The development of molecular biomarker to detect and monitor heavy metal pollution in fresh water: Identification of differentially expressed hepatic genes in response to copper exposure in swordtail fish (*Xiphophorus helleri*)

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Introduction

Copper is one of the essential element required by living organism where it acts as an essential cofactor for a large number of proteins. However, excessive copper is a hazardous toxic. Lethal and sublethal toxicities of copper in have been documented in multiple fish species (De Boeck et al 2003). Among identified source of aquatic copper contamination are mining effluents, copper smelting industries and corrosive pipes, foundries, municipal waste incinerators, burning of coal for power generation, and a variety of copper-based products used in building and construction, in electrical/electronic equipment and in other industries. In addition, contamination of waters, sediments and soils by copper can also arise from the widespread use of copper-based products in agriculture as fertilizers and fungicides. A survey on farmed and wild salmon from Atlantic and Pacific region revealed presence of copper in fish tissues (Foran et al 2004).

Xiphophorus hellleri is a common aquarium species belonging to the *Xiphophorus* genus originating from freshwater habitats of Mexico, Guatemala, Belize, and Honduras. The genus *Xiphophorus* possess the third largest gene maps of teleost fish due to large numbers of fertile interspecific crosses among the 20 species of belonging to this genus (Morizot et al 2001). Furthermore, *Xiphophorus* hybrid models have been utilised study the genetics underlying cancer development as hybrid *Xiphophorus* show an increased susceptibility to cancer development as a result of exposure radiation or exposure to various chemical agents (Walters et al. 2004). Xiphophorus fishes and hybrids have also been utilized in toxicology and contaminant studies.

Metallothionein gene is a well-utilized molecular biomarker to assess the impact of copper toxicity in fish (Schlenk et al 1997). In rainbow trout, Wilson and Taylor (1993) demonstrated that copper not only acts as an ionoregulatory toxicant but also cause secondary pathological disorders. Among disorders reported with copper toxicity in fish are impaired olfactory response, massive haemoconcentration, changes in mitochondrial activity cardiac failure, gill problems and DNA damage (Wilson & Taylor 1993, Baldwin et al 2003, Gabbianelli et al. 2003, Manzl et al. 2003, van Heerden et al. 2004). Therefore, identification of genes induced by copper exposure will provide a better understanding on the mechanisms of copper toxicity.

One of the first and major targets of metal toxicity in vertebrates is liver (De Smet et al., 2001). Liver was identified as the organ showing highest level of Cu accumulation after sub-lethal exposure in rainbow trout (McGeer et al. 2000). Both hepatic metallothionein and copper concentation were proposed as potential robust indicators of copper exposure in fish based (Dethloff et al 1999). However, these authors stressed that these assays may not reveal other deleterious physiological effects. Therefore knowledge of

changes in hepatic transcriptome profile due to copper exposure will provide us with better insights.

The differential display analysis is a useful method and relatively cheaper and faster method to identify induced genes between 2 different biological systems. More relevant to our work, this technique has been used in fish, bryophytes for purpose of identifying heavy-metal induced genes (Carginale et al 2002)

Here, we used the GeneFishing differential expressed technique (DEG) technique (Seegene) to identify copper-response genes in swordtail liver after exposure with sublethal doses of this metal.

Materials and methods

All swordtail fish used in this study were obtained from fish laboratory, University Sains Malaysia. The fish were treated either in the absence (control) or presence of 1μg/ml copper for 24h.

In order to determine the copper-inducable gene(s) in swordtail fish, a method known as GeneFishing DEG (Seegene) approach was used as described by the manufacturer. Briefly, total cellular RNA was isolated from liver of either copper-treated or untreated swordtail fish using Tri-Reagent LS (Molecular Research Center) as described by the manufacturer. Total cellular RNA was then treated with DNase-I to completely remove the DNA contaminants. Reverse transcription of the DNase-treated total cellular RNA from treated and untreated samples was carried out using dT-ACP1 primer (Table 1).

Table 1 The sequence of dT-ACP1 used in synthesising cDNA

Primer	Sequence (5' – 3')
dT-ACP1	CTGTGAATGCTGCGACTACGATXXXXX$(T)_{18}$
dT-ACP2	CTGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$

Subsequently, PCR was carried out using different combinations of dT-ACP2 (Table 2) and an arbitrary ACP primer (Table 2). PCR products were then size-fractionated on a 2% (w/v) agarose gel, stained and visualised under UV light.

Table 2 The sequence of primers used in PCR

Primer	Sequence (5' – 3')
dT-ACP2	CTGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$
ACP1	GTCTACCAGGCATTCGCTTCATXXXXXGCCATCGACC
ACP2	GTCTACCAGGCATTCGCTTCATXXXXXAGGCGATGCC
ACP3	GTCTACCAGGCATTCGCTTCATXXXXXCCGGAGGATG
ACP4	GTCTACCAGGCATTCGCTTCATXXXXXGCTGCTCGCG
ACP5	GTCTACCAGGCATTCGCTTCATXXXXXAGTGCGCTCG
ACP6	GTCTACCAGGCATTCGCTTCATXXXXXGGCCACATCG
ACP7	GTCTACCAGGCATTCGCTTCATXXXXXCTGCGGATCG
ACP8	GTCTACCAGGCATTCGCTTCATXXXXXGGTCACGGAG
ACP9	GTCTACCAGGCATTCGCTTCATXXXXXGATGCCGCTG
ACP10	GTCTACCAGGCATTCGCTTCATXXXXXTGGTCGTGCC
ACP11	GTCTACCAGGCATTCGCTTCATXXXXXCTGCAGGACC
ACP12	GTCTACCAGGCATTCGCTTCATXXXXXACCGTGGACG
ACP13	GTCTACCAGGCATTCGCTTCATXXXXXGCTTCACCGC
ACP14	GTCTACCAGGCATTCGCTTCATXXXXXGCAAGTCGGC
ACP15	GTCTACCAGGCATTCGCTTCATXXXXXCCACCGTGTG
ACP16	GTCTACCAGGCATTCGCTTCATXXXXXGTCGACGGTG
ACP17	GTCTACCAGGCATTCGCTTCATXXXXXCAAGCCCACG
ACP18	GTCTACCAGGCATTCGCTTCATXXXXXCGGAGCATCC
ACP19	GTCTACCAGGCATTCGCTTCATXXXXXCTCTGCGAGC
ACP20	GTCTACCAGGCATTCGCTTCATXXXXXGACGTTGGCG

The candidates of differentially expressed gene fragments were excised from the gel, cloned into pGEM-T plasmid, transformed into *E. coli* JM109, propagated, purified and sequenced. Nucleic acid sequences were then compared to the published sequences in the GenBank/EMBL database using the BLAST command (www.ncbi.nlm.nih.gov/blast).

In order to determine the specificity of the copper-inducable candidate genes identified from GeneFishing DEG method, RT-PCR was carried out. Briefly, total cellular RNA was isolated from liver of swordtail fish either untreated or treated with varying concentrations of copper for presequisite period. For dose response experiment, swordtail fish were treated with 6 different concentrations of copper (0μg/ml, 0.05μg/ml, 0.125μg/ml, 0.25μg/ml, 0.5μg/ml and 1.0μg/ml) for 24h. For time course experiment, swordtail fish were treated with 1.0μg/ml copper for 0h, 8h, 12h, 16h, 20h and 24h. Subsequently, RT-PCR was carried out using the specific internal primers designed against individual candidate genes. The amplified products were then size-fractionated on 1% (w/v) agarose gel, stained and viewed under UV light.

Results

The PCR products from various combinations of primers generated various fragments as observed on 2% (w/v) agarose gel. Four differentially expressed bands with the size of 800 bp, 380 bp, 500 bp and 350 bp were observed when the PCR reaction was carried on using the combination of dT-ACP2 with ACP8, ACP16, ACP28 and ACP33, respectively (Figure 1).

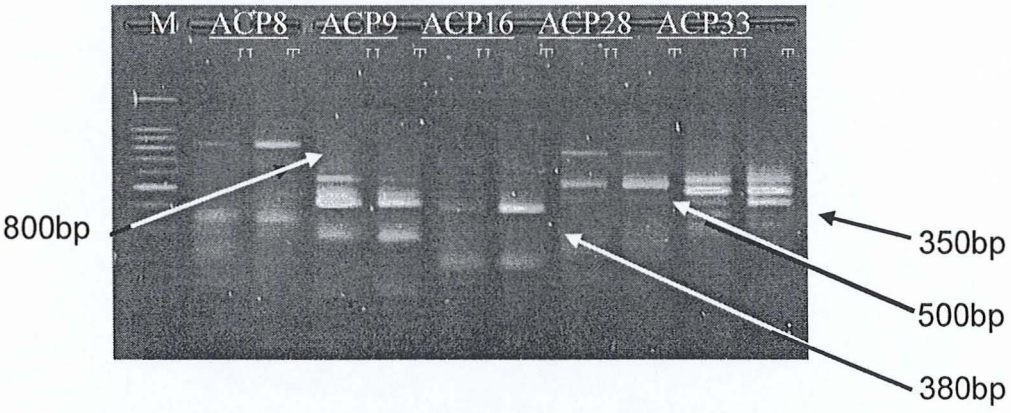


Figure 1 The PCR products of copper-inducable candidate genes using the combination of dT-ACP primer with ACP8, ACP9, ACP16, ACP28 and ACP33. M,100bp DNA ladder; U, untreated sample, T, copper-treated sample

Sequening analysis revealed that 4 copper-inducable candidate gene fragments shared to a certain degree % of identity with the pubished genes in the database i.e. (i) wap65 (warm-temperature-acclimation-related-65kDa-protein-like-protein) gene (also known as hemopexin gene), (ii) mitochondria solute carrier family gene, (iii) heat shock protein70 (HSP70) gene, and (iv) ribosomal protein L19 gene.

In order to confirm the specificity of the copper-inducable candidate gene fragments, dose response and time course experiment were carried out as described in Materials and Methods. As shown in Figure 2, the level of mRNA expression for 3 candidate genes increased gradually when the fish were treated for 24h in increasing concentrations of copper (Figure 2-4). The similar pattern of induction in mRNA expression was also observed in those 3

candidate genes when the treatment period was increased from 0h to 24h in fish treated with 1.0μg/ml copper (Figure 2-4). The results clearly indicate that wap65 (warm-temperature-acclimation-related-65kDa-protein-like-protein) gene (also known as hemopexin gene), mitochondria solute carrier family gene and ribosomal protein L19 gene represent specific copper-inducable genes in swordtail fiah whereas heat shock protein70 (HSP70) gene was a false positive.

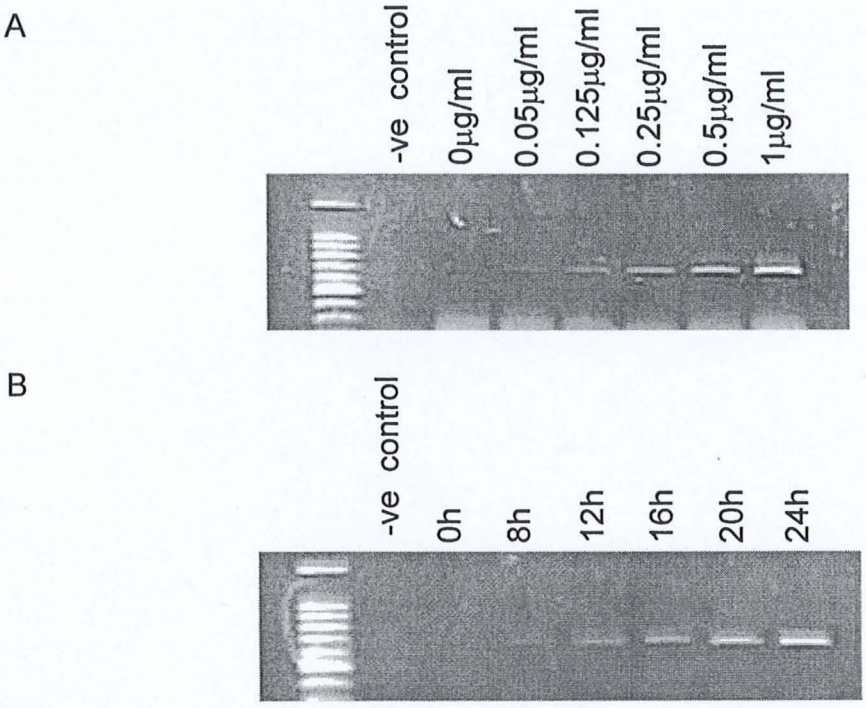
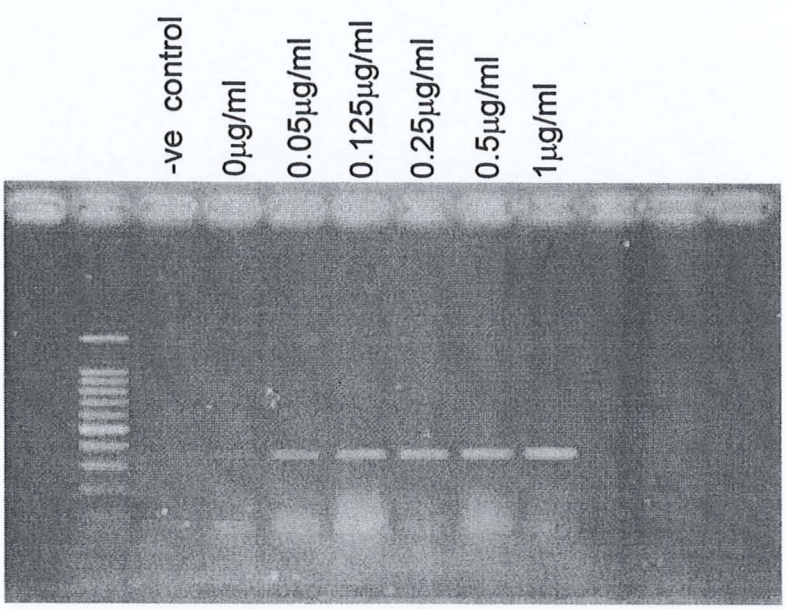


Figure 2 (A) Dose-dependent mRNA expression of wap65 (warm-temperature-acclimation-related-65kDa-protein-like-protein) gene (also known as hemopexin gene. (B) Time-dependent mRNA expression of wap65 (warm-temperature-acclimation-related-65kDa-protein-like-protein) gene (also known as hemopexin gene

A



B

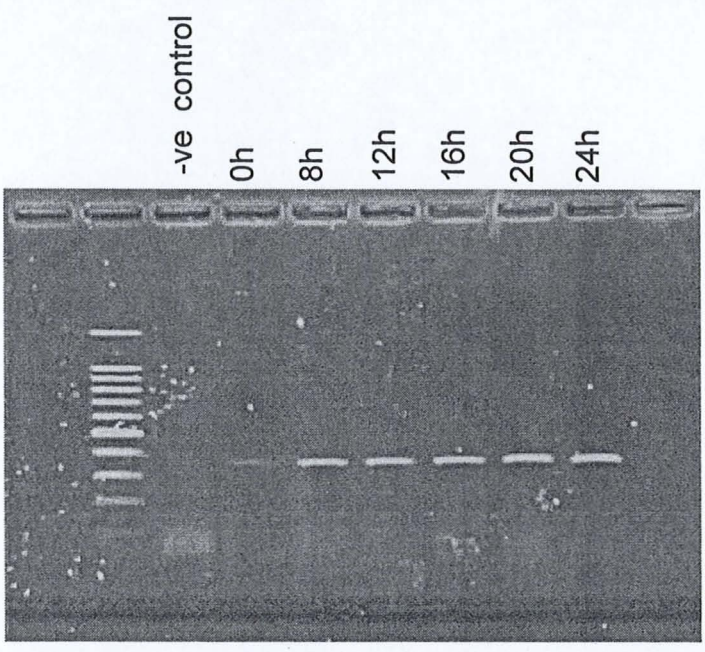


Figure 3 (A) Dose-dependent mRNA expression of mitochondria solute carrier family gene. (B) Time-dependent mRNA expression of mitochondria solute carrier family gene.

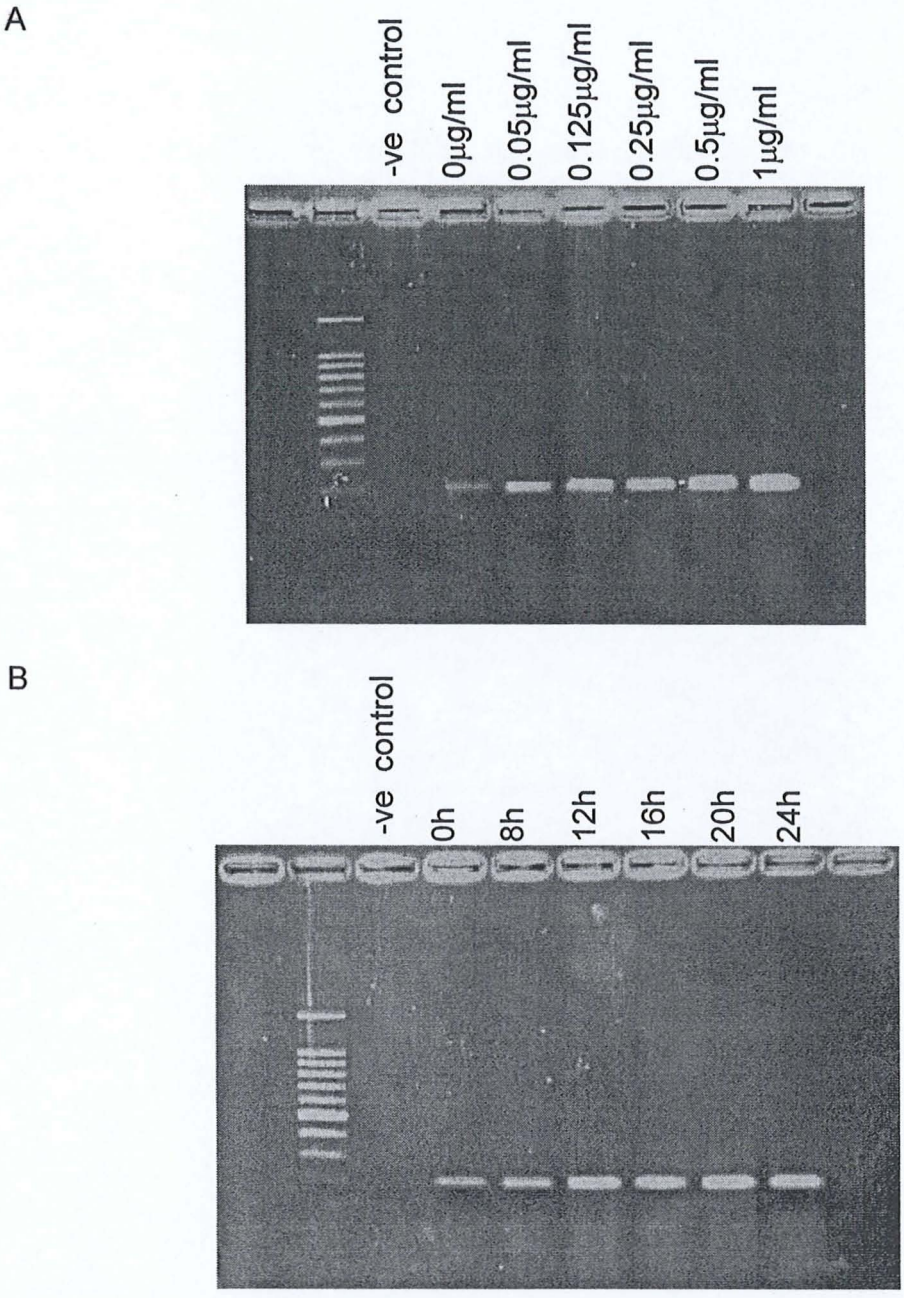


Figure 4 (A) Dose-dependent mRNA expression of ribosomal protein L19 gene. (B) Time-dependent mRNA expression of ribosomal protein L19 gene

GeneFishing DEG approach only allowed the 3'end of a particular gene to be cloned. Therefore, in order to obtain the full length cDNA of the gene of interest (wap65 or hemopexin gene), RLM-RACE was utilised to clone the remaining 5'end of the gene. As shown in Figure 5, a PCR product with the size of 900bp was successfully amplified. The sequence analysis revealed that the amplified fragment represents the 5'end fragment swordtail wap65/hemopexin gene indicating that the full length cDNA of copper-inducable wap65/hemopexin gene from swordtail fish was successfully cloned and charaterised.

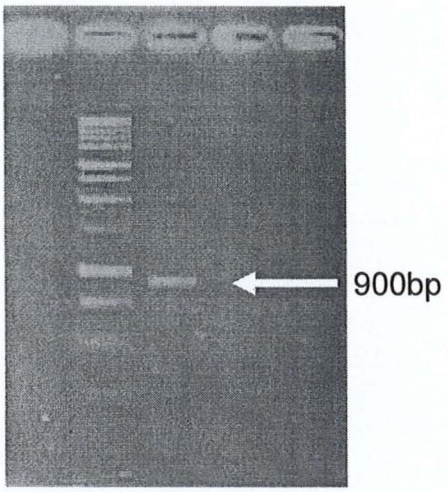


Figure 5 The amplified product of the 5'end of wap65/hemopexin gene from swordtail fish using RLM-RACE

Discussion.

It is widely recognized that metal compounds have a profound effect on gene expression patterns, as demonstrated by the growing number of metal responsive gene in studies involving transcriptomes and proteomes expression patters. Biomarkers such as Cytochrome P4501A, metallothionein, vitellogenin and heat shock proteins have been utilized in numerous toxicity studies. (Tom and Auslander, 2005). There is however a strong interest in large-scale multi-gene screening for novel biomarkers useful for biomonitoring and other toxicogenomic applications (Williams et al 2003). In this context, fish offer many advantages in understanding the relationship between organism response to changes in the environment (Cossins and Crawford 2005). In this study, three hepatic genes representing copper specific-inducable genes in swordtail fish have been cloned and characterised from swordtail fish that were exposed to sublethal copper concentrations.

Our study reveals that one of the upregulated-transcripts showing high homology to the medaka (*Oryzias latipes*) wap65, a warm-temperature-acclimation-related-65 kDa-protein-like-protein (Hirayama et al. 2004). The deduced nucleotide and amino acid sequences of carp and goldfish wap65 showed close homology to mammalian hemopexins, a serum glycoprotein that transports heme from hemolysis to the liver (Kikuchi et al., 1995; Kinoshita et al., 2001). wap65 was first shown to play a role in temperature acclamation. (Watabe et al., 1993; Kikuchi et al., 1993, Kikuchi et al., 1995). Subsequent studies in other teleost species however, suggested a wider role for this protein. Kikuichi et al (1997) further show that goldfish wap65 possess a cytokine response element, suggesting an additional function of in self-defense mechanisms. The upregulation of a hemopexin-like transcript in our present study could be explained by the known role of hemopexin during hypoxia-related stress. A major pathological disorder in fish exposed to sublethal copper concentration is gill damage, resulting in less efficient gas exchange across the gill epithelium, leading to tissue hypoxia (Dalglish and

Novak 2002). More notably, van Heerden et al (2004) showed that reported during short-term exposure of rainbow trout to a sublethal levels of copper, disorders such gill damage, tissue hypoxia and induction of metallothionein gene expression occur. Although our present work did not measure hypoxia levels in fish, the above mentioned facts strongly explain the elevated expression of hemopexin in swordtails. Elsewhere, upregulation of hemopexin-like protein mRNA was detected during hypoxia conditions in goby *Gillichthys mirabilis*. (Gracey et al 2001). In cells, excessive copper can induce oxidative stress, for example, through the catalysis of Fenton-type reactions leading to formation of reactive oxygen species (ROS) which can cause damages to cellular structures, leading to loss of cell integrity and functionality (Gabbianelli et al. 2003).

Thus, in conclusion, 3 cuprum-inducable genes have been successfully characterised in swordtail fish with special emphasis given to hemopexin gene due to its important role in stress conditions.

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