period.
Clear allocation of the fish in the R. Ock to either the 0+ or 1+ year classes was not possible, with overlap in the length frequency distributions evident during both years. The length frequency distributions for fish in the R. Ock suggested that 1+ adults were present throughout the summer months as is the case in some other UK stickleback populations (Allen and Wootton, 1982; O'Hara and Penczak, 1987). The presence of two overlapped year classes, with recruitment of 0+ fish and loss of 1+ individuals through the summer of 2008, presumably accounted for the apparent decline in both mean mass and length of the R. Ock population during this period, a trend that was not evident in 2007 during which mean length and mass increased consistently during the summer months. Inspection of the length frequency distributions for 2008 suggests that 1+ fish may have been present in the R. Ock as late as November. This contrasting profile suggests that some aspect of the population dynamics of fish in the R. Ock (recruitment, adult survival) differed between years. It is tempting to speculate that the different growth profile for fish in the R. Ock during 2008 compared to 2007 was related to the higher levels of chemical exposure during 2008 evidenced by the EROD and CYP1A data for fish in this river.

4.3. RNA:DNA ratio

The difference in size of the fish between rivers was consistent with the time-course of changes in the RNA:DNA ratio data. Whole-body and white muscle RNA:DNA ratios have been widely used to assess the condition and growth of fishes (Richard et al., 1991; Buckley et al., 1999; Zhou et al., 2001) exploiting the fact that the concentration of DNA in somatic tissues is relatively constant whereas the concentration of RNA is proportional to the amount of protein synthesis taking place (Clemmesen, 1994; Chícharo and Chícharo, 2008). In three-spined sticklebacks the somatic RNA:DNA ratio has been shown to be correlated with growth rate (Ali and Wootton, 1998, 2003) and to decline markedly during fasting (Pottinger et al., 2002). The RNA:DNA ratio provides a

useful adjunct to other somatic data by providing information on growth status related to conditions pertaining at the time of capture of the individual. In the present study the RNA:DNA ratios of the two populations tended to vary with the annual temperature cycle and confirmed that fish in the R. Ray experienced a longer growing season than those in the R. Ock. The RNA:DNA ratio for fish in the R. Ray showed a progressive increase between November 2007 until the summer of 2008 whereas for fish from the R. Ock during this period the RNA:DNA ratio did not increase until between March and May 2008. This 4 month period of stasis in anabolic activity in the R. Ock probably reflected a combination of limited food supply in the R. Ock during the winter, and low water temperature, which ranged between daily means of 2.9 – 10.5^{o}C during November – March in the R. Ock in contrast to higher temperatures in the R. Ray of 7.5 – 14.1^{o}C during the same period. The range of mean RNA:DNA ratios observed in this study (~1.0 - ~3.0) was similar to those observed in laboratory studies in which sticklebacks were provided with maintenance rations (Ali and Wootton, 1998) but were lower than those reported for studies in which ration size was larger (Ali and Wootton, 1998, 2003). In the R. Ock, the broad pattern of change in RNA:DNA ratio was similar in both 2007 and 2008, with maximum activity occurring in April/May followed by a steady decline through to November and this pattern was also observed in fish from the R. Ray in 2007. However, during the post-remediation period RNA:DNA ratios did not decline after May in fish in the R. Ray but instead remained at a similar level until November. This observation may be tied closely with the higher specific growth rates exhibited by juvenile fish in the R. Ray during this period in 2008 compared to the same period in 2007.

In this context it should be noted that DO concentrations in the R. Ray prior to the GAC plant coming online, were lower than those in the R. Ock, but markedly improved after installation of the GAC system, possibly due to a reduced biological oxygen demand. The extent to which the DO concentrations at Rodbourne WWTW were representative of those further downstream of the WWTW was not documented but the effluent-induced temperature differential between the R. Ock

and R. Ray was sustained throughout all sample sites. The net effect on the fish population in the R. Ray of increasing DO concentrations is likely to have been beneficial. Hypoxia has been reported to adversely affect reproductive processes in fish (Wu et al., 2003) and although a DO concentration in the order of 70% saturation (~ 6.5 mg / litre O_2 at 20°C) is unlikely to be directly harmful to sticklebacks, the interactive effects of concurrent exposure to chemical contaminants, elevated water temperature and reduced oxygen availability may be of functional significance. The possibility that increased DO had a positive effect on factors indirectly affecting performance of the sticklebacks (e.g. the food supply) must also be considered.

4.4. Biomarkers of chemical exposure

Alterations in the chemical composition of the WWTW effluent may have affected the fish in the R. Ray indirectly, via effects on the abundance of food items. However, direct adverse effects on growth in fish are reported for exposure to sub-lethal concentrations of a wide range of aquatic contaminants (e.g. Alvarez and Fuiman, 2005; Amara et al., 2009) including sewage effluent (Ma et al., 2005) so this cause cannot be excluded. Prior to 2008 the Rodbourne effluent contained a mixture of organic chemicals including steroid estrogens (estrone, estradiol-17β, 17α- ethinylestradiol), anti-androgens, personal care products (triclosan), pharmaceuticals (ibuprofen, naproxen, ketoprofen), phenols (octylphenol, bisphenol-A) and polycyclic aromatic hydrocarbons (PAHs) (Readman et al., unpublished data). No evidence of endocrine disruption or anomalous gonadal structure attributable to estrogenic or androgenic influence was detected among fish in the R. Ray (Katsiadaki et al., unpublished). However, the broader functional consequences for fish of exposure to a complex mixture of chemicals, particularly where most individual components of the mixture are present at levels below those recognised as harmful, is uncertain.

4.4.1. EROD activity

Measurement of the activity of cytochrome P450 isoform CYP1A is widely employed to evaluate the exposure of fish to planar organic contaminants (Whyte et al, 2000). This isoform is induced by dioxins, dibenzofurans, polychlorinated biphenyls (PCBs), PAHs, and other compounds that bind to the aryl hydrocarbon receptor (AhR). Measurement of the activity of the monooxygenase 7-ethoxyresorufin-O-deethylase (EROD) provides a surrogate for direct quantification of CYP1A expression. In the present investigation, assessment of both EROD activity and CYP1A gene expression was conducted to evaluate the status of CYP1A-inducing chemicals in the WWTW effluent after remediation.

EROD activity was quantified in fish from the two rivers immediately prior to and following commissioning of the GAC plant (January and March 2008 respectively). The mean EROD activity levels reported here (1-13 pmol min^{-1} mg $protein^{-1}$) are comparable with the lower range of EROD activities, of between 2.6 and 43.3 pmol min^{-1} mg $protein^{-1}$, that have been reported for sticklebacks exposed to varying degrees of chemical contamination (Sanchez et al., 2007; Wartman et al., 2009). Activity in the range 0.5 - 10 pmol min^{-1} mg $protein^{-1}$ has been reported for laboratory controls and sticklebacks from ostensibly unpolluted sites (Geoghegan et al., 2008; Sanchez et al., 2008a). Although low in comparison to these reported ranges of activity, during January 2008 EROD activity in fish from the R. Ray was nonetheless markedly higher than that in fish from the R. Ock, suggesting a difference in chemical loading between the rivers. Approximately one month following the GAC upgrade on the R. Ray no decline in EROD activity in sampled fish was evident. This may reflect the relatively short period elapsed since commissioning of the GAC plant, or that the WWTW effluent was not the primary cause of the EROD response. If the WWTW was the major source of EROD-inducing chemicals in the R. Ray, and WWTW effluent is certainly documented as an inducer of CYP1A activity in fish (e.g. McArdle et al., 2000), a sustained response might also have arisen if the exposure route of the fish was not primarily via the water column but instead via ingestion of contaminated food items and thus more closely linked to the

chemicals associated with the sediments and detritus (O'Hara and Penczak, 1987; Gewurtz et al., 2000; Rice et al., 2000; Meriläinen and Oikari, 2008). During the period in which fish populations were monitored, sediment PAH concentrations were highest at Elborough Bridge (EB; the first site downstream of the WWTW) lower at Seven Bridges (7B; the site most distant from the WWTW on the Ray) and were lower than both these at Charney Bassett (CB) on the R. Ock (Readman et al, unpublished data). EROD activity in fish from the R. Ock matched or exceeded that observed in fish from the R. Ray during March 2008. This represented an approximately 12-fold increase over values in January 2008. It is unlikely that seasonal variation in enzyme activity alone could account for this change. In an earlier study no substantial seasonal variation in EROD activity was observed in sticklebacks between April and October (Sanchez et al., 2008b). Instead it is more probable that fish in the R. Ock were exposed to a chemical challenge of unknown origin between January and March 2008. This supposition is supported by consideration of the CYP1A expression data.

4.4.2. CYP1A expression

Levels of CYP1A expression showed little change in fish from the R. Ray between the pre- and post-remediation periods, consistent with the pattern of EROD activity. Expression of CYP1A in fish from the R. Ock was higher during 2008 than 2007 and significantly higher overall than that in fish from the R. Ray during 2008 which was again consistent with the EROD results. Expression of CYP1A varied significantly between sites on the R. Ray, exhibiting a similar pattern in 2007 and 2008. If the variation apparent in CYP1A expression was primarily a function of exposure of the fish to chemical contaminants then the higher CYP1A expression levels in fish captured at sites downstream of the WWTW suggests that the primary route of exposure was not to chemicals dissolved in the water column, or that the WWTW was not the only source of CYP1A-inducing chemicals on the R. Ray. Concentrations of chemicals in the water column tended to be highest at the point of discharge but were sustained downstream with relatively little decline as far as the Seven Bridges site 9.5 km from the point of discharge (e.g. bisphenol-A, estradiol-17β; Readman et

al., unpublished data; Grover et al., unpublished data; Balaam et al., 2010). However, the concentrations of most chemicals detected in sediment samples were higher at Elborough Bridge and Seven Bridges, the second and fourth sites downstream of the WWTW, than in samples collected immediately downstream of the WWTW (e.g. PAHs; Readman et al., unpublished data) matching more closely the pattern of CYP1A expression. Persistence of contaminants in the sediment might explain why no decline in EROD and CYP1A activity was detected in fish from the R. Ray after the GAC plant was commissioned, if the primary route of exposure for the fish was via ingestion of contaminated benthic invertebrates (Logan, 2007). It also suggests that exposure of the resident fish populations to contaminants may continue for some time after remediation of the WWTW effluent as the sediment-associated contamination depurates. However, CYP1A induction is a specific response to only a sub-set of the chemical contaminants present in the pre-remediation WWTW effluent and a positive response post-remediation does not mean that exposure to the entire suite of contaminants continued after installation of the GAC plant.

The higher CYP1A expression levels in fish taken from the R. Ock in 2008 compared to 2007 together with the elevated EROD activity detected in fish from the R. Ock during 2008 suggest fish in the R. Ock during this period were exposed to an increased level of chemical contamination. The Charney Bassett (CB), Garford (GAR) and Marcham Mill (MM) sites were on the R. Ock, whereas the Venn Mill (VM) site was on the conjoining Childrey Brook so a localised upstream point source of contamination seems unlikely. It may be the case that sufficient road run-off entered both rivers during periods of high rainfall to raise the levels of PAHs and other chemicals. In sediments collected during 2007 three PAHs identified with road run-off (pyrene, fluoranthene, phenanthrene; Boxall and Maltby, 1997) were quantitatively dominant at sites on the R. Ray (urban) but were present at very much lower concentrations in the R. Ock (Readman et al., unpublished data).

5. Conclusion

Two indicators of performance in the resident stickleback population in the R. Ray, specific growth rate and RNA:DNA ratio, were higher in fish downstream of a municipal WWTW in the period following the installation of a tertiary GAC process than during the equivalent period prior to remediation of the effluent. This improvement in performance may have been related to the elimination of direct effects on the fish of a complex effluent-derived chemical challenge, or may have arisen due to indirect factors such as the increase in dissolved oxygen concentrations following remediation, or effects of effluent remediation on food abundance. The changes in performance detected in fish from the R. Ray were apparent despite evidence from specific biomarkers that exposure to some chemical contaminants, possibly via the ingestion of sediment-associated food items, was sustained into the post-remediation period. It was not possible to directly compare the stickleback population in the R. Ray with that in the R. Ock, a river in close proximity to the study site but unaffected by WWTW discharges, because of the higher water temperature and nutrient loading in the R. Ray brought about by the effluent which resulted in very different population structures and growth profiles for the two rivers. In the R. Ock the population growth profile differed between years and both biomarkers of chemical exposure that were measured indicated that during the second year fish in the R. Ock were exposed to a chemical challenge of a similar magnitude to that experienced by fish in the R. Ray. The findings suggest that remediation of the WWTW effluent had a positive effect on growth in the first post-remediation generation, implying that the pre-remediation effluent was exerting a constraint on performance. This was offset by the broadly positive effects on growth of higher temperatures and nutrient loading. The results suggest that the accurate assessment and attribution of subtle effects of anthropogenic activities on a riverine fish population may be compromised by the complexity and number of interacting factors that must be taken into consideration.

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Figure captions

Fig. 1 The location of the River Ray and River Ock, and the relative positions of the sampling sites on each river. R. Ray: ROD – Rodbourne WWTW; EB – Elborough Bridge; TB – Tadpole Bridge; 7B – Seven Bridges; R. Ock: CB – Charney Bassett; GAR – Garford; VM – Venn Mill; MM – Marcham Mill.

Fig. 2 (a) Daily mean water temperature and (b) daily mean dissolved oxygen (percent saturation) in the River Ray at Rodbourne WWTW (black lines) and in the River Ock at Charney Bassett (grey lines) during the period January 2007 to November 2008. Interruptions in the plots represent periods during which the equipment was removed for servicing. The black bars denotes the period during which the GAC plant was operational on the R. Ray.

Fig. 3. Length frequency distributions for fish in the R. Ray captured during (a) May, (b) July, (c) September, (d) November and for fish in the R. Ock in (e) May, (f) July, (g) September, (h) November during 2007 (pre-remediation; dark bars) and 2008 (post-remediation; light bars).

Fig. 4. Mean body mass (a) and fork length (b) for fish from the R. Ray (solid circles) and R. Ock (open circles) during 2007 and 2008. Each point is the mean ± SEM (n = 8, 19, 80, 90, 89, 73, 58, 38, 83, 98, 100, R. Ray; n = 66, 59, 59, 84, 84, 49, 92, 102, 79, 62, 95, R. Ock). The matched pre- and post-remediation periods are denoted by the grey shaded areas. The period during which the GAC plant was operational on the R. Ray is indicated by the black bars. Water temperature in the R. Ray (solid line) and R. Ock (dashed line) is shown in (a).

Fig. 5. RNA:DNA ratios in fish from (a) the R. Ray (open symbols) and (b) the R. Ock (solid symbols) during 2007 and 2008. Each point is the mean ± SEM (n = 4, 9, 110, 88, 87, 73, 58, 38,

86, 94, 100, R. Ray; n = 44, 59, 43, 89, 85, 46, 94, 102, 80, 62, 75, R. Ock). The matched pre- and post-remediation periods are denoted by the grey shaded areas. The period during which the GAC plant was operational on the R. Ray is indicated by the black bars. Water temperature is shown for (a) the R. Ray (solid line) and (b) the R. Ock (dashed line).

Fig. 6. Specific growth rates for (a) length and (b) mass of fish in the R. Ray between July and November in the pre-remediation (2007; black bars) and post-remediation (2008; grey bars) periods. ROD, Rodbourne WWTW; EB, Elborough Bridge; TB, Tadpole Bridge; 7B, Seven Bridges.

Fig. 7. EROD activity in sticklebacks collected during (a) January 2008 (pre-remediation), and (b) March 2008 (post-remediation). Each bar is the mean + SEM. Sample sizes are indicated by numbers above each bar. R. Ray (solid bars): EB - Elborough Bridge; TB - Tadpole Bridge; 7B - Seven Bridges; R. Ock (open bars): CB - Charney Basset; GAR - Garford; VM - Venn Mill; MM - Marcham Mill. Within each plot, bars sharing the same letter are not significantly different (P>0.05).

Fig. 8. Relative expression of Cytochrome P4501A in liver tissue of sticklebacks collected during (a) September and November 2007, (b) September and November 2008. Each bar is the mean + SEM. Sample sizes are indicated by numbers above each bar. R. Ray (solid bars): ROD – Rodbourne WWTW; EB - Elborough Bridge; TB - Tadpole Bridge; 7B - Seven Bridges; R. Ock (open bars): CB - Charney Basset; GAR – Garford; VM - Venn Mill; MM - Marcham Mill. For each plot, bars sharing the same letter are not significantly different (P>0.05).

Table 1. Locations of sample sites on each river.

River	Site name	Abbreviated name	Grid ref.	Latitude and longitude	Distance from Rodbourne WWTW [km]
Ray	Rodbourne WWTW	ROD	SU128865	51.577 N, -1.817 W	0.2
Ray	Elborough Bridge	EB	SU121872	51.584 N, -1.827 W	1.8
Ray	Tadpole Bridge	TB	SU111896	51.605 N, -1.841 W	5.9
Ray	Seven Bridges	7B	SU119925	51.631 N, -1.829 W	9.5
Ock	Charney Basset	CB	SU381944	51.647 N, -1.451 W	-
Ock	Garford	GAR	SU438962	51.663 N, -1.368 W	-
Childrey Brook	Venn Mill	VM	SU434948	51.650 N, -1.374 W	-
Ock	Marcham Mill	MM	SU448954	51.656 N, -1.354 W	-

Table 2. Water chemistry summary for the periods March-November 2007 (pre-remediation) and March-November 2008 (post-remediation) for the R. Ray and R. Ock. Data were collected by sondes sited at Rodbourne WWTW (R. Ray) and Charney Basset (R. Ock). Each value is the mean ± SEM of daily average measurements (n = 214 –256). * significant differences between rivers within years; superscript a: significant differences between years within rivers.

	R. Ray		**R. Ock**	
Analyte	**2007**	**2008**	**2007**	**2008**
Temperature (^{o}C)	16.2 ± 0.15	15.6 ± 0.2	12.1 ± 0.16*	12.6 ± 0.2*
Dissolved Oxygen (% sat)	63.7 ± 0.9	89.5 ± 1.2[a]	112.6 ± 1.5*	99.3 ± 0.9*[a]
pH	7.17 ± 0.01	7.23 ± 0.01	7.92 ± 0.01*	7.86 ± 0.01*
Ammonium-N (mg/l)	0.40 ± 0.2	1.76 ± 0.15[a]	0.15 ± 0.03*	0.40 ± 0.01*[a]
Conductivity (µS/cm)	802.6 ± 20.7	781.3 ± 9.0	530.5 ± 15.5*	568.5 ± 6.7*
Turbidity (NTU[1])	131.1 ± 15.1	109.1 ± 13.2	48.6 ± 4.6	37.6 ± 3.8*

[1] Nephelometric turbidity units

Table 3. Water temperature recorded at the time of sampling during 2007 and 2008.

		2007				2008				
River	**Site**	**Mar**	**Jul**	**Sep**	**Nov**	**Mar**	**May**	**Jul**	**Sep**	**Nov**
Ray	ROD	11.2	18.8	19.0	18.7	9.8	16.5	18.5	17.2	10.8
	EB	16.6	17.8	18.8	17.1	8.8	14.6	17.3	16.4	9.8
	TB	-	-	-	-	9.5	15.6	17.8	17.2	10.1
	7B	19.1	18.8	17.9	18.7	9.3	14.9	17.8	15.9	9.7
Ock	CB	15.2	15.7	14.9	13.9	6.2	10.1	16.8	14.4	-
	GAR	-	-	-	-	6.3	10.6	16.0	14.3	6.4
	MM	-	-	-	-	6.7	12.7	16.6	14.5	8.2
	VM	-	-	-	-	7.1	12.1	16.9	14.7	8.1

Figure 1.

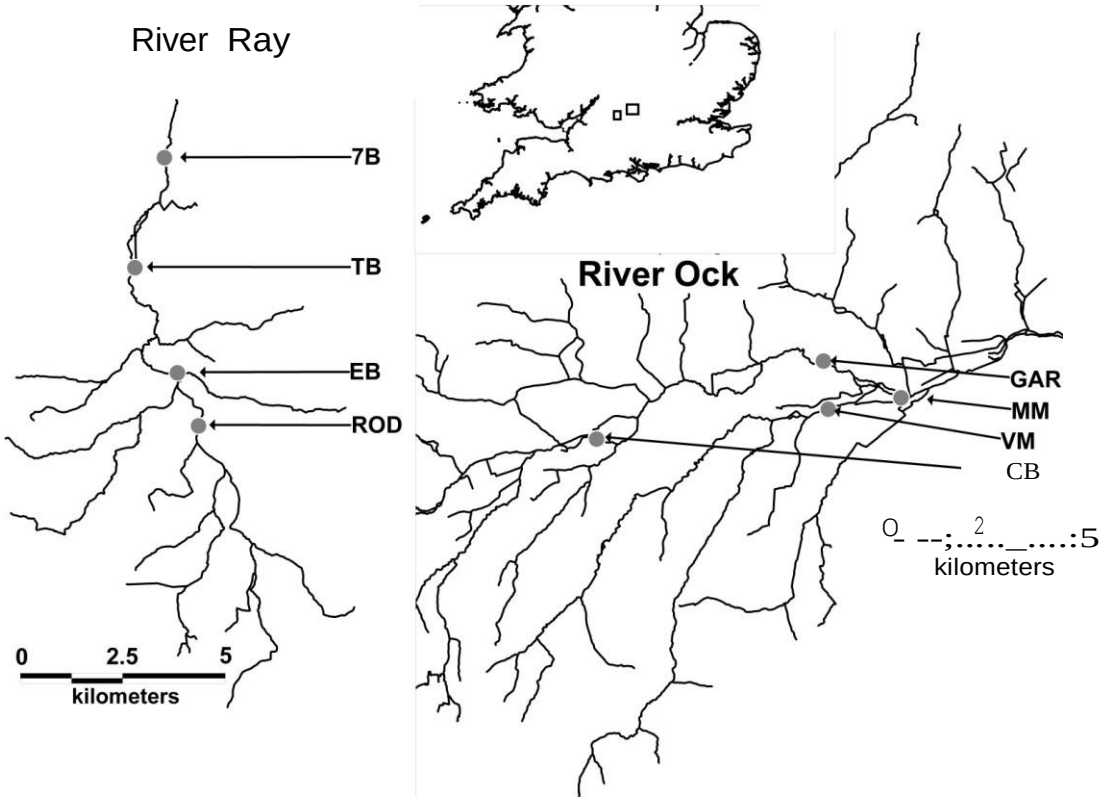

River Ray
7B
TB
EB
ROD
0
2.5
5
kilometers
River Ock
GAR
MM
VM
CB
kilometers

Fig. 2.

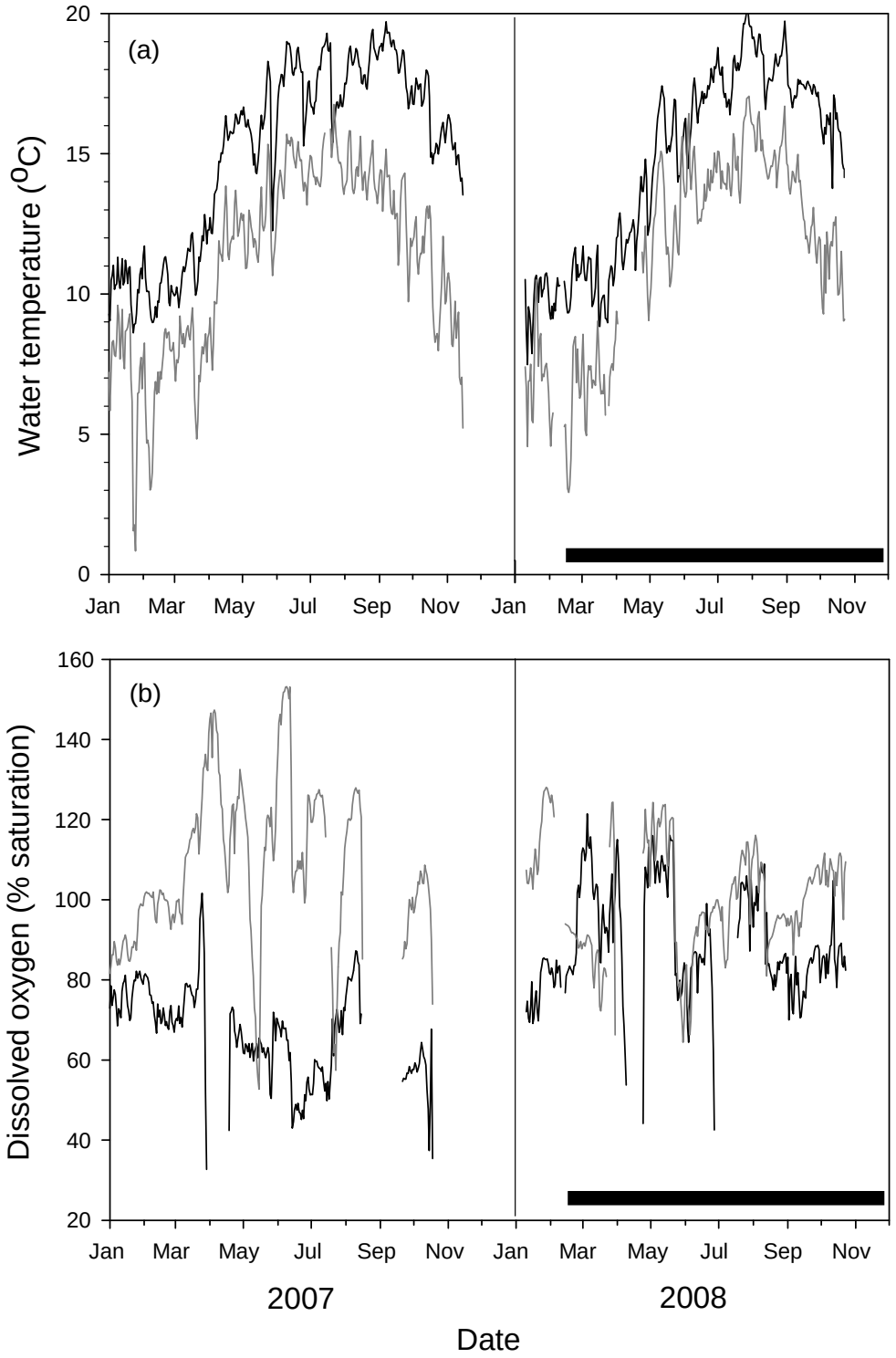

(a)
Water temperature (°C)
0
5
10
15
20
(b)
Dissolved oxygen (% saturation)
20
40
60
80
100
120
140
160
Jan
Mar
May
Jul
Sep
Nov
2007
2008
Date

Fig. 3.

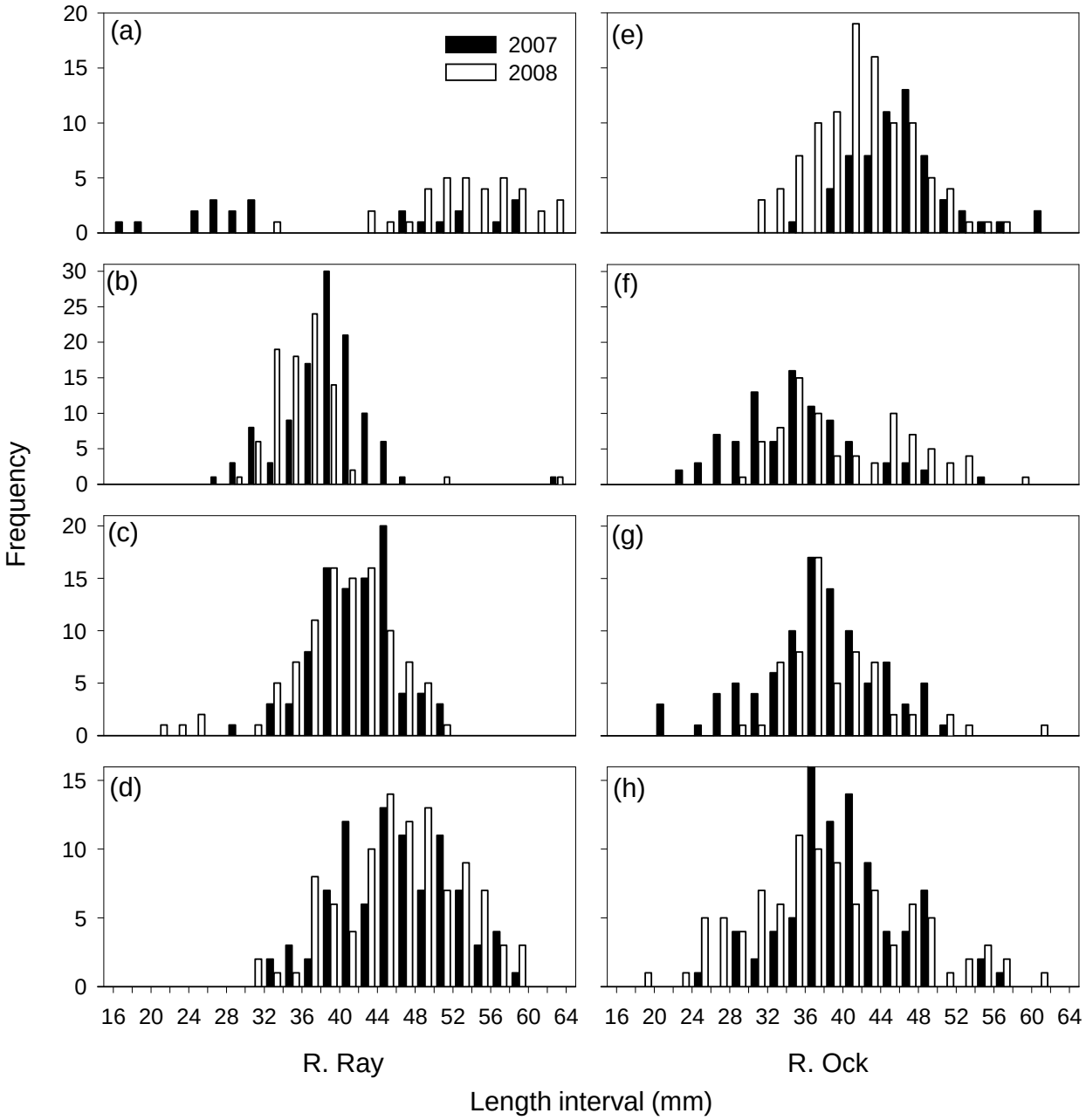


Fig. 4.

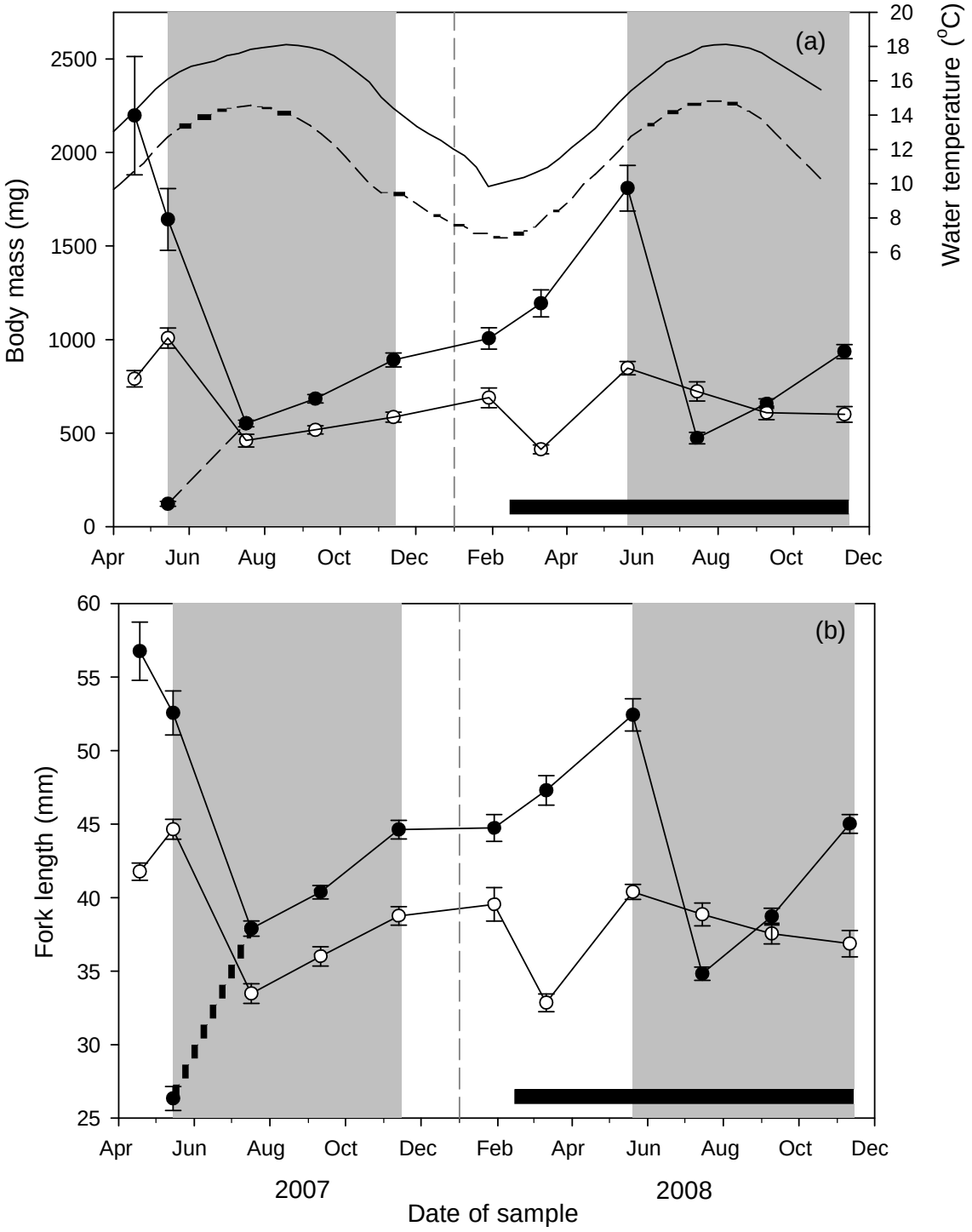

(a)
Body mass (mg)
Water temperature (°C)
0
500
1000
1500
2000
2500
6
8
10
12
14
16
18
20
Apr
Jun
Aug
Oct
Dec
Feb
Apr
Jun
Aug
Oct
Dec
(b)
Fork length (mm)
25
30
35
40
45
50
55
60
2007
2008
Date of sample

Fig. 5.

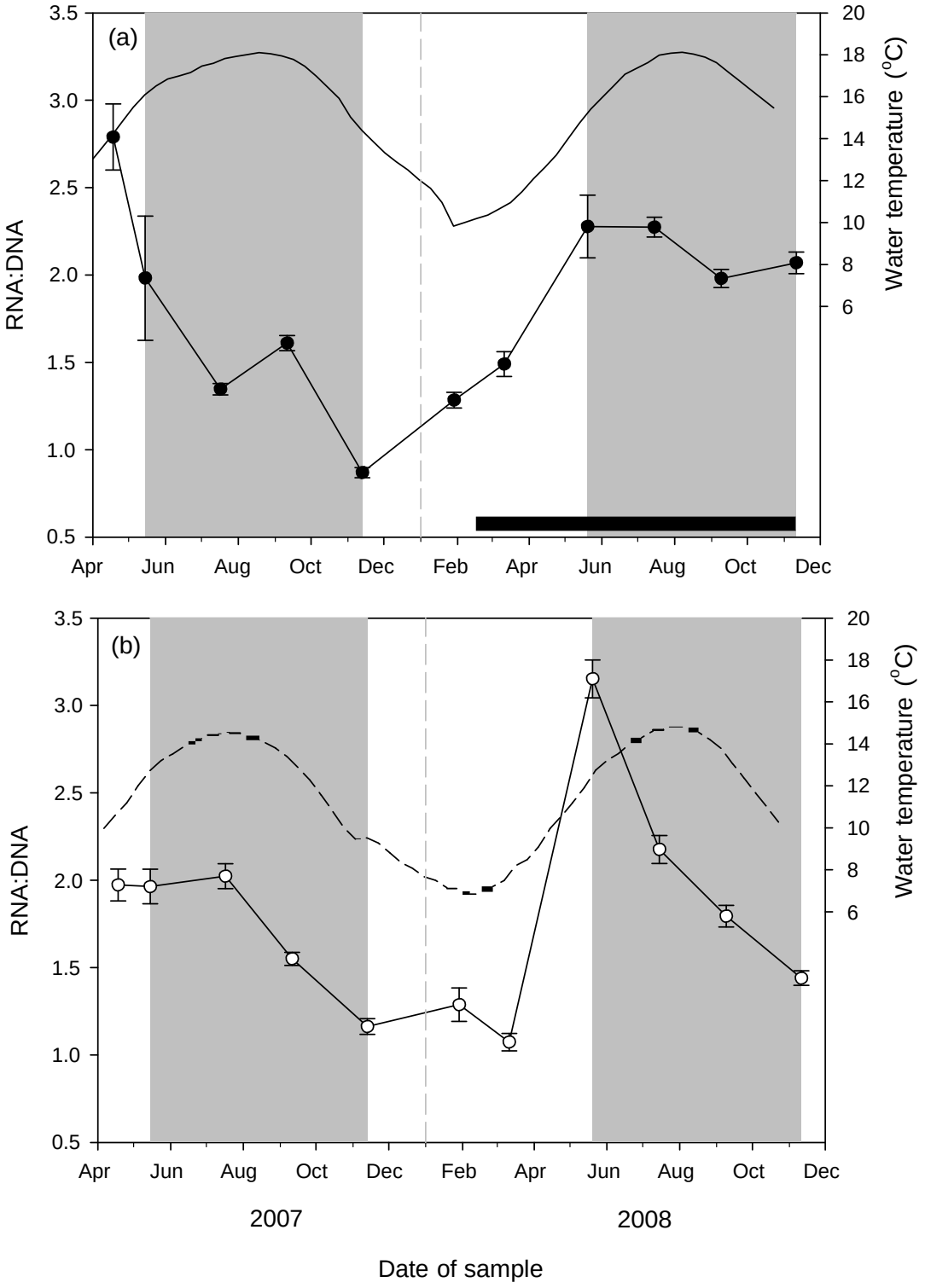

(a)
(b)
RNA:DNA
Water temperature (°C)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
20
18
16
14
12
10
8
6
Apr
Jun
Aug
Oct
Dec
Feb
Apr
Jun
Aug
Oct
Dec
2007
2008
Date of sample

Fig. 6.

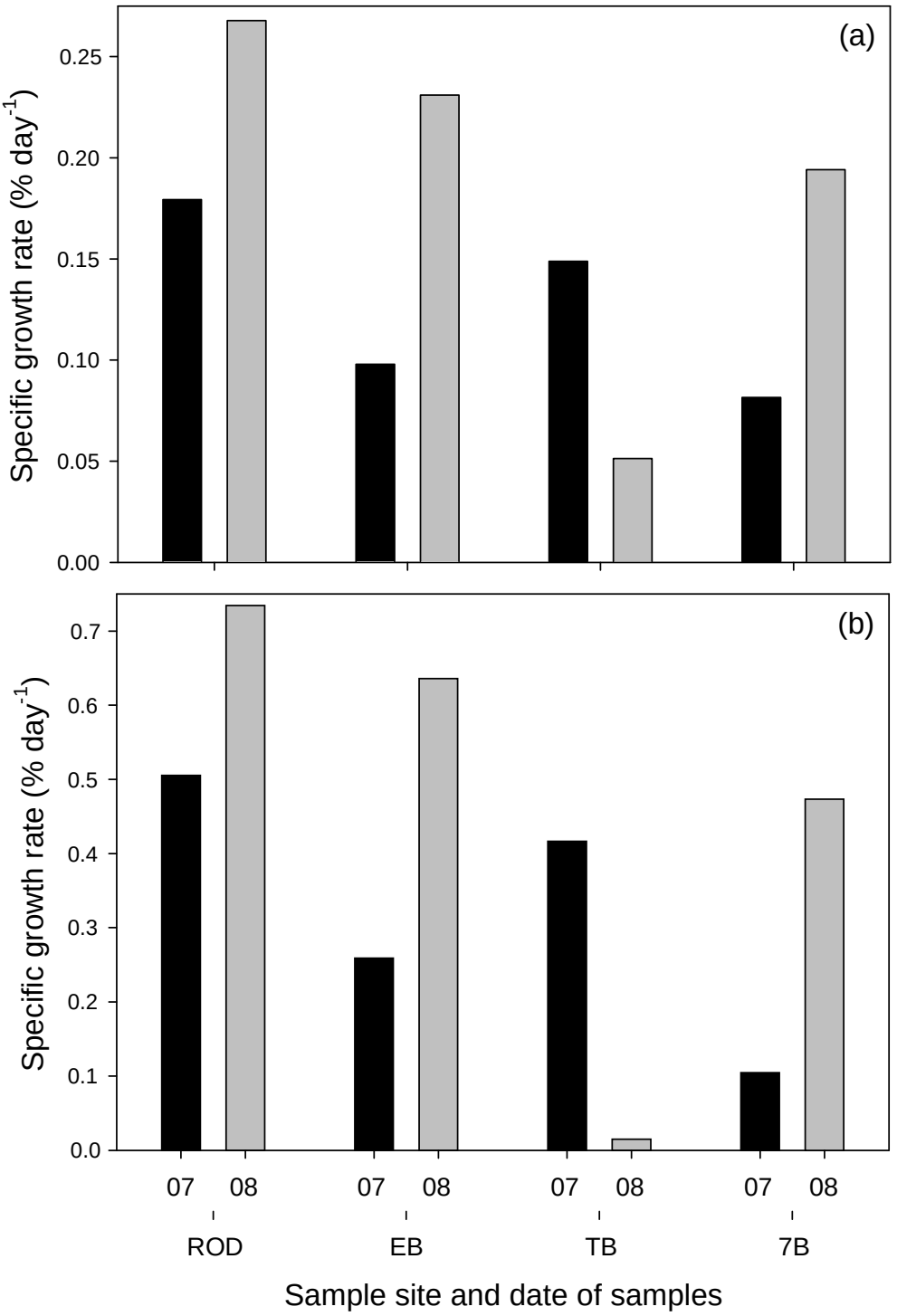

(a)
Specific growth rate (% day-1)
0.25
0.20
0.15
0.10
0.05
0.00
(b)
Specific growth rate (% day-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
07
08
07
08
07
08
07
08
ROD
EB
TB
7B
Sample site and date of samples

Fig. 7.

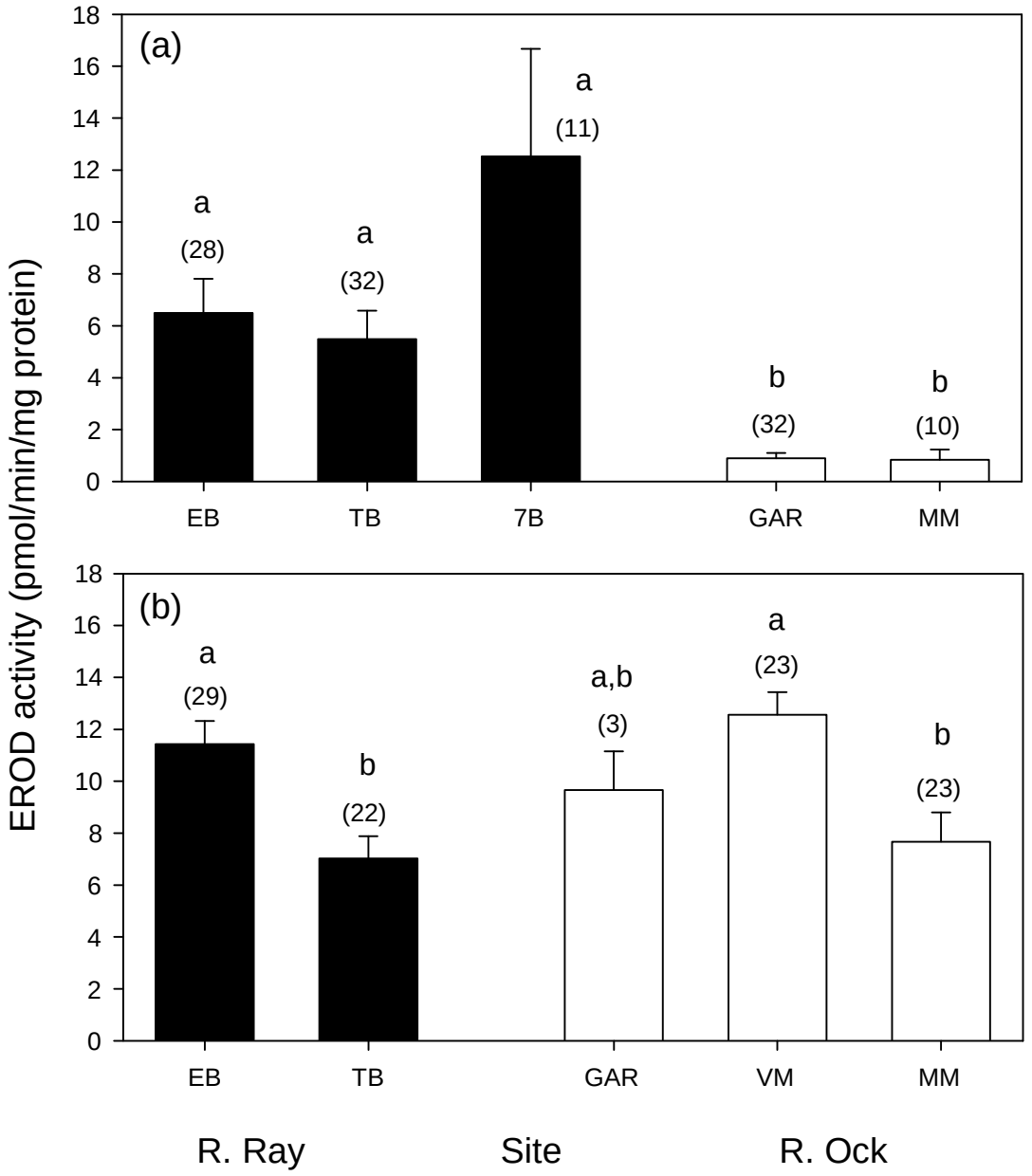
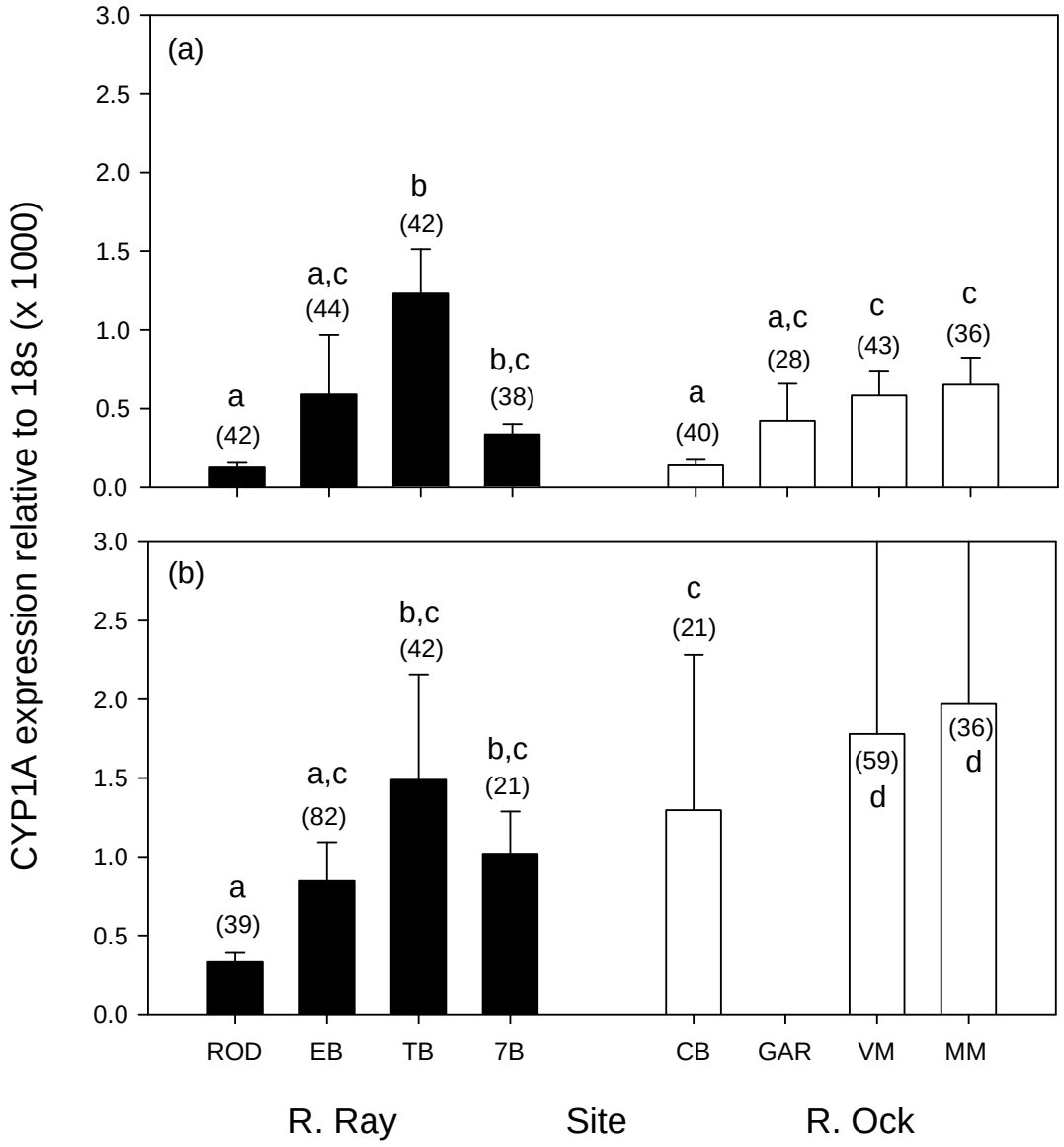

(a)
a
(28)
a
(32)
a
(11)
b
(32)
b
(10)
EB
TB
7B
GAR
MM
(b)
a
(29)
b
(22)
a,b
(3)
a
(23)
b
(23)
EB
TB
GAR
VM
MM
EROD activity (pmol/min/mg protein)
0
2
4
6
8
10
12
14
16
18
R. Ray
Site
R. Ock

Fig. 8.


(a)
(b)
CYP1A expression relative to 18s (x 1000)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a (42)
a,c (44)
b (42)
b,c (38)
a (40)
a,c (28)
c (43)
c (36)
a (39)
a,c (82)
b,c (42)
b,c (21)
c (21)
(59) d
(36) d
ROD
EB
TB
7B
CB
GAR
VM
MM
R. Ray
Site
R. Ock