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Histopathology, vitellogenin and chemical body burden in mosquitofish (*Gambusia holbrooki*) sampled from six river sites receiving a gradient of stressors

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Abstract

There are over 40,000 chemical compounds registered for use in Australia, and only a handful are monitored in the aquatic receiving environments. Their effects on fish species in Australia are largely unknown. Mosquitofish (*Gambusia holbrooki*) were sampled from six river sites in Southeast Queensland identified as at risk from a range of pollutants. The sites selected were downstream of a wastewater treatment plant discharge, a landfill, two agricultural areas, and two sites in undeveloped reaches within or downstream of protected lands (national parks). Vitellogenin analysis, histopathology of liver, kidney and gonads, morphology of the gonopodium, and chemical body burden were measured to characterize fish health. Concentrations of trace organic contaminants (TrOCs) in water were analyzed by *in vitro* bioassays and chemical analysis. Estrogenic, anti-estrogenic, anti-androgenic, progestagenic and anti-progestagenic activities and TrOCs were detected in multiple water samples. Several active pharmaceutical ingredients (APIs), industrial compounds, pesticides and other endocrine active compounds were detected in fish carcasses at all sites, ranging from <4 – 4700 ng/g wet weight, including the two undeveloped sites. While vitellogenin protein was slightly increased in fish from two of the six sites, the presence of micropollutants did not cause overt sexual endocrine disruption in mosquitofish (*i.e.*, no abnormal gonads or gonopodia). A correlation between lipid accumulation in the liver with total body burden warrants further investigation to determine if exposure to low concentrations of TrOCs can affect fish health and increase stress on organs such as the liver and kidneys via other mechanisms, including disruption of non-sexual endocrine axes involved in lipid regulation and metabolism.

Keywords

Australia; Endocrine disruption; GeneBLAzer; micropollutant; pharmaceutical and personal care products; QuEChERS.

1. Introduction

There are many tens of thousands of chemicals in current use, with over 100,000 chemical substances deemed to be in commercial use in the European Union (European Chemicals Agency 2017), more than 84,000 in the United States (U.S. Environmental Protection Agency 2017) and over 40,000 in Australia (Australian Department of Health 2017). Without even taking into account the large number of potential environmental transformation products, an optimistic estimate would suggest that fewer than 1% of these compounds have been monitored in wastewater treatment plant (WWTP) effluents and rivers worldwide to date (Bradley et al. 2017, Kolpin et al. 2002, Leusch et al. 2014b, Loos et al. 2009, Schäfer et al. 2011, Scott et al. 2014a, b, Tousova et al. 2017).

There is both a paucity of monitoring data and a lack of information regarding the toxic effects that these compounds may have on biota in the receiving environments, particularly in Australia (Woods and Kumar 2011). There is some evidence indicating that trace organic contaminants (TrOCs) and endocrine active compounds (EACs) from anthropogenic sources may negatively impact fish in Australian rivers (Table 1); however, the occurrence of endocrine disruption is not always consistent.

Mosquitofish (*Gambusia holbrooki*) is a wide-spread invasive species in Australia that has been particularly useful in studies on endocrine disruption (Table 1) due to a high level of sexual dimorphism, the presence of secondary sexual characteristics that can be affected by exposure to EACs and a well understood endocrinology (Leusch et al. 2006, Rawson et al. 2010). In particular, development of a specialized anal fin in males, the gonopodium, used for sexual reproduction is under hormonal control and can be reduced upon exposure to estrogenic EACs (Doyle and Lim 2002) or elongated upon exposure to androgenic EACs (Angus et al. 2001). Three studies have reported significantly reduced gonopodial length (an androgen-mediated secondary sex characteristic) in male mosquitofish at sites impacted by point (WWTP effluent) and non-point (agricultural and residential) sources (Table 1), indicating exposure to estrogenic or anti-androgenic EACs. At the same time, no significant effects were reported in mosquitofish sampled at different sites impacted by WWTP effluent, industrial contamination, and residential effluents (Table 1).

To help determine the significance of endocrine disruption in Australian rivers, this study prioritized six sites in Southeast Queensland for *in situ* sampling based on recent monitoring and *in vivo* exposure data (Scott et al. 2014a, b, Scott et al. 2017). The sites were chosen to represent a selection of point (*e.g.*, WWTP, landfill) and non-point sources (*e.g.*, agricultural activities) of potential EAC contamination.

Mosquitofish and grab water samples were collected from four impacted sites and two sites in undeveloped reaches of the catchment within or downstream of protected lands (national parks) to assess endocrine disruption using the following techniques: 1) histopathology of gonads, liver and kidneys, 2) measurement of gonopodium length and vitellogenin (Vtg) concentration in adult males, 3) chemical analysis of TrOC and EAC body burden in males and females, and 4) chemical and *in vitro* analysis of grab water samples.

Table 1. Summary of *in situ* studies in Australia assessing endocrine disruption in aquatic environments. WWTP = Wastewater Treatment Plant.

Endpoint(s)	Species	Result(s)	Influence(s)	Environment	Reference
Aromatase mRNA	*Lates calcarifer*	Slight increase	Agricultural	River/estuary	Kroon et al. (2015)
Gonadal histology	*Carassius auratus*	No effect	Urban and WWTP	River	Kellar et al. (2014)
	Crocodylus johnstoni	No effect	Agricultural	River	Yoshikane et al. (2006)
	Cyprinus carpio	No effect	WWTP effluent	River	Hassell et al. (2016)
	Gambusia holbrooki	No effect	Urban and WWTP	River	Kellar et al. (2014)
	Gambusia holbrooki	No effect	WWTP effluent	River	Leusch et al. (2006)
	Melanotaenia fluviatilis	Suppression of spermatogenesis	100% WWTP effluent	Mobile lab	Vajda et al. (2015)
	Rutilis rutilis	No effect	WWTP effluent	River	Hassell et al. (2016)
	Saccostrea glomerate	No effect	WWTP effluent	Marine	Andrew-Priestley et al. (2012)
	Saccostrea glomerate	No effect	WWTP effluent	River	Anderson et al. (2010)
Gonopodium morphology	*Gambusia holbrooki*	No effect	Agricultural (rural)	River	Chinathamby et al. (2013)
	Gambusia holbrooki	No effect	Residential/industrial	River/estuary	Rawson et al. (2009)
	Gambusia holbrooki	No effect	Urban	River	Chinathamby et al. (2013)
	Gambusia holbrooki	No effect	Urban and WWTP	River	Kellar et al. (2014)
	Gambusia holbrooki	No effect	WWTP effluent	River	Leusch et al. (2014a)
	Gambusia holbrooki	No effect	WWTP effluent	River	Chinathamby et al. (2013)
	Gambusia holbrooki	Significantly reduced length	Agricultural	Lake	Game et al. (2006)
	Gambusia holbrooki	Significantly reduced length	Agricultural / residential	Lake	Game et al. (2006)
	Gambusia holbrooki	Significantly reduced length	WWTP effluent	River	Batty and Lim (1999)
	Gambusia holbrooki	Significantly reduced length	WWTP effluent	River	Doyle et al. (2003)
	Gambusia holbrooki	Slightly increased length	WWTP effluent	River	Leusch et al. (2006)
Hormones (plasma)	*Crocodylus johnstoni*	No effect	Agricultural	River	Yoshikane et al. (2006)
Morphometrics	*Gambusia holbrooki*	Slightly reduced testes weight	WWTP effluent	River	Doyle et al. (2003)
Phenotype	*Morula granulata*	Significant increase in imposex	Industrial (shipping)	Marine	Reitsema and Spickett (1999)
	Morula marginalba	Significant increase in imposex	Industrial (shipping)	Marine	Andersen (2004)
Reproductive output	*Gambusia holbrooki*	No effect on spermatozeugmata	WWTP effluent	River	Batty and Lim (1999)
Sex ratio	*Gambusia holbrooki*	Decrease in mature males	WWTP effluent	River	Rawson et al. (2008)
Skeletal morphology	*Gambusia holbrooki*	No effect	WWTP effluent	River	Rawson et al. (2008)
Vitellogenin (plasma)	*Carassius auratus*	No effect	Urban and WWTP	River	Kellar et al. (2014)
	Cyprinus carpio	No effect	WWTP effluent	River	Hassell et al. (2016)
	Gambusia holbrooki	No effect	WWTP effluent	River	Leusch et al. (2014a)
	Rutilis rutilis	No effect	WWTP effluent	River	Hassell et al. (2016)

	Tetractenos glaber	Significantly increased	Agricultural / residential	River/estuary	Booth and Skene (2006)
	Tetractenos glaber	Significantly increased	WWTP effluent	River/estuary	Booth and Skene (2006)
Vitellogenin (protein/mRNA)	*Melanotaenia fluviatilis*	No effect	100% WWTP effluent	Mobile lab	Vajda et al. (2015)
Vitellogenin mRNA	*Lates calcarifer*	Significantly increased	Agricultural	River/estuary	Kroon et al. (2015)
	Plectropomus sp.	Significantly increased	Agricultural	Coastal lagoons	Kroon et al. (2015)
	Saccostrea glomerate	Significantly increased	WWTP effluent	Marine	Andrew-Priestley et al. (2012)
	Saccostrea glomerate	Significantly increased	WWTP effluent	River	Anderson et al. (2010)

2. Experimental section

2.1. Site identification

Historical chemical and *in vitro* (endocrine activity) monitoring data and catchment analysis (Scott et al. 2014a, b) were used to identify four sites in Southeast Queensland (sites labelled AGR1, AGR2, WWEF, LNDF) with some of the highest chemical concentrations and estrogenic activity from a previous year-long survey (2011-2012 data in Table 5), and one site with low anthropogenic contaminants in an undeveloped part of the catchment as a comparison site (UND1) Catchment analysis was used to identify another undeveloped comparison site (UND2), which had not previously been monitored. Basic physico-chemical measurements and more details about the sites are provided in the supplementary material (SI Table S1 and kml geolocation file). Site designation was assigned based on the most significant land-use in the catchment at each sampling location: AGR1 and AGR2 were located within areas of agricultural land-use, WWEF was influenced primarily by wastewater effluent, and LNDF was a short distance downstream of a landfill site. Sites UND1 and UND2 were located in undeveloped areas within or downstream of national parks that experience some recreational use, but otherwise no known anthropogenic pollution source.

2.2. Sampling

Mosquitofish were sampled between April and May 2013 using an electro-fishing unit set to 100 Hz, 20% duty cycle and 225 V, until a sufficient number of fish were obtained for analysis, or until fishing effort dropped below one fish per 10 min (whichever came first) at all six sites. A total of 190 fish were captured for this study, ranging from 14 (AGR2) to 47 (WWEF) with a median of 29.5 across all sites (Table

2). All animal handling was conducted with respect and in accordance with Department of Science, Information Technology and Innovation (DSITI) animal ethics permit. Fish were euthanized on site in 80 mg/L Aqui-S anesthetic (Lower Hutt, New Zealand). Wet weight, standard length and gonopodium length (from the body following the curve of the gonopodium to the tip) were then immediately recorded to the nearest tenth of a mg or mm, respectively (Table 2). Condition factor (K) was calculated using the following equation: $K=10^5 \times W/L^3$, where W is the wet weight (in g) and L is the standard length (in mm). The gonopodium index was calculated as gonopodium length (mm) divided by standard length (mm).

Fish carcasses were split into three roughly equivalent groups by sex for each one of the three separate analyses: Vtg, body burden and histology. As a limited number of fish were captured at site AGR2, biochemical analyses were prioritized and fish were split into two groups only for Vtg and body burden analysis. Male and female whole fish were then placed in 50 mL of Davidson's Fixative (pure ethanol, 10% neutral buffered formalin, glacial acetic acid and deionized water at a ratio of 3:2:1:3) for histological analysis. Fish were transferred to 50 mL pure ethanol after 48 h in Davidson's Fixative until slide preparation (Section 2.3). Fish selected for whole body Vtg analysis (Section 2.4) or body burden analysis (Section 2.5) were wrapped in aluminium foil and frozen in liquid nitrogen, and then stored at -80°C. A 2-L water grab sample was also collected when fish were sampled at each site, adjusted to pH 2 using 12 M HCl, stored at 4°C and extracted within 24 h for chemical and *in vitro* analyses (Section 2.6).

2.3. Histopathology

2.3.1. Slide preparation

Mosquitofish were removed from their ethanol solution, decapitated with a sharp blade, sliced along the sagittal plane (longitudinally) to fully expose the internal cavity and immediately fixed in 100% ethanol, then triple rinsed in 100% ethanol for 24 h, cleared in histolene for 4 h and then impregnated with paraffin for 3 h. Wax impregnated sections were then transferred to disposable molds and embedded in paraffin. Tissue was sectioned to 5 µm thickness using a microtome (Micron HS 355S) and stained with standard hematoxylin and eosin (H&E).

2.3.2. Pathology analysis

Gonads, liver and kidneys were all visually inspected for pathology under a microscope (Nikon Eclipse 80*i*, 4×, 10×, and 20× magnification; Nikon, Sydney, NSW). The classification of "healthy" testes was based on the presence of spermatocytes in all stages of development (SI Fig. S1A), while ovaries were considered to be of good reproductive health if they consisted of several stages of follicular development (SI Fig. S1B), as proposed by Hou et al. (2011). Livers were assessed for fat storage ("lacy liver" or "fatty liver"), granulomas, liquification/haemorrhage, and degenerative fatty necrosis. Kidneys were examined for liquification/haemorrhage and inclusions. Liquification was defined as liquid produced through cellular degeneration and haemorrhage was defined as blood breaching the circulatory system and spreading into tissues (liver and/or kidneys). Inclusions were defined as the separation of kidney tissues from the renal corpuscles, beyond that of the Bowman's space (space between parietal and visceral layers of the Bowman's capsule; Genten et al. 2009). Liver and kidney pathology was described quantitatively by calculating the percentage of affected area relative to total area of the visible organ. Preliminary analysis determined significant effect variation resulting from using smaller cross-sections/higher magnification, and sections were therefore selected based on the largest cross-section of targeted organ available in order to obtain the most representative section and minimize variation. With the exception of liver fat storage, all tissue areas were calculated using Nikon NIS-Elements BR software (Tokyo, Japan) (SI Fig. S2A). Liver fat storage area was determined using a combination of Adobe Photoshop CS 6 (California, USA) and FIJI (Image J version 1.48o for OSX, National Institutes of Health, USA). Colour images (SI Fig. S2B) were first converted to binary images in Photoshop CS 6 using the threshold function (SI Fig. S2C), followed by area analysis using the threshold and analyse particle functions in FIJI.

2.4. Vitellogenin analysis by LC-MS/MS

Vitellogenin (Vtg) protein content was determined by liquid chromatography as described in Scott et al. (2017). In brief, frozen whole fish were homogenized on ice using a tissue homogenizer in 1:4 (mass/volume ratio) buffer of 3 mM Tris and 0.1 µM phenylmethylsulfonyl fluoride (PMSF). Thereafter the homogenate was centrifuged at 12000 g for 98 min at 4˚C and the supernatant was stored at -80˚C until analysis. The homogenate protein content was 10.5 ± 2.9 mg/mL, as quantified using the Bradford method (Bradford, 1976).

Tryptic digestions were conducted using the In-Solution Tryptic Digestion and Guanidination Kit (Thermo Fisher Scientific, Victoria, Australia) following the manufacturer's protocol. Briefly, 1 µL of sample was added to ammonium bicarbonate digestion buffer and dithiothreitol reducing buffer, along with *Gallus gallus* lysozyme, which was spiked into each sample at a final concentration of 16.1 µg/mL as a loading control. Samples were incubated at 95°C for 5 min, after which iodoacetamide alkylation buffer was added and the samples were incubated at room temperature for 20 min in the dark. After incubation, 1 μL of activated trypsin was added to each sample, giving a final concentration of 3.2 μg/mL. The sample was incubated at 37°C for 3 h, then at 30°C overnight.

All chromatographic separations were performed using a 5 μL injection volume onto an Agilent 1290 HPLC, fitted with an Phenomenex Aeris C8 column (column dimensions 2.1 × 100 mm with 1.8 μM particle size, 100 Å pore size), and high resolution mass spectral data were acquired on an Agilent 6530 QTOF using an ESI source fitted with Agilent Jetstream technology, as described in Scott et al. (2017).

2.5. Body burden analysis

2.5.1. Extraction of organic compounds from whole fish

A "quick, easy, cheap, effective, rugged and safe" (QuEChERS) method was applied to extract organic compounds from whole male and female mosquitofish for chemical analysis. Originally developed for pesticide residue analysis in vegetable produce (Anastassiades et al. 2003), QuEChERS has recently been validated for extraction of TrOCs in fish (Lopes et al. 2012, Munaretto et al. 2013, Norli et al. 2011). Frozen fish were sectioned finely (using a razor blade) and placed into 1 mL deionized water with an internal standard consisting of isotope-labelled analytes (details in SI Table S2). The tissue was homogenized for 30 s using an Ultra-Turrax (IKA, Malaysia) at 4000 rpm. The fish homogenate was transferred to a 50 mL centrifuge tube with 8 mL of 1% glacial acetic acid in acetonitrile and the contents of one QuEChERS extraction packet (6 mg $MgSO_4$, 1.5 mg sodium acetate; Agilent, Victoria, Australia). The tube was mixed at 400 rpm with a platform mixer (Ratek, Victoria, Australia) for 1 min and then centrifuged at 3000 g for 5 min (Hercules Multifuge X3R, Thermo

Scientific, Victoria, Australia). The supernatant was transferred to a pre-made 15 mL dispersive solid phase extraction (SPE) centrifuge tube (50 mg primary secondary amine, 50 mg graphitized carbon black, 150 mg $MgSO_4$; Agilent, Victoria, Australia), mixed vigorously again for 30 s and centrifuged again at 3000 g for 5 min. The supernatant was collected and evaporated under a gentle nitrogen stream and reconstituted into the equivalent volume of methanol (in μL) corresponding to the original wet weight of the fish (in mg) (*e.g.* extract from a fish weighing 150 mg was reconstituted into 150 μL methanol).

2.5.2. Chemical analysis of body residue

Fish whole body homogenates were analyzed for 38 compounds including 1 industrial compound, 1 personal care product, 19 active pharmaceutical ingredients (APIs), 5 pesticides, 10 steroids and 2 synthetic hormones (Table 4). Chemical analysis was performed using LC-MS/MS and GC-MS/MS following previously detailed methods (Scott et al. 2014a, b, Trinh et al. 2011, Vanderford and Snyder 2006). Concentrations were corrected to account for any losses during extraction by adding an internal standard prior to extraction (see SI Table S2).

2.6. Analysis of water samples

2.6.1. Solid phase extraction and chemical analysis

Grab water samples obtained concurrently with fish sampling were concentrated using SPE (Oasis HLB SPE cartridges; 500 mg sorbent, 6 cc; Waters, New South Wales, Australia) for chemical analysis and *in vitro* bioassays. The SPE was performed as previously described in Scott et al. (2014b). Chemical analysis of water extracts was performed using LC-MS/MS and GC-MS/MS as described in Section 2.5.2 except for ethinylestradiol (EE2), which was measured by a commercially available enzyme-linked immunosorbent assay (ELISA; Takiwa Chemical Industries, Japan) as detailed in (Scott et al. 2014a).

2.6.2. Bioassay of water samples

Endocrine activity was measured using three CellSensor GeneBLAzer assays (Invitrogen, ThermoFisher Scientific, New South Wales, Australia) to test for estrogenic (ERα), androgenic (AR) and progestagenic (PR) receptor induction (Wilkinson et al. 2008), in both agonist and antagonist modes. The assays were performed as previously described in Escher et al. (2014).

2.7. Statistical analysis

Chemical occurrence and morphometric data were not normally distributed, and thus non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test were used to determine significant differences ($\alpha = 0.05$) between sample sites. All statistics were performed using IBM SPSS Statistics 21 (New York, USA). Analysis of Vtg LC-MS data was performed as previously described in Scott et al. (2017). As there is no pure Vtg protein standard for mosquitofish, whole body Vtg protein concentration was expressed as fold Vtg expression compared to unexposed laboratory reference males as described in Scott et al. (2017).

Table 2. Sample sizes, sex ratio and morphological measurements for mosquitofish (*Gambusia holbrooki*) collected at six sites in Southeast Queensland. Mass, standard length and gonopodium index data are presented as average ± standard error of the mean.

Site label	**AGR1**	**AGR2**	**WWEF**	**LNDF**	**UND1**	**UND2**
Downstream of ...	Agricultural	Agricultural	WWTP	Landfill	Undeveloped	Undeveloped
Sample size for						
Histology (n) [1]	9 (2/7)	0 (0/0)	15 (9/6)	7 (5/2)	6 (2/4)	22 (11/11)
Vitellogenin (n) [2]	6	5	11	10	5	10
QuEChERS (n)	13 (5/8)	9 (4/5)	21 (9/12)	14 (7/7)	13 (3/10)	14 (6/8)
Male (n)	**13**	**9**	**29**	**22**	**10**	**27**
Mass (mg)	198.6 ± 11.1	208.0 ± 9.1	164.5 ± 6.7	296.9 ± 84.2	177.0 ± 20.6	173.6 ± 8.1
Standard length (mm)	23.9 ± 0.4	24.1 ± 0.6	24.0 ± 0.3	24.8 ± 0.4	22.9 ± 1.0	22.7 ± 0.4
Condition factor (K) [3]	1.45 ± 0.03	1.48 ± 0.05	1.34 ± 0.02	1.94 ± 0.58	1.48 ± 0.04	1.53 ± 0.09
Gonopodium index [4]	0.31 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.30 ± 0.02	0.32 ± 0.01
Female (n)	**15**	**5**	**18**	**9**	**14**	**19**
Mass (mg)	340.3 ± 29.7 ab	321.2 ± 39.0 ab	189.4 ± 13.1 b	569.0 ± 63.9 a	311.6 ± 56.2 b	277.1 ± 35.0 b
Standard length (mm)	27.5 ± 0.7 ab	27.5 ± 1.3 ab	23.5 ± 0.5 b	32.9 ± 1.0 a	26.2 ± 1.6 b	24.9 ± 1.2 b
Condition factor (K) [3]	1.58 ± 0.05	1.53 ± 0.07	1.42 ± 0.02	1.56 ± 0.08	1.53 ± 0.04	1.83 ± 0.22

[1] total n (male n/ female n); [2] male fish only; [3] Condition factor (K) calculated as $K = 100{,}000 \times W / L^3$, where W is the wet weight (in g) and L is the standard length (in mm); [4] Gonopodium index = gonopodium length (in mm) / standard length (in mm); Abbreviations: "WWTP" = Wastewater treatment plant. Different letters indicate statistically different groups for each measure (Kruskal-Wallis ANOVA on ranks).

3. Results and discussion

3.1. Morphological measurements and histopathology

A total of 190 mosquitofish were sampled, ranging from 14 (site AGR2) to 47 (site WWEF). Female fish were larger and longer downstream of the landfill site (LNDF) compared to those at the undeveloped (UNDF1 and UNDF2) site and downstream of the wastewater discharge (WWEF) (Kruskall-Wallis ANOVA on rank, $p < 0.05$), but all female fish had a similar condition factor ($p = 0.148$; Table 2). There were no significant differences in morphological measures for male fish (Kruskall-Wallis ANOVA on ranks, $p > 0.05$; Table 2). Mosquitofish from each site (with the exception of site AGR2, due to the small sample size) were used for histological analysis of gonads, liver and kidney tissues (Table 3). There was no evidence of ovotestis in any of the specimens. Furthermore, there was no significant difference ($p>0.05$) in gonopodium elongation (calculated as gonopodial index) between male fish from the different sites (Table 2).

Percent liver fat was highly variable with mosquitofish from undeveloped sites (UND1 and UND2) exhibiting anywhere between 1 and 55% liver fat (relative to total liver area), and livers from mosquitofish from impacted sites (AGR1, WWEF and LNDF) exhibiting between 7 and 72% fat. The only statistically significant result was a higher liver fat content at the wastewater effluent downstream site (WWEF) compared to that at sites AGR1 and UND2 (Kruskal-Wallis; $p=0.020$ and $p<0.001$, respectively). Haemorrhage and liquification in liver tissue was minimal (average of $4.3 \pm 0.9\%$), and there were no significant differences between fish from the various sites ($p>0.05$).

The average area affected by haemorrhages and liquification in kidney tissue of fish from all sites was $23.0 \pm 2.4\%$. At impacted sites, averages varied from 15.0% (site AGR1) to 23.1% (site WWEF), while those at the two undeveloped sites displayed higher affected areas ($32.9 \pm 11.1\%$ and $24.9 \pm 3.4\%$ for UND1 and UND2, respectively); however, they were not significantly different (Kruskal-Wallis; $p>0.05$). Mosquitofish sampled at UND1 and UND2 exhibited less kidney tissue damage from inclusions ($1.3 \pm 0.7\%$ and $1.8 \pm 0.5\%$, respectively) compared to that in fish from the other sites (3.1% to 5.8%), but again the data were quite variable and there were no statistically significant differences between fish from the various sites (Kruskal-Wallis; $p>0.05$). Based on histological analysis (Table 3), mosquitofish from impacted sites (sites AGR1, WWEF and LNDF) were not dissimilar to fish from undeveloped locations (UND1 and UND2), suggesting that there were no gross adverse effects on this fish species at the sites monitored in this study.

The histological analysis of the gonads did not produce any evidence of endocrine disruption in mosquitofish. All gonads inspected were healthy and there was no evidence of ovotestis tissue. This is consistent with previous Australian studies, which have usually found no evidence of ovotestis tissue in fish exposed to treated municipal sewage effluent (Table 1).

Table 3. Analysis of histopathology in mosquitofish gonads, livers and kidneys from three impacted and two undeveloped sites. Data (average ± SEM) are expressed as percent area affected relative to total organ area. Histopathology was not carried out at site AGR2 because the few fish collected (Table 2) were only sufficient for biomarker and chemical analysis.

	Impacted sites			Undeveloped sites	
Pathology	**AGR1**	**WWEF**	**LNDF**	**UND1**	**UND2**
Gonads					
n (male/female)	5 (2/3)	12 (9/3)	4 (2/2)	4 (2/2)	21 (11/8)
Condition	Healthy	Healthy	Healthy	Healthy	Healthy
Liver					
n (male/female)	8 (2/6)	15 (9/6)	7 (5/2)	6 (2/4)	19 (10/9)
Fatty (lacy) % *	25.6 ± 4.8 bc	50.0 ± 3.8 ab	36.0 ± 6.9 abc	38.7 ± 2.8 abc	20.3 ± 3.7 bc
Haem/Liq %	6.4 ± 3.6	2.9 ± 0.6	4.1 ± 1.7	4.5 ± 1.7	4.7 ± 1.9
Kidney					
n (male/female)	8 (2/6)	14 (8/6)	5 (4/1)	6 (2/4)	17 (9/8)
Haem/Liq %	15.0 ± 4.7	23.1 ± 5.2	17.9 ± 2.7	32.9 ± 11.1	24.9 ± 3.4
Inclusion %	3.1 ± 1.1	4.2 ± 0.8	5.8 ± 1.5	1.3 ± 0.7	1.8 ± 0.5

"Haem/Liq" = percentage of area of kidney affected by haemorrhage and liquification. * Letters indicate statistically different groups (Kruskall-Wallis, $p<0.05$).

3.2. Biomarker analysis

Low concentrations of Vtg protein were detected in all male fish (Fig. 1A). This was not unexpected, as trace concentrations of Vtg in males are not uncommon due to low levels of circulating natural estrogens in the blood (Bowman et al. 2000). Fish from UND1 had the lowest Vtg protein, while fish from AGR1 and the other undeveloped site (UND2) had significantly elevated Vtg protein compared to those from unexposed laboratory reference male fish (NC; Mann-Whitney test, p=0.041 and p=0.044, respectively). It is worth noting that chemical analysis likewise suggests that site UND2 may not be as "pristine" as expected, with EE2 detected at 0.07 ng/L (Table 5) in a water grab sample and one fish from the site with 25 ng/g EE2 (Table 4). Vtg protein levels in fish from all other sites were not significantly different from unexposed laboratory reference males (p>0.05). Fish from the AGR1 site had the highest Vtg protein concentration (up to 2.97-fold the concentration of unexposed laboratory reference male fish). With the exception of site UND2, there was good agreement between Vtg protein level and estrogenic activity in the water (determined by *in vitro* bioassay; p = 0.032, R^2 = 0.828, Fig. 1B and Table 5). The discrepancy between estrogenic activity (from a snapshot grab sample) and Vtg protein level (the result of long-term exposure to estrogenic stimulation) suggests prior but intermittent estrogenic exposure at this site.

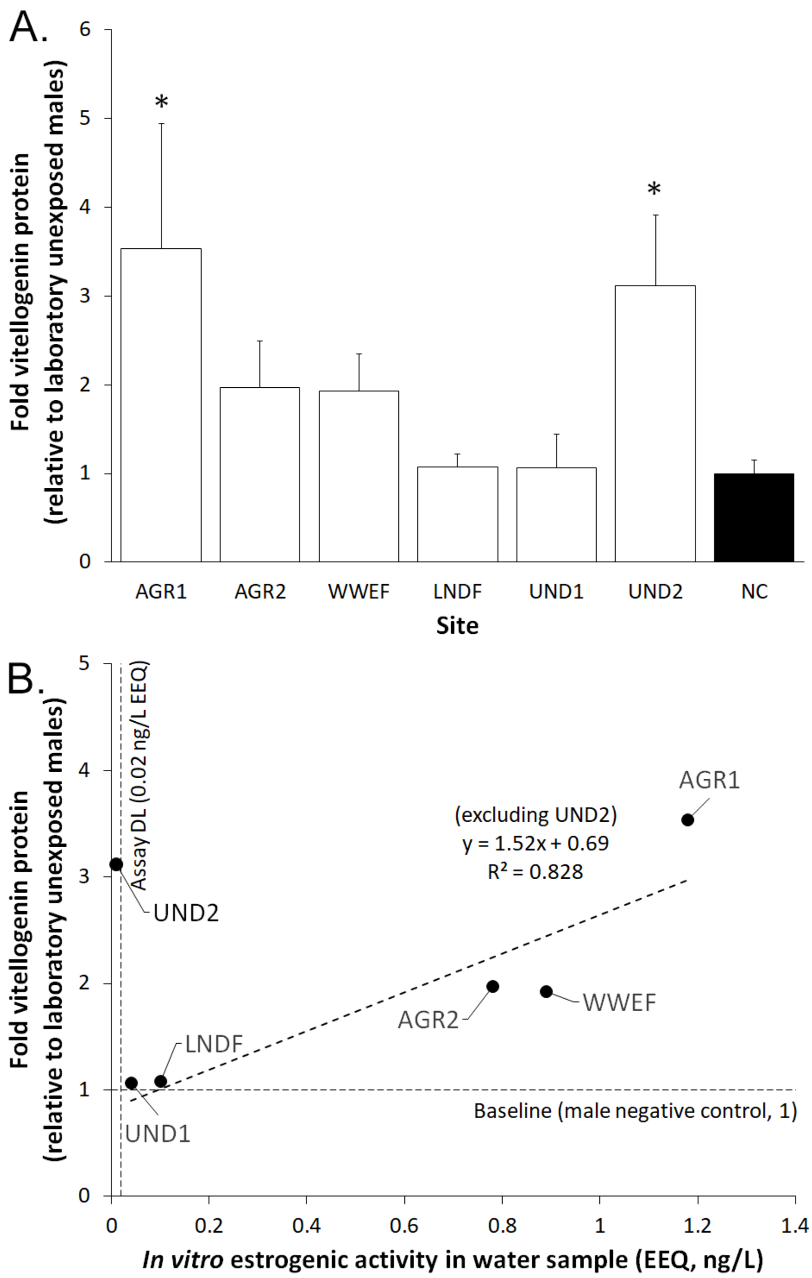

Fig. 1. Vitellogenin (Vtg) protein levels in male mosquitofish sampled at four impacted and two undeveloped sites in Queensland, Australia. A) Vtg protein was significantly induced in mosquitofish sampled at the first agricultural site (AGR1) and the second undeveloped site (UND2) compared to unexposed laboratory reference male (NC), but not other sites. * indicates statistically significant difference from male negative control (Mann-Whitney test, $p<0.05$). B) *In vitro* estrogenic activity was correlated with Vtg protein levels in most samples, except for UND2.

3.3. Body burden

Limits of quantification (LOQ) for the 38 analyzed compounds ranged from 4 to 61 ng/g wet weight (ww; Table 4), in the same range reported in other studies (Munaretto et al. 2013, Togunde et al. 2012). The majority of fish tissues analyzed (52/87, or 60%) contained at least one TrOC, with a maximum of eight (out of 38) compounds in one mosquitofish from site WWEF, which was also the site with the highest liver fat content (Table 3). Fish from site WWEF had the highest detection frequency of synthetic compounds in fish homogenates (*i.e.* number of fish with at least one quantifiable TrOC other than the natural hormones; 81%) and the highest average number of compounds per fish (2.6 ± 0.4). The analytical method used here was developed specifically for wastewater-derived TrOCs (Vanderford and Snyder 2006), and thus this result is not unexpected. This was followed by UND1 (77%; 2.5 ± 0.7), AGR2 (56%, 1.2 ± 0.4), UND2 (43%; 1.4 ± 0.6), LNDF (21%; 1.1 ± 1.4) and AGR1 (15%; 0.4 ± 0.3). In terms of chemical complexity, site WWEF again had the highest total number of different TrOC detected in fish homogenates at 49% (16/38), followed by UND2 at 34% (13/38), LNDF and UND1 at 29% (11/38), AGR2 at 21% (8/38) and AGR1 at 11% (4/38).

The industrial compound tris(2-chloroethyl) phosphate (TCEP) was detected in 14% of fish carcasses (12 of 84) and had the highest overall concentration (4703 ng/g ww in a fish from site WWEF). It was detected in fish at site UND1 (46%; maximum of 1191 ng/g ww) and fish at site WWEF (29%) only. While TCEP was not detected in grab water samples from site UND1 at the time of sampling (Table 5; Apr/May 2013), it had been detected in the grab water samples at that site on previous occasions (Table 5; 2011-2012). The result illustrates that even “remote” sites can be intermittently contaminated by human TrOCs in developed regions of the world.

Active pharmaceutical ingredients (APIs) were detected in 40% of all fish samples, with a maximum of five APIs detected in one fish (at site LNDF). Clozapine, an antipsychotic drug, was the most commonly detected API with an average detection frequency of 17% and a maximum concentration of 155 ng/g ww. It was detected most frequently in fish downstream of the wastewater discharge (WWEF, 43%). Other commonly detected APIs include the anti-histamine hydroxyzine and the anxiety medication meprobamate (13 and 11% of all fish carcasses, respectively). Omeprazole, a proton pump inhibitor, was detected at the highest concentrations (1017 ng/g ww) in one fish from site WWEF, but was otherwise only detected in a few samples (5% of total fish carcasses; Table 4). Fluoxetine, a selective serotonin reuptake inhibitor, was detected at a maximum concentration of 240 ng/g ww at WWEF (Table 4). Fluoxetine was identified in white sucker (*Catostomus commersonii*) liver at a maximum concentration of 80 ng/g ww in a US study (Ramirez et al. 2009).

The personal care product caffeine was detected in 10% of fish carcasses, with a maximum concentration of 74 ng/g wet weight. It was not detected in fish from site AGR1 or AGR2 (Table 4).

At least one of five pesticides analyzed (atrazine, chlorpyrifos, diazinon, linuron and simazine) was detected in 26% of mosquitofish samples. Chlorpyrifos, one of the most widely used insecticides in Australia (ATSE 2002), was the most commonly detected compound overall and quantified in 19% (16 of 84) of carcasses. It was detected only in fish from site WWEF (48%; 10 of 21) and UND1 (46%; 6 of 13). The herbicide results confirm that urban wastewater treatment plant contribute to a great extent to herbicide pollution of surface water (Nitschke and Schüssler 1998).

Nine natural and three synthetic steroid hormones were measured in whole body homogenate with LOQs ranging from 4-61 ng/g ww (Table 4). Natural hormones were detected in 30%

(25 of 87) of mosquitofish sampled. The only hormones not detected were 17α-estradiol, estriol and etiocholanolone. The natural estrogen estrone was the most frequently detected hormone (13%; 11 of 87), with a maximum concentration of 105 ng/g. The androgen androstenedione was detected in 19% of mosquitofish from WWEF, with maximum concentration of 88 ng/g ww. Androsterone was detected twice at concentrations exceeding 1000 ng/g wet weight. Testosterone and dihydrotestosterone were also detected at their maximum concentrations (357 and 119 ng/g wet weight, respectively) in fish from site WWEF. Mosquitofish from WWEF and AGR2 had the highest number of different hormones detected (5 of 12), and concentrations were typically highest in WWEF. Only one of the three synthetic hormones measured was detected: the synthetic hormone 17α-ethinylestradiol (EE2) was detected in two fish (sites AGR2 and UND2, at 37 and 25 ng/g ww, respectively). Site UND2 has no known WWTP influence and is in a rural area so the presence of this compound may be due to defective septic systems, leaking or overflowing sewers or recreational activities.

It is difficult to relate any of the body burden concentrations to possible adverse effects, as most studies to that end measure water concentration, not body burden. To put body burden concentrations for APIs in relative context, an approach could be to compare body residues to the human daily therapeutic dose, assuming a 70-kg adult. While APIs do not necessarily produce similar effects in humans and aquatic wildlife (Rand-Weaver et al. 2013), this comparison is presented here as a means to put aquatic animal exposure in context with intentional human therapeutic dosing. If this is done, only two APIs occur within the range of human daily therapeutic dose. At 1017 ng/g ww, omeprazole was present at 2× the equivalent human daily therapeutic dose to treat ulcers (40 mg/day converts to 571 ng/g for a 70-kg adult). Fluoxetine was detected at 240 ng/g ww, comparable to the equivalent daily human therapeutic dose to treat depression (20 mg/day converts to 286 ng/g for a 70-kg adult). Other detected APIs, such as dilantin, hydroxyzine, meprobamate and metformin, were present at concentrations that were one to three orders of magnitude lower than the equivalent human therapeutic dose in a 70-kg adult.

Depending on octanol-water partition coefficients, the concentrations of compounds that were found in fish tissue and not in water samples may decrease without sustained exposure. For example, carbamazepine was detected in liver and muscle tissue of bluntnose minnows after the first day of a 28 d exposure to 298 μg/L, but decreased within a day of depuration and had returned to a baseline concentration after 14 d depuration (Garcia et al. 2012).

Some concentrations of compounds present in the tissue samples were very high (*e.g.*, TCEP, omeprazole, chlorpyrifos, androsterone, dihydrotestosterone, fluoxetine; Table 4). Although histological analysis did not identify endocrine disruption of sexual axes as a problem, increased body burden of these TrOCs may induce organism stress in other ways and on other endocrine functions (*e.g.*, glucocorticoid). For instance, WWEF was the most polluted site (the fish had higher liver fat content and greater body burden, and the water was chemically more complex compared to that of other sites, Table 5). This could indicate a correlation between water chemistry, chemical body burden and ultimately organism stress such as alteration in lipid metabolism (Fig. 2). While fish showed no observable effects of sexual endocrine disruption, further studies should investigate whether TrOCs in their environment are inducing stress on exposed organisms in different ways, such as via other modes of action, oxidative stress, inflammation, etc.

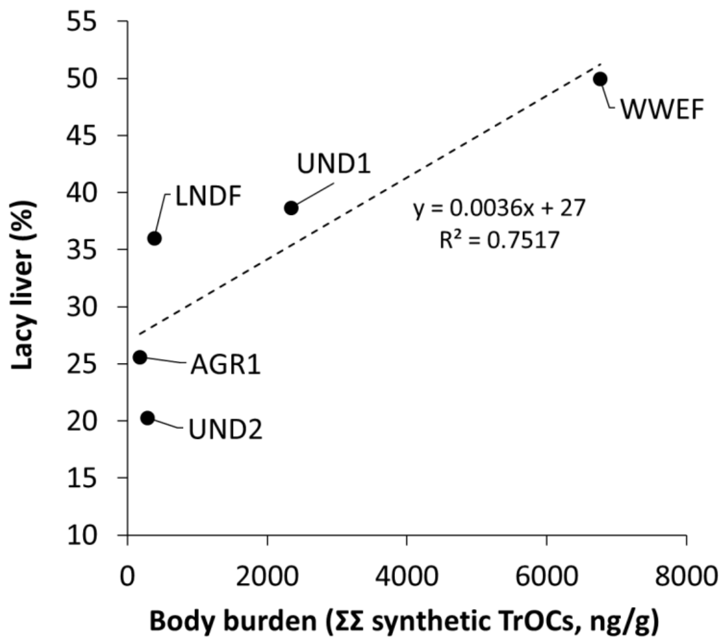

Fig. 2. Correlation between body burden as the sum of all trace organic contaminants (TrOCs) detected in fish carcasses *vs.* lacy liver in mosquitofish captured from 6 sites in Southeast Queensland.

Table 4. Body burden analysis (ng/g wet weight) of mosquitofish from six rivers across South East Queensland, Australia.

		Impacted sites (ng/g wet weight)												**Undeveloped sites (ng/g wet weight)**					
	LOQ	**AGR1 (n = 13)**			**AGR2 (n = 9)**			**WWEF (n = 21)**			**LNDF (n = 14)**			**UND1 (n = 13)**			**UND2 (n = 14)**		
	(ng/g)	**DF (%)**	**90th**	**Max**	**DF (%)**	**90th**	**Max**	**DF (%)**	**90th**	**Max**	**DF (%)**	**90th**	**Max**	**DF (%)**	**90th**	**Max**	**DF (%)**	**90th**	**Max**
Industrial compound																			
TCEP	38	0%	<38	<38	0%	<38	<38	**29%**	**653**	**4703**	0%	<38	<38	**46%**	**1148**	**1191**	0%	<38	<38
Pharmaceutical ingredients																			
Amtriptyline	19	0%	<19	<19	**11%**	**13**	**63**	0%	<19	<19	0%	<19	<19	0%	<19	<19	**7%**	<19	**88**
Atenolol	19	**8%**	<19	**150**	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Carbamazepine	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Clozapine	19	0%	<19	<19	0%	<19	<19	**43%**	**102**	**155**	**7%**	<19	**33**	**31%**	**67**	**113**	0%	<19	<19
Diazepam	19	**8%**	<19	**27**	0%	<19	<19	0%	<19	<19	**7%**	<19	**32**	0%	<19	<19	0%	<19	<19
Dilantin	19	0%	<19	<19	0%	<19	<19	**10%**	<19	**141**	0%	<19	<19	0%	<19	<19	**7%**	<19	**118**
Enalapril	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Fluoxetine	19	0%	<19	<19	0%	<19	<19	**5%**	<19	**240**	0%	<19	<19	0%	<19	<19	0%	<19	<19
Hydroxyzine	19	0%	<19	<19	**33%**	**53**	**56**	**14%**	**41**	**64**	**7%**	<19	**46**	**23%**	**73**	**121**	**7%**	<19	**81**
Meprobamate	19	**8%**	<19	**92**	**11%**	<19	**68**	**14%**	**70**	**164**	0%	<19	<19	**8%**	<19	**155**	**21%**	**89**	**133**
Metformin	38	0%	<38	<38	0%	<38	<38	0%	<38	<38	**14%**	**85**	**214**	0%	<38	<38	**7%**	<19	**74**
Paracetamol	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Primidone	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Risperidone	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Omeprazole	19	0%	<19	<19	0%	<19	<19	**5%**	<19	**1017**	**7%**	<19	**38**	**8%**	<19	**95**	**7%**	<19	**97**
Sulfamethoxazole	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	**7%**	<19	**26**	**8%**	<19	**49**	0%	<19	<19
Triamterene	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	**14%**	<19	**38**
Trimethoprim	19	0%	<19	<19	0%	<19	<19	**5%**	<19	**33**	**14%**	<19	**32**	0%	<19	<19	0%	<19	<19
Verapamil	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Σ pharmaceuticals	--	***15%***	***73***	***177***	***44%***	***71***	***131***	***71%***	***249***	***1017***	***21%***	***140***	***292***	***46%***	***208***	***321***	***36%***	***209***	***250***
Personal care product																			
Caffeine	38	0%	<38	<38	0%	<38	<38	**14%**	**52**	**74**	**14%**	<38	**56**	**15%**	<38	**54**	0%	<38	<38
Pesticides																			
Atrazine	19	0%	<19	<19	0%	<19	<19	**14%**	**33**	**183**	0%	<19	<19	**15%**	**43**	**72**	**14%**	**42**	**82**

		Impacted sites (ng/g wet weight)												Undeveloped sites (ng/g wet weight)					
	LOQ	AGR1 (n = 13)			AGR2 (n = 9)			WWEF (n = 21)			LNDF (n = 14)			UND1 (n = 13)			UND2 (n = 14)		
	(ng/g)	DF (%)	90th	Max	DF (%)	90th	Max	DF (%)	90th	Max	DF (%)	90th	Max	DF (%)	90th	Max	DF (%)	90th	Max
Chlorpyrifos	38	0%	<38	<38	0%	<38	<38	**48%**	**357**	**1557**	0%	<38	<38	**46%**	**533**	**713**	0%	<38	<38
Diazinon	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Linuron	19	0%	<19	<19	0%	<19	<19	**10%**	<19	**78**	**14%**	**35**	**54**	0%	<19	<19	0%	<19	<19
Simazine	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Σ pesticides	--	*0%*	--	--	*0%*	--	--	***52%***	***364***	***1740***	***14%***	***35***	***65***	***54%***	***533***	***785***	***14%***	***42***	***82***
Natural hormones																			
Androstenedione	19	0%	<19	<19	0%	<19	<19	**19%**	**63**	**88**	0%	<19	<19	0%	<19	<19	0%	<19	<19
Androsterone	4	0%	<4	<4	**22%**	**341**	**1012**	**5%**	<4	**1251**	0%	<4	<4	0%	<4	<4	0%	<4	<4
Dihydrotestosterone	61	0%	<61	<61	**11%**	<61	**134**	**5%**	<61	**357**	0%	<61	<61	0%	<61	<61	**7%**	<61	**82**
17α-Estradiol	4	0%	<4	<4	0%	<4	<4	0%	<4	<4	0%	<4	<4	0%	<4	<4	0%	<4	<4
17β-Estradiol	4	0%	<4	<4	**11%**	**11**	**53**	0%	<4	<4	0%	<4	<4	0%	<4	<4	**7%**	<4	**54**
Estriol	12	0%	<12	<12	0%	<12	<12	0%	<12	<12	0%	<12	<12	0%	<12	<12	0%	<12	<12
Estrone	4	**15%**	**27**	**42**	**11%**	**13**	**63**	**5%**	<4	**104**	0%	<4	<4	**31%**	**91**	**105**	**21%**	**42**	**148**
Etiocholanolone	23	0%	<23	<23	0%	<23	<23	0%	<23	<23	0%	<23	<23	0%	<23	<23	0%	<23	<23
Testosterone	19	0%	<19	<19	0%	<19	<19	**14%**	**58**	**119**	**7%**	<19	**63**	**23%**	**64**	**85**	0%	<19	<19
Σ natural hormones	--	***15%***	***27***	***42***	***44%***	***384***	***1012***	***33%***	***104***	***1608***	***7%***	--	***63***	***54%***	***92***	***105***	***29%***	***90***	***148***
Synthetic hormones																			
17α-Ethinylestradiol	4	0%	<4	<4	**11%**	**7**	**37**	0%	<4	<4	0%	<4	<4	0%	<4	<4	**7%**	<4	**25**
Levonorgestrel	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Mestranol	19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19	0%	<19	<19
Σ synthetic hormones		*0%*	--	--	***11%***	***7***	***37***	*0%*	--	--	*0%*	--	--	*0%*	--	--	***7%***	--	***25***
ΣΣ synthetic TrOCs [(1)]	--	***15%***	***73***	***177***	***56%***	***71***	***131***	***81%***	***1123***	***6761***	***21%***	***225***	***384***	***77%***	***1837***	***2341***	***43%***	***237***	***281***

DF = detection frequency; 90^{th} = 90^{th} percentile concentration; Max = maximum concentration; LOQ = Limit of quantification. See Table 2 fo sample size. [(1)] ΣΣ synthetic TrOCs was calculated as the sum of the concentration of all chemicals except the natural hormones.

3.4. Water analysis

3.4.1. Chemistry

Site WWEF had the most chemically complex water sample with 12% of TrOCs (6/51) detected (Table 5). Three compounds were detected at sites AGR1 and UND2, one compound detected at sites LNDF and UND1, while no TrOCs were detected in the sample from AGR2. Historically, site WWEF has had more chemically complex water (using this analytical method) compared to the other sites, with 37% of compounds detected in at least one grab sample over a 12-month monitoring period at that site, compared with 22% for AGR1, 16% for AGR2, 14% for UND1 and 8% for LNDF (2011-2012, Table 5).

All compounds detected at site WWEF were APIs (clozapine, gemfibrozil, paracetamol, and salicylic acid) or personal care products (caffeine and triclosan), with no known estrogenic properties. The synthetic hormone EE2 was detected at two sites (LNDF and UND2) in Apr/May 2013 during fish sampling. The sample from site LNDF had a concentration of 0.11 ng/L, slightly above the predicted no-effect concentration (PNEC) of 0.1 ng/L for 17α-EE2 proposed by Caldwell et al. (2012), while that from UND2 had a concentration of 0.07 ng/L. Further monitoring is required to determine the temporal variation and persistence of EE2 at these sites.

Table 5. Chemical and *in vitro* monitoring data of 38 trace organic pollutants (TrOCs) from water extracts from the present study (Apr and May 2013) and historical data (May 2011 – Feb 2012) adapted from Scott et al. (2014a, 2014b). All values are in ng/L, except where indicated for bioanalytical equivalent concentrations.

Compound	**AGR1**		**AGR2**		**WWEF**		**LNDF**		**UND1**		**UND2**
	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**Apr 2013**	**Apr 2013**
Industrial compounds											
Bisphenol A	**16 - 82**	NA	**<10 - 106**	NA	**15 - 22**	NA	**12 - 25**	NA	**12 - 50**	NA	NA
TCEP	**<10 - 15**	<10	**<10 - 15**	<10	**<10 - 11**	<10	<10	<10	**<10 - 17**	<10	<10
4-t-Octylphenol	<10	<20	<10	<20	<10	<20	<10	<20	<10	<20	<20
Pharmaceutical ingredients											
Amtriptyline	<10	<10	<10	<10	**<10 - 15**	<10	<10	<10	<10	<10	<10
Atenolol	<5	<5	<5	<5	**<5 - 9**	<5	<5	<5	<5	<5	<5
Carbamazepine	**<5 - 7**	<5	<5	<5	**<5 - 166**	**41**	<5	<5	<5	<5	<5
Clozapine	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Diazepam	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Dilantin	<5	<5	<5	<5	**<5 - 22**	<5	<5	<5	<5	<5	<5
Enalapril	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Fluoxetine	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Gemfibrozil	**<5 - 5**	<5	<5	<5	**<5 - 95**	**11**	<5	<5	<5	<5	<5
Hydroxyzine	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Ibuprofen	**<5 - 10**	<5	<5	<5	**<5 - 44**	<5	<5	<5	<5	<5	<5
Ketoprofen	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Meprobamate	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Metformin	NA	<5	NA	<5	NA	<5	NA	<5	NA	<5	<5
Naproxen	**<5 - 6**	<5	<5	<5	**<5 - 15**	<5	<5	<5	<5	<5	<5
Omeprazole	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Paracetamol	**<5 - 5**	**128**	**<5 - 314**	<5	**7 - 28**	**460**	<5	<5	**<5 - 8**	<5	<5
Primidone	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Risperidone	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Salicylic acid	**<20 - 91**	**20**	**<20 - 92**	<20	**<20 - 88**	**75**	**<20 - 97**	<20	**<20 - 46**	**29**	**23**
Simvastatin	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5

Compound	AGR1		AGR2		WWEF		LNDF		UND1		UND2
	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**Apr 2013**	**Apr 2013**
Simvastatin-hydroxyacid	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Sulfamethoxazole	<5	<5	<5	<5	**<5 - 5**	<5	<5	<5	<5	<5	<5
Triamterene	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Trimethoprim	<5	<5	<5	<5	**<5 - 25**	<5	<5	<5	<5	<5	<5
Verapamil	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Personal care products											
Caffeine	**<10 - 186**	**15**	**<10 - 234**	<10	**20 - 285**	**333**	**<10 - 142**	<10	**<10 - 29**	<10	**11**
Propylparaben	**<10 - 34**	<10	**<10 - 33**	<10	<10	<10	<10	<10	**<10 - 20**	<10	<10
Triclocarban	<10	<10	<10	<10	**<10 - 55**	<10	<10	<10	<10	<10	<10
Triclosan	<10	<10	<10	<10	**<10 - 43**	**7**	<10	<10	<10	<10	<10
Pesticides											
Atrazine	<5	<5	**<5 - 8**	<5	<5	<5	<5	<5	<5	<5	<5
Chlorpyrifos *	<5	<10	<5	<10	<5	<10	<5	<10	<5	<10	<10
Diazinon *	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Linuron	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
2-Phenylphenol	<10	<10	<10	<10	**<10 - 20**	<10	<10	<10	**<10 - 59**	<10	<10
Simazine	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Hormones											
Androstenedione	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Androsterone	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Dihydrotestosterone	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16
17α-Estradiol	<1	<1	<1	<1	**<1 - 4**	<1	<1	<1	<1	<1	<1
17β-Estradiol	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Estriol	<3	<5	<3	<5	<3	<5	<3	<5	<3	<5	<5
Estrone	**<1 - 3**	<1	**<1 - 2**	<1	**<1 - 10**	<1	**<1 - 1**	<1	**<1 - 2**	<1	<1
Etiocholanolone	<6	<6	<6	<6	<6	<6	<6	<6	<6	<6	<6
Mestranol	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Testosterone	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Synthetic hormones											
17α-Ethinylestradiol	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	**0.11**	<0.05	<0.05	**0.07**
Levonorgestrel	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
In vitro *endocrine activity*											

Compound	**AGR1**		**AGR2**		**WWEF**		**LNDF**		**UND1**		**UND2**
	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**May 2013**	2011-2012	**Apr 2013**	**Apr 2013**
Estrogenic (EEQ, ng/L)	**<0.1 - 0.52**	**1.18**	<0.1	**0.78**	**<0.1 - 1.16**	**0.89**	**<0.1 - 0.28**	**0.1**	<0.1	**0.04**	<0.02
Antiestrogenic (TMXEQ, µg/L)	<20	**2.44**	<20	<1	<20	<1	<20	**1.46**	<20	<1	**2.74**
Androgenic (DHTEQ, ng/L)	<7	<9	<7	<9	<7	<9	<7	<9	<7	<9	<9
Antiandrogenic (FluEQ, µg/L)	<60	**96**	<60	**80**	<60	**90**	<60	**83**	<60	**90**	**73**
Progestagenic (LevoEQ, ng/L)	<5	**0.09**	<5	<0.06	<5	<0.06	<5	**0.14**	<5	<0.06	**0.11**
Antiprogestagenic (MifEQ, ng/L)	<8	**4.2**	<8	<1.8	<8	**2.2**	<8	**3.5**	<8	**1.8**	**2.1**

* Only measured in one sampling event of four (May 2011 – Feb 2012). NA = Not analyzed; TCEP = Tris(2-chloroethyl) phosphate.

Bioanalytical equivalents are: EEQ = 17β-estradiol equivalents; TMXEQ = tamoxifen equivalents; DHTEQ = dihydrotestosterone equivalents; FluEQ = flutamide equivalents; LevoEQ = levonorgestrel equivalents; MifEQ = mifepristone equivalents.

3.4.2. In vitro *activity*

Five of six sites had detectable estrogenic activity, with values ranging from 0.04 to 1.18 ng/L EEQ (Table 5). Site UND2 was the only site without detectable estrogenic activity. *In vitro*-specific safe EEQs (EEQ-SSE) between 0.5 and 2 ng/L EEQ for short-term and between 0.1-0.4 ng/L EEQ for longer-term exposures have been proposed by Jarošová et al. (2014). Estrogenic activity in samples from AGR1, AGR2 and WWEF (0.78 to 1.18 ng/L EEQ) exceeded the most conservative EEQ-SSE of 0.1 ng/L EEQ, while samples from LNDF, UND1 and UND2 did not (Table 5). The least conservative short-term exposure EEQ-SSE of 2 ng/L EEQ was not exceeded in any of the samples. While there was a correlation between *in vitro* estrogenic activity and Vtg protein levels (with higher Vtg levels at higher EEQ; Fig. 1B), Vtg protein levels in fish at most sites were not significantly elevated compared to unexposed laboratory reference males, except for fish at AGR1 and UND2 (Fig. 1A). A recent study suggests that mosquitofish may not be as sensitive as other native species, such as rainbowfish for example (Scott et al. 2017), and further studies with more endocrine-sensitive and/or sedentary fish or invertebrate species would help determine whether these levels of estrogenic activity constitute a risk of endocrine disruption in other organisms in chronic exposure conditions.

Slight anti-estrogenic activity was detected at sites AGR1, LNDF and UND1, with a maximum concentration of 2.44 µg/L tamoxifen equivalents (TMXEQ). Anti-estrogenic activity has not previously been reported at these sites in the 2011 to 2012 study; however, our LOQ was more sensitive in the current analysis (1 µg/L compared to 5 µg/L TMXEQ; Scott et al. (2014a)). A similar inconstant picture is apparent in the literature, with Leusch et al. (2014b) reporting anti-estrogenic activity in two of nine WWTP effluents examined (up to 4.4 µg/L TMXEQ), while Roberts et al. (2015) did not detect anti-estrogenic activity in wastewater of a large Australian WWTP. Quantification of antagonistic activity, while technically possible (Neale and Leusch 2015), is difficult to accurately perform *in vitro* due to the presence of competing agonist and the possible interference from natural organic matter (Neale et al. 2015).

Androgenic activity was not detected in any samples, but anti-androgenic activity was detected at all six sites ranging from 73 to 96 μg/L flutamide equivalents (FluEQ). Previous studies of Australian WWTP effluent (Leusch et al. 2014b, Roberts et al. 2015) have generally not detected anti-androgenic activity, but this could be due to the high LOQ in those other studies (*e.g.*, 250 μg/L FluEQ compared to 25 μg/L FluEQ in the present study).

Progestagenic activity was detected at three sites, up to a maximum of 0.14 ng/L levonorgestrel equivalents (LevoEQ) at LNDF (Table 5). Progestagenic activity has previously been reported in Dutch sewage effluent at a concentration up to 2.2 μg/L LevoEQ (Van der Linden et al. 2008) and in Australian WWTP effluent up to 5.4 ng/L LevoEQ, and was hypothesized to be associated with human APIs (Leusch et al. 2014b). Progestagenic activity was not detected at any river sites in a previous Australian national survey (73 sites; Scott et al. (2014a)), although the LOQ in that study was much higher than that of the present study (5 *vs.* 0.06 ng/L LevoEQ, respectively). The potent synthetic progestin levonorgestrel, often used in combination with the synthetic estrogen EE2 in birth control pills, is currently difficult to measure by chemical analysis, with LOQs comparable to the 5 ng/L achieved in the current study. A recent study has calculated a predicted concentration in wastewater ranging from 0.2 to 0.6 ng/L (King et al. 2016). If present at these concentrations, levonorgestrel would likely explain a significant portion of the progestagenic activity detected here. A provisional PNEC of 0.1 ng/L has been derived for levonorgestrel (King et al. 2016). The progestagenic activity at sites LNDF and UND2 was slightly above this concentration (0.14 and 0.11 ng/L LevoEQ, respectively), indicating a potential risk if all the activity is caused by levonorgestrel. Improvements in chemical analysis methods and refinements of the provisional PNEC value are necessary to more firmly quantify the risk that this potent progestin poses to the receiving environment. Anti-progestagenic activity was detected at most sites and ranged from <1.8 to 4.2 μg/L mifepristone equivalents (MifEQ; Table 5). The maximum concentration in the present study was much lower than that in a recent Australian study, which reported anti-progestagenic activity in 16% of Australian rivers sampled (73 in total) at concentrations as high as 32 μg/L MifEQ (Scott et al. 2014a). The concentrations in the current study were also lower than those measured in Chinese WWTP effluent (29 μg/L MifEQ measured with a yeast based bioassay) (Li et al. 2011). The compounds responsible for the anti-progestagenic activity measured in the current study are unidentified, although nonylphenol, which has been shown to significantly inhibit the binding of progesterone to the human progesterone receptor in a yeast-based bioassay (Viswanath et al. 2008), is a potential suspect. Unfortunately, due to analytical complications regarding the quantification of nonylphenol in the environmental samples (as detailed in Scott et al. (2014a)), nonylphenol was not analyzed in this study.

3.5. Conclusions

This study found no overt evidence of endocrine disruption of sexual axes: there was no evidence of abnormal secondary sexual characteristic (gonopodium) or gonadal development (including incidence of intersex) in mosquitofish from any of the sites sampled. *In vitro* bioassays however indicated slight estrogenic and anti-androgenic activity at most sites, and Vtg protein (a sensitive biomarker of exposure to estrogenic EACs) was elevated at two sites (AGR1 and UND2). This suggests that while fish at the sites samples are exposed to low concentrations of EACs, these concentrations are too low to produce significant organism-level disruption, in agreement with recent suggestions that endocrine disruption in Australian freshwaters is unlikely to be widespread (Hassell et al. 2016, Vajda et al. 2015).

Several TrOCs were detected in fish carcasses, confirming that fish are exposed to and ingest a wide range of TrOCs; however only a few TrOCs were detected in grab water, the discrepancy likely illustrating the high variability of concentrations of these TrOCs over time. TrOCs were detected at the two undeveloped sites, suggesting that even areas relatively removed from populated areas may still exhibit the chemical traces of human activity.

Concentrations of TrOCs and EACs in water samples were typically not cause for concern, with one exception at site LNDF where EE2 was detected slightly above the PNEC of 0.1 ng/L. (Anti)estrogenic, anti-androgenic, and (anti)progestagenic activities were all quantified in water samples from at least three (and up to five of six) sites. Estrogenicity ranged from 0.1 – 1.18 ng/L EEQ, in excess of the *in vitro*-specific safe estrogenicity (EEQ-SSE) value of 0.1 ng/L for chronic exposure proposed in Jarošová et al. (2014) at all sites except the two undeveloped sites (Table 5). In a prior study, WWEF had the highest estrogenicity (1.16 ng/L EEQ) compared to 18 other Queensland sites, and the fifth highest estrogenicity out of 73 sites across mainland Australia (Scott et al. 2014a).

While the results of this study indicate a low risk of disruption of sexual endocrine systems in fish, chemical body burdens were correlated with lipid accumulation in liver (hepatic steatosis), which may indicate that other effects, including hormonal regulation of lipid synthesis and storage and other subtle mechanisms of toxicity may be of concern downstream of wastewater discharges and dense human activity. It should be noted that the low sample size at some sites, particularly when split across three analyses (body burden, Vtg and histology), meant limited statistical power to identify subtle differences across sites for some endpoints, and these results should be treated with caution.

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