ORIGINAL RESEARCH ARTICLE

Basal Expression Profile of Seven Glutathione-S-Transferases (GSTs) Genes in Six Tissues of Tilapia (*Oreochromis niloticus*) for Polymerase Chain Reaction Array

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ABSTRACT

Because aquatic habitats are being destroyed and biodiversity is declining, xenobiotic pollution of water is a critical environmental problem that has garnered a lot of attention in the past few decades. This study looked at the expression patterns of *Glutathione-S-transferases* isoforms *(GSTMA, GSTO1LA, GSTA, GSTR1, GSTK, GSTT1, and MGST)*, which are genes involved in xenobiotic metabolism, in Nile tilapia. From adult Nile tilapia, six tissues—liver, spleen, intestines, gills, heart, and muscle—were chosen, and seven genes that were optimised via quantitative polymerase chain reaction (qPCR) were used for validation. Gene expressions were assessed using a PCR array that included duplicate tissues in 96-well qPCR plates, with each gene/plate being assayed six times. Water is used in the remaining wells as no template control (NTC). As reference genes, Pan Ribosomal Protein L3 (RPL3) and Pan 18S ribosomal RNA (18S RNA) were employed. The genes' basal tissue mRNA expression was measured in each tissue. The muscle was utilised to renormalise the expression levels of all genes throughout the remaining tissues since, under assay's circumstances the muscle showed very low signal for most of the genes. Using quantitative real-time PCR (qPCR), the result demonstrated widespread differential expression patterns of the seven GST mRNA in the liver, spleen, intestine, gills, heart, and muscle of an untreated fish. However, it was clear that the highest significant expression level of most genes (different GST's isoforms) was in the liver, followed by the spleen and gut, then the heart and gills compared to muscle (P<0.05). The findings demonstrated widespread differential expression of the GST's isoforms at basal level and highlighted their utility as biomarkers for xenobiotic stress in aquatic environments.

INTRODUCTION

Different organic and trace pollutants, persistent polyaromatic and polyhalogenated hydrocarbons, such as PAHs, PCBs, Dioxins, heavy metals, pesticides like organochlorine and organophosphate, and other emerging pollutants of xenobiotic metabolism are continuously contaminating aquatic ecosystems (freshwater or marine), potentially destroying the physiological functions of individual organisms and the integrity of the biological ecosystem (Van der Oost et al., 2020; Gwenzi et al., 2018).

The aquatic ecosystem's environmental quality cannot be justified solely by quantifying the external levels of a chosen pollutant or combination of pollutants owing to exposure to aquatic species (Environmental monitoring). Because of this, determining the internal dose of the pollutants in the organism is crucial since it determines how the contaminants will behave in terms of biotransformation, accumulation, and bioavailability

would be elucidated in the aquatic environment. However, it is impossible to monitor all pollutants that pose a hazard to the aquatic environment, whether they are man-made (halogenated hydrocarbons, for example) or natural (polycyclic aromatic hydrocarbons, heavy metals, etc.) (Everaart et al., 1994; Bussolaro, 2019).

To monitor the effects of xenobiotic metabolism with other persistent chemicals in fish, a variety of strategies have been implemented. These strategies involve changing the chemical configuration of contaminants that are foreign to the organism's normal bodily processes, such as alterations in the activity of various mRNA transcripts or enzyme levels that are involved in cellular defence against the insult of a xenobiotic chemical. These mRNA/enzyme changes could occur in two different ways: either by altering the phenotypic activities of the exposed organism in comparison to the non-exposed

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organism (activation or inhibition) or by influencing the quantity of the mRNA/enzymes through induction or suppression of protein synthesis (Karaca et al., 2014). Glutathione S-transferases (GSTs) are dimers with many functions.

All organisms include GSTs, which are members of a multigene family of isoenzymes with a wide range of structural variations that allow them to conjugate a wide variety of chemicals (Franco et al., 2020). Lipid peroxidation can be caused by environmental contaminants such as the heavy metal Cd, and the compounds that arise are substrates for some GSTs.

Polyaromatic hydrocarbons (PAH), pesticides, and reactive intermediates generated by phase I biotransformation and other biochemical reactions are among the environmental toxicants detoxified by glutathione S-transferase isoenzymes (GSTs). For this reason, GST expression is significant when assessing susceptibility to toxicity by environmental chemicals and their expression can be used as an early warning biomarker for xenobiotic contamination in aquatic environments (Dasari et al., 2019). Ecotoxicological research has employed GSTs as a biomarker in multiple instances. By catalysing the conjugation of reduced glutathione (GSH) and/or reducing peroxides with electrophilic compounds and by binding toxin to make it more hydrophilic, which aids in excretion in the detoxification process.

Glutathione S-transferases (GSTs) are responsible for maintaining redox homeostasis during the phase II detoxification phase. Fish and other species have shown the function of glutathione S-transferases (GST) as molecular indicators of chemical pollution and related toxic symptoms. Numerous environmental pollutants, reactive intermediates, and subsequent products of oxidative damage are all detoxified by it. Currently, several new groups of GST sequences from marine non-mammalian creatures have been recognised and categorised (Masoud et al., 2023).

Furthermore, fish exhibit variability in their responses to GST expression, necessitating meticulous validation investigations among different species. Three primary families of GST have been found in mammals, primarily because of differences in immunological characteristics, kinetics, isoelectric points, and subunit architectures. The mitochondrial GSTs (Kappa class), microsomal GST, and the cytosolic GSTs (containing seven classes: *Alpha, Mu, Pi, Theta, Sigma, Omega, and Zeta*).

Nevertheless, the characterisation of GSTs in Nile tilapia has been reported seldom, and not much is known about their basal expression profiles (Paul et al., 2019). Therefore, the purpose of this study was to compare gene expression patterns of different target tissues using a quantitative polymerase chain reaction (qPCR) and to identify a tissue optimally expressing different target genes of interest in Nile tilapia for aquatic environmental monitoring to be used in wild tilapia sampling and to ascertain which tissue respond best to GST gene and how these genes are responsive to various tissues at the basal level.

MATERIALS AND METHODS

Fish Harvest and Studied Tissue Processing

Five young Nile tilapias were taken out of the tropical aquarium at the University of Stirling. The fish were put to death by adding 3 millilitres of benzocaine to a litre of water. The fish were measured for total length, weight, and sex before the tissue was removed through dissection. Every fish had six tissue samples taken from it. Samples of tissue were obtained from the colon, liver, spleen, gills, heart, and white muscle. The samples were taken to the laboratory freezer for storage (-20 °C) for additional analysis after being collected in microtubes containing 1 ml RNA for preservation in accordance with the manufacturer's guidelines (Sigma). Every protocol that was followed complied with UK regulations and the AWERB of the University of Stirling.

RNA Extraction and CDNA Synthesis

Following the manufacturer's instructions, tissues (< 100 mg) from the liver, spleen, gills, muscle, heart, and intestine (six samples per tissue) were chopped into around 50 mm² pieces and placed in 2 ml screw cap microtubes (Alpha Labs) holding 1 ml of TriReagent (SIGMA, UK), extraction buffer. Using a Precision Nanoscript 1 reverse transcription kit (PRIMER DESIGN) and following the manufacturer's instructions, 1µg of RNA was reverse transcribed to yield cDNA.

RNA Purification and Quality Assessment

RNA integrity and quality using NanoDrop, and gel electrophoresis viewed using Syngene transilluminator was carried out. RNA concentration and purity were determined by measuring the natural absorbance of light at 260 nm (DNA and RNA) using a NanoDrop ND-1000 spectrophotometer (LABTECH INTERNATIONAL LTD, UK). Usually, a concentration of 70 ng/ μl and above with an absorbance ratio of 260 nm to 280 nm (1.67- 2.00 purity) of RNA is required. The RNA was then standardised to a uniform concentration of 300 ng/μl. RNA quality was evaluated using 1% agarose gel electrophoresis (in Tris-acetate-EDTA-TAE-buffer) stained with ethidium bromide (EtBr) and visualised using Syngene UV illuminator bioimaging (for possible degradation) to check for the banding intensity of 28S: 18S rRNA by electrophoretic separation and visualisation. Prior to the loading, 2 μl of RNA was taken, and 2 μl of 2x gel loading dye was added into a PCR tube and was heated to 75 °C for 2 minutes in a thermocycler for

denaturation, 2 μl to 4 μl (about 500ng) incubated RNA were loaded into the wells of the 12 minigel, and it was run at 70 voltage for 45-60 minutes.

Tissue Distribution Assay

Using TOptical PCR equipment (BIOMETRA, GREMANY), real-time PCR was carried out. Each 10μl reaction contained a mastermix reaction that included 2μl of the diluted cDNA samples, 5μl of SYBR Green Luminaris supermix (THERMOSCIENTIFIC), 2.6μl of MiliQ water, and 0.2μl of 10μM forward and reverse primers (EUROFINS GENOMICS, GERMANY). The purpose of this work was to examine the tissue distribution patterns of the GSTs genes in the gut, heart, spleen, liver, and white muscle, which are the six organs of O. niloticus harvested. To activate the DNA polymerase in the mixture, the following cycle conditions were used: first heating at 50 °C for 2 minutes, then initial incubation at 95 °C for 10 minutes. After that, denaturation was carried out for 10 seconds at 95 °C, 10 seconds at 60 °C, and 15 seconds at 72 °C. The assay was run in triplicate in a 96-well plate to ensure biological reliability. Control lacking cDNA templates was included to determine the specificity of the target cDNA amplification as a no-template control (NTC).

Statistical Analysis

All the analysis of the results was calculated using the delta-delta Ct method (Pfaffl model equation, 2001) in Excel spreadsheets and IBM SPSS version 25 (IBM, Armonk, NY, USA). *18S ribosomal RNA* and *Ribosomal Protein L3* housekeeping genes were used to normalise the expressions of the target genes. The geometric means of two housekeeping genes were used to calculate the differences between the target gene ct values of each sample, and later, the (ΔCt) differences (ΔΔCt) between the two samples were calculated (target and reference). Subsequently, the expression of each gene was normalised for each tissue against muscle individually, making all the results relative to muscle expression = 1, using the Pfaffl equation $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). The basis for the normalisation of muscle tissue is that because muscle had the lowest level of expression of the genes tested, this tissue could be used to normalise other tissue expression values to scale expression similarly across all genes. All quantitative values were presented as the mean ± standard deviation of the normalised expression values relative to muscle expression. The resulting data passed normalisation tests after log transformation, and therefore, the parametric one-way ANOVA and Turkey's post hoc tests were used to compare values of gene expression between tissues ($p < 0.05$).

Table 1 Transcript, oligo name, sequences and melting temperature used in the Real-time Quantitative -PCR.

Transcript	Oligo Name	Sequences	T_m (°C)
Glutathione S-transferase Mu (a)	QtnGSTMAf	CTGTGGGGAAGCTCCAAACT (20)	59.4
	QtnGSTMAr	TGTAGCACAGCCTCACGAAC (20)	59.4
Glutathione S-transferase omega 1LA	QtnGSTO1LAf	TGTGGCCATGGTTTGAGAGG (20)	59.4
	QtnGSTO1Lar	AAAGGGACGGTTGAGGGTTTT (21)	57.9
Glutathione S-transferase Rho 1	QtnGSTR1f	CTCTTGTCGTAGTCGGGAGC (20)	61.4
	QtnGSTR1r	GTGTCCAGACTGTACGAGGTAGC (23)	59.4
Glutathione S-transferase Alpha	QtnGSTAf	ACTGCACACTCATGGGAACA (20)	57.3
	QtnGSTAr	TCCCGAGTTGTCAGAAGCAC (20)	59.4
Glutathione S-transferase Kappa	QtnGSTKf	CACACGCTGCGTTAGGTTTT (20)	57.3
	QtnGSTKr	CAAACCAGGAGGCTTGTTGC (20)	59.4
Glutathione S-transferase Theta 1	QtnGSTT1f	GGAGAGTGAAGCCCGTTTGA (20)	59.4
	QtnGSTT1r	GTAGAGCTCCAGCGCCATAG (20)	61.4
Microsomal Glutathione-S transferase	QtnMGSTf	ACTGGGTGACAGGTGAGATTC (21)	59.8
	QtnMGSTr	TGCTGAAAGCCCTCACTACC (20)	59.4
Pan 18s ribosomal RNA	18sf	ACCACATCCAAGGAAGGCAG (20)	59.9
	18sr	CACCAGACTTGCCCTCCAAT (20)	59.9
Pan Ribosomal protein L3	Rpl3f	GGCAAGAAGCAGCTGGAGAA (20)	60.6
	Rpl3r	TTACGCAGACCACGATGGGT (20)	61.54

RESULTS

Tissue distribution of GST transcripts

Distribution of GST transcripts on tissue of five separate male and female tilapia were used to determine the basal mRNA expression of each gene in six distinct organs. Every individual had quite different levels of gene expression, and both genes' levels of relevance varied widely among all tissues. But when compared to muscle, it was evident that the liver had the highest significant expression level of most genes, followed by the gut and spleen, then the heart and gills (Figure 1). The liver showed significant expression of the mRNAs for *GSTMA, GSTO1LA, GSTA, GSTK, GSTT1,* and *MGST*. Except for the spleen, which has a significant *GSTMA* and *GSTR1* expression, and the intestine with a significant expression of *GSTR1* and *MGST*. The remaining tissues had remained unchanged with the genes except *GSTMA* in the heart and *GSTR1* in the gills. There was a variable significant difference in the level of expression across different tissues in different genes (Table 3). An ANOVA

indicated significant differences in most of the GST's isoform expressions between the liver and other tissues except in *MGST,* where intestine-heart was significant ($p<0.05$). This confirms the role of liver as the primary site in the detoxification process.

Table 2. Relative mRNA expression of each gene/tissue and their significance difference compared to muscle using Turkey's Post Hoc test.

Genes/ Tissues	Fold Values	Turkey's post-Hoc Test P ≤ 0.05
GSTA		
Gills	10.3	1.000
Heart	20.5	0.690
Intestine	1.94	0.730
Liver	14.9	**0.040**
Spleen	16.6	1.000
GST01LA		
Gills	10.0	0.690
Heart	2.24	0.310
Intestine	19.9	0.151
Liver	49.9	**0.045**
Spleen	8.7	0.841
GSTMA		
Gills	12.0	0.151
Heart	5.22	**0.032**
Intestine	4.4	0.056
Liver	50.9	**0.008**
Spleen	24.4	**0.032**
GSTR1		
Gills	15.0	**0.008**
Heart	12.7	0.056
Intestine	22.7	**0.008**
Liver	17.8	0.690
Spleen	11.6	**0.008**
GSTK		
Gills	10.0	0.690
Heart	17.5	0.690
Intestine	2.7	0.175
Liver	14.9	**0.040**
Spleen	16.6	1.000
GSTT1		
Gills	10.0	0.151
Heart	52.6	0.016
Intestine	22.6	0.056
Liver	44.2	**0.008**
Spleen	36.1	0.222
MGST		
Gills	16.4	0.048
Heart	20.5	0.690
Intestine	8.6	**0.008**
Liver	94.4	**0.040**
Spleen	16.6	1.000

Table 3. Comparative expression levels among other tissue in different genes using one-way ANOVA and Turkey's Post Hoc test.

| Genes/Tissues	Turkey's Post HocTest P ≤ 0.05
GSTA	
Gills-Spleen	1.000
Gill-Intestine	1.000
Gill-Heart	1.000
Gill-Liver	0.284
Spleen-Intestine	1.000
Spleen-Heart	1.000
Spleen-Liver	0.077
Intestine-Heart	1.000
Intestine-Liver	0.088
Heart-Liver	**0.011**
GST01LA	
No significant difference in general data	0.061
GSTMA	
Gills-Spleen	1.000
Gill-Intestine	1.000
Gill-Heart	1.000
Gill-Liver	0.254
Spleen-Intestine	1.000
Spleen-Heart	1.000
Spleen-Liver	**0.007**
Intestine-Heart	1.000
Intestine-Liver	0.143
Genes/Tissues	**Turkey's Post HocTest Test P ≤ 0.05**
GSTR1	
No significant difference in general data	0.238
GSTK	
No significant difference in general data	0.271
GSTT1	
Gills-Spleen	1.000
Gill-Intestine	0.856
Gill-Heart	1.000
Gill-Liver	**0.023**
Spleen-Intestine	0.352
Spleen-Heart	1.000
Spleen-Liver	**0.006**
Intestine-Heart	1.000
Intestine-Liver	1.000
Heart-Liver	**0.035**
MGST	
Gills-Spleen	1.000
Gill-Intestine	1.000
Gill-Heart	0.937
Gill-Liver	1.000
Spleen-Intestine	0.352
Spleen-Heart	1.000
Spleen-Liver	0.481
Intestine-Heart	**0.008**
Intestine-Liver	1.000
Heart-Liver	0.127
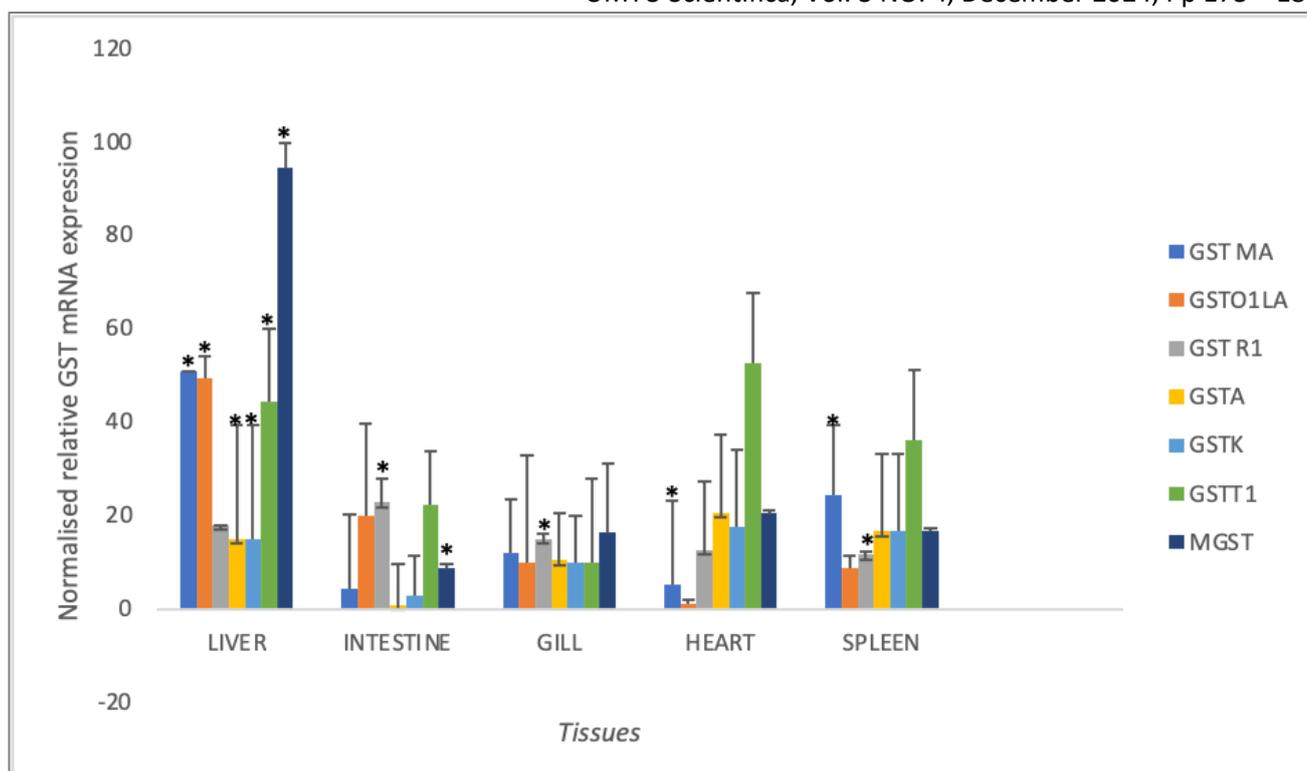
Figure 1: Basal expression of *Oreochromis niloticus GSTs* genes in different tissues (Liver, intestine, gills, heart, and spleen). The asterisk indicates a significance difference at p<0.05 level. Error bars represent the standard deviations of the mean across all the tissues for each gene.

DISCUSSION

GSTs perform two important functions to deal with xenobiotics. Firstly, they take part in detoxification and, secondly, provide an antioxidant defence to xenobiotically induced oxidative stress. Because of heavy pollution loading of freshwater and marine ecosystems and its likely impact on fish health, xenobiotics activation and detoxification and antioxidant systems of many fish species have been researched. Therefore, it is important to observe the expression of GSTs isoforms in a fish at a basal level so that it may provide valuable information on detoxification and defense of antioxidants in fish when exposed to pollutants. There are certain isoforms of GSTs in this study significantly expressed distinctly in numerous tissues. It has been shown that certain fish species have elevated expression of distinct *GST* isoforms in their livers and other tissues (Leaver et al., 1993; Yu et al., 2008; Kim et al., 2010; Ibor et al., 2019). It has been found that GSTs are highly expressed in the total soluble protein that makes up 4% of the liver in mammals. *GSTR1* was found to be significantly expressed in the intestine (23-fold) and the Gill (15-fold).

On the other hand, *MGST* was found to be significantly expressed in the intestine (9-fold), and *GSTMA* was found to be significantly upregulated in the heart (5.2-fold) and spleen (24.4-fold). There is no modular cavity in the bone of teleost fish; therefore, the spleen serves as a primary hemopoietic organ. The spleen also functions in a critical role of trapping, processing, and presentation of antigens

to lymph nodes due to melanomicrophages centre. It may not be surprising if it serves as a storage facility for pollutants and degrades any cellular products. When the melano-macrophages centre is exposed to pollutants, it intensifies detoxification activity immune responses and increases in size (Sun et al., 2019). Spleen, due to its immune-related activity in trapping and removing exogenous compounds as well as maintenance of homeostasis, its structure and functions could be regarded as important in assessing xenobiotic metabolism through gene expression analysis and important in biomonitoring studies (Sun et al., 2019). Lee et al. 2006 demonstrated that differences in tissue distribution of GST are associated with differential susceptibility to antioxidant damage or the presence of other detoxification enzymes, such as aldehyde metabolising enzymes. Landi (2000) also confirmed higher expression of the mammalian GST-Theta class in several tissues, including the intestine. When xenobiotics are absorbed and distributed into different organs, they are usually biotransformed, and the products are transported via the gall bladder to the intestine. Thus, the intestine might be a repository of products of xenobiotic breakdown. In this study, Nile Tilapia GSTA transcripts were moderately distributed throughout the tissues, showing the highest level of mRNA expression in the liver, followed by the intestine, due to its high and ubiquitous expression patterns in all tissues.

All the remaining genes were significantly expressed in the liver except *GSTR1*. This demonstrated that the liver's

function in xenobiotic metabolism could be principally linked to storage, detoxification, and excretion. This shows that variations in the tissue distribution of *GSTs* are related to oxidative damage or the presence of another detoxifying enzyme in Nile tilapia tissues that lack *Glutathione -S- transferase Alpha (GSTA)*. It has been established that mammals express *GSTR1* more highly in a variety of organs, including the intestine (Kim et al., 2010). Cellular epithelia in the intestine serve as a gatekeeper and function in regulating the interaction between an organism and its environment. They regulate food uptake as well as the provision of protection against environmental pollutants. When an intestine is exposed to environmental contaminants, this tissue suffers both morphological and physiological alterations. Such changes include the expression of genes related to such pollutants. Because of this, the intestine could be regarded as a tissue worthy of study in molecular ecotoxicology through qPCR gene expression. When foreign substances are absorbed and dispersed inside fish tissues, they undergo biotransformation, which then delivers the product to the gut via the gall bladder.

Thus, the intestine may store these xenobiotics' broken-down products. The gut may, therefore, be used as a biomarker for PCB and PAH based on the identification of several *GST* expression isoforms, particularly *GSTR1* at the basal level. Ibor et al. (2019) documented the expression of *GSTR1* in the sampled liver and intestine of *Tilapia guinensis* in Eleyele dam compared to the reference site in Nigeria. Ladner et al. (2004) also demonstrated high expression levels of GSTR1 in the Gill of fish. Although the gill is the most likely site of absorption of toxicants, there are few reports on GST expression in the gill of fish. The modest expression of various *GST* isoforms in this study's organs, particularly the liver, may be related to their roles as housekeeping genes, hepatoprotective factors against endogenous oxidative stress, and foreign contaminants at a basal level. Furthermore, Hayes et al. (2005) demonstrated that knockout of GSTT and GSTR leads to overexpression of transferases in the Alpha, Mu, and Pi classes, suggesting that GSTT and GSTR are part of an adaptive mechanism that responds to endogenous chemical cues. Interestingly, the gills of Nile Tilapia showed the second-highest level of GSTK expression.

The endogenous antioxidants, including GSTs, play a pivotal role in mitigating oxidative stress in all forms of life. Although in several fish species GSTA is the dominant class of GST highly expressed in most of the tissues. Other GSTs also have a role in the overall antioxidant detoxification system. Moreover, the upkeep of regular cellular processes may be linked to additional GSTs in the heart, spleen, and gills. Based on the considerable expression of several GST isoforms in the tissues, our study suggests that Nile tilapia are better suited for xenobiotic detoxification processes (Piazza et al., 2019). This is because *GST* catalyses the conversion of some xenobiotics to reduced GSH; hence, after xenobiotics induce GST-catalyzed conjugation, its expression is likely to be activated because of cytosolic GSH exhaustion (Xing et al., 2012). The seven *GSTs* isoforms' basal mRNA levels were thus shown to be tissue-specific, unevenly expressed in different tissues, and potentially changeable in abundance within a single tissue. The differential expression observed in Nile tilapia supports the use of GSTs as reliable biomarkers for xenobiotic contamination and may indicate an adaptive response of the Nile tilapia to maintain homeostasis during exposure to pollutants, particularly in developing regions with limited environmental monitoring frameworks, especially in Northern Nigeria where this approach is limited (Adeogun et al., 2019)

CONCLUSION

The study concludes that, depending on the basal expressions on various tissues in the laboratory exposure experiment/research, GSTs could be employed as a biomarker for various contaminants. Taken together, our results showed that Nile Tilapia mRNA at the basal level caused differential expressions and changes in the content of GST isoforms in different tissues. The various regulations of GST gene expression highlighted the fact that these isoforms may probably played divergent physiological roles in the detoxification and antioxidant functions of different toxins in Nile Tilapia. Coupled with the oxidative stress and peroxidation injury in Nile Tilapia induced by different pollutants and as was reported by previous literature, the changes in GSTs expression suggest that GSTs might play an important role in protecting cells from oxidative stress induced by different xenobiotics. However, further studies should be addressed to confirm these findings. Nevertheless, our results might form a basis for further in-depth analysis of GSTs in detoxification in Nile Tilapia in exposure assay or a polluted site.

RECOMMENDATION(s)

I. It is advised that the GSTs isoform be employed as a biomarker to provide crucial details on the detoxification of xenobiotics in fish, particularly Nile tilapia.

II. Additional investigation is necessary to verify these results.

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