

## AN ABSTRACT OF THE THESIS OF

Tyler S. Norby for the degree of Master of Science in Toxicology presented on June 15, 2007.

Title: Antioxidant Enzyme Response in Rainbow Trout (*Oncorhynchus mykiss*) After Subchronic Exposure to an Environmentally-Relevant Polycyclic Aromatic Hydrocarbon Mixture

Abstract approved:

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Lawrence R. Curtis

Polycyclic aromatic hydrocarbons (PAHs) are significant pollutants in aquatic environments. Many are carcinogenic and lead to DNA fragmentation and adduct formation in marine and freshwater organisms. Previous research demonstrates that rainbow trout (*Oncorhynchus mykiss*) compensate to long-term PAH dietary exposures and reduce the DNA damage.

The aim of this study was to assess whether upregulation of detoxifying/antioxidant enzymes in rainbow trout at least partially explains compensation to PAH exposure that allows for this reduction in DNA damage. Rainbow trout were fed a diet that contained a mixture of nine high molecular weight PAHs in environmentally relevant ratios and concentrations. Two doses were used: a lower dose of 20 parts per million

(ppm) and a higher dose of 200 ppm. Two other treatment formulations were also used containing 20 ppm and 200 ppm concentrations of benzo( $\alpha$ )pyrene. A control group of rainbow trout was also used. 80 rainbow trout per PAH treatment and control were used in this experiment. The fish were fed PAH treatment diets or a control diet daily beginning when their average weight was 20 g. At days 7, 14, 28 and 42 enough fish were sacrificed from each of the four treatment groups plus control to obtain four 1 g pooled liver samples. Body and liver weights were recorded for each sacrificed fish.

Various biomarkers and detoxifying/antioxidant enzyme activities were measured. Lipid peroxidation was estimated for all sample groups using a thiobarbituric acid-reactive substances (TBARS) assay. NAD(P)H:quinone oxidoreductase 1 [DT-diaphorase], also known as quinone reductase (QR), activities were determined for all sample groups using a modified photometric assay that defines QR levels as the dicoumarol-inhibitable reduction of 2,6-dichlorophenolindophenol (DCPIP). Glutathione peroxidase (GPOX) activities were determined for all sample groups using a modified photometric assay that defines GPOX levels as the reduction of NADPH in cytosolic liver fractions. Catalase (CAT) activities were determined for all sample groups using an assay kit that utilized the reaction CAT has with methanol in the presence of hydrogen peroxide.

CAT showed the strongest significant ( $P < 0.05$ ) response, rising and falling over the time-course of the study in a dose-dependent sinusoidal decay pattern as the system moved toward optimal steady-state activity. GPOX activities increased significantly ( $P < 0.05$ ) over the time-course of treatments, also showing a sinusoidal decay pattern similar to that seen with CAT, although the GPOX activity followed a slower decay pattern than CAT and was not dose-dependent. QR activities were low overall. This indicated that this detoxifying enzyme was not a major component of the trout's adaptive response. The TBARS assay was determined to be an insensitive assay for fish tissues due to the harshness and unspecific nature of the test. The mass-spectroscopy-based  $F_2$ -isoprostanes assay was determined to be a much more appropriate assay for the determination of lipid peroxide levels in fish tissues, based on previous research.

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ANTIOXIDANT ENZYME RESPONSE IN RAINBOW TROUT  
(*ONCORHYNCHUS MYKISS*) AFTER SUBCHRONIC EXPOSURE TO  
AN ENVIRONMENTALLY-RELEVANT POLYCYCLIC AROMATIC  
HYDROCARBON MIXTURE

by  
Tyler S. Norby

A THESIS

submitted to

Oregon State University

in partial fulfillment of  
the requirement for the  
degree of

Master of Science

Presented June 15, 2007  
Commencement June 2008

Master of Science thesis of Tyler S. Norby  
presented on June 15, 2007

APPROVED:

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Tyler S. Norby, Author

## ACKNOWLEDGEMENTS

I, Tyler S. Norby, would like to express my unwavering gratitude towards Dr. Lawrence Curtis for his unmatched guidance, compassion, friendship and, most importantly, patience. I would also like to thank Dr. Michael Burgett and Dr. Joseph Beatty, two men who have been there for me and continually believed in me and my abilities for years, even when I did not. If it were not for these three men, you would not be reading this thesis today.

I would also like to thank two groups of people for helping me through this journey. First is my committee (Dr. Kim Anderson, Dr. Joseph Beatty, Dr. Michael Burgett, Dr. Lawrence Curtis and Dr. Barbara Taylor). Second is my labmates (Claudia Bravo, Junga Lee and Rick Scheri), who have always put up with the fact that I often had no idea what I was doing.

A very special thanks goes out to Tania Porter and Jaylene Seeley, two ladies that were nice enough to always have warm smiles, funny jokes and a plethora of kindness for me whenever I needed a boost. Being the black sheep of the department wasn't easy, but these two ladies always made me feel like black was in fashion.

Thanks to Dr. Juan Trujillo and Oregon State University's Department Foreign Language and Literatures for giving me a job that paid for two out of the four years

of schooling this took on top of being the coolest boss I've ever had. Graduate school is expensive, so this was most appreciated.

Also, thanks go out to Dr. Robert Tanguay and the staff of SARL for handling every aspect of growing and caring for our test fish. Thanks are also due to Dr. William Baird and his lab, Dr. Sam Bennett and his lab and Dr. David Williams and his lab for allowing Dr. Curtis and myself to use their lab equipment whenever we needed it.

Lastly, I would like to thank my family; my best friends, Andy Needham, Ben Rader and Jake Stutevoss; Robert Alberg; Thomas Bass; Terrance Bollea; Jack Burton; Randy Castillo (R.I.P.); Randy Couture; Robert Deal; Lee Jun Fan; Frank Ferrano, Jr.; Warren Griffin; Nate Hale; Lincoln Hawk; Toriano Jackson; Jesse Katsopolis; Ian Kilmister; Carlos Norris; Nico Toscani; Robert Van Winkle and Vincent Wharton.

Most important, I thank my woman, Rhiannon Simpson. All these people helped out in one way or another, and it was their love and support and the inspiration that I took from them that acted as my rock throughout the turbulent experience I called graduate school.



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# **Antioxidant Enzyme Response in Rainbow Trout (*Oncorhynchus mykiss*) After Subchronic Exposure to an Environmentally-Relevant Polycyclic Aromatic Hydrocarbon Mixture**

## **INTRODUCTION**

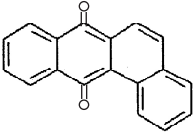
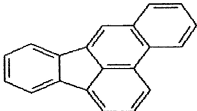
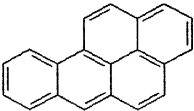

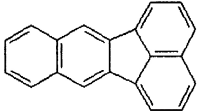
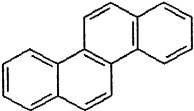
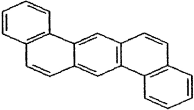
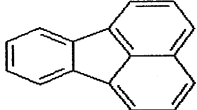
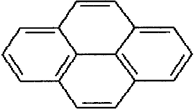
Polycyclic aromatic hydrocarbons (PAHs) represent a major chemical class of interest in environmental and molecular toxicology. PAHs are a major component in soot, a complex mixture that surgeon Sir Percivall Pott, the father of modern toxicology, linked as the causative agent behind the rise in scrotal cancer among chimney sweeps in 1775 (Power, 1969). PAHs are planar, relatively highly conjugated hydrocarbons that contain multiple fused aromatic rings. PAHs containing 4 or fewer aromatic rings are considered “light PAHs,” and those with more than 4 aromatic rings are labeled as “heavy PAHs.” They are non-polar, highly lipophilic, cross biological membranes well and easily bioaccumulate (McCain *et. al.*, 1990). Some common PAHs include: anthracene, benzo( $\alpha$ )pyrene, chrysene, fluoranthene and pyrene (**Table 1**).

PAHs are predominantly formed during petroleum and natural gas production and use, along with incomplete combustion (between 300 °C and 600 °C) of organic matter. These specific processes include, but are not limited to: tar production, oil drilling and refinement, combustion engine exhaust, industrial metal manufacturing and coke oven emissions (Xue and Warshawsky, 2005). PAHs enter aquatic ecosystems via pollution-contaminated dust and rainwater, urban and agricultural runoff (often bound

**Table 1. Polycyclic Aromatic Hydrocarbons (Lijinsky, 1991)**

<b>3-Ring</b>	<b>4-Ring</b>	<b>5-Ring</b>	<b>6-Ring</b>
Acenaphthene Acenaphthylene Anthracene Flourene Phenanthrene	Benz( $\alpha$ )anthracene Benzo( $\alpha$ )fluorene Benzo( $\beta$ )fluorene Benzo(c)fluorene Benzo(c)phenanthrene Chrysene Fluoranthene Naphthacene Pyrene Triphenylene	Benzo( $\beta$ )chrysene Benzo( $\beta$ )fluoranthene Benzo(j)fluoranthene Benzo(k)fluoranthene Benzo( $\alpha$ )pyrene Benzo(e)pyrene Dibenz( $\alpha$ ,h)anthracene Dibenz( $\alpha$ ,j)anthracene Dibenz( $\alpha$ ,c)anthracene Cyclopenta(c,d)pyrene Pentacene Pentaphene Perylene Picene	Anthanthrene Benzo(g,h,i)perylene Dibenzo( $\alpha$ ,e)fluoranthene Dibenzo( $\alpha$ ,e)pyrene Dibenzo( $\alpha$ ,h)pyrene Dibenzo( $\alpha$ ,i)pyrene Dibenzo( $\alpha$ ,l)pyrene Indeno(1,2,3-cd)pyrene

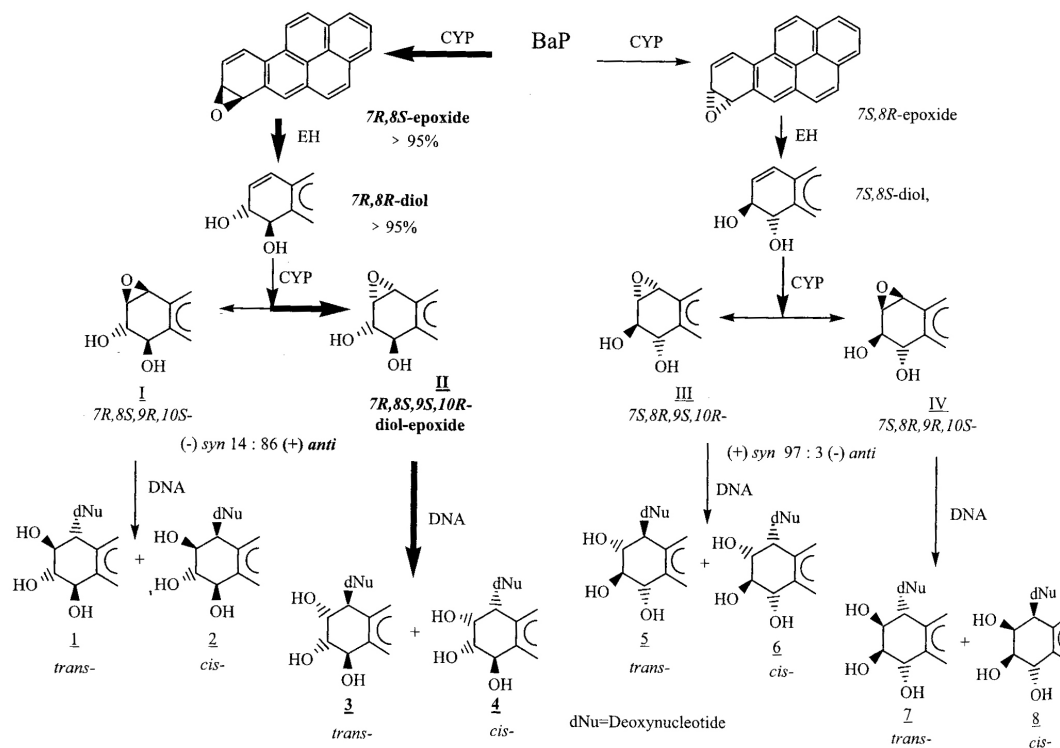
**Table 2. Structures of PAHs Utilized in this Study (Mackay *et. al.*, 1992)**

 <p><b><u>Benz(<math>\alpha</math>)anthracene-7,12-dione</u></b>            MW: 258.3 g/mol            Log <math>K_{ow}</math>: 4.40</p>	 <p><b><u>Benzo(<math>\beta</math>)fluoranthene</u></b>            MW: 252.3 g/mol            Log <math>K_{ow}</math>: 6.29</p>	 <p><b><u>Benzo(<math>\alpha</math>)pyrene</u></b>            MW: 252.3 g/mol            Log <math>K_{ow}</math>: 6.04</p>
 <p><b><u>Benzo(g,h,i)perylene</u></b>            MW: 276.3 g/mol            Log <math>K_{ow}</math>: 7.00</p>	 <p><b><u>Benzo(k)fluoranthene</u></b>            MW: 252.3 g/mol            Log <math>K_{ow}</math>: 6.00</p>	 <p><b><u>Chrysene</u></b>            MW: 228.3 g/mol            Log <math>K_{ow}</math>: 5.86</p>
 <p><b><u>Dibenz(<math>\alpha</math>,h)anthracene</u></b>            MW: 278.4 g/mol            Log <math>K_{ow}</math>: 6.75</p>	 <p><b><u>Fluoranthene</u></b>            MW: 202.3 g/mol            Log <math>K_{ow}</math>: 5.22</p>	 <p><b><u>Pyrene</u></b>            MW: 202.3 g/mol            Log <math>K_{ow}</math>: 5.18</p>

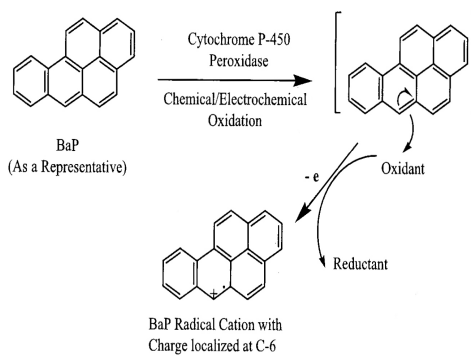
to soil particles) and industrial and sewage effluent (often bound to dust and soil particles) (Potter *et. al.*, 1994; Strmac and Braunbeck, 2001). PAHs pose a serious threat in aquatic ecosystems due to persistence, trophic transfer and toxicities (Fogels and Sprague, 1977; Rowe *et. al.*, 1983; Potter *et. al.*, 1994; Karrow *et. al.*, 1999; Xue and Warshawsky, 2005).

There are three major biotransformation pathways that convert PAHs into reactive intermediates (Xue and Warshawsky, 2005). The first is the bay region dihydrodiol epoxides pathway, in which cytochrome P4501A and epoxide hydrolase convert PAHs into their respective diol epoxide intermediates (**Fig. 1**). The epoxides of these intermediates are strongly electrophilic, allowing intercalation into DNA by covalent bonding, which often occurs at the exocyclic amino groups of the dGuo and dAdo residues. This distorts the helix and interferes with proper transcription and induces apoptosis and error-prone repair. The second pathway is the radical cation pathway, in which cytochrome P450 peroxidase catalyzes the one-electron oxidation of PAHs, thereby generating reactive oxygen species (**Fig. 2**). Reactive oxygen species can cause cell injury by oxidizing lipids, proteins and DNA. These effects can lead to membrane damage, enzymatic dysfunction and tumor formation, respectively (Sturve *et. al.*, 2005). The third pathway is the PAH ortho-quinone pathway, in which dihydrodiol dehydrogenase competes with cytochrome P450 to oxidize the non-K-region diol (**Fig. 3**). This biotransformation eventually leads to the formation of PAH ortho-quinones, which can form DNA adducts or reactive oxygen species.

**Figure 1. The Bay Region Dihydrodiol Epoxides Pathway**  
(Xue and Warshawsky, 2005) (used with permission)

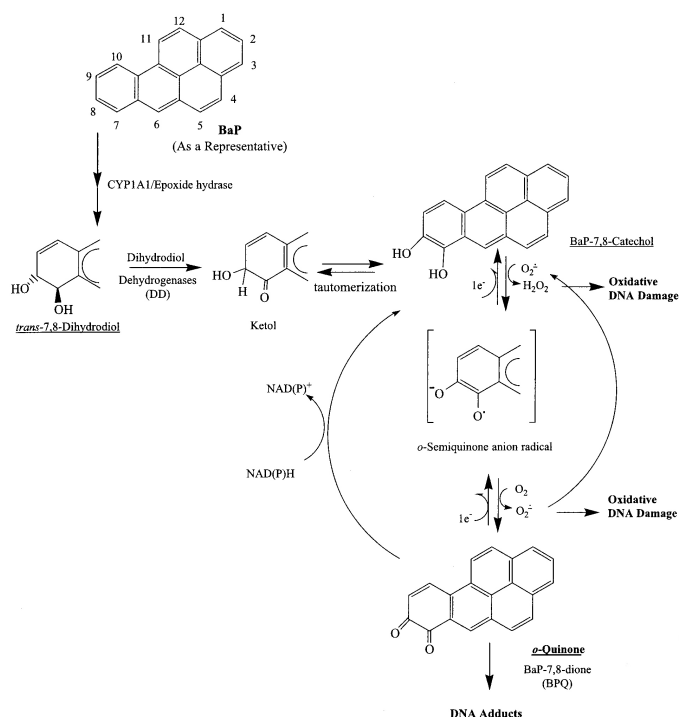


**Figure 2. The Radical Cation Pathway**  
(Xue and Warshawsky, 2005) (used with permission)





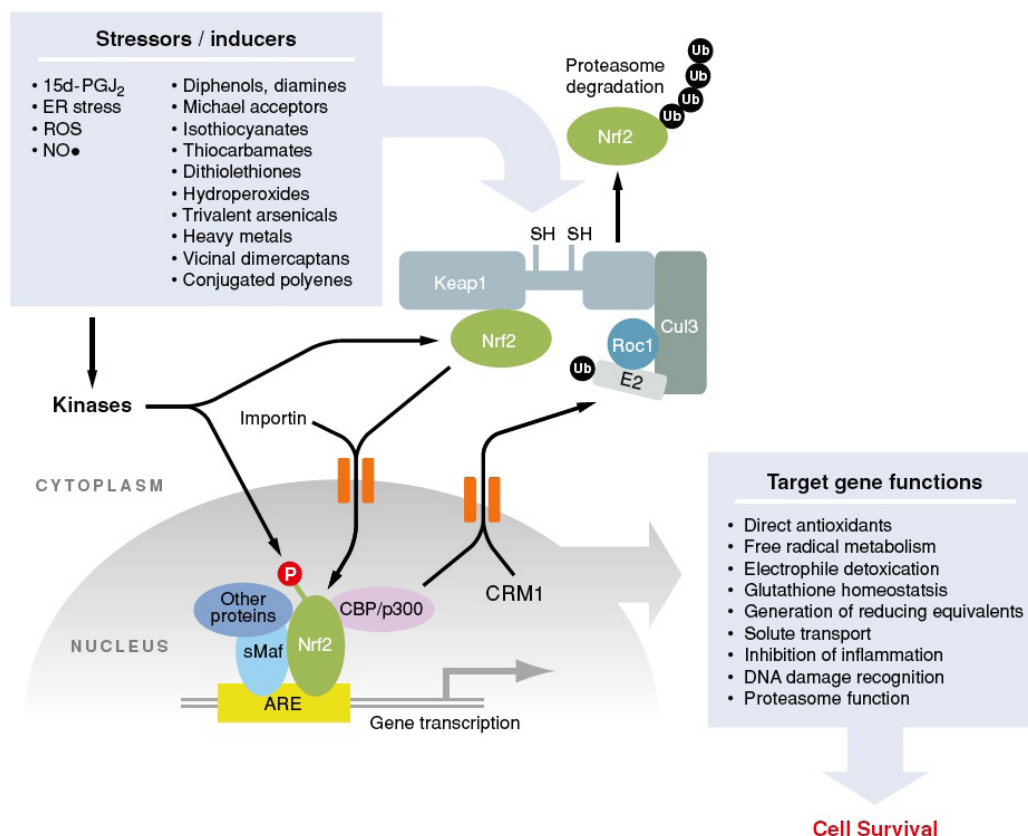
**Figure 3. The PAH Ortho-Quinone Pathway**  
(Xue and Warshawsky, 2005) (used with permission)



The Keap1-Nrf2-ARE pathway is a key factor in protecting cells from both endogenous and exogenous stresses (**Fig. 4**). Nrf2 (nuclear factor erythroid 2-related factor) is a basic leucine-zipper transcription factor regulating expression of genes affecting xenobiotic metabolism (Kensler *et. al.*, 2007). Nrf2 is located in the cytoplasm where it is bound to the actin-anchored repressor protein Keap1 (Kelch ECH associating) during quiescent conditions. Keap1 promotes proteosomal degradation of Nrf2 under these conditions, keeping Nrf2 transcription at a

minimum (Itoh *et al.*, 1999; Wakabayashi *et al.*, 2003; Kang *et al.*, 2004). Several cysteine residues on Keap1 serve as primary stress signaling mechanisms, and their modification leads to conformation changes on the protein, thereby liberating Nrf2 (Kensler *et al.*, 2007). The liberated Nrf2 enters the nucleus and together with Maf proteins, another family of basic leucine-zipper proteins, binds to and activates ARE (antioxidant response element) (Igarashi *et al.*, 1994). This binding activates ARE and leads to the upregulation of multiple detoxifying/antioxidant defense mechanisms (Primiano *et al.*, 1998; Thimmulappa *et al.*, 2002; Kwak *et al.*, 2003; Rangasamy *et al.*, 2004; Yates *et al.*, 2006; Kensler *et al.*, 2007).

**Figure 4. The Keap1-Nrf2-ARE Pathway**  
(Kensler *et al.*, 2007) (used with permission)



One of the prominent antioxidant systems induced by the Keap1-Nrf2-ARE pathway is the glutathione (GSH) system. GSH is one of the most important antioxidant defenses in rainbow trout (*Oncorhynchus mykiss*). GSH acts either non-enzymatically as a hydrogen donor or as a cofactor for glutathione peroxidases, which reduce hydrogen peroxide (Stephensen *et. al.*, 2002). Most intracellular GSH is in its active, reduced form until it is oxidized into inactive glutathione disulfide (GSSG) in the presence of oxidative stress. Research shows that exposure to various prooxidants/oxidants similar in action to PAHs, such as copper sulfate, creates a quantifiable increase in GSH and GSH transferase, and that GSH has a significant protective effect against oxidative stress in rainbow trout cell lines (Maracine and Segner, 1998; Stephensen *et. al.*, 2000; Rau *et. al.*, 2004).

Glutathione peroxidase (GPOX) uses GSH as a cofactor for action as a scavenger for damaging ROS. This inhibits lipid peroxidation by metabolizing both  $H_2O_2$  and organic hydroperoxides. Both GPOX and overall lipid peroxidation demonstrate quantifiable increases after exposure to prooxidants/oxidants, making GPOX a candidate biomarker for oxidative stress (Rau *et. al.*, 2004). Lipid peroxidation is also a biomarker for oxidative stress (Yu and Sinnhuber, 1957; Sinnhuber and Yu, 1958). There was a fifteen-fold increase in detectable lipid peroxidation levels in rainbow trout cell lines after being exposed to 50  $\mu M$  copper sulfate for 24 hours (Rau *et. al.*, 2004).

Catalase (CAT) is another antioxidant enzyme that is regulated by the Keap1-Nrf2-ARE pathway. CAT is a common enzyme found in most living organisms. It is a peroxisomal tetramer protein of four polypeptide chains, which are each over five hundred amino acids long. The enzyme contains four porphyrin heme groups, which allow for the binding of the enzyme to various peroxides, breaking them down into either water or alcohol.

NAD(P)H:quinone oxidoreductase 1 [DT-diaphorase], also commonly known as quinone reductase (QR), is a non-Keap1-Nrf2-ARE regulated detoxifying enzyme that is widely distributed throughout the animal kingdom, although its activity can vary from one species to another (Marki and Martius, 1960; Ernster, 1987; Bayney *et. al.*, 1989). It is found at highest concentrations within the liver, specifically in the cytosol. QR is a flavoenzyme that catalyzes the two-electron reduction of quinones to relatively stable hydroquinones, which can then be subjected to glucuronidation. QR is categorized by almost equal affinity of NADH and NADPH as electron donors. This two-electron reduction is favored over a one-electron reduction, such as that of NADPH-cytochrome P450 reductase (Chesis *et. al.*, 1984). In rainbow trout PAHs act as inducers of QR via two different mechanisms. The first is through direct interaction with the aryl hydrocarbon receptor acting on the xenobiotic response element on the NQO1 gene. The second is through electrophilic metabolites from the cytochrome P4501A1 Phase 1 reaction acting on the ARE (Prochaska and Talalay, 1988).

CAT, GPOX and QR are all important enzymes for the detoxification of xenobiotics in rainbow trout. All three of these enzymes, along with various measures of lipid peroxidation, have been shown to be excellent biomarkers for the upregulation of prooxidant/oxidant detoxification. CAT activities increase significantly after exposure to prooxidant compounds (Stephensen *et al.*, 2000). Glutathione-dependent enzymes are diverse, and there is tight regulation of intracellular GPOX concentration in response to environmental pollutants (Rodriguez-Ariza *et al.*, 1993; Hasspieler *et al.*, 1994; Petrivalsky *et al.*, 1997; Stephensen *et al.*, 2000). Bagnasco *et al.* (1991) suggest that although there are few studies of QR in fish, it may be suitable for quantitative measurement and use as a novel biomarker (Bagnasco *et al.*, 1991; Sturve *et al.*, 2005)

This work addressed the hypothesis that subchronic dietary PAH exposures increase activities of antioxidant enzymes in association with a decrease in lipid peroxidation in rainbow trout liver. To test this hypothesis, we performed a 42 day time-course exposure experiment utilizing five different treatments where rainbow trout were fed diets that contained two different levels and combinations of PAHs. The treatments were: control, 20 ppm PAH mixture (MIX), 200 ppm PAH mixture (MIX), 20 ppm benzo( $\alpha$ )pyrene (BAP), 200 ppm benzo( $\alpha$ )pyrene (BAP). These exposures represented PAH mixture concentrations measured in local aquatic environments that were moderately or heavily contaminated (Arkoosh *et al.*, 2001). The relative proportions of constituents also reflected those measured in local aquatic

environments (Arkoosh *et. al.*, 2001). Liver samples from the trout were taken from all five treatments after days 7, 14, 28 and 42 of dietary exposures. Measurements of CAT, GPOX, QR and lipid peroxidation were performed on the collected samples. Liver and body weights were also measured during sampling.

In summary, the results of the present study support the hypothesis that PAH exposure triggers an antioxidant enzymatic response in rainbow trout. Namely, CAT responds significantly in a dose-dependent manner, GPOX significantly responds in a non-dose-dependent manner and QR is not a significant factor in this enzymatic response. The thiobarbituric acid-reactive substances (TBARS) assay used for detection of lipid peroxidation is an insensitive assay in the rainbow trout model system.

## METHODS

### Chemicals:

2,6-Dichlorophenolindophenol (98%) was purchased from Alfa Aesar (Ward Hill, MA). NADPH tetrasodium salt (99%) was purchased from Calbiochem (La Jolla, CA). Acetic acid glacial (100%); acetonitrile (99.8%); iso-butanol (99.8%); dichloromethane (99%) and pyridine (99%) were purchased from Fisher Chemicals (Fair Lawn, NJ). Benzo( $\alpha$ )pyrene (98%) and dicoumarol (crystalline) were purchased from MP Biomedicals (Solon, OH). Benz( $\alpha$ )anthracene-7,12-dione (97%); benzo( $\beta$ )fluoranthene (98%); benzo(k)fluoranthene (98%); benzo(g,h,i)perylene (98%); chrysene (98%); dibenz( $\alpha$ )anthracene (99%); fluoranthene (99%); glucose-6-phosphate (99%); D-glucose-6-phosphate dehydrogenase (100%); L-glutathione, reduced (99%); glutathione reductase (99%); menadione (98%); pyrene (98%); sodium azide (99.5%); sodium hydroxide (98%); 1,1,3,3-tetramethoxypropane (99%); thiobarbituric acid (98%); trizma base (99.9%) and tween-20 were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide [MTT] (99%) and flavin adenine dinucleotide (99%) were purchased from TCI America (Portland, OR). Bovine serum albumin (98%) and hydrogen peroxide (30%) were purchased from VWR (West Chester, PA).

### Experimental Treatments:

This experiment employed four dietary treatments of various PAH formulations

and one control treatment. The four PAH formulations were 20 and 200 ppm benzo( $\alpha$ )pyrene (20 BAP and 200 BAP) and 20 and 200 ppm of a mixture of various PAHs (20 MIX and 200 MIX) (**Table 3**). Both the concentrations (20 and 200 ppm) and the relative ratios of the various PAHs used in the MIX formulation are based on PAH concentrations seen in actual polluted urban estuaries (Arkoosh *et. al.*, 2001).

**Table 3. PAH Mixture Formulation** (Mackay *et. al.*, 1992; Arkoosh *et. al.*, 2001)

Chemical	M.W.	Percentage of Total Mixture
Benz( $\alpha$ )anthracene-7,12-dione	258.3	7.3
Benzo( $\beta$ )fluoranthene (Benz(e)acephenanthrylene)	252.3	17.7
Benzo( $\alpha$ )pyrene	252.3	6.3
Benzo(g,h,i)perylene	276.3	5.2
Benzo(k)fluoranthene	252.3	6.3
Chrysene	228.3	13.5
Dibenz( $\alpha$ ,h)anthracene	278.4	1.0
Fluoranthene	202.3	21.9
Pyrene	202.3	20.8



The trout were exposed to the experimental treatments via their daily diet. Rangen, Inc.'s (Buhl, ID) commercial trout feed #4 was used throughout the experiment. This commercial feed was selected due to it having the lowest fat content (~ 15%) of any available commercial feed, thereby mimicking a rainbow trout's natural diet's fat content (~ 10%) closer than the average commercial feed, which has an average fat content of 20-25% (Bravo *et. al.*, 2006). The nutritional value of the feed was as follows: crude protein (min) 44.0%, crude fat (min) 15.0%, crude fiber (max) 3.0%, ash (max) 12.0%, phosphorus (min) 1.0%. The treatment chemicals were deposited on the food pellets previous to the fish being exposed to them.

The individual PAHs were dissolved in dichloromethane to make concentrated PAH MIX and BAP stock solutions. Both concentrated stocks were sonicated in a VWR Aquasonic 150HT sonicator for 15-30 minutes until all treatment chemicals had thoroughly dissolved into the dichloromethane. The concentrated stocks were then measured out and diluted with the appropriate volumes of dichloromethane to create 20 and 200 ppm formulations of the PAH MIX and BAP treatments when combined with 3.3 kg of feed pellets, which was the calculated amount of feed needed for the duration of the experiment. The solutions and feed pellets were combined in stainless steel bowls in a way that allowed all the feed pellets to be completely submerged under the PAH/dichloromethane solutions. The control diet was treated with only dichloromethane. The five resulting saturated food solutions were stored under a fume hood and thoroughly stirred daily for one week to ensure proper evaporation of the

dichloromethane and even distribution of the PAHs. Once the dichloromethane had completely evaporated, the feed was frozen until needed.

All labware and storage jars were autoclaved prior to use and rinsed with dichloromethane immediately before being used. Yellow lighting was used during all chemical handling and diet preparation due white light's ability to degrade PAHs via photolysis.

Treatment chemicals and fish diet were weighed out using Mettler analytical digital scales.

### Experimental Animals:

Four hundred juvenile rainbow trout were raised from eggs at Oregon State University's Sinnhuber Aquatic Research Laboratory (SARL). The fish were randomly divided into 10 groups (40 fish per group) once their individual weight was approximately 20 g, and each group was placed in a 1 m diameter fiberglass tank. Two tanks were assigned to each of the five treatment conditions. The animals were maintained at a constant water temperature of  $11.5 \pm 1$  °C and kept on a daily cycle of 12hrs./12hrs. light/darkness. The water in the tanks was local well water and was under constant filtration and aeration. All trout were acclimated in treatment tanks approximately three weeks prior to the start of the experiment.

The trout were fed once per day in the morning Monday through Friday and fasted Saturday and Sunday. The amount of feed given per tank was calculated by the SARL staff based on 2% of total body weight of housed fish. The daily mass of feed given per tank was recalculated and adjusted every Monday after the weekend fast.

#### Treatment Exposure:

The feed for each tank was thawed the morning of use and any remaining food was refrozen after feeding. Feed portions for each of the ten tanks were measured using a digital scale and immediately poured over the water surface. The type of feed pellets used immediately sink through the water column. The contact the feed had with white light was kept at a minimum.

#### Sampling:

Samples were taken 7, 14, 28 and 42 days after initiation of dietary treatments.

Each sample was taken in the morning prior to daily feeding. For each of the five treatments, fish were selected and removed randomly from the two replicate tanks. Selected fish were killed by an overdose of tricaine methanesulfonate (220 mg/L). Fish were then immediately weighed and their livers excised. The excised livers were immediately weighed and placed in ice-cold isotonic (1.1%) potassium chloride solution. At least 1 g of liver tissue was pooled from a treatment group for each sample. Livers were then removed from the isotonic potassium chloride and homogenized in a 4:1 ratio (v/w) with a potassium phosphate/potassium chloride

buffer solution (pH 7.5). The homogenate was quickly placed in a sealed tube and packed in ice. Four sample homogenates were prepared for each of the five treatment groups.

Samples were centrifuged in a Sorvall RC-5B centrifuge for at 10,000×g and 4 °C for 20 minutes. The resulting supernatant was removed and placed in polycarbonate centrifuge tubes. The centrifuge tubes were placed on ice, balanced with ice-cold potassium phosphate/potassium chloride buffer solution (pH 7.5) and centrifuged in either a Beckman Optima LE-80K or a Beckman L7-55 at 100,000×g and 4 °C for 90 minutes. The resulting supernatant representing the cytosolic fraction of the samples was transferred into individual aliquots and immediately stored in a -80 °C freezer (Palace *et. al.*, 1990). The remaining microsomal pellet was resuspended in 5:1 ratio (w/v) with a potassium phosphate/glycerol-based microsomal resuspension buffer solution (pH 7.25). The resuspension was also transferred into individual aliquots and immediately stored in a -80 °C freezer (Palace *et. al.*, 1990).

Whole livers were also collected for each of the five treatment groups during each of the four sampling days. Four fish (two from each tank) were randomly selected for each of the five treatment groups. The selected fish were killed with an overdose of tricaine methanesulfonate (220 mg/L). The fish weights were immediately recorded and their livers excised. The excised livers were weighed and recorded and placed in aliquots suited for cryogenic freezing, which were immediately placed in liquid

nitrogen. The samples were removed from the liquid nitrogen and stored in a -80 °C freezer (Palace *et. al.*, 1990).

#### Determination of Catalase:

CAT activities were determined using an assay kit from Cayman Chemicals (Ann Arbor, MI). The assay is a 96-well plate spectrophotometric assay that utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol to create formaldehyde, which then reacts with purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as the chromophore (Johansson and Borg, 1988). Two independent microsomal samples were run for each treatment, and each sample was run in duplicate. Each sample well was scanned twice, with the two scans being less than 90 seconds apart. The samples were scanned with a SpectraMax 250 96-well plate reader using SOFTmax PRO 3.1.1 analysis software.

#### Determination of Glutathione Peroxidase:

GPOX activities were determined using a modified photometric assay that defines GPOX levels as the reduction of NADPH in cytosolic liver fractions (Gunzler and Flohe, 1985; Stephensen *et. al.*, 2002). Previously frozen cytosolic liver samples were thawed and vortexed in preparation for assay. Two independent samples were run for each treatment, and each sample was run in duplicate. The test solution consisted of 2 mL 50 mM potassium phosphate buffer (pH 7.5), 100

$\mu\text{L}$  6.0 mM NADPH, 100  $\mu\text{L}$  1.0 mM sodium azide, 2 units (100 mL) glutathione reductase and 50  $\mu\text{L}$  cytosol. The reaction was then initiated by adding 100  $\mu\text{L}$  0.8 M hydrogen peroxide. The depletion of NADPH was measured photometrically at 340 $\lambda$  over a period of 2 minutes post initiation in a Shimadzu Pharmaspec UV-1700 spectrophotometer. GPOX levels were calculated using the extinction coefficient for NADPH oxidation ( $\epsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$ ).

#### Determination of Quinone Reductase:

QR activities were determined using a modified photometric assay that defines QR levels as the dicoumarol-inhibitable reduction of 2,6-dichlorophenolindophenol (DCPIP) in cytosolic liver fractions (Ernster *et. al.*, 1962; Sturve *et. al.*, 2005).

Previously frozen cytosolic liver samples were thawed and vortexed in preparation for assay. Two independent samples were run for each treatment, and each sample was run in duplicate with and without dicoumarol (blanks containing distilled water in place of dicoumarol). The test solution consisted of 450  $\mu\text{L}$  50 mM Tris-HCl (pH 7.6), 150  $\mu\text{L}$  40  $\mu\text{M}$  DCPIP, 50  $\mu\text{L}$  0.1 mM dicoumarol in 0.15% NaOH and 50  $\mu\text{L}$  of cytosol. The reaction was then initiated by adding 50  $\mu\text{L}$  6 mM NADPH solution. The decay of DCPIP was measured photometrically at 600 $\lambda$  over a period of 2 minutes post initiation in a Shimadzu Pharmaspec UV-1700 spectrophotometer. QR levels were calculated using the extinction coefficient for DCPIP ( $\epsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

### Determination of Lipid Peroxidation:

Lipid peroxidation levels were determined using a photometric assay that measures TBARS and relies on hepatic malondialdehyde to form the chromophore. Previously cryogenically frozen whole livers were thawed and homogenized in a 9:1 (w/v) ice-cold 1.15% potassium chloride (KCl) solution. Test solutions were prepared with the following formulations: 300  $\mu$ L 1.15% KCl, 40  $\mu$ L 8.1% sodium dodecyl sulfate (SDS), 600  $\mu$ L 20% acetic acid glacial, 600  $\mu$ L 0.6% thiobarbituric acid (TBA) and 100  $\mu$ L vortexed liver homogenate. 100  $\mu$ M 1,1,3,3-tetramethoxypropane (TMP), a source of malondialdehyde bis, was used to create a set of standards of concentrations ranging from 0.25-16 nmol/mL. These standard solutions were buffered in 1.15% KCl. The standards were each mixed in the following formulation in order to produce a standard curve: 400  $\mu$ L standard solution (varying ratio of TMP:KCl), 40  $\mu$ L 8.1% SDS, 600  $\mu$ L 20% acetic acid glacial and 600  $\mu$ L 0.6% TBA. Liver and standard samples were run in quintuplicate. Once formulated, both the liver and standard samples were vortexed and incubated in a 95 °C water bath for 60 minutes. After this incubation period, the liver and standard samples were quickly removed and placed in a -80 °C freezer for 3-4 minutes to stop the reaction. 400  $\mu$ L distilled water and 2 mL 15:1 iso-butanol:pyridine were added to each liver and standard sample, which were then vortexed thoroughly. The liver and standard samples were then centrifuged in a Beckman Model TJ-6 centrifuge at 2,000 $\times$ g and 4 °C for 5 minutes. The resulting butanol layer, which contained the active chromophore, was siphoned out of each liver and standard sample and placed into individual cuvettes. Once loaded, the cuvettes

were allowed to stand undisturbed for approximately 8-10 minutes to allow unseen particulate matter in the sample to settle to the bottom of the cuvette and under the light beam path of the spectrophotometer. Each cuvette was scanned at 532 $\lambda$  in a Shimadzu Pharmaspec UV-1700 spectrophotometer.

#### Determination of Protein Concentrations:

Hepatic protein concentrations were measured using the BCA Protein Assay Kit by Pierce Chemical Company (Rockford, IL). This is a detergent-compatible formulation based on bicinchoninic acid (BCA) that allows for the colorimetric (562 $\lambda$ ) detection and quantification of total protein levels in a sample.

#### Statistical Analysis:

The raw data results for each enzyme-detection assay were submitted to two-way ANOVA with replication and a post-hoc Bonferroni multiple comparison test.  $P < 0.05$  was considered significant in all statistical analyses performed for this study.



## RESULTS

### Mortality and Body Weight:

No mortality occurred in the experimental animals throughout the duration of this experiment. Body weights were not significantly different ( $P > 0.05$ ) in any of the five treatment groups throughout the duration of the experiment (**Table 4**). Average relative growth rate was also calculated (**Table 5**).

**Table 4. Average Body Weights**

	Day 7	Day 14	Day 28	Day 42
CON	Body Weight: 36.24 g Liver Weight: 0.45 g n = 10 SD= 8.58	Body Weight: 44.44 g Liver Weight: 0.55 g n = 8 SD= 13.83	Body Weight: 55.22 g Liver Weight: 0.72 g n = 6 SD= 6.75	Body Weight: 69.47 g Liver Weight: 0.68 g n = 6 SD= 16.21
20 MIX	Body Weight: 25.75 g Liver Weight: 0.34 g n = 12 SD= 5.65	Body Weight: 37.50 g Liver Weight: 0.46 g n = 8 SD= 12.42	Body Weight: 44.75 g Liver Weight: 0.55 g n = 8 SD= 14.76	Body Weight: 60.24 g Liver Weight: 0.59 g n = 7 SD= 15.13
200 MIX	Body Weight: 31.05 g Liver Weight: 0.38 g n = 12 SD= 7.79	Body Weight: 34.84 g Liver Weight: 0.41 g n = 12 SD= 10.56	Body Weight: 49.99 g Liver Weight: 0.66 g n = 7 SD= 18.39	Body Weight: 56.46 g Liver Weight: 0.59 g n = 7 SD= 18.53
20 BAP	Body Weight: 29.48 g Liver Weight: 0.38 g n = 12 SD= 9.07	Body Weight: 34.61 g Liver Weight: 0.48 g n = 12 SD= 12.05	Body Weight: 57.47 g Liver Weight: 0.67 g n = 7 SD= 14.40	Body Weight: 65.81 g Liver Weight: 0.67 g n = 7 SD= 17.42
200 BAP	Body Weight: 27.06 g Liver Weight: 0.34 g n = 12 SD= 4.88	Body Weight: 35.51 g Liver Weight: 0.44 g n = 9 SD= 10.66	Body Weight: 53.45 g Liver Weight: 0.61 g n = 8 SD= 17.20	Body Weight: 52.10 g Liver Weight: 0.56 g n = 9 SD= 18.26

**Table 5. Average Relative Growth Rate**

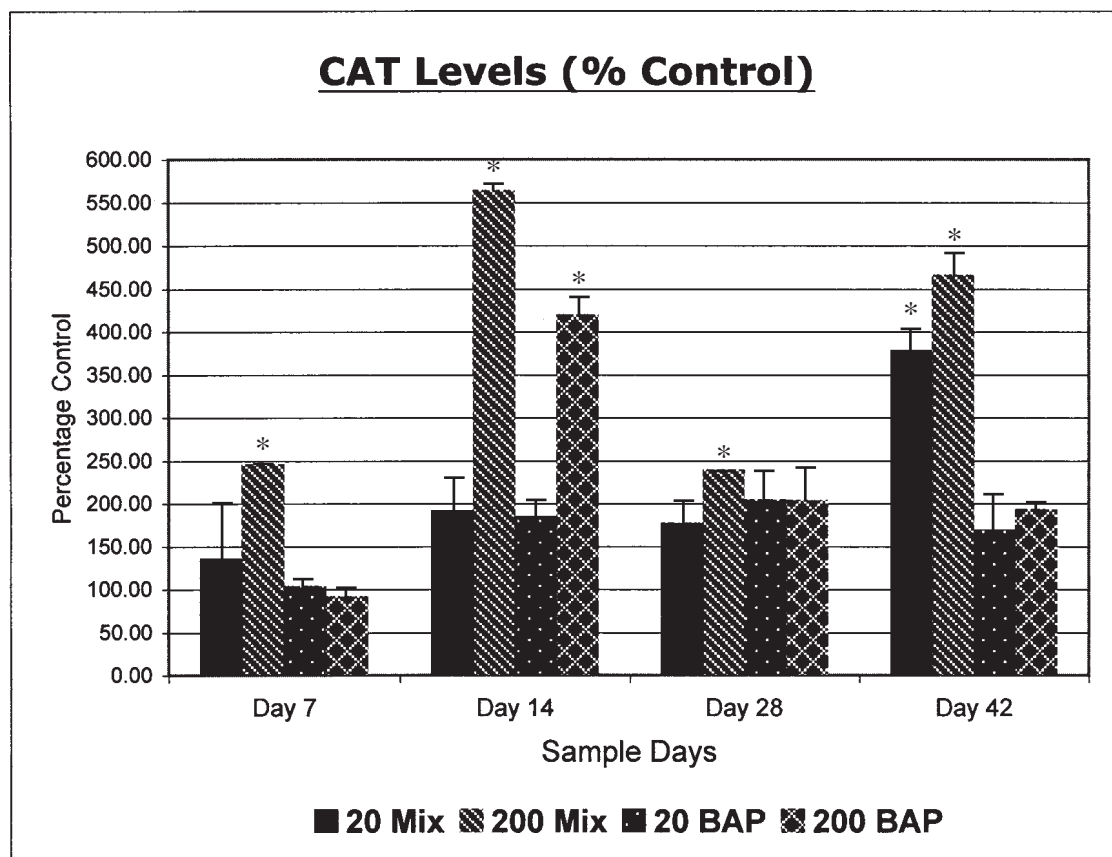
	BODY	LIVER
CON	17.96 mg/g/day	11.63 mg/g/day
20 MIX	22.92 mg/g/day	15.36 mg/g/day
200 MIX	16.59 mg/g/day	12.37 mg/g/day
20 BAP	21.79 mg/g/day	15.78 mg/g/day
200 BAP	18.08 mg/g/day	13.97 mg/g/day

### Antioxidant Enzymes:

#### *Catalase:*

CAT activities were calculated as nmol/mg protein/min using a catalase-specific assay kit from Cayman Chemicals (Ann Arbor, MI), which was designed around previous research (Johansson and Borg, 1988). Results are reported as percent of control. 20 MIX, 200 MIX, 20 BAP and 200 BAP levels for day 7: 136.20%, 245.50%, 104.90% and 92.00% (respectively); day 14: 192.16%, 564.13%, 184.87% and 420.02% (respectively); day 28: 177.72%, 239.75%, 204.86% and 204.12% (respectively); day 42: 378.93%, 466.26%, 169.11% and 193.32% (respectively). Statistical analysis indicates that there was a significant treatment effect on CAT activities ( $P < 0.05$ ) and that the treatment effect significantly changed over time ( $P < 0.05$ ). The Bonferroni multicomparison test indicates that day 42 of 20 MIX; days 7, 14, 28 and 42 of 200 MIX and day 14 of 200 BAP are significant when compared to control ( $P < 0.05$ ) (**Fig. 5**).

**Figure 5. Catalase Activities Measured in Rainbow Trout Exposed to Various PAH Treatments Over a 42 Day Time-Course**



**Solid:** 20 ppm PAH mixture formulation

**Single-hatch:** 200 ppm PAH mixture formulation

**Stipple:** 20 ppm benzo(α)pyrene formulation

**Cross-hatch:** 200 ppm benzo(α)pyrene formulation

\*Statistically significant within time-point when compared to control ( $P < 0.05$ )

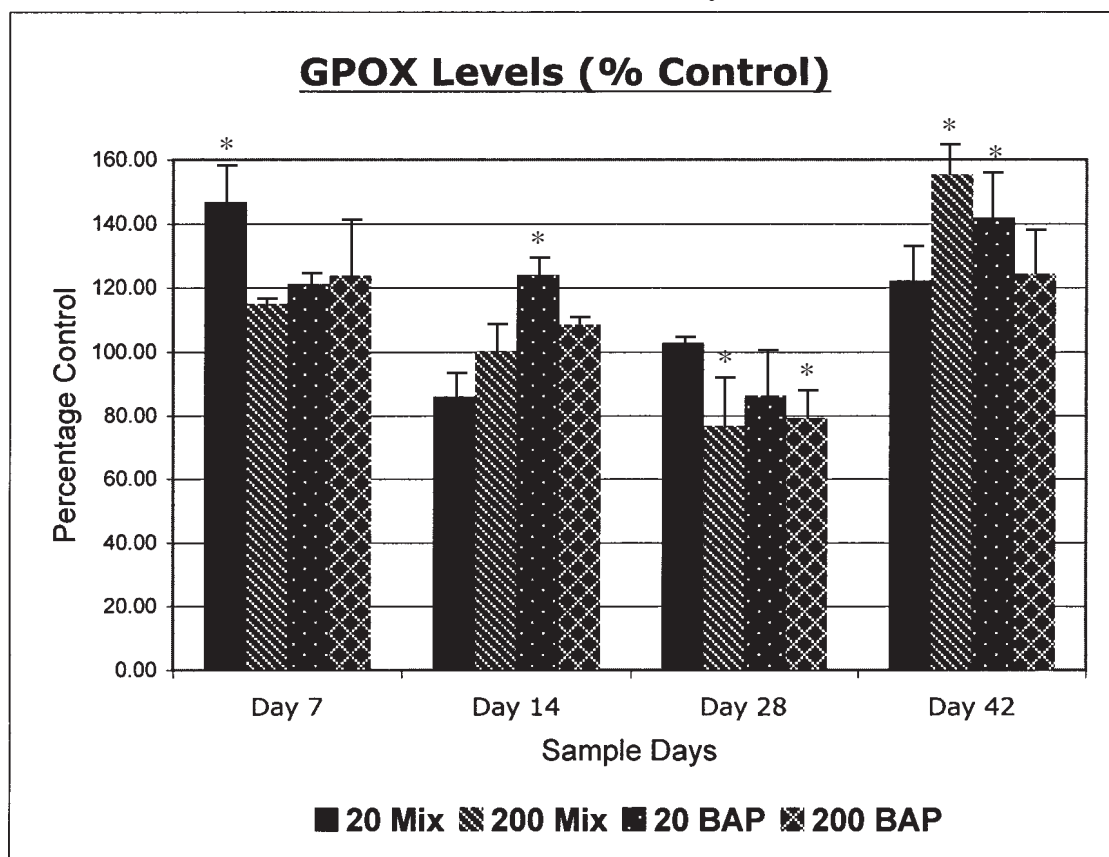
Glutathione peroxidase:

GPOX activities were calculated as  $\mu\text{mol/mg protein/min}$  using previously described methodology (Gunzler and Flohe, 1985; Stephensen *et. al.*, 2002). Results are reported as percent of control. 20 MIX, 200 MIX, 20 BAP and 200 BAP levels for day 7:

146.87%, 114.95%, 120.97% and 123.56% (respectively); day 14: 86.03%, 100.14%, 123.74% and 108.33% (respectively); day 28: 102.76%, 76.61%, 86.20% and 79.09% (respectively); day 42: 122.26%, 155.29%, 141.72% and 124.25% (respectively).

Statistical analysis indicates that there was a significant treatment effect on GPOX activities ( $P < 0.05$ ) and that the treatment effect significantly changed over time ( $P < 0.05$ ). The Bonferroni multicomparison test indicates that day 7 of 20 MIX, days 28 and 42 of 200 MIX, days 14 and 42 of 20 BAP and day 28 of 200 BAP are significant when compared to control ( $P < 0.05$ ) (**Fig. 6**).

**Figure 6. Glutathione Peroxidase Activities Measured in Rainbow Trout Exposed to Various PAH Treatments Over a 42 Day Time-Course**



**Solid:** 20 ppm PAH mixture formulation

**Single-hatch:** 200 ppm PAH mixture formulation

**Stipple:** 20 ppm benzo(α)pyrene formulation

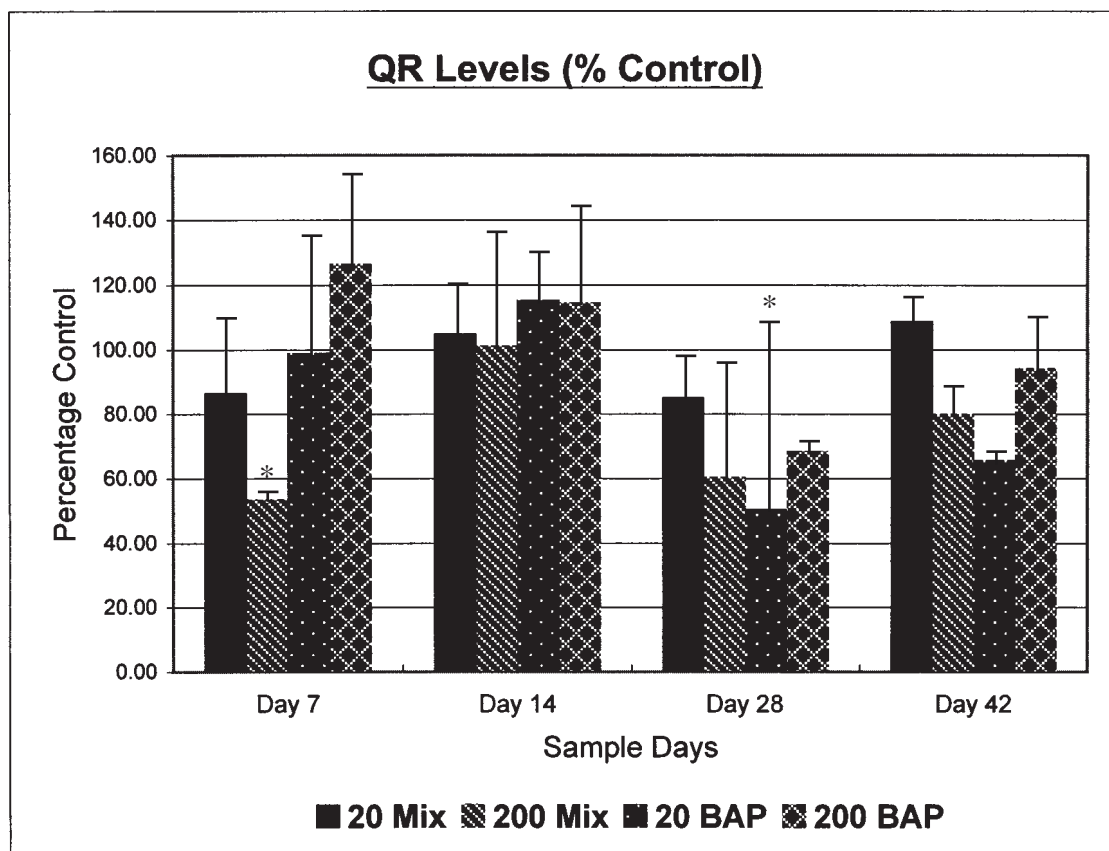
**Cross-hatch:** 200 ppm benzo(α)pyrene formulation

\*Statistically significant within time-point when compared to control ( $P < 0.05$ )

Quinone reductase:

QR activities were calculated as nmol/mg protein/min using previously described methodology (Sturve *et. al.*, 2005). Results are reported as percent of control. 20 MIX, 200 MIX, 20 BAP and 200 BAP levels for day 7: 86.66%, 53.39%, 98.86% and 126.53% (respectively); day 14: 104.45%, 101.20%, 115.31% and 114.45% (respectively); day 28: 85.16%, 60.48%, 50.50% and 68.34% (respectively); day 42: 108.99%, 79.91%, 65.62% and 94.23% (respectively). Statistical analysis indicates that there was not a significant treatment effect on QR activities, and that the treatment effect did not significantly change over time ( $P > 0.05$ ). The Bonferroni multicomparison test indicates that Day 7 of the 200 MIX treatment and day 28 of the 20 BAP treatment are significant ( $P < 0.05$ ) when compared to control (**Fig. 7**).

**Figure 7. Quinone Reductase Activities Measured in Rainbow Trout Exposed to Various PAH Treatments Over a 42 Day Time-Course**



**Solid:** 20 ppm PAH mixture formulation

**Single-hatch:** 200 ppm PAH mixture formulation

**Stipple:** 20 ppm benzo(α)pyrene formulation

**Cross-hatch:** 200 ppm benzo(α)pyrene formulation

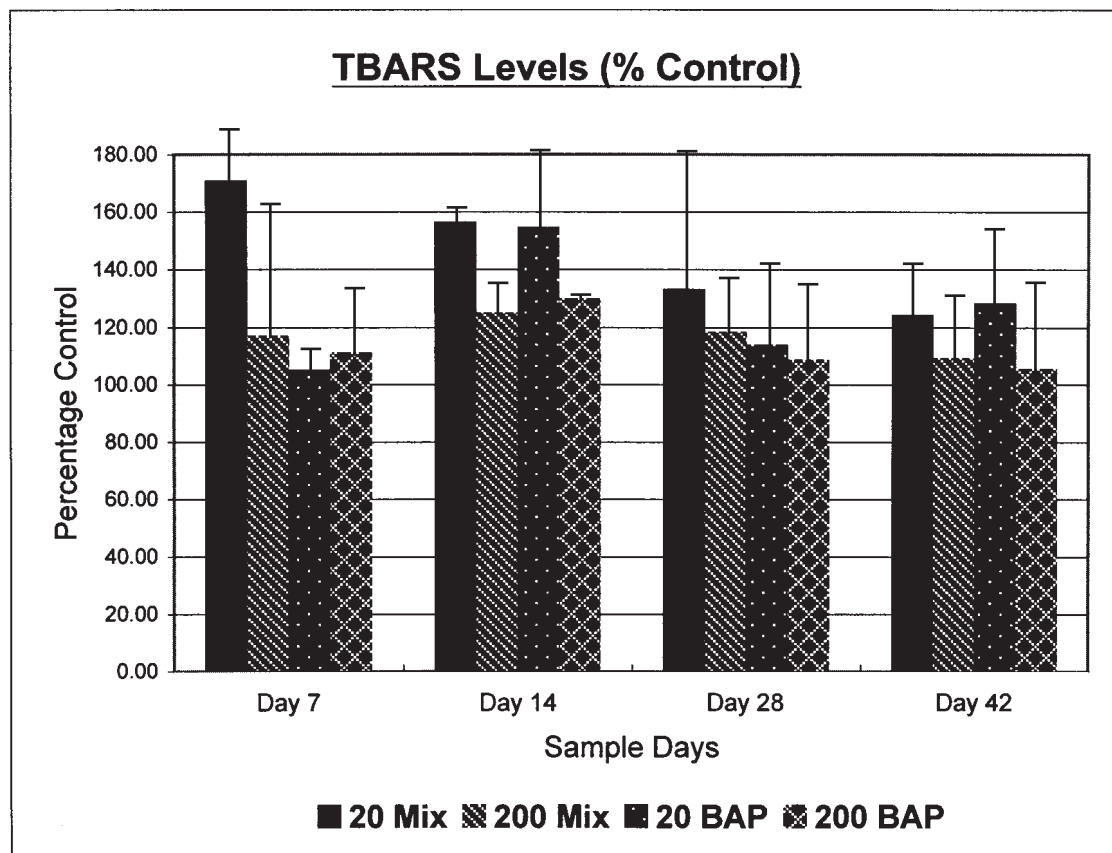
\*Statistically significant within time-point when compared to control ( $P < 0.05$ )

### Thiobarbituric Acid-Reactive Substances (TBARS):

There were no statistically significant differences between TBARS between any treatment group and control at any time (**Fig. 8**). Previous research showed that the mass-spectroscopy-based F<sub>2</sub>-isoprostanes assay detected increased lipid peroxidation in head kidney from rainbow trout fed 40 and 400 ppm of a similar PAH mixture and 160 ppm of benzo( $\alpha$ )pyrene for 50 days. (Bravo *et. al.*, 2006).



**Figure 8. Thiobarbituric Acid-Reactive Substances Levels (TBARS) Measured in Rainbow Trout Exposed to Various PAH Treatments Over a 42 Day Time-Course.**



**Solid:** 20 ppm PAH mixture formulation

**Single-hatch:** 200 ppm PAH mixture formulation

**Stipple:** 20 ppm benzo(α)pyrene formulation

**Cross-hatch:** 200 ppm benzo(α)pyrene formulation

\*Statistically significant within time-point when compared to control ( $P < 0.05$ )

## DISCUSSION

DNA damage in blood cells, as measured by comet assay, increased markedly and then declined in rainbow trout fed a diet containing an environmentally relevant PAH mixture in the same ratio used in this study over a 50 day time-course (Bravo *et. al.*, 2006). The percentage of DNA damage peaked after 14 days of dietary exposure for fish fed both 40 ppm and 400 ppm of the PAH mixture, with the higher dose peaking only 5% higher than the lower dose. This indicates that the exposures approached saturation of the trout's capacity for biotransforming PAHs into their respective reactive intermediates (Bravo *et. al.*, 2006). After day 14, the percent of DNA damage steadily declined for both concentrations until reaching <5% at day 50 (the lowest observed levels in the study). It was unlikely that the source of PAH reactive intermediates was the blood, so we focused on the liver as the most likely source for the majority of PAH metabolism to toxic intermediates (Zhong *et. al.*, 1994; Rau *et. al.*, 2004; Bravo *et. al.*, 2006).

No fish mortality occurred during this experiment. This was expected, as past experiments showed that rainbow trout were highly tolerant to chronic PAH exposures at 1000 ppm (Hendricks *et. al.*, 1985). No gross hepatic lesions were observed during sampling in the current study. This was also expected. Data suggests that rainbow trout are less susceptible to hepatic lesions relative to other fish species popular for toxicological research, such as sole (Varanasi *et. al.*, 1986; Bravo *et. al.*, 2006).

Although a complex hydrocarbon mixture rich in PAHs reduced growth rate of rainbow trout in previous research, there was no significant ( $P > 0.05$ ) effect on body weight of the fish in any of the five treatments (**see Table 4**) (Rowe *et. al.*, 1983).

CAT is a tetrameric antioxidant enzyme that contains four porphyrin heme groups which allow the enzyme to react with peroxides. CAT was selected as a likely antioxidant enzyme active in PAH exposure compensation due to conservation throughout living organisms and also having one of the highest turnover rates for all enzymes (Jones *et. al.*, 1976; Vainshtein *et. al.*, 1981). CAT was measured in hepatic microsomal fractions. A two-way ANOVA analysis of measured CAT levels indicated a significant ( $P < 0.05$ ) interaction with the various treatments over time. A multicomparison test showed the following samplings were significant ( $P < 0.05$ ): day 42 20 MIX; days 7, 14, 28 and 42 200 MIX; day 14 200 BAP.

CAT activities markedly increased throughout the course of the experiment in all four treatment groups. The 20 MIX and 200 MIX treatments were both above control levels on day 7, continuing to rise by day 14. Activities declined after 28 days, representing the lowest measured value for 200 MIX. Both rose again by day 42, creating a pattern of rising, falling and rising again in both treatments. CAT activities in 20 BAP and 200 BAP treatments increased from day 7 to day 14, which represented a peak for 200 BAP, similar to that of 200 MIX. The 20 BAP treatment rose slightly higher and peaked at day 28, whereas 200 BAP declined, putting the

two at almost identical levels (0.74% of control difference). Both declined slightly at day 42. CAT activities for all groups were above control at all sample times, save day 7 200 BAP. The higher doses of each formulation followed very similar patterns during the beginning of the time-course, with 200 MIX peaking above 200 BAP. This was expected since in equal doses the PAH mixture used in this study were more toxic than benzo( $\alpha$ )pyrene (Arkoosh *et. al.*, 2001; Bravo *et. al.*, 2006). From day 28 to day 42 the 20 and 200 ppm treatments trend together in each formulation, with the PAH mixture treatments dramatically rising again, although 200 MIX was not as high as its previous peak, and the BAP treatments both drop slightly, appearing to level off. These data indicated CAT was definitely a major contributing factor in adaptation to PAH-induced toxic insult in rainbow trout. The rise and fall pattern for CAT activity in all four treatment groups suggests an adaptive response toward a steady-state in a decaying sinusoidal wave pattern. An extended time-course experiment would be necessary for testing this hypothesis. Similar sinusoidal decay was reported in other fish models exposed to carcinogens. DNA-adduct levels in zebrafish (*Danio rerio*) exhibited a sinusoidal decay pattern over time after exposure to aflatoxin B<sub>1</sub> (Troxel *et. al.*, 1997).

GPOX is a selenium-dependent tetrameric glycoprotein that reduces hydroperoxides generated by superoxide dismutase, UV radiation and organic peroxides to water and alcohol (Stephensen *et. al.*, 2002). GPOX was selected as a likely antioxidant enzyme active in PAH exposure compensation due to it being a primary mechanism

for the removal of lipid peroxides in cells, which has a positive correlation with PAH exposure in rainbow trout (Bravo *et. al.*, 2006). GPOX was measured in hepatic cytosolic fractions. A two-way ANOVA analysis of measured GPOX levels indicated a significant ( $P < 0.05$ ) interaction with the various treatments over time. A multicomparison test showed the following samplings were significant ( $P < 0.05$ ): day 7 20 MIX; days 28 and 42 200 MIX; days 14 and 42 20 BAP; day 28 200 BAP.

GPOX activity followed a similar trend to CAT activity, showing a definite rise in levels relative to that of control followed by decline below control activities between day 14 and day 28 and rising again above control by day 42. All treatments, save 20 MIX, were above control at day 7, decreased below control by day 28 and then peaked at their highest observed levels at day 42. 20 MIX also was above control at day 7, but differed from the other three treatments with the low point at day 14 instead of day 28. GPOX activity in 20 MIX did rise between day 14 and day 42, but the level at day 42 was not the highest measured level as with the other treatments. This distinctive increase in hepatic GPOX activity relative to control throughout the time-course of the study indicate a positive response to the PAH insult. The rise and fall patterns exhibited by GPOX in all four treatment groups are, like those of CAT, indicative of a sinusoidal decay pattern. The sinusoidal decay patterns of GPOX stabilization for the various treatments that would be seen if the time-course were to be extended appear less extreme and generally more stable than those predicted to be seen with CAT judging by the relatively smaller amplitudes and lower frequencies

of the sinusoidal waves themselves. The lower frequencies is perhaps indicative of GPOX requiring a longer period of time than CAT to stabilize into steady-state levels.

GPOX activities were approximately twice as high as those reported in comparable past research involving similar fish models and redox cycling compounds, which supports the idea that GPOX can be induced to the relatively high levels seen with other common antioxidant enzymes when exposed to toxic insult (Stephensen *et. al.*, 2000; Stephensen *et. al.*, 2002). However, there is conflicting data on the general response of GPOX in fish after exposure to carcinogenic compounds, with some studies indicating induction and some indicating inhibition (Gabryelak and Klekot, 1985; Rodrigues-Ariza *et. al.*, 1993; Hayes and McLellan, 1999; Stephensen *et. al.*, 2002).

The time-course data for hepatic CAT suggested that upregulation of CAT due to PAH exposure was dose-dependent. The highest exposure treatments used in this experiment, 200 BAP and 200 MIX, created the most dramatic responses— more than 200% and 350% above the next highest response on that day (day 14), respectively. These results agree with previous research that demonstrated a PAH mixture elicited a stronger response than comparable concentrations of benzo( $\alpha$ )pyrene (Bravo *et. al.*, 2006). The hepatic GPOX fluctuations due to the PAH treatments showed no evidence of dose-dependence. One of the 20 ppm treatments exhibited higher levels of GPOX activity than its respective 200 ppm counterpart at all four

time points, and both treatment series exhibited this on day 28. Of the three instances where the 200 ppm dose elicited a stronger response than the 20 ppm dose, the differences between the two were low overall: 2.59% of control (BAP) on day 7; 14% of control (MIX) on day 14; 33% of control (MIX) on day 42.

QR is a widely distributed flavoprotein oxidoreductase that protects against quinone toxicity by promoting obligatory two-electron reductions that divert quinones from oxidative cycling or direct interactions with critical nucleophiles (Benson *et. al.*, 1980; De Long *et. al.*, 1986). QR was identified as a possible detoxifying enzyme active in PAH exposure compensation due to high basal activities in rainbow trout compared to other fish (Sturve *et. al.*, 2005). Previous work demonstrated QR was not elevated significantly in rainbow trout after single BAP doses below 15 mg/kg (Sturve *et. al.*, 2005). QR was measured in hepatic cytosolic fractions. A two-way ANOVA analysis of measured QR activities revealed no significant ( $P > 0.05$ ) interaction with the various treatments over time. These results indicate that QR played no major role in the enzymatic compensatory response to PAHs in this experiment. However, the overall low QR activities during this experiment, especially with the most toxic of treatments, 200 MIX, support the possibility that the PAHs caused toxicity-induced degradation of the enzyme. It is also a possibility that the already high levels of QR seen in rainbow trout were high enough to detoxify the quinones generated by the PAHs and concentrations the fish were exposed to during this study.

The TBARS assay estimated oxidative stress in the liver samples, as increases in lipid peroxidation have been associated with oxidative stress (Bravo *et. al.*, 2006). This research found no significant increases in hepatic TBARS due to the PAH treatments. It is possible that adaptation suppressed PAH-induced oxidative stress at the time-points sampled. However, previous research on rainbow trout detected a positive correlation between PAH exposure and oxidative stress as estimated by the comet and protein nitration assays (Bravo *et. al.*, 2006).

The TBARS assay was extremely variable for fish liver, with the variability being to the extent that each sample had to be run in quintuplicate. The harsh preparation conditions, specifically the prolonged incubation procedure (95 °C for 60 minutes), probably contributed to this variability. Fish tissue is much more sensitive to oxidation at high temperatures than mammalian tissue due to high lipid unsaturation in fish reared at cold temperatures (Curtis *et. al.*, 1995). High background due to lipid peroxidation, specifically to lipid peroxides, was probable.

Previous research concurs that the TBARS assay is a poor choice for the determination of specific levels of lipid peroxidation in vivo due to malondialdehyde not being a specific marker for lipid peroxidation (Halliwell and Grootveld, 1987; Morrow *et. al.*, 1990; Longmire *et. al.*, 1994; Roberts and Morrow, 2000). The experience of this study with the TBARS assay indicates that it is a poor choice for toxicological research using fish models. A much more specific and stable assay, such as the



mass-spectroscopy-based F<sub>2</sub>-isoprostanes assay, would be a much more appropriate assay for use with fish models in future research (Bravo *et. al.*, 2006).

## CONCLUSIONS

Detoxifying and antioxidant capacity in rainbow trout is an amalgam of various enzymes and co-factors that work in concert, and this study has only scratched the surface of this complex network. In summary, previous research showed rainbow trout adapt to oxidative stress induced by subchronic PAH exposure in environmentally-relevant formulations as indicated by the gradual reduction of PAH-induced DNA damage over time (Bravo *et. al.*, 2006). This study examined enzymes considered potentially important in the initiation of this observed adaptive response. CAT was the most prominent enzyme detected, showing a significant, dose-dependent increase that peaked at over 550% of control levels in high-dose PAH exposures. The MIX treatments, a formulation of various PAHs in ratios seen in heavily contaminated urban estuaries, elicited stronger responses than the BAP treatments in hepatic CAT levels (Arkoosh *et. al.*, 2001). This was to be expected, as these differences in response to various treatment concentrations had been documented in previous research (Bravo *et. al.*, 2006). GPOX showed a significant, non-dose-dependent response that did not show a significant difference between the two formulations (MIX and BAP). CAT and GPOX, both enzymes of the Keap1-Nrf2-ARE antioxidant pathway, demonstrated a continuous rising and falling pattern during the time-course of the study that indicates that both enzymes were following sinusoidal decay courses. This decay course is due to decreased antioxidant enzyme activity that followed peak induction, perhaps to find the steady-state level for

each enzyme that allows for maximum protection against the toxic assault while simultaneously preserving optimal systemic stability and health. The detoxifying enzyme QR was also considered as a possible alternative to Keap1-Nrf2-ARE pathway enzymes, but no significant increases in QR activities throughout the time-course of this study were detected. Lipid peroxidation was measured using the TBARS assay. This assay did not produce conclusive results. A more specific and reliable assay, such as the mass-spectroscopy-based  $F_2$ -isoprostanes assay, would be a much more appropriate choice for similar research in the future.

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