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Biomarkers in tigerfish (*Hydrocynus vittatus*) as indicators of metal and organic pollution in ecologically sensitive subtropical rivers

R. Gerber[a,*], N.J. Smit[a], Johan H.J. van Vuren[a], Y. Ikenaka[a,b], Victor Wepener[a]

[a] Water Research Group, Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa
[b] Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan

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ABSTRACT

Studies have shown high levels of contamination of both metals and organochlorine pesticides (OCPs) in aquatic systems of the world renowned Kruger National Park, South Africa. With effects evident in top predators, including, unexplained *Crocodylus niloticus* deaths and organ level and histological changes in *Hydrocynus vittatus*. A suite of biomarkers reflecting exposure and were selected to evaluate biological responses of *H. vittatus* to anthropogenic stressors as well as to evaluate whether the chosen suite of biomarkers could successfully distinguish between the different pollution profiles present in the selected rivers. During this study a clear relationship was found between exposure to environmental contaminants and the concomitant responses of *H. vittatus* to these stressors. The ensuing biomarker responses indicated that there is a physiological attempt to deal with, and mitigate the deleterious effects that metals and OCPs may induce. In the Luvuvhu River there is a clear indication in *H. vittatus* of the stimulation of anti-oxidant protective mechanisms in response to internal OCP exposure. This is reflected by the increasing cytochrome P-450, superoxide dismutase, and more specifically reduced glutathione, which resulted in decreased lipid and protein breakdown (reflected in decreased lipid peroxidation and protein carbonyl levels). Consequently *H. vittatus* populations of the Luvuvhu River are under greater cumulative stress and this is reflected in the lower energy budgets. Our results further show the integrated application value of the current suite of biomarkers in assessing responses of subtropical fish to metal and OCP exposure as the entire suite of biomarkers when used in conjunction were able to explain 100% of the variation in the data.

1. Introduction

Globally aquatic ecosystems are continuously being contaminated with toxic stressors from a wide range of human activities, including agricultural, domestic and industrial sources (Matos et al., 2007). The acute and chronic exposures of aquatic organisms to these anthropogenic pollutants may result in adverse effects caused by increased tissue burdens of these pollutants (Amin and Hashem, 2012). Aquatic organisms may be affected at all levels of biological organisation by these contaminants (Wepener, 2008; Gerber et al., 2017). In most situations, stressors, such as pollutants indirectly affect higher levels of the ecosystem hierarchy (populations/communities), but directly affect the molecular and cellular (sub-organism) level processes (Downs et al., 2001). This is evident in the aquatic ecosystems of South Africa's largest and most notable, ecologically sensitive conservation area, the Kruger National Park (KNP). For example, the still unexplained deaths of hundreds of Nile crocodiles (*Crocodylus niloticus*) from the Olifants River system within the borders of the KNP (Huchzermeyer et al., 2011; du Preez and Wepener, 2016), as well as organ level changes and histological alterations to important organs of predatory tigerfish, *Hydrocynus vittatus*, populations from the Olifants and Luvuvhu Rivers within the KNP (Gerber et al., 2017). Recent studies in the KNP also demonstrated bioaccumulation of high levels of metals and organochlorine pesticides (OCPs) in *H. vittatus* (Gerber et al., 2016a, 2016b). The sources of these xenobiotics and subsequent exposure of aquatic organisms within the borders of the conservation area originate from the multiple activities outside of the Park (Gerber et al., 2015a, 2015b).

However, very often these bioaccumulation studies do not link pollutant exposure to biological effects in the test organisms and therefore the ecological significance of the levels are not known (Wepener et al., 2011). The sub cellular effects of metals and OCPs have not been assessed in any aquatic species from these ecologically sensitive aquatic ecosystems. Any biological response to a pollutant that can be measured at the sub-individual level, that indicates a deviation from the normal status which cannot be detected in the intact organism, is known as a biomarker (van der Oost et al., 2003). Thus, biomarkers

* Corresponding author.

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also examine whether normal detoxification or repair capacities have been exceeded (Martin and Black 1998). Biomarkers have successfully been applied to assess biological responses of various fish species to pollutant exposure e.g. *Astyanax aeneus* (Trujillo-Jiménez et al., 2011), *Labeo capensis* (Wepener et al., 2011), *Astyanax altiparanae* (Vieira et al., 2014), *Cyprinus carpio* (Schoenaers et al., 2016) and *Clarias gariepinus* (du Preez and Wepener, 2016). The rationale behind the measurement of biomarker responses is to demonstrate that an organism has been exposed to pollutants (i.e. that these stressors have entered the organism and have been distributed within the tissue) and are eliciting a toxicological effect on biological structures and functions (Walker, 1998). The biochemical parameters found in fish serve as indicators of damage due to pollutants (Matos et al., 2007). A suite of biomarkers reflecting biotransformation (Cytochrome P450 – CYP450), oxidative stress (Catalase – CAT; Superoxide dismutase – SOD; Reduced glutathione – GSH), susceptibility (Acetylcholine esterase – AChE), Metallothionein – MT and energetics (Cellular Energy Allocation – CEA) were selected to evaluate biological responses of a top aquatic predator to contaminants in the aquatic environment.

The selected study organism i.e. *H. vittatus* more commonly known as tigerfish are the top piscine predators in southern African aquatic ecosystems and therefore are at the greatest risk to the adverse effects caused by pollutants found in aquatic systems. It was shown that *H. vittatus* populations from the Olifants and Luvuvhu River systems within the KNP are exposed to- and bioaccumulate a wide range of anthropogenic stressors, specifically metals and organochlorine pesticides (OCPs) (Gerber et al., 2016a, 2016b). The usefulness of fish as bioindicators and specifically what makes *H. vittatus* an ideal bioindicator is well outlined and discussed in Gerber et al. (2017), and includes several key aspects. Where *H. vittatus* occupy a prominent position in the food web, are long lived (20 years Gerber et al., 2009) and although considered a migratory species, their range is limited to the study area through dams and weirs which act as migratory barriers, thus reflecting the status quo of the aquatic systems associated with the study area.

This paper tests the hypothesis that biological responses, using a suite of biomarkers, will reflect the anthropogenic stressors that *H. vittatus* are exposed to in two subtropical river systems, i.e. Olifants and Luvuvhu Rivers. The aim of this study was to use a suite of biomarkers to determine the biological response of *H. vittatus* populations to the various anthropogenic stressors they are exposed to in the Olifants and Luvuvhu Rivers. The purpose of this paper is not only to compare and expand on spatial and temporal differences in biomarker responses, but also to apply uni- and multivariate statistical procedures to determine whether a relationship could be detected between pollutant exposure (bioaccumulation) and the concomitant responses of *H. vittatus* to these stressors. Thereby providing important information regarding the use of biomarkers in native non transplanted fish species, as a tool to identify key polluted areas within ecologically sensitive subtropical systems. The research further contributes to the limited knowledge base on linking environmental exposure to biological effects in ecologically sensitive subtropical systems.

2. Materials and methods

2.1. Study area and sampling

Prior to data sampling, ethical clearance for fish collection and processing were obtained from the University of Johannesburg, Faculty Ethics Committee. Sampling of *H. vittatus* by rod and line angling techniques took place during the period September 2009 to June 2011 at sites along the Olifants (OLI) (S23° 59' 25.2" E31° 49' 33.3") and Luvuvhu (LUV) (S22° 27' 04.3" E31° 04' 47.7") rivers within the KNP (Fig. 1). *Hydrocynus vittatus* were sampled during two surveys in each the OLI and LUV rivers, the OLI sampling incorporated a single high flow (HF; Austral winter – June 2011) and a low flow (LF; Austral spring – November 2009) survey whilst two LF surveys were conducted to the LUV (Austral spring – October 2009 and 2010). Captured fish were transported to a nearby field laboratory for processing, effort was made to keep the time between capture and processing to under one hour. Body mass (g) and total length (mm) of each fish were recorded and subsequently killed by severing the spinal cord anterior to the dorsal fin. Axial muscle and the liver were then dissected out and approximately 1 g of each were placed in cryotubes, mixed with Hendrickson stabilising buffer (Wepener et al., 2011) and frozen in liquid nitrogen for biomarker analyses. All dissection tools were rinsed with 99.8% ethanol between dissections. The flash frozen samples were transported back to the laboratory and kept at − 80 °C in a freezer (Snijders Scientific, Model No. UF440-86E) until further analyses. Axial muscle tissue samples were also removed for metal and organochlorine pesticide determination as described in Gerber et al. (2016a) and Gerber et al. (2016b), respectively.

2.2. Biomarker analyses

The activities of the different biomarkers of exposure and effect were measured in the hepatic cytosolic fraction. The protein content was determined separately for each of the different biomarkers using the Bradford (1976) method and involves the binding of Coomassie Brilliant Blue G250 to protein, where the absorbance was measured at 630 nm and bovine serum albumin (BSA) used as a standard. Protein content was determined to express biomarker concentrations in terms of its activity per milligram protein. Cellular energy allocation (CEA) was determined in the cytosolic fraction of the muscle tissue. The biomarkers selected for this study were acetylcholinesterase (AChE – pesticide and metal exposure), metallothionein (MT - metal exposure), cytochrome P450 (CYP450 - organic compounds e.g. OCPs), catalase (CAT – oxidative stress), superoxide-dismutase (SOD – oxidative stress), lipid peroxidation (LP – lipid breakdown) and protein carbonyls (PC – protein breakdown), as well as the non-enzymatic reduced glutathione (GSH – oxidative stress). The cellular energy allocation biomarker is an indication of cellular energy utilization during stress conditions. Refer to Table 1 for the diagnostic nature of the biomarker responses and their interpretation.

2.3. Tissue preparation for biochemical analyses

For each fish, approximately 0.2 g liver samples were placed in each of the Eppendorf tubes, labelled A, B and C, and 0.2 g of muscle tissue samples were placed in Eppendorf tubes labelled D and E. All samples were homogenised on ice and centrifuged using a Madell Technology Corporation centrifuge (Model No. TGL-16M). Sample A was homogenised in 1000 µL of Tris-sucrose Buffer (Tris – 0.05 M; Sucrose – 0.25 M), centrifuged at 5590*g* for 10 min at 4 °C and aliquots of the supernatant taken for AChE and LP activity analysis. Sample B was homogenised in 2000 µL General Homogenising Buffer (GHB – 0.1 M), centrifuged at 5590*g* for 10 min at 4 °C and aliquots of the supernatant taken for CAT, SOD, GSH, PC and Cyp-450 analysis. Sample C was homogenised in 1000 µL Homogenising Buffer (consisting of normal Tris buffer – 0.02 M, with the addition of 0.006 mM Leupeptine, 0.0005 M Phenylmethylsulphynolflouride and 0.01% b-Mercaptoethanol), centrifuged at 30000*g* and the supernatant used solely for MT analysis. Samples D and E were homogenised in 1000 µL deinonised water and 1000 µL electron transport system (ETS) Homogenising Buffer (consisting of 0.1 M Tris-HCL buffer (pH 8.5) with the addition of 0.2% Triton X-100, 15% Polyvinyl pyrolidone and 0.153 mM $MgSO_4$) respectively, centrifuged at 3000*g* for 10 min and the supernatant used for the respective analyses. Aliquots were taken from Sample D for carbohydrate, protein and lipid determination, whilst the supernatant from Sample E was used for the determination of electron transport activity.