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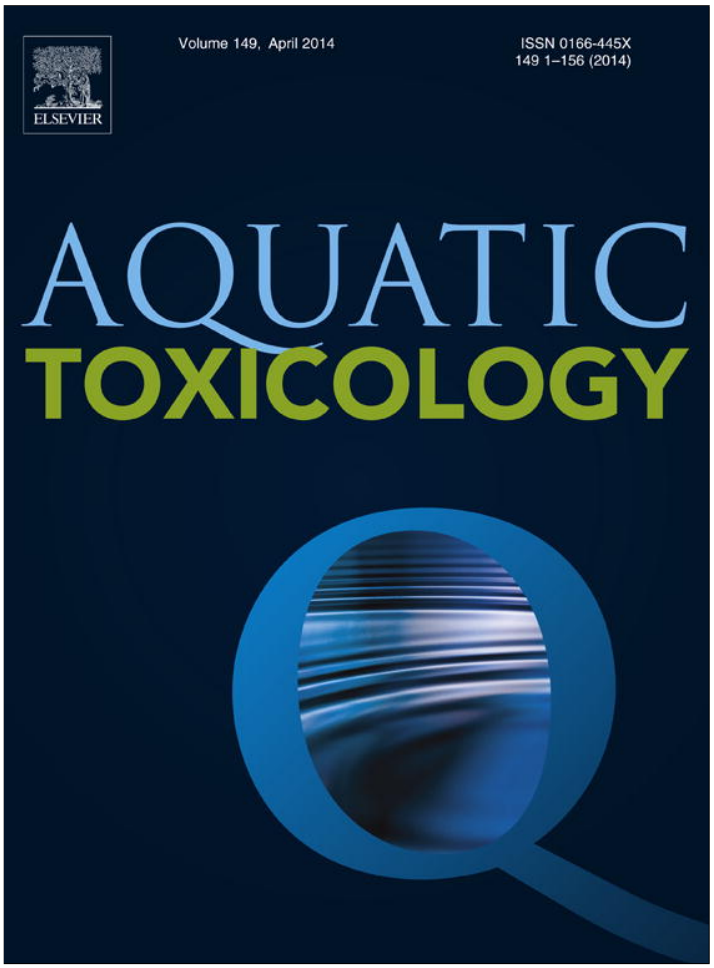

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Altered reproductive behaviours in male mosquitofish living downstream from a sewage treatment plant

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ABSTRACT

Freshwater environments are common repositories for the discharge of large volumes of domestic and industrial waste, particularly through wastewater effluent. One common group of chemical pollutants present in wastewater are endocrine disrupting chemicals (EDCs), which can induce morphological and behavioural changes in aquatic organisms. The aim of this study was to compare the reproductive behaviour and morphology of a freshwater fish, the mosquitofish (*Gambusia holbrooki*), collected from two sites (wastewater treatment plant (WWTP) and a putative pristine site). The mosquitofish is a sexually dimorphic livebearer with a coercive mating system. Males inseminate females using their modified anal fin as an intromittent organ. Despite this, females are able to exert some control over the success of male mating attempts by selectively associating with, or avoiding, certain males over others. Using standard laboratory assays of reproductive behaviour, we found that mosquitofish males living in close proximity to WWTP showed increased mating activity compared to those inhabiting a pristine site. More specifically, during behavioural trials in which males were allowed to interact with females separated by a transparent divider, we found that WWTP-males spent more time associating with females. Concordant with this, when males and females were subsequently allowed to interact freely, WWTP-males also spent more time chasing and orienting towards the females. As a result, females from both sites showed more interest towards the WWTP-site males. Male anal fin morphology, however, did not differ between sites. Our study illustrates that lifetime exposure to WWTP-effluents can greatly affect male behaviour. The results underscore the importance of behaviour as a potential tool for investigating unknown contaminants in the environment.

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Introduction

Urban and peri-urban waters can contain a large number of organic chemicals from a diverse range of sources, such as roadside run-off, waste water treatment plant (WWTP) inputs, stormwater and agricultural enterprises. Some of these pollutants may prove directly toxic to aquatic organisms (*e.g.* Guillette et al., 1995), while others may elicit more subtle effects (Vandenberg et al., 2012). For example, pollutants such as endocrine disrupting chemicals (EDCs) present in WWTP effluents and surface waters (Johnson and Sumpter, 2001; Viganò et al., 2008; Thorpe et al., 2009; Allinson et al., 2010), can interfere with the endocrinology of aquatic and terrestrial organisms (Scott and Sloman, 2004; Waring and Harris, 2005; Kidd et al., 2007; Ankley et al., 2009). Intense research on the topic during the last two decades has shown that EDCs can cause a wide range of physiological and morphological changes (Oppen-Berntsen et al., 1992; Jobling et al., 1998; Christensen et al., 1999; Ankley et al., 2003). Nevertheless, there has been a growing number of studies in recent years looking at the impacts of EDCs

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on behaviour (see reviews: Zala and Penn, 2004; Clotfelter et al., 2004; Scott and Sloman, 2004; Söffker and Tyler, 2012). Behaviour has proven to be a sensitive early-warning indicator of contamination (Bell, 2001; Martinovic et al., 2007; Saaristo et al., 2009b; Hallgren et al., 2011; Shenoy, 2012) and, most importantly, altered sexual behaviours are predicted to have important ecological and evolutionary consequences (Candolin and Wong, 2012)

So far, only a few studies have explored the impact of industrial and WWTP effluents on the reproductive behaviour of fish – and the results have been equivocal. While some studies have detected impacts on behaviour, others have not. For instance, fathead minnow (*Pimephales promelas*) males failed in competing for nest sites and mates after being exposed to estrogenic WWTP effluent for three weeks (Martinovic et al., 2007), while three-spined stickleback (*Gasterosteus aculeatus*) males showed altered reproductive behaviour after short-term exposure to anti-androgenic sewage effluents (Sebire et al., 2011). By contrast, in two separate studies on eastern mosquitofish (*Gambusia holbrooki*), no differences were found in male behaviours between contaminated and reference sites, even though morphological indicators (anal fin length, testis size, liver weight) showed clear impact of EDCs (Toft et al., 2003, 2004). Clearly, the causal link between behavioural responses and WWTP effluents warrants further investigation.

Our study species, the eastern mosquitofish (*G. holdbrooki*) is an excellent model system for studying contaminants in WWTP effluents. The species has a widespread, cosmopolitan distribution in shallow freshwater habitats in both urban and agricultural areas (Pyke, 2005). The mosquitofish is a small (size 20–40 mm) sexually dimorphic livebearer, with males inseminating females using their gonopodium (a modified anal fin), as an intromittent organ (Constantz, 1984). Male mosquitofish do not court females but, instead, attempt forced copulations by thrusting their gonopodia towards the female's genital pore (McPeek, 1992; Bisazza and Marin, 1995). Despite the coercive mating system, female mosquitofish have been shown to be choosy (Bisazza et al., 2001; Kahn et al., 2010) and are able to exert some control over the success of male mating attempts by selectively approaching certain males over others (Bisazza et al., 2001). Due to their internal mode of fertilisation, male mosquitofish need to be in close proximity to females before any mating attempts can be made (Martin, 1975). Females prefer males with larger gonopodia, and so male genitalia appears to be a sexual ornament that is under the influence of sexual selection (Kahn et al., 2010). Morphologically, mosquitofish present a valuable local indicator species for exposure to EDCs because the development of the male gonopodium is androgen dependent. Indeed, previous research has found that embryonic exposure to androgenic hormones can increase the length of gonopodium of males in relation to body size (Angus et al., 2001; Leusch et al., 2006), and induce gonopodial development in females (Turner, 1942; Bortone and Davis, 1994; Angus et al., 2001).

The aim of our study was to examine the effects of aquatic contaminants on male and female reproductive behaviour and morphology of wild collected fish. Specifically, we compared male and female reproductive behaviours and anal fin morphology in mosquitofish that had been living in close proximity to a WWTP and a comparatively more pristine (*i.e.* reference) site. Laboratory-based studies of EDC exposure are often criticised for lacking ecological relevance (see review by Zala and Penn, 2004), as they tend to be conducted using single compounds or laboratory reared model species rather than individuals from wild populations. On the other hand, field observations alone, without follow up experimental studies, are often insufficient to establish cause and effect. By bringing wild fish into the laboratory, our approach combines field exposure with laboratory-based behavioural assays.

Material and methods

Sampling sites and collection of fish

The study was carried out in Victoria, Australia, during March–April 2011, which corresponds to the late breeding season of mosquitofish (Haynes and Cashney, 1995). The collection sites Brodies Lake (reference site) and Jackson's Creek (WWTP-site) were chosen according to an EDC-monitoring program being carried out by The Centre of Aquatic Pollution, Identification and Monitoring (CAPIM) in 2010–2011 (Chinathamby et al., 2013). Brodies Lake is a relatively pristine site (total estrogen equivalent concentration: 0.5 ng/EEQ/L: Chinathamby et al., 2013) located adjacent to a reservoir that supplies drinking water to parts of suburban Melbourne. Jackson's Creek, by contrast, was 100 m downstream from the outlet of a WWTP (total estrogen equivalent concentration: 12.5 ng/L/EEQ: Chinathamby et al., 2013). We collected 300 fish from each site and took water samples to determine the concentration of estrone (E1) estradiol (E2) and ethinyl estradiol (EE2); and the androgen androstenedione. Fish were caught with dip nets and brought back to the laboratory where they were separated by sex and acclimated to laboratory conditions (12:12 h light regime) for two weeks in 54 L tanks (20 fish per tank), before the commencement of the behavioural trials (Fig. 1). Fish were kept in filtered freshwater tanks during the acclimation period. Water temperature in the tanks was monitored daily and ranged from 19 to 23 °C. Fish were fed *ad libitum* with commercial fish flakes (Otohime Hirame, Aquasonic) once a day.

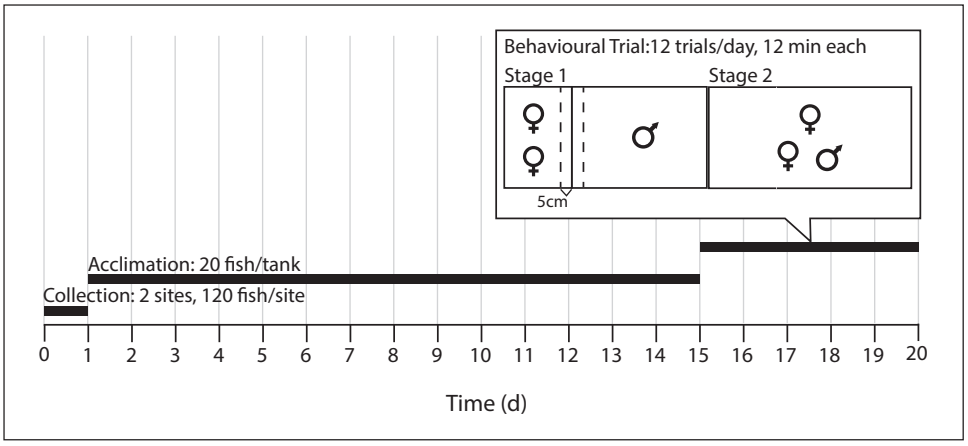

Fig. 1. Experimental timeline showing the time of fish collection, acclimation period and behavioural assay. A detailed description of the behavioural assay is also presented.

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Table 1
Total length of females and males (mean ± SE) used in the behavioural trials. Trial combinations were: (1) control male and control female; (2) WWTP-male and control female; (3) WTTP-male and WTTP-female.

Trial combination	*n*	Mean total length (mm)	S.E.
Female			
C-male and C-female	20	35.4	0.50
WTTP-male and C-female	19	38.7	0.50
WTTP-male and WTTP-female	21	34.5	0.70
Male			
C-male and C-female	20	27.0	0.40
WTTP-male and C-female	19	26.7	0.48
WTTP-male and WTTP-female	21	26.4	0.47

Behavioural assays

The trial tanks (60 cm × 30 cm × 24 cm) had a 3-cm layer of gravel on the bottom and an air-driven filter in each corner (Fig. 1). The behavioural assay consisted of two stages. During stage one, the trial tank was divided into two compartments by a transparent divider, which had several holes for water change and chemical communication. The experiment started by placing two sexually mature females (both from either the reference or WWTP-site) into a randomly chosen compartment of the tank, while a male (from either the reference or WWTP-site) was placed into the other compartment. This resulted in three treatment combinations: (1) A male from the reference site with females from the reference site ($n = 20$), (2) a male from the WWTP-site with females from the WWTP-site ($n = 19$), and (3) a male from the WWTP-site with females from the reference site ($n = 21$). There was no difference in the total length of either males (ANOVA: $F = 0.588$, df = 15, $n = 60$, $p = 0.869$) or females (ANOVA: $F = 0.748$, df = 15, $n = 60$, $p = 0.724$) between the treatment combinations (see Table 1 for details). Behavioural observations were conducted at the same time every day (10:00–15:00). First, the fish were allowed to settle for 5 min before the video recording started. Male and females behaviours were recorded for 12 min (Camcorder HDC-SD40, Panasonic). For scoring purposes, each of the compartments of the experimental tank was divided into zones. The zone closest to the divider (5 cm) was designated as the 'preference zone', and the zone outside the preference zone was designated as the 'neutral zone'. For females, the proportion of time both females were simultaneously in the preference zone was used to indicate the females' interest in the males (Houde, 1997). Association time is a viable measure of mating intentions in poeciliid fishes (*e.g.* Dosen and Montgomerie, 2004; Wong et al., 2005) and is a standard measure of mating preferences in mosquitofish (Wong and McCarthy, 2009; Kahn et al., 2010; Saaristo et al., 2013).

During stage 2, the dividers separating the male and female compartments were removed, thus allowing males to interact freely with the females. Interactions between the male and females were then video recorded for a further 12 min. From the videos, we quantified whether or not the male was showing an interest in the female by orienting towards her (within 5 cm of her body), chasing her, or engaging in gonopodial thrusts (Saaristo et al., 2013). Due to the difficulties of keeping track of individual, size-matched females, only male behaviours were quantified in stage 2.

Behavioural data from both assays was analysed using JWatcher software, which calculates duration (ms) and frequency of each quantified behaviour during the recording period. Behavioural data was scored blind to the treatment. After the behavioural assays, fish were caught and euthanised with an overdose (40 mg/L) of anaesthetic clove oil (Cunha and Rosa, 2006). Fish were weighed and measured (to the nearest mm), and males preserved in 70% ethanol for further morphological measurements.

Anal fin measurements

We measured the key features of the gonopodial anatomy in males following the methods of Doyle and Lim (2002). The anal fin was photographed using a moticam 3.0 mounted on a Motic SMZ-168 stereomicroscope. From these images using DigiLab-II software, we obtained the following measurements: the length of the fourth (R4) and sixth (R6) rays in the anal fin, body length, and the number of serrae and hooks on the gonopodium tip.

Water chemistry

Collection sites were sampled twice: at the time of fish collection, and two months later. Methodology for measurement of E1, E2 and 17 alpha-ethinyl estradiol using commercial enzyme linked immunosorbent assays (ELISA) is explained in detail in Allinson et al. (2010). The levels of these estrogens were supported by liquid chromatography–mass spectrometry (LC–MS/MS) analysis of the extracts prepared for ELISA analysis using the method of Yamamoto et al. (2006).

As a proxy for androgen contamination, measurement of androstenedione was also included using LC–MS/MS using the method of Yamamoto et al. (2006).

Ethical note

The methods for animal housing, handling and experimental protocols were assessed and approved by the Biological Sciences Animal Ethics Committee at Monash University (permit number: BSCI/2011/07). Because mosquitofish are a noxious species under State laws, the terms of the collecting permit (Department of Primary Industries Victoria, permit number NP191) did not allow them to be returned to the wild and hence fish were euthanised at the end of the study.

Statistical analysis

Data were checked for normality and heterogeneity of variance. Male behavioural and morphological data were not normally distributed and we therefore tested for differences between treatment groups using the Kruskall–Wallis test, followed by Mann–Whitney tests for *post hoc* comparisons. Female behavioural data was normally distributed and analysed with one-way ANOVA followed by *post hoc* Tukey tests. Tank (exposure and behavioural trial) and time of the behavioural assay were factored into the tests and were found to be non-significant ($p > 0.05$). Behavioural data were analysed as total time (ms) and frequency counts. All statistical analyses were performed using SPSS 20.0.

Results

Behavioural assays

In stage 1, reference site males spent significantly more time in the non-preference zone compared to the WWTP-site males (Kruskall–Wallis, Chi-Square = 13.036, df = 2, $p < 0.001$, $n = 60$, Fig. 2a). Females presented with WWTP males also spent more time in the preference zone than females presented with reference males (ANOVA, $F = 3.35$, df = 2, $p = 0.01$, $n = 60$; Fig. 2b).

In stage 2, when fish were allowed to freely interact, WWTP-site males showed significantly more interest in the females than did the reference-site males (Kruskall–Wallis: Chi-Square = 21.22, df = 2, $p < 0.001$, $n = 60$, Fig. 3a). Specifically, WWTP-site males spent more time orientating towards females irrespective of female origin (Chi-Square = 22.974, df = 2, $p < 0.001$, $n = 60$, Fig. 3b), chased reference site females more (Mann–Whitney: $U = 97.50$, $p = 0.009$,

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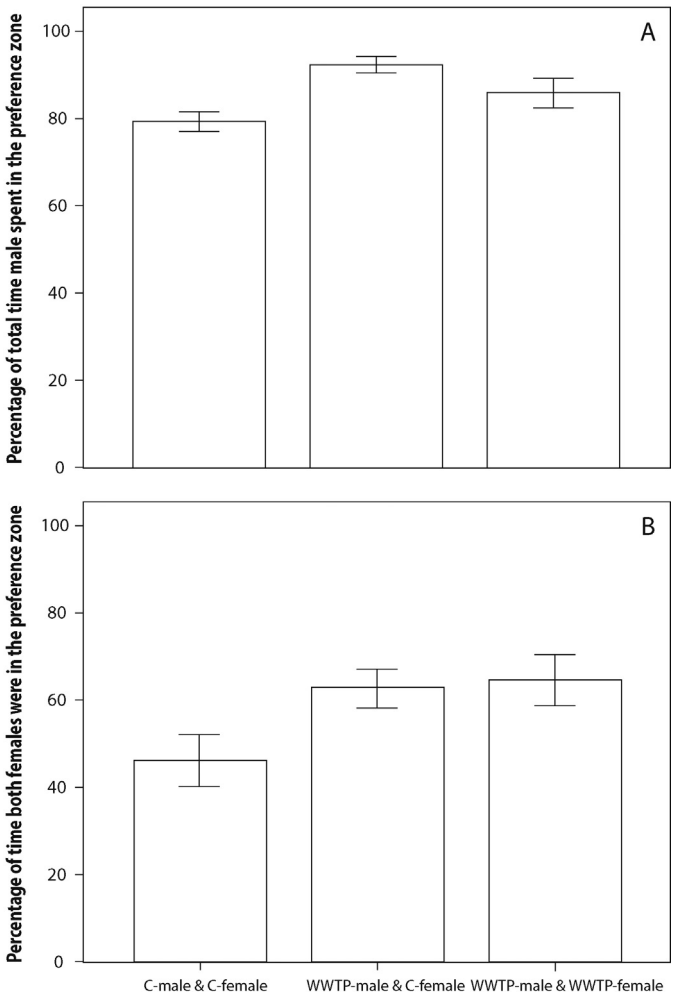

Fig. 2. Mean percentage of time (A) a male spent in the preference zone, and (B) both females were simultaneously in the preference zone. Error bars present standard error of mean. The three treatment combinations are: (1) A male from the reference site with females from the reference site ($n = 20$), (2) a male from the WWTP-site with females from the WWTP-site ($n = 19$), and (3) a male from the WWTP-site with females from the reference site ($n = 21$).

$n = 40$, Fig. 3c), and performed gonopodial thrusts more frequently than reference site males ($U = 116.50$, $p = 0.039$, $n = 40$, Fig. 3d).

Morphological measurements

Male anal fin morphology did not differ between WWTP- and reference site. Specifically, there were no statistically significant differences in the ray 4: body length ratio (Mann–Whitney: $U = 262$, $p \geq 0.05$, $n = 60$), R4: R6 ratio ($U = 341$, $p \geq 0.05$, $n = 57$) or the number of serrae ($U = 277$, $p \geq 0.05$, $n = 60$).

Water chemistry

The measured estrogen levels (ELISA) and androstenedione concentrations (LC–MS/MS) are listed in Table 2.

Discussion

Reproductive behaviour

Our study demonstrated that male mosquitofish living in close proximity to a wastewater treatment plant (WWTP) showed increased mating activity. Specifically, WWTP-males spent more time orienting towards females (both WWTP and reference site). They also chased and performed gonopodial thrusts more frequently towards the reference site females. The majority of studies looking at the impacts of estrogenic EDCs show that such pollutants can impair reproductive behaviours (Baatrup and Junge, 2001; Bell, 2001; Bayley et al., 2002; Zala and Penn, 2004; Kristensen et al., 2005; Martinovic et al., 2007; Larsen et al., 2008; Saaristo et al., 2009a,b, 2010a,b; Söffker and Tyler, 2012) but a few have found no impact on mating behaviours (Bortone et al., 1989; Nash et al., 2004). In the current study, comparable levels of estrogenic EDCs were measured at the two sites. Thus, it seems unlikely that estrogenic EDCs, on their own, are responsible for the behavioural differences. However, they could have played a role through additive effects – estrogens together with androgenic/anti-estrogenic EDCs might have had stimulating effects on behaviour (see review by Kortenkamp, 2007). Our behavioural measurements, therefore, suggest that other compounds present at the WWTP-site were likely responsible for the underlying differences in behaviour.

One possibility is that the altered behaviours observed in our study were due to the exposure of fish to androgenic EDCs. The male-typical steroid testosterone is known to mediate aggression and courtship behaviour in males (Lephart, 1996). Several laboratory studies have shown that male fish have higher androgen levels during courtship (Knapp et al., 1999; Pankhurst et al., 1999; Rodgers et al., 2006) and male-male competition (Whoriskey and FitzGerald, 1994). In this regard, the observed increase in mating activity (*i.e.* more frequent gonopodial thrusts and chasing) in WWTP-site males is consistent with an earlier study on the closely related western mosquitofish (*Gambusia affinis*). In that study, Howell et al. (1980) showed that males collected from a site downstream from paper mill effluent discharge were more aggressive in their mating attempts than males from a reference site (Howell et al., 1980). So, could androgens underlie the behavioural differences reported in our study?

We measured the androgen androstenedione, which is an important precursor of male and female sex hormones (Achermann and Hughes, 2008) and has been detected in sediments and rivers receiving effluents from various sources (Jenkins et al., 2001, 2003; Durhan et al., 2002). Here, we only observed approximately 1 ng/L of androstenedione in the samples. However, it is important to bear in mind that this is likely to be an underestimate since re-processing of the ELISA extracts for LC–MS/MS analysis through additional chemical and purification separation steps will have resulted in significant loss of analytes. Although it is unknown what other androgenic and anti-androgenic compounds were present (and at what levels) at the time of sampling for this study, we do know that testosterone and androstenedione were observed previously in the WWTP effluent at similar levels to the steroid estrogen concentrations in this study (Allinson et al., 2008). This would suggest that androgenic pollutants at the WWTP site may actually have been much higher than those observed in the re-processed samples. Such a possibility warrants further investigation but it nevertheless confirms that behaviour can be a sensitive biomarker of exposure to pollutants, especially when the specific contaminants are not explicitly known.

Fin morphology

Male mosquitofish, like other poeciliids, rely on their modified anal fin (*i.e.* gonopodium) for the successful transfer of sperm to females during copulation. Because anal fin development is dependent on androgens, gonopodium length has been widely used to test for EDC exposure (Angus et al., 2001; Turner, 1942; Bortone and Davis, 1994; Batty and Lim, 1999; Dreze et al., 2000; Doyle and Lim, 2005, Leusch et al., 2006). For example, male mosquitofish sampled from sewage and WWTP sites were found to

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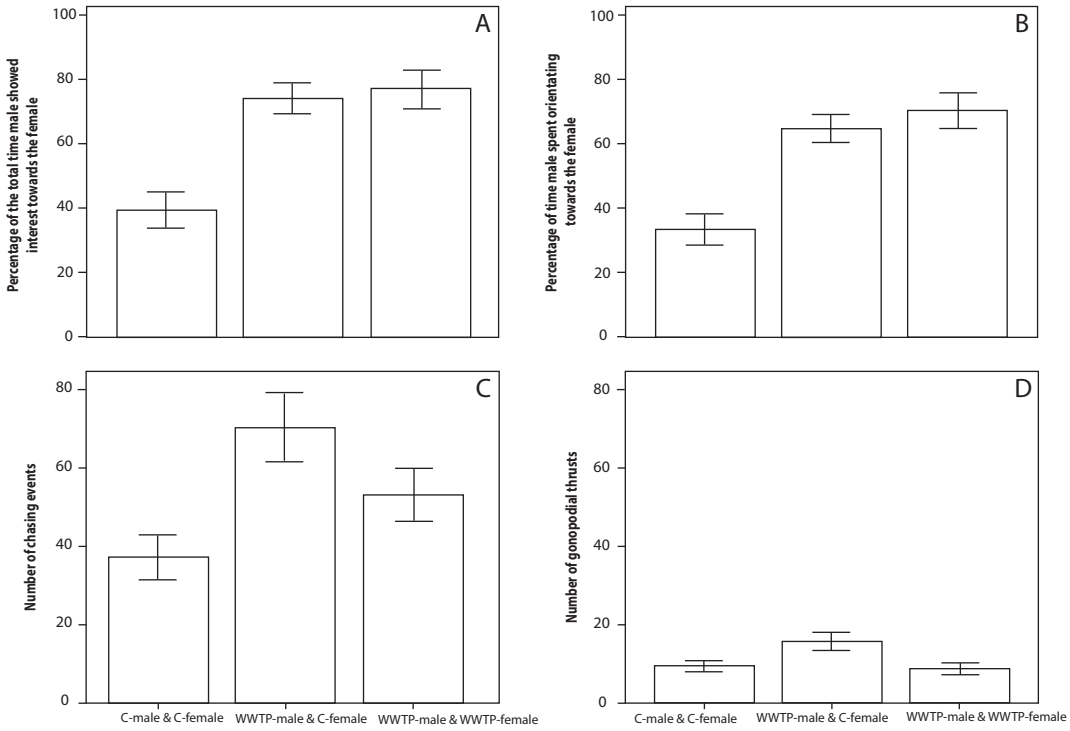

Fig. 3. Percentage of time a male spent (A) showing interest towards the female, (B) orientating towards the female. Mean frequency male spent (C) chasing the females and (D) performing gonopodial thrusts. Error bars present error of mean. The three trial combinations are: (1) A male from the reference site with females from the reference site ($n = 20$), (2) a male from the WWTP-site with females from the WWTP-site ($n = 19$), and (3) a male from the WWTP-site with females from the reference site ($n = 21$).

possess shorter gonopodia than mosquitofish sampled at reference sites (Batty and Lim, 1999), indicating the presence of estrogenic and/or anti-androgenic chemicals (Doyle and Lim, 2002). In the current study, however, we did not observe any abnormal anal fin development in the mosquitofish males collected from the WWTP site, and, further, found no other morphological differences between the collection sites. This is consistent with Leusch et al. (2006) who reported that undiluted tertiary-treated WWTP effluent had no measurable effects on morphological indicators of exposure in mosquitofish. Together with our results, it would appear that gonopodium length is not necessarily a comprehensive, single indicator of exposure and should be accompanied by a suite of other biomarkers, including non-lethal measures such as behaviour. Indeed, behaviour appears to be a much more sensitive indicator of chemical contamination and, as a result, should be used more widely in ecotoxicological risk assessments.

Water chemistry: estrogenic activity in the sampling sites

Estrogenic activity was observed at both the reference and WWTP effluent impacted sites (Table 2). The presence of estrogens at the WWTP site was unsurprising, since estrogens and estrogenic activity has been observed in the final effluent from the plant and the site waters previously (Allinson et al., 2010; Chinathamby et al., 2013). However, the presence of estrogens at the reference site was unexpected given the lack of obvious sources in that catchment (*i.e.* no WWTP or intensive animal husbandry). Although immunoassay tests seldom give false negatives, they may give false positives (*i.e.* detection by immunoassay but not by more traditional chromatographic-mass spectrometry laboratory analysis; Morozova et al., 2005), and so the effects of cross-reactivity and matrix interference in the samples on the ELISA system producing erroneous readings at the reference site cannot be discounted. However, in that context, low levels of E1 and E2 were confirmed using LC–MS/MS (data not reported) and Allinson et al. (2010, 2011) have shown that, when used with the appropriate sample preparation methods, the use of commercial ELISA kits is an accurate way to cost-effectively rapidly screen water samples.

In Australia, there have been few published studies of steroid concentrations in waterways, but in that context, the total estrogen (ES) levels observed in this study are comparable with previous information released on the sites investigated (Chinathamby et al.

Table 2
Summary of the water samples analysed from the two sampling sites. Methods used for the quantification were enzyme-linked immunosorbent assay (ELISA) and liquid chromatography–mass spectrometry (LC–MS–MS).

Sampling site and date	Methodology				
	ELISA				LC–MS–MS
	E1 (ng/L)	E2 (ng/L)	EE2 (ng/L)	Total estrogens (ng/E2eq/L)	Androstenedione (ng/L)
Brodies Lake					
15/2/2011	9.52	3.01	0.26	7.82	1.1
22/3/2011	3.68	2.47	0.14	6.52	
Jackson's Creek					
25/03/11	4.40	2.75	0.43	8.97	1.0
16/05/11	9.48	3.35	0.51	13.19	

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(2013). In Queensland, up to 11.8 ng/L estradiol equivalents (EEQ) has been reported in receiving river waters downstream of an effluent discharge point (Ying et al., 2009). Internationally, the E1 and E2 levels observed in this study are well within the wide range of concentrations reported in waterways: China: 2.3–7.4 ng/E2/L (Wang et al., 2011, 2012a,b); 15.6–55.8 ng/E1/L (Wang et al., 2011, 2012a,b); France: up to 2.5 ng/L (EEQ) in the River Seine; Italy: 5.0 ± 6.1 ng/L EEQ and 6.7 ± 7.4 ng/L EEQ upstream and downstream of a WWTP (Schilirò et al., 2009); USA: widely varying estrogenic activity in agricultural and peri-urban rivers in California (<0.15–56 ng/L EEQ respectively) (Lavado et al., 2009).

Conclusions

Our study demonstrates that mosquitofish males coming from proximity to a WWTP showed increased mating activity. More specifically, males collected from a WWTP-impacted site spent more time orienting towards the females (both WWTP and reference site), and chased reference site females more frequently. These changes can have important fitness consequences, especially for females, which are likely to be subjected to increased harassment in a species whose mating system is dominated by coercive mating attempts. Unnecessary copulations come at a cost as harassed females produce smaller and less attractive offspring (Clutton-Brock and Parker, 1995; Pilastro et al., 2003; Gasparini et al., 2012). Our study therefore underscores the importance of using behaviour as a sensitive tool in assessing the impact of mixtures of unknown contaminants, including EDCs, present in the environment.

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References

Achermann, J.C., Hughes, I.A., 2008. Disorders of sex development. In: Larsen, P.R., Kronenberg, H.M., Melmed, S., Polonsky, K.S. (Eds.), Williams Textbook of Endocrinology. , 11th edition. Saunders, Philadelphia, PA, pp. 783–848.

Allinson, M., Shiraishi, F., Allinson, G., 2011. A comparison of recombinant receptor-reporter gene bioassays and a total estrogen enzyme linked immunosorbant assay for the rapid screening of estrogenic activity in natural and waste waters. Bull. Environ. Contam. Toxicol. 86, 461–464.

Allinson, M., Shiraishi, F., Salzman, S.A., Allinson, G., 2010. In vitro and immunological assessment of the estrogenic activity and concentrations of 17β-estradiol, estrone and ethinyl estradiol in treated effluent from forty-five wastewater treatment plants in Victoria, Australia. Arch. Environ. Contam. Toxicol. 58, 576–586.

Allinson, G., Allinson, M., Shiraishi, F., Salzman, S.A., Myers, J.H., Hermon, K.M., et al., 2008. Androgenic activity of effluent from forty-five municipal wastewater treatment plants in Victoria, Australia. WIT Trans. Ecol. Environ. 110, 293–304.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, W., et al., 2003. Effects of the androgenic growth promoter 17β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22, 1350–1360.

Ankley, G.T., Bencic, D., Breen, M.S., Collette, T.W., Conolly, R.B., Denslow, N.D., et al., 2009. Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanism of action. Aquat. Toxicol. 92, 168–178.

Angus, R.A., McNatt, H.B., Howell, W.M., Peoples, S.D., 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): a quantitative study using computer image analysis. Gen. Comp. Endocrinol. 123, 222–234.

Baatrup, E., Junge, M., 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*). Environ. Health Pers. 109, 1063–1070.

Batty, J., Lim, R., 1999. Morphological and reproductive characteristics of male mosquitofish (*Gambusia affinis holbrooki*) inhabiting sewage-contaminated waters in New South Wales, Australia. Arch. Environ. Contam. Toxicol. 36, 301–307.

Bayley, M., Junge, M., Baatrup, E., 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat. Toxicol. 56, 227–239.

Bell, A.M., 2001. Effects of an endocrine disrupter on courtship and aggressive behavior of male threespined stickleback, *Gasterosteus aculeatus*. Anim. Behav. 62, 775–780.

Bisazza, A., Vaccari, G., Pilastro, A., 2001. Female mate choice in a mating system dominated by male sexual coercion. Behav. Ecol. 12, 59–64.

Bisazza, A., Marin, G., 1995. Sexual selection and sexual size dimorphism in the eastern mosquitofish *Gambusia holbrooki* (Pisces Poeciliidae). Ethol. Ecol. Evol. 7, 169–183.

Bortone, S.A., Davis, W.P., 1994. Fish intersexuality as indicator of environmental stress. BioScience 44, 165–172.

Bortone, S.A., Davis, W.P., Bundrick, C.M., 1989. Morphological and behavioral characters in mosquitofish as potential bioindication of exposure to kraft mill effluent. Bull. Environ. Contam. Toxicol. 43, 370–377.

Candolin, U., Wong, B.B.M., 2012. Behavioural Responses to a Changing World: Mechanisms and Consequences. Oxford University Press, Oxford.

Chinathamby, K., Allinson, M., Shiraishi, F., Lopata, A.L., Nugegoda, D., Pettigrove, V., et al., 2013. Screening for potential effects of endocrine-disrupting chemicals in peri-urban creeks and rivers in Melbourne, Australia using mosquitofish and recombinant receptor–reporter gene assays. Environ. Sci. Pollut. Res. 20, 1831–1841.

Christensen, L.J., Korsgaard, B., Bjerregaard, P., 1999. The effect of 4-nonylphenol on the synthesis of vitellogenin in the flounder *Platichthys flesus*. Aquat. Toxicol. 46, 211–219.

Clotfelter, E.D., Bell, A.M., Levering, K.R., 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Anim. behav. 68, 665–676.

Clutton-Brock, T.H., Parker, G.A., 1995. Sexual coercion in animal societies. Anim. Behav. 49, 1345–1365.

Constantz, G.D., 1984. Sperm competition in poeciliid fishes. In: Smith, R.L. (Ed.), Sperm Competition and the Evolution of Animal Mating Systems. Academic Press, Orlando, pp. 465–485.

Cunha, F.E.A., Rosa, I.L., 2006. Anaesthetic effects of clove oil on seven species of tropical reef teleosts. J. Fish Biol. 69, 1504–1512.

Dosen, L.D., Montgomerie, R., 2004. Mate preferences by male guppies (*Poecilia reticulata*) in relation to the risk of sperm competition. Behav. Ecol. Sociobiol. 55, 266–271.

Doyle, C.J., Lim, R.P., 2002. The effect of 17β-estradiol on the gonopodial development and sexual activity of *Gambusia Holbrooki*. Environ. Toxicol. Chem. 21, 2719–2724.

Doyle, C.J., Lim, R.P., 2005. Sexual behavior and impregnation success of adult male mosquitofish following exposure to 17β-estradiol. Ecotoxicol. Environ. Saf. 61, 392–397.

Dreze, V., Monod, G., Cravedi, J.P., Biagianti-Risbourg, S., Le Gac, F., 2000. Effects of 4-nonylphenol on sex differentiation and puberty in mosquitofish (*Gambusia holbrooki*). Ecotoxicology 9, 93–103.

Durhan, E.J., Lambright, C., Wilson, V., Butterworth, B.C., Kuehl, D.W., Orlando, E.F., et al., 2002. Evaluation of androstenedione as an androgenic component of river water downstream of a pulp and paper mill effluent. Environ. Toxicol. Chem. 21, 1973–1976.

Gasparini, C., Devigili, A., Pilastro, A., 2012. Cross-generational effects of sexual harassment on female fitness in the guppy. Evolution 66, 532–543.

Guillette Jr., L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodward, A.R., 1995. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102, 680–688.

Hallgren, S., Volkova, K., Reyhanian, N., Olsen, K.H., Hällström, I.P., 2011. Anxiogenic behaviour induced by 17α-ethynylestradiol in male guppies (*Poecilia reticulata*). Fish Phyosiol. Biochem. 37, 911–918.

Haynes, J.L., Cashney, R.C., 1995. Life history and population dynamics of the western mosquitofish: a comparison of natural and introduced populations. J. Fish Biol. 46, 1026–1041.

Houde, A.E., 1997. In: Krebs, J.R., Clutton-Brock, T. (Eds.), Sex, Color and Mate Choice in Guppies. Princeton University Press, Princeton, New Jersey.

Howell, W.M., Black, D.A., Bortone, S.A., 1980. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masculinization. Copeia 4, 676–681.

Jenkins, R., Angus, R.A., Mcnatt, H., Howell, W.M., Kemppainen, J.A., Kirk, M., et al., 2001. Identification of androstenedione in a river containing paper mill effluent. Environ. Toxicol. Chem. 20, 1325–1331.

Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., 2003. Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol. Sci. 73, 53–59.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual distribution in wild fish. Environ. Sci. Technol. 32, 2498–2506.

Johnson, A.C., Sumpter, J.P., 2001. Removal of endocrine-disrupting chemicals in activated sludge treatment works. Environ. Sci. Technol. 35, 4697–4703.

Kahn, A.T., Mautz, B., Jennions, M.D., 2010. Females prefer to associate with males with longer intromittent organs in mosquitofish. Biol. Lett. 6, 55–58.

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Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., et al., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U.S.A. 104, 8897–8901.

Knapp, R., Wingfield, J.C., Bass, A.H., 1999. Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*). Horm. Behav. 35, 81–89.

Kortenkamp, A., 2007. Ten years of mixing coctails: a review of combination effects of endocrine-disrupting chemicals. Environ. Health Persp. 115 (Suppl. 1), 98–105.

Kristensen, T., Baatrup, E., Bayley, M., 2005. 17β-Ethinylestradiol reduces the competitive reproductive fitness of the male guppy (*Poecilia reticulata*). Biol. Reprod. 72, 150–156.

Lavado, R., Loyo-Rosales, J.E., Floyd, E., Kolodziej, E.P., Snyder, S.A., Sedlak, D.L., Schlenk, D., 2009. Site-specific profiles of estrogenic activity in agricultural areas of California's inland waters. Environ. Sci. Technol. 43, 9110–9116.

Larsen, M.G., Hansen, K.B., Henriksen, P.G., Baatrup, E., 2008. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminizing effects of 17α-ethinyloestradiol - morphological sexual characteristics do not. Aquat. Toxicol. 87, 234–244.

Leusch, F.D., Chapman, H.F., Kay, G.W., Gooneratne, S.R., Tremblay, L.A., 2006. Anal fin morphology and gonadal histopathology in mosquitofish (*Gambusia holbrooki*) exposed to treated municipal sewage effluent. Arch. Environ. Contam. Toxicol. 50, 562–574.

Lephart, E.D., 1996. A review of brain aromatase cytochrome P450. Brain Res. Rev. 22, 1–26.

Martin, R.G., 1975. Sexual and aggressive behavior, density and social structure in a natural population of mosquitofish, *Gambusia affinis holbrooki*. Copeia 3, 445–454.

Martinovic, D., Hogarth, W.T., Jones, R.E., Sorensen, P.W., 2007. Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. Environ. Toxicol. Chem. 26, 271–278.

McPeek, M.A., 1992. Mechanisms of sexual selection operating on body size in the mosquitofish (*Gambusia holbrooki*). Behav. Ecol. 3, 1–12.

Morozova, V.S., Levashova, A.I., Eremin, S.A., 2005. Determination of pesticides by enzyme immunoassay. J. Anal. Chem. 60, 202–217.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., et al., 2004. Long-term exposure to environmental concentration of the pharmceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Perspect. 112, 1725–1733.

Oppen-Berntsen, D.O., Gramjensen, E., Walther, B.T., 1992. Zona radiata proteins are synthesized by rainbow-trout (*Oncorhynchus mykiss*) hepatocytes in response to estradiol-17-beta. J. Endocrinol. 135, 293–302.

Pankhurst, N.W., Hilder, P.I., Pankhurst, P.M., 1999. Reproductive condition and behavior in relation to plasma levels of gonadal steroids in the spiny damselfish *Acanthochromis polyacanthus*. Gen. Comp. Endocrinol. 115, 53–69.

Pilastro, A., Benetton, S., Bisazza, A., 2003. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. Anim. Behav. 65, 1161–1167.

Pyke, G.H., 2005. A review of the biology of *Gambusia affinis* and *G. holbrooki*. Rev. Fish Biol. Fisher. 15, 339–365.

Rodgers, E.W., Earley, R.L., Grober, M.S., 2006. Elevated 11-ketotestosterone during paternal behavior in the Bluebanded goby (*Lythrypnus dalli*). Horm. Behav. 49, 610–614.

Saaristo, M., Craft, J.A., Bjork, H., Lehtonen, K.K., Lindström, K., 2009a. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17α-ethinyl estradiol, an endocrine disruptor. Horm. Behav. 55, 530–537.

Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2009b. Sand goby (*Pomatoschistus minutus*) males exposed to an endocrine disrupting chemical fail in nest and mate competition. Horm. Behav. 56, 315–321.

Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010a. An endocrine disrupting chemical changes courtship and parental care in the sand goby. Aquat. Toxicol. 97, 285–292.

Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010b. Exposure to 17α-ethinyl estradiol impairs courtship and aggressive behaviour of male sand gobies (*Pomatoschistus minutus*). Chemosphere 79, 541–546.

Saaristo, M., Tomkins, P., Allinson, M., Allinson, G., Wong, B.B.M., 2013. An Androgenic agricultural contaminant impairs female reproductive behaviour in a freshwater fish. PLoS ONE 8, e62782.

Schilirò, T., Pignata, C., Rovere, R., Fea, E., Gilli, G., 2009. The endocrine disrupting activity of surface waters and of wastewater treatment plant effluents in relation to chlorination. Chemosphere 75, 335–340.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.

Shenoy, K., 2012. Environmentally realistic exposure to the herbicide atrazine alters some sexually selected traits in male guppies. PlosOne 7, e30611.

Sebire, M., Katsiadaki, I., Taylor, N.G.H., Maack, G., Tyler, C.R., 2011. Short-term exposure to a treated sewage effluent alters reproductive behaviour in the three-spined stickleback (*Gasterosteus aculeatus*). Aquat. Toxicol. 105, 78–88.

Söffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish – a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42, 653–668.

Thorpe, K.L., Maack, G., Benstead, R., Tyler, C.R., 2009. Estrogenic wastewater treatment works effluents reduce egg production in fish. Environ. Sci. Technol. 43, 2976–2982.

Toft, G., Edwards, T.M., Baatrup, E., Guillette Jr., L.J., 2003. Disturbed sexual characteristics in male mosquitofish (*Gambusia holbrooki*) from a lake contaminated with endocrine disruptors. Environ. Health Perspect. 111, 695–701.

Toft, G., Baatrup, E., Guilletter Jr., L.J., 2004. Altered social behavior and sexual characteristics in mosquitofish (*Gambusia holbrooki*) living downstream of a paper mill. Aquat. Toxicol. 70, 213–222.

Turner, C.L., 1942. Morphogenesis of the gonopodial suspensorium in *Gambusia affinis* and the induction of male suspensorial characters in the female by androgenic hormones. J. Experim. Zool. 91, 167–193.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee Jr., D.-H., et al., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endoc. Rev. 33, 378–455.

Viganò, L., Benfenati, E., van Cauwenberge, A., Eidem, J.K., Erratico, C., Goksøyr, A., et al., 2008. Estrogenicity profile and estrogenic compounds determined in river sediments by chemical analysis, ELISA and yeast assays. Chemosphere 73, 1078–1089.

Wang, L., Ying, G.G., Zhao, J.L., Liu, S., Yang, B., Zhou, L.J., Tao, R., Su, H.-C., 2011. Assessing estrogenic activity in surface water and sediment of the Liao River system in northeast China using combined chemical and biological tools. Environ. Pollut. 159, 148–156.

Wang, G., Ma, P., Zhang, Q., Lewis, J., Lacey, M., Furukawa, Y., O'Reilly, S.E., Meaux, S., McLachlan, J., Zhang, S., 2012a. Endocrine disrupting chemicals in New Orleans surface waters and Mississippi Sound sediments. J. Environ. Monit. 14, 1353–1364.

Wang, L., Ying, G.G., Chen, F., Zhang, L.J., Zhao, J.L., Lai, H.J., Chen, Z.-F., Tao, R., 2012b. Monitoring of selected estrogenic compounds and estrogenic activity in surface water and sediment of the Yellow River in China using combined chemical and biological tools. Environ. Pollut. 165, 241–249.

Waring, R.H., Harris, R.M., 2005. Endocrine disruptors: a human risk? Mol. Cell. Endocrinol. 244, 2–9.

Whoriskey, F.G., FitzGerald, G.J., 1994. Ecology of the threespined stickleback on the breeding grounds. In: Bell, A.M., Foster, S.A. (Eds.), The Evolutionary Biology of the Threespine Stickleback. Oxford University Press, Oxford, pp. 188–206.

Wong, B.B.M., McCarthy, M., 2009. Prudent male mate choice under perceived sperm competition risk in the eastern mosquitofish. Behav. Ecol. 20, 278–282.

Wong, B.B.M., Fisher, H.S., Rosenthal, G.G., 2005. Species recognition by male swordtails via chemical cues. Behav. Ecol. 16, 818–822.

Yamamoto, A., Kakutani, N., Yamamoto, K., Kamiura, T., Miyakoda, H., 2006. Steroid hormone profiles of urban and tidal rivers using LC/MS/MS equipped with electrospray ionization and atmospheric pressure photoionization sources. Environ. Sci. Technol. 40, 4132–4137.

Ying, G.G., Kookana, R.S., Kumar, A., Mortimer, M., 2009. Occurrence and implications of estrogens and xenoestrogens in sewage effluents and receiving waters from South East Queensland. Sci. Total Environ. 407, 5147–5155.

Zala, S.M., Penn, D.J., 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Anim. Behav. 68, 649–664.