Screening for the potential effects of endocrine disrupting chemicals in peri-urban creeks and rivers in Melbourne, Australia using mosquitofish and recombinant receptor-reporter gene assays

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Abstract

Sexually mature male mosquitofish (*Gambusia holbrooki*) were collected from various sites around Melbourne in 2009 to evaluate the performance of gonopodial indices as a biomarker for endocrine disruption in Melbourne's waterways. The mosquitofish indices assessed were body length, gonopodial length/body length ratio, ray 4: 6 ratio, and the absence or presence of hooks and serrae, and these varied between sites. The study was complemented by measurements of the estrogenic, retinoid, thyroid and aryl hydrocarbon receptor activity of the water. Male mosquitofish were 16.3 - 21.5 mm in length, and, although there was a statistically significant positive relationship showing that bigger fish had longer gonopodia than small fish (r^2 = 0.52, p< 0.001), there were few significant differences in GL/BL ratio of fish between sites. Measured estrogenic activity was mostly in the range 0.1 - 1.7 ng/L EEQ, with one site having much higher levels (~ 12 ng/L EEQ). Aryl hydrocarbon receptor activity was observed in all water samples (7-180 ng/L βNF EQ), although there was no consistent pattern in the level of AhR activity observed, i.e. 'clean' sites were as likely to return a high AhR activity response as urban or WWTP impacted sites. There was no correlation between measurements of receptor actvity and gonopodium length: body length ratio and body length. We conclude that the mosquitofish gonopodia only fulfills part of the criteria for biomarker selection for screening. The mosquitofish indices assessed were cheap and easy to perform procedures, however there is no baseline data from the selected sites to evaluate whether differences in the morpholical indices observed at a site were a result of natural selection in the population or due to estrogenic exposure.

Keywords

Male mosquitofish, gonopodium, two-hybrid yeast recombinant receptor-reporter gene assay activity, Australia

Introduction

In the late 1990s, Jobling et al. (1998) reported an unusually high incidence of intersex in wild populations of the roach (*Rutilus rutilus*) in English rivers. This was arguably the first well-documented example of a widespread sexual disruption in wild populations of aquatic vertebrates, with the reproductive abnormalities being broadly consistent with exposure to hormonally active substances associated with discharges from municipal wastewater treatment plants (WWTPs). Since then, although the occurrence of endocrine disrupting chemicals (EDCs) in WWTP discharges and their impacts on aquatic wildlife have generated a significant amount of scientific and public interest, there has been little relevant Australian information produced. Selecting a suitable fish species for environmental monitoring in Australia can be limited by scientific factors, such as the abundance and spatial distribution of a species, and social factors, such as limitations placed on collection of native fish by institutional animal ethics committees. For such pragmatic reasons, the introduced and ubiquitous mosquitofish, *Gambusia holbrooki*, has been suggested as a candidate species for screening for the effects of endocrine disrupting chemicals in peri-urban creeks and rivers in Australia.

The mosquitofish (*Gambusia holbrooki*) is a small, short lived, sexually dimorphic species introduced into Australia in 1925 as biological control to curb mosquito populations (Morgan et al. 2004; Ayres et al. 2010). Now considered a noxious species, the mosquitofish is commonly found in freshwater streams and creeks in Victoria, where it inhabits shallow, still or slow moving reasonably vegetated water in a wide range of environments from apparently pristine creeks to highly disturbed urban environments (Pyke 2008). Both the eastern and western mosquitofish show a preference for warmer water temperatures between 25°C and 31°C (Winkler 1979; Lada et al. 2006). Although aggressive in behaviour, mosquitofish often

move around in groups locally in a small area (Pyke 2005). Male mosquitofish are typically much smaller than females.

Born with undifferentiated anal fins (gonopodia) which elongate during development, male mosquitofish use their gonopodia during copulation to transfer sperm into the female (Angus et al. 2001). In juvenile mosquitofish, anal fin ray numbers 3 through 6 are not different in either sex. However, in mature adult males, rays 3, 4 and 5 elongate progressively until they are twice as long as the other rays in the fin. Angus et al. (2001) reported that the ratio of the length of rays 4 and 6 is an average of 2.5 times for a normal adult male mosquitofish (see Figure 1). The fully developed gonopodium also has a number of serrae and hooks at the tip which help the male secure the female during copulation. Gonopodial development normally takes between 30 to 50 days to complete, and, because its development is androgen dependant fin growth and ray ratios can be inhibited by exposure to estrogenic chemicals (Angus et al. 2005; Doyle and Lim 2002). In the wild, estrogenic effects on mosquitofish gonopodial morphology have been suggested by Batty and Lim (1999), Toft et al. (2003) and Game et al. (2006). In the laboratory, modifications to the gonopodial length have been observed when juvenile male fish were exposed to estrogenic chemicals (Dreze and Monad 2000; Doyle and Lim 2002; Angus et al. 2005), however, once the gonopodium is fully developed, exposure to estrogenic compounds does not seem to have an effect on the fish. These hormone-dependant morphologic attributes have, in part, led to the mosquitofish being a valuable candidate biomonitor for endocrine disruptors (Hou et al 2011; Norris and Burgin 2011, Xie et al 2010).

The wide structural variety of EDCs for the most part restricts the applicability of single-compound chemical analysis of complex matrices, such as found in many peri-urban waterways. Several *in vitro* assays have been developed to screen the hormonal activity of

compounds in natural waters, including ligand-binding assays, recombinant receptor-reporter gene assays, assays based on the measurement of cell proliferation, and enzyme-linked immunosorbent assays (ELISA; Streck 2009; Kinnberg 2003). Recombinant receptor-reporter gene assays, such as the yeast two-hybrid bioassays used in this study, measure the activation of receptor, and allow for quantification of hormonal activity, without having to know the precise chemical make up of the sample. They have, however, been little utilised on natural water samples in Australia, with the only Victorian information published to date that of Allinson et al (2011a) who reported negligible estrogenic (ER), retinoid (RAR) and thyroid (TR) receptor activity, but measurable aryl hydrocarbon (AhR) receptor activity in the main stem of the Yarra River Victoria.

In recognition of the potential risks that EDCs pose to aquatic ecosystems, and the lack of robust information on the levels of such compounds in Victorian freshwaters, this study was initiated to assess the gonopodia of mosquitofish as a suitable biomarker of effect for estrogenic contamination. To that end, *Gambusia holbrooki* were collected from 16 urban, peri-urban and rural waters around Melbourne between February 9 and April 1, 2009. One of the challenges in environmental monitoring is the lack of a combined approach using chemical measurements of contaminants and bioanalytical tools to investigate health of ecosystems as a parallel study (Sumpter and Johnson 2008). To address this challenge, grab water samples were obtained from the sites, and samples prepared for a number of bioanalytical tests, including measurement of sample 'hormonal' activity using human and medaka (*Oryzias latipes*) estrogen (hERα and medERα), retinoic acid (RAR), aryl hydrocarbon (AhR), and thyroid (TR) receptor assay activity using a suite of yeast-based bioassays. The two sampling approaches were used to identify sites that may be most impacted by endocrine disrupting chemicals.

Materials and Methods

Study sites

Sexually mature adult male mosquitofish with elongated gonopodia (see Figure 1) were collected using a dip net from streams, lakes and wetlands in and around Melbourne (Figure 2) between February 9 and April 1, 2009; streams were grouped according to surrounding land use types such urban (U), rural (R) wastewater treatment plant impacted (WWTP) and reference sites (Ref), although in some case this grouping is somewhat arbitrary since some creeks flow through both residential and industrial or agricultural land and may contain a combined source of pollutants. In the field, fish were euthanized (blow to the head), transported on ice to the RMIT Ecotoxicology Laboratory and stored in 70% ethanol for later morphometric measurements.

Fish morphometrics

Body length was measured using a caliper to the nearest 0.01 mm from snout to caudal peduncle. Gonopodial length (GL) defined as the length from the anterior base of the anal fin to the gonopodial tip was measured under a Wild M3Z Heerbrugg stereozoom microscope using an objective micrometer to the nearest 0.01mm. The length of rays 4 and 6 were also taken as a measure of the elongation index of the ratio of rays 4:6 (R4:6). The presence and absence of hooks and serrae at the tip of the gonopodia indicate maturity and was also noted. Fish deemed immature were not used in the study. Fish whole bodies were weighed to the nearest 0.001 mg; because the liver and gonads were removed for other measurements (*data not reported*), the eviscerated mass reported. Dependent variables included standard body lengths (SBL), gonopodium length : body length ratio (GL/BL), elongation ratio (R4:6) and body mass.

Water sampling

Water samples were collected as 'grab' or spot samples from the16 urban, peri-urban and rural waters (Figure 2). Samples were directly collected in glass bottles, stored on ice, and then at 4°C until processed (generally within 36 h of collection). For each site, an aliquot of the effluent (1L) was extracted for the measurement of receptor (hormonal) activity using a yeast-based bioassay. The sample preparation methods for these tests are described elsewhere (Shiraishi et al. 2000; Allinson et al. 2007, 2008), but, in short, involved filtration and adding buffer solution to the sample to ensure an acid pH (according to JEA 1998), filtration through GF/C filters to remove particulate matter, and then solid phase extraction (for bioassays: SPE; Octadecyl C18 disk (Empore; 47 mm; 3M, MN, USA); for ELISA: Discovery DSC-18 cartridge (6 mL 500 mg, Supelco, PA USA)). After elution of analytes with methanol and evaporation, the sample was re-suspended in a mixture of 3:1 hexane: dichloromethane (1 mL), and loaded onto a florisil column (Varian Bond Elut-FL, 500 mg, 3mL; CA, USA). For all samples, elution protocols separated the extract into three fractions, first a 3:1 hexane:dichloromethane fraction (H/D), second a 1:9 acetone:dicholoromethane fraction (A/D), and finally a methanol fraction (MeOH). The A/D fraction contained the steroid hormones, and the separation was undertaken to minimise the effects of matrix components on the ELISA and bioassay systems.

Measurement of estrogenic and retinoic acid activity was undertaken with a yeast two-hybrid recombinant receptor-reporter gene bioassay system in accordance with the method of Shiraishi et al. 2000 (described in English in Allinson et al. 2008) using yeast cells (*Saccharomyces cerevisiae* Y190) into which the human estrogen receptor ERα or the estrogen receptor from

Japanese medaka (*Oryzias latipes*) had been inserted (hERα and medERα, respectively; Nishikawa et al. 1999), and the method of Kamata et al. (2008) using the same strain of yeast into which the human RARγ receptor had been inserted. Measurement of AhR activity was undertaken in accordance with the method of Kamata et al. (2009) using yeast cells (YCM3) carrying the response element for the AhR complex, XRE5 (Miller 1999). Measurement of TR activity was undertaken according to Shiraishi et al. (2003). Positive controls were used with all assays: hERα and medERα assays, 17β-estradiol and estrone (Wako Pure Chemical Industries Ltd, Osaka, Japan); RAR assay, all-trans-retinoic acid (a-t-RA; Wako Pure Chemical Industries Ltd, Osaka, Japan); AhR, β-naphthoflavone (βNF; Wako Pure Chemical Industries Ltd, Osaka, Japan); and TR, triiodothyronine (T3; Wako Pure Chemical Industries Ltd, Osaka, Japan). A solvent (vehicle) control (DMSO, Nacalai Tesque Inc., Kyoto, Japan) was used in all cases. The agonist activities of the H/D, A/D and MeOH fractions of the sample extracts were measured, and unless otherwise stated data is reported as the sum of the activity observed in all three fractions. The bioassay method's limits of reporting (LOR) for the hERα and medERα systems were 0.1 and 0.4 ng/L 17β-estradiol equivalents (EEQ), respectively. For the RAR, AhR, and TR bioassays, they were 0.4 ng/L a-t-RAEQ, 0.4 ng/L βNFEQ, and 10 ng/L T3EQ, respectively.

Measurement of total estrogens (ES) was undertaken using commercial ELISA kits in accordance with the manufacturer's instructions (Ecologiena® Estrogenes (E1/E2/E3) ELISA Kit (Tokiwa Chemical Industries, Japan)). In order to verify calibration accuracy, check standards (i.e. standards from the kit run as samples) were run in duplicate on each ELISA plate during each ELISA test. The ratio of nominal concentrations and result values were 105% for total estrogen. The ELISA kit manufacturer claims that the ES monoclonal antibody binds

"exclusively with E1, E2, and estriol (E3) and does not show cross-reaction with other chemicals of similar structures." Consequently, to assess the relative response of the ELISA kit, the kits were challenged with E1, E3, and both 17α- and 17β-E2. The average ratio of nominal concentrations and result values were: E1, 70%; E3, 50%; 17α-E2, 10%; 17β-E2, 100%. The ELISA method LOR was 0.1 ng/L EEQ.

Statistical analysis

All statistical analyses were performed using PASW ver. 18.0 for Macintosh. Significance was set at $\alpha = 0.05$ for all tests. For statistical comparisons each measurement data was tested for normality and homoscedasity. If data met assumptions of heterogeneity and normality required for parametric methodologies, one-way analysis of variance (ANOVA) was used to test for differences between sites; else the data was transformed before testing. Specifically, body length was log transformed, whereas gonopodium length: body length ratio was arcsin transformed before testing. A post hoc Dunnet's test was used to identify differences between means. Pearson's correlation coefficient was used to test for differences between total estrogenic levels and mean gonopodium length: body length ratio and mean body lengths at the collection sites. Data for measured biological endpoints are presented as mean ± standard error.

Results and Discussion

The factors that influence the size at which male mosquitofish reach sexual maturity are somewhat varied. A number of studies have reported that mosquitofish size and gonopodial indices are influenced by factors such as population density and the social environment as well as environmental factors such as temperature. For instance, Meffe (1992) demonstrated that

mosquitofish grew faster and larger at 25°C than at 32°C; Site 11 (a reservoir) had the highest temperature (at 25°C) and also significantly larger fish compared to all other sites (Table 1). Zulian et al. (1995) found that the mean size of mature males is dependent on population density, with males attaining larger sizes and maturing later in high density populations, and that small size is favoured in populations with female-biased sex ratios or low density (Zulian et al. 1995). Bisazza and Marin (1995) found that small males take less time to mature sexually. Maturing faster lengthens reproductive life and reduces mortality before reproduction. In this study, the mean size of male mosquitofish ranged from 16.3 mm at Site 1 (U) to 21.5 mm at Site 11 (Ref); the latter were significantly larger than fish from all other sites ($P < 0.01$) (Table 1). Fish from Site 1 (U) were significantly smaller than fish from Sites 11 (Ref), 8 (WWTP), 15 (R), 2 (U), and 3 (U) ($P < 0.01$). Fish from Site 11 (Ref) also had the largest body mass compared to fish from all sites except from five rural sites (Sites 14, 15, and 16), and two urban sites (Sites 2 and 3) ($P < 0.05$). The variation in body size among sites in this study can be attributed to mosquitofish exhibiting natural developmental plasticity to take advantage of varying environmental conditions (Zulian et al. 1993).

Gonopodial length:body length ratio (GL/BL)

There was a positive and statistically significant relationship showing that bigger fish had longer gonopodia than small fish ($r^2 = 0.52$, $p< 0.001$). There were no significant differences in GL/BL ratio of fish when comparing all the reference sites to the WWTP sites with one exception; fish from Site 5 (WWTP) had a significantly larger GL/BL ratio compared to fish from Sites 11 (Ref), 2 (U) and 3 (U) ($P < 0.05$). The only significant difference in R4:6 ratio observed was between fish from Site 5 (WWTP) and Site 15 (R) ($P < 0.05$). This trait provides a more accurate measure of elongation in that Ray 6 does not elongate while Ray 4 lengthens

during gonopodial development (Angus et al. 2001), and therefore is not being subjected to allometry (Angus et al. 2005).

The elongation of rays 3,4 and 5 of the mosquitofish gonopodium are androgen dependent and their outgrowths have been induced in juvenile males with the administration of the androgens ethynl testosterone (Ogino et al 2004) and 17β–trenbolone (Sone et al 2005). In that context, Angus et al (2001) demonstrated an increase in R4:6 ratio in fins of female mosquitofish exposed to 11-ketotestosterone for 77 days. A similar androgenic response in both male and female mosquitofish exposed to paper mill effluents showed a significant increase in segments of ray 3, which also elongates, like ray 4 in male fish (Hou et al 2011). Angus et al. (2001) reported that R4:6 ratio averages 2.5. In this study, the highest mean R4:6 ratio observed in fish from Site 5 (2.86, WWTP) even though these fish were some of the smallest in size, perhaps suggesting that fish from this site may have been exposed to androgenic pollutants. Moreover, the data from Site 5 is similar to results reported by both Angus et al (2002) and Leusch et al. (2006) where fish captured downstream of a treatment plant had longer gonopodia.

Many of the hormonally active chemicals found in contaminated waters are also directly toxic to aquatic organisms, and may affect the bioassays used in this study. Unfortunately, we were unable to assess toxicity or genotoxicity of the water samples collected during fish surveys, so we cannot evaluate the potential impact of known point-sources, land-use or in some cases, the bush-fires that raged through the upper Yarra catchment in the summer of 2009, on the performance of the bioassays (Table 2). However, the results of the positive controls run at the same time as the samples suggest the bioassays were performing to expectation, and thus capable of measuring low ng/L levels of hormonal activity. In that context, on the whole low levels of estrogenic activity were observed was low using the yeast-based bioassay (generally

below 2 ng/L, with the exception of Site 7, 12.0 ng/L EEQ; Table 2). A similar pattern was observed using ELISA. Consequently, lack of any observed bioassay response is most likely due to lack of receptor-inducing compounds in the samples, rather than a toxic effect on the yeast cells. Estrogenic activity was observed at all four WWTP effluent impacted sites, but only sporadically in the reference, rural and urban sites (Table 2). Specifically, reference sites (in the order lowest to highest ES, sites 11, 12, 9 and 10) had, for the most part lower ES than rural sites (Sites 13, 14, 16, 15 and 4). ES at rural sites was not noticeably different from that observed at the urban sites (Sites 1, 2, and 3) or the majority of the WWTP sites (Sites 5, 6 and 8). The one stand-out site was Site 7 (ES, 12.5 ng/L EEQ). There was a very good correlation between estrogenic activity and the measured total estrogen concentrations for these freshwater samples for both the hERα bioassay (r^2 = 0.98), and the medERα bioassay (r^2 = 0.97; Allinson et al. 2011b).

The low estrogen agonist activity observed in this study is broadly comparable with many international reports. For instance, Inoue et al. (2009) reported less than 0.3 ng/L EEQ in six rivers of central Japan; Jugen et al. (2009) observed higher estrogenic activity in the River Seine downstream of two WWTP discharge points compared to upstream control sites (up to 2.5 cf. < 1.2 ng/L EEQ, respectively); Schilirò et al. (2009) reported that the estrogenic activity was 5.0 ± 6.1 ng/L EEQ upstream of a WWTP on the Dora Riparia River (one of the tributaries of the River Po) in north-west Italy, but that estrogenic activity did not increase downstream of the WWTP (6.7 ± 7.4 ng/L EEQ); and Lavada et al. (2009) who examined estrogenic activity in agricultural and peri-urban rivers in California, reporting that most sites had low (<10 ng/L EEQ) activity but that the highest in vitro bioassay responses were mot consistently observed at sites associated with agriculture on the Sacramento, Tuolumne and Merced rivers (<0.15-164; 24.6-242; and <0.15-56.1 ng/L EEQ respectively; Table 3).

There was no correlation observed between the physiological indices used in this study and measured levels of estrogen receptor activity, despite the estrogenic activity observed at some of the sites in principle being capable of inducing effects in fish (e.g. 0.2 – 2 ng/L EEQ; Burkhardt-Holm et al. 2008). We do, however, need to be somewhat cautious in making this conclusion. First, the yeast-based recombinant receptor-reporter gene assays used in this study provide an indication of external exposure to estrogenic compounds through an integrated measure of estrogenic activity in the water. This may not reflect internal exposure, and since it is the internal exposure at the site(s) of action that drives a response in the gonopodium biomarker, the lack of any correlation between observed gonopodial responses and bioassay-derived estrogenic activity in the water may simply reflect the difference between external and internal estrogenic exposure. One of the challenges in environmental monitoring is the lack of a combined approach using chemical measurements of contaminants and bioanalytical tools to investigate health of organisms as a parallel study (Sumpter and Johnson, 2008). So, in this study, grab water samples were obtained from the mosquitofish sites at the time of fish sampling and measurements of estrogenic activity made. However, for the effects of estrogens to be observable via the gonapodial biomarker, juvenile male fish would have had to be exposed to estrogenic pollutants during development as once developed the gonopodia will not be affected (Angus et al. 2005), and so again we must be cautious in using the exposure 'snapshots' provided by the grab water sampling when trying to correlate the physiological indices used in this study with measured levels of estrogen receptor activity, because of the potential for temporal variation in estrogen concentrations. However, the current world wide consensus is that natural steroid estrogens usually provide the majority of the estrogenic activity rivers, with a range of anthropogenic chemicals being the dominant in certain specific locations (Sumpter and Johnson, 2008). Natural steroid estrogens, tend to be continually

discharged into rivers rather than being one-off occasional events. Although easily degraded in river water (with half-lives of around a few days), and with limited evidence of reservoir-effects resulting from sorption to organic materials in sediment, the natural steroidal estrogens are 'pseudo-persistent,' a concept that was conceptualised by Sumpter and Johnson (2008) as, "a perpetual plume, whose dimensions are modified by dilution, but remain … like an 'eternal flame'." In that context, the yeast assay and ELISA measurements of total estrogenic activity in the grab water samples provide a reasonable estimate of the background exposure to estrogens in the sampled rivers. However, to more fully address this challenge, there is a need to monitor estrogenic, and other hormonal activities, at sampling sites for several months prior to sampling the fish in conjunction with an internal biomarker of exposure, such as VTG expression.

Retinoid activity (<0.4 – 8.1 a-t-RA EQ; Table 2) was observed at most clean and rural sites, but only half of the WWTP effluent impacted and one urban site. There were no consistent patterns in the level of retinoid activity observed, i.e. the reference sites were as likely to return a high RAR activity response as urban or WWTP impacted sites. There are few studies with which to compare our results, but the data is consistent with that Gardiner et al. (2003) who reported RAR agonistic activity in lake and pond water samples from Minnesota and California, respectively, where frog malformations were routinely found; and that of the RAR agonist activity reported by Inoue et al. (2010) in samples collected from 20 sites on the Ina and Lake Biwa–Yodo Rivers in central Japan (1.1 to 23.5 ng/L atRA EQ). Vitamin A (retinol) and its biologically active metabolites (collectively known as retinoids) play an important role in the development and maintenance of vertebrates, including being necessary for visual development, control of growth, and differentiation of embryonic cells (Inoue et al. 2010; Janošek et al. 2006, 2008). Although the precise mechanisms by which retinoid signalling

pathways are disrupted by xenobiotic chemicals are not understood, it is well known that both a deficiency and a surplus of endogenous retinoids, most notably retinoic acids (RAs), can cause physiological impacts. The highest RAR agonistic activity was 8.1 ng/L, which is two orders of magnitude lower than the a-t-RA concentration reported to cause RAR-mediated adverse effects (dysmorphogenesis of embryonic structures) in *Xenopus laevis* (0.6 μg/L; Degitz et al. 2003), although this does not mean that negative effects by the putative environmental retinoids are not occurring in the freshwater environment we targeted.

None of the samples produced a positive response on the TR assay, which is consistent with Jugen et al. (2009) who reported no TR agonist activity in river water extract at sites on Seine River upstream of any WWTP influence, and Inoue et al. (2009) who reported minimal TR activity in six rivers of central Japan except at two sites downstream of WWTPs.

Aryl hydrocarbon receptor activity was observed in all water samples (7 – 180 βNF EQ; Table 2), although again there was no consistent pattern in the level of AhR activity observed, i.e. the 'clean' reference sites were as likely to return a high AhR activity response as urban or WWTP impacted sites (Table 2). Bioassays based on the binding to the AhR have previously been used only once for the evaluation of contamination of peri-urban waters in Victoria (Allinson et al 2011a) who reported a progressive increase in AhR agonist activity upstream-downstream in the main stem of the Yarra River (10 - 27 ng/L β-NF EQ). It is, however, difficult to compare our results directly with the few other studies reporting AhR activity in river waters, because of differences in bioassay systems incubation protocols, cellular type (e.g. yeast cells cf. human cell lines), expression of response (e.g. chemiluminescence cf. luciferase expression) and reporting units (e.g. βNF equivalents cf. TCDD equivalents). However, with respect to the latter, Kamata et al. (2009) has suggested that the AhR affinity of βNF is about the same as that

385 of TCDD in the YCM3 cell assay, especially at the relatively low concentrations that are significant for data analysis in this assay. Therefore, the numerical activities of the 16 river waters samples relative to TCDD are roughly the same as reported for βNF. Consequently, even though TCDD EQ activity was not assessed directly (for laboratory safety reasons), we are able to say that this study's results are higher than those of Rawson et al. (2009), who
390 observed up to 0.032 ng/L TCDDEQ in water samples from 10 wetlands and 24 creeks and rivers in Sydney using the H4IIE bioassay. Moreover, if we assume that the AhR agonist behaviour of all the AhR ligands in our samples is additive and can be represented by the effects of 2,3,7,8-TCDD, the observed AhR activity (7-180 ng/L βNF EQ) would represent concentrations of 2,3,7,8-TCDD that would be at least one order of magnitude lower than those
395 reported to cause jaw malformation in zebra fish embryos via AhR-mediated down-regulation of the chondrogenic transcription factor, sox9b (Xiong et al., 2008; 1 μg/L).

Although there is considerable understanding that many chemicals act as agonists at the estrogenic receptor, there is much less understanding of the impact of anti-estrogenic
400 chemicals, i.e. compounds that antagonize or inhibit estrogen-dependent processes, and of cross-talk between receptor mediated cellular functions (Sumpter 2008; Kirby et al, 2007; Navas and Segner 2000). The majority of known AhR ligands are coplanar aromatic dioxin-like compounds. For instance, Hilscherová et al. (2000) reported that the three potential classes of compounds with dioxin-like properties that can bind to the AhR were (1) planar
405 hydrophobic aromatic compounds (such as planar congeners of PCBs and PCDD/PCDFs, polychlorinated naphthalenes (PCNs), and several high molecular weight PAHs); (2) poly- and mixed halogenated and alkylated analogues of class (1) compounds, chlorinated xanthenes and xanthones, and polychlorinated diphenyl toluenes, anisols, anthracenes, fluorenes); and (3) a wide range of non-planar, non-aromatic, lipophilic compounds that are transient inducers and

weak AhR ligands (includes natural compounds like indoles, heterocyclic amines, and some pesticides and drugs). In that context, a number of coplanar aromatic compounds are known to bind to the AhR, induce the biotransformation enzyme cytochrome P450 1A (CYP 1A), and cause anti-estrogenic effects, such as reduced vitellogenin (VTG) synthesis or impaired gonad development (Navas and Segner 2000). CYP1A1 is the terminus of the mixed function oxygenase (MFO) system and plays a key role in the detoxification of contaminants such as PAHs. In this study, there was no evidence of any correlation between the physiological indices used and measured levels of AhR receptor activity, but it is possible that PAHs from known point-sources, land-use or in some cases, the bush-fires that raged through the upper Yarra catchment in the summer of 2009, may have affected the physiological indices through anti-estrogenic mechanisms mediated through the AhR. Further research on the extent to which the ER and AhR systems can influence each other in the development of mosquitofish when both are being stimulated - so-called 'crosstalk' – is required.

Biomarkers are often considered as early warning systems (den Besten 1998; Hanson 2009), although the usefulness of the selected biomarker to trigger further research depends on the suitability of the biomarker. Van der Oost et al. (2003) states that a biomarker should be reliable, relatively cheap, easy to perform, and sensitive to pollution exposure/effects. Baseline data should be available to determine that the effect is pollution induced and not due to natural variability. For screening purposes, the gonopodium of the male mosquitofish was selected as a cost effective and simple biomarker to detect the effects of estrogenic contaminants. Measuring mosquitofish gonopodia is cheap and easy to perform with a microscope. Mosquitofish are ubiquitous and have been used as a bioindicator organism for different types of contaminants in many studies all around the world. However, the data obtained in our study is inconclusive and

suggests that the selected mosquitofish indices may be influenced by natural variability and other environmental factors.

Conclusions

In the laboratory, the investigation of changes in the male mosquitofish gonopodium appears to be a sensitive morphological biomarker of estrogenic exposure if exposure occurs during the period of sexual maturation for male fish. However, this does not appear to be the case in the field, where mosquitofish are highly plastic and adaptable to a variety of water conditions making interpretations of morphological characteristics complicated. Confounding factors such as temperature, individual site history, predators and other factors may have influenced indices selected for this study. The estrogenic activities observed in water samples at some of the sites are capable of inducing effects in fish, however no correlations with morphological effects were observed in this study. It is recommended that baseline data be obtained from a range of relevant field sites to better understand natural variability in the gonopodia of the mosquitofish in order for it to be used as a reliable biomarker in Australian conditions. It is also recommended that a multi-assessment method using various biomarkers be used to evaluate estrogenic and other receptor effects in contaminated waters, i.e combining external measurements of chemical exposure with internal bioindicators of exposure (such as VTG expression). Periodic measurements of receptor activity or levels of known receptor agonists prior to sampling of fish are necessary in order to identify sites that have recurring high levels of contaminants such as natural steroidal estrogens, and to understand fluctuations in estrogenic levels and effects in fish.. Although, there is substantial variability in structure, most potent AhR ligands have high log Koc and log Kow partition coefficients, and as such might reasonably be expected to be found at higher concentration in sediments than in the water column. In this study, the receptor activity of sediments was not examined, and consequently there is a need to do so, to assess the ecological significance of the bioassay data.

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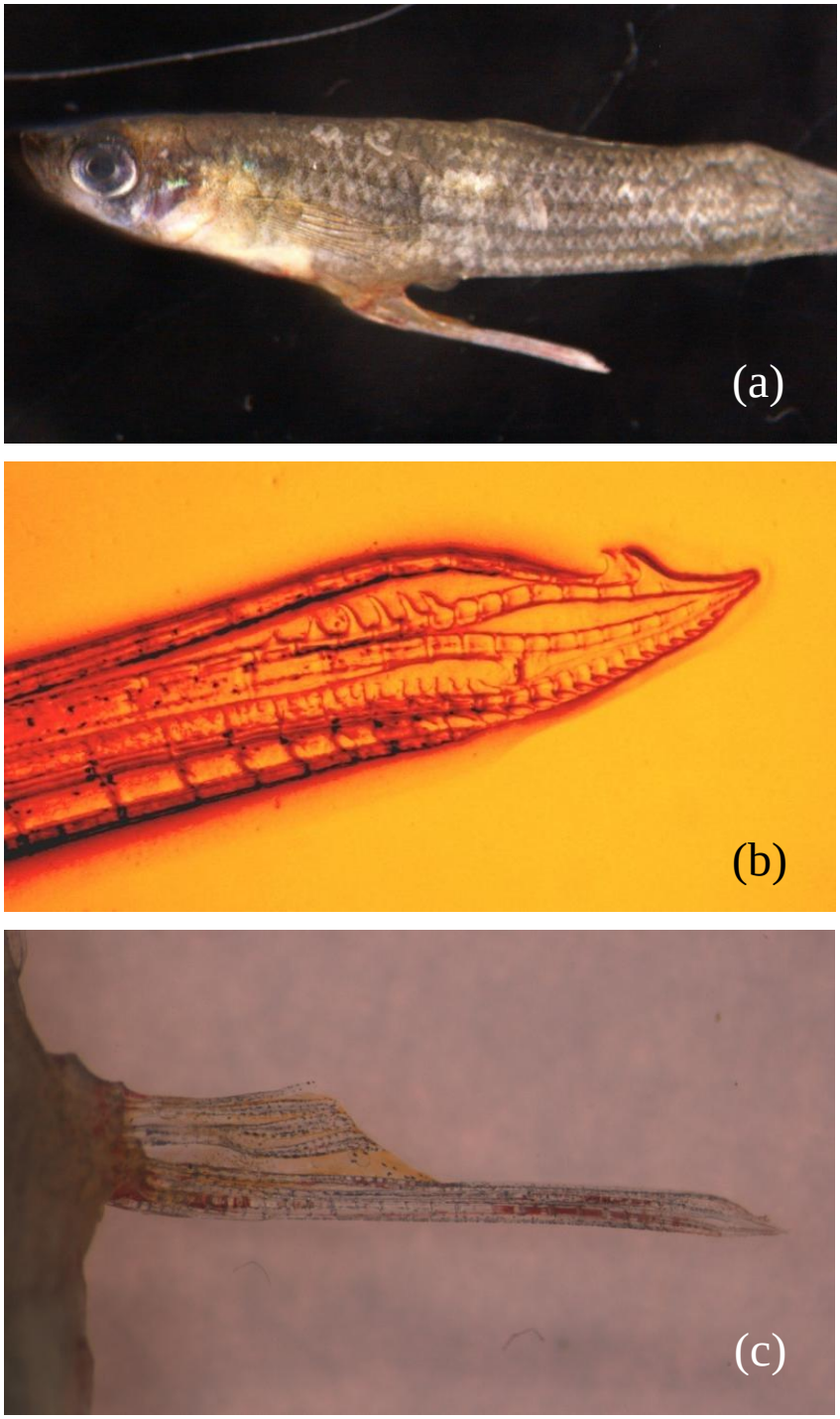

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690 Figure 2

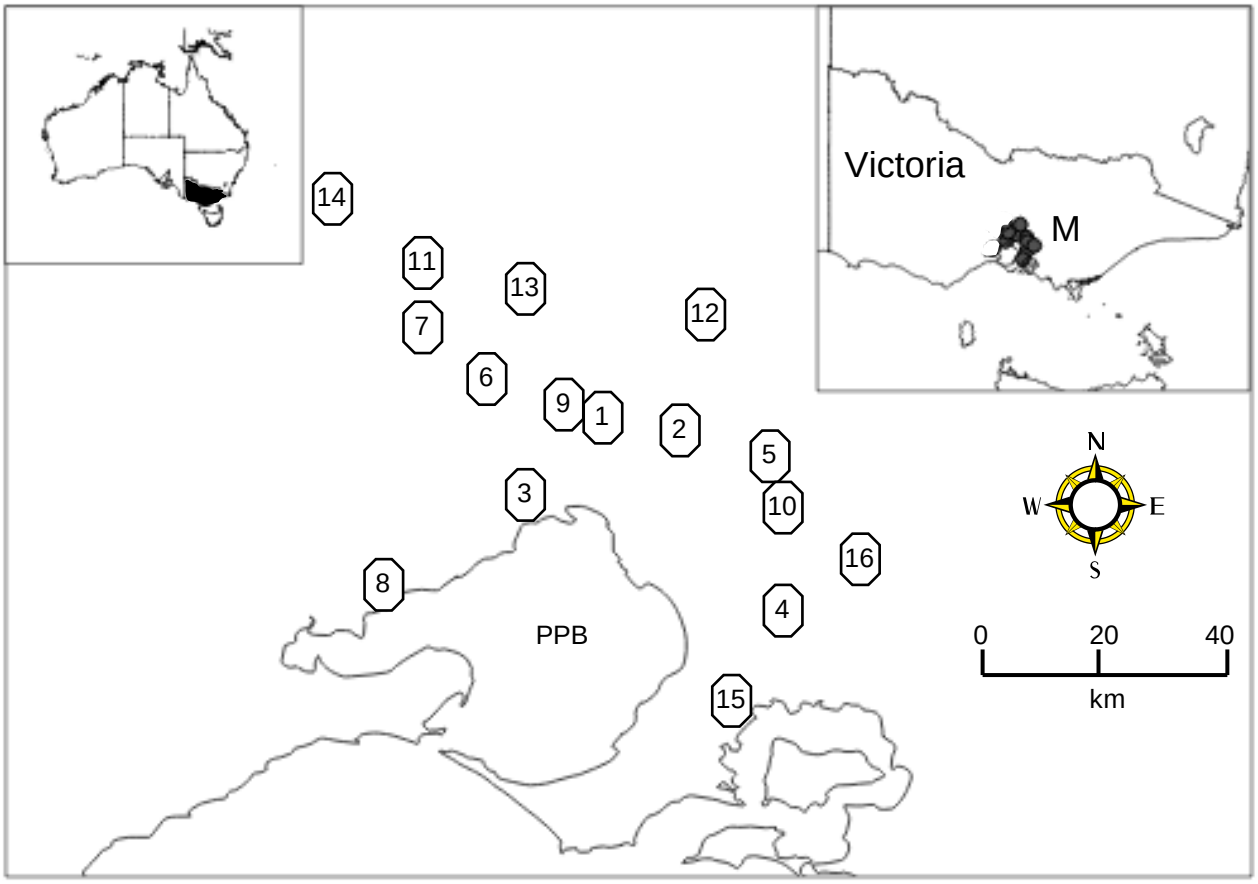

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Table 1

Group	Site #	Description	n	Standard length (mm)		Gonopodium length (mm)		GL/BL ratio		R4:6 ratio		Mass (g)	
Ref	10	Lake	15	17.88^{ac}	±0.44	6.11	±0.13	0.35^{ad}	±0.01	2.51^{a}	±0.07	0.101^{b}	±0.007
	9	Lake	16	17.88^{ac}	±0.33	6.45	±0.11	0.36^{acd}	±0.01	2.71^{a}	±0.06	0.105^{b}	±0.006
	12	Lake	15	17.87^{ac}	±0.48	6.67	±0.17	0.37^{acd}	±0.01	2.71^{a}	±0.05	0.107^{b}	±0.009
	11	Lake	15	21.47^{b}	±0.26	7.61	±0.11	0.35^{bd}	±0.00	2.65^{a}	±0.03	0.160^{a}	±0.009
Rural	13	Creek	14	17.72^{ac}	±0.34	6.41	±0.13	0.36^{ad}	±0.00	2.60^{a}	±0.04	0.113^{b}	±0.007
	14	River	17	18.85^{ac}	±0.30	6.78	±0.14	0.36^{ad}	±0.01	2.73^{a}	±0.08	0.118^{a}	±0.008
	*16	Lake	15	18.37^{ac}	±0.36	7.11	±0.14	0.39^{ac}	±0.00	2.78^{a}	±0.05	0.118^{a}	±0.007
	15	Creek	14	19.36^{a}	±0.27	6.98	±0.09	0.36^{ad}	±0.00	2.55^{a}	±0.05	0.124^{b}	±0.006
	*4	Stormwater drain	16	17.22^{ac}	±0.24	6.55	±0.10	0.38^{acd}	±0.01	2.67^{a}	±0.05	0.086^{b}	±0.005
Urban	*1	Creek	15	16.27^{c}	±0.32	6.50	±0.12	0.40^{ac}	±0.01	2.72^{a}	±0.05	0.078^{b}	±0.004
	2	Wetland	10	18.75^{a}	±0.44	6.48	±0.15	0.35^{bd}	±0.00	2.73^{a}	±0.06	0.124^{a}	±0.006
	*3	Creek	14	18.93^{a}	±0.41	6.55	±0.16	0.35^{bd}	±0.01	2.53^{a}	±0.08	0.155^{a}	±0.007
WWTP	5	Creek	18	17.06^{ac}	±0.5	6.57	±0.11	0.39^{a}	±0.01	2.86^{b}	±0.06	0.090^{b}	±0.008
	6	Creek	15	16.93^{ac}	±0.40	6.27	±0.17	0.37^{acd}	±0.01	2.79^{a}	±0.07	0.093^{b}	±0.007
	7	Creek	14	17.33^{ac}	±0.37	6.31	±0.12	0.34^{acd}	±0.02	2.68^{a}	±0.06	0.087^{b}	±0.006
	8	Drain	15	18.67^{a}	±0.35	6.92	±0.13	0.37^{a}	±0.00	2.64^{a}	±0.05	0.117^{b}	±0.006

* includes sites that could be classified as either rural or urban (sites 4 and 16) and urban or industrial (sites 1 and 3); n, number of fish in sample; GL, gonapodium length; BL, body length; R4:6, rap 4/6 ratio; Ref, reference site; WWTP, wastewater treatment plant influenced sites; Means not sharing the same letters are significantly different from each other

Table 2

Group *	Site #	Temp	pH	EC	TOC	ES	Receptor activity				
							hERα	medERα	RAR	TR	AhR
		(°C)		(μS/cm)	(mg/L)		EEQ		a-t-RA EQ	T3 EQ	βNF EQ
									(ng/L)		
Ref	10	21.7	7.1	940	n/a	0.6	1.7	1.6	5.2	< 14	26
″	9	20.6	7.8	2910	13.8	0.5	<0.1	< 0.4	< 0.4	< 14	7
″	12	18.7	6.0	80	25.7	0.2	<0.1	< 0.4	0.9	< 14	11
″	11	25.0	6.5	160	7.2	0.1	<0.1	< 0.4	< 0.4	< 14	19
Rural	13	20.3	9.1	2000	12.2	0.2	<0.1	< 0.4	< 0.4	< 14	8
″	14	25.1	9.0	1250	14.7	0.7	0.3	< 0.4	< 0.4	< 14	16
″	16	21.4	7.6	160	5.9	0.8	<0.1	< 0.4	0.9	< 14	14
″	15	18.0	6.9	620	8.2	1.0	0.1	< 0.4	0.8	< 14	12
″	4	24.5	6.3	610	19.7	1.6	1.4	1.1	4.5	< 14	180
Urban	1	18.8	7.7	1920	n/a	0.6	<0.1	< 0.4	< 0.4	< 14	27
″	2	18.6	7.5	2370	8.9	1.0	<0.1	0.8	0.5	< 14	14
″	3	20.0	7.7	3580	11.9	0.1	<0.1	< 0.4	< 0.4	< 14	35
WWTP	5	19.4	6.8	717	9.5	1.5	0.3	< 0.4	0.4	43	130
″	6	19.3	8.6	3050	8.9	1.7	0.9	< 0.4	2.2	< 14	31
″	7	15.3	6.8	813	8.8	12.5	12.0	11.0	8.1	< 14	19
″	8	19.0	8.0	519	42.8	1.2	0.6	< 0.4	< 0.4	43	24

715 * includes sites that could be classified as either rural or urban (sites 4 and 16) and urban or industrial (sites 1 and 3); n, number of fish in sample

Table 3

Location	Assay	Estrogenic activity			Reference
		u/s WWTP	d/s WWTP	Other (ng/L EEQ)	
China	YES			0.2 - 78.8	Wang et al, 2011
France	MELN	< 1.2	≤ 2.5		Jugan et al, 2009
	MELN	<0.3 – 2.8			Miège et al, 2009
Ireland	YES	0.5 – 2.7	0.9 - 1.5		Kelly et al. 2010
Italy	E-screen	5.0 ± 6.1			Schilirò et al, 2009
Japan	Y2H				
South Korea	E-screen	0.08	0.03 – 0.7	0.1 – 0.3	Oh et al, 2009
Taiwan	MVLN			0.3 – 4.4	Shue et al, 2010
USA				<0.15 - 242	Lavado et al, 2009
			Australia		
Queensland	E-screen			<0.2 – 0.95*	Leusch et al, 2010
Vic	Y2H			<0.5	Allinson et al, 2011a
Victoria	Y2H		<0.3 - 11	< 0.3 – 1.6	This study

u/s WWTP, upstream of a WWTP discharge; d/s WWTP, downstream of a WWTP discharge; Other, agricultural or urban site, or no data on nature of site; MELN, assay using transformed MCF-7 human breast cancer cells; Y2H, yeast two-hybrid assay using the recombinant yeast *Saccharomyces cerevisiae* Y190; E-screen, assay with human estrogens receptor-positive MCF-7 BUS breast cancer cells; * estimated from author figures