ENERGETIC COSTS OF PYRENE METABOLISM IN ISOLATED HEPATOCYTES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

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ABSTRACT

The biotransformation of xenobiotics by fish can be important to their survival while living in contaminated environments. However, the energetic costs of such detoxification are unknown. This study examined the respiratory costs of pyrene biotransformation and excretion in isolated hepatocytes of adult rainbow trout, *Oncorhynchus mykiss*. Baseline oxygen consumption rates measured at an acclimation temperature of 7.5°C and during an acute temperature increase to 15°C were 10.1±0.1 and 22.6±0.4 ng O_2 min^{-1} mg $cells^{-1}$, respectively. Hepatoctyes exposed to 1, 5 and 10 µg/ml pyrene at 7.5°C exhibited concentration-dependent increases in oxygen consumption (ng O_2 min^{-1} mg $cells^{-1}$): 18.8±0.7% (increase compared to acclimated baseline), 31.1±0.8% and 40.6±0.7%, respectively. Exposure of cells to pyrene at 15°C also elevated oxygen consumption, however, the relationship with pyrene concentration was biphasic. Oxygen consumption rates of hepatocytes exposed to 1, 5 and 10 µg/ml pyrene at 15°C increased 13.2±1.6%, 34.1±1.7%, and 21.7±0.8%, respectively. The major metabolite identified was conjugated 1-hydroxypyrene. At 7.5°C, increased pyrene metabolism correlated with increased hepatocyte respiration rates. At 15°C, however, pyrene metabolism reached a maximum at 5 µg/ml, suggesting saturation of detoxification enzymes, which correlated with maximum respiration rates at this concentration. Measures of respiration by isolated mitochondria indicated that changes in hepatocyte oxygen consumption were not through direct effects of pyrene or 1-OH pyrene on mitochondria. Furthermore, cytotoxicity tests *via* cell viability and LDH assays showed that the pyrene concentrations selected in this experiment did not significantly affect the integrity of the cells. Overall, this study indicates that significant respiratory costs may be accrued by teleost hepatocytes actively metabolizing and secreting xenobiotic compounds.

DEDICATION

This thesis is dedicated to the members of my family: my father (Gian), my mother (Joginder) and my brothers (Sukhjit and Sunny).

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LIST OF ABBREVIATIONS AND ACRONYMS

1-OH	1-hydroxy
AB	Assay buffer
ADP	adenosine 5'-diphosphate
BSA	Bovine serum albumin
^{14}C	Carbon-14
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
DDT	1,1,1-trichloro-2,2 bis(p-chlorophenyl)ethane
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetate
F	Farad
g	Gram
g	Acceleration of gravity
G.I.	Gastrointestinal
H_2SO_4	Sulfuric acid
HB	Homogenization buffer
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
hr	Hour
HSS	Hanks' salt solution
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen orthophosphate
KOH	Potassium hydroxide

L	Litre
M	Molar
m	Metre
$MgSO_4$	Magnesium sulfate
min	Minute
mm Hg	Millimeters of mercury
MS-222	3-Aminobenzoic acid ethyl ester methane sulphonate
N	Normality
NADH	β-nicotinamide adenine di-nucleotide
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
Na_2HPO_4	di-Sodium hydrogen orthophosphate
NaOH	Sodium hydroxide
O_2	Oxygen
P_b	Barometric pressure
P_{H2O}	Vapor pressure
Pa	Pascal
PAH	Polycyclic aromatic hydrocarbon
PO_2	Partial pressure of oxygen
RCR	Respiratory control ratio
SA	Succinic acid
SEM	Standard error of the mean
SFS	Synchronous fluorometric spectroscopy
TLC	Thin layer chromatography
Tris	Tris[hydroxymethyl]aminomethane
TX-100	Triton X-100

v.	Versus
v/v	Volume to volume
Ω	Ohm

Prefixes for units of measurement:

n	nano
μ	micro
c	centi
m	milli
k	kilo

INTRODUCTION

Energetic Costs and Detoxification

Quantitative relationships between the acquisition and allocation of energy to an array of biological, biochemical and physiological processes within an organism are of importance in bioenergetics (Xie and Sun 1993). Individual animals require energy from their food and utilize it in these processes, such as those related to metabolism, growth, reproduction and excretion (Xie and Sun 1993). The majority of energy harnessed from food is directed towards metabolism: standard/basal and active. Standard and basal metabolic rate both refer to the energy utilized by a non-stressed, fasting organism at rest as a means of maintaining normal bodily functions (Campbell et al. 1999). Basal metabolic rate is associated with endotherms, organisms like birds and mammals that maintain a constant body temperature even if the environmental temperature fluctuates, while standard metabolic rate is found to occur in ectotherms, organisms like reptiles and amphibians that change their internal temperature with respect to their surroundings (Campbell et al. 1999). Active metabolic rate relates to the amount of energy utilized by an organism performing its normal daily activities, for instance, catching prey and avoiding predators. Any energy remaining after metabolism is then divided to several remaining processes, including growth and reproduction.

All organisms tend to have a relatively fixed energy budget (Maryański et al. 2002), i.e., an increase in energy demands by one of the biological, biochemical or physiological processes would mean that one or several other processes may be compromised. This idea is linked to the principle of energy allocation, which states that if an organism can only acquire a limited amount of materials and energy for which two processes compete directly, then an increase in allocation to one must result in a decrease in allocation to another (Kitchell 1983). Although energy is diverted to the processes that need immediate attention to maintain survival, other processes may be negatively impacted.

Energetic costs have been demonstrated in numerous studies, especially those associated with reproduction and sexual displays, swimming and diving, immunity as well as xenobiotic exposure. With respect to reproduction and sexual displays, a study by Kotiaho et al. (1998) demonstrated that in the wolf spider (*Hygrolycosa rubrofasciata*), sexual signaling (drumming) is costly in terms of energy expended. During sexual signaling, metabolic rate was 22 times higher than at rest. Drumming was also 4.5 times more demanding than just movement of the spider, and in most cases the metabolic rate during drumming seemed to be even higher than the maximal sustainable metabolic rate. Energy expenditure in the polygynandrous millipede (*Alloporus uncinatus*) during copulation was 30% and 14% above resting levels in males and females, respectively (Telford and Webb 1998). The greater energetic costs incurred by males may be due to the physical force required to subdue females. From the study, the measured energetic costs of copulation in male and female *A. uncinatus* represent a significant energetic investment in reproduction, especially considering that individuals of both sexes could potentially mate in excess of 100 times over a four month breeding season. Additional costs of frequent mating are presumably a risk of suffocation for females if copulation is prolonged and the loss of time for both sexes which could otherwise be invested in maintenance activities such as feeding. In the study by Nicol et al. (1995), it was shown that female Antarctic krill (*Euphausia superba*) lose approximately 34% of its body mass when they lay eggs. This represents a considerable input of energy which was estimated using a combination of measured mass and energy differences in female krill and from the measured energetic content of ovarian tissue. Large female krill would lose 2.9 to 3.8 kJ each time a batch of eggs was laid.

Energetic costs in organisms have also been associated with activities such as swimming and diving. Butler (2000) showed that significant increases in oxygen consumption due to diving and surface swimming at maximum sustainable speeds (both ~3.5 times higher than the resting value) were found to be energetically costly in tufted ducks (*Aythya fuligula*). In another study by Webber et al. (2000), researchers were able to demonstrate that the cost of vertical swimming in the squid (*Loligo forbesi*) was more than four times that of *Nautilus pompilus* with the actual cost being 324 mg O_2/kg and 85 mg O_2/kg, respectively, for a 240 m climb. If assuming that the daily cycle of these species involved 12 hr resting and 12 hr hovering in the water column, then the costs of vertical swimming would be 12% (squid) and 10% (*Nautilus*) of their total daily energy budgets (Webber et al. 2000).

Energetic costs have also been noted in studies dealing with immunity challenges. In one study, C57BL/6J mice injected with keyhole limpet hemocyanin mounted significant immunoglobulin G response and consumed more oxygen compared to mice injected with saline (Demas et al. 1997). In Henken and Brandsma (1982), chickens injected with sheep red blood cells (SRBC) consumed more food but gained less weight than control chickens injected with saline. The increase in energy intake may likely be directed at the increased energetic demands required for mounting an anti-SRBC antibody response. Research on bighorn sheep (*Ovis canadensis*) has provided evidence for energetic trade-offs between immune and reproductive function, as seen by increased parasitic infection in fecal samples of lactating bighorn ewes compared to non-lactating ewes (Festa-Bianchet 1989). This may be due to a reduced immune function since lactation requires substantial energy. In Ots et al. (2001), wintering male great tits (*Parus major*) injected with a non-pathogenic antigen (sheep red blood cells) demonstrated that activation of the immune system altered the metabolic activity and profile of immune cells in these birds during the week subsequent to antigen injection. Individual birds mounting an immune response had nearly 9% higher basal metabolic rates than sham-injected control birds. Also, these birds exhibited a 3% (0.5 g) loss of their body mass following the immune challenge. In a study by Martin II et al. (2003), house sparrows (*Passer domesticus*) were injected with phytohaemagglutinin (PHA), a commonly used mitogen that activates cell-mediated immunity, into the wing web. PHA injection significantly elevated the resting metabolic rate (RMR) of challenged sparrows versus those injected with saline. The total cost of this immune activity was approximately 4.20 kJ per day (29% of RMR).

There have been a variety of published reports showing energetic costs associated with the introduction of a xenobiotic to terrestrial and aquatic organisms. In relation to terrestrial organisms, Patnaik (2002) found that exposure to 8 ppm of copper resulted in ~66% decrease in soil consumption, ~97% decrease in egestion, ~80% decrease in growth and ~31.5% increase in maintenance costs of the earthworm (*Lampito mauritii*) when compared to the control. Maryański et al. (2002) were able to show significant decreases in body caloric value as well as morphological changes (decrease in elytraie, bibiae and rear femora) in carabid beetles (*Poecilus cupreus*) after feeding them cadium or zinc contaminated housefly pupae during their lifetime.

Besides terrestrial organisms, numerous experiments have documented energetic costs in aquatic organisms after exposure to a xenobiotic. Lake chubsuckers (*Erimyzon sucetta*) were exposed for 124 days to coal ash-polluted sediments, which resulted in substantial decreases in growth (seen within 60 days of exposure) compared to control fish (Hopkins et al. 2000). Another study by Berntssen and Lundebye (2001) examined the energetics in Atlantic salmon parr (*Salmo salar L.*) fed with elevated dietary cadmium (Cd). These fish were reared for four months on experimental diets supplemented with 0.5, 5, 25, 125, or 250 mg Cd/kg, which led to significant decreases in protein concentration (for the 250 mg Cd/kg treatment group), as well as lipid (for the ≥ 125 mg Cd/kg treatment groups) and glycogen (for the ≥ 125 mg Cd/kg treatment groups) concentrations when compared to the control group. Growth rates were significantly decreased in fathead minnow (*Pimephales promelas*), which were fed a diet spiked with 20 and 30 ppm of selenium (Ogle and Knight 1989). Finally, caloric content was significantly decreased in freshwater amphipods (*Gammarus pseudolimnaeus*) after 15 days exposure to 0.77 mg/L of pentachlorophenol (Graney and Giesy Jr. 1986). In these studies, the energetic costs of xenobiotic exposure include those involved in detoxification/excretion of the compound as well as costs involved in maintaining homeostasis or repairing toxicant-induced damage. Due to the complicating factor of toxic effects, isolating detoxification costs is not possible.

The primary focus of the research presented in this thesis was the energetic costs of xenobiotic detoxification. Limited research on the costs of detoxification exists, and has mainly been focused on host plant specialization in insects and in vertebrate foraging behavior. In herbivorous insects, plant secondary metabolites can exact significant nutritional costs to herbivores by affecting either nutrient utilization (Glick and Joselyn 1970; Lindroth and Batzli 1984; Robbins et al. 1987; Robbins et al. 1991), or by increasing the energetic cost of detoxification post-absorption (Dash 1988; Remington 1990). Efforts to quantify the direct costs of plant allelochemical detoxification have for the most part proven equivocal because in the majority of these studies, measurements of metabolic costs are confounded with toxicological impact; the xenobiotic itself may inhibit feeding and reduce the digestive efficiency of food, therefore, the effects seen are due to reduced total energy assimilation. This has been the case with the few fish studies to date. It is well documented that xenobiotics such as dioxins and organochlorines can affect fish growth (Helder 1981; Krisfalusi et al. 1998). Due to the toxicological effects of these chemicals, it is unclear if the energetic costs of detoxification are significant and result in an energy reallocation from growth, or if growth reduction was due to appetite suppression, reduced assimilation efficiency, the costs of physiological stress, repair of damaged systems, or all of the above (Beyers 1999a, 1999b).

Cresswell et al. (1992) were the first to demonstrate unequivocally that the presence of a dietary toxin resulted in an increase in the allocation of assimilated food to energy metabolism in the southern armyworm (*Spodoptera eridania*) which was not related to any decrease in the amount of food digested. Specifically, dietary nicotine (0.5%), a substrate of the polysubstrate monooxygenase detoxification system, was found to have significant negative effects on the weight of food ingested, weight gained, relative growth rate and conversion efficiency of digested food by *S. eridania* larvae on a nutrient-rich artificial diet. Furthermore, in Berenbaum et al. (1994), it was shown that the detoxification of the plant allelochemical xanthotoxin was maintained, at the expense of growth, in the parsnip webworm (*Depressaria pastinacella)* fed nutrient deficient diets. Moreover, recent work in birds (Guglielmo et al. 1996), marsupials (Dash 1988; Foley 1992), and mammals (Lindroth and Batzli 1984) have directly measured metabolite excretion and estimated the associated energy losses. In ruffed grouse (*Bonasa umbellus*), the detoxification of coniferyl benzoate assimilated from the diet, resulted in an estimated energetic cost of 10% to 14% of metabolizable energy intake (Guglielmo and Karasov 1996).

As suggested by these studies, the costs of detoxification may be detrimental to other processes like growth and reproduction since there is the likelihood of a decrease in the energy allocation to these processes. The costs of xenobiotic detoxification may be related to (1) increases in energy used in enzyme conversions, (2) maintenance of acid-base balance modified by the production of acidic metabolities, and (3) nutrient drains from conjugation reactions with endogenous substrates (Foley 1992).

Hepatic Biotransformation

Although lower vertebrates such as fish have been found to have high levels of xenobiotic metabolizing enzymes in the kidney, gastrointestinal tract and the gill, most of the information regarding xenobiotic detoxification has been derived from studies on the liver (Andersson and Forlin 1992). In the present study, trout hepatocytes were used since they are considered to be important sites in the biotransformation of foreign compounds by converting lipophilic xenobiotics into more-water soluble metabolites for excretion. This is accomplished by two detoxification processes, namely Phase I and II reactions.

Phase I reactions usually take place in the smooth endoplasmic reticulum of hepatic parenchymal cells (Ioannides 1996). These reactions tend to produce metabolites containing hydroxyl groups, which are necessary for most, but not all, of the subsequent conjugation reactions which constitutes Phase II reactions. Phase I reactions use oxidation, reduction and hydrolysis catalyzed by either cytochrome P450-dependent monooxygenases for hydroxylation of carbon atoms or flavin-containing monooxygenases for hydroxylation of N- or S-heteroatoms (George 1994; Buhler and Williams 1988). Cytochrome P-450 monooxygenase enzymes comprise a widely distributed super family of more than 400 individual isoforms and is expressed in a diverse array of organisms, including bacteria, plants, fungi and animals (Zapata-Perez et al. 2002). Alternatively, pre-existing functional groups can be exposed by a variety of hydrolytic enzymes, namely epoxide hydrolases, esterases and amine oxidases (Buhler and Williams 1988). Also, there are redox enzymes, like alcohol and aldehyde dehydrogenases, that are able to alter the oxidation state of a carbon atom, thereby, facilitating the excretion or biotransformation of the parent compound (George 1994). Modification of functional groups on the parent molecule by Phase I reactions usually leads to a decrease in toxicity and an increase in water solubility for excretion.

Phase II reactions normally involve conjugation reactions in the cytosol of hepatocytes where the metabolites of the Phase I reactions are bound to a highly polar endogenous molecule leading to an even more water soluble xenobiotic that is able to be easily excreted from the organism. These conjugation reactions are catalyzed primarily by either UDP glucuronosyl-transferases, glutathione-transferases, or sulpho-transferases (Buhler and Williams 1988; Walker et al. 1997). There are also other minor Phase II pathways in fish that involve other enzymes including acetyltransferases for organic xenobiotics containing an amino group and amino-acyl transferases for carboxylic acids such as 2,4-D and pyrethroid insecticides (George 1994). Some xenobiotic compounds, however, possess the required functional groups for direct metabolism by Phase II enzyme systems, which suggest that they can bypass Phase I reactions (George 1994).

Polycyclic Aromatic Hydrocarbons and Pyrene

Polycyclic aromatic hydrocarbons (PAHs) are a class of xenobiotics usually made up of two or more fused benzene rings in linear, angular or cluster arrangements (Namdari 1998). There are hundreds of PAHs ranging in molecular weight from 128.16 (naphthalene) to 300.36 (coronene) (Namdari 1998). PAHs are ubiquitously present throughout the environment in water, soil, air and sediments.

Due to the carcinogenic properties of many PAH compounds, and their widespread diffusion from various sources such as domestic heating, forest fires, traffic, power plants and creosoted wood, PAHs are regarded as a major environmental threat (Ariese et al. 1997; Gabos et al. 2001). In relation to the aquatic environment, PAHs are normally released *via* sewage effluents, surface runoff and accidental releases of petroleum and its products (Law et al. 1994). Usually, medium and high molecular weight PAHs such as pyrene are highly hydrophobic, which allows these compounds to adsorb to suspended inorganic and organic particulates (Hinkle-Comm et al. 1998). Typically, sediments in aquatic environments contain approximately 1000 times greater concentrations of PAHs than the overlying water (Lin et al. 1994). Therefore, fish can be exposed to PAHs through dermal contact with contaminated sediments (Saarikoski et al. 1986), consumption of contaminated prey (Ariese et al. 1997), and absorption across the gills (Namdari and Law 1996). It has been suggested that the gills are the most important uptake route for waterborne PAHs in fish and that the gastrointestinal tract and the skin do not contribute significantly to the systemic absorption of PAHs (Balk et al. 1984; Jimenez et al. 1987; Kennedy and Law 1990). For example, Namdari and Law (1996) showed that pyrene concentrations in the blood of trout after branchial exposure were significantly higher than those of the dermally exposed trout, indicating that the gills were the most important route of absorption of waterborne pyrene. The gastrointestinal tract is not considered to be an important route of waterborne pyrene absorption as freshwater trout are not likely to drink water and only low levels of PAHs are absorbed after oral exposure (Kennedy and Law 1990; Namdari and Law 1996).

In the present study, pyrene was selected as a model xenobiotic because (i) it is extensively metabolized in trout via Phase I and II reactions, and (ii) it has no measurable toxic effects even at relatively high concentrations (Nisbet and LaGoy 1992). Pyrene is lipid soluble (log K_{ow}: 5.23; water solubility: 130 µg/L) and is present in high concentrations in waters contaminated by PAHs (EPA 1975) as well as in sediments (Youngblood and Blumer 1975; Johnson et al. 1985; Pelletier et al. 1997).

Synchronous fluorescence spectroscopy (SFS) is a highly sensitive method that is suitable for detecting pyrene metabolites, particularly 1-hydroxypyrene (1-OH pyrene) (Ariese et al. 1993; Stroomberg et al. 1996; Ariese et al. 1997). It is a cost-efficient method for screening large numbers of samples for exposure to PAHs in fish (Lin et al. 1994). Furthermore, this method requires little instrumentation and consumes no solvents, making it rather inexpensive to use.

Numerous studies devoted to occupational PAH exposure have reported 1-OH pyrene to be a useful biomarker (Levin 1995). Furthermore, 1-OH pyrene has been detected in fish bile, showing a good correlation with the degree of pollution of the area (Krahn et al. 1987; Ariese et al. 1993). Regardless of the exposure to other PAHs, 1-OH pyrene was found to be the predominant PAH metabolite in bile in those surveys.

Objectives of Study

All of the literature available suggests that there is a quantifiable cost to organisms which must detoxify xenobiotics. The significance of the cost of detoxification in terms of overall energy budgets is unknown. Therefore, the main objective of this study was to begin to directly measure the costs of xenobiotic transformation in fish utilizing isolated hepatocytes and respirometry. Isolated hepatocyte preparations represent a unique experimental system for the study of detoxification costs as they allow repeated measures on the same population of cells from an individual fish, eliminating confounding factors that arise in using whole animals such as the variation in the concentration of the xenobiotic delivered to the sites of biotransformation.

Several other objectives were also investigated in this study, including the effects of temperature on detoxification costs in hepatocytes and a measurement of pyrene's cytotoxicity at the concentrations used in the study.

MATERIALS & METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss*) of both sexes weighing 560 to 650 g were obtained from Sun Valley Trout Farms (Mission, BC) and kept in 1200 L tanks at Simon Fraser University (Burnaby, BC). They were acclimated for two weeks in a well-oxygenated flow-through system of dechlorinated water at 7.5°C and a photoperiod of 12 hr light: 12 hr dark. The hardness of the water ranged from 6.6 to 6.8 mg $CaCO_3$/L and pH of 6.67±0.30. During acclimation, tanks were partially covered with black tarp in order to minimize disturbance. Fish were fed with Pro-Form Floating Koi Food (3.0 mm pellets) *ad libitum* until 48 hours before an experiment.

Purification of Pyrene

Pyrene was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and ground to fine powder before purification. A glass column was packed with a 75 ml mixture containing hexane and Silica gel-60 (mesh size 230 to 400) (EM Science, Gibbstown, NJ). Pyrene was dissolved in a minimal amount of hexane and added to the column. The column was run with hexane and 10 ml fractions were collected. Fractions were then spotted on aluminum-backed thin layer chromatography (TLC) Silica gel-60 F_{254} plates (EM Science, Gibbstown, NJ). The TLC plates were run using a mixture of ethyl acetate and hexane (1:1) and exposed to ultra-violet light as a means of detecting which fractions contained pyrene. The pyrene fractions were combined and evaporated using a Buchi RE 111 rotary evaporator (Gen Tech Inc., Arcade, NY). The purity of pyrene was found to be greater than 99%. All purified pyrene standards used in this experiment were dissolved in dimethyl sulfoxide (DMSO, Caledon Laboratories Ltd., Georgetown, ON).

Hepatocyte exposure to pyrene and respiration measurements

Chemicals

N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), KCl, DL-lactic acid (sodium salt), $MgSO_4$ were from Sigma Chemical Co. (St. Louis, MO). Anhydrous D-glucose, Na_2HPO_4, NaCl were obtained from BDH Inc. (Toronto, ON). KH_2PO_4 and ethyl acetate were acquired from Caledon Laboratories Ltd. (Georgetown, ON). $CaCl_2$ and $NaHCO_3$ were from Fisher Scientific Company (Fair Lawn, NJ), and Merck KGaA (Darmstadt, Germany), respectively.

Three types of Hank's salts solutions (HSS) were required for this experiment as these were the same as those used by Moon et al. (1985). For each HSS, 176 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, and 10 mM HEPES were dissolved in ultrafiltered water and stored at 4°C. HSS A comprised of these chemical components and was gassed with a mixture of 1% CO_2 and 99% O_2 (Praxair Products Inc., Mississauga, ON) for 30 minutes at room temperature. Following this, 6 mM of $NaHCO_3$ was added as well as D-glucose and DL-lactic acid to final concentrations of 3 mM and 1 mM, respectively (Gill and Walsh 1990). The pH was adjusted to approximately 7.6 at 20°C by the addition of either 1 N H_2SO_4 or 1 N NaOH. During experimentation, HSS A was kept on ice.

HSS B and C were prepared in a similar manner; however, oxygenation of HSS B took place at either 7.5°C (acclimated temperature of the trout) or 15°C (non-acclimated temperature). Following the pH adjustment, 0.11 g/L $CaCl_2$ and 20 g/L BSA were added to HSS B while 0.3 g/L of Type IV collagenase was added to HSS C. During experimental use, HSS C was kept on ice while HSS B was placed into a water bath at either the acclimated temperature or non-acclimated temperature. Prior to each respiration rate measurement, the oxygen concentration (mg/L) of HSS B was recorded using a YSI Dissolved Oxygen Meter (Yellow Springs Instrument Co., Yellow Springs, OH).

For metabolite analysis, pyrene and 1-hydroxypyrene (1-OH pyrene) were supplied by the Aldrich Chemical Co. (Milwaukee, WI) while anhydrous ethanol was obtained from Commercial Alcoholics Inc. (Brampton, ON).

Hepatocyte Isolation

For each experimental run, a trout was anesthetized in dechlorinated water containing 0.2 g/L MS-222 buffered with 0.2 g/L $NaHCO_3$. Liver biotransformation rates in trout treated with MS-222 have not been found to be significantly different than the rates in controls (Kolanczyk et al. 2003); hence, using this method of anesthetization was adequate in this experiment. Once all opercular movement had ceased, the fish was euthanized with a blow to the head and place on ice ventral side up. The fork length and mass of the trout were recorded.

Hepatocyte isolation was a performed according to a modified procedure of Moon et al. (1985). A ventral incision was made from the gills to the pectoral fins and then another behind each pectoral fin exposing the liver. Blood vessels leading from the liver to the heart were cut, followed by the cannulation of the hepatic portal vein. The syringe head used in cannulation was attached to a Peristatic pump (Cole-Parmer Instrument Co.). The liver was first perfused with HSS A at a flow rate of 2 ml min^{-1} g^{-1} of liver until the blood in the liver was cleared. In order to set the flow rate, the mean liver mass of four trout was used (4.65 g). Following perfursion with HSS A, the liver was subjected to a 35 min perfusion with HSS C while gently massaged by hand. The syringe was then removed and the liver carefully cut away from the fish and gall bladder and then placed on a watch glass on ice. At this point, the liver was rinsed with HSS A and then coarsely minced with a razor blade. Any poorly perfused areas were removed while the remainder was gently pushed through a screen of nylon mesh (253 μm) followed by washing through a further two screens (73 μm) with HSS A. Following this, the hepatocyte solution was poured into a 15 ml polypropylene conical tube (Becton Dickinson Labware, Franklin Lakes, NJ) and kept on ice.

The hepatocytes were collected by low speed centrifugation (125x*g*) for 2 min at 4°C using a Centra MP4R centrifuge (International Equipment Co., Needham Heights, MA). The resulting supernatant was decanted and the cells were resuspended in HSS B (on ice). The decanted supernatant was subjected to a second centrifugation for 2 min at 125x*g* with the purpose of obtaining more hepatocytes. Next, all the cells were combined and washed two times with HSS B using low speed centrifugation. Following this, the cells were kept on ice in a HSS B suspension. The final concentration of hepatocytes was determined as in Siebert (1985). A 1 ml aliquot of the suspension was placed into a pre-weighed 1.5 ml microcentrifuge tube and centrifuged using a biofuge (Heraeus Instruments, Hanau, Germany) for 2 min at a speed of 3700x*g*. The supernatant was removed and cells were weighed to determine the concentration of hepatocytes in mg/ml.

Pyrene Exposure

Six 20 ml glass scintillation vials were labeled with the following treatments: Control 1, Control 2, 0 μg/ml pyrene, 1 μg/ml pyrene, 5 μg/ml pyrene and 10 μg/ml pyrene. Into each scintillation vial, 3 ml of HSS B and approximately 75 mg viable cells were added and subjected to a 30 minute acclimation period in a water bath set at the acclimation temperature of 7.5°C. Afterwards, pyrene in DMSO (5 μl) was added to the appropriately labeled vials to achieve final concentrations of 1, 5 and 10 μg/ml (two control vials without pyrene were run consectively; the 0 μg/ml pyrene vial contained 5 μl of DMSO as a solvent control). All treatments then underwent a 30 minute incubation period before oxygen consumption rates were measured to ensure that cells were actively metabolizing and excreting pyrene during respiration measurements (Johnston et al. 1999).

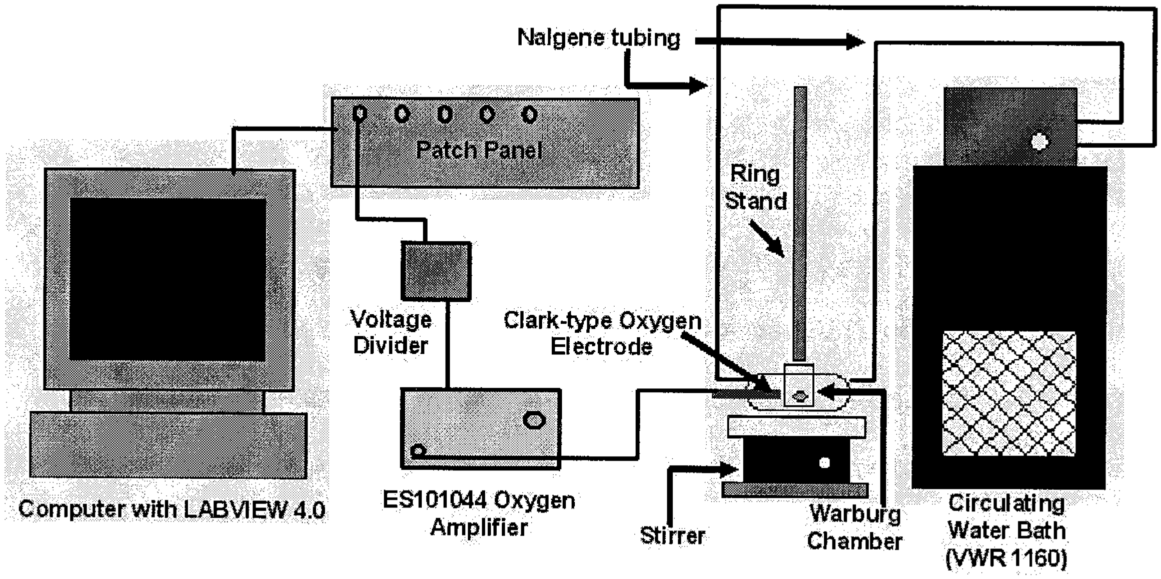
Hepatocyte Respiration Measurements

The experimental set up for respiration measurements is shown in Figure 1. Oxygen consumption rates of suspended rainbow trout hepatocytes were measured in a Warburg chamber equipped with a Clark-type polarographic oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The probe was placed into a Warburg chamber consisting of a water jacket and a 2 ml chamber where the suspended hepatocytes were placed. The water jacket was attached to a circulating water bath (VWR 1160, VWR International, Mississauga, ON). The circulating water bath maintained constant incubating temperatures at either the acclimated (7.5°C) or non-acclimated (15°C) temperature.

Before adding the cells to the chamber, the probe was subjected to electrode calibration and polarization with well-oxygenated HSS B (2 ml), which was thoroughly mixed with a magnetic stirbar (7x2 mm). After calibration, 1 ml of the solution in the chamber was removed and replaced with 1 ml of the cell suspension to achieve a final concentration of 12.5 mg/ml viable cells. The LABVIEW 4.0 program (National

Instruments Corporation, Austin, TX) was used for recording oxygen consumption. The slope of the rate of oxygen consumption by hepaocytes was used in calculating hepatocyte respiration rate in ng O_2 min^{-1} mg $cells^{-1}$ based on Equations 1 and 2, along with the barometric pressure (kPa), vapor pressure of water at 7.5°C or 15°C (1.037 and 1.705 kPa, respectively) (Totten 2000), and the solubility of oxygen in HSS B at each temperature (mg/L) (Doug Wilson, pers. comm.)

Figure 1. Apparatus for measuring oxygen consumption rates of hepatocytes. A fixed polarizing voltage from the ES101044 oxygen amplifier is applied between the platinum cathode and silver anode of the oxygen electrode. As oxygen diffuses through the membrane to the cathode, it is reduced ($O_2 + 2\ H_2O + 4e^- \rightarrow 4\ OH^-$). The oxidation taking place at the reference electrode (anode) is Ag° (metal) $+\ Cl^- \rightarrow AgCl + e^-$. Accordingly, a current will flow that is proportional to the rate of diffusion of oxygen, and in turn to the concentration of dissolved oxygen in the sample. The voltage divider is responsible for scaling the voltage output signal from the oxygen amplifier and is connected to the patch panel by a BNC connector. The patch panel also allows for connection of a multi-wire cable ribbon from the computer so that readings of the dissolved oxygen concentration in the chamber can be displayed on Labview.


Nalgene tubing
Patch Panel
Ring Stand
Voltage Divider
Clark-type Oxygen Electrode
Computer with LABVIEW 4.0
ES101044 Oxygen Amplifier
Stirrer
Warburg Chamber
Circulating Water Bath (VWR 1160)

$$\text{Oxygen Consumption Rate (mg/L*min)} = \frac{\{A * B * [0.209(P_b-P_{H2O})]\}}{[0.209(101.3-P_{H2O})]} \qquad (1)$$

$$\text{Respiration Rate (ng } O_2 \text{ min}^{-1} \text{ mg cells}^{-1}) = \frac{O_2 \text{ consumption rate} * C}{D} \qquad (2)$$

where P_b is the barometric pressure, P_{H2O} is the vapor pressure of water, A is the oxygen concentration of HSS B after being gassed with 1% CO_2/99% O_2, B is the oxygen consumption rate from LABVIEW, C is volume of the Warburg chamber (0.002 L), and D is the total mass of cells in chamber (25 mg). After recording the oxygen consumption rates, samples were frozen at -80°C.

Pyrene Metabolite Analysis

The method described in Seubert and Kennedy (1997) was used to extract pyrene metabolites from hepatocyte suspensions. Hepatocyte samples from each treatment group (Control 1, Control 2, 0 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml pyrene) were thawed and extracted three times with ethyl acetate (3:1) in order to separate phase I metabolites as well as any unmetabolized parent compound. The extracts were combined, dried with nitrogen, resuspended in 1 ml of an ethanol/water solution (50:50% v/v), covered in Parafilm and then stored at 4°C for 24 hours prior to synchronous fluorometric spectroscopy (SFS).

The remaining aqueous layer, containing conjugated phase II metabolites, was subjected to acid hydrolysis (1 N H_2SO_4; pH 1 to 2) for 24 hrs at 80°C in a water bath (Precision Scientific Co., Chicago, IL). This was done to hydrolyze 1-OH pyrene conjugates to 1-OH pyrene for extraction. The samples were then extracted three times with ethyl acetate (3:1). These extractions were treated in the same manner as the extractions containing the phase I metabolites and parent compound.

Synchronous Fluorometric Spectroscopy (SFS)

SFS spectra of resuspended extracts were measured according to Ariese et al. (1993) in 1.5 ml quartz cuvettes (Fisher Scientific) using a Perkin-Elmer LS 50 fluorescence spectrophotomer (Perkin-Elmer Ltd., Beaconsfield, England), scanning both monochromators simultaneously with a constant wavelength difference of 37 nm, and with excitation/emission bandwidths of 5 nm. For quantitation of the amount of 1-OH pyrene, the area underneath the fluorescence intensity trace was measured from 335-356 nm. Samples with spectral readings off-scale were diluted with an ethanol/water solution. A standard curve was created using a range of 1-OH pyrene concentrations (0.114 to 11.4 ng/ml) and areas underneath the fluorescence intensity traces corresponding to these concentrations. Following this, the areas underneath the traces for each treatment group (Control 1, Control 2, 0 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml pyrene) were recorded and incorporated into the standard curve equation to determine the actual concentration of 1-OH pyrene present (ng/ml). Rates of pyrene metabolism (ng 1-OH pyrene produced min^{-1} mg cells^{-1}) were calculated using the total amount of 1-OH produced, the incubation period (min) and the total mass of cells in the chamber.

Mitochondrial Respiration

Chemicals

Tris[hydroxymethyl]aminomethane base (Tris), KCl, fatty-acid free bovine serum albumin (BSA), adenosine 5'-diphosphate (sodium salt) (ADP), succinic acid (SA) and Bradford protein dye reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium phosphate, sucrose and disodium ethylenediaminetetraacetate (EDTA) were obtained from Caledon Laboratories Ltd. (Georgetown, ON), Merck KGaA (Darmstadt, Germany) and Fisher Scientific (Napean, ON), respectively.

Two buffers were used for mitochondrial isolation and respiration recordings. The homogenization buffer (HB) consisted of 10 mM potassium phosphate, 0.25 M sucrose, and 0.5 mM disodium EDTA in ultrafiltered water. This buffer was subjected to air saturation, followed by a pH adjustment of 7.4 using either 1 N H_2SO_4 or 1 N NaOH. This buffer was kept on ice for the duration of this experiment

The assay buffer (AB) consisted of 25 mM potassium phosphate, 10 mM Tris, 100 mM KCl and 2.7 mg/ml of BSA in ultrafiltered, deionized, distilled water. This buffer was air saturated and the pH was adjusted to 7.4 using 1 N H_2SO_4 or 1 N NaOH. This buffer was kept at the temperature used to conduct mitochondrial respiration rates (15°C). The AB was treated in the same manner as the HSS B from the hepatocyte respiration measurement following its preparation. Before each mitochondrial respiration measurement, the oxygen concentration (mg/L) of the AB was recorded using the dissolved oxygen meter.

Preparation of Mitochondria

Mitochondria were prepared according to Suarez and Hochachka (1981). Fish were anesthetized with 0.2 g/L of MS-222 and 0.2 g/L $NaHCO_3$, followed by euthanization by a sharp blow to the head. The liver was quickly dissected out, free from the gall bladder and connective tissue, placed onto a watchglass (on ice), washed in ice-cold HB, weighed and minced with a razor blade. A series of washings with HB were required to remove blood and any bile. Following this, the minced liver was placed into a Potter-Elvehjem homogenizer with 10 volumes of HB and homogenized by hand with a loose-fitting Teflon pestle. The homogenate was subjected to differential centrifugation as a means of separating the mitochondria from the rest of the hepatocyte cell contents. First, the homogenate was spun at 600x*g* for 15 min in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge (Production Engineering-Medical Equipment Division, Denver, CO). The supernatant from the first spin was decanted and spun at 9000x*g* for 10 min. The supernatant from the second spin was discarded and the pellet was resuspended in approximately 10 volumes of HB by gentle aspiration with a Pasteur pipette. The dark, pigmented material at the bottom of the pellet was left undisturbed while the rest of the resuspended pellet (containing the mitochondria) was spun in another centrifuge tube at 9000x*g* for 10 minutes. Mitochondrial preparations were washed in this manner three times before final resuspension in a small volume of HB containing 1 mg/ml of fatty acid-free BSA. All mitochondrial preparations were kept on ice and were documented to be stable for approximately 8 hrs (Suarez and Hochachka 1981).

Mitochondrial respiration measurements

Oxygen consumption was monitored using the LABVIEW program as described for hepatocyte respiration (Figure 1). Two ml of air saturated AB was added to a water-jacketed Warburg Chamber at 15°C to calibrate and polarize the oxygen probe. Approximately 150 µl of AB was replaced with 150 µl of mitochondrial suspension in the chamber (containing approximately 1.1 mg mitochondrial protein). The contents in the chamber were mixed thoroughly using a magnetic stirrer and a Teflon-covered stir bar (7 mm length * 2 mm diameter) and the mitochondrial oxygen consumption rates were recorded. These consumption rates were used in Equations 1 and 2 for calculating mitochondrial respiration rates (ng O_2 min^{-1} mg mitochondrial $protein^{-1}$). The differences in using these equations for mitochondrial respiration measurements compared to the hepatocyte respiration measurements are that A is the oxygen solubility of the AB (mg/L) and D is the total amount of mitochondrial protein present in the chamber. The method of determining the total amount of mitochondrial protein will be addressed later on in this chapter.

Seven treatment groups were used for this experiment: (1) Succinic Acid (SA) /ADP 1, (2) Succinic Acid/ADP 2, (3) 0 µg/ml, (4) 1 µg/ml, (5) 5 µg/ml, (6) 10 µg/ml pyrene and (7) 500 ng/ml 1-OH pyrene metabolite. The 1-OH pyrene concentration was selected to be higher (5-fold greater) than the concentration of metabolite produced by hepatocytes of acclimated and non-acclimated trout determined earlier (Dr. Russell Nicholson, pers. comm.).

To determine if the mitochondrial preparation was functioning according to parameters reported in the literature (Suarez and Hochachka 1981), the SA/ADP 1 and SA/ADP 2 polargraphic recordings were created prior to and after subjecting the mitochondria to the pyrene treatments, respectively. The SA/ADP 1 and 2 recordings involved measurement of baseline respiration when stimulated by the addition of 10 µmoles of SA (dissolved in AB and neutralized with 1 N KOH) using a 100 µl Hamilton syringe. State III respiration was initiated with 0.4 µmoles of ADP (dissolved in AB and neutralized with 1 N KOH) using a 10 µl Hamilton syringe (Suarez and Hochachka 1981). During this state of respiration, ADP is converted to ATP via oxidative phosphorylation in the mitochondria (Campbell et al. 1999). Once all the ADP is consumed, State IV respiration is initiated. Respiratory control ratios (RCR) and ADP: O ratios were calculated according to Estabrook (1967) as seen in Equations 3 and 4:

$$\mathrm{RCR} = \frac{\text{State III respiration (ng O2 min}^{-1}\text{ mg mitochondrial protein}^{-1}\text{)}}{\text{State IV respiration (ng O2 min}^{-1}\text{ mg mitochondrial protein}^{-1}\text{)}} \tag{3}$$

$$\mathrm{ADP{:}O} = \frac{\text{ATP formed (µmol)}}{\text{Oxygen atoms consumed (µatoms)}} \tag{4}$$

Where

- ATP formed = Amount of ADP injected into the chamber
- Oxygen atoms consumed = Decrease in O_2 from State III (mM) * Chamber Volume (2 ml) * 2 (depicts 2 atoms of oxygen in an O_2 molecule)

The direct effects of pyrene (0 [DMSO control], 1, 5, and 10 µg/ml) and 1-OH pyrene (500 ng/ml) on mitochondrial oxygen consumption were examined following stimulation with 10 µmoles of SA. Pre-exposure rates (SA-stimulated mitochondrial oxygen consumption rate without the addition of pyrene) and post-exposure rates (with addition of pyrene) were recorded on LABVIEW. These rates were compared to see if mitochondrial respiration rates without pyrene were significantly different than mitochondrial respiration rates with pyrene present. Each of the treatments as well as samples of AB were saved and placed in a 0°C freezer until they were needed for mitochondrial protein determination.

Mitochondrial Protein Measurements

The amount of mitochondrial protein used for each treatment was determined using the Bradford reagent (Bradford 1976). This assay was performed in a 96 well plate based on the protocol from Sigma. Protein standards of BSA ranging from 0 to 1 mg/ml were prepared in ultrafiltered water. An aliquot of 5 µl from protein standards, AB and the pyrene treatment groups were added to separate wells. To each well being used, 200 µl of Bradford reagent was added and mixed. The samples were incubated at room temperature for 30 minutes before recording their absorbance values at 595 nm using a Spectra Max 420 microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The BSA protein standards were used in creating a Bradford assay curve needed in ascertaining the quantity of mitochondrial protein in the chamber during mitochondrial respiration measurements.

The mitochondrial protein concentration (mg/ml) for each treatment group was calculated *via* a two-step process. First, absorbance values from the pyrene/1-OH pyrene treatment groups were subtracted from the absorbance of AB, leaving the absorbance of mitochondrial protein only. Next, the mitochondrial protein concentration corresponding to this new absorbance value was determined using the Bradford assay standard curve.

Lactate Dehydrogenase Assay

Chemicals

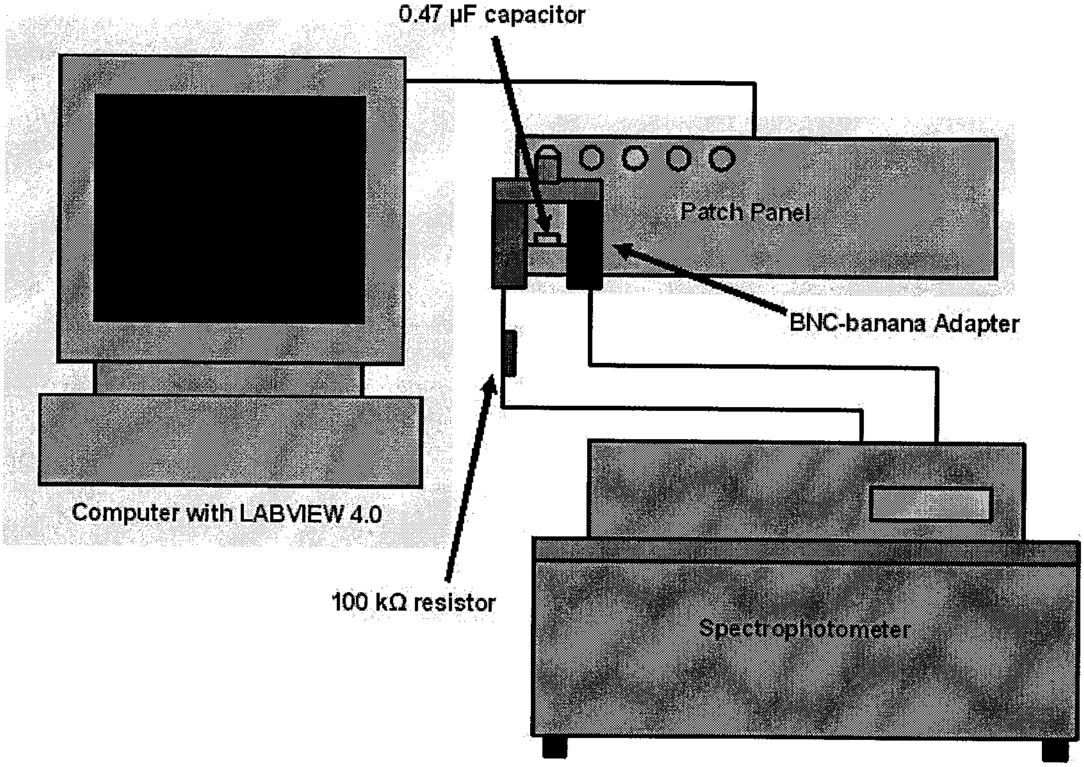
For this assay, pyruvic acid, the reduced form of β-nicotinamide adenine dinucleotide disodium salt (NADH) and Triton X-100 were obtained from Sigma (St. Louis, MO). All other reagents used for this section were described previously.

LDH Assay

Lactate dehydrogenase (LDH) activity was measured in four separate fish as an indicator of cell membrane damage (Pesonen and Andersson 1992) which may have occurred due to pyrene exposure. Assays were conducted by modifying and combining protocols from Nicholson (1994) and Pesonen and Andersson (1992). LDH activity was measured in cell incubations (25 mg/ml viable cells) in HSS B containing pyrene (0, 1, 5, 10 μg/ml) or 1% Triton X-100 as a positive control at 0, 2, 4 and 6 hrs. The blank consisted of HSS B without cells. Approximately 3 ml of each treatment was placed in appropriately labeled scintillation vials and placed on ice. The first LDH reading was taken at 0 hr with the treatment on ice and then 2 hrs, 4 hrs and 6 hrs after incubating the samples at 10°C in a water bath, which was the acclimation temperature of the trout during the time this experiment was performed.

The equipment set up for the LDH assay is shown in Figure 2. The absorbance of the Milton Roy Spectronic 301 spectrophotometer (Mahin & Associates, Reno, NV) was set to 340 nm and autozeroed with 3 ml of reference solution (HSS B). Three ml of HSS B and 50 μl of one of the treatments, except for the Triton X-100 treatment (where only 35 μl was added), was placed into the spectrophotometer for recording of absorbance. 100 μl NADH was added to the cuvette to give a achieve concentration of

Figure 2. Experimental set up of the LDH assay. The low-pass resistor-capacitor (RC) filter, comprising of the 100 kΩ resistor and 0.47μF capacitor connected in series, allows low frequency voltages changes from the spectrophotometer to pass through, thereby reducing the noise produced by the spectrophotometer. The RC filter is connected to the patch panel with a BNC-banana adapter. The patch panel also allows for connection of a multi-wire cable ribbon from the computer so that absorbance readings from the spectrophotometer can be displayed on Labview.


0.47 µF capacitor
Patch Panel
BNC-banana Adapter
Computer with LABVIEW 4.0
100 kΩ resistor
Spectrophotometer

0.1 mM. This was followed by the addition of 100 µl pyruvic acid (final concentration of 0.4 mM). Both NADH and pyruvic acid were added using a 100 µl Hamilton syringe. LDH catalyzes the reaction of pyruvic acid to lactate with NADH as a co-enzyme (Campbell et al. 1999). Therefore, as LDH is released from damaged hepatocytes, NADH is used up in converting pyruvic acid to lactate and the absorbance levels of NADH detected by the spectrophotometer decreases. If the cells are intact, then NADH levels outside of the hepatocytes will remain unchanged. The rate of decline in absorbance/min after injecting pyruvic acid was used to measure LDH activity. The greater the decline of absorbance with time suggests higher LDH activity (van Beek et al. 2000).

The rate of decline was substituted into Equation 5, which gave the final LDH activity in the cuvette as nmole min^{-1} mg cells^{-1} (Douglas Wilson, pers. comm.):

$$\text{LDH activity} = ([(A/6.22) * 3.11] / B) * 1000 \qquad (5)$$

where A is the rate of decline in absorbance (absorbance/min) and B is the mass of cells (mg) in the cuvette (1.25 mg for all treatment groups except for the Triton X-100 treatment group, in which 0.875 mg was used). The absorptivity of NADH is 6.22 mM^{-1} cm^{-1} while the pathlength of light through the cuvette is 3.11 cm.

Cell Viability

Cell viability was assessed in acclimated and non-acclimated trout hepatocytes in each treatment. Viability was determined 1 hr post incubation. 50 µL of 0.4% sterile-filtered Trypan Blue solution (Sigma Chemical Co., St. Louis, MO) and 50 µl of the treatment were mixed thoroughly. Approximately 9 µl of this Trypan Blue/ cell suspension mixture was added into the counting chamber of a hemacytometer (American Optical Scientific Instrument Division, Buffalo, NY). The number of stained and unstained cells was counted and the percent cell viability was calculated as in Gill and Walsh (1990).

Statistical Analysis

Statistical analyses were performed using the JMP IN 4.0.3 program (SAS Institute Inc., Cary, NC). Results were expressed as means±SE. The sections of the experiment involving hepatocyte respiration rates, concentrations of 1-OH pyrene produced and rates of pyrene metabolism at either their acclimated (7.5°C) or non-acclimated temperature (15°C) were compared using ANOVA and Tukey-Kramer HSD tests in a single-factor randomized complete block design. However, when each of the previously mentioned sections involved the statistical analysis of temperature effects, a two factor split-plot design was utilized. Mitochondrial respiration rates between exposure treatment groups (0, 1, 5 and 10 µg/ml pyrene) were compared using ANOVA and Tukey-Kramer HSD tests in a single-factor randomized complete block design. LDH activity between treatments was compared using ANOVA and Tukey-Kramer HSD tests in a two-factor split-plot in time design with the two factors being incubation period (0, 2, 4, and 6 hrs) and exposure treatment groups (HSS B control, 1% Triton X-100 as well as 0, 1, 5 and 10 µg/ml pyrene). Cell viability was compared similarly as a two factor split-plot design with the factors being exposure treatment groups (HSS B control as well as 0, 1, 5 and 10 µg/ml pyrene) and incubation temperatures (7.5°C and 15°C). Differences were considered significant at $p \leq 0.05$.

RESULTS

Lactate Dehydrogenase Assay and Cell Viability

A typical LDH absorbance reading is shown in Figure 3. This figure is associated with hepatocytes exposed to 5 µg/ml pyrene after 2 hrs of incubation. Mean LDH activities for each treatment are shown in Figure 4. No significant difference was detected between incubation periods (0, 2, 4, 6 hrs) or between the following treatment groups: (i) HSS B + cells, as well as (ii) HSS B + cells + pyrene (0, 1, 5, 10 µg/ml). This suggests that the pyrene concentrations used in this study did not cause cell membrane damage in hepatocytes and the release of LDH. In this set of treatments, LDH activities are found to be in the range of 4.70±0.90 to 7.80±0.93 nmole min^{-1} mg cells^{-1}.

Significant differences were noted between the positive control (1% Triton X-100) and the negative control (HSS B + 10 µg/ml pyrene + no cells). The negative control was found to have no LDH release since no cells were added to this treatment group. The Triton X-100 treatment had the highest level of LDH activity in all incubation periods, ranging from 56.78±1.60 to 66.35±7.60 nmole min^{-1} mg cells^{-1}. These values are approximately 10-fold higher compared to the other treatment groups.

Figure 5 shows the mean cell viability of acclimated (7.5°C) and non-acclimated (15°C) hepatocytes at two incubation periods (1 and 3 hr) in the various treatment groups (Control 1, Control 2, 0 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml pyrene). Significant differences in viability were not seen between treatments and incubation periods, indicating that the pyrene concentrations used in this study do not cause overt membrane damage of the hepatocytes. However, significant differences were noted

Figure 3. A typical polarographic recording of an LDH assay illustrating zeroing of electrode as well as the absorbances of HSS B and cells treated with 5 µg/ml pyrene, 0.1 mM NADH, and 0.4 mM pyruvic acid.

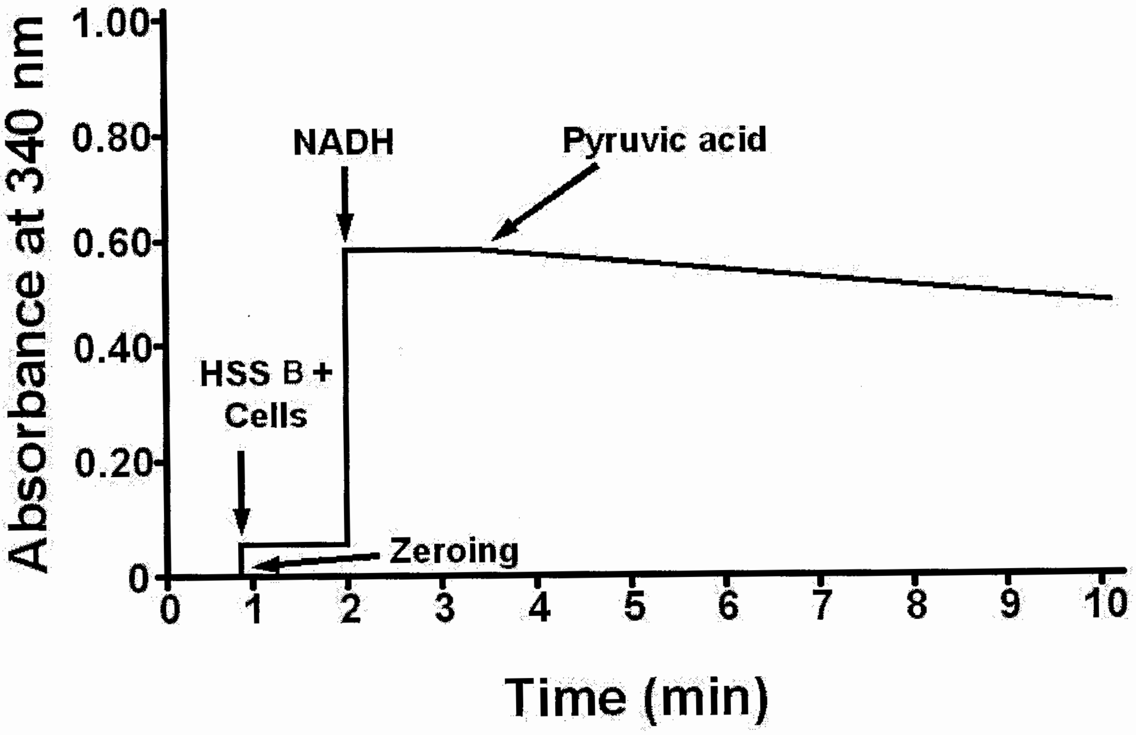

Absorbance at 340 nm
1.00
0.80
0.60
0.40
0.20
0
NADH
Pyruvic acid
HSS B +
Cells
Zeroing
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Figure 4. LDH enzyme activities in isolated hepatocytes of various treatment groups. The positive control is indicated by the cells in HSS B exposed to 1% Triton X-100 while the negative control comprises of only HSS B and 10 µg/ml pyrene (no cells). The remaining treatment groups contained HSS B, cells and pyrene (0, 1, 5, 10 µg/ml). LDH activities were taken 0, 2, 4, and 6 hrs post incubation. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=4).

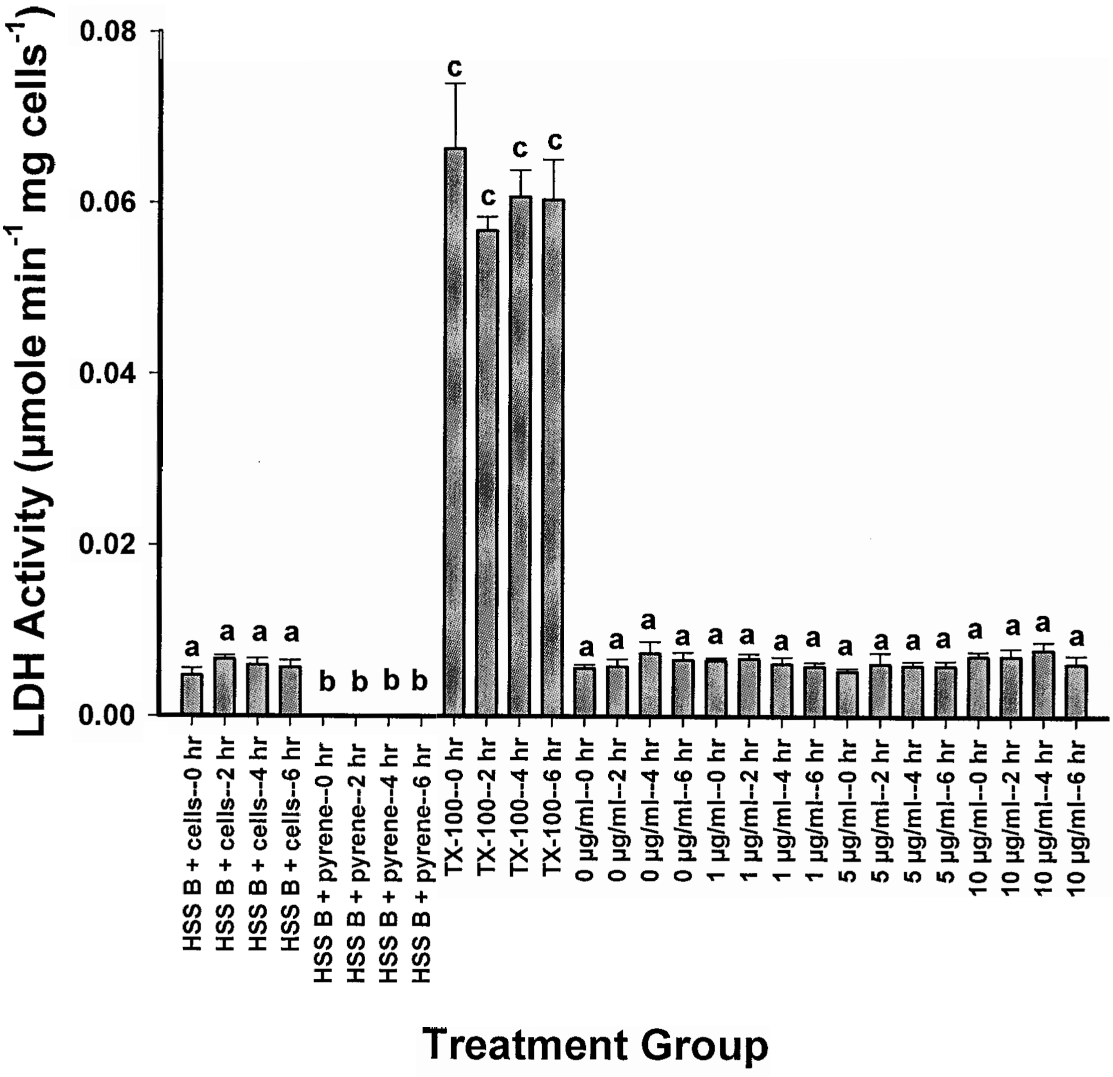
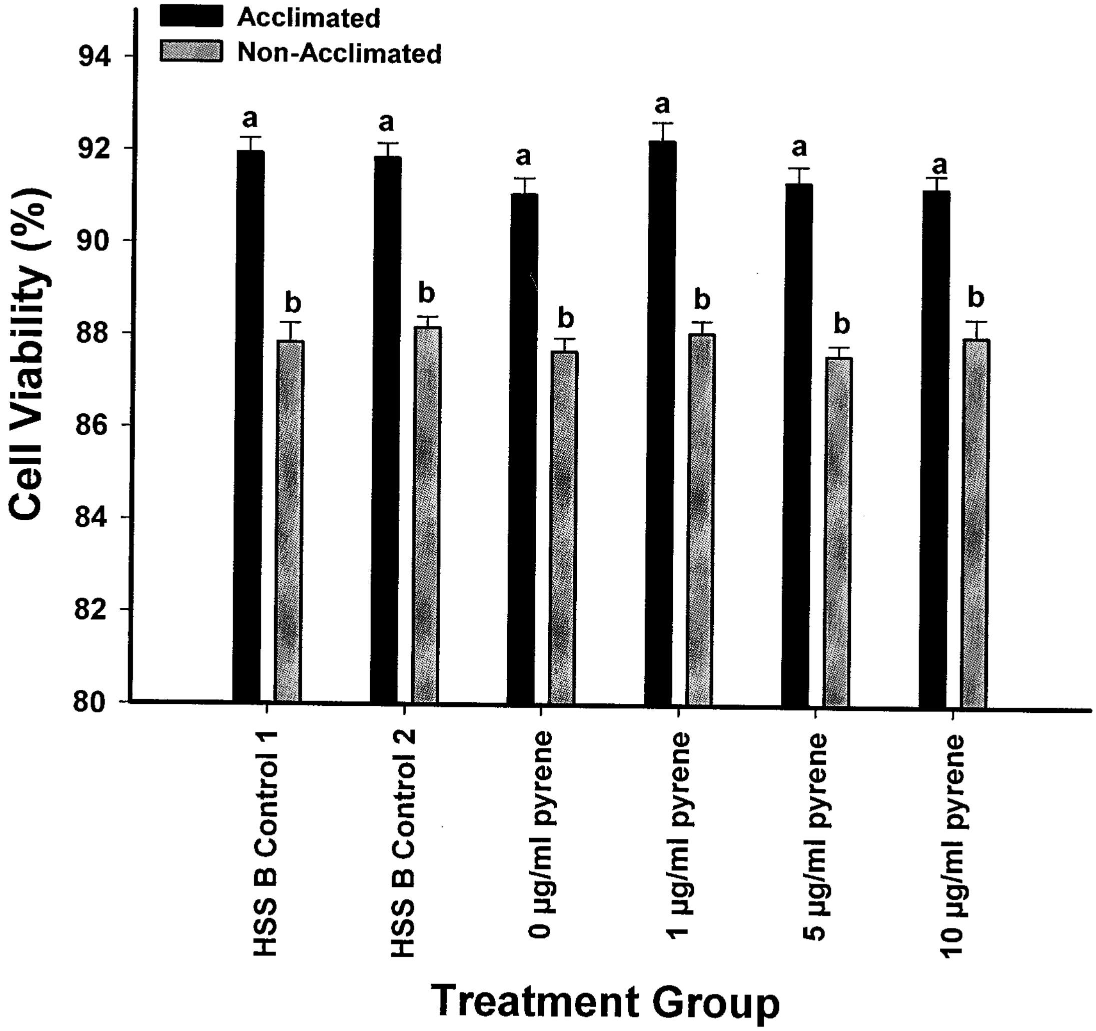

LDH Activity (µmole min^{-1} mg cells^{-1})
0.08
0.06
0.04
0.02
0.00
a
a
a
a
b
b
b
b
c
c
c
c
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
HSS B + cells--0 hr
HSS B + cells--2 hr
HSS B + cells--4 hr
HSS B + cells--6 hr
HSS B + pyrene--0 hr
HSS B + pyrene--2 hr
HSS B + pyrene--4 hr
HSS B + pyrene--6 hr
TX-100--0 hr
TX-100--2 hr
TX-100--4 hr
TX-100--6 hr
0 µg/ml--0 hr
0 µg/ml--2 hr
0 µg/ml--4 hr
0 µg/ml--6 hr
1 µg/ml--0 hr
1 µg/ml--2 hr
1 µg/ml--4 hr
1 µg/ml--6 hr
5 µg/ml--0 hr
5 µg/ml--2 hr
5 µg/ml--4 hr
5 µg/ml--6 hr
10 µg/ml--0 hr
10 µg/ml--2 hr
10 µg/ml--4 hr
10 µg/ml--6 hr
Treatment Group

Figure 5. Cell viability of hepatocytes from acclimated (n=5) and non-acclimated (n=6) trout in the various treatment groups at 1 hr post incubation as determined by Trypan blue dye exclusion. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM.


Acclimated
Non-Acclimated
Cell Viability (%)
94
92
90
88
86
84
82
80
a
a
a
a
a
a
b
b
b
b
b
b
HSS B Control 1
HSS B Control 2
0 µg/ml pyrene
1 µg/ml pyrene
5 µg/ml pyrene
10 µg/ml pyrene
Treatment Group

between the acclimated (90.73±0.31 to 92.23±0.41%) and non-acclimated (85.98±0.18 to 88.18±0.24%) groups, with a higher cell viability for hepatocytes incubated at their acclimated temperature.

Mitochondrial Respiration

Figure 6 shows an example of a SA/ADP polarographic recording used in determining mitochondrial respiration, respiratory control ratios (RCR) and ADP:O ratios. RCR and ADP:O ratios ranged from 2.48 to 2.73 and 1.43 to 1.58, respectively. For each pyrene treatment group (0, 1, 5, and 10 μg/ml), pre-exposure rates (SA-stimulated mitochondrial respiration) and post-exposure rates (respiration in the presence of the selected pyrene treatment or 500 ng/ml 1-OH pyrene) were recorded on LABVIEW such as the one seen in Figure 7 and 8, respectively. Figure 9 shows the mean pre- and post- exposure mitochondrial respiration rates for each pyrene treatment group (except for the 500 ng/ml 1-OH pyrene treatment group). Pre-exposure mitochondrial respiration rates ranged from 220.9±2.2 to 230.2±3.4 ng O_2 min^{-1} mg protein^{-1} while post-exposure mitochondrial respiration rates ranged from 220.1±2.3 to 229.6±2.6 ng O_2 min^{-1} mg protein^{-1}. The pre- and post-exposure rates of mitochondria to the 500 ng/ml 1-OH pyrene metabolite ranged from 208.0±2.9 to 221.55±2.1 ng O_2 min^{-1} mg protein^{-1}. No significant differences were detected between the pre- and post-exposure rates of pyrene and 1-OH pyrene, indicating that the pyrene concentrations used and the metabolites produced from detoxification did not directly affect mitochondrial respiration.

Figure 6. A typical polarographic recording of trout mitochondrial oxygen consumption without pyrene: succinic acid (SA; 10 µmoles) was added where indicated. State III respiration was initiated with 0.4 µmoles ADP. Return to State IV respiration is also indicated.

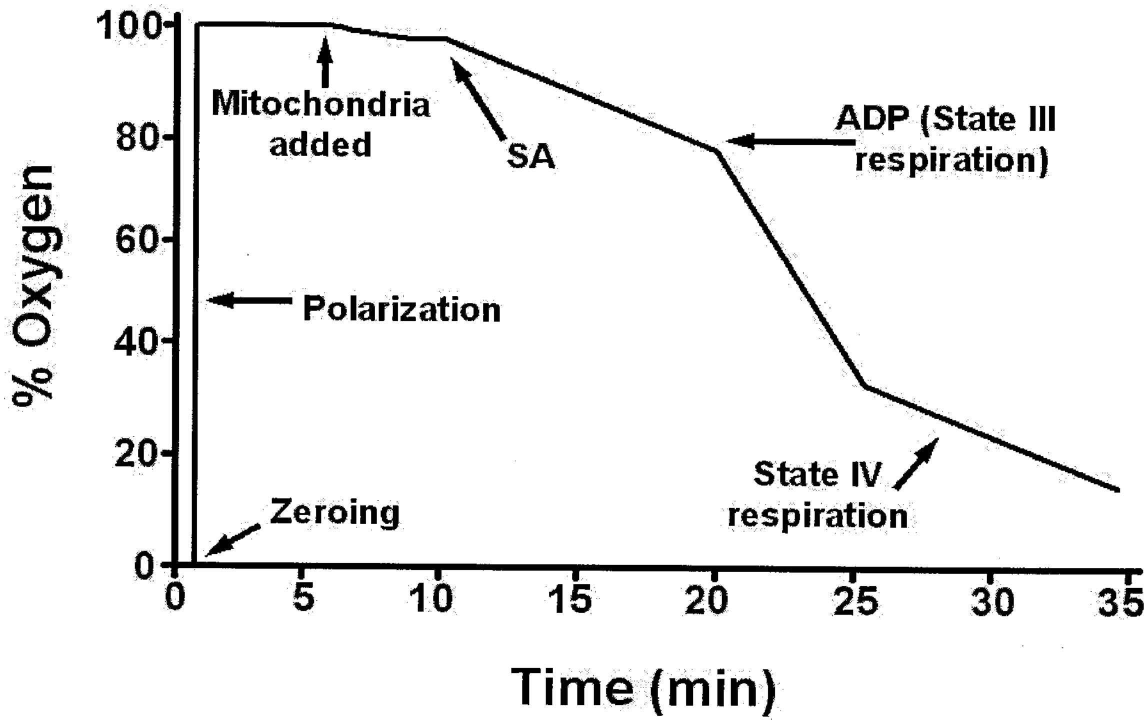

100
80
60
40
20
0
% Oxygen
0
5
10
15
20
25
30
35
Time (min)
Mitochondria added
SA
ADP (State III respiration)
Polarization
Zeroing
State IV respiration

Figure 7. A typical polarographic recording of trout mitochondrial oxygen consumption with pyrene: succinic acid (SA; 10 µmoles) and pyrene (10 µg/ml) were added where indicated.

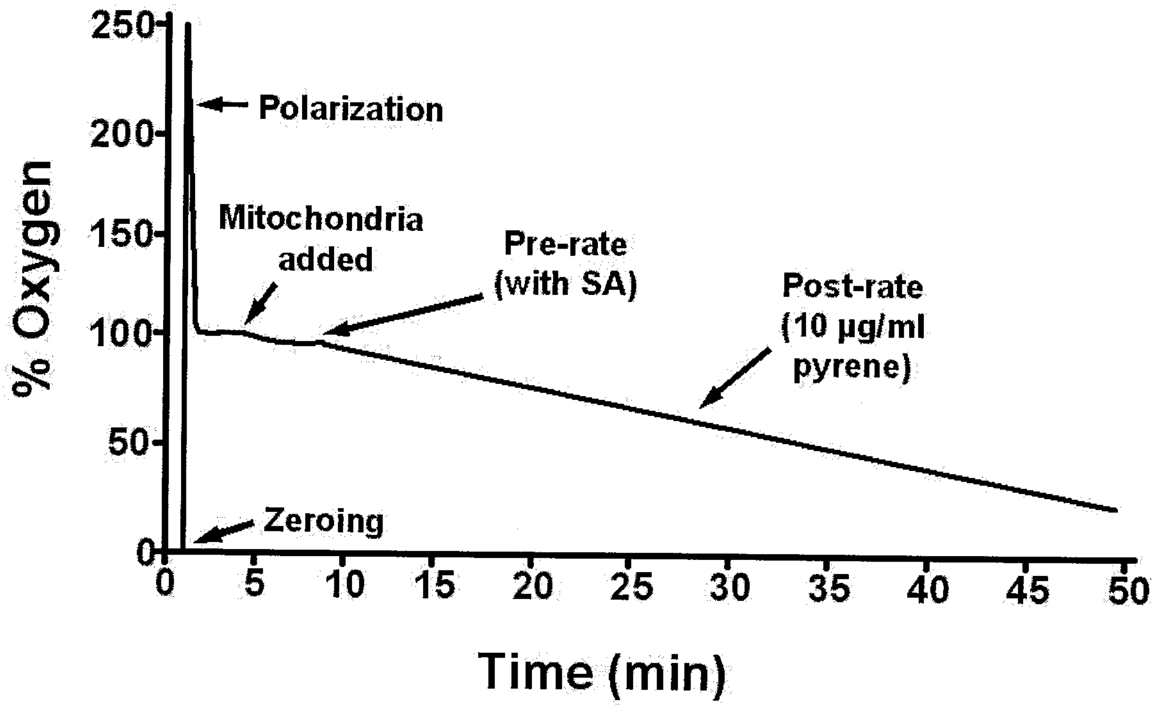

% Oxygen
250
200
150
100
50
0
Polarization
Mitochondria added
Pre-rate (with SA)
Post-rate (10 µg/ml pyrene)
Zeroing
0
5
10
15
20
25
30
35
40
45
50
Time (min)

Figure 8. A typical polarographic recording of trout mitochondrial oxygen consumption with 1-OH pyrene: succinic acid (SA; 10 µmoles) and 1-OH pyrene (500 ng/ml) were added where indicated.

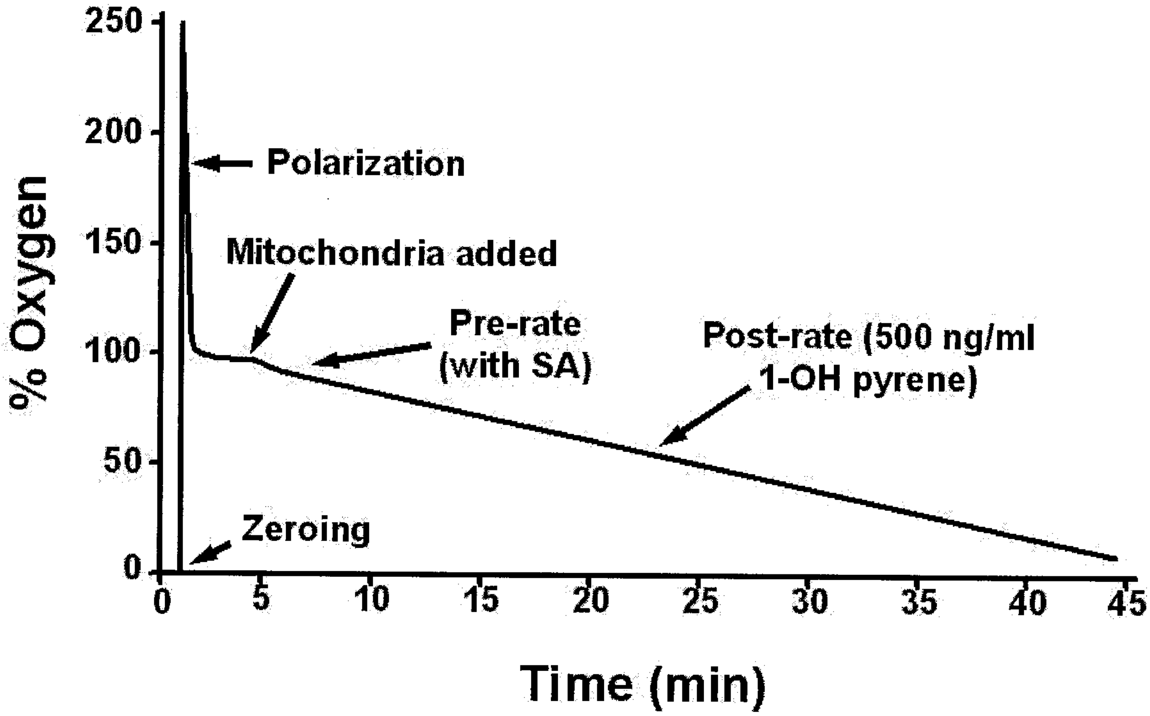
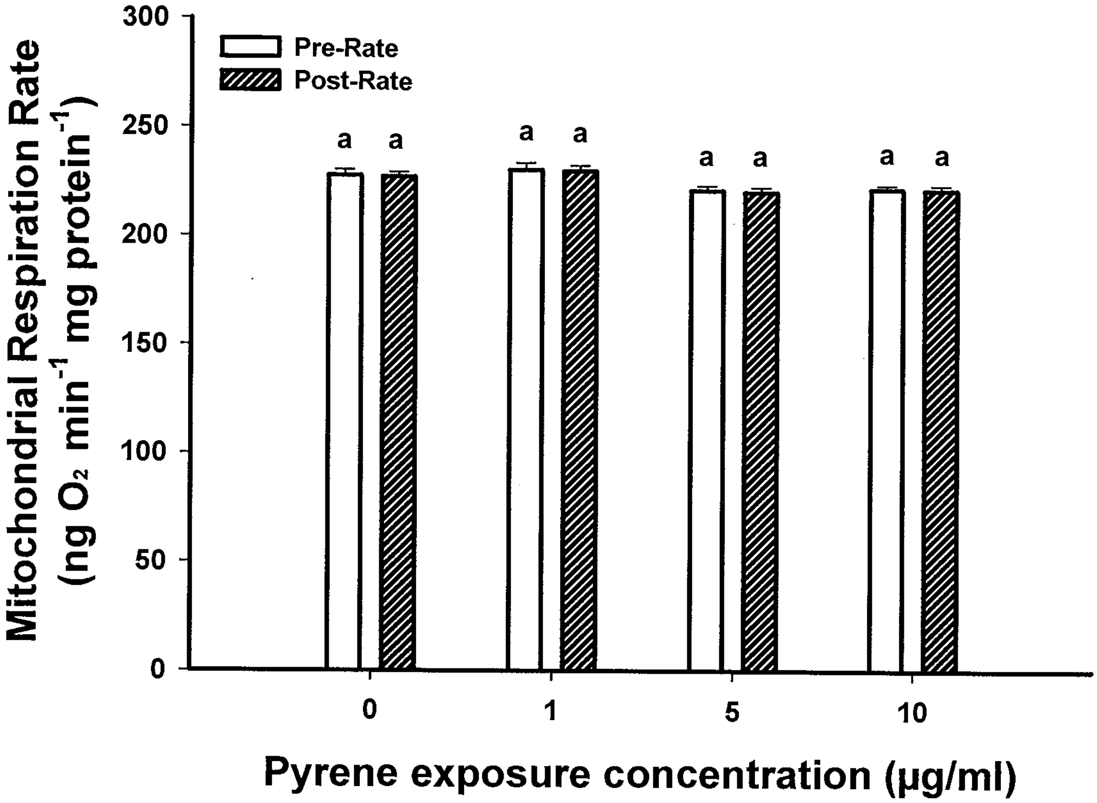

250
200
150
100
50
0
% Oxygen
0
5
10
15
20
25
30
35
40
45
Time (min)
Polarization
Mitochondria added
Pre-rate
(with SA)
Post-rate (500 ng/ml
1-OH pyrene)
Zeroing

Figure 9. Pre- and post- mitochondrial respiration rates for each treatment: 0 (DMSO), 1, 5, and 10 μg/ml pyrene. The substrate used was SA (10 μmoles). Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=4).



Mitochondrial Respiration Rate
(ng O2 min-1 mg protein-1)
300
250
200
150
100
50
0
Pre-Rate
Post-Rate
a a
a a
a a
a a
0
1
5
10
Pyrene exposure concentration (µg/ml)

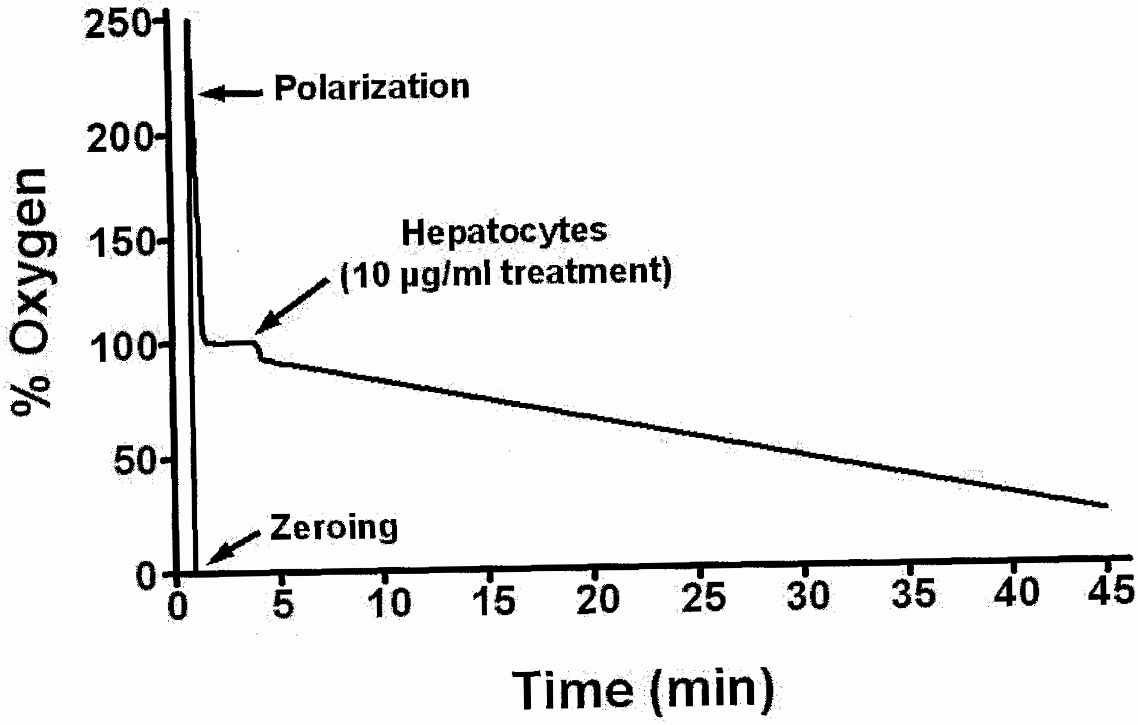
Acclimated Trout Hepatocytes and Pyrene Metabolism

For each of the treatment groups (Control 1, Control 2, 0 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml), a Labview recording of oxygen consumption was recorded as seen in Figure 10. In all cases, the addition of hepatocytes to the respirometry chamber resulted in a linear decline in the concentration of oxygen in the medium from which hepatocyte respiration rate was calculated (ng O_2 min^{-1} mg $cells^{-1}$).

Figure 11.1 shows the respiration rates of hepatocytes from five adult rainbow trout exposed at their acclimation temperature in the following treatments: Control 1 (10.13±0.13 ng O_2 min^{-1} mg $cells^{-1}$), Control 2 (10.21±0.20), 0 µg/ml (10.24±0.10), 1 µg/ml (12.48±0.11), 5 µg/ml (14.72±0.13) and 10 µg/ml (17.09±0.20). The respiration rates of the controls and 0 µg/ml treatment group (DMSO control) were not significantly different from each other and were significantly lower compared to the treatment groups with pyrene. Exposure to pyrene at 7.5°C resulted in concentration- dependent increases in hepatocyte respiration rates. Also, the respiration rates of the pyrene-exposed hepatocytes were significantly different from each other. Pyrene-exposed hepatocyte respiration rates increased 18.8±0.7, 31.1±0.8, and 40.6±0.7% for the 1, 5, and 10 µg/ml treatment groups, respectively (Figure 11.2), over controls.

In addition to examining hepatocyte respiration rates, the metabolism of pyrene by these cells was also examined in each treatment group. Following the first set of solvent extractions, synchronous fluorometric spectroscopy revealed that the only parent compound (pyrene) without detectable Phase I metabolites. Therefore, only the second set of ethyl acetate extractions were used to determine the extent of pyrene metabolism since only the Phase II 1-OH pyrene metabolites were present. An example of fluorescence intensity traces *via* synchronous fluorometric spectroscopy (SFS) for several pyrene concentrations is illustrated in Figure 12. Figure 13 shows the standard

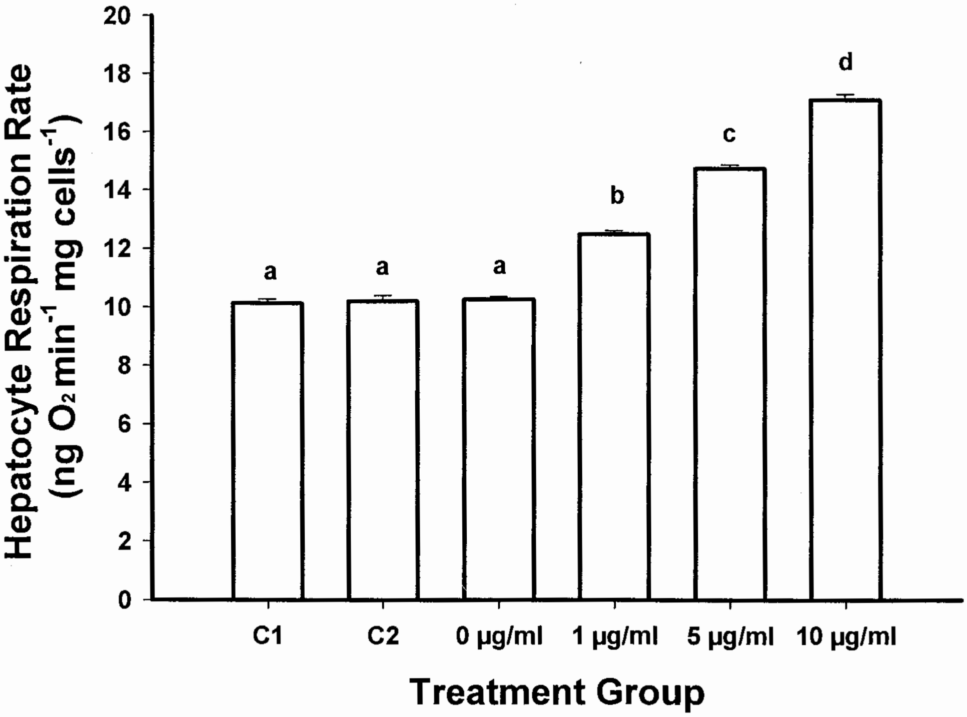
Figure 10. A typical polarographic recording illustrating zeroing of the electrode, polarization, and oxygen consumption by hepatocytes from a fish acclimated to 7.5°C. Hepatocytes were exposed to pyrene at 10 μg/ml.



250
200
150
100
50
0
% Oxygen
Polarization
Hepatocytes
(10 µg/ml treatment)
Zeroing
0
5
10
15
20
25
30
35
40
45
Time (min)

Figure 11.1. Respiration rates of hepatocytes from acclimated trout incubated in the following treatments: Control 1 and 2 (no pyrene or DMSO), 0 µg/ml pyrene (DMSO control), as well as 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=5).

Figure 11.2. Percent increase in respiration rate of hepatocytes from acclimated trout incubated with pyrene (1, 5, and 10 µg/ml). Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=5).


Hepatocyte Respiration Rate
(ng O_2 min^{-1} mg $cells^{-1}$)
20
18
16
14
12
10
8
6
4
2
0
a
a
a
b
c
d
C1
C2
0 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
Treatment Group

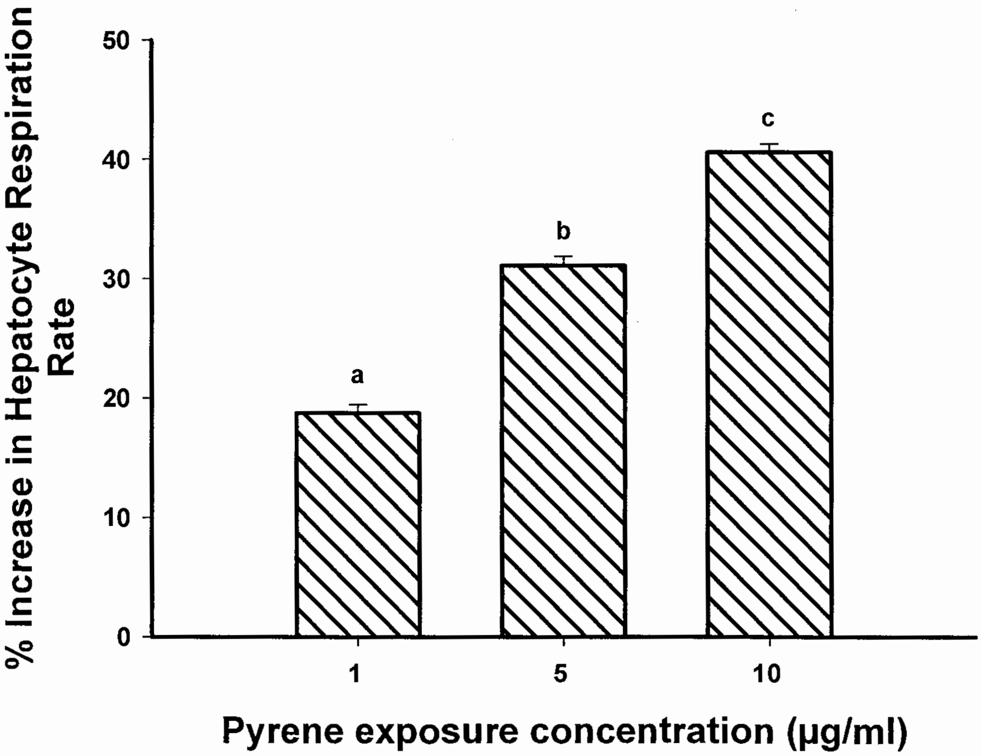

% Increase in Hepatocyte Respiration Rate
50
40
30
20
10
0
a
b
c
1
5
10
Pyrene exposure concentration (µg/ml)

Figure 12. An overlay of the SFS fluorescent intensity peaks for Phase II 1-OH pyrene from an acclimated fish. Areas under the fluorescent intensity curves between 338-356 nm are used in determining the concentration of 1-OH pyrene produced (ng/ml) and metabolite rate (ng 1-OH produced min^{-1} mg cells^{-1}).

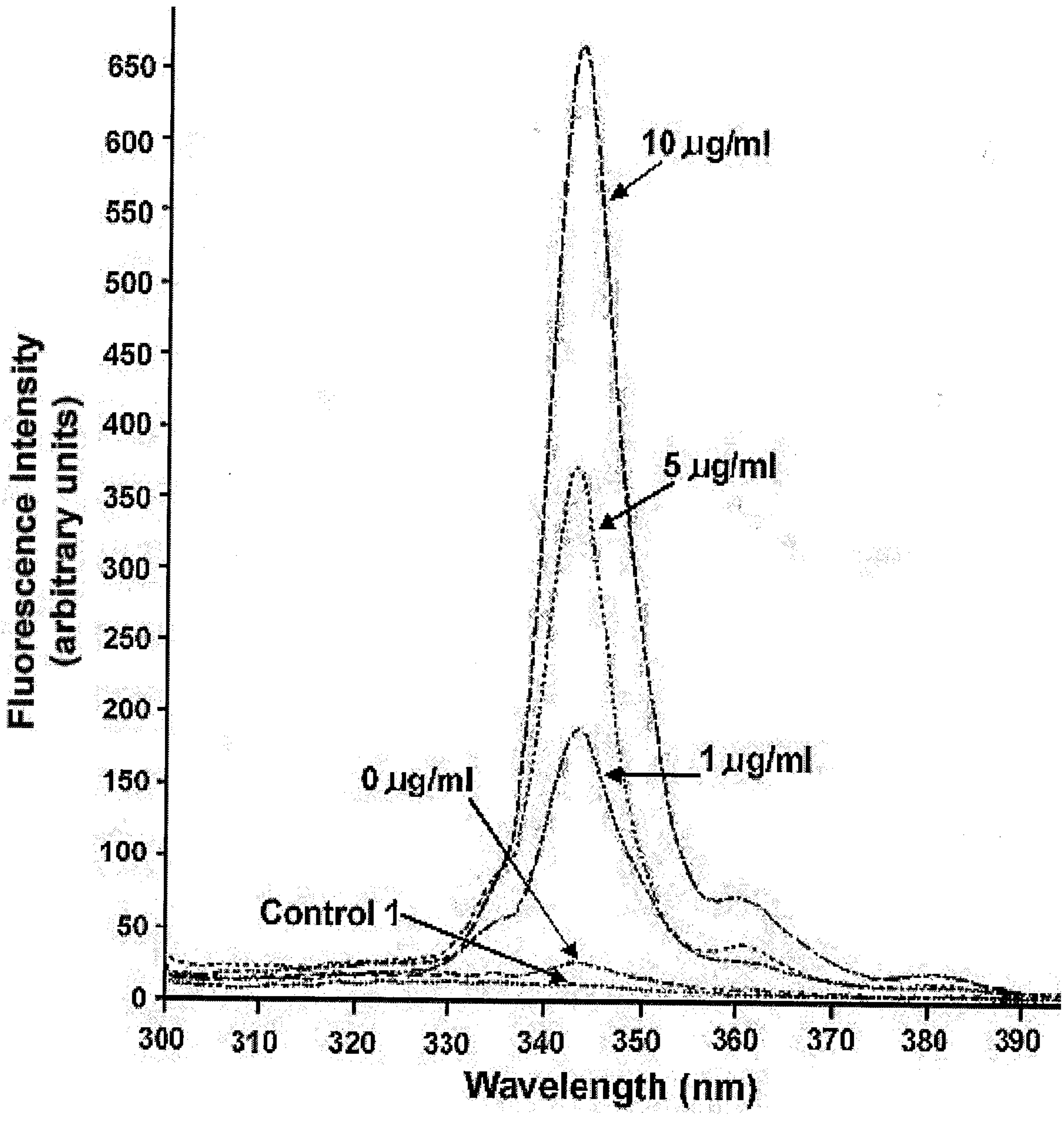
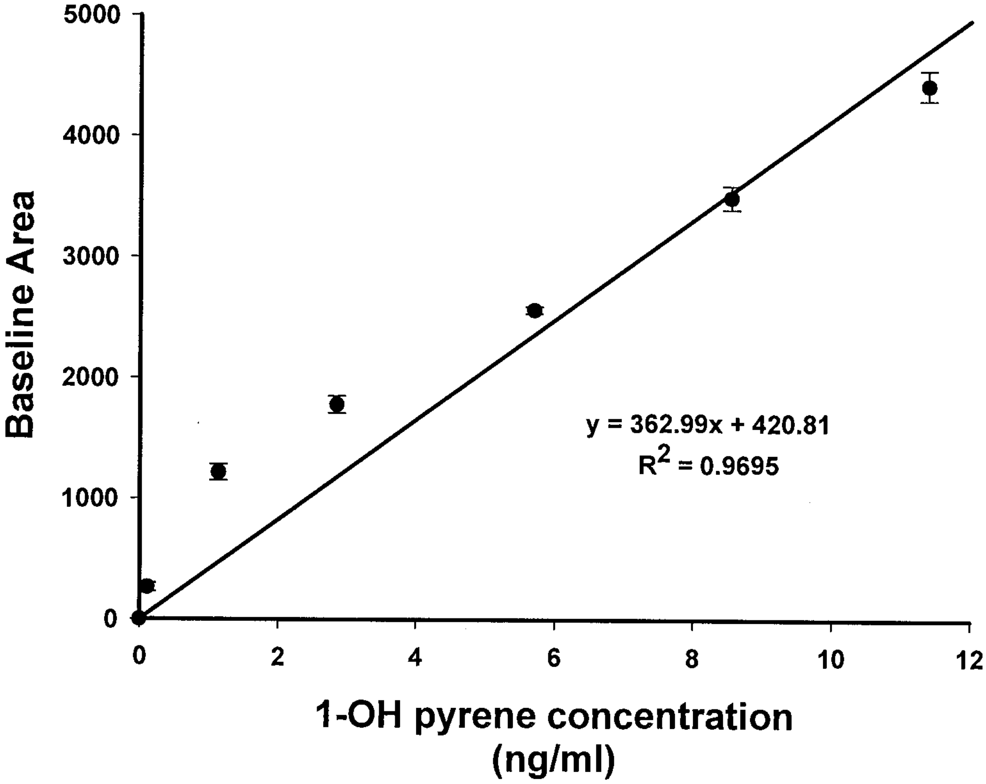

650
600
550
500
450
400
350
300
250
200
150
100
50
0
Fluorescence Intensity (arbitrary units)
300
310
320
330
340
350
360
370
380
390
Wavelength (nm)
10 µg/ml
5 µg/ml
1 µg/ml
0 µg/ml
Control 1

Figure 13. Metabolite standard curve used for determining the concentration of 1-OH pyrene in cell extracts.


5000
4000
3000
2000
1000
0
Baseline Area
0
2
4
6
8
10
12
1-OH pyrene concentration (ng/ml)
y = 362.99x + 420.81
R^2 = 0.9695

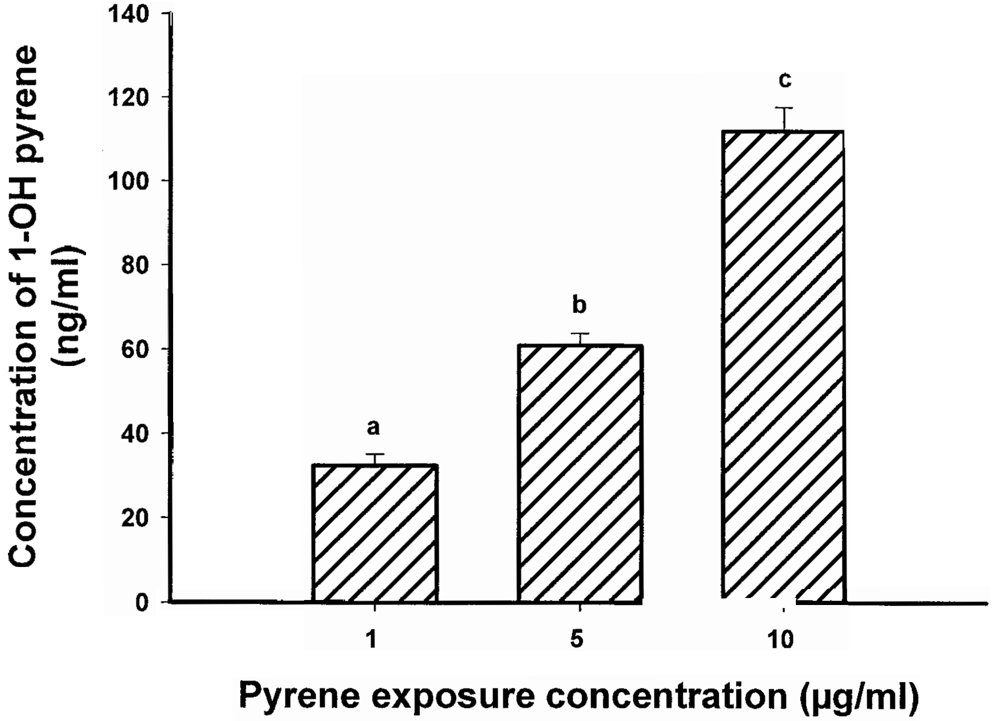
curve constructed for determining the actual concentration of metabolite present using SFS. The concentration of the standards ranged from 0.114 to 11.40 ng/ml 1-OH pyrene. Each set of each concentration were made and measured *via* SFS in triplicate. Baseline areas calculated from fluorescent intensity peaks representing 1-OH pyrene were used to determine the concentration of metabolite produced by hepatocytes. Figure 14.1 shows the mean concentration of 1-OH pyrene produced in the Warburg chamber by hepatocytes exposed to 1, 5 and 10 µg/ml pyrene: 32.54±2.78, 60.95±2.90, and 111.94±5.71 ng/ml, respectively. Significant differences were seen between metabolite concentrations in each pyrene exposure group. As the concentration of pyrene exposed to the hepatocytes increased from 1 to 10 µg/ml, the amount of metabolites produced increased accordingly.

The rate of pyrene metabolism (ng 1-OH pyrene min^{-1} mg $cells^{-1}$) in each treatment is shown in Figure 14.2. The rates of metabolism were 0.037±0.003 for the 1 µg/ml treatment, 0.069±0.006 at 5 µg/ml and 0.125±0.005 at 10 µg/ml, respectively. Significant differences among treatments were also observed. Higher pyrene exposure concentrations resulted in increased rates of pyrene metabolism.

Correlations between hepatocyte respiration rate and (i) concentration of 1-OH pyrene conjugates formed (Figure 15.1) as well as (ii) rate pyrene metabolism (Figure 15.2) were found. When hepatocytes were exposed to pyrene at 7.5°C, respiration rates increased in proportion to the amount of 1-OH pyrene produced in a concentration-dependent manner. Similarly, pyrene metabolism rates also increased as the concentration of pyrene exposure was increased from 1 to 10 µg/ml.

Figure 14.1. Concentration of 1-OH pyrene (ng/ml) produced by hepatocytes from acclimated trout incubated with 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=5).

Figure 14.2. Rate of pyrene metabolism (ng 1-OH produced min^{-1} mg $cells^{-1}$) of hepatocytes from acclimated trout incubated with 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=5).


Concentration of 1-OH pyrene (ng/ml)
140
120
100
80
60
40
20
0
a
b
c
1
5
10
Pyrene exposure concentration (µg/ml)

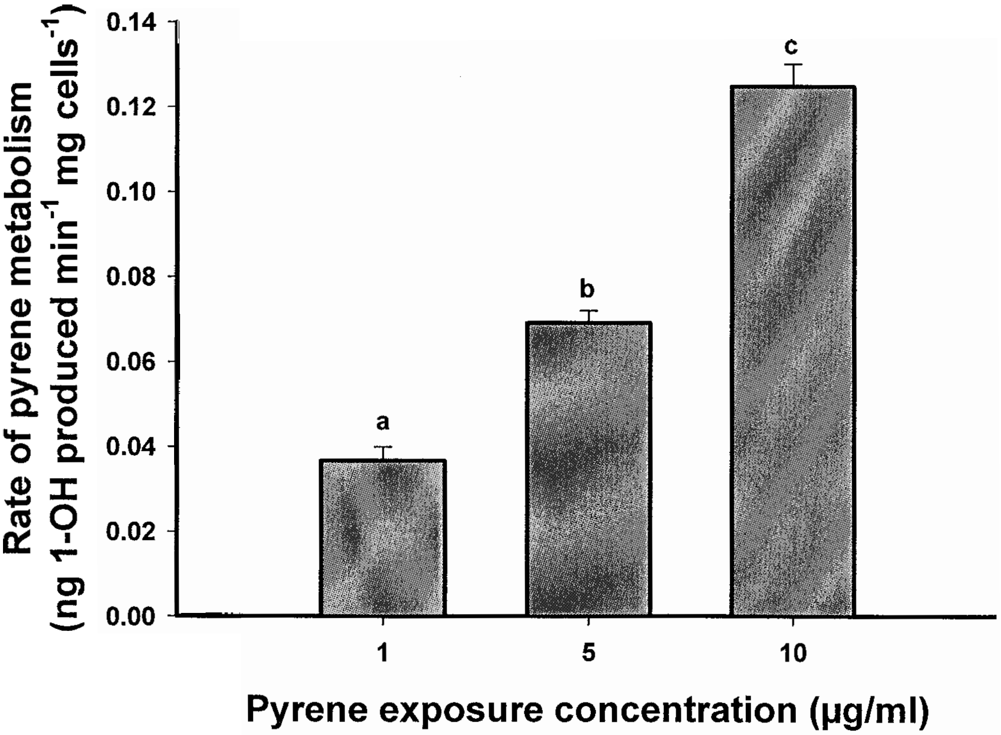
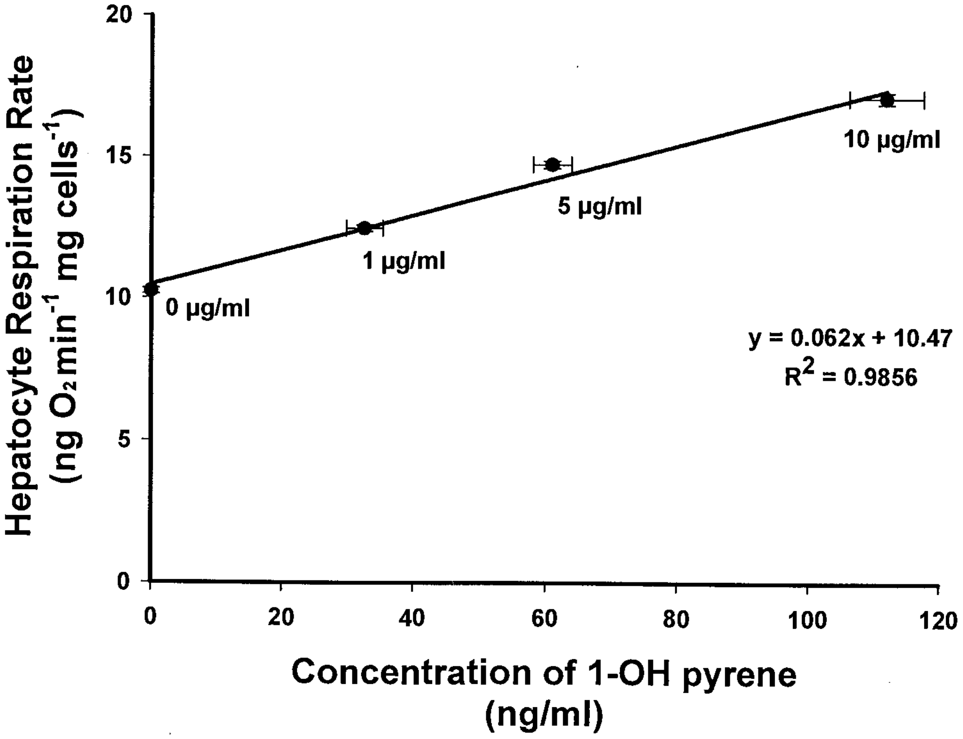
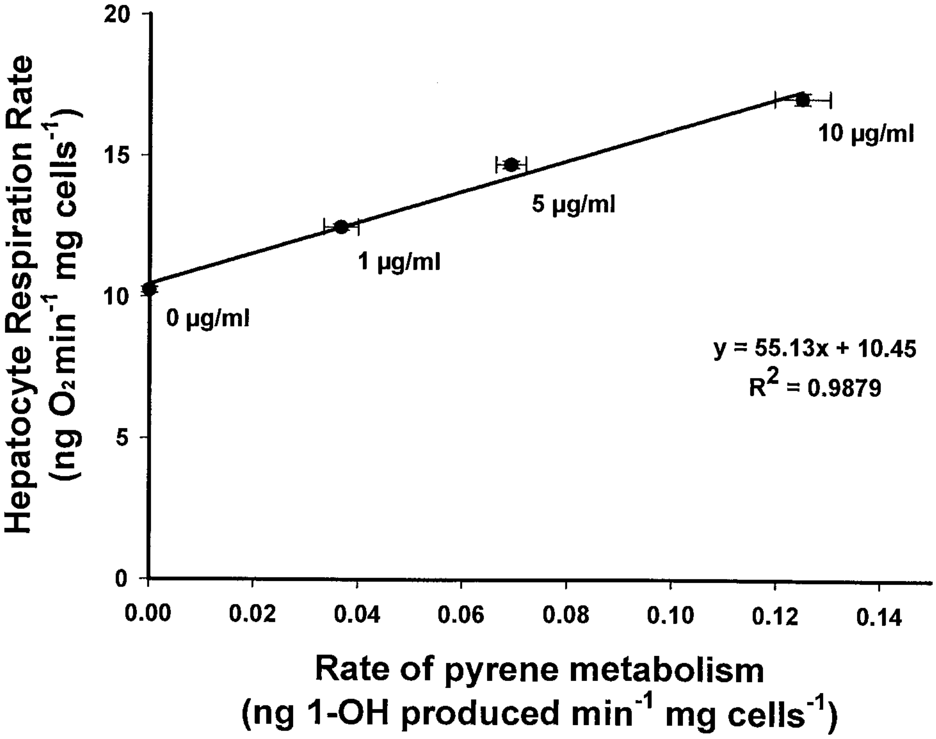

Rate of pyrene metabolism (ng 1-OH produced min^{-1} mg cells^{-1})
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
a
b
c
1
5
10
Pyrene exposure concentration (µg/ml)

Figure 15.1. Correlation of hepatocyte respiration rate and concentration of 1-OH produced from acclimated trout. Error bars represent means±SEM (n=5).

Figure 15.2. Correlation of hepatocyte respiration rate and rate of pyrene metabolism from acclimated trout. Error bars represent means±SEM (n=5).



Hepatocyte Respiration Rate
(ng O₂ min⁻¹ mg cells⁻¹)
20
15
10
5
0
0
20
40
60
80
100
120
Concentration of 1-OH pyrene
(ng/ml)
0 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
y = 0.062x + 10.47
R² = 0.9856



Hepatocyte Respiration Rate
(ng O₂ min⁻¹ mg cells⁻¹)
20
15
10
5
0
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Rate of pyrene metabolism
(ng 1-OH produced min⁻¹ mg cells⁻¹)
0 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
y = 55.13x + 10.45
R² = 0.9879

Non-Acclimated Trout Hepatocytes and Pyrene Metabolism

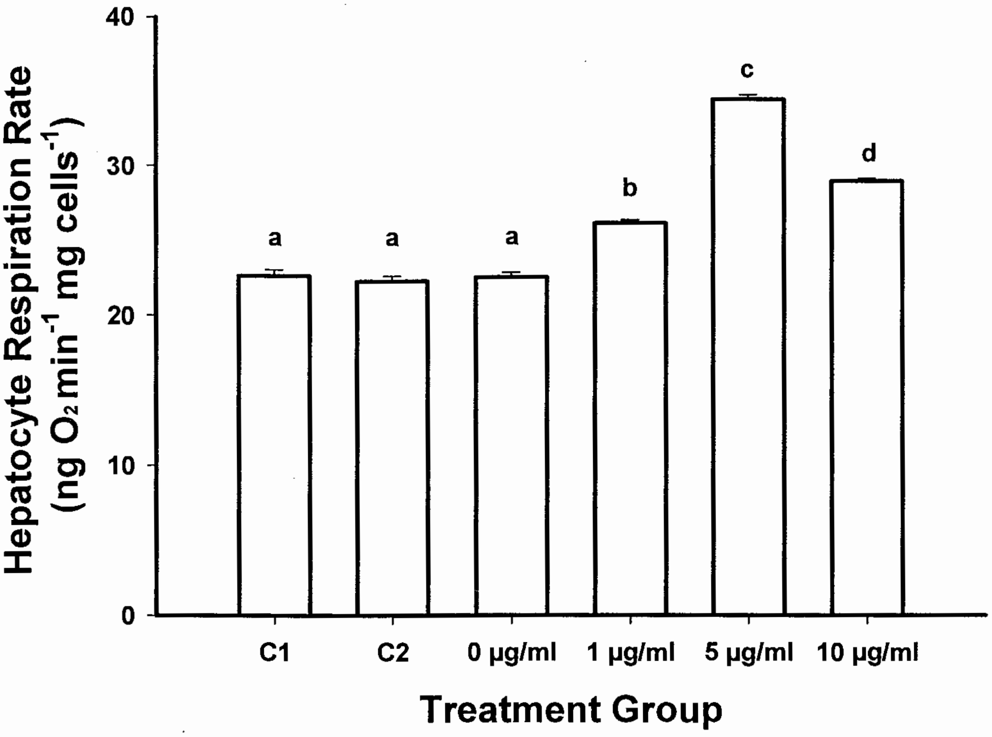
Typical polarographic recordings for oxygen consumption in non-acclimated hepatocytes (i.e. at 15°C) were similar to that depicted in Figure 6 for acclimated cells. Figure 16.1 shows the mean respiration rates of non-acclimated hepatocytes in the following treatments: Control 1 (22.64±0.41 ng O_2 min^{-1} mg $cells^{-1}$), Control 2 (22.30±0.33), 0 µg/ml (22.53±0.31), 1 µg/ml (26.13±0.21), 5 µg/ml (34.41±0.30) and 10 µg/ml (28.94±0.16). The respiration rates of the controls and the 0 µg/ml treatment group (DMSO control) were not significantly different from each other and were significantly lower than those of pyrene-treated hepatocytes. An increase in respiration rates occurred in cells as the concentration of pyrene increased from 1 (13.2±1.6% increase over controls) to 5 µg/ml (34.1±1.7% increase). However, respiration rates in cells exposed to pyrene at 10 µg/ml (21.7±0.8% increase), were significantly lower than at 5 µg/ml (Figure 16.2).

The production of 1-OH pyrene in hepatocytes was also examined for each treatment. 1-OH pyrene liberated from Phase II conjugates was measured *via* SFS. Figure 17.1 shows the amount of 1-OH pyrene produced in the Warburg chamber by non-acclimated hepatocytes incubated with 1, 5 and 10 µg/ml pyrene (74.82 ± 6.20, 145.49 ± 4.49, and 117.86 ± 4.29 ng/ml, respectively). Significant differences were seen in the concentration of metabolites produced. Figure 17.1 shows that the amount of metabolite increased as the concentration of pyrene exposure was increased from 1 to 5 µg/ml. A decrease in concentration of metabolites produced was seen in the 10 µg/ml treatment group. No 1-OH pyrene was present in the controls and 0 µg/ml treatment group (DMSO control).

The rate of pyrene metabolism (ng 1-OH min^{-1} mg $cells^{-1}$) is depicted in Figure 17.2 (0.086±0.007 for the 1 µg/ml treatment group, 0.168±0.010 for the 5 µg/ml

Figure 16.1. Respiration rates of hepatocytes from non-acclimated trout incubated in the following treatments: Control 1 and 2 (no pyrene or DMSO), 0 µg/ml pyrene (DMSO), as well as 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=6).

Figure 16.2. Percent increase in respiration rate of hepatocytes from non-acclimated trout incubated with pyrene (1, 5, and 10 µg/ml) at their non-acclimated temperature. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM with (n=6).


Hepatocyte Respiration Rate
(ng O_2 min^{-1} mg cells^{-1})
40
30
20
10
0
a
a
a
b
c
d
C1
C2
0 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
Treatment Group

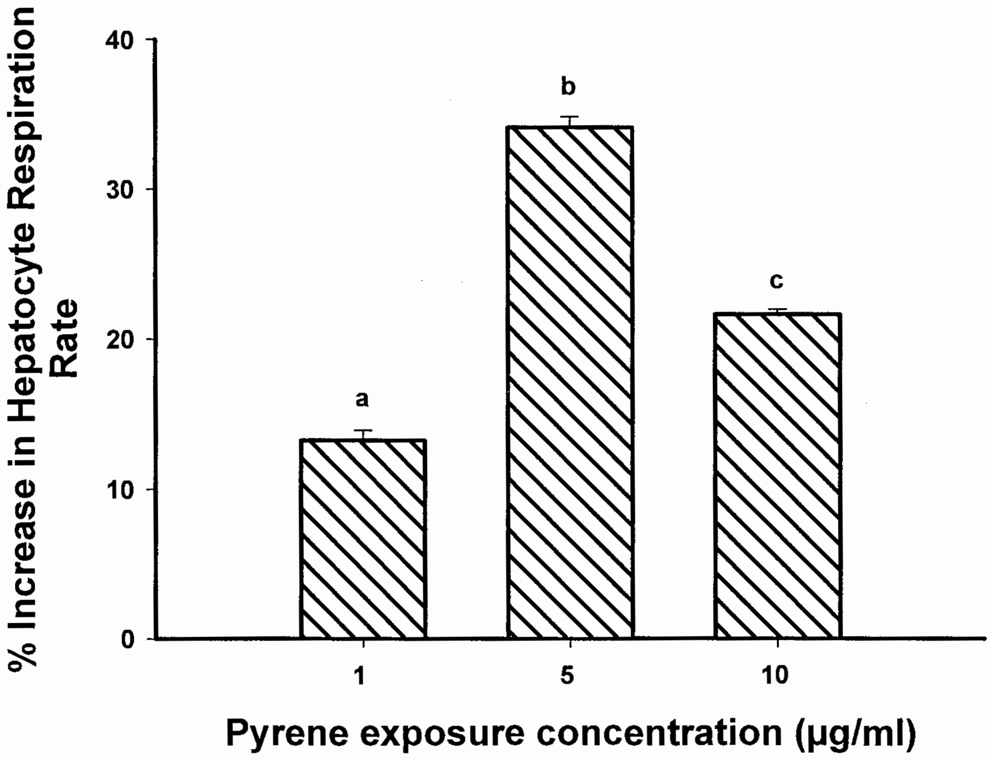
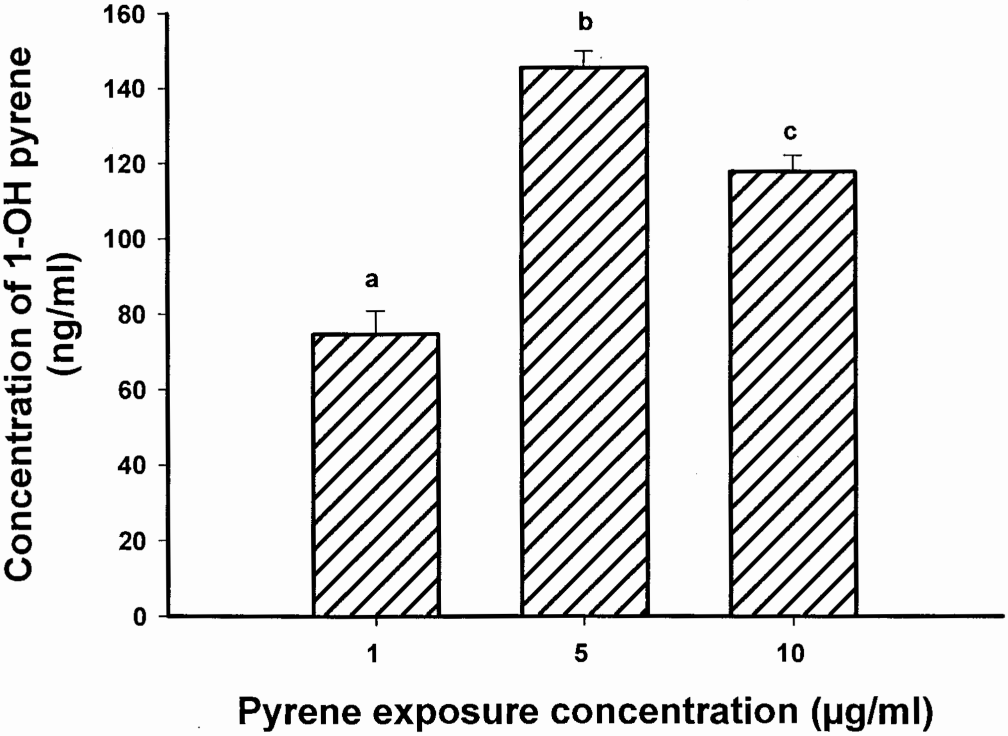
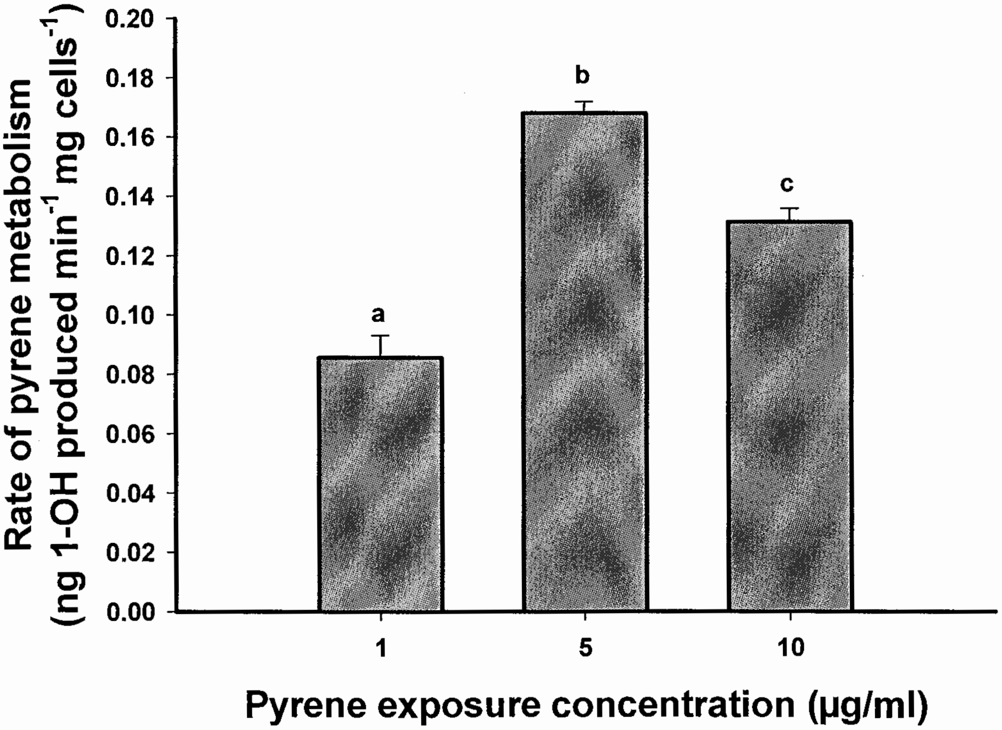

% Increase in Hepatocyte Respiration Rate
40
30
20
10
0
a
b
c
1
5
10
Pyrene exposure concentration (µg/ml)

Figure 17.1. Concentration of 1-OH pyrene (ng/ml) produced by hepatocytes from non-acclimated trout incubated with 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=6).

Figure 17.2. Rate of pyrene metabolism (ng 1-OH produced min^{-1} mg $cells^{-1}$) of hepatocytes from non-acclimated trout incubated with 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM (n=6).


Concentration of 1-OH pyrene (ng/ml)
0
20
40
60
80
100
120
140
160
a
b
c
1
5
10
Pyrene exposure concentration (µg/ml)


Rate of pyrene metabolism (ng 1-OH produced min^{-1} mg cells^{-1})
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
a
b
c
1
5
10
Pyrene exposure concentration (µg/ml)

treatment group and 0.131±0.005 for the 10 µg/ml treatment group). Significant differences were found among treatment groups, i.e. as the exposure concentration was elevated from 1 to 5 µg/ml, the rate of metabolism increased accordingly; however, when the exposure concentration increased from 5 to 10 µg/ml, the rate of metabolism decreased.

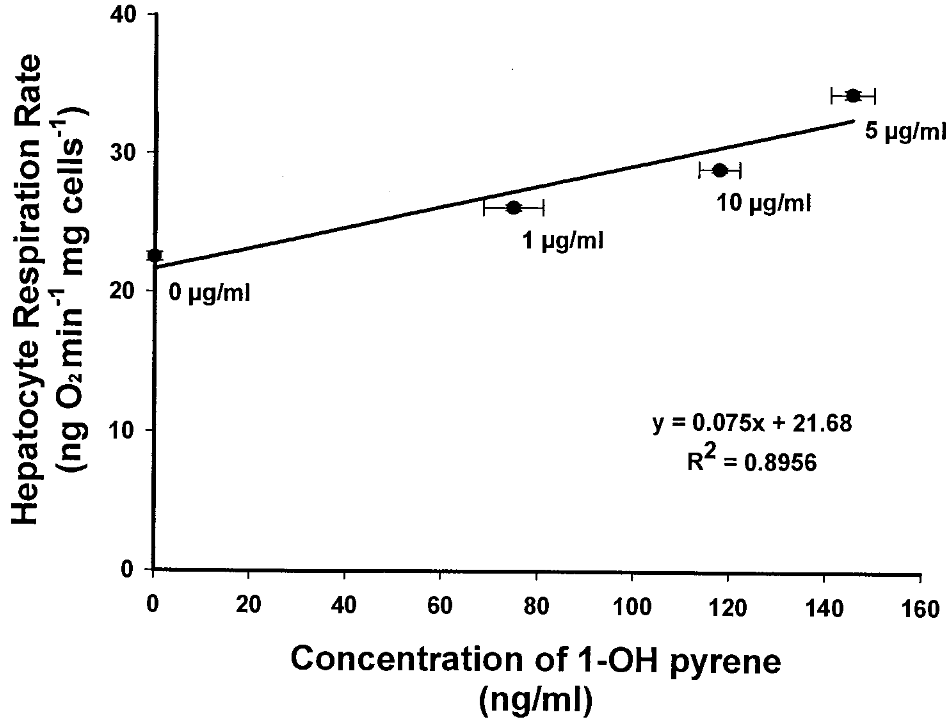
Correlations between hepatocyte respiration rates and the concentration of 1-OH pyrene produced (Figure 18.1) as well as the rate of pyrene metabolism (Figure 18.2) were made. When hepatocytes were exposed to pyrene at 15°C, respiration rates increased in accordance to the amount of 1-OH pyrene produced and rate of pyrene metabolism, however, the respiration rates did not increase in a dose-dependent manner. For example, the respiration rate for the 10 µg/ml treatment was significantly lower than the 5 µg/ml treatment due to a decrease in pyrene metabolism and 1-OH pyrene produced. Therefore, increases in respiration rates of hepatocytes are likely a function of the amount of metabolism that was occurring within the suspensions, and not a function of pyrene exposure concentrations.

Temperature Effects

When the incubation temperature was raised from 7.5°C to 15°C, respiration rates for cells in all treatment groups were significantly higher by approximately 2-fold when compared to the same treatments for cells exposed at their acclimation temperature (Figure 19.1). However, in Figure 19.2, the % increase in respiration rate for pyrene-treated hepatocytes at each temperature varied. No significant difference in % increase in acclimated and non-acclimated groups in the 1 and 5 µg/ml treatment groups, but a significant decrease of approximately 45% was noted for the 10 µg/ml pyrene-treated hepatocytes at 15°C *v.* the cells at 7.5°C.

Figure 18.1. Correlation of hepatocyte respiration rate and concentration of 1-OH produced from non-acclimated trout. 0, 1, 5 and 10 µg/ml signify the pyrene exposure concentrations. Error bars represent means±SEM (n=6).

Figure 18.2. Correlation of hepatocyte respiration rate and rate of pyrene metabolism from non-acclimated trout. 0, 1, 5 and 10 µg/ml signify the pyrene exposure concentrations. .Error bars represent means±SEM (n=6).


Hepatocyte Respiration Rate
(ng O_2 min^{-1} mg cells^{-1})
40
30
20
10
0
0
20
40
60
80
100
120
140
160
Concentration of 1-OH pyrene
(ng/ml)
0 µg/ml
1 µg/ml
10 µg/ml
5 µg/ml
y = 0.075x + 21.68
R^2 = 0.8956

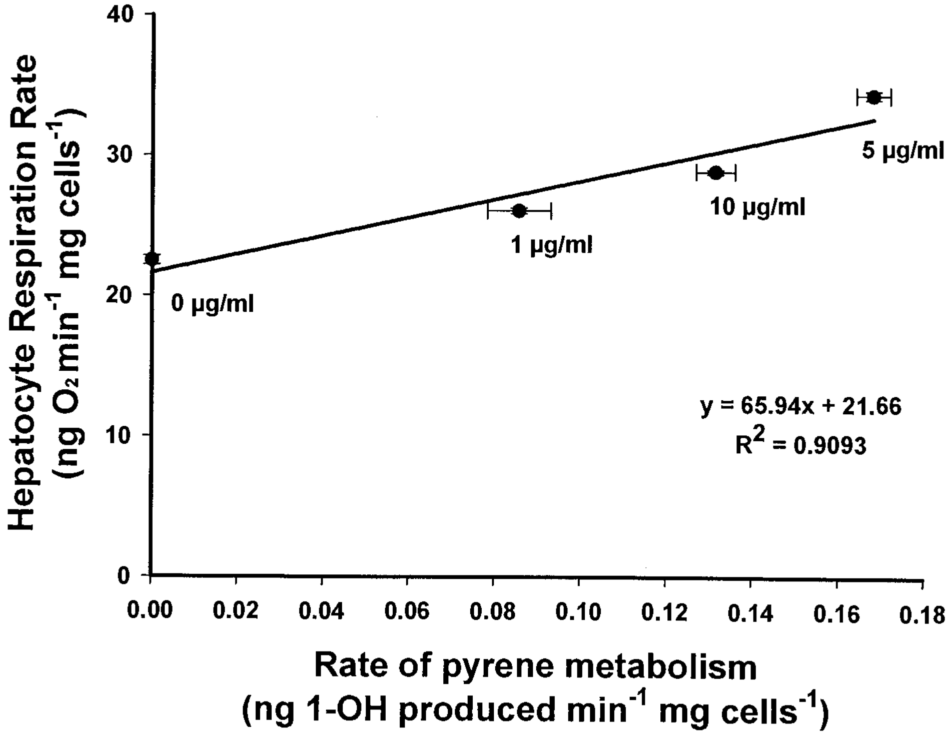
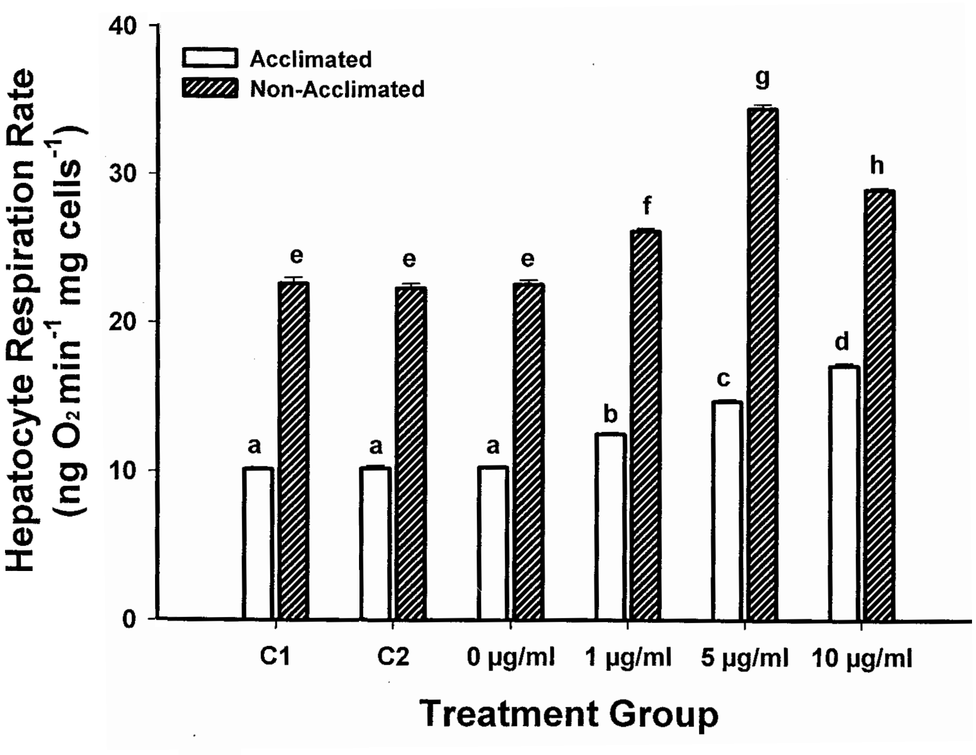
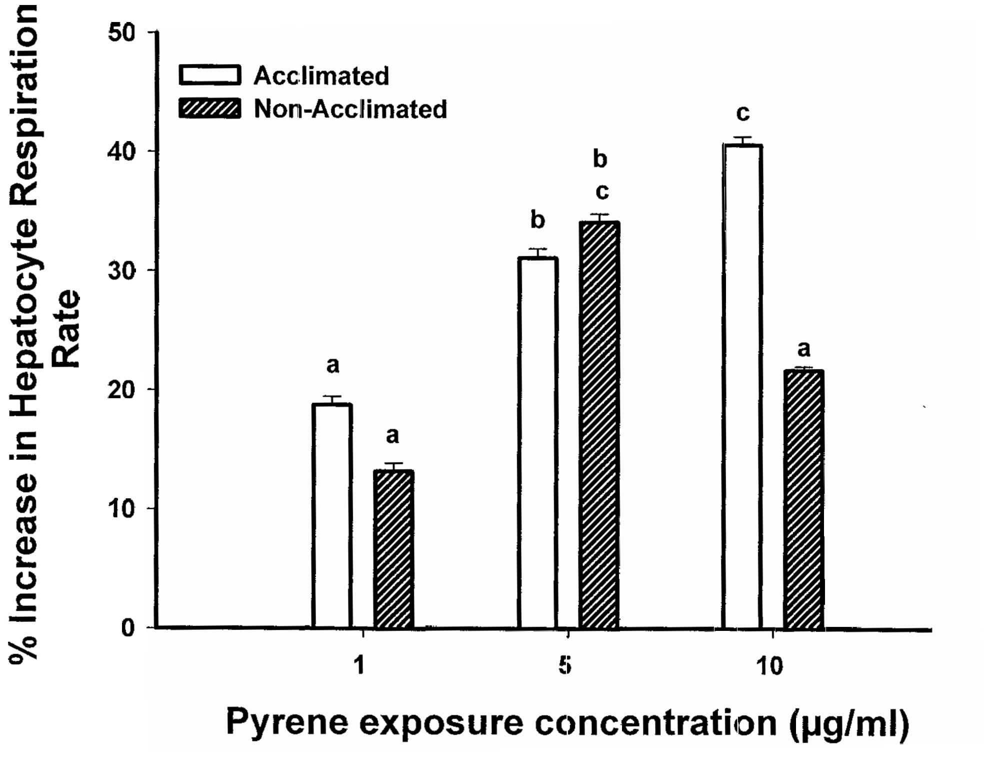

Hepatocyte Respiration Rate
(ng O_2 min^{-1} mg cells^{-1})
40
30
20
10
0
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
Rate of pyrene metabolism
(ng 1-OH produced min^{-1} mg cells^{-1})
0 µg/ml
1 µg/ml
10 µg/ml
5 µg/ml
y = 65.94x + 21.66
R^2 = 0.9093

Figure 19.1. Comparing respiration rates between acclimated (n=5) and non-acclimated (n=6) trout hepatocytes incubated with DMSO (Control 1 and 2), 0 µg/ml pyrene (DMSO), as well as 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM.

Figure 19.2. Comparing % increase in respiration rates between acclimated (n=5) and non-acclimated (n=6) trout hepatocytes incubated with 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM.

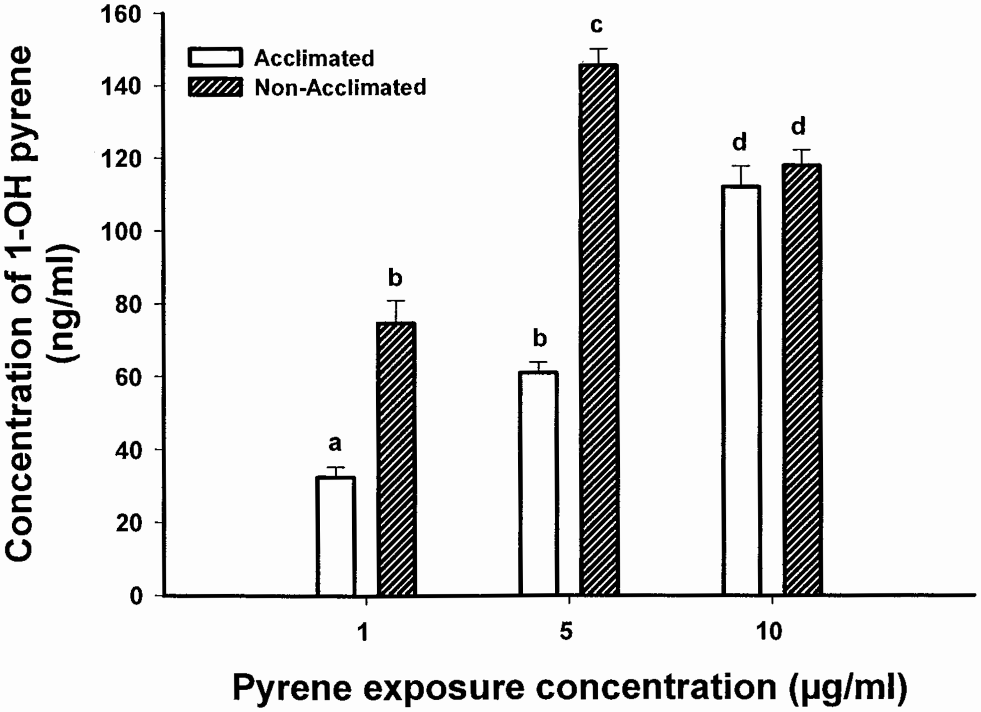

Hepatocyte Respiration Rate
(ng O_2 min^{-1} mg cells^{-1})
0
10
20
30
40
Acclimated
Non-Acclimated
a
e
a
e
a
e
b
f
c
g
d
h
C1
C2
0 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
Treatment Group

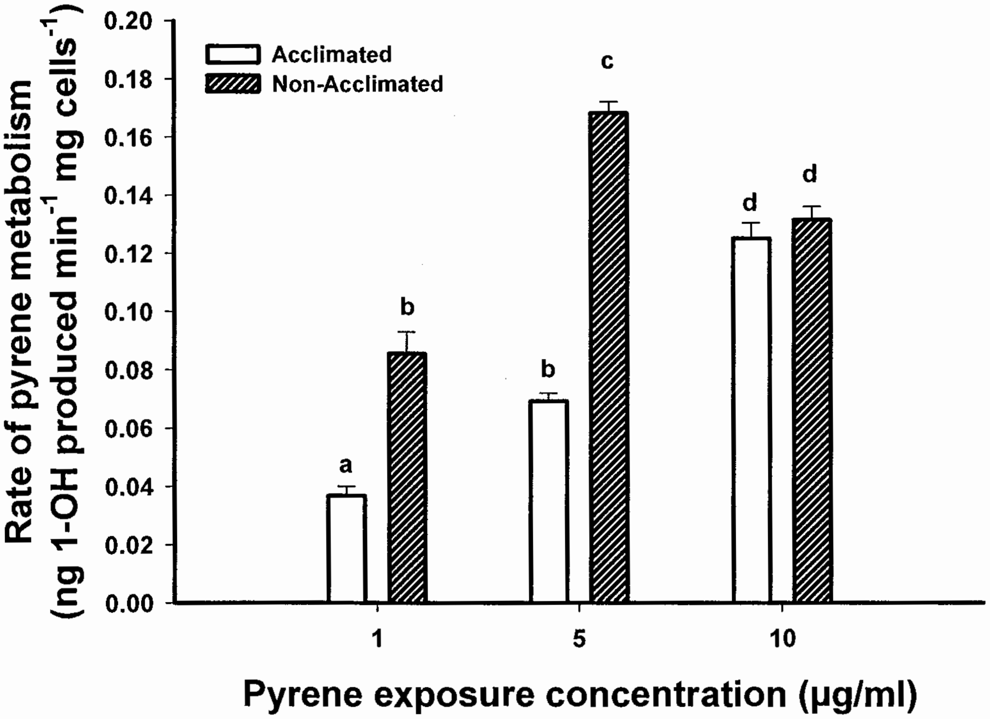

% Increase in Hepatocyte Respiration Rate
0
10
20
30
40
50
Acclimated
Non-Acclimated
a
a
b
b
c
c
a
1
5
10
Pyrene exposure concentration (µg/ml)

Furthermore, there were varied results in the concentration of 1-OH pyrene produced (Figure 20.1) and rate of pyrene metabolism (Figure 20.2) between acclimated and non-acclimated hepatocytes. The amount of 1-OH pyrene produced as well as the rate of metabolism in non-acclimated hepatocytes in the 1 and 5 µg/ml treatment groups were significantly higher (approximately 2-fold) compared to acclimated cells. However, in the 10 µg/ml treatment groups, no significant difference between the temperature groups in either the amount of 1-OH pyrene produced or rate of pyrene metabolism was seen.

Figure 20.1. Comparing 1-OH pyrene concentrations between acclimated (n=5) and non-acclimated (n=6) trout hepatocytes incubated 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM.

Figure 20.2. Comparing rates of pyrene metabolism between acclimated (n=5) and non-acclimated (n=6) trout hepatocytes incubated 1, 5, and 10 µg/ml pyrene dissolved in DMSO. Treatments with similar letters were not significantly different from each other at $p<0.05$. Error bars represent means±SEM.


Concentration of 1-OH pyrene (ng/ml)
0
20
40
60
80
100
120
140
160
Acclimated
Non-Acclimated
a
b
b
c
d
d
1
5
10
Pyrene exposure concentration (μg/ml)


Rate of pyrene metabolism (ng 1-OH produced min^{-1} mg cells^{-1})
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
Acclimated
Non-Acclimated
a
b
b
c
d
d
1
5
10
Pyrene exposure concentration (μg/ml)

DISCUSSION

Cytotoxicity

In this study, the respiratory costs of pyrene exposure and metabolism were measured using isolated hepatocyte preparations from rainbow trout. A prerequisite to accurate measurement of respiration in these cells is that they remain functionally intact through isolation and in the presence of pyrene. The viability of hepatocytes using this isolation technique was typically high for both the acclimated (ranging from 90.73 to 92.23%) and non-acclimated (ranging from 85.98 to 88.18%) cells. These values were in accordance with studies conducted by Moerland et al. (1981) and Rissanen et al. (2003), which found viability to be in the range of 85 to 90% in isolated hepatocytes of killifish (*Fundulus heteroclitus*) and greater than 90% in rainbow trout hepatocyte preparations, respectively. Furthermore, a study by Doblander et al. (1996) used a trypan blue exclusion test to show that cell viability of trout hepatocytes incubating at 20°C were in the range of 86-90%. Incubation temperature had the most significant effect on viability; hepatocytes incubated at 15°C had significantly lower viability values than cells incubated at 7.5°C. This is presumably due to both the direct effects of temperature on membrane characteristics and the isolation procedure since pyrene exposure did not further decrease cell viability at the higher temperature.

The results of the LDH assay showed no significant difference in LDH release from hepatocytes incubated at the concentrations of pyrene chosen for incubation when compared to controls. The LDH activity in the pyrene-treated cells was found to be considerably lower (ranging from 4.70 ± 0.86 to 7.80 ± 0.93 nmoles NADH consumed min^{-1}

mg cells^{-1}) in relation to the Triton X-100 exposed cells, which ranged from 56.78±1.60 to 66.35±7.66 nmoles NADH consumed min^{-1} mg cells^{-1}. Therefore, cell viability as determined by trypan blue exclusion and LDH leakage indicated that pyrene exposure was not cytotoxic at these concentrations, and did not affect the integrity of hepatocytes. Given this, pyrene proved to be an excellent model compound for examining the costs of exposure and metabolism in this *in vitro* system.

Mitochondrial Respiration

The majority of oxygen consumption by hepatocytes occurs in mitochondria (Porter and Brand 1995). For example, mitochondrial oxygen consumption accounts for approximately 85% of the resting oxygen consumption of rat hepatocytes. Therefore, it was imperative to determine if pyrene affected mitochondrial oxidative function directly *via* changes in the respiratory rate of isolated mitochondria.

The baseline values for RCR and ADP:O ratios in this study (RCR ranged from 2.48 to 2.73; ADP:O ratios ranged from 1.43 to 1.58) were similar to those reported in Suarez et al. (1981), which were 3.7 and 1.8, respectively. The RCR was also comparable to the value reported in Chamberlin et al. (1991) in another teleost fish (*Salvelinus namaycush*) (3.62±0.32). The ratios obtained were adequate to conduct this experiment as this data indicates that mitochondria were well-coupled.

Pre- and post-exposure mitochondrial respiration rates were not significantly different from each other for all pyrene and 500 ng/ml 1-OH pyrene treatment groups, indicating that that the pyrene concentrations used in this experiment and its metabolites were not toxic to mitochondria and that increases in hepatocyte respiration in other experiments could not be attributed to direct effects on mitochondria. The pre- and post-exposure mitochondrial respiration rates were similar to the average value found by

Suarez and Hochachka (1981), which was 278.05 ng O_2 min^{-1} mg mitochondrial $protein^{-1}$ in the presence of 5 mM succinate.

Hepatocyte Respiration

Several studies have reported increases in the respiration rate of hepatocytes after exposure to a toxicant. For example, in the experiment performed by Rissanen et al. (2003), it was established that dehydroabietic acid (DHAA), a resin acid and aquatic toxicant found in wood industry effluents, increased rainbow trout hepatocyte oxygen consumption rates in a dose-dependent manner, with maximal significant increases of 6.1±2.1%, 10.7±2.2%, and 16.8±2.2% in comparison to the control for hepatocytes exposed to 25 μM, 50 μM, and 100 μM DHAA, respectively. Furthermore, increased oxygen consumption rates were noted in isolated rat hepatocytes exposed to the naphthohydroquinone rifamycin SV (Saez et al. 1991). In all of these studies, however, distinguishing between the costs of detoxification and those incurred through toxic effects are difficult.

In the present study, the confounding effects of toxic injury and its associated costs are likely eliminated, since pyrene is relatively non-toxic to fish, particularly at the concentrations used in this study (Kennedy and Law 1990; Law et al. 1991; Namdari and Law 1996) and no direct effects on isolated liver mitochondria were detected.

Baseline values determined for respiration in hepatocytes showed good agreement with other studies. For example, the average value for controls at 7.5°C in the present study was 10.17±0.17 ng O_2 min^{-1} mg $cells^{-1}$, which is similar to the rate reported for trout hepatocytes (13.8 ± 0.6 ng O_2 min^{-1} mg $cells^{-1}$) acclimated to 10°C (Siebert 1985).

Significant increases in respiration rate (up to 41%) were noted in the presence of varying concentrations of pyrene (1, 5 and 10 µg/ml). This may be attributed to the costs associated with the detoxification of xenobiotics, which accrues from several processes including increases in energy and molecular oxygen used in enzymatic conversions, nutrient drains from conjugation reactions with endogenous substrates, the maintenance of cellular acid-base balance altered by the production of acidic metabolites (Foley 1992), and membrane transport of conjugated metabolites.

When hepatocytes were exposed at 15°C, respiration rates were approximately two-fold higher than at 7.5°C. This increase can be attributed to the direct effects of temperature on biochemical reaction rates (Hochachka and Somero 1984). Understandably, increases in temperature have been shown to increase respiration rates in organisms. For example, oxygen consumption increased with temperature in laboratory produced *Penaeus califoniensis* post-larvae from 4.5 µg O_2 g^{-1} min^{-1} at 19°C to 14.2 µg O_2 g^{-1} min^{-1} at 35°C (Villarreal et al. 1993). In a study by Fernandes et al. (1995), the oxygen consumption measured in 25°C-acclimated prochilodontid fish, *Prochilodus scrofa,* (79.99±2.84 ml O_2 kg^{-1} hr^{-1}) significantly increased as the temperature was raised to 30°C (129.38±7.12 ml O_2 kg^{-1} hr^{-1}) and 35°C (183.63±8.73 ml O_2 kg^{-1} hr^{-1}).

Increases in hepatocyte oxygen consumption correlated with pyrene concentration and rate of pyrene metabolism in cells exposed at 7.5°C. However, this was not the case at 15°C where a biphasic relationship was observed. These results can be interpreted by an examination of pyrene metabolism in this *in vitro* system.

Acute temperature changes, such as the one in this study from 7.5°C to 15°C, can represent a realistic situation, for example, in aquaculture facilities where temperature may act as a stressor, particularly due to accentuated diurnal temperature cycles in shallow ponds or tanks, or due to accidental temperature shocks during water replacement activities (Dalla Via et al. 1998). Fish may also often encounter acute changes in environmental temperature as a result of short-term migrations during foraging or spawning activities. In addition, the ambient temperature of some aquatic microenvironments, including the intertidal zone, riffle pools, shallow reaches, and areas receiving water from reservoirs or thermal pollution inputs, can be quite variable over a relatively short time period (Black et al. 1991). Hence, fish in these environments may experience sudden or intermittent temperature changes over a shorter time span than the 2-3 weeks necessary for them to acclimate to a new temperature. Since the body temperature of aquatic poikilotherms conforms to their external thermal environment, acute changes in water temperature can affect vital biochemical and physiological functions of fish living in these environments (Black et al. 1991).

Pyrene Metabolism

As stated previously, metabolism of organic xenobiotics, such as pyrene, in many aquatic organisms is accomplished by the mixed-function oxidase (MFO) system, a multienzyme complex that metabolizes both foreign and endogenous compounds (such as steroids and fatty acids) through a series of oxidation reactions (Jimenez et al. 1989). Previous mammalian and microbial biotransformation studies have shown that the major oxidation metabolite of pyrene is 1-hydroxypyrene, which may be metabolized further by conjugation reactions to form highly water-soluble metabolites (Law et al. 1994; Pelletier et al. 1997). Besides 1-hydroxypyrene, small amounts of 1,6-dihydroxypyrene and 1,8-dihydroxypyrene were discovered as metabolites in the study conducted by Law et al. (1994). After analyzing the amount of metabolites formed, no Phase I 1-hydroxypyrene was detected, but rather the predominant existence of the conjugated form (Phase II) with glucuronic acid. This result is similar to the study by Namdari (1998) where the percentages of ^{14}C-pyrene excreted as a conjugated metabolite constituted the majority of the radioactivity in the bile (97%) of starry flounder, *Platichthys stellatus*, following *i.a.* administration. Similarly, Law et al. (1994) reported that 1-hydroxypyrene was primarily conjugated with glucuronic acid or sulphate in the bile of rainbow trout (free 1-hydroxypyrene: 4.05±1.47 µg/ml; β-glucuronide: 779±270 µg/ml; sulphate: 270±49.4 µg/ml). In Varanasi et al. (1981), metabolites in bile from two groups of starry flounder (4 and 12°C) at 24 and 168 hr after feeding were characterized by high percentages (>85%) of the conjugates, of which glucuronides were the major components. The reason for a greater production of conjugated metabolites could be associated with the concept that stability of conjugates is often higher than free metabolites, especially in the case of 1-hydroxypyrene (Ariese et al. 1997).

At the higher incubation temperature of 15°C, more 1-OH pyrene metabolites were produced (especially in the 1 and 5 µg/ml treatments) than at 7.5°C. This is likely due to an increase in the amount of pyrene accumulating in the cells, and an increased rate of biotransformation reactions. Acute temperature changes are known to affect membrane permeability (White et al. 1982) with higher temperatures resulting in biological membranes being more fluid and permeable to passively diffusing molecules such as oxygen and other lipophilic xenobiotics (Black et al. 1991). Increased fluidity and uptake of xenobiotics have been previously studied. In Kennedy et al. (1994), it was shown that the accumulation of benzo[a]pyrene was higher in isolated gill cells of gulf toadfish, *Opsanus beta*, when cells were exposed to the PAH during an acute temperature increase. In another study by Black et al. (1991), accumulation of 1,1,1-trichloro-2,2 bis(p-chlorophenyl)ethane (DDT) by mosquito fish (*Gambusia affinis*) was three times greater at 20°C compared with exposures at 5°C. Researchers in the study by Jimenez et al. (1987) looked at how bluegill sunfish (*Lepomis machrochirus*) experienced a two-fold increase in body burden of benzo[a]pyrene with a 10°C increase in exposure temperature.

Links between biotransformation rates and acute temperature changes have also been reported in previous research. For example, a study by Gill and Walsh (1990) demonstrated that at a higher benzo[a]pyrene exposure concentration, there were significant effects on the rates of metabolism in toadfish (*Opsanus beta*) hepatocytes with increasing incubation temperature, especially when hepatocytes were incubated at temperatures higher than the acclimation temperature. For toadfish hepatocytes acclimated at incubated at 18°C, the Phase II metabolite production rate was approximately 60 $\mu g\ g^{-1}\ hr^{-1}$ while hepatocytes acclimated at 18°C but incubated at 28°C exhibited a metabolite production rate of approximately 150 $\mu g\ g^{-1}\ hr^{-1}$. Kennedy et al. (1989) found significant effects of all temperature treatments on the amount of [^{14}C] benzo[a]pyrene metabolized and excreted into the bile of gulf toadfish. For toadfish acclimated and exposed at 18°C, the amount of benzo[a]pyrene metabolized and excreted to the bile in 24 hrs was significantly lower (1.40±0.04 µg/fish) than the amount metabolized in toadfish acclimated at 18°C but exposed to 28°C (2.51±0.09 µg/fish).

In the present study, the accumulation of pyrene metabolites increased with increasing pyrene exposure at 7.5°C. Thus, respiration rates correlated well with pyrene metabolism as well as pyrene exposure concentration. At 15°C, cellular respiration rates did not increase linearly with increasing pyrene concentration. However, pyrene metabolism at each exposure concentration did correlate with increasing respiration rates (Figure 3b). This indicates that increases in hepatocyte respiration were not due to pyrene exposure concentration or any toxic effects, but likely related to the energetic costs of metabolizing pyrene and excreting its metabolites.

Two factors may explain why there was no increase in hepatocyte respiration rate and concentration of 1-OH pyrene produced beyond the 5 µg/ml treatment group for the non-acclimated hepatocytes, (i) fluidity of the membrane, and (ii) excess pyrene that has not undergone detoxification. Since the hepatocytes were incubated at 15°C rather than at their acclimated temperature of 7.5°C, the membrane tends to become more fluid. This fluidity as well as the excess pyrene may have disrupted protein-lipid interactions, especially the interaction between the cytochrome P-450 and the smooth endoplasmic reticulum. Alternatively, the cytochrome P-450 reached maximum catalytic capacity in non-acclimated hepatocytes exposed to 5 µg/ml pyrene and any pyrene concentration greater than 5 µg/ml.

Conclusion

This is the first study to quantify the direct costs of xenobiotic metabolism and excretion in hepatocytes. The increases in cellular respiration seen in this study are substantial (up to 40% of basal rates), yet it would be difficult to extrapolate these *in vitro* results to those occurring *in vivo*, or to predict consequences and trade-offs when energy is limiting. It has been suggested that the biosynthetic energy costs of producing and maintaining a high titer of detoxification enzymes might constitute a significant component of the energy budget of an organism smaller than a rodent (Brattsen 1979, 1988). Indeed, Cresswell et al. (1992) determined that a reallocation of assimilated food energy occurred in the southern army worm (*Spodoptera eridania*) when a dietary toxin was present, with potential consequences to other physiological processes. Several studies designed to examine the costs of detoxification in insects (Waldbauer 1968; Schoonhoven and Meerman 1978; Scriber 1981), and two studies in fish (Beyers 1999a, 1999b), have manipulated chemical exposure and then monitored indirect measures of detoxification and effects, such as changes in relative growth rate, efficiency of conversion of digested food to biomass, and the subsequent quantification of growth.

The design of those studies, however, does not allow the direct assessment of tradeoffs between detoxification and growth. The dietary toxins may have affected food intake and energy assimilation, so it is unclear if reduced growth was the result of an increase in energy allocation to detoxification.

The costs of detoxification estimated in hepatocytes and the potential trade-offs that could arise, may be higher than those seen in insects due to the fact that pyrene metabolism involves conjugation reactions which are more energetically and nutritionally expensive than the functionalization (Phase I) reactions used to detoxify many plant allelochemicals.

The results of this study therefore suggest that there is a quantifiable cost to organisms that must detoxify xenobiotics. The significance of this cost in terms of the overall energy budgets of animals are unknown, however, this study suggests that these costs may be substantial. Assessing the energetic cost of detoxification is an important step in identifying selective and evolutionary forces that involve xenobiotics including foraging behaviour, predator-prey relationships, and habitat selection. This knowledge can also be extremely valuable in understanding sublethal toxicological impacts on organism energy budgets, a concept that is not species-specific, and has broad ecological application to all organisms.

LITERATURE CITED

Andersson, T., Forlin, L., 1992. Regulation of the cytochrome P450 enzyme system in fish. Aquat. Toxicol. 24, 1-20.

Ariese, F., Burgers, K., Rutten, T., Stroomberg, G., and Vethaak, D., 1997. Comparison of analytical approaches for PAH metabolites in fish bile samples for marine and estuarine monitoring. Institute for Environmental Studies, vrije Universiteit, Amsterdam, Netherlands.

Ariese, F., Kok, S.J., Verkaik, M., Gooijer, C., Velhorst, N.H., and Hofstraat, J.W. 1993. Synchronous fluorescence spectrometry of fish bile: A rapid screening method for biomonitoring of PAH exposure. Aquat. Toxicol. 26, 273-286.

Berenbaum, M.R., Zangerl, A.R., 1994. Costs of inducible defense: Protein limitation, growth, and detoxification in parsnip webworms. Ecology. 75(8), 2311-2317.

Berntssen, M.H.G, Lundebye, A., 2001. Energetics in Atlantic salmon (*Salmo salar* L.) parr fed elevated dietary cadmium. Comp. Biochem. Physiol . 128C(3), 311-323.

Beyers, D.W., Rice, J.A., Clements, W.H., Henry, C.J., 1999a. Estimating physiological cost of chemical exposure: Integrating energetics and stress to quantify toxic effects in fish. Can. J. Fish. Aquat. Sci. 56(5), 814-822.

Beyers, D.W., Rice, J.A., Clements, W.H., 1999b. Evaluating biological significance of chemical exposure to fish using a bioenergetics-based stressor-response model. Can. J. Fish. Aquat. Sci. 56(5), 823-829.

Black, M.C., Millsap, D.S., McCarthy, J.F., 1991. Effects of acute temperature change on respiration and toxicant uptake by rainbow trout, *Salmo gairdneri* Richardson. Physiol. Zool. 64(1), 145-168.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brattsen, L.B., 1979. Biochemical defense mechanisms in herbivores against plant allelochemicals. In: Rosental, G.A., Janzen, D.H. (Eds.), Herbivores, their interaction with secondary plant metabolites. Academic Press, New York, pp. 200-269.

Brattsen, L.B., 1988. Enzymic adaptations in leaf-feeding insects to host-plant allelochemicals. J. Chem. Ecol. 14(10), 1919-1940.

Buhler, D.R., Williams, D.E., 1988. The role of biotransformation in the toxicity of chemicals. Aquat. Toxicol. 11(1-2), 19-28.

Butler, P.J., 2000. Energetic costs of surface swimming and diving of birds. Physiol. Biochem. Zool. 73(6), 699-705.

Campbell, N.A., Reece, J.B., Mitchell, L.G., 1999. Biology. Benjamin/Cummings, Menlo Park.

Chamberlin, M.E., Glemet, H.C., Ballantyne, J.S., 1991. Glutamine metabolism in a holostean (*Amia calva*) and teleost fish (*Salvelinus namaycush*). Am. J. Physiol. 260, R159-R166.

Cresswell, J.E., Merritt, S.Z., Martin, M.M., 1992. The effect of dietary nicotine on the allocation of assimilated food to energy metabolism and growth in fourth-instar larvae of the southern army worm, *Spodoptera eridania* Lepidoptera Noctuidae. Oecologia. 89(3), 449-453.

Dalla Via, J., Villani, P., Gasteiger, E., Niederstätter, H., 1998. Oxygen consumption in sea bass fingerling *Dicentrarchus labrax* exposed to acute salinity and temperature changes: metabolic basis for maximum stocking density estimations. Aquaculture. 169, 303-313.

Dash, J.A., 1988. Effects of dietary terpenes on glucuronic acid excretion and ascorbic acid turnover in the brushtail possum, *Trichosurus vulpecula*. Comp. Biochem. Physiol. 89B(2), 221-226.

Demas, G.E., Chefer, V., Talan, M.I., Nelson, R.J., 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. Am. J. Physiol. 273, R1631-R1637.

Doblander, C., Lackner, R., 1996. Metabolism and detoxification of nitrite by trout hepatocytes. Biochim. Biophys. Acta. 1289, 270-274.

Environment Protection Agency (EPA), 1975. Scientific and technical assessment report on particulate polycyclic organic matter (PPOM). EPA Publ. EPA-600/6-75-001. Washington, DC.

Estabrook, R.W., 1967. Mitochondrial respiratory control and the polargraphic measurement of ADP:O Ratios. In: Estabrook, R.W., Pullman, M.E. (Eds.), Methods in enzymology, 10. Academic Press, New York, pp. 41-48.

Fernandes, M.N., Barrionuevo, W.R., and Rantin F.T., 1995. Effects of thermal stress on respiratory responses to hypoxia of a South American prochilodontid fish, *Prochilodus scrofa*. J. Fish Biol. 46, 123-133.

Festa-Bianchet, M., 1989. Individual differences, parasites, and the cost of reproduction for bighorn ewes (*Ovis Canadensis*). J. Anim. Ecol. 58, 785-795.

Foley, W.J., 1992. Nitrogen and energy retention and acid-base status in the common ringtail possum, *Pseudocheirus peregrinus*, evidence of the effects of absorbed allelochemicals.. Physiol. Zool. 65(2), 403-421.

Gabos, S., Ikonomou, M.G., Schopflocher, D., Fowler, B.R., White, J., Prepas, E., Prince, D., Chen, W., 2001. Characteristics of PAHs, PCDD/Fs and PCBs in sediment following forest fires in northern Alberta. Chemosphere. 43(4-7), 709-719.

George, S.G., 1994. Enzymology and molecular biology of Phase II xenobiotic-conjugating enzymes in fish. In Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology: Molecular, biochemical and cellular perspectives. Lewis Publishers, Boca Raton, Florida, pp. 37-85.

Gill, K.A., Walsh, P.J. 1990. Effects of temperature on metabolism of benzo[a]pyrene by toadfish (*Opsanus beta*) hepatocytes. Can. J. Fish. Aquat. Sci. 47, 831-837.

Glick, Z., Joslyn, M.A., 1970. Food intake depression and other metabolic effects of tannic-acid in the rate. J. Nutr. 100(5), 509-515.

Graney, R.L., Giesy Jr., J.P., 1986. Effects of long-term exposure to pentachlorophenol on the free amino acid pool and energy reserves of the freshwater amphipod *Gammarus pseudolimnaeus* Bousfield (Crustacea, Amphipoda). Ecotoxicol. Environ. Saf. 12, 233-251.

Guglielmo, C.G., Karasov, W.H., Jakubas, W.J., 1996. Nutritional costs of a plant secondary metabolite explain selective foraging by ruffed grouse. Ecol. 77(4), 1103-1115.

Helder, T., 1981. Effects of 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD) on early life stages of rainbow trout (*Salmo gairdneri*, Richardson). Toxicol. 19(2), 101-112.

Henken, A.M., Brandsma, H.A., 1982. The effect of environmental temperature on immune response and metabolism of the young chicken. 2. Effect of the immune response to sheep red blood cells on energy metabolism. Poult. Sci. 61, 1667-1677.

Hinkle-Conn, C., Fleeger, J.W., Gregg, J.C., Carman, K.R., 1998. Effects of sediment-bound polycyclic aromatic hydrocarbons on feeding behavior in juvenile spot (*Leiostomus xanthurus* Lacepede: Pisces). J. Exp. Mar. Biol. Ecol. 227(1), 113-132.

Hochachka, P.W., Somero, G.N., 1984. Biochemical Adaptation. Princeton University Press, Princeton, New Jersey.

Hopkins, W.A., Snodgrass, J.W., Roe, J.H., Jackson, B.P., Gariboldi, J.C., Congdon, J.D., 2000. Detrimental effects associated with trace metal element uptake in lake chubsuckers (*Erimyzon sucetta*) exposed to polluted sediments. Arch. Environ. Contam. Toxicol. 39, 193-199.

Ioannides, C., 1996. Cytochromes P450: metabolic and toxicological aspects. CRC Press Inc., Boca Raton, Florida.

Jimenez, B.D., Burtis, L.S., 1989. Influence of environmental variables on the hepatic mixed-function oxidase system in bluegill sunfish, *Lepomis macrochirus*. Comp. Biochem. Physiol. 93C(1), 11-21.

Jimenez, B.D., Cirmo, C.P., McCarthy, J.F., 1987. Effects of feeding and temperature on uptake, elimination and metabolism of benzo-a-pyrene in the bluegill sunfish *Lepomis macrochirus*. Aquat. Toxicol. 10(1), 41-58.

Johnson, A., Larsen, P.F., Gadbois, D.F., Humason, A.W., 1985. The distribution of polycyclic aromatic hydrocarbons in the surficial sediments of Penobscot Bay (Maine, USA) in relation to possible sources and other sites worldwide. Mar. Environ. Res. 15, 1-16.

Johnston, B.D., Alexander, G., Kennedy, C.J., 1999. Thermal modulation of the toxicokinetics of benzo[a]pyrene in isolated hepatocytes of sablefish (*Anoplopoma fimbria*), black rockfish (*Sebastes melanops*), and chub mackerel (*Scomber japonicus*). Comp. Biochem. Physiol. 124C, 157-164.

Kennedy, C.J., Law, F.C.P., 1990. Toxicokinetics of selected polycyclic aromatic hydrocarbons in rainbow trout following different routes of exposure. Environ. Toxicol. Chem. 9(2), 133-140.

Kennedy, C.J., Gill, K.A., Walsh, P.J., 1991. Temperature acclimation of xenobiotic metabolizing enzymes in cultured hepatocytes and whole liver of the gulf toadfish, *Opsanus beta*. Can. J. Fish. Aquat. Sci. 48(7), 1212-1219.

Kennedy, C.J., Walsh, P.J., 1994. The effects of temperature on the uptake and metabolism of benzo(a)pyrene in isolated gill cells of the gulf toadfish, *Opsanus beta*. Fish Physiol. Biochem. 13(2), 93-103.

Kitchell, J.F., 1983. Energetics. In Webb, P.W., Weihs, D. (Eds.), *Fish Biomechanics*. Praeger Publishers, New York, pp. 312-338.

Kolanczyk, R.C., Fitzsimmons, P.N., McKim, Sr., J.M., Erickson, R.J., Schmieder, P.K. 2003. Effects of anesthesia (tricane methanesulfonate, MS-222) on liver biotransformation in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 64, 177-184.

Kotiaho, J.S., Alatalo, R.V., Mappes, J., Nielsen, M.G., Parri, S., Rivero, A., 1998. Energetic costs of size and sexual signalling in a wolf spider. *Proc. R. Soc. Lond.* 265B, 2203-2209.

Krahn, M.M., Burrows, D.G., MacLeod Jr., W.D., Malins, D.C., 1987. Determination of individual metabolites of aromatic compounds in hydrolyzed bile of English sole (*Parophrys vetulus*) from polluted sites in Puget Sound, Washington. Arch. Environ. Contam. Toxicol. 16, 511-522.

Krisfalusi, M., Eroschenko, V.P., Cloud J.G., 1998. Exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to methoxychlor results in a dose-dependent decrease in growth and survival but does not alter male sexual differentiation. Bull. Environ. Contam. Toxicol. 60(4), 659-666.

Law, F.C.P., Abedini, S., Kennedy, C.J., 1991. A biologically based toxicokinetic model of pyrene in rainbow trout. Toxicol. Appl. Pharmacol. 110(3), 390-402.

Law, F.C.P., Meng, J.X., He, Y.T., Chui, Y.C., 1994. Urinary and biliary metabolites of pyrene in rainbow trout (*Oncorhynchus mykiss*). Xenobiotica. 3, 221-229.

Levin, J.O., 1995. First international workshop on hydroxypyrene as a biomarker for PAH exposure in man—summary and conclusions. Sci. Total Environ. 163(1-3), 164-168.

Lin, E.L.C., Cormier, S., Racine, R.N., 1994. Synchronous fluorometric measurement of metabolites of polycyclic aromatic hydrocarbons in the bile of brown bullhead. Environ. Toxicol. Chem. 13, 707-715.

Lindroth, R.L., Batzli, G.O., 1984. Plant phenolics as chemical defenses effects of natural phenolics on survival and growth of prairie voles, *Microtus orchrogaster*. J. Chem. Ecol. 10(2), 229-244.

Martin II, L.B., Scheuerlein, A., Wikelski, M., 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc. R. Soc. Lond.* 270B, 153-158.

Maryański, M., Kramarz, P., Laskowski, R., Niklińska M., 2002. Decreased energetic reserves, morphological changes and accumulation of metals in carabid beetles (*Poecilus cupreus* L.) exposed to zinc- or cadmium-contaminated food. Ecotoxicology. 11, 127-139.

Moerland, T.S., Sidell, B.D., 1981. Characterization of metabolic carbon flow in hepatocytes isolated from thermally acclimated killifish, *Fundulus heteroclitus*. Physiol. Zool. 54(3), 379-389.

Moon, T.W., Walsh, P.J., Mommsen, T.P., 1985. Fish hepatocytes: A model metabolic system. Can. J. Fish. Aquat. Sci. 42(11), 1772-1782.

Namdari, R., Law, F.C.P., 1996. Toxicokinetics of waterborne pyrene in rainbow trout (*Oncorhychus mykiss*) following branchial or dermal exposure. Aquat. Toxicol. 35(3-4), 221-235.

Namdari, R., 1998. A physiologically based toxicokinetic model of pyrene and its major metabolites in starry flounder (*Platichthys stellatus*). Ph.D. Thesis, Simon Fraser University, B.C., Canada.

Nicholson, R.A., 1994. Excitatory actions of dehydroabietic acid on mammalian synaptosomes. Pharmacol. Toxicol. 75(5), 274-279.

Nicol, S., De La Mare, W.K., Stolp, M., 1995. The energetic cost of egg production in Antarctic krill (*Euphausia superba* Dana). Antarctic Sci. 7(1), 25-30.

Nisbet, I.C.T., Lagoy, P.K., 1992. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Reg. Toxicol. Pharmacol. 16, 290-300.

Ogle, R.S., Knight, A.W., 1989. Effects of elevated foodborne selenium on growth and reproduction of the fathead minnow (*Pimephales promelas*). Arch. Environ. Contam. Toxicol. 18, 795-803.

Ots, I., Kerimov, A.B., Ivankina, E.V., Ilyina, T.A., Hõrak, P., 2001. Immune challenge affects basal metabolic activity in wintering great tits. Proc. R. Soc. Lond. B. 268, 1175-1181.

Patnaik, A., 2002. Impact of copper application on soil metabolism, energy budget and formation of stable aggregates by anecic earthworm in tea plantations. Indian J. Exp. Biol. 40(2), 216-219.

Pelletier, M.C., Burgess, R.M., Ho, K.T., Kuhn, A., McKinney, R.A., Ryba, S.A., 1997. Phototoxicity of individual polycyclic aromatic hydrocarbons and petroleum to marine invertebrate larvae and juveniles. Environ. Toxicol. Chem. 16(10), 2190-2199.

Pesonen, M., Andersson, T., 1992. Toxic effects of bleached and unbleached paper mill effluents in primary cultures of rainbow trout hepatocytes. Ecotoxicol. Environ. Saf. 24, 63-71.

Porter, R.K., Brand, M.D., 1995. Causes of differences in respiration rate of hepatocytes from mammals of different body mass. Am. J. Physiol. 269, R1213-1224.

Remington, T. 1990. Ph.D. Dissertation. University of Wisconsin.

Rissanen, E., Krumschnabel, G., Nikinmaa, M., 2003. Dehydroabietic acid, a major component of wood industry effluents, interferes with cellular energetics in rainbow trout hepatocytes. Aquat. Toxicol. 62, 45-53.

Robbins, C.T., Hanley, T.A., Hagerman, A.E., Hjeljord, O., Baker, D.L., Schwartz, C.C., Mautz, W.W., 1987. Role of tannins in defending plants against ruminants reduction in protein availability. Ecology. 68(1), 98-107.

Robbins, C.T., Hagerman, A.E., Austin, P.J., MacArthur, C., Hanley, T.A., 1991. Variation in mammalian physiological responses to a condensed tannin and its ecological implication. J. Mammal. 72, 480-486.

Saarikoski, J.R., Lindstrom, M.T., Viluksela, M., 1986. Factor affecting the absorption of phenolic and carboxylic acids in the guppy (*Poecilia reticulate*). Ecotoxicol. Environ. Saf. 11, 158-173.

Saez, G.T., Valls, V., Cabedo, H., Iradi, A., Bannister, W.H., Bannister, J.V., 1991. Effect of metal ion catalyzed oxidation of rifamycin SV on cell viability and metabolic performance of isolated rat hepatocytes. Biochim. Biophys. Acta. 1092, 326-335.

Schoonhoven, L., Meerman, J., 1978. Metabolic cost of changes in diet and neutralization of allelochemics. Entomol. Exp. Appl.. 24, 489-493.

Scriber, J.M., 1981. Sequential diets metabolic costs and growth of *Spodoptera eridania* (Leidoptera : noctuidae) feeding on dill, lima bean and cabbage. Oecologia, 51(2), 175-180.

Seubert, J.M., Kennedy, C.J., 1997. The toxicokinetics of benzo[a]pyrene in juvenile coho salmon, *Oncorhynchus kisutch*, during smoltification. Fish Physiol. Biochem. 16(5), 437-447.

Siebert, H., 1985. Viability control and oxygen consumption of isolated hepatocytes from thermally acclimated rainbow trout (*Salmo gairdnerii*). Comp. Biochem. Physiol. 80B(4), 677-683.

Stegeman, J.J., 1993. The cytochromes P450 in fish. In: Hochachka, P.W., Mommsen, T.P. (Eds.), Molecular Biology Frontiers. Biochemistry and molecular biology of fishes, 2. Elsevier, Amsterdam, pp. 137-158.

Stroomberg, G.J., Reuther, C., Kozin, I., Van Brummelen, T.C., Van Gestel, C.A.M., Gooijer, C., Cofino, W.P., 1996. Formation of pyrene metabolites by the terrestrial isopod *Porcellio scaber*. Chemosphere. 33(10), 1905-1914.

Suarez, R.K., Hochachka, P.W., 1981. Preparation and properties of rainbow trout Liver mitochondria. J. Comp. Physiol. 143B, 269-273.

Telford, S.R., Webb, P.I., 1998. The energetic cost of copulation in a polygynandrous millipede. J. Exp. Biol. 201(11), 1847-1849.,

Totten, G.E., 2000. Handbook of hydraulic fluid technology. Marcel Dekker, New York.

Varanasi, U., Gmur, D.J., Reichert, W.L., 1981. Effect of environmental temperature on naphthalene metabolism by juvenile starry flounder (*Platichthys stellatus*). Arch. Environ. Contam. Toxicol. 10, 203-214.

van Beek, L.M., Mulder, M., van Haeringen N.J., Kijlstra A., 2000. Topical ophthalmic beta blockers may cause release of histamine through cytotoxic effects on inflammatory cells. Br. J. Ophthalmol. 84, 1004-1007.

Villarreal, H., Rivera, J.A., 1993. Effect of temperature and salinity on the oxygen consumption of laboratory produced *Penaeus californiensis* post-larvae. Comp. Biochem. Physiol. 106A(1), 103-107.

Waldbauer, G.P., 1968. The consumption and utilization of food by insects. In: Beament, J.W.L., Treherne, J.E., Wigglesworth, V.B. (Eds.), Advances in Insect Physiology, 5. Academic Press, New York, pp. 229-288.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 1997. Principles of ecotoxicology. Taylor & Francis Ltd., London.

Webber, D.M., Aitken, J.P., O'Dor, R.K., 2000. Costs of locomotion and vertic dynamics of cephalopods and fish. Physiol. Biochem. Zool. 73(6), 651-662.

White, F.N., Somero G., 1982. Acid-base regulation and phospholipid adaptations to temperature: time courses and physiological significance of modifying the milieu for protein function. Physiol. Rev. 62(1): 40-90.

Xie, X.J., Sun, R., 1993. Pattern of energy allocation in the southern catfish (*Silurus meridionalis*). J. Fish Biol. 42(2), 197-207.

Youngblood, W.W., Blumer, M., 1975. Polycyclic aromatic hydrocarbons in the environment: homologous series in soil and recent marine sediments. Geochim. Cosmochim. Acta. 39, 1303.

Zapata-Perez, O., Gold-Bouchot, G., Ortega, A., Lopez, T., Albores, A., 2002. Effect of pyrene on hepatic cytochrome P450 1A (CYP1A) expression in nile tilapia (*Oreochromis niloticus*). Arch. Environ. Contam. Toxicol. 42(4), 477-485.