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**THE EFFECTS OF THE XENOESTROGEN, OCTYLPHENOL (OP), AND UV-B
RADIATION ON SOMATIC DEVELOPMENT AND HYPOTHALAMIC GENE
EXPRESSION OF THE LEOPARD FROG (*RANA PIPIENS*)**

DOUGLAS CRUMP

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology



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0-612-57102-5

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Abstract

Statistical meta-analysis of large and diverse data sets has indicated that amphibians have been declining worldwide since the 1960s. Exposure to UV-B radiation and endocrine-disrupting chemicals (EDCs) have been considered as possible hypotheses to explain the observed declines. Newly-hatched leopard frog (*Rana pipiens*) tadpoles were exposed for ten days to one of three concentrations of octylphenol (OP) (10 μM , 1 μM , 1 nM) plus a 0.01% ethanol vehicle control, with and without two levels (7 $\mu\text{W}/\text{cm}^2$, 25 $\mu\text{W}/\text{cm}^2$) of UV-B radiation. The LC50 for water borne OP was also determined. OP is an EDC which has been shown to elicit responses similar to 17 β -estradiol (E_2). There was no significant effect of low levels of OP (1 μM , 1 nM) or UV-B radiation (7 $\mu\text{W}/\text{cm}^2$) on survivorship and the LC50 was 2.8 μM OP. Levels of UV-B (25 $\mu\text{W}/\text{cm}^2$) that were approximately 10% of summer ambient levels caused an increase in tadpole deformity and mortality and the interaction between OP/UV-B did not result in increased mortality.

The RNA-arbitrarily primed PCR (RAP-PCR) differential display strategy was employed to isolate candidate genes differentially regulated in the tadpole diencephalon which were affected by various OP and UV-B treatments. A reverse Northern multiple-gene dot blot was used to verify expression patterns of specific cDNA transcripts cloned from differential display. Homology cloning was performed to obtain *R. pipiens* GAD65 and GAD67, enzymes responsible for γ -aminobutyric acid (GABA) synthesis, and these were included on the dot blot. Environmentally relevant levels of OP (1 nM) and UV-B (7 $\mu\text{W}/\text{cm}^2$), alone and in combination, modulated the expression of molecules implicated in GABA synthesis, angiogenesis, cellular energy conversions, and signal transduction. For

example, Nck, Ash and phospholipase gamma-binding protein 4 (NAP4), a protein involved in signal transduction pathways, was increased by 3-fold as a result of OP exposure.

Tadpoles exposed early in life to a combination of OP and UV-B displayed accelerated development with respect to overall growth and hindlimb emergence. This study demonstrates for the first time that *R. pipiens* tadpoles are vulnerable to environmentally relevant levels of OP and UV-B, alone and in combination, with respect to survivorship, premetamorphic development, and gene expression in the brain.

Résumé

Des analyses statistiques de grandes envergures effectuées à partir de plusieurs bases de données ont démontré qu'il y a eu un déclin des populations amphibiens depuis les années 1960. L'exposition aux UV-B et à des perturbateurs endocriniens (EDCs) a été proposée comme hypothèse possible afin d'expliquer les déclins observés. Des têtards de grenouille léopard (*Rana pipiens*) nouvellement éclos ont été exposés pour 10 jours à une des trois concentrations d'octylphenol (OP) (10 μM , 1 μM , 1 nM) et à 0.01% d'éthanol servant de véhicule, avec ou sans deux niveaux de radiation UV-B (7 $\mu\text{W}/\text{cm}^2$, 25 $\mu\text{W}/\text{cm}^2$). La CL50 pour OP dans l'eau a également été déterminée. OP est un EDC ayant démontré des effets similaires au 17 β -estradiol (E_2). Il n'y avait pas d'effet significatif à de faibles niveaux d'OP (1 μM , 1 nM) ou de radiation UV-B (7 $\mu\text{W}/\text{cm}^2$) sur la survie et la CL50 était de 2.8 μM OP. Les niveaux d'UV-B correspondant à 10% du niveau ambiant d'une journée d'été (25 $\mu\text{W}/\text{cm}^2$) ont causé une augmentation des malformations et de la mortalité chez les têtards et l'interaction OP/UV-B n'a pas entraîné une augmentation de la mortalité.

La méthode de "differential display" avec l'utilisation d'amorces arbitraires (RAP-PCR) a été employée comme stratégie pour isoler des gènes dont l'expression dans le diencéphale des têtards est affectée par différents traitements à OP et aux UV-B. Un Northern inversé comportant plusieurs gènes a été utilisé pour vérifier le patron d'expression des gènes clonés par "differential display". Un clonage par homologie a été effectué afin d'obtenir GAD65 et GAD67 de *R. pipiens*, des enzymes responsables de la synthèse d'acide γ -aminobutyric (GABA), et ceux-ci ont été inclus sur le "dot blot". Des niveaux d'OP (1 nM) et d'UV-B (7 $\mu\text{W}/\text{cm}^2$) que l'on retrouve dans l'environnement ont modifié, seuls ou en

combinaison, l'expression de molécules impliquées dans la synthèse de GABA, l'angiogenèse, la transformation d'énergie cellulaire et la signalisation cellulaire. Par exemple, l'exposition à OP a triplé l'expression de Nck, Ash et "phospholipase gamma-binding protein 4" (NAP4), une protéine impliquée dans la signalisation cellulaire. Les têtards exposés en début de vie à une combinaison d'OP et UV-B ont démontré un développement accéléré caractérisé par une croissance et une émergence des pattes arrières plus rapides. Cette étude démontre pour la première fois que les niveaux d'OP et d'UV-B que l'on retrouve dans l'environnement peuvent affecter, seuls ou en combinaison, la survie, le développement pré-métamorphique et l'expression de gènes dans le cerveau des têtards de *R. pipiens*.

ACKNOWLEDGMENTS

Somewhat like winning an Oscar, there are probably individuals and events that I might leave out and I apologize ahead of time for that. I'd like to acknowledge funding from a variety of sources that have made the last two years of research possible. NSERC grants to Dr. David Lean, Dr. Vance Trudeau, and a PGSA NSERC scholarship to support my day to day life have helped out tremendously. In addition, Dr. Vance Trudeau's CNTC grant has contributed funds to the amphibian project. I would like to thank family members and friends who have provided external incentive to continue with this project. Also the band, Trucker Billy, for providing an escape from science and a chance to develop musically. Colleagues in both Dr. Vance Trudeau's lab and Dr. David Lean's lab have made this master's experience exceptional (especially the redheads). Summer students are acknowledged as they provided a different tempo in the lab. I would also like to thank the BGSA for putting faith in me as the president for the last year. I thank Bill Fletcher for all his aid in the aquatic care facility and this research could not have been possible without the leopard frogs.

Technical expertise in molecular biological techniques (especially differential display) was provided by Suzanne Chiu and this is graciously acknowledged. I also wish to acknowledge the help of Dr. Craig Parfett and Raymond Pilon of the Mutagenesis Branch of Health Canada for their patience and expertise with the reverse Northern technique. Their help relieved potential stressful times substantially. Of course a thesis is difficult to carry out without an incredible supervisor and fortunately, I had two. Dr. David Lean and Dr. Vance Trudeau have provided continuous opportunities for me to develop as a scientist. They have

also supported me at various conferences which allowed me to gain exposure in the scientific community. Defending a thesis is also difficult without some form of committee and I thank Dr. Iain Lambert from Carleton University and Drs. Tom Moon and Jules Blais from the University of Ottawa for being on my committee and for reading the thesis during busy times. I would like to thank Dr. Michael Berrill (Trent University) for first turning me on to the study of amphibian biology/toxicology. He has been and remains a mentor. Bruce Pauli is also acknowledged as a fellow herpetologist who was always interested in the status of this research. Thank you to Christian Doyon for his translation help with the abstract. Dr. Scott Findlay (University of Ottawa) was extremely helpful in getting me through a statistical maze and I'd like to acknowledge Jeff Houlihan's (University of Ottawa) Nature publication which gives all herpetologists studying amphibian decline a purpose.

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LIST OF ABBREVIATIONS

APEO - Alkylphenol polyethoxylate
BAF - Bioaccumulation factor
BAI - Brain-specific angiogenesis inhibitor
BCF - Bioconcentration factor
C/L - carbon/liter
CA - Cellulose acetate
CRH - Corticotropin-releasing hormone
DOC - Dissolved organic carbon
E₂ - 17 β-estradiol
EDC - Endocrine-disrupting chemical
ER - Estrogen receptor
GABA - γ-aminobutyric acid
GAD - Glutamate decarboxylase
GnRH - Gonadotropin-releasing hormone
GVBD - Germinal vesicle breakdown
HLE- Hindlimb emergence
LC50 - Lethal concentration 50%
NAP4 - Nck, Ash and phospholipase C gamma-binding protein 4
NOEC - No observable effect concentration
nm - nanometer
NP - Nonylphenol
OP - Octylphenol
PAH - Polycyclic aromatic hydrocarbons
PAR - Photosynthetically-active radiation
PCB - Polychlorinated biphenyl
PRL - Prolactin
RAP-PCR - RNA-arbitrarily primed polymerase chain reaction
STP - Sewage treatment plant
T- Testosterone
T₃ - Thyroid hormone
TRH - Thyrotropin-releasing hormone
TSH - Thyrotropin
UV - Ultraviolet radiation
VTG - Vitellogenin

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Chapter 1

1.0 THE EFFECTS OF UV-B RADIATION AND ENDOCRINE-DISRUPTING CHEMICALS (EDCs) ON THE BIOLOGY OF AMPHIBIANS

1.1 Introduction

The First International Herpetological Congress held in Canterbury, England in 1989 represented an important step in addressing the potentially vulnerable status of amphibians worldwide. Statistical meta-analysis of large and diverse data sets has indicated that amphibians have been declining worldwide since the 1960s (Houlahan *et al.* 2000). Habitat destruction is a prime candidate linking these declines. However, cases of extinction have been reported in areas apparently protected from human influence suggesting that other factors including acid precipitation, drought, pesticides, and increased UV-B radiation (280-320 nm), might be plausible causes (Phillips 1994). Although little evidence of reproductive toxicity of xenobiotics in amphibians is available, early exposure to endocrine-disrupting chemicals (EDCs) could cause abnormal development of the amphibian reproductive system, inhibit vital hormone messages that drive metamorphosis, and ultimately contribute to the decline of some amphibian populations (Colborn *et al.* 1997; Clark *et al.* 1998; Kloas *et al.* 1999; Lutz and Kloas 1999; Pickford and Morris 1999).

Declining populations are not the only concern. Increasing occurrences of severely deformed frogs and toads have been reported recently in widespread areas of the United States and Canada. Ouellet *et al.* (1997) have documented 25 distinct types of deformities in natural populations of green frogs (*Rana clamitans*), northern leopard frogs (*R. pipiens*), American toads (*Bufo americanus*), and bullfrogs (*R. catesbeiana*). Common deformities

include ectrodactyly (missing digits) and ectromelia (missing limbs), supernumerary limbs and digits (polymelia, polydactyly), ectopic limb growth, and eye and central nervous system malformations (Ouellet *et al.* 1997; Ankley *et al.* 1998a). At present, the ultimate cause of this phenomenon is not fully understood. However, retinoid pesticides (e.g. methoprene), which are derived from vitamin A, are known to produce birth defects in humans and other vertebrates by acting as agonists of the retinoid receptor(s) (Ankley *et al.* 1998a).

With a scope as broad as amphibian decline, individual studies must be carried out to elucidate single or synergistic causes of some of the observed deformities and declines in native amphibian species such as the leopard frog (*R. pipiens*). Few studies have addressed the importance and impact of EDCs on amphibian species and in fact, the U.S. EPA stated recently that, "...[amphibians] might represent a unique sentinel animal model for laboratory and field exposure studies" (Pickford and Morris 1999, p. 285). The data that exists regarding the effects of UV-B on amphibian development is controversial and inconsistent. Thus, a study which focuses on the possible synergy between UV-B and the xenoestrogen octylphenol (OP) on the development of *R. pipiens* is extremely important. Additionally, analyzing the effects of these stressors at the level of gene expression will provide insights into the subtle, molecular changes that may impact reproductive success and normal development of this species. By exposing *R. pipiens* to environmentally relevant doses of OP and UV-B, alone and in combination, we can begin to address the complexity behind endocrine disruption and combinations of xenobiotic and biotic factors.

The review of relevant literature which follows provides a framework for the UV-B hypothesis, endocrine disruption and combinations of xenobiotic and biotic factors as they

relate to amphibians.

1.1.2 UV-B Radiation (280-320 nm)

Since the early 1970s, predictions have been made that human activities would lead to a depletion of the stratospheric ozone layer (Stolarski *et al.* 1992). The discovery of the Antarctic ozone hole in 1985 verified these concerns (Stolarski *et al.* 1992). Kerr and McElroy (1993) documented a trend which linked ozone depletion with a concurrent increase in ambient UV-B radiation (280-320 nm) (Figure 1.1). Increases in UV-B radiation have been on the order of 10-20% in north-temperate regions from 1979-1992 (Madronich 1994). Kerr and McElroy (1993) calculated that, annually, UV-B radiation was increasing by 35% during the winter months and 6.7% during the summer months in Toronto, Ontario. The 35% increase is disproportionately less intense than the 6.7% calculated for summer months because the intensity of UV-B is at a maximum between May and August. The flux between winter and summer intensities results in a maximal, seasonal change of UV-B reaching the earth's surface in the early spring. This is critical because several amphibians undergo important developmental processes which are potentially UV-sensitive in the early spring.

The pronounced shift in the spectral balance between UV-B, UV-A (320-400 nm) and photosynthetically-active radiation (PAR; 400-700 nm) due to ozone depletion represents a key determinant of how much UV-B induced photodamage will occur relative to UV-A and PAR photoreactivation (DeFabo 1992). The shift is disproportionately weighted towards the lower wavelength UV-B which has a greater capacity to induce biological damage. Biologically effective doses have been calculated for several factors including generalized DNA damage, photosynthesis, erythematous (or sunburn) references and, cyclobutane

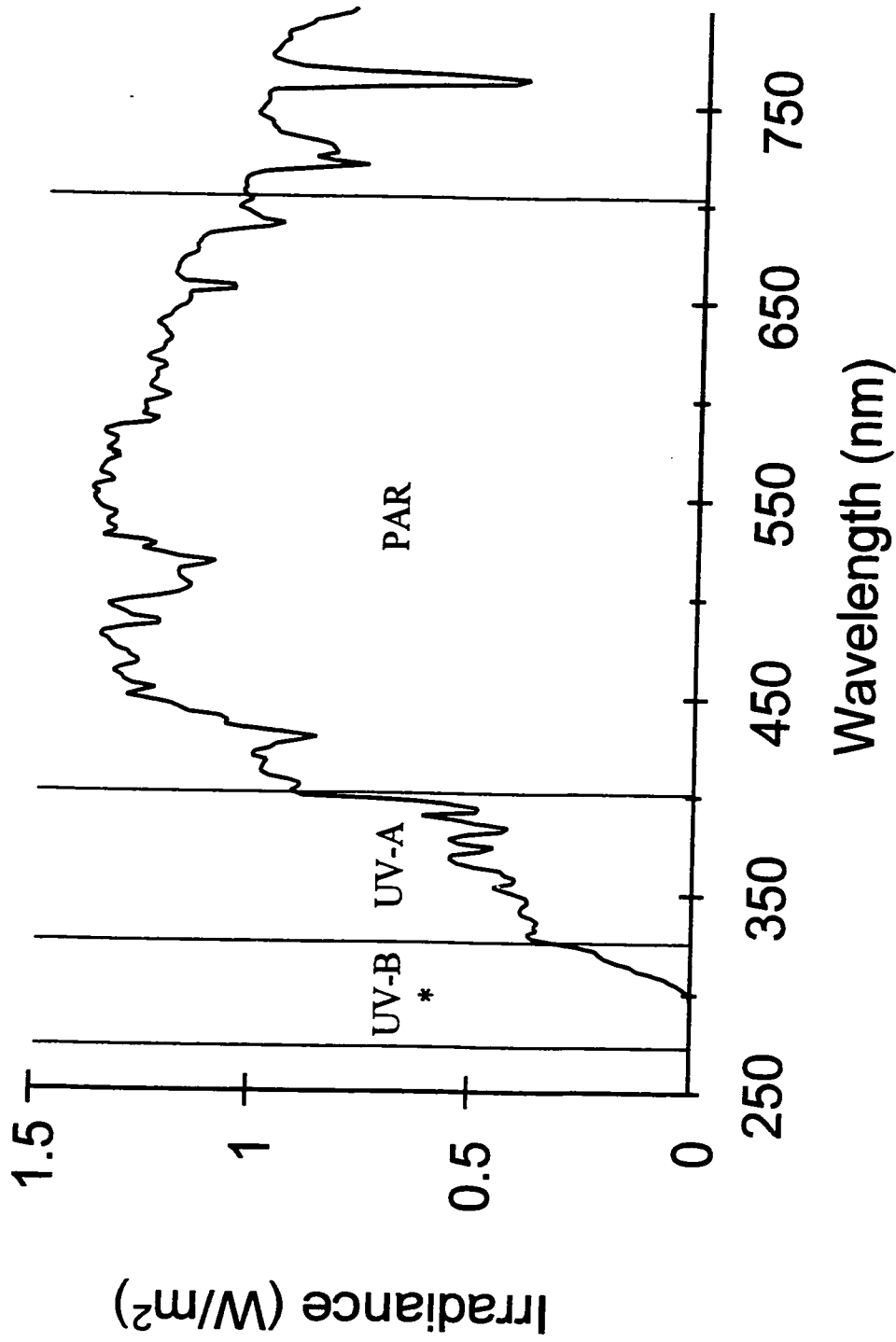


Figure 1.1. Spectral composition of ambient summer sunlight as measured by a spectroradiometer (Crump unpublished results). The spectrum has been divided based on spectral portion as follows; UV-B (280-320 nm), UV-A (320-400 nm), and photosynthetically-active radiation (PAR; 400-700 nm). * Denotes the portion of the spectrum most affected by ozone depletion.

pyrimidine dimer formation. In all cases, the lower the wavelength (into the UV-B region), the greater the biological impact (Madronich 1994).

1.1.3 UV-B and Amphibian Development

Several studies have shown that amphibian embryos and larval stages display increased abnormality and mortality when exposed to both ambient and enhanced UV-B radiation (Worrest and Kimeldorf 1976; Blaustein *et al.* 1994; Blaustein *et al.* 1995; Grant and Licht 1995; Hays *et al.* 1996; Blaustein *et al.* 1997; Nagl and Hofer 1997; Ovaska *et al.* 1997; Anzalone *et al.* 1998; Lizana and Pedraza 1998; Crump *et al.* 1999a). Assuming ambient UV-B continues to increase as a result of stratospheric ozone depletion, additional research with respect to UV-B and amphibian development is crucial. A comprehensive review of the recent findings concerning UV-B and amphibian development is presented in Table 1.1.

Laboratory Studies

Higgins and Sheard (1926) exposed leopard frog (*R. pipiens*) embryos to a quartz mercury lamp daily and observed abnormalities such as the twisting of neural ridges. They found that the jelly layer surrounding developing embryos absorbed UV-B and thus the size and thickness determined the negative effect. Blum and Matthews (1952) exposed salamander larvae to UV-B alone, and in the presence of UV-A, and found that UV-A was extremely important for the process of photorecovery. Under UV-B alone, substantial mortality occurred.

Table 1.1. Summary of recent field and lab exposures of aquatic stages of amphibians to incident and enhanced levels of UV-B radiation.

Species	Ref.	Altitude (m)	UV-B Level		Placement of Containers			UV-B sensitivity			
			Inc	Enh	In Lab	Outdoors	In Pond	Embryos		Tadpoles	
								Inc	Enh	Inc	Enh
<i>Hyla regilla</i>	1	1200	X				X	N			
	2	90	X	X		X		N	N	N	Incr
	9	290	X				X	N			
<i>H. cadaveria</i>	9	290	X				X	Decr			
<i>Rana cascadae</i>	1	1200	X				X	Decr			
<i>Bufo boreas</i>	1	1200	X				X	Decr			
<i>R. aurora</i>	2	90	X	X		X		N	Decr	N	Incr
	3	76	X			X		N			
<i>Ambystoma gracile</i>	4	183	X				X	Decr			
<i>A. macrodactylum</i>	5	2000	X				X	Decr			
<i>Taricha alpestris</i>	6	—	(X)	X	X					Incr	Incr
		580	X			X				Incr	Incr
<i>T. torosa</i>	9	290	X				X	Decr			
<i>R. pipiens</i>	7	250	X	X		X		N	N		
	13	100	X	X			X	N	N	N	Incr
<i>H. versicolor</i>	8	—	(X)	X	X			N	N		
	13	100	X	X			X	N	N	N	Incr
<i>R. clamitans</i>	8	—	(X)	X	X			N	N		
	13	100	X	X			X	N	N	N	Incr
<i>R. sylvatica</i>	8	—	(X)	X	X			N	N		
	13	100	X	X			X	N	N	N	Incr
<i>B. americanus</i>	8	—	(X)	X	X			N	N		
	13	100	X	X			X	N	N	N	Incr
<i>B. bufo</i>	10	1920	X				X	Decr			
	11	2810	X				X	N			
<i>B. calamita</i>	10	1920	X				X	N			
<i>R. temporaria</i>	12	10	X	X		X		N	N		
<i>A. maculatum</i>	13	100	X	X			X	N	N	N	Incr
<i>A. laterale</i>	13	100	X	X			X	N	N	N	Incr
<i>R. catesbiana</i>	13	100	X	X			X	N	N	N	Incr

References: 1, Blaustein *et al.* 1994; 2, Ovaska *et al.* 1997; 3, Blaustein *et al.* 1996; 4, Blaustein *et al.* 1995; 5, Blaustein *et al.* 1997; 6, Nagl and Hofer 1997; 7, Grant and Licht 1995; 8, Long *et al.* 1995; 9, Anzalone *et al.* 1998; 10, Lizana and Pedraza 1998; 11, Corn 1998; 12, Cummins *et al.* 1999; 13, Crump *et al.* 1999a. (Abbreviations: (X), incident level approximated under lab conditions; N, no difference from controls; Inc, Incident; Enh, Enhanced; Decr, Decreased; Incr, Increased)

Worrest and Kimeldorf (1976) were the first to address concerns regarding ozone depletion in the context of amphibian development. Boreal toad (*B. boreas*) tadpoles were exposed to enhanced UV-B and abnormal development of the presumptive cornea, curvature of the spine and increased mortality were observed. Abnormalities occurred predominantly during limb-bud and foot-paddle stages of metamorphosis. The authors suggested that increased UV-B exposure might result in an increased frequency of abnormalities and a hindered competitive ability of metamorphs. Worrest and Kimeldorf (1976) also found that exposure to UV-A and visible light, preceding or following the exposure to UV-B, mitigated the lethal effects of UV-B. The disproportionate increase in UV-B as a result of ozone depletion may limit the processes of photorepair and recovery by UV-A and visible light.

American toad (*B. americanus*), grey treefrog (*H. versicolor*), green frog (*R. clamitans*), and wood frog (*R. sylvatica*) tadpoles were exposed to simulated ambient and enhanced UV-B and all displayed complete mortality under enhanced UV-B. At ecologically relevant doses, however, no significant effect was observed on any metamorphic parameter or larval survivorship (Grant and Licht 1995). *R. sylvatica* embryos hatched successfully at ecologically relevant levels of UV-B but experienced acute lethality at the extreme of the artificial intensities. These artificial intensities would not be realized in the wild.

Increased larval mortality and developmental irregularities including lordosis (curvature of the spine), bloating/distension and abnormal development of the cornea were observed for *R. cascade* and *H. regilla* tadpoles and metamorphs that were chronically UV-treated (Hays *et al.* 1996). Long *et al.* (1995) reported no detectable effect of UV-B, even at the highest UV-B level (9507 eff J/m²/day), on embryonic mortality of *R. pipiens*. Similarly,

Zaga *et al.* (1997) exposed *H. versicolor* and *Xenopus laevis* embryos and tadpoles to 0.65 W/m² UV-B and observed minimal effects. When we exposed newly-hatched *R. pipiens* tadpoles to 0.65 W/m² and 0.25 W/m² in the laboratory for 6 hours per day, approximately 90% mortality was observed in the high exposure after 5 days and 80% after ten days for the low exposure (unpublish. results). The tadpoles were exposed in clear water in shallow petri dishes allowing for no attenuation of UV-B. This is unlikely representative of typical breeding areas where dissolved organic carbon (DOC) attenuates ambient UV-B levels rapidly (Crump *et al.* 1999a,b).

Ankley *et al.* (1998a) linked UV-B directly with hindlimb deformities of *R. pipiens* tadpoles when they were exposed to 0.44 W/m² UV-B. They found that this level of irradiance induced bilateral, often symmetrical, hindlimb ectromelia and ectrodactyly. The effect of this sub-ambient UV-B level was stage specific as tadpoles exposed to UV-B before 2-3 weeks (stage 23-25) of age did not exhibit significant deformities. Prior to stage 23, developing amphibians are relatively immobile and restricted to the depth in the water column at which the eggs are laid. As mobility of the tadpoles increases (approximately stage 24), there is more potential to avoid extreme UV regimes which could mitigate the effects seen by Ankley *et al.* (1998a) in the field.

Adults of three amphibian species were exposed to ten times the normal outdoor level of UV-B and the two species that had stable populations, *X. laevis* and the Pacific tree frog (*H. regilla*), avoided the high UV-B regime (Phillips 1994). The Cascade frog (*R. cascade*), which is a declining species, did not. Van de Mortel and Buttemer (1998) carried out a similar experiment with adults and tadpoles of the green and golden bell frog (*Litoria aurea*),

a species, noted for basking, which has disappeared completely from high altitude sites in New South Wales. *L. dentata* and *L. peronii* were also included in the study as they have stable populations and are not noted for basking. They found that tadpoles of all three species might be able to detect and avoid high levels of UV-B. *L. aurea* and *L. peronii* adults did not show a preference for areas under UV-B blocking filters, however, the statistical power was very low and the authors suggested that differences in population stability between these species was unlikely due to a differential ability to detect peaks in UV-B. (van de Mortel and Buttemer 1998).

Field Experiments

Field exposures are extremely important as they provide an indication of the vulnerability of amphibians with respect to UV-B in their natural habitat. Studies of this nature are more complex than laboratory studies as variables such as water chemistry (dissolved organic carbon (DOC) content, water hardness, pH), spatial and temporal shading, altitude, latitude, and depth in the water column where eggs are deposited must be taken into account. The underwater spectral properties are greatly modified in frog ponds due to DOC content as the chromophores of DOC are extremely efficient for the attenuation of UV-B radiation (Scully and Lean 1994; Crump *et al.* 1999b). In ponds with high DOC, amphibian species that deposit their eggs deeper in the water column would be protected due to rapid attenuation.

A pioneering *in situ* study was conducted by Blaustein *et al.* (1994) in the Cascade mountain region of Oregon (1200-2000m). Using enclosures in natural oviposition sites subjected to ambient and UV-B deficient sunlight, the authors found that *H. regilla* embryos

were highly resistant to UV-B whereas *B. boreas* and *R. cascade* displayed greater hatching success under sunlight lacking UV-B. Upon measuring the enzymatic activity of photolyase, *H. regilla* was found to have the highest photolyase activity whereas, *B. boreas* and *R. cascade* had the lowest levels of activity (Blaustein *et al.* 1994). Photolyase repairs cyclobutane pyrimidine dimers (CBPDs) which form between adjacent thymine residues in DNA due to UV-B exposure (Kim and Sancar 1993).

It was previously determined that the northwestern salamander (*Ambystoma gracile*) had low photolyase activity (Blaustein *et al.* 1994). When exposed *in situ* to UV-B regimes, as described above, embryos that were shielded from UV-B suffered significantly less mortality than those exposed to ambient sunlight (Blaustein *et al.* 1995). The long-toed salamander (*A. macrodactylum*) has low photolyase activity (Blaustein *et al.* 1994) and lays its eggs in shallow open water leaving them potentially vulnerable to high UV-B regimes during embryonic development. Ambient levels of UV-B were found to adversely affect development and induce deformities contributing directly to embryo mortality and perhaps reduced fitness after hatching (Blaustein *et al.* 1997).

By employing a similar *in situ* strategy, Lizana and Pedraza (1998) found a detrimental effect of ambient UV-B radiation on embryonic survival of *B. bufo*. However, *B. calamita*, that spawn in very shallow water and thus might be expected to have a greater resistance to high levels of UV-B hatched successfully when exposed to UV-B. Neither hatching success nor rate of development of *H. regilla* embryos were affected by UV-B whereas, *H. cadaverina* and *Taricha torosa* embryos displayed high rates of mortality under ambient UV-B (Anzalone *et al.* 1998). This study showed intraspecific consistency with the

results obtained by Blaustein *et al.* (1994) with respect to the resistance of *H. regilla* and perhaps provides support for the photolyase hypothesis.

Photolyase activity in three Australian tree frogs (*L. aurea*, *L. dentata*, and *L. peronii*) was compared and the species that had suffered marked population declines (*L. aurea*) had the lowest photolyase activity of the three. A non-significant trend of reduced hatching success under UV-B was observed (van de Mortel *et al.* 1998). However, when compared to species monitored in the photolyase study by Blaustein *et al.* (1994) several discrepancies arose. *L. aurea* had a dimer repair rate similar to *A. gracile* (Blaustein *et al.* 1994; 1995) but relative hatching success was substantially higher for *L. aurea* embryos (van de Mortel *et al.* 1998). Additionally, *L. peronii* embryos had a repair rate similar to that of *R. cascade*, a declining species that experienced high mortality under ambient UV-B. In contrast, *L. peronii* did not appear to be declining and did not show susceptibility to UV-B (van de Mortel *et al.* 1998). Finally, the hatching success of *L. peronii* was similar to *L. dentata*, although the photolyase activity of the latter was double. Thus differences in photolyase activity may not necessarily manifest themselves as differences in hatching success.

B. boreas had relatively low photolyase activity, displayed decreased hatching success under UV-B, and is reported to be in decline (Blaustein *et al.* 1994). In a field exposure, Corn (1998) found no differences in *B. boreas* hatching success related to UV-B exposure. Corn (1998) suggested several possible causes for the discrepancy. The water mold *Saprolegnia ferax* which acts synergistically with UV-B with respect to amphibian survival (Kiesecker and Blaustein 1995) was not found in his study site. In addition, neither the level of UV-B nor water chemistry parameters such as DOC and turbidity that affect the

penetration of UV-B were quantified. The size of enclosures used varied between the two studies and Corn (1998) used saran wrap as a UV-B transmitting filter whereas Blaustein *et al.* (1994) used cellulose acetate. Cellulose acetate (CA) is a plastic that has been widely used in UV-B studies as it excludes wavelengths less than 290 nm which is an environmentally relevant cut-off point for the ultraviolet spectrum (Middleton and Teramura 1993).

Three studies have emerged recently that address the issue of ozone depletion by exposing developing amphibians to enhanced UV-B in the field. Crump *et al.* (1999a) reported no effect of ambient or enhanced UV-B on the hatching success of eight amphibian species. Even under enhanced UV-B supplied by lamps, attenuation was so great due to the high DOC content (13.0 mg C/L) that the enhanced levels at 3 cm (depth of the enclosures) were similar to incident values at 1.36 cm (Figure 1.2). Differential sensitivity was predicted based on where embryos were typically laid in the water column. However, hatching success was in excess of 82% in all cases indicating a previously unpredicted tolerance of a wide range of species (Crump *et al.* 1999a). Larvae of all eight species, including *R. pipiens*, were negatively affected by enhanced UV-B with later stages being more sensitive than newly hatched.

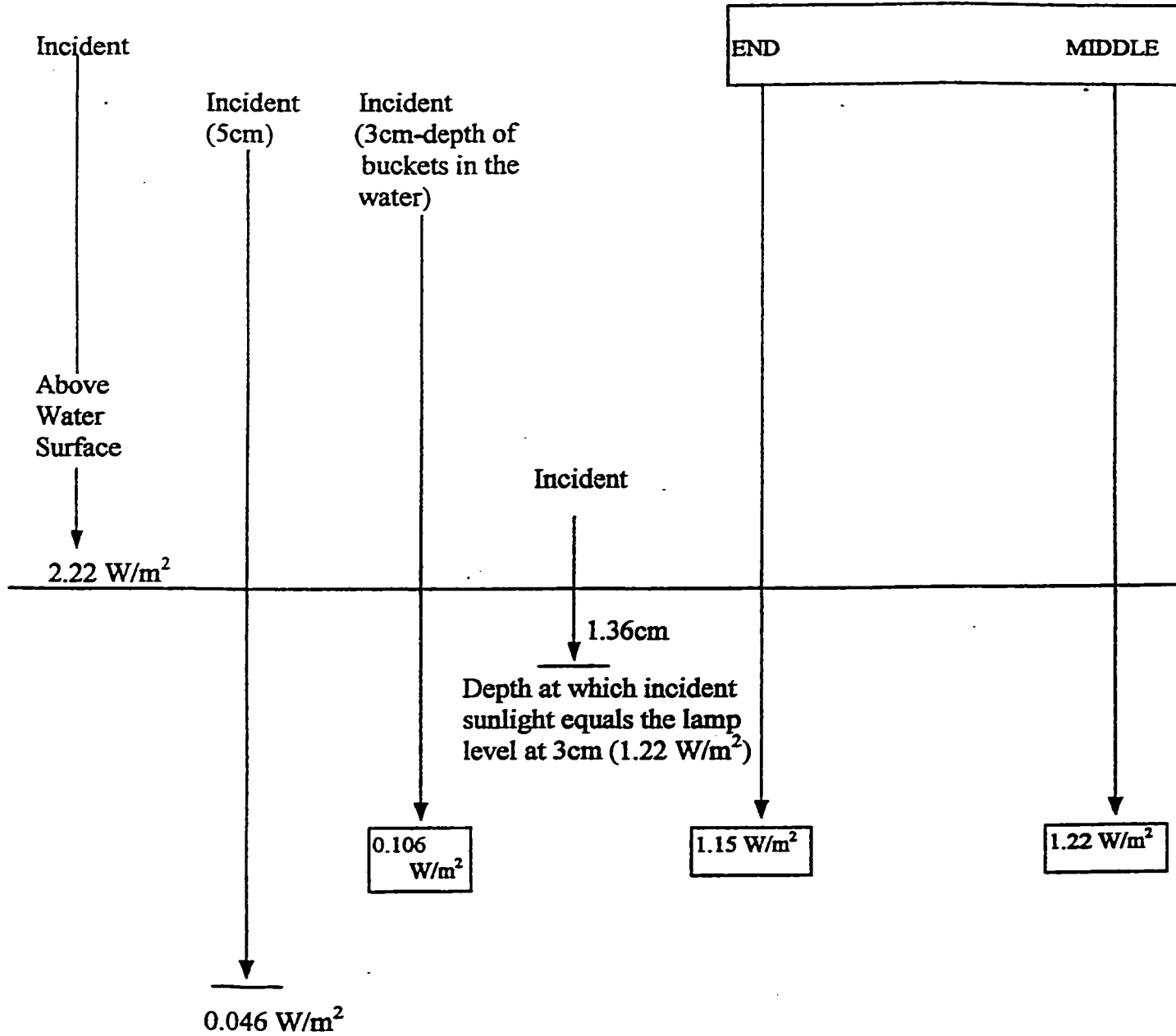


Figure 1.2. Relative UV-B irradiance (W/m^2) under enhanced and ambient conditions at a natural pond with high dissolved organic carbon (DOC) content (13.0 mg C/L). Enhanced levels at 3 cm (depth of the enclosures) were similar to incident values at 1.36 cm due to the rapid attenuation (adapted from Crump *et al.* 1999a).

Ovaska *et al.* (1997) achieved UV-B enhancement levels of 15 and 30% above midday ambient levels and found hatching success of *H. regilla* did not differ between ambient, filtered or enhanced UV-B whereas, *R. aurora* experienced reduced hatching success under enhanced UV-B. The larvae of both species displayed increased mortality under enhanced conditions and for both species, the hatching success and larval survivorship were not affected by ambient UV-B. Cummins *et al.* (1999) exposed common frog (*R. temporaria*) embryos outdoors to supplemental UV-B. The effective dose was approximately double that experienced by embryos under ambient conditions. No increase in either mortality or incidence of overt abnormality was observed compared to ambient conditions.

1.1.4 The UV-B hypothesis questioned

The discrepancies between studies are intriguing. Oddly, in experiments where UV-B is enhanced both in the field and in the lab, hatching success is rarely affected (exception is *R. aurora* in Ovaska *et al.* 1997), whereas in certain field studies, especially those at high altitude sites (Blaustein *et al.* 1994; Blaustein *et al.* 1995; Blaustein *et al.* 1997; Anzalone *et al.* 1998; Lizana and Pedraza 1998), ambient UV-B causes increased mortality (Table 1.1). The UV-B hypothesis is thus more complicated than originally believed.

When making an overall comparison of both lab and field studies it is important to note that in several studies UV-B was not measured (Blaustein *et al.* 1994; Blaustein *et al.* 1995; Hays *et al.* 1996; Corn 1998; Anzalone *et al.* 1998; Lizana and Pedraza 1998) and where it was measured, inconsistencies in units exist (Table 1.2).

Table 1.2. A summary of UV-B levels measured, type of instrument used, and type of material used as a UV-B screen in amphibian studies.

UV-B Intensity	Instrument Used	UV-B Screen	Placement of screens	Reference
0.8 W/m ² ; 0.15 W/m ²	N/A	CA M	On containers	Worrest and Kimeldorf
N/A	Optronics OL 752	CA M	On containers	Blaustein <i>et al.</i> 1994
N/A	N/A	CA M	On containers	Blaustein <i>et al.</i> 1995
4341 and 9507 <i>eff</i> J/m ² /day	Optronics OL 752	CA M	Around lamps	Long <i>et al.</i> 1995
0.05-0.255 W/m ²	Solar Light PMA 2100 UV meter	CA M	On containers	Blaustein <i>et al.</i> 1997
0.67 W/m ²	UVX radiometer (310 nm)	CA	Around lamps	Licht and Grant 1997
0.8 W/m ² ; 2.0 W/m ²	Delta-T Devices Ltd. UV meter (312nm)	M	On containers	Nagl and Hofer 1997
0.04 W/m ² ; 0.65 W/m ²	Optronics OL 754	N/A	Around lamps	Zaga <i>et al.</i> 1997
N/A	N/A	Acrylic M	On containers	Anzalone <i>et al.</i> 1998
0.44 W/m ²	IL 1700 Radiometer	N/A	N/A	Ankley <i>et al.</i> 1998a
N/A	N/A	Saran Wrap M	On containers	Corn 1998
N/A	N/A	Llumar Film PVC	On containers	Lizana and Pedraza 1998
0.106 W/m ² ; 1.22 W/m ²	Optronics OL 752	CA M	Around lamps (CA); on containers (M)	Crump <i>et al.</i> 1999a

(N/A = not available; CA = cellulose acetate; M = Mylar)

For example, Grant and Licht (1995) report exposing amphibians to an ecologically relevant dose of 0.961 W/cm^2 which is equivalent to $0.961 \times 10^{-4} \text{ W/m}^2$. In a more recent review paper, Licht and Grant (1997) cite the ecologically relevant dose of UV-B used in their first paper as 0.961 J/cm^2 over 4 hours. This is equivalent to 0.67 W/m^2 which falls close to the range established by other studies. Kirk *et al.* (1994), Scully and Lean (1994), Zaga *et al.* (1997), Ankley *et al.* (1998a), Crump *et al.* (1999a), and Crump *et al.* (1999b) report summer levels of ambient UV-B between 1.0 and 2.5 W/m^2 . The issue of radiation units is therefore extremely important as is the instrument used to collect the radiation data.

Blumthaler and Ambach (1990) report that the influence of tropospheric ozone is reduced at high-altitude stations but give no numerical comparisons of total UV-B irradiance at high and low altitude sites. Only one high altitude amphibian study to date has documented UV-B levels and reported values of $4.77\text{-}25.5 \mu\text{W/cm}^2$ ($0.05\text{-}0.25 \text{ W/m}^2$) at 2000 m (Blaustein *et al.* 1997). This range is much less than recorded ambient UV-B at several low elevation sites indicated above. At an altitude of approximately 2000 m in the Canadian Rocky mountains, Scully (unpublished data) recorded ambient UV-B ranging from $0.8\text{-}4.6 \text{ W/m}^2$ depending on the intensity of cloud cover. Because limited studies exist and reliable UV-B measurements are difficult to find, interstudy comparisons can prove to be difficult. Thus, a main component of any UV-B study should clearly be reliable UV-B measurements.

The toxicity of plastics used as UV filters in the field and lab must also be addressed since this may indirectly impact results. Berrill and Lean (1998) reported that CA from two different suppliers was toxic to developing amphibians when the plastic was submerged in the field. In a lab toxicity test, tadpoles were exposed to 1.3 grams of the acetate (enough to

cover the bottom of a 1 L beaker), and there was 100% mortality within 18 hours (Berrill and Lean 1998). Thus, experiments that utilize CA as a UV-B screen should address the possibility of plastic toxicity as well as UV-B effects.

Especially for *in situ* studies, water quality parameters such as temperature and DOC are extremely important to acknowledge. Temperature can affect developmental rate and high DOC concentrations may result in minimal exposure to UV-B depending on the depth at which embryos are laid. As Carey (1999, p. 1) states "...[it is important to] evaluate how these disparate conclusions have been reached and whether the data currently support the contention that UV-B has played a causal role in amphibian declines."

1.1.5 Endocrine-disrupting chemicals (EDCs) in the environment

The Chemical Manufacturers Association (CMA) in the USA has stated that more than \$500 million is expected to be spent to test 15,000 chemicals for hormone-mimicking properties (ECME 1999). Within the past decade, the knowledge base surrounding EDCs has evolved extremely rapidly (Colborn 1995). The underlying reason behind this interest lies in the view that, "humans now live in an environment that can be viewed as a virtual sea of estrogens" (Sharpe and Shakkebaek 1993, p. 1392). To provide a basis for discussion, it is important to have a working definition for the term EDC. At an EPA workshop in April 1995, the following definition of an EDC was proposed: "...an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock and Ankley 1996, p. 732). This definition is extremely broad and multi-faceted, as are the potential mechanisms of action of these

substances.

Both natural and synthetic EDCs are ubiquitous in the environment. Natural estrogens, commonly referred to as phytoestrogens, are found in many plants and fungi (mycoestrogens). Soya is the richest source of phytoestrogens and its consumption has increased over the past two decades as it commonly substitutes meat protein in vegetarian diets (Sharpe and Shakkebaek 1993). Hormone mimics can also be found in parsley, sage, garlic, wheat, oats, rye, barley, potatoes, alfalfa sprouts, coffee, bourbon whiskey, beer and in the leaves of marijuana plants (Colborn *et al.* 1997; Nimrod and Benson 1998).

Phytoestrogens, which include compounds such as genistein, equol, coumestrol and enterolactone, have been shown to induce an estrogenic response in several *in vitro* and *in vivo* assays (Nimrod and Benson 1998).

A number of anthropogenic environmental pollutants including *o,p'*-DDT, *p,p'*-DDE, bisphenol A, octylphenol (OP), nonylphenol (NP), TCDD, PCBs, methoxychlor, endosulfan, and benzo[a]pyrene, are known to disrupt the endocrine systems of wildlife and humans (Colborn *et al.* 1997; Nimrod and Benson 1998). These compounds can mimic the effects of endogenous sex steroids such as testosterone (T) and 17 β -estradiol (E_2), by interacting with androgen or estrogen receptors. However, the chemical structures of several compounds that have displayed estrogenic activity are remarkably dissimilar compared to the structure of 17 β -estradiol (E_2) (Figure 1.3). Because of the structural dissimilarity of environmental estrogens, bioassays must be based on biomarkers of estrogenicity that use a predictable estrogenic response as an indicator of exposure.

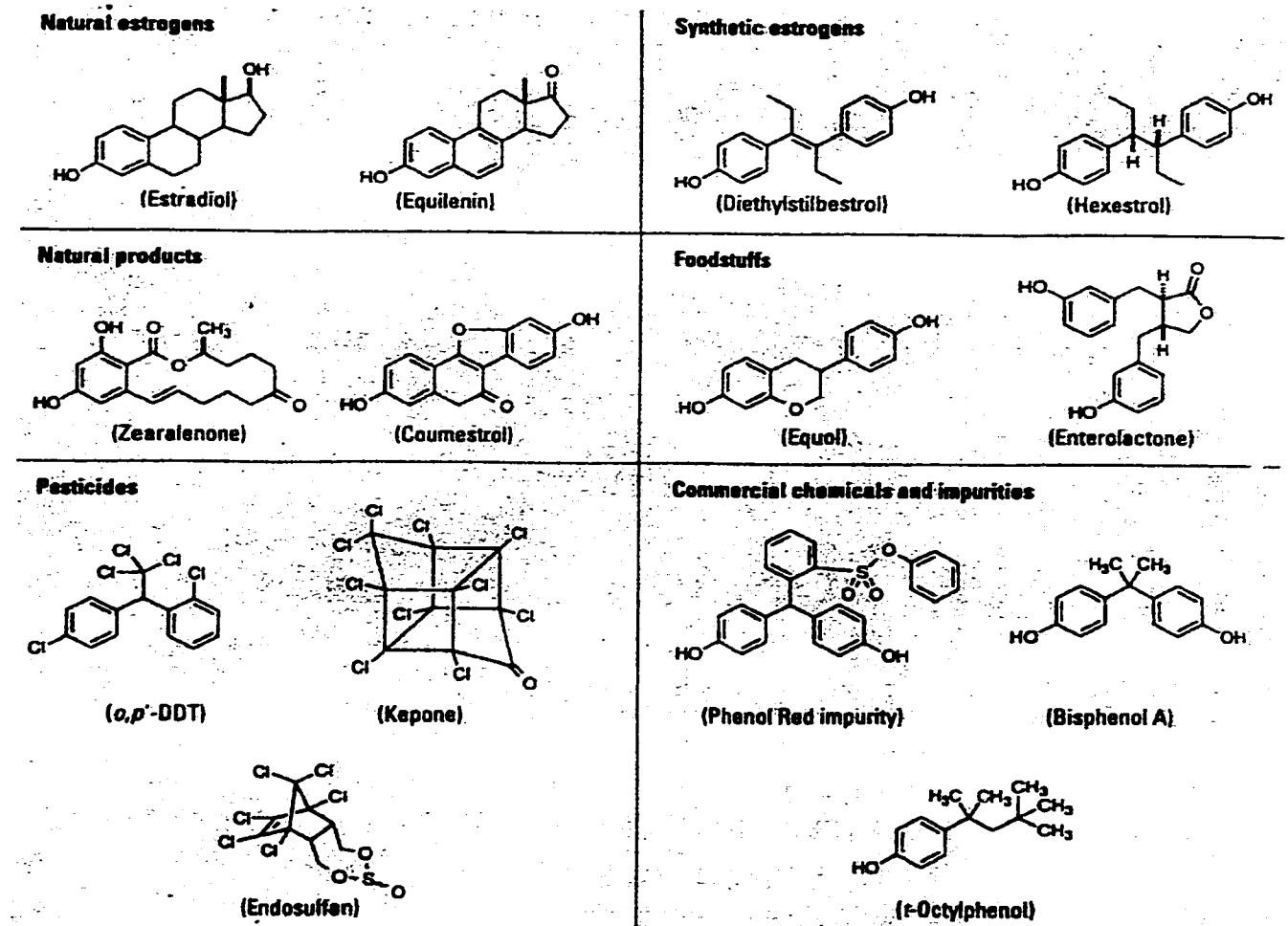


Figure 1.3. A variety of dissimilar structures of compounds that have estrogenic activity displayed according to their various sources (adapted from Katzenellenbogen 1995).

1.1.6 Alkylphenol Polyethoxylates

U.S. domestic exports of OP, NP and their isomers to Canada amounted to 1.8×10^6 kilograms in 1998 (www.ita.doc.gov). The primary use of these technical grade products is in the preparation of alkylphenol polyethoxylates (APEOs). There are two main forms of domestic and industrial APEOs: nonylphenol polyethoxylates (NPEOs) and octylphenol polyethoxylates (OPEOs). NPEOs account for 85% of the market (>250,000 tonnes worldwide per year) and OPEOs make up the remaining 15% (Bennett and Metcalfe 1998). APEOs are a major group of surfactants and their formulations are employed as detergents, emulsifiers, dispersants, antifoamers, dyeing assists, stabilizers, lubricants, spermicides and pesticide adjuvants (Bennie *et al.* 1998). Five sectors are responsible for the bulk of the environmental discharges including, pulp and paper manufacturing, petroleum production, household/industrial/institutional cleaning, textile manufacturing, and leather manufacturing (Maguire 1999). These products are also used to a lesser extent in building and construction, paint and protective coating, metal processing, plastic and elastomer manufacturing, and food and beverage sectors (Bennie 1999). NP has been used directly in aminocarb insecticide sprays to control spruce budworm. Consequently, there are many routes for these substances to make their way into the environment.

NP and its ethoxylates were added to the Priority Substances List 2 (PSL2) in December 1995 (Maguire 1999). OP and its ethoxylates were not directly included on the list as they account for only a small portion of the market usage. In addition, worldwide, very little data are available for OPEOs and their degradation products. However, OP is not only a degradation product of OPEOs but is also found as a minor component in NPEO preparations

(Bennie *et al.* 1998).

The following recommendation was made regarding NPEOs by the Ministers' (of Health and of the Environment) Expert Advisory Panel:

“NPEs are discharged into the environment primarily from textile and pulp and paper production facilities. They are also used in coal processing, latex paints, grease and lubricating oils, pesticides and industrial detergents. Acute adverse effects have been reported in invertebrates, fish, mammals and algae. There are also concerns that these substances may interfere with endocrine function. An assessment is required to determine exposure levels and the risk they may pose to the environment and human health in Canada.” (Bennie 1999, p. 81; Maguire 1999, p. 39; Servos 1999, p. 125)

The biological degradation pathway of APEOs is shown in Figure 1.4. Under aerobic and anaerobic conditions, oxyethylene chains are cleaved and this primary biodegradation yields the more toxic and estrogenic metabolites known as alkylphenols (OP and NP) (Jobling and Sumpter 1993). These degradation products are hydrophobic (K_{ow} OP = 4.12), relatively resistant to further microbial degradation to H_2O and CO_2 , and tend to be associated with sludge and sediments (Bennie 1999). Approximately 60% of APEO discharge to the environment from sewage treatment plants (STPs) occurs as alkylphenols in the sludge (Bennie 1999). Therefore, these metabolites have the potential to accumulate in sediments and lead to toxic effects on vertebrates such as amphibians or turtles which spend a great deal of their lives associated with sediments. In areas close to human habitation and potential discharge, the impacts could be quite significant, however amphibian toxicity data does not exist for OP at present.

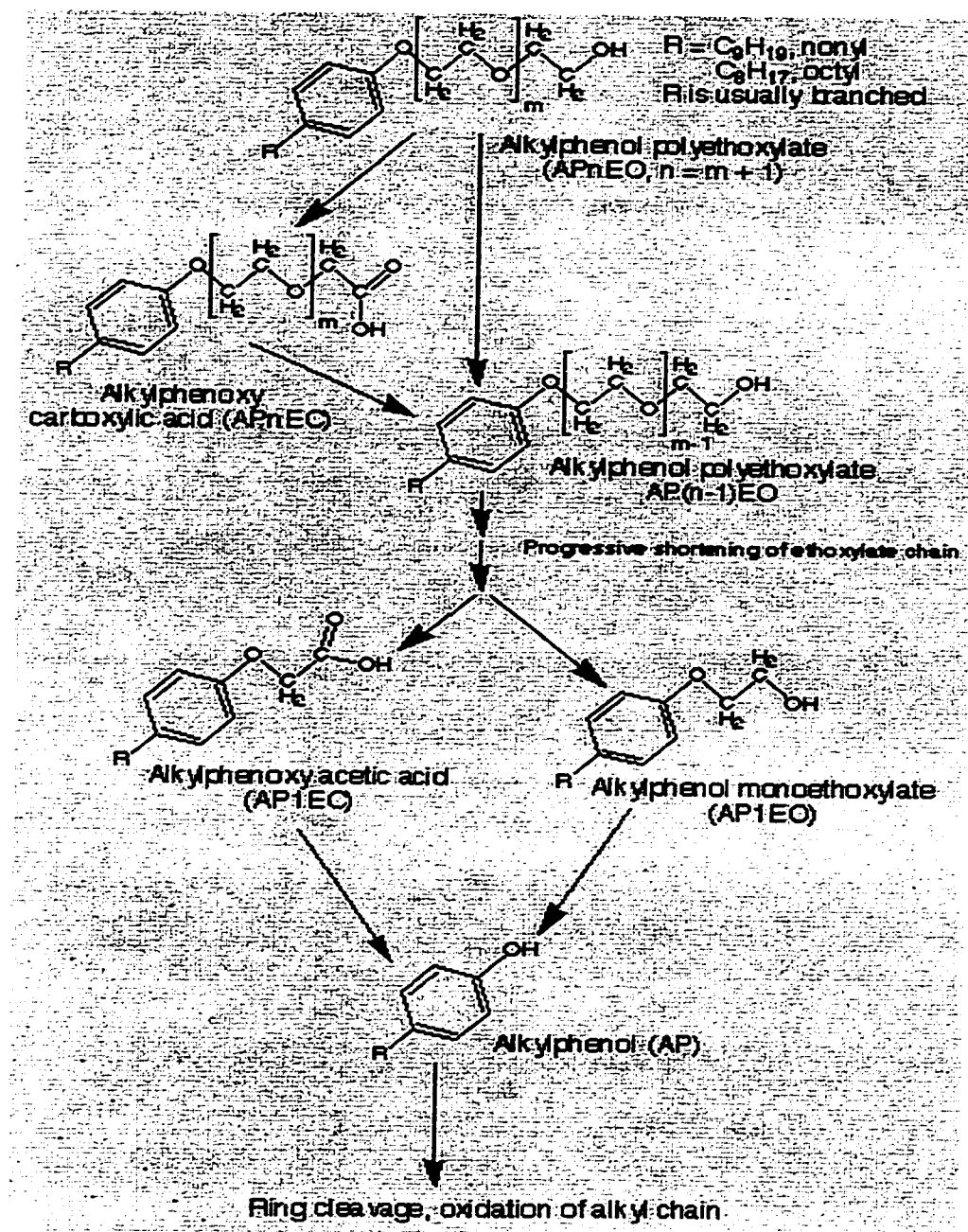


Figure 1.4. The biological degradation pathway of alkylphenol polyethoxylates (APEOs). The R group is either a branched octyl or nonyl group and the degradation product of concern is the alkylphenol (AP), either octylphenol (OP) or nonylphenol (NP). (adapted from Maguire 1999)

In the mid 1990s, studies began emerging indicating that detergent components in sewage effluents were weakly estrogenic to fish. These compounds resulted in the synthesis of vitellogenin (VTG) by hepatocytes (*in vitro*) and male fish (*in vivo*) (Jobling and Sumpter 1993; Sumpter and Jobling 1995; Folmar *et al.* 1996). Folmar *et al.* (1996) found that male carp collected downstream from an STP had significantly elevated VTG levels and significantly reduced serum testosterone levels compared to control fish. Jobling and Sumpter (1993) and Sumpter and Jobling (1995) identified the APEOs, specifically NP and OP, as the major contributors in the observed VTG induction. *In vitro* VTG assays with trout hepatocytes revealed that 2.11 μM OP had a similar effect on VTG synthesis as 1.81 nM E_2 (Jobling and Sumpter 1993) (Table 1.3). Jobling *et al.* (1996) determined that OP was the most estrogenic alkylphenol. OP inhibited testicular growth by 50% and at 0.15 μM caused a million-fold increase in VTG concentrations in the plasma of rainbow trout. At concentrations of 10 μM OP, the amount of VTG secreted was almost equal to that secreted by hepatocytes exposed to 10 nM E_2 (White *et al.* 1994) (Table 1.3).

Abraham and Frawley (1997) found that 1 μM OP stimulated the expression of the prolactin (PRL) gene (Table 1.3). PRL is an anterior pituitary hormone which is critical for the initiation of lactation in mammals and for the development of the immune system of infants during early neonatal stages (Abraham and Frawley 1997). In amphibians, the secretion of PRL antagonizes metamorphosis and promotes larval growth leading to larger, more fit animals at metamorphosis (Tata 1996; Denver 1998). Female rats which were exposed to OP as neonates were found to be in persistent estrous when they reached adulthood and those exposed as adults experienced induced persistent estrous (Blake and

Ashiru 1997). A physiologically based approach to study the effects of OP on the size of reproductive organs, daily sperm production, and behaviour revealed significant reductions in both daily sperm production and efficiency for males whose mothers had consumed a 2 ng/g dose of OP (Vom Saal *et al.* 1998). This finding lends support to the hypothesis that exposure to environmental estrogens during testicular differentiation in the womb could account for the observed decreased sperm count in men over the last 50 years (Sharpe and Shakkebaek 1993).

The stimulatory effects of OP on two estrogen-responsive human breast cancer cell lines, MCF-7 and ZR-75, revealed that the effect was 10,000 times less than stimulation with E_2 (White *et al.* 1994) (Table 1.3). These authors found that OP displaced E_2 from trout and mouse estradiol receptors in a competitive manner with an approximate K_d of 11 μ M.

Thus, whether one looks at *in vivo* or *in vitro* studies, it is apparent that OP is a xenoestrogen of great concern. It is important to have reliable environmental measurements to compare with concentrations found to illicit estrogenic responses. Additionally, we must determine whether OP is ubiquitous in the environment or whether it is solely associated with point source effluents of STPs and other industries.

Table 1.3. A summary of the relative potency of octylphenol (OP) with respect to 17 β -estradiol (E₂) for several assays used to determine estrogenicity.

[E ₂] required to elicit response	[OP] to elicit comparable response	Multiplication factor for OP to obtain same response	Assay	Reference
10 nM	10 μ M	1000	<i>In vitro</i> VTG induction	White <i>et al.</i> 1994
0.1 nM	1 μ M	10000	MCF-7; ZR-75 cell line proliferation	White <i>et al.</i> 1994
1 nM	1 μ M	1000	Stimulation of PRL gene expression	Abraham and Frawley 1997
1.81 nM	2.11 μ M	1160	<i>In vitro</i> VTG induction	Jobling and Sumpter 1993
10-100 nM	10-100 nM	0	Feminization of <i>Xenopus</i>	Kloas <i>et al.</i> 1999

Freshwater in the Delaware River was found to contain concentrations of OP ranging from N.D. to 3 µg/L (14 nM) and drinking water samples from a treatment facility upstream from a chemical plant and a STP, contained 0.01 µg/L (0.05 nM) OP (Bennie 1999). In the Great Lakes basin and upper St. Lawrence River area, freshwater samples from various lakes and rivers had concentrations of OP ranging from <0.005 - 0.47 µg/L (<2.0 pM-2.5 nM) (Bennie *et al.* 1997). Sediment concentrations from the same sites were in the range of <0.010 - 1.8 µg/g dry weight with the highest levels reported in Hamilton Harbour (Bennie *et al.* 1997). Lee and Peart (1995) determined concentrations of OP in effluent and sludge from STPs in Ontario and found OP ranging from 0.12 - 2.5 µg/L (0.6 nM-12 nM) in the effluent and from <0.005-12.1 µg/g dry weight in the sludge and sediments. Bennett and Metcalfe (1998) found that sediments with high concentrations of OP (> 1 µg/g dry weight) were not widespread throughout the lower Great Lakes and that high levels were mainly detected in sediment samples collected near STPs and industries. Thus, OP would appear to be mainly a point source chemical of concern. However, background levels do occur at a distance from point sources.

1.1.7 EDCs, reptiles and amphibians

Reptiles and amphibians often inhabit areas on the boundaries of human habitation and can thus be exposed to many different chemicals released due to human activity. Many of these chemicals may not have direct toxicological risks but instead have the capacity to disrupt the function of the endocrine system. Many reptiles have temperature-sensitive sex determination (TSD). That is to say, reptiles do not have heteromorphic sex chromosomes, and thus, environmental temperature modifies sex steroid production during critical periods

(e.g. gonadal development) (Crews *et al.* 1995; Hayes 1999). Male-producing incubation temperatures up-regulate androgen receptor genes and female-producing temperatures up-regulate estrogen receptor genes (Crews *et al.* 1995; Hayes 1999). Crews *et al.* (1995) incorporated temperature-sensitive sex determination into a reptilian assay for estrogenic pollutants and found that minute amounts of exogenous estrogenic ligands resulted in female hatchlings from eggs incubated at male-producing temperatures.

Amphibians differ from reptiles in that, in general, larval sexual development is not temperature dependent and despite a genotypic distribution of 50% males and 50% females, treatments with E₂ may shift the offspring to phenotypic feminization (Lutz and Kloas 1999; Kloas *et al.* 1999). Kloas *et al.* (1999) have used this information regarding genotypic distribution as an assessment of the estrogenic potencies of endocrine disruptors *in vivo*. Control groups treated only with solvent consisted of approximately 60% males whereas, tadpoles raised in media containing E₂ (10⁻⁷ and 10⁻⁸ M), NP (10⁻⁷ M), OP (10⁻⁷ and 10⁻⁸ M), and bisphenol-A (10⁻⁷ M) expressed significant feminization (i.e. 70-90% females) (Table 1.3).

Other amphibian assays used to assess estrogenic potencies of endocrine disruptors include: (1) binding to the liver estrogen receptor (ER); (2) estrogenic activity *in vitro* by inducing vitellogenin (VTG) synthesis in primary cultured hepatocytes; and (3) *in vivo* induction of serum VTG (Palmer and Palmer 1995; Ankley *et al.* 1998b; Palmer *et al.* 1998; Lutz and Kloas 1999; Kloas *et al.* 1999).

In amphibians, like in all other vertebrates, estrogenic effects of compounds are mediated by binding to nuclear ERs in target cells leading to the dimerization of the hormone-

receptor complex (Lutz and Kloas 1999; Kloas *et al.* 1999). In the nucleus, the hormone-receptor complex binds to specific estrogen responsive elements (EREs) of the DNA to induce transcription of specific genes (Nimrod and Benson 1998). Both the ERs and ARs (androgen receptors) in *X. laevis* have been cloned and thus binding assays could be performed (Ankley *et al.* 1998b). Competitive displacement experiments using radiolabelled [³H]E₂ were performed by Lutz and Kloas (1999) for *X. laevis* and estrogenic compounds showed a decreasing affinity compared to E₂ with decreasing estrogenicity. The main advantage of the radioreceptor assay is that it can be used to screen potential estrogenic activities of pure substances and complex mixtures such as sewage effluents (Lutz and Kloas 1999).

Although the expression of the vitellogenin (VTG) gene and the subsequent induction of VTG protein is under multihormonal control, E₂ plays the predominant role in all egg laying vertebrates and the main organ of expression is the liver (Palmer *et al.* 1998; Lutz and Kloas 1999; Kloas *et al.* 1999). The ability of amphibian hepatocytes to stimulate VTG-mRNA after exposure to E₂ was determined using semiquantitative RT-PCR (Kloas *et al.* 1999). The authors found that VTG-mRNA induction was E₂ specific and that estrogenic contaminants (NP and bisphenol A) stimulated VTG expression in the following ranking: E₂>NP>Bisphenol A.

In vivo bioassays for xenobiotic estrogens based on VTG induction have been developed for amphibians (Palmer *et al.* 1998). *X. laevis* tadpoles and adults were exposed by immersion to dieldrin, toxaphene, and chlordane, and serum VTG was determined using a diethylaminoethyl (DEAE) chromatography procedure. Toxaphene and dieldrin-treated frogs

showed levels of serum VTG that were significantly greater than controls (Palmer *et al.* 1998). Ramsdell *et al.* (1998) reported a dose-dependent increase in feminization of *X. laevis* tadpoles exposed to 30-500 nM NP and serum VTG levels were significantly greater in males and females compared to control groups. Serum VTG induction was reported in male reared slider turtles exposed to E₂, diethylstilbestrol and DDT (Palmer and Palmer 1995). The VTG assay is currently being adapted to test water for estrogenicity and assess the exposure of wild amphibian populations to environmental estrogens (Palmer *et al.* 1998).

Xenobiotics that affect germinal vesicle breakdown (GVBD) in the oocyte can be assessed using both *in vivo* or *in vitro* approaches (Ankley *et al.* 1998b). As progesterone-induced oocyte maturation is a prerequisite for subsequent fertilization of the released ova, disruption of this process could have considerable consequences for female amphibian reproduction and population stability. Pickford and Morris (1999) found that a variety of EDCs (octylphenol, *o,p'*-DDT, di-*n*-butyl phthalate and bisphenol A) did not antagonize *in vitro* progesterone-induced GVBD, however, methoxychlor was a potent inhibitor of GVBD. It was determined that the mechanism of action for methoxychlor was unlikely direct competition with progesterone for the progesterone receptor (PR) (Pickford and Morris 1999). This indicates that endocrine disruption is not necessarily mediated by direct competitive binding which highlights the need for a broad-based approach for screening EDCs which acknowledges nongenomic effects such as noncompetitive binding or membrane disruption resulting in altered Ca²⁺ fluxes (Pickford and Morris 1999).

Clark *et al.* (1998) determined the estrogenicity of DDT and DDE in larval tiger salamanders (*A. tigrinum*) by monitoring gonaduct growth. Müllerian ducts were stimulated

by E₂ and dihydrotestosterone (DHT), whereas Wolffian ducts were stimulated only by DHT. The predicted estrogenic actions of DDT and antiandrogenic actions of DDE were not observed and in fact, DDT had an antiestrogenic effect and DDE an estrogenic effect for this assay (Clark *et al.* 1998). Species variability, tissue type and stage of development must all be considered when looking at the effects of these and other EDCs on the reproductive biology/toxicology of amphibians.

Although these assays are an important tool for looking at specific estrogenic effects, the various physiological processes that influence responsiveness to an estrogenic compound in the context of the whole animal are often overlooked. Nishimura *et al.* (1997) raised *X. laevis* in water containing different concentrations of E₂, DES, progesterone and dihydrotestosterone (DHT) and found that concentrations of 10 µM E₂ caused malformations of the head and abdomen and resulted in suppressed organogenesis including crooked vertebrae. Additionally, mRNAs for the estrogen receptor were present in embryos, tadpoles, and the adult liver indicating the likelihood that ERs are involved in the induction of developmental defects in estrogen treated *X. laevis* (Nishimura *et al.* 1997).

1.1.8 Synergistic studies and amphibians

It is becoming increasingly clear that a single factor cannot explain widespread amphibian declines in remote areas around the world. Scientists need to take an integrated approach and conduct research in which they manipulate several variables at once (Hileman 1998a). The multi-faceted approach has been attempted by a limited number of researchers thus far.

Kiesecker and Blaustein (1995) demonstrated an interaction between UV-B radiation

and a pathogenic fungus (*Saprolegnia ferax*) that increased the mortality of amphibian embryos compared to either factor alone. Incorporating the concerns of both decreasing pH and the increase in ambient UV-B, Long *et al.* (1995) found that neither enhanced UV-B nor a low pH (4.5) had a significant effect on the survival of *R. pipiens* eggs alone. However, the combination of the two factors reduced embryonic survival.

A number of polycyclic aromatic hydrocarbons (PAHs) can absorb UV radiation resulting in excited singlet and triplet state molecules. The process can yield singlet O₂ which is produced when the energy associated with excited state PAHs is transferred to molecular O₂ (Ankley *et al.* 1995). Singlet O₂ production can occur in the external media (i.e. water) and within biological tissues leading to oxidative stress and toxicity (Ankley *et al.* 1995). *R. pipiens* larvae were exposed to clean water or five concentrations (0.89, 2.18, 6.99, 12.0 and 30.6 µg/L) of the PAH fluoranthene and then exposed to UV radiation ranging from 0.61-1.53x10² µW/cm² (Monson *et al.* 1999). No mortality was observed during the uptake period but low concentrations (in the range of 2-10 µg/L) were lethal when combined with UV. Hatch and Burton (1998) assessed the photoinduced toxicity of fluoranthene on newly hatched larvae of *A. maculatum*, *R. pipiens* and *X. laevis* and all species were affected by low concentrations in the sunlight (Hatch and Burton 1998). The skin of bullfrog larvae (*R. catesbeiana*) was sensitive to the phototoxic effects of fluoranthene and exhibited signs of necrosis and structural alterations at sublethal levels (10 µg/L) and low levels (3-6 µW/cm²) of UV-B (Walker *et al.* 1998).

Certain pesticides can be photomodified by UV-B radiation to yield structures that are more teratogenic or acutely toxic than the parent molecule. For example, Carbaryl, a

carbamate insecticide, was photoactivated with as little as 1.5% of ambient solar UV-B, leading to increased mortality for embryos and tadpoles of *X. laevis* and *H. versicolor* (Zaga *et al.* 1997). Methoprene, an insect growth regulator, has been implicated in recent reports of amphibian deformities (Hileman 1998b) as it is a known retinoic acid receptor agonist. Retinoic acid, a derivative of vitamin A, normally binds to this receptor and when it is in excess or supplied exogenously, limb defects can be induced (Ankley *et al.* 1998a). Methoprene is known to absorb light in the UV region and then undergo photoisomerization (LaClair *et al.* 1998). Ankley *et al.* (1998a) found that methoprene, in the absence or presence of UV radiation, did not result in abnormalities of developing *R. pipiens* similar to those reported in wild amphibian populations. The highest concentration of methoprene (500 µg/L) did cause lethal developmental effects but the significance of such a finding is unclear because it is an unrealistic concentration far in excess of those found in the environment (Ankley *et al.* 1998a).

A 50:50 mixture of two commonly mixed field-grade herbicide formulations, atrazine and alachlor, was found to have a synergistic effect with respect to mortality of *R. pipiens* and *B. americanus* larvae (Howe *et al.* 1998). Estrogenic chemicals have also been shown to elicit greater responses in combination. Dieldrin, endosulfan or toxaphene alone, weakly inhibited the binding of [³H] 17 β-estradiol to the human estrogen receptor *in vitro* (Arnold *et al.* 1996). However, a mixture of any two of the compounds resulted in a potency two hundred times greater than the individual potency. This interaction is apparently mediated by the interaction of the ER with more than one chemical simultaneously (Arnold and McLachlan 1996). There are profound environmental implications if xenobiotic estrogens

have synergistic effects because these compounds typically occur in combination within aquatic environments.

1.1.9 UV-B and Octylphenol: Possible synergistic effects on development and gene expression in the brains of *R. pipiens*

The potential individual effects of both UV-B and OP have been discussed. Additionally, the importance of incorporating more than one variable into studies which try to elucidate possible factors responsible for amphibian population declines has been addressed. Ahel *et al.* (1994) determined the kinetics of photochemical degradation of OP in natural waters and calculated a photolysis half-life of approximately 10-15 hours. The half-life was dependent upon the DOC content of the water and the depth of incubation. At 20-25 cm, photolysis was 1.5 times slower. However, it was found to be faster in the presence of natural organic matter. Breeding ponds of amphibians are often shallow and contain high concentrations of DOC so photolysis could be a significant degradation pathway for OP. Photochemical degradation can generate photointermediates which are more toxic to organisms than the parent compound. Thus, the first objective of the study was to determine whether a synergy existed between UV-B and OP with respect to survivorship of *R. pipiens* tadpoles.

Extensive literature exists on the physiology, endocrine system and normal development of amphibian embryos and larvae. Thus, on a gross level, morphological abnormalities and mortality were monitored for newly-hatched *R. pipiens* tadpoles exposed to environmentally relevant levels of OP with and without supplemental UV-B radiation. The individual effects of UV-B were also quantified. It was hypothesized that a combination of

UV-B plus OP at environmentally relevant concentrations would result in decreased survivorship of newly-hatched *R. pipiens* tadpoles compared to either stressor alone.

In the context of endocrine disruptors, Colborn (1995) suggests that we must move beyond acute toxicity and incorporate a molecular approach which addresses changes in gene expression. The RNA-arbitrarily primed PCR (RAP-PCR) differential display strategy was employed to isolate candidate genes differentially regulated in the frog hypothalamus which were affected by various treatments. The hypothalamus is a main target for sex steroid action in the brain and also controls the endocrine axis responsible for amphibian metamorphosis (Blázquez *et al.* 1998a; Denver 1998). The primary objective of the molecular approach was to determine whether the hypothalamus can be used as an effective environmental sensor for neurotoxicity. A reverse Northern multiple-gene dot blot technique was used to verify the expression patterns of structurally and functionally important cDNA transcripts cloned from differential display. By developing a specific dot blot array of several candidate brain molecules, it will be possible to screen populations in laboratory and field experiments for altered gene expression due to exposure. This method is extremely sensitive even for low levels of contaminants and therefore may lead us to a greater understanding of the mechanisms of action of biotic factors such as UV-B and abiotic factors such as estrogenic contaminants.

Chapter 2

2.0 THE EFFECTS OF THE XENOESTROGEN, OCTYLPHENOL (OP), AND UV-B RADIATION ON LEOPARD FROG (*RANA PIPIENS*) TADPOLES

2.1 Introduction

The alarming disappearance and decline of several amphibian species worldwide has resulted in intensified efforts to determine possible causes. The influence of UV-B on amphibians has gained considerable attention from many researchers although the ultimate implications of this stressor remains questionable. It is known that the maximal, seasonal change of UV-B reaching the earth's surface occurs in the early spring (Kerr and McElroy 1993) which coincides with the amphibian breeding season in north temperate latitudes. Both laboratory and field experiments have been conducted for a wide range of species and variable sensitivity of embryos and tadpoles to UV-B has been observed (Worrest and Kimeldorf 1976; Blaustein *et al.* 1994; Blaustein *et al.* 1995; Grant and Licht 1995; Hays *et al.* 1996; Blaustein *et al.* 1997; Nagl and Hofer 1997; Ovaska *et al.* 1997; Anzalone *et al.* 1998; Corn 1998; Lizana and Pedraza 1998; Crump *et al.* 1999a). Recently, the role of endocrine-disrupting chemicals (EDCs) in amphibian decline has been addressed. It has been hypothesized that exposure to pollutants may disrupt endocrine systems controlling metamorphosis and sexual development (Cheek *et al.* 1999; Kloas *et al.* 1999; Tietge *et al.* 1999).

Alkylphenol polyethoxylate (APEO) degradation products, including OP and NP, have been found to elicit both estrogenic and toxic effects in several vertebrates (Jobling and

Sumpter 1993; White *et al.* 1994; Sumpter and Jobling 1995; Folmar *et al.* 1996; Jobling *et al.* 1996; Nimrod and Benson 1998; Ramsdell *et al.* 1998; Servos 1999). There are two main forms of domestic and industrial APEOs: nonylphenol polyethoxylates (NPEOs) and octylphenol polyethoxylates (OPEOs). NPEOs account for 85% of the market (>250,000 tonnes worldwide per year) and OPEOs make up the remaining 15% (Bennett and Metcalfe 1998). APEOs are a major group of surfactants and their formulations are employed as detergents, emulsifiers, dispersants, antifoamers, dyeing assists, stabilizers, lubricants, spermicides and pesticide adjuvants (Bennie *et al.* 1998).

Kloas *et al.* (1999) reported a significantly higher number of female phenotypes for *X. laevis* when exposed to 10 nM OP and Ramsdell *et al.* (1998) found a dose-dependent increase in the proportion of *X. laevis* exhibiting female sexual morphological characteristics when exposed to 30-500 nM NP. At four weeks after metamorphic climax, serum vitellogenin (VTG) levels were significantly higher in both male and female frogs exposed to high levels of NP (Ramsdell *et al.* 1998). Amphibian toxicity data have been recorded for NP. *R. catesbeiana* tadpoles had a 30 day LC50 of 260 mg/kg (260 ppm) and a no observable effect concentration (NOEC) of 115 mg/kg (115 ppm) in sediments (Servos 1999). The 14 day LC50 based on interstitial water concentrations in the sediments was 0.36 μ M compared to 0.6 μ M based on dosed water concentrations (Servos 1999). *B. boreas* had a reported LC50 of 0.6 μ M in water (Servos 1999). At present, amphibian toxicity data for OP does not exist. This is a concern as OP has been found at high levels in freshwater (2.5 nM) and sediments (1.8 μ g/g dry weight) throughout the Great Lakes basin and upper St. Lawrence River (Bennie *et al.* 1997; Bennie 1999).

UV-B radiation has been shown to interact with other stressors (e.g. pesticides, PAHs, low pH) resulting in decreased survivorship for several amphibian species (Long *et al.* 1995; Zaga *et al.* 1997; Ankley *et al.* 1998a; Hatch and Burton 1998; LaClair *et al.* 1998; Monson *et al.* 1999). Ahel *et al.* (1994) determined the kinetics of OP photolysis in natural waters and calculated a half-life of approximately 10-15 hours. Photolysis or photomodification can generate reactive photometabolites including excited singlet and triplet state molecules (e.g. singlet O₂) which have a greater negative effect than the parent compound. The purpose of the present study was to determine whether an interaction existed between environmentally relevant levels of UV-B and the xenoestrogen OP with respect to survival of *R. pipiens*. We also report the LC50 of water borne OP to *R. pipiens* tadpoles. This value has not been previously reported and it provides an important toxicological benchmark with respect to amphibian survivorship and potential vulnerability in the wild. It was hypothesized that we would observe decreased survivorship when newly-hatched *R. pipiens* tadpoles were exposed to a combination of the two stressors than either factor alone at environmentally relevant levels.

2.2 Materials and Methods

2.2.1 Egg collection

Two naturally fertilized *R. pipiens* egg masses were collected on April 29, 1999 within 24 hours of being laid from a typical breeding pond on the campus of Trent University, Peterborough, Ontario (44° 22' N 78°17' W). The eggs were maintained in aerated filtered water from the Aquatic Care Facility at the University of Ottawa which had

the following characteristics: pH 7.0, dissolved oxygen 8.4-10 mg/L, phosphate <0.01 mg PO₄/L, nitrate <0.01 mg NO₃/L, and temperature 16.5-18°C. Upon hatching, stage 20-21 (Gosner 1960) tadpoles were removed from the main aquarium and placed in the respective treatment exposures.

In addition, 500 *R. pipiens* embryos were purchased from Carolina Biological (April 20, 2000) in order to conduct the LC50 experiment. The rearing conditions were as above.

2.2.2 Conditions of Exposure

The exposure experiment commenced on May 2, 1999 and lasted for ten days. Tadpoles were exposed to one of three concentrations of OP (10 µM, 1 µM, 1 nM) plus a 0.01% ethanol solvent control, with and without two levels (7 µW/cm², 25 µW/cm²) of supplemental UV-B radiation. A range of OP concentrations between 1 µM (no lethal effects) and 10 µM (100% mortality after one day) were used in the seven-day LC50 study which began on April 25, 2000. Tadpoles were exposed in a controlled environmental chamber (18°C; 12:12 h light:dark photoperiod) in 100 x 15 mm (diameter/height) Pyrex petri dishes which had a capacity of approximately 100 ml (Figure 2.1). Fifteen tadpoles were assigned to each treatment dish. The mean loading density calculated at the beginning of the experiment was 0.6 g/L based on a mean wet weight (specimens blotted on paper towel) of 0.004 g/tadpole. After 10 days, tadpole weight increased to 0.02 g and the mean loading density was 3.0 g/L. The experiment was performed with four replicates of each treatment group and solvent-exposed controls.

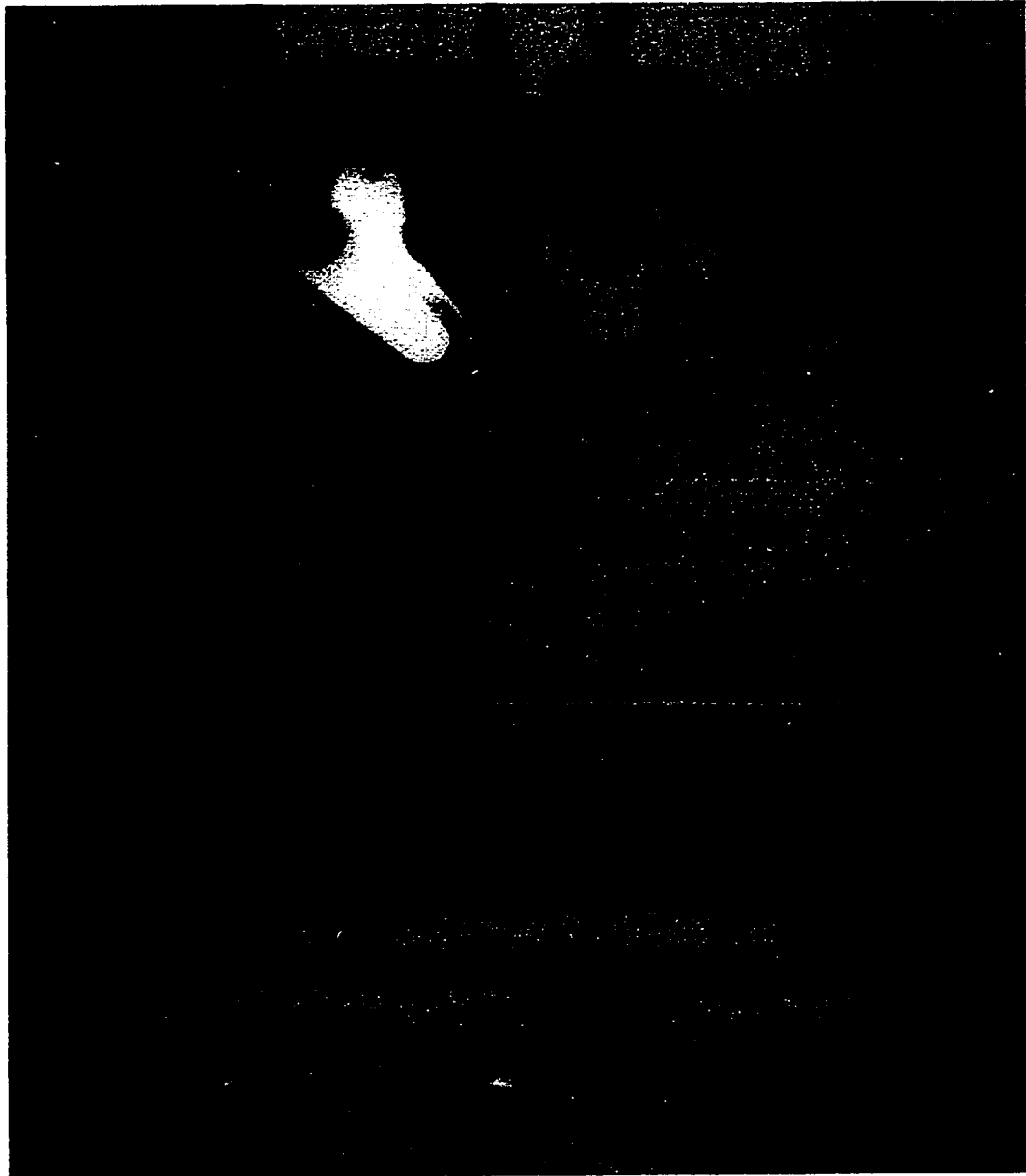


Figure 2.1. The solar simulator design with UV-A and UV-B lamps positioned at a particular height above the exposure petri dishes to attain the desired UV-B regime. Cellulose acetate was placed around the UV-B lamp to block out any wavelengths less than 290 nm. Fifteen tadpoles were placed in each petri dish with replicates of four dishes per treatment.

2.2.3 4-OP Exposure

In a preliminary experiment, tadpoles were exposed to nominal concentrations ranging from 1 pM to 10 μ M and 100% mortality was observed in the high exposure. We thought this was a good representative value for the lethal threshold limit, whereas the two other levels chosen for the main experiment were consistent with environmental levels reported for sewage sludge (1 μ M) and freshwater (1 nM). Stock solutions of 20.6 mg/ml, 20.6 μ g/ml, and 20.6 ng/ml were prepared by dissolving 99% pure technical grade 4-OP (Aldrich Chemical Co.; F.W. 206.33) in 95% ethanol. The nominal concentrations were prepared by adding 100 μ l of the appropriate OP stock solution to 1 L of Aquatic Care Facility filtered water which was then distributed to the respective petri dishes. For control and UV-B-only groups, 100 μ l of 95% ethanol was added to 1 L of filtered water before distribution to the containers. The static renewal system operated on a water renewal every 48 hours for all treatments to maintain consistency. The OP formulations in the LC50 experiment were prepared as above.

2.2.4 Solar simulator design

The supplemental lighting in the solar simulator consisted of UV-B (UBL FS20T12/UVB-BP) and UV-A (combination of Cool White Sylvania F20T12 CW and NEC T10 Black Light Blue) lamps as well as background laboratory lighting supplied by full spectrum bulbs (Verilux F40T12VLX). Two light panels (4 feet long), which housed the UV-B and UV-A bulbs, were mounted above the two UV-B chambers at distinct heights above the exposure containers to attain the desired UV-B/UV-A regimes (Figure 2.1). Mylar

D screens were positioned between all chambers to block any wavelengths <320 nm (Middleton and Teramura 1993) from reaching adjacent chambers. The full spectrum bulbs were on a consistent 12:12 h light:dark photoperiod whereas the UV lamps were on for 6 hours per day (10:00 am - 4:00 pm) to approximate the natural daily fluctuation pattern of the solar spectrum.

UV measurements were carried out in each of the three experimental chambers using an Oriel GOLDILUX UV meter (Model # 70217) with interchangeable UV-B (Model # 70221) and UV-A probes (Model # 70219). Measurements were taken at several different positions under the lamps in order to characterize the variation of UV within the solar chambers. Cellulose acetate was placed around the UV-B lamps in order to block out wavelengths less than 290 nm (Middleton and Teramura 1993), an environmentally relevant cut-off. Lower UV-B wavelengths (<290 nm) would cause excessive damage and are not realistic with respect to present day conditions.

2.2.5 UV levels

During preliminary trials using a UV-B regime ranging from 43.5 (end of the lamp) to $64.6 \mu\text{W}/\text{cm}^2$ (middle of the lamp) and a UV-A regime ranging from 128-194.4 $\mu\text{W}/\text{cm}^2$, 90% mortality of newly-hatched tadpoles was observed after only a few hours. As $64.6 \mu\text{W}/\text{cm}^2$ UV-B was clearly above the lethal threshold in this experiment, two lower levels were chosen; 25 $\mu\text{W}/\text{cm}^2$ (ranging from 20.6-28.5 $\mu\text{W}/\text{cm}^2$) and 7 $\mu\text{W}/\text{cm}^2$ (ranging from 4.6-7.1 $\mu\text{W}/\text{cm}^2$) UV-B. UV-A levels ranged from 60-88.2 and 34-90 $\mu\text{W}/\text{cm}^2$, respectively. The range of UV was relatively large and thus a daily rotation was carried out in order to

decrease within group variation based on positioning under the lights alone. The control and OP-only groups, although not exposed to UV lamps, were also rotated daily so as to keep the slight external stimuli of shifting petri dishes consistent among all treatments.

2.2.6 Tadpole condition

Five days into the ten-day static renewal experiment feeding began. Tadpoles were fed Nutrafin® fish flakes daily. The overall condition of tadpoles was monitored daily after the UV lamps were switched off. A five point severity scale was established which characterized tadpole condition in response to prodding with a plastic pipet tip and all tadpoles were assigned a number each day. Condition was monitored by more than one researcher to avoid bias. The five conditions were as follows; 1) avoidance behaviour is normal; darting away when prodded; 2) rapid, erratic tail movements in response to stimulus; irregular or sporadic swimming; 3) reduced reaction to stimulus; avoidance behaviour decreases; 4) bloating (distension/swelling) of the head; abnormal growth and development of tail and spinal chord (i.e. lordosis or curvature of the spine) leading to a circular swimming pattern; and 5) paralysis or mortality; no response to stimuli. Dead tadpoles were removed daily and the petri dishes were washed thoroughly during the water renewal to avoid waste accumulation.

2.2.7 Statistical analysis

A two-way non-parametric ANOVA was used to assess the effect of treatment on tadpole condition. The H statistic was calculated based on the sum of squares generated by

the ANOVA for the OP, UV and UV/OP treatments compared to the control. The H statistic result was then compared to the Chi-Squared distribution and considered significant if $p < 0.05$. Linear interpolation was used to estimate the LC50 value. The line between the two nearest concentration values that produced mortality levels above and below the specified percentage (50%) was used in the calculation. Systat 7.0.1[®] software was used for all statistical tests except for the H statistic which was calculated manually with the sum of squares values generated by the two-way ANOVA.

2.3 Results

Both the individual and combined effects of OP and UV-B on tadpole survivorship were characterized. Tadpoles exposed to 0.01% ethanol were active and had a condition of 1 ± 0 based on the severity scale (Table 2.1). OP, at 1 μM and 1 nM, had no effect on the condition of the tadpoles. However, the highest concentration, 10 μM , was at the lethal threshold limit as 100% of the tadpoles in the test containers died after the first day (Table 2.1). We found OP had a significant effect on tadpole survivorship ($H_{\text{OP}}=29.7$; $\text{df}=3$; $p<0.05$). In addition, the calculated seven-day LC50 value for water borne OP was 2.8 μM (586 $\mu\text{g/L}$) (Figure 2.2). The low level of UV-B, 7 $\mu\text{W/cm}^2$, caused no deformities or mortality (Table 2.1; condition = 1 ± 0) when tadpoles were exposed to this stressor alone. On the other hand, the high level, 25 $\mu\text{W/cm}^2$, resulted in significant mortality and severe abnormalities including lordosis and distension, characteristic abnormalities of tadpoles exposed to UV-B (Table 2.1; $H_{\text{UV}}=10.7$; $\text{df}=2$; $p<0.05$). There was no significant interaction between OP and UV-B (Table 2.1; $H_{\text{UVOP}} = 4.02$; $\text{df}=6$; $p<0.05$).

Table 2.1. The effects of three concentrations of octylphenol (OP) and two levels of UV-B radiation, alone and in combination, on the condition of newly-hatched *Rana pipiens* tadpoles. ^a

	UV- (0.01% ethanol)	7 $\mu\text{W}/\text{cm}^2$	25 $\mu\text{W}/\text{cm}^2$
OP- (0.01% ethanol)	1.0 \pm 0	1.0 \pm 0	4.4 \pm 1.1*
1 nM	1.1 \pm 0.72	1.0 \pm 0	4.4 \pm 1.1*
1 μM	1.2 \pm 0.81	1.12 \pm 0.6	4.8 \pm 0.4*
10 μM	5.0 \pm 0*	5.0 \pm 0*	5.0 \pm 0*

^aTadpole condition was based on a severity scale from one to five (1=healthy; 5=death) and differences were considered significant where $p < 0.05$ (* $H_{\text{OP}}=29.7$, $df=3$; * $H_{\text{UV}}=10.7$, $df=2$; $H_{\text{UVOP}}=4.02$; $df=6$). * Denotes a significant effect.

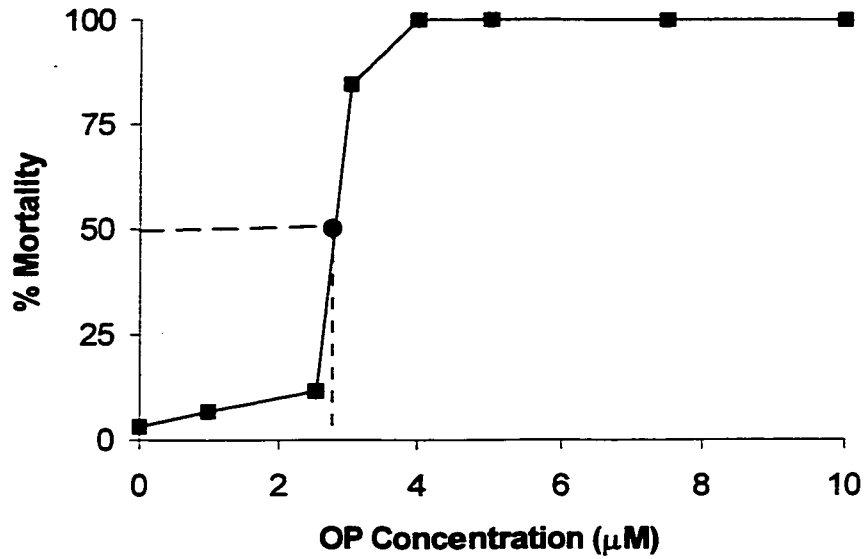


Figure 2.2. The LC50 curve for water borne OP with respect to newly-hatched leopard frog (*Rana pipiens*) tadpoles following exposure to eight different levels of OP (0, 1, 2.5, 3, 4, 5, 7.5 and 10 µM). Linear interpolation was used to estimate the LC50 value where the equation of the line between the two nearest concentration values that produced mortality levels above and below the specified percentage (50%) was used in the calculation.

2.4 Discussion

Our results demonstrate that OP is highly toxic to leopard frog tadpoles at levels ranging from 4 μM -10 μM . Prior to death, we observed gross morphological deformities induced by OP. These abnormalities included curvature of the spine and apparent tissue damage. The LC50 was calculated to be 2.8 μM . We found that sub-ambient levels of UV-B (25 $\mu\text{W}/\text{cm}^2$) caused an increase in tadpole deformity and mortality. From a toxicological standpoint, the predicted interaction of OP and UV-B was not observed in the context of decreased survivorship.

The highest OP concentrations (4-10 μM) were at or above the lethal threshold limit for *R. pipiens* tadpoles based on nominal water concentrations (Figure 2.2). One hundred percent mortality was observed after one day in all cases preceded by severe malformations. Deformities were also observed at 3 μM OP. Nishimura *et al.* (1997) found that E₂-treated *X. laevis* tadpoles displayed malformations of the head and abdomen and suppressed organogenesis, including crooked vertebrae. The latter pathology was most common in our observations with *R. pipiens*. Previous to our study there had been no reports of OP toxicity for anurans. Toxicity data however, does exist for the related compound NP. It is not surprising that the range of 4-10 μM caused 100% mortality when looking at NP toxicity data. *R. catesbeiana* tadpoles had a 14 day LC50 of 0.36 μM based on interstitial water concentrations compared to 0.6 μM based on dosed water concentrations (Servos 1999). *B. boreas* had a reported LC50 of 0.6 μM in water (Servos 1999). Without taking into account species-specific toxic responses, the calculated LC50 for OP, 2.8 μM , is substantially greater

than for NP. It is also apparent from Figure 2.2 that the toxic response is extremely abrupt; at 2.5 μM there is minimal mortality and at 3 μM there is 84% mortality. The *R. pipiens* tadpoles exposed to 1 μM OP in this study displayed no abnormal or lethal effects (Table 2.1; Figure 2.2) even though the level is almost double the LC50 values for NP in *R. catesbeiana* and *B. boreas*. Although the two lower levels of OP had no obvious effects on the morphology of developing tadpoles (Table 2.1), endocrine disruption should not be ruled out as subtle changes at the level of the endocrine system can result in overall stress and may not be overtly apparent.

In the present study, exposure to OP was via the water only. However, in a natural setting, there would be exposure to OP by at least two routes; direct uptake from water, and uptake from dietary sources. Tadpoles of this species often forage in sediments where the highest environmental concentrations of OP have been reported (up to 1.8 $\mu\text{g/g}$ dry weight; Bennie *et al.* 1997; Bennie *et al.* 1998). Thus, depending on the dietary concentration of OP, consumption rates and assimilation efficiencies, significant exposure could occur via this route. For NP, the reported 30 day LC50 for *R. catesbeiana* tadpoles in sediments was 260 mg/kg (260 ppm) and the NOEC was 115 mg/kg (115 ppm) (Servos 1999). Perhaps OP (or NP) is less available when associated with the sediments as the LC50 value for *R. catesbeiana* tadpoles (260 ppm) is greater than the highest dosed water concentration used in this study (10 μM ; 2.06 ppm) which resulted in 100% mortality. Differing sensitivities between species cannot be ruled out and bioavailability should be further explored. In addition, the acute toxicity determined in the lab should be interpreted with caution as lethal concentrations may be lower in nature where uptake is controlled by multiple pathways.

This study indicates that exposure to $25 \mu\text{W}/\text{cm}^2$ UV-B can induce severe developmental abnormalities and high rates of mortality in *R. pipiens* tadpoles. One of the major complications of UV-B studies with amphibians arises because it is difficult to relate levels of experimental UV-B exposure to those of a typical breeding site. Summer levels of ambient UV-B vary between 100 and $250 \mu\text{W}/\text{cm}^2$ at the water surface (Scully and Lean 1994; Zaga *et al.* 1997; Ankley *et al.* 1998a; Crump *et al.* 1999a,b) but are attenuated rapidly in breeding ponds that have high dissolved organic carbon (DOC) levels (Scully and Lean 1994; Crump *et al.* 1999b). Crump *et al.* (1999a) reported a decrease in UV-B intensity from $220 \mu\text{W}/\text{cm}^2$ at the pond surface to $10.6 \mu\text{W}/\text{cm}^2$ at a depth of 3 cm. This value is similar to the low level exposure in this study. The lethal level of UV-B reported here appears to be extremely low and is approximately 10% of ambient levels. However, in a natural environment this level might be realized in the top 2 cm of the water column where tadpoles are often found.

The UV-B-induced developmental abnormalities observed in this study are consistent with several previous reports. Increased larval mortality and developmental irregularities including lordosis (curvature of the spine), bloating/distension and abnormal development of the cornea were observed for *R. cascade* and *H. regilla* tadpoles that were chronically UV-treated (Hays *et al.* 1996). Worrest and Kimeldorf (1976) reported abnormal development of the presumptive cornea, curvature of the spine and increased mortality for Boreal toad (*B. boreas*) tadpoles exposed to enhanced UV-B. Symmetrical, hindlimb ectromelia and ectrodactyly of metamorphs was observed when *R. pipiens* tadpoles were exposed at various stages of development to $44 \mu\text{W}/\text{cm}^2$ UV-B (Ankley *et al.* 1998a). The vulnerable

developmental stage (Gosner Stage 25 +) determined by Ankley *et al.* (1998a) was not tested in this study. Thus, the developmental window chosen for UV-B exposures is extremely important as there is obvious variation in sensitivity based on the stage of exposure. Several authors have reported hindered hatching success of several species as a result of ambient sunlight (Blaustein *et al.* 1994; Blaustein *et al.* 1995; Blaustein *et al.* 1997; Anzalone *et al.* 1998; Lizana and Pedraza 1998) while others regard larval stages as more susceptible to UV-B damage and the embryonic stage as resistant to the detrimental effects of UV-B (Worrest and Kimeldorf 1976; Grant and Licht 1995; Nagl and Hofer 1997; Ovaska *et al.* 1997; Zaga *et al.* 1997; Ankley *et al.* 1998a; Crump *et al.* 1999a).

After having established the individual effects of OP and UV-B on tadpole development we examined potential synergistic interactions. A significant interaction in the form of increased mortality or abnormality was not observed (Table 2.1). Others have found synergistic interactions between UV-B and certain pesticides, low pH and PAHs (Long *et al.* 1995; Zaga *et al.* 1997; Hatch and Burton 1998; LaClair *et al.* 1998; Monson *et al.* 1999). High energy photointermediates of many chemicals are more toxic than the parent compound. Low concentrations (in the range of 2-10 $\mu\text{g/L}$) of the polycyclic aromatic hydrocarbon (PAH) fluoranthene were lethal to *R. pipiens* larvae when combined with UV (Monson *et al.* 1999). Hatch and Burton (1998) found that larvae of *R. pipiens* were significantly affected by 5 and 25 $\mu\text{g/L}$ fluoranthene in the presence of sunlight. As little as 1.5% of ambient solar UV-B was sufficient to cause a ten-fold increase in the toxicity of the insecticide Carbaryl for embryos and tadpoles of *X. laevis* and *H. versicolor* (Zaga *et al.* 1997). Ahel *et al.* (1994) determined the kinetics of photochemical degradation of OP in

natural waters and calculated a photolysis half-life of approximately 10-15 hours.

Photointermediates were not characterized, however, the breakdown pathway of OP under UV-B radiation does not appear to yield toxic photoproducts as the condition of tadpoles co-treated with OP and UV-B was not different from control animals (Table 2.1).

2.4.1 Summary and conclusions

In summary, this study demonstrates a resilience of newly-hatched tadpoles to low levels of both OP and UV-B radiation. We also found that OP was toxic at levels ranging from 4-10 μM , the lower range being close to levels found in sewage sludge and sediments. We observed severe, sub-lethal effects of OP including curvature of the spine and an abrupt toxic response between 2.5 and 3 μM . The calculated LC50 value for OP in water was 2.8 μM . UV-B levels that were 10% of ambient (25 $\mu\text{W}/\text{cm}^2$) caused an increase in tadpole deformity and mortality after the 10-day exposure. The importance of water quality, especially DOC, must be acknowledged in this interpretation with respect to natural, *in situ* exposure regimes. The original prediction that an interaction between OP and UV-B would result in increased mortality of newly-hatched *R. pipiens* tadpoles was not observed.

Chapter 3

3.0 THE EFFECTS OF THE XENOESTROGEN, OCTYLPHENOL (OP), AND UV-B RADIATION ON PREMETAMORPHIC SOMATIC DEVELOPMENT AND GENE EXPRESSION IN HYPOTHALAMIC TISSUE OF THE LEOPARD FROG (*RANA PIPIENS*)

3.1 Introduction

The presence of an increasing number of endocrine-disrupting chemicals (EDCs) in the environment will result in long term, unavoidable chronic exposure to low concentrations of an array of contaminants including pesticides (e.g. DDT, methoxychlor), polychlorinated biphenyls (PCBs), synthetic steroids (e.g. ethinylestradiol) and some alkylphenolic compounds (e.g. octylphenol). These have the capacity to cause deleterious physiological effects to both wildlife and humans. Among wildlife species, the focus has been on animals associated with wetlands or aquatic habitats receiving sewage effluent and agricultural runoff and in which pollutant exposure has been linked to endocrine disruption (Purdom *et al.* 1994; Sumpter and Jobing 1995; Guillette *et al.* 1994; Folmar *et al.* 1996). Exposure to EDCs may also be associated with the recent statistical data which verifies that amphibian decline has been occurring worldwide since the 1960s (Houlahan *et al.* 2000).

The U.S. EPA concluded in a recent report on environmental endocrine disruption that "...amphibians might represent a unique sentinel animal model for laboratory and field exposure studies" (Pickford and Morris 1999, p. 285). Hayes and Menendez (1999) demonstrated the importance of sex steroids with respect to primary and secondary sex differentiation of the sexually dichromatic reedfrog (*Hyperolius argus*). Larvae exposed to

testosterone (T) produced 100% males at metamorphosis whereas, E₂-treated larvae displayed intersex characteristics (i.e. ovarian cavities and/or follicles and testicular tissue). In addition, T induced vocal sac development and E₂ induced dorsal colour change. Several environmental estrogens, including octylphenol (OP), were able to displace [³H]E₂ from the *Xenopus* estrogen receptor (Lutz and Kloas 1999), cause significant feminization at low concentrations (e.g. 10 nM OP), and stimulate vitellogenin-mRNA expression in cultured hepatocytes (Kloas *et al.* 1999). Nishimura *et al.* (1997) raised *X. laevis* in water containing different concentrations of E₂ and observed malformations of the head and abdomen, suppressed organogenesis, and suppressed central nervous system development. Estradiol (Frieden and Naile 1955) and TCDD (Jung and Walker 1997) have been shown to accelerate metamorphosis in *Bufo bufo* and *B. americanus*. Cheek *et al.* (1999) found that the herbicide acetochlor (ACETO), in the presence of exogenous thyroid hormone (T₃), accelerated metamorphosis in *R. pipiens*.

The effect of EDCs on metamorphosis is intriguing as it appears as though they have the capacity to alter the normal rate at which it occurs. The entire process of metamorphosis is under multihormonal control and involves a coordinate series of modifications in essentially every tissue. The hypothalamus can be stimulated by biotic or abiotic factors in the larval habitat (e.g. pond desiccation) to release the neuropeptide corticotropin-releasing hormone (CRH) (Denver 1997). CRH plays a central role to induce metamorphosis by stimulating the pituitary-thyroid endocrine axis, resulting in elevated T₃ levels in the blood (Denver 1997; Denver *et al.* 1997; Denver 1998; Tata 1999). T₃ is thought to control all aspects of metamorphosis by regulating gene expression (Denver *et al.* 1997). Exposure to

stressors also results in the release of corticosteroids from the interrenal glands, which can synergize with T_3 to accelerate metamorphosis (Denver 1997). Inhibition of metamorphosis is mediated by hypothalamic neurosecretion of the tripeptide thyrotropin-releasing hormone (TRH) which controls the biosynthesis and release of the pituitary hormone prolactin (PRL) (Tata 1996; Denver 1998). PRL antagonizes metamorphosis and promotes larval growth leading to larger, more fit animals at metamorphosis (Tata 1996; Denver 1998). Thyroid status is easily altered by the addition of a variety of hormones or T_3 synthesis inhibitors to water (Denver *et al.* 1997) indicating that the hypothalamo-pituitary-thyroid axis is extremely vulnerable to disruption. Metamorphic alterations as discussed above could lead to reduced fitness and survivorship.

Alkylphenol polyethoxylates (APEOs) are a large group of nonionic surfactants in commercial production (approximately 250,000 tonnes produced/year) which enter the aquatic environment predominantly via sewage treatment plants (STPs) and pulp and paper mills (White *et al.* 1994; Sumpter and Jobling 1995; Jobling *et al.* 1996). Upon discharge, APEOs are rapidly degraded to form relatively stable, hydrophobic metabolites; e.g. the alkylphenols, nonylphenol (NP) and octylphenol (OP). These metabolites are estrogenic and stimulate vitellogenin (VTG) gene expression in trout hepatocytes, induce breast cancer cell growth in MCF-7 and ZR-75 cell lines, inhibit testicular growth in rainbow trout, and competitively bind to both trout and mouse estrogen receptors (Jobling and Sumpter 1993; White *et al.* 1994; Jobling *et al.* 1996). Abraham and Frawley (1997) found that 1 μM OP stimulated PRL gene expression in rats. OP is the most estrogenic alkylphenolic compound with effective concentrations ranging from 0.1 μM -10 μM depending on the assay used

(Jobling and Sumpter 1993; Jobling *et al.* 1996). Blázquez *et al.* (1998a) found that 1 μM OP resulted in 50% mortality among immature male goldfish 20 days after the initial, single water exposure. We have also found that OP is a potent toxin as 2.8 μM caused 50% mortality among leopard frog (*Rana pipiens*) tadpoles exposed immediately after hatching (Chapter 2.0). OP is thus a biologically active contaminant and there is reason for concern as OP has been found at significant concentrations in freshwater (<2.0 pM-2.5 nM), sediments (<0.010 - 1.8 $\mu\text{g/g}$ dry weight), and STP effluent (0.6 - 12 nM) and sludge (<0.005 -12.1 $\mu\text{g/g}$ dry weight) in North America (Lee and Peart 1995; Bennie *et al.* 1997, 1998; Bennie 1999).

Exposure to UV-B radiation (280-320 nm) causes hindered hatching success and larval survivorship of many amphibians (Blaustein *et al.* 1994,1995,1997; Ovaska *et al.* 1997; Crump *et al.* 1999a) as well as severe deformities including lordosis (curvature of the spine), bloating/distension, and abnormal development of the presumptive cornea (Worrest and Kimeldorf 1976; Hays *et al.* 1996). Ankley *et al.* (1998a) reported symmetrical, hindlimb ectromelia and ectrodactyly after *R. pipiens* tadpoles were exposed to low levels (44 $\mu\text{W/cm}^2$) of UV-B. Again, metamorphic alterations of this nature in the wild would likely lead to impaired reproductive success, predator avoidance and overall fitness. UV-B can also potentiate the toxicity of several pesticides and polycyclic aromatic hydrocarbons (PAHs) with respect to amphibian survivorship due to the generation of toxic photometabolites (Zaga *et al.* 1997; Hatch and Burton 1998; Monson *et al.* 1999). The end-point of interest in these synergy studies is increased mortality.

A primary focus of toxicology has been to demonstrate a decrease in survivorship following an acute exposure to a particular stressor. For amphibians, overt effects are easily

characterized as much is known about their normal development. The assessment of chemical contaminants and other stressors using the 96-hour LC50 approach is extremely beneficial to toxicologists as species-specific benchmark safety levels or NOECs (no observable effect concentrations) can be established. In most cases, 96-hour LC50 values exceed likely natural environmental exposure levels. LC50 values only address acute toxicity and do not address potential reproductive dysfunction and population recruitment failure as a result of chronic exposure to environmentally relevant levels of persistent EDCs or UV-B radiation. In addition, Colborn (1995) encourages that we move beyond acute toxicity and incorporate a molecular approach which addresses subtle effects including gene expression and differentiation.

We carried out a ten-day acute toxicity study with several levels of OP and UV-B, alone and in combination, and determined lethal levels for leopard frog (*R. pipiens*) tadpoles (Chapter 2.0). In the current study, we used OP and UV-B levels that were previously found to have no overt effects on early tadpole development or survivorship after ten days to identify subtle effects on mRNA expression in the brain and post-exposure effects on aspects of metamorphosis (e.g. hindlimb emergence). The specific region of the brain we chose to focus on was the tadpole diencephalon (preoptic/hypothalamic area) as the gene expression program for neural development of this region of the brain has been characterized and it undergoes dramatic changes throughout metamorphosis (Denver *et al.* 1997). In addition, many aspects of hypothalamic function are modulated by sex steroids (Blázquez *et al.* 1998a) and thus we hypothesized that this brain region would be an effective environmental sensor for neurotoxicological studies.

We identified candidate genes in the hypothalamic region of developing leopard frog (*R. pipiens*) tadpoles that were differentially regulated by OP and/or UV-B treatments. By using the RNA-arbitrarily primed PCR (RAP-PCR) differential display strategy, genes associated with angiogenesis, signal transduction, synaptic plasticity, and structural integrity were isolated. Homology cloning was performed to obtain *R. pipiens* GAD 65 and GAD 67, the enzymes responsible for the synthesis of γ -aminobutyric acid (GABA) from glutamate (Bosma *et al.* 1999). We developed a *R. pipiens*-specific multiple-gene dot blot and used the reverse Northern strategy (Parfett *et al.* 1996; Wan and Erlander 1997) to detect, simultaneously and semiquantitatively, changes in abundance of particular mRNA species in tadpole diencephali and metamorph hypothalami. The reverse Northern technique verified that the expression of genes associated with GABA synthesis, angiogenesis, and signal transduction were differentially regulated in tadpole and metamorph brain tissue. We also show that early exposure to OP and UV-B can affect premetamorphic development by increasing growth rate and decreasing time to hindlimb emergence.

3.2 Materials and Methods

3.2.1 Animals and rearing conditions

Adult northern leopard frogs (*Rana pipiens*) were purchased from a commercial supplier (Charles Sullivan & Co., Nashville, Tennessee, USA) and housed in a flow-through aquarium at 18°C. They were fed mealworms treated with Reptovit (Terrafauna®) vitamin supplement. For males and females, breeding was induced in mid-January 1999 by two injections of des-Gly¹⁰, [D-His(Bzl)⁶]-Luteinizing Hormone-Releasing Hormone Ethylamide

(Sigma) four days apart at a dose rate of 50 ng/g in frog saline (0.6% NaCl). Eggs were maintained in aerated filtered water (pH 7.0; dissolved oxygen 8.4-10 mg/L; 16.5-18°C) in polystyrene containers until hatching (stage 20-21) after which they were distributed to the respective treatment exposures.

In addition, two naturally fertilized *R. pipiens* egg masses were collected on April 29, 1999 from a typical breeding pond on the campus of Trent University, Peterborough, Ontario (44° 22' N 78°17' W) to be used in the second exposure experiment. The rearing conditions were as above.

3.2.2 Conditions of Exposure

Two separate ten day exposure experiments were conducted under the same regimes of OP and UV-B. The first commenced on January 30, 1999 with tadpoles (Gosner stage 21) from the induced breeding event and the second, May 2, 1999 with tadpoles from the naturally fertilized egg masses. Tadpoles were reared in 100 x 15 mm Pyrex petri dishes and exposed in a controlled environmental chamber (18°C; 12:12 h light:dark photoperiod) to one of three concentrations of OP (10 µM, 1 µM, 1 nM) plus a 0.01% ethanol vehicle treatment, with and without two levels (7 µW/cm², 25 µW/cm²) of supplemental UV-B radiation. OP stock solutions (20.6 mg/mL, 20.6 µg/mL, 20.6 ng/mL) were prepared by dissolving 99% pure technical grade 4-OP (Aldrich Chemical Co.) in 95% ethanol. The nominal concentrations were prepared by adding 100 µL of the appropriate OP stock solution to 1 L of filtered water. Control and UV-B-only groups were treated with a 0.01% ethanol vehicle. The static renewal system operated on a water renewal every 48 hours for all

treatments to maintain consistency.

The supplemental lighting consisted of UV-B (UBL FS20T12/UVB-BP) and UV-A (combination of Cool White Sylvania F20T12 CW and NEC T10 Black Light Blue) lamps as well as background laboratory lighting supplied by full spectrum bulbs (Verilux F40T12VLX). Light panels (4 feet long) were positioned above the exposure containers to attain the desired UV-B regime (i.e. $7 \mu\text{W}/\text{cm}^2$, $25 \mu\text{W}/\text{cm}^2$). These are sub-ambient levels as it has been shown that summer levels of ambient UV-B in Eastern Ontario vary between $100\text{-}250 \mu\text{W}/\text{cm}^2$ (Crump *et al.* 1999*a,b*). The UV lamps were on for 6 hours per day (10:00 am - 4:00 pm) and UV measurements were carried out using an Oriel GOLDILUX UV meter with interchangeable UV-B and UV-A probes.

The experiments were performed with four replicates of each group. Each petri dish had fifteen tadpoles and they were fed Nutrafin® fish flakes daily. Following the ten day exposure, the diencephalon, which contains the developing preoptic/hypothalamic area, was rapidly dissected and frozen on dry ice for subsequent isolation of poly (A)⁺RNA. Ten diencephali were combined in each of four overall pools per treatment group to ensure sufficient genetic material. Tadpoles exposed to $10 \mu\text{M}$ OP experienced 100% mortality within one day irrespective of UV-B level and subsequently, were not studied.

3.2.3 Post-exposure developmental effects

After the second ten day exposure (May 12, 1999), 20 tadpoles from the ethanol exposed control, the two low levels of OP ($1 \mu\text{M}$, 1nM) and the low level of UV-B ($7 \mu\text{W}/\text{cm}^2$), alone and in combination, were transferred into clean water in 4.5 L grow-out

containers to monitor premetamorphic growth and development. The tadpoles were kept in a separate environmental chamber with no background UV-B under a 12:12 h light:dark photoperiod supplied by full spectrum lamps (1-2 $\mu\text{W}/\text{cm}^2$ UV-A) and a constant temperature of 19°C. There were two containers per group in order to minimize density stress as the tadpoles developed. Tadpoles were fed *ad libitum* with Nutrafin® fish flakes daily and overall health was monitored. Two months after the initial ten day exposure (July 16, 1999) tadpoles were weighed to assess potential differences in developmental rate. The onset of hindlimb emergence of individual tadpoles was also documented daily. At metamorphic climax (tail resorption), hypothalami were rapidly dissected and frozen on dry ice for isolation of poly(A)⁺RNA.

3.2.4 RAP-PCR and transcript identification

The RAP-PCR differential display strategy used in this study was similar to that of Blázquez *et al.* (1998b). Poly(A)⁺RNA from diencephali from the January exposure and metamorph hypothalami was isolated using the Straight A's™ mRNA isolation system (Novagen) and 100 ng was reverse transcribed using Superscript II (Gibco) to cDNA using one of three single 18-base arbitrary primers (primer A₃: 5'-AATCTAGAGCTCTCCTGG-3'; primer B₃: 5'-CATACACGCGTATACTGG-3'; primer C₃: 5'-CCATGCGCATGCATGAGA-3'). The resulting cDNAs were then PCR-amplified in the presence of high specific activity radiolabelled nucleotides (³²P]dCTP; 10 mCi/ml) and the original primer used for cDNA synthesis. Amplification was carried out in a thermocycler with a single cycle of low-stringency amplification (94°C - 1 min/36°C - 5 min/72°C - 5 min) followed by 40 cycles

with higher stringency (94°C - 1 min/54°C [A₃ and B₃]; 56°C [C₃] - 2 min/72°C - 2 min). PCR products were loaded on a 4% acrylamide/7 M urea sequencing gel (BioRad SequiGel Apparatus) and electrophoresed at 1700 V for 6 hours. All reactions were loaded in two adjacent lanes and this was repeated on the same gel to confirm the accuracy of the banding patterns. Gels were dried and exposed to Blue sensitive x-ray film (Kodak). Of approximately 50 bands on the differential display gel, only those that displayed a presence/absence pattern in the various treatments were considered differentially regulated. These bands were carefully cut from the gel and the DNA was extracted in 50-100 µl of TE buffer at 60°C for 60 min followed by an overnight incubation at room temperature. Reamplification of the transcripts was conducted using 30 cycles of the higher stringency reaction (from above) and the products were run on a horizontal 1% agarose gel in 1 X TBE buffer to quantify the size and to screen for multiple products. The differentially expressed transcripts were cloned into a pCR® II-TOPO® vector and transformed in *E. coli* competent cells using the TOPO TA Cloning Kit® (Invitrogen). Colony screening by PCR using the original primers was carried out to confirm the presence of the cDNA fragments. Upon confirmation, white colonies were picked from X-Gal/ampicillin LB agar plates and grown in LB liquid media overnight at 37°C. Plasmids were purified using the Qiagen Plasmid Mini Kit and the cDNA was sequenced by Canadian Molecular Research Services Inc. (Ottawa, Ontario) using Simultaneous Bi-directional Sequencing (SBS) reactions with the DYEnamic Cycle Sequencing Kit (US79535) from Amersham Pharmacia Biotech and IRD700 and IRD800 labeled vector primers (LiCor) flanking the DNA insertion site. The sequencing reactions were separated on a LiCor 4200L sequencer and analyzed with Sequencher 4.0

(Gene Codes Corporation, Ann Arbor, Michigan). Sequence results were compared to known sequences available in GenBank using the BLAST search accessible through NCBI.

3.2.5 Reverse Northern multiple-gene dot blot

Poly(A)⁺RNA was purified from brain tissue collected during the second exposure using the Straight A's™ mRNA isolation system (Novagen) and 200 ng was reverse transcribed following the methods of Parfett *et al.* (1996) except that Superscript II (Gibco) was used in place of M-MLV reverse transcriptase. For this experiment, only brain tissues from the lowest levels of OP (1 nM) and UV-B (7 μW/cm²), alone and in combination, and the control were utilized. Second-strand cDNA was synthesized in the same salts/buffer mixture as the first-strand and the 50 μl reaction mix contained 1.5 μl fresh dNTPs (10mM), 1 μl BSA (0.1 μg/μl), 0.5 μl RNase H (0.027 U/μl), and 0.5 μl *E. coli* DNA polymerase holoenzyme I (0.1 U/μl). The second-strand cDNA was incubated at 15°C for 1 hour, phenol extracted and precipitated with ethanol. Probe synthesis consisted of labeling 100 ng of double-stranded DNA with [³²P]dCTP using the Amersham Pharmacia random primer/T7 DNA polymerase oligo-labeling kit.

A composite selection of seven candidate genes isolated by differential display from both tadpole and metamorph brains were chosen for the multiple-gene dot blot. Very few brain-specific molecules of any class have been cloned from the leopard frog (*R. pipiens*). Thus the differential display strategy allowed for rapid isolation of candidate genes regulated by UV-B and OP. In addition, the two isoforms of leopard frog (*R. pipiens*) glutamate decarboxylase (Accession Numbers; AF202124 (GAD67); AF202125 (GAD65); Trudeau *et*

al. 2000) and snapping turtle (*Chelydra serpentina*) β -actin (100% identical at the predicted amino acid level with *Xenopus* β -actin) were included. To isolate approximately 30 μ g of purified cDNA insert required for the dot blots, the Qiagen Plasmid Maxi Kit was used. cDNA inserts were digested with EcoRI (Gibco) and extracted from 1% agarose gels using the QIAquick gel extraction kit (Qiagen). Purified cDNA (300 ng/dot prepared in 240 μ l 12X SSC) of each of the ten molecules was dotted, in triplicate, onto HybondTM-N+ membranes (Amersham Pharmacia) resulting in a 10 X 3 gene array. Dot blots were made using a 96-well Schleicher and Schuell apparatus. Dot blots were hybridized (Parfett *et al.* 1996) for 48 hours at 55°C with denatured, [³²P]-labeled double-stranded cDNA synthesized from poly(A)⁺RNA from the various treatment groups in order to detect changes in the relative abundance of the ten mRNA species.

After hybridization, membranes were sequentially washed for 30 min from low (2 X SSC/0.1% SDS at 55°C) to high stringency (0.1 X SSC/0.1% SDS at 55°C) and specific hybridization signals were visualized and quantified using the Quantity One[®] software and the BioRad phosphor-imaging system. This quantification system includes a function which indicates whether signals are saturated and we found that even the heavy signals (i.e. control molecules) were on the linear range for the machine. In order to differentiate light signals from background noise, a background value was calculated for each blot and all data were corrected accordingly. All data were normalized to β -actin as it did not change with OP or UV-B treatments.

3.2.6 Statistical analysis of data

Tadpole weight and age at hindlimb emergence (HLE) were analyzed using a two-way ANOVA. A post-hoc Fisher's Least-Significant-Difference (LSD) multiple comparisons test was performed to identify differences between treatment groups. The average signal intensity of internal triplicates was calculated per dot blot and this value was divided by the average signal intensity for β -actin. Each treatment group had an independent replicate of three or four blots ($n = 3$ or 4). A two-way non-parametric ANOVA was used to test for significant interactions between OP and UV-B with respect to mRNA expression followed by Fisher's LSD test to determine differences between groups. For all statistical procedures, group means were considered different if $p < 0.05$. However, gene expression data was not normally distributed and therefore, non-parametric. The median of each treatment group has been presented (Sokal and Rohlf 1981). Systat 7.0.1[®] software was used for all statistical procedures.

3.3 Results

3.3.1 Post-exposure developmental effects

Developmental rate in the clean water grow-out tanks, as determined by the weight of tadpoles after two months (Gosner stage 26-27; prior to hind-limb emergence), was found to change based on early life-stage exposure. Tadpoles treated with a combination of $1 \mu\text{M}$ OP and $7 \mu\text{W}/\text{cm}^2$ UV-B were significantly larger (Figure 3.1A; $p < 0.05$) than all other treatments. Tadpoles exposed to UV-B alone displayed a significant reduction in weight ($p < 0.05$) compared to the control and both combination groups (Figure 3.1A). Tadpole

weight in the control group was not significantly different than the two OP concentrations alone (Figure 3.1A). A similar trend was observed for the onset of hindlimb emergence (HLE) (Figure 3.1B). Tadpoles treated with 1 μ M OP and 7 μ W/cm² were significantly advanced with respect to day at HLE compared to all other treatments (Figure 3.1B; $p < 0.05$). Tadpoles exposed to UV-B alone displayed a significant delay compared to both combination groups (Figure 3.1B; $p < 0.05$) and day at HLE in the control group was not significantly different than the two OP concentrations alone (Figure 3.1B).

3.3.2 RAP-PCR

Identification of putative treatment-responsive genes from the tadpole diencephalon - The differential display strategy identified three candidate cDNA transcripts regulated by OP, one transcript regulated by UV-B, and two molecules that were equally expressed irrespective of treatment (Table 3.1). A cDNA fragment (774 bp) approximately 66% identical to the 3'-end of human and rat plectin was up-regulated by 1 nM OP. Plectin is an intermediate filament binding protein which provides mechanical strength and structural integrity to the cytoskeleton and is strategically localized at the cytoskeleton-plasma membrane interface (e.g. the cells forming the blood-brain barrier) (Liu *et al.* 1996). Another transcript (798 bp) up-regulated by 1 nM OP was 89% identical to human Nck, Ash and phospholipase C gamma-binding protein 4 (NAP4). NAP4 is implicated in coordinating various signaling pathways including growth factor and cell adhesion receptors (Matuoka *et al.* 1997). One transcript (517 bp) was down-regulated by both 1 nM and 1 μ M OP and was 59% identical to human brain-specific angiogenesis inhibitor 2 (BAI2). BAI2 is a membrane protein which

inhibits neovascularization and acts as a growth suppressor of glioblastoma (primary brain tumor development) (Shiratsuchi *et al.* 1997). A 403 bp transcript was down-regulated by the high UV-B treatment ($25 \mu\text{W}/\text{cm}^2$) and was 56% identical to rat arcadlin. Arcadlin is a novel cadherin molecule expressed at the synapses that may play an important role in activity-induced synaptic reorganization underlying long term memory (Yamagata *et al.* 1999). Finally, cytochrome C oxidase chain I, an important mitochondrial proton-pumping respiratory protein, and NADH dehydrogenase subunit 4, involved in glycolysis and cellular respiration, were equally expressed irrespective of treatment.

Identification of putative treatment-responsive genes from the metamorph hypothalamus - A

567 bp transcript that was 90% identical to human brain-specific angiogenesis inhibitor 3 (BAI3) was determined to be unchanged based on differential display (Table 3.1). BAI3 is part of the same family of angiogenesis inhibitors as BAI2 (Shiratsuchi *et al.* 1997).

Differential display identified a 896 bp fragment that was up-regulated by 1 nM and 1 μM OP, alone and in combination with $7 \mu\text{W}/\text{cm}^2$ UV-B, which was 54% identical to goldfish NADH dehydrogenase subunit 4 (Table 3.1). Note that NADH dehydrogenase subunit 4 was unchanged in the tadpole brain which infers a stage- and treatment-specific shift in expression of this molecule.

3.3.3 Verification of RAP-PCR results using a reverse Northern multiple-gene dot blot

The multiple-gene dot blot was a composite selection of the seven genes discussed above that were cloned via differential display as well as GAD 65, GAD67 and β -actin.

Figure 3.2 provides an example of dot blots probed with RNA isolated from tadpole diencephali from the different treatments. The median expression values have been normalized to β -actin as it did not change with OP or UV-B treatments.

Gene expression in the tadpole diencephalon - Octylphenol (1 nM) induced a 3-fold increase in the expression of NAP4 (Figures 3.2, 3.3), confirming the initial differential display results (Table 3.1). Although BAI3 was originally isolated from the hypothalamus of *R. pipiens* metamorphs, we found that its expression was up-regulated 4-fold in animals previously exposed to UV-B (Figure 3.4). In addition, we verified that cytochrome C oxidase chain I and NADH dehydrogenase subunit 4 expression was not affected by the various treatment exposures (Appendix 1,2). The median values for plectin expression were not greater in the OP treatment and in fact were similar across all treatments (Appendix 3). This observation is inconsistent with the differential display results as are the results for BAI2 and arcadin (Appendix 4,5). Neither of these molecule showed a tendency towards the trend observed with differential display (Table 3.1). Reverse Northern experiments confirmed the expression pattern of three of the six transcripts originally isolated from tadpole diencephalon using differential display. A similar level of confidence has been reported for differential display and reverse Northern blots in other vertebrate systems (Wan and Erlander 1997). The median expression of GAD65 and GAD67 was similar across all treatments in tadpole diencephali (Appendix 6,7).

Gene expression in the metamorph hypothalamus - Early exposure to the combination of OP

(1 nM) and UV-B ($7 \mu\text{W}/\text{cm}^2$) significantly up-regulated GAD67 expression by 2-fold compared to all other treatments (Figure 3.5A). GAD67 expression in the control group was not significantly different than the OP or UV-B group. Early exposure to OP (1 nM) resulted in a significant decrease in gene expression ($p < 0.05$) compared to the UV-B group (Figure 3.5A). The expression pattern of BAI2 in the various treatment groups was very similar to that observed for GAD67 (Figure 3.5A,B). Early exposure to OP (1 nM) resulted in a 2-fold decrease in BAI2 expression compared to control and UV-B groups. The combined OP/UV-B exposure resulted in significant up-regulation of BAI2 compared to all other treatments (Figure 3.5B). Although cytochrome C oxidase chain I was originally isolated from tadpole diencephali, we found that early exposure to UV-B induced a 3-fold increase in its expression in metamorph hypothalami (Figure 3.6). BAI3 was originally determined to be unchanged based on differential display (Table 3.1) but, the median expression values were significantly greater by approximately 3 to 4-fold in both the UV-B and combination groups (Figure 3.7). As in the tadpole diencephalon, BAI3 expression appears to be sensitive to UV-B treatment and the result is significant up-regulation (Figures 3.4,3.7). NADH dehydrogenase subunit 4 expression was not regulated (Appendix 8) as predicted by differential display (Table 3.1).

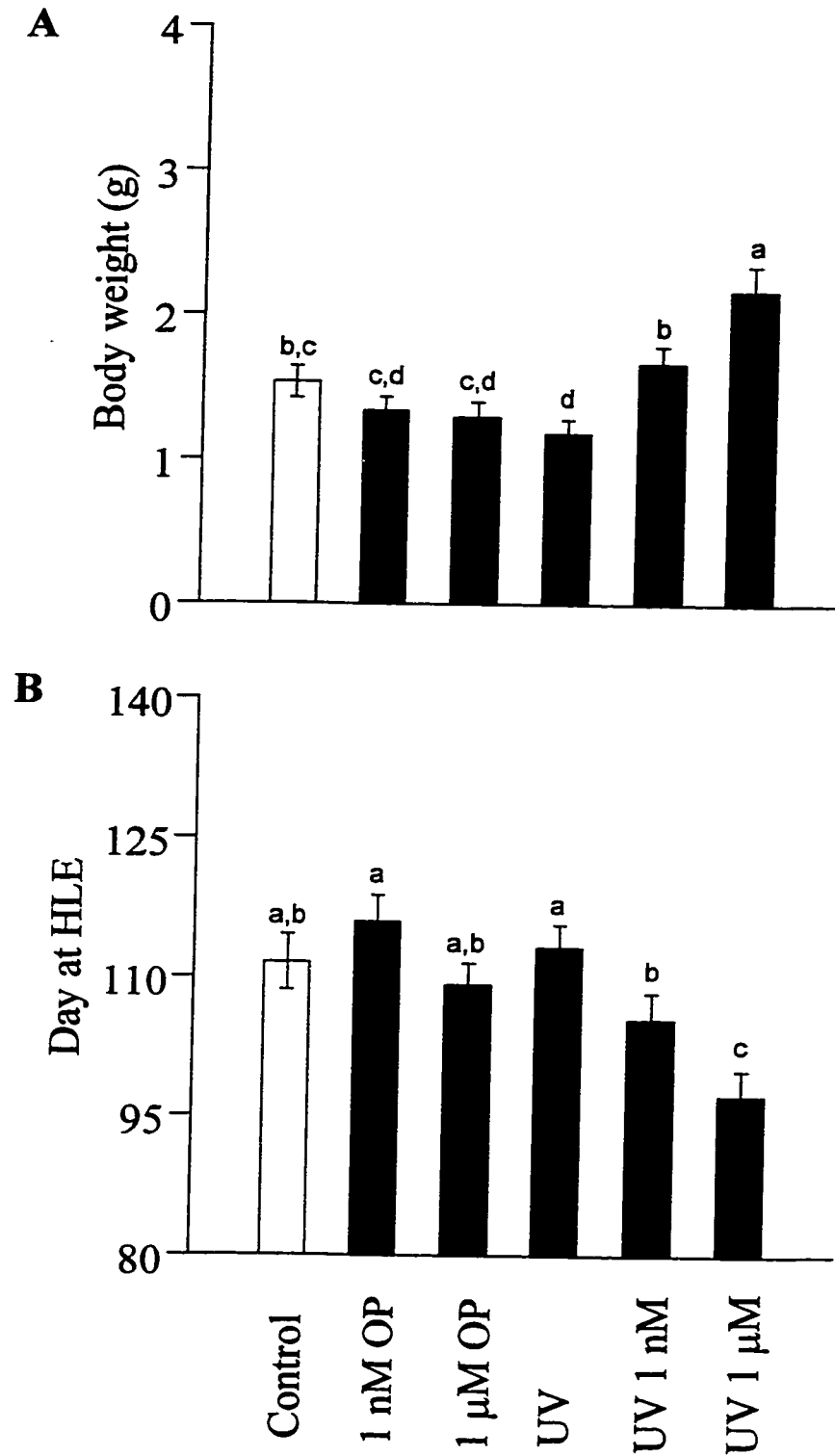


Figure 3.1. The effects of early exposure to octylphenol (OP) (1 nM, 1 μM) and UV-B radiation (7 μW/cm²), alone and in combination, on premetamorphic parameters of the leopard frog (*Rana pipiens*). **A**, Growth in premetamorphic tadpoles based on overall weight ($p < 0.05$). **B**, Day at hindlimb emergence (HLE) as a function of treatment ($p < 0.05$). Different letters represent significant differences.

Table 3.1. mRNA transcripts potentially regulated by octylphenol and UV-B in the hypothalamic tissue of leopard frog (*Rana pipiens*) tadpoles and metamorphs as determined using RAP-PCR.

Stage	Molecule	Functions	cDNA Length	Primer	% Identity ^{a,b}	Treatment Effects
Tadpole	Plectin	Mechanical strength for cytoskeleton; Structural integrity	774 bp	C ₃	66% (Rat; X59601)	OP ₁ ↑
	Nck, Ash and phospholipase C gamma-binding protein (NAP4)	Signal transduction; Growth factor action	798 bp	C ₃	89% (Human; PC4427)	OP ₁ ↑
	Brain-specific angiogenesis inhibitor 2 (BAI2)	Development of glioblastoma	517 bp	A ₃	59% (Human; NP_001694)	OP ₁ ↓ OP ₂ ↓
	Arcadlin	Ca ²⁺ signaling; Synaptic reorganization	403 bp	B ₃	56% (Rat; BAA82442)	UV ₂ ↓
Metamorph	Cytochrome C Oxidase Chain I	Mitochondrial respiratory protein; Brain oxidative phosphorylation	676 bp	B ₃	62% (Alligator; AAD09982)	No change
	NADH Dehydrogenase Subunit 4	Glycolysis; Cellular respiration	898 bp	A ₃	54% (Goldfish; BAA31247)	No change
	Brain-specific angiogenesis inhibitor 3 (BAI3)	see BAI2	567 bp	A ₃	90% (Human; NP_001695)	No change
	NADH Dehydrogenase Subunit 4	Glycolysis; Cellular respiration	896 bp	A ₃	54% (Goldfish; BAA31247)	All treatments ↑ except control

^a% Identity at the predicted amino acid level

^b In brackets is the species and accession number of the GenBank sequence for the first BLAST search hit

Note: Treatments were; OP₁- 1nM; OP₂- 1µM; UV₁- 7 µW/cm²; UV₂- 25 µW/cm². ↑ = increased expression; ↓ = decreased expression

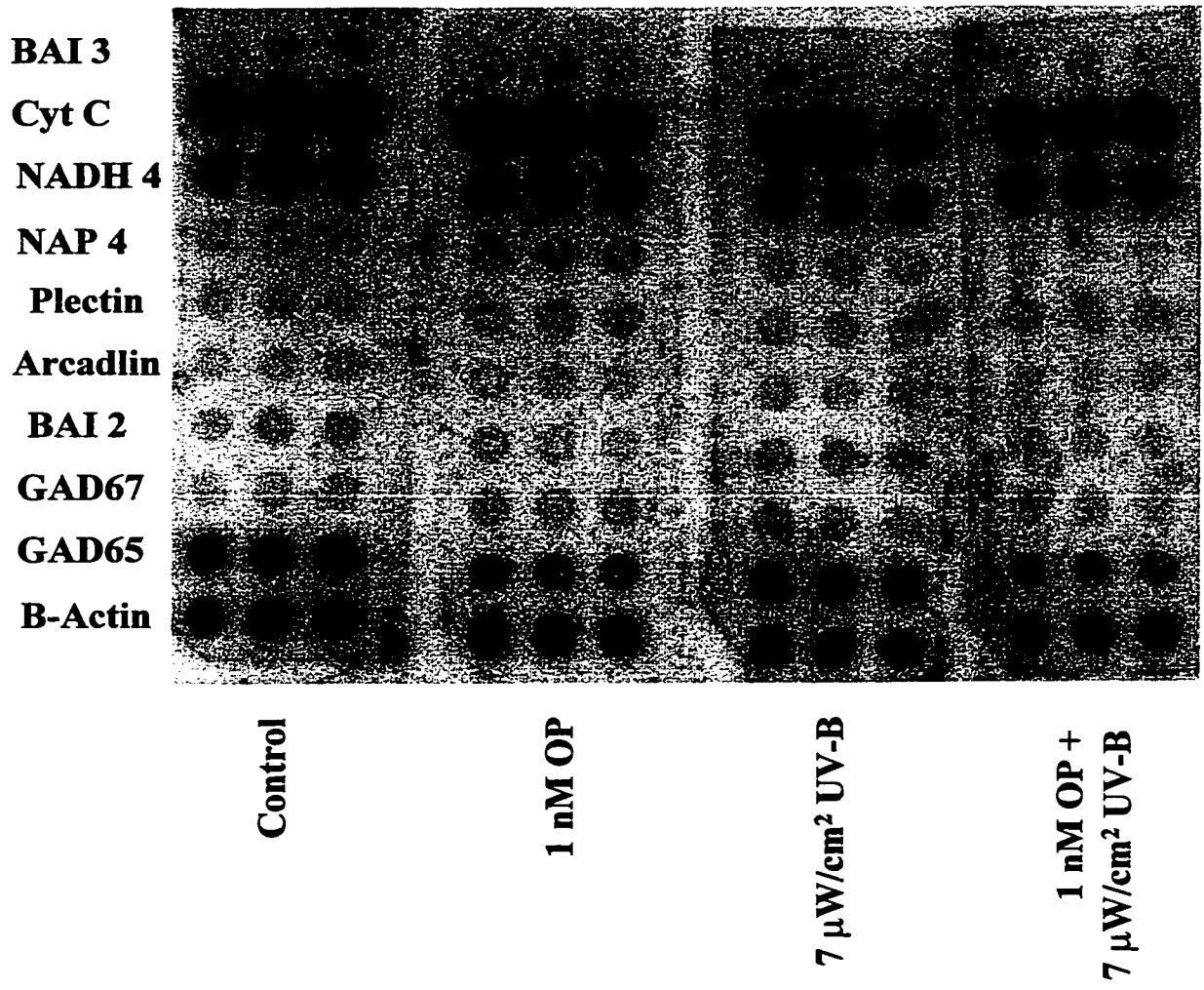


Figure 3.2. Multiple-gene dot blots comprised of seven candidate genes cloned from differential display, the two isoforms of glutamate decarboxylase (GAD 65 & 67), and β -actin that were probed with RNA isolated from the diencephalon of leopard frog (*R. pipiens*) tadpoles from the four treatment groups. (BAI-brain-specific angiogenesis inhibitor; NAP4-Nek, Ash and phospholipase gamma-binding protein 4; NADH 4-NADH dehydrogenase subunit 4; Cyt C-Cytochrome C oxidase chain I)

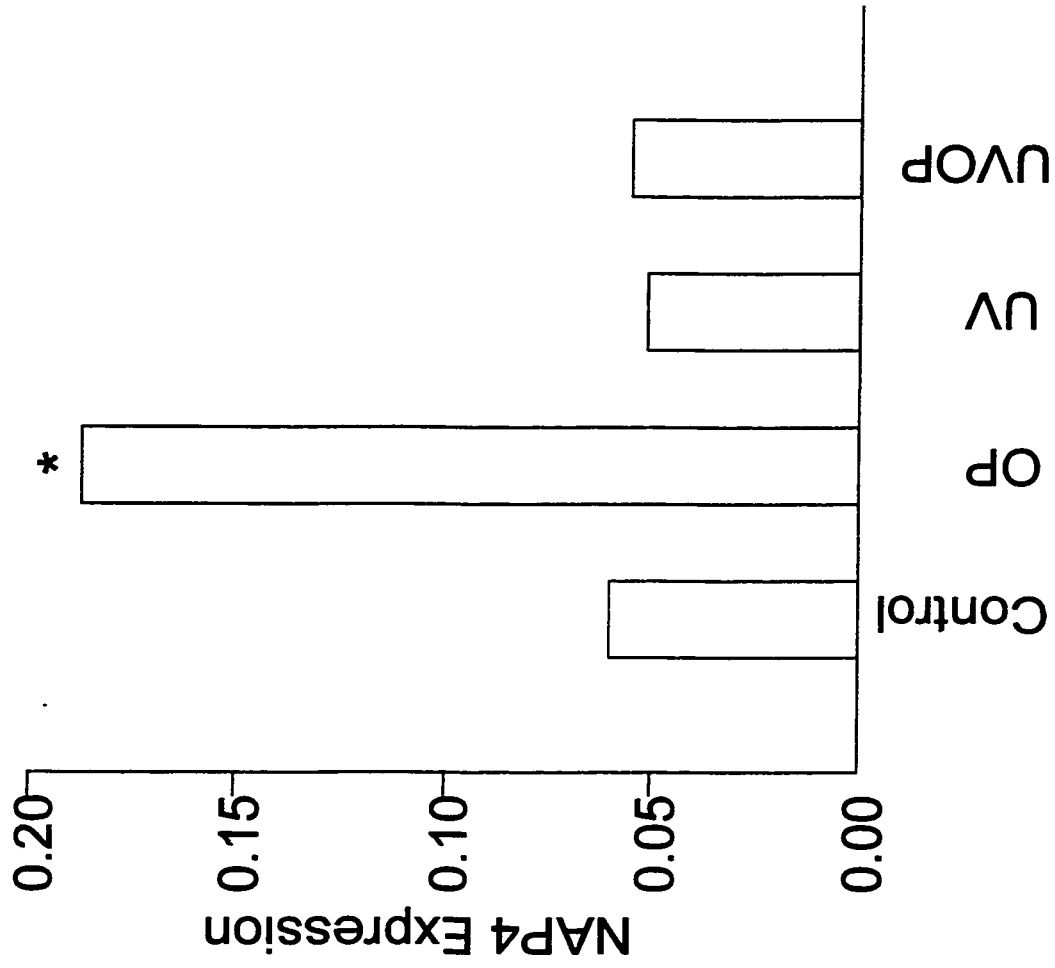


Figure 3.3. The relative expression of NAP4 (Nck, Ash and phospholipase gamma-binding protein 4) in the diencephalon of leopard frog (*R. pipiens*) tadpoles normalized to β -actin as a function of treatment where, OP = 1 nM; UV = 7 μ W/cm²; and UVOP = combination of the two. (differences significant at $p < 0.05$)

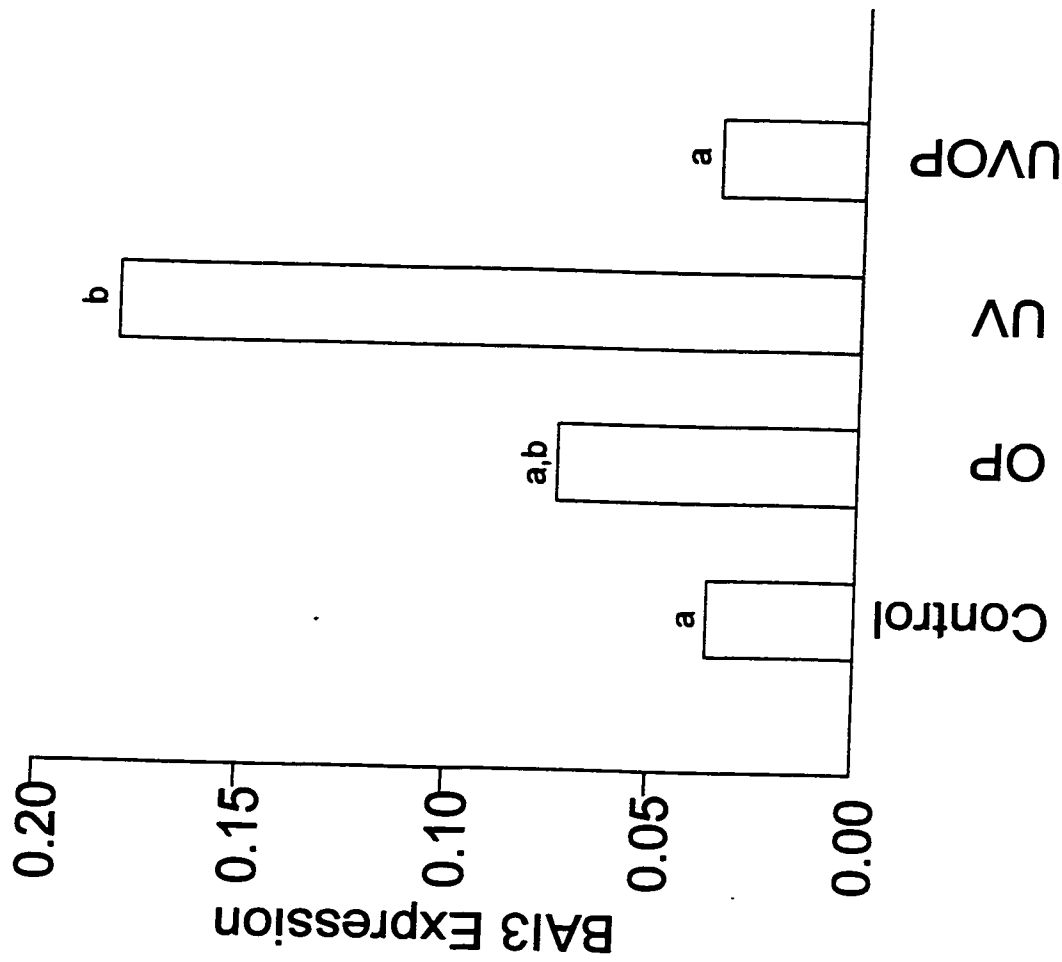


Figure 3.4. Relative mRNA expression of BAI3 (brain-specific angiogenesis inhibitor 3) in the diencephalon of leopard frog (*R. pipiens*) tadpoles normalized to β -actin as a function of four treatments where, OP = 1 nM; UV = 7 μ W/cm²; and UVOP = combination of the two. Different letters are representative of statistical differences ($p < 0.05$).

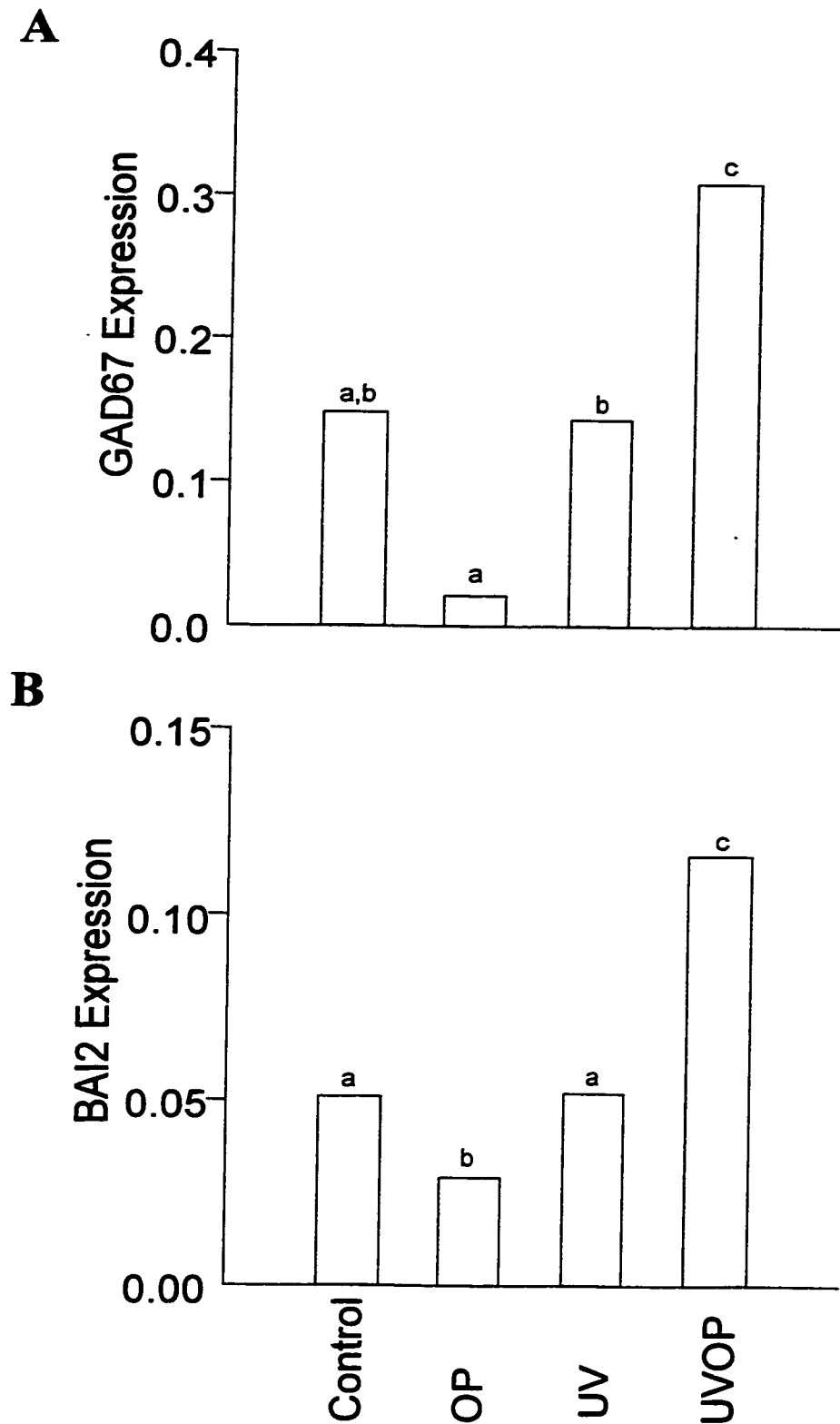


Figure 3.5. The effect of OP (1 nM) and UV-B radiation (7 μ W/cm²), alone and in combination, on the mRNA expression of; **A**, Glutamate decarboxylase (GAD) 67 and; **B**, Brain-specific angiogenesis inhibitor 2 (BAI2) in the hypothalamus of leopard frog (*R. pipiens*) metamorphs. Different letters indicate significant differences ($p < 0.05$) and median values are normalized to β -actin.

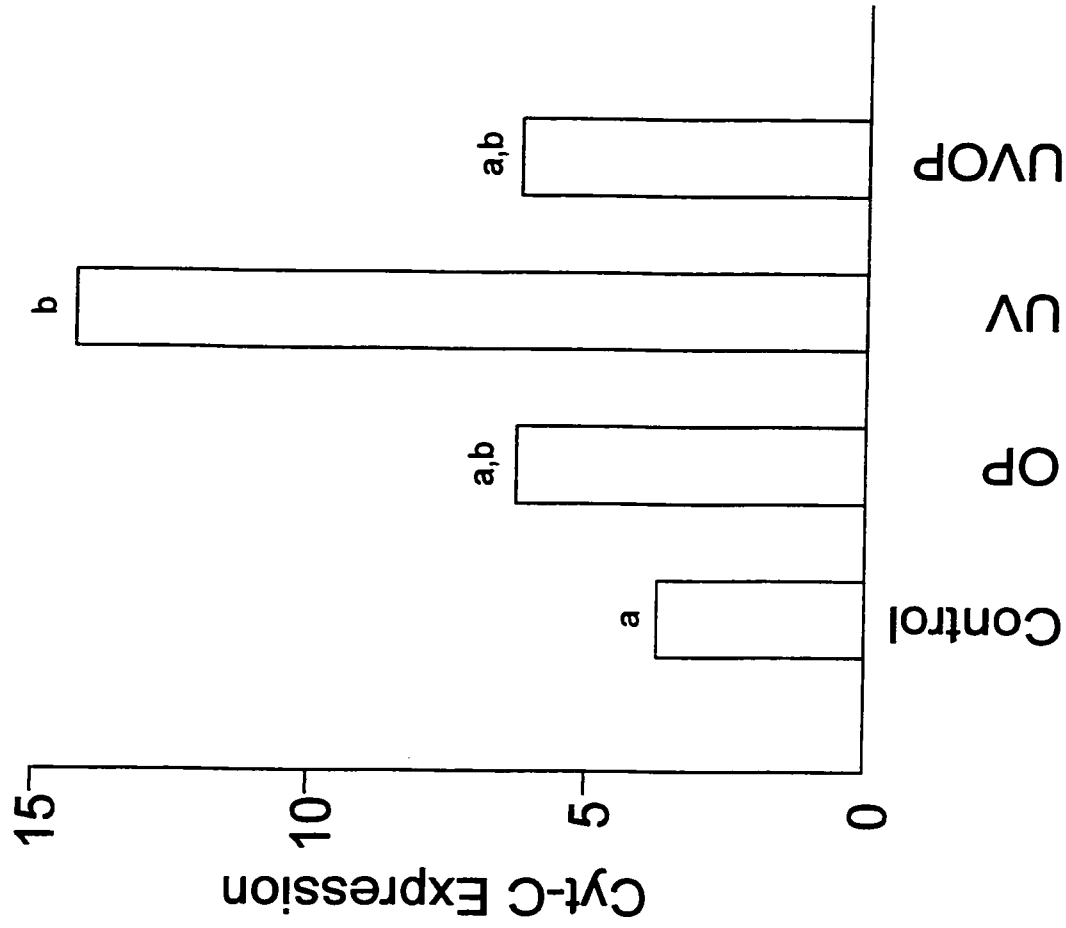


Figure 3.6. The relative mRNA expression of Cyt-C (Cytochrome C oxidase chain I) in leopard frog (*R. pipiens*) metamorph hypothalmi as a result of treatment with OP (1 nM) and UV-B (7 μ W/cm²), alone and in combination. Median values are normalized to β -actin and different letters represent significant differences ($p < 0.05$).

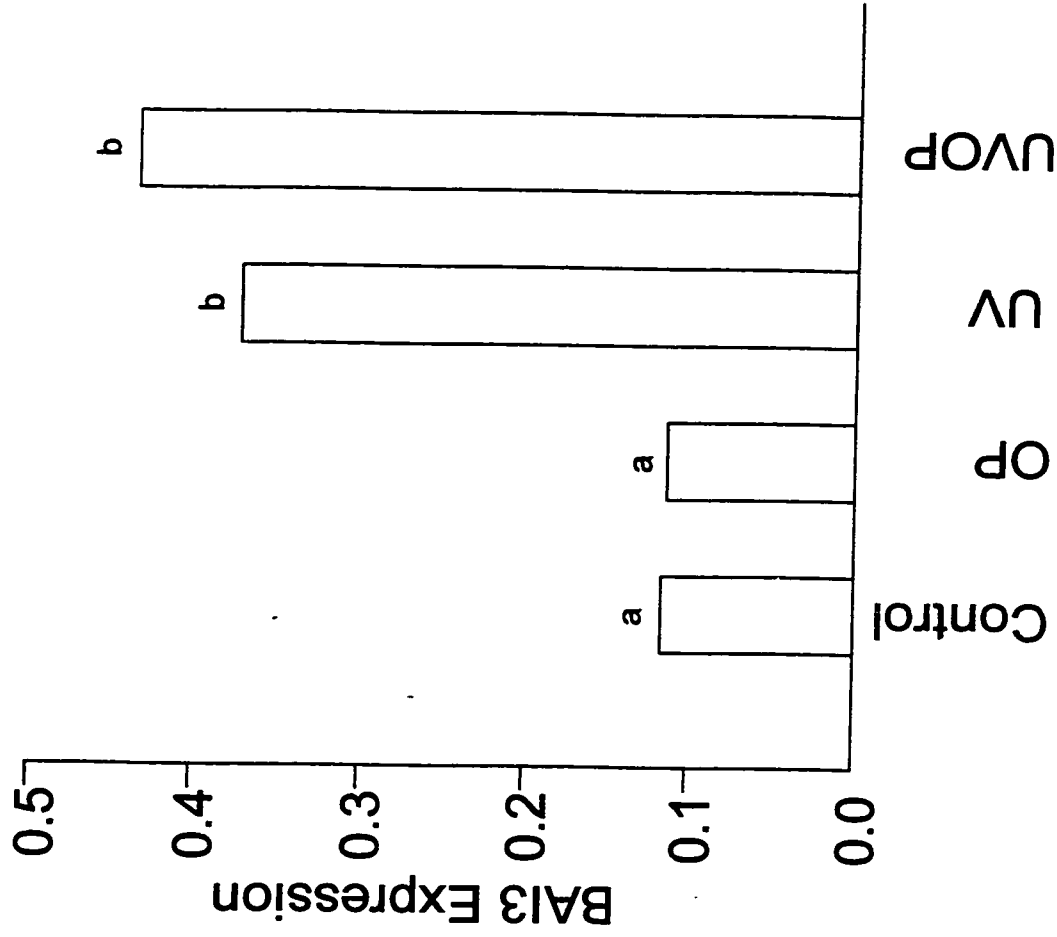


Figure 3.7. The effects of OP (1 nM) and UV-B radiation (7 μW/cm²), alone and in combination, on the relative mRNA expression of BAI3 (brain-specific angiogenesis inhibitor 3) in the hypothalamus of the leopard frog (*R. pipiens*) metamorph. All data have been normalized to β-actin and different letters indicate significant differences (p<0.05).

3.4 DISCUSSION

We demonstrate that an early ten-day exposure of leopard frog tadpoles (*R. pipiens*) to environmentally relevant levels of OP and UV-B radiation has the potential to disrupt normal premetamorphic development. Body weight prior to hindlimb emergence was increased and the day at hindlimb emergence was significantly accelerated by co-treatments of OP and UV-B. By using the differential display strategy, we were able to isolate candidate genes from a species whose genome is not well characterized. The rapid screening technique for treatment related genes identified molecules with structural and/or functional importance (Table 3.1). Gene expression screening techniques are qualitative and therefore, must include a confirmation step which verifies the predicted mRNA expression patterns. We used semi-quantitative reverse Northern multiple-gene dot blot hybridization experiments to detect changes in abundances of particular mRNA species. The advantage of this technique is that analyses can be carried out on several genes simultaneously without requiring excessive mRNA or increasing the number of hybridization reactions necessary (Parfett *et al.* 1996). Here, we focus on four of the RAP-PCR products including, Nck, Ash and phospholipase C gamma-binding protein 4 (NAP4), brain-specific angiogenesis inhibitors 2 & 3 (BAI2,3), and cytochrome C oxidase chain I, and glutamate decarboxylase 67 (GAD67).

NAP4 expression was up-regulated by 1 nM OP in the tadpole diencephalon verifying the pattern observed using differential display (Table 3.1; Figure 3.3). NAP4 is a putative signaling molecule found in adult and fetal human brain, fetal lung fibroblasts, and human leukocytes indicating wide expression in various tissues and developmental stages (Matuoka *et al.* 1997). NAP4 contains a complete Src homology region 2 (SH2) domain which has

been found to interact with the middle SH3 domain of Nck (Matuoka *et al.* 1997). Nck is an adaptor protein which serves to physically bridge activated cell surface receptors to various signal transduction pathways (McCarty 1998). Signal transduction cascades require the formation of multimeric intracellular protein complexes often initiated by adaptor proteins with SH2 and SH3 domains and NAP4 facilitates more efficient tyrosine kinase-mediated signal transduction (Matuoka *et al.* 1997). Epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) are all tyrosine kinase-mediated signal transduction events associated with Nck (McCarty 1998). In addition, Nck is capable of affecting pathways leading to cellular mitogenesis and morphogenesis and has been assigned an oncogenic role as overexpression in fibroblasts leads to their anchorage-independent cell growth and morphological transformation (McCarty 1998). Finally, Nck has been found in the nucleus although it lacks a nuclear localization signal (NLS) which implies that NAP4, which has a putative NLS, facilitates the transport of Nck into the nucleus (Matuoka *et al.* 1997).

As NAP4 has only recently been cloned and characterized, the literature surrounding its functions is limited, although it is clear that this molecule is extremely important in many cellular processes. We show that levels of OP that have been reported in freshwater up-regulate the expression of this molecule by approximately 3-fold (Figure 3.3). During early tadpole development, the diencephalon undergoes substantial restructuring and the regulation of intercellular communication (especially from the brain to the developing thyroid axis) is essential for proper development and survival (Denver *et al.* 1997). In this context, an up-regulation of NAP4 following OP treatment could alter important aspects of tadpole

development, such as tyrosine kinase-mediated signal transduction events involved with growth factors and morphogenesis. However, deregulation of certain signal transduction pathways associated with cellular proliferation (e.g. Ras GTPases; extracellular signal-related kinase (ERK)) can result in the onset of various abnormalities including cancer (McCarty 1998). Nck, and thus NAP4, appear to be functionally involved with the above signaling cascades and therefore a disruption of normal function, due to OP exposure in this case, could lead to changes in cell proliferation in the developing tadpole brain.

The expression of BAI3 was up-regulated in brain tissue of tadpoles and metamorphs exposed to UV-B and was additionally up-regulated by the combination of OP and UV-B in the metamorph hypothalamus (Figures 3.4, 3.7). Another member of the BAI family, BAI2, displayed decreased expression in the OP treatment and was up-regulated by the combined treatment of OP and UV-B in the metamorph hypothalamus (Figure 3.5B). Angiogenesis is controlled by a local balance between stimulators and inhibitors of neovascularization and vasoproliferation (Nishimori *et al.* 1997; Shiratsuchi *et al.* 1997). Tumour cells are potently angiogenic because of increased secretion of inducers (e.g. basic fibroblast growth factors, bFGF; vascular endothelial growth factors, VEGF) and decreased production of endogenous negative regulators (e.g. thrombospondins, glioma-derived angiogenesis inhibitor factors) (Nishimori *et al.* 1997; Shiratsuchi *et al.* 1997). All members of the BAI family, comprised thus far of BAI1, BAI2, and BAI3, contain a 7-span transmembrane region with an extracellular motif recognized by the integrins and sequences corresponding to thrombospondin (TSP) type I repeats that can inhibit experimental angiogenesis (Shiratsuchi *et al.* 1997). Recently, the angiogenesis inhibitors have emerged in the popular media

(Reuters 2000) as potential cancer drugs as they could block the signaling of cancer cell inducers to surrounding blood vessels and inhibit the vasoproliferation necessary for the growth, persistence and metastasis of solid tumors (Nishimori *et al.* 1997).

NAP4 is also associated with signal transduction events involving inducers of vasoproliferation (e.g. VEGF) and has been shown to have an oncogenic role when overexpressed in fibroblasts. Although a decrease in expression of either BAI2 or BAI3 was not observed in the tadpole diencephalon, early exposure to OP did decrease BAI2 expression in the metamorph hypothalamus (Figure 3.5B). NAP4 was significantly up-regulated by OP in tadpole diencephalon (Figure 3.3) which might stimulate signal transduction events that would lead to vasoproliferation and in metamorphs, the endogenous inhibitors of such vasoproliferation appear suppressed. Additionally, UV-B is a known carcinogen and in both the tadpole diencephalon and metamorph hypothalamus, BAI3 expression appears to be sensitive to UV-B treatment resulting in significant up-regulation (Figures 3.4, 3.7). This up-regulation might inhibit vasoproliferation into any potential tumour cells associated with UV-B exposure.

Early exposure to the combination of OP and UV-B significantly up-regulated GAD67 expression in metamorph hypothalami by 2-fold compared to all other treatments (Figure 3.5A). GAD67 expression in the control group was not different than either the OP or UV-B treated animals. Early exposure to OP resulted in a significant decrease in gene expression relative to the UV-B group. Tadpoles exposed early to UV-B showed a significant decrease in expression compared to the combination group (Figure 3.5A). Both GAD65 and GAD67 mediate the synthesis of GABA but their distribution in the brain

suggest divergent functional roles (Pinal and Tobin 1998). GABA is the primary inhibitory neurotransmitter in the mammalian central nervous system and acts to induce membrane hyperpolarizations by binding to postsynaptic GABA_A receptors and presynaptic GABA_B receptors (Pinal and Tobin 1998). GABA also has a stimulatory role in the control of hypothalamic GnRH and pituitary gonadotropic hormone release indicating an involvement with central reproductive control (Blázquez *et al.* 1998b). GABA is also known to act as an important neurotrophic and neurodifferentiating signal molecule during early brain development (Waagepetersen *et al.* 1999). GABA neurons can be phasically or tonically active. GAD67 mRNA predominates in tonically firing neurons and is subject to regulation at the transcriptional level where denervation or neuronal injury results in changes in the expression of GAD67 mRNA (Pinal and Tobin 1998).

GABA is found throughout the hypothalamus and regulates most aspects of hypothalamic function (McCann and Rettori 1988). An intriguing pattern emerges upon observation of GAD67 expression in the metamorph hypothalamus (Figure 3.5A) and the trend observed for both tadpole weight and age at hindlimb emergence (Figure 3.1A,B). Although no changes in weight or HLE were observed with OP (1 nM), it can be postulated that the hypothalamic gene expression changes in the combined treatment of OP (1 nM) and UV-B (7 μ W/cm²) likely precede the changes in metamorphosis since the high OP (1 μ M) combined with UV-B (7 μ W/cm²) actually resulted in a quantitative difference in weight and HLE (Figure 3.1A,B). Therefore, the expression pattern of GAD67 appears somewhat linked to the changes observed in premetamorphic parameters. Although speculative, it may be that GABA is involved in metamorphosis as the alteration of development, post-exposure,

corresponds to changes in the synthesizing enzyme GAD67.

Physiological control of metamorphosis is predominantly associated with corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH), neuropeptides of the preoptic/hypothalamic brain region (Tata 1996; Denver 1998). The former stimulates the secretion of the pituitary hormone thyrotropin (TSH) which controls T_3 production whereas, TRH stimulates the secretion of prolactin (PRL) which inhibits metamorphosis and leads to larval growth (Tata 1996; Denver 1998). In mammals, GABA has been found to have both stimulatory and inhibitory effects on TSH release (McCann and Rettori 1988). In ovariectomized rats, GABA lowered plasma TSH via the dopamine system however, TSH was lowered less effectively in rats treated with E_2 and progesterone (McCann and Rettori 1988). Tonic stimulation of TSH release by GABA in both males and ovariectomized female rats has also been reported (McCann and Rettori 1988). Perhaps a co-treatment with OP (1 μ M) and UV-B (7 μ W/cm²) resulted in GABA-mediated tonic stimulation of TSH release. Alternatively, the inhibitory role of GABA on plasma TSH levels may have been less effective following exposure to an estrogenic contaminant (OP) in combination with UV-B radiation.

Prunet *et al.* (1993) found that GABA inhibited PRL release from trout pituitary cells *in vitro*. The authors showed both GABA_A and GABA_B-receptor mediated suppression of PRL release from the pituitary. The inhibitory control of PRL release by GABA has also been reported for mammals (McCann and Rettori 1988). For metamorphosis, hypothalamic neurosecretion becomes extremely important during tadpole growth as TRH synthesis leads to the secretion of PRL which antagonizes metamorphosis and promotes larval growth

leading to larger, more fit animals at metamorphosis (Tata 1996; Denver 1998). Derby (1975) found that PRL did not affect hindlimb growth indicating that although the increase in weight observed in the combination group (Figure 3.1A) may have been related to pituitary secretion of PRL, the acceleration of hindlimb emergence (Figure 3.1B) was likely T_3 -mediated as the earliest responding organ to T_3 is the limb bud (Brown *et al.* 1996). The role of GABA, OP or UV-B in this apparent disruption of hypothalamic secretion is not well understood at present.

We found that the expression of cytochrome C oxidase chain I was up-regulated by approximately 3-fold in the metamorph hypothalamus as a result of early exposure to UV-B (Figure 3.6). This molecule has been shown to be T_3 -regulated in the *Xenopus* gene expression program for neural development and thus, is associated with changes in the diencephalon important for metamorphosis (Denver *et al.* 1997). Cytochrome C oxidase is an important mitochondrial proton-pumping respiratory protein which catalyzes the transfer of electrons from cytochrome C to oxygen (Purves *et al.* 1992; Denver *et al.* 1997). The extensive brain restructuring involved in metamorphosis and the corresponding metabolic demands during cell proliferation and differentiation would require considerable energy. Thus, enzymes involved with cellular energy conversions would be required. Denver *et al.* (1997) proposed that the increased expression of cytochrome C oxidase subunit I in tadpoles might be correlated with changes in brain oxidative phosphorylation. From the data in this study, it appears as though UV-B exposure during early development results in an increased energy demand as the leopard frog brain is undergoing extensive restructuring prior to metamorphosis.

The literature surrounding the above molecules relates predominantly to functional proteins, the downstream result of mRNA expression. It is extremely important to recognize that changes at the mRNA level which were determined here using reverse Northern dot blots may not accurately predict changes in the functional protein. Thus, further analyses of expression of each gene at the protein level would be ideal. The functional analyses of protein expression would help determine the precise roles of these molecules in the context of amphibian neural development, metamorphosis and overall physiology. The differential display and reverse Northern strategies provided a means to begin assessing the impacts of low levels of contaminants at the subtle level of gene expression. It is important to develop extensive gene expression programs specific to contaminant exposure to be used on wild amphibian populations and address the question “What implications does a 3-4 fold increase or decrease in one or more structural and functional brain genes have in the context of the whole animal?” This will aid in determining the overall impact contaminants may have in the context of survival and development of amphibians and other species.

In conclusion, we have developed the first gene expression screen for *R. pipiens* with respect to two relevant environmental stressors. It is apparent that differential display provides us with a rapid screening technique to isolate candidate genes from a highly uncharacterized species and that the reverse Northern is a sensitive method which can identify subtle shifts in gene expression. The *R. pipiens*-specific multiple-gene dot blot represents a step towards pollutant-sensitive cDNA arrays which could be used as a diagnostic tool for amphibian populations in the field. Addressing the impacts of exposure at the level of gene expression in the brain enables us to quantify subtle changes that cannot be

observed in a developmental response study. Differential gene expression occurs in all phases of life and if we can identify these genes, our understanding concerning the molecular mechanisms involved with a particular biological system will improve substantially. Postembryonic development of amphibians involves intensive gene switching and structural and functional remodelling (Tata 1996). Connecting the post-exposure developmental effects on premetamorphic characteristics with particular genes represents an important step for the future. We show here that low levels of both OP and UV-B, alone and in combination, have the capability of altering gene expression and development.

Chapter 4

4.0 AREAS OF FUTURE RESEARCH INTERESTS

This work illustrates for the first time that *R. pipiens* tadpoles are vulnerable to environmentally relevant levels of OP and UV-B, alone and in combination, with respect to survivorship, premetamorphic development, and gene expression. As with most studies, the findings here provoke additional questions.

An important aspect of synergistic studies with UV-B is to characterize potential photointermediates produced as a result of the interaction with a chemical; OP, in this case. The photolysis of OP in natural waters has been characterized (Ahel *et al.* 1994) but intermediates have not been. The preceding experiments were conducted in a laboratory setting where the photolysis rate is likely different than in natural waters as a result of varying water chemistry parameters (most importantly dissolved organic carbon). Initially a time course experiment should be conducted to determine the rate constant for photochemical degradation of OP. An HPLC analysis technique for OP has been well established by Chris Metcalfe's lab at Trent University so quantifying water levels could be done easily. HPLC may also aid in the identification of possible photointermediates.

As OP is commonly associated with sediments and this is where *R. pipiens* tadpoles forage for food and escape predation, a study which focuses on bioavailability of OP from the sediments would be beneficial. An exposure experiment could be conducted in aquaria with treated sediments and the concentration of OP in the interstitial water layer and the standing water could be measured to determine the potential release from sediments. The bioaccumulation of OP in tadpole tissues could also be characterized and frog-specific

bioconcentration factors (BCFs) could be calculated. BCFs in the lab and bioaccumulation factors in the field (BAFs) have been calculated for NP in a number of algae, plants, invertebrates and fish species (Servos 1999). BCFs and BAFs in these biota ranged from 0.9-3400 which is considered low to moderate (Servos 1999). Very few data are available for OP but the data suggests that OP would have BCF/BAFs slightly higher than NP. Another reason the bioavailability question is so intriguing is that, the reported 30 day LC50 for *R. catesbeiana* tadpoles in sediments of 1 mM (260 mg/kg dry weight) (Servos 1999) is greater than the highest dosed water concentration used in this OP toxicology study (10 μ M; 2060 μ g/L) which resulted in 100% mortality.

The ten-day exposure of newly-hatched tadpoles should be extended to include a long term, continuous exposure to the sub-lethal levels of OP and UV-B that were determined in this study. As the classical toxicology has already been done, the study could focus solely on tadpole development with a primary interest being the alteration of premetamorphic parameters. In addition, continuous exposure to UV-B, especially during hindlimb emergence, might result in the kinds of hindlimb deformities observed by Ankley *et al.* (1998a). Further experiments are necessary in order to quantify plasma T₃ levels in developing tadpoles and relate these levels back to the observed differences in premetamorphic parameters observed in the combination groups post-exposure.

Although the mechanism is at present unclear, the gene expression pattern of BAI2 is similar to that of GAD67 in metamorph hypothalami (Figure 3.5A,B) suggesting possible relationships between angiogenesis and GABA neurons during metamorphic development of the hypothalamus. The inclusion of an immersion study looking at the effect of GABA

agonists and antagonists on tadpole metamorphosis would help us understand the role of GABA in metamorphosis as we now have some evidence that there is likely a connection between GABA synthesis (apparent through the regulation of GAD67 in metamorph hypothalami) and metamorphosis. Including the high OP (1 μ M) and UV-B (7 μ W/cm²) combination group in the gene expression study would allow us to further understand the relationship between GAD67, BAI2, and the differences observed in premetamorphic parameters. We observed a quantitative difference in weight and HLE (Figure 3.1A,B) in this treatment group but did not quantify the gene expression of GAD67 and BAI2. If it followed the observed gene expression trend in the low OP (1 nM) and UV-B (7 μ W/cm²) combination group, we would have more confidence associating the subtle changes in gene expression with the gross morphological changes.

Now that we have established a working method for verifying mRNA expression via the reverse Northern strategy, additional molecules should be included on the multiple-gene dot blots. UV-B exposure causes the formation of cyclobutane pyrimidine dimers in cellular DNA and this effects the gene expression of DNA photolyase (photoreactivating enzyme) (Kim and Sancar 1993). The primers are available for this enzyme so a *R. pipiens* clone would be ideal. The cloning of *R. pipiens* CRH and subsequent inclusion on the dot blot would allow further insight into this physiological mediator of metamorphosis under varying conditions of stress. The overall idea is to develop extensive gene expression programs specific to contaminant exposure to be used on wild amphibian populations as a diagnostic tool. This move, from the laboratory to the field, represents one of the most important aspects of amphibian toxicology in the broad context of population decline.

As a follow up to the dot blot work, further analyses of expression of each gene at the protein level would be ideal. mRNA expression does not necessarily manifest itself as protein expression. Functional analyses of the particular proteins would help determine the precise roles of these molecules in the context of amphibian neural development, metamorphosis and response to environmental toxicants.

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APPENDIX

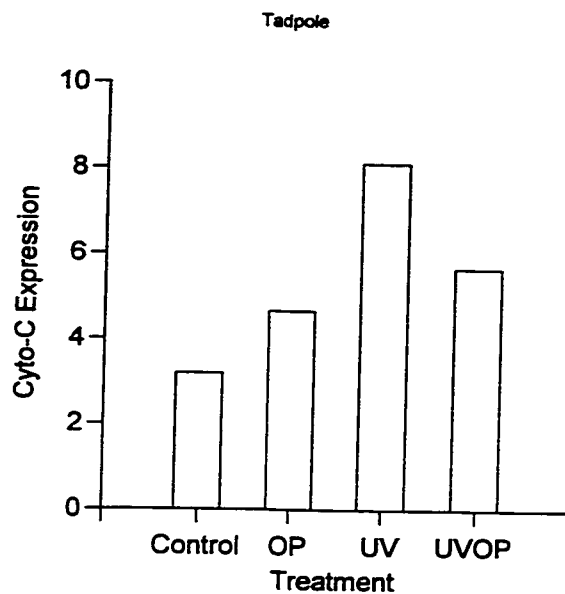


Figure 1. Cytochrome C oxidase chain I mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

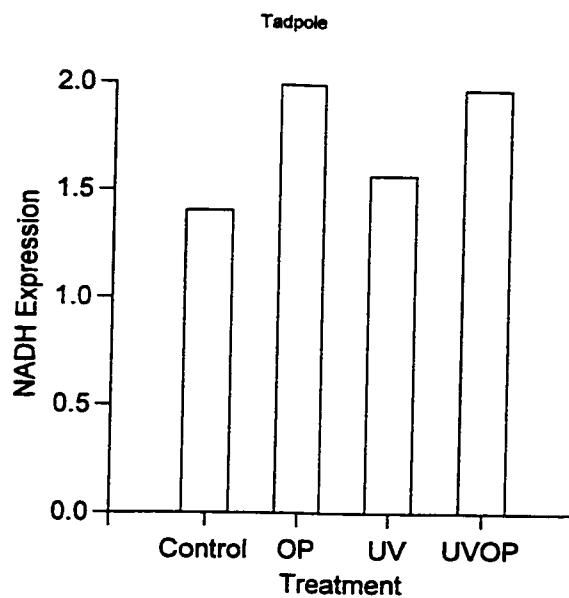


Figure 2. NADH dehydrogenase subunit 4 mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

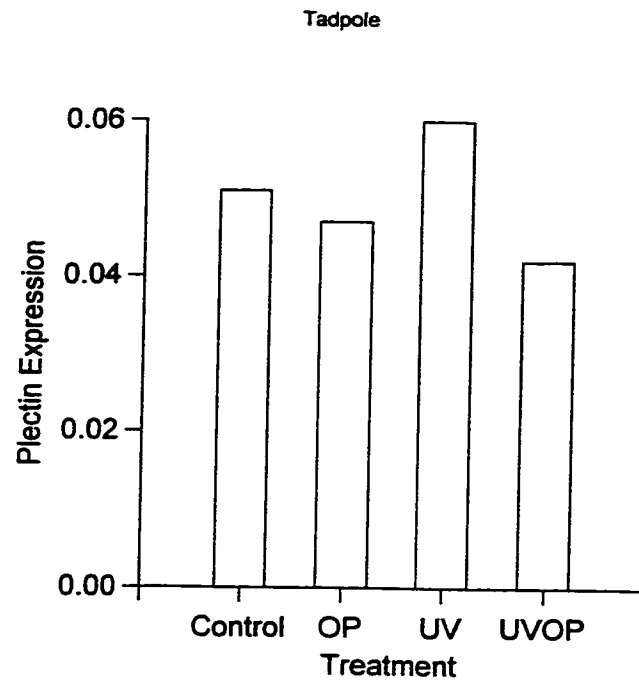


Figure 3. Plectin mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

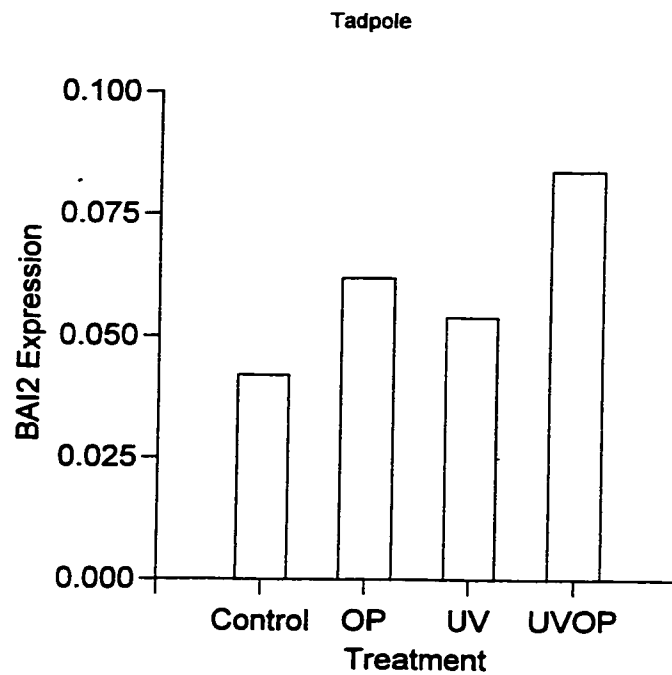


Figure 4. Brain-specific angiogenesis inhibitor 2 (BAI2) mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

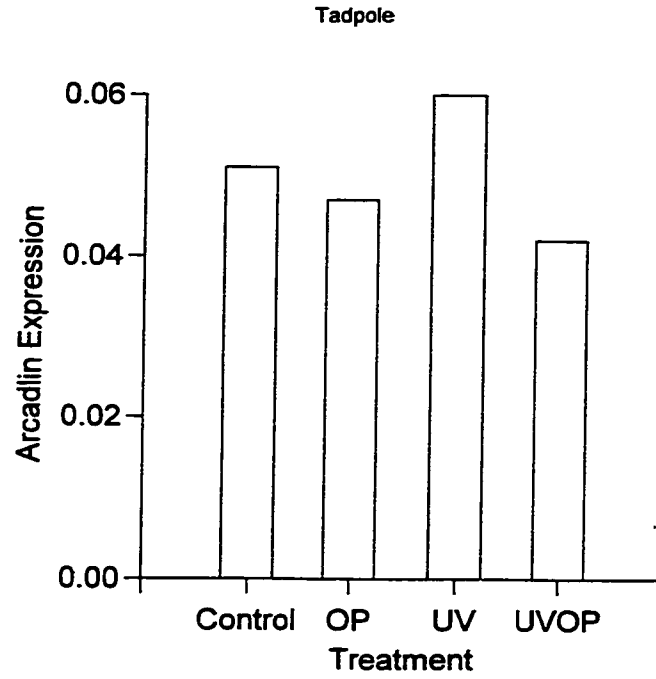


Figure 5. Arcadlin mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

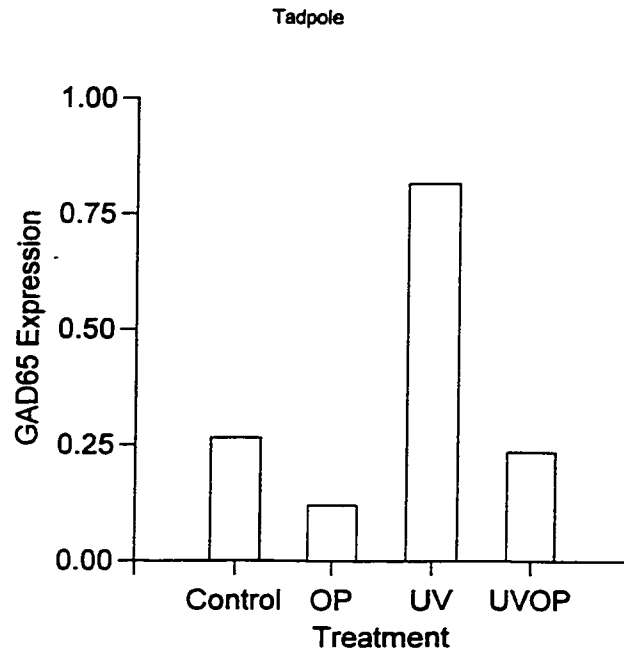


Figure 6. Glutamate decarboxylase 65 (GAD65) mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

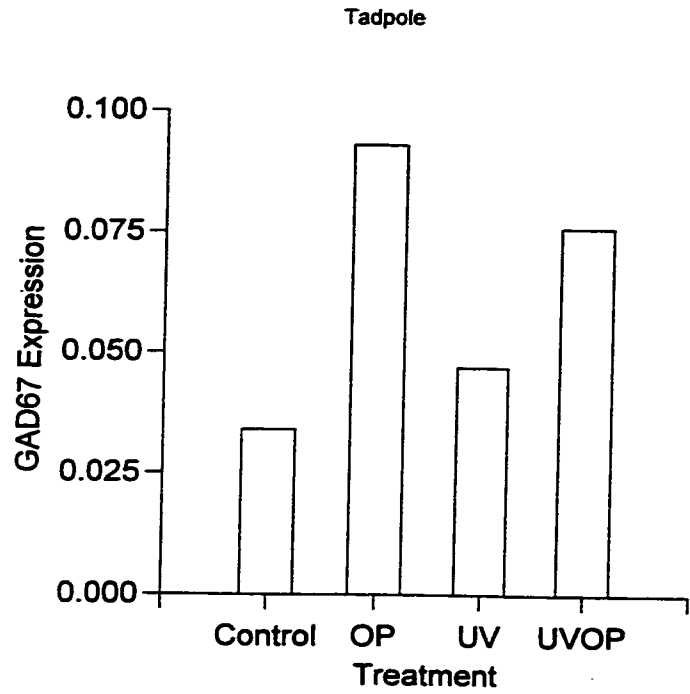


Figure 7. Glutamate decarboxylase 67 (GAD67) mRNA expression in the tadpole diencephalon following treatment. Data normalized to β -actin.

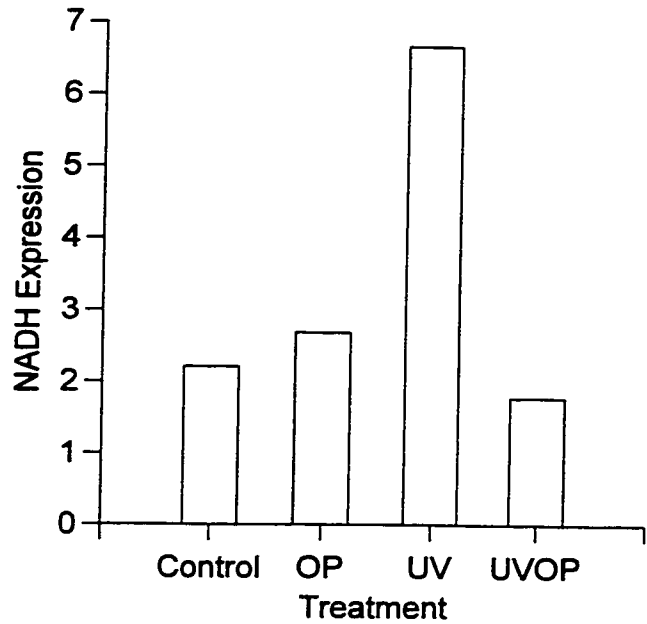


Figure 8. NADH dehydrogenase subunit 4 mRNA expression in the metamorph hypothalamus following treatment. Data normalized to β -actin.