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Gene Expression Response in Early Developmental Stages of Rainbow Trout Exposed to Ecologically Relevant Concentrations of Malathion

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GENE EXPRESSION RESPONSE IN EARLY DEVELOPMENTAL STAGES OF RAINBOW TROUT EXPOSED TO ECOLOGICALLY RELEVANT CONCENTRATIONS OF MALATHION

by

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ABSTRACT
GENE EXPRESSION RESPONSE IN EARLY DEVELOPMENTAL STAGES OF RAINBOW TROUT EXPOSED TO ECOLOGICALLY RELEVANT CONCENTRATIONS OF MALATHION

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Abstract. Understanding the early life stage toxic effects of environmental organophosphate exposure on organism health is crucial to identifying biomarkers that can be used for preventative care. Malathion, a potent organophosphate, is one of the most widely used organophosphates in agriculture and pest eradication. Due to its widespread use, pesticide runoff into area bodies of water poses a great threat to aquatic life and human inhabitants. Acute exposure to high concentrations of malathion causes neurological abnormalities and can result in respiratory failure, muscle spasms, and mental confusion in humans.

In the present study, the effects of malathion are observed following acute, low-level exposure; however, most diagnostic tests require sustained exposure to concentrations high enough to induce acetylcholinesterase inhibition. There is a direct relationship between AChE inhibition and acute exposure to malathion, that results in systematic disturbances in neural function and elicits overt toxicity. While disrupted AChE activity serves as a biomarker, it is not sensitive to low

levels of malathion exposure. Therefore, it is necessary to identify novel biomarkers that are more sensitive to malathion exposure so that steps can be taken to ensure the safety of humans and aquatic life before the neurological complications can develop.

Here were we report the discovery of two new potential biomarkers that are expressed following acute, low-concentration malathion exposure. We used two early life stages of rainbow trout (sac fry and swim-up fry) exposed to varying malathion levels (3-100 parts per billion, ppb) over 48 hours. At these levels, swim-up fry had heightened mortality rates compared to sac fry, indicating that they may exhibit change in gene expression. To identify transcriptional biomarkers, a 16K salmonid cDNA microarray was used; 349 genes were found to be differentially expressed at concentrations as low as 10 ppb. Additionally, we further analyzed malathion responsive genes using qPCR, network and ontologic analysis. The results from qPCR revealed that the gene encoding for a small heat shock protein, *HSP30*, was robustly upregulated. A second gene put forth as a candidate biomarker in this study is cytochrome P4501A3 (*CYP1A3*); this study also shows paralog CYP1A1 was not found to be malathion inducible in either life stage. Network and ontologic analysis suggested changes in expression of genes involved in metal ion binding, catalytic activity, transport, oxidation-reduction, metabolism and stress response. The novel findings of this study contribute to the construction of a repertoire of predictive biomarkers, induced by malathion exposure, that may also be used as tools to survey fish population health.

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Introduction

The usage of pesticides, fungicides and herbicides in standard agricultural practice results in a high probability of contamination of aquatic habitats and ground water reservoirs due to pesticide run-off and ground infiltration. The toxicity of prevalently used organophosphate (OP) agrochemicals is well studied; in humans, symptoms of acute poisoning include respiratory failure, muscle spasms, and mental confusion (Haque et al., 1987; Sweeney and Lyon, 1999). Unfortunately, a dichotomy often exists between pesticide effectiveness and safety; this provides for challenges in management and regulation of such pesticides. The OP malathion [1,2-Di(ethoxycarbonyl)ethyl *O,O*-dimethyl phosphorodithioate], a widely used chemical compound classified as a broad spectrum insecticide, exemplifies such a challenge. Malathion poses a moderate to high toxicity in fish species, where concentrations as low as 4 parts per billion (ppb) have been shown to adversely affect trout species (U.S. EPA, 2000); yet, water concentrations as high as 583 ppb have been reported to be found in bodies of water near pesticide application sites (Ando, 1994). Thus, prevalence of malathion contamination in biologically significant amounts presents a toxic hazard toward freshwater eco-systems.

The mechanisms that underlie malathion's adverse effects in fish are beginning to be understood. One well-studied adverse effect is acetylcholinesterase inhibition in both target and non-target species (Chambers and Carr, 1995). AChE is essential for cholinergic neurotransmission and functions to catalyze the hydrolysis of the neurotransmitter acetylcholine (ACh).

Sustained high levels of AChE inhibition induced by acute exposure to malathion systemically disturb neural function. In fish, aberrant behaviors associated with acute OP toxicity include erratic swimming, hyperexcitability, loss of equilibrium, and caudal bending (Patil and David, 2010). Malathion also manifests pro-oxidative properties (Banerjee et al., 1999) capable of eliciting lipid peroxidation in rodent erythrocytes, liver and brain tissues (Hazarika et al., 2003) and mitogenic effects (Moore et al., 2010). Importantly, changes at the level of gene expression provide clues as to the molecular mechanisms of malathion toxicity in organisms ranging from fish to humans, yet this has not been well investigated. Due to this, we hypothesized that genes found to be consistently dysregulated by malathion are suitable biomarkers to detect low-level malathion exposure.

In the present study, we investigated the molecular consequences of malathion low-level exposure using *Oncorhynchus mykiss* (rainbow trout). Rainbow trout are uniquely suited to this analysis because they exhibit a heightened sensitivity to environmental OPs and can serve as a biological detector. In our investigation, we profiled malathion-mediated changes both *in vivo* and using gene expression analysis to identify candidate biomarkers. Malathion responsive genes were identified from two distinct early developmental stages of rainbow trout fry at environmentally relevant concentrations. We found two robust biomarkers that had dramatic changes in expression levels that occurred during malathion-induced morbidity. Moreover, we used network analysis to identify other potential biomarkers of malathion toxicity when the fish were exposed to very low levels.

Methods

Procurement of Oncorhynchus mykiss eggs

Rainbow trout eyed eggs were obtained from U.S. Fish and Wildlife Service Ennis National Fish hatchery (Ennis, MT). Embryos were group housed in a flow-through tank system where dissolved oxygen levels (> 10 mg/L) and temperature (10.0 ± 1.0 °C) were maintained with a 12 h light:12 h dark photoperiod. All embryo and fish rearing and use were conducted in accordance to the guidelines established by the Animal Care and Use Committee (ACUC) of University of Wisconsin-Milwaukee.

Malathion exposure regimen

Two developmental stages (One-day post-hatch sac fry and 14-days post-hatch swim-up fry) of rainbow trout were used, all arising from the same hatchery-reared cohort of embryos. These life stages of fish were subjected to a malathion exposure for 48 hours. Ten fish per beaker were exposed to either a single waterborne malathion (Chem Service Inc., West Chester, PA) treatment or ethanol vehicle following a 24-hour acclimation period. On the day of exposure, malathion was dissolved in ethanol (the lowest volume was used so as to be miscible in water and was calculated to be 0.0001% of total exposure volume) before being mixed with water. Fish were exposed by static renewal to one of five different concentrations of malathion: 3, 10, 30, 100, 300 ppb, or an ethanol vehicle control. Each treatment was tested in triplicate. Mortality and water quality data (dissolved oxygen, temperature, pH and ammonia concentration)

were recorded three times: prior to commencement of exposure, and after 24 and 48 hours from onset of exposure. Water quality parameters of exposure regimens were not of significant difference.

Toxicity of the exposure medium used in fish experiments (referred to as "malathion spent water") was confirmed with *Daphnia* magna (*n*=10) where mortality was assessed at 24 and 48 hours after exposure. A parallel *Daphnia* toxicity test was performed using medium with freshly mixed malathion from original stock at 3, 10, 30, 100, 300 ppb with an ethanol vehicle control; all testing was performed in triplicate. Mortality data from both types of *Daphnia* exposures (malathion spent water *vs.* fresh water) were compared and evaluated for significant differences, where none were found.

RNA Isolation

At 48 hours, fish from each treatment group were collected (by random computer-generated assignment), and anesthetized using 0.01g of tricaine methane sulfonate (MS-222), (Western Chemical, Ferndale, Washington). Individual fish were immediately flash frozen in liquid nitrogen and stored at -80 °C until retrieval for RNA isolation. Total RNA was prepared from individuals flash-frozen in liquid nitrogen (*n*=5 per treatment group) and ground to a fine dust using pre-baked (at 300 °C) mortars and pestles that were pre-cooled with liquid nitrogen directly before use. Total RNA from each individual was extracted using TRIzol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA) followed by further homogenization with a Qiashredder kit (Qiagen, Valencia,

CA). Total RNA samples were treated with DNase and cleaned using RNeasy MinElute Cleanup kits (Qiagen). Pre-cleaned and cleaned RNA samples were quantified by spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA) and quality-checked with agarose gel electrophoresis.

Transcriptional analysis

A salmonid 16K cDNA array was used that was obtained from the consortium for genomic research on all salmon project (cGRASP of University Victoria, B.C.). The experimental design incorporated the use of a common reference (vehicle control) with two-color labeling of pooled samples (*n*=5 per treatment group) and three technical replicates (including one dye-swap) per malathion concentration tested (10 ppb, 30 ppb, and 100ppb), resulting in a total of nine chips. Hybridizations were performed using Genisphere 900 (3DNA, Hatfield, PA) labeling kits and methods specified by the manufacturer. For each microarray, 2 µg of pooled total RNA from both control and treated samples were reverse-transcribed using the supplied oligo primers with 3' or 5' unique capture sequences (used in the Cy3 or Cy5 labeling reactions, respectively) and reverse-transcriptase kit (Invitrogen, Carlsbad, CA). Microarray slides were prepared for hybridization using cDNA in formamide-based hybridization buffer applied to pre-warmed microarray slides and hybridized overnight (16 h) at 50 °C. After the cDNA hybridization, microarray slides were washed and dried by centrifugation. This was followed by the labeling hybridization step performed (3 hours at 50 °C) using the Genisphere 3DNA Cy 3 and Cy 5 capture reagents in formamide

hybridization buffer. Each of the fluorescent capture reagents hybridize to a corresponding capture sequence on the Cy 3 or Cy 5 oligo primers bound to the microarray (Cooper et al.). Post-hybridization washing and drying were performed in the same manner as previously detailed (Rise et al. 2004a).

Real-time RT-Quantitative PCR (qPCR)

Total RNA was reverse transcribed using Affinity Script reverse transcriptase (Stratagene, Cedar Creek, TX) as previously performed (Rise et al., 2004). Brilliant SYBR Green kits (Stratagene) were used along with two PCR primers either for target gene of interest (GOI), or the housekeeping normalizer gene Guanine Nucleotide-Binding Protein Subunit Beta-2-Like 1 (*GNB2L1*, more commonly known as *RACK 1*). All qPCR primer design and methods followed. Each 25 µl qPCR reaction contained diluted template from individual fish (n=5 per treatment group) and run in duplicate for 40 cycles using a Mx3000p (Stratagene) thermocycler. A standard curve was completed on each plate for each gene. No template controls were included for each reaction. Normalized Ct values from malathion-exposed and vehicle control samples were compared using Mx3000p software and the relative delta-delta Ct quantification method of Pfaffl (2001). Melting curves for qPCR products were evaluated to show single peaks, and end-point agarose gel electrophoresis was performed to show single bands of expected size.

Microarray analyses

Microarray slides were scanned using a ScanArray Express (Perkin-Elmer, Waltham, MA) to acquire fluorescent images with settings of 10 um resolution, laser power of 90%, and a wavelength of 543 nm and 633 nm (10 nm bandwidth) to excite the Cy 3 and Cy 5 fluorophores, respectively. Fluorescence intensity data for each channel were extracted from TIFF images using ImaGene 7.0 software (Biodiscovery, Santa Monica, CA) and further analyzed in Genespring (Agilent, Santa Clara, CA) software; data were background corrected and Lowess normalization was applied. LIMMA's (Linear Models for Microarray Data package), found as a part of Bioconductor (http://bioconductor.org), lmFit function was used to fit linear models to the gene expression data generated in Genespring. A list of genes showing significant differential expression was then generated using a moderated F-statistic and composed a final gene list used in all subsequent transcriptomic secondary analysis. Submission of microarray data and experimental design abides by standards set forth according to minimum information about a microarray experiment (MIAME) standards (Brazma et al., 2001).

Secondary Analysis

Candidate malathion-responsive genes identified in LIMMA were involved in a secondary analysis to include network, cluster, and ontologic analysis. Graphic representations of data were generated using the graph and gplot

applications of Bioconductor. Gene interaction networks of significantly dysregulated genes were generated through the use of igraph open-source software package and incorporated the use of the algorithm described by Newman (2006). Network data was visualized using Cytoscape (Shannon et al., 2003), an open-source bioinformatics software platform for visualizing molecular interaction networks. Clustering of dysregulated transcripts was performed using agglomerative hierarchical clustering without performing mean centering of expression values, and using Ward's method of merging clusters (Eisen et al., 1998).

Results

Malathion 48- hour exposure

The ability to rapidly determine exposure to dangerous and harmful levels of malathion is challenging. Most studies have focused on malathion toxicity and its mechanism of action, yet to the best of our knowledge no studies have identified accurate and sensitive transcriptional biomarkers that can be used to determine fish health status in exposed environments. Specifically, malathion is highly toxic to all salmonids, of which the most sensitive species include rainbow trout, lake trout, brown trout and coho salmon. Levels as low as 2 ppb (U.S. EPA, 2002) cause overt signs of toxicity in rainbow trout, though levels ranging from 3-300 ppb (U.S. EPA, 2006; Kahn, 1992; Ando, 1994) are frequently reported in lakes and streams located in close proximity to agricultural sites. Therefore, we first quantified malathion toxicity to rainbow trout sac fry and swim-

up fry by using a broad spectrum range of malathion concentrations (3 - 300 ppb) at 24- and 48-hour time points.

Swim-up and sac fry exhibit differential mortality profiles to malathion in a dose- and time-dependent manner (Figure 1). At 24 hours, mortality in swim-up fry was not seen at 3, 10 and 30 ppb malathion concentrations, with a slight increase to 3% at 100 ppb. However, mortality dramatically increased to 93% at 300 ppb. In sharp contrast, sac fry exhibited a small increase in mortality to 3% at 100 ppb and displayed 0% mortality with all other malathion treatments. The differences in sensitivity between swim-up and sac fry continued at 48 hours, specifically in 100 and 300 ppb malathion treatment groups. We observed a 40% mortality in swim-up fry at 100 ppb and 100% at 300 ppb. Sac fry remained relatively resistant to malathion, with a modest mortality of 3% at both 100 and 300 ppb treatments.

Qualitative analysis of malathion-induced mortality was positively correlated with increased morbidity revealed by the accompanying behavioral changes. We video recorded lethargy (difficulty maintaining an upright position), increased respiratory distress, presence of dorsally located mid-tail hematomas, and slowed or absent reflex response to tail touch with a forceps (suggestive of paralysis). Morbidity observations were exclusive to the swim-up fry and could be detected by 24 hours in 100 and 300 ppb treatment groups.

Identification of novel transcriptional biomarkers

Next, we sought to identify transcriptional dysregulation resulting from malathion exposure in the more sensitive life stage of swim-up fry using microarray analysis. We identified 349 dysregulated genes at concentrations as low as 10 ppb. Network and enrichment analysis of the microarray data revealed changes in expression of wide-ranging 'high trust' genes that included small ribosomal protein (*RPL23A*), glucose catabolic process gene (*ALDOB*), glycoprotein metabolism (*ST3GAL5*) and several transporter genes (*G6PT*, *ATP5L*, *SLCO1B2*) (Table 4). As expected, most of the dysregulated genes did not exhibit similar expression patterns across broad malathion exposure and time points. Therefore, we used several approaches, including qPCR, cluster, ontological and enrichment analyses to identify those genes that consistently differed from non-treated controls. In principle, these consistently dysregulated genes may be more suitable biomarkers. We discovered two compelling genes during our analysis that showed consistent dysregulation, cytochrome P4501A (*CYP1A*) and heat shock protein 30 (*HSP30*) (Figs. 2, 3), warranting further investigation.

We more fully explored the role of *CYP1A* and *HSP30* as potential malathion biomarker genes 48 hours after treatment (3, 10, 30, 100 ppb). We quantified transcript expression levels of *CYP1A* in both sac fry and swim-up fry to determine if these were inducible by malathion. To further investigate this we used the *CYP1A1* and *CYP1A3* paralogs, which share a 96% amino acid identity in rainbow trout (Cao et al., 2000), in qPCR analysis. Transcript analysis of

CYP1A3 showed a modest, 3.3-fold up-regulation at 100 ppb compared to controls (Fig. 2). Interestingly, expression of *CYP1A1* did not differ across malathion treatment groups or from controls of either life stage, while transcript expression levels for both *CYP1A1* and *CYP1A3* did not differ in sac fry across all experimental trials (Fig. 2).

In looking at the other candidate biomarker *HSP30,* we quantified transcript expression levels in swim-up fry, which showed the greatest change in malathion-induced mortality and morbidity. We observed changes in transcript expression levels for all *HSP30* paralogs (lettered A-D) analyzed by qPCR, where expression was most strongly up-regulated compared to non-treated controls in the 100 ppb malathion treatment group. The most pointed changes occurred with *HSP30C* and *HSP30D* transcripts, with a dramatic 24.02- and 46.26-fold increase in expression level, respectively (Figure 3). These data indicate the potential value of *HSP30*, particularly *HSP30C* and *HSP30D* as robust biomarkers of malathion exposure in aquatic ecosystems.

Taken together, our data suggest that the two independent biomarkers *CYP1A1* and *HSP30* can be employed to determine malathion toxicity in sensitive rainbow trout swim-up fry.

Network, gene ontology, and cluster analyses suggest additional biomarkers

Our robust gene expression patterns of *HSP30* led us to ask whether we could identify genes that change at very low malathion concentrations (≤10 ppb). To address this we performed a network analysis of the microarray data, which

revealed 26 distinct gene communities. We followed this using hubness (hub) scores to confer network connectivity, community association and biological importance of a gene (Table 1). The top 50 genes, based on hub score belonged to relatively few (7) communities within the network, suggesting potentially identifiable biological pathways in malathion toxicity. Moreover, the direction of dysregulation at 10 ppb resulted in 45 down-regulated genes of the 50 total highest hub score genes (Table 1). These results suggest that malathion toxicity elicits a gene regulatory response at low levels.

We then used ontological analysis of the 349 malathion-responsive genes used in network analysis, and placed them into biological and molecular functional gene ontology (GO) categories (Tables 2, 3). Biological processes most affected by malathion exposure included metabolism (31 genes), oxidative stress (11 genes) and signal transduction (20 genes) (Table 2). Our analysis also revealed diverse molecular function categories that included metal ion binding (41), catalytic and hydrolase activity (39 genes), cell transport (12 genes), oxidation-reduction (11 genes) and transferase activity (9 genes). These broad but identifiable changes hold great promise for identification of molecular mechanisms of malathion-induced toxicity.

Discussion

The present study suggests tremendous value in using early life stages of fish to identify potentially harmful pollution of both the aquatic environment and its derived food sources in human consumption. Specifically, we show that swim-

up fry are highly sensitive to these environmental changes. These fish exhibit a substantial increase in mortality compared to sac fry and non-exposed controls. Importantly, we identified two novel biomarkers expressed in swim-up fry that are upregulated within two days of exposure to malathion at 100 ppb. Our additional network analysis suggests that further investigation will yield additional biomarkers that can be identified at even lower malathion exposure concentrations.

Observation of early developmental stages of fish have long been used as indicators of organophosphate pesticide contamination in water, yet the ability to identify robust genomic biomarkers has been relatively non-existent despite the potential impacts on humans and wildlife. Using acute exposure to fenitrothion, Hobden et al. suggested that rainbow trout juveniles were most sensitive to exposure due to an inability to convert the compound into a benign metabolite (Hobden and Klaverkamp, 1977). Similarly, walleye swim-up fry are highly sensitive to low concentrations of chlorpyrifos, where changes in gill structure related to age have been suggested as the basis for enhanced sensitivity (Phillips et al., 2002). In agreement with our study, both rainbow trout and Japanese medaka (*Oryzias latipes*) sac fry were the least sensitive to OP exposure (Hobden and Klaverkamp, 1977; Takimoto et al., 1987). The promise of using these early stage fish to identify organophosphate exposure highlights the need to better identify biomarkers that are concomitantly expressed.

The biomarkers (*CYP1A3, HSP30C*, and *HSP30D*,) identified in our current study are novel for their use in identifying malathion toxicity at lower levels than

has been used with AChE inhibition, which has traditionally been employed for this purpose. Induced by pesticides in fish, Cytochrome P450 1A (CYP1A) enzymes are known to mediate biotransformation of a wide range of xenobiotics (Haluzova et al., 2009). Specifically, cytochrome P450 isoforms (along with other flavin-containing monooxygenases) play an influential role in the toxicity of OPs since they catalyze the formation of oxon metabolites; malaoxon is the bioactivated metabolite of malathion and provides for a dramatically enhanced potency of AChE inhibition (Fukuto, 1990). Separate hydrolytic processes are required for detoxification of malathion where AChE remains unaffected (Maxwell, 1992). In the present study, we found *CYP1A3* to be inducible in swim-up fry but not in sac fry. This finding suggests the possibility of swim-up fry possessing a greater catalytic efficiency of oxon formation, and could explain the difference in susceptibility to malathion toxicity between the life stages. To our knowledge, this is the first time that malathion has been reported as an inducer of *CYP1A3* in trout.

Transcriptional analysis

In this study we sought to use varied methods of network data analysis to provide biologically meaningful insight into malathion-responsive genes. Several gene annotations were found to have both high hub scores and over-representation among community ontologies supporting their likely involvement in malathion toxicity. Moreover, several unifying biological themes were represented in these analyses. Genes encoding metabolism, metal ion

homeostasis, oxidation-reduction reactions, ATP binding, proteolysis, apoptosis, and neurodevelopment appear to correlate with a distinctive phenotype of subtle malathion toxicity. In combination, many of these processes have been linked to neuromuscular disorders as previously mentioned, due to a cellular disturbance in energetic processes or homeostasis (including metal ion homeostasis, mitochondrial respiration, and lipid metabolism).

Analyzing malathion-induced dysregulated genes revealed 40 potential candidates involved in metal ion binding. Not only does this present potential biomarkers, but it also provides insight into the type of malathion-induced tissue damage. Genes involved in metal ion binding were of specific interest since metal ion homeostasis is crucial for proper physiologic function in all living organisms. Genes encoding zinc ion binding activity were most commonly represented followed by calcium, iron, copper, and magnesium. These catalytic cofactors are required for numerous cellular functions and maintenance activities. Loss of metal ion homeostasis has been shown to clinically develop into disorders such as retinal degeneration, diabetes mellitus, and neurological disturbances (Hellman and Gitlin, 2002; Hochstrasser et al., 2005).

Organophosphates are known to induce oxidative stress leading to lipid peroxidation and DNA single-strand breaks. Malathion exposure purportedly leads to the formation of reactive oxygen species and subsequent induction of oxidative stress causing tissue impairment through lipid peroxidation and DNA damage (Jomova and Valko, 2011). Here we found a dramatic upregulation of two heat shock protein (HSP) transcripts, *HSP30C* and *HSP30D*, following

malathion exposure. An upregulation of HSPs correlate with the adaptation to stress by reprogramming cellular metabolic activity so as to protect cells from further oxidative stress (Parsell and Lindquist, 1993). These structurally conserved proteins are largely considered to be molecular chaperones, functioning in proper protein folding and the maintenance of protein homeostasis.

In addition to identifying *HSP30* paralogs and *CYP1A3* as a quality candidate biomarker of exposure to malathion and generating a clearer view of malathion toxicity at the molecular level, our research contributes to the small amount of existing literature that shows age-dependant sensitivity differences where an early life stage is far more resilient to toxic exposure compared to a later life stage. Here we emphasize that acetylcholinesterase inhibition should not be the sole relied upon parameter for the evaluation of toxicity, due the wide range of cellular events adversely altered by malathion concentrations below the threshold required for AChE inhibition. Additionally, the lack of data that relates AChE inhibition to malathion exposure scenarios of ecologically relevant concentrations is problematic and compromises the reliable use of AChE inhibition as a primary biomarker of exposure for OP pesticides. Thus, with the ever-expanding use of malathion in standard agricultural practices, we highlight the need for more suitable and sensitive biomarkers of malathion exposure. We demonstrated that malathion exposure alters gene expression at concentrations as low as 10 ppb, and where such subtle changes potentially precede adverse effects on cellular function including metabolism, metal ion homeostasis, oxidation reduction, and signal transduction events. Therefore, a new class of

biomarkers in fish expanded beyond AChE, may transform detection methods and augment risk assessment in such a way so as to mitigate harmful organophosphate exposure leading to health consequences in diverse living organisms that use water resources.

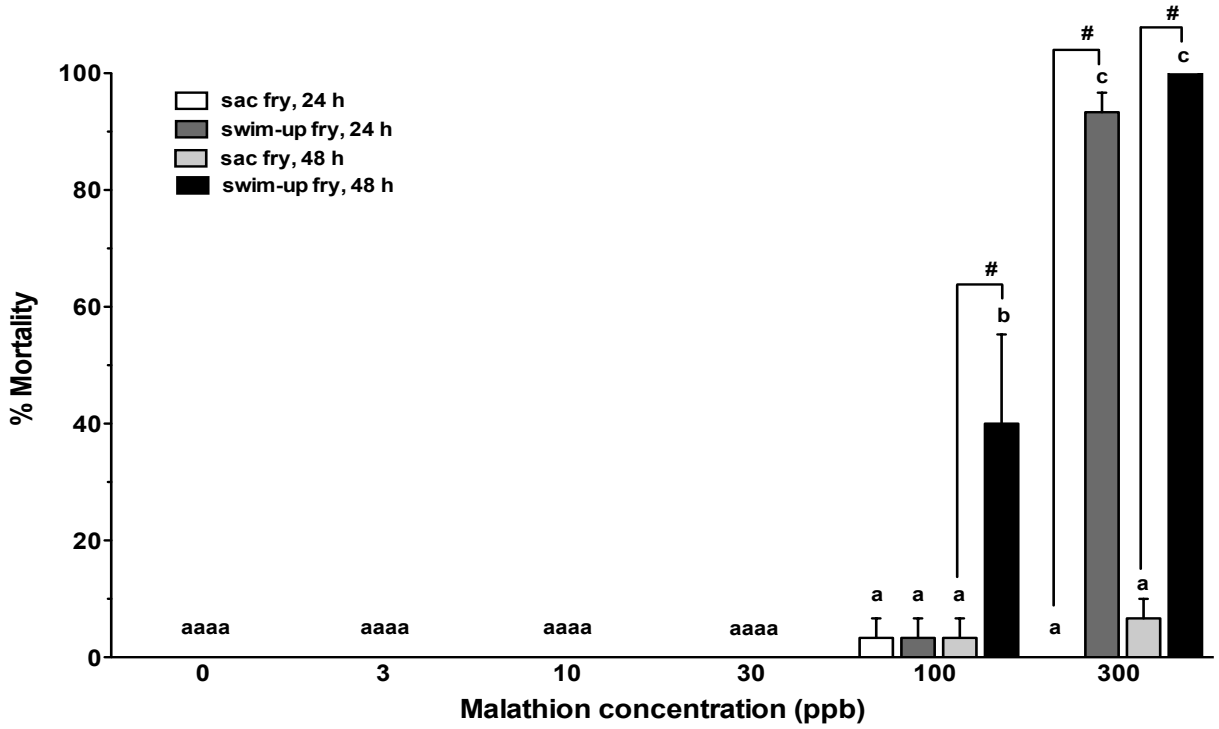

Figure 1. Swim-up fry exhibit a greater malathion-induced mortality rate compared to sac fry. Rainbow trout sac fry and swim-up fry were exposed to malathion for 24 and 48 hours and the percentage mortality recorded. At 100 ppb, swim-up fry displayed 3% mortality after 24 hours and 40% mortality after 48 hours. At 300 ppb swim up fry mortality was at 93% at 24 hours and 100% at 48. Sac fry had substantially lower mortality rates of 3% after both 24 and 48 hours with 100 ppb, and 300 ppb after 48 hours. All other mortality rates were at 0%. Values are reported as mean ± S.E.M. (n = 10). Different letters differ significantly (p<0.05) between treatment group or exposure time, and # (p<0.05) designates difference between life stage. Significance was determined by a repeated-measures 2-way ANOVA and Bonferroni post-test.

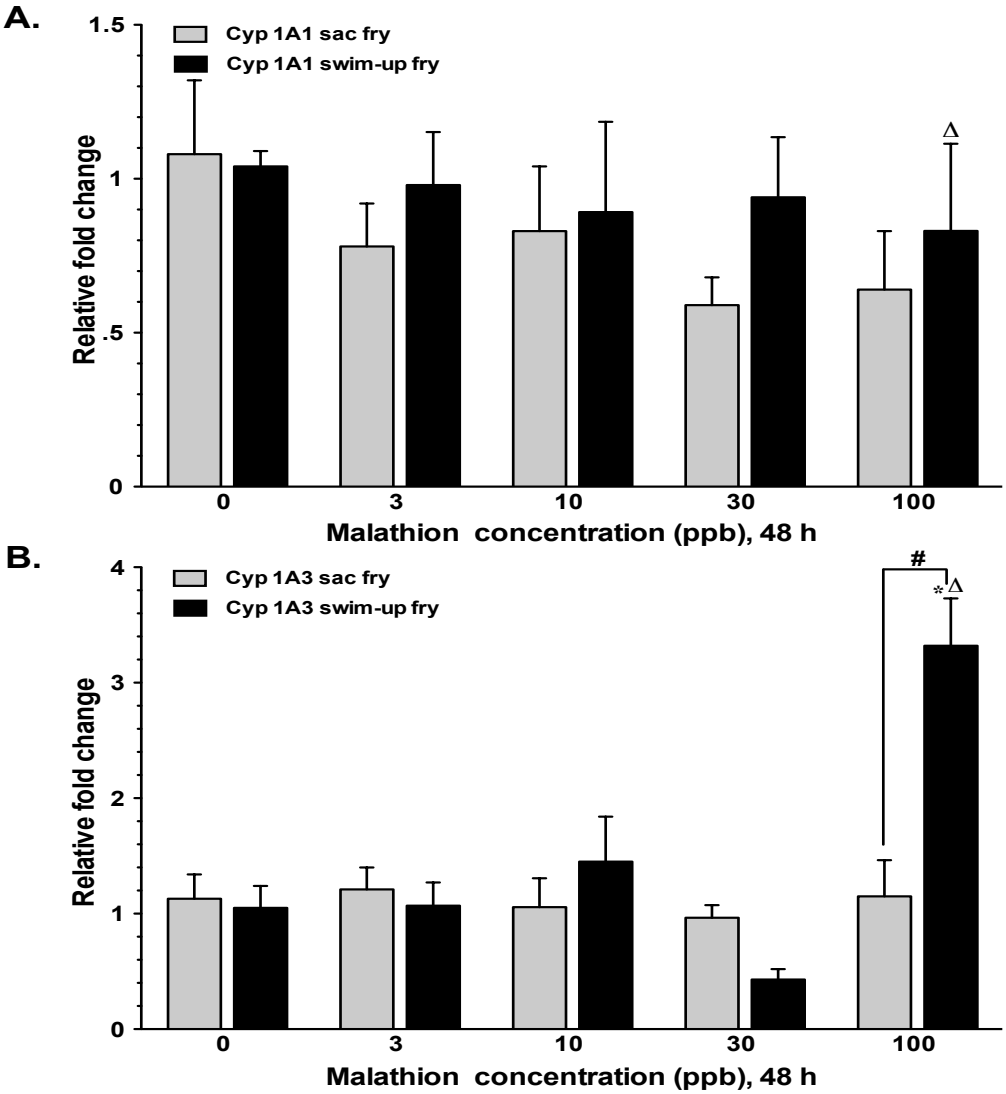

Figure 2. Malathion induces cytochrome P4501A (*CYP1A*) gene expression in a life stage-specific and paralog-specific manner. Transcript expression levels of *CYP1A* paralogs (*CYP1A1, CYP1A3*) were compared from qPCR analysis on rainbow trout sac fry and swim-up fry life stages following a 48 h malathion exposure. **(A)** Expression of transcript *CYP1A1* was neither induced nor differed significantly in sac fry nor swim-up fry treated by malathion. **(B)** Swim-up fry of the 100 ppb treatment group showed a 3.3-fold up-regulation of *CYP1A3* mRNA levels compared to non-treated controls after a 48-hour exposure. *(p<0.05) indicates a significant difference compared to control (0 ppb), and Δ(p<0.05) compared between paralogs *CYP1A1* and *CYP1A3, and* #(p<0.05) compared between life stages. Values are expressed as mean ± SEM (n = 5). Significance was determined by repeated-measures 2-way ANOVA and Bonferroni post-test.

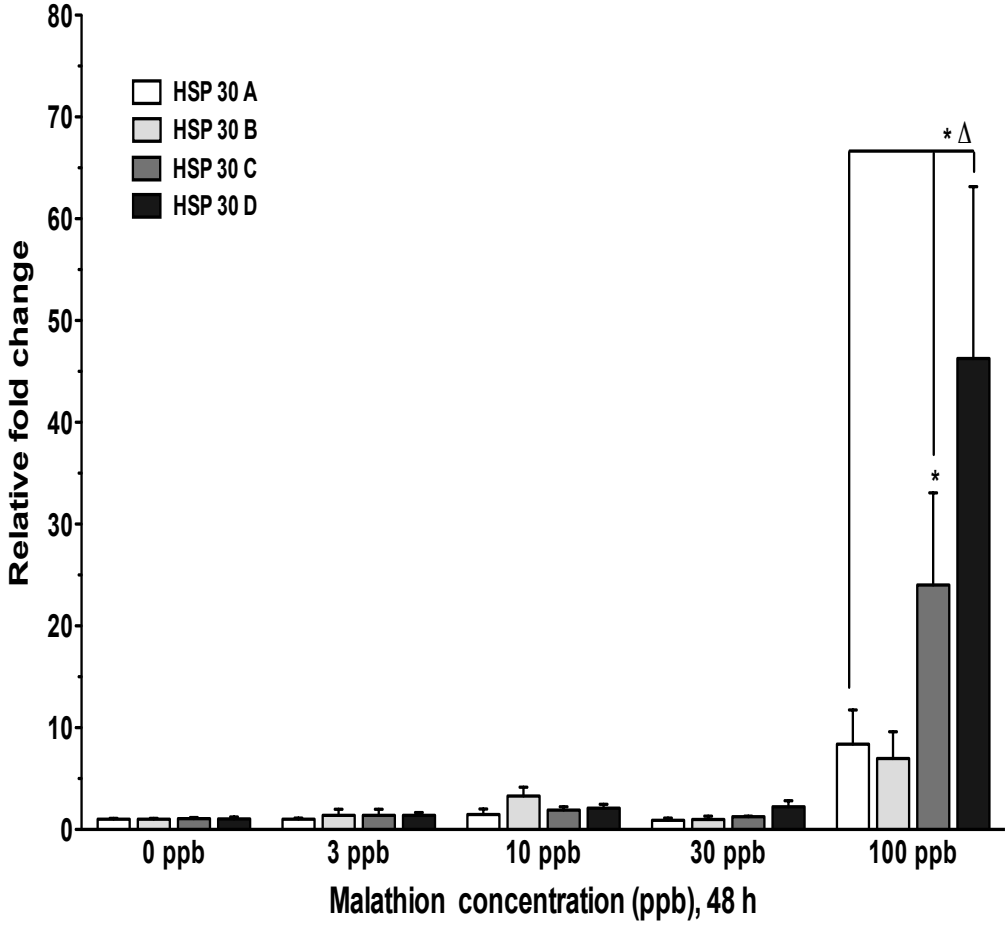

Figure 3. The candidate biomarker HSP30 transcript expression levels are dramatically upregulated in swim-up fry exposed to 100 ppb malathion. Relative gene expression of four *HSP30* paralogs (A-D) was measured by qPCR, in rainbow trout swim-up fry following a 48 h malathion exposure. Swim-up fry of the 100 ppb treatment group show a 24-fold up-regulation of *HSP30 C*, and 46-fold up-regulation of *HSP30 D* transcript levels compared to non-treated controls. Values are expressed as mean ± SEM (n = 5). *(p<0.05) indicates significant difference compared to control (0 ppb), whereas Δ(p<0.05) indicated a significant difference between paralogs. Significance was determined by a repeated-measures 2-way ANOVA and Bonferroni post-test.

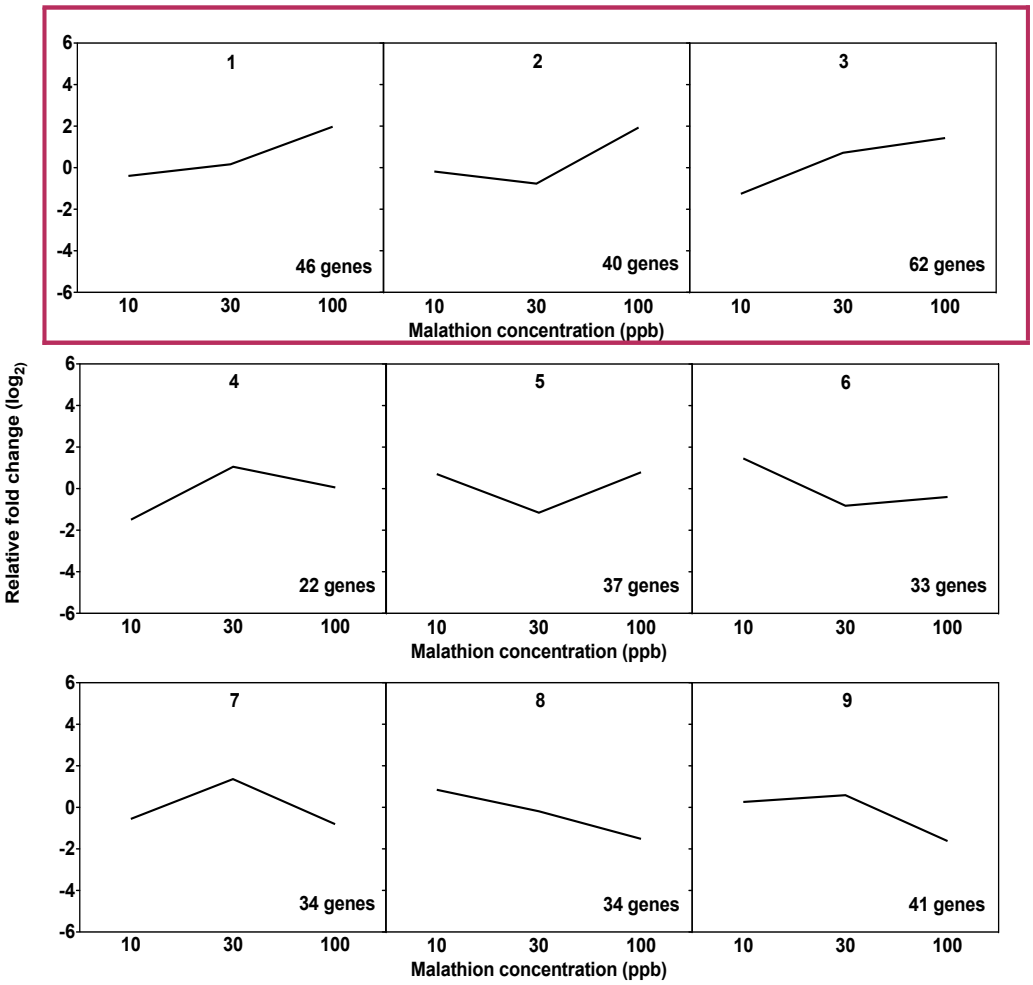

Figure 4. Dynamic expression cluster patterns (9), of genes dysregulated by malathion in 48-hour exposed swim-up fry; 349 dysregulated genes were identified by microarray for 10, 30, and 100 ppb malathion treatment groups. The number of genes that correspond to each cluster is shown in the bottom right corner. Clusters numbered 1-3 are highlighted by a red box due to being the only expression patterns represented among high hubness score genes (top 50 genes with highest hubness scores of network analysis).

Table 1. 50 highest hubness score genes (of network analysis) dysregulated by malathion in rainbow trout swim-up fry.

Accession #/Gene Name	Hubness Score	Community	Cluster #	10 ppb	30 ppb	100 ppb
CB496918 60S ribosomal protein L23a	1.00	1	1	-1.07	1.44	2.60
CA053629 Glucose-6-phosphate translocase	0.95	24	3	-2.22	**-1.12**	1.94
CB497649 Nucleoside diphosphate kinase B	0.82	1	1	-1.25	1.56	4.35
CB497960 Rainbow trout (S.gairdneri) cytochrome P450IA1 mRNA, complete cds	0.79	1	3	-1.90	1.78	4.07
CA058532 UNKNOWN	0.74	1	1	-1.10	1.05	3.58
CB497826 Collagen alpha-3(VI) chain precursor	0.73	1	1	-1.02	1.44	3.24
CK990568 Protein S100-A1	0.70	1	2	-1.31	**-1.42**	3.08
CA059260 UNKNOWN	0.68	1	1	-1.79	1.19	3.67
CA054628 UNKNOWN	0.68	1	2	-1.32	**-1.29**	7.75
CB509793 CDC5L, KIAA0432, PCDC5RP: Cell division cycle 5-like protein	0.63	1	3	-1.60	2.56	8.14
CA058261 Beta-sarcoglycan	0.62	25	3	-2.52	1.18	1.99
CB494032 Carbonic anhydrase	0.61	1	3	-2.29	1.48	3.65
CK990774 UNKNOWN	0.60	22	2	**1.02**	1.06	6.16
CB506607 liver-expressed antimicrobial peptide 2 isoform A precursor	0.58	25	3	-4.71	1.31	2.33
CA062887 Cysteine and glycine-rich protein 1	0.56	1	3	-1.27	1.99	2.70
CB492677 Cyclin-L1	0.56	25	3	-1.90	1.27	2.68
CK991122 Heat shock protein 30	0.56	1	1	**1.18**	2.73	17.64
BU965792 Myosin heavy chain, fast skeletal muscle	0.55	1	3	-1.30	2.66	5.66
CB511687 Ceruloplasmin precursor	0.55	1	2	-1.06	**-1.89**	5.85
CA063484 F-box only protein 46	0.54	25	3	-1.31	1.75	4.12
CB518072 Ubiquitin	0.54	22	2	-1.18	**-1.50**	2.79
CA063520 Signal peptide peptidase-like 2A	0.54	1	1	-2.69	**-1.26**	6.40
BU965792 Myosin heavy chain, fast skeletal muscle	0.54	21	1	-1.99	**-1.49**	2.50
CA054279 UNKNOWN	0.53	21	1	-1.30	1.51	5.48
BU965780 Integral membrane protein 2B	0.53	1	2	-1.96	**-2.92**	7.27
CA044571 Salmo salar caspase 3B gene, complete cds	0.53	1	3	-1.65	2.53	4.11
CA059497 AN1-type zinc finger protein 2B	0.50	24	1	-2.86	**-2.00**	**-2.33**
CA054811 Solute carrier organic anion transporter family member 1B2	0.49	24	3	-1.27	2.01	4.86
CB488319 Uncharacterized protein C8orf76	0.48	21	1	-1.88	**-1.10**	2.63
CB514220 ATP synthase subunit g, mitochondrial	0.48	24	3	-14.10	1.45	3.51
CA057297 G-rich sequence factor 1	0.45	24	3	-4.56	**-1.00**	2.45
CA050885 Cleavage and polyadenylation specificity factor 6	0.45	22	1	-1.44	1.22	5.14
CA061046 UNKNOWN	0.43	18	2	**1.27**	1.07	3.02
CA050836 UNKNOWN	0.42	18	2	-2.14	**-2.54**	2.05
CB492951 DNA-binding protein inhibitor ID-2	0.42	24	3	-3.00	1.43	4.47
CA045746 Actin, cytoplasmic 1	0.41	25	3	-2.58	1.65	2.61
CA038556 Tumor suppressor candidate 4	0.41	18	1	-1.09	1.10	5.37
CA061907 UNKNOWN	0.40	24	3	-2.36	1.11	**-1.47**
CB492545 Transcription factor HES-1	0.39	1	1	-1.19	1.21	5.98
CA064263 U6 snRNA-associated Sm-like protein LSm8	0.39	1	2	-1.08	**-1.13**	16.61
CB492414 T-complex protein 1 subunit delta	0.39	21	1	**1.08**	1.54	2.57
CB489812 Oncorhynchus kisutch 5S ribosomal RNA gene, partial sequence	0.37	1	2	-2.05	**-2.39**	2.60
CB509708 Ferritin, heavy subunit	0.36	21	1	-1.92	1.11	3.55
CB515915 Lactosylceramide alpha-2,3-sialyltransferase	0.35	22	1	-2.39	**-1.49**	2.58
CA063464 UNKNOWN	0.33	21	3	-3.50	1.46	6.35
CA058089 Probable arylformamidase	0.33	1	2	**1.21**	1.19	5.54
CB492432 UNKNOWN	0.33	21	1	-1.60	**-1.40**	3.92
CB493026 Thioredoxin	0.32	19	1	-1.43	**-1.00**	3.23
CA767684 H-2 class II histocompatibility antigen gamma chain	0.31	18	2	-1.27	**-1.39**	2.68
CA061400 COP9 signalosome complex subunit 2	0.31	22	1	-1.45	**-1.22**	3.29

Table 2. Biological process gene ontologies described for 349 genes identified by microarray.

GO[1] Biological Process (BP) Term	GO ID #	# Genes[2]
Metabolism	GO:0044237	31
Response to Stimulus	GO:0050896	22
Stress response	GO:0033554	11
Signaling/Signal transduction	GO:0023052	20
Anatomical structure development	GO:0048856	16
Transport	GO:0006810	13
Transcription	GO:0045449	11
Apoptosis	GO:0006915	10
Cell differentiation/cell cycle	GO:0030154	10
Immune system	GO:0006955	9
Cell adhesion	GO:0007155	7
Neurological	GO:0050877	7
Cell proliferation	GO:0008283	6
Homeostasis	GO:0033554	6
Cytoskeleton organization	GO:0007010	6
Proteolysis	GO:0006508	6
Translation	GO:0006412	5
Ribosome	GO:0042254	5
Cell migration	GO:0016477	3
Developmental process	GO:0032502	3
Oxidation-reduction	GO:0055114	3

[1] GO= gene ontology of biological process classification described on website: http://www.geneontology.org/

[2] Number of genes refers to the number out of 349 genes (identified by microarray analysis and found to be significant using a combined p value, $p < 0.05$) described by the listed GO BP term and GO ID #.

Table 3. Molecular function gene ontologies described for 349 genes identified by microarray.

GO[1] Molecular Function (MF) Term	GO ID #	# Genes[2]
Protein binding	GO:0005515	52
Nucleic acid binding	GO:0003676	51
Metal ion binding total	GO:0046872	41
Calcium	GO:0005509	9
Zinc	GO:0008270	18
Copper	GO:0005507	3
Iron	GO:0008199	6
Magnesium	GO:0000287	2
Other metal (unspecified)	GO:0046872	3
ATP binding	GO:0005524	17
Receptor binding activity	GO:0004872	3
Catalytic/hydrolase activity	GO:0003824	39
Transport	GO:0005215	12
Oxidation-Reduction	GO:0016491	11
Transferase activity	GO:0016740	9
Ribosome	GO:0003735	6
Peptidase activity	GO:0008233	5
Signal-Transduction	GO:0004871	5
Cell cycle	GO:0007049	4
Transcription	GO:0030528	4
Enzyme inhibitor	GO:0004857	3
Protein Dimerization	GO:0046983	3

[1] GO= gene ontology classes of molecular function and described on website: http://www.geneontology.org/

[2] Number of genes refers to the number out of 349 genes (identified by microarray analysis and found to be significant using a combined p value, p <0.05) described by the listed GO MF term and GO ID #.

Table 4. GO terms found to be enriched within network communities (having >10 members).

Community ID	Ontology	GO ID	GO Term	*P* Value	# Genes	Total Genes/Comm
1	MF[1]	GO:0003774	motor activity	0.012	2	33
1	MF	GO:0005198	structural molecule activity	0.049	3	33
3	MF	GO:0008233	peptidase activity	0.018	6	39
4	MF	GO:0016491	oxidoreductase activity	0.038	4	43
4	BP[2]	GO:0044281	small molecule metabolic process	0.048	5	43
16	BP	GO:0044255	cellular lipid metabolic process	0.032	3	43
16	MF	GO:0008234	cysteine-type peptidase activity	0.023	2	43
16	BP	GO:0061024	cell membrane organization	0.026	2	43
18	BP	GO:0043170	macromolecule metabolism	0.016	7	21
18	MF	GO:0022857	transporter activity	0.027	2	21
21	BP	GO:0006091	generation of precursor metabolites and energy	0.018	2	12
22	BP	GO:0043170	macromolecule metabolism	0.003	4	18
24	MF	GO:0005215	transport activity	0.001	4	21

[1]MF=Molecular Function
[2]BP=Biological Process

Chapter 1.

Extended Thesis Introduction

Malathion (1,2-di(ethoxycarbonyl)ethyl *O,O*-dimethyl phosphorodithioate) is a chemical compound classified as an organophosphate (OP) broad spectrum insecticide with widespread use. It is commonly employed in agricultural practice as a pesticide, in public health sanitation efforts for pest eradication (such as those aimed at mosquito abatement, boll weevil eradication, and medfly control), and in residential use as a herbicide/fungicide. Estimated annual application in the U.S. is approximated 16.7 million pounds, where 12.5 million pounds are applied exclusively to food crops (ATSDR, 2003). Consequently, a high probability of contamination exists for aquatic habitats in close proximity to agricultural land due to pesticide run-off and infiltration of ground water.

According to the U.S. EPA, malathion poses a moderate to high toxicity in fish species; specifically, rainbow trout show extreme sensitivity to exposure and correspond to a 96-h LC50 of 4 ppb (U.S. EPA, 2000). While limited data are available for malathion concentration levels of specific freshwater environments, water concentrations as high as 583 ppb have been reported to occur downstream from agricultural application sites and have resulted in fish-kills (Ando, 1994). More recently, the National Marine Fisheries Service (NMFS) investigated registered malathion usage (as established by EPA Registration Eligibility Decision, 2006) and the degree of exposure risk on 28 Pacific salmonid species. NMFS concluded that registered malathion usage would jeopardize the

continued existence of 27 Pacific salmonid species, and destroy or adversely modify critical aquatic habitat (NMFS Biological Opinion, 2008). Thus, prevalence of malathion contamination in biologically significant amounts is of extreme relevance and poses potential hazards for freshwater eco-systems.

While malathion is highly effective as a multi-use OP insecticide, concern arises due to it's indiscriminate mode of action via acetylcholinesterase inhibition in both target and non-target specie (Chambers and Carr, 1995). AChE is essential for cholinergic neurotransmission and functions to catalyze the hydrolysis of the neurotransmitter acetylcholine (Ach). Malathion acts to target the enzyme AChE with the resultant effect of ACh accumulation, subsequent overstimulation of cholinergic receptors, and ensuing excitotoxicity. Sustained high levels of AChE inhibition induced by acute exposure to an organophosphate such as malathion, can disturb function of both the central and peripheral nervous systems. In humans, symptoms of acute OP poisoning are dependent upon severity and length of exposure, but often include respiratory failure, muscle spasms, and mental confusion (Haque et al., 1987; Sweeney and Lyon, 1999). In severe exposures, coma and death can result (Warren et al., 1985). In fish, aberrant behavior associated with acute toxicity may include erratic swimming, hyper- excitability, loss of equilibrium, and caudal bending (Patil and David, 2010). Such behavioral alterations adversely affect fish performance and survival.

It is well accepted that malathion-induced neurotoxicity is primarily elicited via inhibition of acetylcholinesterase; however, malathion exposure elicits

toxicologically relevant noncholinergic outcomes even at concentrations that are below the threshold for AChE inhibition. Malathion has pro-oxidative properties (Banerjee et al., 1999) capable of eliciting lipid peroxidation in rodent erythrocytes, liver and brain tissues (Hazarika et al., 2003). Malathion-induced oxidative stress also suppresses anti-oxidant enzyme expression, and has demonstrated ability to damage DNA of rat hippocampal tissue (Brocardo et al., 2007; Franco et al., 2009). In vitro studies have demonstrated noncholinergic concentrations of malathion initiate apoptosis via caspase-dependant pathways (Masoud et al., 2003). In contrast, malathion also exerts mitogenic effects at low levels in vitro (Moore et al.). Collectively, these studies support the idea that toxicological action of malathion does not arise solely from the inhibition of AChE, while emphasizing the importance of considering additional molecular targets of action. The noncholinergic effects of OPs have received significantly renewed attention in the scientific literature. However, few recent publications focus on the identification of additional mechanisms of toxicity for low-dose malathion exposure. Moreover, few publications explore how gene expression is altered by malathion and the corresponding biological implications of such alterations.

In the present study, we directed our efforts toward gaining insight into the molecular consequences of low-level exposure using rainbow trout as a model system. We were able to accomplish this objective through analysis of differential gene expression assessed by microarray. Malathion-responsive genes were identified from two distinct early developmental stages of rainbow trout fry at environmentally relevant concentrations. The objective of this study was to

identify candidate biomarkers of exposure to malathion, while deriving biological meaning from induced gene expression alterations in rainbow trout. The present study aims to improve our understanding of the molecular basis for malathion induced physiologic dysfunction apart from acetylcholinesterase inhibition. The establishment of malathion-induced transcriptional biomarkers could improve and transform current wildlife health risk assessment practice by allowing for early detection and quantification of toxic exposure in aquatic environments.

Appendix

Transcriptional analysis

Analyzing the GO functions associated with the genes that were dysregulated by malathion revealed 40 genes involved in metal ion binding. Genes involved in metal ion binding were of specific interest since metal ion homeostasis is crucial for proper physiologic function in all living organisms. Genes encoding zinc ion binding activity were most commonly represented followed by calcium, iron, copper, and magnesium. Metal ions such as zinc, iron, copper, and magnesium play roles as catalytic cofactors to a variety of metalloproteins and enzymes that are required for numerous cellular functions and maintenance activities. Zinc and copper are capable of modulating gene expression through interference of signal transduction pathways and have the capacity to activate transcription factors involved in cell cycle progression and apoptosis (Evan and Vousden, 2001). Similarly, calcium is a cell signaling molecule integral to signal transduction pathways as a secondary messenger. Metal ion homeostasis is a tightly regulated event vital for tissue health, where disruption negates such status through impairment of cell viability. Loss of metal ion homeostasis has been shown to be causative in a wide range of disease including cardiac arrhythmia, myocardial infarction, and chronic neurodegeneration (Stohs and Bagchi, 1995; Valko et al., 2005). Disruption of metal ion homeostasis could result in dramatic physiologic effects following exposure to malathion exposure.

Not surprisingly, metal ion binding (specifically zinc, iron, and copper) genes were well represented among the top 50 hubness (hub) scores (Table 1). Carbonic anhydrase 1 (CA 1, hubness score = 0.61) utilizes zinc as a co-factor while promoting osteoclast differentiation and bone resorption. Defects in the gene CA 1 often cause ostepetrosis, a disease characterized by abnormally dense bone due to disrupted resorption of immature bone. Cysteine and glycine-rich protein 1 (CSRP1) is involved in zinc ion binding (hub score = 0.56) and potentially plays a role in neuronal development (Swiss Prot). Cerulosplasmin (CP, hub score = 0.55) is known to be involved in both copper metal ion binding and cellular iron homeostasis via ferroxidase activity. Defects in CP are responsible for iron metabolism disorders that potentially lead to iron accumulation in brain tissue and other visceral organs; clinical manifestations of such disorders include retinal degeneration, diabetes mellitus, and neurologic disturbances (Hellman and Gitlin, 2002; Hochstrasser et al., 2005). Additionally, Hochstrasser (2005) and colleagues suggest that altered CP activity caused by gene mutation may play a role in neurodegeneration (as exhibited in Parkinson's disease), due to iron-induced oxidative injury to the brain.

Remaining genes that encode metal ion binding proteins identified by high hubness scores included ZFAND2B and ferritin heavy subunit. Ferritin heavy subunit is important for cellular iron ion homeostasis and plays a role in immune response and negative regulation of cell proliferation. Defects in ferritin proteins have been shown to be associated with neurodegenerative diseases (Bush and Tanzi, 2008; Huang et al., 2004; Jiang et al., 2009). Ferritin in included under the

GO terms category of oxidoreductase activity (Tables 2, 3) and was found to be significantly over-represented in network community 21 (Table 4). This allows us to consider that other genes of community 21 could potentially share involvement in the oxidoreductase pathway. The network and gene ontology analyses strongly indicate that metal ion binding is altered by malathion exposure where normal physiology is dramatically impaired.

In order to maintain a holistic view of physiologic response, it is important to consider potentially related functional categories of responsive genes. It is not surprising that genes associated with the GO category of oxidation-reduction responded to malathion exposure. It has been recently reported that disruption of metal ion homeostasis (related to redox homeostasis), may be responsible for formation of reactive oxygen species and subsequent induction of oxidative stress, causing tissue impairment through lipid peroxidation and DNA damage (Jomova and Valko, 2011). Similarly, malathion metabolism has been reported to cause oxidative damage.

It has been shown that zinc attenuates malathion-induced ‘depressant behavior’ and affords a neuroprotective effect in the brain (Brocardo et al., 2007). This study investigated the imbalance of antioxidant status of the brain, liver, and red blood cells after rats were exposed to concentrations of malathion below the threshold for acetylcholinesterase inhibition. Malathion is considered to have a pro-oxidant effect; however, zinc has been shown to play a crucial role in affording protection against oxidative damage by attenuating lipid peroxidation (Brocardo et al., 2007).

Malathion exposure induced several genes associated with oxidation and reduction activities (see Table 3). Organophosphates are known to induce oxidative stress leading to lipid peroxidation and DNA-single strand breaks. Genes having the biological process gene ontology 'response to stimulus' comprised the second largest category elicited by malathion exposure (Table 2). Interestingly, half of the genes involved in 'response to stimulus' specifically functioned in stress response, which includes oxidative stress as a sub-category.

Several heat shock proteins (HSPs) are expressed at elevated levels under a wide variety of stress conditions. HSPs support the adaptation to stress by reprogramming cellular metabolic activity so as to protect cells from further oxidative stress (Parsell and Lindquist, 1993). For this reason, heat shock proteins and response are sometimes referred to as 'stress proteins' and 'stress response' in literature. Heat shock proteins are structurally conserved and have been identified in both prokaryotic and eukaryotic organisms indicating a potentially crucial role in cellular physiology and response to stress. HSPs are considered to be molecular chaperones and function in proper protein folding and the maintenance of protein homeostasis of the cell. HSP families are designated by their deduced molecular mass in kilodaltons. The most commonly studied HSPs are HSP90, HSP70, HSP65, and HSP27. The family of small heat shock proteins (sHSPs) is defined as comprising a molecular mass of 15-30 KDa that contain a highly conserved region of approximately 90 amino acid residues in the C-terminal region. sHSPs have been identified in both in-vitro and in-vivo studies as molecular chaperones where they bind unfolded proteins, suppress

aggregation, and promote refolding of proteins to their functional state. Small HSPs are inducible and increase tolerance to cytotoxic stressors.

Small heat shock protein 30 (HSP30) genes were found to be the most highly up-regulated genes in our microarray experiments. Probes for two Hsp30 paralogs were present on the microarray (HSP30A and HSP30B) and two additional paralogs were subsequently identified (HSP30C, HSP30D). Expression levels for all four paralogs were examined by qPCR (Figure 3). Of the HSP paralogs investigated, HSP30C and HSP30D showed strong induction of gene expression at the highest concentration of malathion with HSP30D up-regulated >45 fold. This suggests that both genes are strong candidate biomarkers for exposure to high environmentally relevant malathion concentrations. This is a novel finding since HSP30 has not been identified in fish as a potential biomarker of exposure to any stressor.

According to the literature, chronic exposure to malathion has been observed to be associated with metabolic disorders (Montgomery et al., 2008) and genes associated with GO terms related to metabolism were the largest category of dysregulated genes. In terms of gene expression, one study found that ‘sub-threshold levels’ of malathion suppressed the gene encoding for hypothalamic corticotropin-releasing hormone (CRH) in Wister rats (Rezg, 2008). CRH is the primary neurohormone that regulates the hypothalamic-pituitary-adrenal (HPA) axis that influences lipid and carbohydrate metabolic pathways. Rezg (2008) compared the malathion induced suppressed hypothalamic CRH expression to the diabetes mellitus model of CRH expression. It is suggested by

both Rezg (2008) and Montgomery (2008), that chronic exposure to malathion (even at sub-threshold levels) is responsible for alteration of metabolic events that potentially result in a significantly increased risk for the development of type II diabetes (Rezg, 2008; Montgomery, 2008). Malathion has also been found to alter expression of genes involved in mitochondrial metabolism, specifically cytochrome c I and III (Salazar et al., 2007).

Glucose-6-phosphate translocase had the second highest hubness score (0.9458) out of the 349 genes analyzed and functions in glucose homeostasis. Defects in the glucose-6-phosphate translocase gene result in glycogen storage disease, biochemical abnormalities such as hypoglycemia and hyperlipidemia, and kidney enlargement. Fructose-bisphosphate aldolase B (hub score = 0.536) is involved in glycolysis where defects cause fructose intolerance and hypoglycemia.

Another well-represented category of biological process gene ontology is anatomical structural development. COL6A3 (hub score = 0.733) is a cell-binding protein, and functions in several areas of structure development that include involvement in axon guidance and muscle organ development. Defects in the COL6A3 gene in humans may result in Bethlem myopathy or Ulrich congenital muscular dystrophy (Lampe and Bushby, 2005), both of which are characterized by muscle weakness and early childhood onset of joint contractures (Demir et al., 2002; Jobsis et al., 1999). ID2 T (hub score = 0.417) is a transcription regulator (Sun et al., 1991) that plays many roles in the cell including the negative regulation of neuronal differentiation, leukocyte

differentiation, and osteoblast formation (Swiss prot www.expasy.com). ID-2 is a positive regulator of smooth muscle cell proliferation and astrocyte differentiation. DNA-binding protein inhibitor is also involved in digestive tract formation and morphology, as well as lymph node development. Unrelated to these functions, ID-2 also plays a role in the synchronization of circadian rhythms to environmental cues (such as light).

Still, other anatomical structural development genes that were found to be dysregulated (but do not possess high hubness scores) include those that regulate skeletal muscle development (prostate stem cell antigen precursor-like gene); limb development (transcription factor Sp8); cardiac muscle morphology (bifunctional protein NCOAT); and brain, retina and lens development (ceroid-lipofuscinosis neuronal protein 5). Defects in the CLN5 gene result in neurodegeneration and are clinically characterized by seizures, dementia, visual loss, and cerebral atrophy. Lastly, the anatomical development GO category included several genes outside of the top 50 highest hubness scores that all function in angiogenesis and include: collagen alpha-1(XV) chain precursor, CpG-binding protein, glutamyl aminopeptidase (where two separate features on the microarray representing this gene were both found to be dysregulated), and focal adhesion kinase 1. Focal adhesion kinase 1 has several functional annotations in addition to angiogenesis that include negative regulation of neuron axonogenesis, signal transducer activity, and cytoskeleton development. Interestingly, focal adhesion kinase has additionally been found to be involved in hepatic cell proliferation and induction of hepatic cell apoptosis via the caspase-3

pathway (Zhang, XL, 2008). This is important to note since in the present study, the caspase-3B gene is among the top 50 highest hubness score (hub score: 0.525) genes, and apoptosis is represented as a category of BP GO terms (Table 2) mentioned below.

Genes having roles in apoptosis and found to be among dysregulated high hubness score genes include caspase 3B, integral membrane protein 2B, tumor suppressor candidate 4, and thioredoxin. Aside from apoptosis, caspase 3B is thought to be involved in cleavage of amyloid-beta 4A precursor protein, and associated with neuronal death in Alzheimer's (Swiss prot). Thioredoxin specifically possesses an anti-apoptotic functional role and is potentially involved in resistance to anti-tumor agents (Chen et al., 2002), while also playing a role in mitochondrial membrane potential (Damdimopoulos et al., 2002). Consistent with our findings, previous studies report apoptosis following organophosphate exposure. For example, an in vitro study that described the effects of malathion exposure at non-cholinergic doses, demonstrated activation of caspase 3 protein expression and suggests that cytoxicity of malathion is mediated through caspase-dependent apoptosis (Masoud et al., 2003). Another study that related apoptosis to OP exposure reported alteration of cell cycle events, proliferation, and cell death just one hour after an initial acute exposure to soman (Dillman et al., 2009). Additionally, apoptosis has been cited as a non-genomic biomarker of effect in rats exposed to the OP pesticides malathion and chlorpyrifos (Aluigi et al., 2005).

Several communities in the network were enriched for genes with specific functional annotations such as proteolysis, ATP binding, metal ion binding, oxidoreductase activity, apoptosis, cell cycle, metabolism, neurological function, immune response, protein synthesis and cytoskeletal organization (Table 4). Derivation of biological functions from enrichment analysis required investigating cellular role and disease involvement of individual genes per community (comm). To begin, two genes encoding for myosin heavy chain, and two ribosomal genes (RPL23A & RPL19) were found to be over-represented in comm 1 and were additionally among high hub score genes displayed in Table 1. While it was expected that motor activity genes would be responsive in acetylcholinesterase inhibition, the enrichment status of the gene encoding a small ribosomal protein (RPL23A) and 1st place listing (hub score =1.0) amongst other high hub scores seemed puzzling. However, of recent, ribosomal proteins have garnered appreciation outside of their predominant role in protein synthesis. The literature contains studies that suggest RPL23A potentially plays a role in drug clearance via participation as a drug carrier protein (Dinman, 2007). Interestingly, RPL23A has also been implicated in conferring drug resistance due to interaction with p53 and subsequent apoptotic suppression (Singh, 2006).

Four of six genes responsible for comm 3's enrichment status were found to play a role in both proteolysis and metal ion binding activity. Of particular interest was the gene TPP1, due to participation in lipid metabolic processes, zinc metal ion binding, and nervous system development (Table 4). Defects in TTP1 have been documented to be the cause of neuronal ceroid lipofuscinosis

type 2, a neurodegenerative lysosomal storage disease characterized by seizures, dementia, visual loss, and/or cerebral atrophy. Also found in comm 3 was ROM 4, a gene that encodes for protease activity, but additionally acts to control apoptosis by preventing release of mitochondrial cytochrome c, which would otherwise respond to apoptoptic signals.

In community 4, five genes associated with over-represented annotations are involved in calcium ion homeostasis, ATP binding and/or regulation of ATPase activity, and zinc ion binding. Of those linked to disease, bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) plays an essential role in early development by providing for cell adhesion, signal transduction and ATP binding, although it is also involved in tumorigenicity and metastatic behavior of malignant cells. Interestingly, a defect in GNE provides for metabolic defects that often result in sialuria a disease that induces developmental delay of variable degrees. GNE defects can also cause body myopathy type 2, a neuromuscular disorder affecting the leg muscles. GTPase KRas (KRAS) is also a gene of comm 4 with over-represented ontology status that participates in the activation of MAPKK activity, Ras protein signal transduction, and axon guidance. Defects in this gene have been shown to be a cause of acute myelogenous leukemia, characterized by cardiac anomalies, deafness, motor delay, and also gastric cancer. Furthermore, KRAS gene defects have been shown to cause glial cell derived tumors of the brain and spinal cord with the potential to be malignant. Lastly, the disease cardio-facio-cutaneous syndrome (CFS) is the result of defects in the KRAS gene and is

associated with heart defects (such as cardiomyopathy and atrial septal defects) and mental retardation.

Community 16 genes of enrichment status include proactivator polypeptide (PSAP) which is involved in lipid metabolism. Gene defects in PSAP cause a fatal lipid storage disorder, and consequent severe neurologic dysfunction. Furthermore, PSAP gene defects are also responsible for the disease Krabbe, a disorder of galactosylceramide metabolism characterized by progressive encephalopathy and abnormal myelination in the cerebral white matter**.** A second gene, UNC13D, regulates excocytosis in lymphocytes. Intercepted gene function of UNC13D elicits immune system dysregulation, while a range of clinical manifestations may be exhibited including neurologic abnormalities such as seizures, cranial nerve deficits, and ataxia. Yet, another community 16 gene that functions in lipid transport and apoptosis encodes for cell death protein 6 homolog (CED6). CED6 may play an additional role in providing for internalization and endosomal trafficking of the previously mentioned proactivator polypeptide (PSAP); this suggests that communities may be partitioned into functionally related, co-expressed gene groups. Lastly, prostate stem cell antigen (PSCA) of comm 16 is a proteolytic enzyme, dependant on zinc or calcium metal ions for it's catalytic mechanisms. In the brain, PSCA provides a critical role in modulation of excitatory neurotransmission. However, there is literature support for PSCA having a role in cell proliferation regulation (Sakamoto, 2010). PSCA is used often as a diagnostic indicator of prostate cancer, but has additional use as a marker for

neurological disorders such as schizophrenia, Alzheimer's, and Huntington disease.

Solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1) is a gene of enrichment status and interest community 18. The main role of SLC2A1 lies in regulation of insulin secretion. Defects in this gene directly cause defective blood-brain barrier glucose transport and induced neurologic disorders. The prevalent phenotype displayed in these severe neurologic disorders includes epileptic encephalopathy associated with delayed development, uncoordinated motor ability, and spasticity. Additionally, SLC2A1 defects cause a range of cognitive impairment spanning the onset of learning disabilities to severe mental retardation. Additional clinical symptoms indicative of defects in this glucose transporter gene include ataxia, confusion, lethargy, sleep disturbance, and headaches. Lastly, the disease GLUT1 deficiency syndrome type 2 (GLUT1DS2), is also caused by SLC2A1 gene defects featuring abnormal involuntary movements of the limbs, epilepsy, and/or mild mental retardation.

Four genes of community 21 associated with over-represented ontologies include involvement in oxidoreductase activity, gluconeogenesis, ATP binding, and iron ion homeostasis. Amongst these is NADH-ubiquinone oxidoreductase. Of important note is this gene's role in mitochondrial respiration and electron transport. Gene defects are associated with mitochondrial respiratory chain disorders and are the basis of many prevalent neurodegenerative diseases such as Parkinson's. Observed phenotypes include encephalopathy, cardiomyopathy, myopathy, liver disease, Leber hereditary optic neuropathy, and Parkinson's

disease variations. Most notable is the fact that 3 of the 4 enriched comm 21 genes also possess high hub scores.

Community 22 genes that hold enrichment status include 26S proteasome non-ATPase regulatory subunit 8 (PSMD8) known to be involved in the apoptotic process and DNA damage response, the gene properdin (involved in innate immunity), and high hub score gene lactosylceramide alpha-2,3-sialyltransferase (ST3GAL5) involved in glycosylation. Defects in ST3GAL5, are known to be the cause of infantile epilepsy syndrome characterized by developmental stagnation and blindness.

The gene encoding thioredoxin-interacting protein is of community 24, and involved in both innate immune response and suppression of tumor cell growth through transcriptional repression. Glucose-6-phosphate translocase (part of the solute carrier family, SLC37A4) of comm 24 and also corresponds to a high hub score, is part of the solute carrier family and central to cellular blood sugar homeostasis by gluconeogenesis involvement. The 3rd gene of comm. 24 is ATP synthase, (involved in the catabolic process of ATP and maintaining proper mitochondrial membrane potential) which also corresponds to a high hub score (Table 1). Notably, impaired mitochdrial respiration caused by decreased ATP production, has been reported in neurodegenerative diseases such as parkinson's. ATP synthase mutations cause mitochondria dysfunction where neuromuscular impairment is exhibited (Kim, 2011). Loss of ATP synthase activity is followed by muscle degeneration and accumulation of tubulin protein; previous studies show this to be the equivalent of the *parkin* mutant phenotype

(Celotto, 2006; Mayr, 2010).

Finally, community 25 genes of enrichment status include actin-like protein 3 (involved in ATP and actin binding, while also involved in ciliogenesis), and beta-sarcoglycan (SGCB) involved in cytoskeletal development. Of functional interest, is the relationship between SGCB gene defects and muscular dystrophy type 2E, where muscles of the limbs and pelvic girdle are adversely affected.

Varied methods of data analysis in this study, from both network and enrichment analysis, provided biologically meaningful insight to malathion-responsive genes. Several gene annotations were found to have both high hub scores and over-representation amongst community ontologies supporting their likely involvement in malathion toxicity. Moreover, several unifying biological themes were represented in these analyses. Genes encoding for proteins involved in metabolism, metal ion homeostasis, oxidation reduction reactions, ATP binding, proteolysis, apoptosis, and neurodevelopment appear to correlate with a distinctive phenotype of subtle malathion toxicity. In combination, many of these processes have been linked to neuromuscular disorders as previously mentioned, due to a cellular disturbance in energetic processes or homeostasis (including metal ion homeostasis, mitochondrial respiration, or lipid metabolism).

This research shows a snapshot of the transcriptional response to malathion treatment and the impact of low, persistent environmental levels on early life stages of development in fish. It has been shown that malathion exposure results in dysregulation of transcripts involved mainly in metabolism, oxidoreductase activity, anatomical structure development, metal ion

homeostasis, apoptosis, signal transduction, cytoskeleton development and cell cycle events. Among dysregulated genes, HSP30 paralogs C and D showed strong malathion responsiveness and are put forth as candidate biomarkers of exposure to environmentally relevant high doses of malathion. Additionally, we found induction patterns to differ between *CYP 1A1* and *CYP 1A3*, where *CYP 1A3* expression was found to be malathion inducible but *CYP 1A1* was not. Interestingly, CYP 1A3 induction by malathion was found to be specific to a later developmental life stage (swim-up fry), which could explain differences in toxic susceptibility between life stages as observed through mortality and morbidity.

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