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Intersex in feral indigenous freshwater *Oreochromis mossambicus*, from various parts in the Luvuvhu River, Limpopo Province, South Africa

I.E.J. Barnhoorn [a,*], J.C. van Dyk [a], G.M. Pieterse [b], M.S. Bornman [a]

[a] *Andrology: Department of Urology, School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa*
[b] *Department of Zoology, University of Johannesburg, South Africa*

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ABSTRACT

This study reports on intersex in *Oreochromis mossambicus*, an indigenous fish species inhabiting most aquatic systems throughout South Africa (SA). Male fish were collected from three sites in the Luvuvhu River, Limpopo Province, SA: Albasini Dam (AD), Nandoni Dam (ND), and Xikundu Weir (XW). The latter two sites are situated in a currently dichloro-diphenyl-trichloroethane (DDT) sprayed area. A laboratory-bred reference group (Aq R) were included for a histological comparison. 48% of the fish at AD were intersex individuals compared with 63% at ND, and 58% at XW. The Aq R fish had no cases of intersex. *o,p′*- and *p,p′*-DDT and metabolites dichlorodiphenyldichloroethane (DDD) and -dichlorodiphenyldichloroethylene (DDE) were detected in fat samples, indicative of contamination of the aquatic environment and subsequent exposure of fish to these chemicals. Although some of the fat samples contained levels of DDTs no association could be established between intersex and chemical contaminants in fish.

1. Introduction

The occurrence of intersex as a result of embryonic exposure to endocrine disrupting chemicals (EDCs), in various wild fish species, is a widely discussed topic globally (Van Aerle et al., 2001; Gercken and Sordyl, 2002; Hinck et al., 2009; Sun and Tsai, 2009). Intersex, in naturally gonochoristic (separate sexes) species, is defined as the presence of both male and female reproductive features within the same individual. Intersex is commonly known as a condition that originates during embryogenesis, assuming the fish has been exposed to pollutants at that time (Jobling and Tyler, 2003). Therefore, intersex should not be confused with natural hermaphroditism or the process of sex determination that can be influenced by environmental factors such as photoperiod, temperature, pH, nutrients, and social interactions (Hurley et al., 2004). Most intersex cases in gonochoristic fish species reported feminization of the reproductive ducts in male fish (Gray and Metcalfe, 1997; Metcalfe et al., 2001) due to exposure to EDCs during embryonic development (Jobling and Tyler, 2003). The degree of intersex in a population may pose a risk for reproductive potential and thus survival of that specific species (Jobling et al., 2002). Furthermore, Sun and Tsai (2009) speculated that 50% of the feminized tilapia species found in Era-Jiin River, Taiwan, was caused by EDCs present in the river system.

Over the last couple of years, data became available on the presence of EDCs in South African (SA) fresh water sources (Barnhoorn et al., 2004). Estrogenic activity (Aneck-Hahn, 2002; Timmerman, 2003) was also reported from various water sources. Following the international reports on intersex in various fish species (Van Aerle et al., 2001; Gercken and Sordyl, 2002; Hinck et al., 2009; Sun and Tsai, 2009), the first cases of intersex in SA were found in feral sharptooth catfish, *Clarias gariepinus* (Barnhoorn et al., 2004). The fish inhabited an urban impoundment, Rietvlei Dam (RVD), which receives contaminated industrial and farming wastes and also supplies drinking water to 15% of municipalities in the Tshwane district, Pretoria (Rietvlei Nature Reserve: Scientific Information, 2009). Target chemical analyses of water, sediment, and fish fat showed the presence of DDT and metabolite residues (Barnhoorn et al., 2004). A recent pilot study conducted in a currently DDT-sprayed area in the north of SA, also indicated that several aquatic and terrestrial biota had noteworthy levels of DDT and metabolites (Bornman et al., 2009). There is also concern about possible health effects in humans living in this area as high levels of DDT and metabolites were detected in human serum samples (Bornman et al., 2009).

The Luvuvhu River is the main source of fresh water that flows through the Vhembe district in the Limpopo Province of SA. The agricultural activities along the river produces citrus, mangos, bananas, and macadamia nuts while the downstream catchment area is dominated by rural villages including community gardens

* Corresponding author. Fax: +27 12 329 5152.
E-mail address: Irene@med.up.ac.za (I.E.J. Barnhoorn).

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and farming with livestock (State of the Rivers Report, 2001). Subsistence farming represents about a third of the total agricultural component along the Luvhuvu River. Other than being a drain and therefore a sink for run-off water, water transfers and extractions, this river is also an important recreational site and is used by locals for household activities such as washing of clothes, bathing, and by entrepreneurial car wash businesses serving local communities along the river. The north-eastern parts of the Limpopo Province, are medium- to high-risk malaria areas and subsequently has a history of dichlorodiphenyltrichloroethane (DDT)-spraying since 1945 (Mbaso et al., 2004; Sadasivaiah et al., 2007). DDT is still sprayed in this area in accordance with the interim recommendations of the Stockholm Convention (Bouwman, 2004). As a result, DDT residues are most likely ending up in the nearby Luvuvhu River via rainwater runoff and atmospheric movement (Barnhoorn et al., 2009).

Oreochromis mossambicus is a gonochoristic teleost species, indigenous to South Africa and inhabits the Luvuvhu River. The species prefers standing waters with higher temperatures (above 22 °C). They feed on algae; especially diatoms but the mature animals may also take insects and other invertebrates. *O. mossambicus* spawn during the summer season and a female may raise multiple broods every three to four weeks (Skelton, 1993). We report on testicular oocytes in *O. mossambicus* and detected levels of selected EDCs in mesenteric fat, water, and sediment samples. The findings in a reference group (Aq R) *O. mossambicus* were compared with the wild fish.

2. Materials and Methods

2.1. Tissue sampling

Fish were collected from three sites within the Luvuvhu River catchment area as indicated in Fig. 1. Aquarium reared fish were included for comparison from a supposedly EDC-free water system.Six field surveys were done over a period of five years to collect tissue samples for histology as well as fat samples, water, and sediment samples for EDC analysis. One hundred and twenty-nine male *O. mossambicus* were collected using gill nets. To verify the gonochoristic characteristics of these fish, laboratory-bred fish (Aq R) were included for comparison of gonadal histology. Ten males were collected from this group. All fish were sexed according to the secondary urogenital papilla, upper-lip, and breeding colors. Fish were sacrificed where after the testes were macroscopically examined, and sampled for histology. Mid-sections of the testes were fixed in Bouin's fixative, dehydrated in graded ethanol, and embedding in paraffin wax; sections (5 μm) were cut and stained with Haematoxylin and Eosin (van Dyk, 2006). The slides were examined using light microscopy.

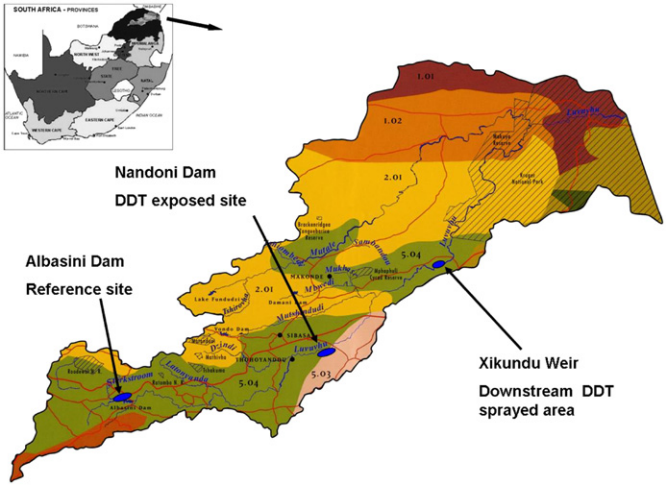

Fig. 1. A map indicating the three sites where sampling was done in the Luvuvhu River catchment (Map adapted from State of the Rivers Report, WRC report no.: TT165/01).

Available fat was collected from each fish, wrapped in aluminum foil, and stored at −20 °C before target chemical analysis.

2.2. Target chemical analysis

The compounds selected for target chemical analysis included the organochlorine pesticides (OCs) including (Alpha- hexachlorocyclohexane (HCH), gamma (γ)-HCH (lindane), heptachlor, aldrin, dieldrin, beta (β)-HCH, delta (δ)-HCH, heptachlor epoxide, endosulfan I, endosulfan II, endosulfan sulfate, alpha (α)-chlordane, gamma (γ)-chlordane, o,*p*′- and *p*,*p*′- dichloro-diphenyl-trichloroethane (DDT), -dichlorodiphenyldichloroethane (DDD) and -dichlorodiphenyldichloroethylene (DDE), endrin, endrin aldehyde, endrin ketone, methoxychlor), and polychlorinated biphenyl (PCB) 153 as representative of PCBs (Spano et al., 2005).

2.2.1. Extraction and analyses from fat samples

Extractions were done using solid phase C_{18} cartridges (Waters-Microsep) conditioned with petroleum ether followed by acetone and methanol (Cacho et al., 1995). The extracts were loaded onto the cartridge and allowed to flow through. The collected eluate was rinsed with acetonitrile and allowed to elute from the cartridges. The samples were evaporated under nitrogen at 35 °C and reconstituted into 2 ml of hexane. A florisil cartridge was placed on the manifold and after conditioning with 10 ml of hexane, the samples were loaded. The samples were eluted with 10 ml of petroleum ether–diethyl ether (98:2, v/v) and 12 ml of petroleum ether–diethyl ether (85:15, v/v). The two fractions were combined and evaporated under nitrogen to dryness.

2.2.2. Organochlorine pesticide analysis in fat

The OC residues were analyzed by a gas chromatography-mass spectrometer (GC-MS) (Agilent 7890 A) equipped with a 5975C mass spectrometer and an Equity 1701 fused silica capillary column (Supelco). The column temperature was increased from 90 to 200 °C at a rate of 40 °C/min. The temperature of injector and detector was 250 and 200 °C, respectively. High purity helium was used as carrier gas at a flow rate of 0.84 ml/min. Samples were injected under splitless injection mode (Bordet et al., 2002; Villaverde et al., 2008). The mass spectra were collected in the electron impact mode at 70 eV and the mass-to-charge ratios (*m*/*z*) of the ions were used for quantification in SIM mode. The confirmation of the OCs was done using selected ion monitoring (SIM) mode with three selective ions. The quantification was done with one of the selective ions. The detection limit is too low for a full spectrum.

2.2.3. Alkylphenols analyses in fat

The APs were analyzed by a GC-MS (similar to OC analysis) according to a method by Croce et al. (2003). Data processing was done by the Agilent MSD ChemStation E.01.00.232 software. The mass spectrometer was operated in selected ion monitoring mode (SIM) with pulse splitless injection mode (680 kPa). The phenols were analyzed as the pentafluoropropionic anhydride (PFPA) derivatives.

2.3. Water and sediment analysis

Water from the aquaria used for the laboratory-bred fish, and surface water (from the selected sites in the Luvuvhu River catchment) ±45 cm deep was collected in 1 L glass bottles, pre-washed with ethanol. Sediment samples were collected on the same day, approximately 1 m below the surface, in clean wide-mouth glass flasks. The water and sediment collection points were close to where fish were collected. All water and sediment samples were kept at 4 °C in the laboratory, prior to sample preparation for OC, AP analyses. Phthalates were analyzed in sediment and included dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) benzyl butyl phthalate (BBP), and di-(2-ethylhexyl) phthalate (DEHP). In water, hormone analyses of ethinylestradiol, estradiol, estrone, and estriol were done.

2.3.1. Extraction from water

The extraction method for water used was based on the method described by Cacho et al. (1995) where 1 L water was filtered into an acid washed reagent bottle. C_{18} solid phase cartridges (SPE) (Waters-Microsep) were conditioned with water, methanol, and water. The samples were then allowed to pass through the cartridge and it was dried and eluted with 12 mL of hexane-diethylether (85:15, v/v). The samples were evaporated under nitrogen to dryness and reconstituted in 200 μL methanol, before 1 μL was injected. The OCs were analyzed and quantified by gas chromatography-mass spectrometry (GC-MS). A Hewlett Packard (HP7890) Gas Chromatography system equipped with a HP 7683 auto injector and HP5975 mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separation and recording of mass spectra.

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2.3.2. Extraction from sediment samples

Sediment extraction was based on the method by Naudé et al. (1998). Sediment samples were dried at 110 °C in an oven. 5 g samples were weighed, 100 mL hexane-petroleum ether (50:50, v/v) added and extracted via reflux for a period of 12 h. The samples were allowed to cool and evaporate to dryness under vacuum at 35 °C. SPE clean-up was performed on C_{18} cartridges as mentioned under the section for water samples.

2.3.3. Organochlorine pesticides analyses, water and sediment

The OC pesticide residues were analyzed by a GC-MS. The column temperature was increased from 90 to 200 °C at a rate of 40 °C/min. The temperature of the injector and detector was 250 and 200 °C, respectively. Carrier gas (high pure helium) at a flow rate of 0.84 mL/min were used. Samples were injected under splitless injection mode (Bordet et al., 2002; Villaverde et al., 2008). Confirmation was done using selected ion monitoring (SIM). The mass spectra were collected in the electron impact mode at 70 eV and the mass-to-charge ratios (m/z) of the ions were used for quantification in SIM mode.

2.3.4. Alkylphenols analyses, water and sediment

The APs were analyzed by using a GC-MS (Agilent 7890A) equipped with a 5975C mass spectrometer and an Equity 1701 fused silica capillary column (Supelco) Croce et al. (2003). Data processing was done by the Agilent MSD ChemStation E.01.00.232 software. The mass spectrometer was operated in selected ion monitoring mode (SIM) with pulse splitless injection mode (680 kPa). The phenols were analyzed as the pentafluoropropionic anhydride (PFPA) derivatives (Croce et al., 2003).

2.3.5. Phthalates analyses, water and sediment

The phthalates were analyzed by a GC-MS (Agilent 7890A) equipped with a 5975C mass spectrometer and an Equity 1701 fused silica capillary column (Supelco). Data processing was done with Agilent MSD ChemStation E.01.00.232 software. The mass spectrometer was operated in electron impact (EI) mode with SIM detection. Pulse splitless injection mode (680 kPa) was used to enhance the detection of the phthalates (Petrovicè et al., 2002).

2.3.6. Hormones analyses in water

The hormones were analyzed by liquid chromatography mass spectrometry (LC MS) method using a Shimadzu LC 20 consisting of a binary pump, vacuum degasser, autosampler, column heater, and mobile phase switching valve. An Applied Biosystems/MDX Sciex 4000 Q–Trap Tandem Mass Spectrometer was used for the detection system. Data processing was done by Analyst 1.4.2 Software. A Synergi Fusion C_{18} HPLC-column: 2.0×150 mm^2, 4 μm particle size, was used to separate the individual compounds. A gradient elution of water and acetonitrile was used to elute the hormone from the column. The mass spectrometer was operated in the multiple reaction monitoring mode (MRM). Two MRM transitions were used for identification purposes and one MRM per hormone for quantification (Diaz-Cruz et al., 2003; Isobe et al., 2003).

3. Results

Fish were sexed according to the external features, such as breeding colors and the lip morphology and the presence of normal male gonads after laparotomy. Only male fish according to the above characteristics were used during this study.

3.1. Testis histology

Macroscopically, the testes of all specimens showed no structural abnormalities. The histological assessment of the testes of fish from the AD, ND, XW, and the Aq R showed normal lobular structure of the seminiferous tissue and all stages of spermatogenesis were present in most of the samples assessed. However, the histological assessment revealed the presence of primary oocytes within the testicular tissue (ovotestis) (Fig. 2) in 49% of male fish from AD, 63% of fish from ND, and 58% of fish from the XW (Fig. 3). No ovotestis was found in any of the Aq R specimens.

3.2. Fish fat OC levels

DDT and its metabolites (*o,p′*-DDT, *p,p′*-DDT, *o,p′*-DDD, *p,p′*-DDD, *o,p′*-DDE, and *p,p′*-DDE) as well as lindane (γ-HCH) were detected in the fish fat samples from ND and XW (Table 1).

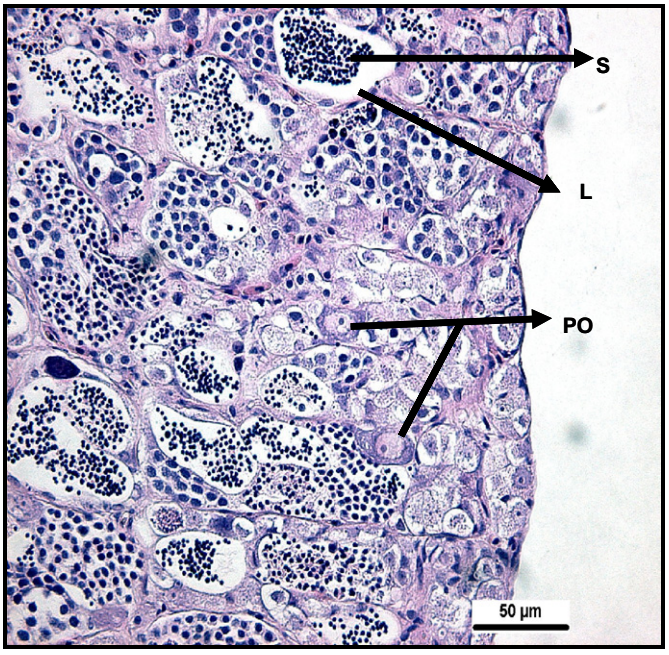

Fig. 2. Transverse sections through the testis of a mature *O. mossambicus* male showing intersex gonads with primary oocytes (PO) scattered through the testicular tissue (natural population). (S) Spermatozoa; (L) Lumen. Scale bar 50 μm, 10 × magnification.

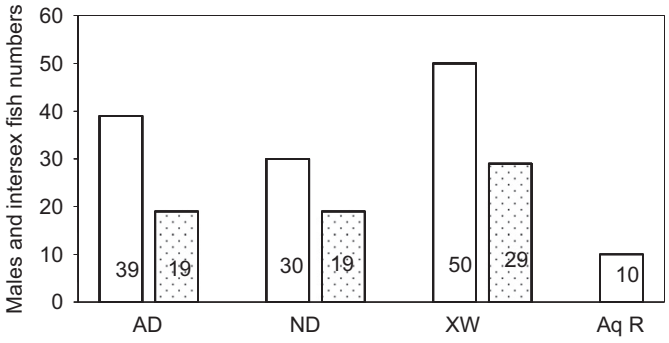

Fig. 3. Number of intersex *O. mossambicus* collected at the three sites in the Luvuvhu River. The white bar indicating the number of males at the location and the dotted bars indicates the number of intersex individuals.

The major metabolite detected was *p-p′*-DDE in 97% of samples at ND and 35% at XW, but the levels at XW were higher compared with ND (1764 ± 3244 vs 7909 ± 5546 μg/kg fat). The intermediary metabolite *p-p′*-DDD was present in 71% and 35% of samples, respectively, from ND and XW. Fish fat samples from ND also contained endosulfan I, endosulfan sulfate, and PCB153 while fish fats from XW had levels of dieldrin and endrin aldehyde. Unfortunately the fish from AD and the Aq R had insufficient fat for target chemical analyses.

3.3. Water and sediment

In the water samples analyzed (Table 2), all six DDT isomers were present in one or more samples from the three sites, but not during the same survey and at levels just above the detection limit. During one survey, dieldrin residues were present at all three sites. No *p*-NP or OcP residues, ethinyl estradiol, estradiol, estrone, estriol, or any of the phthalates were detected in the water samples. The water from the laboratory-bred fish had no levels of the selected EDCs tested for.

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Table 1
Mean levels (μg/kg ± SD) of EDCs in *O. mossambicus* fat, sampled at three sites within the Luvuvhu River catchment.

	ND	=n analyzed	OC detected (%)	XW	=n analyzed	OC detected (%)
***o,p′*-DDT**	110 ± 81	35	66	4035 ± 2867	35	33
***p,p′*-DDT**	948 ± 1562	35	46	5889 ± 3036	35	26
***o,p′*-DDD**	114 ± 67	35	34	159 ± 143	35	35
***p,p′*-DDD**	860 ± 1027	35	71	847 ± 1166	35	35
***o,p′*-DDE**	94 ± 49	35	32	171 ± 45	35	17
***p,p′*-DDE**	1764 ± 3244	35	97	7609 ± 5546	35	35
Lindane	48 ± 45	20	50	18 ± 7	17	8
Dieldrin	*	*	*	14 ± 1	17	2
Endrin aldehyde	*	*	*	2661 ± 1035	17	9
Endosulfan I	42 ± 22	20	30	*	*	*
Endosulfan sulfate	21 ± 6	20	25	*	*	*
PCB 153	119 ± 36	20	15	*	*	*

*=below detection limit of 10 μg/kg.

Table 2
Levels of the different DDT isomers and dieldrin (μg/L) detected in the water samples at the different localities.

	o,p′-DDE	*p,p′*-DDE	*p,p′*-DDD	*p,p′*-DDT	ΣDDT	Dieldrin
Survey 1						
Albasini Dam	*	*	*	*	*	*
Nandoni Dam	*	*	*	0.12	*	*
Xikundu Weir	*	*	*	*	*	*
Survey 2						
Albasini Dam	*	*	*	*	*	*
Nandoni Dam	*	0.3	*	*	*	*
Xikundu Weir	*	0.4	*	*	*	*
Survey 3						
Albasini Dam	*	1	1	0.3	2.3	*
Nandoni Dam	*	1.1	*	*	*	*
Xikundu Weir	*	*	*	*	*	*
Survey 4						
Albasini Dam	*	1	*	*	*	2
Nandoni Dam	*	*	*	*	*	2.4
Xikundu Weir	1.2	1	*	*	2.2	4

*=below detection limit of 0.1 μg/L.

Table 3
Levels of the different DDT isomers (μg/Kg). detected in the sediment samples at the different localities.

	p,p′-DDT	*o,p′*-DDE	*p,p′*-DDE	PCB 153
Survey 3				
Albasini Dam	*	*	*	*
Nandoni Dam	*	*	*	*
Xikundu Weir	*	*	*	*
Survey 4				
Albasini Dam	*	*	*	*
Nandoni Dam	1.4	*	3.7	*
Xikundu Weir	4.1	*	13	*

*=below detection limit of 0.1 μg/Kg.

Sediment from both ND and XW contained residues of *p,p′*-DDT (1.4 and 4.1 μg/kg) and *p,p′*-DDE (3.7 and 12.8 μg/kg), respectively, during Survey 4 (Table 3).

4. Discussion

Research on intersex fish indicates that various EDCs have negative reproductive effects (Jobling et al., 2002; Denslow and Sepúlveda, 2007). OCs, including the DDTs, chlordane, natural and synthetic hormones, herbicides, fungicides, industrial chemicals, and pharmaceuticals may act as endocrine disruptors in fish as well as other organisms (Hinck et al., 2009). In SA, DDT was introduced for malaria vector control in 1945 and is still being used annually in parts of the country, including the Limpopo Province (Mabaso et al., 2004). In 1974, SA banned DDT for agricultural use, but farmers could use their stockpiles until 1976 (van Dyk et al., 1982). DDT used for indoor residual spraying (IRS) (World Health Organization (WHO), 2002)) can through various routes contaminate the environment. Once in the environment, DDT can persist for as long as 15 years, or is broken down into its main metabolites dichlorodiphenylchloroethane (DDE) and dichlorodiphenyldichloroethane (DDD) (Agency for Toxic Substances and Disease Registry (ATSDR), 2002). DDT binds to particles in surface water that precipitate to the sediment from where it is ingested by fish and other aquatic organisms. DDT is lipophylic and bioaccumulate in fatty tissue. Bottom feeder fish are particularly vulnerable in this regard. Fatty tissue therefore provides an ideal matrix for chemical analyses as it reflects the overall exposure of the individual to DDT over time.

According to Hunter and Donaldson (1983), fish embryos exposed to estrogens or estrogen mimicking chemicals during development may induce intersex individuals. Although the use of water and sediment samples was inadequate to identify the presence of DDT and metabolites, the mesenteric fatty samples tissue indicated that the aquatic environment was indeed contaminated by DDT. In the United States of America, intersex in a variety of wild fish species is commonly associated with exposure to DDT and other insecticides such as chlordane, toxaphene, aldrin, and dieldrin but especially dieldrin and DDT that mimic the hormone, estrogen (Denslow and Sepúlveda, 2007) even though most of these chemicals have been banned for years. Estrogenic activity measured in the aquatic environment of all three sampling sites was within the range 0–10 ng/L estradiol equivalents (EEs) (Bornman et al., 2009). Also Matthiessen et al. (2006) concluded that long-term EEs > 1 ng/l, if bioavailable, are likely to cause ovotestis and other estrogen-induced intersexual abnormalities (e.g. vitellogenin induction) in fish. Therefore, the presence of intersex in fish should be regarded as an indicator of EDC exposure until proven otherwise. It should be emphasized that EDC exposure not only leads to intersex in fish species, but are responsible for a number of reproductive, developmental (Fujimoto et al., 2004), behavioral (Allen et al., 1999), and immunological anomalies (Allen et al., 1999; Fujimoto et al., 2004), as well as cancers (Cook et al., 1997) found in both wildlife and humans (Baker, 2001; Spanò et al., 2004).

It also seems likely that the high number of intersex fish at the different sites in the Luvuvhu River may imply possible adverse effects in both terrestrial animals and humans exposed to the same pollutants. From the Great Lakes (North America) the

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reproductive effects of DDT in wildlife indicated that metabolite *p,p′*-DDE inhibited the reproductive success of the Bald Eagles (*Haliaeetus leucocephalus*) by thinning of the egg shells (Bowerman et al., 1998). *p,p′*-DDE induced eggshell thinning caused crushed eggs and breeding failure of fish eating birds (Fry, 1995). Therefore, fish eating birds and fish eagles in the study area may be similarly affected. Furthermore, local people from the surrounding area consume the fish from the Luvuvhu River, and use the water for household purposes such as washing clothes and bathing themselves. Although exposure to DDT through contaminated drinking water is not considered problematic due to the low solubility in water, DDT contamination of food may pose a risk to human health. Further research on the edibility of fish from these sites is necessary.

5. Conclusions

Following the report by Barnhoorn et al. (2004) on the first histological evidence of intersex in a freshwater fish in South Africa, similar gonadal abnormalities have now been identified in a second indigenous freshwater fish species, *O. mossambicus*. The fish was collected within the Luvuvhu River catchment that runs through a currently DDT-sprayed area. Within the entire sample group, 49% of testes contained testicular oocytes. Chemical analysis of fat samples showed bioaccumulation of DDT and its metabolites, indicative of historical DDT contamination of the aquatic environment. DDT and its metabolites are known EDCs and have most likely contributed to the gonadal abnormalities identified. However, further investigation is necessary to confirm the exact causative agents responsible for intersex in the gonochoristic species, *O. mossambicus* from the Luvuvhu River. Furthermore, fat tissue maybe an alternative to water or sediment sampling when assessing exposure over time.

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