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

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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.

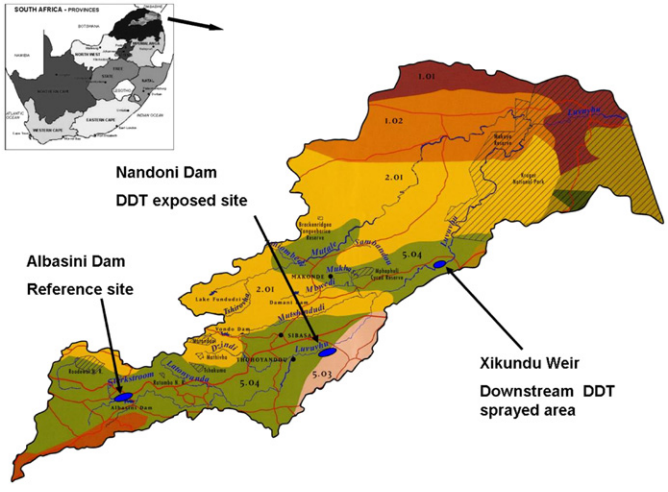

Fig. 1. A map indicating the three sites where sampling was done in the Luvuvhu River catchment (Map adapted from State of the Rivers Report, WRC report no.: TT165/01).

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.