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Chronic exposure to UVB radiation and 4-tert-octylphenol disrupts metamorphosis and the thyroid
system of Northern leopard frog (*Rana pipiens*) tadpoles

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**Chronic exposure to UVB radiation and 4-tert-octylphenol disrupts metamorphosis
and the thyroid system of Northern leopard frog (*Rana pipiens*) tadpoles**

Maxine Carmen Croteau

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ABSTRACT

Ultraviolet B radiation (UVBR) and chemical pollutants are environmental stressors that may affect amphibian populations. While adverse effects of UVBR and chemicals on amphibians are documented, few studies examined the effects of interactions between stressors on amphibian health. Gosner stage 25 *Rana pipiens* tadpoles were chronically exposed to environmentally relevant levels of UVBR (0.15-0.22 W/m²) and the estrogenic chemical 4-*tert*-octylphenol (OP) (0.01 nM or 10 nM) alone and in combination. Tadpoles exposed to UVBR and/or OP exhibited significantly delayed development at stage 29. Significantly fewer UVBR-treated tadpoles developed past stage 34 and metamorphosed. Effects of UVBR/OP on the thyroid system were assessed because it is the main regulator of amphibian metamorphosis. Stage 29 and 34 tadpoles were collected for gene expression analysis in tail and T3 (triiodothyronine) whole body levels (minus tail). Moreover, we examined whether pre-exposure to UVBR/OP affected the molecular and morphological responses of tadpoles to exogenous T3. OP increased the expression of several genes in tail and brain and/or their response to T3 (*e.g.*, deiodinase 2), and the addition of UVBR can alter the effect of OP. Although UVBR had no observable effect on gene expression, developmentally delayed UVBR-treated stage 31 tadpoles exposed to T3 exhibited slowed tail resorption and accelerated hind limb development. UVBR and/or OP did not affect T3 levels of stage 29 and 34 tadpoles. However, a decrease and increase in deiodinase 2 and 3 (D2 and D3) mRNA levels, respectively, were observed in groups of tadpoles with slowed developmental rates at those stages. Since D2 activates and D3 inactivates thyroid hormones (TH), UVBR/OP mediated disruptions in development are likely caused by dysfunctions in the localized

metabolism of THs. These results indicate that environmental levels of UVBR and/or OP can affect metamorphosis, potentially by disrupting the biological action of T3 and deiodinases in peripheral tissues, which could contribute to population declines.

RÉSUMÉ

Les rayons ultraviolets B (UVBR) et les produits chimiques polluants sont des facteurs de stress environnementaux qui affectent possiblement les populations d'amphibiens. Bien que les effets néfastes de l'exposition aux UVBR et produits chimiques sur les amphibiens soient bien documentés, peu d'études ont examiné les effets des interactions entre des facteurs environnementaux sur la santé d'amphibiens. Des têtards *Rana pipiens* au stade de développement Gosner 25 ont été exposés de manière chronique à des niveaux de UVBR (0.15-0.22 W/m²) et de polluant ostrogénique 4-*tert*-octylphénol (OP) (0.01 nm ou 10 nm) représentatifs aux niveaux retrouvés dans l'environnement, seul ou en combinaison. Le développement des têtards exposés aux UVBR et/ou OP est devenu retardé au stade 29. Beaucoup moins de têtards traités aux UVBR ont développé passé le stade 34 et ont métamorphosé. Nous avons évalué les effets de UVBR/OP sur le système thyroïdien, car il est le principal régulateur de la métamorphose chez les amphibiens. Des têtards aux stades de développement 29 et 34 ont été recueillis pour l'analyse de l'expression des gènes dans la queue et des niveaux de T3 (triiodothyronine) dans le corps (moins la queue). En outre, nous avons examiné si une pré-exposition à UVBR/OP a affecté les réponses moléculaires et morphologiques des têtards au T3 exogène. OP a causé une augmentation de l'expression de plusieurs gènes dans le cerveau et la queue et/ou leur réponse au T3 (par exemple, le gène de D2), et l'ajout de UVBR peut modifier l'effet de l'OP. Bien que les animaux exposés aux UVBR n'ont pas subi de changements d'expression génique, leur développement était retardé. La résorption de la queue de ces têtards était ralentie et le développement de leurs pattes et orteils était d'accélééré lorsque exposés au T3 exogène. L'exposition des têtards au stade de développement 29 et 34 aux

UVBR et/ou OP n'a pas eu d'effet sur leurs niveaux de T3. Toutefois, la diminution et l'augmentation des niveaux d'ARNm de D2 et D3, respectivement, se sont produites dans les groupes de têtards avec des taux de développement ralenti à ces stades de développement. Étant donné que D2 active et D3 inactive les hormones thyroïdiennes (TH), les perturbations dans le développement des têtards exposés UVBR/OP sont probablement causées par des dysfonctionnements localisés dans le métabolisme des TH. Ces résultats indiquent alors que UVBR et/ou OP peuvent influencer le taux du développement de têtards ainsi que leur métamorphose, potentiellement en perturbant l'action biologique de T3 et des déiodinases dans les tissus périphériques. Cette recherche démontre que les niveaux d'UVBR et/ou OP semblables à ceux dans l'environnement peuvent affecter le développement des têtards et peuvent potentiellement contribuer à la diminution de populations d'amphibiens.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APEO	alkylphenol polyethoxylate
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
CRH	corticotropin-releasing hormone
D2	deiodinase type II
D3	deiodinase type III
DOC	dissolved organic carbon
E2	17 β -estradiol
EDC	endocrine disrupting chemical
EtOH	ethanol
FLE	forelimb emergence
HPT axis	hypothalamo-pituitary-thyroid axis
mRNA	messenger ribonucleic acid
NP	nonylphenol
OP	octylphenol
PRL	prolactin
PRLR	prolactin receptor
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
SEM	standard error of the mean
SVL	snout vent length
TH	thyroid hormone
T3	triiodothyronine
T4	thyroxine
TR	thyroid receptor
TR alpha	thyroid receptor alpha
TR beta	thyroid receptor beta
TRE	thyroid response element
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
TTR	transthyretin
UVAR	ultraviolet A radiation (320-400 nm)
UVBR	ultraviolet B radiation (280-320 nm)
UVCR	ultraviolet C radiation (200-280 nm)
VIS	visible light (400-750 nm)

CHAPTER 1

General Introduction*

1.1 Thesis Rationale

Amphibian populations have been declining worldwide over the past 40 years. In light of anthropogenic contributions to global environmental disturbances, it is important to investigate how our actions affect the health and survival of amphibians. This is especially essential when one considers the importance of amphibians in the food chain of various ecosystem communities and as biological indicators of environmental health. Since they inhabit both aquatic and terrestrial habitats and have unshelled eggs and permeable skin, they are vulnerable to changes in environmental conditions and habitat quality. Many studies have considered endocrine disrupting chemicals (EDCs) polluting the environment and recent increases in UVB radiation (UVBR) as possible contributing factors to the reduction in amphibian populations. However, amphibians in the wild are exposed to multiple environmental stressors simultaneously. While adverse effects of exposure to UVBR and EDCs have been documented, few studies have assessed the impact(s) of interactions between UVBR and EDCs on amphibian development and health.

Preliminary results in our lab have shown that chronic exposure of Northern leopard frog (*Rana pipiens*) tadpoles to UVBR, combined with or without the estrogenic pollutant 4-*tert*-octylphenol (OP), inhibited the metamorphosis of animals compared to

*Adapted from: Croteau M.C., M.A. Davidson, D.R.S. Lean and V.L. Trudeau. 2008a. Global increases in UVB radiation: potential impacts on amphibian development and metamorphosis. *Physiological and Biochemical Zoology*. 81(6):743-761

those in the control group or exposed to OP only (Croteau *et al.*, 2001). This result is significant because the supplemental UVBR given was equivalent to present day, environmentally relevant levels as measured midday underwater in a typical pond in the Ottawa region on a sunny day. In addition, *R. pipiens* is a species native to North America that has suffered from enigmatic population declines in a number of regions. During this study (Croteau *et al.*, 2001) we did not however determine the molecular mechanism(s) behind the metamorphic disruption, which is required to fully understand the nature and significance of the effects of environmental changes on amphibian metamorphosis. For my Ph.D. research, I combined the use of morphological, biochemical, molecular biological and histological techniques to study the mechanisms behind the UVBR and/or OP mediated disruption of metamorphosis, since this had not yet been investigated. In particular, the aim of this thesis was to assess the thyroidal status of exposed animals, since metamorphosis in amphibians is controlled by the thyroid system.

The following hypotheses were tested in this study.

Hypotheses

1. UVBR and OP exposure affects the rate of tadpole development and number of tadpoles that metamorphose.
2. Exposure of tadpoles to UVBR and OP together affects the rate of development and number of tadpoles that metamorphose more than exposure to either factor alone.
3. UVBR and OP exposure affects the thyroid system of exposed tadpoles.
4. Exposure of tadpoles to UVBR and OP together modifies the effect that either factor alone has on the thyroid system.

To test these hypotheses, I examined the effects of UVBR and/or OP on somatic growth, development and metamorphosis in relation to effects on thyroid gland histology, thyroid hormone levels (T3) and expression levels of key genes regulating metamorphosis.

In the following sections, global increases in UVBR and endocrine disrupting chemicals (EDCs) with regard to amphibian exposure are discussed. Specifically, studies on the effects of UVBR and estrogenic EDCs on amphibian development and metamorphosis are reviewed, and the endocrine control of metamorphosis is discussed to establish the context under which these studies were conducted.

1.2 Global Amphibian Population Declines

Amphibian populations have been declining on a global scale since the 1960s (Houlahan *et al.*, 2000) and are more threatened and are declining faster than populations of birds or mammals (Stuart *et al.*, 2004). For example, 32.5% of all amphibian species are threatened worldwide (*i.e.*, listed in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List Categories of Vulnerable, Endangered or Critically Endangered) and 7.4% are listed as Critically Endangered (IUCN category of highest threat), as compared to 12% and 1.8% of bird species and 23% and 3.8% of mammalian species, respectively. These percentages are likely underestimates since there is an inadequate amount of information to determine the status of approximately one quarter of all amphibian species (Stuart *et al.*, 2004).

Since the 1990s, several comprehensive reviews on amphibian population declines have been published (*e.g.*, Stebbins and Cohen, 1995; Alford and Richards,

1999; Houlahan *et al.*, 2000; Corn, 2000; Blaustein and Kiesecker, 2002; Collins and Storfer, 2003; Blaustein *et al.*, 2003; Blaustein *et al.*, 2004; Lannoo, 2005) and there has been a dramatic increase in research on the effects and mechanisms behind the different anthropogenic factors that may be negatively affecting individuals in the wild. However, the causes of population declines in several regions of the world remain to be elucidated. The main stressors that have been postulated as possible contributors of reductions in amphibian populations include: (1) introduced/invasive species; (2) over-exploitation; (3) habitat modification/loss; (4) global environmental change (including increasing UVB radiation and global climate change); (5) environmental contamination; (6) disease; and (7) interactions among various factors (see reviews listed above). Since it is likely that a number of stressors are interacting in the environment to affect amphibian populations, it has been difficult for researchers to establish what specifically is causing the observed declines. Stuart *et al.* (2004) recently stated that habitat loss and over-exploitation have caused the loss of many of the 435 “rapidly declining” amphibian species identified using the IUCN red list; however, 48% of these are “enigmatic decline species”, which are declining quickly where suitable habitat remains, due to unknown causes.

Over the past three decades, there have been concerns regarding the biological consequences of enhanced UVBR levels reaching the Earth’s surface and contaminants polluting the environment. Although it remains controversial, many scientists believe that increased exposure to these environmental stressors may have contributed to the decline of several populations worldwide; however, it is very difficult to confirm that wild populations are affected since it is impossible to assess the harm caused to populations that have already declined or that no longer exist. A variety of agents may be acting alone

or in combination to cause current amphibian losses, therefore future research programs must consider the potential of combined effects between multiple factors polluting the aquatic environment on the health of amphibians.

1.3 Endocrine Disrupting Chemicals (EDCs)

There are approximately 100 000 chemical compounds in commercial use today, with about 25 000 to 30 000 new substances synthesized annually. Surprisingly, only 10% of all the compounds currently in use were subject to detailed toxicity and ecotoxicological tests and tests for carcinogenicity and mutagenicity (Ostroumov, 2005). An increasing number of scientists worldwide are concerned about the health of wildlife and humans since several of the synthetic chemicals currently polluting the aquatic environment possess the ability to interfere with reproduction and development by disrupting the endocrine system (*e.g.*, Danzo, 1997; Nishimura *et al.*, 1997; Danzo, 1998; Maruyama *et al.*, 1999; Pickford and Morris, 1999).

The World Health Organization has defined an endocrine disruptor as: “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (Damstra *et al.*, 2002). Endocrine disrupting chemicals (EDCs) are exogenous agents that may mimic or block the actions of natural hormones by: (1) competing with endogenous hormones at receptors sites, either acting as agonists or antagonists; (2) inducing changes in the level of circulating hormone by altering steroid synthesis, metabolism or binding to plasma proteins; (3) disrupting the binding of endogenous hormones to hormone receptors; or (4) altering receptor populations and

affinity (Campbell *et al.*, 1994; Crews *et al.*, 2000; McLachlan, 2001). A wide variety of EDCs and their metabolites are commonly detected in aquatic environments (Kolpin *et al.*, 2002), with many originating from urban sources such as sewage treatment plants (STP) (Metcalf *et al.*, 2003; Kolpin *et al.*, 2004). Numerous field and laboratory studies have demonstrated that exposure to EDCs produces adverse effects in some wildlife species and populations, with aquatic species at the top of the food chain most affected. These effects range from subtle physiological and behavioural changes to permanently altered sexual differentiation (Damstra *et al.*, 2002).

1.4 Effects of Estrogenic Chemicals on Amphibian Development

EDCs that elicit estrogenic responses by mimicking endogenous estrogen are referred to as environmental estrogens. This is currently the largest group of known and studied EDCs (Arcand Hoy *et al.*, 1998; Welshons *et al.*, 2003). Examples of synthetic estrogenic chemicals include pesticides such as methoxychlor and lindane, polychlorinated biphenyls (PCBs), dioxins, plastic ingredients including bisphenol A, nonylphenol (Maruyama *et al.*, 1999) and octylphenol (Arnold and McLachlan, 1996; Huang *et al.*, 2005), pharmaceuticals such as ethinylestradiol (Denslow *et al.* 2001; Folmar *et al.* 2002; Andersen *et al.* 2003; Thorpe *et al.* 2003; Nash *et al.* 2004) as well as certain surfactants (Tyler *et al.*, 1996). Although many estrogen mimics discovered to date are only weakly estrogenic (Jobling *et al.*, 1998), their effects on humans and wildlife must be fully assessed since many of these compounds are lipophilic and can bioaccumulate in organisms (Tyler *et al.*, 1996).

Exposure to estrogenic chemicals has been shown to induce demasculinization and feminization in fish (*e.g.*, Papoulias *et al.*, 1999; van Aerle *et al.*, 2002; Weber *et al.*, 2003; Andersen *et al.*, 2003), amphibians (*e.g.*, Kloas *et al.*, 1999; Hayes *et al.*, 2002; Hayes *et al.*, 2003; Mackenzie *et al.*, 2003), reptiles (*e.g.*, Guillette *et al.*, 1994), birds and mammals (*e.g.*, Crain and Guillette, 1997). Exposure to environmental estrogens is also associated with decreased fertility, precocious puberty, disruption of sexual differentiation, teratogenicity and carcinogenicity in a wide variety of species ranging from invertebrates to mammals, including humans (Cooper and Kavlock, 1997, Lai *et al.*, 2002). As such, most studies involving the effects of EDCs on amphibians have consisted of experiments focussed on examining reproductive endpoints. However, there is increasing data indicating that estrogenic chemicals can have detrimental effects on amphibian development and metamorphosis.

Amphibians are considered excellent test organisms to assess the impacts of EDCs on the endocrine system. Their morphological change from water breathing larvae to air breathing frogs makes them particularly sensitive to the action of EDCs, since metamorphosis is a highly coordinated process that is controlled by complex hormonal pathways. In particular, thyroid hormones (THs) are required for amphibian metamorphosis to occur. Brucker-Davis (1998) summarized the effects of a multitude of synthetic chemicals on thyroid function in vertebrates, which mostly consisted of pesticides and industrial chemicals. Some of the synthetic chemicals identified as having thyroid disruption properties in this review were also identified as estrogenic chemicals in other studies (*e.g.*, DDT, dieldrin, toxaphene etc.).

It has been demonstrated that the endogenous hormone 17 β -estradiol (E2) has the potential to affect the rate and timing of amphibian metamorphosis. Most published studies indicate that E2 inhibits tadpole development (Richards and Nace, 1978; Gray and Janssens, 1990; Hayes, 1997a). For example, E2 exposure inhibited tail resorption in *Xenopus laevis* (African clawed frog) tadpoles, a process that is normally mediated by TH (Hayes, 1997a). Exposure of *X. laevis* tadpoles to E2 antagonized the metamorphic effects of exogenous T3 (Gray and Janssens, 1990). Vandorpe and Kuhn (1989) demonstrated that E2 implants in adult *Rana ridibunda* (Eurasian marsh frog) frogs caused a decrease in plasma T4 and T3 levels and a reduction in deiodinase type 2 activity (*i.e.*, enzyme that converts THs to their active form) in the kidneys. Hayes (1997a) suggested that E2 potentially regulates TH activity by affecting the hypothalamo-pituitary-thyroid axis. It was postulated that the most likely mechanisms of E2 inhibition of metamorphosis are through: (1) a decrease in TH levels; (2) an increase in prolactin levels (*i.e.*, a pituitary hormone that can inhibit metamorphosis); and (3) interactions with TH in peripheral tissues (Hayes, 1997). Numerous studies have demonstrated that estrogenic EDCs can also affect development and metamorphosis in various amphibian species (*e.g.*, Cheek *et al.*, 1999; Iwamuro *et al.*, 2003; Christensen *et al.*, 2005; Fort *et al.*, 2004a and 2004b; Hogan *et al.*, 2008a). However, the mechanism(s) of action behind the effects of estrogenic chemicals on amphibian metamorphosis have not been completely elucidated, partly because the influence of E2 on the metamorphic process is not entirely understood.

1.5 Alkylphenol Polyethoxylates and Octylphenol

The discharge of synthetic surfactants into the environment is rapidly increasing worldwide, with an annual increase of ~2 to 5% (Ostroumov, 2006). Alkylphenol polyethoxylates (APEOs) have been used since the 1940s and are the second largest group of non-ionic surfactants in commercial production (White *et al.*, 1994). These compounds are used as detergents, dispersants, wetting agents, emulsifiers, solubilizers and foaming agents, and are important to a variety of industrial applications including pulp and paper, textiles, coatings, agricultural pesticides, lube oils and fuels, metals and plastics (Ying *et al.*, 2002). After they are used, these substances are mainly discharged into municipal and industrial wastewaters that enter STPs before they are released into the aquatic environment. A complex biodegradation process of APEOs takes place during the different steps of sewage treatment, forming several metabolites (Thiele *et al.*, 1997). These metabolites are less biodegradable, more hydrophobic, more persistent, more lipophilic, and more toxic than the parent compound and exhibit estrogenic properties (Bennie, 1999).

Two of the most significant commercial APEO surfactants are Octylphenol Polyethoxylates (OPEOs) and Nonylphenol Polyethoxylates (NPEOs) (Bennie *et al.*, 1998). NPEOs account for approximately 80% of the total APEO market, whereas OPEOs make up most of the remaining 20% (White *et al.*, 1994; Naylor, 1998). Analysis of natural waters, industrial effluents, sediments, municipal wastewater effluents, soil and sludge has demonstrated that 4-tert-octylphenol (OP) is the dominant metabolite of OPEOs present in the Canadian environment (Donald Bennie, pers. comm.; Figure 1.1). Although studies have shown that OP is more estrogenic and lipophilic than the related

metabolite nonylphenol (NP) (reviewed in Thiele *et al.*, 1997; Servos *et al.*, 2001), OP is often not considered in environmental assessments because OPEOs are not used as frequently. Sludge concentrations were measured and ranged from 1.5 to 297 $\mu\text{g/g}$ d.w. OP and final effluent samples contained concentrations ranging from 0.005 to 0.37 $\mu\text{g/L}$ in 16 samples collected from STPs across Canada (Bennie *et al.*, 1998). Sediment samples collected from 28 sites in industrialized and pristine locations of the lower Great Lakes in Canada were analyzed for concentrations of OP. Results showed mean concentrations ranging from <0.001 to 23.7 $\mu\text{g/g}$ dry weight (Bennett and Metcalfe, 1998). Bennie *et al.* (1997) collected sediment and water samples from sites in the Laurentian Great Lakes and the upper St-Lawrence river in Canada and analysis showed mean concentrations ranging from <0.01 to 1.8 $\mu\text{g/g}$ d.w. and <0.005 to 0.084 $\mu\text{g/L}$ OP, respectively.

APEO metabolites such as OP are widespread in the aquatic environment because of its broad use. OP is one of numerous APEO metabolites that exhibits estrogenic properties (Bennie, 1999) and has been shown to cause significant endocrine disrupting effects in a variety of organisms (OSPAR commission, 2003) including amphibians (*e.g.*, Lutz and Kloas, 1999; Kloas *et al.*, 1999; Crump *et al.*, 2002; Mayer *et al.*, 2003; Suzuki *et al.*, 2004). However, the majority of studies on the sublethal effects of OP on amphibians have been related to reproduction. For these reasons, OP is a good model compound to assess the mechanisms behind disruptions in amphibian development by similar estrogenic compounds alone and combined with a large-scale environmental stressor such as UVBR.

1.6 Stratospheric Ozone Depletion

Stratospheric ozone (O₃) shields the planet from damaging UV radiation (UVR) emitted from the sun. UVR is subdivided into three spectral regions: ultraviolet-C radiation (UVCR; 200-280 nm), ultraviolet B radiation (UVBR; 280-320 nm) and ultraviolet-A radiation (UVAR; 320-400 nm). UVCR is not environmentally relevant at the Earth's surface because it is completely absorbed in the atmosphere by oxygen (mostly) and ozone, whereas most UVAR passes through the ozone layer and reaches the Earth's surface (Perin and Lean, 2004). However, since UVBR is only partially absorbed by ozone, changes in the concentration of stratospheric ozone affect the amount of UVBR that reaches the surface of the planet. UVBR is the portion of the spectrum that is of most biological concern because higher wavelengths (*i.e.*, UVAR) are less efficiently absorbed by certain biomolecules such as DNA and RNA (Vincent and Neale, 2000).

The anthropogenic destruction of stratospheric ozone has been caused by the emission of persistent industrial chemicals such as chlorofluorocarbons (CFCs) and other gases containing chlorine and bromine. The first quantitative evidence for a downward trend in stratospheric ozone was reported in 1985. Results demonstrated that springtime ozone levels between 1975 and 1984 had dropped by approximately 40% over Antarctica (Farman *et al.*, 1985), forming a massive hole in the ozone layer. The Antarctic ozone hole has been occurring every spring since the mid-1970s, and since 1985 is generally growing larger, forming earlier, lasting longer and showing greater depletion, with over 60% depletion when compared to pre-1980 values (Jones and Shanklin, 1995).

It is believed that climate change has played an important role in recent ozone depletion (Perin and Lean, 2004; McKenzie *et al.*, 2007; Aucamp, 2007). Strong stratospheric winds and temperatures below -78°C create a “polar vortex” that allows the formation of polar stratospheric clouds (PSCs) in the ozone layer. PSCs enhance the destruction of ozone by providing a reaction site to chemically convert halogen source gases such as CFCs into reactive free radicals. Since minimum winter temperatures in the Arctic stratosphere are approximately 10°C warmer than in that of the Antarctic, the Arctic polar vortex is not as strong and PSCs do not always form. However, ozone depletion over sub-Arctic and Arctic regions has recently become comparable to the loss of ozone over Antarctica. Greenhouse gases trap heat in the troposphere and radiate energy and heat away from the stratosphere. Therefore, the temperature at the Earth’s surface is expected to continue increasing due to climate change, whereas the temperature of the stratosphere is expected to cool. A small cooling of the lower stratosphere has been taking place since the 1970s and several Arctic winters in the 1990s were characterized by record low stratospheric temperatures and a stronger polar vortex. As the atmosphere naturally produces the same amount of new stratospheric ozone each year (although the total amount of ozone destroyed is higher), the ozone-depleted air from Polar Regions mixes with the ozone-rich air from outside the poles, leaving the entire (average) ozone supply of the Earth slightly more diminished every year. Stratospheric ozone depletion also takes place directly at latitudes between the equator and Polar Regions, but to a lesser degree (Perin and Lean, 2004 and references therein).

Several studies have demonstrated that UVBR levels have increased in regions where stratospheric ozone has decreased (*e.g.*, Blumthaler and Ambach, 1990; Kerr and

McElroy, 1993; Herman *et al.*, 1996; McKenzie *et al.*, 1999). For example, Kerr and McElroy (1993) measured daily ozone levels and the intensity of UVBR near 300 nm from 1989 to 1993 in Toronto, Canada and found evidence that the observed increase in UVBR (35% and 7% per year for winter and summer, respectively) coincided with the decreasing ozone levels measured within this same time period. It is estimated that for each 1% decrease in stratospheric ozone, there is a 1 to 2% increase in UVBR transmitted to the Earth (Kerr and McElroy, 1993; Lubin and Jensen, 1995; Hanelt *et al.*, 2001). Therefore, even slight decreases in stratospheric ozone can have considerable impacts on living organisms and ecosystems through increased exposure to UVBR.

1.7 Underwater UVR Levels

UVBR levels measured in the air are not representative of levels present within aquatic habitats because the depth of penetration of solar radiation in freshwater systems is dependent on the concentration of dissolved organic carbon (DOC) (see Scully and Lean, 1994; Lean, 1998a and references therein). DOC is the organic residue of decaying organic matter and contains chemical structures or chromophores that absorb light and UVR (Xenopoulos and Schindler, 2001). Shorter wavelengths of solar irradiance are attenuated more rapidly by DOC than are longer wavelengths (Lean, 1998a). Consequently, UVBR is highly attenuated in aquatic environments with high concentrations of DOC. For example, measurements taken under midday sun in June at a pond with high DOC content (13 mg C/L) in Ontario indicated a UVBR intensity of 2.22 W/m² above the water surface and 0.046 W/m² at a depth of 5 cm inside the water

column (Crump *et al.*, 1999a). On the other hand, UVBR intensities can reach depths of several meters in clear (low DOC) freshwater ecosystems (Lean, 1998b).

Interactions among global atmospheric stressors such as stratospheric ozone depletion, climate change, and acidification can modify DOC levels in aquatic environments, thereby increasing underwater UVBR levels (*e.g.*, Schindler *et al.*, 1996; Yan *et al.*, 1996; Lean, 1998a and 1998b) and increasing the risks associated with UVBR exposure. Increasing levels of UVBR penetrating the water column as a result of ozone depletion can lead to the enhanced photobleaching of light absorbing chromophores of DOC, making them less efficient in absorbing UVBR. Changes in global precipitation patterns are expected due to climate change, and as a consequence some regions will receive significantly more or less precipitation (Caldwell *et al.*, 2007). With less rain, water is retained in lakes for longer periods and there is increased photodegradation of DOC (Lean, 1998a and 1998b). Warmer, drier conditions may reduce the contribution of DOC from terrestrial runoff into aquatic ecosystems, also leading to greater penetration of UVBR (Lean, 1998b; Aucamp, 2007). Acidification of lakes and rivers caused by anthropogenic emissions of sulphur and nitrogen oxides lowers the pH in precipitation and in poorly buffered aquatic systems. This results in increased DOC aggregation and sedimentation thus removing DOC from the water column. Increased acidity also results in the mobilization of metals (*e.g.*, aluminum), which also leads to increased aggregation and sedimentation of DOC (Lean, 1998b). Climate warming may cause further acidification of aquatic habitats by decreasing water levels. The exposed littoral sediments that contain reduced sulphur can be reoxidized and can remobilize acid into the water, thus further diminishing DOC levels (Yan *et al.*, 1996).

Twenty years of data collected at the Experimental Lakes Area in Northwestern Ontario (Canada) demonstrated that in clear, shallow aquatic ecosystems, decreases in DOC caused by climate warming and/or acidification can be more effective in increasing the exposure of aquatic organisms to UVBR than changes to incident UVBR as a result of stratospheric ozone depletion (Schindler *et al.*, 1996). Although the amount of UVBR reaching terrestrial and aquatic ecosystems can be attenuated by several other factors such as plant canopy, surface albedo, cloud cover, atmospheric pollutants, aerosols, (Xenopoulos and Schindler, 2001) and water turbidity (Ovaska *et al.*, 1997; Aucamp, 2007), even small increases in UVBR exposure may be harmful to organisms at sensitive stages of development or to organisms that do not possess adequate defence mechanisms in preventing UVBR-induced damage.

1.8 UVBR and Amphibians

A variety of behaviours exhibited by different amphibian species can contribute to increased exposure of animals to present day UVBR levels. Since many amphibian species oviposit at or near the surface of the water, this may increase harm to eggs and embryos because of greater UVBR exposure (Murphy *et al.*, 2000). Species that breed in the spring when UVBR levels are high are especially at risk, as this coincides with early and sensitive stages of embryo and tadpole development (Kerr and McElroy, 1993). Amphibians inhabiting shallow or clear aquatic environments are especially susceptible to increased UVBR exposure because of the deep penetration of sunlight, which can sometimes reach the bottom of the water column depending on its depth and DOC levels (Morris *et al.*, 1995). Exposure to enhanced levels of UVBR as a result of (1) ozone depletion; (2) modifications of

DOC levels in aquatic systems (Schindler *et al.*, 1996); and (3) clear cutting of vegetation or habitat destruction (eliminating shelter from solar radiation), make basking in sunlight to thermoregulate a greater threat to amphibians than in the recent past because of the many detrimental effects caused by current UVBR levels (Murphy *et al.*, 2000).

Numerous laboratory and field experiments have shown that amphibian embryos and/or larvae can experience reduced survivorship when exposed to ambient or enhanced levels of UVBR (*e.g.*, Worrest and Kimeldorf, 1975; Worrest and Kimeldorf, 1976; Blaustein *et al.*, 1994 and 1997; Ovaska *et al.*, 1997; Anzalone *et al.*, 1998; Ankley *et al.*, 2000 and 2002; Belden *et al.*, 2000; Flamarique *et al.*, 2000; Häkkinen *et al.*, 2001; Tietge *et al.*, 2001; Belden and Blaustein, 2002a; Weyrauch and Grubb, 2006). Sublethal effects such as disruptions of growth (*e.g.*, Pahkala *et al.*, 2000 and 2001; Belden *et al.*, 2000; Belden and Blaustein, 2002a and 2002b), developmental rate (*e.g.*, Smith *et al.*, 2000; Pahkala *et al.*, 2002a and 2002b), metamorphosis (*e.g.*, Pahkala *et al.*, 2001 and 2003), and behaviour (*e.g.*, anti-predator behaviour, activity level) (*e.g.*, Kats *et al.*, 2000; Blaustein *et al.*, 2000) can occur as a result of UVBR exposure. Although UVBR is not likely the cause of the high incidence of gross limb malformations recently observed in amphibians in nature (Blaustein and Johnson, 2003; Ankley *et al.*, 2004), several other types of developmental malformations and abnormalities have been observed in experiments exposing embryos and tadpoles to UVBR. These malformations and abnormalities include spinal curvature (*e.g.*, Worrest and Kimeldorf, 1975; Worrest and Kimeldorf, 1976; Hays *et al.*, 1996; Blaustein *et al.*, 1997; Bruggeman *et al.*, 1998; Pahkala *et al.*, 2001; Weyrauch and Grubb, 2006), edema or bloating (*e.g.*, Hays *et al.*, 1996; Blaustein *et al.*, 1997), eye abnormalities (*e.g.*, Worrest and Kimeldorf, 1975 and 1976; Fite *et al.*, 1998; Bruggeman *et al.*, 1998; Flamarique *et al.*, 2000;

Ankley *et al.*, 2000 and 2002), and hind limb malformations (*e.g.*, Ankley *et al.*, 1998, 2000 and 2002; Pahkala *et al.*, 2001). Such developmental anomalies may cause direct mortality or may significantly disrupt the normal activities of individuals in nature, by preventing animals from effectively foraging for food or evading predators.

1.9 Effects of UVBR on the Rate of Amphibian Development and Metamorphosis

Numerous experiments conducted outdoors or in the laboratory have shown that UVBR emitted from sunlight or artificial lights can influence the rate of amphibian development and metamorphosis. Summarized in Table 1.1 are the results of 14 published studies demonstrating that amphibians subjected to UVBR can either exhibit delays, accelerations or no change in developmental and/or metamorphic rates.

Eight of the studies presented in Table 1.1 reported a delay in the development and/or metamorphosis of embryos or tadpoles exposed to UVBR for either short or long time periods (*e.g.*, from a few days to several months), in at least one test species examined. One study demonstrated that exposure to UVBR can result in inhibited or blocked development and metamorphosis (Grant and Licht, 1995). For example, *Rana clamitans* (Green frog) tadpoles chronically exposed to UVBR in the laboratory did not develop past Gosner stage 34 (early development of digits) and only reached early stages of paddle foot and digit development (Gosner stages 31-33) by 22 months. Exposure to UVBR not only delayed the development of these animals, but also completely inhibited or blocked metamorphosis. However, animals were exposed for short time intervals (minutes per day) to very high irradiances in relation to environmental UVBR levels (Grant and Licht, 1995). Although most studies reporting delayed development and/or

metamorphosis consisted of lab exposures (5/9 studies; Table 1.1), three studies consisted of outdoor exposures of amphibians to sunlight in natural aquatic habitats (Blaustein *et al.*, 1997) or in containers/enclosures filled with pond or lake water (Smith *et al.*, 2000; Belden and Blaustein, 2002b). This demonstrates that the developmental rate of amphibians subjected to present day UVBR levels can be negatively affected and that some populations or species may not be able to cope with exposure to rising UVBR levels expected in the future. It should be noted that all outdoor studies reporting developmental delays focused on embryonic development and exposed animals to UVBR for short time periods. Future outdoor studies should consider exposing animals to UVBR throughout all life stages including metamorphosis, in order to assess delays in development in the wild and to better reflect natural exposure conditions. Of six studies performed outdoors in ambient or filtered sunlight, three showed no effect of UVBR on development and/or metamorphosis (Anzalone *et al.*, 1998; Merilä *et al.*, 2000; Ankley *et al.*, 2002). This may indicate a difference in species/population sensitivity to UVBR exposure or may be a consequence of differences in experimental approaches and design.

Delayed or arrested development could be fatal to animals that have not metamorphosed before their habitat freezes or dries, and this is especially important for amphibian species living in temporary habitats (*e.g.*, *Ambystoma macrodactylum*, Long-toed salamander). Since these species must develop quickly before their habitat disappears, they seek sunlight to enhance their developmental rate (Blaustein and Belden, 2003) and thus are likely to be exposed to more damaging UVBR levels. Since amphibian eggs, larvae and newly metamorphosed tadpoles are typically the life stages that are most vulnerable to predation (with the egg stage being most vulnerable of all) (Pough *et al.*,

2001), the delayed development or metamorphosis of embryos and tadpoles subjected to UVBR could result in increased predation at these life stages. Two short-term laboratory exposure experiments conducted by Pahkala *et al.* (2002a and 2002b) reported an acceleration in development after exposure to UVBR in at least one of several *Rana temporaria* (European common frog) populations studied (Table 1.1). Although all four studies conducted by this group used the same species and UVBR levels (normal and enhanced), each study produced a different outcome. For example, Pahkala *et al.* (2002a) examined the effects of UVBR on eight different populations of *R. temporaria* in Sweden and found that UVBR did not affect the developmental rate of animals in two of the populations examined. However, they did observe that one population exhibited accelerated development in the normal and enhanced UVBR treatments and that five populations exhibited developmental delays in the enhanced treatment only. This underscores the importance of taking into consideration population sensitivity differences when conducting or interpreting the results of UVBR exposure experiments. Acceleration in development may also point to a physiological disruption, and it should be acknowledged that the exposure of the same species to similar exposure regimes could manifest itself in different sublethal responses.

1.10 Interactions between UVBR and Other Environmental Stressors

Although many studies have demonstrated the negative effects of UVBR on amphibians, studies examining single stressors may not provide adequate information on how amphibians are coping with exposure to several stressors in the wild. Even if underwater UVBR levels remain the same or only increase slightly, interactive effects

between UVBR and other stressors could produce significantly adverse effects on amphibians (Lannoo, 2005). Blaustein *et al.* (2003) reviewed the effects of interactions between UVBR and numerous environmental stressors present in the aquatic environment on amphibian health. The environmental stressors included: (1) the pathogenic fungus *Saprolegnia ferax*; (2) low pH; (3) nitrates; (4) pesticides (*e.g.*, carbaryl); and (5) PAHs (polycyclic aromatic hydrocarbons). Interactions between two stressors may occur when animals have a reduced capacity to respond to one agent in the presence of another (Blaustein *et al.*, 2001). Exposure to UVBR can compromise the immune system of amphibians, making them more vulnerable to the effects of pathogens (reviewed in Blaustein and Kiesecker, 2002). Interactions may also occur between UVBR and contaminants, when one factor enhances the toxicity of the other factor (Blaustein *et al.*, 2001). Other recent studies on amphibians have demonstrated that interactions between UVBR and various environmental stressors including: (1) sodium nitrite (Macías *et al.*, 2007); (2) bisphenol A (Koponen and Kukkonen, 2002); (3) fire retardant chemicals (Calfee and Little, 2003); (4) 4-octylphenol (Crump *et al.*, 2002); and (5) copper chloride (Baud and Beck, 2005) produce adverse effects on exposed amphibians (Table 1.2). For example, a 10-day exposure of newly hatched *R. pipiens* tadpoles to UVBR or OP alone had no effect on hind limb emergence (Gosner stage 36), although exposure to the combination of UVBR+OP accelerated hind limb emergence (Crump *et al.*, 2002). More research is needed to determine whether UVBR alone or combined with multiple stressors in the environment such as estrogenic chemicals can have a direct effect on amphibian populations.

1.11 Endocrine Control of Amphibian Development and Metamorphosis

The actual mechanism(s) by which UVBR and/or OP disrupt amphibian development remain to be elucidated. Crump *et al.* (2002) hypothesized that the developing hypothalamus may be a potential environmental sensor for neurotoxicologic studies because of its role in the endocrine control of metamorphosis. They demonstrated that a 10-day exposure of *R. pipiens* tadpoles to subambient levels of UVBR and/or 4-octylphenol (OP) altered the expression of important hypothalamic genes (*e.g.*, glutamate decarboxylase 67) at metamorphic climax as well as genes in the tadpole diencephalon (*e.g.*, brain-specific angiogenesis inhibitor 3). However, this study did not examine the effects of UVBR and/or OP on the expression of specific genes that control amphibian metamorphosis. Several possible mechanisms of action whereby UVBR and OP may suppress development and metamorphosis may exist. Since amphibian metamorphosis is a highly coordinated process controlled by the thyroid hormones, decreased developmental rates in tadpoles exposed to UVBR and/or OP suggest a disruption of the thyroid system. Below is a brief overview of the hormonal pathways that control amphibian metamorphosis, which have been extensively described by several authors (*e.g.*, Dodd and Dodd, 1976; Denver, 1996; Tata, 1996; Shi, 2000; Shi and Ishizuya-Oka, 2001; Denver *et al.*, 2002; Brown, 2005; Tata, 2006; Brown and Cai, 2007; Fort *et al.*, 2007).

Amphibian metamorphosis is regulated by the hypothalamo-pituitary-thyroid (HPT) axis (Figure 1.2; based on Denver, 1997 and 1998 and Shi, 2000). Environmental factors and internal processes (hormonal) stimulate the hypothalamus to secrete corticotropin-releasing hormone (CRH), which in turn stimulates the secretion of

pituitary thyrotropin (thyroid stimulating hormone, TSH) and adrenocorticotrophic hormone (ACTH) (Denver, 1997). TSH stimulates the production and secretion of thyroid hormones (TH) T4 (thyroxine) and T3 (3,5,3'-triiodthyronine) from the thyroid gland into blood circulation (Denver, 1997; Tata, 1999). A group of enzymes called deiodinases are responsible for the conversions of TH to active and/or inactive forms in the thyroid gland and within target tissues, regulating TH levels in various tissues at different stages of tadpole development (Denver *et al.*, 2002). T3 controls metamorphosis by regulating the expression of genes required to induce the coordinated morphological changes that are expected during the transition of tadpoles to frogs. Thyroid hormone receptors (TRs) (alpha and beta) are localized within the nucleus of cells. TH binds the TR, forming a heterodimer with liganded 9-*cis*-retinoic acid receptors (RXR). This complex then binds the TRE (TH response element) within the promoter to alter the expression of genes involved in development and metamorphosis (Bucholtz *et al.*, 2006; Fort *et al.*, 2007). Although amphibian metamorphosis is dependent on TH (Denver *et al.*, 2002; Brown, 2005), corticosteroid levels released from the interrenal glands rise concurrently with plasma TH levels during late prometamorphosis and metamorphic climax and may synergize with TH to accelerate metamorphosis (Kikuyama *et al.*, 1993; Shi, 2000) (Figure 1.2). In contrast, thyrotropin-releasing hormone (TRH) stimulates the secretion of prolactin (PRL) from the anuran pituitary gland, which inhibits amphibian development and accelerates larval growth (Figure 1.2).

UVBR levels have increased globally over the past few decades, yet very few studies have examined mechanisms whereby UVBR may alter endocrine function in vertebrates. Belden *et al.* (2003) conducted the only study to our knowledge on the effect

of UVBR exposure on part of the endocrine axis controlling amphibian development. They found that *Rana cascadae* (Cascades frog) tadpoles did not avoid low levels of UVBR, and that whole body corticosterone levels were unaffected by exposure. However, rates of development were not measured in these animals, as the focus of this study was to investigate UVBR avoidance behaviour and survival in exposed tadpoles. Most studies examining the sublethal effects of OP on amphibians have focussed on the mechanisms of action behind reproductive impairment and not on metamorphic disruptions. Since developmental changes are correlated with distinct and specific hormonal cues, it is warranted to assess the effects of UVBR and OP on the thyroidal status of amphibians.

1.12 Thesis Outline

The main objective of this thesis was to assess the effects of UVBR and/or OP on somatic growth, development and metamorphosis in relation to effects on the thyroid system of exposed animals. TH level measurements in combination with histological, morphological and molecular test methods are useful and frequently used approaches to measure thyroid dysfunction in organisms (Fort *et al.*, 2007). Moreover, the incorporation of endpoints at central (*i.e.*, within HPT axis), peripheral (*i.e.*, at tissue level) and TR-mediated (*i.e.*, examining biological actions of T3 *in vivo*) levels of the thyroid system could provide insight into the mechanisms behind morphological or physiological responses of animals to environmental stressors (Eales *et al.*, 1999). Figure 1.3 illustrates the approach taken in this thesis to assess the effects of UVBR and/or OP on the thyroidal status of exposed tadpoles.

This thesis is a collection of manuscripts that are either published (Chapter 2), in press (Chapter 1) or in preparation (Chapters 3 and 4) for publication. There was an effort to reduce redundancies throughout the thesis where and if possible. It is organized into three data chapters that include results of one or more *in vivo* chronic exposure experiments of *R. pipiens* tadpoles to UVBR and/or OP (Figure 1.3). The first experiment involved treating tadpoles to UVBR and/or OP for eight months and examining effects on somatic growth, development, metamorphosis and survival (Chapter 2). The effects of UVBR and/or OP exposure on the biological response of tadpoles to T3 were examined in Chapter 3. Alterations in the expression of genes in tail and/or whole brain tissue were measured after 24h exposure to T3, in tadpoles pre-exposed to UVBR and/or OP. A subsequent experiment was conducted to measure changes in morphometric endpoints expected from the precocious induction of metamorphosis, after seven days of exposure to T3 (Chapter 3). To assess specific effects of UVBR and/or OP on the thyroid system of tadpoles, the last data chapter examines the effects of chronic exposure to these stressors on T3 levels and the expression of genes important for development (Chapter 4). A second experiment in Chapter 4 was conducted to determine the effects of chronic exposure to UVBR on the morphology of the thyroid gland of exposed animals.

Table 1.1 Summary of studies on the effects of UVBR on amphibian development and metamorphosis.

Species	Type of Exposure	UVBR ^a Levels	W/m ²	Filters	Developmental Stage Exposed	Length of Exposure	Effect on		Ref ^b
							Developmental Rate	Metamorphosis	
<i>Ambystoma macrodactylum</i>	O ^c	94% of ambient UVBR removed (ambient UVBR ranged from 4.77 to 25.5 $\mu\text{W}/\text{cm}^2$) ^d 90% of ambient UVBR transmitted	NA ^e	Mylar	Embryo (<24hrs old)	Until hatch or death	Delayed	NA	1.
<i>Hyla cadaverina</i> , <i>Hyla regilla</i> and <i>Taricha torosa</i>	O	Less than 10% UVBR transmittance ^d	NA	Mylar	Embryo	Until embryos hatched or died			2.
		Ambient sunlight	NA	None			No effect	NA	
		~80% UVBR transmittance	NA	Acrylic			No effect	NA	
<i>Hyla versicolor</i>	L	Daylight fluorescent lights ^d	NA	None	Tadpole (Gosner stage 21)	Until metamorphosis			3.
		0.113 W/cm ²	1130			Until metamorphosis; 2 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.170 W/cm ²	1700			Until metamorphosis; 3 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.113 W/cm ²	1130	Acetate (varying layers)		Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.961 W/cm ²	9610			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	

Table 1.1 Continued

Species	Type of Exposure	UVBR Levels	W/m ²	Filters	Developmental Stage Exposed	Length of Exposure	Effect on		Ref
							Developmental Rate	Metamorphosis	
<i>Bufo americanus</i>	L	Daylight fluorescent lights ^d	NA	None	Tadpole (newly hatched)	Until metamorphosis			3. (cont'd)
		0.113 W/cm ²	1130			Until metamorphosis; 2 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.170 W/cm ²	1700			Until metamorphosis; 3 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.961 W/cm ²	9610			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		1.152 W/cm ²	11520			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		1.440 W/cm ²	14400			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
<i>Rana clamitans</i>		0.961 W/cm ²	9610	None	Tadpole (newly hatched)	Until metamorphosis; 240 min/day for 5 consecutive days weekly	No effect	No effect	
		Daylight fluorescent lights ^d	NA			Until metamorphosis			
		0.113 W/cm ²	1130			Until metamorphosis; 2 min/day, 3 times weekly on alternate days	Delayed	Blocked	
		0.170 W/cm ²	1700			Until metamorphosis; 3 min/day, 3 times weekly on alternate days	Delayed	Blocked	

Table 1.1 Continued

Species	Type of Exposure	UVBR Levels	W/m ²	Filters	Developmental Stage Exposed	Length of Exposure	Effect on		Ref
							Developmental Rate	Metamorphosis	
<i>Rana sylvatica</i>	L	Daylight fluorescent lights ^d	NA	None (varying layers)	Tadpole (newly hatched)	Until metamorphosis			3. (cont'd)
		0.113 W/cm ²	1130			Until metamorphosis; 2 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.170 W/cm ²	1700			Until metamorphosis; 3 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.113 W/cm ²	1130			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.961 W/cm ²	9610			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		1.056 W/cm ²	10560			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		1.152 W/cm ²	11520			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		1.440 W/cm ²	14400			Until metamorphosis; 240 min/day, 3 times weekly on alternate days	No effect	No effect	
		0.961 W/cm ²	9610			Until metamorphosis; 240 min/day for 5 consecutive days weekly	No effect	No	
<i>Rana aurora</i>	O	UVBR blocked ^d	NA	Mylar	Embryo (Gosner stages 2-6)	6 weeks; until Gosner stages 19-21	Delayed	NA	4.
<i>Rana blairi</i>	O	~80% UVBR from ambient sunlight	NA	Acetate	Embryo (~3 days old, ≤ Gosner stage 9)	8 days; until embryos hatched			5.
		Filtered sunlight (~58% UVBR transmittance)	NA	Petri dish cover			No effect	NA	
		Filtered sunlight (~84% UVBR transmittance)	NA	Saran wrap			Delayed	NA	

Table 1.1 Continued

Species	Type of Exposure	UV/BR Levels	W/m ²	Filters	Developmental Stage Exposed	Length of Exposure	Effect on		Ref
							Developmental Rate	Metamorphosis	
<i>Rana pipiens</i>	L	Constant day/light or darkness ^d	NA	NA	Embryo	3 days			6.
		Quartz mercury lamp ^e + constant daylight or darkness				3 days; 1 min/day	Delayed (day/light treatment)	NA	
		Quartz mercury lamp + constant day/light or darkness				3 days; 2 min/day	Delayed (day/light and darkness treatments)	NA	
<i>Rana pipiens</i>	L	Normal laboratory fluorescent light ^e	Not detected	None	Embryo (2-3hrs post-fertilization)	113 days; UV/BR/VIS provided for 12h of a 16h light period	No effect	No effect	7.
		UVBR at air water interface (44 $\mu\text{W}/\text{cm}^2$) attenuated by ~76% at a water depth of 3cm	NA						
<i>Rana pipiens</i>	O	Ambient sunlight	NA	None	Embryo (stages 5-7)	Until metamorphosis	NA	No effect	8.
		Filtered sunlight (various levels of UVR)		Nitex and stainless steel screen (with or without glass or acrylamide)			NA	No effect	
<i>Rana pipiens</i>	L	Full spectrum laboratory lighting ^d	NA	None	Tadpole (Gosner stage 21)	10 days; 12:12 hr light:dark photoperiod	No effect	NA	9.
		7 $\mu\text{W}/\text{cm}^2$	0.07			10 days; 360 min/day			

Table 1.1 Continued

Species	Type of Exposure	UVBR Levels	W/m ²	Filters	Developmental Stage Exposed	Length of Exposure	Effect on		Ref
							Developmental Rate	Metamorphosis	
<i>Rana temporaria</i>	O	UVBR and shorter wavelengths removed ^d	NA	Mylar	Embryo (~1 day old)	12 days; until the majority of embryos hatched (Gosner stage 25)			10.
		Ambient sunlight	NA	None			No effect	NA	
		Wavelengths shorter than UVBR removed	NA	Pre-burned acetate			No effect	NA	
<i>Rana temporaria</i>	L	UVBR blocked ^c	NA	Mylar	Embryo (<2 hrs old)	Until the majority of larvae reached Gosner stage 25; 137 min/day			11.
		1.254 kJ/m ²	0.1526	Acetate			NA	No effect	
		1.584 kJ/m ² (enhanced by 26%) ^g	0.1927				NA	Delayed	
<i>Rana temporaria</i>	L	UVBR blocked ^d	NA	Mylar	Embryo (<2 hrs old)	Until larvae reached Gosner stage 25; 137 min/day			12.
		1.254 kJ/m ²	0.1526	Acetate			No effect/accelerated ^h	NA	
		1.584 kJ/m ² (enhanced by 26%)	0.1927				Delayed/no effect/accelerated ^h	NA	
<i>Rana temporaria</i>	L	UVBR blocked ^d	NA	Mylar	Embryo (<2 hrs old)	~12 days; 137 min/day			13.
		1.254 kJ/m ²	0.1526	Acetate			Accelerated	NA	
		1.584 kJ/m ² (enhanced by 26%)	0.1927				No effect	NA	
<i>Rana temporaria</i>	L	UVBR blocked ^d	NA	Mylar	Embryo (<2 hrs old)	Until metamorphosis; 137 min/day			14.
		1.254 kJ/m ²	0.1526	Acetate			NA	Delayed	
		1.584 kJ/m ² (enhanced by 26%)	0.1927				NA	Delayed	

^a UVBR levels have been shown to range between 1-2.5 W/m² in ambient sunlight above water in Ontario, Canada (Crump *et al.*, 1999a, 1999b; Croteau *et al.*, 2008b) and 0.0042-0.72 W/m² in various types of aquatic environments in North America (at a depth of 10 cm; Barron *et al.*, 2000; Calfee and Little, 2003), depending on environmental factors.

^b References: 1) Blaustein *et al.*, 1997; 2) Anzalone *et al.*, 1998; 3) Grant and Licht, 1995; 4) Belden and Blaustein, 2002b; 5) Smith *et al.*, 2000; 6) Higgins and Sheard, 1926; 7) Ankley *et al.*, 1998; 8) Ankley *et al.*, 2002; 9) Crump *et al.*, 2002; 10) Merilä *et al.*, 2000; 11) Palkkala *et al.*, 2001; 12) Palkkala *et al.*, 2002a; 13) Palkkala *et al.*, 2002b; 14) Palkkala *et al.*, 2003.

^c O, outdoors; L, laboratory.

^d Control treatment for the indicated experiment.

^e NA, not applicable.

^f Quartz mercury lamps emit both UVBR and UVCR.

^g The 26% enhanced UVBR level was obtained using a computer model that calculated the daily increase in UVBR that would follow from 15% ozone depletion under clear-sky conditions, which is within the observed daily variation in ozone in central Sweden in April.

^h This study examined the effects of UVBR on 8 different populations in Sweden. UVBR did not affect development in two populations. One population exhibited accelerated development in the normal and enhanced UVBR treatments. Five populations exhibited developmental delays in the enhanced treatment only.

Table 1.2 Examples of studies demonstrating adverse effects of interactions between UVBR and various stressors on amphibians.

Species	Stressor and Effect of Interaction	Reference
<i>Pseudacris crucifer</i>	With copper chloride reduces survival	Baud and Beck 2005
<i>Rana perezi</i> and <i>Bufo bufo</i>	With sodium nitrite reduces survival	Macías <i>et al.</i> 2007
<i>Rana pipiens</i>	With 4-octylphenol increases weight, accelerates hind limb emergence and affects hypothalamic gene expression	Crump <i>et al.</i> 2002
<i>Rana sphenocephala</i>	With certain fire retardant chemicals with YPS ^a reduces survival	Calfee and Little 2003
<i>Rana temporaria</i>	With bisphenol A produces developmental anomalies and reduces survival	Koponen and Kukkonen 2002

^a YPS, Yellow prussiate of soda (sodium ferrocyanide) is a corrosion inhibitor that is added to fire fighting chemicals.

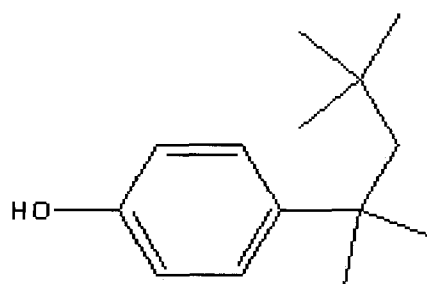


Figure 1.1 Chemical structure of 4-*tert*-octylphenol (OP).

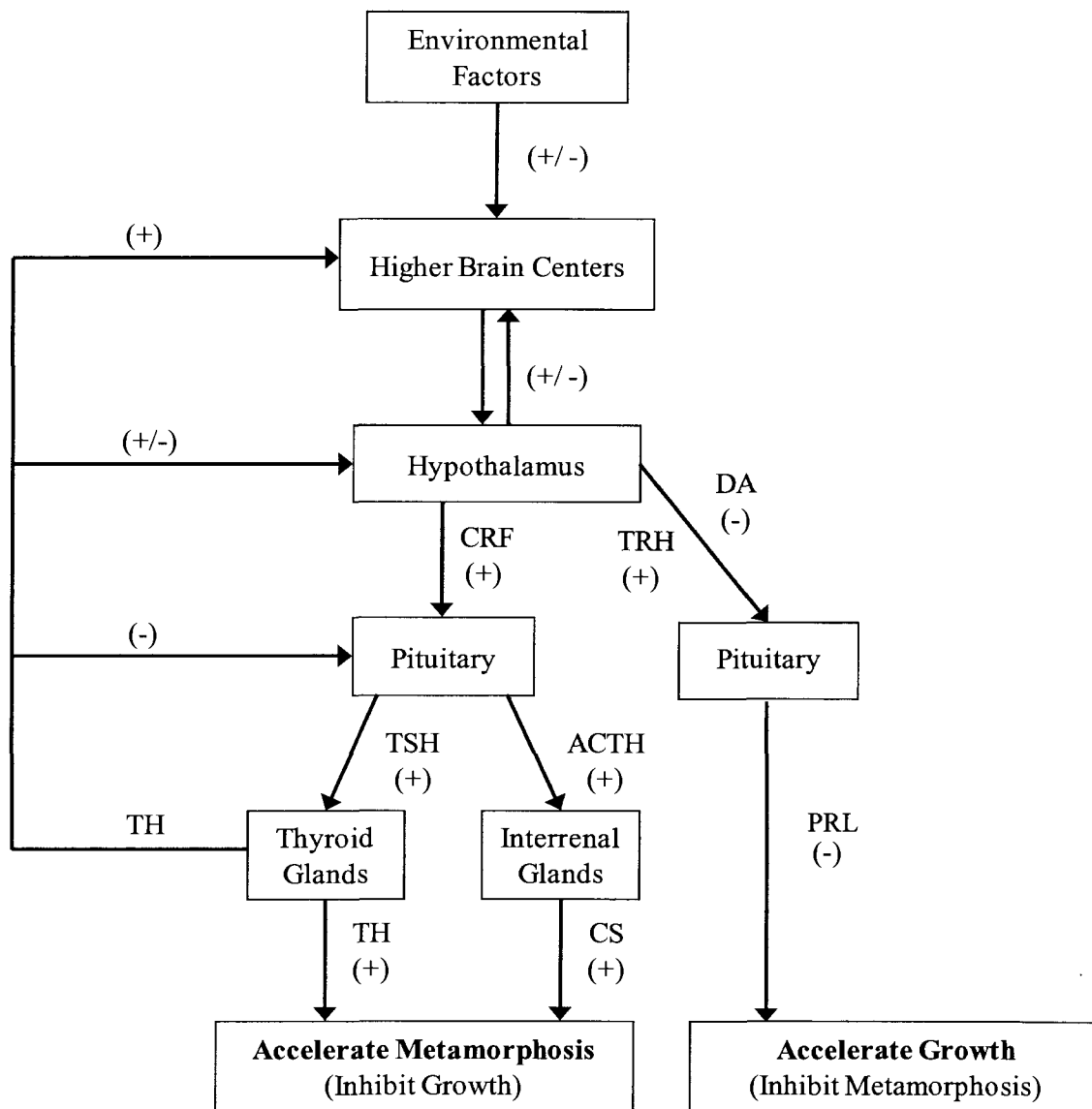


Figure 1.2 Schematic overview of the endocrine systems controlling amphibian metamorphosis. A plus sign indicates a positive or stimulatory action and a minus sign indicates a negative or inhibitory action. Thyroid hormone and corticosteroids stimulate metamorphosis whereas prolactin inhibits metamorphosis. Hormonal pathways are under complex neuroendocrine regulation involving both positive and negative feedbacks depending on developmental stage or physiological state (feedback loops for corticosteroids are not shown). CRF, corticotropin-releasing factor; TRH, thyrotropin-releasing hormone; TSH, thyrotropin; ACTH, adrenocorticotropin; CS, corticosteroids; TH, thyroid hormone; PRL, prolactin; DA, dopamine. Adapted from Denver (1997, 1998) and Shi (2000).

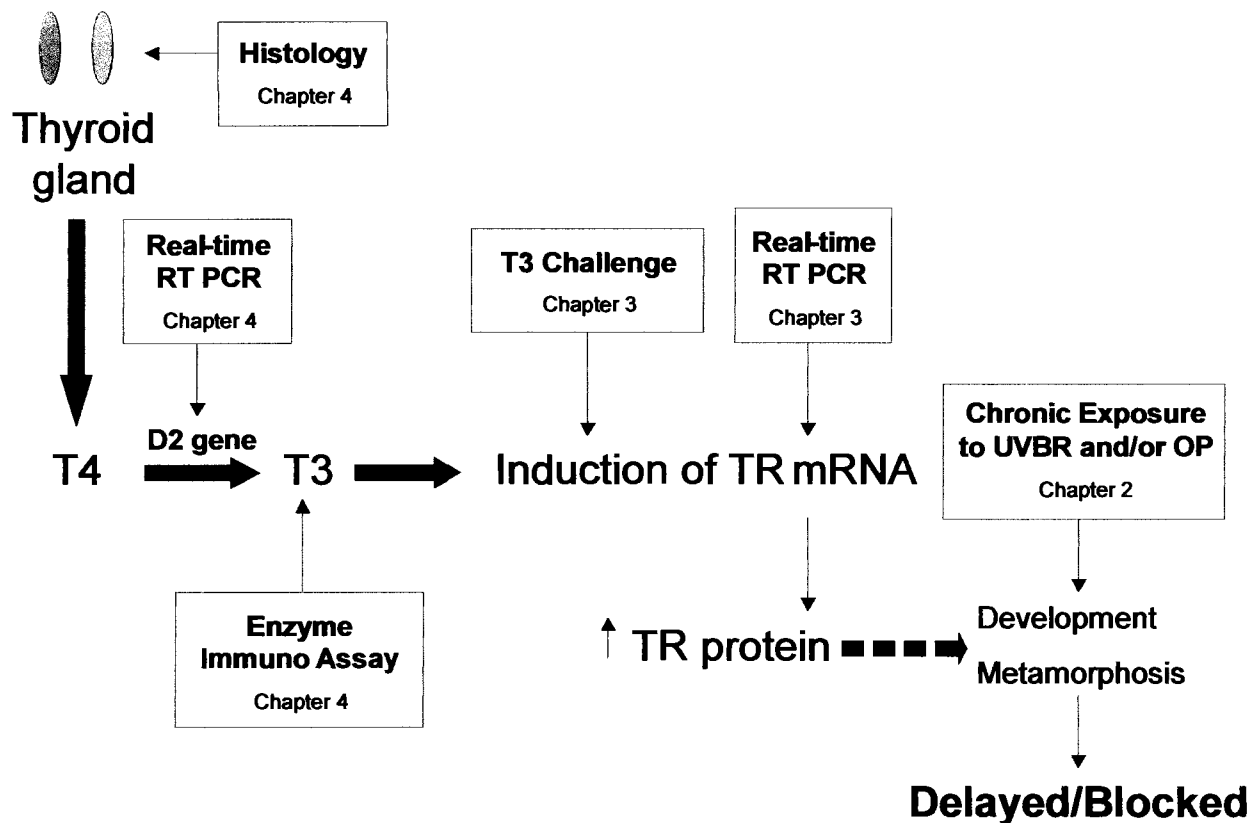


Figure 1.3 A basic depiction of the thyroid system that regulates amphibian development and metamorphosis. The techniques that were used in this project to assess various endpoints along this axis and the corresponding thesis chapters are indicated. PCR, polymerase chain reaction; T4, thyroxine; D2, deiodinase type II; T3, triiodothyronine; TR, thyroid hormone receptor; UVBR, UVB radiation; OP, 4-*tert*-octylphenol.

CHAPTER 2

Chronic exposure of *Rana pipiens* tadpoles to UVB radiation and the estrogenic chemical 4-*tert*-octylphenol*

2.1 INTRODUCTION

With amphibian populations declining worldwide (Houlahan *et al.*, 2000; Stuart *et al.*, 2004), it is essential to identify the anthropogenic factors that may be involved. These may include alien species invasions, over-exploitation, land use changes, emerging infectious diseases, toxic chemical exposure, global climate change, and increased ultraviolet-B radiation (UVBR), which all may be acting alone or in concert to affect amphibians (Collins and Storfer, 2003). In addition, there are increasing observations of malformed amphibians in the wild (Ouellet *et al.*, 1997; Sower *et al.*, 2000; Meteyer *et al.*, 2000); exposure to UVBR, contaminants and parasitic infections are also believed to be causal factors (Stocum, 2000; Loeffler *et al.*, 2001). Exposure of amphibians to endocrine disrupting chemicals is of particular concern because these pollutants disrupt the functioning of endocrine systems involved in reproduction, development and metamorphosis (Crump, 2001).

Alkylphenol polyethoxylates (APEO) are widely used surfactants in domestic detergents, pesticide formulations, and industrial products. These compounds are (1) directly released into the environment or discharged from sewage treatment plants (STP), and (2) rapidly degraded into metabolites such as the alkylphenols nonylphenol (NP) and

*Adapted from: Croteau M.C., C.J. Martyniuk, V.L. Trudeau and D.R.S. Lean. 2008. Chronic exposure of *Rana pipiens* tadpoles to UVB radiation and the estrogenic chemical 4-*tert*-octylphenol. Journal of Toxicology and Environmental Health A. 71:134-144

octylphenol (OP). The metabolites are generally more toxic, lipophilic, persistent and estrogenic than their parent compounds (Bennie, 1999). Available amphibian toxicity data for OP includes a 7-day LC50 value of 2800 nM 4-OP for *Rana pipiens* (Northern leopard frog) tadpoles (Crump *et al.*, 2002), as well as species sensitivity differences, as the 14-day LC50 for *R. pipiens* tadpoles is 1420 nM 4-*tert*-OP compared to 740 nM for *Rana sylvatica* (Wood frog) (Hogan *et al.*, 2006). Levels of 4-*tert*-OP in the aquatic environment in Canada and the United States range from <0.024 nM to 2.3 nM in freshwater, <0.024 nM to 8.25 nM in STP final effluents, and <0.005-1.8 µg/g dry weight in sediment (Bennie, 1999). In the United Kingdom, levels as high as 63 nM were reported in the outer Teas estuary (Blackburn and Waldock, 1995). Although environmental levels are generally below acute toxicity thresholds, there is cause for concern with regard to endocrine effects following chronic OP exposure on amphibians.

White *et al.* (1994) found that a number of alkylphenolic compounds such as 4-OP exerted estrogenic effects since these chemicals are capable of stimulating (1) transcription of an estrogen response element driven reporter gene in transfected cells; (2) vitellogenin gene expression in trout hepatocytes; and (3) growth of human breast cancer cell lines. *In vitro* binding assays for *Xenopus laevis* (African clawed frog) estrogen receptor (ER) demonstrated that 4-OP displaced radiolabeled 17 β-estradiol from the ER (Lutz and Kloas, 1999) and that *p*-OP has a relatively high binding capacity to ERα (Suzuki *et al.*, 2004). Sublethal effects of low-level OP exposure were reported in some amphibian species, such as feminization of *X. laevis* tadpoles following exposure to 10 to 100 nM 4-OP from hatch to metamorphosis (Kloas *et al.*, 1999), and accelerated gonadal differentiation of female and male *Rana catesbeiana* (Bullfrog) tadpoles exposed to 1 nM

4-*tert*-OP for 24hr (Mayer *et al.*, 2003). However, little research has been conducted on the effects of chronic exposure to OP on the survival, development, growth, and metamorphosis of amphibians.

Decreases in stratospheric ozone, climate warming, and acidification can produce a reduction of dissolved organic carbon (DOC) levels in natural waters, which in turn results in increased penetration of solar radiation and enhanced exposure of aquatic organisms to UVBR (Schindler *et al.*, 1996; Yan *et al.*, 1996; Lean, 1998b). Field and lab studies have shown that exposure of amphibians to ambient or enhanced levels of UVBR produced an increased incidence of developmental abnormalities and/or reduced survivorship (Blaustein *et al.*, 1997; Ankley *et al.*, 1998, 2000, 2002; Tietge *et al.*, 2001; Belden and Blaustein, 2002a; Weyrauch and Grubb, 2006). UVBR also affects amphibian development (Smith *et al.*, 2000; Belden and Blaustein, 2002b), metamorphosis (Pahkala *et al.*, 2001 and 2003), growth (Pahkala *et al.*, 2000 and 2001; Belden and Blaustein, 2002b) and behavior (Kats *et al.*, 2000).

Although many studies have demonstrated the adverse effects of UVBR and chemicals on amphibians, studies examining single stressors may not provide adequate information on how amphibians are coping with exposure to numerous stressors in the wild. Exposure of polycyclic aromatic hydrocarbons (PAH) and certain pesticides to UV radiation enhances the toxicity of these chemicals by generating photometabolites that are highly toxic to developing amphibians (Blaustein *et al.*, 2003). Moreover, intermediate photodegradation products of NP, including phenol, 1,4-dihydroxylbenzene and 1,4-benzoquinone, were generated after 80 hr exposure in a solar UVR simulator (Neamtu and Frimmel, 2006), of which 1,4-benzoquinone was shown to induce a toxic response

and cell death in *Oncorhynchus mykiss* (Rainbow trout) (Schmieder *et al.*, 2003). Brand *et al.* (2000) studied the degradation of 4-OP photoinduced by the photolysis of iron III aqua complexes, and identified benzoquinone, 4-octylpyrocatechol and 4-hydroxyacetophenone as photoproducts. Although the effects of these photometabolites on amphibians are unknown, UVBR combined with OP may exert indirect effects on the health of aquatic animals through the generation of toxic photoproducts.

Few studies have examined the possible interactive effects of UVBR and estrogenic compounds on developing amphibians. This study presents data resulting from the chronic exposure of *R. pipiens* tadpoles to UVBR in combination with an estrogenic compound, 4-*tert*-octylphenol (OP), at environmentally relevant levels. The objective was to investigate the effects of UVBR and OP, alone and in combination on tadpole (1) survival; (2) development (measured by the incidence and types of developmental anomalies and rate of development); (3) growth; and (4) metamorphosis.

2.2 MATERIALS AND METHODS

2.2.1 Animal Collection and Care

A sub-set of four naturally fertilized *R. pipiens* egg masses were collected in April 2002 from a pond in Old Chelsea, Québec, Canada (45° 30' 50" N; 75° 47' 50" W) with no known pollution sources other than possible atmospheric inputs. Our lab has followed a healthy population of *R. pipiens* at this location since 1999. The egg masses were held together and allowed to hatch in the lab. Eggs and hatchlings and were maintained in 13-L tanks containing aerated, carbon filtered water from the Aquatic Care Facility at the University of Ottawa (pH 6.5 to 7, dissolved oxygen 9.2 to 10 mg/L, <0.01 mg PO₄/L, <0.01 mg NO₃/L, and temperature 18 to 20°C). Water was changed three times weekly

until the animals reached Gosner stage 25 (beginning of independent feeding; Gosner, 1960), after which tadpoles were randomly distributed to different exposure treatments. All animal experimentation followed the guidelines and standards of the Animal Care Committee (University of Ottawa) and the Canadian Council on Animal Care.

2.2.2 Conditions of Exposure

Tadpoles were exposed to one of two environmentally relevant sublethal concentrations of 4-*tert*-octylphenol (OP) (0.01 or 10 nM), or a 0.01% ethanol vehicle control, with or without a sub-ambient level of UVBR. The average UVBR intensity was 0.22 W/m², which simulates underwater levels and represents approximately 10% of ambient UVBR expected for the Ottawa region at this time of year. In total, 1200 animals were exposed, 200 per treatment group. The water used in the experiment was as described above at time of water renewal, with the exception of the water temperature (20 to 21°C). The static renewal exposure regimen began on May 10, 2002, and included water renewal 6 times weekly for the duration of the exposure period. The animals were then transferred to a clean water grow-out system on January 10, 2003 (*i.e.*, exposure to OP, ethanol and UVBR was terminated) and water was renewed every 48 h. The experiment was terminated on April 10, 2003.

To avoid overcrowding and to maintain appropriate water quality levels during the experiment, rearing densities were adjusted by reducing the number of tadpoles per litre and increasing the volume of water used in the experiment, according to guidelines recommended by the U.S. National Research Council (1974) and Edginton (2001). For week one of the experiment, animals weighing 0.01-0.02 g were divided among four Pyrex bowls (1.5 L volume; 20 X 8 cm diameter/height) per treatment (50

tadpoles/bowl), each filled with 1 L water (water depth of 3.5 cm). After three weeks of exposure, numbers of animals per bowl were reduced (by adding replicate bowls) and the volume of water in each was increased to 1.3 L (water depth of 4.5 cm). The rearing density was gradually reduced to 8 to 10 tadpoles/L by month four of the experiment and to 5 to 8 tadpoles/L by the end of the experiment. Dissolved O₂ and pH were measured over the course of the study using an Oxyguard® Handy MK III O₂ meter (Birkerød, Denmark) and Carolina Biological Supply Co. pH water test strips (Burlington, NC), and were constant between 6 and 9 mg/L and at approximately 6.5, respectively. Ammonia levels were less than 250 µg/L throughout the experiment, the detection limit of the Ammonia-Nitrogen water test kit (Lamotte®; Chestertown, MD). Tadpoles were fed *ad libitum* with Nutrafin® fish flakes (Rolf C. Hagen Inc., Montréal, QC) and tadpole food (Carolina Biological Supply Co., Burlington, NC) (approximately equal amounts) once daily.

2.2.3 Controlled Environmental Chambers

Tadpoles were kept in two controlled environmental chambers (Convion model CMP 3023 and Convion model S10/S10H; Winnipeg, MB) for the duration of the experiment. The solar simulators were composed of fluorescent and incandescent lighting as sources of UVAR (320-400 nm), UVBR (280-320 nm) and visible light (400-750 nm) (VIS). The Convion model S10/S10H contained four incandescent bulbs (40-W soft white longlife General Electric incandescent lights; Cleveland, OH) and twelve fluorescent lights (112 cm length; Cool White General Electric F48T12/CW/HO). The Convion model CMP 3023 contained eight incandescent bulbs (40-W soft white longlife

General Electric incandescent lights) and ten fluorescent lights (173 cm length; Duro-test® Vita lite® F72T12 Ultra hi output 1500 CW HO 85W; Philadelphia, PA). The lights followed a 12:12 hr light:dark photoperiod, with the light cycle occurring from 7 a.m. to 7 p.m. Shelves were positioned at 65 cm (model S10/S10H) and 43.5 cm (model 3023) below the lamps to attain the desired UVAR, UVBR and VIS intensities. UVBR was obtained by removing the protective cover that usually shields the lamps.

To approximate existing water temperature conditions that these tadpoles would normally be exposed to in our region, the temperature of the air inside both environmental chambers was set at 16°C so that the temperature of the water gradually increased to 20±1°C during the light cycle and gradually decreased to 16°C during the dark cycle (with maximum 1°C change per hour). Temperature was monitored daily. Water was renewed during the light cycle with Aquatic Care Facility water as described earlier, with a temperature of 20-21°C to avoid water temperature fluctuations.

2.2.4 UVR Levels

An Optronics OL-754 spectroradiometer (Orlando, FL) calibrated using National Institute of Standards and Technology traceable standard was used to measure UVBR, UVAR, and VIS levels inside the environmental chambers. All non-UVBR bowls were also in the chambers but were covered with a piece of Mylar to block the transmission of UVBR (*i.e.*, only wavelengths of 320nm and above are transmitted through the filter; Worrest and Kimeldorf, 1976). Holes were cut in Mylar filters to permit gas exchange but were sufficiently small (<0.5 cm diameter) to block transmission of UVBR. The perforated Mylar filter permitted the penetration of UVAR and VIS but efficiently

blocked UVBR, as the UVBR irradiance measured under the filter was negligible, ranging from 7.8×10^{-4} to 3.5×10^{-7} W/m². Figure 2.1A shows the entire (250 to 800 nm) spectral irradiance for both environmental chambers at the height of the shelves, under the perforated Mylar filters (measured inside CMP 3023 chamber) as well as the spectrum of incident sunlight measured in Ottawa in late May 2002. Figure 2.1B provides details of the UVR region only (280 to 400 nm) inside both chambers. Measurements were also carried out regularly using the Oriel GOLDILUX UVR and light meter (Model #70217; Stratford, CT) with interchangeable UVBR probe (Model 70219), to ensure the Mylar covers were efficiently blocking UVBR in the non-UVBR treatments.

The irradiances measured inside the Conviron S10/S10H and Conviron CMP 3023 environmental chambers were 0.3 and 0.13 W/m² UVBR, 1.44 and 1.93 W/m² UVAR and 78.30 and 59.40 W/m² VIS, respectively. The average irradiances for both incubators were 0.22 W/m² UVBR, 1.69 W/m² UVAR and 68.85 W/m² VIS. The irradiance for incident sunlight was 2.02 W/m² for UVBR, 41.39 W/m² for UVAR and 415.02 W/m² for VIS. Since the attenuation in frog ponds is rapid (Crump *et al.*, 1999b), these levels correctly simulate underwater conditions. Underwater measurements of solar radiation (at 10 cm depth) taken in a variety of aquatic habitats in the western United States (Calfee and Little, 2003) were comparable. Bowls were randomly rotated daily within and between incubators to account for variations in temperature and UVR/VIS levels.

2.2.5 Exposure to 4-tert-Octylphenol

Two environmentally relevant levels of OP were chosen for the tadpole exposure experiment, 0.01 nM (2.06 ng/L) and 10 nM (2.06 µg/L); these were labelled OP1 and

OP2, respectively. Stock solutions were prepared biweekly (100 nM and 100 μ M) by dissolving 99% pure technical grade 4-*tert*-octylphenol (Aldrich Chemical Co., Milwaukee, WI) in 95% ethanol. The nominal concentrations were prepared by pipetting 130 μ L of the appropriate stock solution into 1.3 L water. Treatments that did not include OP (control and UVR treatments) received 130 μ L of 95% ethanol. Solutions were thoroughly mixed prior to use and protected from light. The water was changed six days per week to maintain the desired nominal concentrations. Water renewal occurred just prior to the dark cycle of the photoperiod to reduce photodegradation of OP. It was found that the related compound NP has a photolysis half-life of 10 to 15 h in natural surface waters under summer sunlight at noon (Ahel *et al.*, 1994).

2.2.6 Endpoints

Tadpoles were observed six times each week at the time of water renewal to determine the effects of treatment on survival and to identify different types of developmental malformations and abnormalities (following Meteyer, 2000). A malformation represents (1) intrinsic errors in development; (2) errors in chemical communication; or (3) translation error of genetic information (Meteyer, 2000). An abnormality is defined here as any abnormal developmental finding for which one has little or no information. The developmental stages of all animals were identified regularly under a dissection microscope using the Gosner staging system (Gosner, 1960) to determine the effects of treatment on the rate of development. A sub-set of tadpoles (n=50 animals) per treatment was randomly weighed at week 42, the developmental stages of these animals were recorded, and the animals were returned to the experiment.

From the time the first tadpole reached metamorphosis (forelimb emergence [FLE]; stage 42), containers were observed twice daily for metamorphosed animals. Metamorphosed tadpoles were weighed and snout vent length (SVL) was measured using electronic callipers. The age of individuals at metamorphosis (number of days elapsed from hatch to metamorphosis) and percentage of metamorphosing tadpoles were also recorded to determine if the treatments had effects on metamorphosis.

2.2.7 Statistical Analyses

The sample size at the beginning of the experiment was 200 tadpoles/treatment. Sample sizes were corrected throughout the experiment for tadpole mortality not attributable to treatment (*e.g.*, accidental mortality). The corrected sample size by the end of the experiment (week 48) ranged from 126 to 172 tadpoles/treatment. Cumulative percentages of mortalities and developmental anomalies were calculated using the corrected sample size per treatment at a given time point. Percent metamorphosis was calculated two different ways: (1) Percent tadpoles to metamorphose was calculated cumulatively throughout the experiment using the initial sample size corrected as described earlier; and (2) total percent tadpoles to metamorphose after 48 weeks was calculated using the initial sample size corrected as described above as well as by correcting for mortality attributable to treatment after 48 weeks. Sample size ranged from 40 to 61 tadpoles/treatment and was corrected for mortality attributed to treatment to ensure that mortality differences between treatments did not influence percent metamorphosis data.

Results from a preliminary experiment (Croteau *et al.*, 2001) indicated that a significant majority of *R. pipiens* tadpoles exposed to similar levels of UVBR did not

develop past stage 34 (early digit development), suggesting that the development of these animals was blocked. Therefore, to determine the effects of UVBR and OP on the rate of development, it was decided to analyze the percent tadpoles remaining at week 45 that had developed past stage 34 (including animals that survived to metamorphosis). The median developmental stage of tadpoles in the control treatment was stage 34 at this time, and the sample size ranged from 46 to 80 tadpoles/treatment. Log linear models were used to determine the effects of UVBR, OP, and UVBR+OP on percent mortality, percent metamorphosis, percent abnormalities and malformations and percent tadpoles developed past stage 34. In each log linear model, a variable with a $p < 0.05$ was considered statistically significant.

Data for weight of tadpoles at week 42 (sample size of 50 tadpoles/treatment), as well as weight, SVL and age of tadpoles at metamorphosis (sample size ranged from 1 to 16 tadpoles/treatment) were normally distributed and homoscedastic. These are expressed as the mean $\pm$ SEM (standard error of the mean). Two-way analysis of variance (ANOVA) was used to test whether UVBR, OP1 and UVBR+OP1 had a significant effect on weight, SVL and age at metamorphosis. Importantly, only one tadpole metamorphosed in the UVBR+OP2 group. Therefore, t-tests were performed between the UVBR or OP2 treatments and the control group to determine if these factors had an effect on weight, SVL and age at metamorphosis. A two-way analysis of covariance (ANCOVA) model was used to determine if there was a significant effect of treatment on body weights at week 42. The developmental stage of all tadpoles was recorded when they were weighed and this was used as the covariate in this analysis. For all ANOVAs

and ANCOVAs, a factor with a $p < 0.05$ was considered statistically significant. All data were analyzed using SYSTAT software (Version 10; San José, CA).

2.3 RESULTS

2.3.1 Mortality

After only one week of exposure, mortality of animals in the UVBR+OP groups was considerably higher (22-30%) than in the control group (9%) (Figure 2.2). Mortality rates increased considerably during the first two months of exposure, after which the increase in mortality in all treatment groups became more gradual until the end of the experiment. A significant effect of UVBR on mortality was found after 48 weeks ($p = 0.002$), which signifies that the mortality of all tadpoles exposed to UVBR, regardless of the presence of OP, was affected compared to those in non-UVBR treatments. Percent mortality in the UVBR and UVBR+OP treatments were 73% and 77 to 78%, respectively, compared to 62 to 68% in non-UVBR treatments (Figure 2.2). Tadpoles in the UVBR+OP treatments had the highest incidence of mortality at week 48 with approximately 10 to 15% more mortality compared to animals in control and OP groups respectively (Figure 2.2), but the interaction term (UVBR+OP) did not achieve statistical significance. There was also no significant effect of OP on mortality.

2.3.2 Developmental Malformations and Abnormalities

A similar trend was observed for the frequency of tadpoles with developmental malformations and abnormalities, as the first week of exposure to UVBR alone and UVBR+OP led to a rapid increase in the incidence of animals affected (Figure 2.3). Both levels of OP as well as all UVBR treatments affected tadpoles, as malformations and

abnormalities after 48 weeks were 38% for both 0.01 and 10 nM OP, 42% for UVBR only and 50 to 59% in the UVBR+OP treatments compared to 27% in the control group (Figure 2.3). Statistical analyses indicated a significant effect of UVBR ($p < 0.0001$) and OP alone on the frequency of affected tadpoles. To determine if there was an effect of OP concentration, OP1 (0.01 nM) and OP2 (10 nM) were systematically removed from the log-linear model. The effects of both OP1 ($p = 0.0003$) and OP2 ($p = 0.0077$) were statistically significant, indicating that the two concentrations of OP affected percent malformations and abnormalities observed at week 48. A significant effect of the interaction term UVBR+OP was not observed on the number of animals with malformations and abnormalities.

A total of 13 different tadpole malformations and abnormalities were identified (Table 2.1). The categories of malformations observed include (following Meteyer, 2000): spinal curvature (describes both lateral and dorsal ventral deviations in the normal straight line of the spine, which are differentiated as scoliosis or kyphosis in Meteyer, 2000); polydactyly (duplications of metatarsal bones with or without a complete set of phalanges); ectrodactyly (completely missing digit); microphthalmia (small eye); amelia (no evidence of a limb); polyphalangy (duplicate sets of phalanges); and rotation (distortion of the direction of bone growth which makes the orientation of the limb and foot abnormal). The categories of abnormalities observed include: different types of edema (excessive accumulation of fluid, swelling); abdominal bloating (enlargement of the stomach, which was filled with compacted stomach contents upon dissection); and other (various types of abnormalities that have not been documented in the literature).

Throughout the experiment, no more than one developmental anomaly per animal was detected. The most frequently observed malformation in this study was spinal curvature (Figure 2.4A). Figures 2.4, B, C, and D consist of pictures of unaffected tadpoles for comparison. Animals with spinal curvature swam in circles and were unable to forage for food. The degree of spinal curvature observed ranged from moderate tail flexure to clearly curved spines, with the majority of the observations in the later category. After 48 weeks, percentages of tadpoles affected by spinal curvature in the UVBR (30%) and UVBR+OP (39%) treatments were approximately twice as high as in the control group (16%) (Table 2.1). Over 75% of all cases of spinal curvature occurred within the first month of the experiment and none of the tadpoles with this condition survived. Abdominal bloating and different types of edema were also common in this study and were more prevalent after the third month of exposure. These abnormalities were also fatal to animals. Tadpoles with abdominal edema (Figure 2.4B) would float at the surface of the water column and were unable to forage for food that had settled at the bottom of the bowl. The stomachs of tadpoles with abdominal bloating (Figures 2.4, E and F) were filled with compacted stomach contents. Tadpoles with abdominal bloating were fed and transferred to clean water for three days to observe if defecation would occur in these animals; they did not defecate during this period. More cases of abdominal bloating occurred in the UVBR (5.2%), OP1 (4.8%) and UVBR+OP1 (9.2%) treatments than in the control treatment (1.6%) (Table 2.1). The various limb and digit malformations were generally not as common; these were not fatal to animals and were observed throughout months four to eleven.

2.3.3 Rate of Development, Metamorphosis and Growth

The development of tadpoles exposed to UVBR and OP alone was approximately two to three weeks delayed relative to control animals at stage 29 (hind limb bud development). Additionally, the development of animals treated to UVBR+OP was approximately three to six weeks delayed as compared to those in the control group. By week 45, all UVBR treated animals were approximately two to four months delayed in development compared to those in the control and OP treatments. The median developmental stage of tadpoles in UVBR treatments at week 45 was Gosner 31 and 32 (foot paddle development) whereas tadpoles in OP and control treatments were at stages 33 and 34 (early development of digits). There was a significant effect of UVBR on the frequency of remaining tadpoles developing past stage 34 at week 45 ($p < 0.0001$). For example, percentages of tadpoles developing past stage 34 in the UVBR and UVBR+OP groups were 10 to 17%, whereas those in the control, OP1 and OP2 treatments were 29%, 41%, and 26%, respectively (Figure 2.5). There was no significant effect of OP or the interaction term UVBR+OP on the number of animals developed past stage 34 at week 45.

By the end of the experiment, only 0.5 to 2% of tadpoles exposed to UVBR+OP and 2% of those exposed to UVBR alone metamorphosed, compared to 6 to 10% in other treatments including controls (Figure 2.6A). Although a significant effect of OP or UVBR+OP was not found on the incidence of metamorphosed animals, the log linear model indicated a significant overall effect of UVBR on this endpoint ($p < 0.0001$). The percentage of tadpoles to reach metamorphosis out of those that survived until the end of the experiment was also analyzed (by correcting the sample size for mortality attributable

to treatment). The same trend was observed, as 2 to 8% of tadpoles exposed to UVBR+OP and 7% of those exposed to UVBR alone metamorphosed, compared to 17 to 30% in other treatments (Figure 2.6B). Statistical analyses also indicated that there was a significant effect of UVBR on percentage of surviving tadpoles to metamorphose by week 48 ($p < 0.0001$). However, there was no effect of treatments on the weights of animals at week 42 or on the age, snout-vent length (SVL) and weights of tadpoles at forelimb emergence (FLE) (Table 2.2).

Table 2.1 Total percentages of 13 different categories of malformations and abnormalities observed per treatment during the 48-week experiment.

Categories	Treatments ^a					
	C	OP1	OP2	UVBR	UVBR + OP1	UVBR+ OP2
	(126) ^b	(165)	(162)	(153)	(153)	(172)
Malformations ^c						
Spinal Curvature ^d	15.9	18.1	21.6	30	38.6	39.0
Polydactyly	0.8	1.8	1.9	0	0.7	0.6
Ectrodactyly	0.8	0.6	1.9	0	0	0.6
Microphthalmia	0	0.6	0.6	0	0.7	0
Amelia	0	0	0	0	0	1.2
Polyphalangy	0	0	0	0	0.7	0
Rotation	0	0.6	0	0	0	0
Abnormalities ^e						
Abdominal edema ^f	6.3	7.3	4.9	3.3	7.2	5.2
Abdominal bloating ^g	1.6	4.8	1.2	5.2	9.2	2.3
Edema of legs	0	2.4	2.5	1.8	0	0
Other ^h	0.8	0.6	2.5	2.0	0.7	0
Edema of branchial chambers	0.8	0.6	1.2	0	0	0
Edema of head	0	0	0	0	1.3	0.6
Total	27.0	37.4	38.3	42.3	59.1	49.5

^a C, Control; UVBR, UVB radiation; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol.

^b Numbers in parentheses represent sample size.

^c Represent intrinsic errors in development, errors in chemical communication or in the translation of genetic information. Malformations listed above were classified following Meteyer (2000).

^d This term includes both lateral and dorsal ventral deviations in the normal straight line of the spine (which are differentiated as scoliosis or kyphosis in Meteyer, 2000) (Figure 2.4A).

^e Represent any abnormal developmental finding for which we have little or no information.

^f Edema, excessive accumulation of fluid (swelling) (Figure 2.4B).

^g Enlargement of the stomach, which was filled with compacted stomach contents upon dissection (Figures 2.4, E and F).

^h Various types of abnormalities that have not been documented in the literature.

Table 2.2 Average weight of *Rana pipiens* tadpoles at week 42 and age, snout-vent length and weight of tadpoles at forelimb emergence.

Treatments ^a	Weight (g) week 42 ^b	Age (days) FLE ^c	SVL (mm) FLE	Weight (g) FLE
C	0.89 ± 0.07 ^d	287.1 ± 11.9 (12) ^e	18.3 ± 0.24	1.06 ± 0.06
OP1	1.01 ± 0.06	276.1 ± 13.3 (16)	19.3 ± 0.44	1.24 ± 0.06
OP2	0.90 ± 0.02	279 ± 12.3 (10)	18.6 ± 0.62	1.22 ± 0.14
UVBR	0.74 ± 0.04	277.7 ± 9.5 (3)	19.4 ± 0.32	1.17 ± 0.05
UVBR+OP1	0.73 ± 0.04	300 ± 33.3 (3)	18.0 ± 0.28	1.14 ± 0.01
UVBR+OP2	0.80 ± 0.06	366 (1)	23.71	1.93

^a C, Control; UVBR, UVB radiation; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol.

^b Sample size is 50 tadpoles per treatment.

^c FLE, forelimb emergence (Gosner stage 42).

^d Standard error of the mean.

^e Sample size at FLE is given in parentheses.

Note. Statistical analyses indicated no effect of treatments on these endpoints ($p \geq 0.05$).

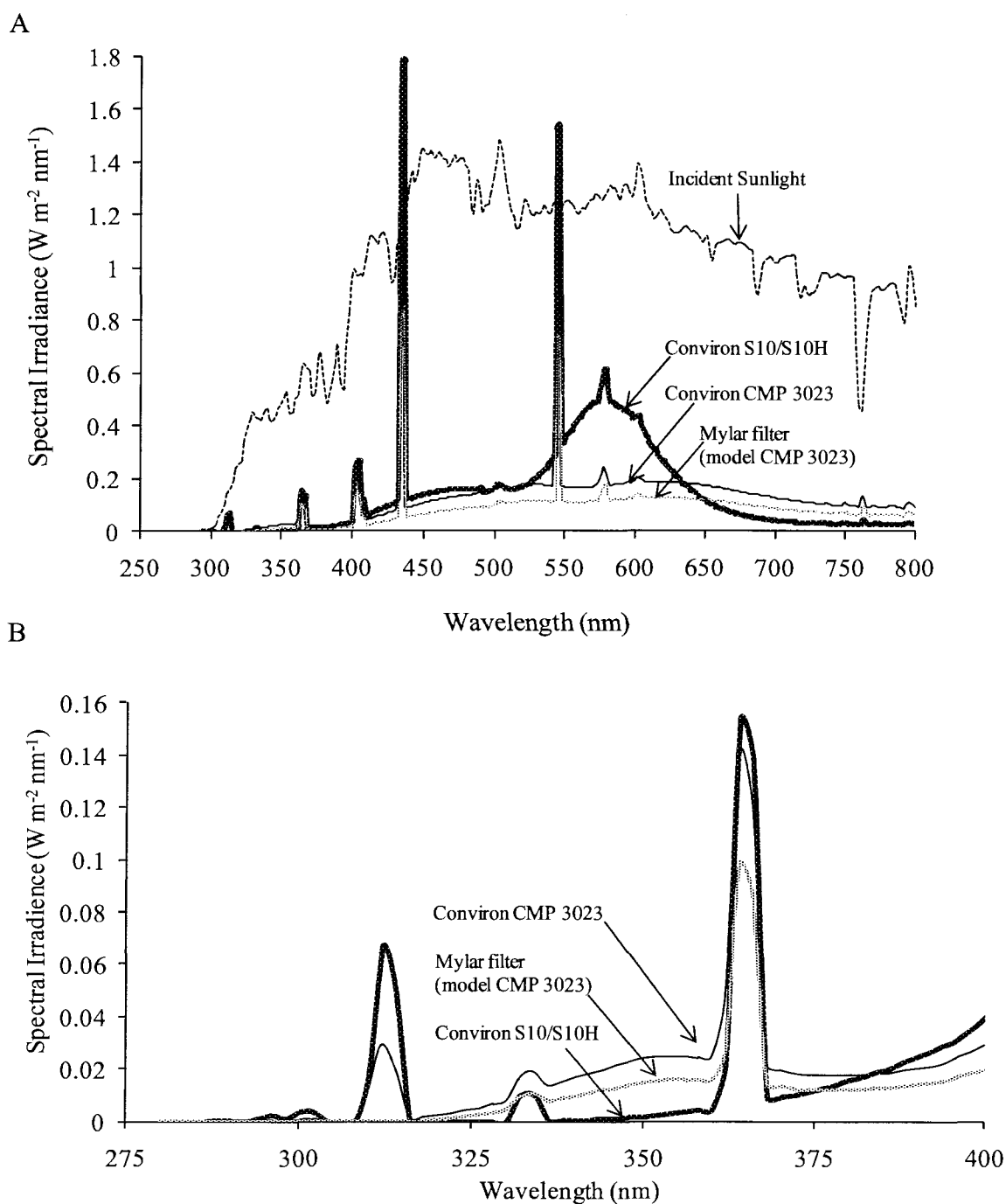


Figure 2.1 (A) Spectral irradiance inside environmental chambers and with Mylar filter (inside model CMP 3023) compared to incident sunlight spectral irradiance measured in Ottawa on May 22, 2002 from 12:37-12:50 h with the Optronics OL754-C spectroradiometer. (B) Spectral irradiance of the UVR region only (280 to 400 nm) inside the environmental chambers.

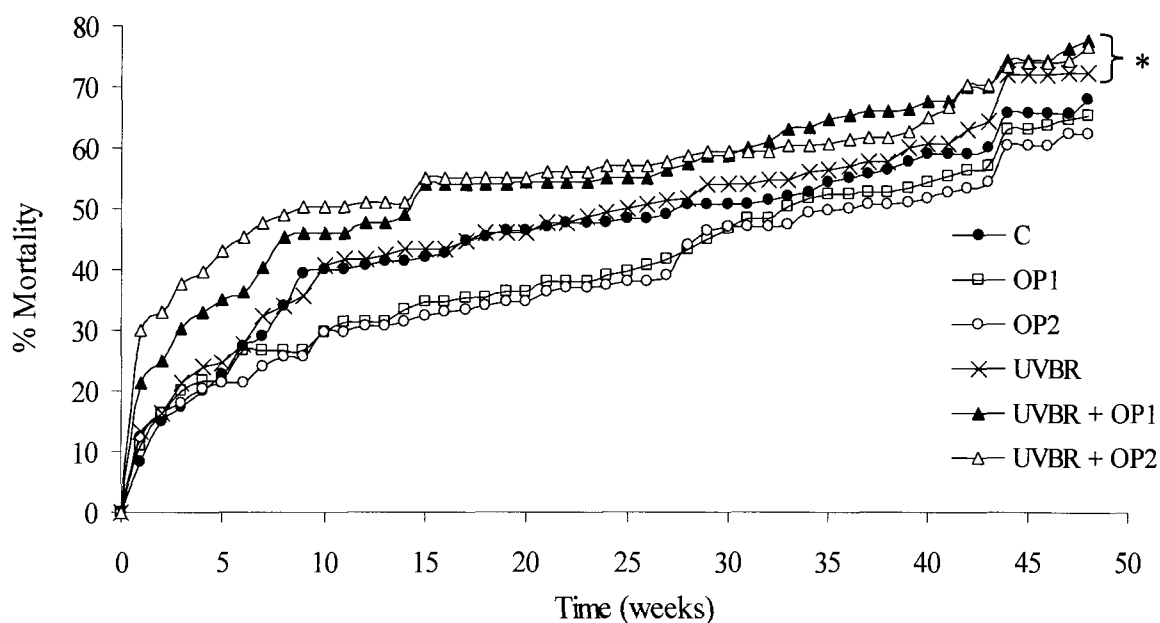


Figure 2.2 Cumulative percentages of tadpole mortalities per treatment with time. C, Control; UVBR, UVB radiation; OP1, 0.01 nM 4-tert-octylphenol; OP2, 10 nM 4-tert-octylphenol. Sample size at week 48 ranged from 126 to 172 tadpoles per treatment. Asterisk (*) denotes a significant effect of UVBR ($p=0.002$) on mortality at week 48.

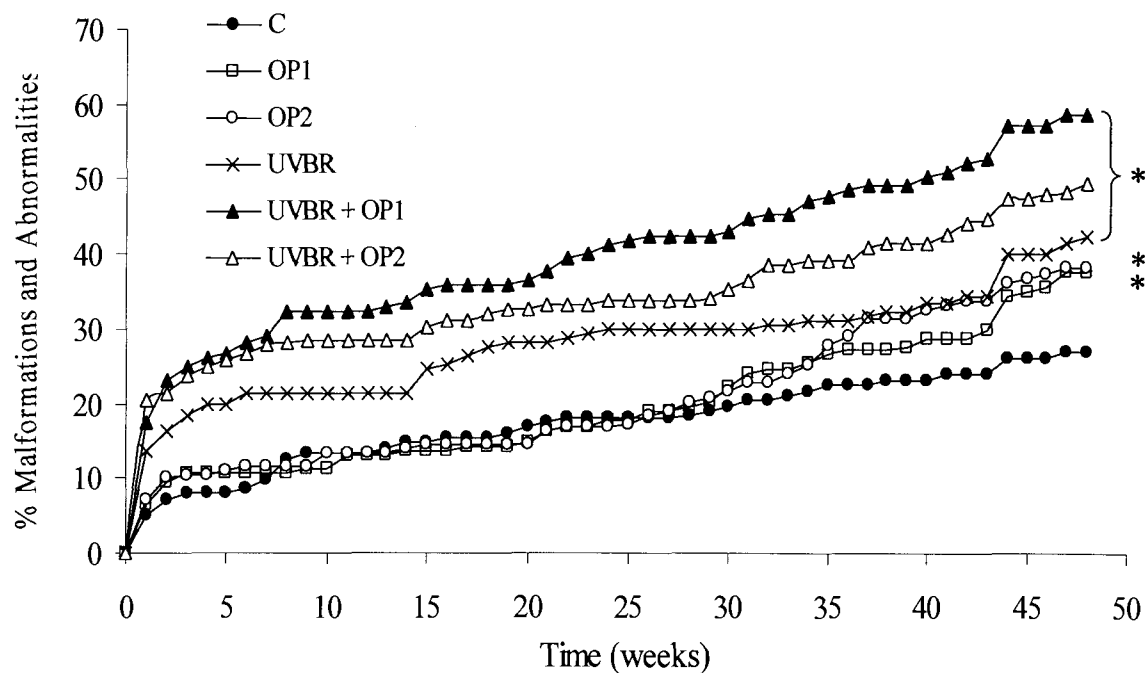


Figure 2.3 Cumulative percentages of tadpole malformations and abnormalities per treatment with time. C, Control; UVBR, UVB radiation; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol. Sample size at week 48 ranged from 126 to 172 tadpoles per treatment. Asterisk (*) denotes a significant effect of UVBR ($p < 0.0001$) and OP1 ($p = 0.0003$) and OP2 ($p = 0.0077$) on the frequency of abnormalities and malformations at week 48.

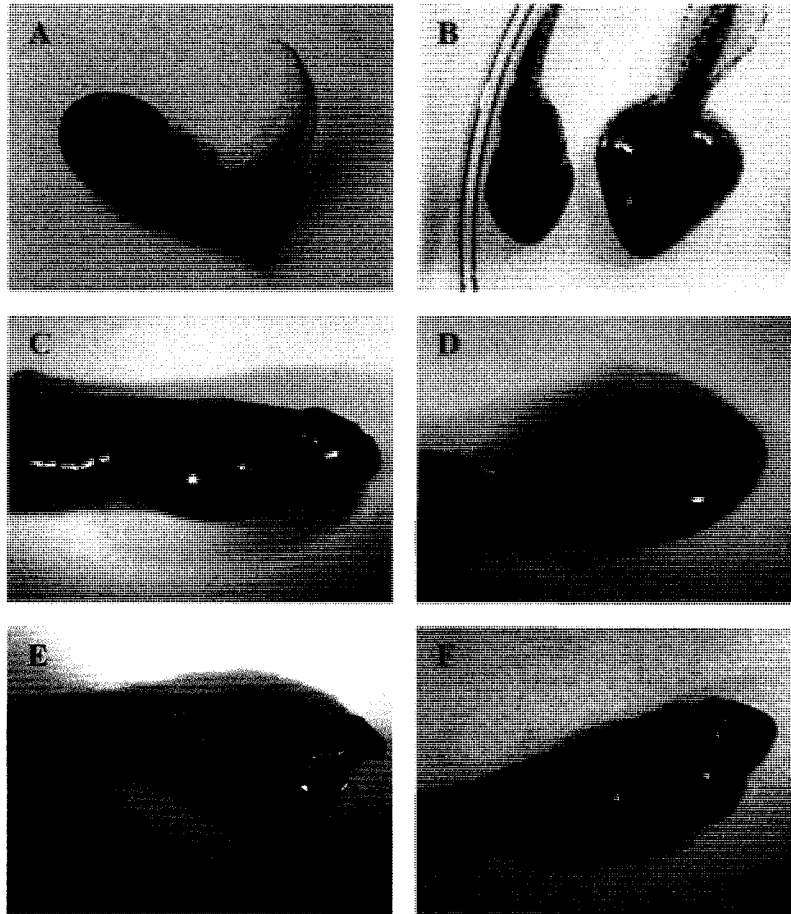


Figure 2.4 Most frequently observed tadpole malformations and abnormalities in this study: (A) curvature of the spine; (B) unaffected tadpole (left) and tadpole with abdominal edema (right); (C, D) side and ventral view of an unaffected tadpole; and (E, F) side and ventral view of a tadpole with abdominal bloating, consisting of an enlarged stomach filled with compacted stomach contents upon dissection.

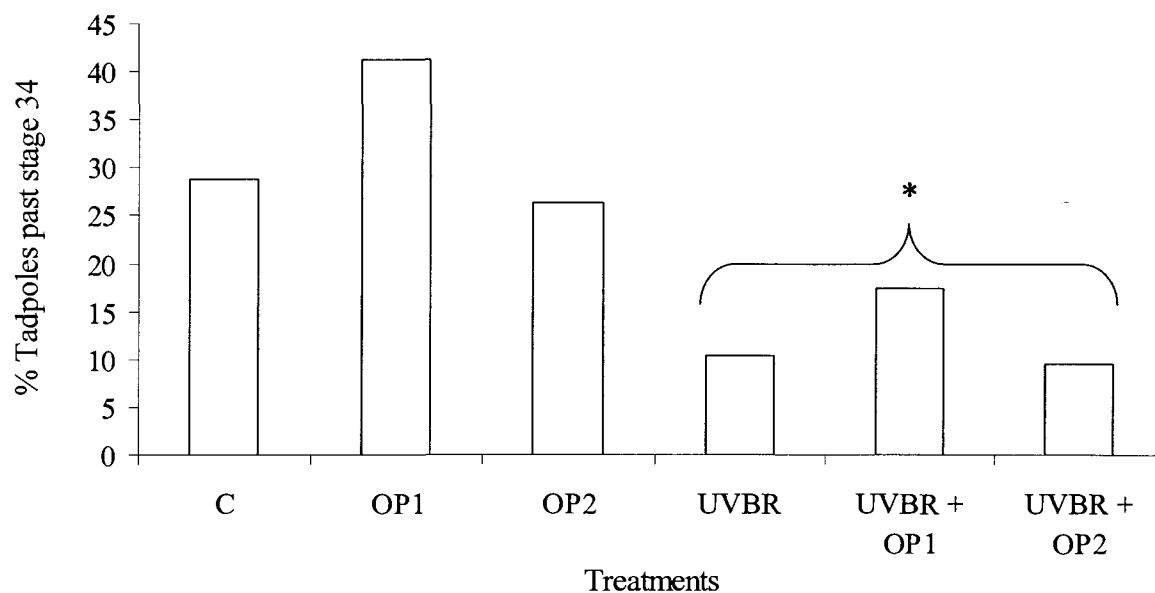


Figure 2.5 Tadpole development after 45 weeks, measured by the percentage of remaining tadpoles developed past Gosner stage 34 per treatment (including animals that survived to metamorphosis). C, Control; UVBR, UVB radiation; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol. Sample size ranged from 46 to 80 tadpoles per treatment. Asterisk (*) denotes a significant effect of UVBR ($p < 0.0001$) on the frequency of tadpoles developed past stage 34 at week 45.

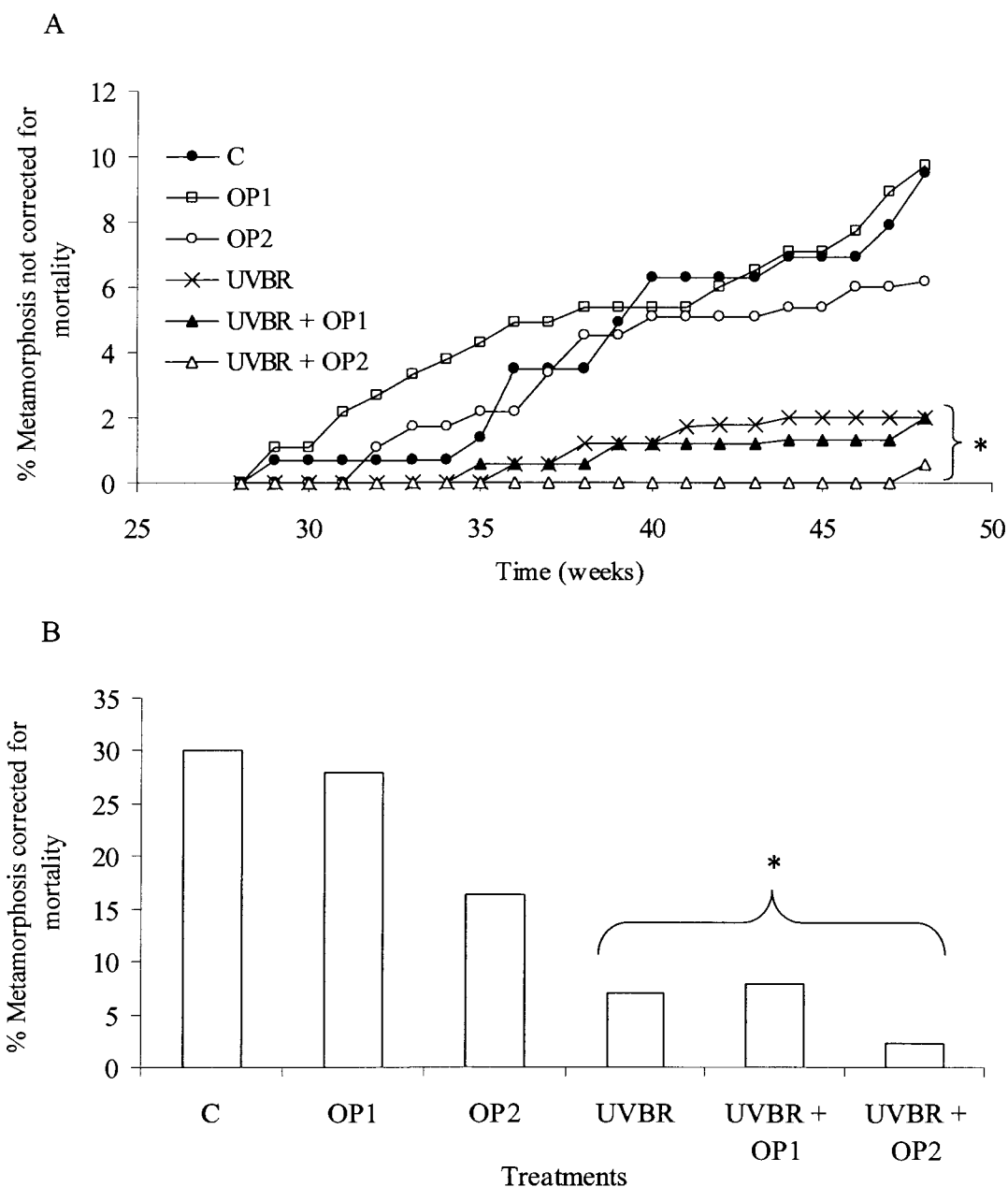


Figure 2.6 (A) Cumulative percentages of tadpoles to metamorphose per treatment throughout the 48-week experiment, without correcting for mortality of tadpoles attributable to treatment. Sample size at week 48 ranged from 126 to 172 tadpoles per treatment. Asterisk (*) denotes a significant effect of UVBR on the frequency of tadpoles to metamorphose at week 48 ($p < 0.0001$). (B) Percentage of tadpoles to metamorphose by week 48, with a correction for mortality of tadpoles attributable to treatment. Sample size ranged from 40 to 61 tadpoles per treatment. Asterisk (*) denotes a significant effect of UVBR on the frequency of tadpoles to metamorphose at week 48 ($p < 0.0001$). C, Control; UVBR, UVB radiation; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol.

2.4 DISCUSSION

Tadpoles were slower to develop in this study compared to other lab studies with *R. pipiens* from more southerly North American populations (*e.g.*, 52 days for the first tadpole to reach FLE in Ankley *et al.*, 2000) (Ankley *et al.*, 1998 and 2000). To approximate existing water temperatures to which these tadpoles would typically be exposed to in our region, the incubators were set so that the water temperature gradually increased to $20\pm 1^{\circ}\text{C}$ during the light cycle and gradually decreased to 16°C during the dark cycle. Since temperature (Dodd and Dodd, 1976) and overcrowding (Gromko *et al.*, 1973) are factors that may negatively affect amphibian development, it was originally considered that the low water temperature during the dark cycle may have caused the long development times and low metamorphic rates observed in all treatments. It is unlikely that rearing density was a factor involved in the slow development of tadpoles, as the density was kept at recommended levels and water quality remained high throughout the experiment. Our lab used the same healthy population of *R. pipiens* in other experiments and observed similar developmental rates and mortality levels when using lower rearing densities and/or constant water temperatures (unpublished results). It is well known that anuran larval development exhibits remarkable plasticity such that the variance in the timing of metamorphosis can be up to 90% within species, depending largely on genetics and environmental conditions (Crespi and Denver, 2005).

High mortality levels in our controls were not unexpected given the length of the experiment in the environmental chambers. The percentage of tadpole mortality in control treatments can be highly variable in a lab setting, such as 26% (Ankley *et al.*, 2000), 38% (Howe *et al.*, 2004) and 25 to 50% (Harris *et al.*, 1998) observed with *R.*

pipiens, and 79% observed with *Rana clamitans* (Green frog) (Coady *et al.*, 2004). Many reported mortality levels are lower than those reported in the present study, but mortality is often only calculated for much shorter exposure periods and therefore does not always reflect the number of mortalities that may have occurred if the experiment had lasted for several months, or what might be typical in the wild.

In our experiment, chronic exposure of tadpoles to sub-ambient levels of UVBR exerted adverse effects on tadpole survival, development, and metamorphosis. All animals exposed to UVBR alone and in combination with OP had increased levels of mortality and developmental anomalies, which were most substantial after one week of exposure when tadpoles were at stage 25, and continued for the first two months of the experiment (when stages ranged from 25 to 31). Previous studies on *R. pipiens* demonstrated a similar developmental period of enhanced sensitivity to UVBR (extending from hatch to stages 25 to 30), with observations of increased mortality and incidences of hind limb malformations (Ankley *et al.*, 1998, 2000 and 2002). Although UVBR was shown to adversely affect survival and/or development in different species of amphibian embryos and larvae (Crump, 2001), research also demonstrated that larvae are more sensitive to UVBR exposure than embryos in terms of decreased survival and increased occurrence of malformations (Tietge *et al.*, 2001; Ankley *et al.*, 1998, 2000 and 2002, Weyrauch and Grubb, 2006). For example, the outdoor exposure of *Rana septentrionalis* (Mink frog), *R. clamitans* and *R. pipiens* embryos and larvae to ambient solar UVBR only produced significant mortality of early-stage tadpoles and not of embryos or late larval stages, indicating a period of enhanced sensitivity during early larval stages (Tietge *et al.*, 2001). This underscores the importance of investigating the

effects of UVBR not only on the developing embryo but also on early stages of larval development, to ensure that the adverse effects of UVBR on amphibians are not underestimated.

Low environmental levels of OP did not produce significant mortality in chronically exposed tadpoles. However, both levels of OP (0.01 and 10 nM) were sufficient to produce an increase in the occurrence of various types of developmental anomalies. Rohr *et al.* (2003) reported similar findings after a 37-day exposure of *Ambystoma barbouri* (Streamside salamander) to 24.3 nM OP; this higher level of OP had no effect on mortality but induced a significant number of limb malformations.

Very few studies examined and categorized in detail the normal and abnormal development in *R. pipiens* tadpoles over the extensive time period of this lab study. Our study documents the type and timing of all malformations and abnormalities observed throughout the experiment, making it relatively novel. Most studies only state the types of developmental anomalies observed in embryos or tadpoles without quantification, choose one type of anomaly to quantify, or focus on gross malformations in adult amphibians. Spinal curvature accounted for much of the significant difference in total percent developmental anomalies observed between control and UVBR treated tadpoles. Other studies also reported the occurrence of spinal curvature in amphibians exposed to UVBR (Hays *et al.*, 1996; Blaustein *et al.*, 1997; Pakkala *et al.*, 2001; Weyrauch and Grubb, 2006). To our knowledge, abdominal bloating has not been reported previously in the literature. Although the significance of these results in terms of amphibian malformations in the wild remains uncertain, they indicate that OP and UVBR at levels currently found

in the natural habitat of amphibians might have a negative impact on the development of tadpoles.

Exposure to UVBR with or without OP produced a significant delay in the rate of tadpole development (a two to four month delay) and a decrease in percent tadpoles to reach metamorphosis. The delay in development occurred when the median development stage of tadpoles in the control group was stage 29. After 45 weeks, we did not see a high percentage of tadpoles exposed to UVBR develop past stage 34 compared to the controls. These findings corroborate a preliminary study conducted in our lab on the chronic effects of environmental levels of UVBR and OP on *R. pipiens* tadpoles (Croteau *et al.*, 2001), which demonstrated a delay in development, inhibition of metamorphosis (none metamorphosed in UVBR treatments after six months of exposure) and none of the animals exposed to UVBR alone and in combination with OP developing past stage 34. Other studies also reported that exposure to UVBR delays amphibian development and/or delays or blocks metamorphosis under laboratory conditions (Pahkala *et al.*, 2001 and 2003) and in the field (Smith *et al.*, 2000; Belden and Blaustein, 2002b; Blaustein *et al.*, 1997). It is the thyroid system that mediates amphibian development and metamorphosis. Interestingly, the UVBR exposed tadpoles rarely developed past stage 34, which is approximately the time when the thyroid gland is functional and thyroid hormone (TH) levels start increasing (Shi, 2000). DNA damage induced by exposure to UVBR may result in a shift of energetics towards repair, which might reduce energy availability for other physiological processes such as development and metamorphosis. While it is clear that UVBR may delay development and metamorphosis in *R. pipiens*, the mechanisms involved remain to be investigated.

There still exists conflicting data in the literature on the effects of UVBR on amphibian health. For example, some studies found that exposure of amphibian embryos or larvae to UVBR had no effect on mortality (Pahkala *et al.*, 2000 and 2003b; Smith *et al.*, 2000) or the occurrence of developmental anomalies (Pahkala *et al.*, 2000 and 2003a). However, the effects of UVBR on amphibians may vary between species or between populations of the same species. For example, embryos of species that were most resistant to UVBR in field exposures (*e.g.*, *Hyla regilla* [Pacific treefrog], *Rana aurora* [Red-legged frog], *R. pretiosa* [Oregon spotted frog], and *R. luteiventris* [Columbia spotted frog]) had higher cyclobutane pyrimidine dimer (CPD) photolyase activity (enzyme that repairs DNA damage) than the embryos of species more susceptible to the effects of UVBR (*e.g.*, *Bufo boreas* [Western toad], *Ambystoma macrodactylum* [Long-toed salamander], *Rana cascadae* [Cascades frog]) and *A. gracile* [Northwestern salamander] (Blaustein *et al.*, 2003). Populations that are naturally exposed to elevated levels of UVBR, such as those living in elevated regions (*e.g.*, mountain populations), may be more adapted to cope with the effects of radiation and have higher survival rates (Belden and Blaustein, 2002a). Recently, Weyrauch and Grubb (2006) exposed 12 populations of *R. sylvatica* to UVBR and demonstrated that populations with low genetic diversity experienced higher tadpole mortality rates than those with high genetic diversity. Differences in larval tolerance to UVBR may also exist within the same population, as suggested by the survival and metamorphosis of some exposed tadpoles in the present study. Consequently, the use of different species, different populations of the same species or different methodologies (*e.g.*, different equipment, laboratory vs. field

exposures) are important factors to consider when interpreting the results of studies regarding the effects of UVBR on amphibians.

This study is the first to examine the effects of chronic exposure of developing amphibians to the combination of UVBR and the estrogenic chemical 4-*tert*-octylphenol. Although other studies have identified photodegradation products of NP and 4-OP exposed to visible light and/or UVR in aqueous solution under various conditions (Brand *et al.*, 2000; Neamtu and Frimmel, 2006), a statistically significant effect of the combination of UVBR and OP was not observed on the endpoints measured in this study. However, it was found that simultaneous exposure to these two stressors was frequently more harmful to tadpoles than exposure to one single stressor, suggesting that the severity of combined exposure may have biological significance. A previous study exposing *R. pipiens* tadpoles for 10 days demonstrated an interaction between UVBR and OP producing increased body weight and earlier hind limb emergence (Crump *et al.*, 2002). Other APEO metabolites and phenolic compounds may interact with UVBR to interfere with amphibian survival, development and metamorphosis. For example, a 20-day exposure of *Rana temporaria* (Common frog) embryos to bisphenol A (BPA) and UVBR showed that 1000 µg/L BPA exerted a significant negative effect on the survival of embryos in both the UVBR and no-UVBR treatments, with the effect being greater with the addition of UVBR (Koponen and Kukkonen, 2002). Studies on multiple stressors are essential, because it is likely that numerous anthropogenic factors are acting in concert to contribute to current amphibian losses in the wild.

In conclusion, our results show that exposure to UVBR or OP exerts a negative effect on amphibian survival and/or development. The most frequently observed

malformation in this study consisted of spinal curvature, which mainly occurred during the first month of the experiment. The observed delay in development at stages 29/34 and significantly delayed metamorphosis induced by UVBR suggest a disruption of the thyroid axis. This was independent of any effect on growth since tadpoles at the end of the study were of similar size and weight in all groups. Likely mechanisms whereby UVBR may suppress metamorphosis that have not been investigated in any amphibian species include decreased production of TH or disruption of the expression of gene coding for key proteins regulating TH action (*e.g.*, TH receptors, deiodinases).

CHAPTER 3

Molecular and morphological responses to 3,5,3'-triiodothyronine (T3) in *Rana pipiens* tadpoles exposed to UVB radiation and 4-*tert*-octylphenol

3.1 INTRODUCTION

Previous studies from our lab have demonstrated that exposure of *Rana pipiens* (Northern leopard frog) tadpoles to environmental levels of UVBR and/or the estrogenic chemical 4-*tert*-octylphenol (OP) can alter developmental rates and metamorphosis (Croteau *et al.*, 2001; Crump *et al.*, 2002; Chapter 2, Croteau *et al.*, 2008b). For example, chronic exposure (eight months) of *R. pipiens* tadpoles to UVBR and/or OP resulted in a developmental delay of animals apparent at approximately Gosner developmental stage 29 (end of hind limb bud development; Gosner, 1960). By the end of the experiment, a significantly smaller percentage of animals exposed to UVBR (combined with or without OP) had developed past Gosner stage 34 (early development of digits) and a significantly lower percentage of animals reached metamorphosis when compared to those exposed to OP alone or in the control group (Chapter 2, Croteau *et al.*, 2008b). A preliminary study indicated similar results, as all animals exposed to UVBR (combined with or without OP) were delayed in development and none metamorphosed or developed past Gosner stage 34 after six months of exposure (Croteau *et al.*, 2001). Crump *et al.* (2002) found that a 10-day exposure of *R. pipiens* tadpoles early in development to UVBR and 4-OP together caused an acceleration of hind limb emergence (Gosner stage 36); exposure to UVBR or 4-OP alone however had no effect on this endpoint (Crump *et al.*, 2002).

Disruptions in the rate of development and metamorphosis of amphibians subjected to environmental or enhanced levels of UVBR have been reported in a number of published studies (reviewed in Croteau *et al.*, 2008a). Several estrogenic chemicals have also been shown to affect the timing of development and/or metamorphosis in different amphibian species (*e.g.*, diethylstilbestrol (DES), Frieden and Naile, 1955; bisphenol A, Iwamuro *et al.*, 2003; ethinylestradiol, Hogan *et al.*, 2008a; nonylphenol, Fort *et al.*, 2000 and Christensen *et al.*, 2005; methoxychlor, Fort *et al.*, 2004a and 2004b). Acceleration in the development and metamorphosis of animals in the wild could result in a decreased size at metamorphosis, which in turn could affect fitness and lead to a decrease in population size (Crump, 2001). Conversely, a decrease in developmental rates could impact individuals by increasing the risk of predation and the amount of time that animals are in contact with, and are therefore affected by environmental stressors such as UVBR and OP.

Metamorphosis in anurans, which includes the transition of tadpoles to frogs, involves the transformation of most tadpole organs to their adult forms, such as the development of hind limbs, remodeling of the brain and the complete resorption of the tadpole tail during late metamorphosis. The morphological changes of different organs and tissues occur at distinct developmental stages mediated by the action of thyroid hormones (Shi, 2000). Corticotropin-releasing hormone (CRH) is a neuropeptide released from the hypothalamus that stimulates the pituitary to release thyroid stimulating hormone (TSH) (Denver, 1988; Denver and Licht, 1989), a hormone that stimulates the synthesis of the thyroid hormones T4 (thyroxine) and T3 (3,5,3'-triiodthyronine) (Denver, 1997; Tata, 1999; Figure 1.2). Deiodinase enzymes D1 and D2 (or type 1 and 2)

convert T4 to the biologically active T3, whereas D1 and D3 (deiodinase type 3) convert T4 and T3 to inactive forms of TH (to reverse T3 and T2, respectively) (St. Germain, 1994; Denver *et al.*, 2002; Dubois *et al.*, 2006; Kuiper *et al.*, 2006). TH levels are relatively low during premetamorphosis (hind limb bud growth) and gradually rise during prometamorphosis (to promote the rapid development of digits and hind limb growth), reaching peak levels at metamorphic climax (forelimb emergence and tail resorption) (Etkin, 1968; Dodd and Dodd, 1976; OECD, 2004). In *Xenopus laevis* (African clawed frog), TR alpha genes are activated shortly after hatch and their expression remains at high levels throughout metamorphosis. The TR beta genes are lowly expressed early in tadpole development (reviewed in Shi, 2000). However, T3 positively regulates the expression of its own receptor (autoregulation) and there is a rapid upregulation of TR beta gene expression coinciding with increasing T3 levels (Tata, 1999; Ulisse *et al.*, 1996; Shreiber *et al.*, 2001; Opitz *et al.*, 2006; Buchholtz *et al.*, 2006).

The endocrine control of amphibian development and metamorphosis through the hypothalamo-pituitary-thyroid (HPT) axis is very complex, and is regulated by external environmental factors and internal hormonal cues (Denver, 1997; Hayes, 1997b; Rose, 2005; Figure 1.2). As such, an upset in the hormonal balance of developing tadpoles by endocrine disrupting chemicals such as OP or environmental stressors such as increased exposure to UVBR could potentially cause a disruption in developmental and metamorphic processes. Although it is clear that UVBR and/or OP (as well as other estrogenic chemicals) have the potential to disrupt the coordinated development of tadpoles through to metamorphosis, the mechanism(s) by which UVBR with or without

OP exert these sublethal effects, which may include disruptions of TH-mediated processes, have not yet been examined.

Tadpoles are competent to respond to exogenous TH shortly after hatching, even though natural metamorphosis does not occur until endogenous TH has been produced (Shi *et al.*, 1996). Exposure to both T3 and T4 can activate TH-dependent target genes and induce precocious metamorphosis (Shi, 2000). Therefore, the examination of TR-mediated biological effects of T3 such as amphibian metamorphosis may be useful in assessing the potential of a stressor such as UVBR and OP in disrupting the thyroid system (Eales *et al.*, 1999). Since premetamorphic *R. pipiens* tadpoles chronically exposed to UVBR/OP exhibited delays in development prior to the expected rise in TH levels (at approximately stages 29-34) (Croteau, 2001; Croteau *et al.*, 2008b), exposure to exogenous T3 to induce TH-dependent gene expression or precocious metamorphosis would assess how animals exposed to UVBR/OP could respond to increased levels of TH.

The present study investigates the effects of pre-exposure to UVBR/OP on premetamorphic *R. pipiens* tadpoles subsequently challenged to T3, to determine if UVBR and/or OP exposure disrupts the molecular and morphological responses of tadpoles to T3. TH-responsive genes and genes involved in the endocrine control of development and metamorphosis were targeted, to determine if UVBR/OP alone affected the expression of these genes as well as their TH-induced response. Changes in the expression of TR (alpha and beta), deiodinase (D2 and D3) and CRH genes in tail and/or whole brain tissue were measured after 24h exposure to T3, in tadpoles pre-exposed to UVBR and/or OP. Changes in morphometric endpoints expected from the precocious

induction of metamorphosis including body weight and SVL, tail length, development and forelimb emergence, were also measured after seven days of exposure to T3.

3.2 MATERIALS AND METHODS

3.2.1 Animal Collection and Care

Portions of five naturally fertilized *R. pipiens* egg masses were collected in May 2005. Information on the location from which the animals were collected and laboratory rearing conditions are described in Chapter 2 (section 2.2.1).

3.2.2 Experimental Design

Figure 3.1 illustrates the experimental design and timeline of the study. On May 18, a total of 640 tadpoles (Gosner stage 25, beginning of independent feeding; Gosner, 1960) were randomly placed in four different exposure treatments (n=160 animals/treatment group). The treatments included: (1) a 0.01% ethanol solvent control; (2) an environmentally relevant sublethal concentration of 4-*tert*-octylphenol (OP) (10 nM); (3) a subambient level of UVBR (average of 0.15 W/m²); and (4) OP and UVBR combined. Animals were continuously exposed to these treatments until a subset was either used in Experiment 1 (n=125) or in Experiment 2 (n=110). In both experiments, animals pre-exposed to UVBR and/or OP were then exposed to triiodothyronine (T3) to determine if UVBR and OP could disrupt the endocrine control of development and metamorphosis by affecting molecular and morphological responses of tadpoles to T3. The developmental stages and body weights (wet weight) of tadpoles were also measured to determine if UVBR and/or OP affected these endpoints (Figure 3.1).

3.2.3 Conditions of Exposure to UVBR and OP

The water used in the exposure was as described in Chapter 2 (section 2.2.1) at time of water renewal, with the exception of the water temperature (21 to 22°C) and pH (6.7 to 7.5). The water was renewed six times weekly for the duration of the exposure period and animals were fed *ad libitum* with Nutrafin® fish flakes (Rolf C. Hagen Inc., Montréal, QC) and tadpole food (Carolina Biological Supply Co., Burlington, NC) (approximately equal amounts) on the mornings the water was changed. Tadpole rearing densities were adjusted by adding replicate bowls to reduce the number of animals per litre of water. For the first week of exposure, 160 animals weighing 0.03-0.04 g were divided among eight Pyrex bowls per treatment (20 tadpoles/bowl), each filled with 1L of water. The density was gradually reduced to 8 to 10 tadpoles/L by the second month of the experiment and to 5 to 7 tadpoles/L by the end of the experiment. Dissolved O₂ and pH were measured over the course of the study using an Oxyguard® Handy MK III O₂ meter (Birkerød, Denmark) and Accumet Basic pH meter (Fisher Scientific Company, Ottawa, ON), and levels were maintained between 6 to 8 mg/L and 6.7 to 7.5, respectively. Ammonia levels were measured throughout the experiment using the QuickChem method 10-107-06-1-J (Ammonia (phenolate) in potable and surface waters) and were below the detection limit of 0.002 mg N/L as NH₃ (Lachat Instruments, QuickChem 8000 flow injection analysis; Loveland, CO).

3.2.4 Controlled Environmental Chambers

Tadpoles were kept in two controlled environmental chambers (Conviron model CMP 4030; Winnipeg, MB) for the duration of the experiment. The solar simulators were composed of black, fluorescent and incandescent lights as sources of UVAR, UVBR and

visible light (VIS). Incubators 1 and 2 contained ten and twelve fluorescent lights (Philips F72T8/TL841/HO 65W U.S.A.), respectively. Both incubators contained nine incandescent bulbs (Country 60W/130V Mexico) and two black lights (Sylvania 350 Blacklight F20T12/350BL 20W Canada). UVBR was obtained by removing the protective cover that usually shields the lamps. Shelves were positioned at 46 to 53 cm below the lamps to attain the desired UVAR, UVBR and VIS intensities. The photoperiod was the same as in Chapter 2 (section 2.2.3). The temperature of the air inside incubators 1 and 2 was set at 16 and 17 °C during the light cycle and 23 and 24°C during the dark cycle, respectively, to maintain the water temperature at $21 \pm 1^\circ\text{C}$.

3.2.5 UVR Levels

An Oriel GOLDILUX (Newport Corp., Stratford, CT) radiometer/photometer GRP-1 (Model 70237) with interchangeable UVAR probe GAP-1 (Model 70237), UVBR probe GBP-1 (Model 70238) and cosine probe GLP-1 (illuminance/VIS; Model 70235) was used to measure UVR and VIS levels inside the environmental chambers. Measurements were taken in the ambient air at 18 different locations within the incubators to calculate average levels per incubator. The average irradiances measured inside incubators 1 and 2 were 0.14 W/m^2 and 0.16 W/m^2 UVBR, 1.73 W/m^2 and 1.82 W/m^2 UVAR and 30.8 klx and 38.24 klx VIS, respectively. The average irradiances for both incubators were 0.15 W/m^2 UVBR, 1.78 W/m^2 UVAR and 34.52 klx VIS. UVBR levels used in this study are considered subambient because levels have been shown to range between 1 and 2.5 W/m^2 in ambient sunlight above water in May and June in Ontario, Canada (Crump *et al.*, 1999a,b; Croteau *et al.*, 2008b) and 0.0042 to 0.72 W/m^2 in various types of aquatic environments in North America (at a depth of 10 cm; Barron

et al., 2000; Calfee and Little, 2003), depending on environmental factors. Bowls were randomly rotated daily within and between incubators to account for variations in temperature and UVR/VIS levels. Mylar filters were placed on bowls that did not include UVBR exposure, as described in Chapter 2 (section 2.2.4).

3.2.6 Exposure to 4-*tert*-Octylphenol

The level of OP used in this study is the same as in previous studies (Croteau *et al.*, 2001 and Chapter 2, Croteau *et al.*, 2008b). Tadpoles were exposed to a sublethal environmental concentration of 4-*tert*-octylphenol (OP) (10nM or 2.06 µg/L) representative of that measured in some aquatic systems [*e.g.*, 63nM in the outer Teas estuary in the United Kingdom (Blackburn and Waldock, 1995); up to 14nM in freshwater of the Delaware river (Bennie, 1999)]. Details regarding the preparation of solutions are described in Chapter 2 (section 2.2.5), with the exception of the addition of 100 µL of the stock solution (100 µM) to 1L of water in this study. Figure 3.2A shows the absorption spectrum for OP (dissolved in 95% EtOH vehicle) diluted in aquatic facility water and demonstrates an absorption peak in the UVBR range (CARY 100 BIO UV-Visible Spectrophotometer; Varian, Mulgrave, Australia).

3.2.7 Exposure to Triiodothyronine (T3)

In this study, we tested the effects of UVBR and/or OP exposure on the expression of genes involved in metamorphosis (Experiment 1) as well as morphological responses of tadpoles to T3 exposure (Experiment 2). Tadpoles pre-exposed to UVBR and/or OP were exposed to either 0, 5 or 50 nM triiodothyronine (T3) during Experiments 1 or 2. The 5 nM concentration is physiologically relevant and mimics circulating T3

levels in the blood of different amphibian species at metamorphic climax (5 to 10 nM) (reviewed in Shi, 2000 and Brown, 2005). The 50 nM concentration was chosen to induce a maximal physiological response. Stock solutions were prepared (50 and 500 μ M) by dissolving 3,5,3'-triiodo-L-thyronine (thyroid hormone or T3; Sigma Canada Ltd., Oakville, ON) in 0.1N NaOH. The nominal concentrations (5 and 50 nM) were prepared by pipetting 100 μ L of the appropriate stock solution to 1L of water. Treatments that did not include T3 received 0.01% NaOH. Stock solutions were stored at 4 °C, protected from light and thoroughly mixed prior to use. The characteristics of the water used in Experiments 1 and 2 were as described above (section 3.2.3).

Shown in Figure 3.2B is the absorption spectrum for T3 (dissolved in 0.1N NaOH vehicle) diluted in aquatic facility water (pH adjusted to 7). It demonstrates that T3 predominantly absorbs in the UVBR range (and can thus be potentially degraded by it). Tadpoles exposed to T3 in both experiments were therefore not simultaneously exposed to UVBR and OP. It is important to avoid variations in the levels of T3 between UVBR and non-UVBR groups due to photodegradation, as this could potentially cause differences in the T3-dependent responses measured throughout the experiments. All bowls were kept in incubator 1 during both experiments, covered with a Mylar filter (*i.e.*, UVBR was blocked but not UVAR and VIS), and rotated.

3.2.8 Experiment 1

3.2.8.1 Exposure

The stages of tadpole development were regularly identified under a dissection scope according to the Gosner staging system (Gosner, 1960) to assess the rate of tadpole development during the experiment. On August 24, the developmental stages of most

animals (n= 86 to 103 animals/treatment) were assessed to determine the number of tadpoles at stage 29 (end of hind limb bud development) for use in Experiment 1. We chose to expose stage 29 tadpoles to T3 because animals exposed to OP and/or UVBR in previous experiments began exhibiting delayed development at approximately this stage when compared to those in the control treatment (Croteau *et al.*, 2008b). Although animals in this study were not yet developmentally delayed (see Results section), we were interested in the response of tadpoles to T3 just prior to the expected disruption in the rate of development.

A subset (n=125 total) of stage 29 animals was randomly chosen for this experiment (Figure 3.1). Tadpoles from the four pre-exposure treatments (*i.e.*, Control, OP, UVBR and UVBR+OP) were exposed to 0, 5 or 50 nM T3 for 24h, resulting in a total of 12 exposure treatments. Exposure of the tadpoles (*i.e.*, n=125) to previous treatments was terminated as soon as exposure to T3 began. On September 2 and 14, tadpoles were exposed to T3 or 0.01% NaOH vehicle in 1 bowl/treatment containing 4 to 7 tadpoles/bowl. Exposures were conducted on two separate dates so as to have a sufficient number of animals collected at stage 29 for the subsequent gene expression analysis. The developmental stages of most animals exposed to UVBR/OP were identified on September 13 (70 to 86 animals/treatment) to ensure that the developmental rate of tadpoles had not changed between the two T3 exposure dates (the rate was still the similar in all treatment groups; see Results section). T3 stocks were made fresh on both exposure dates. The pH of the water was monitored to ensure that fluctuations due to the addition of the NaOH vehicle were minimal. The pH only increased by 0.1 to 0.2 of a unit in the 24h exposure period.

3.2.8.2 Tissue Collection

Tadpoles were anaesthetised by immersion in 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich; 1 g/L in water) after 24h of exposure to T3. Tails and whole brains were immediately dissected on ice, frozen on dry ice and stored at -80°C .

3.2.8.3 RNA Extraction, DNase Treatment and cDNA Synthesis

Sample sizes for real-time RT-PCR analysis were 7 to 9 tails and 6 to 8 whole brains/treatment, chosen randomly between the two exposure dates. The acid guanidinium thiocyanate (GITC)–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) as modified by Chomczynski and Mackey (1995), was used to extract total RNA from tail samples. Briefly, frozen tail samples were homogenized in a mixer mill (Retsch® MM301, Haan, Germany) in a GITC solution (solution D, pH 4.0) until the solution was homogenous. Sodium acetate (pH 4), phenol and chloroform were added to the sample, which was then mixed vigorously and centrifuged at 12000g for 20min. at 4°C . The aqueous supernatant was transferred to another tube, the RNA was precipitated with high salt buffer and isopropanol at room temperature for 10min., and a pellet was formed by centrifugation at 12000g for 15min at 4°C . The RNA pellet was washed twice with 80% ethanol for 10min, and was then air-dried for ~5-10min. Samples were then resuspended in RNase-free water and DNase treated using the RNeasy® Micro Kit as per the manufacturer's protocol (Qiagen, Mississauga, ON). Total RNA was extracted and DNase treated from whole brain using the RNeasy® Micro Kit (Qiagen). The DNase-treated RNA from both tissues was resuspended in RNase-free water, the concentration of total RNA in each sample was measured spectrophotometrically using GeneQuant®

(Amersham Pharmacia Biotech, Piscataway, NJ) and samples were stored at -80°C .

cDNA was synthesized with approximately 1 μg (brains) or 2 μg (tails) of RNA and 200 ng random hexamer primers using Superscript[™] II RNase H⁻ reverse transcriptase as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). The 20 μL cDNA sample was diluted to a final concentration of 2.5 ng/ μL (brain) or 5 ng/ μL (tail) before real-time RT-PCR amplification, and stored at -20°C .

3.2.8.4 Cloning of CRH

A partial cDNA sequence for corticotropin-releasing hormone (CRH) was cloned from *R. pipiens* whole brain. Degenerate primers were designed using conserved regions of amphibian (*Rana catesbiana* [Bullfrog], *Phyllomedusa sauvagei* [Waxy monkey tree frog] and *Spea hammondi* [Western spadefoot toad]) CRH gene sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) and constructed using ClustalW (<http://www.ebi.ac.uk/clustalw/>) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) programs. Primers used to clone CRH were: forward primer 5'-GCNANGAGTGCAGAGCNTTC-3'; reverse primer 5'-GTATCAGGGCAGCNCNATGG-3'. The Mastercycler[®] gradient Thermal Cycler (Eppendorf, Westbury, NY) was used for PCR amplifications. Each 50 μL PCR reaction contained the following reagents: 2 μL of cDNA template in 36.3 μL DEPC water; 1X PCR reaction buffer; 1.5 mM MgCl_2 ; 0.2 mM dNTPs; 0.4 mM forward (F) and reverse (R) primers; and 0.02 U Taq[®] DNA Polymerase (Invitrogen). PCR conditions were as follows: an initial enzyme activation step at 95°C for 4 min, followed by 35 cycles with a denaturation step at 95°C for 45 s, an annealing step at 50°C for 30 s, and an extension

step at 72°C for 1 min. After the last amplification cycle, 10 min final extension step was performed at 72°C and samples were immediately cooled to 4°C.

The amplification product was ligated into the 2.1 TOPO[®] cloning vector (TOPO TA cloning kit; Invitrogen), transformed into One Shot TOPO 10[®] chemically competent *Escherichia coli* cells (Invitrogen) and plated onto LB-agar plates containing ampicillin and X-gal. Positive colonies were selected and used as templates in a PCR using M13 primers to ensure that the correct insert size was present before sequencing. The positive colonies were then grown overnight in LB broth containing ampicillin, and plasmids were purified using the Wizard[®] Plus SV Minipreps DNA Purification System (Promega, Madison, WI). All procedures were conducted according to the manufacturer's protocols. Purified plasmid (10 µL) was sequenced by the Centre for Advanced Research in Environmental Genomics (CAREG, University of Ottawa, Ottawa, ON) core facility. The cDNA sequence of 508 bp for *R. pipiens* CRH has been deposited in the GenBank database (accession no. DQ779202).

3.2.8.5 Real-Time RT-PCR

All real-time RT-PCR analyses of relative gene expression in tail and whole brain samples were carried out using an Mx4000 real-time polymerase chain reaction system (Stratagene, La Jolla, CA). A total of 4µL of diluted tail cDNA (*i.e.*, 20 ng) or 5µL of diluted brain cDNA (*i.e.*, 12.5 ng) was added to the real-time RT-PCR reactions.

Multiplex real-time RT-PCR assays for deiodinase and thyroid receptor genes followed validated procedures as described by Hogan *et al.* (2007), and were used to measure the relative mRNA expression of these genes in whole brain and tail samples.

Dual-labelled fluorescent probes and primers sets were designed for two triplex assays, one for thyroid receptor (TR alpha and TR beta) and L8 (ribosomal protein L8) genes and the other for deiodinase (type 2 or D2 and type 3 or D3) and L8 genes. L8 was used as a reference gene in all real-time RT-PCR assays (see Hogan *et al.*, 2007 for information on L8 primers) since it has been demonstrated that the expression of this gene is ubiquitous, relatively constant during development, and not affected by the treatment of tadpoles to thyroid hormone (Shi and Liang, 1994 and references therein).

The relative mRNA expression of CRH and L8 in whole brain was measured in a simplex real-time RT-PCR SYBR Green I-based assay. Primers were designed for the simplex assay of CRH based on the previously described *R. pipiens* CRH partial cDNA sequence (accession no. DQ779202). Primer sequences were: forward primer 5'-GCCGACTCCTCTCCATTC-3'; reverse primer 5'-TGTCCATCAGTTTCCTATTGCT-3' and amplified a sequence of 172 bp. Primers were tested by RT-PCR using *R. pipiens* whole brain cDNA and the resulting amplicons were cloned and sequenced to confirm primer specificity. Each 25 µL PCR reaction contained the following reagents: 12.5 ng (brain) cDNA template; 1X PCR buffer (Qiagen); 3.5 mM MgCl₂ (Qiagen); 150 nM primer (Invitrogen); 0.25X SYBR Green I dye (Invitrogen); 200 µM dNTPs (Invitrogen); 1.25 U HotStarTaq (Qiagen); and 100 nM ROX reference dye (Stratagene). The thermal cycling parameters were as follows: an enzyme activation step at 95°C for 15 minutes, followed by 40 cycles with a denaturation step at 95°C for 15 s, an annealing step at 60°C for 5 s, an extension step at 72°C for 30 s, and a detection step at 80°C for 8 s. After the amplification was complete, a dissociation curve was generated starting from 55°C (+1°C/30 seconds) to 95°C.

The relative standard curve method (Applied Biosystems, 1997) was used to interpolate relative mRNA levels of reference and target genes within each sample. Standard curves were produced by serial dilutions of a pool of cDNA generated from experimental samples. All real-time PCR reactions included a no template control (*i.e.*, RNase-free water added to the PCR instead of cDNA template), and a no reverse transcriptase control (*i.e.*, RNase-free water added to the cDNA synthesis reaction instead of the enzyme). Samples were run in triplicate, and the data generated for each sample were averaged and normalized to L8. Normalized data for all samples within a treatment group were then averaged and are expressed as the mean relative mRNA expression (+SEM) relative to the 0 nM T3-treated control group. The expression of L8 did not change in response to treatment, in both the simplex and multiplex assays.

3.2.8.6 Data Analysis

Real-time RT-PCR data were tested for normality and homoscedacity and were log transformed to meet these criteria. As samples were randomly chosen between the two exposure dates (September 2 and 14) for PCR analysis, the effect of exposure date on gene expression data was examined. We performed Student's t-tests on randomly chosen data from treatments with a sample size of $n \geq 3$ per exposure date (*i.e.*, 7 of the 12 treatments for both tail and brain samples), for all genes and both tissues. There was no effect of exposure date on this data. Data were then analyzed by three-way analysis of variance (ANOVA), and comparisons between treatments were based on examination of Least Squares Means (LSM) and multiple comparisons using Bonferroni's adjusted post-

hoc tests. The results of full models are reported in this study given that results obtained with reduced models were almost identical (*e.g.*, p values were approximately the same).

To determine the effects of UVBR and OP on the rate of development at different time points before and during Experiment 1, the percentage of tadpoles that had developed past the median developmental stage of animals in the control group was calculated. The median developmental stage of tadpoles in the control group on August 24 and September 13 was Gosner 28 and Gosner 29, respectively. Log linear models were used to determine the effects of treatments on the number of tadpoles that developed past the developmental stage indicated above. Data were analyzed using SYSTAT (Version 10; San José, CA) software and a variable with a p value of <0.05 was considered statistically significant.

3.2.9 Experiment 2

3.2.9.1 Exposure

On October 13, the developmental stages of most of the remaining tadpoles were identified (n=52 to 68 animals/treatment) and a sub-set of tadpoles (n=40 animals/treatment) was randomly weighed to determine the effects of UV/OP on these endpoints before the start of Experiment 2 (Figure 3.1). A developmental delay of tadpoles was observed in the UVBR-treated animals at approximately stage 31 (paddle foot stage) (see Results section). We were interested in measuring the morphological response of developmentally delayed tadpoles to T3, to determine if pre-treatment to UVBR/OP affected the TR-mediated response of tissues to T3 and thus the morphological changes that are typically induced in animals exposed to this hormone.

A subset (n=110) of stage 31 animals was used for this experiment, which began on October 19 (Figure 3.1). Stage 31 animals were randomly assigned to four or six bowls/treatment containing five tadpoles/bowl (*i.e.*, n=20 for Control, and n=30 for UVBR, OP and UVBR+OP treatments). Endpoints measured before the start of the experiment (Time 0) included: (1) weight; (2) snout-vent length (SVL); and (3) tail length. Data recorded at Time 0 was collected within 24h prior to the exposure of tadpoles to T3. Time 0 data was log transformed to normality and homoscedacity and was analyzed by two-way nested ANOVA. The analysis revealed that there was no bowl effect for any of the endpoints. Therefore, half of the bowls per treatment were randomly assigned to 0 nM T3 (*i.e.*, 0.01% NaOH vehicle) or 5 nM T3 exposure groups, resulting in a total of eight treatments for this experiment (*i.e.*, Control, UVBR, OP and UVBR+OP, with and without T3 exposure). Sample size ranged from 10-15 tadpoles/treatment. Exposure of tadpoles to previous treatments (*i.e.*, UVBR/OP) was terminated as soon as exposure to T3 began. Animals were fed daily and water was changed every 24 h.

3.2.9.2 Morphological Endpoints and Data Analysis

A log linear model was used to determine the effects of UVBR and/or OP on the number of tadpoles developing past stage 31 (*i.e.*, median stage of animals in the control treatment), measured on October 13. Two-way analysis of covariance (ANCOVA) was used to determine if there was a significant effect of UVBR and/or OP on body weights, also measured on October 13. Weight data was tested for normality and homoscedacity

and log transformed to meet these criteria. The developmental stage of all tadpoles was recorded when they were weighed and this was used as the covariate in this analysis.

Weight, SVL, tail length and stage of development were measured in tadpoles exposed to T3 for seven days. Sample size ranged from 9 to 15 animals/treatment and varied from the original sample size due to the accidental mortality of three tadpoles during the experiment; however none died due to treatment during the 7-day experiment. Since there was no bowl effect for all data collected at Time 0, ANOVAs were performed again without the nested factor (*i.e.*, bowl) to determine the effects of UVBR and/or OP on weight, SVL and tail length at Time 0. Statistical analyses indicated that there was no effect of UV/OP treatments on tail length at Time 0. Therefore, Day 7 data was log transformed to normality and homoscedacity, and a three-way nested ANOVA was performed to determine whether any of the treatments affected tail length. There was no bowl effect; therefore the ANOVA was performed again without the nested factor (*i.e.*, bowl) followed by multiple comparisons using Bonferroni's adjusted post-hoc tests. However, analysis of data collected at Time 0 revealed that UVBR had a slight effect on SVL and weight in this subset of animals (see Results section). Weight or SVL change was therefore calculated and represents the difference between the average weight or SVL of animals at Time 0 and Day 7. This calculation corrects for the fact that UVBR had a slight effect on the SVL and weight of tadpoles measured at Time 0, before the tadpoles were exposed to T3. Time to forelimb emergence (FLE) and first visible changes in tail regression (*i.e.*, tail tip dark and shrunken), hyperactivity and emaciation were also recorded throughout the experiment.

The stage of development of all animals was measured after seven days of exposure to T3. The number and percentage of animals that developed past stage 34 was calculated. Stage 34 was the median stage of animals calculated for the T3-treated control group (*i.e.*, C + T3) on Day 7. Data of treatments with and without T3 exposure (*e.g.*, OP no T3 vs. OP + T3) were tested by Fisher's Exact test (2X2 contingency tables) to determine the effects of pre-exposure to UVBR and/or OP on the number of tadpoles developing past stage 34 after exposure to T3. Data were analyzed using SYSTAT (Version 10; San José, CA) and S Plus (Version 8.0; Seattle, WA) software, and statistical results of full models are reported, with a $p < 0.05$ considered as statistically significant.

3.3 RESULTS

3.3.1 Weight and Stage of Development

There was no significant effect of treatments on the number of animals developing past stage 28 or 29, before or during Experiment 1 (August 24 and September 13, respectively). On these dates, the median developmental stage of tadpoles was the same in all four treatments (Table 3.1). However, statistical analysis indicated a significant effect of UVBR on the number of animals developing past stage 31 before the start of Experiment 2 (October 13; $p = 0.001$), with ~27-31% of animals in the control and OP groups developed past stage 31, compared to ~10-15% of those exposed to UVBR and UVBR+OP. This signifies that the development of all tadpoles exposed to UVBR, regardless of the presence of OP, was delayed compared to those not exposed to UVBR. The median developmental stage of tadpoles in non-UVBR treatments was stage 31 (paddle foot stage) at this time, whereas the median in the UVBR and UVBR+OP

treatments was stage 30.5 and 30 (end of hind limb bud development), respectively (Table 3.1). There was no effect of treatments on the weight of a random subset of animals collected at various developmental stages and measured on October 13.

3.3.2 Experiment 1: Real-time RT-PCR

3.3.2.1 Whole Brain

The relative mRNA levels of TR alpha in whole brain samples are shown in Figure 3.3A. There was no effect of T3 on the expression of TR alpha. A moderate increase in the abundance of TR alpha mRNA was caused by exposure to OP alone ($p=0.001$), and was independent of T3 exposure. There was no effect of UVBR alone on TR alpha. However, the interaction term UVBR+OP was significant ($p=0.014$), as UVBR exposure modified the effect of OP to decrease the expression of TR alpha (as seen on Figure 3.3A and from the examination of LSM). Values for TR alpha in the UVBR+OP combination group were midway between the values in the UVBR and OP alone groups.

As seen in Figure 3.3B, T3 had a concentration dependent effect on TR beta mRNA levels ($p<0.0001$) in whole brain. The 5 nM concentration of T3 had a moderate stimulatory effect in all treatments (~45% overall increase in TR beta expression based on the LSM analysis), but this response was not statistically different in the post hoc test from that of animals not exposed to T3. However, the 50 nM concentration of T3 stimulated the expression of TR beta (~171% overall increase based on the LSM), and the response was statistically different than that of 0 nM and 5 nM T3 for all treatments. OP produced a minor but statistically significant induction of TR beta mRNA levels ($p=0.011$) (Figure 3.3B). This response represented a ~12% increase in expression

compared to the effect of treatments without OP, derived from examinations of LSM.

UVBR alone had no effect and did not modify the response of TR beta to T3 or OP.

Figure 3.3C shows the relative mRNA abundance of D2 in whole brain samples. There was a significant concentration dependent effect of T3 on the expression of D2 ($p < 0.0001$). Examination of LSM indicated an overall increase of ~27% and ~83% in D2 expression by 5 nM and 50 nM T3, respectively. The animals in the control and UVBR treatments were however not as responsive to T3, as Bonferroni's adjusted post hoc test showed no difference in the expression of D2 with and without T3 treatment for these groups (Figure 3.3C). OP had a significant effect ($p = 0.001$) on the expression of D2, which represents an overall increase in expression of ~28% based on the LSM analysis. The post hoc tests revealed differences in D2 levels between OP + 0 nM T3 and OP + 50 nM T3 as well as UVBR+OP + 0 nM T3 and UVBR+OP + 50 nM T3 (Figure 3.3C). These results indicate that OP, even in presence of UVBR, enhances the responsiveness of D2 to 50 nM T3. It was not possible to accurately measure relative mRNA levels of D3 in the brain because this gene is lowly expressed in this tissue at this stage of development (Hogan *et al.*, 2007).

There was no effect of T3 on relative mRNA levels of CRH in whole brain samples (Figure 3.3D). The ANOVA indicated a minor but significant effect of OP ($p = 0.048$) on the expression of CRH. However, this should be interpreted with caution because the overall percent increase in expression due to OP was only ~9% based on the observation of LSM, indicating a minor effect of exposure to OP.

3.3.2.2 Tail

T3 had a significant effect on TR alpha mRNA levels in tail tissue ($p=0.013$) (Figure 3.4A). Both concentrations of T3 produced an overall ~29% increase in the expression of TR alpha, derived from the examination of LSM. The ANOVA identified a significant three-way interaction between UVBR, OP and T3 ($p=0.028$), suggesting that OP and UVBR affected this moderate T3 induced response in TR alpha. The only statistically significant difference in expression revealed in the post hoc analysis was between Control+ 0 nM T3 vs. UVBR+OP+ 5 nM T3 treatments. However, this response was moderate and variable as seen by the size of the error bar for the UVBR+OP+ 5 nM T3 treatment, and was not seen in the UVBR+OP+ 50 nM T3 treatment (Figure 3.4A).

Figure 3.4B illustrates the relative mRNA abundance of TR beta in the tail, and demonstrates a concentration dependent effect of T3 ($p<0.0001$) on mRNA levels. The 5 nM concentration of T3 caused a ~4 to 9 fold induction in the expression of TR beta, whereas the 50 nM concentration produced a ~8 to 12 fold increase in expression when compared to levels in the Control+ 0 nM T3 treatment (Figure 3.4B). There was no effect of OP or UVBR alone on the expression of TR beta. However, the three-way interaction between UVBR, OP and T3 was significant ($p=0.022$) and indicates that OP and UVBR modified the response of animals to T3. Both concentrations of T3 increased the expression of TR beta by ~9 fold in the UVBR+OP group, while 5 nM T3 only produced a ~4 to 6 fold increase and 50 nM T3 a ~8 to 12 fold induction of TR beta in the other treatment groups (Figure 3.4B). Bonferroni's adjusted post hoc test indicated a significant difference in the response of TR beta to 0 nM and 5 nM T3 and to 0 nM and 50 nM T3 exposure for all treatments. There was, however, only a significant difference in the

response of TR beta to 5 nM and 50 nM T3 exposure in the OP only and UVBR only treatments.

T3 had an effect on mRNA abundance of D2 in tail tissue ($p=0.026$; Figure 3.4C), resulting in a concentration dependent effect that is most evident when examining the LSM calculated from this data set. The LSM indicate that exposure to 5 nM and 50 nM T3 increased the overall expression of D2 in tail tissue by ~36% and ~66%, respectively, compared to tails not exposed to T3. An effect of OP on D2 was also detected ($p=0.027$), and observation of LSM indicated an overall increase in D2 expression of ~35% in OP-treated groups. The variability in mRNA levels in OP-treated animals is high compared to those in the control treatment (Figure 3.4C). A number of animals in OP treatments had very high relative mRNA levels, which ranged from 0.41 to 25.67 in the OP + 0 nM T3 group, 0.42 to 60.37 in the OP + 5 nM T3 group and 1.08 to 72.55 in the UVBR+OP + 50 nM T3 group. Variable relative mRNA levels were also observed in tadpoles from the UVBR + 50 nM T3 group, ranging from 0.87 to 28.16; however, there was no main effect of UVBR alone on the expression of D2.

The levels of D3 mRNA in tail tissue are shown on Figure 3.4D. There was a concentration dependent effect of T3 on mRNA levels of D3 ($p<0.0001$). The expression of D3 in tail tissue is more responsive to T3 than D2, as the LSM indicates that exposure to 5 nM T3 increased the overall expression of D3 by ~225% and 50 nM T3 induced expression by ~335% compared to 0 nM controls (as opposed to ~36-66% increase in D2 expression by T3 exposure based on the LSM). The average fold change induction in D3 mRNA levels by both concentrations of T3 ranged from ~9 to 93 when compared to levels in the C + 0 nM T3 treatment (Figure 3.4D). Bonferroni's adjusted post hoc test

identified a difference in the effects of 0 nM and 50 nM T3 on D2 in all treatment groups, and a difference between 0 nM and 5 nM T3 in C and UV+OP treatments specifically (Figure 3.4D). Similar to the response of D2 in tail tissue, a number of animals exposed to UVBR and/or OP with or without T3 had high D3 mRNA levels when compared to those measured in the control groups. For example, relative mRNA abundances ranged from 0.29 to 173.72 in the OP + 0 nM T3 group, 1.22 to 362.66 in the OP + 5 nM T3 group, 24.58 to 410.75 in the UVBR+OP + 50 nM T3 group. However, responses of D3 to UVBR/OP pre-treatments were generally variable and there was no significant effect of OP or UVBR alone or combined on the expression of D3.

3.3.3 Experiment 2: Effects of T3 on Morphological Endpoints

3.3.3.1 Weight and SVL Change

Although there was no effect of UVBR and/or OP on the weight of animals measured on October 13 from a random subset collected at different stages of development (Table 3.1), there was a detectable effect of UVBR on the weight ($p=0.008$) and SVL ($p=0.048$) of a smaller subset of stage 31 tadpoles measured at Time 0 (*i.e.*, prior to the exposure to T3). In this subset of animals, there was a small increase in SVL and weight (Table 3.2). These differences were taken into account by reporting the weight and SVL change between Time 0 and Day 7 of T3 exposure. An effect of T3 on the difference in weight change of tadpoles was evident (Table 3.2), as the weight change in groups not exposed to T3 ranged from 0.08 to 0.12 g (11-15% weight loss) compared to 0.29 to 0.37 g (42-47% weight loss) in T3-treated animals. The same trend was observed for the SVL change data (Table 3.2). Figure 3.5 illustrates the effect of T3 on the weight and SVL of tadpoles from the control treatments (exposed to 0 nM and 5 nM

T3). Animals in these treatments were all at stage 31 and of similar weights at the beginning of the experiment (Time 0), yet the tadpole exposed to 5 nM T3 is visibly smaller and shows signs of emaciation by the end of the experiment that is typical of precocious or induced metamorphosis (Figure 3.5). After five to six days, all tadpoles exposed to 5 nM T3 were emaciated and hyperactive. None of the tadpoles in the 0 nM T3 treatments exhibited these characteristics. Although there was an apparent effect of T3 on the weight and SVL of tadpoles after seven days, there is no clear indication of an effect of pre-exposure treatments (*i.e.*, UVBR and/or OP) on weight and SVL change (Table 3.2).

3.3.3.2 Tail Length

Statistical analysis indicates that there were no overall significant effects of pre-exposures to UVBR and/or OP on the tail length of tadpoles exposed to T3 for seven days (Table 3.2). The tips of the tails of animals in T3-treated groups were noticeably black after four days of exposure, indicating apoptosis and the beginning of tail resorption. As expected, the tails of tadpoles exposed to T3 for seven days had shortened considerably compared to those not exposed to T3 (T3 $p < 0.0001$). The reduction of tail length in T3-treated animals after seven days ranged from 13-19%, while the tail length of animals not treated with T3 hardly changed during the experiment (Table 3.2). Bonferroni's adjusted post-hoc analysis identified a differential effect of T3 in some pre-treatment groups. In the control group ($p = 0.048$) and in the group pre-exposed to OP ($p = 0.029$), there were significant reductions in tail length in response to the T3 exposure (Table 3.2). In contrast, there was no significant reduction in tail length in response to T3 in animals pre-exposed to UVBR regardless of whether they were also treated with OP. These results

demonstrate that the tails of controls or animals pre-exposed to OP were capable of responding to exogenous T3, but that pre-exposure to UVBR affected this response when compared to that of other groups.

3.3.3.3. Stage of Development

As was observed for weight change, SVL change and tail length, there was a clear effect of T3 on the number of tadpoles developing past stage 34 after seven days of exposure. The median developmental stage of animals not exposed to T3 did not change during the 7-day experiment and all tadpoles remained at stage 31 (paddle foot stage). However, the median stages of tadpoles exposed to T3 were 34 in control and OP groups, 34.5 in the UVBR+OP group and 35 (early development of digits) in the UVBR group (Table 3.2). An increase of 50% and 60% in the number of tadpoles developing past stage 34 was observed in the UVBR + T3 and UV+OP +T3 groups, respectively, whereas this percentage was only ~21 and 40% in OP + T3 and Control + T3 groups (Table 3.2). Fisher's Exact tests (2X2 contingency tables) were used to determine if pre-treatments had an effect on the response of tadpoles to T3 (*i.e.*, number of tadpoles developing past stage 34). Statistical analyses revealed a significant difference between UV+OP and UV+OP +T3 ($p=0.0022$) groups and between UVBR and UVBR + T3 ($p=0.0007$) groups. There were no statistical differences between C and C + T3 groups and OP and OP + T3 groups ($p\geq 0.05$; Table 3.2). All animals had undergone forelimb emergence (FLE) after seven or eight days of exposure to T3 (many animals had only one emerged forelimb when last observed on Day 7), whereas none of the tadpoles in the 0 nM T3 treatments underwent FLE as expected. In this experiment, the developmental stage of tadpoles was based on

the development of the hind limb and digits as per Gosner (1960) and not based on the emergence of the forelimbs (FLE) due to induced metamorphosis, which usually represents Gosner stage 42. Overall, these results show that animals pre-exposed to UVBR and/or OP responded to exogenous T3, as development was advanced over the 7-day exposure period. However, animals pre-exposed to UVBR responded differently to T3 exposure (*i.e.*, were more advanced) than animals not exposed to UVBR.

Table 3.1 Median stage, percentage of tadpoles developing past the median stage of the control group, and average weight (g) $\pm$ SEM of animals measured at various time points during the exposure to UVBR and/or OP.

Treatments ^a	August 24		September 13		Weight ^c (g)	October 13	
	Median Stage	% Past Median ^b	Median Stage	% Past Median		Median Stage	% Past Median
C	28	45.3% (39/86)	29	38.6% (27/70)	0.62 $\pm$ 0.063 ^d	31	30.7% (16/52)
OP	28	44.8% (43/96)	29	43.0% (34/79)	0.72 $\pm$ 0.056	31	27.0% (17/63)
UVBR	28	34.0% (35/103)	29	34.9% (30/86)	0.64 $\pm$ 0.059	30.5	14.7% * (10/68)
UVBR+OP	28	42.6% (43/101)	29	35.2% (25/71)	0.70 $\pm$ 0.050	30	9.7% * (6/62)

^a C, Control; OP, 10 nM 4-*tert*-octylphenol; UVBR, UVB radiation.

^b The median stage used for this endpoint was that of the control group on the specified date. For example, the % Past Median for October 13 was calculated by counting the number of tadpoles that had developed past Gosner stage 31, the median stage of the control group on that date (*i.e.*, by counting animals at stages 32+). The numerator in parentheses represents the number of tadpoles that developed past the median stage and the denominator is the total number of animals measured.

^c Sample size is 40 tadpoles/treatment.

^d SEM, standard error of the mean.

*Indicates a significant effect of UVBR on the number of tadpoles developing past Gosner stage 31 ($p < 0.001$).

Table 3.2 Morphological endpoints measured in tadpoles during Experiment 2, at Time 0 (before exposure to T3 began) and after seven days of exposure to T3.

Treatments ^a	Time 0 ^b				Day 7				
	Weight (g)	SVL (mm)	Tail Length (mm)	Median Stage	Weight ^c Change (g)	SVL ^c Change (mm)	Tail Length (mm)	Median Stage ^d	% Past Median ^e
C	0.68 ±0.08 ^f	15.5 ±0.76	31.3 ±1.5	31	-0.08	-1.6	32.0 ±2.0	31	11.1% (1/9)
OP	0.72 ±0.06	15.7 ±0.56	32.3 ±1.1	31	-0.08	-1.1	32.6 ±1.1	31	0% (0/15)
UVBR	0.84* ±0.09	16.2* ±0.60	32.7 ±1.1	31	-0.11	-1.0	32.8 ±1.0	31	0% (0/15)
UVBR+OP	0.78* ±0.07	16.3* ±0.50	32.0 ±1.2	31	-0.12	-1.1	31.8 ±1.3	31	0% (0/15)
C+ T3	0.62 ±0.06	14.7 ±0.72	30.7 ±1.4	31	-0.29	-3.2	24.8** ±1.6	34	40% (4/10)
OP + T3	0.71 ±0.07	15.6 ±0.58	31.7 ±1.2	31	-0.32	-3.4	26.4** ±1.3	34	21.4% (3/14)
UVBR + T3	0.84* ±0.08	16.2* ±0.64	32.5 ±1.2	31	-0.37	-3.8	28.0 ±1.5	35	60%*** (9/15)
UVBR+OP + T3	0.85* ±0.07	16.3* ±0.72	32.9 ±1.1	31	-0.36	-3.1	28.7 ±1.5	34.5	50%*** (7/14)

^a C, Control; OP, 10 nM 4-*tert*-octylphenol; UVBR, UVB radiation; T3, triiodothyronine.

^b Data presented for Time 0 were collected within the 24h prior to the exposure of tadpoles to T3. T3 is therefore not a factor that is included in the statistical analyses of data collected at Time 0.

^c Weight or SVL (snout vent length) change represents the difference between the average weight or SVL of animals at Time 0 and Day 7. This calculation corrects for the fact that UVBR had a slight effect on the SVL and weight of tadpoles measured at Time 0, before the tadpoles were exposed to T3. The negative symbol represents a weight loss or reduction in SVL.

^d In this experiment, the developmental stage of the tadpole is based on the development of the hind limb and digits as per Gosner (1960) and not based on the emergence of the forelimbs (FLE) due to induced metamorphosis, which occurred in all T3-treated tadpoles on Day 7 or 8 (Gosner stage 42 usually represents FLE).

^e The median stage used for this endpoint was that of the T3-treated control group (*i.e.*, C + T3). The % Past Median for October 13 was therefore calculated by counting the number of tadpoles that had developed past stage 34 (*i.e.*, by counting animals at stages 35+). The numerator in parentheses is the number of tadpoles developed past stage 34 and the denominator is the total number of animals measured.

^f SEM, standard error of the mean.

*Indicates a significant effect of UVBR on weight ($p=0.008$) and SVL ($p=0.048$).

**Indicates a significant effect of T3 ($p<0.0001$) on tail length, specifically between C no T3 and C + T3 and between OP no T3 and OP + T3.

***Indicates a significant difference in the number of tadpoles developing past stage 34 between UVBR no T3 and UVBR + T3 ($p=0.0007$) and between UV+OP no T3 and UV+OP +T3 ($p=0.0022$).

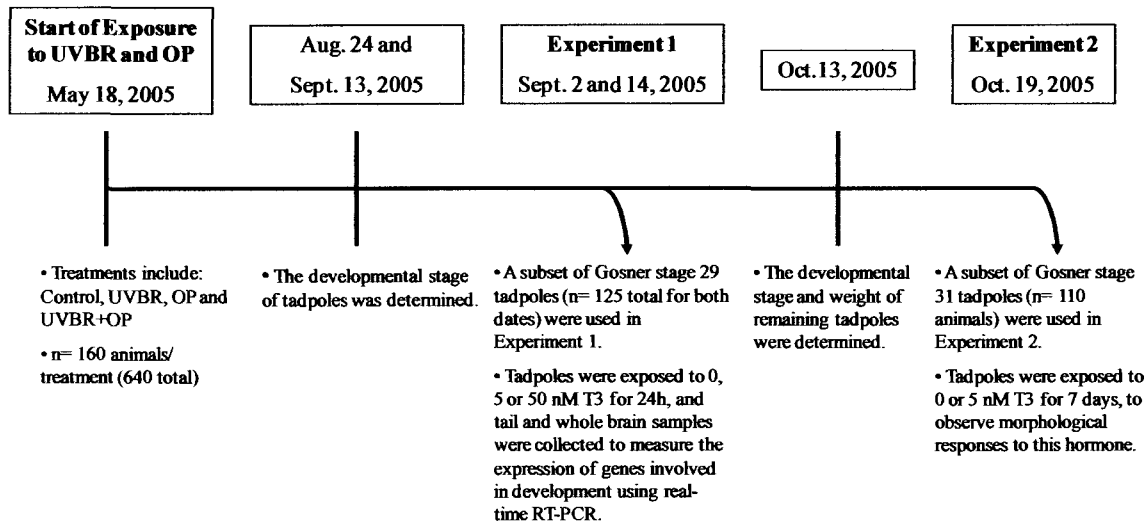


Figure 3.1 Schematic overview of the timeline of the study, with details on endpoints measured and sample sizes used. UVBR, ultraviolet B radiation; OP, 4-*tert*-octylphenol; T3, triiodothyronine; RT-PCR, reverse transcriptase-polymerase chain reaction.

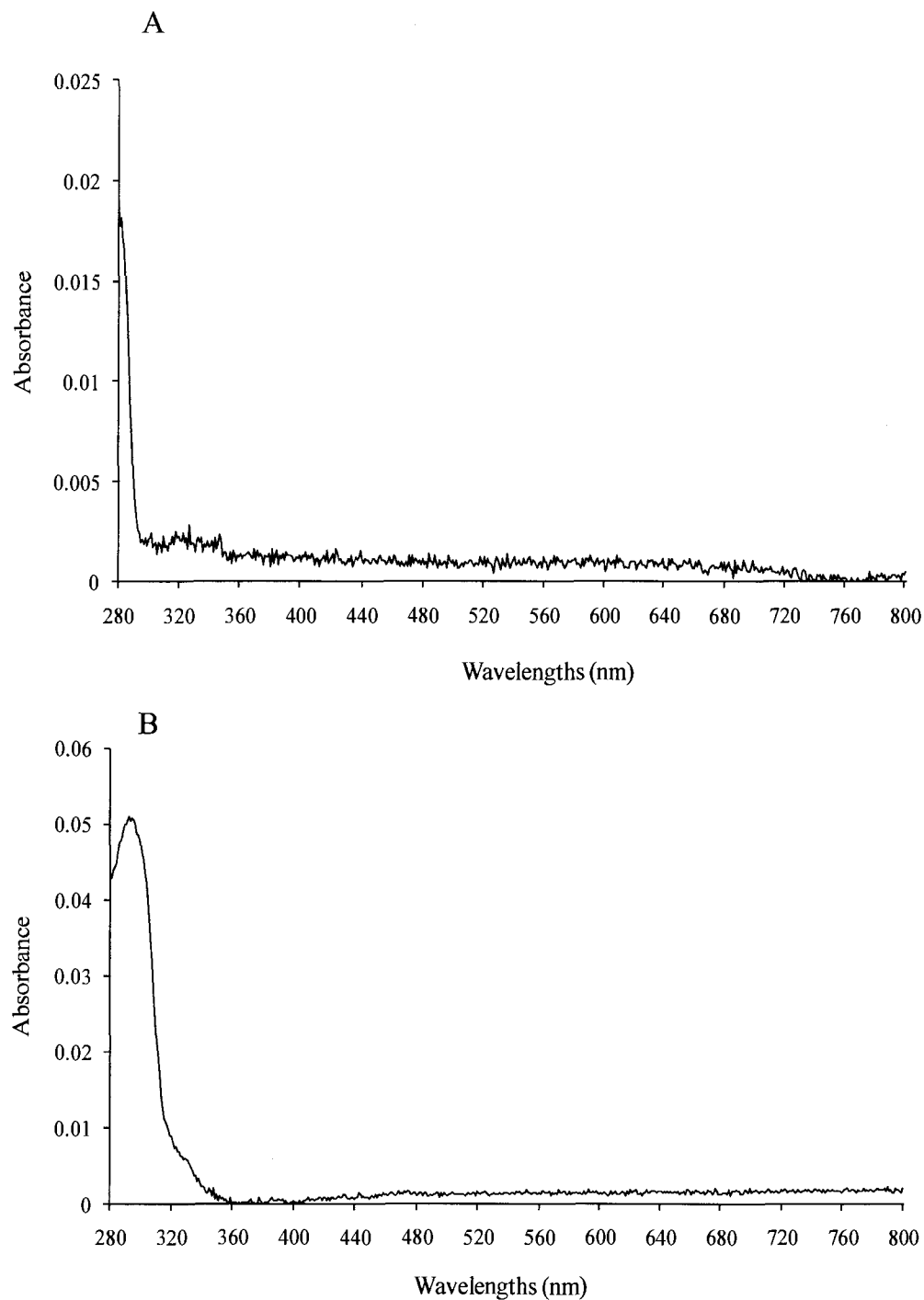


Figure 3.2 Absorption spectra of (A) 500 μ L of 100 μ M OP (dissolved in 95% EtOH) in 3 mL of aquatic facility water and (B) 100 μ L of 500 μ M T3 (dissolved in 0.1N NaOH) in 3.5 mL of aquatic facility water (pH adjusted to $\sim$ 7.0, to simulate conditions during the T3 exposures), measured using a CARY 100 BIO, UV-Visible Spectrophotometer (Varian, Mulgrave, Australia).

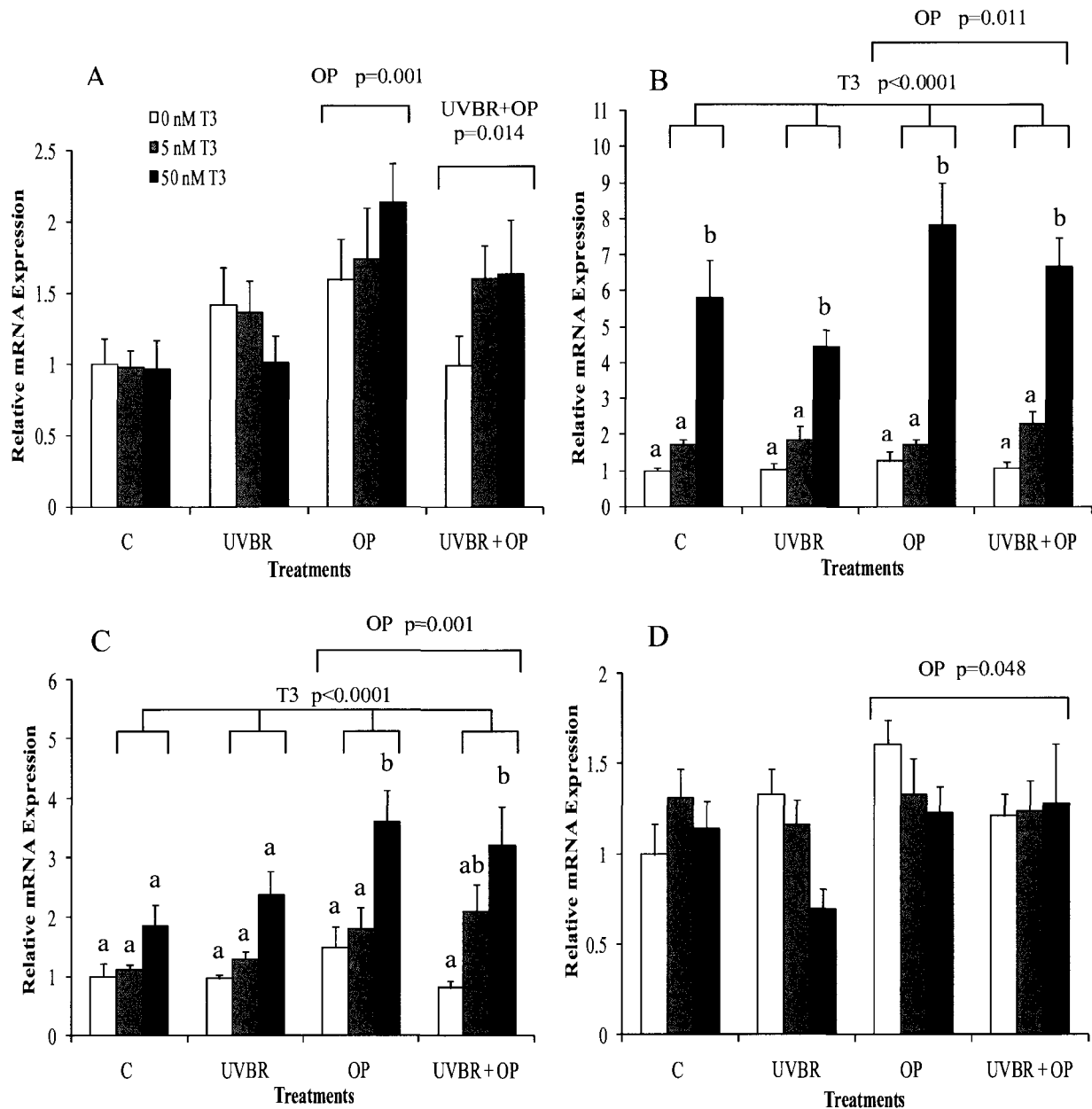


Figure 3.3 Effects of exposure to UVBR and/or OP on T3-induced relative mRNA expression of thyroid hormone receptor (TR) alpha (A), TR beta (B), deiodinase type II or D2 (C), and corticotropin-releasing hormone (D) in the brain of *Rana pipiens* tadpoles. Data were determined by multiplex (A-C) or simplex (D) real-time RT-PCR, normalized to L8 ribosomal protein, and presented as the arithmetic mean + standard error of the mean relative to the 0 nM T3-treated control group (n=6-8 individuals/treatment). Data were assessed by 3-way analysis of variance (ANOVA), and comparisons between treatments were based on examination of Least Squares Means and multiple comparisons using Bonferroni's adjusted post hoc tests (significance at $p \leq 0.05$). Brackets specify statistically significant factors in the ANOVA, with corresponding p values. Different letters represent statistically significant differences in the three concentrations of T3 within a pre-treatment group (i.e., C, OP, UVBR, UVBR+OP), based on the outcome of the post hoc analyses. C, Control; UVBR, UVB radiation; OP, 10 nM 4-tert-octylphenol; T3, triiodothyronine.

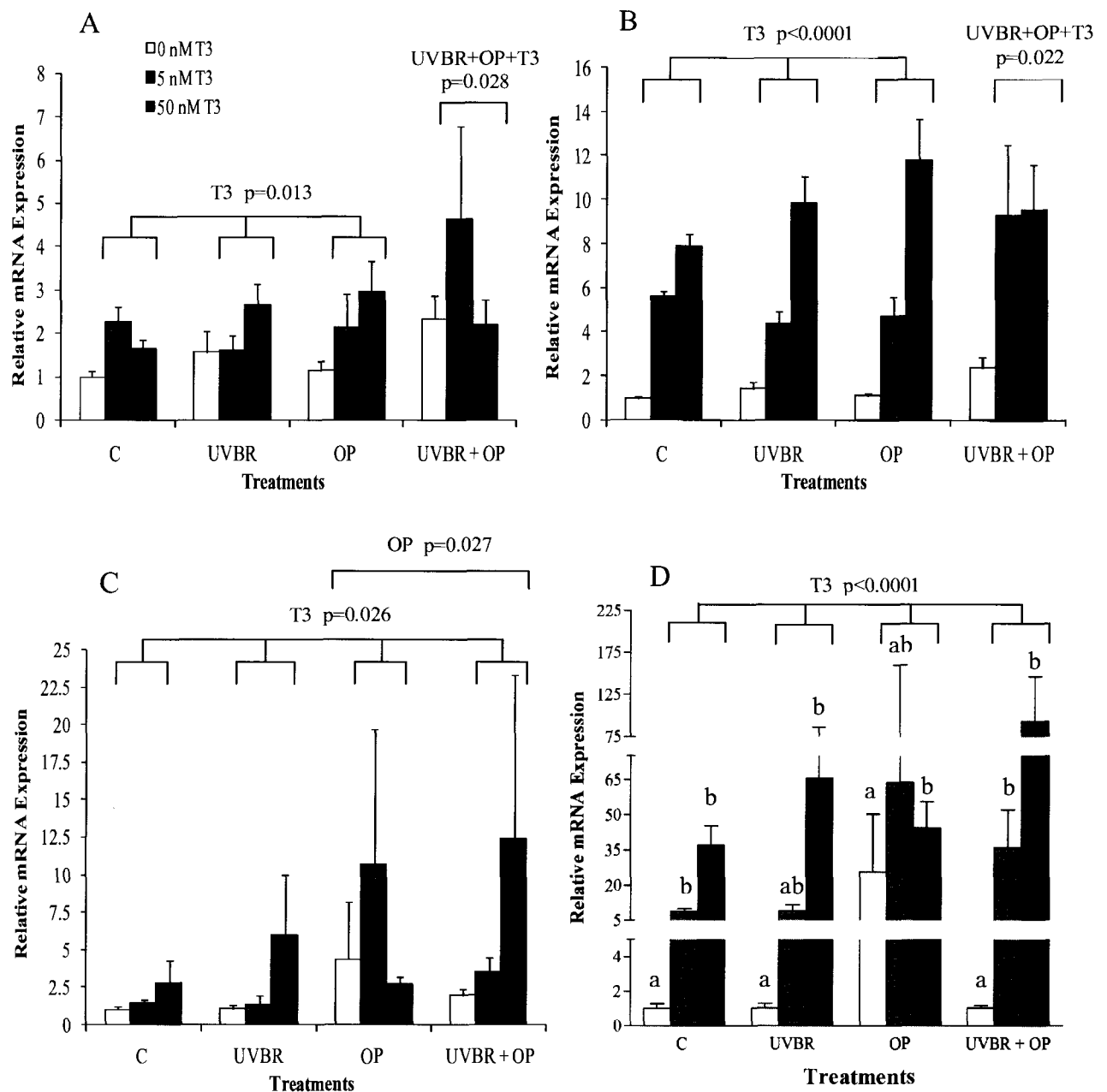


Figure 3.4 Effects of exposure to UVBR and/or OP on T3-induced relative mRNA expression of thyroid hormone receptor (TR) alpha (A), TR beta (B), deiodinase type II or D2 (C), and deiodinase type III or D3 (D) in the tail of *Rana pipiens* tadpoles. Data were determined by multiplex real-time RT-PCR, normalized to L8 ribosomal protein, and presented as the arithmetic mean + standard error of the mean relative to the 0 nM T3-treated control group (n=7-9 individuals/treatment). Data were assessed by 3-way analysis of variance (ANOVA), and comparisons between treatments were based on examination of Least Squares Means and multiple comparisons using Bonferroni's adjusted post hoc tests (significance at $p \leq 0.05$). Brackets specify statistically significant factors in the ANOVA, with corresponding p values. Different letters represent statistically significant differences in the three concentrations of T3 within a pre-treatment group (i.e., C, OP, UVBR, UVBR+OP), based on the outcome of the post hoc analyses. C, Control; UVBR, UVB radiation; OP, 10 nM 4-tert-octylphenol; T3, triiodothyronine.



Figure 3.5 Tadpoles from the 0 nM and 5 nM T3-treated control groups, at the end of the experiment: (Right) Tadpole not exposed to T3; (Left) Tadpole exposed to 5 nM T3. The tadpole exposed to T3 is visibly smaller and shows signs of emaciation, which is typical of precocious or induced metamorphosis. T3, triiodothyronine.

3.4 DISCUSSION

To the best of our knowledge, this is the first report on the effects of UVBR, in combination with a chemical stressor (*i.e.*, OP), on thyroid system-related disruptions in developing amphibians. Tadpoles exposed to an environmental level of UVBR, regardless of the presence of OP, exhibited a delay in development in the present study. *R. pipiens* tadpoles collected from the same population were chronically exposed (starting at stage 25) to similar levels of UVBR (0.14-0.22 W/m²) in three separate experiments in our laboratory (Croteau *et al.*, 2001 and 2008b and the present study). Delays in hind limb development and/or delays or inhibition of metamorphosis were observed in all three experiments. Notably, the results of the present study further corroborate those of a previous study (Chapter 2, Croteau *et al.*, 2008b), indicating that the onset of developmental delays observed in UVBR-exposed tadpoles occurs during mid to late premetamorphosis (*i.e.*, stages 29-31). Moreover, previous exposures to UVBR have resulted in a lower incidence of tadpoles developing past stage 34 (*i.e.*, start of prometamorphosis) (Chapter 2, Croteau *et al.*, 2008b) or none developing past stage 34 (Croteau *et al.*, 2001) compared to those exposed to OP alone and from the control group. Since late premetamorphosis is the time when the thyroid gland becomes functional in many species of anurans (at approximately Gosner stages 31-33; Shi, 2000; OECD, 2004) and prometamorphosis is the time when TH levels start to increase and are important for TH-dependent morphogenesis such as the development of digits (Etkin, 1968; Dodd and Dodd, 1976; Fort *et al.*, 2007), this window of sensitivity to UVBR (*i.e.*, stages 29-34) indicates that a delay in the development of tadpoles may be the result of a disruption at the level of the thyroid system. Moreover, developmental stages in late premetamorphosis

and early prometamorphosis are suggested for use in assays that assess disruptions on the thyroid hormone axis of amphibians (*i.e.*, agonist and antagonist activity) because this is a transitional phase in which the thyroid is acquiring activity (OECD, 2004). Although we did not observe an effect of OP alone on the developmental rate of tadpoles in this study, we did observe a transient delay in animals exposed to OP alone (0.01 and 10 nM) that also began during mid-premetamorphosis (stage 29) in a previous experiment (Chapter 2, Croteau *et al.*, 2008b). There remains the possibility that a disruption in development could have occurred later in this study had we continued with the exposure to OP for a longer period of time (*i.e.*, past stage 31).

The delayed developmental and metamorphic rates observed in *R. pipiens* tadpoles chronically exposed to UVBR and/or OP (Croteau *et al.*, 2001 and 2008b) have led us to the examination of potential mechanism(s) of action producing these sublethal effects, such as a disruption at the level of the thyroid system. Given that numerous studies have shown the importance of TRs and DIs in metamorphosis (reviewed in Shi, 2000), we used real-time RT-PCR to assess the ability of UVBR and/or OP to alter the expression of these target genes and their responsiveness to T3 in whole brain and tail tissue (Experiment 1). Additionally, the role of CRH in amphibians is extremely important to metamorphosis as it stimulates the secretion of both thyroid stimulating hormone (TSH) and adrenocorticotrophic hormone (ACTH) from the pituitary into blood circulation (Denver, 1997). TH is essential for the normal development of the vertebrate brain, influencing various processes such as neuronal migration, axonal maturation and dendritic outgrowth (Denver *et al.*, 1999; Porterfield, 2000; Zoeller *et al.*, 2002). An alteration in the expression of genes or their responsiveness to T3 by UVBR/OP exposure

in whole brain could be detrimental to brain development and neuroendocrine function. Crump *et al.* (2002) found that a 10-day exposure of *R. pipiens* tadpoles to UVBR, OP and UVBR+OP modified the expression of genes in the brain that are involved in the structural remodeling of the developing CNS and neurotransmitter synthesis. In addition to examining gene expression in the brain, we examined genes in the tail because it is a TH-responsive tissue that is available at all stages of tadpole development and is extensively used to assess disruption of metamorphosis in amphibians.

Results demonstrate that TR beta, D2, and D3 genes were all responsive to T3 in both whole brain and/or tail tissue, as they exhibited an overall increase in mRNA levels after exposure to T3 for 24h. CRH and TR alpha in brain however did not respond significantly to T3 exposure. Hogan *et al.* (2007) observed that TR beta, D2 and D3 genes in the brain of stage 33-34 *R. pipiens* tadpoles were responsive to a 48 h exposure to 50 nM T3 and also report no effect of T3 (0.5 nM and 5 nM or 50 nM) on TR alpha in the brain, confirming that TR alpha is relatively insensitive to T3 in this tissue. In tail, we observed a moderate effect of T3 on TR alpha mRNA levels, but this response was low compared to the fold change increase in TR beta mRNA levels after T3 exposure in both brain and tail tissues. A similar fold change increase in TR beta mRNA levels was detected in the brain *R. pipiens* tadpoles at metamorphic climax and after exposure to 50 nM T3 (Hogan *et al.*, 2007), which is expected given that TR beta expression is autoregulated (Tata, 1999; Tata, 2000).

Results of the ANOVA indicate that OP significantly increased the expression of TR alpha and beta in the brain; exposure to UVBR+OP together diminished the induction in TR alpha expression produced by OP. TR alpha may act as a transcriptional repressor

of genes important for metamorphosis to allow tadpoles to grow when TH is absent during premetamorphosis, and as an activator of transcription once the thyroid gland starts secreting TH (Buchholz *et al.*, 2006). The TR alpha bound to TH recruits coactivators to induce the expression of target genes such as TR beta. TR beta also interacts with coactivators that are present with TH, and its main role in gene regulation is in activation because TR beta is expressed primarily only when T3 is present (Buchholz *et al.*, 2006). T3 positively regulates the expression of TR beta (autoregulation), and this facilitates the transcription of other TH-responsive genes leading to tissue-specific development based on metamorphic requirements (Tata, 1999; Tata, 2000). Basic transcription element binding protein (BTEB) and basic region leucine zipper transcription factor (TH/bZIP) are two of five T3-responsive genes in amphibians with characterized TREs in their promoter (Buchholz *et al.*, 2006). The regulation of BTEB and TH/bZIP by T3 is important for brain development, as these proteins play an important role in the growth of neurons and astrocytes (McEwan and Alves, 1999; Denver *et al.*, 1999) and in transcriptional regulation in the tadpole brain (Denver *et al.*, 1997), respectively. Therefore, if genes such as TR alpha and TR beta are not responding in the brain as they typically should, this could negatively impact brain development by upsetting the balance of gene products in the brain to subsequently impact endocrine pathways that regulate metamorphosis. The combination of UVBR+OP significantly modified the response of TR alpha and TR beta to T3 in the tail, as the expression of these genes increased in certain UVBR+OP+T3 treatments. Results also demonstrated that OP induced the expression of TR alpha in the brain and that the presence of UVBR altered this response (by reducing gene expression), emphasizing the importance of

considering the impact of multiple stressors on sublethal endpoints such as gene expression. Hogan *et al.*, (2008b) found that exposure to ethinylestradiol (EE2) decreased the expression of TR alpha and suppressed the stimulatory effect of T3 (5 nM and 50 nM) on TR beta expression in the brain of *R. pipiens* tadpoles. Similarly, the estrogenic chemical bisphenol A (BPA) reduced the expression of TR beta almost completely and suppressed the T3-induced increase in TR beta mRNA expression in a dose-dependent manner in the tails of *X. laevis* tadpoles (Iwamuro *et al.*, 2003). Thus, the overall increases in TR alpha and TR beta mRNA levels by OP exposure observed in this study are not consistent with the typical responses of these genes to other estrogenic chemicals. OP does not appear to be acting as a weak estrogen, but rather it is increasing the expression of multiple thyroid-dependent genes.

Localized activity of deiodinase enzymes regulates the concentration of T3 in specific cells and tissues and is one of the most simple and elegant ways to control gene expression (Brown, 2005). Results demonstrate that OP, even in the presence of UVBR, had a significant effect on D2 mRNA levels in the brain and enhanced the responsiveness of D2 to 50 nM T3 compared to treatments not exposed to OP. OP also induced the expression of D2 in tail with fold change increases reaching up to ~12.5 fold difference when compared to samples in the control group, whereas the fold change increase of D2 in the brain only reached a maximum of ~3.5 fold in OP-treated animals. Relative D2 mRNA levels in the tail were also much more variable in the UVBR, OP and UVBR+OP groups than in the brain (*i.e.*, data set contained many outliers). The lack of variability in D2 levels in brain samples is not surprising, given the fact that neural gene expression is tightly regulated, thus controlling homeostasis. Although the only factor that significantly

affected D3 expression in the tail was T3, the response of D3 was extremely variable in UVBR, OP and UVBR+OP groups. These results demonstrate that D2 levels in the brain and tail and D3 levels in the tail of many animals either exposed to OP, UVBR and UVBR+OP were greatly affected. Since D3 inactivates TH by converting T3 to T2 and T4 to reverseT3, the increases in D3 mRNA levels in some animals may reflect their ability to maintain homeostasis and metabolize the excess T3 in their system. Since D2 activates TH (by converting T4 to the biologically active T3) and D3 inactivates TH, such alterations in deiodinase levels can disrupt local conversions of TH that are necessary for the coordinated timing of important developmental processes. Hogan *et al.* (2008b) demonstrated that exposure of Gosner stage 36 *R. pipiens* tadpoles to EE2 suppressed the expression of D2 in the brain and D3 in the tail, but increased D3 mRNA levels in the brain and potentiated the effect of 50 nM T3 on D2 expression in the tail. Again, the overall increases in D2 and D3 expression following OP exposure observed in this study are not the same as the response seen when tadpoles were exposed to EE2.

Since this experiment was conducted at a time when the developmental rates of tadpoles were not yet affected, it is difficult to relate the effects of UVBR and OP on mRNA levels to the developmental delays later observed in UVBR-treated tadpoles. However, these results clearly point to a disruption of the normal endocrine cascade that regulates the coordinated process of metamorphosis and show that exposure to OP with and without UVBR could potentially affect other TH-dependent processes such as brain development or metamorphic climax.

To assess the effects of pre-exposure to UVBR and/or OP on TH-induced morphological changes associated with metamorphosis, we measured the body weight,

SVL, tail resorption and hind limb development of tadpoles after seven days of exposure to T3 (Experiment 2). There was no change in the weight of a randomly chosen subset of tadpoles at various stages of development when it was measured days prior to Experiment 2. However, SVL and body weights measured at Time 0 increased moderately in tadpoles exposed to UVBR (regardless of the presence of OP), in a subset of stage 31 animals used in Experiment 2. A 10-day exposure of *R. pipiens* tadpoles to 0.07 W/m² UVBR alone and in combination with 206 µg/L OP respectively reduced and increased the body weight of stage 29 tadpoles measured two months after the exposure (Crump *et al.*, 2002). The use of wet weight as an indicator of somatic growth may lead to false conclusions because tadpoles exposed to many stressors such as UVBR and OP can often exhibit edema in various tissues (Chapter 2, Croteau *et al.*, 2008b). This is an important point to keep in mind when interpreting these results. It would be important to measure body dry weight, DNA and/or total protein contents to accurately assess growth. Nevertheless, it is the pituitary hormone prolactin (PRL) that stimulates larval growth in amphibians (Shi, 2000) thus it would be of interest to examine the possible link between the effects of UVBR on tadpole weight and PRL levels.

Results of the 7-day T3 challenge demonstrated that all animals exposed to T3 were responsive to treatment, as indicated by forelimb emergence in all tadpoles after seven days of exposure. However, the tail and hind limbs of all tadpoles pre-exposed to UVBR did not display the same response to T3 as those in the Control or OP only pre-treatments. Tadpoles pre-exposed to UVBR (with and without OP) exhibited reduced responsiveness to T3 in tail tissue, as measured by a smaller reduction in tail length when compared to the response of animals in the OP or Control group to the hormone. On the

other hand, the hind limbs of all tadpoles exposed to UVBR exhibited heightened sensitivity to T3, as there were significantly more tadpoles developing past stage 34 in UVBR and UVBR+OP pre-treatments after exposure to T3 compared to those in the control and OP groups. The inhibitory effect of UVBR on tail resorption and stimulatory effect on hind limb development is relevant since a developmental delay became apparent in these same UVBR-treated animals days prior to the experiment. These data indicate that UVBR disrupts the normal TH-dependent cascade of expected morphological changes. Morphological transformations at metamorphosis are mediated by changes in the expression of TH-dependent genes in various tissues, at various times (Shi, 2000). UVBR exposure may ultimately be affecting the expression of key genes involved in inducing specific morphological changes during development (*e.g.*, TRs, deiodinases), which in turn could alter the responsiveness of tissues to T3. Although these results do not specifically demonstrate that the delay in development of UVBR-treated tadpoles was the direct result of the effects of UVBR on the response of tissues to T3, they point to a clear disruption in TR-mediated processes at the level of the hind limbs and tail. The degree of TH responsiveness in various tissues depends upon the number of TRs present, TR binding affinities for TH, and the availability of free TH in the tissue (*e.g.*, through the activity of deiodinase enzymes) (Galton, 1992). Therefore, the effects of UVBR on tail and hind limb sensitivity to T3 could be attributable to an alteration in one of these factors. Until this work, no experiments on the effects of UVBR on the thyroid system had been conducted on amphibians; therefore there are no published results to which we can compare our results.

Although OP did not significantly affect T3-mediated tail resorption or the developmental rate of the hind limbs in this study, the PCR results reported above demonstrate that OP altered the expression of thyroid system-related genes, which could produce detrimental effects later in development. Fort *et al.* (2000) exposed *X. laevis* tadpoles to different concentrations of nonylphenol (NP), a metabolite of alkylphenol polyethoxylates surfactants similar to OP, for 14 days at metamorphic climax. NP stimulated the rate of tail resorption and increased whole body T3 levels in exposed animals. Exposure to NP with and without propylthiouracil (PTU, inhibits the synthesis of TH) demonstrated that tadpoles were responsive to PTU and that the stimulatory effect of NP on tail resorption could be reversed (Fort *et al.*, 2000). In another experiment, *R. catesbiana* tadpoles at developmental stages equivalent to Gosner 35-37 were exposed to NP (234 µg/L, 468 µg/L or 936 µg/L) for seven days with or without the addition of exogenous T3 (Christensen *et al.*, 2005). In treatments including T3, increasing concentrations of NP produced a significant decrease in the rate of cranial transformation, and the rate of tail resorption in the 936 µg/L NP treatment was significantly lower than in the controls. These results suggest an overall inhibitory effect of NP on metamorphic progression and tail resorption. However, the levels of NP used by Christensen *et al.* (2005) were typically greater than what is found in the environment and much higher than the level of OP used in the present study (2.06 µg/L), which may explain why we did not observe a similar morphological response to T3 in tadpoles pre-exposed to OP.

In conclusion, our results demonstrate that chronic exposure of tadpoles to the estrogenic chemical OP (regardless of the presence of UVBR or T3) produced an overall increase in the expression of all genes examined in the brain and of D2 in the tail. The

combination of UVBR+OP affected the expression of TR alpha in the brain as well as the responses of TR alpha and TR beta to T3 in tail tissue, demonstrating that exposure to multiple stressors can produce different gene expression profiles than those observed after exposure to a single stressor. Although UVBR alone did not alter the expression of any of the genes examined, analyses revealed that UVBR and UVBR+OP affected T3-dependent morphological changes in the hind limbs (*i.e.*, increased developmental rate) and tails (*i.e.*, decreased rate of tail resorption) of animals exhibiting delayed development. Additional research is required to elucidate the specific mechanism(s) of action of these stressors on the thyroid system. Assessing potential impacts at the onset of and during developmental delays at central and peripheral levels of the thyroid system such as disruptions in thyroid gland morphology, TH levels and mRNA levels of genes at the tissue level (*e.g.*, TRs, deiodinases) will provide a better understanding of the effects of these environmental stressors on the thyroidal status of exposed animals.

CHAPTER 4

Assessment of thyroid system disruption in *Rana pipiens* tadpoles chronically exposed to UVB radiation and 4-*tert*-octylphenol

4.1. INTRODUCTION

Metamorphosis in amphibians is a morphological and physiological transformation that is regulated by complex hormonal pathways. External environmental stressors and input from higher brain centers stimulate the secretion of hormones from the hypothalamus that regulate pituitary gland thyroid stimulating hormone (TSH) secretion. This in turn stimulates the production of thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3), from the thyroid gland (Denver, 1997, 1998; Shi, 2000). The thyroid gland is histologically and functionally similar in all vertebrates (Porter, 1972) and consists of follicles of varying sizes that contain colloid produced by the surrounding follicular cells, also called epithelial cells (Capen, 1999; Figures 4.1 and 4.2F). Epithelial cells respond to TSH by actively sequestering iodine and by releasing thyroid globulin (TG), thyroid peroxidase and iodine into the follicle lumen. Iodinated TG is then pinocytosed from the lumen and enzymatically cleaved to form T4 and T3 (Capen, 1999). Once synthesized, THs are released from follicular cells and are transported through the blood to target tissues by serum binding proteins (Denver *et al.*, 2002).

The abundance of TRs, the concentration of TH, and the localized activities of deiodinase enzymes that synthesize (*e.g.*, type 2 or D2) and inactivate (*e.g.*, type 3 or D3) THs are important factors that influence the timing of metamorphic events (Brown and

Cai, 2007). The main bioactive form of TH is T3, and most T3 is derived from localized metabolism of T4 to T3 by the type 2 deiodinase (D2) in peripheral tissues (Kuiper *et al.*, 2006). The expression of D3 allows tissue-specific regulation of intracellular T3 and T4 through its actions to convert T4 to inactive rT3 and active T3 to inactive T2 (Fort *et al.*, 2007) and is believed to protect cells from rising hormone levels (Brown and Cai, 2007). The local activity of both enzymes controls the levels of T4 and T3 available in different tissues (Dubois *et al.*, 2006). T3 controls metamorphosis by regulating a cascade of genes controlling morphogenesis and development. THs bind the TRs (alpha and beta) located within the nucleus of cells in target tissues to induce the transcription of genes involved in development (Shi, 2000). In contrast, prolactin (PRL), a pituitary hormone controlled by the hypothalamic neurosecretion of thyrotropin-releasing hormone (TRH), inhibits amphibian development and accelerates larval growth (Figure 1.2).

The morphology and histology of the thyroid gland reflect the amount of TH produced and stored in the follicular lumen (François-Krassowska, 1978; François-Krassowska, 1989; Jennings and Hanken, 1998), as well as the activity of the gland and the intensity of the stimulation by TSH (Capen, 1999). For example, when the thyroid axis is disrupted in a manner that TSH stimulation is sustained for a long period of time, there is an increase in epithelial cell height and the colloid becomes smaller with a higher incidence of vacuoles due to endocytosis. There may be a progressive depletion of colloid and partial collapse of follicles because endocytosis of colloid usually proceeds at a greater rate than synthesis (Capen, 1999). Changes in thyroid gland morphology by environmental stressors are therefore used to assess potential impacts on TH production during development and metamorphosis.

The thyroid gland becomes fully functional in several species of anurans at approximately Gosner stages 31-33 (Dodd and Dodd, 1976; OECD, 2004). The absence of the thyroid gland in *Xenopus laevis* (African clawed frog) has been shown to produce giant tadpoles that have hind limb buds that morphologically resemble buds at NF stage 51 (Nieuwkoop and Faber, 1956), which corresponds to Gosner stage 29 (Shi, 2000; Gosner, 1960). Coincidentally, this represents the approximate time that the onset of delays in development caused by UVBR has occurred in previous experiments using *R. pipiens* (Gosner stages 29-31; Chapters 2 and 3). It has been demonstrated that exposure to chemicals such as methoxychlor, TCDD, organochlorines and metals affects the thyroid gland in rainbow trout (Cooley *et al.*, 2001), amphibians (Fort *et al.*, 2004a; Fort *et al.*, 2004b) deer mice (Thuett, 2002) and rats (Wade *et al.*, 2002; Nishimura *et al.*, 2003). In contrast, there is a very limited amount of information in the literature on the effects of UVBR on thyroid gland morphology. Rabbits exposed to solar radiation showed a high proportion of small follicles and reduced or lack of colloid in numerous follicles (reviewed in Milne, 1974). Dose-dependent DNA fragmentation and apoptosis occurred in cultured rat thyroid cells irradiated with ultraviolet C radiation (UVCR) (Del Terra *et al.*, 2001). However, UVCR is not environmentally relevant, making these results difficult to interpret in terms of a possible outcome following exposure of the thyroid gland to environmental levels of UVBR. Research is needed to determine whether present day levels of UVBR can affect the thyroid gland of amphibians.

Production of T3 and the expression of TH-response genes play an important role in the coordinated development of tadpoles towards metamorphic climax. Exposure to chemicals can affect TH levels and the expression of genes involved in amphibian

development. For example, exposure of premetamorphic *Pseudacris regilla* (Pacific tree frog) tadpoles to tetrabromobisphenol-A altered TR alpha mRNA levels in the brain (Veldhoen et al., 2006). A 30-day exposure of *X. laevis* to methoxychlor slowed hind limb development during the transition from pre to prometamorphosis (*i.e.*, around Gosner stage 33-34) and caused follicular hyperplasia of the thyroid gland. Exposure to methoxychlor at metamorphic climax inhibited the rate of tail resorption in a concentration dependent manner and reduced and delayed the T3 surge during climax compared to control animals (Fort *et al.*, 2004b). In Chapter 3, it was demonstrated that exposure to OP alone and combined with UVBR could significantly affect gene expression and/or the response of genes and tissues to T3. Although tadpoles exposed to UVBR were developmentally delayed when morphological responses of tissues to T3 were measured, the rate of tadpole development was not yet affected when samples were collected for the gene expression work. Here, we examine tadpoles at the onset of and during the delays in hind limb development to elucidate the specific mode of action of UVBR and OP to disrupt amphibian development.

The objectives of this study were to: (1) determine the effects of chronic exposure to environmental levels of UVBR on the morphology of the thyroid gland in *R. pipiens* tadpoles; and (2) determine the effects of chronic exposure to environmental levels of UVBR and/or OP on T3 levels and the expression of genes important for development at three significant developmental stages. Histomorphological endpoints including the number of follicles, density of colloid and area, roundness, and perimeter of colloidal space were measured. Changes in the expression of TR (alpha and beta), deiodinase (D2

and D3) and prolactin receptor (PRLR) genes were measured in the tail of tadpoles exposed to UVBR and/or OP and bodies (minus the tail) were used to measure T3 levels.

4.2. MATERIALS AND METHODS

4.2.1 Overview

This chapter includes the results of two separate experiments. The first involves samples collected during the exposure experiment described in Chapter 2. *R. pipiens* tadpoles were exposed to UVBR ($\sim 0.22 \text{ W/m}^2$) alone and in combination with either 0.01 nM (OP1) or 10 nM (OP2) of OP for eight months and then were kept in a clean water grow-out system for three additional months. Results demonstrated that the onset of developmental delays occurred at stage 29 in all treatments compared to control. There was also a significantly lower percentage of tadpoles that developed past stage 34 and that reached metamorphosis (stage 42) in all tadpoles exposed to UVBR (refer to Chapter 2 for details on the exposure and results). For these reasons, samples were collected at stages 29, 34 and 42 during this experiment for analyses of relative mRNA levels of genes that influence metamorphosis and to measure T3 (triiodothyronine) levels. Details on tissue collection, protocols for these molecular assays and results are described below.

The results of a separate exposure experiment conducted in 2004 are also presented in this study, examining the effects of UVBR exposure on the morphology of the thyroid gland of stage 31 *R. pipiens* tadpoles. This stage was chosen because it is during this development period (stages 29-31) that the onset of developmental delays in tadpoles treated to UVBR was observed in previous experiments (see Chapters 2 and 3).

Relative mRNA levels of genes involved in metamorphosis were also measured in tail samples collected from stage 26 animals during this exposure.

4.2.2 Thyroid Gland Histology

4.2.2.1 Animal Collection and Care

Portions of four naturally fertilized *R. pipiens* egg masses were collected in late April, 2004. Information on the location from which the animals were collected and laboratory rearing conditions is described in Chapter 2 (section 2.2.1).

4.2.2.2 Conditions of Exposure

The water used in the exposure was as described in Chapter 2 (section 2.2.1) at the time of water renewal, with the exception of the water temperature (21 to 22°C). Gosner stage 25 (beginning of independent feeding; Gosner, 1960) tadpoles were randomly assigned to either the control or UVBR treatment. At the beginning of the experiment, 210 tadpoles/treatment weighing on average approximately 0.03 g were divided among seven Pyrex bowls filled with water (*i.e.*, 30 tadpoles/bowl). Tadpoles were maintained at a constant density by adjusting water levels in the bowls and by adding replicate bowls to account for tadpole growth, mortality and sampling throughout the exposure. For example, after approximately one month of exposure there were 7 to 9 tadpoles/bowl. The water was renewed six times weekly for the duration of the exposure period and animals were fed *ad libitum* with Nutrafin® fish flakes (Rolf C. Hagen Inc., Montréal, QC) and tadpole food (Carolina Biological Supply Co., Burlington, NC) (approximately equal amounts) on the mornings the water was changed. Dissolved oxygen, pH and ammonia levels were measured with the same equipment and maintained

at the same levels as in Chapter 3 (see section 3.2.3), with the exception of ammonia levels which ranged between the detection limit (0.002 mg N/L as NH₃) and 0.0092 mg N/L as NH₃.

4.2.2.3 Controlled Environmental Chambers

Tadpoles were kept in a controlled environmental chamber (Conviron model CMP 3023; Winnipeg, MB) for the duration of the experiment. The solar simulators were composed of fluorescent and incandescent lights as sources of ultraviolet A radiation (UVAR; 320-400 nm), ultraviolet B radiation (UVBR; 280-320 nm) and visible light (VIS; 400-750 nm). The incubator contained a combination of 14 fluorescent lamps (12 Sylvania Cool White F72T12/CW/HO 85W Canada H398 and two F24T12/CW/HO 35W Cool White USA) and seven incandescent bulbs (Sylvania 60W 6000hrs 120-125V).

The photoperiod was the same as reported in Chapter 2 (section 2.2.3). Shelves were positioned at 45.5 cm below the lamps to attain the desired UVAR, UVBR and VIS intensities. UVBR was obtained by removing the protective cover that usually shields the lamps. The temperature inside the incubator was set at 18 °C during the light cycle and 23°C during the dark cycle of the photoperiod. Under these conditions the water temperature was maintained relatively constant between 21-22°C during the entire day.

4.2.2.4 UVR Levels

The same photometer and probes were used to measure UVR/VIS levels inside the environmental chamber, as described in Chapter 3 (refer to section 3.2.5). The average irradiances measured were 0.21 W/m² UVBR, 1.17 W/m² UVAR and 31.45 klx VIS. The control bowls were also in the environmental chamber but were covered with

Mylar shields, as described in Chapter 2 (section 2.2.4). Bowls were randomly rotated daily within the incubator to minimize the effects of fluctuations in UVR/VIS levels, temperature and external stimuli.

4.2.2.5 Tissue Collection

The stages of development of tadpoles were recorded regularly during the experiment according to the Gosner staging system (Gosner, 1960). After 14 days of exposure, stage 26 (beginning of hind limb bud growth) tadpoles were collected for real-time RT-PCR analyses. Stage 31 (foot paddle stage) tadpoles were collected for histological analyses of the thyroid gland after 84 days of exposure. Stage 26 animals were anaesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS222, Sigma-Aldrich; 1 g/L in water), weighed and the tails were immediately dissected on ice, frozen on dry ice and stored at -80°C . Stage 31 animals were also anaesthetized and the bodies (minus the tail) were fixed in modified Davidson's fix (see Appendix A for protocol) for 3.5 days. The heads were then trimmed, placed in individual plastic vials and transferred to 70% ethanol until histological processing.

4.2.2.6 Histological Processing and Analysis

An automated tissue processor (Tissue-Tek® VIP™ Vacuum Infiltration Processor E150/E300 Series; Sakura Finetek U.S.A. Inc., Torrance, CA) was used for dehydration (in a series of increasing graded ethanols), clearing (in xylene), and infiltration of tissues in paraffin (see Appendix A for protocol). Using a rotatory microtome (Microm HM350; Microm, Heidelberg, Germany), specimens were sectioned serially using transverse step sections ($5\mu\text{m}$) through the area of the eyes from caudal-

rostrally. Sections were spread in a water bath at 49°C containing STA-On[®] tissue section adhesive (Surgipath; Richmond, IL), and the ribbons were mounted on precleaned glass slides (Surgipath). Slides were dried at 57°C for 30 min and the sections were then deparaffinized, rehydrated, stained with Periodic Acid Schiff (PAS) stain, and cover slips were mounted with Permount (Fisher) (see Appendix A for further details).

The PAS stain is used because the colloid inside the thyroid follicles becomes an intense purple (magenta) color that can be easily identified (Wade *et al.*, 2002). The stained sections were examined under a light microscope and the treatment to which the sample was exposed remained unknown to the observer. Since the right lobe of the paired thyroid gland is considered to be the same as the left lobe, we based our observations on the right lobe. Images of the right lobe of every second section per gland were captured using a digital camera (Qimaging Micro Publisher 3.3 RTV) connected to a Leitz light microscope. All images were taken using the same calibrations (light adjustments and white balance) and on the same day to minimize differences in natural light.

4.2.2.7 Histomorphological Endpoints and Data Analysis

Histomorphological analysis of the thyroid gland (4 tadpoles/treatment; 8 tadpoles total) was conducted using the images captured as described above. At this stage of tadpole development (Gosner 31), the thyroid gland was not completely developed and the follicles were still rudimentary. Epithelial cell height was not measured in this study, as many thyroid follicles examined did not possess an organized layer of epithelial cells surrounding the colloidal space. Therefore, only the colloidal space of each follicle in the images was selected and used to calculate the area, mean density, roundness, and

perimeter using the digital image analysis software ImagePro Plus v. 5.0 (Media Cybernetics, Bethesda, MD). Area and mean density were measured to estimate follicle volume and the amount of colloid inside the follicles, respectively. Perimeter was measured to estimate the size of the follicles. As hyperactive follicles can collapse as the colloid found inside the follicle is depleted, roundness was calculated to estimate the extent to which the follicle cross section deviated from the normal round shape. A perfect circle would be attributed a value of 1, and the value would increase as the perimeter of the colloidal space became less circular (Wade *et al.*, 2002). Additionally, the number of follicles was measured to estimate the size of the gland by counting every follicle present in all images taken for each tadpole.

Data was assessed for normality and homoscedascity and was analyzed using Student's t-tests if these criteria were met. When normality and homoscedascity could not be achieved using standard data transformations, Mann-Whitney U tests were used to analyze the data. The accepted level of statistical significance was set at $p < 0.05$. SYSTAT (Version 10; San José, CA) and MS Excel 2000 software (Seattle, WA) were used to conduct the statistical analyses.

4.2.3 RNA Extraction, DNase Treatment and cDNA Synthesis

Tissue collection of animals from the UVBR/OP exposure conducted in Chapter 2 was based on a stage-matched comparison to account for differences in hormone concentrations between developmental stages. Therefore, stage 29, 34 and 42 animals were weighed, tails were dissected, and tails and bodies were immediately placed in

separate tubes and frozen on dry ice, regardless of their age. The tails were used for real-time RT-PCR analysis and the bodies were used for T3 measurements.

Stage 26 tails collected during the 2004 histology experiment (7 tadpoles/treatment) and stage 29, 34 and 42 tails (n=6 animals/treatment) were processed for RNA isolation. TRIzol Reagent[®] (Invitrogen Life Technologies, Carlsbad, CA, USA) was used as described by the manufacturer to extract total RNA from tail samples. The RNA of stage 26 animals was DNase treated using the RNeasy[®] Micro Kit as per the manufacturer's protocol (Qiagen, Mississauga, ON). The RNA of stage 29, 34 and 42 tails was treated with RQ1 RNase-Free DNase as per the manufacturer's protocol (Promega, Madison, WI). DNase treated RNA was purified by organic extraction using phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform as described by Sambrook *et al.* (1989). Ethanol (99%) and sodium acetate (0.3 M) were used to precipitate the treated RNA and the RNA was resuspended in RNase-free water. The concentration of total RNA in all samples was measured spectrophotometrically using GeneQuant[®] (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and samples were stored at -80 °C.

cDNA was synthesized with approximately 1 µg of RNA and 200 ng random hexamer primers using Superscript[™] II RNase H⁻ reverse transcriptase as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). The 20 µL cDNA sample was diluted to a final concentration of 0.5 ng/µL to 5 ng/µL before real-time RT-PCR amplification, and stored at -20 °C.

4.2.4 Cloning of Prolactin Receptor (PRLR)

A partial cDNA sequence for PRLR was cloned from *R. pipiens* tail tissue. Degenerate primers were designed using conserved regions of amphibian (*Xenopus laevis*, *Rana catesbeiana* [Bullfrog], *Xenopus tropicalis* [Western clawed frog]) PRLR gene sequences obtained from GenBank and constructed using ClustalW and Primer3 programs. Primers used to clone PRLR were: forward primer 5'-TTTCNCCTAACCTCNGACTNTG-3'; reverse primer 5'-GCTAGGGCGTNNTTTTGGNT-3'. All cloning procedures were conducted as described in Chapter 3. The cDNA sequence of 662 bp for *R. pipiens* PRLR has been deposited in the GenBank database (accession no. EU030184).

4.2.5 Real-Time RT-PCR

All real-time RT-PCR analyses of relative gene expression in tail samples were carried out using an Mx4000 real-time polymerase chain reaction system (Stratagene, La Jolla, CA). A total of 4 µL of diluted cDNA (*i.e.*, 2 to 20 ng) was added to the real-time multiplex RT-PCR reactions. Refer to Chapter 3 for information on the multiplex assays used to measure relative mRNA levels of TR alpha, TR beta, D2 and D3.

The relative mRNA levels of PRLR and L8 in tail samples was measured in a simplex real-time RT-PCR SYBR Green I-based assay. Primers were designed for the simplex assay of PRLR based on the previously described *R. pipiens* PRLR partial cDNA sequence (accession no. EU030184). Primer sequences were: forward primer 5'-AGGTGGATGACAGCAAGGAG-3'; reverse primer 5'-GGGCATGTAGTTGCAGAAGG-3' and amplified a sequence of 220 bp. Primers were

tested by RT-PCR using *R. pipiens* tail cDNA and the resulting amplicons were sequenced to confirm primer specificity. The simplex real-time PCR SYBR Green I-based assay was performed the as described in Chapter 3, with the exception of the annealing temperature (58° C), the primer concentration (100 nM) and that 5µL of diluted cDNA template (*i.e.*, 8 ng) was added to the reaction.

Relative standard curves were generated as described in Chapter 3 (section 3.2.8.5). All real-time PCR reactions included a no template control and a no reverse transcriptase control. Samples were run in triplicate, and the data generated for each sample were averaged and normalized to L8. Normalized data for all samples within a treatment group were then averaged and are expressed as the mean relative mRNA expression + standard error of the mean (SEM) relative to the control group. The expression of L8 did not change in response to treatment in both the simplex and multiplex assays.

PCR data were tested for normality and homoscedacity and if these criteria were not met, the data were log transformed. Data for stage 26 tail samples were analyzed by Student's t-tests. Two-way ANOVAs were used to analyze stage 29 and 34 data, and comparisons between treatments were based on examination of Least Squares Means (LSM) and multiple comparisons using Tukey's honestly significant difference (HSD) post-hoc tests. Given that a small number of tadpoles exposed to UVBR reached metamorphosis (stage 42; refer to results section 2.3.3), the data collected for stage 42 animals in all UVBR treatments could not be analyzed since the sample size was two or less. Therefore, t-tests were performed between the OP1 or OP2 treatments and the control group to determine if these factors had an effect on relative mRNA levels.

4.2.6 Thyroid Hormone Extraction

Thyroid hormone was extracted from the bodies (minus the tail) of stage 29 and 34 tadpoles (n=8 tadpoles/treatment) using methods adapted from Denver (1993) and references therein. Briefly, samples were homogenized in three volumes of methanol (MeOH) containing 1 mM propylthiouracil (PTU). Samples were homogenized for 20 s using a Polytron homogenizer (Brinkmann Instruments Ltd., Mississauga, ON) and sonicated for 20 s using a Branson Sonifier (Branson Ultrasonics Co., Danbury, CT). A 200 μ L aliquot of the homogenate was removed and stored at -20°C for subsequent protein determination (see section 4.2.8 below). To estimate hormone extraction efficiencies, [125 I]- T3 (radioactive 3,5,3'-triiodothyronine; Perkin Elmer Inc., Waltham, MA) was used as a radioactive tracer. Free iodine was removed from the T3 label prior to use with a Sep-Pak C18 cartridge (Waters Co., Milford, MA). The cartridge was activated with 1 mL MeOH and rinsed with 1 mL of 0.02 M phosphate-buffered saline (PBS; pH 7.4). The [125 I]T3 was loaded onto the cartridge in a volume of 0.1 mL PBS, washed four times with 1 mL PBS and eluted in a volume of 3 mL MeOH. The eluted tracer was diluted with MeOH-PTU to give 1000 cpm/200 μ L. This volume was added to each sample homogenate and incubated for 15 min at room temperature. Samples were vortexed (VWR VX-2500, Mississauga, ON) for 12 min and centrifuged (IEC 3000R, Worcester, MA) for 20 min (1300 x g; 4°C). After centrifugation, the supernatant was decanted into tubes containing 5 mL chloroform (CH₃Cl) and extracted into an aqueous phase with 0.5 mL 2 N ammonium hydroxide (NH₄OH). The aqueous phase was removed and the extraction was repeated twice with the same CH₃Cl but new NH₄OH. The tissue pellet obtained above was kept in 1 mL of MeOH-PTU and was extracted

three times as previously described. All aqueous phases were pooled and extracted a final time with an equal volume of CH₃Cl. The final extract was loaded onto Bio-Rad Poly-Prep ion exchange chromatography columns (Mississauga, ON), which were prepared with Bio-Rad AG 1X2 chloride resin (200-400 mesh; 1.5 mL bed volume) in 0.2 M acetate buffer (pH 7) and equilibrated with 0.2 M acetate buffer (pH 7). The columns were then washed with the following series of solutions: (1) 2 mL acetate buffer (pH 7); (2) 2 mL of 1 mM ethanol (EtOH)-PTU; (3) 4 mL acetate buffer (pH 7); (4) 2 mL 1 mM EtOH-PTU; (5) 2 mL acetate buffer (pH 7); (6) 2 mL acetate buffer (pH 4); (7) 2 mL acetate buffer (pH 3); (8) 2 mL 1% acetic acid; and (9) 2 mL 35% acetic acid. Samples were eluted from columns in 1.5 mL 70% acetic acid, dried in a Savant AE2010 Speedvac (Holbrook, NY), and resuspended in 200 µL of human steroid-free serum (MP Biomedicals Inc., Orangeburg, NY). Extraction efficiencies were based on the recovery of radiolabeled T3 tracer. Samples were counted in a gamma counter (Packard Cobra™ auto-gamma®, Hewlett Packard Co., Mississauga, ON) and recoveries ranged from 30 to 70%.

4.2.7 Enzyme-Immuno Assay (EIA)

T3 levels were measured with enzyme immunoassay (EIA) test kits for human serum (MP Biomedicals Inc., Orangeburg, NY), as per the manufacturer's instructions. Steroid-free serum in which the samples were reconstituted was initially tested by EIA and no thyroid hormone was detected (<0.2 ng T3/mL). The standard curve and samples were assayed in duplicate. Serial dilutions of sample extracts were parallel to the curve produced by standards supplied in the EIA test kits. Samples that were non-detectable by the kit (*i.e.*, seven Gosner stage 29 tadpoles) were ascribed an absorbance value that

equaled 95% of the absorbance value of the zero standard supplied in the EIA test kits. This value was then plotted on the standard curve to determine the T3 level in these samples. T3 levels measured were corrected for volume and recoveries and were normalized using tadpole body weight (wet weight) or protein level. Data were tested for normality and homoscedacity and were log transformed to meet these criteria. Data were then analyzed by two-way ANOVA using SYSTAT software, and statistical significance was set at $p < 0.05$.

4.2.8 Protein Determination

The 200 μL aliquot collected following tissue homogenization and sonification was used in the Bio-Rad Protein assay to measure protein levels. The microassay procedure for microtiter plates was performed as per the manufacturer's instructions. Due to the incompatibility of the methanol solvent in which the samples were homogenized with this protocol, samples were dried in a Savant AE2010 Speedvac and reconstituted in 800 μL ddH₂O. Samples were vortexed for approximately 60 s and centrifuged for 15 min (15000 $\times$ g; 4°C) and the supernatant was used in the assay. Bovine serum albumin (BSA) was serially diluted in ddH₂O to provide the standard curve, and both the standard curve and samples were assayed in duplicate. The protein concentration measured was used to calculate the amount of protein per animal in mg.

4.2.9 Weight and Stage of Development

Data for developmental stages are not reported for the 2004 experiment because samples were collected frequently for histological or PCR analyses and therefore the

effect of UVBR on the rate of development could not be accurately assessed. However, we did take note of the maximum developmental stage reached by tadpoles in the control and UVBR treatments during the exposure.

The effect of treatments on the number of tadpoles that developed past stage 29 (*i.e.*, median stage of the control group) at week 9 of the UVBR/OP exposure (Chapter 2) was analyzed using a log linear model (n= 94 to 142 tadpoles/treatment). The body weight data of stage 29 (n= 9 to 11 tadpoles/treatment) and 34 tadpoles (n= 10 to 11 tadpoles/treatment) that were exposed to UVBR/OP were tested for normality and homoscedacity and met these criteria. Two-way ANOVAs were used to analyze the data, and comparisons between treatments were based on examination of LSM and multiple comparisons using Tukey's HSD post-hoc test. Weight and developmental data were analyzed using SYSTAT and MS Excel 2000 software. Statistical significance was set at $p<0.05$.

4.3. RESULTS

4.3.1 Thyroid Gland Histology

The histomorphological endpoints measured in the right lobe of the thyroid gland are summarized in Table 4.1. The thyroid gland in tadpoles from both the control and UVBR treatments contained follicles of varying size, with an average of 85 to 90 follicles per animal (when observing every second slide). Each follicle contained colloid (stained magenta), surrounded by follicular cells (stained blue) (Figure 4.2). Vacuoles were equally observed in the colloid of follicles in the control and UVBR groups (data not shown), indicating similar activity in the thyroid glands of both groups. Statistical

analyses of the data collected for all endpoints measured in Table 4.1 indicate that there are no differences in the morphology of the thyroid gland of stage 31 tadpoles exposed to UVBR when compared to those in the control group. By the end of the experiment, it was noted that none of the tadpoles exposed to UVBR had developed past stage 31 (foot paddle stage) while the maximum developmental stage reached by tadpoles in the control group was stage 34 (early digit differentiation).

4.3.2 Weight and Stage of Development

The body weight of tadpoles used for real-time PCR and T3 measurements was determined and analyzed to assess if exposure to UVBR and/or OP affected this endpoint. Stage 29 animals exposed to OP (regardless of the presence of UVBR) had increased body weights (OP $p < 0.0001$) when compared to animals not exposed to OP (Table 4.2). Based on the examination of the Least Squares Means (LSM), OP1 and OP2 produced an overall increase in tadpole body weight of ~38% and 47%, respectively. There was no effect of treatments on the body weight of stage 34 tadpoles (Table 4.2).

Statistical analysis indicated a significant effect of UVBR ($p < 0.0001$) and OP ($p < 0.0001$) alone on the number of animals developing past stage 29 after 9 weeks of exposure (Table 4.2). Approximately 36% of tadpoles in the control group had developed past stage 29 at this time, compared to 29% of those exposed to UVBR alone, 17-19% of those exposed to OP1 and OP2 and 5-7% of those exposed to UVBR+OP1 and UVBR+OP2. This signifies that the development of all tadpoles exposed to UVBR and OP was delayed compared to animals in the control group. The median developmental

stage of tadpoles from the control group was stage 29 on week 9, whereas the median stage in all other treatments was stage 28 (Table 4.2).

4.3.3 Real-time RT-PCR

4.3.3.1 Gosner stage 26 tadpoles

Figure 4.3 shows the relative mRNA levels of TR alpha, TR beta, D2, D3 and PRLR in the tail of Gosner stage 26 animals exposed to UVBR or from the control group. Statistical analysis revealed that UVBR had a significant 1.8 fold increase in D3 mRNA abundance ($p=0.039$) compared to levels in control animals (Figure 4.3B). There was no effect of UVBR exposure on the expression of TR alpha, TR beta, D2 or PRLR.

4.3.3.2 Gosner stage 29 tadpoles

The mRNA levels of TR alpha, TRbeta, D2, D3 and PRLR in the tail of Gosner stage 29 animals exposed to UVBR and/or OP is illustrated in Figure 4.4. UVBR had an overall effect on TR alpha mRNA levels ($p=0.030$), corresponding to a ~17% increase derived from the examination of Least Squares Means (LSM) (Figure 4.4A). The term UVBR+OP was also significant ($p=0.022$), which signifies that an interaction between UVBR and OP produced a response that was statistically different from that of UVBR or OP alone. For example, Tukey's HSD post hoc analysis indicated a significant difference between OP2 and UVBR+OP2 groups ($p=0.016$), as OP2 caused a small decrease in TR alpha mRNA level and UVBR+OP2 produced the opposite effect (Figure 4.4A).

Although OP had no effect on TR alpha levels, it produced a minor but significant ~11% increase in TR beta mRNA levels ($p=0.048$) in treatments including either OP1 or

OP2 based on the LSM analysis (Figure 4.4A). UVBR exposure produced a significant ~14% increase in TR beta expression ($p=0.005$) compared to the effect of treatments without UVBR based on the examinations of LSM (Figure 4.4A). The UVBR+OP treatments had no effect on TR beta expression.

Figure 4.4B illustrates the relative mRNA abundance of D2 and D3 in the tail of stage 29 tadpoles exposed to UVBR and/or OP. UVBR ($p<0.0001$) and UVBR+OP ($p=0.043$) were identified as statistically significant factors in the ANOVA, causing a ~3.5 fold decrease in the expression of D2. Although OP as a main factor was not identified as statistically significant ($p=0.080$), exposure to OP2 produced a 3-fold decrease in D2 mRNA levels (Figure 4.4B). Tukey's HSD post hoc test detected a difference in the expression of D2 between the effects of the control group and the OP2 group ($p=0.016$). This is likely a result of the relatively low p value of OP as a main factor in the ANOVA, which was higher than 0.05 but close enough to statistical significance that one of the concentrations of OP could be identified as statistically significant in the pairwise comparisons. The post-hoc test also indicated that the D2 mRNA levels measured in the UVBR ($p=0.002$), UVBR+OP1 ($p=0.004$) and UVBR+OP2 ($p=0.003$) groups were different than that of the control treatment (Figure 4.4B).

Relative D3 mRNA levels were affected by exposure to OP ($p=0.008$). However, exposure to the two concentrations of OP resulted in opposing effects on D3 mRNA levels (Figure 4.4B). Exposure to OP1 resulted in a ~3 fold induction of D3 expression, whereas exposure to OP2 and UVBR+OP2 caused a decrease in D3 mRNA levels (1.67

and 1.37 fold reduction, respectively) (Figure 4.4B). An interaction between UVBR+OP was also identified as a factor affecting the expression of D3 in the ANOVA ($p=0.016$).

Statistical analysis revealed an effect of OP ($p=0.044$) on PRLR mRNA abundance (Figure 4.4C). Examination of LSM indicated that all treatments that included OP1 and OP2 exposure caused a ~45% and ~43% increase in the expression of this gene, respectively. However, UVBR alone and UVBR+OP were not statistically significant terms in the ANOVA ($p\geq 0.05$).

4.3.3.3 Gosner stage 34 tadpoles

The mRNA abundance of TR alpha, TRbeta, D2, D3 and PRLR in the tail of Gosner stage 34 tadpoles exposed to UVBR and/or OP is shown in Figure 4.5. There was a general decrease of UVBR on TR alpha mRNA abundance ($p=0.0030$) in animals exposed to UVBR. Exposure to OP also had an overall effect on the expression of TR alpha ($p<0.0001$). Exposure to OP1 and OP2 alone produced a concentration dependent effect, with 1.9 and 2.5 fold inductions in TR alpha mRNA levels, respectively (Figure 4.5A). Tukey's HSD post hoc analysis indicated that the effect of OP2 on TR alpha mRNA abundance was statistically different from that of all other treatments except OP1. The post hoc test also detected a significant difference in TR alpha mRNA levels between OP1 and the control group and between OP1 and the UVBR group (Figure 4.5A).

There was an effect of OP ($p<0.0001$), UVBR ($p<0.0001$) and UVBR+OP ($p=0.004$) on the expression of TR beta (Figure 4.5A). There was a concentration dependent effect of OP alone on the expression of TR beta (Figure 4.5A), which caused a 2.3- and 3.6-fold induction in mRNA levels in OP1 and OP2 treatments, respectively

(Figure 4.5A). Results of the post hoc analysis indicated that the effect of OP2 on TR beta mRNA levels was statistically different from that of all other treatments except OP1. Post-hoc comparisons also detected a significant difference in mRNA levels between OP1 and all other treatments, except OP2 and UVBR+OP2 (Figure 4.5A).

The mRNA levels of D2 and D3 in stage 34 tadpole tails is shown in Figure 4.5B. Statistical analysis indicated that there was no effect of treatments on the expression of D2. However, UVBR exposure produced a significant overall 140% increase in D3 mRNA levels based on the analysis of LSM ($p=0.001$). OP and UVBR+OP were not statistically significant factors in the ANOVA.

Figure 4.5C illustrates the relative mRNA abundance of PRLR, and shows that treatments that included OP exposure caused a general increase in mRNA expression ($p=0.016$) when compared to the control and UVBR only exposure groups. Observation of LSM indicated that treatments that included OP1 and OP2 exposure produced a ~68% and ~100% overall increase in the expression of this gene, respectively. However, UVBR alone and UVBR+OP were not statistically significant terms in the ANOVA ($p\geq 0.05$).

4.3.3.4 Gosner stage 42 tadpoles

There was no effect of OP1 or OP2 on TR alpha, TR beta, D2 and D3 relative mRNA levels ($p\geq 0.05$; Figure 4.6) in Gosner stage 42 tadpoles. There is no data for UVBR, UVBR+OP1 and UVBR+OP2 treatments in Figure 4.6 because the sample size for these groups was two or less.

4.3.4 T3 Levels

Whole body (minus the tail) T3 levels were measured in stage 29 (Figure 4.7) and 34 (Figure 4.8) tadpoles exposed to UVBR and/or OP. Statistical analysis of T3 levels corrected for body mass and protein level in tadpoles at both developmental stages revealed no significant differences between treatment groups.

Table 4.1 Histomorphological endpoints measured in the right lobe of the thyroid gland of control and UVBR-treated Gosner stage 31 *Rana pipiens* tadpoles (n=4/treatment).

Endpoints ^a	Control	UVBR
Area of colloidal space (μm^2)	202.7 (191.0-244.3) ^c	223.2 (153.2-228.6)
Roundness ^b of colloidal space	1.23 (1.22-1.40)	1.29 (1.24-1.31)
Perimeter of colloidal space (μm)	51.85 $\pm$ 1.83	52.39 $\pm$ 1.96
Mean density of colloid	2113.0 (2042-2425)	2074.6 (1968-2252)

^a These endpoints were measured in every second slide. Statistical analyses indicate that there was no effect of UVBR on the endpoints measured ($p \geq 0.05$).

^b Roundness is used to estimate the degree to which the follicle cross section deviates from the normal round shape (value equal to 1).

^c Means ($\pm$ standard error of the mean) of the endpoints are presented for data assessed using the Student's t-tests and medians (including the range of data in parentheses) are presented for data assessed using Mann-Whitney U tests.

Note. Epithelial cell height was not measured in this study since most of the thyroid follicles examined did not have an organized layer of epithelial cells surrounding the colloidal space.

Table 4.2 Median stages, percentage of tadpoles developing past Gosner stage 29, and average body weight (g $\pm$ SEM) of stage 29 and 34 animals exposed to UVBR and/or OP.

Treatments ^a	Median Stage ^b	% Past Median ^c	Weight (g) Stage 29 ^d	Weight (g) Stage 34 ^d
C	29	36.2% (34/94)	0.38 $\pm$ 0.027 ^e (xz)	0.89 $\pm$ 0.057
OP1	28	18.6% * (22/118)	0.47 $\pm$ 0.027 (xy)	1.02 $\pm$ 0.063
OP2	28	16.9% * (24/142)	0.49 $\pm$ 0.020 (xy)	0.91 $\pm$ 0.058
UVBR	28	28.8% * (34/118)	0.30 $\pm$ 0.033 (z)	0.97 $\pm$ 0.066
UVBR+OP1	28	4.9% * (5/103)	0.48 $\pm$ 0.036 (xy)	0.86 $\pm$ 0.059
UVBR+OP2	28	6.9% * (7/102)	0.53 $\pm$ 0.041 (y)	1.10 $\pm$ 0.126

^a C, Control; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol; UVBR, UVB radiation.

^b Median developmental stage of all remaining tadpoles after 9 weeks of exposure.

^c The median stage used for this endpoint was that of the control group at week 9 (Gosner stage 29). The % Past Median was calculated by counting the number of tadpoles that had developed past stage 29 (*i.e.*, by counting animals at stages 30+) at week 9. The numerator in parentheses represents the number of tadpoles that developed past the median stage and the denominator is the total number of animals measured.

^d Sample size for stage 29 and 34 tadpoles is 9-11 and 10-11 tadpoles/treatment, respectively.

^e SEM, standard error of the mean.

*Indicates a significant effect of UVBR ($p < 0.0001$) and OP ($p < 0.0001$) on the number of tadpoles developing past Gosner stage 29.

Note. There was a significant effect of OP on the body weights of Gosner stage 29 tadpoles ($p < 0.0001$). Different letters in parentheses (x,y,z,) represent statistically significant differences between treatments, as determined by Tukey's HSD post hoc test. There were no statistical differences between treatments on the body weights of stage 34 tadpoles.

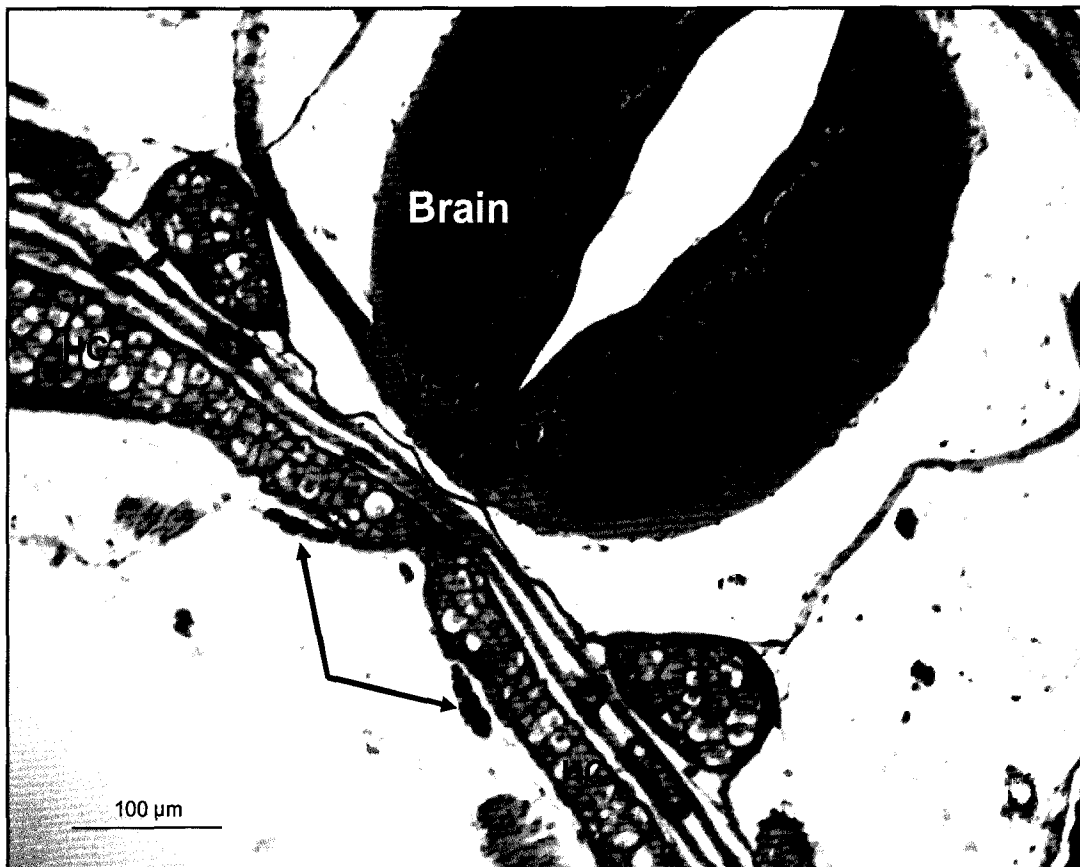


Figure 4.1 Cross section (5 μ m) of a Gosner stage 27 *Rana pipiens* tadpole head (PAS stained) with arrows indicating paired thyroid glands. HC, hyoid cartilage.

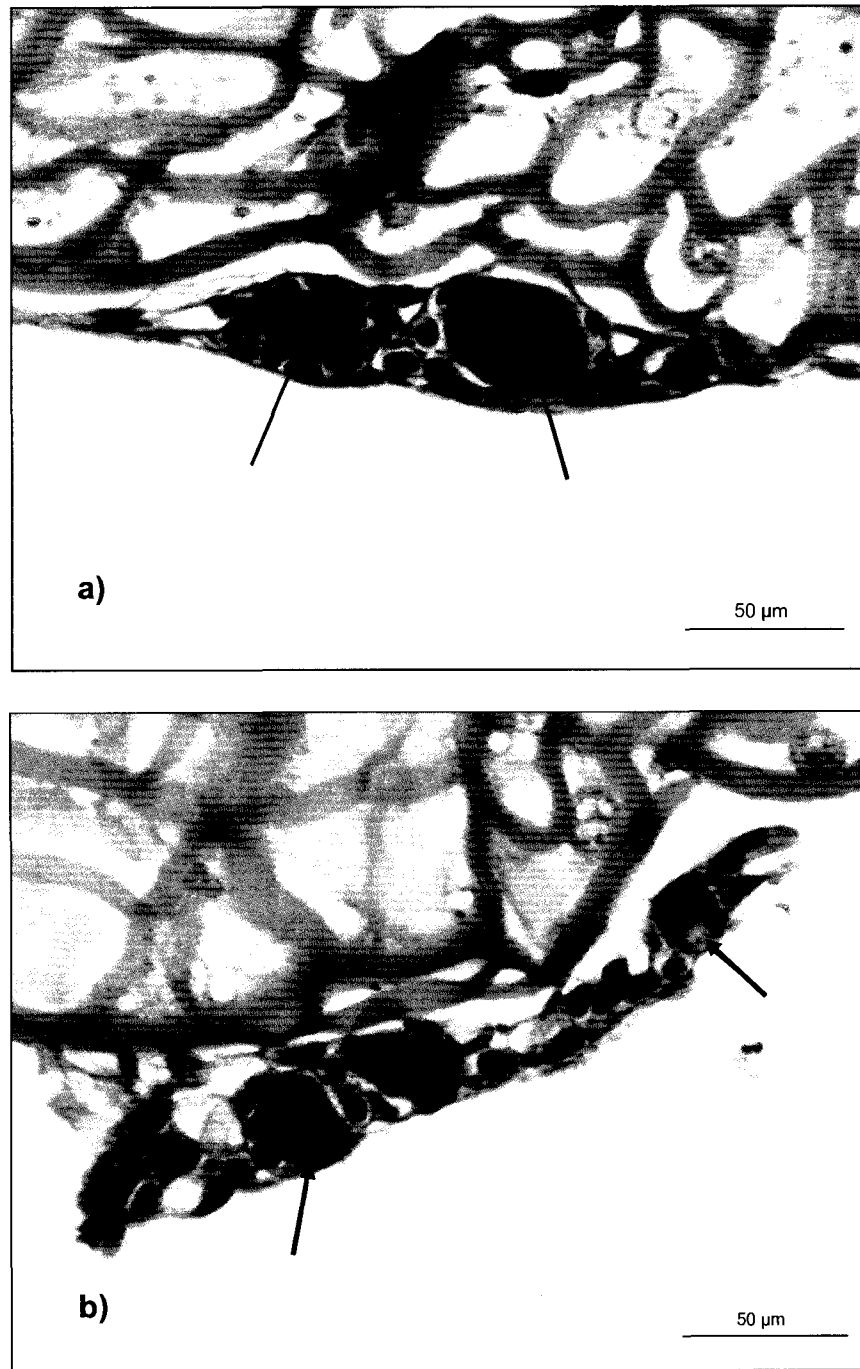


Figure 4.2 Cross section (5µm) of the right lobe of the thyroid gland in Gosner stage 31 *Rana pipiens* tadpoles from a) the control group and b) the UVBR-treated group. The arrows indicate follicles filled with homogenous colloid (stained magenta) and surrounded by epithelial cells (stained blue); the arrow to the right in panel b points to a vacuole inside the colloid.

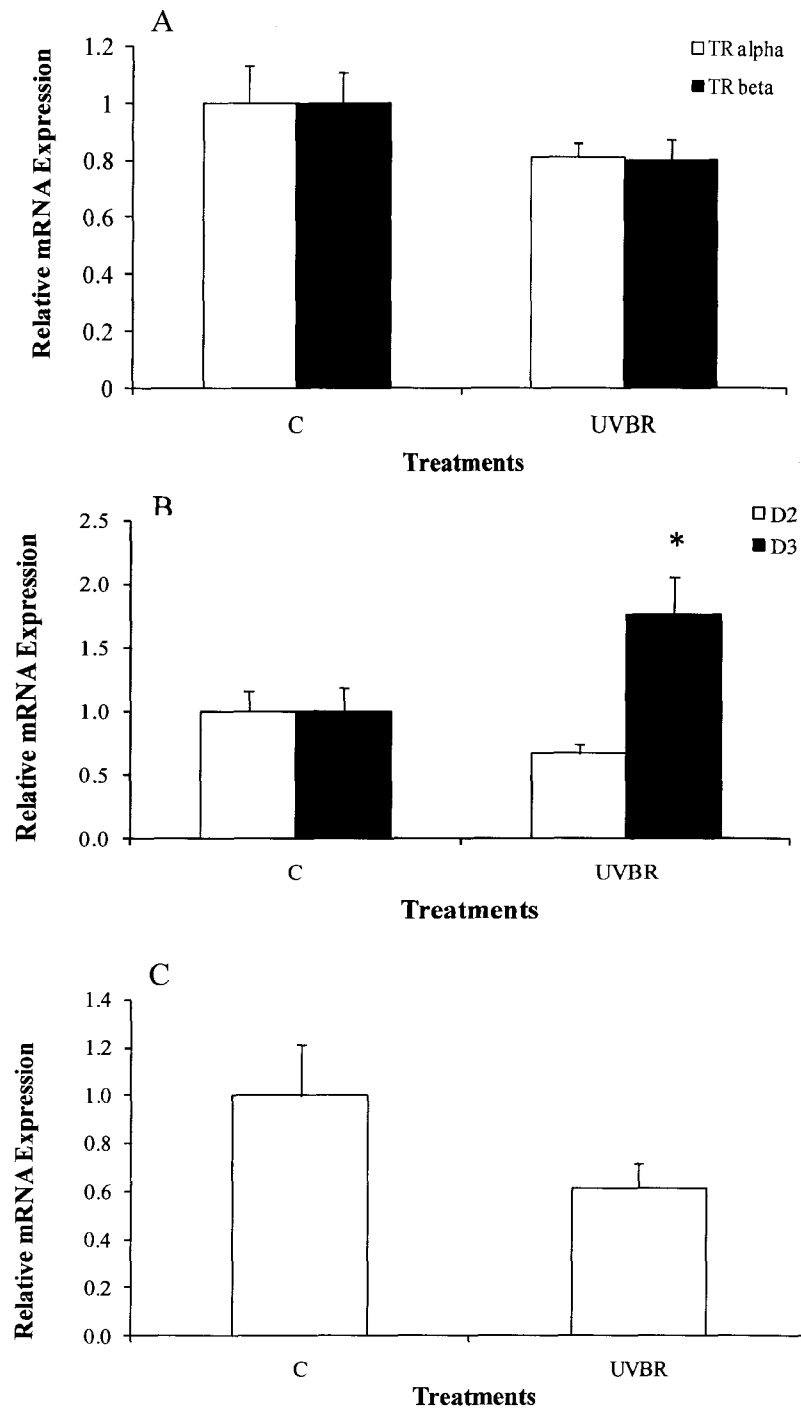


Figure 4.3 Effects of exposure to UVBR on relative mRNA expression of thyroid hormone receptor (TR) alpha and beta (A), deiodinase type II and III (D2 and D3) (B) and prolactin receptor (C) in the tail of Gosner stage 26 *Rana pipiens* tadpoles. Data were determined by multiplex (A and B) or simplex (C) real-time RT-PCR, normalized to L8 ribosomal protein, and presented as the arithmetic mean + standard error of the mean relative to the control group (n=7 individuals per treatment). Data were assessed by Student's t-tests (significance at $p < 0.05$). The asterisk (*) in panel B indicates a statistically significant result ($p = 0.039$). C, Control; UVBR, UVB radiation.

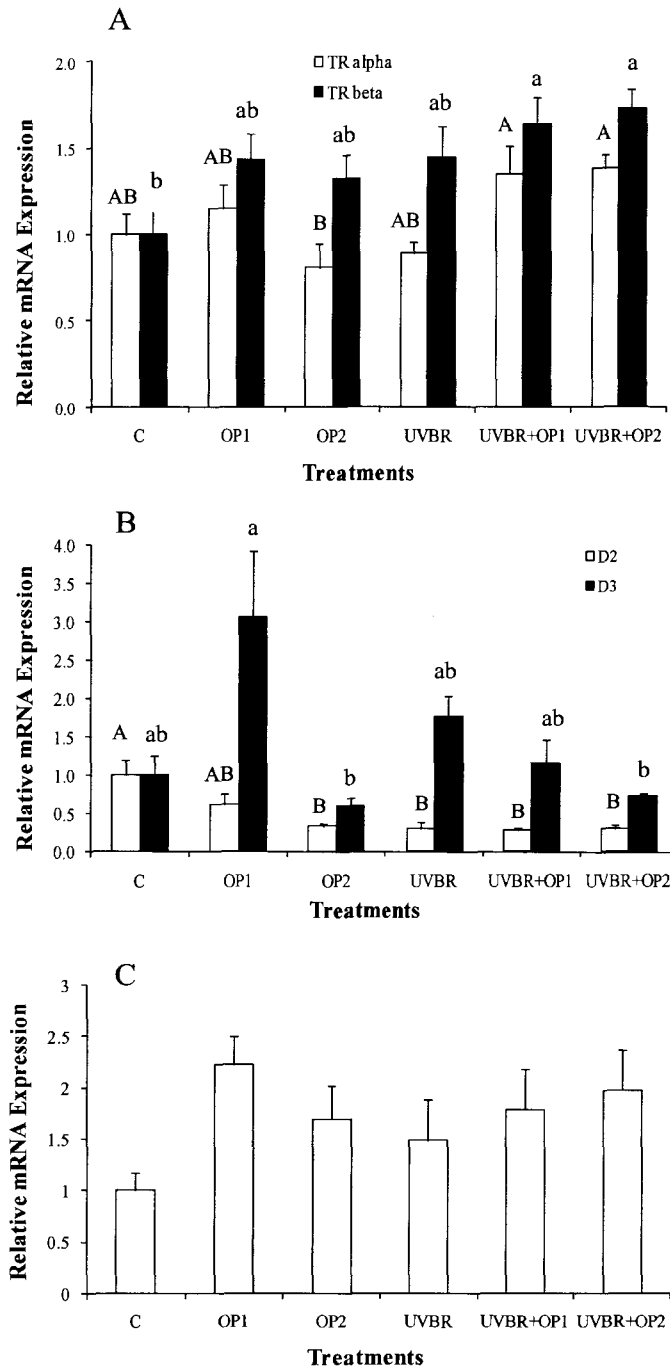


Figure 4.4 Effects of exposure to UVBR and/or OP on relative mRNA expression of thyroid hormone receptor (TR) alpha and beta (A), deiodinase type II and III (D2 and D3) (B) and prolactin receptor (C) in the tail of Gosner stage 29 *Rana pipiens* tadpoles. Data were measured by multiplex (A and B) or simplex (C) real-time RT-PCR, normalized to L8 ribosomal protein, and presented as the arithmetic mean $\pm$ standard error of the mean relative to the control group ($n=6$ individuals/treatment). Data were assessed by 2-way ANOVA, and comparisons between treatments were based on examination of Least Squares Means and multiple comparisons using Tukey's HSD post hoc tests (significance at $p<0.05$). Statistically significant factors in the ANOVAs were: TR alpha, UVBR $p=0.030$, UVBR+OP $p=0.022$; TR beta, OP $p=0.048$, UVBR $p=0.005$; D2, UVBR $p<0.0001$, UVBR+OP $p=0.043$; D3, OP $p=0.008$, UVBR+OP $p=0.016$; PRLR, OP $p=0.044$. Different letters represent statistically significant differences between treatments, as determined by the post hoc tests. C, Control; OP1, 0.01 nM 4-tert-octylphenol; OP2, 10 nM 4-tert-octylphenol; UVBR, UVB radiation.

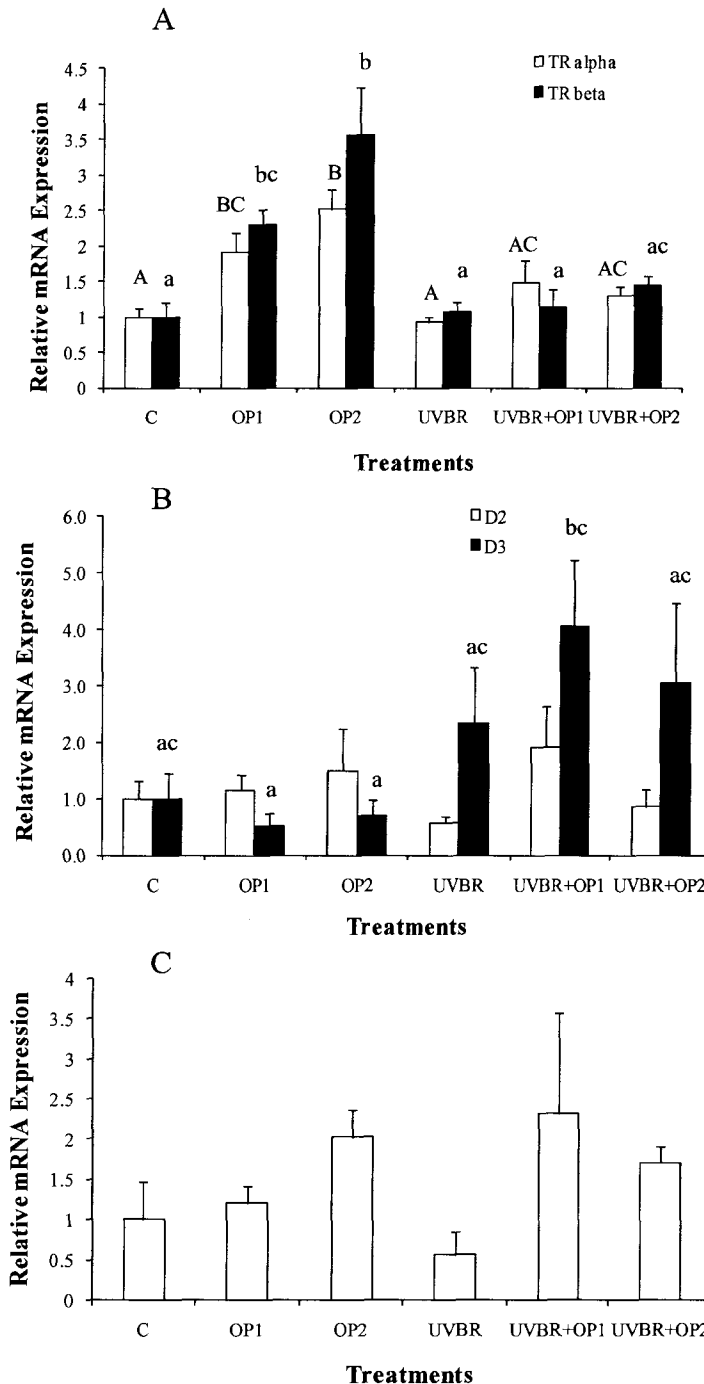


Figure 4.5 Effects of exposure to UVBR and/or OP on relative mRNA expression of thyroid hormone receptor (TR) alpha and beta (A), deiodinase type II and III (D2 and D3) (B) and prolactin receptor (C) in the tail of Gosner stage 34 *Rana pipiens* tadpoles. Data were measured by multiplex (A and B) or simplex (C) real-time RT-PCR, normalized to L8 ribosomal protein, and presented as the arithmetic mean $\pm$ standard error of the mean relative to the control group ($n=6$ individuals/treatment). Data were assessed by 2-way ANOVA, and comparisons between treatments were based on examination of Least Squares Means and multiple comparisons using Tukey's HSD post hoc tests (significance at $p<0.05$). Statistically significant factors in the ANOVAs were: TR alpha, UVBR $p=0.0030$, OP $p<0.0001$; TR beta, OP $p<0.0001$, UVBR $p<0.0001$, UVBR+OP $p=0.004$; D2, none; D3, UVBR $p=0.001$; PRLR, OP $p=0.016$. Different letters represent statistically significant differences between treatments, as determined by the post hoc tests. C, Control; OP1, 0.01 nM 4-tert-octylphenol; OP2, 10 nM 4-tert-octylphenol; UVBR, UVB radiation.

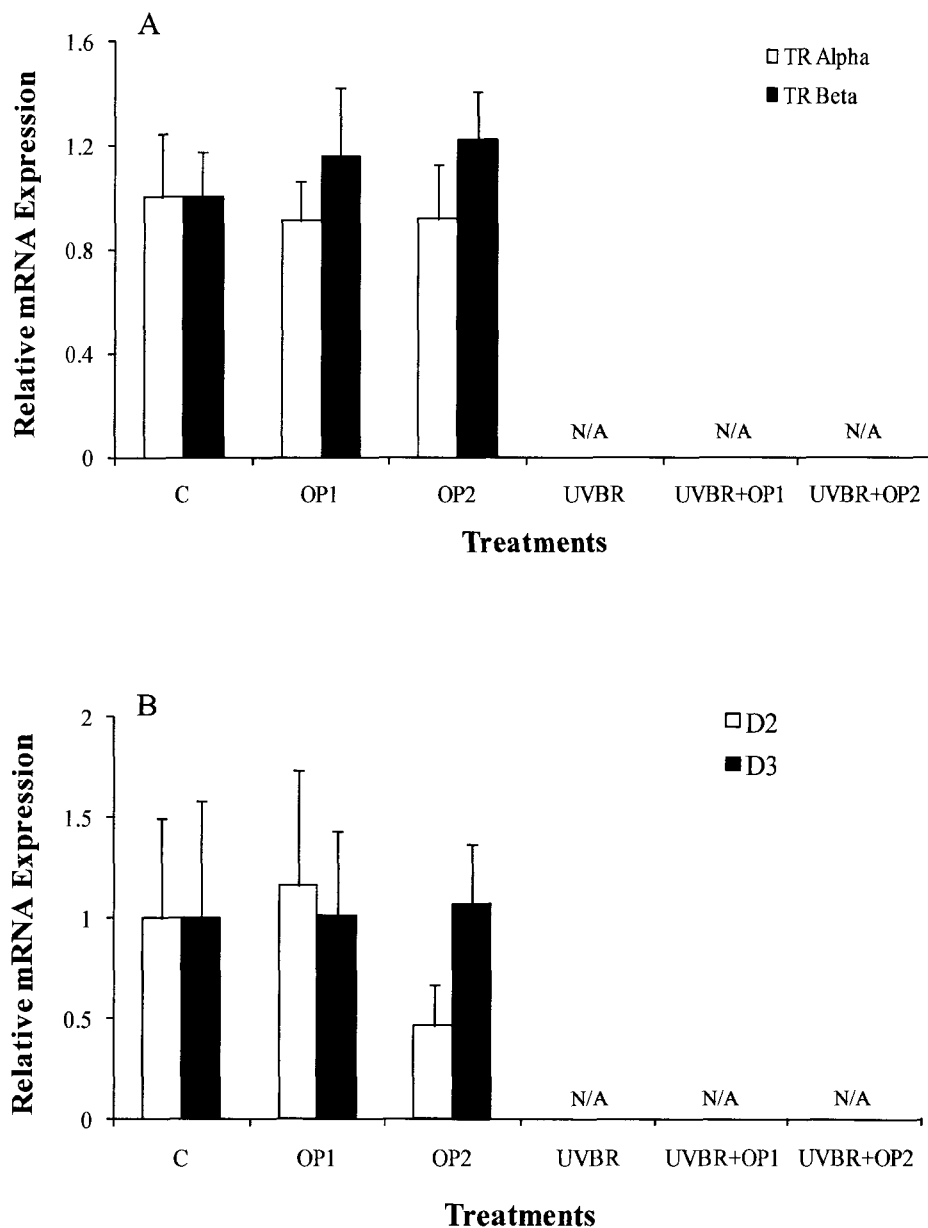


Figure 4.6 Effects of exposure to UVBR and/or OP on relative mRNA expression of thyroid hormone receptor (TR) alpha and beta (A) and deiodinase type II and III (D2 and D3) (B) in the tail of Gosner stage 42 *Rana pipiens* tadpoles. Data were measured by multiplex real-time RT-PCR, normalized to L8, and presented as the arithmetic mean + standard error of the mean relative to the control group (n=6 individuals per treatment). There is no data for UVBR, UVBR+OP1 and UVBR+OP2 treatments because n≤2. Data were assessed by t-tests and there were no significant differences between treatments (p≥0.05). C, Control; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol; UVBR, UVB radiation; N/A, not available.

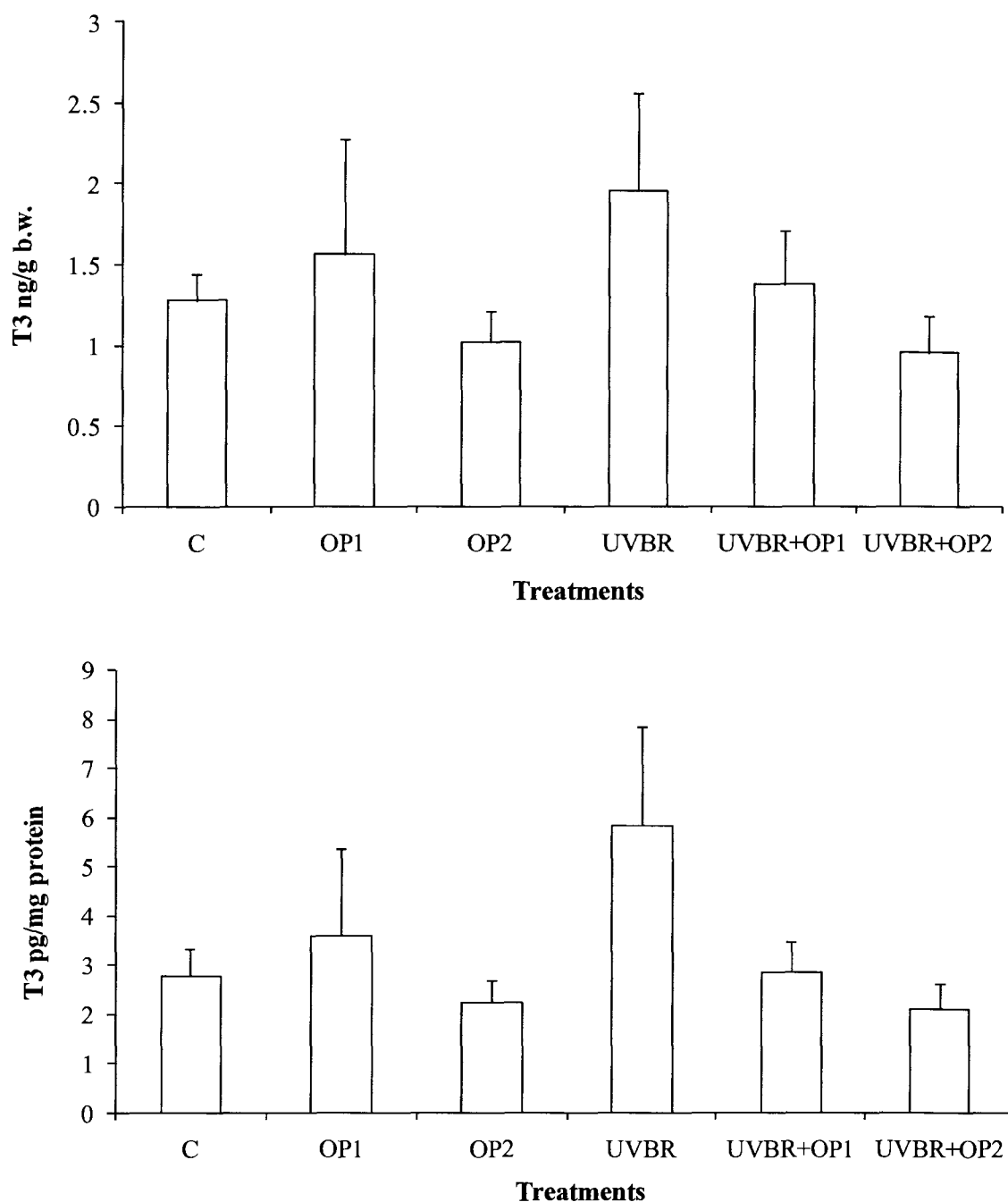


Figure 4.7 Whole body (minus the tail) T3 levels normalized for body weight (g) (A) and protein level (mg) (B), in Gosner stage 29 *Rana pipiens* tadpoles chronically exposed to UVBR and/or OP. Values represent arithmetic mean + standard error of the mean (n=8 individuals/treatment). Data were assessed by 2-way ANOVA (significance at $p < 0.05$). There was no effect of treatment on T3 levels. C, Control; OP1, 0.01 nM 4-*tert*-octylphenol; OP2, 10 nM 4-*tert*-octylphenol; UVBR, UVB radiation; T3, triiodothyronine.

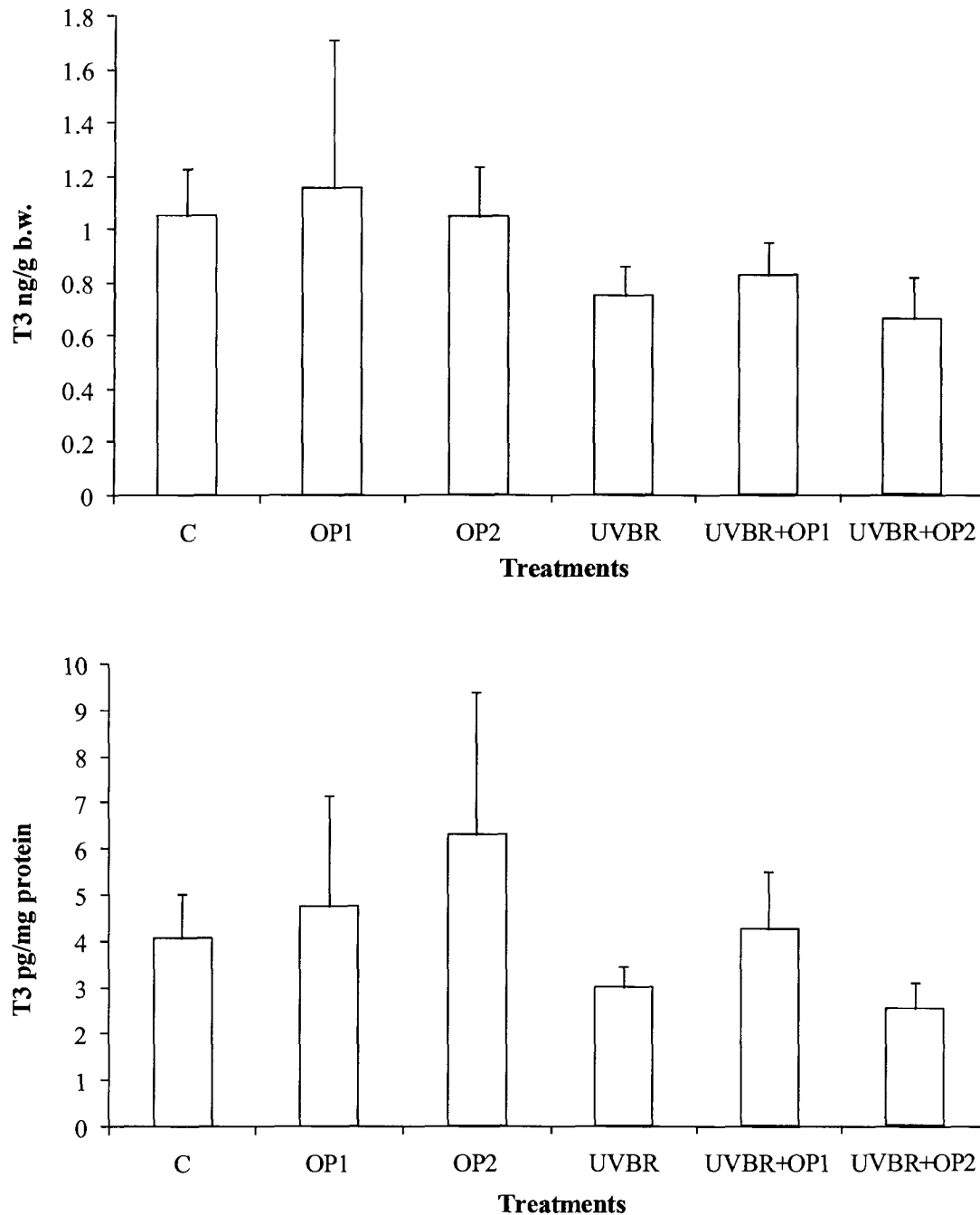


Figure 4.8 Whole body (minus the tail) T3 levels normalized for body weight (g) (A) and protein level (mg) (B), in Gosner stage 34 *Rana pipiens* tadpoles chronically exposed to UVBR and/or OP. Values represent arithmetic mean +standard error of the mean (n=8 individuals per treatment). Data were assessed by 2-way ANOVA (significance at $p<0.05$). There was no effect of treatment on T3 levels. C, Control; OP1, 0.01 nM 4-tert-octylphenol; OP2, 10 nM 4-tert-octylphenol; UVBR, UVB radiation; T3, triiodothyronine.

4.4 DISCUSSION

In this study, we determined the effects of chronic exposure to environmental levels of UVBR and/or OP on the morphology of the thyroid gland, on T3 levels and on the expression of key genes involved in amphibian development in *R. pipiens* tadpoles. There was no effect of UVBR on thyroid gland morphology of stage 31 tadpoles, which represents the approximate stage of development when the onset of developmental delays was observed in UVBR-treated tadpoles in previous experiments (Chapters 2 and 3). These results demonstrate that the activity of the thyroid gland in UVBR-treated animals was considered the same as in animals from the control group, and may suggest that some aspects of the hypothalamo-pituitary-thyroid (HPT) axis were not affected by UVBR exposure. However, stage 31 may be too early a stage to detect effects on the thyroid system by observing the morphology of the thyroid gland, since the gland is just becoming functional at this stage and TH levels are still relatively low (OECD, 2004). Since many tadpoles exposed to UVBR in past experiments did not develop to later stages and a very small percentage reached metamorphosis (Croteau *et al.*, 2001; Chapter 2, Croteau *et al.*, 2008b), the examination of the thyroid gland at later stages of development was not possible. Interestingly, none of the tadpoles exposed to UVBR in the present study developed past stage 31 (foot paddle stage) by the end of the experiment, while the maximum developmental stage reached by tadpoles in the control group was stage 34 (early digit differentiation). This observation is in agreement with previous results, demonstrating that either no tadpoles or significantly fewer tadpoles exposed to UVBR (with or without OP) developed past stage 34 (Croteau *et al.*, 2001; Chapter 2, Croteau *et al.*, 2008b).

The effects of UVBR, with or without a chemical stressor such as OP, on TH levels in amphibians have not been previously assessed to our knowledge. The few studies to date examining the effects of UVBR on TH levels have been conducted in mammals. Some studies have shown that UVBR exposure of mammals has an effect on TH levels or other key hormones that influence development, while others have not. Exposure of calves to artificial UVBR twice daily for either 8, 9 or 10 days increased the activity of the thyroid gland; the circulating concentration of T4 in exposed animals was significantly increased, but no significant change in T3 was observed (Brouček *et al.*, 1987). Altmeyer *et al.* (1983) found a significant decrease in the average plasma cortisol level measured in 49 healthy test persons after 60 min. of whole-body UV irradiation. Irradiation also caused a decrease in the concentrations of T4 and T3 (13.1% and 22.1% respectively, only statistically significant for T3), but levels of ACTH (adrenocorticotropin) and TSH remained unchanged. On the other hand, a group of 24 healthy men exposed to UVBR did not exhibit significant changes in serum levels of several hormones including TSH, PRL, T4 and T3 (Falkenbach *et al.*, 1997).

Chronic exposure to UVBR and/or OP did not affect whole body (minus tail) T3 levels in stage 29 and 34 tadpoles, after normalization with either body weight or protein level. Whole body TH extractions were performed because of the small size of premetamorphic *R. pipiens* tadpoles and since it is not possible to collect a sufficient amount of blood to measure circulating TH levels. TH measurements from whole body extracts have been conducted in other studies using tadpoles and were successful in identifying numerous agents that cause alterations in TH levels (*e.g.*, Goleman *et al.*, 2002; Fort *et al.*, 2000; Fort *et al.*, 2004b). Effects of UVBR and/or OP on tadpole T3

levels were not detected, which further suggests that the HPT axis may not be affected by UVBR and/or OP exposure. However, whole body T3 levels do not reflect subtle changes in local T3 levels or in circulating T3. Deiodinase enzymes are responsible for regulating the local levels of THs in peripheral tissues. Therefore, even if UVBR and/or OP do not affect whole body T3 levels, it is important to consider whether T3 levels in peripheral tissues are affected by changes in deiodinase levels or activity.

Real-time RT-PCR results for stage 26 tadpoles exposed to UVBR demonstrate a significant increase in D3 mRNA expression in the tail. Given that D3 is an enzyme that converts T4 and T3 to inactive forms of TH, a subsequent increase in D3 enzyme levels after exposure to UVBR could reduce the amount of T4 and T3 available at the tissue level. Nevertheless, the hind limb bud in *Rana pipiens* tadpoles appears at Gosner stage 26 (Gosner, 1960) and its growth occurs independently of TH (Shi, 2000). Therefore, D3 levels (and thus TH levels) could be affected in some of the tissues of stage 26 tadpoles without an accompanying delay in hind limb development, as observed in previous experiments. An effect on D3 mRNA levels may not affect hind limb development, but could potentially affect other TH-dependent processes at this time (*e.g.*, metabolism, brain development).

Stage 29 tadpoles exposed to UVBR and/or OP exhibited a significant delay in development, as measured by the number of tadpoles developed past stage 29 at week 9 when compared to the control group. Results demonstrate a significant ~3.5 fold decrease in D2 mRNA levels (*i.e.*, enzyme that converts inactive T4 to active T3) in tadpole tails from all treatments except OP1. There was also an overall significant effect of OP and UVBR+OP on the expression of D3, with OP1 producing a ~3 fold induction in D3

mRNA levels compared to control. Similarly, Lehigh Shirey *et al.* (2006) demonstrated that exposure of *X. laevis* tadpoles to polychlorinated biphenyls (PCBs) affected the timing of metamorphosis as well as mRNA levels of transthyretin, D2 and D3 genes. Such decreases in D2 and increases in D3 mRNA abundance by exposure to UVBR and/or OP could result in major localized changes in the availability of the biologically active T3. Thyroidectomy in *X. laevis* produces giant tadpoles with hind limb buds that morphologically resembled buds at NF stage 51 (equivalent to Gosner stage 29) (Shi, 2000), emphasizing the importance of THs in hind limb development at this stage. Our results indicate that a dysfunction in local conversions of THs by D2 and D3 may explain the delay in hind limb development observed in UVBR and/or OP treated tadpoles.

Treatments including OP and UVBR also produced an overall significant induction in TR beta mRNA abundance in stage 29 tadpoles. The expression of this gene increased by ~1.5 fold in all treatments compared to levels in the control group. This increase is physiologically important given that a developmental profile of TR beta mRNA levels in the brain of *R. pipiens* tadpoles has shown no change in expression between Gosner stages 30 and 36 tadpoles (Hogan *et al.*, 2007). Howe *et al.* (2004) also observed an increase in TR beta mRNA levels in the tail of *R. pipiens* tadpoles chronically exposed to formulations containing glyphosate and polyethoxylated tallowamine surfactant. It was suggested that the delays in metamorphosis that were observed in these tadpoles could have been caused, in some part, by disruption of hormone signaling because TR beta mRNA transcript levels in tails were unusually elevated. However, an increase in TR beta mRNA levels is not the result that one would expect to observe in developmentally delayed tadpoles. Since TRs mediate the actions of

THs during development, it would be expected that a decrease in TR beta mRNA abundance would result in a reduced rate of development. For example, exposure of *X. laevis* tadpoles to the estrogenic chemical bisphenol A caused a delay in metamorphosis, as well as a near complete reduction in TR beta mRNA transcript levels in the tail (Iwamuro *et al.*, 2003). Nevertheless, the results obtained in the present study indicate an alteration in TR beta mRNA transcript levels. The induction in the expression of TR beta in response to UVBR and/or OP exposure may reflect a compensatory mechanism to attempt to accelerate the rate of development of these severely delayed and disrupted tadpoles.

In Chapter 3, UVBR exposure delayed the developmental rate and caused an increase in the body weight of tadpoles. The pituitary hormone prolactin (PRL) is generally a more potent growth promoter than growth hormone in tadpoles (see Dodd and Dodd, 1976; Kikuyama *et al.*, 1993; Denver, 1996; Takada and Kasai, 2003). PRL stimulates larval growth and has also been found to possess anti-metamorphic activity (Denver 1997 and 1998; Shi, 2000; Tata, 2006). The inhibition of metamorphosis by PRL may be mediated through the blockage of the autoinduction of TR gene expression (Baker and Tata, 1992; Tata *et al.*, 1993) or by inducing the expression of D3 mRNA (Shintani *et al.*, 2002). The only study to our knowledge on the effects of UVBR on PRL was conducted in humans and demonstrated that UVBR did not significantly affect serum levels of several hormones including PRL in 24 healthy men (Falkenbach *et al.*, 1997). However, we thought it would be of interest to examine the possibility of a link between effects of UVBR and/or OP on tadpole weight, PRL levels, and the observed delays in tadpole development. Since it was not possible to collect the pituitary gland from

tadpoles at such early stages of development, we assessed the effects of UVBR and/or OP exposure on PRLR. Both PRL and PRLR mRNA levels are low during premetamorphosis and increase at metamorphic climax, thus the expression of PRLR could be an indirect measure of PRL status (Li *et al.*, 2006).

Our results demonstrate that the body weight of stage 29 tadpoles increased in all treatments that included OP, as did PRLR mRNA levels. The same pattern was not observed in stage 34 animals. There was no effect of either UVBR or OP on the body weight of stage 34 tadpoles, but an increase in PRLR mRNA abundance was still observed in OP-treated animals. However, animals exposed to OP alone were no longer developmentally delayed at stage 34; therefore the increase in PRLR does not appear to be linked to an anti-metamorphic effect or change in body weight. As we observed effects of UVBR on tadpole body weight in Chapter 3 and moderate effects of OP on body weights in this study, the impact of UVBR/OP on weight are variable and thus weight is not a useful parameter in explaining the consistently observed developmental delays. Body weight may provide unreliable results because of edema in various tissues, which has been shown to occur in tadpoles exposed to UVBR and/or OP (Chapter 2, Croteau *et al.*, 2008b). It will be important to assess growth in future studies by measuring the dry weight of tadpoles or by conducting whole body measurements of total RNA or total protein levels.

OP had a transient effect on the rate of hind limb development. Only UVBR-treated tadpoles (regardless of the presence of OP) were developmentally delayed by the end of the experiment, as measured by the number of tadpoles developed past stage 34 and the low number of tadpoles to reach metamorphosis (Chapter 2, Croteau *et al.*,

2008b). The experimental design was such that tadpoles were exposed to UVBR and/or OP for 8 months and then were placed in a clean water grow out system for an additional three months. Since we were staging the tadpoles regularly to monitor the developmental rate of the population, we noticed that tadpoles exposed to OP alone were able to recover within this three-month period and were no longer delayed at the time we collected stage 34 tadpoles (*i.e.*, near the end of the experiment). The percentage of tadpoles developing past stage 34 in the UVBR and UVBR+OP groups was 10 to 17%. In contrast, the percentage of tadpoles developing past stage 34 in the control and OP1 and OP2 treatments was 29%, 41%, and 26%, respectively (Croteau et al., 2008b). UVBR-treated animals were not able to recover, which indicates that exposure had a permanent detrimental effect on development and metamorphosis. UVBR-treated tadpoles were delayed in development at stage 34 and exhibited an overall increase in D3 mRNA transcript levels, ranging from ~2-4 fold induction. This increase is likely to be important and physiologically relevant, since a study on the developmental profile of D3 mRNA levels in the brain of *R. pipiens* tadpoles indicated no change in D3 expression between Gosner stages 30 and 36 (Hogan *et al.*, 2007). Therefore, we hypothesize that a major but localized increase in D3 in the tail would contribute to delayed metamorphosis. As tadpoles exposed to OP only were no longer developmentally delayed at stage 34, D3 mRNA levels measured in these groups of tadpoles were not significantly different from controls. In addition, TR alpha and beta mRNA levels increased significantly in OP only treatments, producing ~2 to 4-fold inductions compared to the control group. This is a physiologically relevant effect that may reflect a compensation mechanism to accelerate the rate of development, as was observed in stage 29 animals. Since the expression of

deiodinase genes was no longer affected in stage 34 tadpoles exposed to OP only, this could have increased the amount of local T3 available to induce TR mRNA levels and stimulate the development of tadpoles. These results further suggest that the observed disruptions in the rate of development are not likely caused by a disruption in PRLR or TR mRNA levels, but by an increase in local transformation of THs by the metabolic enzymes D2 and D3. Exposure to OP only had no effect on the number of animals reaching stage 42 (forelimb emergence; Chapter 2, Croteau *et al.*, 2008b), or on TR alpha, beta, D2 and D3 mRNA levels in stage 42 tadpoles.

This study is one of the first attempts at examining the mechanism(s) of action behind the sublethal effects of UVBR and/or OP exposure on the developmental rate of any anuran species. Based on results that the morphology of the thyroid gland and T3 levels are normal, the disruption in the rate of tadpole development is likely a problem with endogenous localized metabolism of THs through alterations in D2 and D3 expression in peripheral tissues. The coordinated metabolism of THs by deiodinase enzymes in tissues is essential to control the timing of morphological transformations that occur throughout tadpole development (Cai and Brown, 2004). Metabolic enzymes were clearly affected in developmentally delayed animals to possibly decrease the synthesis and increase the local degradation of T3. For example, D2 and D3 mRNA levels were affected when tadpoles exposed to OP only were developmentally delayed, but were unaffected once the development of these tadpoles recovered. After the eight months of exposure to UVBR was terminated, UVBR-treated animals were still developmentally delayed after the three months and deiodinase mRNA levels were still affected. This

study links exposure to UVBR and/or OP to specific alterations in the expression of thyroid system genes and severe developmental delays in *R. pipiens*.

CHAPTER 5

General Discussion

5.1 Thesis summary

5.1.1 Rate of tadpole development and metamorphosis

The first objective of my research was to assess the effects of UVBR and/or OP exposure on the developmental rate and metamorphosis of *Rana pipiens* tadpoles. I hypothesized that: (1) UVBR and OP exposure reduces the rate of tadpole development and number of tadpoles that metamorphose; and (2) Exposure of tadpoles to UVBR and OP together causes a significantly greater reduction in the rate of development and number of tadpoles that metamorphose than exposure to either factor alone. Figure 5.1 summarizes the overall effects of UVBR and OP on metamorphosis and the rate of development. Collectively, results clearly demonstrate that exposure to environmental levels of UVBR (with and without OP) consistently causes a delay in the developmental rate of tadpoles (Chapters 2 and 3), such that a lower number of individuals metamorphose compared to control animals or those exposed to OP only (Chapter 2; Figure 5.1). The effect of OP on development was however not as consistent, as tadpoles exposed in Chapter 2 exhibited a transient delay in development, whereas those exposed in Chapter 3 were not affected by stage 31 (*i.e.*, when the exposure was terminated). The recovery of animals treated to OP only in Chapter 2 occurred during the three months in a clean water grow-out system (Figure 5.1), which suggests that the effect of OP on the rate of development was reversible and the effect of UVBR was irreversible. Exposure of tadpoles to UVBR and OP together did not cause a greater delay in the rate of

development and number of tadpoles that metamorphose than exposure to either factor alone, as the data analyses indicated that the effects of UVBR+OP were not statistically significant. Therefore, the overall results of this research only support the first hypothesis, although the effect of OP was not as consistent as the effect of UVBR.

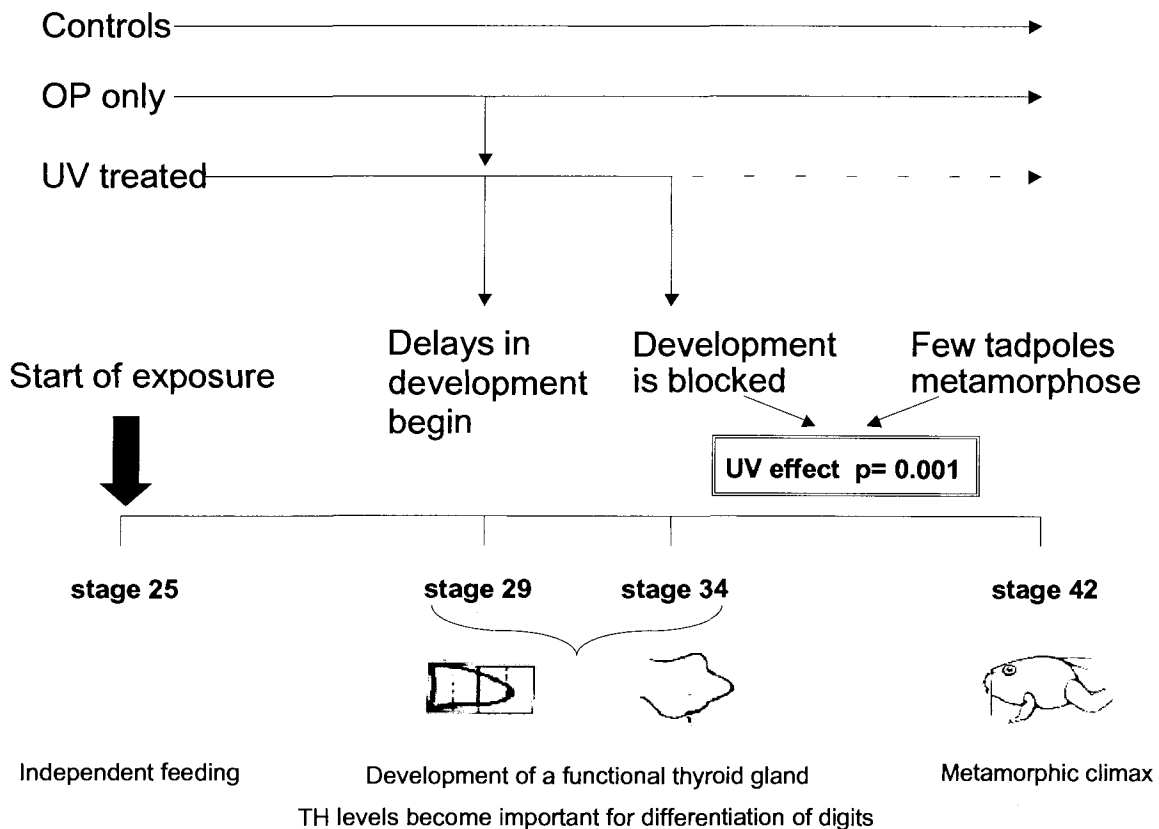


Figure 5.1 Schematic of the window of developmental sensitivity of *Rana pipiens* tadpoles exposed to environmental levels of UVBR and/or OP in Chapter 2. The onset of delays in tadpole development occurred at Gosner stage 29 in all OP and UVBR-treated animals, a time when the thyroid gland becomes functional and when the hind limbs are soon to develop digits. However, near the end of the experiment, Gosner stage 34 tadpoles exposed to OP only were no longer developmentally delayed whereas those exposed to UVBR were delayed and few tadpoles reached metamorphosis (Gosner stage 42). The recovery of animals treated to OP only occurred during the 3 months in a clean water grow-out system, which suggests that the effect of OP on the rate of development was reversible and the effect of UVBR (with and without the presence of OP) was irreversible.

Although several studies have demonstrated that the exposure of amphibians to UVBR or estrogenic chemicals can result in a disruption of developmental rates and/or metamorphosis (refer to Table 1.1 and section 1.4), few if any have identified a specific “window” of developmental sensitivity to UVBR and OP. The onset of slowed tadpole development in Chapters 2 and 3 (*i.e.*, stages 29-31) coincided with the time that the thyroid gland becomes functional (Shi, 2000; OECD, 2004; Fort *et al.*, 2007) and that TH begins to exert influence on development of the hind limbs and digits (Figure 5.1). For example, the hind limbs of *Xenopus* tadpoles in which TH synthesis is blocked by methimazole develop a limb bud structure that is equivalent in size and shape to that of a NF stage 52 tadpole (*i.e.*, Gosner stage 30), but do not develop beyond this stage (Cai and Brown, 2004). In addition, few tadpoles exposed to UVBR develop past Gosner stage 34 (Chapter 2 and Chapter 4, histology experiment), which marks the beginning of prometamorphosis and a time when TH levels increase to induce metamorphosis (Figure 5.1; OECD, 2004; Fort *et al.*, 2007). This “window” of developmental sensitivity of tadpoles to OP and/or UVBR clearly points to a disruption at the level of the thyroid system.

5.1.2 Potential mechanisms of action behind the delayed rate of tadpole development

Ultimately, whatever the signal is that accelerates or slows the rate of tadpole development, the response must result in an alteration of the hormonal systems that control metamorphosis. Therefore, the second objective of this research was to identify potential mechanisms by which UVBR and/or OP could affect the thyroid system of exposed tadpoles to produce the observed delays in development. I hypothesized that: (3)

UVBR and OP exposure affects the thyroid system of exposed tadpoles; and (4)

Exposure of tadpoles to UVBR and OP together significantly modifies the effect of either factor alone on the thyroid system. A multi-parameter approach was taken to determine whether UVBR and/or OP have an adverse effect on the central hypothalmo-pituitary-thyroid axis, on the thyroid cascade in peripheral tissues or on the biological action of T3.

This is the first study to report potential disruptions of the thyroid system of amphibians caused by UVBR and/or OP exposure. Results of Chapter 3 indicate that pre-exposure of premetamorphic *R. pipiens* tadpoles to environmental levels of UVBR/OP disrupts the thyroid system by affecting molecular and morphological responses of tadpoles to exogenous T3. OP induced the expression of several of the genes examined in the brain and tail and/or their response to T3 (*e.g.*, D2), and the addition of UVBR can alter the effect of OP (*e.g.*, TR alpha in brain). The combination of UVBR+OP also affected the response of TR alpha and TR beta to T3 in the tail, demonstrating that exposure to multiple stressors can produce different gene expression profiles than that of single stressors. UVBR alone did not have a statistically significant effect on the expression of genes or their response to T3 exposure *per se*; however, the expression of D2 and D3 was very variable in certain groups of tadpoles exposed to UVBR, OP and UVBR+OP. These results indicate that D2 and D3 mRNA levels of many animals exposed to OP, UVBR and UVBR+OP were greatly affected. The tails and hind limbs of developmentally delayed UVBR-treated tadpoles were less and more responsive, respectively, to exogenous T3 exposure than in tadpoles from other treatments, as measured by tail resorption and Gosner stage of development. These data indicate that UVBR disrupts the normal TH-dependent cascade of expected morphological changes.

Together, findings from Chapter 3 indicate that exposure of tadpoles to UVBR and/or OP can disrupt the expression of genes that are important in amphibian development and the biological action of T3 in peripheral tissues.

In Chapter 2, tadpoles that were collected at stage 29 for gene expression and whole body (minus tail) T3 level analyses exhibited slowed development in all treatments compared to the control group. Stage 34 animals were also collected, although only those exposed to UVBR (with and without OP) were delayed in development at this time. Interestingly, the decrease in expression of D2 in the tail of stage 29 tadpoles and increase in D3 mRNA levels in stage 34 tadpoles occurred only in animals from treatments that exhibited a slowed developmental rate at those specific stages (Chapter 4). Given that D2 activates and D3 inactivates THs, UVBR/OP mediated disruptions in development are likely caused by dysfunctions in the localized metabolism of THs. The results of Chapter 3 described above support these findings, as they also pointed to a disruption in the expression of D2 and D3 in the tail.

The findings of this research support the third and fourth hypotheses. All treatments disrupted the thyroid system in one way or another, and exposure to UVBR+OP did cause a greater effect than exposure to UVBR or OP alone in some cases. Although these data were obtained using tail and brain, they suggest that similar effects could potentially occur in other tissues such as the hind limbs. Overall, these results provide a better understanding of the sensitive developmental period of tadpoles to UVBR and/or OP exposure and the mechanism(s) by which the disruptions in development may occur.

5.2 Multiple Stressors: UVBR and OP

Results of this research revealed that exposure of tadpoles to the combination of UVBR and OP did not have a statistically significant effect on the incidence of mortality, developmental malformations or abnormalities, tadpoles developing past a specified Gosner stage or tadpoles reaching metamorphosis. In all experiments, UVBR was the overall factor that most affected these endpoints. Nevertheless, the effects that UVBR+OP produced were often greater than that caused by UVBR exposure alone. For example, by the end of the experiment presented in Chapter 2, the percentage of tadpole mortalities in the UVBR and UVBR+OP treatments were 73% and 77 to 78%, respectively, compared to 62 to 68% in non-UVBR treatments (Figure 2.2). Although the effect of UVBR+OP was not statistically significant, these findings may have biological consequences. In nature, a 4-5% increase in tadpole mortality (compared to UVBR alone) may not adversely impact a population per se. However, given that the exposure of tadpoles to UVBR+OP also produced a greater incidence of developmental deformities and lower number of tadpoles to reach metamorphosis (Chapter 2), the biological impacts of UVBR+OP on amphibians in the wild are conceivable. There is no one cause for amphibian declines. Multiple stressors are likely working in concert, contributing to the problem. It is therefore imperative that the impacts of multiple stressors be considered in future experimental approaches assessing the effects of environmental changes on amphibians.

There is currently no consideration of the impacts of multiple stressors in risk assessments of chemical pollutants in Canada. My research demonstrates that interactions between stressors are important to consider, even if effects are not synergistic or additive.

UVBR levels are increasing on a global scale, and it is predicted that exposure to UVBR in aquatic ecosystems will continue to increase in the future due to interactions between climate change, ozone depletion and acid rain (refer to section 1.7). It is therefore important to take into account the interactions between UVBR and chemicals in toxicological assessments and to incorporate other environmental stressors in lab and field exposures, to mimic realistic environmental conditions.

5.3 Future Research and Perspectives

My research has revealed a potential mechanism of action for the delay in development exhibited by tadpoles exposed to UVBR and/or OP. A disruption in the expression of D2 and D3 genes was observed in the brain and/or tail of exposed tadpoles. Since the delays in development are exhibited at the level of hind limb morphology (*i.e.*, when the digits begin to develop), it would be warranted to examine T3 levels and deiodinase gene expression patterns in the hind limbs. The measurement of circulating T3 levels would also better indicate disruptions in TH synthesis and secretion than whole body T3 levels, as were measured in the present study. However, these approaches were not possible using *Rana pipiens* because premetamorphic tadpoles are too small to collect a sufficient amount of blood to measure circulating T3 levels and the hind limbs are too small to obtain enough tissue to conduct the RNA isolations and cDNA synthesis required to measure gene expression by real-time RT-PCR. These measurements may be possible by using a different amphibian model such as *Rana catesbiana* (Bullfrog), because tadpoles of this species are of much greater size. Other approaches that could improve our understanding of the overall effects of UVBR/OP on deiodinase enzymes

include *in situ* hybridizations to localize deiodinase mRNA levels in the hind limbs of developmentally delayed animals and deiodinase enzyme activity assays to further assess effects on the peripheral metabolism of THs.

In addition to disruptions of TH levels and deiodinase mRNA levels and activity, one of the most commonly reported ways that chemicals affect the thyroid system is by interfering with the binding of TH to transthyretin (TTR) proteins (Brucker-Davis, 1998; Kloas, 2002). Since THs are lipophilic, they are transported through the blood by TTR (Kloas, 2002). Interestingly, many of the EDCs that are capable of displacing labelled T3 from TTR are compounds including ethynylestradiol (EE2), diethylstilbestrol (DES), bisphenol A, *p*-*t*-OP and 4-NP (Yamauchi *et al.*, 2001), which also exhibit estrogenic activities. Since TTR has a higher affinity for T3 than T4 in amphibians, chemicals that interact with TTR may influence the concentration of T3 in the blood (Yamauchi *et al.*, 2001). A problem at the level of TTR binding may represent an additional mechanism of action of UVBR/OP that occurs concurrently with the other TH system disruptions reported in this study. Therefore, the potential disruption of TTR provides a further reason why measuring circulating T3 levels in affected tadpoles is warranted.

Future UVBR/OP exposure experiments should include a time course to collect tadpoles at various stages of development and study the profile of TH levels and mRNA levels of several genes throughout development. When collecting tadpoles at specific developmental stages for molecular analysis, we are examining the effects of stressors at one point in time. By only observing tadpoles at a few developmental stages, it is possible that we may overlook other UVBR/OP effects that may be occurring throughout development. In addition, it would be of interest to expose tadpoles to different levels of

UVBR to determine what threshold or percent increase in UVBR is needed to cause a physiological disruption such as a delay in tadpole development.

5.4 Species and population sensitivity differences to UVBR exposure

A wide range of laboratory and outdoor experiments examining the impacts of UVBR exposure on amphibians have documented disruptions in the rate of development and metamorphosis of exposed animals (Table 1.1). These studies support a causal association between UVBR exposure and developmental disruptions that have been lacking in large-scale field studies of wild populations. However, it is important to recognize that not all species or populations of amphibians are equally sensitive to UVBR. Determining which populations or species are particularly affected by increased UVBR exposure is difficult given the wide range of factors that contribute to the exposure of amphibians to UVBR. For example, studies described in Table 1.1 either observed (1) effects of UVBR on metamorphosis and developmental rates; (2) no effect of UVBR; or (3) both effects and no effects when using different amphibian species or UVBR levels. Amphibians possess several defence mechanisms that allow them to cope with the harmful effects of UVBR by either limiting UVBR-induced damage or repairing it after it has occurred (Blaustein and Belden 2003). It has been demonstrated that the effectiveness of the different defence mechanisms employed by amphibians to cope with UVBR exposure can vary greatly between species and populations, which could influence the extent of UVBR to which they are subjected as well as subsequent detrimental effects. For example, Little *et al.* (2003) found that UVBR exposure affected the survival of *Bufo woodhousii* (Woodhouse's toad) and not of *Bufo boreas* (Boreal toad)

tadpoles, and this was not attributed to differences in the UVBR-absorbing substances of the egg jelly or non-melanin photoprotective substances in the skin but potentially due to a greater amount of melanin in the skin of *B. boreas*. However, other studies have found that melanin does not protect exposed animals from the damaging effects of UVBR (e.g., Belden and Blaustein, 2002c). It is therefore difficult to extrapolate results from one study or situation to another, even when using the same species or population.

Differences in sensitivity to UVBR exist due to differential exposure (e.g., amphibians from a low DOC vs. a high DOC pond) and differences in the physiological capacity of animals to withstand it. It is therefore very challenging to assess or predict the actual exposure of amphibians to UVBR and the resulting consequences for populations and species. Despite the various coping mechanisms that can help protect amphibians against UVBR, numerous laboratory and field studies have determined that exposure to present day environmental levels of UVBR can have harmful effects on developmental rates and metamorphosis.

5.5 Concluding Remarks

Multidisciplinary approaches must be used to fully understand how organisms respond to environmental stressors. We must move beyond solely examining overt death of amphibians and begin to understand that subtle effects of environmental stressors can have profound effects on development, metamorphosis and other physiological processes (e.g., sexual development, reproduction, somatic growth). Results of this study demonstrate that UVBR and/or OP exposure delays the larval development of a native amphibian, *Rana pipiens* (Northern leopard frog) and affects the number of tadpoles that

metamorphose. This research also provides the first results on the possible mechanisms by which UVBR and/or OP affect the rates of tadpole development, which consist of a disruption of the biological action of T3 and of local TH metabolism by deiodinase enzymes. Determining the mechanisms of action behind a sublethal effect can help us understand the extent to which the environmental stressor is a risk to amphibian populations and the likelihood that this sublethal effect can also occur in other organisms in the wild.

While it is clear that there exist regions on Earth where UVBR is increasing and where chemical pollution is a problem, much remains to be known about the extent to which this will affect amphibian populations. Key questions remain, for example, regarding the minimum increase in UVBR that would impact a given population or species. There are certainly major differences in species and population sensitivity to UVBR and chemical pollution that remain to be elucidated, not only by identification of the most sensitive species, but also by determining which life history stages are most susceptible to exposure. Arrested metamorphosis caused by a disruption of the thyroid system may cause the complete loss of a generation and result in the declines of amphibian populations, especially in regions where aquatic habitats can freeze or dry up (Kloas, 2002). There is no one cause for enigmatic amphibian declines, and the impacts of multiple stressors must be considered in future experimental approaches.

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APPENDIX A: Histological Methods

A. Schedule for Tissue Processing and Embedding in Paraffin

Modified Davidson's fix preparation

The fix was prepared using the protocol from Latendresse et al. (2002):

- 30% of a 37-40% solution of formaldehyde
- 15% ethanol
- 5% glacial acetic acid
- 50% distilled water

The Tissue-Tek® VIP™ Vacuum Infiltration Processor (Sakura E150/E300 Series) was used for dehydration, clearing and infiltration of tissues in paraffin:

Dehydration of tissues (45 minutes for each step)

1. 70% Ethanol at 40°C
2. 80% Ethanol at 40°C
3. 90% Ethanol at 40°C
4. 95% Ethanol at 40°C
5. 100% Ethanol at 40°C
6. 100% Ethanol at 40°C

Clearing tissues of ethanol

7. Xylene (three 45 min. cycles)

Infiltration of tissues in paraffin

8. Paraffin at 58°C (four 45 min. cycles)

Embedding of tissues in paraffin

9. Processed heads were embedded in paraffin for transverse caudal to rostral sectioning.

B. Periodic Acid Schiff (PAS) Staining Procedure for Thyroid Paraffin Sections

Deparaffinize and hydrate sections

1. Xylene (three 3 min. cycles)
2. 100% Ethanol (three 1 min. cycles)

3. 95% Ethanol (1 minute)
4. Tap water (30 seconds)
5. Rinse sections in tap water

Stain sections using Periodic Acid Schiff (PAS)

1. Place sections in Periodic Acid Solution (5 minutes)
2. Rinse sections in distilled water
3. Schiff reagent (15 minutes)
4. Rinse sections in luke warm running tap water (10 minutes)
5. Hematoxylin (1 minute)
6. Rinse sections in distilled water (30 seconds)
7. Bluing reagent (1 minute)
8. Rinse sections in distilled water (30 seconds)

Dehydrate and clear sections

1. 100% Ethanol (two 1 min. cycles)
2. Xylene (three 1 min. cycles)
3. Mount slides with Permount (Fisher)

Magenta: carbohydrates, glycogen, basement membranes

Blue: nuclei

Light purple: background

APPENDIX B: List of Manuscripts (published or in press)

Croteau M.C., M.A. Davidson, D.R.S. Lean and V.L. Trudeau. 2008. Global increases in UVB radiation: potential impacts on amphibian development and metamorphosis. *Physiological and Biochemical Zoology*. 81(6):743-761 (Chapter 1)

Croteau M.C., C.J. Martyniuk, V.L. Trudeau and D.R.S. Lean. 2008. Chronic exposure of *Rana pipiens* tadpoles to UVB radiation and the estrogenic chemical 4-*tert*-octylphenol. *Journal of Toxicology and Environmental Health*. 71:134-144 (Chapter 2)

Croteau M.C., V. Renner, F. Archibald, V. Langlois, J. Cahn, J. Ridal, V. Trudeau and D. Lean. 2007. Investigation of pathogenic *Escherichia coli* and microbial pathogens in pulp and paper mill biosolids. *Water Environment Research* 9:1050-1056

Croteau M.C., N. Hogan, J. Gibson, D. Lean and V.L. Trudeau. 2008. Toxicological threats to amphibians and reptiles in urban and suburban environments. Chapter 13 *In: Herpetological Conservation* vol. 3. *Urban Herpetology: Ecology, Conservation and Management of Amphibians and Reptiles in Urban and Suburban Environments*. R.E. Jung Brown J.C. Mitchell and B. Bartholomew (eds.). p. 197-209