Effects of Hypoxia and 4-tert-octylphenol on Gene Expression Profiles of the Sheepshead Minnow (Cyprinodon variegatus)

Arthur Alan Karels
University of Southern Mississippi

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EFFECTS OF HYPOXIA AND 4-\textit{TERT}-OCTYLPHENOL ON
GENE EXPRESSION PROFILES OF THE SHEEPSHEAD
MINNOW (\textit{CYPRINODON VARIEGATUS})

by

Arthur Alan Karels

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

May 2012
ABSTRACT
EFFECTS OF HYPOXIA AND 4-TERT-OCTYLPHENOL ON GENE EXPRESSION PROFILES OF THE SHEEPSHEAD MINNOW (CYPRINODON VARIEGATUS)

by Arthur Alan Karels

May 2012

Hypoxia occurs in estuaries of northern Gulf of Mexico and world-wide, with increasing frequency/severity via eutrophication and anthropogenic influences. Hypoxia inducible factors (HIFs) form transcriptional complex and bind DNA at hypoxia responsive elements (HREs) in promoter regions of genes needed for systemic and cellular adaptation of fish to low dissolved oxygen (hypoxia, DO <2.0 mg/ml). Hypoxia-induced activation of HIF-αs can lead to a cascade of downstream activation, such as erythropoietin (EPO). Return to normal DO levels (normoxia), prolyl hydroxylases (PHDs) are activated to degrade HIF-αs back to baseline. Fish are affected by environmental estrogen mimics, like 4-tert-octylphenol (4tOP), binding estrogen receptor alpha (ERα) at estrogen responsive elements (EREs) and activating genes vitellogenin (VTG). Previous research showed overlap or crosstalk between these two mechanistic pathways. Hypoxia triggers unknown factors regulating ERE-mediated ERα signaling pathway, and stressor combinations could increase/decrease hypoxic or endocrine pathway. Research examined molecular/physiological effects of hypoxia (acute and chronic, moderate and severe) and 4tOP (~60µg/L) on adult male and/or female sheepshead minnow (Cyprinodon variegatus). Three genes identified, cloned, and sequenced (HIF-1α, HIF-2α, and PHD3), plus previously identified genes EPO and VTG,
were examined in liver/testes exposed to hypoxia and/or 4tOP for cellular/physiological changes. Endpoints examined included mRNA expression from real-time PCR of HIF-1α, HIF-2α, PHD3, EPO, and VTG using cDNA from total RNA extracts, and microarray analyses of genes expressed during the transition from hypoxia back to normoxia. Phylogenetic analyses confirmed isolation of two HIF-α isoforms (HIF-1α and HIF-2α) and the PHD3 isoform. Significant up-regulation of PHD3 occurred within 10 hrs of chronic hypoxia, and persisted when severe (1.5 mg/L) and declined when moderate (~2.5mg/L). Significant up-regulation of HIF-1α and EPO occurred within 30 minutes to 2 hours of onset of acute severe and very severe (~1.08mg/L) hypoxia. Hypoxia acted similar to an estrogen mimic, with huge up-regulation of VTG gene expression in males, and increased VTG levels (additive effect) when hypoxia was combined with 4tOP. Microarray analyses showed 125 genes with significant transcriptional change, with up- or down-regulation from transitions of: (1) hypoxia (72 hrs) to normoxia (74 hrs) and (2) hypoxia+4tOP (72 hrs) to normoxia+4tOP (74 hrs).
The University of Southern Mississippi

EFFECTS OF HYPOXIA AND 4-TERT-OCTYLPHENOL ON GENE EXPRESSION PROFILES OF THE SHEEPSHEAD MINNOW (CYPRINODON VARIEGATUS)

by

Arthur Alan Karels

A Dissertation
Submitted to the Graduate School of The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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May 2012
DEDICATION

I would like to dedicate my dissertation to my wife, Diane Louise Ehrman, for all her love, support, encouragement, and teamwork over the years, working and sharing all things together, through ups and downs, in our journey together from Minneapolis and St. Paul, Minnesota to Hattiesburg, Mississippi to Ocean Springs, Mississippi. I feel in my heart all of her smiles, hear all of her giggles, see her bright brown eyes, and feel the warmth of her heart. After God, this New Orleans uptown woman is my best friend, my compass, and she is indeed my better half! My love goes back to you, Diane!
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LIST OF ABBREVIATIONS

acute hypoxia = short-term decline in DO (hours) that leads to gradually declining pO₂ levels in cells, tissues, and organs

ANOVA = Analysis of Variance

chronic hypoxia = long-term decline in DO (days) that leads to low pO₂ levels in cells, tissues, and organs

Cyprinodon variegatus = sheepshead minnow

Ct = Cycle threshold

DO = dissolved oxygen

ds cDNA = double-stranded complementary deoxyribonucleic acids

ECs = Estrogenic Chemicals

EDCs = Endocrine Disrupting Chemicals

EPO = Erythropoetin

ERα = Estrogen Responsive Alpha

ERE = Estrogen Responsive Element

E2 = 17β-estradiol

FDR = False Discovery Rate

Hypoxia = oxygen deficiency event

HIF-1α = Hypoxia Inducible Factor one alpha

HIF-2α = Hypoxia Inducible Factor two alpha

PES = partial eta squared

PHD3 = Prolyl Hydroxylase 3

ODDD = oxygen-dependent degradation domain
qRT-PCR = quantitative Real-Time Polymerase Chain Reaction

ROS = reactive oxygen species

SAM = Significant Analysis of Microarrays

ss cDNA = single-stranded complementary deoxyribonucleic acids

VTG = vitellogenin

4tOP = 4-tert-octylphenol
CHAPTER I
INTRODUCTION

Many aquatic organisms are directly or indirectly impacted by hypoxia in a variety of ways, including survival, reproduction, alterations in behavior, and changes in food web and habitat utilized. Impacts from hypoxia are particularly stressful for organisms that are sessile or habitat-specific, such as estuaries. Impacts of hypoxia on cells, tissues, and organs of aquatic organisms include systemic and molecular responses promoting adaptations to decreased oxygen levels. This introduction has three parts covering the pertinent scientific literature to date: I) information about hypoxia in coastal waters and impacts on fish reproduction, II) mechanism of HIF activation during hypoxia and HIF inactivation upon return to normoxia, III) interaction between hypoxia and estrogenic chemicals (ECs). The research of this dissertation revolves around impacts of exposure to hypoxia (low dissolved oxygen) and/or the EC, 4-tert-Octylphenol (4tOP), on the aquatic species *Cyprindon variegatus* (Sheepshead minnow), in terms of how several genes (HIF-1α, HIF-2α, EPO, PHD3, and VTG) are transcriptionally activated to compensate for the stress placed on these adult fish.

Hypoxia

Many studies have defined 2.0 mg/L as the start of hypoxia, and as the threshold level of dissolved oxygen for fish movement (Eby and Crowder, 2002; Sagasti et al., 2003). Other studies indicate that 2.0 mg/l dissolved oxygen level may not act as a universal threshold level for hypoxia, but instead this level is dependent upon the species, system, and time of year, such as interaction of temperature and oxygen preferences (Eby and Crowder, 2002). During summer, hypoxia (low levels of dissolved oxygen) occurs
when water becomes stratified from warmer, calm, less saline surface waters, along with cooler more saline bottom waters. Stratified water forms a pycnocline, and prevents these two layers of water from mixing, and thus prevents the ability of bottom waters from being re-oxygenated. This stratified water is often accompanied by the addition of nutrients, such as nitrogen, which stimulates algal and/or phytoplankton bloom. Excess fertilizer washed from the land, along with untreated overflow sewage entering into the water are the largest sources for this excess of nutrients. When algae or phytoplankton die, they sink into the bottom waters where they are decomposed by aerobic microorganisms, which use up the available bottom dissolved oxygen. Depletion of oxygen in bottom water causes fish kills if there is no place to escape the hypoxic area. Hypoxic events can be especially harmful to young or juvenile fish that require estuarine waters, in terms of food, protection from predators, and appropriate salinity for osmoregulation.

Many human activities, within and near coastal zones, modify the physical and chemical characteristics within estuaries. Anthropogenic activities in US estuaries cause changes in dissolved oxygen, salinity, current velocity, temperature, sedimentation, depth, and nutrient loading (Eby and Crowder, 2002), where changes in hydrology affect oxygen budgets of estuaries in southeastern U.S., causing hypoxia (Eby and Crowder, 2002). Because of increasingly greater anthropogenic eutrophication, hypoxia and anoxia are becoming more widespread and persistent and many coastal and estuarine communities around the world are hypoxic (Sagasti et al., 2003; Diaz and Rosenberg, 2008). Watershed nutrient management strategies have been prompted in many of the major estuaries in the U.S. and the world, because of concerns from the effects of low
dissolved oxygen and its real or potential impacts on the fish and shellfish populations (Figs 1-2) (Borsuk et al., 2002; Rabalais and Turner, 2001; Rabalais, 2006).

**Figure 1.** Frequency of occurrence of midsummer hypoxia over the 60–80-station grid, 1985–2001. (Fig. 4; Rabalais and Turner, 2001).

**Figure 2.** Similar size and expanse of bottom water hypoxia in mid-July 2002 (shaded area) & in mid-July 2001 (outlined with dashed line) (N. Rabalais, LUMCON). Asterisk indicates location of high frequency temporal data. (Fig. 1; Rabalais, 2006)

The more severe hypoxic events are often caused or exacerbated by human activity, and they have the potential disrupt food webs, and they can bring fish and
invertebrates to their physiological limits. Environmental stress and recruitment are major regulators of community structures and community processes, because stress can alter recruitment at numerous stages, decrease fecundity of adults, and/or survival of propagules by altering settlement patterns, changing growth or predation rates on recent settlers (Sagasti et al., 2003). The most serious effects of hypoxia on fisheries are long-term weakening of species stressed by over-fishing, economic loss, habitat loss, and long-term changes in ecology from a shift in the dominant species of algae and/or a change the dominant fish species especially if there is limited recruitment back into the area (Sagasti et al., 2003).

Different types of aquatic organisms have varying degrees of ability to cope or survive through hypoxic events. Fish are more sensitive to hypoxia than crustaceans, followed by annelids, and with bivalves being the most tolerant (Mistri, 2004). Tolerance experiments using 0.2 mg/l of O$_2$ at 10 °C have shown that *Mytilus edulis* survived for more than 1000 hrs, *Nereis diversicolor* survived for about 200 hrs, and *Carcinus maenas* for less than 100 hrs (Mistri, 2004). Mobile organisms, such as fish and blue crabs (*Callinectes sapidus*), will tend to use avoidance behavior to escape or bypass these hypoxic waters, whereas sessile organisms and infaunal species initiate a series of responses to hypoxia, based on its severity, to try to survive through this temporal period. Fish distributions have been shown to shift away from hypoxic zones to available oxygenated habitats in a number of estuaries, including Chesapeake Bay, Long Island Sound, Gulf of Mexico, and Neuse River Estuary, but few studies have shown effects of hypoxia on metabolic costs, feeding rates, and species interactions (Eby and Crowder, 2002). There are costs associated with sub-lethal effects from milder (duration or
severity) hypoxic events to these various types of aquatic organisms. The costs to fishes remaining in hypoxic waters have been considered strictly a physiological response; however, external conditions (predation risk, prey availability, and temperature) influence behavior, and behavioral avoidance of low oxygen concentrations may be context-dependent (Eby and Crowder, 2002; Woodley and Peterson, 2003). Aquatic organisms physiologically contend with the onset of hypoxia and the limitations to aerobic metabolism in one of two strategies. Aquatic organisms using the first strategy are called oxygen conformers. They decrease their oxygen consumption uniformly or linearly as their partial pressure of molecular oxygen (pO$_2$) decreases from the air-saturated value (Virani and Rees, 2000). Aquatic organisms using the second strategy are called oxygen regulators. They maintain relatively constant oxygen consumption levels even as the pO2 decreases over time until they reach a critical partial pressure of oxygen (P$_c$). At this point, oxygen regulators decrease their oxygen consumption with further drops in pO$_2$ (Virani and Rees, 2000). The estuarine fish, Fundulus grandis (bull minnow), has been shown use the oxygen conforming strategy, as determined by blood lactate levels under different levels of hypoxic stress (Virani and Rees, 2000). Both strategies involve physiological and biochemical adaptations to maintain aerobic metabolism and allow for a transition to anaerobic metabolism (glycolysis) during a hypoxic event, as with the Amazonian cichlids in the seasonally oscillating DO in the waters of the Amazon River (Chippari-Gomes et al., 2005).

Recent research has shown that hypoxia in estuaries impairs the reproductive system of aquatic organisms as much as any of the known endocrine disrupting chemicals. For example, chronic exposure to hypoxia has been shown to decrease serum
levels of testosterone, estradiol, and triiodothyronine in carp (*Cyprinus carpio*), which lead to retarded gonadal development in males and females, and reduced spawning success, sperm motility, fertilization success, hatching rate, and larval survival (Wu *et al*., 2003). Other research involving the Atlantic croaker (*Micropogonias undulatus*) demonstrated suppressed ovarian and testicular growth during chronic environmental hypoxia, with supporting lab studies showing that this endocrine disruption was associated with impairment of reproductive neuroendocrine function and decreases in hypothalamic serotonin (5-HT) content and the activity of the 5-HT biosynthetic enzyme, tryptophan hydroxylase (Thomas *et al*., 2007). Hypoxia reduced growth and reproduction in the estuarine gulf killfish (*Fundulus grandis*), with a 50% reduction in 17β-estradiol (E2) levels in females and 50% reduction in 11-ketotestosterone (11KT) levels in males (Landry *et al*., 2007).

**Hypoxia Inducible Factor (HIF)**

Multicellular aerobic organisms are dependent upon atmospheric oxygen for their survival, and they are affected or impacted by changes in O\textsubscript{2} concentration because it is a physiological stimulus. Animals maintain a narrow range of intracellular O\textsubscript{2} concentration to avoid detrimental effects of metabolic damage caused by a lack of O\textsubscript{2} from hypoxia or oxidative damage from excess O\textsubscript{2} from hyperoxia and potentially increase the level of reactive oxygen species (ROS) due to attenuation of cytochrome c oxidase and a build-up of electrons early in the electron transport chain (Semenza, 2001; Zagórska and Dulak, 2004). Varying oxygen concentrations present a fundamental physiological challenge that requires the coordinated regulation of extensive arrays of genes (Epstein *et al*., 2001). Higher eukaryotes have adopted specialized mechanisms for
oxygen homeostasis, and the conserved oxygen-dependent responsive pathways are expressed in almost every mammalian cell (Lee et al., 2004). Regulation of O\textsubscript{2} homeostasis, in terms of delivery and adaptation to low O\textsubscript{2}, for animals occurs via the hypoxia-inducible factor 1 or HIF-1 (Semenza, 2001), which plays a central role in both local and systemic responses to hypoxia (Epstein et al., 2001; Lee et al., 2004). The HIF-1 is a transcriptional complex that plays an essential role in cellular and systemic oxygen homeostasis (Lee et al., 2004).

As a master regulator of the hypoxia response, HIF-1 undergoes conformational changes in response to varying oxygen concentrations (Lee et al., 2004). HIF-1 is a αβ-heterodimer composed of two subunits, HIF-1α and HIF-1β (Lee et al., 2004; Pugh and Ratcliffe, 2003). HIF-1β subunit (also known as the aryl hydrocarbon receptor nuclear translocator or ARNT) is constitutively expressed, and the HIF-1α subunit is expressed and transcribed in precise regulation to cellular O\textsubscript{2} concentration (Semenza, 2001; Lee et al., 2004). The alpha (α) subunit of HIF-1 is the primary site of regulation for the activity of this protein, which includes protein stabilization, post-transcriptional modifications, nuclear translocation, dimerization, transcriptional activation, and interaction with other proteins (Zagórska and Dulak, 2004). HIF-1α appears to be correlated with HIF-1 activity, whereas HIF-1β is present in the nucleus regardless of oxygen levels (Zagórska and Dulak, 2004). Activation of the HIF-1 heterodimer triggers a cascade of target genes that become up- or down-regulated within the cells of the affected tissues (Zagorska and Dulak, 2004; Lee et al., 2004). Both the HIF-1α and the ARNT subunits of HIF-1 have variations in mRNA expression due to alternative splicing of exons (Zagórska and Dulak, 2004).
More than 60 proteins are induced by HIF-1, including vascular endothelial growth factor (VEGF) and erythropoietin. The processes known to be up-regulated by HIF-1α are the control of vascular system (angiogenesis and vasomotor control), maturation of red blood cells (erythropoiesis and iron transport), energy metabolism (glycolysis, glucose transport, and the multifunctional enzyme glyceraldehydes-3-phosphate dehydrogenase), cell proliferation and viability (arrest of cell cycle, apoptosis, and growth factors), pH regulation, nucleotide metabolism, matrix metabolism, catecholamine synthesis, and negative feedback regulation of HIF-1 transactivation (Zagórska and Dulak, 2004).

Both of the HIF-1 subunits have a basic helix-loop-helix (bHLH) domain that mediates dimerization and DNA binding (Semenza, 2001). A second dimerization motif is also found within the HIF-1 subunits, and it is called the PAS (PER-ARNT-SIM) domain based on its original identification in the PER (period circadian protein), ARNT (aryl hydrocarbon receptor nuclear translocator protein), and SIM (single-minded protein) proteins (Semenza, 2001; Lee et al., 2004). Each subunit contains two PAS domains, known as PAS-A and PAS-B (Lee et al., 2004). The bHLH and PAS domains of these HIF-1 transactivator are required for heterodimer formation between the α and β subunits and for DNA binding (Lee et al., 2004; D’Angelo et al., 2003). The PAS domain proteins are a superfamily, and the majority of these proteins are prokaryotic signal transduction molecules involved in responding to environmental stimuli such as light, O₂, concentration, and redox state (Semenza, 2001). Because PAS domains of several prokaryotic proteins bind prosthetic groups such as heme, it was initially thought that HIF-1 might be directly regulated by O₂, but that has not been found to be the case.
Instead the two folded PAS domains of HIF-α contain an empty cavity where a heme pocket is located for these other PAS domain proteins that use it as a signal sensor for O$_2$-regulated expression. The HIF-1 O$_2$ signal transduction for the regulation of HIF-1 levels is not well understood, and it is possibly connected to upstream O$_2$ binding hemoproteins or the generation of ROS (Semenza, 2001).

The O$_2$-regulated activity of HIF-1α is mediated by a functional domain of about 200 amino acids located C-terminal to the PAS domain (Semenza, 2001). The HIF-1α subunit contains TAD-N and TAD-C (N- and C-terminal transactivation domains, respectively) bridged by an inhibitory domain (Lee et al., 2004). The TAD-N is continuous with stability, and overlaps with the ODDD, and TAD-C interacts with coactivator such as p300/CBP (central integrating factor composed of p300 or E1A binding protein and CBP or CREB or cAMP-response element-binding protein) via a cysteine-histidine-rich domain of HIF-1α, independent of protein stability and is required for full HIF activity (Lee et al., 2004; Zagórska and Dulak, 2004). The p300/CBP-TAD complex recruits accessory coactivators like histone acetyltransferases SRC-1, TIF-2, and redox factor Ref-1 (Zagórska and Dulak, 2004). The p300/CBP interacts with C-TAD only when asparagine (Asn) is non-hydroxylated which enables the assembly of transcriptional coactivator complex, whereas the hydroxylation of Asn during normoxia silences TAD-C domain by preventing its interaction with p300/CBP (Zagórska and Dulak, 2004).

HIF-1 binds to a core pentanucleotide DNA sequence 5′-A/(G)CGTG-3′ within hypoxia response elements (HREs) as a heterodimer of basic helix-loop-helix PAS proteins designated HIF-1α and HIF-1β subunits (Epstein et al., 2001; Masson and
The HIF consensus binding site (HBS, HIF-1 binding site) is found within the hypoxia response element (HRE) (Takahashi et al., 2000; Zagórska and Dulak, 2004). HREs are located within either the promoter or enhancer regions, which are either 5’-flanking or 3’-flanking or intervening (Zagórska and Dulak, 2004). HIF-1 ancillary sequences (HAS) are found in most hypoxia-induced genes, which are located 8-9 nucleotides down- or up-stream of the HBS, and it is necessary for HIF-1-mediated transcription activation (Zagórska and Dulak, 2004). HAS is an imperfect inverted repeat of HBS, thus the secondary structure of HRE seems to be crucial for its activatory function, and the HAS recruits protein complexes distinct from HIF-1 (Zagórska and Dulak, 2004).

Assembly of an active HIF complex is a multi-step process involving regulated synthesis, processing and stabilization of HIF-α, nuclear localization, dimerization, and interaction with transcriptional coactivators (Masson and Ratcliffe, 2003) (Fig. 3). Frequently, efficient gene activation requires binding multiple HIF-1 (such as genes for glycolytic enzymes, glucose transporter 1, and transferrin) or binding additional transcriptional factors, which are not hypoxia-dependent (Zagórska and Dulak, 2004). Additionally, there is a binding site for ATF-1/CREB-1 factor (activating transcription factor-1/c-AMP-response element-binding protein-1) in the HRE of lactate dehydrogenase A gene, for AP-1 (activator protein-1) binding factor in VEGF, and the HNF-4 (orphan receptor hepatic nuclear factor-4) in the erythropoietin gene (Zagórska and Dulak, 2004). Additional transcription factors implies that hypoxic response amplification occurs in particular conditions, allows for varied response of different tissues, and enables diverse induction of distinct target genes (Zagórska and Dulak,
Additionally, studies of erythropoiesis and erythropoietin showed that induction of HIF and HIF-target genes by hypoxia is closely mimicked by exposure of cells to cobaltous ions and by exposure to specific iron chelators (Masson and Ratcliffe, 2003). Interestingly, HIF is also activated by growth factors, oncogenes and tumour suppressor mutations that promote cell survival or proliferation, thus effecting a potential link between the growth of metabolizing tissues and the provision of an oxygen supply (Masson and Ratcliffe, 2003).

Under normoxic conditions, HIF-1α is subject to ubiquitination and proteasomal degradation (Lee et al, 2004; Martin et al, 2005; Pugh and Ratcliffe, 2003; Semenza, 2001) (Fig. 3). Biochemical studies have shown that the von Hippel-Lindau (VHL) tumor-suppressor protein is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for degradation, and that interaction with VHL requires the O₂- and iron-dependent hydroxylation of proline residue 564 in HIF-1α by an enzymatic activity distinct from the known procollagen prolyl hydroxylases (Lee et al, 2004; Martin et al, 2005; Semenza, 2001; Zagórska and Dulak, 2004) (Fig. 3). Proline residues Pro-402 and Pro-564 of HIF-1α are constitutively hydroxylated under normoxic conditions, and this hydroxylation allows for the binding of the VHL protein (Huang et al, 2002) (Fig. 3). Additionally, ubiquitinated HIF-1α is degraded by 26S proteasome proteolysis (Zagórska and Dulak, 2004) (Fig. 3).

Other members of the HIF-1α family have been discovered with distinct gene loci. These multiple isoforms with different biological properties include: HIF-2α, also known as endothelial PAS protein (EPAS1), and HIF-3α (Masson and Ratcliffe, 2003; Zagórska and Dulak, 2004). These two HIF forms heterodimerize with members of the ARNT
Figure 3. HIF-1α dimerization with HIF-1β and its degradation via prolyl hydroxylation and ubiquitination when exposed to normoxia, hypoxia, and an EC. (HIF = Hypoxia Inducible Factor), (PHD3 = Prolyl Hydroxylase), (EDC = Endocrine Disrupting Chemical), (ODDD = Oxygen Dependent Degradation Domain), (P = HIF1-α Proline), (P300 = E1a binding protein), & (CBP = CREB binding protein). pVHL = von Hippel-Lindau tumor-suppressor protein is the recognition component of E3 = ubiquitin-protein ligase.
family, that include ARNT (HIF-1β), ARNT2, or ARNT3 (BMAL/MOP3) (Zagórska and Dulak, 2004). There is similarity between function, structure, and regulation of HIFs, but HIF-2α, HIF-3α, ARNT2, and ARNT-3 are tissue-specific with a more restricted pattern of expression (Semenza, 2000). High levels of HIF-1α and HIF-2α mRNAs were shown in the brain, heart, liver, and gonads, with lower levels found in muscle tissue, by northern blot analysis of the Atlantic croaker (*Micropogonias undulatus*) exposed to hypoxic conditions (Rahman and Thomas, 2007). The HIF family members, excluding HIF-1α, are thought to play specialized roles in the organisms, because of their tissue-specificity (Zagórska and Dulak, 2004).

Considerably less is known about the molecular responses of non-mammalian vertebrates and invertebrates to hypoxic exposure, and the physiological responses linking them to HIF are less well-developed (Nikinmaa and Rees, 2005). The diversity of fish presents many opportunities to evaluate if inter- and intra-specific variation in HIF structure and function correlate with hypoxia tolerance, while also offering an opportunity to examine the interactions between hypoxia and other stressors, including pollutants, common in aquatic environments (Nikinmaa and Rees, 2005). Adult zebrafish (*Danio rerio*) have been studied for long-term adaptive responses to hypoxia, and these studies have identified 367 out of 15,532 differentially expressed genes in the respiratory organs (the gills), using cDNA microarrays, of which 117 showed hypoxia-induced and 250 hypoxia-reduced expressions (van der Meer et al., 2005). Metabolic depression was indicated by repression of genes in the TCA cycle in the electron transport chain and of genes involved in protein biosynthesis, whereas enhanced expression of the monocarboxylate transporter and of the oxygen transporter myoglobin is indicated by
activation of genes in the TCA cycle and protein biosynthesis (van der Meer et al., 2005). Some cDNAs encoding HIF subunits from the estuarine fish *Fundulus heteroclitus* (mummichog) include a HIF-2α homolog and ARNT2alt, which is a splice variant of ARNT2 containing an additional exon encoding 16 amino acids near the amino terminus (Powell and Hahn, 2002). HIF-2α, ARNT2, and ARNT2alt mRNAs are expressed in all organs examined, and the HIF-2α combines with *Fundulus* ARNT2 splice variant or murine ARNT1 (Powell and Hahn, 2002). There is a 53-54% identity between HIF-2α and mammalian and avian orthologs, but the oxygen-dependent degradation domain (ODDD) exhibits substantial divergence from mammalian sequences and thus potentially important functional differences affecting its response to hypoxia (Powell and Hahn, 2002).

**Prolyl Hydroxylation**

Site-specific hydroxylation of the proline residues in HIF is catalyzed by a recently described family of enzymes, PHD1/HPH3/EGLN2/HIF-PH1, PHD2/HPH2/EGLN1/HIF-PH2, and PHD3/HPH1/EGLN3/HIF-PH3, which appear to have arisen by gene duplication and are represented by a single gene in the nematode *Caenorhabditis elegans* (Eg9) and the fruit fly *Drosophila melanogaster* (Fatiga) (Appelhoff et al., 2004; Aravind and Koonin, 2001; Berra et al., 2003; Huang et al., 2002; Metzen et al., 2002). The PHDs, or the prolyl hydroxylase domains, are the mammalian versions of these hydroxylation enzymes (Appelhoff et al., 2004; Berra et al., 2003). Hypoxia reduces the activity of PHDs that hydroxylate specific proline residues in the ODDD of hypoxia-inducible factor 1α (HIF-1α) (Berra et al., 2003; D’Angelo et al., 2003; Masson et al., 2001). The ODDD has been shown to have two independent
regions, and reinforces the role of prolyl hydroxylation as an oxygen-dependent
destruction signal (Masson et al., 2001) (Fig. 3). Since PHD activity is dependent on
oxygen and ferrous iron, HIF-1 mediates not only oxygen- but also iron-regulated
transcriptional gene expression (Martin et al., 2005). HIF-1 dependent promoter
activation via a hypoxia responsive element (HRE) also serves as a sensory system for
copper metabolism by induction of the plasma copper-binding transport protein
ceruloplasmin (a known HIF-1 target gene) in the presence of hypoxia and/or CuCl₂, as
shown in reporter gene assays (Martin et al., 2005). PHD1 was found exclusively within
the nucleus of osteosarcoma cells (U2OS) when using three-dimensional 2-photon
confocal fluorescence microscopy of fused hydroxylases with enhanced green fluorescent
protein (EGFP) within cultured osteosarcoma cells (U2OS), whereas PHD2 was mainly
located in the cytoplasm and PHD3 was homogenously distributed in cytoplasm and
nucleus (Metzen et al., 2002).

HIF hydroxylation is not an equilibrium reaction and the extent of modification at
a given oxygen concentration will also be affected by the quantity of available enzyme
(Masson and Ratcliffe, 2003). Prior exposure of cells to hypoxia enhances the HIF prolyl
hydroxylase activity found in cell extracts, and the rate of HIF-α degradation following a
return to normoxia (Masson and Ratcliffe, 2003). Small interfering RNA (siRNA)
techniques have shown a dominant role for PHD2 in controlling the low steady-state
levels of HIF-1α in normoxia in a range of cell types, with little or no observed effect
with PHD1 and PHD3 (Appelhoff et al., 2004; Berra et al., 2003), and PHD2 acting as the
critical oxygen sensor (Berra et al., 2003). However it is unclear whether this
predominance of PHD2 is related to a lack of precise knowledge of protein abundance or
because of the existence of tissue-specific expression patterns, as suggested by the analysis of mRNA expression for the PHDs (Appelhoff et al., 2004). Interestingly, estrogen has been shown to have the ability to induce PHD1 mRNA. Thus, the combination of hypoxia and estrogen exposure has the potential to alter the both the relative abundance of the PHDs and their relative contribution to the regulation of HIF within different cell types (Appelhoff et al., 2004).

Many prolyl hydroxylases (PHDs) enzymes have been found, with different functions in a variety of cells and tissues. Vertebrate prolyl 4-hydroxylases (PH4s) are $\alpha_2\beta_2$ tetramers in which the $\beta$-subunit is identical to the enzyme and chaperone protein disulfide isomerase (PDI) (Nissi et al., 2001). There are two PH4 families that catalyze the formation of 4-hydroxyproline by the hydroxylation of proline residues in peptide linkages (Hieta et al., 2003). One of these PHD4 (or PH4) families have homology to the collagen hydroxylases (C-P4Hs) that catalyze the hydroxylation of proline residues in collagen and to a larger family of iron-containing dioxygenases that catalyze a variety of hydroxylation reactions using both protein and non-protein substrates (Huang et al., 2002; Hieta et al., 2003). The other PHD4 (or PH4) family is the hypoxia-inducible factor (HIF) P4Hs, which are cytoplasmic enzymes that play a key role in the response of cells to hypoxia by catalyzing hydroxylation of the alpha ($\alpha$) subunit of HIF (Hieta et al., 2003). The C-P4Hs act on -Xaa-Pro-Gly- triplets in collagens and more than 15 other proteins with collagen-like sequences, whereas the HIF-P4Hs hydroxylate -Leu-Xaa-Xaa-Leu-Ala-Pro-Tyr- and –Leu-Xaa-Xaa-Leu-Ala-Pro-Ala- sequences (Hieta et al., 2003). There are two known isozymes of the collagen prolyl 4-hydroxylases (C-P4Hs), which are Type I and Type II. Prolyl hydroxylase actively catalyze the oxygen-
dependent hydroxylation of proline residue in procollagen, and it is up-regulated by hypoxia via HIF-1 transcription factor complex (Takahashi et al., 2000). The difference in these two isozymes is based on having a different isoforms of the alpha (α) subunit (Nissi et al., 2001). The two isoforms of the alpha (α) subunit do not appear to co-localize within a single molecule of prolyl 4-hydroxylase, and therefore do not produce a mixed tetramer form of the enzyme (Nissi et al., 2001).

Unlike the C-P4Hs, there is only one known isoform of the HIF-P4H α subunit. Therefore HIF-P4Hs appear to consist of only one type of monomer, the size of which ranges from 239 to 426 residues in the three human isoenzymes (Hieta et al., 2003). Molecular oxygen (O₂) and 2-oxoglutarate (2OG) are substrates, along with hydroxylacceptor proline residue, are utilized by these PHD dioxygenase enzymes (Huang et al., 2002; Masson et al., 2001), and iron acts as a co-factor (Berra et al., 2003). The stoichiometric decarboxylation of 2OG is coupled to hydroxylation of the proline substrate of these PHD enzymes (Huang et al., 2002). Proline hydroxylation occurs constitutively and promotes the VHL-mediated degradation of HIF under normoxic conditions, whereas this hydroxylation does not occur under hypoxic conditions, which allows for HIF to not be degraded (Huang et al., 2002). HIF-α is therefore part of a large set of cellular regulators whose activity is determined by tightly controlled proteolysis (Masson et al., 2001). Sometimes, phosphorylation of particular residues provides a specific recognition signal that targets the substrate to ubiquitin ligase complexes (Masson et al., 2001). Some enzymes of this class require ascorbate for full catalytic activity, providing an alternative oxygen acceptor in uncoupled decarboxylation cycles (Huang et al., 2002; Masson and Ratcliffe, 2003).
Mutagenesis studies have shown that PHDs require only a short stretch of HIF amino acids for selective recognition, with the HIF-1α peptides being as short as 20 residues (Huang et al., 2002). These short peptides support both site-specific proline hydroxylation with subsequent binding to VHL, and the sites of the proline hydroxylation (within the ODDD) occur within an LXXLAP (where X means any amino acid and P indicates the hydroxylacceptor proline) sequence motif that is strongly conserved between the two hydroxylation target sites of HIF-1α and HIF-2α and between HIF isoforms from different species (Berra et al., 2002; Huang et al., 2002). Research using COS-1 cells indicates that PHD2 has the highest specific activity toward primary hydroxylation site of HIF-1α (Huang et al., 2002). Mutations within the LXXLAP motif that still maintain its functionality can occur at the 5-, 2-, and 1- positions, relative to proline, which indicates that only the hydroxylacceptor proline is strictly required (Huang et al., 2002).

Oxygen-regulated transcription, via the use of HIF, occurs with at least two distinct types of 2-oxoglutarate-dependent oxygenases, which includes prolyl hydroxylase and asparaginyl hydroxylase in a dual regulation (Masson and Ratcliffe, 2003; Pugh and Ratcliffe, 2003). Prolyl hydroxylases are within the highly conserved portion of the 2-oxoglutarate-Fe(II) dioxygenase superfamily of proteins, or the EGL-9 family (animals and pathogenic proteobacteria). These enzymes share a region or domain of specific extended conservation amino terminal to the core double-stranded β-helix (DSBH) fold containing a HX[DE] dyad (where X is any amino acid) and a conserved carboxyterminal histidine, which together chelate a single iron atom (Aravind and Koonin, 2001). The closest relatives of the EGL-9 family are the proline
hydroxylases with which they share a region of specific extended conservation amino terminal to the core DSBH domain (Aravind and Koonin, 2001). This relationship, along with the combination to the intracellular MYND domain and the lack of signal peptides, suggests that the EGL-9 family proteins are prolyl hydroxylases that modify intracellular proteins, unlike the classic prolyl hydroxylases that have been implicated primarily in the modification of collagens in the endoplasmic lumen (Aravind and Koonin, 2001). The common involvement of prolyl and asparaginyl hydroxylation by distinct Fe(II)- and 2-OG-dependent oxygenases in different modes of HIF regulation suggests that such enzymes may be well suited to a role in cellular oxygen sensing (Aravind and Koonin, 2001; Masson and Ratcliffe, 2003). PHD2 and PHD3 are up-regulated by hypoxia, providing an HIF-1-dependent auto-regulatory feedback mechanism, via the HIF-1α subunit, driven by the oxygen tension (Berra et al., 2003; Marxsen et al., 2004). The interesting aspect of the EGL-9 family is its presence in *Vibrio cholerae* and *Pseudomonas aeruginosa*, which have apparently acquired these genes by horizontal transfer from eukaryotes (Aravind and Koonin, 2001). There is a possibility that the bacterial EGL-9-like proteins modify host proteins in a manner that favors the survival and spread of the pathogen, which might be especially pertinent if the host down-regulates the endogenous ortholog in response to the infection (Aravind and Koonin, 2001).

Interaction between Hypoxia and Estrogenic Chemicals (ECs)

Since the early 1980s, scientific data have shown that man-made chemicals released in the environment have adverse effects on endocrine system of humans and wildlife (Cooper and Kavlock, 1997). Some endocrine-disrupting chemicals (EDCs)
affect the endocrine system because of their ability to mimic natural estrogen, whereas others may function as an antiestrogen.

Estrogen contamination of waterways is a concern because low concentrations (10–100 ng L\(^{-1}\)) of these chemicals in water can adversely affect the reproductive biology of fish, turtles, and frogs by disrupting the normal function of their endocrine systems (Hanselman et al., 2004). Estrogen (20 ng/ml estradiol) has been shown to have the ability to induce PHD1 protein in the BT-474 cell line (Appelhoff et al., 2004), and the combination of hypoxia and estrogen exposure has the potential to alter both the relative abundance of PHDs and their relative contribution to regulation of HIF within different cell types. By contrast, prolyl hydroxylase enzymes PHD2 and PHD3 did not show any up-regulation from estrogenic (20 ng/ml estradiol) exposure (Appelhoff et al., 2004).

Estrogen up-regulates HIF-1α in rat uterus resulting in up-regulation of VEGF (Kazi and Koos, 2007) and potentiates the induction of EPO mRNA in the oviduct of mice (Masuda et al., 2000). Studies on breast cancer lines (MCF-7 and HEK293 cells) have demonstrated that combined estrogen (10 nM E2) and hypoxia up-regulates ERα transcriptional activity in a synergistic manner, while also degrading ERα protein in a synergistic and proteasome-dependent manner (Jinhyung et al., 2009). Similarly, hypoxia induces proteasomal degradation of ER (Stoner et al., 2002).

Along with recruitment of both ERα and HIF-1α to the VEGF (vascular endothelial growth factor) gene promoter (Kazi et al., 2005; Kimbro and Simons, 2006), estradiol has been shown to induce a later or delayed increase (2-4 hrs versus 0-1 hr of exposure) in HIF-1α mRNA and protein expression in the rat uterus versus HIF-1β mRNA and protein, which suggests a possible need for it in the uterus for longer term
The increased protein expression of HIF-1α in the rat uterus appears to be, at least in part, based on the increasing levels of transcription from 0-4 hrs (Kazi et al., 2005). Research has demonstrated a possible link or communication between hypoxia and the estrogen receptor 1 (ESR1) protein and key proteins in the hypoxic response of rat lung (Wu et al., 2008). Other research showed synergistic effects of estrogen and hypoxia on ERα-mediated transactivation in breast cancer cells (Jinhyung et al., 2009). Taken together, all these studies show that estrogen affects transcription of HIF-1α, HIF-1 target genes, and PHDs, and that hypoxia affects ERα mRNA and protein, clearly demonstrating the possibility of cross-talk between the hypoxic and estrogenic pathways.

ECs (foreign or man-made estrogens), in a manner similar to estradiol (E2), can activate E2-regulated genes by forming a complex with the ER (Yamamoto, 1985). DNA binding of this complex activates expression of specific target genes or gene networks implicated in growth and differentiation of female reproductive tissues (Flouriot et al., 1996), including transcription of the ER-encoding gene (autoregulation) (Flouriot et al., 1996; Pakdel et al., 1991) and the vitellogenin (VTG)-encoding gene in fish, amphibians, reptiles, and birds (Flouriot et al., 1995, 1997).

Environmental estrogens have affinity for the ER of 0.02 to 0.0001 that of the natural hormone E2 (Arnold and McLauchlin, 1996). Concern is that adult animals can bioaccumulate (1,000–3,000 times) these chemicals (Ekelund et al., 1990) and that exposure could occur at a critical time in the organism’s development (Gillesby and Zacharewski, 1998). Because of the bioaccumulation potential of many of these chemicals, long-term EDC exposure at low concentrations could adversely affect an
organism, influence the success of future progeny, and lead to changes in population levels (Daston et al., 1997).

Sewage treatment plants can release large amounts of estrogenic chemicals in aquatic environments in the form of alkylphenols. These are products of microbial breakdown of alkylphenol-polyethoxylates (APEs), which are widely employed as industrial and household nonionic surfactants. More than 300 million kilograms of APEs are produced annually (Talmadge, 1994). Following sewage treatment, about 60% of the APEs are released into the aquatic environment as short-chain APEs, including nonylphenol and octylphenol. The alkylphenol, 4-tert-octylphenol (4tOP), was found to be about 5-20 times more estrogenic than 4-nonylphenol and between 100 and 10 000 times less estrogenic than estradiol-17β in the in vitro systems employed (Soto et al., 1992; Jobling and Sumpter, 1993; White et al., 1994), causing the feminization of male *Cyprinodon variegatus* with the presence of VTG in the blood (Karels et al., 2003) via downstream gene activation of its induced ERα receptor (Karels and Brouwer, 2003). Alkylphenols, in turn, also have been shown to be slowly biodegradable (Gaffney, 1976; Sundaram and Szeto, 1981). These chemicals have a strong tendency to bioconcentrate (Ekelund et al., 1990), bind to the estrogen receptor of fish and mammals (Flouriot et al., 1995; White et al., 1994), induce transcriptional activation of estrogen-responsive genes, produce detectable VTG in fish hepatocyte cell cultures, and produce VTG in male rainbow trout (*Oncorhynchus mykiss*) at concentrations of 4.8 μg/L (White et al., 1994; Jobling and Sumpter, 1993; Ren and Lech, 1996; Jobling et al., 1996). Of the alkylphenols examined, 4tOP appears the most biologically active. Rainbow trout exposed to 30 μg/L of 4tOP show reduction in testicular growth (Jobling et al., 1996),
and male Japanese medaka (*Oryzias latipes*) exposed to 50 to 100 μg/L nonylphenol developed true oocytes in the testes (Gray and Metcalfe, 1997).

Vitellogenin is an egg yolk precursor protein synthesized in the liver, transported in the blood, and taken up by growing oocytes during vitellogenesis in fish, amphibians, reptiles, and birds (Tyler *et al.*, 1996), and it is used as a food supply for the embryo and larval stages of fish. The production of VTG is estrogen dependent; therefore, it is normally found in significant concentrations only in females (Tyler *et al.*, 1996). The presence of elevated levels of VTG in males is therefore a good indication of estrogenic chemicals in the environment, and VTG expression may be interpreted as a warning of reproductive consequences (Cheek *et al.*, 2001). Laboratory studies have shown that VTG in plasma of male Japanese medaka (*Oryzias latipes*) exposed to 4tOP is correlated to reproductive impairment (Gronen *et al.*, 1999). Exposure to estrogens can lead to feminization of male fish, as indicated by VTG in their blood, and interfere with sperm production and thus reproduction.

There appears to be an induction/activation of at least some PHD genes by estrogen, and possibly by estrogen-mimic pollutants, with possible cross-talk between estrogenic and hypoxia pathways, using ERE and HRE DNA segments, respectively, for downstream activation of target genes. 4tOP, an environmental estrogen shown to affect VTG production and spermatogenesis in male sheepshead minnows (Karels *et al.*, 2003), will be used as the compound for the estrogenic exposure study.

**Fish Model**

Sheepshead minnow (*Cyprinodon variegatus*) is a small teleost fish (2-4 g) that is an obligate resident in estuaries along most of the east coast of the United States and the
northern Gulf of Mexico, from Florida to Texas (Fig. 4). *Cyprinodon variegatus* was used as the model fish for the hypoxia experiments (exposures) of my dissertation. Because this fish species is estuarine, it is evolutionarily adapted to survive severe and rapid changes or fluctuations in hydrological parameters, including rapidly declining oxygen levels during hypoxia.

![Image of Cyprinodon variegatus](image)

*Figure 4. Cyprinodon variegatus* (sheepshead minnow) is a small teleost fish. These fish are ~2-4 g in weight, 4-5 cm in length, and indigenous to estuaries of coastal Mississippi.

**Research Objectives**

Cellular and physiological mechanisms involved in HIF gene activation and the cascade of events that follow the initiation of hypoxia are well understood in mammals, but they are not as well understood in fish. Additionally, it is even less understood how this cascade of events occurs when compounded by the addition of an endocrine disrupting chemical 4-tert-octylphenol (4tOP). My dissertation focused on following the
transcriptional expression of several genes, whose transcription is hypothesized to be altered in response to exposure to hypoxia followed by a shift back to normoxia. The genes I have chosen for this study include hypoxia inducible factors HIF-1α, HIF-2α, prolyl hydroxylase 3 enzyme PHD3, erythropoietin EPO, and vitellogenin VTG. HIF genes (such as HIF-1α and HIF-2α) are upstream genes directly impacted by the presence of hypoxia, HIF-1α and HIF-2α, and they are the trigger for activation of a multitude of genes (downstream effect) which includes the EPO gene (indirect measure of HIF activation). The activation of EPO demonstrates a longer-term physiological activation or change within the cells, and thus an initiation of a physiological response beginning in the tissues and organs of the body. The research involved chronic (long-term) or acute (short-term) exposure of male and/or female adult sheepshead minnow (C. variegatus) to hypoxia (both moderate and severe) and/or 4tOP (additive or synergistic effects) to obtain liver and testes for isolation of RNA (total RNA extraction) for the preparation of cDNA. The prepared cDNA was used for the isolation, cloning, and sequencing of these genes of interest. All cDNA samples were tested for gene expression levels via the use of real-time PCR. Additionally, microarray analyses were done for large-scale gene expression profiling under the various exposure treatment conditions of hypoxia and 4tOP.

My dissertation studies focused on the up- and down-regulation of HIF-1α, HIF-2α, PHD3, EPO, and VTG, along with the differential expression of these genes induced by the environmental stressor of hypoxia and/or 4tOP under laboratory conditions, with the expression levels measured and compared across experiments and over time. Results of my microarray research were interfaced with the results of a previously prepared cDNA library of C. variegatus (within this lab) under hypoxic and normoxic states and
used suppressive subtractive hybridization (SSH), to identify genes differentially expressed.

The specific research objectives that needed to be accomplished for this project include: (1) Obtain partial nucleotide sequences of three genes of interest from liver samples of *Cyprinodon variegatus*, which are cypHIF-1α, cypHIF-2α, and PHD3; (2) PCR, cloning, and sequencing for use in qPCR primer design; (3) Design qPCR primers for four genes of interest, which include: HIF-1α, HIF-2α, PHD3, EPO (sequence and primers already known), and VTG, for which highly conserved & primers can be designed from known species; (4) Run PHD3 gene expression from moderate chronic hypoxia exposure (~2.5 mg/L) and from severe chronic hypoxia exposure (~1.5 mg/L); (5) Run a severe acute hypoxia exposure (~1.5 mg/L) with transition back to normoxia; (6) Run a four-part exposure with adult *C. variegatus* based using two stress factors of dissolved oxygen (DO) and the endocrine disrupting chemical (4tOP). The hypoxia is a very severe acute hypoxia exposure (~1.0 mg/L). Each treatment factor had two levels, to assess gene expression in the five target genes from liver samples: DO—normoxia vs hypoxia, Treatment—Control versus 4tOP; (7) For each exposure, assess gene expression in one or all of five target genes using real-time PCR; (8) Assess overall gene expression of all known genes based on the use of microarrays and comparing it with a cDNA library of known genes of *C. variegatus*

Objectives and Hypotheses

1. Phylogenetic Relationships of the Isoforms of the HIF-α and PHD Protein Families

HIF-1α and HIF-2α nucleotide and amino acid sequences are the most closely related HIF-α isoform in the HIF-α family, and they need to be distinguished from each
other. PHD3 isoform is very closely related to the PHD1 and PHD2 isoforms of the PHD superfamily, and they need to be distinguished from each other.

Hypothesis 1: Phylogenetic analyses can be used to identify the cloned HIF and PHD isoforms.

2. Effects of Hypoxia vs Normoxia on Adult Cyprinodon Variegatus

HIF-1α and HIF-2α mRNAs levels are scaled up during onset of hypoxia, along with the downstream target gene EPO, to adjust to the physiological shift from aerobic to anaerobic metabolism (Fig. 5). Decreasing dissolved oxygen levels to ~1.5 mg/L O₂ (hypoxia) will trigger up-regulation of PHD3 mRNA levels (Fig. 5). PHD3 mRNA is transcribed in advance of the next period of normoxia, in order to quickly translate more PHD3 enzyme for the hydroxylation of HIF proteins during the period of normoxia. Additionally, hypoxia acts like an EDC and inhibits transcription of ERα and thus mRNA levels of ERα, and this is followed by inhibition of target gene VTG mRNA (Fig. 5).

Hypothesis 2: Hypoxia will enhance transcription of HIF-1α, HIF-2α, EPO and PHD3 and represss transcription of VTG.

3. Effects of 4tOP on Adult Cyprinodon Varietatus

Because 4tOP acts as an estrogen mimic, mRNA levels followed by protein levels of ERα will increase, thus activating the downstream VTG (target gene) by increasing transcription (Fig. 5). Increasing 4tOP levels will trigger up-regulation of HIF-1α and HIF-2α transcription factor mRNA levelss to increase vascularization, and thus will increase EPO mRNA transcription levels (Fig. 5).

Hypothesis 3: 4tOP will enhance of VTG, HIF-1α, HIF-2α and EPO.
4. Effects of Combination (Hypoxia and 4tOP) on Adult Cyprinodon Variegatus

Combining 4tOP (estrogen mimic) and hypoxia (EDC-mimic) will result in the up-regulation of HIF-1α, HIF-2α, and EPO transcription levels (Fig. 5). Based on the literature, I also postulated 4tOP will activate PHD3 transcription of mRNA, which will cause some HIF-1α and HIF-2α hydroxylation, as hypoxia inhibits PHD activity (Fig. 5). Overall, the anaerobic state from severe hypoxia should be the dictating factor, causing deactivation of any PHD enzyme activity, and an accumulation of HIF-1α and HIF-2α mRNA, along with an accumulation of EPO mRNA. The combined effects of hypoxia and 4tOP should give intermediate results for VTG mRNAs, because the gonads are being hormonally supported by 4tOP and adversely affected by hypoxia (EDC-like).

Hypothesis 4: 4tOP + hypoxia will enhance transcription of PHD3, HIF-1α, HIF-2α and EPO relative to hypoxia exposure and 4tOP exposure; 4tOP will enhance VTG transcription relative to its estrogenic biochemical properties.

Hypothesis 5: Microarray analysis will help to identify metabolic pathways altered by the transition from hypoxia to normoxia.
Figure 5. Hypotheses of mRNA and expression levels of HIF-1α, HIF-2α, PHD3, EPO and VTG based on the presence or absence of hypoxia and/or 4-tert-octylphenol (4tOP). Upon hypoxic release, HIF-1α, HIF-2α, and PHD3 recover relatively rapidly (hours to days) when returning to normoxia (reference state).
CHAPTER II
CLONING, SEQUENCING, AND PHYLOGENY OF HIF-1α, HIF-2α, AND PHD3

Abstract

Partial nucleotide sequences of hypoxia inducible factor alphas HIF-1α, HIF-2α, and prolyl hydroxylase PHD3 were isolated, using degenerate and kit primers to create gene specific primers to isolate these sequences from total RNA liver extracts of sheepshead minnow (Cyprinodon variegatus) to assess differentiation in gene expression profiles of hypoxia and treatment (4tOP) exposed fish. Phylogenetic analyses was needed for each of the three amino acid sequences derived from the isolated and sequenced nucleotide sequences in order to confirm that the sequences being transcriptionally monitored were indeed the correct genes, and more specifically the correct isoform within these two large families of proteins. The large nucleotide sequences of HIF-1α, HIF-2α, and PHD3 that were isolated from C. variegatus were cloned and sequenced in order to design primers of a small internal sequence for future use in gene expression profiling via real-time PCR. Phylogenetic analyses comparing the isolated sequences to known sequences from NCBI using the software PHYLIP confirmed that two different HIF isoforms were identified (HIF-1α and HIF-2α) and the isoform PHD3. ClustalW comparisons of the these NCBI sequences to these identified sequences also identified the portion of the entire sequences identified, and thus allowed discovery of the protein domains (PAS-A, PAS-B, PAC, and ODDD in the HIF-α isoforms and 2OG Fe(II) Oxy Superfamily domain in PHD3 present with determination of how conserved these regions were within C. variegatus.
Introduction

The alpha (α) subunit of HIF-1 is the primary site of regulation for the activity of this protein, which includes protein stabilization, post-transcriptional modifications, nuclear translocation, dimerization, transcriptional activation, and interaction with other proteins (Zagórska and Dulak, 2004). HIF-1α appears to be correlated with HIF-1 activity, whereas HIF-1β is present in the nucleus regardless of oxygen levels (Zagórska and Dulak, 2004). Activation of the HIF-1 heterodimer triggers a cascade of target genes that become up- or down-regulated within the cells of the affected tissues (Zagorska and Dulak, 2004; Lee et al., 2004).

Other members of the HIF-1α family have been discovered with distinct gene loci. Functional activity of these HIF-α isoforms (gene duplication) are very similar, but they are active in different organs of the body or in different vertebrate and invertebrate species. These additional HIFs (multiple isoforms with different biological properties) include: HIF-2α, also known as endothelial PAS protein (EPAS1), and HIF-3α (Masson and Ratcliffe, 2003; Zagórska and Dulak, 2004). These two HIF forms heterodimerize with members of the ARNT family, which include ARNT (HIF-1β), ARNT2, or ARNT3 (BMAL/MOP3) (Zagórska and Dulak, 2004). There is similarity between function, structure, and regulation of HIFs, but HIF-2α, HIF-3α, ARNT2, and ARNT-3 are tissue-specific with a more restricted pattern of expression (Semenza, 2000). Additionally, there is also a HIF-4α isoform, which is even more specialized in its use in certain organs of the body during hypoxia. The HIF family members, excluding HIF-1α, are thought to play specialized roles in the organisms, because of their tissue-specificity (Zagórska and Dulak, 2004). High levels of HIF-1α and HIF-2α mRNAs were shown in the brain,
heart, liver, and gonads, with lower levels found in muscle tissue, by northern blot
analysis of the Atlantic croaker (*Micropogonias undulatus*) exposed to hypoxic
conditions (Rahman and Thomas, 2007).

Site-specific hydroxylation of the proline residues in hypoxia-inducible factor, or
HIF, is catalyzed by a recently described family of enzymes, PHD1 (also known as
HPH3/EGLN2/HIF-PH1), PHD2 (also known as HPH2/EGLN1/HIF-PH2), and PHD3
(also known as HPH1/EGLN3/HIF-PH3), which appear to have arisen by gene
duplication and are represented by a single gene (gene duplication) in *Caenorhabditis
elegans* (Egl9) and *Drosophila melanogaster* (Fatiga) (Appelhoff et al, 2004; Aravind
and Koonin, 2001; Berra et al, 2003; Huang et al, 2002; Metzen et al, 2002). The PHDs,
or the prolyl hydroxylase domains, are the mammalian versions of these hydroxylation
enzymes (Appelhoff et al, 2004; Berra et al, 2003). Hypoxia reduces the activity of
PHDs that hydroxylate specific proline residues in the oxygen-dependent degradation
domain (ODDD) of hypoxia-inducible factor 1α (HIF-1α) (Berra et al, 2003; D’Angelo et
al, 2003; Masson et al, 2001). The ODDD has been shown to have two independent
regions, and reinforces the role of prolyl hydroxylation as an oxygen-dependent
destruction signal (Masson et al, 2001).

Three genes, hypoxia inducible factor alphas 1 and 2 (HIF-1α and HIF-2α) and
prolyl hydroxylase 3 (PHD3), that are involved in the physiological responses of fish to
adjust to hypoxia were chosen to be identified, cloned, and sequenced. These sequences
were used to design gene-specific primers for real-time PCR analyses to measure the
level of changes in transcriptional responses to hypoxia and 4tOP over time.

Phylogenetic analyses was done for each of the three amino acid sequences derived from
the nucleotide sequences in order to confirm that the genes being transcriptionally monitored were indeed the correct gene, and more specifically the correct isoform within these two large families of proteins.

Materials and Methods

Preparation of cDNA

First-strand cDNA synthesis (Invitrogen protocol) was used to convert RNA into single-stranded cDNA: (1) combined 2 µg of total RNA with 2 µl of 50 ng/µl random hexamers, 1 µl of 10 mM dNTP mix, and diethylpyrocarbonate (DEPC)-treated or nuclease-free water up to 10 µl, (2) incubated samples at 65°C for 5 minutes, (3) incubated on ice for at least 1 minute, (4) created a mastermix containing 2 µl of 10X reverse transcription (RT) Buffer, 4 µl of 25 mM MgCl₂, 2 µl of 0.1 M DTT, and 1 µl of RNaseOUT for each sample and then added 9 µl of this reaction mix to each RNA mixture, (5) incubated the full RNA mixture for 2 minutes, (6) added 1 µl (50 units) of SuperScript II RT, (7) incubated at 25°C for 10 minutes, (8) transferred the tubes to 42°C incubator or heating well plate and incubate for 50 minutes, (9) terminated the reactions at 70°C for 15 minutes and chill on ice, (10) collected the reactions by brief centrifugation, (11) added 1 µl of RNase H to each sample tube and incubated for 20 minutes at 37°C, (12) and these tubes were either kept on ice for short-term storage or placed at -20°C.

Second-strand cDNA was prepared from the first-strand cDNA by combining 20 µl of each sample with 80 µl of a reaction mastermix that contained 8 µl of 10X Reaction Buffer for DNA Polymerase I, 0.2 µl (1 unit) of RNase H, E coli, 3 µl (30 units) DNA
Polymerase I, E coli (10,000 units/ml), and 68.8 µl of nuclease-free water to 100 µl per reaction.

RNA Extractions

Several adult *Cyprinodon variegatus* fish were sampled from culture, and they were euthanized with Tricaine Methanesulfonate (MS-222) at a dosage of ~80 mg/L for ~1 minute, cervical dislocation, or brain pithing. These fish were dissected for liver for RNA extraction to obtain total RNA. A sample of liver or testes (40 mg) was placed into 1.5 ml centrifuge tubes with 750 µl of RNA STAT-60 (Tel-Test, Inc) if processed directly or placed in 3:1 volume:mass ratio of RNA later (Ambion) and kept at -20°C. Later the sample was thawed and placed in RNA STAT-60, homogenized with a battery-powered centrifuge tube (1.5 ml) homogenizer, centrifuged at 2700 X g for 5 minutes at 4°C, and the homogenate (minus large pieces of tissue) was transferred to clean sterile 1.5 ml centrifuge tubes with 150 µl of phenol:chloroform. The homogenate was vortexed for 15 seconds and incubated at room temperature for 2-3 min, centrifuged at 12,000 X g for 15 min at 4°C, producing (1) a lower red phenol:chloroform phase (protein), (2) a middle white interface (DNA), and (3) a upper aqueous phase (RNA). The aqueous phases were pipetted off and placed into a new 1.5 ml centrifuge tube containing 750 µl RNA STAT-60 and 150 µl chloroform, vortexed for 15 seconds, incubated for 2-3 min, and centrifuged at 12,000 X g for 15 minutes at 4°C. The aqueous phases were added to 750 µl of isopropanol in 1.5 ml centrifuge tubes, the tubes were inverted to mix them and incubated for 2 hrs to overnight at 20°C to form a white RNA precipitate. The supernatant was carefully discarded and the pellets were washed in 750 µl of 75% ethanol, flicking off the pellet from the bottom of the tube to allow it float free in
solution. These pellets were centrifuged at 7,500 X g for 5 minutes at 4°C, and the supernatant was carefully discarded. The pellets were air-dried for ~10 min at room temperature and resuspended in 25-50 µl of RNA Storage Solution (Ambion). These resuspended RNA samples were put through an Ambion DNA-free treatment by adding 0.1 volume of 10X DNase I Buffer and 1 µl TURBO DNase I, mixed, and incubated at 37°C for 30 minutes. A second aliquot of 1 µl TURBO DNase I was added to each RNA sample, mixed, and incubated for 30 minutes. Afterwards, 0.1 volume or 2 µl of DNase Inactivation Reagent was added to the sample and mixed, incubated for 2 minutes at room temperature, and centrifuged at room temperature at 20,817 X g for 2 minutes. The RNA solution was placed into a clean sterile centrifuge tube with 0.1 volumes of 3M NaOAc and 3 volumes of 100% EtOH, mixed, and incubated at -20°C for a minimum of 2 hrs to overnight. These solutions were centrifuged at 12,000 X g for 1 hr at 4°C. The supernatant was carefully poured off, the pellets were washed in 750 µl of 75% ethanol (twice) and flicked free into solution, and then centrifuged at 7,500 X g for 5 minutes at 4°C. After carefully pouring off the supernatant, the samples were air-dried for 10 minutes and resuspended in 25-50 µl of RNA Storage Solution (Ambion).

Sample quality (rRNA ratios of 28S/18S) was analyzed using the Agilent 2100 Bioanalyzer (Firmware Version A.01.16) and RNA 6000 Nano Chip kit (Agilent, Palo Alto, CA) to determine the extent of DNA contamination and RNA degradation. Sample quantity/purity of the RNA samples were assessed using the ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) for a 260/280nm ratio of less than 1.8 (relatively protein-free). All total RNA samples were stored at -80°C until use.
**Gene Cloning and Sequencing**

1: Isolation and purification of total RNA. 2: RNA → cDNA 3: amplification cDNA-primary PCR followed by secondary PCR. 4. Gel purification 5: cloning and sequencing

Isolated and purified total RNA from RNA extraction was reversed transcribed into cDNA using first strand cDNA synthesis (Invitrogen). This product was then made double-stranded via second strand cDNA synthesis (Invitrogen). Degenerate primers for cDNA amplification were chosen by assessing known nucleotide sequences of the genes of interest from other species, based upon sequence data from NCBI. The species sequences were aligned via ClustalW to isolate the most conserved regions of these genes to use a starting point to create these primers via Primer 3 and the Beacon Designer program for qRT-PCR. A partial prolyl hydroxylase EST sequence, isolated from previously completed cDNA *C. variegatus* suppression subtraction hybridization libraries and identified within GenBank using NCBI’s basic local alignment search tool BLASTX (http://ncbi.nih.gov/BLAST/), was also used in primer design. These primers were used to assess the presence of the correct size of the amplicon of the gene in question via amplification.

The large nucleotide sequences of HIF-1α, HIF-2α, and PHD3 that were isolated from *C. variegatus* were cloned and sequenced in order to design forward and reverse primers of a small internal sequence (~150 – 400 base pairs) for use in gene expression profiling via real-time PCR. The longer an amplicon, or sequence fragment, of a gene of interest used in real-time PCR, the longer a period of time (30 seconds – 2 minutes)
needed to guarantee replication, and thus amplification, of this sequence during the polymerase chain reaction (PCR) using the iCycler thermocycler.

In order for a pair of forward and reverse primers to be successful for cloning purposes, they have to (1) be unique for the gene of interest and (2) reflect the highly conserved regions or domains of these transcriptional proteins and enzymes. Thus, there was a need for utilizing a unique sequence within a conserved region of a protein to prevent cloning of unwanted proteins or isoforms of this same family of proteins. Use of a specific primer set, along with the presence of only one amplicon within the melting curve at the end of the PCR (each double-stranded cDNA isoform melts or comes apart with increasing temperature), ensured that only one isoform was being copied or amplified. A list of the sequences of the forward and reverse primers for each of the three genes (PHD3, HIF-1α, and HIF-2α) isolated and sequenced is shown in Table 1.

The PCR parameters for each of the two pairs of forward and reverse primers set up for 40 cycles on Perkin Elmer thermocyclers were as follows: pre-PCR of 2 min at 95°C; denature for 1 min at 95°C; anneal for 1 min at 54°C for HIF-1α and HIF-2α and 56°C for PHD3; extend for 2 min at 72°C; and a final 10-min extension at 72°C.

The 5’ RACE (Rapid Amplification of cDNA Ends) kit (Invitrogen Life Technologies) was used to obtain the entire open-reading frame of the prolyl hydroxylase cDNA fragment isolated from the subtractive hybridization. A degenerate kit primer for the 5’ end and a non-degenerate (specific) primer for the 3’ end from a conserved portion of the original cDNA fragment was used to isolate and amplify the entire open-reading frame of this sequence via PCR using a Gene Amp PCR System 2400 (Perkin Elmer and Applied Biosystems). After PCR amplification and 1% agarose gel electrophoresis to
Table 1

**Degenerate, Kit, and Gene-Specific Primers for Sequence Identification.**

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>HIF-1α</td>
<td>5’-GTCAAGGAACCGAGCACAGAGCGG-3’</td>
<td>5’- CGTNTGTGNNAGCNCTCCNNTANGG-3’</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>5’-GAGGATGAAATGCACGCTGAC-3’</td>
<td>5’-TGAGCTGTAGTAGAACCTCGG-3’</td>
</tr>
<tr>
<td>HIF-2a</td>
<td>5’-NGANCTGNAGTAANCACNNNGGC-3’</td>
<td>5’-CTCCCTGGAGCAGACGGAGGCCATG-3’</td>
</tr>
<tr>
<td>HIF-2a</td>
<td>5’-GACCGCTACCTGCTGGCCCTTGTTGTTG-3’</td>
<td>5’-CAACAGAGGCGCAGCTGTTAACCTC-3’</td>
</tr>
<tr>
<td>PHD3</td>
<td>5’-ACGCTATCAGGTTTGGATTTTGAC-3’</td>
<td>5’-GCAGGNACANAANNCAATGNAATCNG-3’</td>
</tr>
<tr>
<td>Invitrogen 5’ RACE Kit Primer (Abridged Universal Amplification Primer—AUAP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD3</td>
<td>Reverse primer</td>
<td>5’- GGCTCGCTCCTCAAGAGGCATAAAAATACC-3’</td>
</tr>
</tbody>
</table>

Separate out the appropriate PCR product, the isolated cDNA segments were located via low energy UV light, cut out of the 1% agarose gel, gel-purified using the QIAquick gel extraction kit (Quiagen, USA), and these PCR products were quantified with a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan). The double-stranded (ds) cDNA was cloned with the Promega pGEM T-Easy Vector System according to manufacturer’s instructions. Plasmids from transformed JM109 *E. coli* cells were purified using the Promega Wizard Plus Minipreps DNA purification system, and they were screened for inserts by agarose gel electrophoresis after a 3-hr, 37°C restriction digestion using EcoRI. Plasmids with the correct size of the entire sequence of PHD3 obtained from 5’ RACE and cloning were sequenced on an automated sequencer, using the Sanger method (Sanger et al., 1977), by the DNA Sequencing Center at the University of Maine.
HIF-1α and HIF-2α, prepared in the same manner, were sequenced in our lab using a Beckman Coulter CEQ 8000 Genetic Analysis System. These sequences were put into Blast searches using NCBI’s basic local alignment tool search tool (BLASTX, http://ncbi.nih.gov/BLAST/) to determine what type of gene sequence that it was most closely related to in GenBank.

**Phylogenetic Analyses**

Each of the amino acid sequences (HIF-1a, HIF-2a, and PHD3) that were deduced from the nucleotide sequences were compared to other amino acid sequences of these same genes from other species, both from other fish species and from species in other vertebrate classes, obtained from the National Center for Biotechnology Information (NCBI) at the website http://www.ncbi.nlm.nih.gov/. These amino acid sequences were then compared to each other, using the phylogenetic software program PHYLogeny Inference Package PHYLIP 3.69 produced by Joe Felsenstein of the Department of Genome Sciences and the Department of Biology at the University of Washington, to determine the phylogenetic relationships between them and form an unrooted tree with a specified outgroup. SEQBOOT was the algorithm used to prepare 1000 bootstrapped data sets from the resampling of the originally inputted sequence data set. PROTPARS was the algorithm used to estimate the phylogeny of the inputted data sets, based on the most parsimonious method. CONSENSE was the algorithm used to prepare a majority-rule consensus tree into a tree file program to demonstrate the most likely relationships between these amino acid sequences produced from SEQBOOT. The most parsimonious phylogram (consensus tree) produced from the PHYLIP 3.69 tree file was displayed using Neighbor Joining Plot (NJPlot) Windows 95, which was useful for displaying a
rooted tree, based upon outgroup inputted, from an unrooted tree produced by PROTPARS. The use of parsimony is the idea of adopting the simplest assumption in tree-branching to derive the phylogram.

ClustalW was used to align these same amino acid sequences to compare number and location of identical, conserved, and semi-conserved amino acids. These aligned amino acid sequences were also used to define the level of similarity, and thus the amount of conservation, of the major functional domains found within these transcription factors and enzymes.

Results

Sequences

The sequencing results from cloning three different genes are shown in Appendix A. The three C. variegatus genes are hypoxia inducible factors one alpha and two alpha (HIF-1α and HIF-2α) and prolyl hydroxylase 3 (PHD3). The results of each cDNA sequence are shown in two forms: (A) nucleotide sequences and (B) amino acid sequences. The two partial HIF-1α and HIF-2α amino acid sequences, and the entire PHD3 amino acid sequence, from C. variegatus were then compared to other known vertebrate species sequences in phylograms to confirm they represented the correct isoform, and then a direct amino sequence comparison to determine the extent of the conserved nature of the domains present for the functionality of these proteins. Each of these sequences are missing their 3’ end and their 5’ ends. Therefore both the HIF-1α and the HIF-2α cDNA sequences do not contain their entire open-reading frame for these transcription factor proteins, but they do contain a part of the PAS-A domain, the PAS-B domain, and the majority of the ODDD.
Phylograms

The partial *C. variegatus* HIF-1α and HIF-2α amino acid sequences, determined from the cloned and sequenced nucleotide sequences, were compared against a variety of other HIF-1α, HIF-2α, and HIF-3α amino acid sequences from a variety of other species across different vertebrate classes, based on sequences obtained from National Center for Biological Information (NCBI) database from the website http://www.ncbi.nlm.nih.gov/. The idea was to confirm that these two HIF-α sequences would indeed group along with the other HIF-1α and HIF-2α amino acid sequences, respectively. A most parsimonious phylogram (Fig. 6) showed that the two HIF sequences cloned are indeed different from each other and group with other HIF-1α or HIF-2α isoforms.

Figure 6 phylogram was constructed with the use of boot-strap analysis which compared 1000 trees or assimilations of 38 other species to come to the overall best consensus tree, in terms of the simplest branching pattern explanation. Truncated HIF-α sequences (absent their 5’ end) were used to compare the two partial sequences (HIF-1α and HIF-2α) to all the other species to determine the correct HIF-α isoform of these two sequences and then also to determine the closest phylogenetic relationship to other species within that isoform. Daggerblade grass shrimp (*Palaemonetes pugio*) HIF (isoform A) was the basis for the outgroup of the HIF-α isoforms (Fig. 6), since this sequence is from a crustacean and its evolution predates vertebrate species being compared.

A most parsimonious unrooted phylogram distinguished *C. variegatus* prolyl hydroxylase 3 (PHD3) from the six isoforms (PHD1, PHD2, PHD3, PHD4, CoPH, and LysH) of the prolyl hydroxylase family of enzymes (Fig. 7), based upon a sequence
Figure 6. Phylogram of gene similarity of the putative *Cyprinodon variegatus* HIF-1α and HIF-2α to the four isoforms of HIF-α from other species and classes of vertebrates. Accession numbers of each species, relative to phylogram, are in Appendix B.
Figure 7. Phylogram of the gene similarity of the putative *Cyprinodon variegatus* PHD3 to the six isoforms of prolyl hydroxylase from other species of organisms and classes of vertebrates. The accession numbers of each species, relative to phylogram, are in Appendix B.
comparison to other species found in the NCBI databank. The PHD phylogram itself was built using boot-strap analysis of 1000 trees to come to the overall best consensus of tree branching. Because of the highly conserved portion of the 2-oxoglutarate-Fe(II) dioxygenase superfamily, the four PHD enzyme isoforms have close sequence similarity and enzyme function (hydroxylase), with some unique differences because of their tissue-specific activity (Fig. 7). The most parsimonious phylogram (Fig. 7) demonstrated that the C. variegatus PHD sequence does indeed group with all the other PHD3 sequences from all other species in different vertebrate classes. The PHD3 amino acid sequence that was obtained for Cyprinodon variegatus was compared to other amino acid sequences from all four isoforms of PHD, which are PHD1 – PHD4, obtained from the NCBI database.

Appendix B contains the PHD3 nucleotide and amino acid sequence generating from cloning and sequencing. By comparison, the PHD3 nucleotide sequence (Fig. B3a) is nearly complete, with only the extreme 5’ end of the sequence missing, and it does contain the entire open-reading frame with the start and stop codons highlighted. Therefore the PHD3 amino acid sequence in Fig B3b represents the entire protein or enzyme.

Comparison of Functional Domains of Cyprinodon Variegatus HIF-1α, HIF-2α, and PHD3 with Those from Other Vertebrates

A comparison of HIF-1α protein domains (Fig. 8) displays identical and conserved amino acids between the various vertebrate species from the Fig. 6 phylogram. Amino acid sequence comparison in Fig. 8 shows that the PAS-A and PAS-B domains, along with the PAC region and the sequences connecting the domains together are highly
Figure 8. HIF-1α amino acid comparison of Cyprinodon variegatus to Fig. 6 species.
**Figure 8** (cont). HIF-1α amino acid comparison of *Cyprindon variegatus* to Fig 6 species. Black bars are identical and grey bars are conserved or semi-conserved amino acids. conserved across species, whereas the ODDD amino acid sequences are much less highly conserved.
Figure 9 displays a generic map of a HIF amino acid sequence, identifying the location of the primary domains that it contains. The N-terminal half of this amino acid sequence is the conserved portion of the protein and the C-terminal half of this sequence is the variable portion of the HIF transcription factor protein. Portions of the HIF-1α and HIF-2α amino acid sequences obtained from *C. variegatus* are displayed at the bottom of Fig. 9. The N-terminal portion of the HIF-α amino acid sequence contains the bHLH, PAS-A domain, PAS-B domain, and the PAC, whereas the C-terminal domain contains the ODDD, the pVHL, N-TAD, and C-TAD.

The partial HIF-1α amino acid sequence obtained was compared to the generic HIF-α amino acid sequence in Fig. 9. The conserved N-terminal half of *C. variegatus* HIF-1α has 77.9% total conservation (identical, conserved, and semi-conserved amino acids).

---

**Figure 9.** Map of domains and their locations within a generic HIF-α amino acid sequence, and the identification of the conserved and variable regions of these transcription factors. Terms include: bHLH = basic helix-loop-helix (needed for dimerization of HIF-α and HIF-β); PAS-A domain = Per-Arnt-Sim domain A; PAS-B domain = Per-Arnt-Sim domain B; PAC domain = PAS-associated C-terminal domain, which is a conserved region of 40-45 amino acids situated carboxy-terminal to any PAS repeat which can contribute to the PAS structural domain; ODDD = Oxygen Dependent Degradation Domain; pVHL = von Hippel-Lindau tumor-suppressor protein, which is a recognition component of E3 ubiquitin-protein ligase; N-TAD = N-terminal transactivation domains; C-TAD = C-terminal transactivation domains; Cyp = *C. variegatus*. 

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acids combined) with all the other HIF-1α amino acid sequences. The partial PAS-A domain of *C. variegatus* showed 71.7% total conservation, the complete PAS-B domain of *C. variegatus* showed 96.3% total conservation, and the complete PAC region of *C. variegatus* showed 85.4% total conservation with all the other amino acid sequences from the vertebrate classes of fish, mammals, and birds (Fig. 8). By comparison, the ODDD of HIF-1α *C. variegatus* displayed only a 33.3% total conservation of amino acids compared with all the other fish species and only a 24.7% total conservation of amino acids with all the other sequences (fish, mammals, and birds) (Fig. 8). Therefore it appears that replacement in the conserved and semi-conserved amino acids of the PAS and PAC domains along with the regions that connect them together, which make up over 50% of the total conservation in these regions, account for the primary changes in HIF-α evolution. Conserved amino acid changes in the ODD domain are much more sparse, and thus this region is under less functional pressure to maintain as precise a three-dimensional shape. The remaining C-terminal region, other than pVHL region that is identical between species, is even less conserved or more variable a region, in terms of the amino acid sequence.

Figure 9 shows the positioning of the partial *C. variegatus* HIF-2α amino acid sequence relative to the positioning of the domains in a generic HIF-α amino acid sequence, while Fig. 10 displays an amino acid sequence comparison between HIF-2α *C. variegatus* with these other vertebrate species. The N-terminal portion of the partial *C. variegatus* HIF-2α amino acid sequence encompasses the conserved PAS-B domain and the PAC region directly after it, while the C-terminal portion contains the variable ODDD excluding the N-TAD region (Fig. 10). The N-terminal conserved region of the HIF-2α
Specifically, the total conservation of the PAS-B domain is 59.2% and the PAC region is

![Amino acid sequence comparison](image)

*Figure 10. HIF-2α amino acid comparison of Cyprinodon variegatus to Fig. 6 species. Black bars are identical and gray bars are conserved or semi-conserved amino acids.*
63.8%, whereas the ODDD has only 17.9% total conservation, when *C. variegatus* is compared to amino acid sequences of other vertebrate species (Fig. 10).

The four HIF-α amino acid isoform sequences can be distinguished from each other based upon the size and relative positioning of their two PAS 3 domains, ODDD, along with their unique C-terminal end of their sequence (Fig. 9). The most conserved locations of the HIF-α amino acid sequences are located at the N-terminal end and the middle of the amino acid sequence, and this highly conserved region contains the basic helix-loop-helix (bHLH) domain, the PAS A domain, and the PAS B domain (Fig. 9). Although the PAS B domains do vary between the HIF-α isoforms, the variation is small and between conserved amino acids of similar properties. The largest and most distinct differences in amino acid sequence occur between the ODDD of species within an isoform as well as between the isoforms themselves. This variation is also displayed within and between the two putative *C. variegatus* HIF-1α and HIF-2α isoforms.

The PHD3 isoform of *C. variegatus* and other fish species is most closely related to the PHD1 and PHD2 isoforms. Figure 11 shows that the isolated *C. variegatus* PHD sequence demonstrated that it is a PHD3 isoform by the size and location of the 2OG-Fe2+ Oxy Superfamily domain in the amino acid sequence relative to the PHD1 and PHD2 isoforms. The PHD3 isoform is distinctly smaller than all the other PHD isoforms, only approximating 240 amino acids. Fig. 11 compares a generic PHD3 isoform to the two most closely related isoforms (PHD1 and PHD2), which are the three PHD isoforms used for oxygen sensing. All the other isoforms have an additional and unique N-terminal addition to their amino acid sequence, which distinguishes each isoform from the others.
Figure 11. Comparison of domains, variable regions, and conserved regions within the three oxygen sensing PHD amino acid isoforms (PHD1, PHD2, and PHD3).

The PHD3 amino acid isoform of *C. variegatus* was compared to other PHD3 amino acid sequences from several other species (Fig. 12). There is 51.5% amino acid sequence identity and 58.9% total conservation (identity, conserved, and semi-conserved amino acids) between *C. variegatus* PHD3 and the other vertebrate species, while there is 59.6% amino acid sequence identity and 84.8% total conservation over the 2OG Fe(II) Oxy Superfamily domain. Within the 241 amino acids of the *C. variegatus* PHD3 isoform, most of its sequence is part of the 2OG-Fe^{2+} Oxy Superfamily domain (amino acids ~43-213), thus this domain contains ~170 amino acids (Fig. 12).
The PAS-B domain is an important component in the dimerization of HIF-1α or HIF-2α (PAS-B domain and Oxygen Degradation Domain (PAS-B domain and ODDD, respectively)).

### Comparison of PHD3 amino acid sequences

<table>
<thead>
<tr>
<th>Organism</th>
<th>PHD3 Sequence</th>
<th>1OG Fe(Oxy) Superfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. variegatus Rhesus macaque</td>
<td>LAGSIPIVSSERIGDKNVYVGSGGCEAISSFPLNLDRLLSVCASRLDKATQREKSHKAVCTFQNGAGYV</td>
<td>1.5% sequence identity over entire amino acid sequence of PHD3</td>
</tr>
<tr>
<td>C. variegatus house mouse</td>
<td>LAGROYVSSERIGDKNVYVGSGGCEAISSFPLNLDRLLSVCASRLDKATQREKSHKAVCTFQNGAGYV</td>
<td>1.5% sequence identity over entire amino acid sequence of PHD3</td>
</tr>
<tr>
<td>C. variegatus human</td>
<td>LAGROYVSSERIGDKNVYVGSGGCEAISSFPLNLDRLLSVCASRLDKATQREKSHKAVCTFQNGAGYV</td>
<td>1.5% sequence identity over entire amino acid sequence of PHD3</td>
</tr>
<tr>
<td>C. variegatus European cattle</td>
<td>LAGROYVSSERIGDKNVYVGSGGCEAISSFPLNLDRLLSVCASRLDKATQREKSHKAVCTFQNGAGYV</td>
<td>1.5% sequence identity over entire amino acid sequence of PHD3</td>
</tr>
<tr>
<td>C. variegatus African clawed frog</td>
<td>LAGROYVSSERIGDKNVYVGSGGCEAISSFPLNLDRLLSVCASRLDKATQREKSHKAVCTFQNGAGYV</td>
<td>1.5% sequence identity over entire amino acid sequence of PHD3</td>
</tr>
</tbody>
</table>

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**Discussion**

Figure 6 phylogram does confirm that the two identified HIF-α sequences for *C. variegatus* are unique isoforms (HIF-1α and HIF-2α), based upon separate groupings amongst the different isoforms from other species and classes of vertebrates. Both *C. variegatus* HIF-α isoforms have relatedness to these two different HIF-α isoform groups of species by the presence of two conserved domains unique to them, (Per-Arnt-Sim Domain and Oxygen Degradation Domain (PAS-B domain and ODDD, respectively)).

The PAS-B domain is an important component in the dimerization of HIF-1α or HIF-2α with HIF-1β or HIF-2β, respectively (Semenza, 2001; Lee et al., 2004). The ODDD is **Figure 12.** Comparison of *C. variegatus* PHD3 amino acid sequence with several different vertebrate species (51.5% sequence identity over entire amino acid sequence of all species and 59.6% sequence identity over the 2OG Fe (II) Oxy Superfamily domain).
the region of the HIF-1α where two proline residues become hydroxylated for the degradation of the HIF-1α protein during normoxia (Huang et al., 2002).

For C. variegatus HIF-1α, the closest relationship to another amino acid sequence within the phylogram was to *Epinephelus coioides* (orange-spotted grouper), followed by *Micropogonias undulatus* (Atlantic croaker) and then by *Oryzias melastigma* (Indian medaka) (Fig. 6). Like *C. variegatus*, these fish species most closely associated with it are from the Superorder Acanthopterygii, which includes the Order Perciformes (*Epinephelus coioides, Micropogonias undulatus, Notothenia coriiceps, and Perca fluviatilis*), Order Gasterosteiformes (*Gasterosteus aculeatus*), and Order Beloniformes (*Oryzias melastigma*), which are all more evolutionarily advanced or recent forms of ray-finned fish (Froese and Pauly, 2006; Nelson, 2006; and ITIS, 1999). The remaining fish associated with *C. variegatus* in the HIF-1α isoform are from the Superorder Ostariophysi, in the Order Cypriniformes, or the carp (*Ctenopharyngodon idella* or grass carp), and the Superorder Protacanthopterygii, or salmon, trout, and pike (*Salmo salar, Oncorhynchus mykiss, and Esox lucius*, respectively), which are all older and more primitive ray-finned fish within the Class Actinopterygii (Froese and Pauly, 2006; Nelson, 2006; and ITIS, 1999). Thus the relationship between closely related HIF-1α sequences matches the evolutionary relationship found between these fish species.

For HIF-2α, the closest relationship to another amino acid sequence within the phylogram (Fig. 6) was found to be to *Tetraodon nigroviridis* (spotted green pufferfish) of the Order Perciformes, followed by *Fundulus heteroclitus* (mummichog) of the Order Cyprinodontiformes (a close relative to the pupfish *C. variegatus*) and the sciaenid *Micropogonias undulatus* (Atlantic croaker) of the order Perciformes. The Order
Cyprinodontiformes, and especially the Order Perciformes, are evolutionarily more advanced and more recently derived, within the ray-finned fish of the Superorder Acanthopterygii within the Class Actinopterygii (Froese and Pauly, 2006; Nelson, 2006; and ITIS, 1999). The rest of the fish representatives from this HIF-2α isoform came from the Superorder Ostariophysi, in the Order Cypriniformes, which includes *Danio rerio* (zebrafish) and *Ctenopharyngodon idella* (grass carp), and the Order Siluriformes for *Ictalurus punctatus* (channel catfish), which are all older and more primitive ray-finned fish within the Class Actinopterygii (Froese and Pauly, 2006; Nelson, 2006; and ITIS, 1999). Therefore the relationship between closely related HIF-2α sequences matches the evolutionary relationship found between these fish species.

The branching of the PHD phylogram shows the separation of all four PHD isoforms into their own unique group, where the *C. variegatus* PHD3 is clearly grouped with other PHD3 isoforms of a variety of other species and classes of vertebrates. It is most closely aligned with *Danio rerio* (zebrafish) of the Superorder Ostariophysi (Fig. 7), which are older and more primitive ray-finned fish within the Class Actinopterygii (Froese and Pauly, 2006; Nelson, 2006; and ITIS, 1999). The four isoforms of PHD, along with closely related procollagen and the lysyl hydroxylase all group within separate branches, where it appears that the procollagen and lysyl hydroxylase groups originate from PHD4. There is also a clear separation, and thus deviation in the amino acid sequence between PHD3 isoforms in aquatic vertebrates (fish) versus non-aquatic vertebrates, including amphibians and mammals (*Xenopus laevis, Mus musculus, Bos taurus, Macaca maculata, and Homo sapiens*), with distinct progression of amino acid variation within these species sequences as it progresses up the evolutionary ladder.
The PHD1 isoforms are composed of ~410 amino acids, and the 2OG-Fe\(^{2+}\) Oxy Superfamily domain is located between approximately amino acid positions #198-375. The PHD2 isoforms are composed of ~425 amino acids, and the 2OG-Fe\(^{2+}\) Oxy Superfamily domain is located at approximately amino acid positions 220-390. The PHD4 isoforms are composed of ~565 amino acids, and the 2OG-Fe\(^{2+}\) Oxy Superfamily domain is broken into two parts located at approximately amino acid positions 244-324 and 420-520. An EFh superfamily (calcium binding) domain is also located at amino acid positions ~190-250 in the PHD4 isoforms. The sequence of CoPH isoforms is ~540 amino acids long, and the 2OG-Fe\(^{2+}\) Oxy Superfamily domain is located at approximately amino acid positions 355-525. A P4Ha_N Superfamily is also located at amino acid positions ~30-160 in the CoPH isoforms, and this is part of the Prolyl 4 Hydroxylase enzyme that is most closely related. The LysH isoforms are composed of ~730 amino acids, and the 2OG-Fe\(^{2+}\) Oxy Superfamily domain is located at approximately amino acid positions 560-730. The distinctions in the overall size of the amino acid sequences of PHD 1-3 isoforms, along with the size and location of the 2OG Fe(II) Oxy Superfamily domain, demonstrated that the amino sequence of *C. variegatus* was a part of the smallest and evolutionarily oldest PHD3 isoform. PHD1-PHD3 isoforms are all similar in size and relative positioning of the 2OG Fe(II) Oxy Superfamily domain, with only the N-terminal segments of PHD1 and PHD2 being larger and more variable.

Phylogenetic analyses does confirm that three sequences obtained (HIF-1\(\alpha\), HIF-2\(\alpha\), and PHD3) are indeed the three sequences needed for the gene expression analyses of adult *C. variegatus* under the two stressors of low DO (hypoxia) and an estrogenic chemical (4tOP). The phylograms demonstrate the sequence similarity of HIF-1 and
HIF-2 to each other because of the longer and thus closer evolutionary relationship between them. PHD3 was shown to be closely related to the PHD1 and PHD2 isoforms in terms of nucleotide and amino acid sequence similarity. Based upon the phylogram, it also appears that PHD3 is the shortest and the evolutionarily the oldest of these three PHD isoforms, and apparently acts as the ancestor gene to the radiation of the larger PHD1 and PHD2 isoforms, as well as the longer and more highly modified amino acid sequences of the collagen PHDs.
CHAPTER III

CHRONIC, MODERATE AND SEVERE, HYPOXIA OF ADULT MALE AND FEMALE SHEEPSHEAD MINNOW (CYPRINODON VARIEGATUS)

Abstract

Research examined and compared the molecular and physiological effects of moderate chronic hypoxia versus severe chronic hypoxia. Analysis focused on answering one major hypothesis, which was that hypoxia would increase mRNA levels for the catabolic enzyme PHD3 in liver cells. Two exposures were used to test this hypothesis. The first exposure involved a moderate chronic hypoxia exposure (~2.5 mg/L) of adult male and female sheepshead minnow (Cyprinodon variegatus) for 7 days, and the second exposure involved a severe chronic hypoxia exposure (~1.5 mg/L) of adult male and female C. variegatus for 7 days. Results showed that moderate chronic hypoxia (exposure 3.1) significantly up-regulated PHD3 at 10 hrs for adult male C. variegatus liver samples and then both genders declined to near baseline by the end of the 168 hr exposure. Severe chronic hypoxia (exposure 3.2) also significantly up-regulated PHD3 initially (10 hrs) and continued to increase in severity over the course of the 168 hr exposure in the C. variegatus liver samples. These results confirm the up-regulation of PHD3 transcript levels with the onset of hypoxia presumably to translate them into functional proteins following return to normoxia to facilitate shift back from anaerobic metabolism back into aerobic metabolism.

Introduction

Impacts from hypoxia are particularly hard or stressful for organisms that are sessile or habitat-specific, such as estuaries. Impacts of hypoxia on cells, tissues, and
organs of aquatic organisms include systemic and molecular responses promoting adaptations to decreased oxygen levels. Many studies have defined hypoxia as occurring at 2.0 mg/L, and the threshold level of dissolved oxygen for fish movement (Eby and Crowder, 2002; Sagasti et al, 2003). However, the onset of hypoxia is dependent upon the species, system, and time of year, such as interaction of temperature and oxygen preferences and stressors between species (Eby and Crowder, 2002). Varying oxygen concentrations present a fundamental physiological challenge that requires the coordinated regulation of extensive arrays of genes (Epstein et al., 2001). Higher eukaryotes have adopted specialized mechanisms for oxygen homeostasis, and the conserved oxygen-dependent responsive pathways are expressed in almost every mammalian cell (Lee et al, 2004). Regulation of O$_2$ homeostasis, in terms of delivery and adaptation to low O$_2$, for animals occurs via the hypoxia-inducible factor 1 or HIF-1 (Semenza, 2001), which plays a central role in both local and systemic responses to hypoxia (Epstein et al, 2001, Lee, 2004). The HIF-1 is a transcriptional complex that plays an essential role in cellular and systemic oxygen homeostasis (Lee, 2004), which presents a fundamental physiological challenge that requires the coordinated regulation of extensive arrays of genes (Epstein, 2001).

Site-specific hydroxylation of the proline residues in hypoxia-inducible factor, or HIF, is catalyzed by a recently described family of enzymes, PHD1/HPH3/EGLN2/HIF-PH1, PHD2/HPH2/EGLN1/HIF-PH2, and PHD3/HPH1/EGLN3/HIF-PH3, which appear to have arisen by gene duplication and are represented by a single gene in Caenorhabditis elegans (Egl9) and Drosophila melanogaster (Fatiga) (Appelhoff et al, 2004; Aravind and Koonin, 2001; Berra et al, 2003; Huang et al, 2002; Metzen et al, 2002). The PHDs,
or the prolyl hydroxylase domains, are the mammalian versions of these hydroxylation enzymes (Appelhoff et al, 2004; Berra et al, 2003). Hypoxia reduces the activity of PHDs that hydroxylate specific proline residues in the oxygen-dependent degradation domain (ODDD) of hypoxia-inducible factor 1α (HIF-1α) (Berra et al, 2003; D’Angelo et al, 2003; Masson et al, 2001). The ODDD has been shown to have two independent regions, and reinforces the role of prolyl hydroxylation as an oxygen-dependent destruction signal (Masson et al, 2001).

HIF hydroxylation is not an equilibrium reaction, and the extent of modification at a given oxygen concentration will also be affected by the quantity of available enzyme (Masson and Ratcliffe, 2003). Prior exposure of cells to hypoxia enhances the HIF prolyl hydroxylase activity found in cell extracts, and the rate of HIF-α degradation following a return to normoxia (Masson and Ratcliffe, 2003). Small interfering RNA (siRNA) techniques have shown a dominant role for PHD2 in controlling the low steady-state levels of HIF-1α in normoxia in a range of cell types, with little or no observed effect with PHD1 and PHD3 (Appelhoff et al, 2004; Berra et al, 2003), and PHD2 acting as the critical oxygen sensor (Berra et al, 2003). However it is unclear whether this predominance of PHD2 is related to a lack of precise knowledge of protein abundance or because of the existence of tissue-specific expression patterns, as suggested by the analysis of mRNA expression for the PHDs (Appelhoff et al, 2004). Involvement of prolyl hydroxylation by distinct Fe(II)- and 2-OG-dependent oxygenases in different modes of HIF regulation suggests that such enzymes may be well suited to a role in cellular oxygen sensing (Aravind and Koonin, 2001; Masson and Ratcliffe, 2003). PHD2
is up-regulated by hypoxia, providing an HIF-1-dependent auto-regulatory feedback mechanism driven by the oxygen tension (Berra et al., 2003).

Hypoxia is usually considered below ~4 mg/L D.O. (start of physiological stress) down to ~2 mg/L D.O. (visible stress before a threshold leading to death) for most fish, and both of these hypoxia exposures fall into this range. However, for C. variegatus, which is an estuarine fish that can deal with hypoxia quite well and based on previous experience with this fish model, the range of hypoxia in which fish will survive goes all the way down to ~1.0 mg/L DO. Both adult male and female C. variegatus were used in a pair of chronic (7-day) hypoxia exposures, at two different low DO levels. These two hypoxia exposures were used to assess the difference in the magnitude of prolyl hydroxylase 3 (PHD3) gene expression level, and then follow how this response changed over the course of one week (168 hours). The chronic moderate hypoxia exposure 3.1 averaged DO levels to 2.5 mg/L, and the chronic severe hypoxia exposure 3.2 averaged DO levels to 1.5 mg/L. Both of these exposures maintained DO in the lower half of the hypoxia range to try to guarantee a physiological response from the estuarine-hardy C. variegatus. It is possible that some of the genes responding to a hypoxic event, such as PHD3, could be used as a biomarker of hypoxic stress.

Materials and Methods

Fish Culture

Sheepshead minnow (Cyprinodon variegatus) served as the experimental test animal in the described studies, following University of Southern Mississippi Institutional Animal Care and Use Committee protocols and Guidelines for Use of Fishes in Research proposed by the American Fisheries Society detailed at the website
Approved IACUC protocol and number for all aquatic toxicological studies with small fish species in the William Shoemaker Toxicology building at the Department of Coastal Sciences of the University of Southern Mississippi is found in Appendix C.

Sexually mature *C. variegatus* from existing multi-generational lab-reared stocks at the Gulf Coast Research Laboratory (a mix of fish from EPA, Gulf Breeze, FL and Aquatic BioSystems Inc, Fort Collins, CO) were exposed to hypoxia via an intermittent flow-through system in the William Shoemaker Toxicology building. Flow rate was sufficient to provide ~4.0 volume additions/day in each test chamber. These fish were kept on a 16:8 hour light:dark daily photoperiod supplied via fluorescent bulbs, with a 30-minute transition period simulating dawn and dusk. Fish were maintained in filtered artificial seawater prepared from synthetic sea salt (Fritz Super Salt Concentrate) diluted to 15 g/L with well water. Fish were fed twice daily, once with AquaTox Special dry flake food (Zeigler, Gardner, PA, USA) and once with brine shrimp nauplii (*Artemia franciscana*). Test aquaria were housed within a central water bath and were maintained at 27±1°C. Duration of each experiment was 7 days, with pH, temperature (°C), salinity (g/L), and DO (mg/L) measured continuously in one hypoxic aquarium and twice daily for all treatment aquaria with a multi-parameter water quality monitor (600 XLM data sonde, YSI Environmental Monitoring Systems).

**Hypoxia Exposures**

During all of the hypoxia exposures to adult *Cyprinodon variegatus*, compressed nitrogen gas, using a regulator to control flow, was bubbled into the individual aquaria in order to drive off the additional dissolved oxygen above the pre-set dissolved oxygen
(DO) concentrations called for in the experiments. The size of the aquaria were 35L tanks (48.3 cm length X 48.3 cm width X 20.3 cm height with a 15 cm high overflow drain). The oxygen levels within the exposure aquaria were regulated by the AquaController III unit (Neptune Systems, San Jose, CA) to continuously monitor and maintain the DO level within the aquaria to within ±0.3 mg/L (Appendix D), initiating more bubbling of nitrogen gas into the tanks as needed.

Two chronic hypoxia exposures were run using sexually mature male and female C. variegatus, where each exposure lasted for 168 hrs. Exposure 3.1 was a moderate chronic hypoxia exposure, with average hydrological parameters of ~2.5 mg/L DO, ~26.9°C temperature, and ~15 ppt salinity (Appendix D). Exposure 3.2 was a severe chronic hypoxia exposure, with average hydrological parameters of ~1.5 mg/L DO, ~27.1°C temperature, and ~15 ppt salinity (Appendix D). These two hypoxia exposures (8 tanks/exposure) were run with sexually mature male and female C. variegatus, with both hypoxia and normoxia treatments. In exposure 3.1, there were 96 fish (48 males and 48 females) exposed to moderate, chronic hypoxia (~2.5 mg/L DO) or normoxia (~7 mg/L DO). In exposure 3.2, another 96 fish (48 males and 48 females) were exposed to severe, chronic hypoxia (~1.5 mg/L DO) or normoxia (~7 mg/L DO). Both experiments had four sampling events at time points (10, 48, 96, and 168 hrs), with 12 fish/tank (6 males and 6 females) maintained at each specified hypoxic or normoxic treatment. For each exposure, four replicates (n = 4) were sampled at each of the four time points, with one male and one female C. variegatus removed from each aquaria to dissect liver samples.
RNA Extractions

One adult *Cyprinodon variegatus* fish was randomly sampled from each tank replicate, and they were euthanized with Tricaine Methanesulfonate (MS-222) at a dosage of ~80 mg/L for ~1 minute, cervical dislocation, or brain pithing. These fish were dissected for liver and testes for RNA extraction to obtain total RNA. RNA extraction was performed as described in Chapter 2.

Preparation of cDNA

First-strand cDNA synthesis (Invitrogen protocol) was used to convert RNA into single-stranded cDNA as described in Chapter 2.

QRT-PCR

Differential expression of the selected genes were further validated with use of quantitative real time reverse transcription PCR (qRT-PCR) using the iCycler iQ (BIORAD, ver. 3.1), with the SYBR-Green fluorophore (497 nm excitation peak), a passive fluorescein dye in SYBR-Green I Supermix to detect amplified cDNA made by the thermocycler. First-strand cDNA was used for Real-Time PCR, and it was diluted to 1:25 with sterile water. A mastermix was made with 25 µl of 2X SyBr Green Supermix (Bio-Rad), 1.5 µl of 10 µM Forward Primer, 1.5 µl of 10 µM Reverse Primer (Table 2), and 17 µl DEPC-treated or nuclease-free water per reaction sample, and each mastermix aliquot was combined with 5 µl of a sample for a total of 50 µl. Samples were placed into individual wells of a 96-well plate, mixed gently, covered with optically clear tape, and collected by brief centrifugation, and cDNA amplified on the Real-Time PCR iCycler (Bio-Rad).
Table 2

List of primers used for qPCR

<table>
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<th>Name</th>
<th>Type</th>
<th>Sequence</th>
<th>Amplicon</th>
<th>Efficiency</th>
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<tr>
<td>18S rRNA</td>
<td>Forward</td>
<td>5’-GCTGAACGCCACCTTGTCC-3’</td>
<td>552 bases</td>
<td>95.2%</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5’-ATCCGATAACGAACGAGACTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD3</td>
<td>Forward</td>
<td>5’-CATGATGCACCAGCTCTCAGCTAC-3’</td>
<td>354 bases</td>
<td>96.5%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GCTCTGTGGAGGCTGTAGGGCTCT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The quality and quantity of amplified PCR products were confirmed by visualization of the appropriate size band on a 1% agarose gel using gel electrophoresis (Fisher Biotech Electrophoresis Systems) and a Fluor-S MultImager (BIORAD). Forward and reverse primers were prepared from a more variable area within a conserved domain to identify the correct isoform within a family of genes using quantitative Polymerase Chain Reaction (qPCR). The 18S rRNA was used as the housekeeping gene to normalize the amount of target cDNA found in samples from each of the four time-point series of the two chronic hypoxia exposures 3.1 and 3.2. Table 2 details the forward and reverse primers used for 18S rRNA.

Relative induction levels of the gene of interest (PHD3) were assessed by normalization of the raw Ct (Cycle threshold) data from the experimental treatment (hypoxia) against the housekeeping gene 18S rRNA for calculation of the delta (Δ) Ct values. These ΔCt values were compared with the ΔCt values of the control treatment (normoxia) prepared in the same manner, to calculate the difference between the experimental and control treatments for the negative delta delta (-ΔΔ) Ct values. Relative
induction levels of the treatment samples occurred exponentially on the thermocycler, and these levels were determined by the equation \((1 + \text{percent efficiency of qPCR primer set})^{\Delta\Delta C_{t}}\). Triplicate samples of cDNA prepared from reverse transcription were run along with one replicate of the respective negative control (-RT) of each sample. All male and female samples from one time point were run on a single 96-well iCycler qPCR plate (BIORAD).

The PCR parameters for each of the two pairs of forward and reverse primers set up for 40 cycles on a Bio-Rad iCycler iQ Real-time thermocycler (version 3.1) were as follows: pre-PCR incubation of 2 min at 96°C; denatured for 1 min at 96°C; annealed for 30 sec at 54°C for 18S rRNA and 56°C for PHD3 for 40 cycles; followed by a 1 min denaturation and a 1 min annealing at either 54°C or 56°C for the development of a sequential melt curve from either 54°C or 56°C to 96°C (10 sec per 0.5°C); final indefinite hold temp of 4°C. The cDNA samples from exposure 3.1 and 3.2 were run in triplicate for qPCR. The sizes of the cDNA sequences that were obtained via amplification of the genes of interest on the thermocycler using the specified qPCR primer sets (amplicon) to determine the relative induction levels or changes in mRNA expression of each of the samples is shown in Table 2.

Data Analyses for qPCR

SigmaStat version 3.1 was performed on delta Ct values of samples using three-way and two-way ANOVAs for exposure 3.1 and 3.2 on the qPCR gene (cDNA) expression results, using the independent variables of 1) DO (hypoxia versus normoxia), 2) gender (male versus female) and 3) time points of exposure for the three-way ANOVA and the independent variables of DO and time points for the combined gender two-way
ANOVA. Holm-Sidak pairwise comparisons were used to determine specific points of significance (p<0.05) when the F-value of three-way ANOVA was significant (p<0.05). Assumptions of ANOVA addressed included: (1) independent samples, (2) equal sample sizes (n) amongst groups, (3) normal distribution, and (4) homogeneity of variance. Any limitations to normal distribution or homogeneity of variance, especially when not severe, is compensated by the built-in robustness of the statistical analysis produced from single violations of ANOVA assumptions (Glass, 1972; Johnson, 1993; Harwell, et al. 1992; Kenny and Judd, 1986; Schmider et al., 2010), and it was the strength and power of this test that determined its consistent use in these analyses. Larger or multiple deviations in assumptions would be handled with natural log (ln) transformation of data or ranking the data for use in three-way ANOVA. The experimental hypoxia treatment was compared to the normoxia control over the course of the time series to assess for significance.

Results

The results of activation of PHD3 mRNA expression levels from exposure of adult male and female C. variegatus to moderate chronic hypoxia and to severe chronic hypoxia exposure are shown in Figures 13 and 14, respectively, and the statistical results displayed in Tables 3 and 4, respectively. There was no significant difference in PHD3 transcriptional levels between the male and female C. variegatus based on a three-way ANOVA (Table E3.1). Therefore the two genders were combined for analysis using two-way ANOVA using the treatment factors of DO and time, and this analysis showed significant difference with DO, time duration, and an interaction of DO over time (Table 3). The interaction effect showed that duration time had an impact on mRNA expression
levels during moderate hypoxia treatment. The combined male and female PHD3 mRNA expression levels at all four time points of moderate hypoxia represent statistically significant increases over the companion normoxia samples. Time point 1 (10 hrs) of the hypoxia treatment had significant and the greatest transcriptional expression (lowest mean ΔCt value) versus successive time points (48, 96, and 168 hrs) as compared to lower and similar transcriptional expression found throughout the time course of the normoxia control treatment (Fig 13, Table E3.1). During moderate chronic hypoxia exposure, PHD3 had a significant initial -ΔΔCt value of ~4 (~16-fold up-regulation in mRNA levels) for the combined male and female samples at 10 hrs (time point 1) (Fig. 13).

**Figure 13.** Exp 3.1 PHD3 mRNA expression from the liver of adult male and female *Cyprinodon variegatus* under moderate, chronic hypoxia (~2.5 mg/L DO) exposure (7 days). Statistically significant pts: 1) based on hypoxia versus normoxia, are identified by a star * and 2) based on treatment over time are identified by a number # (with no difference between genders).
There was a progressive decline in the peak -ΔΔCt value of PHD3 starting with time point 2 (48 hrs) until the end of the exposure at time point 4 (168 hrs) (Fig. 13, Table E3.1). At 48 hours, the significant-ΔΔCt value of PHD3 declined to ~3 (~8-fold up-regulation in mRNA levels) for the combined male and female samples, while by 96-hrs -ΔΔCt values had a further decline to ~1.8 (~3.5-fold up-regulation in mRNA levels) for the combined male and female samples (Fig. 13, Table E3.1). By 168 hrs (time point 4), the significant -ΔΔCt levels declined to their lowest point of ~1.5 or near baseline (~2.8-fold up-regulation in mRNA levels) for the combined male and female samples (Fig. 13, Table E3.1).

Similarly, severe chronic hypoxia exposure had no significant difference between the male and female *C. variegatus* based on initial statistical analysis using a three-way ANOVA (Table E3.2). Therefore, the two genders were pooled together for a two-way ANOVA over the two treatment factors of DO and time duration, which showed a significant difference in mRNA expression levels for DO and the interaction of DO over time (Table E3.2). The combined male and female PHD3 mRNA expression levels at all four time points of severe hypoxia represent statistically significant increases over the companion normoxia samples. Statistical analysis showed an increasing disparity in the means of the ΔCt values between normoxia and hypoxia over the duration of the hypoxia exposure and the level of transcriptional expression, with mean normoxia ΔCt values increasing over time and with mean hypoxia ΔCt values decreasing over time (Fig. 14). At time point 1 (10 hrs), PHD3 had a significant -ΔΔCt value of ~3 (~8-fold up-regulation in mRNA levels) for the combined male and female samples (Fig. 14, Table E3.2). During the course of this one week hypoxia exposure, the difference between the
PHD3 mRNA levels of hypoxia and normoxia continued to increase. At time point 2 (48 hrs), the significant -ΔΔCt value of PHD3 increased to ~4 (~16-fold up-regulation in mRNA levels) for the combined male and female samples (Fig. 14, Table E3.2). By time point 3 (96 hrs), the -ΔΔCt value climbed to ~5 (~32-fold up-regulation in mRNA levels) for the combined male and female samples (Fig. 14, Table E3.2). At 168 hrs or the final time point 4, the significant -ΔΔCt value of PHD3 reached ~6 (~64-fold up-regulation in mRNA levels) for the combined male and female samples (Fig. 14, Table E3.2).

\[ \text{Figure 14. Exp. 3.2 PHD3 mRNA expression from the liver of adult male and female } \]
\[ \text{Cyprinodon variegatus under severe, chronic (~1.5 mg/L DO) exposure (1 week).} \]
\[ \text{Statistically significant points are identified by a star * based on hypoxia versus normoxia (with no difference between genders).} \]
Discussion

PHD3 expression of mRNA within the liver cells of the estuarine fish *Cyprinodon variegatus* (sheepshead minnow) appeared to be triggered by the exposure to hypoxia, with the level of PHD3 expression dependent upon not only the severity (moderate or severe) of hypoxia but also the length of its duration (acute or chronic). The production of mRNA for PHD3 translation was initiated within hours of the beginning of hypoxia and the duration can last for a few hours to a few days when the hypoxia persisted.

Moderate chronic hypoxia exposure to adult *C. variegatus* appeared to be manageable, physiologically-speaking, with little difficulty in terms of the length of time it took these fish to produce enough PHD3 mRNA transcripts for translation of enough prolyl hydroxylase enzyme to presumably initiate the degradation of the excess HIF-1α (10 hrs) within the liver cells during the subsequent return to normoxia (Fig. 13). The remaining time points of this moderate, chronic hypoxic exposure (48, 96, and 168 hrs) appeared to indicate there was excess abundance of this transcript. In other words, enough PHD3 enzyme was translated by the 10 hr time point, and the excess levels of PHD3 transcript declined over the remaining time of this hypoxia exposure. The quantity of PHD3 enzyme, and possibly its transcript, is important for the elimination of HIF-1α during normoxia recovery within hepatocytes, and the tissue as a whole, by minimizing the energy diverted into anaerobic metabolism because of HIF-α activation from low pO$_2$ from hypoxia (Koukourakis *et al.*, 2006). Because proteins generally have a longer half-life than mRNA, the PHD3 enzyme would most likely be in greater quantity than its transcript during the duration of hypoxia and its transition back into normoxia, unless their was an immediate need to increase PHD3 protein levels at the onset of normoxia.
Once pO$_2$ levels return to normal, removal of HIF-α allows these liver cells to return to normal functioning via aerobic metabolism and thus maximizing energy (ATP) production (Virani and Rees, 2000).

Severe chronic hypoxia appeared to be difficult to manage physiologically in *C. variegatus*, because these liver cells continued to produce increasing amounts of PHD3 mRNA with increasing lengths of time under hypoxia (Fig. 14). In fact, the level or rate of production of PHD3 mRNA appeared to modestly increase with additional time that these fish were exposed to severe chronic hypoxia. Similarly, previous studies have also shown an increase in PHD3 transcription through exposure to hypoxia (Berra et al., 2003; Mason and Ratcliffe, 2003). Hepatocytes appeared shifting into an anaerobic state, since they were continuing to create more PHD3 enzyme needed in aerobic recovery. Thus under severe chronic hypoxia, these liver cells were not able to regain cellular aerobic homeostasis and appeared to be acting as an oxygen conformer like *Fundulus grandis* by slowing down metabolic activities (Virani and Rees, 2000). Increased PHD3 levels may thus be an indirect indicator of this homeostatic imbalance with *C. variegatus* decreasing it oxygen consumption and shifting into more anaerobic metabolism (Chippari-Gomes et al., 2005) as do the gulf killifish exposed to hypoxia (Virani and Rees, 2000). However, Fig. 14 also showed that there was a change over time in the ΔCt value of the normoxia control, resulting in an increase in the -ΔΔCt value when compared to the experimental hypoxia treatment. Two-thirds of the increase in PHD3 transcription (-ΔΔCt) over time relative the normoxic control appears to be due to decreased levels of PHD3 mRNA levels in the normoxic controls. Further studies are needed to better assess the actual baseline and not over-estimate the magnitude of the actual physiological change that
occurred from a stress response. In the case of this severe, chronic hypoxic exposure (Fig. 14), it is quite apparent that there is a strong transcriptional response of PHD3 up-regulation, greater than that estimated in the initial response during the moderate, chronic hypoxia exposure (Fig. 13), and that this response does persist throughout the entire one week exposure. However it is much less clear whether or not the magnitude of this transcriptional expression actually increased with time and what the implications of the longer term physiological response are.

HIF-1α is known to up-regulate transcription of PHD giving rising to a negative feedback loop in which HIF-1α controls its own level (Berra et al., 2003; Marxsen et al, 2004; Zagórska and Dulak, 2004). In exposure 3.1 with a hypoxia exposure of 2.5 mg/L DO, the decline in DO levels were great enough to trigger a hypoxia response but apparently great enough to only slow down the catabolic activity of PHD3. Therefore the PHD3 presumably marked the HIF-1α for degradation, and this eliminated additional PHD3 mRNA from being produced for the rest of the 168 hr hypoxic exposure. Without the presence of HIF-1α, PHD3 transcriptional levels declined with the remaining PHD3 transcripts apparently degraded away. Therefore in exposure 3.1, the liver cells were able to maintain aerobic metabolism. In exposure 3.2 with the hypoxia exposure of 1.5 mg/L DO, the decline in DO levels were great enough to trigger a hypoxia response and also to stop the catabolic activity of PHD3. With the continued presence of lower DO levels and HIF-1α, the PHD3 enzyme was apparently not able to hydroxylate HIF-1α for degradation and the transcriptional levels of PHD3 continued to increase over the course of the entire 168 hr exposure. Therefore these liver cells did apparently have to switch over to anaerobic metabolism for the duration of exposure 3.2.
These physiological responses of PHD3 at the cellular level for hypoxia exposure of *C. variegatus* could possibly occur under conditions where estuarine fish are unable to escape temporally oxygen-poor waters, by being trapped in an isolated localized area of a given estuary, cut off from access to normoxic water within their salinity tolerance for osmoregulation. Because of the shallow conditions and segmental arrangement of estuarine waters, plus a general increase of nutrients from fertilizers entering into coastal waters, it is quite possible that this hypoxic stressor condition occurring under peak summer conditions can occur with increasing severity. With increased anthropogenic input of fertilizers and chemicals to estuarine waters, it is also possible that other compounds, such as endocrine disrupting chemicals (EDCs) are causing a combined (additive/synergistic) hypoxic effect by affecting some other aspect of the HIF complex.

Many other fish utilize these estuarine waters for some or all of their life cycle, with many of these fish not having the ability to withstand oxygen-deprived waters nearly as well as the estuarine obligate and highly adaptable *C. variegatus*. The results of this research with *C. variegatus* suggests that many other fish, along with invertebrates, utilizing these estuarine waters, could be even more severely impacted by these realistic levels of hypoxia occurring during the summer and which may be more severe from anthropogenic impacts/additions to these estuarine waters. The dramatic up-regulation in the expression of PHD3 under hypoxia conditions also demonstrates its potential use as a biomarker for the status of a variety of fish utilizing these nursery grounds in the summer time. Other organ tissues, such as gills, could also be tested to determine if they show a greater or more consistent response to hypoxic conditions, with the possibility of biopsy (non-lethal) sampling versus lethal sampling (liver dissection) for fisheries/wildlife
management purposes. Sampling fish species over the course of the summer could potentially be used to prospectively determine how these fish in the estuary are physiologically coping with increasing hypoxia.
CHAPTER IV

ACUTE, SEVERE HYPOXIA AND ACUTE, VERY SEVERE, HYPOXIA
COMBINED WITH 4-TERT OCTYLPHENOL OF ADULT MALE SHEEPSHEAD
MINNOW (CYPRINODON VARIEGATUS)

Abstract

Research examined transcriptional effects on adult male and female sheepshead minnow (Cyprinodon variegatus) from two exposures to environmental stressors: (1) exposure 4.1 acute severe hypoxia (~1.5 mg/L) transitioning back to normoxia and (2) exposure 4.2 acute very severe hypoxia (~1.08 mg/L) combined with environmental estrogen 4tOP (~60 µg/L) transitioning back to normoxia. Transcript levels of five genes (HIF-1α, HIF-2α, PHD3, EPO, and VTG) were examined in liver and testes taken over time after initiation of hypoxia and after initiation back into normoxia. Microarrays were prepared on exposure 4.2 samples using previously prepared 25-mer oligos of Cyprinodon variegatus cDNA SSH library, hybridized with Cy3-labeled ds-cDNA target samples from combined acute hypoxia to normoxia + 4tOP exposure for gene expression profiling of hepatocytes. Exposure 4.1 transcript levels of HIF-1α and EPO were significantly up-regulated in first 24 hrs of hypoxia. Transcript levels of HIF-2α and PHD3 were also up-regulated non-significantly. Exposure 4.2 transcript levels of HIF-1α, HIF-2α, and EPO showed significant induction in first 2-7 hrs of exposure and PHD3 showed no significance. Continued oscillation in expression of these four genes showed that a longer period was needed to complete aerobic homeostatic recovery. VTG mRNA levels were up-regulated significantly and exponentially for normoxia + 4tOP and hypoxia + 4tOP treatments within 2-7 hrs, peaked at ~11,585-fold at 72 hrs, and declined
to ∼2048-fold by 24 hrs into normoxia recovery. Increased duration and magnitude of VTG response from hypoxia + 4tOP versus normoxia + 4tOP lent credence to hypoxia cross-talk with reproductive pathway of vitellogenin production. Testes mRNA levels of these same genes demonstrated no significant response. Microarray analysis of hypoxia into normoxia transition demonstrated 125 significant genes up- or down-regulation in transcription levels from the treatment transitions of: (1) H→NC or hypoxia (72 hrs) to normoxia (74 hrs) and (2) H→NOP or hypoxia + 4tOP (72 hrs) to normoxia + 4tOP (74 hrs). Three primary biochemical pathways were affected by presence of hypoxia and/or 4tOP, including significant down-regulation in immunological response, significant increase in detoxification response, and significant but mixed response (up- and down-regulation) in various aspects of cellular metabolism.

Introduction

Hypoxia

Many studies have defined hypoxia as occurring at 2.0 mg/L, and the threshold level of dissolved oxygen for fish movement (Eby and Crowder, 2002; Sagasti et al., 2003). Other studies indicate that 2.0 mg/l dissolved oxygen level may not act as a universal threshold level for hypoxia, but instead this level is dependent upon the species, system, and time of year (Eby and Crowder, 2002), such as interaction of temperature and oxygen preferences and potential increases in prey vulnerability (Eby and Crowder, 2002). Impacts from hypoxia are particularly hard or stressful for organisms that are sessile or habitat-specific, such as estuaries. Impacts of hypoxia on cells, tissues, and organs of aquatic organisms include systemic and molecular responses promoting adaptations to decreased oxygen levels. Varying oxygen concentrations present a
fundamental physiological challenge that requires the coordinated regulation of extensive arrays of genes (Epstein, 2001). Higher eukaryotes have adopted specialized mechanisms for oxygen homeostasis, and the conserved oxygen-dependent responsive pathways are expressed in almost every mammalian cell (Lee et al., 2004).

**HIFs and Target Genes (EPO)**

Regulation of $O_2$ homeostasis, in terms of delivery and adaptation to low $O_2$, for animals occurs via hypoxia-inducible factor 1 or HIF-1 (Semenza, 2001), which plays a central role in both local and systemic responses to hypoxia (Epstein et al., 2001, Lee et al., 2004). The HIF-1 is a transcriptional complex that plays an essential role in cellular and systemic oxygen homeostasis (Lee et al, 2004), which presents a fundamental physiological challenge requiring coordinated regulation of extensive arrays of genes (Epstein et al., 2001). As a master regulator of hypoxia response, HIF-1 undergoes conformational changes in response to varying oxygen concentrations (Lee et al., 2004).

HIF-1 is a αβ-heterodimer composed of two subunits, HIF-1α and HIF-1β (Lee et al., 2004; Pugh and Ratcliffe, 2003). HIF-1β subunit or ARNT (aryl hydrocarbon receptor nuclear translocator) is constitutively expressed, and the HIF-1α subunit is expressed and transcribed in precise regulation to cellular $O_2$ concentration (Semenza, 2001; Lee et al., 2004). HIF-1α is the primary site of regulation for activity of this protein, which includes protein stabilization, post-transcriptional modifications, nuclear translocation, dimerization, transcriptional activation, and interaction with other proteins (Zagórska and Dulak, 2004).

Activation of the HIF-1 heterodimer triggers a cascade of target genes that become up- or down-regulated within the cells of the affected tissues (Zagorska and
Dulak, 2004; Lee et al., 2004). Included within these target genes is erythropoietin (EPO), which is involved in red blood cell production and needed for oxygen transport in the circulatory system, and the vascular endothelial growth factor (VEGF) (Zagorska and Dulak, 2004; Lee et al., 2004). These genes increase oxygen availability by promoting angiogenesis and erythropoiesis, which leads to activation of glucose transport and metabolism (Lee et al., 2004). There are a variety of physiological processes known to be up-regulated by HIF-1α, and they include the control of vascular system (angiogenesis and vasomotor control), maturation of red blood cells (erythropoiesis and iron transport), energy metabolism (glycolysis, glucose transport, and the multifunctional enzyme glyceraldehydes-3-phosphate dehydrogenase), cell proliferation and viability (arrest of cell cycle, apoptosis, and growth factors), pH regulation, nucleotide metabolism, matrix metabolism, catecholamine synthesis, and negative feedback regulation of HIF-1 transactivation (Zagórska and Dulak, 2004).

Considerably less is known about the molecular responses of non-mammalian vertebrates and invertebrates to hypoxic exposure, and the physiological responses linking them to HIF are less well-developed (Nikinmaa and Rees, 2005). The diversity of fish presents many opportunities to evaluate if inter- and intra-specific variation in HIF structure and function correlate with hypoxia tolerance, while also offering an opportunity to examine the interactions between hypoxia and other stressors, including pollutants, common in aquatic environments (Nikinmaa and Rees, 2005). Adult zebrafish have been studied for long-term adaptive responses to hypoxia, and these studies have identified 367 out of 15,532 differentially expressed genes in the respiratory organs (the gills), using cDNA microarrays, of which 117 showed hypoxia-induced and 250 hypoxia-
reduced expressions (van der Meer et al., 2005). Metabolic depression was indicated by repression of genes in the TCA cycle in the electron transport chain and of genes involved in protein biosynthesis, whereas enhanced expression of the monocarboxylate transporter and of the oxygen transporter myoglobin (van der Meer et al., 2005). Some cDNAs encoding HIF subunits from the estuarine fish Fundulus heteroclitus (Atlantic killifish or mummichog) include a HIF-2α homolog and ARNT2alt, which is a splice variant of ARNT2 containing an additional exon encoding 16 amino acids near the amino terminus (Powell and Hahn, 2002). HIF-2α, ARNT2, and ARNT2alt mRNAs are expressed in all organs examined, and the HIF-2α combines with Fundulus ARNT2 splice variant or murine ARNT1 (Powell and Hahn, 2002).

Under normoxic conditions, HIF-1α is subject to ubiquitination and proteasomal degradation (Lee et al., 2004; Martin et al., 2005; Pugh and Ratcliffe, 2003; Semenza, 2001). Biochemical studies have shown that the von Hippel-Lindau (VHL) tumor-suppressor protein is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for degradation, and that interaction with VHL requires the O2- and iron-dependent hydroxylation of proline residue 564 in HIF-1α by an enzymatic activity distinct from the known procollagen prolyl hydroxylases (Lee et al., 2004; Martin et al., 2005; Semenza, 2001; Zagórska and Dulak, 2004). Proline residues Pro-402 and Pro-564 of HIF-1α are constitutively hydroxylated under normoxic conditions, and this hydroxylation allows for binding of the VHL protein (Huang et al., 2002).

**Prolyl Hydroxylases (PHDs)**

Site-specific hydroxylation of the proline residues in hypoxia-inducible factor, or HIF, is catalyzed by a recently described family of enzymes, PHD1/HPH3/EGLN2/HIF-
PH1, PHD2/HPH2/EGLN1/HIF-PH2, and PHD3/HPH1/EGLN3/HIF-PH3, which appear to have arisen by gene duplication and are represented by a single gene in Caenorhabditis elegans (Egl9) and Drosophila melanogaster (Fatiga) (Appelhoff et al., 2004; Aravind and Koonin, 2001; Berra et al., 2003; Huang et al, 2002; Metzen et al., 2002). The PHDs, or prolyl hydroxylase domains, are mammalian versions of these hydroxylation enzymes (Appelhoff et al., 2004; Berra et al., 2003). Hypoxia reduces the activity of PHDs that hydroxylate specific proline residues in the ODDD of hypoxia-inducible factor 1α (HIF-1α) (Berra et al., 2003; D’Angelo et al., 2003; Masson et al., 2001). The ODDD has been shown to have two independent regions, and reinforces the role of prolyl hydroxylation as an oxygen-dependent destruction signal (Masson et al., 2001).

HIF hydroxylation is not an equilibrium reaction, and the extent of modification at a given oxygen concentration will also be affected by the quantity of available enzyme (Masson and Ratcliffe, 2003). Prior exposure of cells to hypoxia enhances the HIF prolyl hydroxylase activity found in cell extracts, and the rate of HIF-α degradation following a return to normoxia (Masson and Ratcliffe, 2003). Small interfering RNA (siRNA) techniques have shown a dominant role for PHD2 in controlling the low steady-state levels of HIF-1α in normoxia in a range of cell types, with little or no observed effect with PHD1 and PHD3 (Appelhoff et al., 2004; Berra et al., 2003), and PHD2 acting as the critical oxygen sensor (Berra et al., 2003). However it is unclear whether this predominance of PHD2 is related to a lack of precise knowledge of protein abundance or because of the existence of tissue-specific expression patterns, as suggested by analysis of mRNA expression for the PHDs (Appelhoff et al., 2004). Involvement of prolyl hydroxylation by distinct Fe(II)- and 2-OG-dependent oxygenases in different modes of
HIF regulation suggests that such enzymes may be well suited to a role in cellular oxygen sensing (Aravind and Koonin, 2001; Masson and Ratcliffe, 2003). PHD2 and PHD3 are up-regulated by hypoxia, providing an HIF-1-dependent auto-regulatory feedback mechanism driven by the oxygen tension (Berra et al., 2003; Marxsen et al., 2004).

**EDCs and ECs**

Some of endocrine-disrupting chemicals (EDCs) affect the endocrine system because of their ability to mimic natural estrogen, whereas others may function as an antiestrogen. Recent research has shown that hypoxia in estuaries impairs the reproductive system of aquatic organisms as much as any of the known endocrine disrupting chemicals. Chronic exposure to hypoxia has been shown to decrease serum levels of testosterone, estradiol, and triiodothyronine in carp (*Cyprinus carpio*), which lead to retarded gonadal development in males and females, reduced spawning success, sperm motility, fertilization success, hatching rate, and larval survival (Wu et al., 2003). Research involving marine teleost Atlantic croaker (*Micropogonias undulatus*) showed suppressed ovarian and testicular growth during chronic environmental hypoxia, with supporting lab studies showing that this endocrine disruption was associated with impairment of reproductive neuroendocrine function and decreases in hypothalamic serotonin (5-HT) content and the activity of the 5-HT biosynthetic enzyme, tryptophan hydroxylase (Thomas et al., 2007). Hypoxia reduced growth and reproduction in the estuarine Gulf killifish (*Fundulus grandis*), with a 50% reduction in E2 levels in females and 50% reduction in 11-ketotestosterone (11KT) levels in males (Landry et al., 2007).

ECs (foreign or man-made estrogens), in a manner similar to 17β estradiol (E2), can activate E2-regulated genes by forming a complex with the ER (Yamamoto, 1985).
DNA binding of this complex activates expression of specific target genes or gene networks implicated in growth and differentiation of female reproductive tissues (Flouriot et al., 1996), including transcription of the ER-encoding gene (autoregulation) (Flouriot et al., 1996; Pakdel et al., 1991) and the vitellogenin (VTG)-encoding gene in fish, amphibians, reptiles, and birds (Flouriot et al., 1995, Flouriot et al., 1997).

Environmental estrogens have affinity for the ER of 0.02 to 0.0001 that of the natural hormone E₂ (Arnold and McLauchlin 1996). Concern is that adult animals can bioaccumulate (1,000–3,000 times) these chemicals (Ekelund et al., 1990) and that exposure could occur at a critical time in the organism’s development (Gillesby and Zacharewski, 1998). Because of the bioaccumulation potential of many of these chemicals, long-term EDC exposure at low concentrations could adversely affect an organism, influence the success of future progeny, and lead to changes in population levels (Daston et al., 1997).

Sewage treatment plants can release large amounts of estrogenic chemicals in aquatic environments as alkylphenols. These are microbial breakdown products of alkylphenol-polyethoxylates (APEs), which are widely employed as industrial and household nonionic surfactants. Over 300 million kilograms of APEs are produced annually (Talmadge, 1994). Following sewage treatment, about 60% of APEs are released into the aquatic environment as short-chain APEs, including nonylphenol and octylphenol. Alkylphenol, 4-tert-Octylphenol (4tOP), was found to be about 5-20 times more estrogenic than 4-nonylphenol and between 100 and 10 000 times less estrogenic than estradiol-17β in the in vitro systems employed (Jobling and Sumpter, 1993; Soto et al., 1992; White et al., 1994), causing feminization of male C. variegatus with the
presence of VTG in the blood (Karels et al., 2003) via downstream gene activation of its induced ERα receptor (Karels and Brouwer, 2003). Alkylphenols, in turn, also have been shown to be slowly biodegradable (Gaffney, 1976; Sundaram and Szeto, 1981). These chemicals have a strong tendency to bioconcentrate (Ekelund et al., 1990), bind to the estrogen receptor of fish and mammals (Flouriot et al., 1995; White et al., 1994), induce transcriptional activation of estrogen-responsive genes, produce detectable VTG in fish hepatocyte cell cultures, and produce VTG in male rainbow trout at concentrations of 4.8 μg/L (Jobling et al., 1996; Jobling and Sumpter, 1993; Ren and Lech, 1996; White et al., 1994). Of the alkylphenols examined, 4tOP appears the most biologically active. Rainbow trout exposed to 30 μg/L of 4tOP show reduction in testicular growth (Jobling et al., 1996), and male Japanese medaka (Oryzias latipes) exposed to 50 to 100 μg/L nonylphenol developed true oocytes in the testes (Gray and Metcalfe, 1997).

Vitellogenin is an egg yolk precursor protein synthesized in the liver, transported in the blood, and taken up by growing oocytes during vitellogenesis in fish, amphibians, reptiles, and birds (Tyler et al., 1996), and it is used as a food supply for the embryo and larval stages of fish. Production of VTG is estrogen dependent; thus normally found in significant concentrations only in females (Tyler et al., 1996). Presence of elevated levels of VTG in males is a good indication of estrogenic chemicals in the environment, and VTG expression may be interpreted as a warning of reproductive consequences (Cheek et al., 2001). Laboratory studies have shown that VTG in plasma of male Japanese medaka (Oryzias latipes) exposed to OP is correlated to reproductive impairment (Gronen et al., 1999). Exposure to estrogens can lead to feminization of male fish, as indicated by VTG in their blood, and interfere with sperm production and thus reproduction.
Two exposures were used to examine gene expression profiles of five genes impacted by hypoxia and/or 4tOP. These genes include prolyl hydroxylase 3 (PHD3), hypoxia inducible factor one alpha (HIF-1α), hypoxia inducible factor two alpha (HIF-2α), erythropoietin (EPO) which is up-regulated after the activation of HIF-αs, and vitellogenin (VTG) which is up-regulated by EDCs and thought to be impacted by hypoxia via cross-talk in biochemical pathways. Since there was no difference between genders impacted by hypoxia in the previous two exposures (Chapter III) on PHD3 mRNA expression levels, only male *C. variegatus* were used in the present hypoxia exposures using two different low DO levels. Exposure 4.1 examined effects from the stressor of acute severe hypoxia (72 hrs.) with a transition back into normoxia. Exposure 4.2 examined effects from two different stressors, the EC 4tOP alone and combined with acute (72 hrs.) very severe hypoxia with a transition back into normoxia. These two hypoxia exposures focus on short time periods of hypoxia and how gene transcription changes with transition into hypoxia and how it changes with the transition back into normoxia, with and without the presence of the additional stressor 4tOP. Microarrays are also used to assess broad-based changes in gene transcription in the lesser studied transition from hypoxia back into normoxia for *C. variegatus*.

**Materials and Methods**

*Fish Culture*

Fish culture was done as described previously in Chapter III.

*Hypoxia Exposures*

During all of the hypoxia exposures to adult *Cyprinodon variegatus*, compressed nitrogen gas, using a regulator to control flow, was bubbled into the individual aquaria in
order to drive off the additional dissolved oxygen above the pre-set DO concentrations called for in the experiments. The size of the aquaria were 35L tanks (48.3 cm length X 48.3 cm width X 20.3 cm height with a 15 cm high overflow drain). The oxygen levels within the exposure aquaria were regulated by the AquaController III unit (Neptune Systems, San Jose, CA) to continuously monitor and maintain the DO level within the aquaria to within ±0.2 mg/L by initiating more bubbling of nitrogen gas into the tanks as needed.

Exposure 4.1, used 80 sexually mature adult male *C. variegatus* in a severe transition hypoxia (~1.5 mg/ml DO, ~26.8 °C temperature, ~15 ppt salinity) exposure (Appendix D). This transition exposure had an initial 30-minute transition from normoxia (~7 mg/ml DO) to hypoxia (~1.5 mg/ml DO), followed by 72 hrs in hypoxia, with a second 30-minute transition from hypoxia back to normoxia, for a total of 72 hrs in normoxia. One male fish/tank were sampled from the five replicate tanks (n = 5) at each of these time points, which included time points of 0, 0.5, 2, 5, 10, 24, 72 hrs into hypoxia and then time points 0.5, 2, 5, 10, 24, and 72 hrs back into normoxia (144 hrs total). Another set of tanks was maintained at normoxia for baseline comparisons, with five replicates (one fish/tank) sampled at these time points. Physiological responses were assessed in exposure 4.1 by determining gene expression levels for PHD3, HIF-1α, HIF-2α, and EPO.

Exposure 4.2 used 200 sexually mature adult male *C. variegatus* in a very severe transition hypoxia (~1.08 mg/L DO, ~27.3°C temperature, and ~15 ppt salinity) exposure (Appendix D), nearer the physiological limits of *C. variegatus*. This transition exposure had an initial 30-minute transition from normoxia to hypoxia (72 hrs), and then a second
30-minute transition from hypoxia back into normoxia (72 hrs), and this exposure was combined with a second stressor, 4tOP (~60 ug/L) (Appendix D, Table D1). There were four treatment combinations: 1) normoxia control (NC), 2) normoxia + 4tOP (NOP), 3) hypoxia to normoxia control (H→NC), and 4) hypoxia to normoxia + 4tOP (H→NOP). Physiological response was assessed in exposure 4.2 by determining gene expression levels of PHD3, HIF-1α, HIF-2α, EPO, and VTG. Individual fish were sampled from each of the five replicate (n = 5) tanks (10 fish/tank) of each treatment at sequential time points, which included time points 0, 2, 7, 24, 72 hrs into hypoxia and then time points 2, 7, 24, and 72 hrs back into normoxia. Physiological responses were assessed in exposure 4.1 by determining gene expression levels for PHD3, HIF-1α, HIF-2α, and EPO, and VTG. Appendix D (Table D1) details the actual concentrations of 4tOP measured from water samples, taken at the beginning and the end of exposure 4.2 from each aquarium, by Micro Methods located in Ocean Springs, MS).

**RNA Extractions**

One adult *Cyprinodon variegatus* fish was randomly sampled from each tank replicate, and they were euthanized with Tricaine Methanesulfonate (MS-222) at a dosage of ~80 mg/L for ~1 minute, cervical dislocation, or brain pithing. These fish were dissected for liver and testes for RNA extraction to obtain total RNA. RNA extraction was performed as described in Chapter II.

**Preparation of cDNA**

First-strand cDNA synthesis (Invitrogen protocol) was used to convert RNA into single-stranded cDNA as described in Chapter II.
**QRT-PCR**

Validation of differential expression of the selected genes, preparation of a mastermix using 2X SyBr Green Supermix, use of 96-well plates for qRT-PCR on the iQCycler iQ, and identification of the appropriate amplified PCR product using 1% agarose gel electrophoresis and Fluor-S MultiImagery were done as they were described in Chapter III. All of the modifications to the qPCR procedure are described next.

Forward and reverse primers (Table 5), prepared from the conserved portion of the cDNA, were used in the quantitative Polymerase Chain Reaction (qPCR). The 18S rRNA forward and reverse primers were used as the housekeeping gene to normalize the amount of cDNA being found in the samples from the nine time-points of the two hypoxia→normoxia transition exposures.

Relative induction levels of the genes of interest (PHD3, HIF-1α, HIF-2α, EPO, and VTG) were assessed by normalization of the raw data Ct (Cycle threshold) values from the thermocycler of the experimental treatments with the Ct values of the housekeeping gene 18S rRNA for calculation of the delta (Δ) Ct values. These ΔCt values were compared to the ΔCt values of the control treatment (normoxia) prepared in the same manner, to calculate the difference between the experimental and control groups for the negative delta delta (–ΔΔ) Ct. Relative induction levels of the treatment samples occurs exponentially on the thermocycler, and these levels were determined by the equation \((1 + \text{percent efficiency of qPCR primer set})^{\Delta\Delta Ct}\).

Duplicate samples of cDNA from reverse transcription (RT) were amplified on the thermocycler for each sample. All male samples from one time point were run on a single 96-well iCycler qPCR plate (BIORAD).
A list of the sequences of the forward and reverse primers for each of the six genes (PHD3, HIF-1α, and HIF-2α, EPO, VTG, and 18S rRNA) isolated and sequenced are shown in Table 3. The PCR parameters for each of the pairs of forward and reverse primers set up for 40 cycles on a Bio-Rad iCycler iQ Real-time thermocycler (version 3.1) were as follows: pre-PCR incubation of 2 min at 96°C; denatured for 1 min at 96°C; annealed for 30 sec at 54°C for EPO, HIF-1α, and HIF-2α, 56°C for PHD3, and 58°C for VTG for 40 cycles; followed by 1 min denaturation and 1 min annealed at 54°C, 56°C, or 58°C for development of a sequential melt curve from 54°C to 96°C (10 sec per 0.5°C); final indefinite hold temp of 4°C. The cDNA samples from exposure 4.1 and 4.2 were run in triplicate for qPCR. Table 3 shows the sizes of the amplicons, or the cDNA Table 3

**List of primers used for qPCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Sequence</th>
<th>Amplicon</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Forward</td>
<td>5’-GCTGAACGCCACCTTGTCG-3’</td>
<td>552 bases</td>
<td>95.2%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ATTCCGATAAAGGAGAGACTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Forward</td>
<td>5’-AAGGAACCGGACAGAG-3’</td>
<td>193 bases</td>
<td>89.7%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TCACAGATCGACCCAGATAG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-2α</td>
<td>Forward</td>
<td>5’-GGCTGAGAGGTGCCTGTTG-3’</td>
<td>82 bases</td>
<td>95.2%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCAGGCGAGGTCGTCAG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD3</td>
<td>Forward</td>
<td>5’-CATGATGCACCCAGCTCTAC-3’</td>
<td>354 bases</td>
<td>96.5%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GCTCTGTGGAGCTGGCTTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VTG</td>
<td>Forward</td>
<td>5’-TGCTCACTGTGAGGTCAACG-3’</td>
<td>330 bases</td>
<td>99.8%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TTTTCAACAGTGGCTAGGTTCT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPO</td>
<td>Forward</td>
<td>5’-GCGCAAATCTGAGCTGCTGA-3’</td>
<td>149 bases</td>
<td>112.3%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TGCTCCGGCTGCTTTTC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
sequences that were obtained via amplification of the genes of interest on the thermocycler using the specified qPCR primer sets, to determine the relative induction levels or changes in mRNA expression of each of the samples. Amplification efficiency of these genes, relative to 100% exponential amplification, is also shown in Table 3.

Microarrays

Second-strand cDNA was prepared from the first-strand cDNA by combining 20 µl of each sample with 80 µl of a reaction mastermix that contained 8 µl of 10X Reaction Buffer for DNA Polymerase I, 0.2 µl (1 unit) of RNase H, E coli, 3 µl (30 units) DNA Polymerase I, E coli (10,000 units/ml), and 68.8 µl of nuclease-free water to 100 µl per reaction. Double-stranded cDNA was used to prepare microarrays, where the double-stranded cDNA was amplified to contain a Cy3 fluorescent dye to identify up- or down-regulated genes via transcription (see subsection VI of Methods on Microarrays).

The BIO-RAD VersArray Chip Writer Compact System was used for the probe-printing of 16 VWR Epoxy 2 Microarray slides (#16001-026) with an in-house annotated single-stranded 25-mer oligonucleotide library of *Cyprinodon variegatus* that was synthesized by Invitrogen, and it was contained within seventeen 96-well amplification plates (Nalge Nunc International, item # 259676), sealed with single tab sealing foil (USA Scientific, item # 2938-4100), and stored at -80°C. After printing with the Chip Writer, each glass slide contained 1632 unique probes, which were printed on the slides in triplicate to create a total of 4896 labeled spots. The probe cDNA (25-mer oligos) were then linked to the glass slides via short UV light exposure from the STRATEGENE UV StrataLinker 1800. These labeled slides were then stored in a slide box in a dessicator at room temperature.
Tri Link Biotechnologies Cy3 (light-sensitive, reactive water soluble fluorescent dye of the cyanine family, excitation of ~550 nm and emission at ~570 nm) random 9-mers (18 µl/sample) were combined with target cDNA (1 µg/sample) and nuclease-free water (Ambion) to obtain a total volume of 39 µl/sample (prepared in low light). The solution was heat denatured at 98°C for 10 minutes and then immediately chilled on ice for 5-10 minutes. A master mix was then prepared that contained the Klenow fragment (exo-) nuclease enzyme (50 ul at 5 units/ul, Thermo Scientific, Fermentas Molecular Biological Tools), which lacks 5’→3’ and 3’→5’ proofreading exonuclease activity and thus is able to incorporate modified nucleotides like the Cy3 random 9-mers along with 50 ul of 50X dNTPs. Then, 6 ul of this prepared master mix was added to these Cy3-cDNA solutions (prepared in low light) and they were incubated for 2 hours at 37°C in order to amplify the original double-stranded target cDNA. At this point, 55 ul of nuclease-free water (Ambion) was added to each of these sample solutions. EDTA (10 µl, 0.5 M) was added to the solution to terminate the amplification reaction by stopping the enzyme activity. NaCl (5 M, 11.5 ul) was then added, along with Isopropanol (110 µl) at room temperature. The solutions were centrifuged at 12,000 X g for 10 minutes to form a pink Cy3-cDNA labeled pellet (in low light). The supernatant was carefully removed from the pellet (via pipetting) so as not to lose the pellet, rinsed in 500 µl of 80% ice-cold ethanol (dislodge the pellet in the ethanol) and centrifuged at 12,000 X g for 2 minutes. The ethanol was removed from pellets via pipetting, air-dried for ~5 minutes in minimal light, and then resuspended in 21.5 µl of nuclease-free water (Ambion). The Cy3-labeled and amplified target cDNA solutions were quantified via the
ND-1000 Spectrophotometer NanoDrop, under the Microarray double-stranded cDNA setting, for the concentration of Cy3 dye incorporated target cDNA using 1.5 µl/sample.

The Cy3-labeled target double-stranded cDNA was prepared for hybridization with the 25-mer oligonucleotide probes attached to the glass slides. The Cy3-labeled target cDNA needed to be double-stranded, because the 25-mer oligonucleotide probes were a mixture of either sense or anti-sense strands, and thus both strands of the target cDNA were needed to ensure a complement strand was available for hybridization to the attached probes. The Cy3-labeled cDNA samples were diluted from 20 µl to 50 µl using nuclease-free water (Ambion), and then these samples were heated to 70°C on a VWR Digital Heat Block for three minutes. Each sample was then placed into 150 µl of pre-hybridization solution (700 µl of 10X PBS, 10% Tween-20, 29 µl Nanopure water, and 20 µl BSA (10 mg/ml). The Miltenyi Biotech a-Hyb Hybridization Station was used for the hybridization of the Cy3-labeled double-stranded target cDNA from two treatments of exposure 4.2 (hypoxia only and hypoxia + 4tOP) at 72 hrs in hypoxia (H and H4OP, respectively) and at 74 hrs after hypoxia to normoxia transition (H \rightarrow N and H4OP \rightarrow N4OP, respectively). These samples were put into pre-hybridization solution (200 µl total volume) and combined with the single-stranded 25-mer oligonucleotide probes fixed to the glass slides. Before hybridization began, the glass slides were blocked with 200 µl of 1X BlockIt Blocking Buffer (ArrayIt Microarray Technology) and then washed with three different 50 ml solutions (2XSSC + 1% sarcosyl, 2X SSC, and 0.2% SSC, each made with DEPC-treated water) to prevent background labeling of the slides. This focused the hybridization on binding to the 1632 unique 25-mer oligonucleotide probes in triplicate (4896 total spots). After hybridization of the target
cDNA (overnight, ~16-17 hrs), the same three washes as noted above were done. After hybridization, the glass slides were rinsed with Nanopure water and then immediately scanned with the BIO-RAD VersArray Chip Reader (10 µm system) to prepare a digital read-out of the 25-mer oligonucleotide probes that did hybridize with the Cy3-labeled target cDNA from the exposures. The 16 microarrays were stored in a slide box dry at room temperature. These digital files were then analyzed (feature extraction) using the software ImaGene 7.0 Standard Edition.

Data Analyses

qPCR analysis. Two-way ANOVA was performed with SigmaStat version 3.1 using a two-way ANOVAs for exposure 4.1 on the qPCR gene (cDNA) expression results, using the independent variables of (1) DO (hypoxia versus normoxia) and (2) time points of exposure (0, 0.5, 2, 5, 10, 24, 72, 72.5, 74, 77, 82, 96, and 144 hrs) for each of the four genes PHD3, HIF-1α, HIF-2α, and EPO. Post-hoc (Holm-Sidak) pair-wise comparisons, was used to determine significant differences in gene expression over successive time points of the hypoxia→normoxia transition treatment and the normoxia control treatment for exposures 4.1. Similarly, two-way ANOVA was used for exposure 4.2 on the qPCR gene (cDNA) expression results, using the independent variables of (1) treatment (NSC, N4OP, Hyp, and H4OP) and (2) time points of exposure (0, 2, 7, 24, 72, 74, 79, 96, and 144 hrs) for each of the five genes PHD3, HIF-1, HIF-2, EPO, and VTG. Post-hoc Holm-Sidak pairwise comparisons were used to determine specific points of significance (p<0.05) when the F-value of a two-way ANOVA was significant (p<0.05) of three experimental treatments hypoxia→normoxia only (Hyp), hypoxia→normoxia plus 4tOP (H4OP), and 4tOP only (N4OP) and the normoxia control treatment (NSC).
The list of the assumptions of ANOVA, and how any limitations or deviations to these assumptions would be handled, was done in the same manner as described in the Methods section of Chapter III. The three experimental treatments that involved hypoxia and/or 4tOP were compared to the normoxia control over the course of the time series to assess for significance.

Microarray analyses. The intensity of labeled probes specifies which genes are present and whether they are up- or down-regulated, and after log₂ transformation and standardization of the data, it was analyzed by the software Jump Genomics 5.0 (JMP 5). ImaGene 7.0 Standard Edition software package assessed the difference in intensity between individual probe or cDNA replicates (in triplicate) as well as between the thousands of genes on each array. This determined a mean intensity and standard deviation for each target cDNA, and displayed the intensity for each spot in terms of a signal to noise ratio to eliminate out the neighboring background noise, where a number above one refers to up-regulation and below one refers to down-regulation. In Jump Genomics 5.0, this original signal to noise ratio data was log₂ transformed to produce a normal distribution or spread of up-regulated genes versus down-regulated genes where the baseline was at zero instead of one. The Fast Ward algorithm was used for hierarchical clustering analysis in Jump Genomics 5.0. Statistical Analysis Software (SAS) standardized samples from each microarray by dividing each sample by the mean standard deviation of all the samples from each microarray. The False Discovery Rate (FDR) (Benjamini and Hochberg, 1995), was used to limit the number of potential false-positives of significant genes, and it was set at 5%.
Two-way ANOVA was used for comparison between microarrays (equal sample sizes between exposures and log$_2$ transformed data for normal distribution), followed by the comparison of the four treatment combinations from exposure 4.2: (Hyp) Hypoxia Control (H→NC) at 72 hrs, (Hyp) Hypoxia Control (H→NC) at 74 hrs, Hypoxia to Normoxia plus 4tOP (H→NOP) at 72 hrs, Hypoxia to Normoxia plus 4tOP (H→NOP) at 74 hrs to analyze simple difference pairwise comparisons that represent all single level differences in independent variables to determine significantly expressed genes (either up-regulated or down-regulated) using SAS. Time point 72 hrs was during hypoxia, and time point 74 hrs was after the transition back to normoxia. These simple difference treatment combinations examined include (H4OP – Hyp), (H4OP – N4OP), (Hyp – NSC), and (N4OP – NSC). Only genes significantly expressed, based upon significant p-value from the F-ratio of either DO (normoxia versus hypoxia), Treatment (control versus 4tOP), or the interaction between these two independent variables, are used for these pairwise comparisons. Alpha level was set at 0.05, with a False Discovery Rate (FDR) of 0.05.

Results

Gene Expression from Real-Time PCR

Exposure 4.1, severe acute hypoxia exposure using liver samples.

EPO. This exposure measured mRNA levels of HIF-1α, HIF-2α, EPO, and PHD3 over 72 hrs following the initiation of severe hypoxia (~1.5 mg/L), and over a second 72 hr period following recovery back into normoxia (Figs. 15-18, Tables E4.1-1 – E4.1.4 in Appendix E). With the onset of hypoxia, liver samples showed a significant -ΔΔCt values of ~2.4 (~5.3-fold up-regulation) in mRNA EPO levels, maintained this
level for 2 hrs after the initiation of hypoxia, and it was followed by a gradual decrease in EPO expression levels (higher hypoxia ΔCt values) over the 72 hrs of hypoxia in the hypoxia treatment (Fig. 15, Table E4.1-1 in Appendix E). During the first 2 hrs of transition back into normoxia in the hypoxia treatment, the EPO mRNA levels declined to baseline and remained there during the rest of the 144 hr exposure (Fig 15). The ΔCt levels of the normoxia treatment stayed relatively more constant throughout the duration of the exposure (Fig 15).

![Figure 15. Exp. 4.1  EPO mRNA expression of adult male Cyprinodon variegatus liver from severe hypoxia (~1.5 mg/L DO) in an acute exposure (3 days) followed by a 30-minute transition back to normoxia and 3 days in normoxia.](image)

**HIF-1α.** Transcriptional expression of HIF-1α was shown to be significant based on DO, time duration, and the interaction of DO over time (Table E4.1-2 in Appendix E). Over the course of the exposure, the mean ΔCt values of the hypoxia treatment showed declines, and thus increased HIF-1α transcription, compared to the normoxia control.
mean ΔCt values which stayed constant over the course of the exposure (Fig. 16).
Therefore, increased duration of the hypoxia treatment caused increased HIF-1α transcription. The mRNA expression levels of HIF-1α started to significantly increase within 30 minutes of the onset of hypoxia with a -ΔΔCt value of 1.2 (~2.3-fold up-regulation), which peaked within 2 hrs with a -ΔΔCt value of 1.7 (~3.2-fold up-regulation), declined back to baseline within 24 hrs, with a second non-significant increase in -ΔΔCt value increase of 0.5 (~1.4-fold up-regulation) at 72 hrs (Fig. 16, Table E4.1-2 in Appendix E). Following the onset of normoxia recovery, there was a third significant increase in -ΔΔCt value of ~1 (~2-fold up-regulation), with a decline back to near baseline by 96 hrs, and a fourth significant increase in -ΔΔCt values of ~1.2 (~2.3-

**Figure 16.** Exp. 4.1 HIF-1α mRNA expression of adult male *Cyprinodon variegatus* liver from severe hypoxia (~1.5 mg/L DO) in an acute exposure (3 days) followed by a 30-minute transition back to normoxia and 3 days in normoxia. Statistically significant pts identified based on trtmt (hypoxia vs normoxia) and over time are shown by a star *. 

fold up-regulation) at 144 hrs or 72 hrs back into normoxia recovery (Fig. 16, Table E4.1-2 in Appendix E).

**HIF-2α.** Transcriptional levels os HIF-2α displayed initial -ΔΔCt values of 0.4 (~1.3-fold down-regulation) in mRNA levels at the onset of hypoxia, declined, and stayed very close to baseline for the remainder of the hypoxia exposure (Fig. 17, Table E4.1-3 in Appendix E). After the onset of normoxia, there was another non-significant decline below baseline with a -ΔΔCt value of 0.4 (~1.3-fold down-regulation) that declined to near baseline by the end of exposure (Fig 17, Table E4.1-3 in Appendix E). However, there is no statistically significant -ΔΔCt values over the time course of the

![Graph](image)

**Figure 17.** Exp. 4.1 HIF-2α mRNA expression of adult male *Cyprinodon variegatus* liver from severe hypoxia (~1.5 mg/L DO) in an acute exposure (3 days) followed by a 30-minute transition back to normoxia and 3 days in normoxia.
exposure, and thus the hypoxia versus normoxia significance involves only a difference in overall magnitude of the two treatment means relative to each other.

**PHD3.** Transcriptional levels of PHD3 from hypoxia had an initially significant expression level decline with a $-\Delta\Delta Ct$ values of $\sim-2.4$ ($\sim$-5.3-fold down-regulation) with onset of hypoxia (Fig 18, Table E4.1-4 in Appendix E). The mRNA levels of PHD3 normoxia started out higher levels and declined over the course of the hypoxia exposure, and slowly declined to the more consistent levels of PHD3 mRNA found in the hypoxia treatment (Fig. 18). Due to the large variability in this data set for both hypoxia and normoxia treatments, there was no significant down-regulation of hypoxia over time.

![Figure 18. Exp. 4.1 PHD3 mRNA expression of adult male Cyprinodon variegatus liver from severe hypoxia (~1.5 mg/L DO) in an acute exposure (3 days) followed by a 30-minute transition back to normoxia and 3 days in normoxia.](image)
Exposure 4.2, very severe acute hypoxia, with /without 4tOP, using liver samples

EPO. The multi-exposure of adult *C. variegatus* assessing all combinations of normoxia versus hypoxia combined with or without 4tOP applying severe hypoxia (~1.08 mg/L) for 72 hrs. followed by 72 hrs. of recovery back into normoxia, displayed a variety of different gene expression responses. EPO showed transcriptional significance in terms of treatment, time duration, and the interaction of treatment and time in exposure 4.2 (Fig 19, Table E4.2-1 in Appendix E). The treatments hypoxia plus 4tOP (H4OP) and hypoxia only (Hyp) displayed the same differential up- and down-regulation relative to the normoxia solvent control (NSC) over the time course of the exposure, with H4OP showing significant increase in the -ΔΔCt values of ~3.0 and ~2.8 (~8.0-fold and ~7.0-fold, respectively) at 2 hrs. and 7 hrs., respectively, after the onset of hypoxia, and remained near baseline for the rest of the time in hypoxia and back into normoxia (Fig 19, Table E4.2-1 in Appendix E). During Hyp, the EPO mRNA peaked with a nearly significant -ΔΔCt value of 1.3 (~2.5-fold up-regulation) at 2 hrs and a -ΔΔCt values of 1.5 (~2.8-fold up-regulation) at 7 hrs. and declined to near baseline by 24 hrs., similar in magnitude and timing to H4OP. Normoxia + 4tOP (N4OP) stayed near baseline and was not significant for the entire exposure (Fig 19, Table E4.2-1 in Appendix E). NSC did not proceed as a straight line, but instead more as a sinusoidal curve (Fig. 19, Table E4.2-1 in Appendix E).

HIF-1α. Transcriptional expression of HIF-1α for exposure 4.2 showed significance relative to time duration and the interaction of time with treatment (Fig. 20, Table E4.2-2 in Appendix E). Only in concert with time, thus interaction, were all three treatments significant (Fig 20, Table E4.2-2 in Appendix E). All three experimental treatments
(H4OP, Hyp, and N4OP) displayed significant transcriptional increases in HIF-1α, relative to NSC, after the onset of hypoxia and/or the presence of 4tOP. After the transition back to normoxia, N4OP and Hyp also had another HIF-1α transcriptional increase relative to NSC (Fig. 20, Table E4.2-2 in Appendix E). NSC did not follow a straight line, and thus this baseline had a small range of fluctuation (Fig. 20, Table E4.2-2 in Appendix E).

**Figure 19.** Exp. 4.2. EPO mRNA expression levels of adult male *Cyprinodon variegatus* liver in very severe acute hypoxia (~1.08 mg/L DO) and 4tOP with a transition back to normoxia over 144 hrs (statistically significant pts are a star * by trtmt over time).

Gene expression of HIF-1α displayed a significant increase in the -ΔΔCt values of ~2.4 (~5.3-fold up-regulation) for the H4OP treatment that peaked at 2 hrs, and declined to baseline by 24 hrs (Fig. 20, Table E4.2-2 in Appendix E). For the rest of hypoxia and
all of normoxia time points, the -ΔΔCt values remained above and near baseline (Fig. 20, Table E4.2-2 in Appendix E).

Similarly, an initial significant up-regulation of HIF-1α occurred in the Hyp exposure with a -ΔΔCt value of ~2.6 (~6.1-fold up-regulation) at 2 hrs, and declined to near baseline by 24 hrs (Fig. 20, Table E4.2-2 in Appendix E). Afterwards, HIF-1α -ΔΔCt values stayed near baseline for the rest of hypoxia and the first couple of hours into normoxia. At 7 hrs into normoxia, there was a significant increase in the HIF-1α -ΔΔCt values to ~2.2 (~4.6-fold up-regulation), after which these levels stayed near baseline for the remainder of normoxia (Fig. 20, Table E4.2-2 in Appendix E).

**Figure 20.** Exp. 4.2 HIF-1α mRNA expression of adult male *Cyprinodon variegatus* liver from very severe acute hypoxia (~1.08 mg/L DO) and 4tOP with a transition back to normoxia over 144 hrs. Statistically significant pTs were identified based on treatment, relative to normoxia solvent control (NSC), over time and shown by a star *. 
The N4OP exposure also had an initial significant peak in HIF-1α -ΔΔCt values of ~2.7 (~6.5-fold up-regulation) by 2 hrs, and then this transcriptional expression declined to and stayed near baseline for the remaining part of hypoxia and the first couple of hours into normoxia, staying near baseline (Fig. 20, Table E4.2-2 in Appendix E). At 7 hrs into normoxia, N4OP had a second significant peak in the -ΔΔCt values of ~2.2 (~4.6-fold up-regulation), and levels declined to near baseline for the rest of the normoxia exposure (Fig. 20, Table E4.2-2 in Appendix E).

HIF-2α. Transcriptional expression of HIF-2α for exposure 4.2 displayed significance relative to treatment, time duration, and the interaction of treatment over time (Fig 21, Table E4.2-3 in Appendix E). After the initiation of hypoxia and/or the presence of 4tOP, there was an oscillating non-distinct change of HIF-2α ΔCt values in all three of the experimental treatments, relative to NSC ΔCt values, at 2 hrs (Fig 21, Table E4.2-3 in Appendix E). NSC did not follow a straight line but instead a sinusoidal curve, with a small fluctuation in this baseline (Fig 21, Table E4.2-3 in Appendix E).

The mRNA expression of HIF-2α displayed significant increase in the -ΔΔCt value of ~1.0 (~2.0-fold up-regulation) at 7 hrs into hypoxia during H4OP relative to NSC (Fig 21, Table E4.2-3 in Appendix E). By 24 hrs into hypoxia, the -ΔΔCt value declined significantly below baseline to ~1.1 (~2.1-fold down-regulation) for the H4OP treatment, and these values stayed below and near baseline for the rest of hypoxia and into the transition into normoxia (Fig. 21, Table E4.2-3 in Appendix E). At 96 hrs and 144 hrs, H4OP had another significant decline in HIF-2α -ΔΔCt value of ~1.2 and ~0.9 (~2.3-fold and ~1.9-fold down-regulation, respectively) (Fig. 21, Table E4.2-3 in Appendix E).
The Hyp treatment of HIF-2α stayed near baseline throughout the 72 hrs of hypoxia, except with a significant -ΔΔCt down-regulation of ~1 (~2-fold down-regulation) at 24 hrs (Fig. 21, Table E4.2-3 in Appendix E). After the onset of normoxia, the Hyp treatment had a significant increase in the -ΔΔCt values of ~0.9 and ~1.1 (~1.9-fold to 2.1-fold up-regulation) of HIF-2α transcription above baseline at 74 to 79 hrs, respectively (Fig. 21, Table E4.2-3 in Appendix E). For the remainder of the normoxia, HIF-2α levels stayed around baseline (Fig. 21, Table E4.2-3 in Appendix E).

![Figure 21](image)

Figure 21. Exp. 4.2 HIF-2α mRNA expression of adult male *Cyprinodon variegatus* liver from very severe acute hypoxia (~1.08 mg/L DO) and 4tOP with a transition back to normoxia over 144 hrs. Statistically significant points were based on treatment, relative to normoxia solvent control (NSC) over time and shown by a star *.

The N4OP treatment showed HIF-2α mRNA expression near baseline at the onset of hypoxia (Fig. 21, Table E4.2-3 in Appendix E). At 24hrs into hypoxia, N4OP displayed a significant decline in the -ΔΔCt value of ~1.0 (~2.0-fold down-regulation)
(Fig. 21, Table E4.2-3 in Appendix E). During the remainder of hypoxia, the onset of normoxia, and throughout normoxia, HIF-2α mRNA expression remained just above or near baseline (Fig. 21, Table E4.2-3 in Appendix E).

**PHD3.** Transcriptional expression in PHD3 of exposure 4.2 displayed only non-distinct transcriptional expression in any of the experimental treatments (H4OP, Hyp, and N4OP) relative to NSC (Fig. 22, Table E4.2-4 in Appendix E). None of these treatment exposures displayed any significant up-regulation of PHD3 mRNA expression (Fig. 22, Table E4.2-4 in Appendix E).

![Graph](https://via.placeholder.com/150)

*Figure 22. Exp. 4.2 PHD3 mRNA expression of adult male *Cyprinodon variegatus* liver in very severe acute hypoxia (~1.08 mg/L DO) and 4rOP with a transition back to normoxia over 144 hrs (no statistically significant points).*

**VTG.** Transcriptional expression of VTG in exposure 4.2 demonstrated significance in terms of treatment, time duration, and the interaction of treatment over time (Fig. 23, Table E4.2-5 in Appendix E). All three experimental treatments (H4OP,
Hyp, and N4OP) displayed increasingly large (exponential) amounts of VTG transcripts over the time course of their respective treatment in comparison to NSC (Fig. 23, Table E4.2-5 in Appendix E). VTG mRNA expression levels were assessed over the 144 hr period for H4OP, N4OP, and Hyp, and they displayed significance throughout most of the exposure (Fig. 23, Table E4.2-5 in Appendix E) with a very rapid increase (within 2 hrs) in VTG mRNA levels. There were very large increases in the mRNA expression of VTG from H4OP treatment above baseline, and they were expressed at time points 7 hrs to 144 hrs, with significant -ΔΔCt values ranging from ~7 to ~13.5 (~128-fold to ~11,585-fold up-regulation, respectively) (Fig. 23, Table E4.2-5 in Appendix E). Large, significant, transcriptional increases in VTG were also displayed in the N4OP treatment during the same time frame of 7 hrs to 144 hrs, with significant -ΔΔCt values similarly ranging from ~7 to ~13.5 (~128-fold to ~11,585-fold up-regulation, respectively) (Fig. 23, Table E4.2-5 in Appendix E). Both of these exposures had exponential increases in VTG from 7 hrs to 72 hrs, as noted by these large -ΔΔCt values, with declines that occurred from 72 hrs to 96 hrs (onset of normoxia), leaving -ΔΔCt value of ~11 (~2048-fold up-regulation), followed by modest increase in the -ΔΔCt values by 144 hrs (end of exposure) (Fig. 23, Table E4.2-5 in Appendix E).

The Hyp treatment had much lower, but significant VTG induction levels than the other two exposures over the entire time-frame of the exposure. The -ΔΔCt values increased rapidly (within 2 hrs) after initiation of exposure to 4tOP, with non-significant values of ~1.5 (~2.8-fold up-regulation) (Fig. 23, Table E4.2-5 in Appendix E). Interestingly, there was a significant decline in VTG mRNA expression at 24 hrs where the -ΔΔCt value were found to be ~2.3 (~4.9-fold down-regulation) (Fig. 23, Table E4.2-
5 in Appendix E). However, the -ΔΔCt values of the hypoxia treatment then increased again becoming significant at 72 and 74 hrs with -ΔΔCt values of ~3.8 and ~2.5 (~13.9-fold and ~5.7-fold up-regulation) respectively (Fig. 23, Table E4.2-5 in Appendix E).

![Graph showing mRNA expression levels](image)

**Figure 23.** Exp. 4.2 VTG mRNA expression of adult male *Cyprinodon variegatus* liver during severe very acute hypoxia (~1.08 mg/L DO) and 4tOP with a transition back to normoxia over 144 hrs (statistically significant pts identified by * for treatment and time).

**Exposure 4.2, very severe acute hypoxia, with/without 4tOP, in testes samples**

**EPO.** Gene transcription was assessed at the transition from hypoxia (72 hrs) back to normoxia (74 and 79 hrs) for the testes tissue samples. The EPO mRNA expression level from testes tissue demonstrated no significance for H4OP, N4OP, and Hyp compared to the normoxia solvent control (NSC) at any of the three time points (72, 74, and 79 hrs) (Fig. 24, Table E4.2-6 in Appendix E). The -ΔΔCt values at 72 hrs
ranged a maximum of ~1.4 (~2.6-fold with both up- and down-regulation) and a relatively large variance (Fig. 24, Table E4.2-6 in Appendix E).

![Figure 24](image_url)  
*Figure 24. Exp. 4.2. EPO mRNA expression of adult male *Cyprinodon variegatus* testes in very severe acute hypoxia (~1.08 mg/L DO) with a transition back to normoxia.*

**HIF-1α.** Transcriptional expression of HIF-1α from testes tissue for the hypoxia exposure showed near baseline, non-significant expression during the time-frame of 72 hrs. to 79 hrs. in the transition from hypoxia to normoxia (Fig. 25, Table E4.2-7 in Appendix E). The maximum -ΔΔCt value found for any of the treatments compared to the normoxia control was ~4 (~16-fold up-regulation) at 74 hrs. for H4OP, but with a large variance (Fig. 25, Table E4.2-7 in Appendix E). There was no significance by treatment, and only by time points.
Figure 25. Exp. 4.2. HIF-1α mRNA expression of adult male *Cyprinodon variegatus* testes in very severe acute hypoxia (~1.08 mg/L DO) with a transition back to normoxia.

*HIF-2α.* Transcriptional expression of HIF-2α for hypoxia plus 4tOP, normoxia plus 4tOP, and hypoxia only for these three treatment exposures at the three time points (72, 74, and 79 hrs.) for the transition from normoxia to hypoxia demonstrated no significance by treatment and only by time point (Fig. 26, Table E4.2-8 in Appendix E). The maximum -ΔΔCt value between any treatment and the normoxia control was found to be ~2.5 (~5.7-fold up-regulation) at 74 hrs. (normoxia), but with a large variance (Fig. 26, Table E4.2-8 in Appendix E).
Figure 26. Exp. 4.2 HIF-2α mRNA expression of adult male *Cyprinodon variegatus* testes in very severe acute hypoxia (~1.08 mg/L DO) with a transition back to normoxia.

**PHD3.** Transcriptional expression of PHD3 for hypoxia plus 4tOP, normoxia plus 4tOP, and hypoxia only exposure had near baseline activity between all treatments compared to the normoxia control at all three time points 72, 74, and 79 hrs., and with a large variance (Fig. 27, Table E4.2-9 in Appendix E). Therefore, no significant change in transcriptional PHD3 expression (-ΔΔCt values) occurred for any of these exposures during this time-frame transitioning from hypoxia to normoxia (Fig. 27, Table E4.2-9 in Appendix E).
Figure 27. Exp. 4.2 PHD3 mRNA expression of adult male *Cyprinodon variegatus* testes in very severe acute hypoxia (~1.08 mg/L DO) with a transition back to normoxia.

**Microarray Analyses**

The 16 microarrays prepared using Cy3-labeling were composed of four replicates at two time points (72 hr. hypoxia and 74 hr. after transition to normoxia) for two different exposure types: (1) hypoxia to normoxia + 4tOP (H4OP→N4OP) at 72 hrs., (2) hypoxia to normoxia control (HC→NC) at 72 hrs., (3) hypoxia to normoxia + 4tOP (H4OP→N4OP) at 74 hrs., and (4) hypoxia to normoxia control (HC→NC) at 74 hrs.

Feature extraction of these scanned arrays (genes prepared in triplicate) generated 4896 genes expression signals. Of this total number of gene expression signals detected, 3349 signals demonstrated a viable signal:noise ratio distinct from the background level on at least 12 of the 16 microarrays. After combining of these triplicate signals and then cross-referencing the signals to the annotated Sheepshead minnow gene expression library
(previously prepared), which was the basis for the microarrays produced, there were 1414 genes identified. Two-way ANOVA of the mean and variances of these compiled and identified 1414 genes showed that 125 of them displayed a significant change in either up-regulation or down-regulation within one or more of the four exposure types listed above.

A 16 X 16 = 256 scatterplot matrix, based on a pairwise (array vs array) method of comparison where the 16 arrays are plotted against each other (multivariate correlation), demonstrated a linear 45 degree angle distribution scatter plot between opposing arrays (Appendix F). This procedure is done in order to confirm a normal distribution of data points between all of the separate arrays, after data standardization by dividing the individual data points for each probe by the mean.

1) **Volcano plots for significant gene expression data.**

Four volcano plots (Figs. 28-31), based on multiple comparison post hoc tests, demonstrate significant genes (p<0.002) which are represented by the dots that are located above the horizontal line of -Log10(p) = -2.814 of each volcano plot. The significant genes of each exposure combination with the other exposure groups are (Normoxia – Control = H→NC (74 hrs.), Normoxia – 4tOP = H→N4OP (74 hrs.), Hypoxia – Control = H→NC (72 hrs.), Hypoxia – 4tOP = H→N4OP (72 hrs.)). The dots to the right of the zero and above the horizontal line refer to genes that have been significantly up-regulated and the dots to the left of the zero and above the horizontal line refer to genes that have been down-regulated. Listings of the significant genes for Figures 28-31, in reference to each of the volcano plots, are detailed in Appendix G (Tables G1 – G4, respectively).
2) Venn diagram to display aggregation and intersection of significant genes.

A Venn Diagram (Fig. 32) displays the 125 genes that demonstrate significant changes in gene expression, in terms of either up-regulation or down-regulation. The Venn Diagram was produced by comparing the significant genes of each exposure combination with the other exposure groups (Normoxia – Control = H→NC (74 hrs.), Normoxia – 4tOP = H→N4OP (74 hrs.), Hypoxia – Control = H→NC (72 hrs.), Hypoxia – 4tOP = H→N4OP (72 hrs.)). The distribution of unique and intersecting significant genes is shown in the Venn Diagram (Fig. 32).

The Venn Diagram displays four unique ovals (A-D), each representing four unique sets of expressed genes (Appendix G, Tables G1-G4), based upon isolating the differences in the genes expressed due to the interaction of two different exposure parameters (DO and Treatment) where the two exposures being compared vary in only one of the two exposure parameters, in order to determine the number of genes expressed due to a single parameter (Fig. 32). The DO factor refers to a condition of either hypoxia or normoxia and the treatment factor refers to either to a condition of control or 4tOP. Each oval in the Venn Diagram represents the number of unique genes expressed in response to one of the two condition of each of the two treatments. The Venn Diagram (Fig. 32) identifies the number of unique genes to each of four ovals (A-D), along with the number of genes common to two or more of these ovals (intersections of common gene expression), with 15 total possibilities (four groups of unique gene expressions, six groups of dual interactions, four groups of triple interactions, and one group of interaction of all four ovals).
Figure 28. Significant genes (black) from post hoc comparison of $H \rightarrow N4OP$ 72 hrs. versus $H \rightarrow NC$ 72 hrs. Dashed line is p-critical value with significant pts above it.

Figure 29. Significant genes (black) from post hoc comparison of $H \rightarrow N4OP$ 72 hrs. versus $H \rightarrow N4OP$ 74 hrs. Dashed line is p-critical value with significant pts above it.
Figure 30. Significant genes (black) from post hoc comparison of H→NC 72 hrs. versus H→NC 74 hrs. Dashed line is p-critical value with significant pts above it.

Figure 31. Significant genes (black) from post hoc comparison of H→N4OP 74 hrs. versus H→NC 74 hrs. Dashed line is p-critical value with significant pts above it.
There are a total of 78 genes (outer two ovals, Fig. 32 A and D of the Venn Diagram) that are expressed based on the presence of 4tOP (difference of DO*Treatment), with 26 of these genes being uniquely expressed in the presence of hypoxia and 26 of these genes being uniquely expressed in the presence of normoxia and another 26 of these genes being commonly expressed genes (intersection) exposed to 4tOP within either the hypoxic or normoxic state. Thus 78 out of 125 (62.4%) of the significant gene expression shown revolves around the presence of 4tOP in either state of DO.

There are a total of 26 genes (inner two ovals, Fig. 32. B and C, of the Venn Diagram) that are expressed based on the presence of hypoxia (difference of DO*Treatment), with 13 of these genes being uniquely expressed in the presence of 4tOP and 13 of these genes being uniquely expressed in the presence of control conditions (no 4tOP) and no genes that are commonly expressed (intersection) with hypoxia with or without 4tOP. Thus 26 out of 125 (20.8%) of the significant gene expression shown revolves around the presence of hypoxia.

Additionally there are other multiple intersections of common gene expression, which represent the remaining 21 out 125 significant genes or 16.8%. Intersection of ovals A and B of the Venn Diagram (common genes from 4tOP under hypoxia conditions and from hypoxia under 4tOP conditions) displays five common genes (4%) (Fig 32). Intersection of ovals C and D of the Venn Diagram (common genes from hypoxia under control conditions and from 4tOP under normoxia conditions) displays two common genes (1.6%) (Fig. 32). Intersection of ovals A and C of the Venn Diagram (common genes expressed from 4tOP under hypoxia conditions and from hypoxia under control
conditions) displays 5 common genes (4%). Intersection of ovals B and D of the Venn Diagram (common genes expressed from hypoxia under 4tOP conditions and from 4tOP

Figure 32. Significant Index for differences in interaction term of the two main variables (DO*Treatment) between different samples or exposure scenarios to isolate the gene expression created from DO (hypoxia vs normoxia) and Treatment (control vs 4tOP).
under normoxic conditions) displays 4 common genes (3.2%). The Venn Diagram also shows four interactions of three of the four ovals, with only one of these interactions showing five common genes (4%) (ovals A, B, and D showing gene expression from 4tOP under hypoxia and under normoxia and gene expression from hypoxia under 4tOP) (Fig. 32). Lastly, the intersection of all four ovals (A-D) shows that there are no common genes expressed amongst all four ovals or the intersection between them (Fig. 32).

*Clustering of gene expression data.* Using hierarchical clustering, Fig. 33 displays two major groupings of related gene expression, in terms of either up-regulation or down-regulation of each respective gene, based upon treatment (control versus 4tOP) and DO (normoxia versus hypoxia). Hierarchical clustering is a method of analyzing the relative degrees of similarities or relatedness in gene expression patterns for the 16 microarrays that are based upon the two main variables of treatment (4tOP versus control) and DO (normoxia versus hypoxia), where the individual microarrays represent the seeds for the clustering analysis and cluster data points based on relative differences in gene expression values between microarrays for each gene. Agglomerative clustering for the 16 microarrays, set up based upon treatment (presence versus absence of 4tOP) and DO (normoxia versus hypoxia), was used to build the dendogram where the tree was built from the bottom up, by putting the most similar genes together first and working upward until all the branches of the tree have been formed. Based upon this analysis, Fig. 33 shows that the 16 microarrays cluster predominantly by treatment (presence or absence of 4tOP). In other words that 4tOP has a larger role in gene expression than the difference in DO levels.
Hierarchical Clustering groups all genes into a dendogram based upon similar patterns of expression, with each array being the basis for the 16 clusters, showing major branches that separate the arrays more strongly by Treatment (4tOP vs Control) than by DO (normoxia vs hypoxia). Increasing intensities of blue represents greater down-regulation of genes, whereas increasing intensities of red represents greater up-regulation of genes. Microarrays cluster predominantly by treatment (presence or absence of 4tOP).

**Gene categorization and gene ontology.** A compilation of all the known and annotated genes from the sheepshead Minnow (*Cyprinodon variegatus*) cDNA library that were identified as significant in their gene expression levels, as previously determined by two-way ANOVA from Jump Genomics 5.0 (Figures 28-31 and Tables E1-E4), is shown in Table 4. These genes were categorized by cellular and metabolic function, based on gene ontological information. Five categories of metabolic functioning were used to categorize this group of genes: (1) Immune Response and Detoxification, (2) Cell Growth/Metabolism and Cell Membrane Transport/Functioning, (3) DNA/RNA Activity, Transcription, and Chromosomal Packaging, (4) Signaling and Receptors, and (5) Reproduction. The first three of these five biochemical pathway
groups contain all but two of these genes, with two other genes placed into the last two categories. With only two exceptions, all of the genes that can be referenced to a particular known gene that are listed as a statistically significant gene for each treatment pairwise comparison are significantly down-regulated (Table 4). Of the remaining genes that cannot be referenced to a known gene, all but just a couple of these are also significantly down-regulated. Thus it appears that most of the physiological impacts that occur from these four treatment combinations involving hypoxia and the EDC 4tOP have negative or slowing effects on the liver cells.

Of the five previously mentioned five significant biochemical pathway categories for C. variegatus liver samples (Table 4), the most commonly expressed category was Cellular Functioning and Metabolism, and these functions or activities seemed to be slowed (down-regulated) under these treatment conditions (Table 4, Appendix G). The second most prominent category listed in Table 4 is the category of most interest, because it refers to various proteins/genes involved in Immune and Detoxification functions, and it applies to approximately one-third of all the genes in this table. Thus there appears to be a consistent stress response occurring in the adult Sheephead minnow affecting, what appears to be, an unusually high percentage of the immune or detoxification genes identified.

For Hypoxia to Normoxia + Control (H→NC) at 72 hrs. – Hypoxia to Normoxia + Control (H→NC) at 74 hrs. (gene expression from control treatment two hours after the return normoxia), there was very little significant change in gene expression. The only named gene with a significant change (down-regulation) was the histone H2A for packaging DNA (supercoiling DNA) (Table 4, Appendix G in Table G3).
Table 4

**Cellular Functions, by Gene Ontology, of the Significant Genes Isolated and Identified through Microarray Analyses of Cyprinodon variegatus Exposures.**

1. **Immune Response and Detoxification**

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<td>AAA92556.1</td>
<td>complement regulatory plasma protein</td>
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<td>BAF43314.1</td>
<td>skin mucus antibacterial l-amino acid oxidase</td>
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<td>lily-type lectin</td>
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<tr>
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2. **Cell Growth/Metabolism and Cell Membrane Transport/Functioning**

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3. **DNA/RNA Activity, Transcription, and Chromosomal Packaging**

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4. **Signaling and Receptors**

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5. **Reproduction**

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<td>VIT1_FUNHE Vitellogenin-1 precursor (Vitellogenin I) (VTG I)</td>
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For Hypoxia to Normoxia + 4tOP (H→N4OP) at 74 hrs. – Hypoxia to Normoxia Control (H→NC) at 74 hrs. (gene expression from 4tOP treatment two hours after the return to normoxia), there was a demonstration of a significant up-regulation in the immune response and detoxification based upon a significant increase in a lily lectin protein, which is involved in the innate immune system, pattern recognition, and pathogen elimination. However, glutathione S-transferase, which is an enzyme involved in glutathione and drug metabolism, along with the protein claudin 25, needed in cellular metabolism and tight junction formation and function, are two of several genes that show a significant up-regulation (Table 4, Appendix G in Table G4).

By comparison there is a down-regulation in the immune response for Hypoxia to Normoxia + 4tOP (H→N4OP) at 72 hrs. relative to Hypoxia to Normoxia Control (H→NC) at 72 hrs. (gene expression from 4tOP treatment under hypoxic conditions) based upon a significant down-regulation of a complement protein involved in the acquired immune system (Table 4, Appendix G in Table G1). Therefore, it appears that hypoxia down-regulates of some components of the immune system during exposure to an endocrine disrupting chemical (EDC), 4tOP. In terms of metabolism, the histone-lysine N-methyltransferase enzyme, along with the paraneoplastic antigen, are significantly down-regulated.

Additionally gene expression from hypoxia under 4tOP conditions Hypoxia to Normoxia + 4tOP (H→N4OP) at 72 hrs. – Hypoxia to Normoxia + 4tOP (H→N4OP) at 74 hrs. (gene expression from 4tOP treatment two hours after return to normoxia) displayed a significant down-regulation in complement regulatory plasma protein (acquired immune system) that regulates complement activation and gives protection
from complement damage itself. Metabolism is altered with a significant down-regulation in the activation of carboxypeptidase A1 enzyme, along with significant up-regulation of the intraflagellar transport protein 20 (Table 4, Appendix G in Table G2). Thus, there is up- and down-regulation of various immune response proteins appears in all these treatment hypoxia and 4tOP combination comparisons.

Discussion

Acute severe hypoxia did initiate an immediate transcriptional response that peaked within 2-7 hrs from all of the genes that were examined in these research studies, which include HIF-1α, HIF-2α, EPO, PHD, and VTG. The largest response came from HIF-1α, EPO and VTG, all of which were statistically significant responses with a 5-7-fold up-regulation in the production of mRNA. VTG was the only transcript that was significantly up-regulated by 4tOP alone.

*Transcriptional Response of Genes to Severe Hypoxia (~1.5 mg/L)*

EPO displayed a minor up-regulation in transcription, and this response declined to near baseline by the end of the hypoxic event and the beginning of the onset of normoxia. This EPO induction showed a minor downstream induction response, relative to the estuarine fish *C.variegatus*. Based on this response, these fish were under only minor hypoxic stress.

HIF-1α expression of mRNA is activated by the onset of hypoxia, and it occurred as an immediate response with increases in transcription beginning within as few as 30 minutes of the initiation of this stressor. These stronger initial responses tended to be followed by muted secondary oscillations of induction later into the hypoxia exposure and even into the 72 hrs of normoxia recovery. These oscillating responses in HIF-1α
transcriptional levels appear to be indicative of modulation in cellular response to adjust to levels based on real-time needs both during and, even more interesting, after hypoxia is over, for at least 3 days of post-hypoxia recovery. In other words, once the activation of HIF-1α had been initiated, the modulation of the cell’s transcriptional activity appears to continue until normal oxygen levels (pO₂) within the tissue have been re-established in the tissues. Thus it appears that the immediate end to the environmental hypoxia does not equate to the immediate end of the physiological impact from the hypoxia, and thus the continued transcriptional activity to produce more HIF-1α to mediate the necessary and energy-saving transition back to aerobic respiration, as demonstrated by rapid declines in blood lactate levels of gulf killifish during the recovery from hypoxia (Nazeem and Rees, 2000).

HIF-2α mRNA levels displayed no significant response to the hypoxic exposure, with induction levels staying near baseline. It appears that HIF-2α plays a very minor role in the induction of downstream hypoxia genes in C. variegatus liver cells, as compared to the well-known, primary transcriptional role of the HIF-1α isoform.

PHD3 showed no significant up- or down-regulation from an acute hypoxic response, which was quite different from the distinct up-regulation in response due to a chronic hypoxic exposures described in Chapter III. It appears that amount of PHD3 produced is produced in smaller and less consistent amounts compared to PHD2 which acts as the primary oxygen sensor for vertebrate organisms. Production and activation of the PHD3 enzyme does not occur until the onset of normoxia recovery (Huang et al., 2002). However, because of the relatively long half-life of proteins, it may be that there
was adequate catabolic enzyme available for HIF-1α degradation without more PHD3 transcripts being produced.

_Transcriptional Response of Genes to Very Severe Hypoxia (~1.08 mg/L)_

_Hyp_. EPO expression of mRNA showed a significant ~3.0-fold up-regulated response from 2 hrs to 7 hrs, before declining to baseline for the remainder of the exposure. Therefore, it appears that a more severe hypoxic response does indeed sufficiently stress _C. variegatus_ sufficiently to induce HIF-1α to initiate downstream activation of target genes such as EPO to help maintain aerobic metabolism within the fish.

HIF-1α expression of mRNA is significantly up-regulated by the onset of hypoxia, and its transcriptional response is quite similar to what was described in the previous section for hypoxia (1.5 mg/L).

HIF-2α expression of mRNA is significantly up-regulated within the first 7 hrs of the hypoxic exposure. This induction is brief and declines to and remains near baseline for the remainder of the exposure. Although a smaller induction than found with HIF-1, under a more severe hypoxic event, there appears to be a supportive or additional role that HIF-2α plays in the activation of the cascade of downstream genes that are used to metabolically cope with a more severe episode of this stressor.

PHD3 showed no significant up- or down-regulation from a more severe acute hypoxic response, very similar to what was described in the last section.

VTG was also activated by hypoxia and gave a significant up-regulation in transcriptional response within 72 hrs of exposure. Induction of VTG by hypoxia does appear to support previous research implying that there is cross-talk or communication
between the biochemical pathways for activation of the HIF response as well as the reproductive pathway, in terms of feminization, for activation of VTG needed as the egg-yolk precursor protein for developing eggs for spawning in fish. This confirms other research that showed a possible communication or cross-talk between hypoxia and the estrogen receptor 1 (ESR1) protein and key proteins in the hypoxic response of rat lung (Wu et al., 2008), and other research that similarly demonstrated synergistic effects of estrogen and hypoxia on ERα-mediated transactivation in breast cancer cells (Jinhyung et al., 2009). Additionally research has also indicated that hypoxia can play a role in endocrine disruption in Atlantic croaker by causing significant impairment of ovarian growth as well as decreased production of fully grown oocytes, thus reducing fecundity by a reduction in viable gametes by limiting the signaling pathway for VTG production needed in developing ovaries (Thomas et al., 2006). The induction of VTG in this present study appears to run counter to these findings.

*4tOP Exposure.* Exposure to 4tOP similarly demonstrated an initial immediate transcriptional up-regulated response for HIF-1α, HIF-2α, EPO, PHD3, and strongly for VTG. The immediate up-regulated responses that occurred for HIF-1, HIF-2, EPO, and PHD3, and mimicked the hypoxic response, were not significant and tended to be more muted in magnitude than with hypoxia exposure.

Additionally, the exposure of the estrogenic chemical 4tOP strongly up-regulated the VTG transcription in male *C. variegatus*, with maximum significant induction levels of 11,585-fold increase in mRNA levels for N4OP within 72 hrs of exposure, and significant inductions as early as 7 hrs at 128-fold increases. This is a clear sign of early feminization at the cellular level, and at more advanced stages can easily be detected in
blood samples from the male fish (Karels et al., 2003). The present results giving distinct indication of large activation of VTG mRNA in male fish, and thus feminization and an early stage of endocrine disruption. Even with continued 4tOP exposure, these induction levels decline to ~2048-fold, and continue to maintain these very levels. The decline in these initially very high mRNA levels of VTG may occur because of more than adequate levels needed to produce adequate levels of the vitellogenic protein (egg-yolk precursor) for egg production. Maintaining excessively high VTG transcriptional levels requires energy (ATP) at the cellular level, and this cannot be maintained without a need or impetus from a long-term physiological change, and thus the dampening of this initial over-shoot in response, as referenced to with gulf killifish (Nazeem and Rees, 2000).

Combined 4tOP plus hypoxia exposure. VTG mRNA levels rapidly increase and become significant within 7 hrs at ~128-fold increases for the H4OP treatment, and these levels continue to increase exponentially to a peak of ~11,585-fold induction levels. A slightly quicker induction, increased magnitude and duration magnitude of the VTG response from the H4OP treatment versus the N4OP treatment lent credence to the idea that hypoxia appears to cross-talk within the reproductive pathway of vitellogenin production. There also appears to be an additive effect in the induction levels produced for VTG with combination of hypoxia and 4tOP. An estrogenic compound in the water can potentially mimic or reinforce a hypoxic response in stimulating HIF-1α expression and strengthen the initial cascade effect on the downstream genes in terms of the cross-talk described by the link between hypoxia and ER in down-regulation of key hypoxia regulated proteins in breast cancer cells (Jinhyung et al., 2009) and rat lung cells (Wu et
In this study, it appears that the hypoxia is reinforcing the estrogenic effects of VTG on male C. variegatus.

There is ambiguous evidence for any additive impact, let alone synergistic impact, from the combination of 4tOP with hypoxia for the other four target genes: HIF-1α, HIF-2α, EPO, and PHD3. Occasionally this combined response was the greatest treatment response, but by a small fraction and also not consistently for any of these genes. There was definitely an additive effect on increasing VTG levels with the combination of 4tOP and hypoxia, in terms of increased magnitude of VTG expression, earlier induction, higher peak levels, and greater longer term response levels.

Transcriptional Response of Genes to Hypoxia and 4tOP in Testes Samples

Testes samples displayed muted non-significant expression levels of HIF-1α, HIF-2α, EPO, and PHD3. Thus the data on the male gonads of C. variegatus did not lend any real insight into a significant physiological response.

Intravariability

These studies re-emphasize the dynamic nature of gene expression in an organism over short periods of time to a stressor like hypoxia. All of the transcriptional expression level data collected does also point to the fact there is indeed a wide range of intravariability among the individual samples and thus between the individual fish. This naturally large range in physiological response lends support to the built-in robustness of the physiological variability within the responses of individual organisms and how this bodes well for the survival of a species from an evolutionary standpoint, because this is the raw material upon which natural selection operates (Virani and Rees, 2000). The overall conclusion from these results also emphasizes the fact that future work should
focus on fewer sampling points with larger numbers of individual adult fish sampled, and 
thus balance the quantity with the quality of the data collected and analyzed and the 
overall cost involved on a per sample basis. Thus, increased biological replicates along 
with the technical replicates analyzed. At a minimum, this strategy would allow for 
easier evaluation of individual responses within a group to determine what is normal 
versus and an outlier, and at a minimum further dampen an extremely low or high 
transcriptional response from one individual. Either way, it would lend further strength 
to the results of the data.

**Gene Categorization and Gene Ontology**

Gene ontology showed that there was a mix of up- and down-regulation in the 
immune response system, as well as genes involved in cellular metabolism from exposure 
to either 4tOP or hypoxia. The immune system has already been shown to be activated 
by exposure to hypoxia, in terms of activating CD3-engaged T-cells biochemically drawn 
to inflammatory sites around hypoxic tumors and activating HIF-1 and its target genes 
(Nakamura et al., 2005). Additionally, HIF-1 induction in liver and in macrophages has 
also been shown to elevate levels of inflammatory cytokines (Wang et al., 2010).

The dynamics of cellular metabolism centers around the idea that there is a 
fundamental shift in the genes turned on or up-regulated for aerobic metabolism (Lee et 
al, 2004; Van der Meer et al., 2005) during a transition back to normoxia versus the 
genes that are up-regulated for anaerobic metabolism (Zagórska and Dulak, 2004) during 
hypoxia. There were 1414 identified genes from the C. variegatus microarrays, with 125 
genesis identified as transcriptionally altered at a significant level, (~9% of these genes), 
and this is compared to 3% significant genes of over 15,000 genes identified as either up-
or down-regulated from the gills of adult zebrafish exposed to hypoxia (Van der Meer et al., 2005). The variety of genes that can be altered between hypoxia and normoxia include the control of vascular system (angiogenesis and vasomotor control), maturation of red blood cells (erythropoiesis and iron transport), energy metabolism (glycolysis, glucose transport, and the multifunctional enzyme glyceraldehydes-3-phosphate dehydrogenase), cell proliferation and viability (arrest of cell cycle, apoptosis, and growth factors), pH regulation, nucleotide metabolism, matrix metabolism, catecholamine synthesis, and negative feedback regulation of HIF-1 transactivation (Zagórska and Dulak, 2004).

The question then becomes whether or not this is a short-term response or pulse in these biochemical pathways, or could these changes lead to a long-term shift in biochemical pathways, leading to a changed physiological state. Based upon the fluctuations over time in some of the gene responses studied in this dissertation, it appears that the individual cells have a lot of flexibility or adaptability to adjust to environmental changes, and that it would take a long-term or chronic exposure to an exogenous or environmental cue to lead to an altered or weakened physiological state. In this case, it seemss that hypoxia and 4tOP both lead to modest to moderate alterations in physiological states. It also does appear that gene expression effects from these exposures seem to be more prominent when these two stressors are combined, in an additive sense. However, it should be noted that the C. variegatus cDNA library used for these studies was not complete, and that this could have some impact on the window of gene expression that could be viewed. Additional exposure studies with a more complete
and robust *C. variegatus* cDNA library would lend support to the findings in this research.
CHAPTER V
CONCLUSIONS AND SUMMARY

Overall Objectives and Significance

The goal of this dissertation research was to gain insight on the physiological responses that occur at the cellular level to the estuarine fish sheepshead minnow (Cyprinodon variegatus) under the multiple environmental stressors of hypoxia and the estrogenic compound 4tOP. From measuring the transcriptional changes in HIF-1α, HIF-2α, EPO, PHD3, and VTG, greater insight was obtained on the strategy that this fish uses to cope with changing DO levels and the influence of anthropogenic compounds mimicking estrogenic hormones. These results lend to insight into the homeostatic responses that aquatic organisms must do to survive and meet their energy needs by shifting from aerobic to anaerobic metabolism and back again, compounded with the insult of a second stressor present. Ultimately, the goal is to gain understanding of these physiological processes that help to understand what occurs in the natural environment with multiple stressors or dynamics occurring at all times.

Conclusions

Overview of Dissertation

Phylogeny of the two distinct cypHIF-α sequences confirm that they represent cypHIF-1α and cypHIF-2α isoforms, which clusters with the respective HIF-α isoform from other fish species that are phylogenetically closely related to each other, and therefore these specific HIFαs show nearly the same evolutionary relatedness as these fish. These two HIF-α isoforms are much more closely related to each other than the HIF-3α & HIF-4α isoforms. The PAS-A and PAS-B domains and the PAC region are
highly conserved among all species sequences, while ODDD, although important for degradation of leftover HIF-α, is a much more variable region in terms of unique base pair sequence and in terms of its size. Thus, the amino acid sequence of ODDD and its 3-D folding pattern are not nearly as conserved as the PAS domains in the evolutionary development of the role of HIF-α gene isoforms.

Phylogeny of cypPHD sequence confirms that it is a member of the 2OG Fe(II) Superfamily. Within this superfamily, the cypPHD clusters firmly with other PHD3 isoforms with a shorter amino acid sequence than the other two closely related isoforms of PHD1 and PHD2. Based on the PHD phylogram (Fig. 7), the PHD3 isoform appears to be evolutionarily the oldest, as well as the shortest, of the PHD1-PHD3 isoforms. Therefore, PHD3 acts as the original prolyl hydroxylase gene for gene duplication of the newer and longer isoforms of PHD1 & PHD2. This additional N-terminal amino acid sequence of PHD1 and PHD2 is much more variable, and it is needed for their specialized function, such as the zinc finger structural motif in PHD2 to coordinate Zn ions to stabilize its 3-D folding pattern.

In Exposure 3.1 (Moderate Chronic Hypoxia, 2.5 mg/L DO), there was no differential expression in PHD3 gene expression between males & females. Initial induction of PHD3 expression (~16-fold up-regulation) peaked at 10 hrs then progressively declined to near baseline by (~2.8-fold up-regulation) by 168 hrs. This suggests that homeostatic balance had been regained early on in the exposure, and that the hepatocytes made more than enough mRNA to translate into prolyl hydroxylase enzyme for HIF-1α degradation. The excess mRNA was gradually degraded during the
majority of the exposure. Physiologically, it appears that the hepatocytes were able to maintain aerobic metabolic functioning, and thus eliminate unneeded PHD3 mRNA.

In Exposure 3.2 (Severe Chronic Hypoxia, 1.5 mg/L DO), there was no differential expression in PHD3 gene expression between males and females. Initial induction of PHD3 expression (~8-fold up-regulation) at 10 hrs was followed by increasing levels of mRNA over the entire 168 hrs (~64-fold up-regulation). It appeared that the liver cells were continually producing more PHD3 mRNA to translate enough of this prolylhydroxylase enzyme in the hepatocytes to degrade the HIF-1α to prevent HIF-1α from accumulating once there was a return to normoxia. The continued increase, or at least maintenance of high levels of PHD3 transcripts, implies the continued presence of active HIF-1α and the inactivity of PHD3 to mark HIF-1α for degradation. This may be an indirect measure of these liver cells losing ground in aerobic metabolism from chronic low pO₂ caused by hypoxia and shifting over to anaerobic metabolism. Thus, severe chronic hypoxic exposure appears to dictate a more extreme physiological response by hepatocytes. Hypoxia initiates an increase in HIF-1α, with HIF-1α transactivating PHD3 transcription, with the continued translation of these PHD3 transcripts during the onset of normoxia, along with the activation of the PHD3 catabolic enzyme acting as the trigger in a negative feedback loop to limit the accumulation of HIF-1α (Berra et al., 2003; Marxsen et al, 2004; Zagórska and Dulak, 2004).

In Exposure 4.1 (Severe Acute Hypoxia with 1.5 mg/ml DO, then back to Normoxia), the initial significant induction of the target genes (HIF-1α, HIF-2α, EPO, and PHD3) occurred within 30 minutes and peaked within 2 hrs, with induction levels of both HIF-1α and EPO mRNA significant. HIF-1α was significantly up-regulated in the
first 24 hrs in hypoxia, declined, and then oscillated up and down and at significant levels at 72 hrs (last hypoxia time point) and 77 hrs (normoxic recovery). EPO mRNA levels gradually increased, and become significant between 24-144 hrs which is the latter part of hypoxia and includes into the normoxic recovery. The liver cells were presumably responding to internal, low pO₂ caused by low DO, which was the physiological trigger to activate or transcribe HIF-1α, and start the cascade effect of downstream multiple gene activation, such as to increase vascularization and increase O₂ binding capacity in blood (EPO), thus the delayed induction response. The oscillations of transcription levels of HIF-1α and EPO appear to indicate a continued homeostatic aerobic recovery as much as 72 hrs into normoxia. PHD3 and HIF-2α target genes in exposure 4.1 had a similar induction, but much more dampened around the baseline level.

Exposure 4.2 (Multi-Treatment set-up) involved the target genes HIF-1α, HIF-2α, EPO, PHD3, and VTG, and it involved an initial 72 hr Very Severe Acute Hypoxia Exposure (1.08 mg/ml DO) followed by return to Normoxia, with and without 4tOP. The four treatment combinations of Normoxia Solvent Control (NSC), Normoxia-4tOP (N4OP), Hypoxia→Normoxia-Control (H→NC), and Hypoxia→Normoxia-4tOP (H→N4OP) appeared to show a biphasic response to hypoxia, with an initial response that occurred within 2-7 hrs of the onset of hypoxia, followed by a decline in mRNA levels back to near baseline. The transition back to normoxia sometimes appeared to be associated with a second induction of mRNA levels for these different genes in the various treatment exposures. The hypoxia treatment consistently caused the greatest second induction for HIF-1α, HIF-2α, EPO, & PHD3. VTG levels were induced by hypoxia + 4tOP and normoxia + 4tOP exposures, with induction levels being 1.5- to 2-
fold greater in Hypoxia + 4tOP (additive effect) relative to Normoxia + 4tOP. Induction began within 2 hrs for Hypoxia +4tOP and Normoxia + 4tOP, exponentially increased and were significant inductions in both exposures by 7 hrs, peaked at 72 hrs or the end point of hypoxia (~15,500-fold and ~10,100-fold respectively), and declined during the normoxia phase of the exposure. Additionally, the Hypoxia only exposure also caused a significant 76-fold increase in VTG levels in liver cells by the 72 hr time point, with a rapid decline to near baseline when transitioning into normoxia. It appears that hypoxia elevated VTG levels in these liver cells, and thus hypoxia appears to be acting as an estrogenic chemical in male *C. variegatus*.

There were 16 microarrays prepared using Cy3-labeling composed of four replicates at two time points (72 hr. hypoxia and 74 hr. after transition to normoxia) for two different exposure types: (1) hypoxia to normoxia + 4tOP (H4OP→N4OP) at 72 hrs., (2) hypoxia to normoxia control (HC→NC) at 72 hrs., (3) hypoxia to normoxia + 4tOP (H4OP→N4OP) at 74 hrs., and (4) hypoxia to normoxia control (HC→NC) at 74 hrs.. In terms of microarray analysis, Jump Genomics 5.0 was used, along with a manual analysis of gene ontology through NCBI. There were a total of 125 significant genes (up- and down-regulated), with a fraction of these genes being known or annotated genes. Many significant genes had no annotation, and this was a limiting factor to distinguish significant metabolic pathways that were up- or down-regulated during these exposures. However, the significantly expressed genes that were identified do appear to give some indication of up- and down-regulation in the areas of (1) immune responses and detoxification, (2) cellular metabolic functioning (anaerobic and aerobic), (3) DNA/RNA
activity, transcription, and chromosomal packaging; (4) signaling and receptors; and (5) reproduction under the different treatment combinations.

Transcriptional activation levels of PHD3 were medium in scale during acute hypoxia (~5-10-fold induction levels), relative to the large increases that occurred during chronic hypoxia (~60-fold). However these large PHD3 transcriptional increases in chronic hypoxia mostly occurred 4-7 days into the hypoxia exposure, in other words only under long-term persistent hypoxia (Figs. 13 and 14). Therefore these very large PHD3 transcriptional increases may only occur under these unusual or extreme circumstances that would only occur for estuarine species that are obligatory to living in these environments with dramatic and sudden changes in their hydrological parameters.

Additionally, there is also an issue of intravariability between individual fish species, which is a natural part of the range in genetic diversity within a species. This range in induction PHD3 induction signifies a need for future research to assess fewer key time points with ten or more individuals per time point to better assess this expression range and more accurately assess outliers.

In summary, the induction of the target genes HIF-1α, HIF-2α, PHD3, and EPO all occur during the initial hours of moderate and severe acute hypoxia and moderate chronic hypoxia exposure, peaked within ~10 hrs or less, declined during the remaining time of hypoxia, and approached baseline in an interrupted fashion or oscillated up and down during the noromoxia recovery, especially during the hypoxia alone exposure. Homeostatic balance appears to take hours to days after the elimination of the hypoxic stressor for complete aerobic recovery. Expression levels appear to take several days to re-establish baseline, commonly with a second milder induction into the transition back
to normoxia. Only during severe chronic hypoxia (more than 4 days) does the ability of
the hepatocytes appear to approach a physiological limit, indicated by continued
increases in the target gene as seen with PHD3, over the entire hypoxia exposure. In
some instances, target genes appears to produce an overshoot of needed mRNA levels,
with the excess transcripts degraded away as the hepatocytes re-establish homeostatic
balance.

The addition of 4tOP appears to have limited influence on the induction rates of
these four target genes. By comparison, VTG induction levels do significantly increase
with the combination of hypoxia along with 4tOP, relative to normoxia + 4tOP. The
removal of hypoxia appears to remove the additional activation of VTG expression.
VTG induction starts out slowly and goes into exponential increase, peaks briefly
(extremely high levels), goes into rapid decline, and stabilizes at an elevated level that is
represented only by the 4tOP. Additionally, the hypoxia only exposure does significantly
increase VTG mRNA levels well above baseline. Therefore, based on the hypoxia only
and the hypoxia + 4tOP exposures, it appears that the presence of hypoxia shows a
feminization response in male *C. variegatus* with the expression of VTG mRNA. There
appears to be an overshoot of the needed VTG mRNA levels in the normoxia +4tOP, the
hypoxia + 4tOP, and the hypoxia only exposures, with the excess transcripts degraded
away over the remainder of the exposures.

*Implications for Field Settings*

Hypoxia does occur naturally, but is commonly exacerbated in severity and
duration by human impact. Laboratory studies give insights into the physiological and
behavioral effects on various aquatic organisms found in bayous, estuaries, and rivers.
However, these control settings only open a small window on the overall impacts to these aquatic organisms. Most organisms are able to tolerate and survive various stressors in the environment, but the real issue is how well these aquatic organisms survive multiple stressors that laboratory studies do not mimic or emphasize. There are multiples field studies that lend insight into the dynamics of aquatic organisms in the wild, and some of these lend insight into how laboratory studies can be of further help of estimation and modeling efforts to better understand and protect these natural habitats and nursery grounds.

Further support of the idea that there is a physiological need for a period of recovery from a hypoxic event for any type of aquatic organism comes from exposure of larval marsh grass shrimp (*Palaemonetes vulgaris*) to both cyclic and constant hypoxia, where it was shown that reduction in growth (total body dry weights) of marsh grass shrimp represented \( \sim 73\% \) of the reduction of the the time-weighted average found in constant hypoxia at the same DO level (Coiró *et al*., 2000). This same growth impairment relationship also occurred in similar hypoxia exposure comparisons for sand mud crab (*Dyspanopeus sayi*) and summer flounder (*Paralichthys dentatus*) (Coiró *et al*., 2000). What was not yet clear was if the impairment imposed by hypoxia was due to one or a combination of parameters, including (1) severity, (2) duration, (3) frequency, and (4) the quickness of onset (acclimation) of hypoxic events. This type of calculation could be very useful to correlate parameters of physiological impact identified under more limited laboratory hypoxic conditions to estimations of possible physiological impacts found under more natural and changing hypoxic conditions in the environment, potentially
not just growth rates but also biochemical responses such as changes in gene transcription.

Obligate estuarine organisms have adapted to the changing environmental parameters of the estuary, but these areas are also nursery habitat for the juveniles of larger predatory fish. Another study has shown declines in growth rates of summer flounder as well as winter flounder (*Pseudopleuronectes americanus*) under sub-lethal hypoxic conditions (~3.5 mg/L) using two temperature regimes comparing laboratory studies with wild caught fish, which gave implications to reduced growth rates of these fish found in estuaries suffering frequent or long-lasting moderate hypoxic events (Stierhoff *et al.*, 2006). Moderate hypoxia events are not strong enough to force the flounder to relocate, but the conditions were sub-optimal enough to reduce their modeled growth rates relative to the lab-reared flounder (Stierhoff *et al.*, 2006).

Overall successful reproduction or spawning success can also be strongly influenced by the presence of daily hypoxia episodes. For Gulf killifish studied in Weeks Bay, AL and Penscola Bay, FL, it was shown that egg production estimates were 50-85% lower in estuarine sites that were impacted by diel hypoxia cycles, and that relative reproductive fitness as measured by the gonadosomatic index (GSI) could be used as a strong predictor of cumulative fertilized egg numbers (Cheek, 2011). Additionally, this study also showed that were correlations between egg production, GSI, body size of the female fish, and the 48 hr mean DO before spawning (Cheek, 2011). These fish are adapted to survive changing estuarine conditions, even changing DO levels, but when compounded with other dynamics stressors such as energy intake before spawning, this
can lead to limitations on how successful spawning can be and thus recruitment into the next generation.

Increased frequency, severity, and size of hypoxic events can also have significant impact on available habitat for larger, predatory fish not obligated to the estuaries. A study on juvenile weak fish (Cynoscion regalis) in the Indian River Bay of Delaware, USA demonstrated distinct moving patterns from the upper, middle, and lower sites of Pepper Creek (Tyler and Targett, 2007). These fish would quickly leave the lower and middle sites of Pepper Creek when hypoxia (<2 mg DO) occurred at these sites and congregated in large numbers at the normoxic upper site on Pepper Creek (Tyler and Targett, 2007). Occasionally, when the upper site was also hypoxic, these fish would also leave here too, but within 2 hours of DO exceeding 2 mg/L DO, they weak fish would return back to the upper site (Tyler and Targett, 2007). This rapid movement back into the upper site seems to give strong indication of the importance of this area for habitat for these weak fish, and it lends insight into possible limitations on good refuge sites for larger mobile fish in general where hypoxia occurs severely and or frequently, especially if exacerbated by human impact such as fertilizer run-off.
APPENDIX A

HIF-1α NUCLEOTIDE AND AMINO ACID SEQUENCES OF *CYPRINODON VARIETATUS*

(a) Cyp_HIF-1α

GTCAAGGAACCGAGCACAGAGCGAAGTTCTTCCTGCGGATGAAATGCACCCTCACCCAGCAGAGGGCGCACCGTTAATGTGAAGTCAGCCACATGGGAAGGTGCTGCATTGCTCGGGTCACGTTCGTGTTCACAGCAGTGAGCAGAGCGCCGACGGCCCTAAGGAGCCACCCGTCCCC

TATCTGTGTCACATTGTGACCCCATCCCCACCCTTCCAACATCGAGGTCCCTCTGGACACCAAGACCTTCCTTAGCCGCCACACCATGGACATGAAGTTCACCTACTGTGATGAGAGGATCACCGAGCTCATGGGTTACGATCCAGAAGACCTGCTGAACCGTTCTGTGTATGAA
TACTACCATGCTCTGACTCAACTCTAGTACAAAGCTCTTGATGCGAGGACAAATGAGTAGAGTCCTGTCTTTGGAGCAGACTGAGGGCGTGGAGCCTGTGAAGGAGCAGCAGCAGGGTGAAGAAGAATCAGCAGCAGAGGCAAACGAGGCTTTGAAGGTGAAGGAAGAGGAGGAGGAGAAGACTCCAGAGCTGGATGTGATAAAGCTCTTCACGGAGGTGGAGATCCAGCCGAAGGACTGTCTGTACAACCTGCTGAAAGGACACCCTGATGCCCTGACCCTGCTGGCCCCGGCAGCCGGGGACACAATCATCTCCCTGGATTTCAGCCGCCCCGGTGCAGAATCAGAGACCCACCTGCTGAAAGATGTCCCTCTCTACAATGACATAATGATGCCGTCCTCGGATGACAAGCTGACGCTGCCCATGTC

(b) Cyp_HIF-1α_AA

VKEPSternFFLRMKCTLTSRGRVTNVKSAWTKVLHCSGHVRVHSVSEQSADGPKFPVPYLVLICDPIPHSNIEVP1.DTKTFLSRHDMKFTYCDERITEGMYEDEDILLNRSYVEYHYLSDYLTKTHHHLFTKGQVTGQYRLAKRGGGFVWETQATVIYNNKNSQPCCV
CVNFVLSGIQEDKLILSLEQTEGEVPKEQQQGEEEEAAEANELKVEEEEEEKTPELDVIKLFTVEV1PDKCNYLKLKHDPALTLLAAPAGDISSLFSPGAESETHLKLKDVPYNDIMPSDDKLLTLPMSPSPLPETPPLDASNSASEEAKPSDFVPTLTTPPNKPSEVDNPSGLFSLSPWKQK

(a) Partial nucleotide sequence of *Cyprinodon variegatus* HIF-1α (1221 bps), with the initial 3’ portion absent.  (b) Partial amino acid sequence of *C. variegatus* HIF-1α (367 amino acids), with the initial 3’ portion absent and the PAS-B domain (first) and the first half of the ODDD (second) highlighted in bold.
HIF-2α NUCLEOTIDE AND AMINO ACID SEQUENCES OF CYPRINODON VARIETATUS

(a) Cyp_HIF-2α_Contig
TGAGCTGTAGTAATCACCTGGGCTGCTAGGCGTGGAGCAGCTGCTGAGGCTCGGAGTAG
CGCTTCCAGGTGGAGCTCTCGTGCATGGTGAAAGCACCAGCCCTTAAAGGCATGTCG
CCTGGAACTGAAGCTGGAGTCTGGGCTGCAGCGGCAGCAGGAGCTGGTTTCTGGCTCTC
GCCTGTCCAAGATGAGATCCAGGAGGTGGCATGGATGAAGAAGACACTGAGGGGAATG
GTGCTGGCTGCTGGGATTCCTCAAACTCAGGACGACCAAAGTCAAGGGAAATGATGGTG
TCTCCAGGTGTCGGAGCGAGCTGAGCCAGGTGCTGCGCTCTCTTTGAAGGGTGGAA
AAGGGAGTCTCCTCCTCCTGCGATCATCCTGCTCCTGCGGATGAGAAGGCTGCTCA
TGTGAGGTGCTGGTGTTAAGACATTGCCCCTGCTGCTGCTGCTGCAAGGAATATCAGCAGTACTCC
AAGGGAGTACCATCAACTCTCTCTACTGTTATGCAAAACATGCACTGAGGCTGAGGCTG
GTGTTAAAATGACAGATTCCTGACTCCAGACACAGATATCCTCCGTCTCAGCAAGCA
TGCGATACGTGACCGTCTACTCCGGCCCCTTGGGTGAACATGTTGCTGGTGCTCTTATG
AGCGCTGTCTGCTAGGGCGGTGGATGAACTCGTAGACCGATCGACCCAGCAGGTCCTC
CAGGAGTAACCCATCAACTCTCTCTGTCCTGACAAGGGCCATCAATGTTGAGGCTGGTGC
GGAGATGTTCGCACATCGAGCACAGACACTGAGGTAGGCTGAGGCTGCTTGTAGCCAGAG
GAACGTGTGACGGGGCCAGCCGTCCTGCTACATCTTCAAGGCTGGCAGTGCACTCCTCC
AGCTCTGCTGACTTGGAGTTAAGGATGCTGCCCTCTTGGTGACCGTACCATCCT

(b) Cyp_HIF-2α_AA
RMKCTVTNRGRTVNLSWKLHLCTGTHLKLMDGCPVRLCGLYKEPPLTCACVLMCEPIP
HPNSIANPLDSDRTFLSRHSMDFKFTYCDNVTEIMYGSPEDLLGRSVYEYHIALDSDV
TKSHNLCTKQAVSQQYRMLAKNGYVWETGTIVYNRSNQSSCPICIVCNYLSDVE
EKSVIIFSLEQTEAMFKEPHMSSEFFTAEGAMTAEDRSLFTSFKEEPDDLQLAPTGD
TIISLDFGRPEFESSQPAPFPSVSSSSMPPPGSSSWTGESQKPAAPAAAAQTPTASVPG
DMPLRAGAFTMQQKPPPSATPSSSCSTPSSPGDYSS

(a) Partial nucleotide sequence of Cyprinodon variegatus HIF-2α (1003 bps), with the initial 3’ portion absent. (b) Partial amino acid sequence of C. variegatus HIF-2α (334 amino acids), with the initial 3’ portion and the final 5’ portion absent, and PAS-B domain (first) and the first half of the ODDD (second) are highlighted in bold.
(a) Cyp_Contig_PHD3

GGGGGGGGGGGGGAGAAATCTATCCTGACAGGAGATCCGGAGCGAAGCCGCCTGTCA
GTCTGATCCACCTGCTCTGACCCACTCCTGCTCTGGCTCGCTCAACTCT
TTATGCCTCTTCTTTTTATTTTGCTGATTTAGTGAATCCACCATTTGTTCTTTAGCTA
AATGCCCTTTATGGAACACACTATCTACTCTGACCCACTGACCTGAGTGCAGCTGCTG
TCGTCCCGCCCTCTGCCTCCACCGCTTCTGCTACGTGGACGGGCTCCTCGGGGAGCTG
GCCGGGAGCGCCGTGCTGCTGAGCTCCAGCAAGCGCTCCAGCAAGCGCTCCAGCAAG
ACCAAAACACCGATGGACGCCATCTTACCTGCATCTACTACCTCAACAAGGACTTG
AAACCAAGGAGCACGGGAGGTCTCAGGCTTCTGCTGAGCGGAGGCGTGGGGGGGCTTGT
GGTCTCCCGTTTTAACCTAAACATGGATTTCTACGTTGCTACCATCTTCTTCTGCTGAC
AGCCAGGAGGCAGCATACGCTTCTTCTGGTTCTCTGCTGACCGGAGGATTCAGGGTGAG
GGGCTGCAGCTC

(b) Cyp_PHD3_AA

MPFTEHIYSDDLALQRLVPALLSHGFCYVDGLLGELAGSVAIVDVQVEMHNHSQGQLD
GRLAGSI0PG5RSLRQDGLNVGSSERGCEAISSFNLIDRILSVCASRLGDKAIQER
SKAVDACLYGAGVYVKHDNPNDGHRLCTIYLYNKDWPKEHGGVLRIFPESKPYV
D1KPLFDRLLVFSDRNPHEVQPSATRYA1TVWYFSDERASDKRFALTASTEQK
GCSSS (Stop—entire amino acid sequence)

(a) Complete nucleotide sequence of *Cyprinodon variegatus* PHD3 (1265 bps), with the start and stop codons highlighted in yellow.  (b) Complete amino acid sequence of *C. variegatus* PHD3 (241 amino acids), with the first and last amino acid in bold.
APPENDIX B

LEGEND OF HYPOXIA INDUCIBLE FACTOR ALPHA ISOFORM PHYLOGRAM

Labels cross-referenced to its name (Latin and common) and Accession # from the NCBI Database. Hypoxia-Inducible Factors 1 – 4 alpha (HIF1-4α):

1. HIF-1α:
   Hif1aCten AAR95697.2 Ctenopharyngodon idella grass carp; hif1aEsox ABO26715.1 Esox lucius northern pike; hif1aOnco NP_001117760.1 Oncorhynchus mykiss rainbow trout; hif1aSaln ACN10960.1 Salmo salar Atlantic salmon; hif1aEpin AAW29027.1 Epinephelus coioides orange-spotted grouper; hif1a?Cypr cDNA in question; hif1aPerc ABO26717.1 Perca fluviatilis European perch; hif1aGast ABO26719.1 Gasterosteus aculeatus three-spined stickleback; hif1aNoto ADC55887.1 Notothenia coriiceps black rockcod; hif1aMicr ABD32158.1 Micropogonias undulatus Atlantic croaker; hif1aDice AAZ95453.2 Dicentrarchus labrax European seabass; hif1aPlat ABO26720.1 Platichthys flesus European flounder; hif1aOryz NP_001116565.1 Xenopus laevis African clawed frog; hif1aGall NP_989628.1 Gallus gallus red junglefowl; hif1aMus NP_034561.2 Mus musculus house mouse; hif1aHomo NP_001521.1 Homo sapiens human.

2. HIF-2α:
   hif2aCotu AAF21052.1 Coturnix coturnix common quail; hif2aMus NP_034267.3 Mus musculus house mouse; hif2aHomo NP_001421.2 Homo sapiens human; hif2aIcta NP_001187163.1 Ictalurus punctatus channel catfish; hif2aCten AAT76668.1 Ctenopharyngodon idella grass carp; hif2aTetr CAG00343.1 Tetraodon nigroviridis spotted green pufferfish; hif2aCypr cDNA in question; hif2aMicr ABD32159.1 Micropogonias undulatus Atlantic croaker; hif2aFund AAL95711.1 Fundulus heteroclitus mummichog; hif2aDani NP_001034895.1 Danio rerio zebrafish.

3. HIF-3α:
   hif3aPan XP_003316502.1 Pan troglodytes common chimpanze; hif3aNoma XP_003277656.1 Nomascus leucogenys northern white-checked gibbon; hif3aEquu XP_001500830.3 Equus caballus wild horse; hif3aBos NP_001098812.1 Bos Taurus European or "taurine" cattle; hif3aHomo BAB69689.1 Homo sapiens human; hif3aRatt NP_071973.1 Rattus norvegicus brown Norway rat; hif3aMus BAF44519.1 Mus musculus house mouse.

4. HIF-4α:
   hif4aEpin AAW29028.1 Epinephelus coioides orange-spotted grouper; hif4aCten AAR95698.1 Ctenopharyngodon idella grass carp; hif4aDani ADF58783.1 Danio rerio zebrafish.

Outgroup:
HIF-1a_Paleo AAT72404.1 Palaemonetes pugio grass shrimp.
LEGEND OF PROLYL HYDROXYLASE ISOFORM PHYLOGRAM

Labels cross-referenced to its species name (Latin and common) along with Accession Numbers from the NCBI Database. Prolyl Hydroxylases:

1. PHD1:
   PHD1-Ratt NP_001004083.1 *Rattus norvegicus* brown Norway rat; PHD1_Mus CAC42516.1 *Mus musculus* house mouse; PHD1_Pong NP_001125777.2 *Pongo abelii* Sumatran orangutan; PHD1_Homo NP_444274.1 *Homo sapiens* human.

2. PHD2:
   PHD2_Dani NP_001002595.1 *Danio rerio* zebrafish; PHD2_Homo NP_071334.1 *Homo sapiens* human; PHD2_Bos NP_001192975.2 *Bos taurus* European or taurine cattle; PHD2_Xeno NP_001086560.1 *Xenopus laevis* African clawed frog.

3. PHD3:
   PHD3_Dani NP_998475.1 *Danio rerio* zebrafish; PHD3_Cypr cDNA in question; PHD3_Xeno NP_001106325.1 *Xenopus laevis* African clawed frog; PHD3_Homo NP_082409.2 *Mus musculus* house mouse; PHD3_Bos NP_001094634.1 *Bos Taurus* European or taurine cattle; PHD3_Homo NP_071356.1 *Homo sapiens* human; PHD3_Maca NP_001181354.1 *Macaca mulatta* Rhesus macaque.

4. PHD4:
   PHD4_Homo NP_808807.2 *Homo sapiens* human; PHD4_Mus NP_083220.3 *Mus musculus* house mouse.

5. Collagen Prolyl Hydroxylase (CoPH):
   CoPH_Dani XP_691737.4 *Danio rerio* zebrafish; CoPH_Mus NP_796135.3 *Mus musculus* house mouse; CoPH_Homo NP_878907.1 *Homo sapiens* human.

6. Lysine Hydroxylase (LysH):
   lysH_Mus NP_036092.1 *Mus musculus* house mouse; lysH_Gall NP_001005618.1 *Gallus gallus* red junglefowl; LysH_Homo AAA60116.1 *Homo sapiens* human.

Outgroup:
PHDA_Dros NP_649525.1 *Drosophila melanogaster* fruit fly.
APPENDIX C

IACUC APPROVED PROTOCOL AND NUMBER FOR AQUATIC TOXICOLOGICAL STUDIES WITH SMALL FISH SPECIES (DEPARTMENT OF COASTAL SCIENCES, UNIVERSITY OF SOUTHERN MISSISSIPPI)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 11092204
PROJECT TITLE: Aquatic Toxicological Studies with Small Fish Species
PROPOSED PROJECT DATES: 10/01/2011 to 09/30/2014
PROJECT TYPE: Renewal/Continuation of a Previously Approved Project
PRINCIPAL INVESTIGATOR(S): Joe Griffitt, Ph.D.
COLLEGE/DIVISION: College of Science & Technology
DEPARTMENT: Coastal Sciences
FUNDING AGENCY/SPONSOR: Departmental
IACUC COMMITTEE ACTION: Full Committee Review
PROTOCOL EXPIRATION DATE: 09/30/2014

Jodlè M. Jawor, Ph.D.
IACUC Chair

DATE
APPENDIX D

DISSOLVED OXYGEN (DO), SALINITY (PPT), TEMPERATURE (°C), AND pH DATA FOR MODERATE, CHRONIC HYPOXIA EXPOSURE 3.1
Dissolved Oxygen (DO), Salinity (PPT), Temperature (°C), and pH data for severe, chronic hypoxia exposure 3.2.

Water Quality Data for Hypoxia Exposure 3.2

![Graph showing water quality data for hypoxia exposure 3.2.](image)
DISSOLVED OXYGEN (DO) AND TEMPERATURE (°C) DATA FOR SEVERE, ACUTE HYPOXIA EXPOSURE 4.1

Water Quality Data for Hypoxia Exposure 4.1

- Temp °C
- DO mg/L

Magnitude

6/14/2009 0:00
6/15/2009 0:00
6/16/2009 0:00
6/17/2009 0:00
6/18/2009 0:00
6/19/2009 0:00
6/20/2009 0:00
6/21/2009 0:00
6/22/2009 0:00
6/23/2009 0:00
6/24/2009 0:00
DISSOLVED OXYGEN (DO) AND TEMPERATURE (°C) DATA FOR VERY SEVERE, ACUTE HYPOXIA EXPOSURE 4.2

Water Quality Data for Hypoxia Exposure 4.2

- **Magnitude**
- **Temp °C**
- **DO mg L**

Date Range: 8/30/2009 to 8/18/2009
Table D1.

Results of 4-Tert-Octylphenol Concentrations from Exposure 4.2, Care of Micro Methods.

<table>
<thead>
<tr>
<th>Tank #</th>
<th>Treatment</th>
<th>Start of Exposure</th>
<th>End of Exposure</th>
<th>Avg--Entire Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(ug/L) Rep Tmt</td>
<td>(ug/L) Rep Tmt</td>
<td>(ug/L) Rep Tmt</td>
</tr>
<tr>
<td>6A</td>
<td>Hyp+4tOP</td>
<td>42.0 38.6</td>
<td>22.3 24.0</td>
<td>32.2 31.3</td>
</tr>
<tr>
<td>6B</td>
<td>Hyp+4tOP</td>
<td>35.1</td>
<td>25.6</td>
<td>30.4</td>
</tr>
<tr>
<td>7A</td>
<td>Hyp+4tOP</td>
<td>70.6 63.9</td>
<td>52.4 48.6</td>
<td>61.5 56.2</td>
</tr>
<tr>
<td>7B</td>
<td>Hyp+4tOP</td>
<td>57.2</td>
<td>44.7</td>
<td>51.0</td>
</tr>
<tr>
<td>8A</td>
<td>Hyp+4tOP</td>
<td>49.3 51.8</td>
<td>20.1 20.1</td>
<td>34.7 35.9</td>
</tr>
<tr>
<td>8B</td>
<td>Hyp+4tOP</td>
<td>54.2</td>
<td>20.0</td>
<td>37.1</td>
</tr>
<tr>
<td>9A</td>
<td>Hyp+4tOP</td>
<td>69.2 64.6</td>
<td>44.5 47.4</td>
<td>56.9 56.0</td>
</tr>
<tr>
<td>9B</td>
<td>Hyp+4tOP</td>
<td>60.0</td>
<td>50.2</td>
<td>55.1</td>
</tr>
<tr>
<td>10A</td>
<td>Hyp+4tOP</td>
<td>61.1 59.4</td>
<td>45.5 44.0</td>
<td>53.3 51.7</td>
</tr>
<tr>
<td>10B</td>
<td>Hyp+4tOP</td>
<td>57.7 55.6</td>
<td>42.4 36.8</td>
<td>50.1 46.2</td>
</tr>
<tr>
<td>11A</td>
<td>Norm+4tOP</td>
<td>74.9 69.4</td>
<td>47.7 46.9</td>
<td>61.3 58.2</td>
</tr>
<tr>
<td>11B</td>
<td>Norm+4tOP</td>
<td>63.9</td>
<td>46.1</td>
<td>55.0</td>
</tr>
<tr>
<td>12A</td>
<td>Norm+4tOP</td>
<td>63.5 63.3</td>
<td>51.5 51.1</td>
<td>57.5 57.2</td>
</tr>
<tr>
<td>12B</td>
<td>Norm+4tOP</td>
<td>63.1</td>
<td>50.7</td>
<td>56.9</td>
</tr>
<tr>
<td>13A</td>
<td>Norm+4tOP</td>
<td>62.7 63.8</td>
<td>55.2 57.3</td>
<td>59.0 60.5</td>
</tr>
<tr>
<td>13B</td>
<td>Norm+4tOP</td>
<td>64.9</td>
<td>59.3</td>
<td>62.1</td>
</tr>
<tr>
<td>14A</td>
<td>Norm+4tOP</td>
<td>63.5 62.2</td>
<td>44.3 45.7</td>
<td>53.9 54.0</td>
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<tr>
<td>14B</td>
<td>Norm+4tOP</td>
<td>60.9</td>
<td>47.1</td>
<td>54.0</td>
</tr>
<tr>
<td>15A</td>
<td>Norm+4tOP</td>
<td>59.5 64.1</td>
<td>52.1 52.1</td>
<td>55.8 58.1</td>
</tr>
<tr>
<td>15B</td>
<td>Norm+4tOP</td>
<td>68.7 64.6</td>
<td>52.0 50.6</td>
<td>60.4 57.6</td>
</tr>
</tbody>
</table>
APPENDIX E

STATISTICAL ANALYSES

Table E3.1

Statistical analysis of exposure 3.1 using 3-way and 2-way ANOVAs.

Three Way Analysis of Variance
General Linear Model
Dependent Variable: Data

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>1</td>
<td>0.754</td>
<td>0.754</td>
<td>1.252</td>
<td>0.272</td>
<td>0.040</td>
</tr>
<tr>
<td>DO</td>
<td>1</td>
<td>71.296</td>
<td>71.296</td>
<td>118.410</td>
<td>&lt;0.001</td>
<td>0.798</td>
</tr>
<tr>
<td>Time Pt</td>
<td>3</td>
<td>36.735</td>
<td>12.245</td>
<td>20.337</td>
<td>&lt;0.001</td>
<td>0.670</td>
</tr>
<tr>
<td>Gender x DO</td>
<td>1</td>
<td>1.133</td>
<td>1.133</td>
<td>1.881</td>
<td>0.180</td>
<td>0.059</td>
</tr>
<tr>
<td>Gender x Time Pt</td>
<td>3</td>
<td>2.767</td>
<td>0.922</td>
<td>1.532</td>
<td>0.227</td>
<td>0.133</td>
</tr>
<tr>
<td>DO x Time Pt</td>
<td>3</td>
<td>13.090</td>
<td>4.363</td>
<td>7.247</td>
<td>&lt;0.001</td>
<td>0.420</td>
</tr>
<tr>
<td>Gender x DO x Time Pt</td>
<td>3</td>
<td>0.883</td>
<td>0.294</td>
<td>0.489</td>
<td>0.692</td>
<td>0.047</td>
</tr>
<tr>
<td>Residual</td>
<td>30</td>
<td>18.063</td>
<td>0.602</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>147.252</td>
<td>3.272</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Two Way Analysis of Variance (males and females combined)
General Linear Model
Dependent Variable: Data

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
<td>1</td>
<td>71.296</td>
<td>71.296</td>
<td>115.091</td>
<td>&lt;0.001</td>
<td>0.752</td>
</tr>
<tr>
<td>Time Pt</td>
<td>3</td>
<td>36.735</td>
<td>12.245</td>
<td>19.767</td>
<td>&lt;0.001</td>
<td>0.609</td>
</tr>
<tr>
<td>DO x Time Pt</td>
<td>3</td>
<td>13.090</td>
<td>4.363</td>
<td>7.044</td>
<td>&lt;0.001</td>
<td>0.357</td>
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<tr>
<td>Residual</td>
<td>38</td>
<td>23.540</td>
<td>0.619</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>147.252</td>
<td>3.272</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: DO

Comparisons for factor: Time Pt
Table E3.1 (cont)

Comparisons for factor: **Time Pt within normoxia**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>168.000 vs. 10.000</td>
<td>0.800</td>
<td>1.761</td>
<td>0.086</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>168.000 vs. 48.000</td>
<td>0.767</td>
<td>1.687</td>
<td>0.100</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>168.000 vs. 96.000</td>
<td>0.117</td>
<td>0.257</td>
<td>0.799</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **Time Pt within hyp2.5**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>168.000 vs. 10.000</td>
<td>3.417</td>
<td>7.519</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>168.000 vs. 48.000</td>
<td>1.050</td>
<td>2.067</td>
<td>0.046</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>168.000 vs. 96.000</td>
<td>0.300</td>
<td>0.660</td>
<td>0.513</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **DO within 10**

**DO within 10**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp2.5</td>
<td>3.917</td>
<td>8.619</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
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Comparisons for factor: **DO within 48**

<table>
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<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp2.5</td>
<td>3.117</td>
<td>6.135</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
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</table>

Comparisons for factor: **DO within 96**

<table>
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<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp2.5</td>
<td>1.717</td>
<td>3.778</td>
<td>0.001</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 168**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp2.5</td>
<td>1.300</td>
<td>2.861</td>
<td>0.007</td>
<td>0.050</td>
<td>Yes</td>
</tr>
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</table>
### Three Way Analysis of Variance

**General Linear Model**  
**Dependent Variable**: DO  
**Normality Test**: Passed (P = 0.508)  
**Equal Variance Test**: Passed (P = 0.538)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>1</td>
<td>2.660</td>
<td>2.660</td>
<td>3.719</td>
<td>0.064</td>
<td>0.121</td>
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<tr>
<td>DO</td>
<td>1</td>
<td>212.700</td>
<td>212.700</td>
<td>297.430</td>
<td>&lt;0.001</td>
<td>0.917</td>
</tr>
<tr>
<td>Time Pt</td>
<td>3</td>
<td>4.271</td>
<td>1.424</td>
<td>1.991</td>
<td>0.139</td>
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</tr>
<tr>
<td>Gender x DO</td>
<td>1</td>
<td>0.0433</td>
<td>0.0433</td>
<td>0.0605</td>
<td>0.808</td>
<td>0.002</td>
</tr>
<tr>
<td>Gender x Time Pt</td>
<td>3</td>
<td>0.935</td>
<td>0.312</td>
<td>0.436</td>
<td>0.729</td>
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<tr>
<td>DO x Time Pt</td>
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<td>13.567</td>
<td>4.522</td>
<td>6.324</td>
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<tr>
<td>Gender x DO x Time Pt</td>
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<td>0.483</td>
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<td>Residual</td>
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<td>19.308</td>
<td>0.715</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td>255.567</td>
<td>6.085</td>
<td></td>
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</tbody>
</table>

### Two Way Analysis of Variance (males and females combined)

**General Linear Model**  
**Dependent Variable**: Data  
**Normality Test**: Passed (P = 0.270)  
**Equal Variance Test**: Passed (P = 0.150)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
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<td>213.587</td>
<td>213.587</td>
<td>319.433</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Time Pt</td>
<td>3</td>
<td>4.858</td>
<td>1.619</td>
<td>2.422</td>
<td>0.082</td>
<td>0.172</td>
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<tr>
<td>DO x Time Pt</td>
<td>3</td>
<td>14.309</td>
<td>4.770</td>
<td>7.133</td>
<td>&lt;0.001</td>
<td>0.379</td>
</tr>
<tr>
<td>Residual</td>
<td>35</td>
<td>23.403</td>
<td>0.669</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>255.567</td>
<td>6.085</td>
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<td></td>
</tr>
</tbody>
</table>

**Multiple Comparisons versus Control Group (Holm-Sidak method):**  
**Overall significance level** = 0.05

**Comparisons for factor: DO**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp1.5</td>
<td>4.500</td>
<td>17.873</td>
<td>3.568E-019</td>
<td>0.050</td>
<td>Yes</td>
</tr>
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</table>

**Comparisons for factor: Time Pt within normoxia**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.000 vs. 168.000</td>
<td>2.540</td>
<td>4.911</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>10.000 vs. 96.000</td>
<td>1.540</td>
<td>3.110</td>
<td>0.004</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>10.000 vs. 48.000</td>
<td>1.073</td>
<td>2.168</td>
<td>0.037</td>
<td>0.050</td>
<td>Yes</td>
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Table E3.2 (cont)

<table>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.000 vs. 96.000</td>
<td>0.708</td>
<td>1.342</td>
<td>0.188</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>10.000 vs. 168.000</td>
<td>0.613</td>
<td>1.239</td>
<td>0.224</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>10.000 vs. 48.000.0.217</td>
<td>0.459</td>
<td>0.649</td>
<td>0.050</td>
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<td>No</td>
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</table>

Comparisons for factor: **DO within 10**

<table>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp1.5</td>
<td>2.827</td>
<td>5.709</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 48**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp1.5</td>
<td>4.117</td>
<td>8.720</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 96**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp1.5</td>
<td>5.075</td>
<td>9.615</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 168**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hyp1.5</td>
<td>5.980</td>
<td>11.563</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table E4.1-1

Statistical analysis of exposure 4.1 (EPO) using two-way ANOVA.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
<td>1</td>
<td>42.522</td>
<td>42.522</td>
<td>9.811</td>
<td>0.002</td>
<td>0.086</td>
</tr>
<tr>
<td>Time Pt</td>
<td>12</td>
<td>19.579</td>
<td>1.632</td>
<td>0.376</td>
<td>0.969</td>
<td>0.042</td>
</tr>
<tr>
<td>DO x Time Pt</td>
<td>12</td>
<td>27.759</td>
<td>2.313</td>
<td>0.534</td>
<td>0.888</td>
<td>0.058</td>
</tr>
<tr>
<td>Residual</td>
<td>104</td>
<td>450.762</td>
<td>4.334</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>540.622</td>
<td>4.191</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>1.144</td>
<td>3.132</td>
<td>0.00225</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table E4.1-2

*Statistical analysis of exposure 4.1 (HIF-1α) using two-way ANOVA.*

Two Way Analysis of Variance

Balanced Design

**Dependent Variable:** Data

**Normality Test:** Passed (P = 0.281)

**Equal Variance Test:** Passed (P = 0.303)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
<td>1</td>
<td>10.389</td>
<td>10.389</td>
<td>33.373</td>
<td>&lt;0.001</td>
<td>0.243</td>
</tr>
<tr>
<td>Time Pt</td>
<td>12</td>
<td>13.621</td>
<td>1.135</td>
<td>3.646</td>
<td>&lt;0.001</td>
<td>0.296</td>
</tr>
<tr>
<td>DO x Time Pt</td>
<td>12</td>
<td>14.096</td>
<td>1.175</td>
<td>3.773</td>
<td>&lt;0.001</td>
<td>0.303</td>
</tr>
<tr>
<td>Residual</td>
<td>104</td>
<td>32.375</td>
<td>0.311</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>70.481</td>
<td>0.546</td>
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</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DO**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.565</td>
<td>5.777</td>
<td>0.00000000799</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **Time Pt**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.995</td>
<td>3.988</td>
<td>0.000124</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 5 hr</td>
<td>0.835</td>
<td>3.346</td>
<td>0.00114</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 77 hr</td>
<td>0.680</td>
<td>2.725</td>
<td>0.00754</td>
<td>0.005</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 10 hr</td>
<td>0.650</td>
<td>2.605</td>
<td>0.0105</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.635</td>
<td>2.545</td>
<td>0.0124</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.510</td>
<td>2.044</td>
<td>0.00435</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 0.5 hr</td>
<td>0.455</td>
<td>1.824</td>
<td>0.0711</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 82 hr</td>
<td>0.220</td>
<td>0.882</td>
<td>0.380</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.215</td>
<td>0.862</td>
<td>0.391</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72.5 hr</td>
<td>0.205</td>
<td>0.822</td>
<td>0.413</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.165</td>
<td>0.661</td>
<td>0.510</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.140</td>
<td>0.561</td>
<td>0.576</td>
<td>0.050</td>
<td>No</td>
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</table>

Comparisons for factor: **Time Pt within hypoxia**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 2 hr</td>
<td>2.030</td>
<td>5.753</td>
<td>0.000</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 5 hr</td>
<td>1.710</td>
<td>4.846</td>
<td>0.000</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>1.340</td>
<td>3.797</td>
<td>0.000</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 77 hr</td>
<td>1.330</td>
<td>3.769</td>
<td>0.000</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 10 hr</td>
<td>1.230</td>
<td>3.486</td>
<td>0.001</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 0.5 hr</td>
<td>1.190</td>
<td>3.372</td>
<td>0.001</td>
<td>0.007</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.950</td>
<td>2.692</td>
<td>0.008</td>
<td>0.009</td>
<td>Yes</td>
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</table>
Table E4.1-2 (cont)

<table>
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<tr>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 82 hr</td>
<td>0.470</td>
<td>1.332</td>
<td>0.186</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.400</td>
<td>1.134</td>
<td>0.260</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72.5 hr</td>
<td>0.340</td>
<td>0.964</td>
<td>0.338</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.280</td>
<td>0.793</td>
<td>0.429</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.0900</td>
<td>0.255</td>
<td>0.799</td>
<td>0.050</td>
<td>No</td>
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</table>

Comparisons for factor: **Time Pt within normoxia**

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 0.5 hr</td>
<td>0.280</td>
<td>0.793</td>
<td>0.429</td>
<td>0.004</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.190</td>
<td>0.538</td>
<td>0.591</td>
<td>0.005</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.0700</td>
<td>0.198</td>
<td>0.843</td>
<td>0.005</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72.5 hr</td>
<td>0.0700</td>
<td>0.198</td>
<td>0.843</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.0700</td>
<td>0.198</td>
<td>0.843</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 10 hr</td>
<td>0.0700</td>
<td>0.198</td>
<td>0.843</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.0500</td>
<td>0.142</td>
<td>0.888</td>
<td>0.009</td>
<td>No</td>
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<tr>
<td>0 hr vs. 5 hr</td>
<td>0.0400</td>
<td>0.113</td>
<td>0.910</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.0400</td>
<td>0.113</td>
<td>0.910</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.0300</td>
<td>0.0850</td>
<td>0.932</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 77 hr</td>
<td>0.0300</td>
<td>0.0850</td>
<td>0.932</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 82 hr</td>
<td>0.0300</td>
<td>0.0850</td>
<td>0.932</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 0 hr**

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.320</td>
<td>0.907</td>
<td>0.367</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **DO within 0.5 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>1.150</td>
<td>3.259</td>
<td>0.002</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 2 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>1.750</td>
<td>4.959</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 5 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>1.430</td>
<td>4.052</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 10 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.840</td>
<td>2.380</td>
<td>0.019</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 24 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.0900</td>
<td>0.255</td>
<td>0.799</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 72 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.560</td>
<td>1.587</td>
<td>0.116</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: **DO within 72.5 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.0500</td>
<td>0.142</td>
<td>0.888</td>
<td>0.050</td>
<td>No</td>
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### Table E4.1-2 (cont)

<table>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.0500</td>
<td>0.142</td>
<td>0.888</td>
<td>0.050</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

<table>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.980</td>
<td>2.777</td>
<td>0.007</td>
<td>0.050</td>
<td>Yes</td>
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<table>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.180</td>
<td>0.510</td>
<td>0.611</td>
<td>0.050</td>
<td>No</td>
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</tbody>
</table>

<table>
<thead>
<tr>
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<th>DO within 96 hr</th>
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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.220</td>
<td>0.623</td>
<td>0.534</td>
<td>0.050</td>
<td>No</td>
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</tbody>
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<table>
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<th>DO within 144 hr</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>1.090</td>
<td>3.089</td>
<td>0.003</td>
<td>0.050</td>
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</table>
Table E4.1-3

*Statistical analysis of exposure 4.1 (HIF-2α) using two-way ANOVA.*

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
<td>1</td>
<td>8.866</td>
<td>8.866</td>
<td>31.714</td>
<td>&lt;0.001</td>
<td>0.234</td>
</tr>
<tr>
<td>Time Pt</td>
<td>12</td>
<td>12.671</td>
<td>1.056</td>
<td>3.777</td>
<td>&lt;0.001</td>
<td>0.304</td>
</tr>
<tr>
<td>DO x Time Pt</td>
<td>12</td>
<td>5.654</td>
<td>0.471</td>
<td>1.685</td>
<td>0.081</td>
<td>0.163</td>
</tr>
<tr>
<td>Residual</td>
<td>104</td>
<td>29.075</td>
<td>0.280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>56.266</td>
<td>0.436</td>
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</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DO**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>0.522</td>
<td>5.632</td>
<td>0.000000153</td>
<td>0.050</td>
<td>Yes</td>
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Comparisons for factor: **Time Pt**

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.925</td>
<td>3.912</td>
<td>0.000164</td>
<td>0.004</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 77 hr</td>
<td>0.475</td>
<td>2.009</td>
<td>0.0472</td>
<td>0.005</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.420</td>
<td>1.776</td>
<td>0.0786</td>
<td>0.005</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.410</td>
<td>1.734</td>
<td>0.0859</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.350</td>
<td>1.480</td>
<td>0.142</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 82 hr</td>
<td>0.275</td>
<td>1.163</td>
<td>0.247</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 0.5 hr</td>
<td>0.220</td>
<td>0.930</td>
<td>0.354</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.0800</td>
<td>0.338</td>
<td>0.736</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72.5 hr</td>
<td>0.0700</td>
<td>0.296</td>
<td>0.768</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.0700</td>
<td>0.296</td>
<td>0.768</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 10 hr</td>
<td>0.0550</td>
<td>0.233</td>
<td>0.817</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 5 hr</td>
<td>0.0200</td>
<td>0.0846</td>
<td>0.933</td>
<td>0.050</td>
<td>No</td>
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</table>
Table E4.1-4

*Statistical analysis of exposure 4.1 (PHD3) using two-way ANOVA.*

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PE S</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO</td>
<td>1</td>
<td>71.262</td>
<td>71.262</td>
<td>26.236</td>
<td>&lt;0.001</td>
<td>0.201</td>
</tr>
<tr>
<td>Time Pt</td>
<td>12</td>
<td>46.237</td>
<td>3.853</td>
<td>1.419</td>
<td>0.169</td>
<td>0.141</td>
</tr>
<tr>
<td>DO x Time Pt</td>
<td>12</td>
<td>26.336</td>
<td>2.195</td>
<td>0.808</td>
<td>0.642</td>
<td>0.085</td>
</tr>
<tr>
<td>Residual</td>
<td>104</td>
<td>282.483</td>
<td>2.716</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>426.318</td>
<td>3.305</td>
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</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DO**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxia vs. hypoxia</td>
<td>1.481</td>
<td>5.122</td>
<td>0.00000140</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table E4.2-1

*EPO* liver analysis of exposure 4.2, two-way ANOVA.

### Two Way Analysis of Variance

General Linear Model

| Normality Test: | Failed (P < 0.050) |
| Equal Variance Test: | Passed (P = 0.438) |

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>9.533</td>
<td>3.178</td>
<td>3.069</td>
<td>0.030</td>
<td>0.062</td>
</tr>
<tr>
<td>Time Pt</td>
<td>8</td>
<td>61.528</td>
<td>7.691</td>
<td>7.427</td>
<td>&lt;0.001</td>
<td>0.299</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>24</td>
<td>53.493</td>
<td>2.229</td>
<td>2.152</td>
<td>0.003</td>
<td>0.271</td>
</tr>
<tr>
<td>Residual</td>
<td>139</td>
<td>143.939</td>
<td>1.036</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>174</td>
<td>269.491</td>
<td>1.549</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

#### Comparisons for factor: Treatment

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>0.344</td>
<td>1.553</td>
<td>0.123</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.310</td>
<td>1.406</td>
<td>0.162</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.0178</td>
<td>0.0807</td>
<td>0.936</td>
<td>0.050</td>
<td>No</td>
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</table>

#### Comparisons for factor: Time Pt

<table>
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<tr>
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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 7 hr</td>
<td>1.181</td>
<td>3.613</td>
<td>0.000422</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>0.882</td>
<td>2.701</td>
<td>0.00778</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.807</td>
<td>2.469</td>
<td>0.0148</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.752</td>
<td>2.303</td>
<td>0.0228</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.555</td>
<td>1.725</td>
<td>0.0868</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.460</td>
<td>1.429</td>
<td>0.155</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.0900</td>
<td>0.275</td>
<td>0.783</td>
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</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.0600</td>
<td>0.186</td>
<td>0.852</td>
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</table>

#### Comparisons for factor: Time Pt within NSC

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<th>Unadjusted P</th>
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<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 79 hr</td>
<td>1.030</td>
<td>1.600</td>
<td>0.112</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.940</td>
<td>1.377</td>
<td>0.171</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.490</td>
<td>0.761</td>
<td>0.448</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.210</td>
<td>0.308</td>
<td>0.759</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.110</td>
<td>0.171</td>
<td>0.865</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.100</td>
<td>0.155</td>
<td>0.877</td>
<td>0.017</td>
<td>No</td>
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<tr>
<td>0 hr vs. 7 hr</td>
<td>0.102</td>
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<td>0.881</td>
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<td>0 hr vs. 2 hr</td>
<td>0.0225</td>
<td>0.0330</td>
<td>0.974</td>
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#### Comparisons for factor: Time Pt within H4OP

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<tr>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 2 hr</td>
<td>1.940</td>
<td>3.014</td>
<td>0.003</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>1.900</td>
<td>2.952</td>
<td>0.004</td>
<td>0.007</td>
<td>Yes</td>
</tr>
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<td>0 hr vs. 144 hr</td>
<td>1.300</td>
<td>2.020</td>
<td>0.045</td>
<td>0.009</td>
<td>No</td>
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</table>
### Table E4.2-1 (cont)

<table>
<thead>
<tr>
<th>Comparison</th>
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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.880</td>
<td>1.367</td>
<td>0.174</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.720</td>
<td>1.119</td>
<td>0.265</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.220</td>
<td>0.342</td>
<td>0.733</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.170</td>
<td>0.264</td>
<td>0.792</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>0.050</td>
<td>0.0777</td>
<td>0.938</td>
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<td>No</td>
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</table>

Comparisons for factor: **Time Pt within N4OP**

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 96 hr</td>
<td>1.460</td>
<td>2.269</td>
<td>0.025</td>
<td>0.006</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>1.100</td>
<td>1.709</td>
<td>0.090</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>1.050</td>
<td>1.631</td>
<td>0.105</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.890</td>
<td>1.383</td>
<td>0.169</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.660</td>
<td>1.025</td>
<td>0.307</td>
<td>0.013</td>
<td>No</td>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>0.480</td>
<td>0.746</td>
<td>0.457</td>
<td>0.017</td>
<td>No</td>
</tr>
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<td>0 hr vs. 79 hr</td>
<td>0.310</td>
<td>0.482</td>
<td>0.631</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.0400</td>
<td>0.0622</td>
<td>0.951</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: **Time Pt within Hyp**

<table>
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<tr>
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<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 79 hr</td>
<td>2.140</td>
<td>3.135</td>
<td>0.002</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>1.620</td>
<td>2.517</td>
<td>0.013</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>1.350</td>
<td>2.098</td>
<td>0.038</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.790</td>
<td>1.227</td>
<td>0.222</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.550</td>
<td>0.855</td>
<td>0.394</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.440</td>
<td>0.684</td>
<td>0.495</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.250</td>
<td>0.388</td>
<td>0.698</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>0.0800</td>
<td>0.124</td>
<td>0.901</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: **Treatment within 0 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
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<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>1.421E-014</td>
<td>2.208E-014</td>
<td>1.000</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>7.105E-015</td>
<td>1.104E-014</td>
<td>1.000</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>3.553E-015</td>
<td>5.520E-015</td>
<td>1.000</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **Treatment within 2 hr**

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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>1.962</td>
<td>2.875</td>
<td>0.005</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.373</td>
<td>2.011</td>
<td>0.046</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.0175</td>
<td>0.0256</td>
<td>0.980</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **Treatment within 7 hr**

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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>1.797</td>
<td>2.633</td>
<td>0.009</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.518</td>
<td>2.223</td>
<td>0.028</td>
<td>0.029</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.997</td>
<td>1.461</td>
<td>0.146</td>
<td>0.050</td>
<td>No</td>
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</table>

Comparisons for factor: **Treatment within 24 hr**

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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>1.540</td>
<td>2.393</td>
<td>0.018</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.410</td>
<td>0.637</td>
<td>0.525</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.230</td>
<td>0.357</td>
<td>0.721</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>
### Table E4.2-1 (cont)

Comparisons for factor: **Treatment within 72 hr**

<table>
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<tr>
<th>Comparison</th>
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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>0.990</td>
<td>1.538</td>
<td>0.126</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.900</td>
<td>1.398</td>
<td>0.164</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.770</td>
<td>1.196</td>
<td>0.234</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **Treatment within 74 hr**

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>0.760</td>
<td>1.113</td>
<td>0.267</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.270</td>
<td>0.396</td>
<td>0.693</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.0100</td>
<td>0.0146</td>
<td>0.988</td>
<td>0.050</td>
<td>No</td>
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</table>

Comparisons for factor: **Treatment within 79 hr**

<table>
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<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>1.110</td>
<td>1.626</td>
<td>0.106</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.980</td>
<td>1.523</td>
<td>0.130</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.720</td>
<td>1.119</td>
<td>0.265</td>
<td>0.050</td>
<td>No</td>
</tr>
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</table>

Comparisons for factor: **Treatment within 96 hr**

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<tr>
<th>Comparison</th>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>0.770</td>
<td>1.128</td>
<td>0.261</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.520</td>
<td>0.762</td>
<td>0.447</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.500</td>
<td>0.732</td>
<td>0.465</td>
<td>0.050</td>
<td>No</td>
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</tbody>
</table>

Comparisons for factor: **Treatment within 144 hr**

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>1.400</td>
<td>2.175</td>
<td>0.031</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.990</td>
<td>1.538</td>
<td>0.126</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.150</td>
<td>0.233</td>
<td>0.816</td>
<td>0.050</td>
<td>No</td>
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</tbody>
</table>
### Table E4.2-2

*HIF-1α liver analysis of exposure 4.2, two-way ANOVA.*

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>10.631</td>
<td>3.544</td>
<td>1.975</td>
<td>0.121</td>
<td>0.044</td>
</tr>
<tr>
<td>Time Pt</td>
<td>8</td>
<td>251.456</td>
<td>31.432</td>
<td>17.515</td>
<td>&lt;0.001</td>
<td>0.523</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>24</td>
<td>69.086</td>
<td>2.879</td>
<td>1.604</td>
<td>0.049</td>
<td>0.231</td>
</tr>
<tr>
<td>Residual</td>
<td>128</td>
<td>229.700</td>
<td>1.795</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>163</td>
<td>569.249</td>
<td>3.492</td>
<td></td>
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</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

#### Comparisons for factor: Time Pt

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 96 hr</td>
<td>2.819</td>
<td>6.554</td>
<td>0.00000000125</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>1.895</td>
<td>4.473</td>
<td>0.0000168</td>
<td>0.007</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>1.784</td>
<td>4.148</td>
<td>0.0000607</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>1.517</td>
<td>3.442</td>
<td>0.000781</td>
<td>0.010</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>1.246</td>
<td>2.826</td>
<td>0.00548</td>
<td>0.013</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>1.144</td>
<td>2.466</td>
<td>0.0150</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.518</td>
<td>1.204</td>
<td>0.231</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>0.397</td>
<td>0.867</td>
<td>0.388</td>
<td>0.050</td>
<td>No</td>
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</table>

#### Comparisons for factor: Time Pt within NSC

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 96 hr</td>
<td>3.240</td>
<td>3.824</td>
<td>0.000</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>1.630</td>
<td>1.924</td>
<td>0.057</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>1.560</td>
<td>1.841</td>
<td>0.068</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>1.480</td>
<td>1.747</td>
<td>0.083</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>1.063</td>
<td>1.087</td>
<td>0.279</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.560</td>
<td>0.661</td>
<td>0.510</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.582</td>
<td>0.648</td>
<td>0.518</td>
<td>0.025</td>
<td>No</td>
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<tr>
<td>0 hr vs. 7 hr</td>
<td>0.163</td>
<td>0.167</td>
<td>0.868</td>
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#### Comparisons for factor: Time Pt within H4OP

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>0 hr vs. 96 hr</td>
<td>3.568</td>
<td>3.970</td>
<td>0.000</td>
<td>0.006</td>
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<tr>
<td>0 hr vs. 79 hr</td>
<td>2.370</td>
<td>2.797</td>
<td>0.006</td>
<td>0.007</td>
<td>Yes</td>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>2.200</td>
<td>2.597</td>
<td>0.011</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>1.858</td>
<td>2.067</td>
<td>0.041</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>1.730</td>
<td>2.042</td>
<td>0.043</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>1.863</td>
<td>1.905</td>
<td>0.059</td>
<td>0.017</td>
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</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.790</td>
<td>0.932</td>
<td>0.353</td>
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</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>0.0800</td>
<td>0.0944</td>
<td>0.925</td>
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<tr>
<td>Comparison</td>
<td>Time Pt within N4OP</td>
<td>Diff of Means</td>
<td>t</td>
<td>Unadjusted P</td>
<td>Critical Level</td>
</tr>
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<td>------------</td>
<td>---------------------</td>
<td>---------------</td>
<td>-------</td>
<td>--------------</td>
<td>----------------</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>3.220</td>
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<td>0 hr vs. 74 hr</td>
<td>2.468</td>
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<td>0 hr vs. 2 hr</td>
<td>1.660</td>
<td>1.959</td>
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<tr>
<td>0 hr vs. 72 hr</td>
<td>1.310</td>
<td>1.546</td>
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</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>1.330</td>
<td>1.359</td>
<td>0.176</td>
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<td>No</td>
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<td>0 hr vs. 79 hr</td>
<td>1.020</td>
<td>1.204</td>
<td>0.231</td>
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<td>No</td>
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<td>0 hr vs. 7 hr</td>
<td>0.340</td>
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<td>0.689</td>
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<td>0.0300</td>
<td>0.0354</td>
<td>0.972</td>
<td>0.050</td>
<td>No</td>
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<table>
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<th>Comparison</th>
<th>Time Pt within Hyp</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>0 hr vs. 96 hr</td>
<td>3.010</td>
<td>3.553</td>
<td>0.001</td>
<td>0.006</td>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>1.910</td>
<td>2.254</td>
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<td>0 hr vs. 2 hr</td>
<td>2.120</td>
<td>2.167</td>
<td>0.032</td>
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<tr>
<td>0 hr vs. 24 hr</td>
<td>1.450</td>
<td>1.711</td>
<td>0.089</td>
<td>0.010</td>
<td>No</td>
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<tr>
<td>0 hr vs. 144 hr</td>
<td>1.440</td>
<td>1.700</td>
<td>0.092</td>
<td>0.013</td>
<td>No</td>
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<tr>
<td>0 hr vs. 79 hr</td>
<td>0.950</td>
<td>1.121</td>
<td>0.264</td>
<td>0.017</td>
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<td></td>
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<td>0 hr vs. 7 hr</td>
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<td>0.634</td>
<td>0.527</td>
<td>0.025</td>
<td>No</td>
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</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>0.180</td>
<td>0.212</td>
<td>0.832</td>
<td>0.050</td>
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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>NSC vs. Hyp</td>
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<td>8.387E-015</td>
<td>1.000</td>
<td>0.017</td>
<td>No</td>
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<tr>
<td>NSC vs. N4OP</td>
<td>3.553E-015</td>
<td>4.193E-015</td>
<td>1.000</td>
<td>0.025</td>
<td>No</td>
<td></td>
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<tr>
<td>NSC vs. H4OP</td>
<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
<td>0.050</td>
<td>No</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Treatment within 2 hr</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>3.183</td>
<td>2.910</td>
<td>0.004</td>
<td>0.017</td>
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<tr>
<td>NSC vs. H4OP</td>
<td>2.921</td>
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<tr>
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<th>Critical Level</th>
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<td>NSC vs. H4OP</td>
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<td>0.783</td>
<td>0.716</td>
<td>0.475</td>
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<td>NSC vs. N4OP</td>
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<th>Critical Level</th>
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<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>0.230</td>
<td>0.235</td>
<td>0.815</td>
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<td>NSC vs. H4OP</td>
<td>0.170</td>
<td>0.201</td>
<td>0.841</td>
<td>0.025</td>
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<td>NSC vs. Hyp</td>
<td>0.110</td>
<td>0.130</td>
<td>0.897</td>
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<td>1.450</td>
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<td>NSC vs. N4OP</td>
<td>0.320</td>
<td>0.378</td>
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<td>0.025</td>
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<td>NSC vs. H4OP</td>
<td>0.233</td>
<td>0.239</td>
<td>0.812</td>
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Table E4.2-2 (cont)

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<td>NSC vs. N4OP</td>
<td>1.908</td>
<td>2.123</td>
<td>0.036</td>
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<td>NSC vs. H4OP</td>
<td>1.640</td>
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<td>1.350</td>
<td>1.593</td>
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Comparisons for factor: Treatment within 74 hr

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<th>Significant?</th>
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<tbody>
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<td>NSC vs. Hyp</td>
<td>2.290</td>
<td>2.703</td>
<td>0.008</td>
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<td>NSC vs. N4OP</td>
<td>2.220</td>
<td>2.620</td>
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<td>0.025</td>
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<tr>
<td>NSC vs. H4OP</td>
<td>0.870</td>
<td>1.027</td>
<td>0.306</td>
<td>0.050</td>
<td>No</td>
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Comparisons for factor: Treatment within 79 hr

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<th>Significant?</th>
</tr>
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<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>2.087</td>
<td>2.323</td>
<td>0.022</td>
<td>0.017</td>
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<tr>
<td>NSC vs. N4OP</td>
<td>1.740</td>
<td>2.054</td>
<td>0.042</td>
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<tr>
<td>NSC vs. H4OP</td>
<td>1.530</td>
<td>1.806</td>
<td>0.073</td>
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</table>

Comparisons for factor: Treatment within 96 hr

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<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>0.858</td>
<td>0.954</td>
<td>0.342</td>
<td>0.017</td>
<td>No</td>
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<tr>
<td>NSC vs. N4OP</td>
<td>0.612</td>
<td>0.682</td>
<td>0.497</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.502</td>
<td>0.559</td>
<td>0.577</td>
<td>0.050</td>
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Comparisons for factor: Treatment within 144 hr
Table E4.2-3

HIF-2α liver analysis of exposure 4.2, two-way ANOVA.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>P&lt;ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>7.258</td>
<td>2.419</td>
<td>7.308</td>
<td>&lt;0.001</td>
<td>0.133</td>
</tr>
<tr>
<td>Time Pt</td>
<td>8</td>
<td>105.599</td>
<td>13.200</td>
<td>39.877</td>
<td>&lt;0.001</td>
<td>0.690</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>24</td>
<td>31.371</td>
<td>1.307</td>
<td>3.949</td>
<td>&lt;0.001</td>
<td>0.399</td>
</tr>
<tr>
<td>Residual</td>
<td>143</td>
<td>47.335</td>
<td>0.331</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>178</td>
<td>191.607</td>
<td>1.076</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: Treatment

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
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<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>0.355</td>
<td>2.907</td>
<td>0.00424</td>
<td>0.017</td>
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<tr>
<td>NSC vs. H4OP</td>
<td>0.346</td>
<td>2.834</td>
<td>0.00526</td>
<td>0.025</td>
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<tr>
<td>NSC vs. Hyp</td>
<td>0.0928</td>
<td>0.760</td>
<td>0.449</td>
<td>0.050</td>
<td>No</td>
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Comparisons for factor: Time Pt

<table>
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<tr>
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<th>Diff of Means</th>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 72 hr</td>
<td>1.490</td>
<td>8.190</td>
<td>1.327E-013</td>
<td>0.006</td>
<td>Yes</td>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>1.420</td>
<td>7.805</td>
<td>1.148E-012</td>
<td>0.007</td>
<td>Yes</td>
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<tr>
<td>0 hr vs. 24 hr</td>
<td>1.248</td>
<td>6.857</td>
<td>0.0000000000196</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.592</td>
<td>3.257</td>
<td>0.00141</td>
<td>0.010</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.349</td>
<td>1.888</td>
<td>0.0611</td>
<td>0.013</td>
<td>No</td>
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<td>0 hr vs. 7 hr</td>
<td>0.343</td>
<td>1.883</td>
<td>0.0618</td>
<td>0.017</td>
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<td>0 hr vs. 79 hr</td>
<td>0.303</td>
<td>1.663</td>
<td>0.0986</td>
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<tr>
<td>0 hr vs. 96 hr</td>
<td>0.0675</td>
<td>0.371</td>
<td>0.711</td>
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Comparisons for factor: Time Pt within NSC

<table>
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<tr>
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<th>Diff of Means</th>
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<th>Unadjusted P</th>
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<tbody>
<tr>
<td>0 hr vs. 74 hr</td>
<td>1.550</td>
<td>4.260</td>
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<td>0.006</td>
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<td>0 hr vs. 72 hr</td>
<td>1.180</td>
<td>3.243</td>
<td>0.001</td>
<td>0.007</td>
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<tr>
<td>0 hr vs. 144 hr</td>
<td>0.820</td>
<td>2.254</td>
<td>0.026</td>
<td>0.009</td>
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<tr>
<td>0 hr vs. 24 hr</td>
<td>0.470</td>
<td>1.292</td>
<td>0.199</td>
<td>0.010</td>
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<tr>
<td>0 hr vs. 2 hr</td>
<td>0.405</td>
<td>1.049</td>
<td>0.296</td>
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<td>0 hr vs. 96 hr</td>
<td>0.290</td>
<td>0.797</td>
<td>0.427</td>
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<td>0 hr vs. 79 hr</td>
<td>0.280</td>
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<td>0 hr vs. 7 hr</td>
<td>0.0900</td>
<td>0.247</td>
<td>0.805</td>
<td>0.050</td>
<td>No</td>
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Comparisons for factor: Time Pt within H4OP

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<th>Unadjusted P</th>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>1.870</td>
<td>5.139</td>
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<tr>
<td>0 hr vs. 24 hr</td>
<td>1.570</td>
<td>4.315</td>
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<td>0.007</td>
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Comparisons for factor: **Time Pt within N4OP**

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<td>0 hr vs. 72 hr</td>
<td>1.800</td>
<td>4.947</td>
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<td>0 hr vs. 74 hr</td>
<td>1.670</td>
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<td>0 hr vs. 24 hr</td>
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<td>0.390</td>
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<td>0 hr vs. 7 hr</td>
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Comparisons for factor: **Time Pt within Hyp**

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<td>0 hr vs. 24 hr</td>
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<td>4.122</td>
<td>0.000</td>
<td>0.009</td>
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<td>0.960</td>
<td>2.638</td>
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<td>0 hr vs. 7 hr</td>
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<td>0.247</td>
<td>0.805</td>
<td>0.025</td>
<td>No</td>
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<tr>
<td>0 hr vs. 96 hr</td>
<td>0.130</td>
<td>0.357</td>
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Comparisons for factor: **Treatment within 0 hr**

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<td>7.105E-015</td>
<td>1.953E-014</td>
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<tr>
<td>NSC vs. N4OP</td>
<td>7.105E-015</td>
<td>1.953E-014</td>
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Comparisons for factor: **Treatment within 2 hr**

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<td>NSC vs. N4OP</td>
<td>0.335</td>
<td>0.868</td>
<td>0.387</td>
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<td>NSC vs. H4OP</td>
<td>0.205</td>
<td>0.531</td>
<td>0.596</td>
<td>0.025</td>
<td>No</td>
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<tr>
<td>NSC vs. Hyp</td>
<td>0.0950</td>
<td>0.246</td>
<td>0.806</td>
<td>0.050</td>
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Comparisons for factor: **Treatment within 7 hr**

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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>0.910</td>
<td>2.501</td>
<td>0.014</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.100</td>
<td>0.275</td>
<td>0.784</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: **Treatment within 24 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>1.100</td>
<td>3.023</td>
<td>0.003</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.070</td>
<td>2.941</td>
<td>0.004</td>
<td>0.025</td>
<td>Yes</td>
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<tr>
<td>NSC vs. N4OP</td>
<td>0.940</td>
<td>2.583</td>
<td>0.011</td>
<td>0.050</td>
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Table E4.2-3 (cont)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Treatment within 72 hr</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>0.620 1.704</td>
<td>0.091</td>
<td>0.017</td>
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<tr>
<td>NSC vs. Hyp</td>
<td>0.560 1.539</td>
<td>0.126</td>
<td>0.025</td>
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<tr>
<td>NSC vs. H4OP</td>
<td>0.0600 0.165</td>
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<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
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<td>0.960 2.638</td>
<td>0.009</td>
<td>0.017</td>
<td>Yes</td>
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<td>NSC vs. H4OP</td>
<td>0.320 0.879</td>
<td>0.381</td>
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<td></td>
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<tr>
<td>NSC vs. N4OP</td>
<td>0.120 0.330</td>
<td>0.742</td>
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<th>Treatment within 79 hr</th>
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<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>1.240 3.408</td>
<td>0.001</td>
<td>0.017</td>
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</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.700 1.924</td>
<td>0.056</td>
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<td></td>
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<tr>
<td>NSC vs. N4OP</td>
<td>0.630 1.731</td>
<td>0.086</td>
<td>0.050</td>
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<th>Critical Level</th>
<th>Significant?</th>
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<tr>
<td>NSC vs. H4OP</td>
<td>1.110 3.050</td>
<td>0.003</td>
<td>0.017</td>
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<td></td>
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<tr>
<td>NSC vs. Hyp</td>
<td>0.420 1.154</td>
<td>0.250</td>
<td>0.025</td>
<td>No</td>
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<td></td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.100 0.275</td>
<td>0.784</td>
<td>0.050</td>
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<table>
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<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>0.940 2.583</td>
<td>0.011</td>
<td>0.017</td>
<td>Yes</td>
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<td></td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.680 1.869</td>
<td>0.064</td>
<td>0.025</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>0.650 1.786</td>
<td>0.076</td>
<td>0.050</td>
<td>No</td>
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</table>
Table E4.2-4

**PHD3 liver analysis of exposure 4.2, two-way ANOVA.**

Two Way Analysis of Variance
General Linear Model
Dependent Variable: Data

<table>
<thead>
<tr>
<th>Normality Test:</th>
<th>Failed ( (P &lt; 0.050) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equal Variance Test:</td>
<td>Passed ( (P = 0.710) )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>8.288</td>
<td>2.763</td>
<td>3.020</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>Time Pt</td>
<td>8</td>
<td>211.408</td>
<td>26.426</td>
<td>28.886</td>
<td>&lt;0.001</td>
<td>0.618</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>24</td>
<td>34.129</td>
<td>1.422</td>
<td>1.554</td>
<td>0.060</td>
<td>0.207</td>
</tr>
<tr>
<td>Residual</td>
<td>143</td>
<td>130.820</td>
<td>0.915</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>178</td>
<td>387.031</td>
<td>2.174</td>
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</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>0.348</td>
<td>1.716</td>
<td>0.0884</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>0.238</td>
<td>1.174</td>
<td>0.242</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.206</td>
<td>1.015</td>
<td>0.312</td>
<td>0.050</td>
<td>No</td>
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</table>

Comparisons for factor: **Time Pt**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 74 hr</td>
<td>3.080</td>
<td>10.183</td>
<td>1.187E-018</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>2.592</td>
<td>8.571</td>
<td>1.508E-014</td>
<td>0.007</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 24 hr</td>
<td>2.260</td>
<td>7.472</td>
<td>7.191E-012</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>2.215</td>
<td>7.323</td>
<td>1.616E-011</td>
<td>0.010</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>1.975</td>
<td>6.530</td>
<td>0.000000000107</td>
<td>0.013</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>1.218</td>
<td>4.025</td>
<td>0.0000919</td>
<td>0.017</td>
<td>Yes</td>
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<tr>
<td>0 hr vs. 7 hr</td>
<td>0.379</td>
<td>1.233</td>
<td>0.220</td>
<td>0.025</td>
<td>No</td>
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<tr>
<td>0 hr vs. 2 hr</td>
<td>0.0625</td>
<td>0.207</td>
<td>0.837</td>
<td>0.050</td>
<td>No</td>
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</table>
Table E4.2-5

*VTG liver analysis of exposure 4.2, two-way ANOVA.*

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<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>2611.875</td>
<td>870.625</td>
<td>375.123</td>
<td>&lt;0.001</td>
<td>0.889</td>
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<tr>
<td>Time Pt</td>
<td>8</td>
<td>542.159</td>
<td>67.770</td>
<td>29.200</td>
<td>&lt;0.001</td>
<td>0.624</td>
</tr>
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<td>24</td>
<td>859.885</td>
<td>35.829</td>
<td>15.437</td>
<td>&lt;0.001</td>
<td>0.724</td>
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<tr>
<td>Residual</td>
<td>141</td>
<td>327.248</td>
<td>2.321</td>
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<td></td>
</tr>
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<td>Total</td>
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<td>4371.005</td>
<td>24.835</td>
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Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Treatment**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>8.409</td>
<td>25.826</td>
<td>2.608E-055</td>
<td>0.017</td>
<td>Yes</td>
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<tr>
<td>NSC vs. N4OP</td>
<td>8.077</td>
<td>24.640</td>
<td>5.960E-053</td>
<td>0.025</td>
<td>Yes</td>
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<td>NSC vs. Hyp</td>
<td>1.162</td>
<td>3.569</td>
<td>0.000490</td>
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Comparisons for factor: **Time Pt**

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<th>Unadjusted P</th>
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<th>Significant?</th>
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<td>5.372</td>
<td>11.152</td>
<td>4.235E-021</td>
<td>0.006</td>
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<tr>
<td>0 hr vs. 72 hr</td>
<td>5.040</td>
<td>10.462</td>
<td>2.588E-019</td>
<td>0.007</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>4.346</td>
<td>8.883</td>
<td>2.734E-015</td>
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<tr>
<td>0 hr vs. 74 hr</td>
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<td>8.827</td>
<td>3.765E-015</td>
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<td>Yes</td>
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<tr>
<td>0 hr vs. 79 hr</td>
<td>4.062</td>
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<td>3.593E-014</td>
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<tr>
<td>0 hr vs. 24 hr</td>
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<td>0 hr vs. 2 hr</td>
<td>3.588</td>
<td>7.334</td>
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<td>0.025</td>
<td>Yes</td>
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Comparisons for factor: **Time Pt within NSC**

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<th>Significant?</th>
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</thead>
<tbody>
<tr>
<td>0 hr vs. 72 hr</td>
<td>2.340</td>
<td>2.429</td>
<td>0.016</td>
<td>0.006</td>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>2.250</td>
<td>2.335</td>
<td>0.021</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>1.460</td>
<td>1.515</td>
<td>0.132</td>
<td>0.009</td>
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<tr>
<td>0 hr vs. 96 hr</td>
<td>1.138</td>
<td>1.113</td>
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<tr>
<td>0 hr vs. 24 hr</td>
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<td>0.013</td>
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<tr>
<td>0 hr vs. 144 hr</td>
<td>0.510</td>
<td>0.529</td>
<td>0.597</td>
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<tr>
<td>0 hr vs. 2 hr</td>
<td>0.462</td>
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<td>0 hr vs. 7 hr</td>
<td>0.400</td>
<td>0.415</td>
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Comparisons for factor: **Time Pt within H4OP**

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</thead>
<tbody>
<tr>
<td>0 hr vs. 72 hr</td>
<td>10.640</td>
<td>11.043</td>
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<td>0.006</td>
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<tr>
<td>0 hr vs. 74 hr</td>
<td>10.260</td>
<td>10.649</td>
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<td>0.007</td>
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Table E4.2-5 (cont)

<table>
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<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 144 hr</td>
<td>10.210</td>
<td>10.597</td>
<td>0.000</td>
<td>0.009</td>
<td>Yes</td>
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<td>0 hr vs. 24 hr</td>
<td>9.970</td>
<td>10.348</td>
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<td>0.010</td>
<td>Yes</td>
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<tr>
<td>0 hr vs. 96 hr</td>
<td>9.450</td>
<td>9.808</td>
<td>0.000</td>
<td>0.013</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>8.780</td>
<td>9.112</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
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<tr>
<td>0 hr vs. 7 hr</td>
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<td>7.597</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>0.580</td>
<td>0.602</td>
<td>0.548</td>
<td>0.050</td>
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Comparisons for factor: *Time Pt within N4OP*

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<th>Critical Level</th>
<th>Significant?</th>
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<td>0 hr vs. 144 hr</td>
<td>11.140</td>
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<td>0.006</td>
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</tr>
<tr>
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<td>11.053</td>
<td>0.000</td>
<td>0.007</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>9.290</td>
<td>9.642</td>
<td>0.000</td>
<td>0.009</td>
<td>Yes</td>
</tr>
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<td>0 hr vs. 74 hr</td>
<td>9.000</td>
<td>9.341</td>
<td>0.000</td>
<td>0.010</td>
<td>Yes</td>
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<td>0 hr vs. 79 hr</td>
<td>8.310</td>
<td>8.625</td>
<td>0.000</td>
<td>0.013</td>
<td>Yes</td>
</tr>
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<td>0 hr vs. 24 hr</td>
<td>7.762</td>
<td>7.596</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>6.770</td>
<td>7.026</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>1.300</td>
<td>1.349</td>
<td>0.179</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: *Time Pt within Hyp*

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr vs. 24 hr</td>
<td>2.670</td>
<td>2.771</td>
<td>0.006</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td>0 hr vs. 7 hr</td>
<td>1.760</td>
<td>1.827</td>
<td>0.070</td>
<td>0.007</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 2 hr</td>
<td>1.450</td>
<td>1.505</td>
<td>0.135</td>
<td>0.009</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 72 hr</td>
<td>1.210</td>
<td>1.256</td>
<td>0.211</td>
<td>0.010</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 144 hr</td>
<td>0.650</td>
<td>0.675</td>
<td>0.501</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 96 hr</td>
<td>0.220</td>
<td>0.228</td>
<td>0.820</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 79 hr</td>
<td>0.190</td>
<td>0.197</td>
<td>0.844</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>0 hr vs. 74 hr</td>
<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: *Treatment within 0 hr*

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>2.132E-014</td>
<td>2.212E-014</td>
<td>1.000</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>7.105E-015</td>
<td>7.374E-015</td>
<td>1.000</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: *Treatment within 2 hr*

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. Hyp</td>
<td>1.912</td>
<td>1.871</td>
<td>0.063</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>1.762</td>
<td>1.725</td>
<td>0.087</td>
<td>0.025</td>
<td>No</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>1.042</td>
<td>1.020</td>
<td>0.309</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: *Treatment within 7 hr*

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>6.920</td>
<td>7.182</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>6.370</td>
<td>6.611</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.360</td>
<td>1.411</td>
<td>0.160</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparisons for factor: *Treatment within 24 hr*

<table>
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<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>10.680</td>
<td>11.084</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>8.472</td>
<td>8.290</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.960</td>
<td>2.034</td>
<td>0.044</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>
### Table E4.2-5 (cont)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>12.990</td>
<td>13.482</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>12.980</td>
<td>13.472</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>3.550</td>
<td>3.684</td>
<td>0.000</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Comparisons for factor: Treatment within 74 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>12.510</td>
<td>12.984</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>11.250</td>
<td>11.676</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>2.250</td>
<td>2.335</td>
<td>0.021</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Comparisons for factor: Treatment within 79 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>10.240</td>
<td>10.628</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>9.770</td>
<td>10.140</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.270</td>
<td>1.318</td>
<td>0.190</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

**Comparisons for factor: Treatment within 96 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. H4OP</td>
<td>10.587</td>
<td>10.360</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. N4OP</td>
<td>10.428</td>
<td>10.203</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>0.918</td>
<td>0.898</td>
<td>0.371</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>

**Comparisons for factor: Treatment within 144 hr**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC vs. N4OP</td>
<td>11.650</td>
<td>12.091</td>
<td>0.000</td>
<td>0.017</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. H4OP</td>
<td>10.720</td>
<td>11.126</td>
<td>0.000</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>NSC vs. Hyp</td>
<td>1.160</td>
<td>1.204</td>
<td>0.231</td>
<td>0.050</td>
<td>No</td>
</tr>
</tbody>
</table>
Table E4.2-6

*EPO tests analysis of exposure 4.2, two-way ANOVA.*

**Two Way Analysis of Variance**  
Balanced Design  
Dependent Variable: Data

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>1.355</td>
<td>0.452</td>
<td>0.083</td>
<td>0.969</td>
</tr>
<tr>
<td>Time Pt</td>
<td>2</td>
<td>14.836</td>
<td>7.418</td>
<td>1.362</td>
<td>0.269</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>6</td>
<td>54.350</td>
<td>9.058</td>
<td>1.664</td>
<td>0.158</td>
</tr>
<tr>
<td>Residual</td>
<td>36</td>
<td>196.026</td>
<td>5.445</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>266.567</td>
<td>5.672</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normality Test: Failed (P < 0.050)  
Equal Variance Test: Passed (P = 0.597)
Table E4.2-7

*HIF-1α testes analysis, exp 4.2, two-way ANOVA.*

Two Way Analysis of Variance
Balanced Design
Dependent Variable: Data

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>7.080</td>
<td>2.360</td>
<td>0.778</td>
<td>0.514</td>
</tr>
<tr>
<td>Time Pt</td>
<td>2</td>
<td>518.095</td>
<td>259.047</td>
<td>85.368</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>6</td>
<td>37.941</td>
<td>6.324</td>
<td>2.084</td>
<td>0.079</td>
</tr>
<tr>
<td>Residual</td>
<td>36</td>
<td>109.241</td>
<td>3.034</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>672.357</td>
<td>14.305</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>Unadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 hr vs. 79 hr</td>
<td>7.297</td>
<td>11.848</td>
<td>5.561E-014</td>
<td>0.025</td>
<td>Yes</td>
</tr>
<tr>
<td>72 hr vs. 74 hr</td>
<td>6.587</td>
<td>10.696</td>
<td>9.985E-013</td>
<td>0.050</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table E4.2-8

*HIF-2α testes analysis of exposure 4.2, two-way ANOVA.*

**Two Way Analysis of Variance**
Balanced Design
Dependent Variable: Data

| Normality Test: | Failed (P < 0.050) |
| Equal Variance Test: | Passed (P = 0.638) |

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>11.957</td>
<td>3.986</td>
<td>2.861</td>
<td>0.050</td>
</tr>
<tr>
<td>Time Pt</td>
<td>2</td>
<td>18.578</td>
<td>9.289</td>
<td>6.667</td>
<td>0.003</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>6</td>
<td>7.366</td>
<td>1.228</td>
<td>0.881</td>
<td>0.518</td>
</tr>
<tr>
<td>Residual</td>
<td>36</td>
<td>50.156</td>
<td>1.393</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>88.057</td>
<td>1.874</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

<table>
<thead>
<tr>
<th>Comparisons for factor: Time Pt</th>
<th>Diff of Means</th>
<th>tUnadjusted P</th>
<th>Critical Level</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 hr vs. 79 hr</td>
<td>1.503</td>
<td>3.602</td>
<td>0.000946</td>
<td>Yes</td>
</tr>
<tr>
<td>72 hr vs. 74 hr</td>
<td>0.534</td>
<td>1.281</td>
<td>0.209</td>
<td>No</td>
</tr>
</tbody>
</table>
Table E4.2-9

*PHD3 testes analysis of exposure 4.2, two-way ANOVA.*

---

**Two Way Analysis of Variance**  
Balanced Design  
Dependent Variable: Data

**Normality Test:** Failed (P < 0.050)  
**Equal Variance Test:** Passed (P = 0.527)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>0.772</td>
<td>0.257</td>
<td>0.396</td>
<td>0.756</td>
</tr>
<tr>
<td>Time Pt</td>
<td>2</td>
<td>1.708</td>
<td>0.854</td>
<td>1.315</td>
<td>0.281</td>
</tr>
<tr>
<td>Treatment x Time Pt</td>
<td>6</td>
<td>3.807</td>
<td>0.635</td>
<td>0.977</td>
<td>0.455</td>
</tr>
<tr>
<td>Residual</td>
<td>36</td>
<td>23.378</td>
<td>0.649</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>29.665</td>
<td>0.631</td>
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</tr>
</tbody>
</table>
Scatterplot Matrix displaying a 16X16 pairwise array comparison of the distribution of data from gene expression to show a normal distribution of the data after standardization of each array.
**APPENDIX G**

**Table G1**

*Significant genes (black dots) from t-test comparison (Hypoxia to Normoxia + 4tOP (H4OP → N4OP) at 72 hrs.) – (Hypoxia to Normoxia Control (HC → NC) at 72 hrs.) from Fig 28 (4tOP genes under hypoxia conditions).*

<table>
<thead>
<tr>
<th>Annotation</th>
<th>F-Value</th>
<th>Organism</th>
<th>Description</th>
<th>F_T3_DO</th>
<th>F_T3_Trtmt</th>
<th>Diff of</th>
<th>t-Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated:</td>
<td></td>
<td></td>
<td>Significant Genes (Hyp+4tOP)-(Hyp+Ctl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>2.18415</td>
<td>9.99873</td>
<td>10.4489</td>
<td>0.890256</td>
<td>4.01994</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>4.4097</td>
<td>10.5614</td>
<td>3.64912</td>
<td>0.56595</td>
<td>3.68997</td>
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<td>3.58203</td>
<td>10.1289</td>
<td>7.91272</td>
<td>0.70855</td>
<td>4.10279</td>
<td></td>
<td></td>
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<tr>
<td>Unknown</td>
<td>0.18195</td>
<td>0.2681</td>
<td>19.8537</td>
<td>0.559005</td>
<td>3.65573</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.76724</td>
<td>8.8539</td>
<td>7.55703</td>
<td>0.86924</td>
<td>3.88696</td>
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<td></td>
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<td>13.3754</td>
<td>6.95994</td>
<td>1.098456</td>
<td>3.45152</td>
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<td></td>
</tr>
<tr>
<td>Contig590</td>
<td>3.00E-12</td>
<td>Equus caballus</td>
<td>ref</td>
<td>XP_001495949.1</td>
<td>PREDICTED: hypothetical protein</td>
<td>0.17852</td>
<td>4.87841</td>
</tr>
<tr>
<td>Down- regulated:</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C02_01_B04</td>
<td>4.00E-14</td>
<td>sp</td>
<td>Q088R8</td>
<td>STIB1B_BRARE</td>
<td>Histone-lysine N-methyltransferase</td>
<td>1.96469</td>
<td>17.2511</td>
</tr>
<tr>
<td>Unknown</td>
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<td>0.62764</td>
<td>-0.97755</td>
<td>-4.2238</td>
<td></td>
<td></td>
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<tr>
<td>Unknown</td>
<td>0.311</td>
<td>26.9984</td>
<td>0.34341</td>
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<td>-4.0879</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
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<td>31.9014</td>
<td>0.846</td>
<td>-0.93522</td>
<td>-4.6442</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.01587</td>
<td>13.8596</td>
<td>7.94139</td>
<td>-0.67494</td>
<td>-4.4006</td>
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<tr>
<td>Unknown</td>
<td>1.74029</td>
<td>14.2298</td>
<td>1.29346</td>
<td>-0.63588</td>
<td>-3.4216</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.63066</td>
<td>11.5881</td>
<td>4.06076</td>
<td>-0.78645</td>
<td>-4.0709</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.00615</td>
<td>14.114</td>
<td>3.42895</td>
<td>-0.76466</td>
<td>-3.8698</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contig476</td>
<td>2.00E-12</td>
<td>Fundulus heteroclitus</td>
<td>gb</td>
<td>AAU50539.1</td>
<td>complement component C3</td>
<td>1.62518</td>
<td>5.45752</td>
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<tr>
<td>Unknown</td>
<td>0.06674</td>
<td>20.8404</td>
<td>2.41591</td>
<td>-0.7813</td>
<td>-4.2842</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.29293</td>
<td>8.41067</td>
<td>6.23389</td>
<td>-0.67245</td>
<td>-3.8162</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C100_01_D03</td>
<td>3.00E-20</td>
<td>Tetraodon nigroviridis</td>
<td>emb</td>
<td>CAG006468.1</td>
<td>unnamed protein product</td>
<td>0.18985</td>
<td>32.0569</td>
</tr>
<tr>
<td>C04_04_B05</td>
<td>2.00E-32</td>
<td>Danio rerio</td>
<td>ref</td>
<td>XP_697147.2</td>
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Table G2

Significant genes (black dots) from t-test comparison (Hypoxia to Normoxia + 4tOP (H4OP → N4OP) at 72 hrs.) – (Hypoxia to Normoxia + 4tOP (H4OP → N4OP) at 74 hrs.) from Fig 29 (Hypoxia genes under 4tOP conditions).

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Table G3

Significant genes (black dots) from t-test comparison (Hypoxia to Normoxia Control (HC → NC) at 72 hrs.) – (Hypoxia to Normoxia Control (HC → NC) at 74 hrs.) from Fig 30 (Hypoxia genes under control conditions).

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- **FDR (0.05)** refers to the False Discovery Rate, a statistical test used to control the rate of false positives.
### Table G4

**Significant genes (black dots) from t-test comparison (Hypoxia to Normoxia + 4tOP (H4OP → N4OP) at 74 hrs.) – (Hypoxia to Normoxia Control (HC → NC) at 74 hrs.) from Fig 31 (4tOP genes under normoxia conditions).**

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REFERENCES


Martin, F., Linden, T., Katschinski, D. M., Oehme, F., Flamme, I., Mukhopadhyay, C.


