ABSTRACT

Title of Document: FACTORS INFLUENCING GLUTATHIONE S-TRANSFERASES IN *D. MAGNA*

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Glutathione S-transferases (GSTs) are an important family of xenobiotic biotransforming enzymes that can make toxicity tests variable. *Daphnia magna* is commonly used in ecotoxicology testing because they are easy to culture, have a short life cycle, and reproduce by parthenogenesis. The specific activities of glutathione S-transferases were determined in *D. magna* of different ages and *D. magna* with eggs and without eggs in their brood chambers and in both cases there was a significant difference in the specific activity between the groups. *D. magna* were exposed to clofibrate and it was determined that clofibrate does not significantly alter the specific activities of GSTs in *D. magna*. Glutathione S-transferases were purified from *D. magna* using affinity chromatography and then the purified sample was run on a SDS-PAGE gel to determine the molecular weight of the subunits of glutathione S-transferases which fell in the range that was consistent with previous studies.

FACTORS INFLUENCING GLUTATHIONE S-TRANSFERASES IN *DAPHNIA MAGNA*

By

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Chapter 1: Introduction

Glutathione S-transferases (GSTs) are an important family of xenobiotic biotransforming enzymes that can make toxicity tests variable. *Daphnia magna,* the water flea*,* is commonly used in ecotoxicology testing because they are easy to culture, have a short life cycle, able to reproduce parthenogenetically, and are sensitive to chemicals (Koivisto, 1995; and Flaherty et al., 2005). *Daphnia* are primary consumers of the aquatic food chain and thus are important to the whole aquatic community (Flaherty et al., 2005). Glutathione S-transferases are phase II biotransformation enzymes which increase the hydrophilicity of a chemical, which then enables the organism to easily excrete the xenobiotic (Parkinson, 2001). GSTs make the chemical less toxic and easily excreted by catalyzing the conjugation of tripeptide glutathione (GSH) to the lipid soluble toxin (Clark, 1989; Vidal et al., 2002). It has been found that the specific activity of glutathione S-transferases are sometimes induced by some chemicals, therefore the organism has increased tolerance of certain chemicals, while other xenobiotics actually inhibit the GSTs (LeBlanc et al., 1985). When there is variability in ecotoxicology testing, it may be due to xenobiotic biotransforming enzymes, such as GSTs.

Pollutants of all types are abundant in the aquatic environment. Pharmaceuticals are newly recognized as aquatic pollutants. Once they leave the human's body or are disposed of down the toilet, they may not be treated by the drinking water treatment facility, therefore some enter the water system unchanged

(Daughton et al., 1999). Some pharmaceuticals are not degraded in sewage treatment plants or are degraded into metabolites and this may lead the pharmaceuticals and metabolites into the aquatic environment (Petrovic et al., 2003). One such human pharmaceutical that has been found in the environment is clofibrate, which lowers the lipid content of the body, and activates peroxisome proliferator activating receptors. Therefore, it is known as a peroxisome proliferator (Nunes et al., 2004; Laville et al., 2004). Clofibric acid is a metabolite of clofibrate and has not only been detected in the aquatic environment but has a high persistence (Daughton et al., 1999). Filter feeders take water in as they feed and then siphon the food out of the water. Therefore if there are pollutants in the water, even in dilute concentrations; it is more than likely the filter feeder will be exposed to it (Sheehan et al., 1999). Enzymes like glutathione S-transferases are important to organisms in a polluted environment, since it is a way that they are able to survive and thrive since glutathione S-transferases convert the chemicals into an easily excreted chemical. Glutathione S-transferases are found in a variety of organisms, ranging from bacteria to humans. Since detoxification enzymes are a way for an organism to deal with xenobiotics, by examining the activities of these enzymes, this enables scientists to determine how the organism will respond to the pharmaceuticals in the environment (Sheehan et al., 1999).

There have been 7 different GSTs found in *D. magna* (Baldwin et al, 1996). They have been broken out into subunits, but the amino acid sequences have not been determined. In this study, I show that *D. magna* do possess glutathione S-transferase by determining that there was activity after running a kinetic assay. An age

progression study was done to determine if there is a difference in specific activity of glutathione S-transferase in *D. magna* depending on the age of the organism. An induction test was also run to determine if clofibrate can induce activity in the organism. One of the samples was purified by affinity chromatography and then an SDS- page electrophoresis gel was run and demonstrated the presence of glutathione S-transferase by the appearance of bands which exhibit the molecular weights of the subunits.

Chapter 2: Literature Review

Introduction

Organisms have evolved mechanisms to deal with toxic substances in their environment, for example, behavioral avoidance, insensitivity, and enzymatic mechanisms, such as, xenobiotic biotransforming enzymes (Newman, 1998). Xenobiotic biotransforming enzymes are usually divided into two groups, phase I and phase II biotransformation enzymes (Sheehan et al., 2001). Glutathione S-transferases are phase II biotransformation enzymes which are extremely important in detoxifying chemicals in organisms (Sheehan et al., 2001). *D. magna*, commonly known as a water flea, is widely used in ecotoxicology testing (LeBlanc et al., 1985). Glutathione S-transferases have been found in *D. magna*, and may be the reason why there is variability in the results when using *D. magna* in toxicity testing(LeBlanc et al., 1985). Because organisms have the ability to get rid of foreign chemicals in their

bodies and this ability may differ in each individual organism, toxicology tests can sometimes be variable (LeBlanc et al., 1985). In this chapter, general xenobiotic biotransforming enzymes, glutathione S-transferases, *D. magna*, glutathione S-transferases in *D. magna*, and pharmaceuticals in the environment will be discussed.

Xenobiotic Biotransforming Enzymes

Xenobiotic biotransforming enzymes are widely distributed throughout the body, they are even found in subcellular compartments. Phase I biotransformation enzymes break down xenobiotics by hydrolysis, reduction, or oxidation. The co-factors for the phase II reactions react with the functional groups that are either present on xenobiotics or introduced or exposed during the phase I biotransformation, therefore the phase II enzymes may or may not follow phase I enzymes.

Phase I enzymes transform the xenobiotic by exposing or introducing a functional group (-OH, -NH2, -SH, or –COOH) to the xenobiotic. After the functional group is added to the xenobiotic, there is a small increase in hydrophilicity of the chemical. The enzymes that are responsible for hydrolysis are carboxylesterases, pseudocholinesterase, and paraoxonase. The different reduction reactions that are used by biotransforming enzymes; are azo and nitro reduction, carbonyl reduction, disulfide reduction, sulfoxide and N-oxide reduction, quinone reduction, and dehydropyrimidine dehydrogenase dehalogenation. Alcohol, aldehyde, ketose oxidation-reduction system, alcohol dehydrogenase, aldehyde dehydrogenase, dihydrodiol dehydrogenase, molybdenum hydrolase, xanthine dehydrogenase – xanthine oxidase, aldehyde oxidase, monoamine oxidase, diamine oxidase, polyamine

oxidase, flavin monooxygenases, and cytochrome P450 are the enzymes responsible for oxidation of xenobiotics.

Phase II xenobiotic biotransformation enzymes greatly increase the hydrophilicity of the drug, which in turn promotes the excretion of the foreign chemical. The processes involved in phase II biotransformations are glucuronide conjugation, sulfate conjugation, glutathione conjugation, amino acid conjugation, acylation, and methylation.

Glutathione conjugation enzymes conjugate the xenobiotic with the tripeptide glutathione; glycine, cysteine, and glutamic acid. Electrophilic xenobiotics or xenobiotics biotransformed to electrophiles are the substrates for glutathione conjugation. The substrates have 3 common features; hydrophobic, contain an electrophilic atom, and are able to react nonenzymatically with glutathione at some measurable rate. The first step in glutathione conjugation is the formation of the peptide bond between cysteine and glutamic acid, which is catalyzed by gamma-glutamylcysteine synthetase. Once the peptide bond is formed, it then forms with glycine, this reaction is catalyzed by glutathione synthetase. The conjugation of xenobiotics with glutathione is catalyzed by glutathione S-transferases. Glutathione S-transferases increase the rate of glutathione conjugation by deprotonating GSH to GS- by the active site (Tyr-O-). **(**The above description of biotransformation enzymes was taken from Chapter 6 of Casarett and Doull's Toxicology The Basic Science of Poisons, 2001**).**

Glutathione S-transferases

Glutathione S-transferases are an important class of phase II enzymes that catalyze the transfer of glutathione to a diverse collection of substrates. Glutathione S transferases are abundant cellular components and they make up 10% of the total cellular protein. GSTs bind, store, and/or transport compounds that are not substrates for glutathione conjugation. Some GSTs are homodimers that consist of 2 identical subunits, which are 23 – 29 kDaltons in size, while some form as heterodimers. There are numerous subunits, which are cloned and sequenced and named based on the nomenclature system of GSTs. Each subunit consists of 200 – 240 amino acids and 1 catalytic site.

Glutathione S-transferases are an important family of detoxifying enzymes that are found in many organisms. These enzymes were first identified more than 40 years ago (Booth et al, 1961). Glutathione S-transferases protect organisms from toxic chemicals once they are ingested or absorbed from the environment (Clark, 1989). GSTs are involved in the first enzymatic step in formation of mercapturic acids (N-acetyl-L-cysteine S-conjugates), which are excreted by mammals. Glutathione S-transferases are in the family of essential multifunctional proteins with important physiological functions (Baldwin et al., 1996; Aceto et al., 1991; Vidal et al., 2002). The main function of these enzymes is to intracellularly detoxify xenobiotics and metabolites (Stenersen et al., 1987; Petrivalsky et al., 1997; Mannervik et al., 1988). Glutathione S-transferases make the chemical less toxic by taking the lipid soluble toxin and converting it into a water soluble, non-reactive conjugate which may easily be excreted (Clark, 1989). The enzymes do this by

catalyzing the conjugation of the tripeptide glutathione (GSH) to the xenobiotics and this in turn makes the xenobiotics more hydrophilic and easily excreted by the organism (Vidal et al., 2002**).** GSTs also act as intracellular carrier proteins of certain organic molecules (Mannervik et al., 1988).

Glutathione S-transferases also protect cells from reactive epoxides and oxygen species (Yuen et al., 2001). Chemical stress on the body may actually cause production of reactive oxygen species which results in oxidative stress on cells, which in turn activates GST activity (Feng et al., 2001). The role of glutathione S-transferase as an antioxidant enzyme is by breaking down the products of lipid peroxides to GSH, they are preventing oxidative damage (Barata et al., 2005). GSTs participate in the defense mechanisms against oxidative stress and in the metabolism of endogenously formed lipid peroxidation products (Sawicki et al., 2003). In rats, an antioxidant responsive element (ARE) has been identified in the 5' flanking region of the glutathione S-transferase Ya subunit gene (Rushmore et al., 1991). Rushmore and associates have found that the ARE is a cis-acting regulatory element that is responsive to oxidative stress reactive oxygen species (Rushmore et al, 1991). This cis-acting regulatory element activates glutathione S-transferase to protect the cells against oxidative stress (Rushmore et al, 1991; Feng et al., 2001).

Since the glutathione S-transferases lack specificity for electrophilic substrates, they are able to catalyze reactions with a wide variety of chemicals, even molecules bearing -OH group, steroid hormones and thyroid hormones, bilirubin, carcinogens, nucleophilic aromatic substitutions, Michael additions to alpha, beta-unsaturated ketones and epoxides ring-opening reactions and numerous organic

anions (Clark, 1989; Hoarau et al., 2001; Listowsky et al., 1988; Pemble et al.,1992; Sheehan et al., 2001).

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is found in most animal cells in relatively high concentrations (0.1 –10 mM) (Meister, 1995). Glutathione is ubiquitous in nature and has two characteristic features, a sulfhydryl group and a γ-glutamyl linkage (Tate et al., 1978). Glutathione is an alpha-amino acid and a tripeptide that protects cells against oxidation and is also involved in metabolism, catalysis and transport (Meister, 1995).

It has been found that glutathione S-transferases are what enables some insects to tolerate pesticides (Clark, 1989). Because of an over-expression of glutathione S-transferase, the organism has a resistance to toxic compounds (Parkinson, 2001). Glutathione S-transferases have been found to be a major reason why certain species react differently when a chemical is introduced into their body (Parkinson, 2001). Glutathione S-transferases may play a direct protective role among organisms exposed to toxic levels of xenobiotics (LeBlanc et al., 1985). The increased cytosolic GST levels may lead to the resistance of insecticides and they then are able to detoxify the insecticide and survive the pesticide treatment (Egaas, 2000).

Two hundred and four Glutathione S-transferases have been identified in many different species, ranging from primitive bacteria to humans (http://www.au.expasy.org; Buetler et al., 1992). Glutathione S-transferases are an important biological feature since there seem to be several enzymes in most species (Stenersen et al., 1987). Glutathione S-transferases are mainly characteristic

components of animal cells, though GSTs have been found in some plants, such as corn and alfalfa (Stenersen et al., 1987). These enzymes have been identified in different organisms by chromatography, enzymatic activity, and immunological methods, such as amino acid sequencing and molecular cloning (Buetler et al., 1992).

It seems that GSTs have a lack of specificity for the substrate that they conjugate, but it may be that individual GST activities may be modulated by specific classes of xenobiotics and this may be why there are different isozymes (Petrivalsky et al., 1997**).** The activities of mammalian GSTs are produced by multiple enzymes (LeBlanc et al., 1987). In the organisms that GSTs have been found, there have been multiple forms, which are different in the catalytic properties, and they possess separate yet complementary activities (Mannervik et al., 1988). There have been 10 classes of GSTs identified from vertebrates and invertebrates, alpha, mu, pi, theta, kappa, omega, delta, zeta, beta and sigma, based on substrate specificity, amino acid/nucleotide sequence and immunological, kinetic and tertiary/quaternary structural properties (Buetler et al., 1992; Vidal et al., 2002; Sheehan et al., 2001). The subunits within each class are about 70% identical and can form heterodimers, while the subunits in the different classes are only about 30% identical and this actually prevents the subunits from forming heterodimers (Parkinson, 2001).

The alpha, mu, pi and theta classification is based on the percentage of N-terminal amino acid identity and immuno-cross –reactivity with the subunits of human GSTs (Pemble et al., 1992). The enzymes found in the classes, alpha, mu, and pi are composed of 2 subunits that have molecular weights of 25,000 (Clark, 1989). Alpha, mu, and pi classes have high affinity for GSH conjugates (Meyer et al., 1995).

It has been found that the C-terminal domains are less similar between alpha, mu and pi than the N-terminal domain (Sheehan et al., 2001). Theta GSTs are heterogeneous; they are widely distributed among all living species (Buetler et al., 1992). Originally when enzymes were classified, many enzymes were put into this class, but have since been put into different classes (Sheehan et al., 2001).

GSTs are composed of subunits of the same class of glutathione S-transferases and are heterodimeric or homodimeric combinations (Baldwin et al., 1996; Sheehan et al., 2001). Each subunit has a characteristic enzyme activity, which is expressed independently of the other subunit (Ostulund Farrants et al., 1987). Alpha, mu, and pi class enzymes have an essential tyrosine in the active site in the N-terminus, while theta class enzymes have an essential serine in the active site in the N-terminus (Sheehan et al., 2001) While in the omega class enzymes, the active site contains cysteine which forms a mixed disulphide bond with GSH (Sheehan et al., 2001).

Nomenclature of Glutathione S-transferases

Glutathione transferases have been assigned the EC number , EC 2.5.1.18, by the Nomenclature Committee of the International Union of Biochemistry, since they are part of the transferases, class number 2, their number starts with a 2. The alternative names for Glutathione transferases are Glutathione S-alkyltransferase, Glutathione S-aryltransferase, S- (hydroxyalkyl) glutathione lyase, and Glutathione S-aralkyltransferase. The nomenclature system for Glutathione S-transferases is a 2-digit number to designate the subunit composition and if the subunits are homodimers of subunits 1 and 2, then they are designated as 1-1 and 1-2, whereas if the subunits

are heterodimers then they are designated as 1-2 (Parkinson, 2001). They are also assigned a capital letter which is determined by what class the particular enzyme is in, A (alpha), M (mu), P (pi), T (theta), K (kappa), O (omega), S (sigma), D (delta), B (beta) and Z (zeta) (Parkinson, 2001). After the letter abbreviations, Arabic numerals are then assigned to designate the different gene products, therefore in dimeric GST isoenzymes, there is a single letter suffix followed by the hyphenated number to signify each of the 2 subunits (Hayes et al., 1995). Glutathione transferases are dimers that have been sequenced from many organisms ranging from bacteria to humans. Classification has been confounded by differential nomenclature but now is standardized into 10 classes.

Class of Glutathione S-transferase	**Organisms found in:**
Alpha (A)	Mammals – human, rabbit, mouse, rat, and chicken (Buetler, 1992)
Beta (B)	Insects
Delta (D)	Referred to as class I GSTs in insects, *D. melanogaster*, *Musca domestica*, *Lucilla cuprina*, *Manduca sexta*, and *Anopheles gambiae* (Lougarre, 1999)
Kappa (K)	Mammals
Mu (M)	Mammals – human, rat, hamster, mouse, guinea pig, *S. japonicum*, *S. mansoni*, *F. hepatica* (Buetler, 1992)
Omega (O)	Referred to as class II GSTs in insects, mammals
Pi (P)	Mammals – human, mouse, rat, bovine, pig, *L. elegans*, salmonids, *Bufo bufo* embryo, mollusks, *Corbicula fluminea* (Buetler, 1992, Fitzpatrick, 1995, Vidal, 2002)
Sigma (S)	Referred to as class II GSTs in insects, mammals, mollusk lens, octopus, and squid lens (Buetler, 1992)
Theta (T)	Referred to as class II GSTs in insects, mammals, yeast, e-coli, bacteria, and plants (Buetler, 1992)
Zeta (Z)	Referred to as class II GSTs in insects, mammals

Table 1: Classification of glutathione S-transferases and what organisms they are found in.

Activity Sites of Different Classes

The subunits of glutathione S-transferases have two active sites, a G-site where the glutathione binds and a H-site where the second substrate binds (Hayes et al., 1995). In the H-site there is a small number of residues which determine substrate specificity (Hayes et al., 1995). Alpha, mu, pi, sigma, and theta have similar activity sites in each subunit, which has two domains, one smaller N-terminal alpha/Beta domain and one larger alpha domain. The smaller domain contains most of the amino

acids of the G-site and the larger domain contains most of the amino acids of the H site. In the alpha, mu, pi, and sigma classes, the G-site is facilitated by a conserved tyrosine, while in the theta class; a serine residue facilitates the G-site (Hayes et al., 1995).

Invertebrate Glutathione S-transferases

In invertebrates, glutathione S-transferase activity towards many different substrates has been found in whole animal homogenates, abdominal homogenates, fat, body, and gut preparations (Tate et al., 1978). Most of the research on GSTs in invertebrates has involved insects. The nature of GSTs may vary from strain to strain of an insect (Clark et al., 1982). Insect GSTs have been classified as theta class due to sequence comparison (Yuen et al., 2001). Two classes of insect GSTs have been identified, class I and class II; they are put into these classes depending on their amino acid sequence homology (>40% identity to other members within the class) and immunological properties (Pongjaroenkit et al., 2001; Yuen et al., 2001). The GSTs are also classified into these two classes by their molecular weights, immunological cross-reactivites, primary sequences and cDNA from gene encoding (Lougarre et al., 1999). Insect class I GSTs are considered part of the delta class, while the class II GSTs are part of the omega/ zeta/ sigma/ theta class (Sheehan et al., 2001).

Multiple GSTs have been found in the fruit fly, *Drosophila melanogaster*, earthworm, *Eisenia foetida*, American cockroach, *Periplaneta Americana* and the housefly, *Musca domestica* (Baldwin et al., 1996; LeBlanc et al., 1987). Class I GSTs have been cloned in several insects, *D. melanogaster*, *M. domestica*, *Lucilla cuprina*,

Manduca sexta, and *Anopheles gambiae* (Lougarre et al., 1999). At least 2 classes of GSTs, both immunologically distinct and containing different molecular weights, have been identified in *D. melanogaster* and *M. domestica* (Baldwin et al., 1996). All the glutathione s-transferases that have been identified in invertebrates have been dimeric (LeBlanc et al., 1987). The molecular weights of this enzyme that have been found in invertebrates have been found to be from 35,000 - 47,000 (LeBlanc et al., 1987). Different studies have shown that there is a wide variation in GST activity in insect species within a group of related organisms (Egaas et al., 1992).

Gammarus italicus, an aquatic macroinvertebrate, was shown to have at least two major isoforms GST II and GST III, one was related to the mammalian pi class immunologically, while the other one showed that it is unrelated to alpha, mu or pi class (Aceto et al., 1991). GST II was found to be a single band with a molecular weight of 28 kDa and has the same antigenic determinant as GSTs' in the pi class, while GST III was isolated as a heterodimeric with subunits that had molecular weights of 27 kDa and 28 kDa (Aceto et al., 1991). *G. italicus* showed a high activity level of glutathione when 1-chloro-2,4-dinitrobenzene (CDNB) was the substrate (Aceto et al., 1991).

Glutathione S-transferases have been isolated in the freshwater clam, *Corbicula fluminea* (Vidal et al., 2002). Three major isoenzymes isolated were GSTc1, which was 64 kDa, GSTc2, which was 55 kDa, and GSTc3, which was 45 kDa, and several subunits were also isolated (Vidal et al., 2002). All three of these major proteins were shown to be more similar to the pi class after the dot-blot analysis (Vidal et al., 2002). The mollusks are an important organism to compare to

Daphnia since they are filter feeders just like *D. magna*. *C. fluminea* are filter feeders, therefore they bioaccumulate contaminants since they filter large amounts of water for nutritional and respiratory requirements (Vidal et al., 2002).

Chironomid larvae's glutathione S-transferases contain 5 subunits and they all possess the same substrate specificity (Yuen et al., 2001). Isoforms II, IV, and V are homodimers, while subunits III and IV could be monomers of same subunit (Yuen et al., 2001). The dimeric weight of the GSTs in Chironomid is 46kDa, which is close to the molecular weight of mammalian GSTs (Yuen et al., 2001).

Glutathione S-transferases have been found in many different insects. Six major GSTs were isolated from the Hebrew character moth, *O. gothica* (Egaas, 2000). These GSTs were separated out into two classes, GST1, which was acidic, and GST2, which was basic (Egaas, 2000). Ten members of the delta class GSTs and 1 member of the epsilon class cluster have been cloned in *Drosophila melanogaster* (Sawicki et al., 2003). The subunits of termites are between 25,900 and 26,800 and the cystolic termite GSTs display broad-based activities, activities toward substrates (Haritos, 1996).

Assays for Glutathione S-transferase Activity

There are 12 model substrates used for analyses of glutathione s-transferase activity and they are 1-chloro-2, 4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ETHA), p-nitrobenzyl chloride (PNBC), 1,2-epoxy-3- (p-nitrophenoxy) propane (ENPP), bromosulfophthalein, 1-menaphthyl sulfate, 4-nitrophenylacetate, 4-nitrophenylbromide, trans-4-phenyl-3-buten-2-one,

styrene-7,8-oxide, and cumene hydroperoxide (Stenersen et al., 1987; Hoarau et al., 2001; Petrivalsky et al., 1997; Hayes et al., 1995). CDNB is known as the universal substrate. DCNB and CDNB are substrates used to determine glutathione s-transferase activity towards an aryl substrate. While p-nitrobenzyl chloride is the substrate used to determine glutathione s-transferase activity towards aralkyl substrate (Tate et al., 1978). 1,2-epoxy-3- (p-nitrophenoxy) propane is the substrate used to determine glutathione s-transferase activity towards the epoxide substrate (Tate et al., 1978). The glutathione S-transferase activity in the blue crab, *Callinectes sapidus*, has been found when using aryl, aralkyl, and epoxide substrates in samples from the hepatopancreas, gill and excretory gland (Tate et al., 1978).

Animals from the following phyla possess glutathione transferase activity; Platyhelminthes, Annelida, Chelicerta, Ciliophora, Porifera, Cnidaria, Mollusca, Crustacea, Uniramia, Echinodermata, Tunicata, and Vertebrata (Stenersen et al., 1987). All of those animals had glutathione transferase activity towards CDNB with one exception, the silica sponge (Stenersen et al., 1987). All phyla displayed activity towards ETHA even though many species did not have any activity or had low activity (Stenersen et al., 1987). Most of the organisms had low activity when DCNB was used as a substrate (Stenersen et al., 1987). Most aquatic organisms lack the ability to conjugate DCNB; DCNB may reflect better fitness to terrestrial life (Stenersen et al., 1987). The common mussel and the cock, *Gallus gallus* displayed the highest glutathione activity among all the organisms (Stenersen et al., 1987). On average, animal cells have similar amounts of transferase proteins (Stenersen et al., 1987). The pi class GST enzymes were found to conjugate PNBC and ETHA in trout

liver (Petrivalsky et al., 1997). The theta class enzymes were found to conjugate ENPP in mammals (Petrivalsky et al., 1997).

Induction of Glutathione S-transferases

The induction of glutathione S-transferases is an important defense mechanism in an organism when tolerating chemical exposure (Baldwin et al., 1996). Glutathione S-transferase activity is induced in mammals, insects and fish from exposure to certain organic compounds (LeBlanc et al., 1985). Glutathione S-transferase activity has been shown to be induced not only by biochemical stresses but also by physical stresses in the spruce budworm, *Choristonerus fumiferana* (Feng et al., 2001). It has been shown that mammalian isozymes have been induced by xenobiotics by the transcription factors acting on specific DNA sequence in promoter regions (Yuen et al., 2001). Some studies have shown that dietary factors and pesticides affect the induction of Glutathione S-transferases (Egaas, 2000). In the Hebrew character moth, *Orthosia gothica*, diet and pesticides did change the GST activities that occurred in the midgut when compared with the control (Egaas, 2000). There was a 10-fold difference in glutathione activity with DCNB in fall armyworms when they were fed on different natural foods (Stenersen et al., 1987). Age, although not dramatically has been shown to influence the amount of glutathione transferase activity in the mouse, rat, mosquito, cabbage fly, mealworm, and pine weevil (Stenersen et al., 1987). Enzyme preparation and method of determination of activity influences the results of specific activity of a given species (Stenersen et al., 1987).

It has been shown that when an organism is in an environment where a chemical is present, the glutathione S-transferase activity increases (Horau et al., 2001). In the liver of the rainbow trout it was shown that when the liver was treated with TCDD, phenobarbital, NO or the highest dose of p, p'DDE the microsomal GST activity was increased (Petrivalsky et al., 1997). When the xenobiotics are metabolized, they create reactive oxygen species that modify the sulfhydryl group of GST cysteine in mammals and this increases the activity of the microsomal GST (Petrivalsky et al., 1997). When analyzing the liver of the Brown Bullhead, it was found that ethoxyquin, which is an antioxidant compound, significantly (2.8 times) increased the activity of glutathione S-transferases (Henson et al, 2001).

Amino Acid Sequences of Glutathione S-transferases

Most amino acid sequences of glutathione S-transferases are deduced sequences from the genome project. The N-terminal sequence of the amino acid sequence of GSTs have been found in Chironomidae larvae, African malaria mosquito, German cockroach, house fly, pig roundworm, *Onchocerca volvulus*, *Caenorhabditis elegans*, *Dermatophagoides pteronyssinus*, *Orcyctolagus cuniculus*, Sloane's squid s-crystalline, Norway rat, and the house mouse (Yuen et al., 2001). These amino acid sequences have been highly conserved when compared with each other (Yuen et al., 2001). The N-terminal amino acid sequence appears to give sufficient evidence for classification of animal GSTs (Mannervik et al., 1988). The homologies of the amino acid sequence within a class are significant and are more

obvious than the similarities between sequences from different classes (Mannervik et al., 1988).

A complete class I GST gene was identified from the genomic library of *Anophales dires* (Pongjaroenkit et al., 2001). This gene structure contains coding sequence for *Anopheles dires* is similar to a sequence from *An. gambiae* (Pongjaroenkit et al., 2001). The putative promoters and possible regulatory elements of this conserved anopheline class I GST gene were identified (Pongjaroenkit et al., 2001). The genomic organizations of insect class I GST have been repeated previously, such as 8 intronless genes in *D. melanogaster*, fusion forms in *M. domestica* and high conservation and type of gene organization suggests this gene is very important and not changed during the course of evolution (Pongjaroenkit et al., 2001).

D. magna

D. magna is one of the standard organisms used for toxicology tests when analyzing possible effects of chemicals on aquatic systems (Doma, 1979). *Daphnia* are used for toxicity tests because they are easy to culture, they have a short life cycle and they are susceptible to toxic effects of many xenobiotics (LeBlanc et al., 1987). They are small (0.2 - 6 mm) aquatic crustaceans in the kingdom of Animalia, the phylum of Arthopoda, the subphylum of Crustacea, the class of Branchipoda (sometimes called Phyllopoda), the order of Anomopoda, the suborder of Cladocera, and the family of Daphnidae, the genus of *Daphnia* and species of *magna* (Dodson et al., 1991). The organisms in this order are characterized together by having flattened

leaf-like legs which produce water currents which allows the organisms to filter feed (Ebert, 2005). *D. magna* is a zooplankton and is a member of the cladocerans that is usually found in quiet freshwater. Daphniidae are known as the dominant organisms of the plankton of freshwater lakes, with the adults measuring at 5 to 6 mm (Fryer, 1991; Koivisto, 1995).

Daphnids are filter feeders that consume phytoplankton by removing it from the water by using their setose thoracic appendages (http://www.science.lander.edu/psfox/daphnia.html). The filtering mechanism in *D. magna* is found on the third and fourth pairs of the trunk limbs (Fryer, 1991). For filtration to work a particle-bearing current is needed to press through a filter, therefore the pump mechanism needs to be rhythmically operating (Fryer, 1991). Not only are the limbs used for feeding, but they are also involved in respiration (Ebert, 2005). Algae and bacteria are the major prey for *D. magna* (Fryer, 1991). *D. magna* can also survive on feeding off the bottom of the lake by bringing the particles on the bottom of the lake into suspension and then filter out the large particles (Fryer, 1991). *D. magna* purposely swim into the bottom deposits in the standing, water and they are able to forage on the bottom since they have a heavy carapace (Fryer, 1991). When feeding, the water continuously flows between the valves of the *daphnia* by the movement of the setose-latent thoracic legs (Ebert, 2005). The food particles are then collected in the median ventral groove at the base of the legs (Ebert, 2005). For the most part, *D. magna* can survive on suspended particles alone (Fryer, 1991). As it can be imagined, *D. magna* also ingest indigestible compounds, such as inorganic particles (Fryer, 1991).

Fryer (1991) has described morphological and physiological aspects of *D. magna* that will be presented here. Swimming is essential in the life of the daphnids, so therefore they have large arborscent antennae armed with long natatory setae that act as oars in the swimming process. *D. magna* have a large heavily built carapace. *D. magna* are strong swimmers and if they are located in deep water with a rich food supply then the daphnids are always in suspension. *D. magna* can tolerate and can be found in aquatic environments that are organically enriched or have high salinity or diverse ionic compositions (Fryer, 1991).

The life cycle of *D. magna* is cyclic parthenogenesis and the population is usually females in non-stressed time and therefore the reproduction is asexual (Doma, 1979). Cyclic parthenogenesis means that both asexual parthenogenesis and gametogenesis maximizes population fitness (Doma, 1979). In cyclic parthenogenesis, two different eggs are produced, a diploid egg and a haploid egg that needs sexual fertilization to develop (Barker et al., 1986). When the population becomes stressed, the daphnid's survival is threatened and then sexual reproduction prevails and males are introduced. A population becomes stressed when the population density becomes too high or if there is a shortage of food or if there is poor water quality or extreme temperatures. During sexual reproduction, a resting egg, ephipia is produced and this egg then "hatches" when the environmental conditions get better, therefore one resting egg can actually start a new population of daphnids (Fryer, 1991). Each brood chamber of the daphnid holds 6-10 eggs, which then turn into embryos and are released within a few days (Ebert, 2005). The dorsal brood pouch is separated from the carapace chamber, which holds the trunk limbs and

the post-abdomen, these two are separate because the feeding mechanism would stop if water leaked in the brood chamber (Fryer, 1991).

A *D. magna* neonate may molt up to seven times before it becomes a primipare, the stage when the daphnid initially produces eggs (Ebert, 2005; Dodson et al., 1991). The stage of *D. magna* between molts is referred to as instar, which lasts for 3 to 4 days (Dodson et al., 1991; Barata et al., 2000). Molting is extremely important in the development of the organism, since with every molt, the juvenile doubles it's volume and increases it's length by approximately 1.26 (Dodson et al., 1991). Adults do molt, but as the organism becomes older, the growth slows down (Dodson et al, 1991).

Glutathione S-transferases and D. magna

Glutathione S-transferases have been found in *D. magna* by different researchers. It has been shown that glutathione S-transferases are accountable for the variability of results of toxicology tests in *D. magna* (LeBlanc et al., 1985). Variability has been observed during repeated acute toxicity tests with a single toxicant when using daphnids as test organism, this may show that there is variability in the chemical detoxification capabilities of *D. magna* (LeBlanc et al., 1985). There was GST activity in *D. magna* towards substrates 1-chloro-2, 4-dinitrobenzene (CDNB) and ethacrynic acid (LeBlanc et al., 1987).

Dierickx and others found 5 major GST isoenzymes when they analyzed *D. magna* for glutathione S-transferase (Dierickx, 1987). Of these 5 major isoenzymes, 4 were anionic and one was neutral, the isoelectric points of these enzymes were 7.12

- 9.51 (Dierickx, 1987). Minor forms of GST were also found and they were cationic (Dierickx, 1987). The neutral isoenzyme had the highest specific activity when they were tested for specific glutathione S-transferase activity (Dierickx, 1987). It was found in the study by Dierickx that the GST composition in the water flea is comparable to that in mammals, since most of the activity is found in cationic isoenzymes and the minor portion is anionic and neutral isoenzymes (Dierickx, 1987). The GST isoenzymes of *D. magna* were inhibited by 2, 4-dichlorophenoxyacetic acid and 1, 4-benzoquinone, just the same as the GST isoenzymes in rat liver (Dierickx, 1987). The reason for multiple GSTs in an organism is because glutathione S-transferases are so ubiquitous and they conjugate numerous types of substances which differ in electrophilic center and their molecular structure so therefore more than one GST is needed to be able to bind with certain types of compounds (Dierickx, 1987; LeBlanc et al., 1987). The study by Dierickx showed that *D. magna* exhibited isoenzyme - specific induction and inhibition, which suggests functional diversity within the GSTs found in *D. magna* (Baldwin et al., 1996).

Baldwin, et al., (1996) found that there are at least 6 GSTs in *D. magna*, which are distinguished with isoelectric points, 4.6, 4.7, 4.8, and 5.6 (Baldwin et al., 1996). They also found that there were 3 other proteins with similar molecular weights to GST subunits that were bound on the glutathione sepharose column (Baldwin et al., 1996). So in the end Baldwin and others found 7 different proteins composed of at least 5 different subunits with molecular weights between 26.9 and 30.2 kDa (Baldwin et al., 1996). Three of the GSTs have similar isoelectric points

and similar molecular weight and similar immunorecognition, which suggest that these 3 GSTs represent a single class of GSTs (Baldwin, et al., 1996). The GST with the isoelectric point of 5.6 can be distinguished from more anionic GSTs based upon the immunoreactivity and the isoelectric point and therefore represent a different class of GSTs (Baldwin, et al., 1996). In 1987, LeBlanc, and others found that 6 proteins in *D. magna* exhibit GST activity (LeBlanc, et al., 1987). When they analyzed the samples by electrophoresis under non-denaturing conditions they identified 6 native protein species, one with molecular weight of 55,000, one with molecular weight of 57,000, three with molecular weight of 58,900 and one with a molecular weight of 61,700 (LeBlanc, et al., 1987). These native proteins are twice the size of denatured enzymes and composed of homo-dimer or hetero-dimer combinations of the 3 subunits (LeBlanc, et al., 1987). The molecular weights of the *D. magna* subunits are similar to the molecular weights of the mammalian subunits than other invertebrates. Mammalian enzymes range from 50,000 to 56,000 MW, other invertebrates' subunits range from 35,000 to 47,000, and the *D. magna* range from 55,000 to 61,700 (LeBlanc, et al., 1987). It has been found that all *D. magna* isoforms are acidic (LeBlanc, et al., 1987).

The fact that when separated into different ammonium sulfate fractions when run on SDS-PAGE proves that there is an existence of multiple enzymes (LeBlanc, et al., 1987). So far the composition of the subunits of GSTs in *D. magna* have not been identified (Baldwin, et al., 1996). It can be seen for the study that Baldwin and others ran, that there are 2 distinct classes of GSTs in *D. magna* (Baldwin, et al., 1996). The amino acid sequence of the individual GSTs found in *D. magna* will have

to be determined to further substantiate the GST class diversity and identify the class distinctions of GSTs in daphnids (Baldwin, et al., 1996).

Pharmaceuticals in the Environment

In recent years, pharmaceuticals of all different types have been found in the aquatic environment. Since pharmaceuticals are chemicals that are manufactured to cause a biological effect, there is a concern for what these drugs are doing to animals in the environment (Sanderson et al., 2004). Neither sewage treatment plants nor drinking water treatment plants actually treat the water for pharmaceuticals, therefore they may slip by and may be found back in the environment or in the drinking water. Clofibrate is one such pharmaceutical which has been found in drinking water, sewage treatment plant effluent, and ground water (Heberer, 2002). Clofibrate was shown to increase reactive oxygen species (ROS) production in fish cells after being treated for 4 hours (Laville et al., 2004). Superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^.$) are examples of reactive oxygen species (Barata et al., 2005).

Chapter 3: Materials and Methods

In this study, the specific activities of glutathione S-transferases in *D. magna* were determined among different age groups of *D. magna*, among different treatments of clofibrate and among presence or absence of eggs in the brood chamber. Mixed age samples of *D. magna* were also tested for specific activity of glutathione S-

transferases and then purified using affinity chromatography. After the sample was purified and then tested again for GST specific activity, a SDS-Polyacrylamide electrophoresis gel was run with a protein ladder to determine the molecular weight of the protein.

Reagents

Reduced glutathione, glutathione-agarose, and clofibrate (2-(4-Chlorophenoxy)-2-methylpropionic acid ethyl ester) were purchased from Sigma. The protein ladder, which included Myosin H-chain, Phosphorylase B, BSA, ovalbumin, carbonic acid, B-lactoglobin, and lysozyme, was purchased from Gibco BRL. All other chemicals were reagent grade and purchased from Fisher and Baker.

D. magna cultures

D. magna was obtained from Carolina Biological. The population of daphnids used has been cultured at the University of Maryland for four years. The aquariums were kept in 20° - 22° Celsius incubators with a 16:8 hour light: dark photoperiod. *D. magna* were grown in twelve 10-gallon aquariums and 10 liters of synthetic water was used as the culture medium. The standard synthetic water was made up of 10 L of deionized water, 96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4$ $2H_2O$, 60mg/L $MgSO_4$, and 4 mg/L KOH, this recipe was from Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms an U.S. Environmental Protection Agency report. The *Daphnia* were fed blended tetrafin^{TM} goldfish food and the solution was 5g of tetrafin^{TM} to 500 ml of

deionized water, which is an adaptation from Baldwin and others (1996). When the aquariums were just cleaned and re-filled, 40 ml of food was given to the daphnia cultures in each aquarium. After the initial feeding, 20 ml of food was given every three days.

Age Dependent Experiment

For the experiments where specific ages of *D. magna* were needed, small 50 ml beakers with 30 ml of culture medium were used. In each of the beakers, one adult daphnid was placed and fed one ml of the food described above every other day. The containers were checked everyday for neonates. These new *D. magna* were kept in 500 ml containers until they were used in the assay. When they were used, they were homogenized in microfuge tubes with a manual homogenizer on ice in 200 ul or 1:4 wet weight/ buffer volume ratio, whichever was greater, of .01 M HEPES buffer pH of 7.4 containing 1 mM EDTA and 10% glycerin (Baldwin et al., 1996; Barata et al., 2005). The sample was then centrifuged in the refrigerator at 4°C at 14,000 x g for 20 minutes. After centrifugation, the supernatant was pipetted from the pellet and the supernatant was stored on ice until the glutathione S-transferase assay was performed that day.

Clofibrate Induction Experiments

D. magna that were 12 +/- 2 days old were used in the clofibrate induction experiments. The *Daphnia*, 30 of them, were placed in 150 ml of the appropriate concentration of clofibrate, control – EPA media, control plus 0.2% ethanol, 0.01

mg/L clofibrate, 0.05 mg/L clofibrate, 0.08 mg/L clofibrate, or 0.10 mg/L clofibrate. The clofibrate solutions were made in the ASTM media with 0.2% ethanol, which helped dissolve the clofibrate into solution (Nunes et al., 2004). The daphnids were in the appropriate media for 48 hours at 20°C in a 16:8 hour light to dark photoperiod. After the 48 hours, the *D. magna* were homogenized by the same process as used in the age experiments. The homogenized sample was centrifuged at 14,000 x g for 20 minutes at 4°C. Again after centrifugation, the supernatant was pipetted from the pellet and the supernatant was stored on ice until the glutathione S-transferase assay was performed that day.

Mixed Age Samples

The samples that were used to be purified were taken right out of the 10 gallon aquarium and were homogenized in a 1:4 wet weight/ buffer volume ratio of the homogenization buffer used by Baldwin and others (1996). These samples were centrifuged the same way as the other samples to prepare for the kinetic assay of glutathione S-transferase.

Glutathione S-transferase Specific Activity Assay

The supernatant was collected, and then the glutathione S-transferase specific activity was determined by a kinetic assay, following the procedure of Grant and others (1989) with some adaptations. Using a 96 well plate, 200 ul of the substrate buffer, 40 ul of 3.5 mM CDNB, and 5 ul of the *Daphnia* supernatant is added to each well. The substrate buffer was made by adding 75 ml glycerol (93.75g) and 6.81

grams KH_2PO_4 to 400 ml deionized water and pH was then adjusted to 6.8 and the volume was then brought to 500 ml. After the plate has reached room temperature (15 minutes), 5 ul of 5 mM GSH, which was made with sodium phosphate buffer as used by Habig and others (1974), was added and then the plate was immediately read in a spectrophotometer at 340 nm, using the kinetic automix feature, and read every 20 seconds for 10 minutes. The measurement of absorbance is due to the conjugation of CDNB with glutathione, which is catalyzed by glutathione S-transferases, to produce a thioether which can be read on the spectrophotometer at the wavelength of 340 nm (Nunes et al., 2006). Protein concentrations of the samples were determined by the method that Bradford established in 1976 with bovine serum albumin as the standard. The concentrations of bovine serum albumin that were used were 0.01 mg/ml, 0.025 mg/ml, 0.050 mg/ml, 0.075 mg/ml, and 0.1 mg/ml. 20 ul of the sample, 40 ul of the Bradford reagent, and 140 ul of deionized water were added to each well in a 96 well plate and were measured on the spectrophotometer at a wavelength of 595 nm. The activity was calculated by using the extinction coefficient of 5.3 based on well volume and the OD amount from the spectrophotometer. An activity unit is the amount of enzyme catalyzing the formation of 1 μmole of product per minute, while the specific activity is the units of enzyme activity per mg of protein as measured by Bradford with BSA as a standard (Habig et al., 1974; Bradford, 1976). Specific activity was calculated by dividing the activity by the total protein, which was found using Bradford reagent.

Glutathione-agarose affinity chromatography

Affinity chromatography was performed on a glutathione-agarose column following the procedure of Simons and Van der Jagt (1981) and Sigma. The glutathione-agarose column used was 1.5 x 20 cm prepared using lyophilized powder of glutathione-agarose which was swelled in 200 ml deionized water/gram of lyophilized powder. Once the swelling occurs, which can be from 30 minutes until the next day, the agarose beads were washed with 10 volumes of water and then poured into the column. After the resin has been poured into the column, wash the column with several column volumes of equilibration buffer. The equilibration buffer used was .01 M HEPES buffer, 1 mM EDTA, at pH of 7.4 (Baldwin, 1996). The sample supernatant was centrifuged at 10,000-x g at 4°C, it is important that the cell supernatant is clear before it is loaded onto the column. Following Sigma protocol, the clear supernatant was loaded onto the column under gravity flow and to improve binding efficiency, the sample was passed through the column 3 more times. The resin was then washed with PBS-T (Phosphate buffered saline: 10 mM phosphate buffer pH 7.4, 150 mM NaCl with 1% Triton X-100, as suggested from Sigma) at 4°C, 4 times. This is to ensure that all non-GST proteins are out of the column. The sample was then eluted into 3 fractions of 1 ml each of elution buffer which was 8mM reduced glutathione in 50 mM Tris pH 9.6. The fractions were then placed into dialysis tubing and put into PBS for 24 hours changing the buffer twice, to remove the free glutathione.

Glutathione S-transferase assay and protein concentration determination was re-run on the sample to determine how much specific activity is in the purified sample as described above.

SDS-PAGE Electrophoresis

The purified and active fraction of glutathione S-transferase was run on the discontinuous SDS-PAGE electrophoresis gel according to the procedure Laemmli described in 1970. The gel was prepared in the EC120 Mini Vertical Gel System by Thermo EC. The gel contained 10% bisacrylamide cross linking which is suitable for proteins from 15 to 150 kDa (Makowsky et al., 1997). Since this was a discontinuous gel, the resolving gel was set first and then the stacking gel was poured. The samples were mixed with the same volume of sample buffer which includes bromphenol blue indicator dye and heated for a few minutes in boiling water. The samples were centrifuged at 12,000 x g for two to five minutes prior to loading on the gel. 10 ul of the sample were loaded into the bottom of the well with a micro liter syringe. A protein ladder was run at the same time that consisted of, Myosin H-chain, Phosphorylase B, BSA, ovalbumin, carbonic anhydrase, B-lactoglobin, and lysozyme. Once the apparatus was set up, the electrophoresis started at 100-125 V, but once the dye moved into the stacking gel, the voltage increased to 200 V. Gel runs typically took one hour, and then the gel was removed and stained with 200 ml of coomasie blue and then destained with destaining solution. The gel was then stored in 7% acetic acid.

Statistical Analysis

When comparing two treatments to determine if there was a statistical difference, the student's t-test was used. When comparing more than two treatments to determine if there was a statistical difference, Analysis of Variance (one-way ANOVA) was performed. These statistical analyses were done on ORIGIN LAB version 7.5. Statistical differences were acknowledged at $P<0.05$.

Chapter 4: Results

Comparison of D. magna with eggs in their brood chambers and without eggs in their brood chambers

D. magna adult females with eggs in their brood chambers were compared with *D. magna* adult females with empty brood chambers to determine if there is a difference in the specific activity of GSTs at this important physiological point of the daphnids' life. See figure 1 for pictures of adult female *D. magna* with eggs in the brood chamber and without eggs in the brood chamber. There was a statistical difference when the data was analyzed with the student's t-test (t-value = -14.0, df =9, P = 0.00001). The specific activity of glutathione S-transferase in the female *D. magna* that have eggs in their brood chamber was much greater than the specific activity of glutathione S-transferase in the female *D. magna* that had empty brood chambers as can be seen in Figure 2.

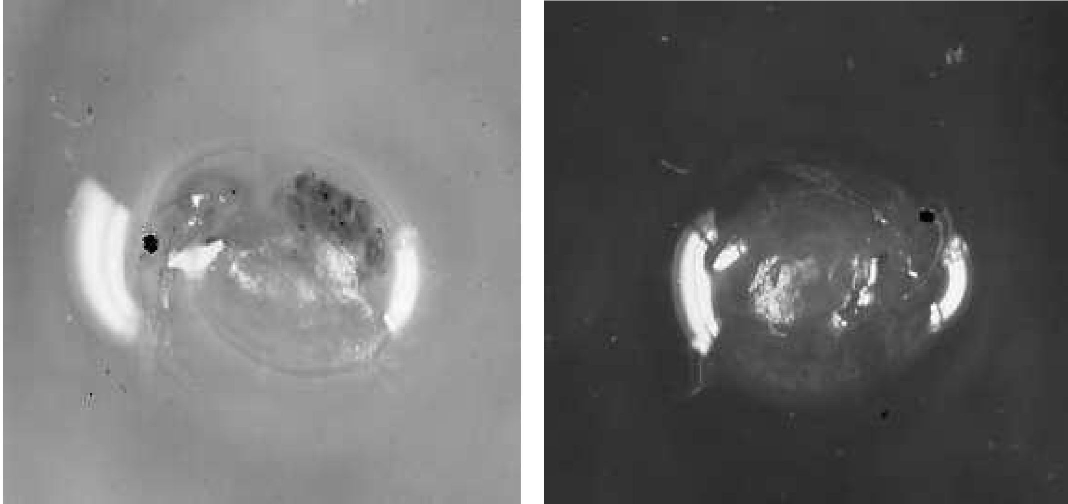

Figure 1: In the picture on the left is a adult female *D. magna* with eggs in her brood chamber while the picture on the right is an adult female with an empty brood chamber.

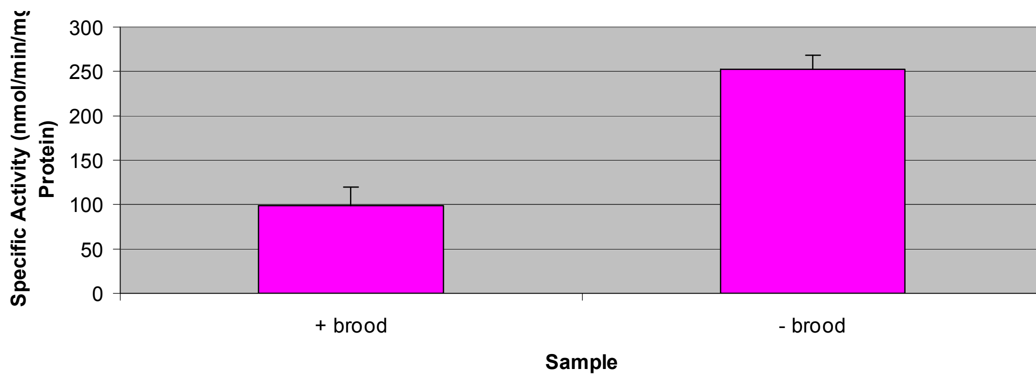

Figure 2: Comparison of specific activity of glutathione S-transferase in *Daphnia magna* with and without eggs in their brood chambers

Clofibrate Induction Experiments

Another test that was done in this study was to determine if clofibrate, a pharmaceutical found in the environment, alters glutathione S-transferases in *D. magna*. This test was done on six different treatments, control (ASTM media), control plus 0.2% ethanol, 0.01 mg/l clofibrate, 0.05 mg/l clofibrate, 0.08 mg/l clofibrate, and 0.10 mg/l clofibrate (see Figure 3). An ANOVA analysis was done on

the six different treatments, and the null hypothesis was accepted (One-Way ANOVA: F=0.936, model df = 5, error df = 59, P = 0.46), which was that there is no difference between the specific activity of glutathione S-transferase of the controls and of the different concentrations of clofibrate. Clofibrate neither induced nor inhibited glutathione S-transferase activity.

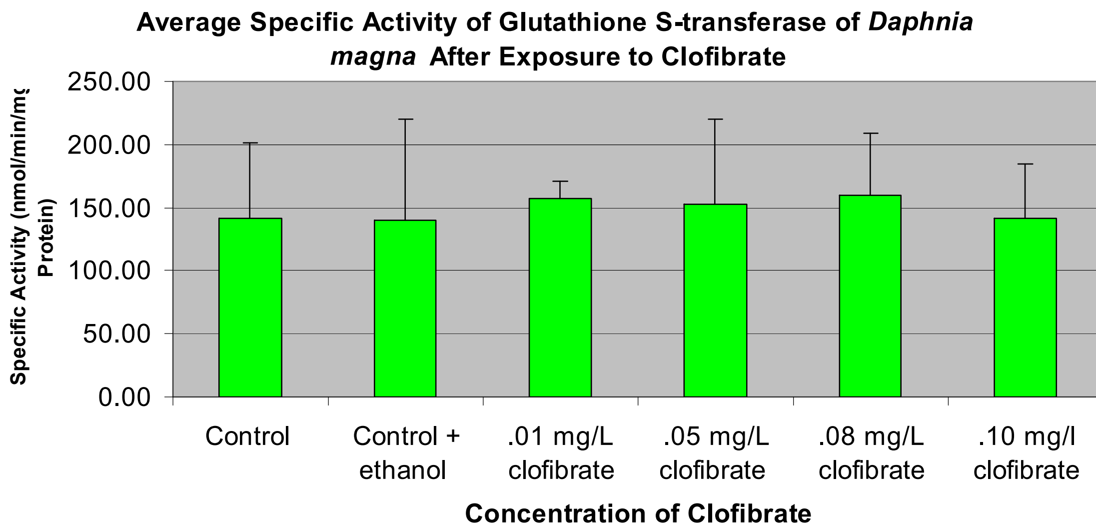

Figure 3: Specific Activity of Glutathione S-transferases in *D. magna* after exposure to clofibrate.

Age Dependent Experiment

Different ages of *D. magna* were tested for specific activity of glutathione S-transferases in *D. magna*. The ages that were tested were 2 days, 3 days, 6 days, less than 7 days, 8 days, 11 days, 14 days, 15 days, 16 days, 19 days, 21 days, 22 days, 23 days, 24 days, 27 days, 28 days, 33 days, 37 days, 38 days, 40 days, 41 days, and 47 days. This gives a broad range of the life cycle of *D. magna* (See Figure 4). When an ANOVA was done to determine if the age of the *D. magna* made a significant difference in the glutathione S-transferase activity, the null hypothesis was rejected

since there was a statistically significant difference (One-Way ANOVA F=10.02, model df = 21, error df = 118, P = 0.001).

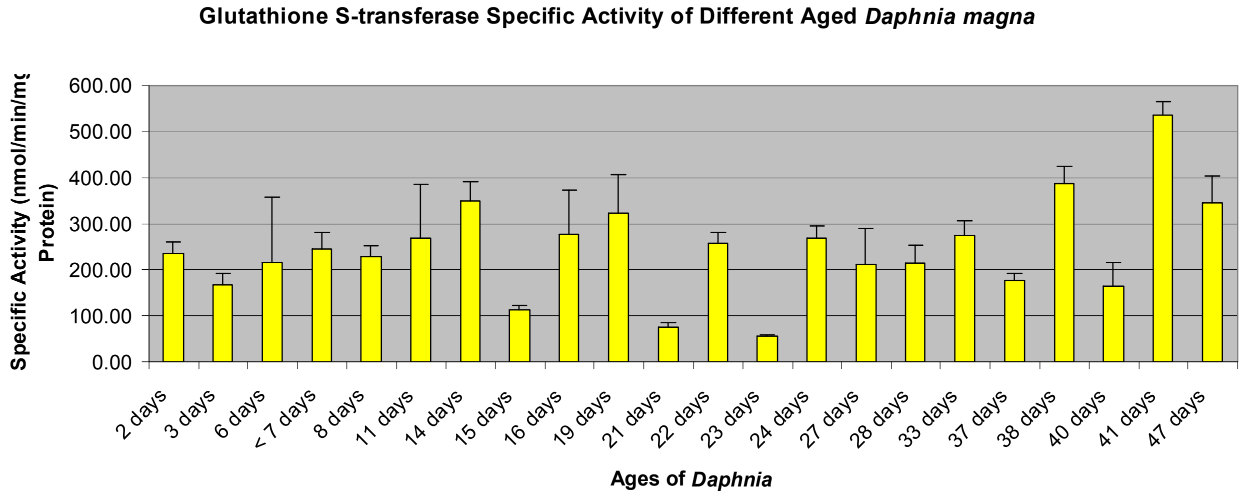

Figure 4: Average Specific Activity of GST of *D. magna* among different age groups

Purified Glutathione-S-transferases Sample

In addition to the comparison tests run on the specific activities of glutathione S-transferases, assays were also performed on mixed age samples of glutathione S-transferase to determine if they did indeed possess glutathione S-transferases (See figure 5). Once that was determined, one of the samples was purified by affinity chromatography (see table 2) and then a denatured SDS-PAGE gel was run to see the molecular weight of the subunits of glutathione S-transferase (See figure 6). As shown in figure 6, the bands show up in the expected range of molecular weights, between 29 kD and 18.4 kD, consistent with previous research done on GSTs in *D. magna*.

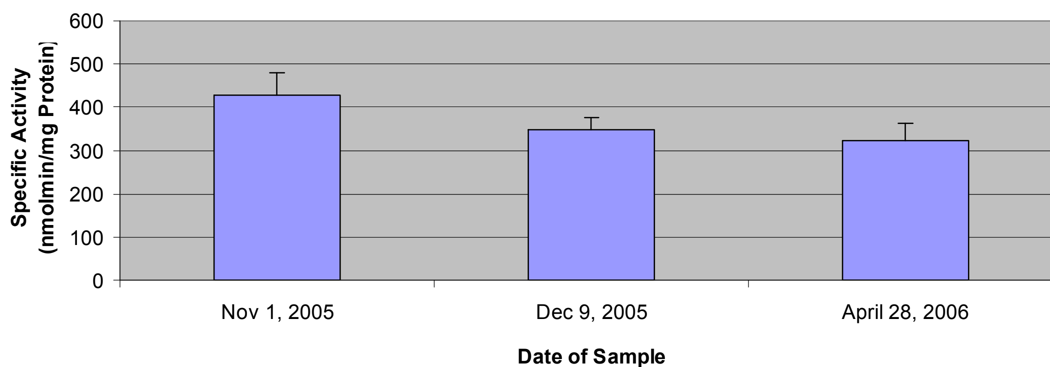

Figure 5: The specific activity of glutathione S-transferases of mixed ages of *D. magna*

Type of Sample	µmol/min/mg +/- SD	nmol/min/mg +/- SD
Mixed Age – Unpurified (Sample 1)	0.13 +/- 0.02	125.79 +/- 21.29
Mixed Age – Purified (Sample 1)	3.19 +/- 0.65	3193 +/- 649
Mixed Age – Unpurified (Sample 2)	0.30 +/- 0.04	324 +/- 39
Mixed Age – Purified (Sample 2)	1.87 +/- 0.42	1871 +/- 417

Table 2: Samples unpurified and then purified by affinity chromatography and their Specific Activity of Glutathione S-transferases.

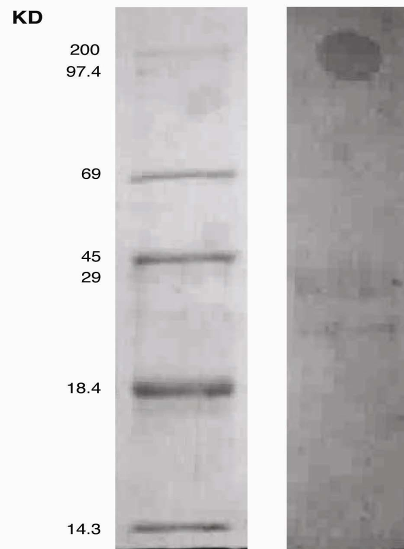

Figure 6: The SDS-PAGE gel, on the left side of figure is the protein ladder, 200 KD is Myosin H-chain, 97.4 KD is Phosphorylase B, 69 KD is bovine serum albumin, 45 KD is ovalbumin, 29 KD is carbonic anhydrase, 18.4 KD is B-lactoglobin, and 14.3 is lysozyme. The column on the right side is the sample of purified GST and there are 2 bands found in the region between Carbonic anhydrase 29 KD and B-lactoglobin 18.4 KD.

Chapter 5: Discussion

In this study, *D. magna* was treated with different concentrations of clofibrate (control, control + 0.2% ethanol, 0.01 mg/l, 0.05 mg/l, 0.08 mg/l, and 0.1 mg/l) for 48 hours and then tested for glutathione S-transferase to determine if clofibrate alters the activity of glutathione S-transferases. The clofibrate concentrations were chosen after an acute toxicity test was done to determine the highest concentration of clofibrate that the *D. magna* would survive in for 48 hours. According to the results it does not statistically significantly induce glutathione S-transferase but nor does it inhibit it when compared to the control data. When inspecting the data, 0.01 mg/L, 0.05 mg/L, and 0.08 mg/L clofibrate appears to induce activity of glutathione S-transferase a

little more than the controls or 0.10 mg/L clofibrate, but the result was not statistically significant.

Many different chemicals have been tested on *D. magna* to determine if glutathione S-transferases are inhibited or induced (Barata et al., 2005; LeBlanc et al., 1987; and LeBlanc et al., 1985). It is important to know that a pharmaceutical that is found in the aquatic environment does not alter one of the mechanisms that the organism uses to detoxify xenobiotics. When the chemicals in question inhibit the detoxifying enzymes then that could have deleterious effects on the organism. Since this is the first study to test clofibrate on glutathione S-transferase specific activity in *D. magna*, it is important to know that it does not inhibit this enzymatic activity.

Clofibrate activates peroxisome proliferator activating receptors when the drug is used on the target organism that it is prescribed (Nunes et al., 2004). In a study by Jones and Neill, it was found that there was a correlation between treatment of clofibrate to mice and the induction of peroxisome proliferation in mice (Nunes et al., 2004). Peroxisome proliferators increase oxidative stress and DNA damage (Cajaraville et al., 2003). This pharmaceutical causes oxidative stress which causes the organism to enhance the production of ROS which in turn increases the organism's antioxidant defenses, one of which is glutathione S-transferase (Barata et al., 2005).

Nunes and others (2004) tested clofibrate on the mosquitoefish, *Gambusia holbrooki*, to determine it's effects on the enzymes, acetylcholinesterase (AChE), lactate dehydrogenaes (LDH) and catalase (CAT). In the acute tests of clofibrate, clofibrate significantly decreased CAT activity (Nunes et al., 2004). Sea bass,

Dicentrarchus labrax, was treated with 70 mg/kg clofibrate for two weeks and glutathione S-transferase was induced in the liver of the sea bass that were treated with clofibrate (Pretti et al., 1999). *Artemia parthenogenetica*, a hypersaline crustacean, was treated with clofibrate, among other pharmaceuticals, and was tested for glutathione S-transferase activity when it was found that there was no statistical difference when clofibrate was used (Nunes et al., 2006). There have been studies done that have found antioxidant enzyme responses, CAT, SOD, GST, and oxidative tissue damage in *D. magna* when exposed to UV and different oxygen concentrations (Barata et al., 2005). Barata and others (2005) studied five prooxidative chemicals, menadione, paraquat, endosulfan, cadmium, and copper and their effects on four antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), total glutathione peroxidase (total GPx) and glutathione S-transferase (GST), the enzymes that responded the greatest were total GPx and GST.

In this study, twenty-two different ages of *D. magna* were tested for glutathione S-transferase activity. It was found that there was a statistical significant difference in the specific activity of glutathione S-transferase between the different ages of *D. magna*. There seems to be an age-dependent pattern in the data that the glutathione S-transferase activity is higher in the first two weeks of life for the daphnids and then it decreases for a while and then as the daphnid approaches 38 days it increases again significantly. Neonates and juveniles may have increased amount of specific activity of glutathione S-transferases since they are still developing. In a study that looked at glutathione S-transferases over time in the yellow-fever mosquito, the concentrations increased up to pupal stage which is 9 days

old and then decreased when the mosquitoes hit senescence (Hazelton et al., 1983). The results in this study during the growth of *D. magna* corresponded to the results of the growth of the mosquito, but the results of the aging *Daphnia* did not correspond to the aging mosquito.

It has been found in other studies that the age of the organism does affect the activity of antioxidant enzymes. It was determined that lactate dehydrogenase activity (LDH) did differ in *D. magna* that were seven days old compared to those that were 14 and 21 days old, but that there was variation with the age of *daphnia* (Diamantino et al., 2001).

The glutathione S-transferase specific activity of *D. magna* was compared with females that had eggs in their brood chambers and females that did not have eggs in their brood chambers. It was found that there was a significant statistical difference between the two groups. The specific activity was actually higher in the females with empty brood chambers. Diamantino and others (2001) found that in *D. magna*, there was a significant statistical difference in lactate dehydrogenase activity when comparing *D. magna* with eggs in the brood chamber and those females without eggs in their brood chamber.

Specific activity of glutathione S-transferases were run on the mixed age samples and then one of the mixed age samples was purified by affinity chromatography and run on a SDS-PAGE gel. The mixed age unpurified samples were a little lower than in other studies, in one study done be LeBlanc and others (1985), the specific activity of glutathione S-transferases in *D. magna* were 584 +/- 99 nmoles/min/mg. Glutathione S-transferases were purified from *D. magna* using

affinity chromatography and then the bands of that sample were seen on SDS-polyacrylamide electrophoresis gel between carbonic anhydrase 29 kDa and B-lactoglobin 18.4 kDa, which is consistent with previous studies that have looked at the molecular weights of GSTs in *D. magna* (LeBlanc et al., 1987). Baldwin and others (1996) found 5 different subunits of GSTs between 26.9 and 30.2 kDa.

Chapter 6: Conclusion

Specific activities of glutathione S-transferases were tested under different conditions and at different physiological aspects of the life cycle of *D. magna*. There was a statistical difference in the specific activity of GSTs in different aged *D. magna*. *D. magna* in different reproductive times of the life cycle showed a statistical difference in the specific activity of glutathione S-transferases. These two factors explain why the activity of glutathione S-transferases in *D. magna* may be different among individual organisms. Clofibrate did not statistically alter the specific activity of GSTs in *D. magna*. This is the first time that clofibrate was tested on glutathione S-transferases in *D. magna* and it is important to know that clofibrate, found in the environment, does not inhibit the activity of GSTs.

The purified glutathione S-transferases were found on the SDS-PAGE gel in the expected range between carbonic anhydrase, 29 KD and B-lactoglobin, 18.4 KD. In future research, the molecular weight of the subunits of glutathione S-transferases in *D. magna* will be determined by RP-HPLC.

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