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Keywords: endocrine disruption, gonad, vitellogenin, biomarker, carp, roach.

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Abstract: Environmental estrogens originate from a variety of sources, however sewage treatment plant (STP) effluents have been identified as one of particular concern, and adverse physiological effects (endocrine disruption) have been observed in several fish species sampled downstream of STP discharges. In this study we examined common carp (Cyprinus carpio) and roach (Rutilis rutilis) for signs of exposure to environmental estrogens in the iconic Yarra River, Melbourne, Australia. The Yarra River flows through the city of Melbourne and more than 2 million people live within the catchment. Two STPs discharge water into the Yarra River within the middle reaches, and the areas immediately downstream of these discharge locations were the focus of this study. Carp and roach were chosen as test species since both have been utilised extensively for endocrine disruption research throughout Europe, North America and Asia, and data from various international studies was used for comparison with the results of the present study.

Neither species showed evidence of exposure to environmental estrogens, with no elevation of plasma vitellogenin levels in males and no incidence of intersex gonads. Most physiological endpoints in both species from this study were within ranges reported in carp and roach from reference sites in other studies. However, 30% of males displayed degenerative changes in testis, including disorganisation of the lobules, atrophy of germinal epithelium, absence of spermatozoa and necrosis, whilst 53% of females showed a slightly increased incidence of oocyte atresia, decreased yolk formation and folding of the oocyte membrane. Estrogenic and androgenic activity in water was measured using the yeast-estrogen screen (YES) and yeast-androgen screen (YAS). Surface water samples showed no estrogenic activity, but did display strong anti-estrogenic and weak androgenic activity. Whilst the results show no evidence of environmental estrogens in the Yarra River, the presence of both anti-estrogenic and androgenic activity in water samples, as well as some

gonadal changes in carp is concerning and indicates that our focus needs to broaden, in order to look for biological impacts in resident fauna that might be due to environmental pollutants other than environmental estrogens.

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Cover Letter

19th December, 2015

R.E. Alcock

Editor-in-Chief, Environment International

Lancaster Environment Centre,

Lancaster University, Lancaster LA1 4YQ, UK

Dear Professor Alcock,

On behalf of my co-authors, I would like to submit a manuscript entitled "No evidence of exposure to environmental estrogens in two feral fish species sampled from the Yarra River, Australia: A comparison with Northern Hemisphere studies" for publishing consideration as a research article in Environment International.

This work presents new information on hormonal activity in surface waters of a large and important waterway in Melbourne, Australia, as well as a comparison of a series of biological endpoints in two fish species that are found in Australia, as well as North America, Europe and Asia. Carp (*Cyprinus carpio*) and roach (*Rutilis rutilis*) have been used extensively in the Northern Hemisphere to monitor waterways and effluent discharge receiving environments for endocrine disrupting chemicals and data from all suitable published literature on these species have been included in this manuscript for comparison with the Australian data we have collected.

We feel this research is suitable for publication in Environment International since it has local relevance, due to the paucity of data currently available on Australian wild fishes, as well as global relevance since the two species tested and the bioassays used are also used widely in the Northern Hemisphere.

This manuscript has not been submitted for publishing consideration elsewhere, and it has been reviewed by all co-authors who have declared no conflict of interest.

I hope you will consider our work for publication in Environment International and I look forward to hearing from you soon.

Kathryn Hassell

No evidence of exposure to environmental estrogens in two feral fish species sampled from the Yarra River, Australia: A comparison with Northern Hemisphere studies.

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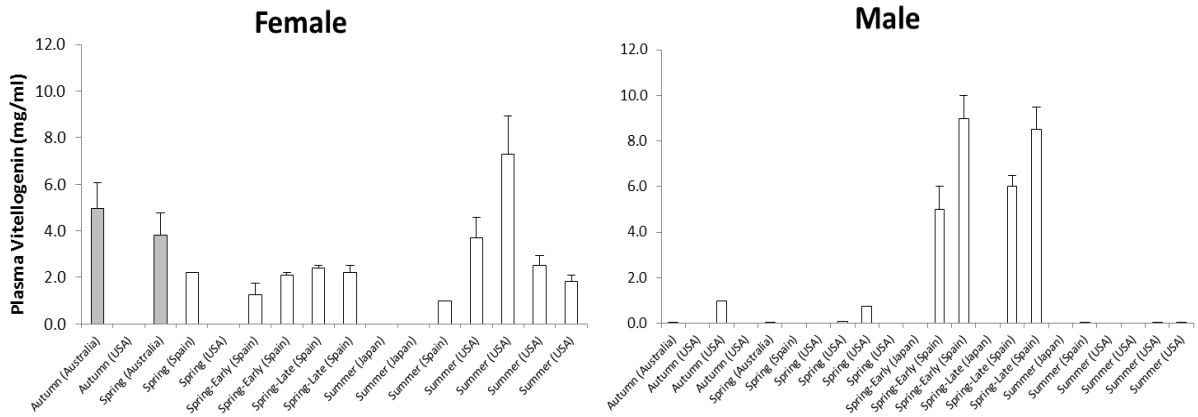
Graphical abstract



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Abstract

Environmental estrogens originate from a variety of sources, however sewage treatment plant (STP) effluents have been identified as one of particular concern, and adverse physiological effects (endocrine disruption) have been observed in several fish species sampled downstream of STP discharges. In this study we examined common carp (*Cyprinus carpio*) and roach (*Rutilis rutilis*) for signs of exposure to environmental estrogens in the iconic Yarra River, Melbourne, Australia. The Yarra River flows through the city of Melbourne and more than 2 million people live within the catchment. Two STPs discharge water into the Yarra River within the middle reaches, and the areas immediately downstream of these discharge locations were the focus of this study. Carp and roach were chosen as test species since both have been utilised extensively for endocrine disruption research throughout Europe, North America and Asia, and data from various international studies was used for comparison with the results of the present study.

Neither species showed evidence of exposure to environmental estrogens, with no elevation of plasma vitellogenin levels in males and no incidence of intersex gonads. Most physiological endpoints in both species from this study were within ranges reported in carp and roach from reference sites in other studies. However, 30% of males displayed degenerative changes in testis, including disorganisation of the lobules, atrophy of germinal epithelium, absence of spermatozoa and necrosis, whilst 53% of females showed a slightly increased incidence of oocyte atresia, decreased yolk formation and folding of the oocyte membrane. Estrogenic and androgenic activity in water was measured using the yeast-estrogen screen (YES) and yeast-androgen screen (YAS). Surface water samples showed no estrogenic activity, but did display strong anti-estrogenic and weak androgenic activity. Whilst the results show no evidence of environmental estrogens in the Yarra River, the presence of both anti-estrogenic and androgenic activity in water samples, as well as some gonadal changes in carp is concerning and indicates that our focus needs to broaden, in order to look for biological impacts in resident fauna that might be due to environmental pollutants other than environmental estrogens.

1.0 Introduction

Endocrine disrupting chemicals (EDCs) are compounds that interfere with an organism's endocrine system by modulating hormone receptors and subsequently affecting the production, storage and uptake of hormones, or the action of a hormone within a specific target tissue or organ (Colborn et al., 1993). There are a number of known or suspected EDCs already present in aquatic environments worldwide, and a major source is sewage treatment plant (STP) effluent discharge (Desbrow et al., 1998; Snyder et al., 1999; Ternes et al., 1999; Tan et al., 2007). These effluents may contain mixtures of chemicals and especially for domestic effluents, compounds such as natural estrogens (17β-estradiol, estrone, estriol), synthetic estrogens (17α-ethinylestradiol) and compounds with estrogenic activity (i.e. alkylphenol ethoxylates, some pesticides, BPA, plasticizers) are commonly reported (Jobling et al., 1995; Meesters and Schroder 2002; Ying et al., 2002a; Ying et al., 2002b) Collectively, these compounds that interact with estrogen receptors are called environmental estrogens or xenoestrogens, and their occurrence in STP effluents has been correlated with adverse physiological effects in resident fishes (Folmar et al., 1996; Lye et al., 1997; Jobling et al., 1998).

A major target of EDCs in fish (and other organisms) is the reproductive axis and a variety of adverse effects have been reported including changes in sex steroid hormone synthesis, induction of the yolk precursor vitellogenin (Vtg), degenerative changes in the ovaries and testes including intersex conditions (oocytes in testicular tissue; spermatogenic cells in ovarian tissue), abnormal gametogenesis, reduced sperm viability, reduced gonad size (and gonadosomatic index -GSI), lowered fertility and changes in the timing of sexual reproduction (i.e. (Jobling and Tyler 2003; Matthiessen 2003; Mills and Chichester 2005). Evidence of endocrine disruption in wild fish has been found in rivers receiving STP discharges (Purdom et al., 1994; Sumpter and Jobling 1995; Folmar et al., 1996), pulp and paper mill discharges (Van der Kraak et al., 1992; Hewitt et al., 2008) and agricultural (pesticide) chemical runoff (Lavado et al., 2004; Schmitt et al., 2005). As such, wild fish have become a valuable monitoring tool for assessing aquatic environments for evidence of endocrine disruption. Two cyprinids, the common carp (*Cyprinus carpio* Linnaeus, 1758) and roach (*Rutilis rutilis* Linnaeus, 1758) are freshwater species that have been studied extensively in Europe, North America and parts of Asia to monitor their responses to EDC exposure.

The common carp is a widely distributed freshwater fish found in several countries. It is long-lived and highly fecund and has been used widely in biomonitoring studies. Downstream of some STPs, male carp have been shown to have elevated plasma Vtg levels, reduced plasma estrogen (17β-estradiol) and androgen levels (testosterone or 11-ketotestosterone) and alterations in estrogen/androgen ratios (Folmar et al., 1996; Sakamoto et al., 2003; Sole et al., 2003b; Mitchelmore and Rice 2006). In addition, histological changes in male gonads, such as intersex, testicular atrophy, macrophage aggregates, necrosis, fibrosis and a change in gonad staging have been reported in carp caught downstream of some STPs (Hassanin et al., 2002; Sole et al., 2003b; Lavado et al., 2004; Mitchelmore and Rice 2006; Stansley and Washuta 2007). Similarly, female carp sampled downstream of some STPs have also shown altered hormone levels (Sakamoto et al., 2003) and histological changes including increased rates of atresia and shifts in gonad staging (Lavado et al., 2004; Mitchelmore and Rice 2006), whilst female carp sampled from areas polluted with diffuse agricultural and industrial chemicals, have displayed histological changes including oocyte atresia, calcified follicles, fibrosis and ovarian tumours (Patiño et al., 2003; Baldigo et al., 2006; Hinck et al., 2008; Hinck et al., 2009). This information demonstrates that carp can be affected by endocrine disruption, and wild carp from several countries have been shown to display Vtg induction, alterations in sex steroid levels and gonadal changes in response to exposure to STP-derived EDCs (Appendix Table A1, A2).

Roach are another long-lived species that have been used widely in biomonitoring studies. A study of juvenile roach exposed to treated sewage effluents in the UK demonstrated dose-dependent induction of plasma Vtg, as well as dose-dependent feminisation of the reproductive ducts in males, however no fish displayed intersex (testicular oocytes) (Rodgers-Gray et al., 2001). In a subsequent study, Beresford et al. (2004) reported high rates (>80%) of reproductive duct feminisation in juvenile roach collected from 5 of 7 rivers sampled in south-west UK, which was attributed to varying contributions of STP effluent to overall flows in the different rivers. As is the case with carp, roach have been shown to be sensitive to environmental estrogens, and assessments of wild fish from various locations have demonstrated adverse physiological effects (Appendix Table A3, A4).

In Australia, only a limited number of studies have measured the concentrations of estrogenic chemicals in STP effluents and surface waters (e.g. Williams et al., 2007; Ying et al., 2009; Ferguson et al., 2013; Scott et al., 2014; Vadja et al., 2015). In studies undertaken in Victoria

between 2003-2007, concentrations of natural estrogens or estrogenic activity (expressed as 17β-estradiol equivalents, EEQ) have been reported in the range of <0.1 – 73 ng/L (Mispagel et al., 2009). Seasonal differences in concentrations have been reported, with the estrogen levels being slightly higher in the summer months than in winter (Allinson et al., 2010). These values are within a similar range to values reported in STP effluents elsewhere in the world (Desbrow et al., 1998; Snyder et al., 1999; Ternes et al., 1999), and the concentrations are within the range known to elicit adverse biological effects in fishes (reviewed by Mills and Chichester, 2005). Few studies have been conducted to assess wild fish populations for evidence of endocrine disruption in Australia (Batty and Lim 1999; Game et al., 2006; Leusch et al., 2006; Codi King et al., 2008; Rawson et al., 2008; Kellar et al., 2014).

In the 1860s, both carp and roach were introduced to Australian rivers for angling purposes and have since established self-sustaining populations (Brumley 1991). Following these introductions, carp have undergone a huge range expansion and are now considered the most abundant large freshwater fish in southern Australia (Koehn 2004). Carp are a declared pest species in several countries, including Australia where they are considered noxious, due to their negative impacts on stream habitat and other species. Roach are also an introduced species, however they are not considered a major threat to native fishes, and therefore are not a formally listed noxious species in Australia. Roach have not undergone a widespread expansion so their distribution tends to be restricted to Victorian inland and coastal waters (Rowe et al., 2008). A major Victorian river in which both species have established populations is the Yarra River.

The Yarra River originates near Warburton, 76 km north-east of Melbourne in the Yarra Ranges National Park, and flows in a south-westerly direction where it eventually drains into the northern end of Port Phillip Bay (Melbourne Water, 2007). The river is 245 km in length, and more than 2 million people live within the 4,078 km^2 Yarra Catchment. Several areas in the upper reaches are reserved for water supply, with numerous major reservoirs and water holding facilities, and these forested and mountainous areas are considered undisturbed and in a pristine condition. Moving downstream, the trend is for a decline in water quality and overall condition, largely due to pollution that is introduced via urbanised tributaries and creeks which join the Yarra River at several points along its length. In addition, there are four STPs that discharge either directly into the Yarra River, or into its tributaries.

The aims of this study were to assess possible endocrine disruption in a Victorian wild fish population, by examining carp and roach from the Yarra River and comparing the results to findings from international studies. In lieu of having reference sites, we compared our findings with published information on the same types of endocrine disruption-related endpoints in wild caught carp and roach from European, North American and Asian studies (Appendix Table A1, A2, A3, A4). There are obviously challenges in trying to directly compare physiological endpoints in fish caught from different environments during different seasons; so rather, we used this information as a guide to determine acceptable ranges of values for fish collected from so-called reference sites.

2.0 Methods

2.1 Sampling sites

Fish, and/or water samples were collected from six different sites along a 30 km section of the Yarra River between Yarra Glen and Warrandyte, throughout which several creeks and tributaries enter the river, and the degree of urbanisation increases (Fig. 1). The furthest most upstream site was Spadoni's Reserve (SPR) near the township of Yering (Appendix Table A5).

Upstream of this site, a total of 4 different STPs discharge wastewater either directly into the Yarra River, or into tributaries of the Yarra River (Table 1). Another potential source of pollution entering the Yarra River around this area is run off from the nearby Yering Meadows golf course. This golf course covers an area of 130 hectares (1.3 km^2), including a 15 hectare walnut plantation, and is situated approximately 1.0 km from the Yarra River. Finally, there are a number of townships in the vicinity of the sampling sites used in this study (Wonga Park, Warrandyte and North Warrandyte) for which sewerage services were not connected at the time of this study, and the residents used septic systems.

For the sampling area relevant to this study, treated wastewater is discharged to the Yarra River or its tributaries from two STPs, those being Lilydale STP (via Olinda Creek), and Brushy Creek STP (via Brushy Creek), whilst Healesville and Upper Yarra STPs discharge treated effluents in to the Yarra River approximately 30 km and 55 km upstream, respectively (Fig. 1) (YVW 2009).

2.2 Water Collection

At the same time as the fish collections, duplicate 1 litre water samples were collected from each site and stored on ice until return to the laboratory. The samples were filtered through 1.2 μm (GF/A Whatman) and then 0.7 μm (GF/F Whatman) glass fibre filter paper, then extracted onto SPE cartridges (Oasis HLB, Waters Corporation, NSW, Australia) and eluted with methanol then used for *in-vitro* assays.

2.3 In-vitro assays

The yeast estrogen screen (YES) and yeast androgen screen (YAS) were used to detect (anti-) estrogenic and (anti-) androgenic activity, in the receiving water samples following standard

procedures as outlined in Routledge and Sumpter (1996) and Sohoni and Sumpter (1998) respectively. Briefly, methanol-extracts of samples and blanks were added in a series of dilutions to multi-well plates, and the plates were dried at room temperature. The estrogen, 17β-estradiol (E2) was included as a positive control for YES assays while the androgen 5α-dihydrotestosterone (DHT) was used for YAS assays. The estrogenicity or androgenicity measured in water and sediment samples was expressed as equivalent E2 or DHT values, respectively.

2.4 Fish Collection

Carp and roach were obtained by means of boat-operated electrofishing, on 20-21st October, 2008 and 8-16th April, 2010. Fish were retained on the boat in aerated tubs until a time (<2 hr) that they could be returned to land for processing.

2.5 Fish sample collection

Carp were captured from 3 sites during 2008 and 2010: Yering Gorge Pumping Station, Wonga Park and Blacks Flat Reserve, whilst Roach were captured from 4 sites during 2010 only: Spadoni's Reserve, Yering Gorge Pumping Station, Wonga Park and Blacks Flat Reserve (Appendix Table A5; Fig 1). Carp were killed with a sharp blow to the head, followed by cervical transection, and roach were killed with an overdose of anaesthetic (50 ppm clove oil - Sigma-Aldrich, Australia), followed by cervical transection. Blood was collected from the caudal sinus in both species using heparinised needles, then centrifuged (5000 rpm, 10 min), and the plasma was snap frozen in dry ice and later transferred to -80^{o}C storage until analysis. Roach plasma had the protease inhibitor aprotinin added (2 TIU/ml – Sigma-Aldrich, Australia) prior to freezing.

The total length (TL) and weight of each fish was recorded, and then different tissues were dissected for analysis. For both species, diffuse liver tissue was removed and weighed for determination of a hepatosomatic index. For carp, the gonads were removed, weighed and then a 2 cm^2 portion of the anterior, middle and posterior regions of both the left and the right gonads were fixed in 10% buffered formalin for histological analysis. For roach, the gonads were removed, weighed and then fixed whole in Bouin's Solution (Sigma-Aldrich, Australia) for 24 h, which was then replaced with 70% ethanol. All roach plasma and gonad samples were sent to the Institute for Environment, Health and Societies, Brunel University for

analysis. Sagittal otoliths were removed from the head cavity in all carp, air dried in paper envelopes and sent to the Fish Ageing Services (FAS) laboratory for age estimation.

A number of indices were calculated for both carp and roach, to provide a general measure of condition. Fulton's K condition index (K) calculates the relationship between the length and the weight of the fish (K = [Total body weight (g) / Fork length $(cm)^3$] x 100) and is used as a general indication of fish health (Ricker 1975). The gonadosomatic index (GSI) is a measure of the gonad size expressed in relation to body weight (GSI = [gonadal weight / body weight] x 100), and similarly, the hepatosomatic index (HSI) provides a measure of the liver weight in relation to body weight (HSI = [Liver weight / total body weight]*100).

2.6 Vitellogenin Analysis of Blood Samples

Carp and roach plasma samples were analysed for Vtg using a commercially available competitive binding carp Vtg enzyme-linked immunosorbent assay (ELISA) kit (Biosense Laboratories, Bergen, Norway).

2.7 Analysis of 17β-estradiol (E2) and 11-ketotestosterone (11-KT) in Blood Samples

Hormone concentrations were determined in the plasma of both species using enzyme immunoassay (EIA) kits (E2 582251 and 11-KT 582751, Cayman Chemicals) by the method proposed by Mills et al. (2010). The method was validated by evaluating the parallelism between the standard curve (E2 or 11-KT) and the hormone concentrations in serially diluted plasma samples (1:2–1:256). The dilution of the sample corresponding to 50% bound-antibody was calculated by regression analysis. This factor was used to dilute the plasma samples for hormone analysis. For female fish, plasma samples (15–20 µL) were diluted 20 times for 11-KT and 100 times for E2 determinations, whilst in males the plasma was diluted 100 times for 11-KT and 20 times for E2 testing. Briefly, 50 µL of the standards or the plasma samples were combined with 50 µL of E2 or 11-KT acetylcholinesterase (AChE) conjugate and 50 µL of E2 or 11-KT specific rabbit antiserum, then applied to an anti-rabbit IgG antibody-coated 96-well plate. The plate was incubated (18 h at 4°C for 11-KT, 60 min at room temperature for E2), then washed multiple times, developed with 200 µL of Ellman's reagent and finally the absorbance was measured at 405 nm (Thermo Labsystems Multiskan Ascent Microplate Photometer). The intensity of the yellow colour represented the amount of (E2 or 11-KT) AChE bound to the well and was inversely proportional to the amount of steroid hormone in the plasma sample.

2.8 Gonad Histology

For both carp and roach, anterior, mid and posterior portions of the pre-fixed left and right gonads were cut into 5-10 mm sections, dehydrated in an ethanol series, embedded in paraffin wax and then sectioned at 5 μm and stained with Haematoxylin and Eosin (H&E). Sections were examined microscopically and assessed for changes in germ cell development, presence of testicular oocytes (intersex) and changes in gonadal staging, following the criteria used for the determination of histopathological alterations in gonads as outlined by Schmitt and Dethloff (2000). Sectioned testicular tissue of both carp and roach was examined for the incidence of different forms of intersex, following procedures described by Nolan et al., (2001).

2.9 Data Analysis

Due to the unrestricted nature of this section of the Yarra River (i.e. no barriers to upstream/downstream movement), as well as the knowledge that both carp and roach are relatively mobile species, data was combined, rather than separating the samples based on different collection sites, and in lieu of having any reference sites, comparisons were made with biological data collected for these two species from several other studies (Appendix Table A1, A2, A3, A4).

3.0 Results

3.1 Water Sampling (In-vitro assays)

The estrogenic activity of all river water samples was below detection however strong anti-estrogenic responses were detected in all of them (LOD YES - 0.09 ng/L). Weak androgenic activity was detected in all samples (1.96-3.13 ng/l DHT EQ), but no anti-androgenic activity was detected (LOD YAS- 1.0 ng/L).

3.2 Fish Sampling

A total of 35 carp were sampled for this study, including 14 females, 20 males and 1 fish that was not yet sexually differentiated, and a total of 42 roach, including 20 females, 14 males as well as 8 fish that were not yet sexually differentiated (Table 2).

3.3 Size, Weight and Age

Carp

Most carp collected during this study were large (>1.5 kg), with the largest female weighing 5.7 kg and the largest male weighing 4.9 kg (Table 2). The age estimates for these two fish were 11 and 39 years, respectively. The smallest carp sampled during this study weighed less than 100 g, had undifferentiated gonads, and was estimated to be less than one year of age (Table 2). One small female carp was collected during the 2008 survey, but was excluded from analysis. It was excluded because it was much smaller than all others collected (830 g compared to >2000 g) and clearly much less reproductively mature, and its inclusion would have skewed the vitellogenin and hormonal data.

Roach

The smallest roach collected was a female which weighed 1.6 g and had a total length of 62 mm (Table 2). The largest roach was a male which weighed 218 g and had a total length of 258 mm. Age estimates could not be determined in roach samples due to processing issues with the extracted otoliths.

3.4 Condition Indices

Carp

The mean GSI values for both female and male carp were higher in spring 2008 than autumn 2010, and for both collection periods females had higher GSI values than males (Table 2). On the contrary, mean HSI values for both females and males were higher in autumn 2010

than spring 2008, and for both years males had higher mean HSI values than females (Table 2). Condition factor values were higher in females in autumn 2010 than spring 2008, whilst in males the values were higher in spring 2008 than autumn 2010 (Table 2).

Roach

In roach, the mean GSI of females was higher than males, and all females with a GSI > 2 had Vtg concentrations of >200 ng/ml (Table 2). The female fish with the highest GSI (6.897) had a Vtg concentration of 2750 ng/ml, and was classified as having Stage III ovaries (mid vitellogenesis). The highest GSI observed in male roach was 2.703, and it was classified as having Stage II testis (early spermatogenesis). No HSI values were determined for roach, due to the small size of the livers, however condition factors were calculated, and males had slightly higher values than females (Table 2).

3.5 Plasma Vitellogenin Analysis

Carp

For carp, low Vtg concentrations (<0.01 mg/mL) were detectable in all male fish. These are considered background levels, and are comparable to the concentrations that have been observed in control male fish reared under controlled laboratory conditions (Jobling et al., 2003). Vitellogenin concentrations above background concentrations were not detected in any male carp collected during the two surveys, whilst female carp exhibited significantly higher plasma Vtg concentrations, ranging from 1.3-8.6 mg/mL (Table 2).

Roach

Only 5 of the male samples had measurable concentrations of plasma Vtg, with the highest one being 0.149 µg/ml, whilst the remaining 4 were all <0.1 µg/ml. Vitellogenin was measured in 13 female roach, with the remaining 7 samples either being below detection limits, or not measurable (Table 2). The highest concentration measured was 4423 µg/ml, in a female with a high GSI (5.71), and Stage III (mid vitellogenesis) ovarian development. All fish <140 mm TL had Vtg concentrations <0.1 µg/ml.

3.6 Analysis of 17β-estradiol (E2) and 11-ketotestosterone (11-KT) in Blood Samples

Carp

Mean E2 plasma concentrations were higher in spring 2008 than autumn 2010 for female carp, and in both seasons were much higher than the concentrations observed in males (Table

2). In contrast, 11-KT values were much higher in males than females, and again, the mean values were higher in spring 2008 than autumn 2010 (Table 2). The E2/11-KT ratios reflect these patterns, with females having values >10, whilst males had values <1.0.

Roach

Mean E2 plasma concentrations were much higher in female roach than males, and interestingly, the E2 concentrations in male roach were much lower (10-fold) than male carp (Table 2). Similar to observed in carp, the mean 11-KT values were much higher in male roach than in females.

3.7 Gonad Histology

Carp

No abnormalities in either morphology or colouration were macroscopically observed in the gonads of any carp collected during this study. Microscopically, the testes were fully mature in most of the males sampled, exhibiting all stages of spermatogenesis; whereby the lumina were filled with spermatozoa and the lobules contained numerous spermatogenic cysts (Fig. 2b, c). For both years, male carp were in Stage III or Stage IV (late spermatogenic, spawning) of gonad development (Fig. 3). None of the sections examined contained any oocytes within testicular tissue, yielding a 0% incidence of intersex. However, 30% of all males sampled during the two surveys displayed some degenerative changes in the testes. Changes included disorganisation and diminished diameters of seminiferous lobules, atrophy of germinal epithelium, inhibited spermatogenesis and/or absence of spermatozoa, vacuoles, fibrous and amorphous eosinophilic tissue and necrosis (Fig. 2).

Most female carp ovaries were observed to be in Stage III and IV of ovarian development (Fig. 3). In 53% of all females sampled during the two surveys, varying degrees of increased oocyte atresia, decreased yolk formation and folding of the oocyte membrane was observed (Fig. 2d).

Roach

No abnormalities in either morphology or colouration were macroscopically observed in the gonads of any roach collected during this study. Males were observed in either Stage I or Stage II of testis development, whilst females were observed in Stage I, Stage II or Stage III

of ovarian development (Fig. 3). No male roach displayed intersex gonads and no male or female samples showed indications of degenerative changes or abnormal histology.

3.8 Comparison with Table 1 data

For all the physiological endpoints we measured (CF, GSI, E2, 11KT, E2/11KT ratio, Vtg and gonad histology), male Yarra River carp were generally within the ranges of values reported for reference site carp elsewhere (Fig. 4, 5; Appendix Table A1, A2). Mean plasma Vtg concentrations up to around 1.0 µg/ml (highest value 2.088 µg/ml) were towards the low end of the range reported in other studies (0.02-49.0 µg/ml), whilst plasma hormone concentrations and estrogen/androgen ratios were within the reported ranges. Male carp GSI values were slightly higher than reported elsewhere (5.14-7.0%) but there may have been a greater incidence of degenerative changes in the gonads. The physiological endpoints measured in female carp in our study were also within the ranges of values measured in carp from elsewhere. Mean plasma Vtg concentrations (~5.0 mg/ml) in female Yarra River carp were within the ranges reported elsewhere (0.2-13.4 mg/ml), yet whilst the plasma E2 and 11KT concentrations were similar to other studies, the mean E2/11KT ratios were much higher (>10) in our study compared to others (generally <7.0) (Fig. 4, 5; Appendix Table A1). It is difficult to accurately gauge histological comparisons, because in most of the studies the histological descriptions (if present at all) were very brief and tended to report only very obvious gonadal changes such as testicular oocyte production (intersex) (Appendix Table A2).

Given the advanced age of some of the fish collected in our study (up to 39 years old), it is likely that some of the degenerative changes we observed in the gonads may be simply related to ageing, however some are consistent with those reported in carp sampled from known polluted sites (degeneration, necrosis, fibrosis and atrophy) (Appendix Table A2).

Male roach were in the general size range as fish reported from other studies, however the fish in our study tended to have higher CF values relative to others, and GSI values were towards the lower end of the range reported elsewhere (Fig. 6; Appendix Table A3). Plasma Vtg concentrations (0.04-0.15 µg/ml) were within the range reported for male roach from reference sites (0.015-126 µg/ml) but mean E2 values (47.9 pg/ml) were low relative to other studies (63-420 pg/ml). Few studies have reported 11KT concentrations for roach from reference sites, however the fish from our study (mean 11KT 3 018 pg/ml) did fit broadly

within the range of values reported elsewhere (39-10 000 pg/ml) (Fig.7). In other studies, male roach collected from polluted environments have displayed reduced milt volume and sperm density, abnormal gonad shape, gonadal duct malformation, delayed spermatogenesis and intersex (Appendix Table A4). In fact, up to 18% of male roach from reference sites have displayed intersex testes in other studies. No intersex or other gonadal changes were observed in male Yarra River roach.

The physiological endpoints measured in female Yarra River roach were generally within the ranges of values reported for reference site female roach in other studies (Fig. 6, 7; Appendix Table A1). Similar to the males, the CF values tended to be higher, yet the GSI values were within the ranges reported elsewhere. Hormone concentrations were within the range of other studies, but due to a lack of similar data, we were unable to compare E2/11KT ratios to other studies. Mean plasma Vtg concentrations (1 574 µg/ml) were at the low end of the range in Yarra River roach relative to other studies (500-15 000 µg/ml). We did not observe any gonadal changes in female Yarra River roach, whereas other studies have reported inhibited gametogenesis, reduced oocyte size, proliferative connective tissue, oocyte atresia and increased incidence of parasitic infections in ovaries of roach collected from polluted sites (Appendix Table A4).

4.0 Discussion

In this study we found no evidence of estrogenic endocrine disruption in two fish species sampled from the Yarra River, Australia. Surface waters collected from the same locations did not exhibit any estrogenic activity either, however we did observe a strong anti-estrogenic response as well as a weak androgenic response in water samples using YES and YAS bioassays.

Brushy Creek and Lilydale STPs both discharge a mean daily flow of >10ML/day into the Yarra River, which increases to 45 and 26.5 ML/day respectively, during peak wet weather flows (YVW 2009). The population equivalent (PE) for these two STPs are 52 000 for Brushy Creek and 57 100 for Lilydale. Based on volume estimates from 2003/04, the contribution that Brushy Creek and Lilydale STP effluents have to Yarra River flows ranges between 0.47-1.30% and 0.29-0.79%, respectively. In a worse-case scenario, the total contribution that these two STPs have on flows within this region of the Yarra River is only 2.09% and if we consider the combined total of all STPs that release effluents into the Yarra Basin, the contribution to overall flows is only 2.85%. Based on this information, the potential risks associated with STP effluents would seem quite low, unless of course the concentrations of chemicals within the effluents were particularly high. However, spot samples collected from Brushy Creek during 2008 were found to have low estrogenic activity (<0.4-0.3 ng/L 17β-estradiol equivalents (EEQ)) and low total estrogen concentrations (1.5 ng/L), indicating a low risk of estrogenic contamination due to STP discharges (Chinathamby et al., 2013). Another study conducted in the Yarra River during 2008 and 2009, in which the estrogenic activity of spot water samples was measured found human estrogen receptor activity bioassay (hERα) measured EEQs of <0.1 ng/L and in the medaka estrogen receptor activity bioassay (medERα) measured EEQs of <0.4 ng/L (Allinson et al., 2011). These findings are further supported by the present study, whereby all water samples had YES activity levels below the limits of detection. These multiple examples indicate that environmental estrogens are not currently an issue of concern within these sections of the Yarra River.

In addition to environmental estrogens, a number of other compounds may be present in river water, including pharmaceuticals (from STPs), pesticides and fertilisers (from golf courses and surrounding agriculture), and metals and urban-use pesticides (from stormwater runoff) (Martinovic-Weigelt et al., 2013). Research targeting the presence and biological effects of

these compounds in the Yarra River needs to be conducted to determine the risks to resident aquatic fauna. We did not observe any evidence of impacts in fish from environmental estrogens, nor did the water samples test positive for estrogenic activity, but we did observe anti-estrogenic activity and weak androgenic activity in surface water. In other recent studies of the Yarra River, both aryl hydrocarbon receptor activity (βNF EQ) and thyroid receptor activity (T3 EQ) have been reported (Allinson et al., 2011; Chinathamby et al., 2013). Clearly this shows that our focus needs to broaden, in order to look for biological impacts in resident fauna that might be due to endocrine disrupting chemicals other than estrogens.

Following exposure to anti-estrogenic compounds, laboratory studies have reported several gonadal changes as well as altered Vtg production in fish. Wester et al. (2003) observed oocyte atresia, accumulation of basophilic granular material, retraction of oocytes from the zona radiata or granulosa cell layer, degenerating eggs in the oviducts, and sharp invaginations of the zona radiata (oocyte membrane folding) in female zebrafish. Associated with these gonadal changes, they also reported a marked decrease in egg production. For the male fish in that study, exposure to anti-estrogens caused proliferation of leydig cells, expansion of the interstitial compartment, edema and asynchronous cell development within individual spermatocysts. Furthermore, males exposed to these compounds also had lower fertilisation rates. Some carp in the present study exhibited changes consistent with some of those mentioned above. Across both surveys, 30% of male carp exhibited some kind of degenerative changes in the gonads, including atrophy, vacuolation, fibrosis, necrosis, disorganisation of the seminiferous lobules and inhibited gametogenesis. In females, 53% displayed varying degrees of atresia, decreased yolk formation and oocyte membrane folding. Admittedly, we only assessed a small number of fish in the present study, and the age variation was quite large, however, the relatively high incidence of gonadal changes in both sexes of carp (but not roach) suggests that there may be compounds within the Yarra River that are affecting their normal functioning, although there is no evidence to suggest that it is due to estrogenic compounds.

Anti-estrogenic responses have been reported in carp hepatocytes co-exposed to E2 and Aryl hydrocarbon receptor (AhR) agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), β-napthoflavone (βNF), polychlorinated biphenyls (PCBs) and benzo(α)pyrene (BαP) (Smeets et al., 1999). Hepatic Vtg production was suppressed in a dose-dependent way, and the authors acknowledged the importance of determining the presence of AhR agonists in

field monitoring studies to ensure that low Vtg levels were 'real' and not the result of suppression due to anti-estrogenic pollutants. This is an interesting point to acknowledge, since the low Vtg levels measured in this study may have been due to anti-estrogenic compounds rather than just the absence of estrogenic activity.

While the current study found no evidence of estrogenic effects in carp or roach, impacts of environmental estrogens on other fauna in the Yarra River cannot be completely ruled out as some species are more sensitive to estrogens than others (Tyler et al., 2005; Lange et al., 2012; Miyagawa et al., 2014). For example, Tyler et al. (2005) reported 10-fold higher vitellogenin responses in rainbow trout exposed to STP effluents than roach, whilst Miyagawa et al. (2014) reported a 9.2 fold difference in the relative potency of bisphenol A to ER ligand binding domains of guppies (*Poecilia reticulata*) compared to carp. In that study they compared the responsiveness of estrogen receptors in 9 different species to natural estrogen (17β-estradiol) and xenoestrogens with both strong and weak (ERα) ligand-binding affinities. They reported a similar degree of response amongst species to E2, but considerable variability in the responsiveness to xenoestrogens, in particular to weak estrogens. Furthermore, species' that were most sensitive to a particular compound were not necessarily the most sensitive to all compounds tested. In general, cyprinids such as roach and carp were the least sensitive of the tested species. From those studies, the authors concluded that the use of single species, or indeed multiple, model species does not always provide a good indication of how a particular estrogenic EDC might affect a particular receiving environment (Lange et al., 2012; Miyagawa et al., 2014). This highlights the need for developing locally relevant bioindicator species that are naturally occurring in the waterways under investigation. In the present study we chose carp and roach as our indicators, because existing data from other studies was available for comparison. There is a marked paucity of data in regards to Australian native fish responses to EDCs, and this needs to be a priority area for future research.

An alternative way to assess fish for signs of exposure to environmental estrogens is to measure internal concentrations of the compounds directly. Bile has been shown to bioconcentrate estrogenic compounds, and is an excellent tissue to directly quantify levels of bioavailable estrogens (Gibson et al., 2005; Fenlon et al., 2010). Using direct methods removes some of the variability associated with fish-based biological assays, such as the need for species-specific methods (i.e. Vtg ELISA) and prior knowledge of normal reproduction

and gonad morphology in the target species. Furthermore, unlike cell-based bioassays that provide a measure of estrogen equivalence (or similar), direct measurements identify specific compounds, so are more informative about the actual compounds that are present in the environment under investigation. Yet, this has its limitations too, since the particular compounds have to be targeted for analysis, and due to the large variety of compounds likely to be present in receiving waters (many of which may be unknown), the cost of analysis becomes prohibitively expensive. Therefore, a combination of direct measurements (surface water, bile, fish tissues and other matrices), cell-based bioassays (to measure activity, or equivalence) and whole animal bioassays/assessments using local, resident species is the most comprehensive way to identify if an environment is polluted with endocrine disrupting chemicals. The most appropriate combination of these methods will be site and situation specific, and of course constrained by logistical, financial and other factors.

Whilst this study indicates that environmental estrogens are not currently an issue in the Yarra River, this may change in the future due to a variety of factors ranging from population growth, water abstraction, changes in sewage treatment technology, potential failures in ageing sewerage infrastructure and septic systems, as well as changing flows and dilution rates due to climate change and drought. Recent hydrological modelling predicts an increase in estrogen concentrations in rivers in both Australia and the United Kingdom by 2050 as a result of the growing populations coupled with reductions in river flow through changing climate (Green et al., 2013). Therefore it is imperative that further investigations are carried out to determine how susceptible Australian fish species are to estrogens (and other EDCs) from all sources, particularly from effluents derived from lower levels of sewage treatment.

5.0 Conclusion

We found no evidence of estrogenic endocrine disruption in either species of fish sampled from the Yarra River. Most physiological variables were within the range of values reported for reference site fish from other studies, although we did observe gonadal changes in both male and female carp that are worthy of further investigation. Both anti-estrogenic and androgenic activity was observed in Yarra River water, and highlights the need for further biological testing and the development of monitoring tools that can identify other types of endocrine disruption effects. The use of exotic species enabled us to compare our results to a good base of background knowledge on carp and roach, however, a future priority will be the

development of tests that utilise local, native species that have greater environmental relevance in Australian waterways.

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TABLES AND FIGURES

Table 1. Information relating to the four Waste Water Treatment Plants that discharge treated effluents into the Yarra River or its tributaries.

STP	Site Code	Mean Daily Flow ML/d	Peak Wet Weather Flow ML/d	Population Equivalent	Secondary Treatment	Tertiary Treatment	Discharge Location	Distance from Sampling Sites	Latitude	Longitude
Upper Yarra	UYSTP	4.3	10	10 700	*IDEA activated sludge process	Sand filtration and UV disinfection	Yarra River	55 km upstream of SPR	37°45'27.29"S	145°33'9.42"E
Healesville	HSTP	1.4	ND	10 000	Oxidation ditch, sidestream reactors and secondary clarification	Upward flow clarification and UV disinfection	Yarra River	30 km upstream of SPR	37°40'9.28"S	145°30'10.87"E
Lilydale	LSTP	12.0	26.5	57 100	Biological nutrient removal (BNR) activated sludge	Alum, caustic soda, sand filtration and UV disinfection	Olinda Creek	7.5 km from Yarra River, immediate vicinity of SPR	37°44'44.43"S	145°21'21.98"E
Brushy Creek	BCSTP	15.5	45	52 000	Alum, caustic soda, extended aeration (EA) activated sludge and secondary clarification	Sand filtration, alum and UV disinfection	Brushy Creek	12.8 km upstream of BFR	37°45'52.84"S	145°17'51.50"E

*IDEA - Intermittently Decanted Extended Aeration

Table 2. Summary of biological measurements (mean ± SE) for two fish species collected from the Yarra River, Victoria, Australia. Values in parentheses show the range of values measured.

Species	Season	Sex	n	Fork Length (mm)	Total Length (mm)	Weight (g)	Condition Factor (K)	HSI (%)	GSI (%)	Vtg (µg/ml)	E2 (pg/mL)	11-KT (pg/mL)	E2/11-KT ratio	Age (years)
Carp	Spring 2008	Female	7	542.86 ± 19.54	ND	3201.43 ± 523.9	1.97 ± 0.23	1.35 ± 0.120	20.95 ± 2.21	3826 ± 950	2792.3 ± 379.12	278.62 ± 47.80	13.71 ± 3.66	ND
										(7, 1299-7323)	(1524.3-4302.2)	(87.9-409.3)	(4.58-29.54)	
		Male	12	495.83 ± 15.25	ND	2527.5 ± 153.0	2.08 ± 0.09	1.75 ± 0.12	8.86 ± 0.58	0.482 ± 0.070	479.51 ± 66.47	4970.6 ± 881.6	0.118 ± 0.02	ND
										(10, 0.103-0.829)	(231.4-959.9)	(1623.5-8599.4)	(0.05-0.26)	
Carp	Autumn 2010	Female	7	547.14 ± 24.09	596.43 ± 28.01	3671.3 ± 515.5	2.16 ± 0.03	1.09 ± 0.12	17.49 ± 2.76	4972 ± 1101	2008.2 ± 347.06	119.97 ± 19.03	17.89 ± 2.80	5.86 ± 1.26
										(7, 1703-8577)*	(1070.6-3450.4)	(63.1-200.8)	(7.907-26.62)	(2-11)
		Male	8	459.38 ± 55.17	499.38 ± 58.35	2262.3 ± 542.5	1.92 ± 0.04	1.55 ± 0.21	8.15 ± 1.64	0.906 ± 0.210	536.14 ± 95.54	1741.6 ± 242.46	0.31 ± 0.03	8.38 ± 4.78
										(8, 0.510-2.088)*	(235.2-1000.4)	(880.3-2743.2)	(0.194-0.462)	(0-39)
		Undiff	1	127	12.7	42	2.05	1.4	ND	0.0376	1255	191.8	6.54	0
Roach	Autumn 2010	Female	20	112.5 ± 7.62	126.45 ± 8.42	25.81 ± 4.64	1.34 ± 0.04	ND	2.94 ± 0.49 *(15)*	1574 ± 450	1676.1 ± 197.90	478.79 ± 48.61	4.19 ± 0.83	ND
										(13, 0.06-4423.1)*	(14, 978-3210)	(14, 235-845)	(1.162-13.66)	
		Male	14	130.36 ± 10.89	145.64 ± 11.91	44.49 ± 15.26	1.40 ± 0.05	ND	1.13 ± 0.20 (*13*)	0.07 ± 0.02	47.92 ± 6.48	3017.8 ± 264.89	0.02 ± 0.00	ND
										(5, 0.04-0.15)*	(12, 20-84)	(12, 1730-4548)	(0.007-0.044)	
		Undiff	8	68.00 ± 3.89	75.88 ± 4.33	4.31 ± 0.94	1.24 ± 0.05	ND	ND	ND	ND	ND	ND	ND

* values in parentheses are n, range of concentrations measured

ND - no data

Figure 1. Map detailing fish collection sites along the Yarra River, and locations of 4 nearby sewage treatment plants (STPs).

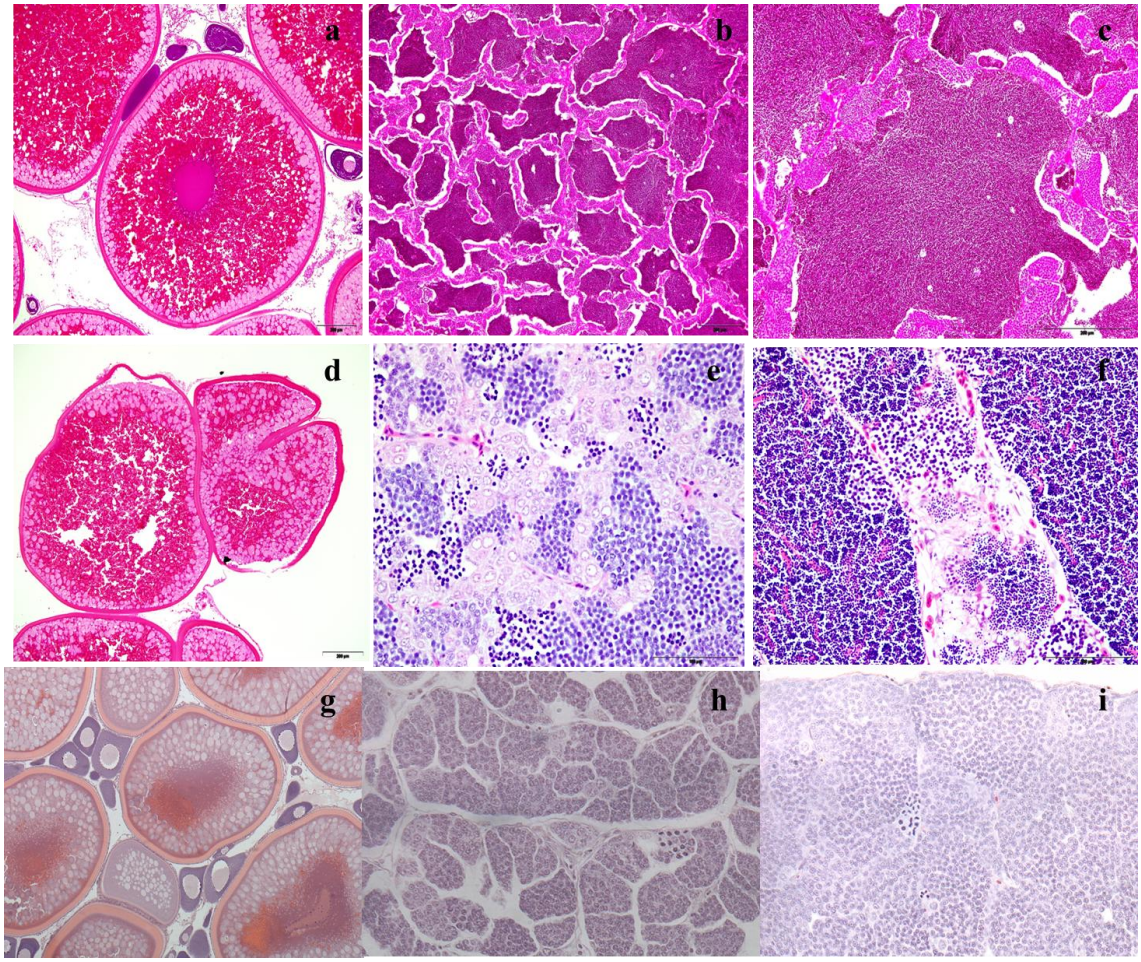

Figure 2 (a-f): Histological appearance of ovaries and testes for carp (*Cyprinus carpio*) sampled from the Yarra River, Australia. a) normal appearance of secondary oocyte, b) normal appearance of mature lobules, densely packed with spermatozoa, c) higher magnification of b), d) membrane folding and detachment of chorion in secondary oocyte, e) lobules containing increased proportion of spermatogonia and no spermatozoa, f) duct containing mixed cell types, including vacuolated and histiocytic cells. Figure 2 (g-i): Histological appearance of ovaries and testes for roach (*Rutilus rutilus*) sampled from the Yarra River, Australia. g) Normal appearance of developing ovary, h, i) Normal appearance of developing testes, showing distinct spermatocysts.

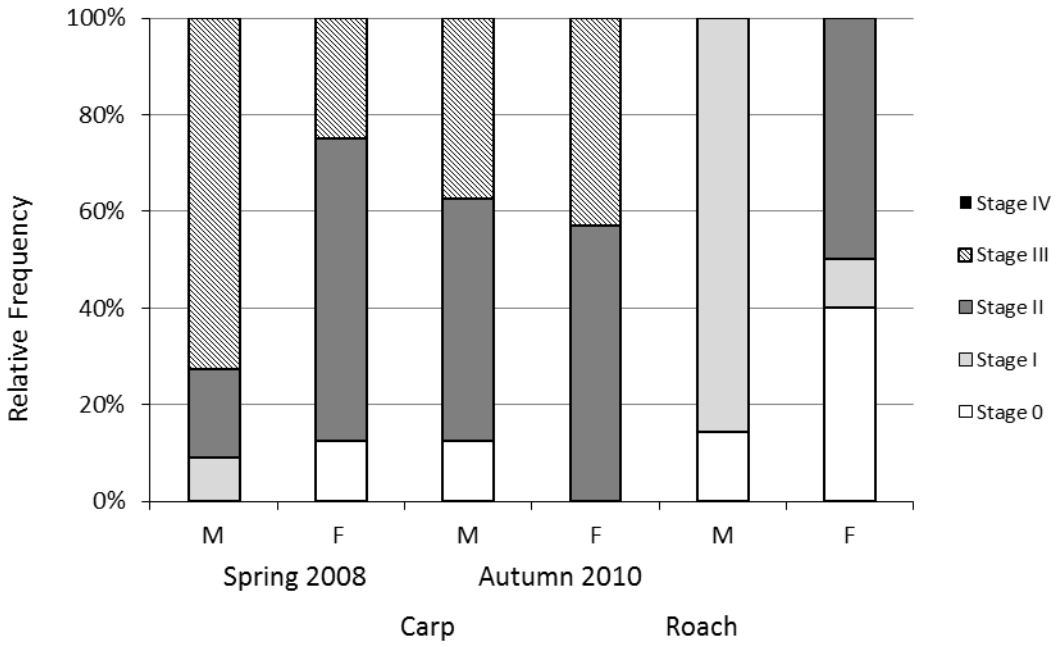

Figure 3. Relative frequencies of different reproductive stages in carp and roach sampled from the Yarra River, Australia. Carp were collected in Spring 2008 (October) and Autumn 2010 (April). Roach were collected in Autumn 2010 (April).

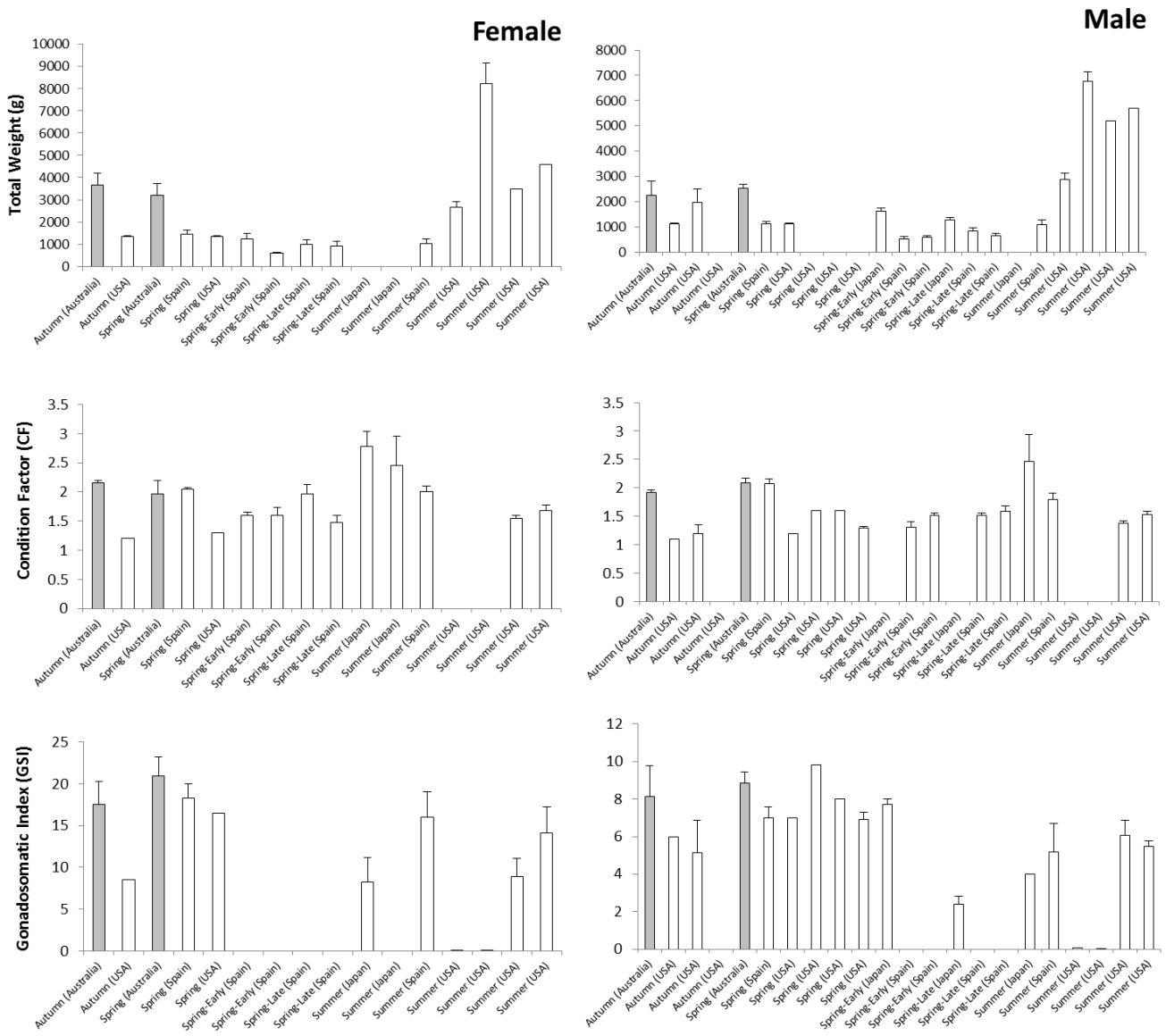

Figure 4. Comparison of biological measurements (mean ± SE) in common carp (*Cyprinus carpio*) collected from the Yarra River, Australia (shaded bars) relative to carp collected from reference sites in other environmental studies in Europe, USA and Japan. Samples are grouped by season then location.

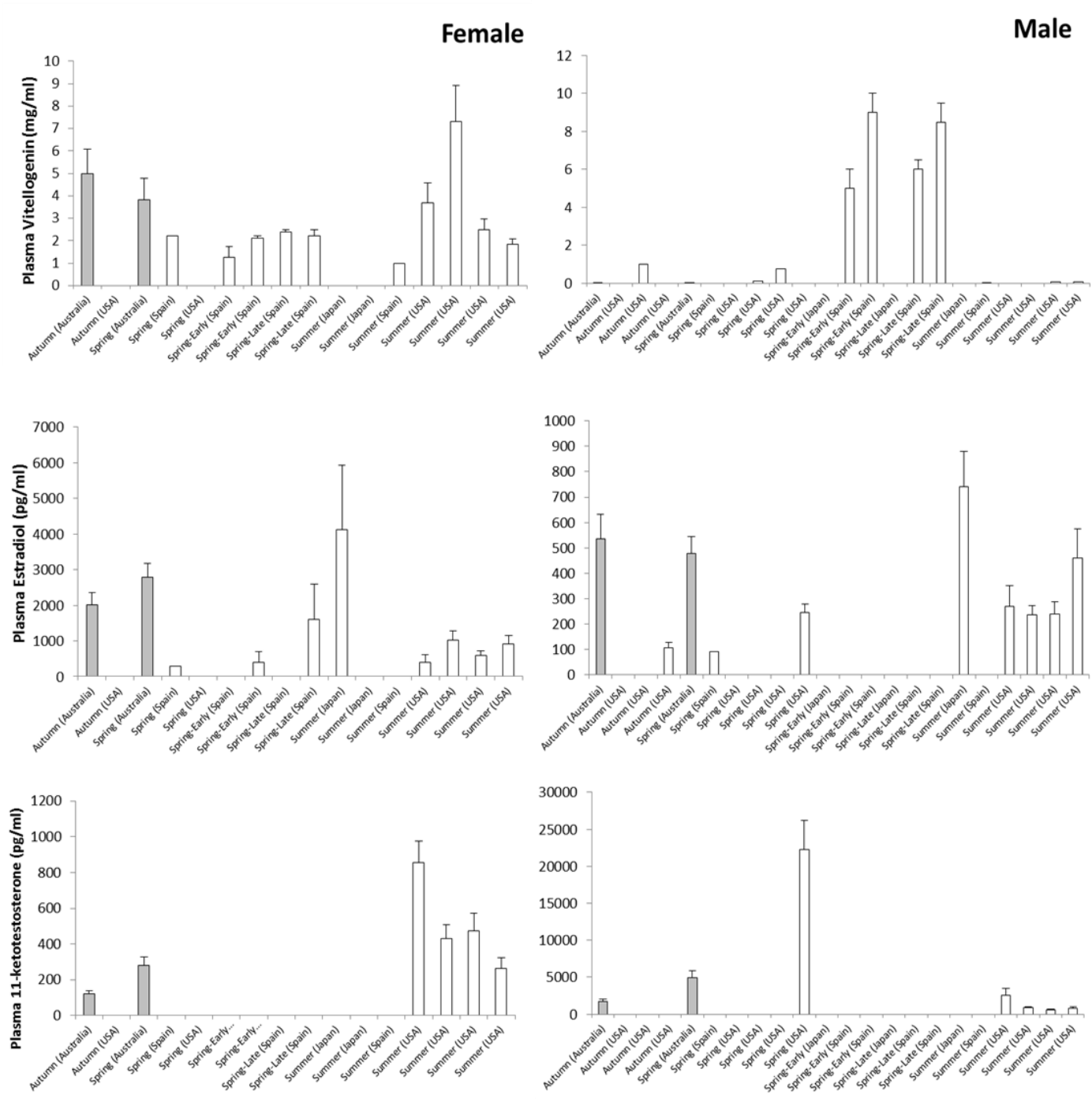

Figure 5. Comparison of biomarkers of endocrine disruption (mean ± SE) in common carp (*Cyprinus carpio*) collected from the Yarra River, Australia (shaded bars) relative to carp collected from reference sites in other environmental studies in Europe, USA and Japan. Samples are grouped by season then location.

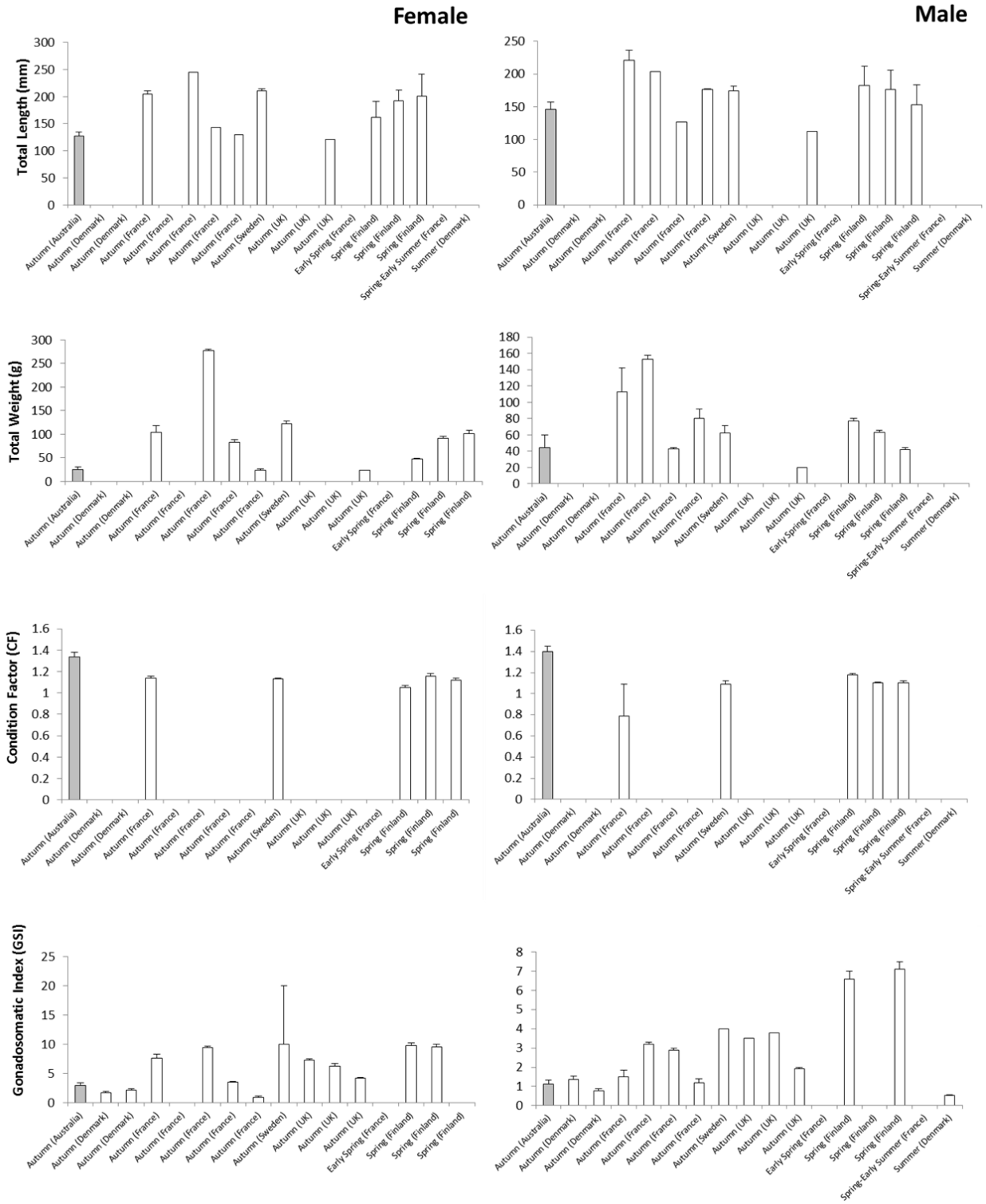

Figure 6. Comparison of biological measurements (mean ± SE) in roach (*Rutilis rutilis*) collected from the Yarra River, Australia (shaded bars) relative to roach collected from reference sites in other environmental studies in Europe. Samples are grouped by season then location.

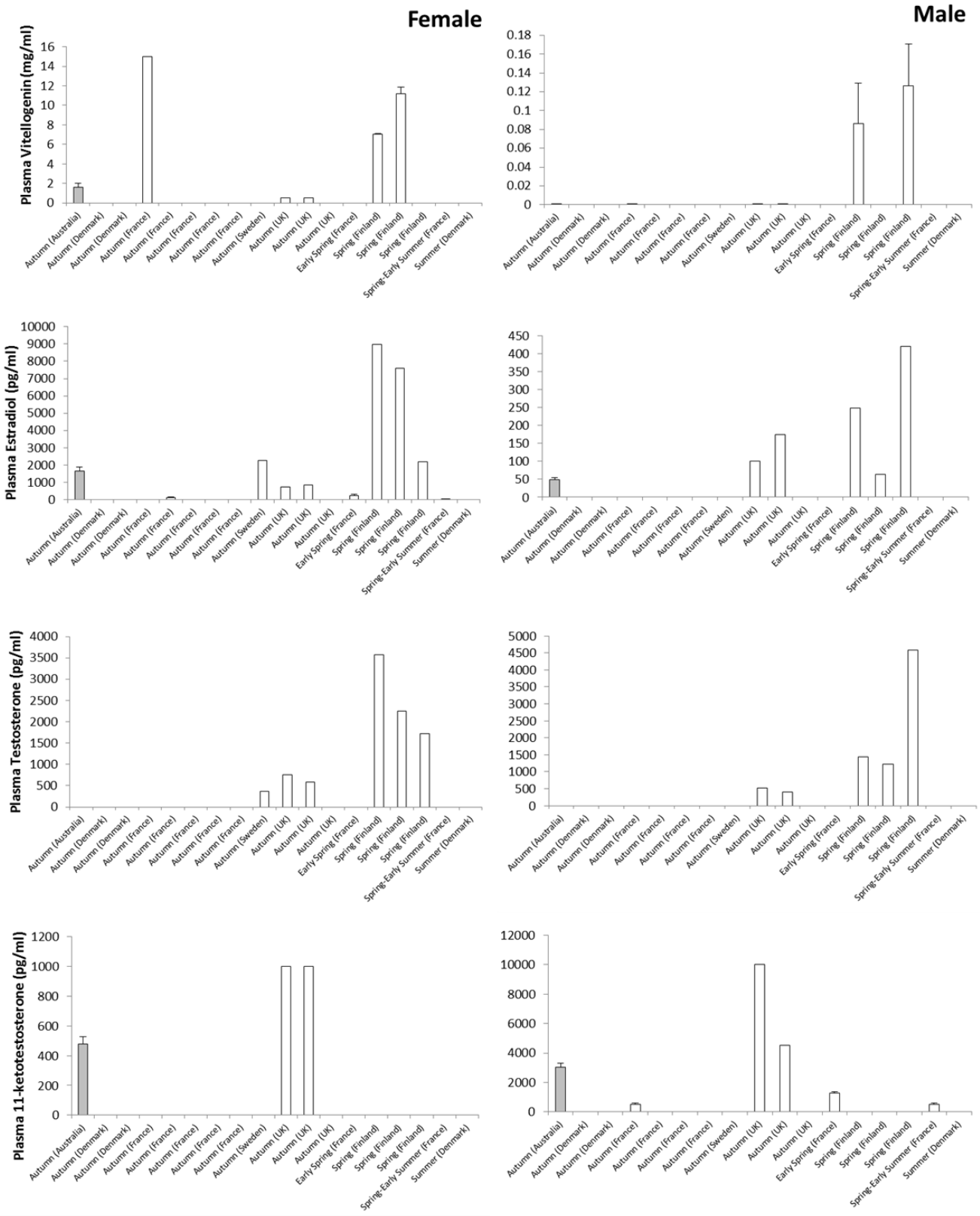

Figure 7. Comparison of biomarkers of endocrine disruption (mean ± SE) in roach (*Rutilis rutilis*) collected from the Yarra River, Australia (shaded bars) relative to roach collected from reference sites in other environmental studies in Europe. Samples are grouped by season then location.

Appendices

Table A1: Summary (mean ±SE) of biological measurements and biomarkers of endocrine disruption in carp from various studies in Europe, USA and Japan. Data from reference sites in this table were used for comparison with carp collected from the Yarra River, Australia as part of the present study.

Table A2. Summary of histological alterations reported in carp from various studies in Europe, USA and Japan. Data from reference sites in this table were used for comparison with carp collected from the Yarra River, Australia as part of the present study.

Table A3: Summary (mean ±SE) of biological measurements and biomarkers of endocrine disruption in roach from various studies in Europe. Data from reference sites in this table were used for comparison with roach collected from the Yarra River, Australia as part of the present study.

Table A4. Summary of histological alterations reported in roach from various studies in Europe. Data from reference sites in this table were used for comparison with roach collected from the Yarra River, Australia as part of the present study.

Table A5: Sampling locations within the Yarra River, and associated features.

Appendices

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