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In many ecosystems, amphibians play a central role in energy flow and nutrient cycling while also acting as keystone species. This role is so vital that its disappearance, or reduction, would cause serious consequences throughout the community, resulting in declines or extinctions of numerous other dependent species. Thus emerging reports about amphibian declines around the world present a serious problem. Increased ultraviolet-B (UV-B) radiation is suspected to be a contributing factor in the declines. Others are habitat loss, climate change, diseases, pesticides and other chemicals. The main objectives of this thesis were to study 1) how UV-B exposure affects toxicokinetics and toxicity of bisphenol A in *Rana temporaria*, 2) how different UV-B doses affect developing *R. temporaria* and *R. arvalis* embryos, and whether there are differences between species and 3) how phytosterols affect reproductive hormones and energy metabolism in postmetamorphic *Xenopus laevis*.

The toxicokinetic study showed that UV-B has no effect on the accumulation and depuration of bisphenol A (BPA) in *R. temporaria* larvae. However, when the accumulation was modeled with growth dilution, the bioconcentration factors, calculated from estimated uptake clearances and elimination rates, were closer to the steady-state BPA concentrations in larvae and water. This finding illustrates that using growth correction is useful and can correct skewness in estimated toxicokinetic parameters. In terms of survival, the two studied *Rana* species were different at larval stages, and it was clear that the UV-B response was cumulative and dependent on UV-B dose in both species. When *R. temporaria* and *R. arvalis* were exposed to UV-B radiation under laboratory conditions for 27 days, it seemed that *R. temporaria* eggs were less tolerant than *R. arvalis* eggs, while *R. temporaria* larvae survived better. Simultaneous exposure to BPA and UV-B caused dramatic mortality after 13 days at all studied BPA concentrations except the highest concentration, where mortality increased after 48 h in both treatments (with or without UV-B). The highest concentration of 1000 µg/l of BPA caused developmental malformations under UV-B exposure. Overall, UV-B increased mortality at all BPA concentrations. The exposure of postmetamorphic *X. laevis* to an environmentally relevant concentration of phytosterols induced physiological changes in frogs. Phytosterols caused a decrease in plasma T<sub>3</sub> and an increase in plasma testosterone concentrations in the exposed females, and exposed individuals of both sexes showed a significant decrease in muscle lipase activity. The muscle phosphorylase activity was lower in the exposed animals, but a statistical difference was seen only when compared to the control females. An interesting finding was a leptin-immunoreactive peptide that has not been found in *X. laevis* before.

The results of this thesis show that the combined effects of UV-B and BPA are greater than the effects of either factor alone. It is important to study multiple stress factors together, because living organisms are surrounded by a myriad of different stress factors and these will act simultaneously.

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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. The publications are referred to in the text by the Roman numerals (I–IV).

- I Koponen, P.S., Tuikka, A. and Kukkonen J.V.K. 2007: Effects of ultraviolet-B radiation and larval growth on toxicokinetics of waterborne bisphenol A in common frog (*Rana temporaria*) larvae. *Chemosphere* 66:1323–1328.
- II Koponen, P.S., Kolehmainen, O., Alho, J. and Kukkonen, J.V.K.: UVB induced mortality in common frog (*Rana temporaria*) and swamp frog (*Rana arvalis*) larvae. Manuscript.
- III Koponen, P.S. and Kukkonen, J.V.K. 2002: Effects of bisphenol A and artificial UVB radiation on the early development of *Rana temporaria*. *Journal of Toxicology and Environmental Health, Part A*, 65:947–959.
- IV Koponen, P.S., Nieminen, P., Mustonen, A-M. and Kukkonen, J.V.K. 2004: Post-metamorphic *Xenopus laevis* shows decreased plasma triiodothyronine concentrations and phosphorylase activity due to subacute phytosterol exposure. *Chemosphere* 57:1683–1689.

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I planned all the studies and was mainly responsible for the sampling, data collection, data analysis and preparation of the manuscripts. The co-authors did the statistical analyses of Article II, while in all other cases I was responsible for the statistical analyses. The processing of the Articles was mainly carried out together with the co-authors.

## 1. INTRODUCTION

The first amphibians, descended from fleshy-finned fish, appeared and colonized the land about 350 million years ago in the Mid-Devonian period. They were the first tetrapods to spend a significant part of their lives on land. Today, the class Amphibia contains about 5900 described species in three existing orders (IUCN, 2006) — Gymnophiona (caecilians, legless amphibians), Caudata (previously Urodela — salamanders, newts) and Anura (frogs, toads) (Campbell and Reece, 2002). There are about 500 species of urodeles, with anurans numbering nearly 4200 species and caecilians about 150 species. New species and even new genera are discovered every year.

The word “amphibian” means “two lives”, referring to the metamorphosis of many frogs. The tadpole is usually an aquatic herbivore with gills, a lateral line system and a long finned tail. During the metamorphosis that leads to the “second life”, legs develop, and the gills and the lateral line system disappear (the latter not in all species). The postmetamorphic young frogs have lungs, external eardrums and a digestive system, which are clear adaptations to terrestrial life and a carnivorous diet. However, many amphibian species do not go through the aquatic tadpole stage, and some species are strictly aquatic or terrestrial.

Amphibians are poikilothermic (cold blooded) animals. In general, metabolic rates in poikilothermic animals are lower than in homeothermic animals. Compared with the metabolic rates of birds and mammals, the lower metabolic rates of amphibians give them substantial advantages in habitat utilization. As poikilotherms, the activity of amphibians varies depending on the thermal optimum. However, many amphibians are active over a relatively broad range of temperatures (Murphy et al., 2000).

Currently, five amphibian species exist in Finland. The species are *Rana*

*temporaria* (grass or common frog), *R. arvalis* (moor or swamp frog), *Bufo bufo* (common toad), *Triturus vulgaris* (smooth newt) and *T. cristatus* (crested newt). In the 1960s, a few populations of *R. ridibunda* (marsh frog), the largest frog in the Europe, were observed in southern Finland. This species was probably introduced earlier, and today all populations have disappeared (Terhivuo, 1998).

In 1989, researchers discovered that amphibians, particularly frogs and toads from many parts of the world, appeared to be declining (Stebbins and Cohen, 1997). There are vital reasons why the loss of amphibians from ecosystems should be of grave concern, the foremost being that amphibians act as keystone species, and also as indicator or sentinel species (Murphy et al., 2000). In architectural terms, the keystone is a wedge-shaped piece at the top of an arch that meshes its two sides and thus supports the entire structure around it. In a similar way, an ecological keystone species is one that has an integral role, stature and position in the ecosystem. This role is so vital to the interconnected web of life that its disappearance, or even reduction, would cause serious consequences throughout the community, resulting in declines or extinctions of numerous other dependent species. By contrast, an indicator or sentinel species is one that is particularly sensitive to changes in the environment.

In many ecosystems, amphibians play a central role in energy flow and nutrient cycling (Stebbins and Cohen, 1997). Amphibians are both predators and prey. Grazing anuran larvae and tadpoles exert important control over the growth of algae and other aquatic plants and transfer energy stored in plants to predatory animals. Adult amphibians in turn are the primary vertebrate predators on invertebrates in many freshwater and moist terrestrial environments. In this way the energy stored in invertebrates is transferred higher up the food chain.

The hypothetical causes of declines in amphibian populations fall into three categories: habitat destruction and alteration, global anthropogenic influences and natural causes (Alford and Richards, 1999). These causes act simultaneously (Blaustein and Kiesecker, 2002). For instance, global warming may change disease dynamics (Pounds et al., 2006), and UV-B radiation itself can suppress the immune system at organism level (Mayer, 1992). A good example is the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). It is suggested that global warming will shift temperatures in many highland localities towards the growth optimum of *Batrachochytrium* (Pounds et al., 2006). At least 30 species of 113 of *Atelopus* frogs have been missing from all known localities for at least 8 years (in 2005) and are probably extinct, and *B. dendrobatidis* is implicated (La Marca et al., 2005). A recent study suggests that climate warming can act on wild temperate zone amphibians by deleteriously affecting their physiology during and after hibernation, causing increased female mortality rates and decreased fecundity in survivors (Reading, 2007).

### 1.1. Objectives

The main focus of this work was to study how UV-B radiation and an endocrine disrupting chemical, BPA, affect the early development of *R. temporaria* and *R. arvalis* larvae under laboratory conditions. BPA was first synthesized in 1905, and its estrogenic properties were discovered in 1936 by Sir Charles Edward Dodds (Dodds and Lawson, 1936). Afterwards it has been shown to cause DNA adducts (Atkinson and Roy, 1995). This term refers to the covalent attachment of the chemical to DNA. DNA adducts are believed to be the initial step in chemical carcinogenesis (reviewed in Poirier et al., 2000).

The secondary aim was to study how phytosterols affect particular parameters of reproduction and energy metabolisms in post-metamorphic *Xenopus laevis* (the

South African clawed frog). A phytosterol mixture, ultrasitosterol, a substance which is extracted from pulp mill effluents, was used in the experiment. Many phytosterols, such as  $\beta$ -sitosterol, which is also the main component of ultrasitosterol, can be identified in pulp mill effluents (Kostamo and Kukkonen, 2003). Thus it can be used in experiments as a model substance for describing pulp mill exposure. *X. laevis* is an excellent model for the study of phytosterols, as it is totally aquatic and the post-metamorphic individuals are carnivorous.

Both of the used chemicals exist as contaminants in natural waters. The endpoints in the experiments were mortality, and the accumulation and depuration of BPA. In the phytosterol study the endpoints were certain hormone concentrations and enzyme activities.

The objectives of this work can be summarized in the following questions.

- 1) How does UV-B exposure affect toxicokinetics (I) and toxicity of bisphenol A in *R. temporaria* (III)?
- 2) How do different UV-B doses affect developing *R. temporaria* and *R. arvalis* embryos, and are there differences between species (II)?
- 3) How do phytosterols affect reproductive hormones and energy metabolism in postmetamorphic *X. laevis* (IV)?

## 2. AMPHIBIANS AND ENVIRONMENTAL THREATS

### 2.1. Ozone and solar UV radiation

Ozone depletion was discovered for the first time in the Antarctic stratosphere in the mid-1980s (Farman et al., 1985). Recent measurements and estimations have shown that global mean total column ozone for the period 1997-2001 was approximately 3% below the pre-1980 average values (UNEP/WMO, 2003). A major

concern regarding the decrease in stratospheric ozone is the consequential increase of ambient solar ultraviolet radiation (UV, 280-400 nm) passing through the atmosphere and reaching the Earth's surface. Ozone absorbs UV strongly, and the presence of ozone and oxygen in the stratosphere results in the absorption of virtually all solar radiation below 290 nm. Ultraviolet-B radiation (UV-B, 280-320 nm) is significantly absorbed by ozone, whereas ultraviolet-A radiation (UV-A, 320-400 nm) is absorbed less than 3%. Annually averaged erythemal irradiance has increased by about 6-14% over the last 20 years. This result is based on pyranometer, total ozone, and other meteorological measurements at several mid- to high latitude sites. There is evidence that long-term UV changes are not driven by ozone alone, but also by changes in cloudiness, aerosols, and surface albedo (UNEP/WMO, 2003). In the Antarctic, ozone depletion has been the dominant factor in the increase of UV irradiance.

The optical quality of water is an important factor when the effects of UV radiation in an aquatic environment are studied. In general, UV is absorbed by water in a wavelength-dependent manner, increasing with decreasing wavelength (Hargreaves, 2003). Penetration of UV into natural waters depends on the concentration and optical qualities of dissolved organic matter (DOM), phytoplankton, other suspended particles and the optical properties of pure water. For instance, in a study in Finland where three lakes were studied, 99% of the solar UV-B radiation attenuation was observed in an approximately half-meter water column in a lake with a DOC concentration of 4.9 mg/l, whereas, in a small humic lake with a DOC concentration of 13.2 – 14.9 mg/l, 99% attenuation was observed in the top 10 cm water column (Huovinen et al., 2003).

## 2.2. Effects of UV radiation on amphibians

The recent increase in UV has been thought to be one stressor responsible for the decline in amphibian populations (Blaustein et al., 1994). In all living cells, the primary site of UV action is DNA, where damage may accumulate and result in cell death or mutation (Mitchell and Karentz, 1993). UV-B is absorbed by DNA, mainly resulting in the creation of cyclobutane pyrimidine dimers (CPDs), but also of other photoproducts. When DNA is exposed to UV radiation approaching its absorption maximum at around 260 nm, adjacent pyrimidines within the same DNA strand may become covalently linked by the formation of mostly four-membered ring structures referred to as CPDs and to a lesser extent of pyrimidine (6-4) pyrimidone photoproducts ((6-4)PPs). The photochemical formation of (6-4)PPs includes the transfer of the hydroxyl group at C(4') of the 3' base, via an oxetane intermediate, to the C(5) position of the 5' base. The (6-4)PPs are almost quantitatively converted to their Dewar isomer form by irradiation with light of 320-350 nm (Weber, 2005). These photoproducts are mutagenic because they block the transcription and translation of DNA. However, many amphibian species have a capacity to repair CPDs (Blaustein et al., 1994), and (6-4) photolyases have been found in *X. laevis* (Kim et al., 1996; Todo et al., 1997). UV-A/blue light (320-500 nm) exposure stimulates photoreactivation (repair of CPDs) (Heelis et al., 1993), which is performed by the enzyme photolyase.

Photolyases are widespread in organisms and are reported in fish, reptiles, amphibians and marsupials (Weber, 2005). The concentration of photolyase varies among amphibian species. Generally, the eggs of species that are exposed to sunlight have greater photolyase activity than the eggs of species that are not typically exposed to sunlight (Blaustein et al., 1994). UV-B radiation can also cause neurobe-

havioral disorders. Häkkinen et al. (2003) reported that UV-B treated larvae of *Esox lucius* (northern pike) were incapable of swimming straight and spun in an uncontrolled manner. This kind of neurobehavioral disorder was also seen in our experiment in *X. laevis*, but not in *R. temporaria* at the same UV-B dose (data not published).

### 2.3. Effects of xenobiotics on amphibians

Many of the man-made substances are harmful to living organisms. Natural waters are the ultimate recipients of most of the toxic substances generated by industrial, agricultural and domestic activities and released into the environment.

The ontogenetic shift from herbivorous tadpoles to carnivorous adults makes amphibians vulnerable to several exposure routes (Duellman and Trueb, 1986). In the real world, organisms are not exposed to a single stressor alone, as is usually the case in laboratory experiments, but rather to mixtures of chemicals. Some chemicals are more toxic than others, and the occurrence of multiple xenobiotics exposes amphibian larvae to potentially synergistic negative impacts. For example, subacute exposure to a pesticide and predation caused a low mortality alone, but heavy mortality in combination (Relyea and Mills, 2001; Relyea, 2003). This type of finding is very alarming, and it is possible that this is only the tip of the iceberg in terms of the synergistic, negative impacts that multiple stressors can have on natural systems (Sih et al., 2004).

Many pesticides have been shown to induce developmental malformations in developing amphibian larvae. Hayes et al. (2003) showed that atrazine exposure of 0.1 mg/l caused gonadal dysgenesis and hermaphroditism in developing *R. pipiens* (the northern leopard frog), and vitellogenesis was discovered in slower developing males. Moreover, gonadal dysgenesis and hermaphroditism were found in field collected individuals. Ankley et al. (1998)

showed that the pesticide methoprene can induce profound developmental malformations (axial distortion) in *R. pipiens* larvae. The effects of methoprene are suggested to be mediated through the retinoic acid (active metabolite of vitamin A) system that controls processes related to cellular differentiation, pattern of development and the establishment of embryonic polarity (Shimeld, 1996; Escriva et al., 2002). Niazi and Saxena (1978) reported that retinyl palmitate caused pattern duplications in regenerating limbs in *B. andersoni* tadpoles, and later a detailed description of the effects of retinoic acid and other retinoids on pattern formation during limb regeneration in the *Ambystoma mexicanum* (axolotl) was described (Maden, 1982).

It is suggested that the metabolites of methoprene interact with one or more retinoid receptors (nuclear receptors related to the steroid and thyroid hormone receptors) (Harmon et al., 1995). A recent study on amphibians has shown that retinoid homeostasis in *R. catesbeiana* was affected by agricultural practices in intensively cultivated areas (Bérubé et al., 2005).

### 2.4. Combined effects of UV radiation and xenobiotics

It is well known that UV radiation may modify some chemicals. Some chemicals photodegrade when exposed to UV radiation and others, like some polycyclic aromatic hydrocarbons (PAHs), exert photoinduced toxicity (Bowling et al., 1983). Photoinduced toxicity or phototoxicity is caused by the absorbance and transfer of UV radiation energy from the excited state of the PAH to molecular oxygen forming superoxide radical anions that cause redox cycling (photosensitization) and subsequent cell death (Diamond, 2003). This is serious phenomenon, because many hydrophobic toxic substances tend to accumulate in the tissues of aquatic animals, such as invertebrates and amphibian larvae. After accumulation, these already toxic substances may be even more

toxic due to photoactivation. Some phototoxic compounds, under certain conditions, may exert toxicity after photochemical modification in the external environment. Most pesticides show UV-Vis absorption bands at relatively short wavelengths, although direct photodegradation of pesticides by sunlight is expected to be less important, since only a small amount of short wavelengths reaches the Earth's surface. Burrows et al. (2002) have written a comprehensive review of reaction pathways and mechanisms of the photodegradation of pesticides.

Photoactivation and photosensitization are not the only phenomena that exert simultaneous adverse effects on organisms. For example, methoprene exposure alone caused developmental malformations in *R. pipiens* larvae, and the presence of UV-induced bilateral and often symmetrical hindlimb malformations (ectromelia and ectrodactyly) (Ankley et al., 1998). The greatest sensitivity to UV radiation was during early limb bud development, corresponding to the formation of the apical ectodermal ridge. This finding indicates that UV radiation should also be considered as a contributing factor to toxic and teratogenic effects.

### 3. MATERIALS AND METHODS

#### 3.1. Study animals

Three anuran species were used in the experiments. *R. temporaria* and *R. arvalis* are domestic species and *X. laevis* is naturally limited to Africa south of the Sahara. *R. temporaria* and *R. arvalis* were collected near the town of Joensuu, Finland. The sampling sites were Hasanniemi (62°35'34''N, 29°44'44''E), Honkaniemi (62°36'23''N, 29°43'06''E), Paskolampi (62°40'03''N, 29°42'58''E) and Niemetönlampi (62°40'06''N, 29°38'34''E).

The *R. temporaria* varies greatly in form, color and pattern, and it is one of the most widely distributed and most abundant amphibian species in Europe

(Grossenbacher, 1997). It breeds in various small waters from northern Spain to North Cape in northern Scandinavia. Its distribution extends throughout Europe east to the Urals, but excluding most of Iberia, much of Italy, and the southern Balkans. *R. temporaria* was introduced into Ireland three hundred years ago. *R. arvalis* inhabits a wide Euro-Asiatic distribution range (Ishchenko, 1997), its distribution being from northeastern France, Belgium, the Netherlands, Germany, Denmark, Sweden, and Finland (about 69°N) south to the Alps, northern Yugoslavia, northern Romania, and east to Siberia (up to Yakutia, 124°E).

Depending on the experiment performed, *R. temporaria* and *R. arvalis* egg clutches were collected directly from nature or by letting captured frogs spawn in buckets in the laboratory in tap water. If the spawns were collected from nature, it was ensured that the spawn was less than 24 h old. This was done by checking a suitable breeding area before dark and by rechecking the area before dawn. If new spawn clutches had emerged between the checks, they were collected and used in the experiments. Eggs were used in the experiments, which were focused on the early stages of development. When experiment was performed using larvae, the embryos were allowed to hatch in buckets and <24 h larvae were used in the experiments. After spawning, the frogs were released at the capture sites.

The origin of the *X. laevis* stock used in Article IV is in the South Africa. A total number of 25 wild male and female frogs were obtained in the year 2000 from Xenopus Express, Inc. USA. And The offspring of these individuals were used in the experiment.

*X. laevis* is a widely used laboratory animal in many parts of the world. It was earlier used for human pregnancy diagnosis (Landgrebe, 1939). The pregnancy was detected by ovulation of *X. laevis* female in response to an injection of pregnant women urine into the dorsal lymph sac.

Today, it has become the most widely used vertebrate species in the research areas of development, cell and molecular biology. The reasons for its popularity are: good strength against infection and disease, simplicity of husbandry, responsiveness to induction of reproduction under seasonal laboratory conditions, and undemanding diet. The ease of maintenance in captivity also made *X. laevis* attractive to the pet trade. The release of captive individuals has led to the formation of feral populations in many parts of the world. Same advantages which made *X. laevis* ideal as laboratory animals then proved to be a considerable advantage for adaptation to new environments (Tinsley and McCoid, 1996).

Pipid frogs are aquatic and apparently more specialized to aquatic life than any other group of amphibians (Trueb, 1996). This is suggested by their wide, flat habitus, dorsal eyes, possession of a lateral line system in the adult, extensive webbing, powerful hind limbs that cannot be folded under the body and specialized aquatic courtship rituals.

All the experimental procedures conformed to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, and the protocols were approved by the Animal Care and Use Committee of the University of Joensuu. The permission to collect frog spawn was given by the North Karelia Regional Environment Center (Joensuu, Finland). The experimental design applied in each paper is summarized in Table 1. A more detailed description is given in the original papers.

### 3.2. Study chemicals

Bisphenol A (BPA, 2,2-bis-(4-hydroxyphenyl)propane, Sigma-Aldrich Co. Ltd. Gillingham, Dorset, UK) was used in the experiments in Articles I and III. BPA is one of the highest-volume chemicals produced worldwide. In 2003 global capacity was 2,214,000 metric tons

(Burrige, 2003). BPA is a relatively small, 228 Da, monomer that is polymerized to produce polycarbonate plastic and the resins used to line metal cans. It is also used as an additive in other types of plastic, such as polyvinyl chloride (PVC) and polyethylene terephthalate (PET), used in medical tubing, toys, water pipes, soda and mineral water bottles, and flame retardants. BPA is also used to make some dental sealants.

BPA is linked by an ester bond in polycarbonate and resins. Heat and contact with either acidic or basic compounds accelerate hydrolysis of the ester bond. Sterilizing food cans by heating, the presence of acidic or basic food or beverages in cans or polycarbonate plastics, and repeated washing of polycarbonate products have all been shown to result in an increase in the rate of leaching of BPA (Krishnan et al., 1993; Brotons et al., 1995). Another potential source of exposure is leaches from landfills. Studies in Japan (Yamamoto et al., 2001; Kawagoshi et al., 2003) and in the United States (Coors et al., 2003) have shown that BPA accounts for most estrogenic activity leaching from landfills into the surrounding ecosystem.

There is convincing evidence of widespread exposure to BPA in humans. In the United States, 95% of urine samples examined by the Centers for Disease Control had measurable BPA concentrations [range: 0.1 µg/l to 5.18 µg/l, mean 1.33 µg/l] (Calafat et al., 2005).

BPA was used in both radioactively labeled and non-labeled form. The non-labeled form was used in Article I, where *R. temporaria* larvae were exposed simultaneously to UV-B radiation and three different concentrations (10, 100, and 1000 µg/l) of BPA. Labeled BPA was used in article II, where the toxicokinetics of BPA [propyl-2-14C-BPA], specific activity 2,074 MBq/mmol, was studied under simultaneous UV-B exposure, and only the trace concentration was needed (1.84 µg/l).

Another chemical used in the experiment was ultrasitosterol. It was obtained

**Table 1.** Summary of the experimental designs of the studies in the different articles of the thesis. A more detailed description of the material and methods are presented in the individual papers (I-IV)

	I	II	III	IV
<b>Test species</b>	<i>Rana temporaria</i>	<i>Rana temporaria</i> <i>Rana arvalis</i>	<i>Rana temporaria</i>	<i>Xenopus laevis</i>
<b>Developmental stage</b>	Embryos <24h old	Embryos <24h old	Larvae <24h hatched	Postmetamorphic
<b>Type of study</b>	Toxicity Toxicokinetic	Survival	Survival	Subacute exposure
<b>Stress factor</b>	Bisphenol A UV-B radiation	UV-B radiation	Bisphenol A UV-B radiation	Ultravioletsterol
<b>Chemical concentration</b>	0, 10, 100, 1000 µg/l		1.84 µg/l	30 µg/l
<b>UV-B dose</b>	2.80 kJ/m <sup>2</sup>	0, 0.81, 1.04, 1.26 kJ/m <sup>2</sup>	1.04 kJ/m <sup>2</sup>	
<b>Experiment length</b>	20 d	27 d	72 h	14 d

from UPM-Kymmene Corporation, Chemical Mill, Lappeenranta, Finland. Ultrasitosterol is a phytosterol mixture (75.7%  $\beta$ -sitosterol, 13% sitostanol, 9% campesterol and campestanol, 0.9% arstenols), and it is extracted from pulp mill effluents. Phytosterols are plant-derived compounds analogous to animal cholesterol.

The solubility of the purified plant sterol mixture is extremely low, and it is therefore mandatory to use solvents for creating exposure waters. In all the Articles, ethanol was used as a solvent for creating the stock solution that was used for creating exposure waters. The most common phytosterols are  $\beta$ -sitosterol, campesterol, and stigmasterol.

Ultrasitosterol was used in Article IV, where postmetamorphic *X. laevis* were exposed to it in flow-through exposure. The concentration used was 30  $\mu\text{g/l}$ , which can be considered to be environmentally relevant (Mattson et al., 2001). The molecular structure of the BPA and the main components of ultrasitosterol are shown in Figure 1.

### 3.3. Ultraviolet-B radiation

The ultraviolet region spans the 10 to 400 nm wavelength range, accounting for less than 9% of the total solar energy output (Madronich, 1993). This wavelength range can be broadly divided into extreme UV (10-120 nm), far UV (120-200 nm), vacuum UV (<240 nm), UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm). Ultraviolet-B radiation was used in Articles I, II and III.

It is essential to calculate the specific UV-B dose in experiments where the effects of UV-B radiation are studied or UV-B is associated as a stress component. Spectral irradiance of the UV-B source at the air-water interface was measured using a calibrated spectroradiometer, which reports the spectral irradiance in units of  $\text{W/m}^2/\text{nm}$ . Philips TL 40W/12 RS UV-B tubes were used as the artificial source of

UV-B radiation. Because these tubes emit UV-C radiation also, it is necessary to use filters to eliminate the ecologically non-relevant UV-C radiation. Filters were also used in control treatments to eliminate both UV-C and UV-B radiation. Cellulose diacetate filters were used for the elimination of UV-C radiation, and polyester filters for UV-B and UV-C radiation. Philips 40W/12 RS UV-B tubes in combination with cellulose diacetate filters give a spectrum that deviates considerably from the daylight spectrum, but it matches the spectrum of increase caused by ozone depletion (Björn and Teramura, 1993). The spectrum of the Philips TL 40W/12 RS UV-B tubes with polyester and cellulose diacetate film and its relation to the modeled sunlight spectrum is shown in Figure 1 in Article III.

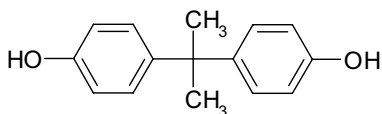
In all the experiments where UV-B was a component, the background laboratory lighting was checked and it was ensured that there was no UV-B radiation present.

### 3.4. Toxicokinetic estimation

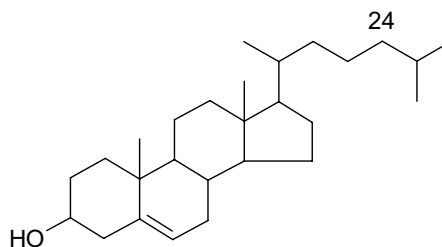
Toxicokinetics is defined as the study and predictive modeling of the internal kinetics of poisons (Newman, 1988). In Article I the accumulation and depuration kinetics of BPA under UV-B exposure were studied in *R. temporaria* larvae. Bioaccumulation is the general term describing the net uptake, biotransformation, and elimination of chemicals within an individual from the environment by any or all of the possible routes (Spacie et al., 1995).

Bioconcentration is a more specific term reserved for describing accumulation from water only. It is a well known fact that new tissue mass dilutes the internal chemical concentration when the body mass of an organism increases due to growth. The apparent elimination rate derived from a growing organism overestimates the actual elimination, as it incorporates both elimination and growth, and therefore underestimates the uptake rate and steady-state tissue residues.

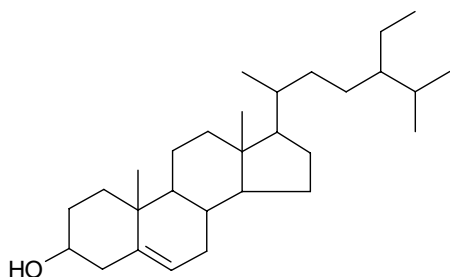
2,2-bis-(4-hydroxyphenyl)propane



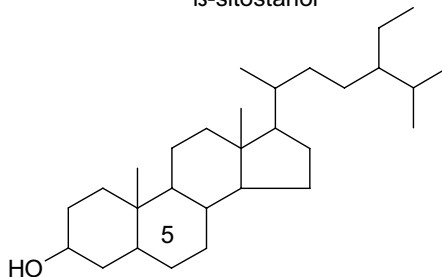
Cholesterol



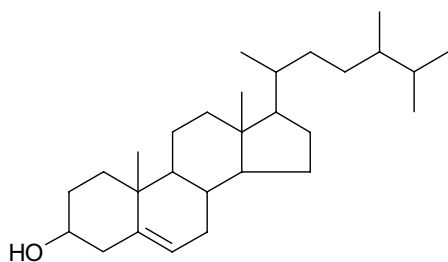
$\beta$ -sitosterol



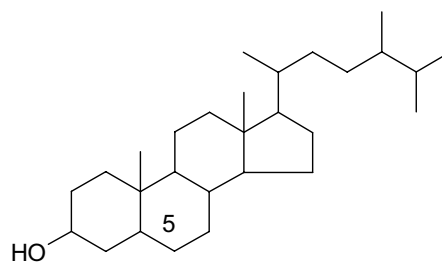
$\beta$ -sitostanol



Campesterol



Campestanol



**Figure 1.** Molecular structure of the chemicals used in the experiments in the thesis. Synonyms: bisphenol A = 2,2-bis-(4-hydroxyphenyl)propane. Ultrasitosterol is a mixture of several phytosterol. The used mixture contained 75.7%  $\beta$ -sitosterol, 13% sitostanol, 9% campesterol and campestanol, 0.9% artenols, and the major components are shown above.

Growth dilution is not a component of elimination, because the total amount of contaminant has not changed as a result of growth (Newman, 1988). However, when modeling accumulation, the growth dilution effect can be avoided if the growth dilution factor ( $g$ ) is incorporated in the

model. Growth dilution influences the results of tissue residue measurements if the studied individuals are growing over time. This could result in a decrease in chemical concentration due to the increased amount of tissue in which the chemical is distributed (Newman, 1988).

In this thesis (I), a one-compartment model was used to study BPA accumulation. The data from the accumulation experiment were fitted by applying an iterative least squares method, with or without the calculated growth dilution factor ( $g$ ), to the following differential equations (Eqn. 1, 2) describing the uptake and elimination of BPA, using the fourth-order Runge-Kutta approach in the Software package MicroMath Scientist® V.2.01 for Windows (MicroMath Inc. Salt Lake City, Utah):

$$\frac{dC_a}{dt} = k_u C_w - k_e C_a \quad \text{Eqn. 1}$$

$$\frac{dC_a}{dt} = k_u C_w - k_e C_a - g C_a \quad \text{Eqn. 2}$$

where  $C_a$  is the concentration of BPA in the larvae ( $\mu\text{g/g}$  wet wt),  $k_u$  is the conditional uptake clearance ( $\text{ml/g/h}$ ),  $C_w$  is the concentration of BPA in the water ( $\mu\text{g/ml}$ ),  $k_e$  is the elimination rate coefficient ( $1/\text{h}$ ),  $t$  is the time, and  $g$  ( $1/\text{h}$ ) is the first-order growth dilution factor. Growth dilution factors and depuration rate ( $kd$ ) were determined by fitting natural-log transformed weight  $W$  ( $\mu\text{g}$  wet wt), tissue concentration  $C$  ( $\mu\text{g/g}$  wet wt) or heat dissipation  $H$  ( $\mu\text{W/mg}$ ) to a first-order curve (Eqns. 3-5).

$$\ln W = \ln W_0 + gt \quad \text{Eqn. 3}$$

$$\ln H = \ln H_0 + gt \quad \text{Eqn. 4}$$

$$\ln C = \ln C_0 - k_d t \quad \text{Eqn. 5}$$

### 3.6. Experimental setup

*R. temporaria* larvae were exposed to three different BPA concentrations, 10, 100 and 1000  $\mu\text{g/l}$ . Both control and solvent control were included (III). This experiment was conducted with and without UV-B radiation. Each treatment combination comprised three 2-litre oval Pyrex dishes with 30 larvae per dish, the total number of

dishes in the experiment being 30. The endpoint of the experiment was mortality. The embryos and larvae were checked daily and dead ones were counted and removed from the dishes. The experiment was terminated after 20 days, since most of the larvae in the UV-B treatment had died.

In Article I the toxicokinetic approach was used. The BPA concentrations in larvae were monitored as a function of time. In the accumulation phase, four *R. temporaria* larvae per sampling time were placed in triplicate beakers and exposed to 1.84  $\mu\text{g/l}$  of [ $^{14}\text{C}$ ]-labeled BPA ( $\sim 1000$  DPM  $\text{ml/l}$ ) with and without UV-B radiation. The daily UV-B dose in the UV-B treatment was 1.04  $\text{kJ/m}^2$ . The depuration of BPA was studied in a separate depuration experiment. The sampling intervals were 3, 6, 12, 24, 48 and 72 h in both experiments. To avoid differences in the developmental stages of the larvae, both accumulation and depuration were started at the same developmental stage (stages 20–22, Gosner, 1960). Additionally, the accumulation was further modeled with correction for growth dilution. The growth dilution factor ( $g$ ) was established by using direct calorimetry and wet weight data. The defined  $g$ -values can be used in the first-order accumulation model.

In Article II, *R. temporaria* and *R. arvalis* larvae were exposed to different UV-B doses (0, 0.81, 1.04 and 1.26  $\text{kJ/m}^2$ ) under laboratory conditions. During the experiment two separate samples were taken for upcoming analyses. However, as a result of being stored too long in Bouin's solution, half of the stored samples were ruined. This experiment thus investigated how different UV-B doses affect the early stages of embryos and larvae in the presence of censoring of the data. The data were grouped by three age segments based on subsampling on days 15 and 27 from the start of the experiment. These removals were treated as survivors in the statistical analyses. The age segments used were: days 0-6, 7-15 and 16-27. Two statistical methods were applied and used in the es-

timations of the results: the log-linear model of relative risk with age (LRR) and the log-linear dose-response model of relative risk with age and UV-B dose (LRRd). By these methods the relative risk of mortality with age and the relative risk with a specific UV-B dose can be estimated.

In Article **IV** postmetamorphic *X. laevis* were exposed for 14 days in a flow-through system to a concentration of 30 µg/l of ultrasitosterol. Both control and solvent control were included. The plasma testosterone, leptin-immunoreactive peptide, thyroxine and triiodothyronine concentrations were measured at the end of the experiment. In addition, glycogen concentration, lipase and phosphorylase activities were determined from liver and muscle tissues, as well as glucose-6-phosphatase activity from liver. This article differs from previous articles in that it focused on investigating adverse physiological responses of phytosterols in carnivorous postmetamorphic individuals.

## 4. RESULTS AND DISCUSSION

### 4.1. Effects of UV radiation on embryos and larvae

Mortality increased with time in *R. temporaria* and *R. arvalis* (**II**), which was an expected outcome. However, mortality differed between the species at different age periods. Moreover, UV-B radiation increased mortality in both species, but at different age periods. When *R. temporaria* and *R. arvalis* were exposed to UV-B radiation under laboratory conditions for 27 days, it seemed that *R. temporaria* was less tolerant in the egg period (period 0-6) (**II**: Fig 1) than *R. arvalis*, but in the middle period (period 7-15) and end period (period 16-27), *R. temporaria* survived better.

Mortality depended on the UV-B dose. In *R. temporaria*, the dose response was significant at period 0-6 with doses of 1.04 and 1.26 kJ/m<sup>2</sup>. For period 16-27, the dose response was significant only at the highest

UV-B dose (1.26 kJ/m<sup>2</sup>). In *R. arvalis*, the dose response was significant at the highest UV-B dose at period 7-15, and at UVB doses 1.04 and 1.26 for the period 16-27 kJ/m<sup>2</sup>. The significance of dose response is that an increased UV-B dose increases the risk of mortality.

In Articles **I**, **II** and **III**, UV-B exposure was carried out without UV-A radiation. Published studies of the effects of UV-B radiation fall into two categories: filtration experiments, where almost all solar ambient UV-B radiation is filtered off, and lamp experiments, where UV-B radiation is added to a background of solar radiation (Cummins et al., 1999). Only a few studies have been conducted under laboratory conditions with supplemental UV-A radiation. For instance, Pahkala et al. (2003) showed that UV-B treatment with the presence of UV-A radiation increased the early growth of *R. temporaria* and *R. arvalis* larvae. Although the authors did not bring it up, this phenomenon could be related to the absorption of UV-B radiation into melanin pigments in the outermost cell layers. The outcome of absorption is heat, which ultimately contributes to growth in the breeding season. The phenomenon was not observed in *Bufo bufo* (common toad) (Pahkala et al., 2003). This makes sense, since both *Rana* species lay their eggs in open, shallow waters, which are often ephemeral, whereas *B. bufo* spawn is laid deeper in double row strings, which are wrapped around aquatic plants or other objects. Therefore, *B. bufo* spawn is more protected against UV-B radiation, and direct adaptation to UV-B radiation is not as well developed as in *R. temporaria* and *R. arvalis*. In *R. temporaria* and *R. arvalis*, both egg clutches and larvae are exposed to full UV-B radiation action, although embryos located in the center of the clutches are more protected than embryos in the outer layer. Elimination of ambient UV-B radiation increased survival of *B. bufo* larvae significantly, but had no effect on *R. temporaria* and *R. arvalis* in the field

experiment (Häkkinen et al., 2001). This finding supports earlier suggestions.

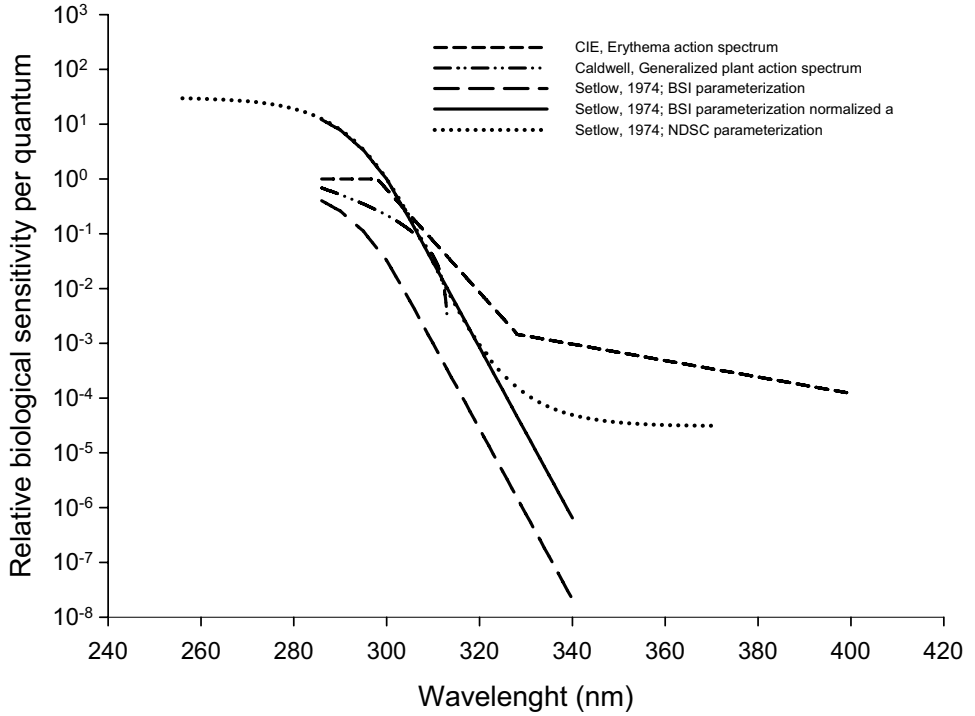
Pahkala et al. (2002a) studied the effects of UV-B radiation on *R. temporaria* embryos originating from eight populations spanning a 1200 km latitudinal gradient across Sweden under laboratory conditions. Their results suggest that the sublethal effects of UV-B radiation on embryonic development up to developmental stage 25 (Gosner, 1960) may differ among populations and that there is no clear latitudinal pattern to UV-B tolerance. They also investigated synergistic effects of UV-B radiation and low pH on *R. temporaria* embryos under laboratory conditions originating from southern and northern Sweden (Pahkala et al., 2002b). The results showed that simultaneous exposure reduced survival rates and increased developmental malformations in the northern but not in the southern population. Also, low pH reduced hatchling size in both populations. Interestingly, embryos in the normal UV-B treatment (1.254 kJ/m<sup>2</sup>, DNA weighted) developed significantly faster than embryos in the enhanced (1.584 kJ/m<sup>2</sup>) or control (no UV-B) treatment. This finding supports suggestion about benefit from the extra heat contributed by UV absorption, but, the results show also that UV-B doses above the normal range have adverse effects. The effects of low pH and UV-B radiation was also studied in the field experiment with *R. arvalis* (Pahkala et al., 2001). These results suggest that low pH reduces survival indeed and increases developmental malformations, but there was no UV-B × pH interaction.

The eggs of many frog species are surrounded by a jelly matrix and vitelline membrane, which may protect developing embryo from UV radiation. For example, the occurrence of UV radiation induced deformities is related to the size and thickness of the jelly matrix when exposed to UV radiation (Grant and Licht, 1995). In addition, specific UV-absorbing substances (UVAS) have also been reported in several invertebrates, algae and the skin of four

fish species (Fabacher and Little, 1995; Dunlap and Shick, 1998; Teai et al., 1998; Sommaruga and Garcia-Pichel, 1999). The jelly matrix and vitelline absorb in the UV-A region, and the UVAS have maximum absorption at approximately 292 nm, depending on the fish species. Hofer and Mokri (2000) have identified UVAS from *R. temporaria* larvae, too. Overall, it seems that *R. temporaria* larvae have some adaptations to UV-B and benefit from the extra heat contributed by UV absorption. Also, the presence of photolyase in *R. temporaria* larvae can be proven indirectly by comparing the results of different publications.

The species-specific dose responses (II) might be due to the different types of breeding areas of these species. *R. temporaria* breeds in small waters, which are open and shallow, and formed by waters from melted snow. *R. arvalis* usually prefers flooding and luxuriant coastal areas of lakes and ponds, which are deeper. The penetration of UV-B in the water in these areas is affected by the vegetation and dissolved organic matter in the water. In this context, the higher resistance of *R. temporaria* larvae to UV-B radiation, compared with that of *R. arvalis*, might be explained by natural selection, but the higher mortality at the pre-hatch stage can not be explained.

The sensitivity of organisms to UV-B radiation is in general a function of wavelength (Madronich, 1993). The wavelength dependence has to be known accurately if an estimate of the biological responses to changes in atmospheric composition is desired. The most common representation of the wavelength dependence of biological effects is through monochromatic action spectra, obtained in laboratory studies by exposing a biological target to various isolated wavelengths of radiation and comparing the responses. The impact of radiation on biological systems is usually described as the integral of the product of spectral irradiance,  $E(\lambda)$ , and a “biological weighting function”  $W(\lambda)$  (Eqn. 6).



**Figure 2.** Different action spectra presented as curves. *CIE*; Erythema action spectrum (McKinlay and Diffey, 1987). *Caldwell*; Generalized plant action spectrum (Caldwell, 1971). *Setlow*; Action spectrum for DNA damage (Setlow, 1974). BSI and NDSC are different parameterizations for Setlow’s original graph, implemented by the NSF UV monitoring network. These can be obtained from their website <http://www.biospherical.com/nsf/>.

$$E_{\text{bio}} = \int_{\lambda_1}^{\lambda_2} E(\lambda)W(\lambda)d\lambda \quad \text{Eqn. 6}$$

$W(\lambda)$  is a dimensionless function and also often denoted as an “action spectrum”. The various action spectra that are normally used in the experiments are expressed in Figure 2.  $E_{\text{bio}}$  is a “biological dose-rate”. Integrating biological dose-rate over time results in a “biological dose” (Eqn. 7).

$$D = \int_{x-h}^{x+h} E(t)dt \quad \text{Eqn. 7}$$

The integral is usually evaluated with the integration limits, 286 and 370 nm, for example. The integration is approximated in this thesis via a sum with  $d\lambda = 1$  nm steps between the integration limits. The dose rate is an instantaneous measure of the biologically weighted UV irradiance, with units of  $\text{W}/\text{m}^2$ . Integration of the dose rate over a full day gives the daily dose and over a full year the yearly dose, in units of  $\text{J}/\text{m}^2$ .  $W(\lambda)$  measures the relative effectiveness of different wavelengths, and thus it is necessary to specify its normalization point in order to compare different calculations of spectral dose rates, dose rates, and doses. In all experiments where

$$W(\lambda) = \frac{1}{0.0326} \times \exp \left[ 13.82 \times \left( \frac{1}{1 + \exp[(\lambda[\text{nm}] - 310)/9]} - 1 \right) \right] \quad \text{Eqn. 8}$$

UV-B radiation was a component, the action spectrum was normalized to unity at 300 nm [ $W(\lambda)$  values were divided by a  $B(\lambda)$  value at 300 nm]. The action spectrum used in all UV-B experiments was Setlow's DNA weighted action spectrum (Setlow, 1974). Setlow did not publish the actual values, only a graph. Thus researchers have made their own parameterization by digitizing Setlow's graph. The parameterization is a formula (Eqn. 8), and the parameterization used in this thesis is published in Bernhard et al. (1997). The original parameterization can be tracked to Steinmüller (1986). This parameterization is also adopted by the Network for the Detection of Atmospheric Composition Change (NDACC) [formerly the Network for the Detection of Stratospheric Change (NDSC)].

All the experiments were conducted without supplementary UV-A radiation, because the full effect of the given UV-B dose was desired. UV-A radiation is needed for photoreactivation. Photoreactivation is a process where dimerized pyrimidines, usually thymines, in DNA are restored by an enzyme (deoxyribodipyrimidine photolyase) that requires light energy from UV-A range.

The results clearly show that the UV-B response of larvae is cumulative and dependent on the UV-B dose (II). The individual fitness is lowered in cases where embryos and larvae have been severely abnormally developed due to UV-B radiation or simultaneous exposure to a xenobiotic (III). If photoactivation is blocked or disrupted, the ultimate outcome is death.

#### 4.2. Toxicokinetic estimations of BPA

When studying the toxicokinetics of BPA at a concentration of 1.84  $\mu\text{g/l}$  in *R. temporaria* (I), the estimated uptake clear-

ance ( $ku$ ) was similar in UV-B and no-UV-B treatments (I: Table 1), being 17.72 ml/g/h and 19.94 ml/g/h, respectively. Elimination rates ( $ke$ ) were also close to each other being 0.152 1/h for UV-B 0.121 1/h and no-UV-B. These values are similar to values obtained by Honkanen et al. (2006) in *R. temporaria* with BPA concentrations of 0.2, 1.5 and 10  $\mu\text{g/l}$  (20.90, 21.34 and 16.53 ml/g/h).

The bioconcentration factors (BCFs) calculated from steady-state concentration in larvae and water,  $\text{BCF}_{\text{Ca/Cw}}$ , varied from  $139.9 \pm 37.5$  (with UV-B) to  $163.7 \pm 13.5$  (without UV-B), but no statistically significant differences were found. When data from the accumulation experiment were fitted with the growth dilution factor ( $g$ ), the  $kes$  decreased (III: Table 1). This influenced the BCFs calculated as  $ku/ke$ . After growth correction, the BCFs calculated from estimates were closer to  $\text{Ca/Cw}$  calculated values. This finding proves that using growth correction in experiments is useful and can correct skewness in estimated parameters, even in an experiment lasting a short period. The obtained BCFs are somewhat higher compared to the values of Honkanen et al. (2006), where  $\text{BCF}_{\text{Ca/Cw}}$  was at all concentrations around 100 at 19°C. They also noticed that the BCF values were higher at lower temperatures.

Interestingly, the variance of replicates was higher in no-UV-B treatments when investigating the scatter plots of accumulation and depuration (I: Fig. 1 and 4). The reason for this is uncertain.

The depuration experiment resulted in a difference of two orders of magnitude between  $kd$  and  $ke$ . This phenomenon is similar to that found by Heinonen et al. (2002), where estimated  $ke$  values were 2-6 times higher than the measured  $kd$  values at lower temperatures (1.8 to 11.6°C). As

mentioned earlier, temperature will affect accumulation and depuration rates. For instance, the influence of low temperatures in the toxicokinetics of BPA have been studied in *Pisidium amnicum* (freshwater clam) (Heinonen et al., 2002), *R. temporaria* (Honkanen and Kukkonen, 2006) and *Salmo salar* m. *sebago* (landlocked salmon) (Honkanen et al., 2001). In all cases, both uptake and elimination were lower at lower temperatures. To my knowledge there is no existing literature about simultaneous UV-B and xenobiotic exposure of organisms at low temperatures. Obviously, this should be investigated, because BCFs are higher at low temperatures and the possible UV-B contribution to adverse effects is more evident, especially in boreal areas in spring.

#### 4.3. Physiological effects of chemicals having estrogenic properties

Many chemicals with estrogenic properties may influence or interfere with the estrogen-dependent reproductive processes. Reproductive failures have been attributed to both pre- and post-natal exposure to environmental endocrine disruptors in a wide range of organisms including reptiles, fish, birds, and mammals (Toppari et al., 1996). For example, significant feminization of *X. laevis* larvae from developmental stage 38/40 was obtained with a concentration of  $10^{-7}$  M (23  $\mu$ g/l) of BPA (Kloas et al., 1999).

Until recently, BPA has been considered a very weak estrogen. Today, there are growing numbers of studies suggesting that BPA is a more potent estrogen than has been believed. The term “weak” estrogen was based on a few assay systems, such as MCF-7 breast cancer cells in culture. The dose of BPA required to stimulate cell proliferation,  $10^{-7}$  M (23  $\mu$ g/l), is roughly 100,000 times higher compared to estradiol, which stimulates cell proliferation at approximately  $10^{-12}$  M (0.23 ng/l) (Welshons et al., 2003). However, the

stimulation of calcium influx by BPA in MCF-7 cells was significant at  $10^{-10}$  M (23 ng/l) (Walsh et al., 2005), and of calcium influx and prolactin secretion in rat pituitary tumor cells at  $10^{-12}$  M (0.23 ng/l), which is similar to the response to estradiol (Wozniak et al., 2005). Usually laboratory tests measure effects solely via the classical genomic pathway for steroid hormone action and they may miss an alternative pathway whereby these chemical may operate (Walsh et al., 2005). A chemical that can alter cell function at a concentration  $<1$  ng/l, cannot be characterized as a “weak” endocrine disruptor (vom Saal and Hughes, 2005).

The exposure of postmetamorphic *X. laevis* to an environmentally relevant concentration of phytoestrogens induced physiological changes in frogs (IV: Table 2). Individuals exposed to ultrasitosterol showed a significant decrease in muscle lipase activity. The most interesting finding was that the phytoestrogen mixture used, ultrasitosterol, caused a decrease in the plasma  $T_3$  concentrations in the exposed females. In adult amphibians, the role of  $T_3$  is more or less unexplored (Hayes, 2000), but the role in metamorphosis is well documented. The metamorphosis is directly stimulated by thyroid hormones ( $T_4$ ,  $T_3$ ) (reviewed in Tata, 2006), and moreover, thyroid hormones are essential for normal brain development in animals (Dussault and Ruel, 1987).

Testosterone plasma concentrations in exposed *X. laevis* females were almost 29 times higher than in unexposed females. Previous studies have shown decrease in plasma testosterone and 17 $\beta$ -estradiol concentrations in fish exposed to pulp mill effluents (Munkittrick et al., 1992). However, the increase in testosterone concentration is similar to the results obtained with the European polecat (*Mustela putorius*) and the field vole (*Microtus agrestis*) where 2-week exposure to ultrasitosterol increased plasma sex steroid concentrations (Nieminen et al., 2002; Nieminen et al., 2003). It is important to deter-

mine the biological significance of the observed increase in testosterone concentration, and further studies are needed.

Several recent studies show that thyroid hormone receptors are targets of industrial chemicals (Zoeller, 2005). In this respect, it is essential to study the effects of phytosterols in larvae and premetamorphic tadpoles. The muscle phosphorylase activity was lower in exposed animals, but a statistically significant difference was seen only when compared to the control females. The liver glycogen concentration was higher in exposed males than in exposed females, but no difference was found between them and their controls. There was no difference in the fat body somatic index and liver somatic index. These changes are not detrimental to adult individuals, but further studies are needed to determine the biological significance of the observed changes in enzyme activities, and especially in plasma T<sub>3</sub> concentrations.

Phytosterols are synthesized by plants. They are in general extracted from by-products of either the pulp and paper industry or the vegetable oil industry by using organic solvents. The product is a mixture of various plant sterols, which vary depending on the plant source. The phytosterol mixture used in this thesis, ultrasitosterol, is a good example. Similar in their appearance, cholesterol and phytosterols have similar structures (Fig. 1). The addition of a methyl or ethyl group at carbon 24 of the cholesterol chain leads to the formation of campesterol or  $\beta$ -sitosterol, respectively. Chemical saturation of the delta 5 double bond of each of the aforementioned plant sterols leads to the formation of 5- $\alpha$ -derivates such as campesterol or  $\beta$ -sitostanol. Phytosterols are present in high concentrations in pulp mill effluents (MacLatchy and Van Der Kraak, 1995). In a study carried out in the United States, the phytosterol concentration in pulp mill effluent ranged from 71  $\mu\text{g/l}$  to 535  $\mu\text{g/l}$ , with  $\beta$ -sitosterol being the major plant sterol component (Cook et al., 1997).  $\beta$ -sitosterol is a highly lipophilic phytosterol

found in both softwood and hardwood. For this reason, it is important to study the effects of phytosterol on the reproduction of fish and other aquatic vertebrates.

Amphibians are particularly vulnerable to xenobiotics because they may be exposed via several routes, e.g. food, their semipermeable skin and water. Exposure via the lungs is also possible, but not to the same extent as in humans. For example, *X. laevis* use 58.5% cutaneous surface and 41.5% pulmonary surface in oxygen exchange, and in carbon dioxide exchange 90.3% and 9.7%, respectively (Duellman and Trueb, 1986). Amphibians use every type of gas exchange (gills, lungs, skin and buccopharyngeal respiration) known in vertebrates. However, the lungless salamanders of the family Plethodontidae use only buccopharyngeal and cutaneous gas exchange. Gills are common in the larval stages of most amphibians, ceasing to function at metamorphosis, but in some neotenic salamanders, gill respiration is retained in the adults.

#### 4.4. Combined effects of UV-B radiation and BPA

The effects of UV-B and BPA were interdependent (III: Fig. 2 A-D). A dramatic increase in mortality was seen in the UV-B treatment after 13 days at all BPA concentrations, except the highest BPA concentration, where mortality increased already after 48 hours (III: Fig 2 E). In the no-UV-B treatment, the mortality was minimal, except in the highest concentration of BPA, where the mortality was high and differed from all the other BPA concentrations including both controls. In the UV-B treatment, statistical differences were found between the control and 10  $\mu\text{g/l}$  of BPA. This difference can be related to increased mean survival time at 10  $\mu\text{g/l}$  of BPA (III: Table 2). The reasons are unclear. The results suggest that BPA concentrations below 100  $\mu\text{g/l}$  are not lethal for *R. temporaria* embryos and larvae. However, the behavioral responses were not studied.

Although the UV-B daily dose used, 2.8 kJ/m<sup>2</sup>, was high compared to estimated ambient UV-B dose during the breeding season at the breeding site (0.8 kJ/m<sup>2</sup> to 1.3 kJ/m<sup>2</sup>), the results clearly show that in all cases UV-B radiation increased mortality significantly (III).

Developmental malformations appeared at the highest BPA concentration with UV-B treatment. During the experiment, a bluish glow was seen in this treatment combination. This suggests accumulation of BPA in the jelly matrix, and this was visible in UV light. A BPA concentration of 1000 µg/l has been shown to induce yolk-sac odema, phlegmatic behavior and changes in skin coloration in *S. salar* m. *sebago* fry (Honkanen et al., 2004), and concentrations of 100 and 1000 µg/l caused histological changes in liver cells. DNA adducts caused by BPA exposure and UVB radiation are the suggested explanation for the developmental malformations that have emerged. Both DNA adducts and CPDs are known to interfere with genome translation and transcription (Poirier et al., 2000; Hemminki et al., 2000; Weber, 2005). A lesion that inhibits genome replication or transcription of essential genes is cytotoxic and suppresses mutation induction.

BPA has been shown to inhibit thyroid receptor mediated transcription by acting as an antagonist (Moriyama et al., 2002). The results suggest that BPA could displace T<sub>3</sub> from the thyroid hormone receptor and recruit a transcriptional repressor, resulting in gene suppression. Dietary exposure of Sprague Dawley rats (*Rattus norvegicus*) to BPA during pregnancy and lactation caused an increase in serum total T<sub>4</sub> in offspring, but serum TSH was not different compared to the controls (Zoeller et al., 2005). This endocrine profile is similar to that observed in thyroid resistance syndrome (Zoeller, 2005; Cheng, 2005).

#### 4.5. Methodological observations and problems in UV exposures

Setlow's action spectrum for DNA damage is frequently used in UV-B dose calculations, and it was also used in this thesis. There are some issues that one should be aware of when using this action spectrum. Setlow's DNA action spectrum refers to unprotected DNA, and this is not the situation in real organisms, where DNA is not unprotected. Other tissues and pigments provide protection by filtering out some UV radiation.

It would have been more appropriate to calculate UV-B doses with several different action spectra in individual papers (I, II, III). This would have made comparisons with different UV-B doses used in other articles easier, because researchers use different action spectra in their studies.

The dose rates of UV-B tubes used are not equal, and filters which remove unwanted wavelengths photodegrade due to UV-B radiation. Therefore more automatic control electronics of lighting should be included in the UV-B exposure systems to achieve a constant dose rate over time. This could be done by incorporating dose rate measurements, dose calculations and UV-B source control in the experiments.

The growth dilution factor was determined by two different methods (I). It is true that heat dissipation measurements are time consuming, a bit difficult to perform and a microcalorimeter is obligatory. In addition, the possible movement of the study subject will affect the results. However, only the steady rate heat dissipation data were used in calculations. This means that at that time the heat dissipation curve is plateau and the larvae were not moving. The heavy fluctuation of the curve indicates movement. Both obtained growth dilution factors are usable and there is no remarkable difference between them.

## 5. CONCLUDING REMARKS

UV-B radiation had no effect on the accumulation and depuration of BPA in *R. temporaria*. The use of growth correction in toxicokinetic studies was shown to be useful as it can correct skewness in estimated parameters, even if the duration of the experiment is short. In terms of survival against pure UV-B radiation, the two studied *Rana* species were different: UV-B increased mortality in both species, but at different age periods. The UV-B response of *R. temporaria* and *R. arvalis* larvae was cumulative and dependent on UV-B dose. Simultaneous exposure to BPA and UV-B caused dramatic mortality after 13 days at all studied BPA concentrations except the highest concentration, where mortality increased already after 48 hours in both treatments (with and without UV-B). UV-B radiation increased mortality at all BPA concentrations. The highest concentration of BPA caused developmental malformations with UV-B radiation. The exposure of postmetamorphic *X. laevis* to an environmentally relevant concentration of phytosterols induced physiological changes in frogs. Phytosterols caused a decrease in plasma T<sub>3</sub> concentrations in the exposed females, and exposed individuals of both sexes showed a significant decrease in muscle lipase activity. The muscle phosphorylase activity was lower in exposed animals, but a statistically significant difference was seen only when compared to control females.

For over a decade, there has been major concern over amphibian decline. Habitat loss is clearly a major cause, and other factors that appear to play a role include pesticides, UV radiation, predators, parasites and disease. In addition to early mortality, in all organisms the most serious effects are those that affect breeding functions. If breeding functions including mating rituals and hormonal functions are disrupted, the whole population is in great danger of extinction. Therefore it is important to study how different stress factors

affect breeding functions and rituals. Moreover, subacute exposure to multiple xenobiotics or stress factors must be studied intensively to detect the overall behavioral response, because there is emerging evidence that subacute exposure to pesticides predisposes anuran larvae to predation. This also concerns other animal groups. In addition, experiments with multiple stressors should be performed at different realistic temperatures.

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