Exposure to synthetic antioxidants disrupt early development in the frogs *Silurana tropicalis* and *Lithobates pipiens*

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Abstract

Many chemicals in commonly used household and industrial products are being released into the environment yet their toxic acute and chronic effects on humans and wildlife are not well understood. Some of these chemicals behave as endocrine disruptors (EDCs), altering development in many wildlife species including amphibians, which are sensitive to these compounds. Producing toxicological information is the first step to evaluate the effects these chemicals may have on the environment and wildlife. Two synthetic phenolic antioxidants, 4,4'-thiobis(6-t-butyl-m-cresol) (CAS 96-69-5; TBBC) and 2,4-di-tert-butylphenol (CAS 96-76-4; DTBP) were chosen to evaluate their potential toxicity and developmental disruption on two amphibian species. Both, TBBC and DTBP, are present in many common products such as rubber and plastic products, they are listed on the National Chemicals Management Plan as potential EDCs and they are potentially toxic to aquatic life. Furthermore, given that their chemical structure resembles the thyroid hormones (TH), the present thesis hypothesis is that they interfere with TH-dependent developmental processes on the frogs *Silurana tropicalis* and *Lithobates pipiens* and affect their early stage development. To test this hypothesis, *S. tropicalis* embryos and *L. pipiens* tadpoles were exposed at the Nieuwkoop-Faber (NF) stage 9-10 and Gosner (G) stage 24-25, respectively, to these synthetic antioxidants' concentrations at several concentrations (0, 0.025, 0.05, 0.075, 0.1, 0.2 and 0.4 mg/l). The FETAX protocol was followed to determine the 96h lethal concentrations and sublethal effects, evaluating survival, growth and development. A chronic exposure on *S. tropicalis* was also conducted exposing tadpoles from stage NF47-48 to three sublethal TBBC concentrations (0, 0.002, 0.1 and 5 µg/l) for 7 weeks to evaluate effects on growth and metamorphosis. The TBBC 96h lethal and effective concentrations (LC_{50} and EC_{50}) were 0.076 and 0.078 mg/l, respectively, for *S. tropicalis.* For *L. pipiens* exposed to TBBC the LC_{50} was 0.17 mg/l and for those exposed to DTBP the lethal concentration was 0.52 mg/l. Acute exposure to all TBBC concentrations affected *S. tropicalis* growth and was lethal at 0.2 and 0.4 mg/l. The TBBC compound also affected *L. pipiens* body

size and was lethal at 0.4 mg/l. All *L. pipiens* tadpoles died at 0.8 mg/l of acute exposure to DTBP. Chronic exposure to sublethal concentrations of TBBC reduced body size by 8% at 5 µg/l and body mass by 17% at 0.002 µg/ when metamorphosis was completed. This study confirms these two synthetic antioxidants are toxic in vivo to these amphibian species. Also, TBBC induces malformations and inhibits tadpole growth at 0.025 mg/l after acute exposure and affects body mass of metamorphs at 0.002 µg/l after chronic exposure. These findings call for further investigations on how these synthetic antioxidants affect growth in *S. tropicalis* and *L. pipiens*.

Résumé

De nombreux produits chimiques contenus dans les produits ménagers et industriels couramment utilisés sont rejetés dans l'environnement, mais leurs effets toxiques sur les humains et la faune sont inconnus. Certains de ces produits chimiques se comportent comme des perturbateurs endocriniens (EDC), altérant le développement de nombreuses espèces sauvages, dont les amphibiens, qui sont sensibles à ces composés. La production d'informations toxicologiques est la première étape pour évaluer les effets que ces produits chimiques peuvent avoir sur l'environnement et la faune. Les antioxydants phénoliques synthétiques 4,4'-thiobis (6-t-butyl-m-crésol) (CAS 96-69-5; TBBC) et 2,4-di-tert-butylphénol (CAS 96-76-4; DTBP) ont été sélectionnés pour une évaluation de leur potentiel toxique et perturbateur endocrinien sur deux espèces d'amphibiens. Le TBBC et le DTBP sont des composés présents dans de nombreux produits d'usage quotidien, tels que les produits en caoutchouc et en plastique. Ces composés figurent dans Le Plan de Gestion de Produits Chimiques (PGPC) en tant que EDC potentiels et pourraient être toxiques pour la vie aquatique. Étant donné que leur structure chimique ressemble aux hormones thyroïdiennes (HT), j'ai émis l'hypothèse qu'elles interféreraient avec les processus de développement dépendants de la TH sur les grenouilles *Silurana tropicalis* et *Lithobates pipiens*, affectant potentiellement leur développement précoce. Des embryons de *S. tropicalis* au stade Nieuwkoop-Faber (NF) 9-10 et des embryons de *L. pipiens* au stade Gosner 24-25 ont été exposés à plusieurs concentrations d'antioxydants synthétiques (0; 0,025; 0,05; 0,075; 0,1; 0,2 et 0,4 mg / l) pour déterminer les concentrations létales et sublétales à 96 h en suivant le protocole FETAX et en évaluant leur survie, leur croissance et leur développement. Une exposition chronique de *S. tropicalis* a également été réalisée en exposant les têtards du stade NF 47-48 à trois concentrations sublétales de TBBC (0; 0,002; 0,1 et 5 µg / l) pendant 7 semaines pour évaluer les effets sur la croissance et la métamorphose. La concentration létale et effective de TBBC à 96 h étaient respectivement de 0,076 et 0,078 mg / l pour *S. tropicalis*. La CL50 pour *L. pipiens* était de 0,17

mg / l. La concentration létale de DTBP pour *L. pipiens* était de 0,52 mg / l. L'exposition à toutes les concentrations de TBBC a affecté la croissance de *S. tropicalis* et a été mortelle à 0,2 et 0,4 mg / l. Le TBBC a également affecté la taille corporelle de *L. pipiens* et s'est révélé mortel à 0,4 mg / l. Tous les têtards de *L. pipiens* sont morts à 0,8 mg / l de DTBP. Après une exposition chronique au TBBC, les têtards de S. tropicalis étaient 8% plus petits à la concentration la plus élevée (5 µg / l) et 17% de poids réduit à la concentration environnementale de 0,002 µg / l par rapport aux témoins lorsque la métamorphose était terminée. Cette étude révèle que ces deux antioxydants synthétiques sont toxiques pour ces amphibiens, le TBBC induit des malformations et inhibe la croissance des têtards après des expositions aiguës à 0,025 mg / l et affecte le poids des métamorphes à 0,002 µg / l. Il s'agit de la première évaluation de la toxicité de ces antioxydants synthétiques largement utilisés sur les amphibiens et elle soutiendra directement la troisième phase du PGPC.

Content

Acknowledgements ii

Abstract iii

Résumé v

Content vii

List of tables ix

List of figures x

Abbreviations xii

Chapter 1 1

General introduction 1

1. Introduction 1

1.1 Amphibian metamorphosis 1

1.2 Exposure to contaminants on amphibian development 3

1.3 Synthetic phenolic antioxidants 3

1.4 The 4,4'-isopropylidenediphenol (BPA) 7

1.5 Study species 7

1.6 Significance and objectives 8

Chapter 2 10

Acute developmental effects of the synthetic antioxidants 4,4-thiobis (6-t-butyl-m-cresol) and 2,4-di-tert-butylphenol on *Silurana tropicalis* and *Lithobates pipiens* 10

2.1 Introduction 10

2.2 Methods 12

2.2.1 Animal husbandry 13

2.2.2 Acute 96h exposure test 13

2.2.3 Chemical analysis 14

2.2.4 Statistical analysis 15

2.3 Results 16

2.3.1 Effects of TBBC acute exposure on early development of S. tropicalis 16

2.3.2 Effects of TBBC acute exposure on early development of L. pipiens 22

2.3.3 Effects of DTBP acute exposure on early development of L. pipiens 26

2.4 Discussion 29

Chapter 3 33

Chronic exposure to 4, 4-thiobis (6-t-butyl-m-cresol; TBBC) affects development of *Silurana tropicalis tadpoles* 33

3.1 Introduction 33

3.2 Methods 35

3.2.1 TBBC chronic exposure 35

3.2.2 Chemical analysis 36

3.2.3 Statistical analysis 36

3.3 Results 37

3.4 Discussion 42

Chapter 4 47

General conclusions 47

5. References 49

List of tables

Table 1. Effects of acute TBBC exposure on abnormalities (head, tail, edemas, gut and heart malformations) in *Silurana tropicalis* tadpoles……..……..18

Table 2. Effects of acute TBBC exposure on body size (snout to vent (SVL), tail length (TaL), and total length (TL)) in *Silurana tropicalis* tadpoles…….……..…...…21

Table 3. Effects of acute TBBC exposure on body size (snout to vent (SVL), tail length (TaL), and total length (TL)) in *Lithobates pipiens* tadpoles in clutch 1 and clutch 2……..…….23

Table 4. Effects of acute TBBC exposure on body size (snout to vent (SVL), tail length (TaL), and total length (TL)) in *Lithobates pipiens* tadpoles……..…28

Table 5. Lethal concentration (LC_{50}) comparison between TBBC and DTBP to other potential endocrine disruptors in amphibians and /or fish……..……....30

Table 6. Effects of chronic TBBC exposure on tadpole survival rates in *Silurana tropicalis*…...38

Table 7. Effects of chronic TBBC exposure on time (days) to metamorphosis in *Silurana tropicalis* NF66 juveniles……..……..…38

Table 8. Effects of chronic TBBC exposure on body size (snout to vent: SVL) and mass at metamorphic climax (NF stages 58-62) in *Silurana tropicalis*…….……..……...41

Table 9. Effects of chronic TBBC exposure on body size (snout to vent: SVL) and mass at the end of metamorphosis (NF66) in *Silurana tropicalis*……..…….……..…41

List of figures

Figure 1. Chemical structure of a) 4, 4-thiobis (6-t-butyl-m-cresol; TBBC; CAS 95 69 5); b) 2,4-di-tert-butylphenol (DTBP; CAS 96 76 4); c) Bisphenol A 4,4'-isopropylidenediphenol (BPA; CAS 80 05 7); and d) Triiodothyronine (T3)……..…6

Figure 2. Dose-response curve after 96h exposure to TBBC (CAS 96 69 5) on *Silurana tropicalis* tadpoles. a) Survival and b) abnormalities, per TBBC log transformed (+1) concentrations……..…….17

Figure 3. Teratogenic effects of 96h acute TBBC exposure on the proportion of a) head, b) tail, and c) edema abnormalities in *Silurana tropicalis* tadpoles. Pictures show tadpoles with abnormalities at different TBBC concentrations and a control tadpole with normal development……..……19

Figure 4. Effect of 96h acute TBBC exposure on a) snout to vent: SVL, b) tail length: TaL, and c) total length: TL, in *Silurana tropicali*s tadpoles……..…..20

Figure 5. Survival dose-response curve after 96h exposure of TBBC on *Lithobates pipiens* tadpoles. TBBC concentrations were log transformed (+1)……..……23

Figure 6. Effects of 96h acute TBBC exposure on a) and d) snout to vent: SVL, b) and e) tail length: TaL, and c) and f) total length: TL, in *Lithobates pipiens* tadpoles for clutch 1 and 2, respectively……..……..……25

Figure 7. Survival dose-response curve after 96h exposure of DTBP (CAS 96 76 4) on *Lithobates pipiens* tadpoles. DTBP concentrations were log transformed (+1). Black circles are the mean of five replicates per concentration with standard error bars……..……26

Figure 8. Effect of 96h acute DTBP exposure on a) snout to vent: SVL, b) tail length: TaL, and c) total length: TL, in *Lithobates pipiens* tadpoles……..……..……27

Figure 9. Effects of chronic TBBC exposure on the proportion of tadpoles in each developmental group (premetamorphosis NF46-55, prometamorphosis NF56-57, metamorphic

climax NF58-62, reaching metamorphosis NF63-65, end of metamorphosis NF66) after 7 weeks of exposure……..…….39

Figure 10. Effects of chronic TBBC exposure on a) snout to vent length (SVL) and b) body mass, at metamorphic climax in *Silurana tropicalis* tadpoles……....40

Figure 11. Effects of chronic TBBC exposure on a) snout to vent length (SVL) and b) body mass, in *Silurana tropicalis* metamorphs (NF66)…….……..……..……40

Abbreviations

ae - Acid equivalent

ai - Active ingredient

AO2246 - 6-tertbutyl- 4-methylphenol

AR - Androgen receptor

BHA – Butylated hydroxyanisole

BHT - Butylated hydroxytoluene

BPA - Bisphenol A

CCAC – Canadian Council on Animal Care

CCIW – Canadian Centre for Inland Waters

CMP - Chemicals Management Plan

COSEWIC – Committee on the Status of Endangered Wildlife in Canada

CRF – Corticotropin-releasing factor

dio - Deiodinase

DMSO – Dimethyl sulfoxide

DTBP - 2,4-di-tert-butylphenol

EC_{50} - Effective concentration

ECHA – European Chemical

EDC - Endocrine disrupting chemical

EPA – Environmental Protection Agency

ER - Estrogen receptor

FETAX - Frog Embryo Teratogenesis Assay-Xenopus

G – Gosner stage

GLM – General linear model

IU - International units

LC_{50} - Lethal concentration

MCIG – Minimum concentration to inhibit growth

MS-222 - Tricaine Methanesulfonate

NF - Nieuwkoop-Faber stage

NP – Nonylphenol

NP12 – Nonylphenol 12

NTP – National Toxicology Program

PFAS – Perfluoroalkyl substances

PFOA – Perfluorooctanoic acid

PFOS – Perfluorooctanesulfonic acid

SP – Sodium perchlorate

SPA - Synthetic phenolic antioxidant

SVL – Snout to vent length

TaL – Tail length

TBBC - 4,4'-thiobis(6-t-butyl-m-cresol)

TCS - Triclosan

TH - Thyroid hormone

TI – Teratogenic index

TL -Total length

TR – Thyroid hormone receptor

TSH – Thyrotropin stimulating hormone

TSHR – Thyrotropic stimulating hormone receptor

T3 – 3,3',5-triiodothyronine (or Triiodothyronine)

T4 – 3,5,3',5-tetraiodothyronine (or Thyroxine)

4NP – 4 nonylphenol

4-tOP – 4-tert-octyphenol

Chapter 1

General introduction

1. Introduction

Industrial, commercial and personal products of daily use contain numerous chemicals, many of which are being released into the environment (Carr and Patiño 2011; Kabir *et al*. 2015). Exposure to these contaminants is considered an important factor in the decline of amphibian populations (Hayes *et al*. 2010; Egea-Serrano *et al.* 2012). Amphibians have a crucial role in an ecosystem, where during their different life stages, they consume large amounts of insects. Amphibians, in turn, are also consumed by many species of arthropods, fish, reptiles, birds and mammals; hence, impacts on amphibian populations in the ecosystem may also affect other species (McDiarmid and Mitchel 2000; Murphy *et al*. 2000). The permeable skin of amphibians makes them very sensitive to environmental contamination as they can easily absorb chemicals, thereby increasing their risk of exposure (Murphy *et al.* 2000; Miyata and Ose 2012). Endocrine disrupting chemicals (EDCs) are chemicals able to change the normal hormone functioning in an organism. These chemicals may affect amphibian metamorphosis and sex differentiation by disrupting thyroid hormones (THs) and estrogens (Carr and Patiño 2011; Kabir *et al*. 2015). Endocrine disruptors may also induce changes in development and survival, thereby affecting population dynamics of amphibians (Heimeier and Shi 2010; Miyata and Ose 2012; Rosenfeld *et al*. 2017).

1.1 Amphibian metamorphosis

Normally, amphibian metamorphosis is controlled by the hypothalamus-pituitary-thyroid (HPT) axis, in which environmental and nutritional factors stimulate key neuroendocrine pathways in the central nervous system (Fort *et al*. 2007). This stimulus produces corticotropin-releasing factor (CRF) in the hypothalamus, which stimulates the secretion of thyrotropin

stimulating hormone (TSH) from the pituitary (Rosenfeld *et al*. 2017). In turn, TSH binds to the TSH receptor (TSHR) in the thyroid gland; in the nucleus of the cell, various thyroid genes are activated by TSH promoting the synthesis of thyroid hormones (TH) by iodination of thyroglobulin (Buchholz *et al*. 2006; Sachs and Buchholz 2017).

There are two types of THs that are released into the blood: 3,5,3',5-tetraiodothyronine (or thyroxine; T4) and 3,3',5-triiodothyronine (or triiodothyronine; T3). In the blood, they are transported mainly by thyroxine-binding globulin and to a lesser extent by transthyretin, but there are also free THs that are active, and these are the ones taken by the target cells (Becker *et al*. 1997; Brown and Cai 2007). Generally, T4 is the main TH released, and subsequently is converted by type II deiodinase (dio2) into the more active T3, by the removal of one iodine atom at 5' position in the aromatic ring of T4. This deionization takes place in the cytoplasm of the target cells. The type III deiodinase (dio3) deactivates T4 and T3 converting them into reverse T3 by removing the iodine atom in position 5 in the aromatic ring. Therefore, dio3 regulates THs intracellular activity when levels are high (Becker *et al*. 1997). After T4 is converted to T3, T3 will bind to thyroid hormone nuclear receptors (TRα and TRβ) inducing heterodimerization and regulating gene expression in the target genes that are involved in metamorphosis (Buchholz *et al*. 2006; Rosenfeld *et al*. 2017; Sachs and Buchholz 2017). This receptor-ligand interaction is very important in the positive-negative regulation of TH-responsive genes involved in metamorphosis (Buchholz *et al*. 2006; Sachs and Buchholz 2017). Responding to TH levels during metamorphosis, amphibian limbs start developing, intestine and skin are remodeled into the adult version, and the tail is completely resorbed when metamorphosis is completed (Buchholz *et al*. 2006). The TRα regulates early development while TRβ is responsible for late metamorphosis changes such as tail resorption (Becker *et al*. 1997; Buchholz 2006; Brown and Cai 2007).

1.2 Exposure to contaminants on amphibian development

Many studies have evaluated the effects of chemical exposures on amphibian metamorphosis, growth, malformations and mortality with different contaminants. For example, the glyphosate-based herbicides exposure to the wood frog tadpoles, *Lithobates sylvaticus,* have increased mortality and decreased growth at metamorphic climax (Lanctôt *et al*. 2014) and have delayed metamorphosis (Navarro-Martin *et al.* 2014). Triclosan, a polychloro phenoxy phenol with a double ring (ECHA 2020); it is a common antibacterial used in hand soap, toothpaste and other consumer products, has been shown to delay metamorphosis of the Pacific tree frog, *Pseudacris regilla* (Marlatt *et al.* 2013) and induce a pre-diabetic state in exposed *Silurana tropicalis* (Regnault *et al*. 2018). Naphthenic acid mixtures are chemicals from oil sand extractions but are also used as preservatives in wood and fabrics (Brown and Ulrich 2015). Exposure to these mixtures increased the mortality in *L. sylvaticus* tadpoles (Melvin and Trudeau 2012) and causes abnormalities in *S. tropicalis* (Gutierrez-Villagomez *et al.* 2019; Melvin and Trudeau 2012b). The adverse outcomes in these studies show that amphibians can be useful study species to determine the potential risk of exposure to chemicals in the aquatic environment.

1.3 Synthetic phenolic antioxidants

Synthetic phenolic antioxidants (SPAs) are additives used to protect plastic and rubber products against oxidation (De la Rie 1988; Rodil *et al*. 2010). One class of SPAs are the hindered phenols which have a phenolic group with at least one bulky group and are considered radical scavengers (De la Rie 1988). They are added to substances that deteriorate due to autoxidation, in which free radicals are formed. Then, hindered phenols interfere in this process, reacting with free radicals such as peroxy (ROO) and alkoxy (RO) to form phenoxy radicals which are more stable (De la Rie 1988). In general, SPAs are present in fuels, lubricants,

adhesives, cosmetics and food products (ECHA 2018). Little is known about the environmental impact of these widely used compounds and basic toxicity information is needed to evaluate the potential risk SPAs might have in nature (Lu *et al*. 2019).

1.3.1 The 4,4-thiobis (6-t-butyl-m-cresol; TBBC)

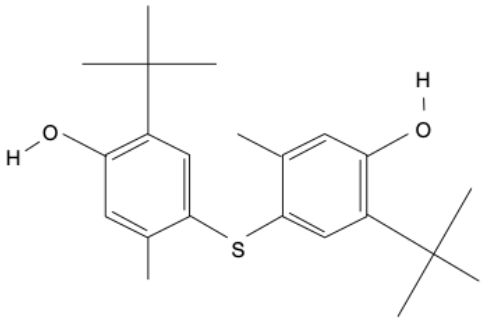
The chemical 4,4-thiobis(6-t-butyl-m-cresol; TBBC; CAS 96 69 5; Fig 1a), is a hindered phenol used in plastic and rubber products such as construction materials, laboratory chemicals, adhesives, sealants, polymers, furniture, computers, cameras, lamps, refrigerators, footwear, paper, cardboard, toys, food packaging and storage containers (NTP 1994; ECHA 2018). The TBBC is a fine, white and crystalline powder with a molecular weight of 358.52, a melting point of 161º C, log P of 5.24, and molecular formula $C_{22}H_{30}O_2S$ (NTP 1994; ECHA 2018). This compound is regarded as a highly produced compound by the European Chemicals Authority (ECHA 2018), with an annual production estimated between 1 000 to 10 000 tons per year in the European Economic Area. In the United States, TBBC production or import is estimated between 500 000 to less than 1 million pounds per year according to the EPA (2010). In Canada, it has been detected in wastewater treatment plants at low concentrations, from non-detected to 8.8 ng/l in wastewater treatment influents, up to 1.6 ng/l in effluents, and up to 8.4 ng/g dry weight in the biosolids (Lu *et al*. 2019). The half-life of TBBC in the environment is reported to be 120 days in soil, 540 days in sediment and 60 days in water, suggesting potential persistence in the environment (Lu *et al*. 2019). This compound is potentially bioaccumulative and with an estimated bioaccumulation factor of 2.5 and is a suspected developmental toxicant (Lu *et al*. 2019). Bioaccumulation together with bioconcentration refer to chemical accumulation from the environment into the organism. While bioaccumulation can occur from sediment exposure or food transfer, bioconcentration refers to the accumulation of contaminants through nondietary routes, mainly respiration and for amphibians through their permeable skin as well (Barron 2003). For tadpoles, bioconcentration might happen when exposed to contaminants in water and transfer through their skin and/or gills (Katagi and Ose 2014). While bioaccumulation

might also happen if microscopic algae is already contaminated with mainly hydrophobic chemicals and tadpoles feeding on these algae (Katagi and Ose 2014).

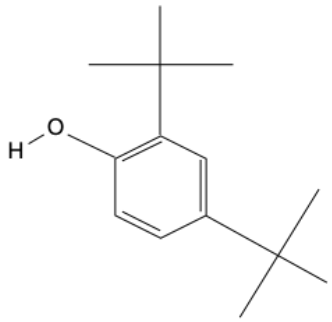
The compound, TBBC, is already identified as very toxic to aquatic life (ECHA 2018). It has been found to cause stomach irritation in rats (Birnbaum *et al*. 1983; NTP 1994), accumulate in liver and adipose tissue of rats and mice (Birnbaum *et al*. 1983; NTP 1994; Munson *et al.* 1988; Takahashi and Oishi 2006), and in the kidneys (NTP 1994) and bone marrow of mice (Munson *et al.* 1988). The ECHA (2018) cautions that TBBC may cause allergic skin reaction and serious eye irritation. In people, Rich *et al.* (1991) found positive test reactions to this compound in two cases of dermatitis linked to the use of latex gloves. Takahashi and Oishi (2006) examined testicular toxicity and estrogenic activity in mice and rats and found that TBBC affected body weight, reproductive accessory organs and liver weight and inhibited the hormone binding of estradiol to the estrogen receptor α in males. After comparing several compounds, it was concluded that TBBC may have even higher risks for humans and the environment than bisphenol A (BPA) because TBBC is slowly absorbed and accumulated in adipose tissue (Takashi and Oishi 2006). Using several types of *in vitro* assays, Satoh *et al*. (2008) found that TBBC had strong androgen receptor (AR) and estrogen receptor (ER) antagonist activity. The toxicity information of TBBC is currently limited to mammal models, there are no studies to date analyzing the potential risk this chemical might pose to aquatic organisms.

1.3.2 The 2,4-di-tert-butylphenol (DTBP)

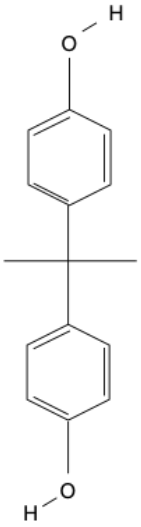
Another hindered phenol, 2,4-di-tert-butylphenol (DTBP; CAS 96 76 4; Fig 1b), is a solid and white phenolic compound (ECHA 2018). It is used in fuel, fuel additives and blends, plastic and rubber additives, and in the production of a variety of other substances (cooling liquids for refrigerators, hydraulic liquids in automotive suspension, lubricants and break liquids; ECHA 2018). The molecular formula of this hindered phenol is $C_{14}H_{22}O$ with a molecular weight of 206.32, a melting point of 56.8º C and a log P of 4.8 (ECHA 2018). The compound DTBP is



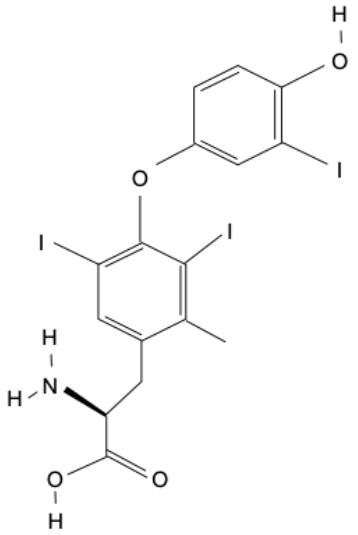
a) 4, 4-thiobis (6-t-butyl-m-cresol) (TBBC)



b) 2,4-di-tert-butylphenol (DTBP)



c) Bisphenol A 4,4'-isopropylidenediphenol (BPA)



d) Triiodothyronine (T3)

Figure 1. Chemical structure of a) 4,4-thiobis (6-t-butyl-m-cresol; TBBC; CAS 96 69 5); b) 2,4-di-tert-butylphenol (DTBP; CAS 96 76 4); c) Bisphenol A 4,4'-isopropylidenediphenol (BPA; CAS 80 05 7); and d) 3,3',5-Triiodothyronine (T3).

considered very toxic to aquatic life and can cause serious eye, skin and respiratory irritation (ECHA 2018). In laboratory investigations, exposure of DTBP in food caused hepatic and renal toxicity in newborn and young rats and decreased their weight (Hirata-Koizumi *et al*. 2005). This compound was found to have AR antagonist activity using *in vitro* assays (Satoh *et al*. 2008). The exposure of DTBP might occur from drinking water that was in contact with plastic piping, inhalation of house dust, or when using domestic electronic products (ECHA 2018), hence, there is higher potential for exposure in humans.

1.4 The 4,4'-isopropylidenediphenol (BPA)

The double ring structure of TBBC (Fig 1a,b) resembles the chemical structure of the THs (Fig 1c). This characteristic is shared with other compounds such as bisphenol A, 4,4'-isopropylidenediphenol (BPA; CAS 80 05 7; Fig 1d), which is known to disrupt hormone pathways in humans (Cantonwine *et al*. 2013) and wildlife (Oehlmann *et al*. 2009). This compound is commonly used in the production of plastics and epoxy resins, where it is found in consumer products such as furniture, toys, electronics, food packaging, appliances and construction material, and there is a risk that this chemical is being released into the environment (ECHA 2018). Bisphenol A is a known endocrine disruptor, it is estrogenic in *Xenopus laevis* (Trudeau *et al*. 2005), and particularly in *S. tropicalis*, BPA at 0.22 mg/l may inhibit metamorphosis (Goto *et al*. 2006). Bisphenol A is used in this thesis as a positive control.

1.5 Study species

Two amphibian species were used in this thesis, a lab model, the western clawed frog *Silurana (Xenopus) tropicalis,* and a Canadian native species, the northern leopard frog, *Lithobates pipiens*. The first study species, *S. tropicalis,* is a fully aquatic species that produces a large number of eggs, with tadpoles large enough to easily evaluate the development between embryos (Amaya *et al.* 1998; Khokha *et al*. 2002). It is recognized as a model species for studies on EDCs (Berg *et al*. 2009), and the Trudeau lab has one of the few *S. tropicalis*

colonies in Canada (Duarte-Guterman and Trudeau 2011). The *S. tropicalis* is the main lab model species for the experiments carried out in this thesis. The second amphibian species used in this project, the northern leopard frog *L. pipiens,* is a local species with wide distribution in Canada, but some local populations have been in decline since the 1970s, especially in the Rocky Mountains (COSEWIC 2009). *L. pipiens* is gaining interest for endocrine disruption studies because this species is sensitive enough for toxicological testing, and results expand our knowledge of how local species and populations can be affected by environmental chemical exposures (Langlois *et al*. 2010; Selcer and Verbanic 2014). By testing both, a lab model and local amphibian species, it will provide a more encompassing range of lethal concentrations for the chemicals being tested, identify sublethal effects and compare species sensitivity that can lead to the development of reliable data for chemicals management.

1.6 Significance and objectives

The two SPAs listed in this project are produced or imported to Canada in large quantities, they are identified as potential EDCs and are on the Government of Canada's Chemicals Management Plan (CMP3) priority list to determine the potential risk they may present to the environment, wildlife and humans. Only one known study has detected TBBC in the environment from non-detected to 1.6 ng/l, whereas DTBP was detected at higher concentrations (276 ng/l) in the effluent of wastewater treatment plants in Canada (Lu *et al*. 2019). The ECHA (2018) has also recommended more investigations into the releases of these chemicals into the water from manufacturing and industrial areas. Evaluating basic toxicity and identifying potential adverse effects in key model organisms is an important step in environmental risk assessments and in drafting regulations to minimize wildlife and human exposure (Miyata and Ose 2012; Scholz *et al.* 2013). It is important to know the potential hazard these substances might have to aquatic organisms and the environment. The overall goal of this thesis was to determine the lethal concentrations and the sublethal developmental effects of 4,4-thiobis(6-t-butyl-m-cresol) and the 2,4-di-*tert*-butylphenol on two amphibian species. Based

on the similarity these two SPAs have with chemical structure of the thyroid hormone, especially TBBC with its two phenolic rings, the present thesis hypothesis is that acute exposure to TBBC and DTBP will affect embryonic development and survival of *S. tropicalis* and *L. pipiens.* Secondly, it is hypothesized that chronic and sublethal TBBC exposure on *S. tropicalis* will affect growth and metamorphosis. This thesis is the first study evaluating basic toxicity knowledge of TBBC and DTBP on amphibians.

Chapter 2

Acute developmental effects of the synthetic antioxidants 4,4-thiobis (6-t-butyl-m-cresol) and 2,4-di-tert-butylphenol on *Silurana tropicalis* and *Lithobates pipiens*

2.1 Introduction

The widespread use and persistence of many chemicals are impacting the environment and biodiversity (Carr and Patiño 2011; Kabir *et al*. 2015). Many of these chemicals are synthetic phenolic antioxidants (SPAs), a group of chemicals used to extend the life of industrial and commercial products (De la Rie 1988). The chemicals TBBC and DTBP are both produced in large volumes (1000 to 10 000 and 100 to 1000 tons per year, respectively) and used worldwide (ECHA 2018). The TBBC compound is present in plastic and rubber products of daily use, and DTBP is used in fuel and intermediate products (ECHA 2018). The TBBC has two phenolic groups in its chemical structure that resembles the TH structure and is suspected to potentially affect endocrine systems in wildlife. They are also on the Government of Canada's CMP3 priority list which determines the potential risk these and other chemicals may present to the environment. Both, TBBC and DTBP are potentially persistent in the environment and predicted to be developmental toxicants (Lu *et al*. 2019).

The adverse effects of some SPAs and other industrial and commercial chemicals have previously been investigated in aquatic organisms (Hogan *et al*. 2006; Yang *et al*. 2018). However, for TBBC and DTBP the basic toxicity knowledge is limited to lab rodent species, while knowledge on aquatic organisms is still lacking. Chemical exposure has been considered an important factor in the decline of amphibian populations (Egea-Serrano *et al.* 2012; Hayes *et al*. 2010), potentially affecting populations of other species in the environment that consume amphibians (McDiarmid and Mitchel 2000; Murphy *et al*. 2000).

Basic toxicity data should be generated for chemicals before the chemicals are produced in high amounts and widely used in common products. The lethal concentration or LC_{50} is a standard measurement used to compare the toxicity of different compounds and the FETAX guidelines (FETAX 2004) are standardized guidelines to produce this information for chemical management. Chemicals might elicit different toxicities for different species. For example, two commercial naphthenic acids have an estimated LC_{50} varying slightly between species where the wood frogs (*Lithobates sylvaticus*) LC_{50} range was 3.04 and 4.76 mg/l (Melvin and Trudeau 2012), while for *Silurana tropicalis* it was 10.4 and 11.7 mg/l (Gutierrez-Villagomez *et al*. 2019). Furthermore, similar chemicals may have differing toxicity on the same species, for example, Yang *et al*. (2018) found different toxicities in four common synthetic antioxidants on the development of zebrafish (*Danio rerio*). For the larvae of *D. rerio*, the compound 6-tertbutyl 4-methylphenol (AO2246), a SPA also used in rubber and plastic products, was the most toxic (LC_{50} of 1.7 mg/l), whereas other compounds used in foodstuffs such as tert-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were considered to be less toxic (9.2, 17.9 and >44 mg/l, respectively). Other commonly used chemicals are pesticides, which have also shown different toxicity levels depending on their formulations. Roundup Ultramax® showed low toxicity with an LC_{50} of 128.2 and 25.8 mg active ingredient (ai)/l in *Discoglossus pictus* and *X. laevis,* respectively (Wagner *et al*. 2017). In the case of Roundup Original® formulation and for the surfactant MON0818 (one of its components), the LC_{50} estimates for *L. pipiens* were 1.8 mg acid equivalent (ae)/l and 0.68 mg/l, respectively (Moore *et al*. 2012). The LC_{50} value provide toxicity comparisons between chemicals for subsequent chemical management.

Bisphenol A (BPA) was used in this study as a positive control, because it is a widely used plasticizer, and it is considered to have moderate toxicity with LC_{50}s ranging between 2 to 8 mg/l in aquatic organisms (Mai *et al*. 2016; Chow *et al*. 2013; Arancio *et al*. 2019). For example, the LC_{50} of BPA on *D. rerio* was 6.6 mg/l (Mai *et al*. 2016) and 8.04 mg/l (Chow *et al*. 2013), and for *X. laevis* the LC_{50} was 2.4 mg/l at the stage NF2 of development (Arancio *et al*.

2019). In our lab, the BPA 96h lethal concentration for *S. tropicalis* exposed at NF9-10 was 7.7 mg/l. Bisphenol A is a well known EDC, however, there is no known LC_{50} reported for *S. tropicalis*.

The lethal concentration and embryonic developmental toxicity for TBBC and DTBP on amphibians are not known. Considering their large production volume, their usage in many common products and their similarity with the TH structure, there is a need for basic ecotoxicological information and investigation into their potential effects on wildlife. In this chapter, the lethal concentration and developmental toxicity effects of TBBC and DTBP were investigated by evaluating morphological responses after acute exposure to these compounds in two amphibian species during embryonic development. The objective of this chapter was to determine the lethal concentration and the embryonic developmental toxicity effects of these two SPAs on *S. tropicalis* and *L. pipiens*.

2.2 Methods

To evaluate the toxicity of TBBC and DTBP, preliminary range-finding experiments were conducted to establish definitive exposure concentrations following the same experimental design used for the definitive lethal tests described below. Once concentrations were established, acute lethality tests were carried out to determine the immediate (acute) toxic effects of these two synthetic antioxidants on *S. tropicalis* and *L. pipiens* tadpoles. The 96h LC_{50} toxicity tests followed the established Frog Embryo Teratogenesis Assay Xenopus (FETAX) protocol guidelines (FETAX 2004). This is a protocol to evaluate developmental toxicity in amphibians by analyzing mortality, malformations and growth in larvae after 96h of exposure. Other aspects of the protocol are the procedures for breeding and rearing conditions, cleaning material, range finding assessment to define the range of concentrations for the definitive test, developmental stage of the embryo to start exposure, renewal of solution every 24h,

temperature and pH of solutions. After 96h, surviving tadpoles are photographed under a microscope to analyze malformations and growth (FETAX 2004).

2.2.1 Animal husbandry

Male and female adult *S. tropicalis* (golden strain) frogs were injected into the dorsal lymph sac with a priming dose of 12.5 international units (IU) keeping them separated for 24 h, and then each frog was injected with a 150 IU boosting dose of human chorionic gonadotropin hormone to induce spawning. After priming and boosting injections, frogs were placed in glass tanks with FETAX solution at a temperature of 25±1ºC with a 12h dark cycle. After deposition, healthy embryos with normal development were selected to proceed with the exposure (FETAX 2004). Adult *L. pipiens* were collected during the spring migration (2019) near Bishop's Mills, Ontario (44.87366 –75.70455). They were induced to breed in captivity following our established protocol (see Trudeau *et al*. 2013). Eggs and embryos were reared at room temperature for about one week until tadpoles reached Gosner stage 24-25, when feeding and exposure to chemicals started. Mortality during rearing time was 0%.

2.2.2 Acute 96h exposure test

Ten *S. tropicalis* embryos (NF, stage 9-10; Nieuwkoop-Faber 1994) were placed in a petri dish per replicate, with five replicates per treatment and eight nominal treatment concentrations: control (FETAX solution, 0 mg/l), solvent control (dimethyl sulfoxide, DMSO at 20 µl/l or 0.002%), positive control (BPA at 5 mg/l), and five TBBC concentrations (0.4, 0.2, 0.1, 0.05 and 0.025 mg/l). Each petri dish was randomly placed in a chamber where the temperature was maintained at 26±1ºC and a 12h dark cycle following FETAX (2004) protocol. This experiment was repeated, with an additional TBBC nominal concentration of 0.075 mg/l. For *L. pipiens*, at Gosner (G) stage 24-25 (Gosner 1960), ten embryos were added to one litre mason jars with 900 ml of dechlorinated tap water and placed in a 21°C chamber with a 12h dark cycle for 96h. Tadpoles were fed with Sera Micron® once a day after each 24h water change. Five replicates per treatment and eight nominal treatment concentrations were used (water control (0

mg/l), solvent control (DMSO 0.002 %), positive control (12 mg/l BPA) and TBBC concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4 mg/l). This experiment was repeated with TBBC concentrations of 0.1, 0.13, 0.17, 0.2 and 0.24 mg/l. For DTBP exposures, the nominal concentrations tested were 0.2, 0.4, 0.6, 0.8 and 1 mg/l and this experiment was conducted simultaneously following the same experimental design as for the TBBC compound.

For all experiments with both species, every 24h survival was analyzed, dead embryos were removed, and the treatment solution was renewed with fresh solution. Water samples of the stock solutions and replicate samples were collected and analyzed to confirm exposure concentrations at the beginning of the 24h and 72h exposure periods. After four water changes (96h), the tadpoles were euthanized with Tricaine Methanesulfonate (MS-222). *S. tropicalis* tadpoles were photographed both dorsally and ventrally using a light stereomicroscope (Nikon SMZ 1500) with a Nikon DS-Fi1 camera and NIS Elements version 3.22.00 software. *L. pipiens* tadpoles were photographed on the dorsal side only using a Google Pixel 2 XL smartphone camera. With these photographs, body measurements, including snout to vent length (SVL), body length (TL) and tail length (TaL) were measured using ImageJ 1.52a software. The Atlas of Abnormalities (Bantle 1991) was used to determine abnormalities, where a tadpole was considered affected if at least one abnormality was evident. The frequency of abnormalities was used to calculate the EC_{50}. To measure developmental hazard, the Teratogenic Index (TI) was calculated by the ratio of survival (LC_{50}) and malformation (EC_{50}), $TI = LC_{50}/EC_{50}$ (FETAX 2004). Experiments were carried out at the University of Ottawa in accordance with the Canadian Council on Animal Care (CCAC).

2.2.3 Chemical analysis

Water samples were analyzed with an ultra-performance liquid chromatography tandem mass spectrometer (UPLC-MS-MS), which consisted of a Waters Acquity LC coupled to Water Xevo TQS triple quadrupole mass spectrometer (Lu *et al*. 2019). The method detection limit (MDL) for TBBC corresponded to 0.006 ng/ml. The average ± standard deviation recovery on

spiked samples was 101 ± 0.87%. The relative standard deviation corresponding to the analysis of three replicates was 11%. The MDL for DTBP corresponded to 8.5 ng/ml. The average recovery on spiked samples was 87 - 89%. The target analyte DTBP was quantified using a 14-level calibration curve ranging from 0.030 to 500 ng/ml DTBP. Sample analysis was conducted by the Environment and Climate Change laboratory of Dr. Amila De Silva at the Canadian Centre for Inland Waters (CCIW), in Burlington, Ontario.

2.2.4 Statistical analysis

To calculate LC_{50} and EC_{50}, survival and abnormalities ratios were normalized, and concentrations were log transformed to perform the nonlinear regression using Prism-Graph program. All other statistical analyses were calculated using the R Statistical Software (R Core Team 2019) version 3.6.1. Survival and abnormality rate were assessed using General Linear Models with binomial distribution. To confirm that the solvent, DMSO, had negligible effects on survival or the occurrence of abnormalities, survival and abnormalities were compared between control and solvent control treatments using a t-test when data followed a normal distribution and homogeneity of variances. When assumptions were not met, the Wilcox-test was used. To determine if clutches were similar between experiments and thus could be combined for further analyses, an independent t-test was run to determine if there were any differences in all body size measurements between clutch 1 and clutch 2 using the solvent control treatments only. Differences in survival, abnormalities and body size between solvent control and positive control were analyzed using a t-test or Wilcox-test depending on distribution and variance homogeneity. To determine if there were any differences in size between solvent control treatments and the test compound treatments for both species, General Linear Models (GLM) were used and the α value was set at 0.05. Model fit was analyzed by plotting residuals vs fitted values to verify homogeneity, a histogram of residuals was used to assess normality and residual vs Treatment was plotted to check independence (Zuur *et al*. 2009).

2.3 Results

2.3.1 Effects of TBBC acute exposure on early development of S. tropicalis

2.3.1.1 TBBC lethal and effective concentrations

The *S. tropicalis* embryos were exposed for 96h to the synthetic antioxidant TBBC. Survival in control and solvent control tadpoles in two clutches was over 90% and there was no difference between the two clutches (t= -0.80, df= 18, p= 0.431). Analyzing the two clutches together, survival and abnormalities rate were not different between control and solvent control (t= 0.26, df= 18, p= 0.794; and t= 1.8, df = 18, p= 0.089, respectively). Therefore, the following analyses were completed using only the solvent control and hereafter referred to as control (Green 2014). After 96h of TBBC exposure of *S. tropicalis* embryos, the average 96h LC_{50} for two clutches was 0.076 mg/l. For clutch 1 it was at 0.081 (log 0.033, R_2= 0.90) and for clutch 2 it was 0.071 mg/l (log 0.029, R_2= 0.84; Fig 2a). Survival for the lowest concentration (0.025 mg/l) was 82%, for the second lowest concentration at 0.05 mg/l survival was 70%, but at 0.075 mg/l survival decreased to 58% and 28% at 0.1 mg/l. Tadpoles from the two highest concentrations (0.4 and 0.2 mg/l) were all dead at 96h. The TBBC EC_{50} values where at least one abnormality was found in *S. tropicalis* tadpoles, was 0.078 mg/l. For clutch 1 the EC50 was 0.093 mg/l (log 16.53, R_2= 0.31) and clutch 2 it was 0.064 mg/l (log 25.77, R_2= 0.54); Fig 2b). The teratogenic index (TI= LC_{50}/EC_{50}) was 0.98.

2.3.1.2 TBBC effects on abnormalities and growth

The abnormalities found in the developing tadpoles exposed to TBBC were tail, head, edemas, gut and heart malformations, though gut and heart malformations were not significantly more frequent than in the controls (Table 1). Head, tail and edema abnormalities were more frequent at the three highest TBBC treatments (0.05, 0.075 and 0.1 mg/l, Fig 3), including the positive control BPA (5 mg/l, Fig 3; Table 1). There was no difference in the size between the two clutches in the controls (SVL, t=1.36, df=88, p=0.178; Total length W= 1 170, *p*= 0.204 ;

Tail length t= 1.29, df= 80 p= 0.199), thus, size was analyzed with the two clutches combined. Exposure to TBBC significantly decreased all body size measurements in surviving tadpoles compared to controls (Fig 4a-c; Table 2). As TBBC concentration increased tadpoles were smaller in the three body measurements (Table 2). The minimum concentration to inhibit growth (MCIG) was 0.025 mg/L TBBC.

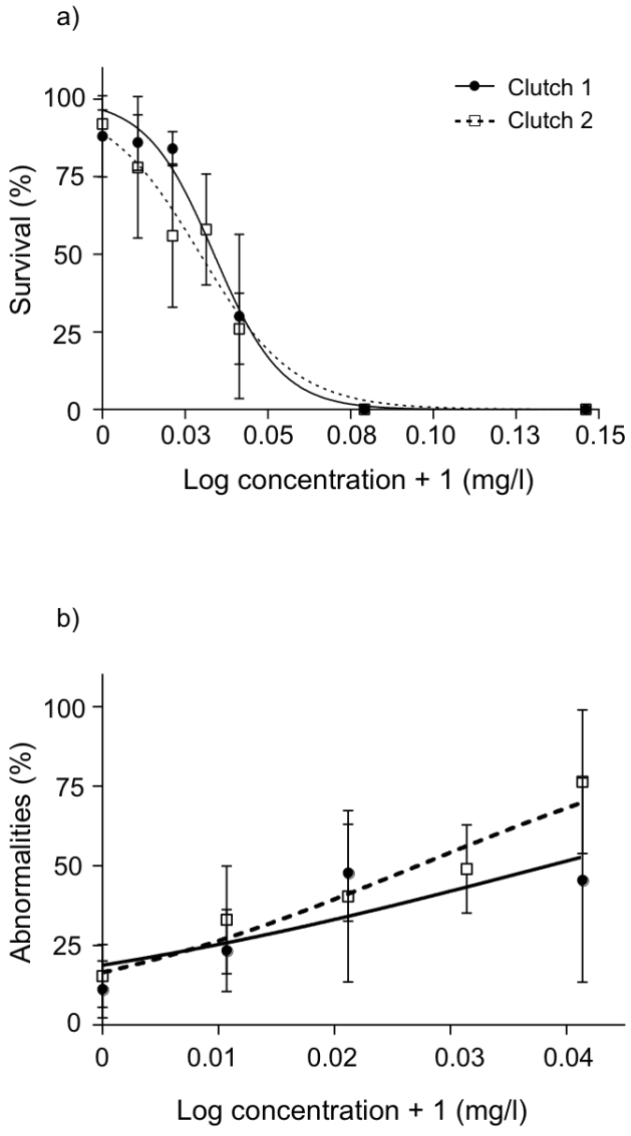


Figure 2. Dose-response curve after 96h exposure to TBBC (CAS 96 69 5) on *Silurana tropicalis* tadpoles. a) Survival and b) abnormalities, per TBBC log transformed (+1) concentrations. Black circles and open squares are the mean of five replicates per concentration with standard error bars. Black circles and lines for clutch 1 and open squares and long dashed lines for clutch 2.

Table 1. Effects of acute TBBC exposure on abnormalities (head, tail, edemas, gut and heart malformations) in *Silurana tropicalis* tadpoles.

	Estimate	Std Error	t value	Pr (> | t |)
glm (Head~Treatment)				
Intercept	-3.40	0.88	-3.86	< 0.001
0.025 mg/)	1.63	0.99	1.65	0.107
0.05 mg/l	2.03	0.96	2.11	0.041
0.075 mg/l	2.67	0.99	2.67	0.011
0.1 mg/l	3.26	0.94	3.46	0.001
Null deviance:	18.33	On 43 degrees of freedom		
Residual deviance:	12.10	On 39 degrees of freedom		
glm (Tail~Treatment)				
Intercept	-4.49	1.41	-3.18	0.003
0.025 mg/l	2.23	1.50	1.49	0.145
0.05 mg/l	3.21	1.46	2.20	0.033
0.075 mg/l	3.62	1.49	2.44	0.019
0.1 mg/l	3.62	1.45	2.49	0.017
Null deviance:	14.77	On 43 degrees of freedom		
Residual deviance:	9.94	On 39 degrees of freedom		
glm (Edema~Treatment)				
Intercept	-3.06	0.66	-4.63	< 0.001
0.025 mg/l	1.19	0.77	1.54	0.131
0.05 mg/l	1.54	0.75	2.05	0.047
0.075 mg/l	1.78	0.81	2.19	0.034
0.1 mg/l	2.67	0.72	3.70	< 0.001
Null deviance:	13.41	On 43 degrees of freedom		
Residual deviance:	9.09	On 39 degrees of freedom		
glm (Gut~Treatment)				
Intercept	-2.09	0.46	-4.51	< 0.001
0.025 mg/l	0.80	0.58	1.39	0.173
0.05 mg/l	0.86	0.58	1.49	0.144
0.075 mg/l	0.21	0.76	0.28	0.778
0.1 mg/l	0.18	0.65	0.28	0.779
Null deviance:	10.33	On 43 degrees of freedom		
Residual deviance:	9.53	On 39 degrees of freedom		

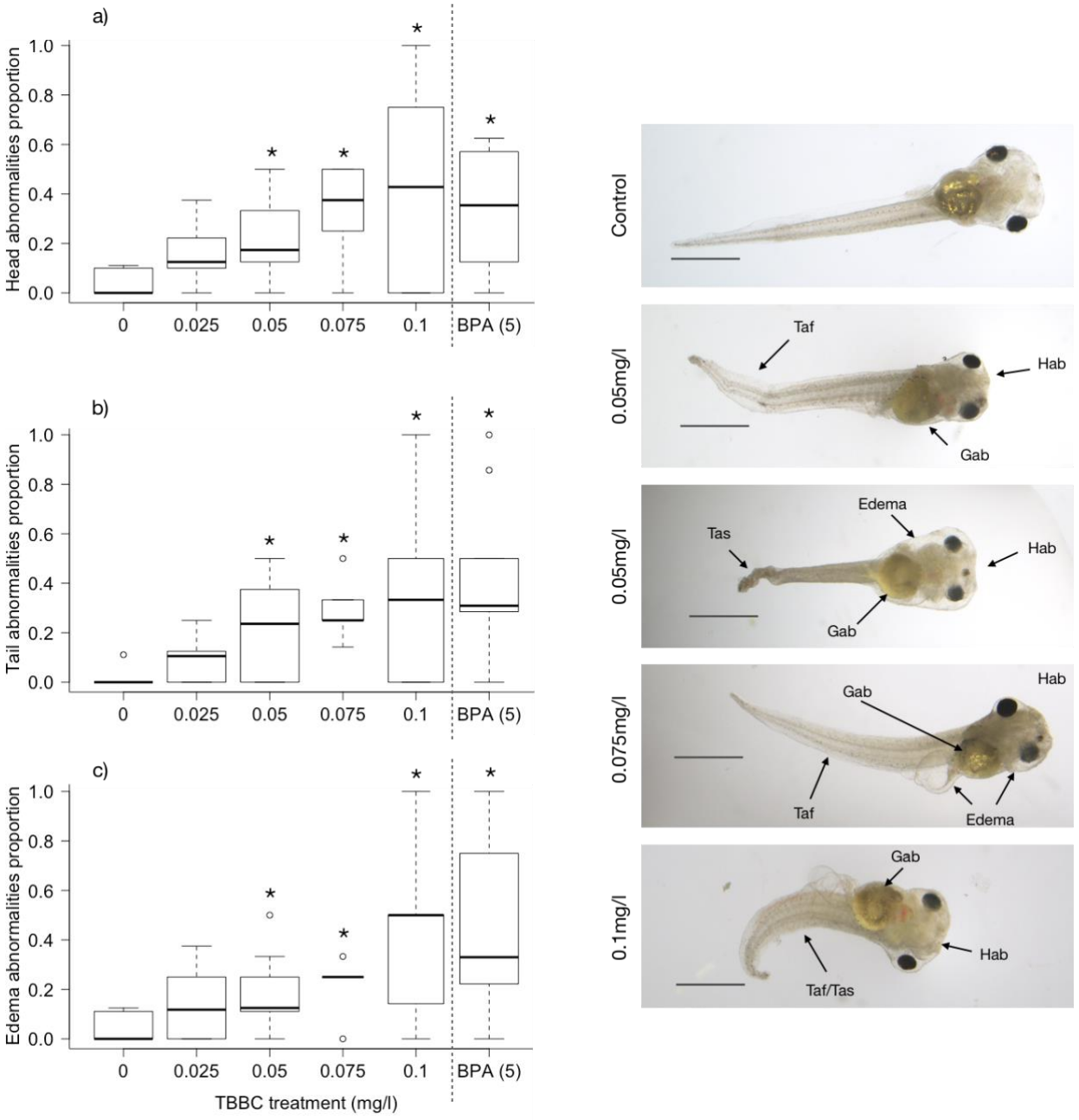


Figure 3. Teratogenic effects of 96 h acute TBBC exposure on the proportion of a) head, b) tail, and c) edema abnormalities in *Silurana tropicalis* tadpoles. The BPA treatment group (positive control) is separated by a dashed line for clarity. Bolded bar represents the mean and standard error in box, thickness of box reflects sample size. The asterisk (*) indicate significance at $p < 0.05$. Pictures on the right show tadpoles with abnormalities as indicated by arrows (Taf= tail flexure, Tas= tail shorted, Hab= head abnormality, Edema, Gab= gut abnormality) at different TBBC concentrations and a control tadpole with normal development.

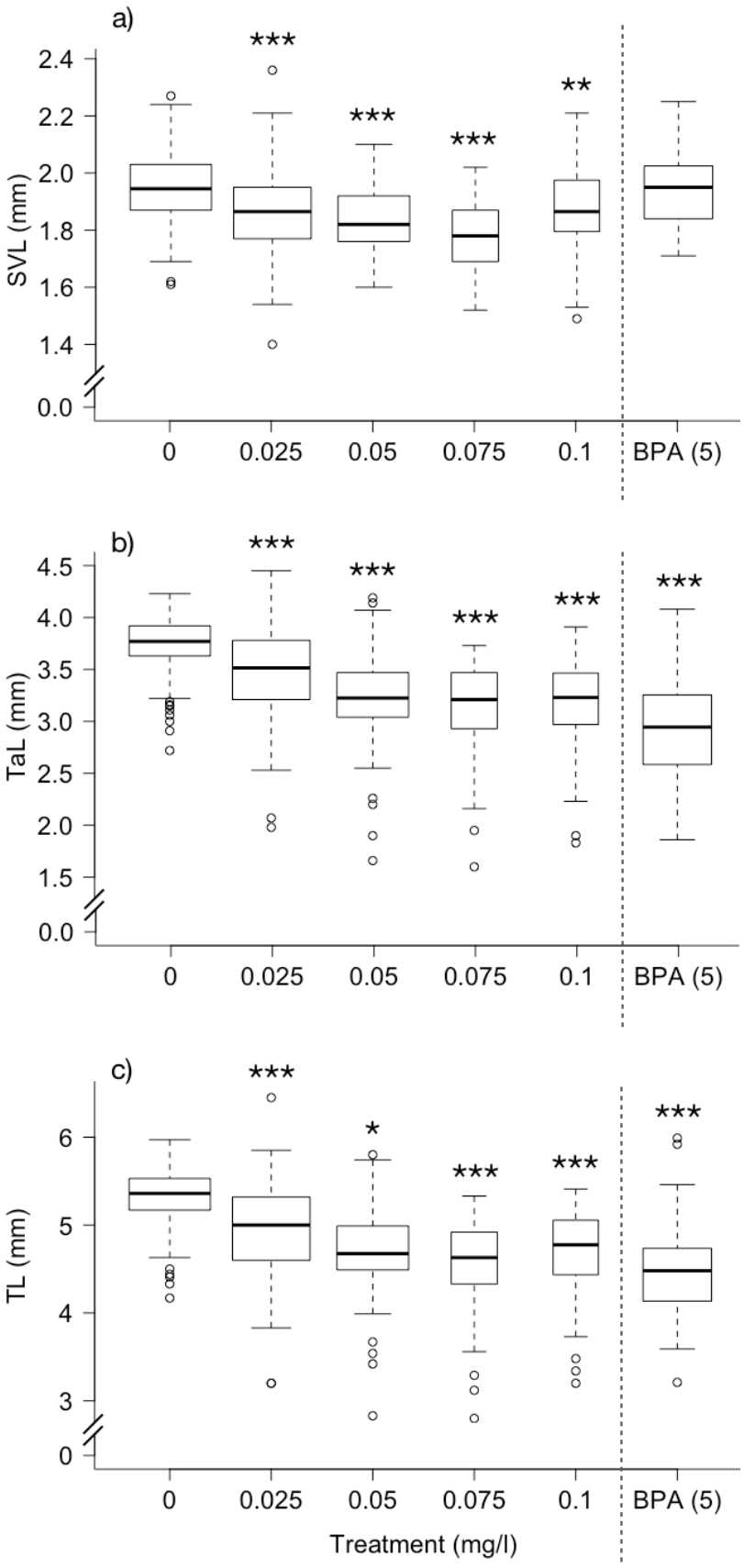


Figure 4. Effect of 96h acute TBBC exposure on a) snout to vent: SVL, b) tail length: TaL, and c) total length: TL, in *Silurana tropicali*s tadpoles. The BPA treatment group (positive control) is separated by a dashed line for clarity. Bolded bar represents the mean and standard error in box, thickness of box reflects sample size, circles are outliers. Asterisks indicate significance at p *** < 0.001, ** 0.01, * 0.05.

Table 2. Effects of acute TBBC exposure on body size (snout to vent (SVL), tail length (TaL), and total length (TL)) in *Silurana tropicalis* tadpoles.

	Estimate	Std Error	t value	Pr (> | t |)
glm (SVL~Treatment)				
Intercept	1.95	0.01	131.33	< 0.001
0.025 mg/l	-0.09	0.02	-4.19	< 0.001
0.05 mg/l	-0.11	0.02	-4.86	< 0.001
0.075 mg/l	-0.17	0.03	-5.72	< 0.001
0.1 mg/l	-0.09	0.03	-2.93	0.004
Null deviance:	6.73	On 298 degrees of freedom		
Residual deviance:	5.84	On 294 degrees of freedom		
AIC: -316.23				
glm (Tail~Treatment)				
Intercept	3.72	0.04	83.77	< 0.001
0.025 mg/l	-0.27	0.06	-4.21	< 0.001
0.05 mg/l	-0.48	0.07	-7.12	< 0.001
0.075 mg/l	-0.63	0.09	-6.97	< 0.001
0.1 mg/l	-0.59	0.09	-6.43	< 0.001
Null deviance:	67.87	On 298 degrees of freedom		
Residual deviance:	52.10	On 294 degrees of freedom		
AIC: 338.12				
glm (TL~Treatment)				
Intercept	5.30	0.05	99.49	< 0.001
0.025 mg/l	-0.36	0.08	-4.64	< 0.001
0.05 mg/l	-0.58	0.08	-7.16	< 0.001
0.075 mg/l	-0.78	0.11	-7.28	< 0.001
0.1 mg/l	-0.63	0.11	-5.80	< 0.001
Null deviance:	97.43	On 298 degrees of freedom		
Residual deviance:	75.13	On 294 degrees of freedom		
AIC: 447.53				

2.3.2 Effects of TBBC acute exposure on early development of L. pipiens

2.3.2.1 TBBC lethal concentration

There was 99 and 100% survival in control and solvent-control, respectively between the two clutches, with no significant difference between the two treatments in survival (t= 1, df= 18, p= 0.331) or size (SVL: t= -0.11, df= 197, p= 0.906; TaL: t= 0.31, df= 197, p= 0.751; TL: t= 0.18, df= 197, p= 0.173). Therefore, the following analyses were completed using only the solvent-control. After 96 h of TBBC exposure on *L. pipiens* tadpoles, the average LC_{50} for the two clutches was 0.177 mg/l; for clutch 1 alone the 96h LC_{50} was 0.182 (log 0.072, R_2 0.99) and for clutch 2 it was 0.172 mg/l (log .068, R_2 0.85; Fig 5). All tadpoles were dead at 0.4 mg/l TBBC. In clutch 1, survival in the three lower concentrations (0.25, 0.05 and 0.1 mg/l) was over 90%, while at 0.2 mg/l there was only one tadpole alive at the end of the experiment. For clutch 2, there was 100% survival at the two lower TBBC concentrations (0.1 and 0.135 mg/l), 80% survival at 0.17 mg/l, whereas, the two highest concentrations (0.2 and 0.24 mg/l) had a survival of 8 and 20% respectively.

2.3.2.2 Effects of TBBC on growth

Control tadpoles from the first clutch were smaller in size than the tadpoles from the second clutch: clutch 1 SVL mean was 7.21 mm, clutch 2 SVL mean was 8.25 mm (W= 109.5, $p < 0.001$). Therefore, the two clutches were analyzed separately. In the first clutch, tadpoles exposed to 0.025 and 0.05 mg/l of TBBC were larger than control tadpoles in body size (SVL, TaL, TL). However, tadpoles exposed to the higher concentrations of 0.1 and 0.2 mg/l of TBBC were significantly smaller than control tadpoles (Fig 6a, b, c; Table 3).

In the second clutch, TBBC exposed tadpoles were smaller compared to control tadpoles, although this decrease was not always significant. Tadpoles exposed to the three lower (0.1, 0.135, 0.17 mg/l) and the highest TBBC concentrations (0.24 mg/l) had SVL and TL significantly smaller than control tadpoles. Only tadpoles exposed to 0.2 mg/l were not significantly smaller than controls in all body measurements (Fig 6d-f; Table 3). Tadpoles

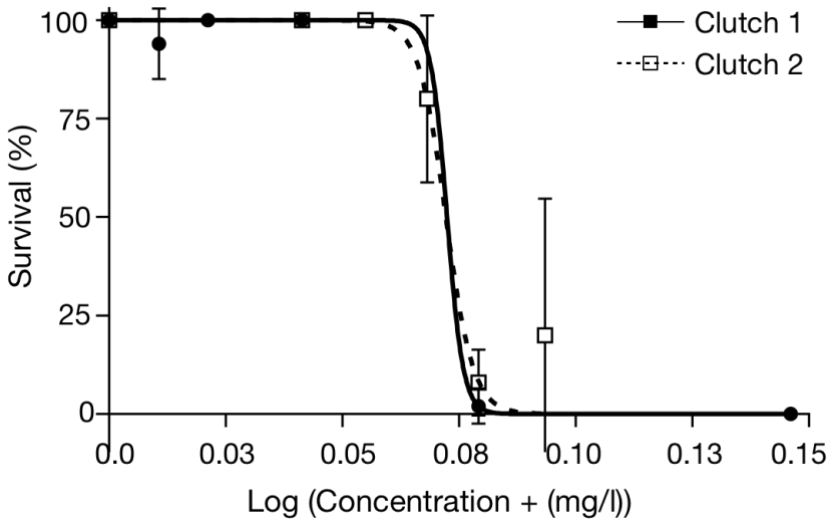


Figure 5. Survival dose-response curve after 96h exposure of TBBC (CAS 96 69 5) on *Lithobates pipiens* tadpoles. TBBC concentrations were log transformed (+1). Black circles and open squares are the mean of five replicates per concentration with standard error bars. Black circles and lines for clutch 1 and open squares and long dashed lines for clutch 2.

Table 3. Effects of acute TBBC exposure on body size (snout to vent (SVL), tail length (TaL), and total length (TL)) of *Lithobates pipiens* tadpoles in clutch 1 and clutch 2.

Clutch 1	Estimate	Std Error	t value	Pr (> | t |)
glm (SVL~Treatment)				
Intercept	7.21	0.07	95.75	< 0.001
0.025 mg/l	0.28	0.11	2.56	0.011
0.05 mg/l	0.42	0.11	3.79	< 0.001
0.1 mg/l	-0.28	0.11	-2.62	0.009
0.2 mg/l	-1.98	0.54	-3.68	< 0.001
Null deviance:	69.83	188 on degrees of freedom		
Residual deviance:	52.15	184 on degrees of freedom		
AIC: 305.02				
glm (TaL~Treatment)				
Intercept	10.07	0.13	78.73	< 0.001
0.025 mg/l	0.29	0.18	1.56	0.120
0.05 mg/l	0.53	0.19	2.76	0.006
0.1 mg/l	-1.17	0.18	-6.44	< 0.001
0.2 mg/l	-3.12	0.91	-3.42	< 0.001
Null deviance:	239.90	on 188 degrees of freedom		
Residual deviance:	150.61	on 184 degrees of freedom		
AIC: 505.45				

glm (TL~Treatment)				
Intercept	17.28	0.19	91.81	< 0.001
0.025 mg/l	0.56	0.27	2.08	0.038
0.05 mg/l	0.95	0.28	3.39	< 0.001
0.1 mg/l	-1.44	0.27	-5.43	< 0.001
0.2 mg/l	-5.10	1.34	-3.80	< 0.001
Null deviance:	507.68	on 188 degrees of freedom		
Residual deviance:	326.02	on 184 degrees of freedom		
AIC: 651.4				
Clutch 2				
glm (SVL~Treatment)				
Intercept	8.25	0.08	99.40	< 0.001
0.1 mg/l	-0.40	0.12	-3.44	< 0.001
0.13 mg/l	-0.35	0.12	-2.98	0.003
0.17 mg/l	-1.10	0.12	-8.86	< 0.001
0.2 mg/l	-0.37	0.30	-1.20	0.231
0.24 mg/l	-1.28	0.20	-6.31	< 0.001
Null deviance:	102.91	on 203 degrees of freedom		
Residual deviance:	68.22	on 198 degrees of freedom		
AIC: 369.48				
glm (TaL~Treatment)				
Intercept	12.37	0.1324	93.450	< 0.001
0.1 mg/l	-0.25	0.1873	-1.350	0.179
0.13 mg/l	-0.21	0.19	-1.14	0.256
0.17 mg/l	-1.66	0.20	-8.36	< 0.001
0.2 mg/l	-0.55	0.49	-1.14	0.256
0.24 mg/l	-2.05	0.32	-6.32	< 0.001
Null deviance:	270.58	on 203 degrees of freedom		
Residual deviance:	173.60	on 198 degrees of freedom		
AIC: 56				
glm (TL~Treatment)				
Intercept	20.63	0.20	102.04	< 0.001
0.1 mg/l	-0.66	0.28	-2.30	0.023
0.13 mg/l	-0.56	0.28	-1.97	0.050
0.17 mg/l	-2.76	0.30	-9.11	< 0.001
0.2 mg/l	-0.92	0.74	-1.24	0.216
0.24 mg/l	-3.33	0.49	-6.73	< 0.001
Null deviance:	648.66	on 203 degrees of freedom		
Residual deviance:	404.49	on 198 degrees of freedom		
AIC: 732.57				

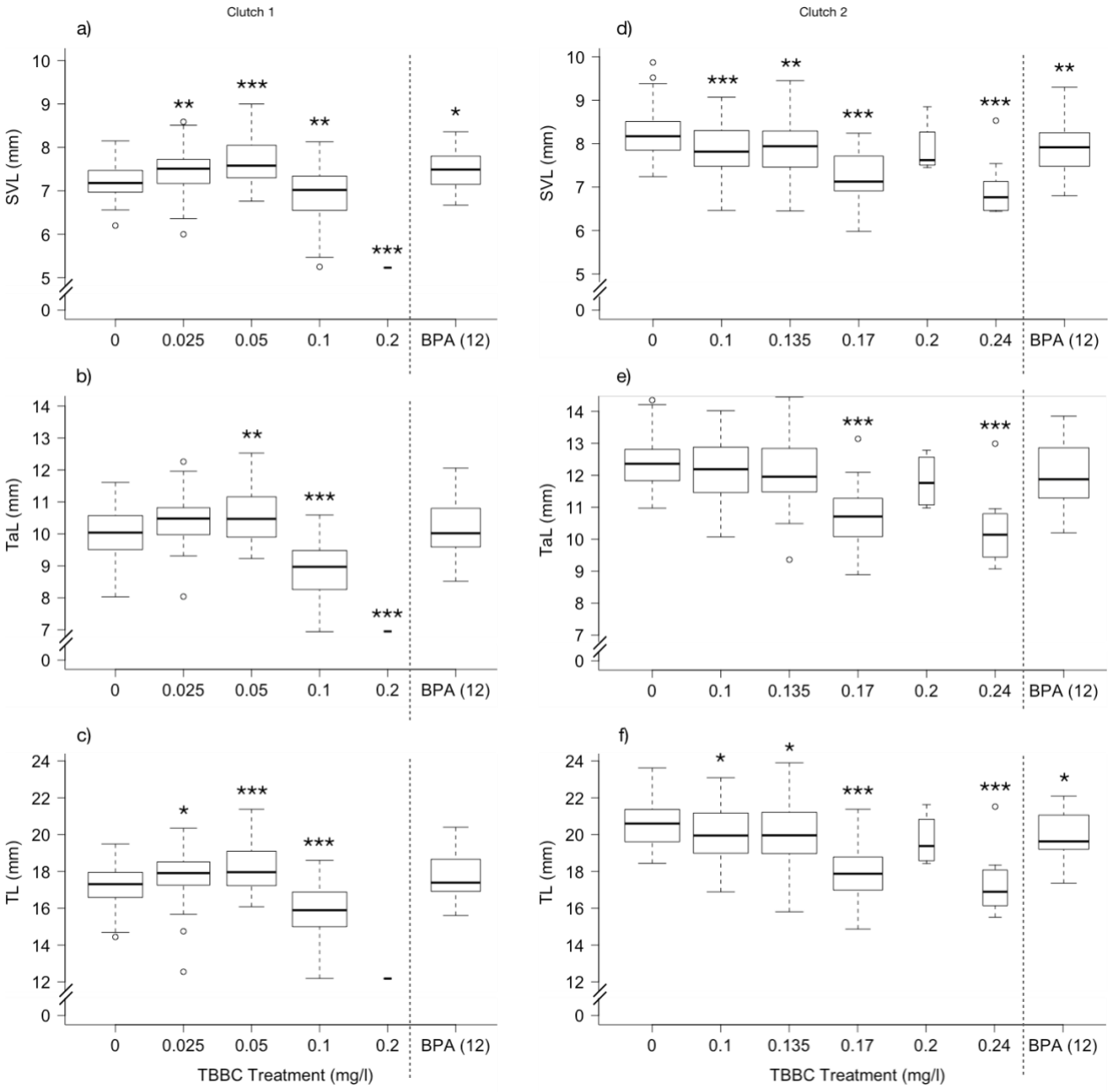


Figure 6. Effects of 96h acute TBBC exposure on a) and d) snout to vent: SVL, b) and e) tail length: TaL, and c) and f) total length: TL, in *Lithobates pipiens* tadpoles for clutch 1 and 2. The BPA treatment group (positive control) is separated by a dashed line for clarity. Bolded bar represents the mean and standard error in box, circles are outliers. Asterisks indicate significance at p *** < 0.001, ** 0.01, * 0.05.

exposed to 0.17 and 0.24 mg/l had a tail length smaller than controls (Fig 6e; Table 3). Body size for *L. pipiens* tadpoles exposed to the positive control of BPA at 12 mg/l was also reduced compared to controls in SVL and TL (Fig 6d-f; Table 3).

2.3.3 Effects of DTBP acute exposure on early development of L. pipiens

2.3.3.1 DTBP lethal concentration and effects on growth

Tadpole survival for *L. pipiens* was 100% for controls and solvent-control treatments during the 96h exposure; therefore, the following analyses were completed using only the solvent control, now referred to as control. After 96h of DTBP exposure on *L. pipiens* tadpoles, 96h LC_{50} was 0.52 mg/l, (log 0.182, R_2 .79). There was 100% mortality of tadpoles at the two highest concentrations (0.8 and 1 mg/l; Fig 7). Body size significantly decreased in a concentration response manner in tadpoles exposed to DTBP compared to control tadpoles, and this decrease was significant at the two highest concentrations (Fig 8a, b, c; Table 4).

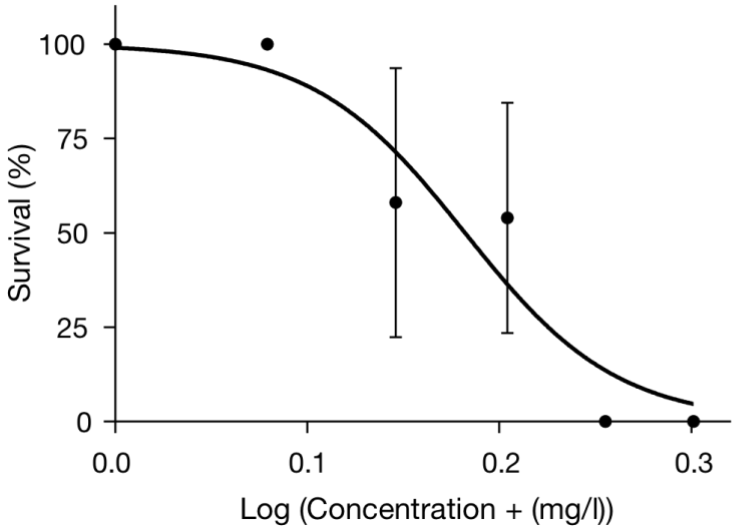


Figure 7. Survival dose-response curve after 96h exposure of DTBP (CAS 96 76 4) on *Lithobates pipiens* tadpoles. DTBP concentrations were log transformed (+1). Black circles are the mean of five replicates per concentration with standard error bars.

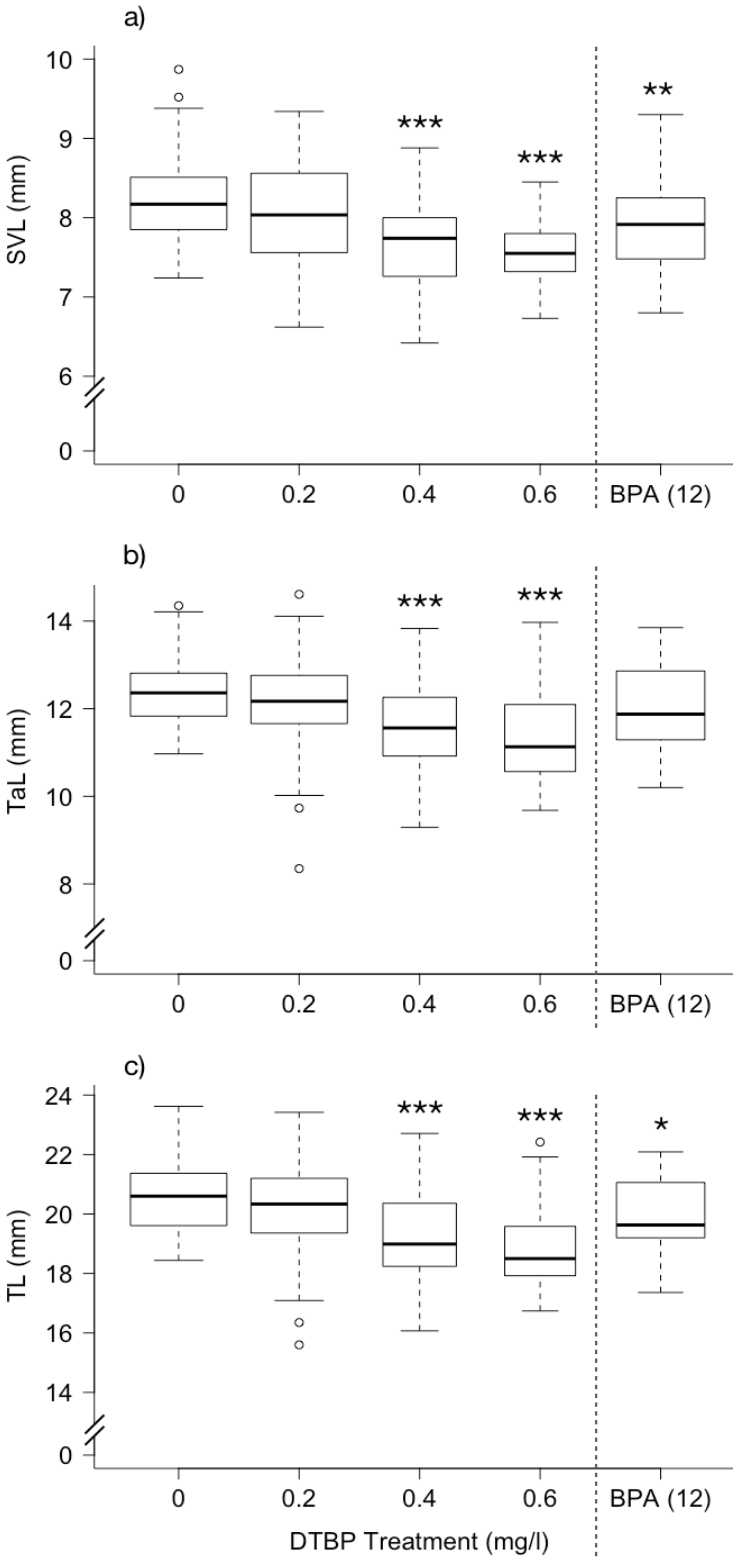


Figure 8. Effect of 96h acute DTBP exposure on a) snout to vent: SVL, b) tail length: TaL, and c) total length: TL, in *Lithobates pipiens* tadpoles. The BPA treatment group (positive control) is separated by a dashed line for clarity. Bolded bar represents the mean and standard error in box, circles are outliers. Asterisks indicate significance at p *** < 0.001, ** 0.01, * 0.05

Table 4. Effects of acute TBBC exposure on body size (snout to vent (SVL), tail length (TaL), and total length (TL)) in *Lithobates pipiens* tadpoles.

	Estimate	Std Error	t value	Pr (> | t |)
glm (SVL~Treatment)				
Intercept	8.25	0.08	103.34	< 0.001
0.2 mg/l	-0.20	0.11	-1.80	0.074
0.4 mg/l	-0.63	0.13	-4.77	< 0.001
0.6 mg/l	-0.71	0.13	-5.25	< 0.001
Null deviance:	61.02	on 155 degrees of freedom		
Residual deviance:	48.46	on 152 degrees of freedom		
AIC: 270.31				
glm (TaL~Treatment)				
Intercept	12.37	0.14	86.76	< 0.001
0.2 mg/l	-0.26	0.20	-1.31	0.191
0.4 mg/l	-0.83	0.23	-3.54	< 0.001
0.6 mg/l	-1.02	0.24	-4.25	< 0.001
Null deviance:	179.44	on 155 degrees of freedom		
Residual deviance:	154.61	on 152 degrees of freedom		
AIC: 451.31				
glm (TL ~ Treatment)				
Intercept	20.63	0.20	101.77	< 0.001
0.2 mg/l	-0.47	0.29	-1.63	0.104
0.4 mg/l	-1.46	0.33	-4.37	< 0.001
0.6 mg/l	-1.73	0.34	-5.06	< 0.001
Null deviance:	384.83	on 155 degrees of freedom		
Residual deviance:	312.16	on 152 degrees of freedom		
AIC: 560.92				

2.4 Discussion

The present study is the first amphibian embryonic developmental toxicity evaluation of the highly produced synthetic antioxidants TBBC and DTBP. Acute exposure to TBBC resulted in LC_{50}s at 0.07 and 0.17 mg/l on *S. tropicalis* and *L. pipiens* respectively, while DTBP lethal concentration was 0.52 mg/l. All together, these lethal concentrations would be considered very toxic. Exposure to TBBC reduced tadpole size in both species at low concentrations and produced several abnormalities in *S. tropicalis*. Exposure to DTBP also reduced *L. pipiens* tadpole body size.

The lethal concentration of TBBC and DTBP was relatively low for *S. tropicalis* and *L. pipiens* compared to other chemicals tested on amphibians, placing these two chemicals in the high toxicity spectrum for these two species (see Table 5). The TBBC LC_{50} was 3 times lower than DTBP for *L. pipiens*, therefore more toxic than the latter. Similar high toxicity levels in amphibians and other aquatic organisms have been found with the 4,-tert-octylphenol (4-tOP; Hogan *et al*. 2006; Saputra *et al*. 2016). Comparing to 4-tOP, exposure to TBBC was 2 to 4 times more toxic for *S. tropicalis*. Exposure to TBBC was also 27 times more toxic to *S. tropicalis* than 6-tert-butyl-4-methylphenol (AO2246) for *D. rerio* (Yang *et al*. 2018). Other SPA compounds found in commercial products have lethal concentrations higher than TBBC and DTBP, therefore they are considered less toxic. For example, lethal concentrations were > 9 mg/L for tert-butyl hydroquinone (TBHQ), the butylated hydroxyanisole BHA and butylated hydroxytoluene BHT, tested on *D. rerio* (LC_{50}= 9.2, 17.9, > 44 mg/l respectively; Yang *et al*. 2018); and nonylphenols (NP, 4-NP and NP12) tested on *X. laevis* when exposed at NF7-9 stages (LC_{50}= 59.14, 10.13 and 14.6 mg/l respectively; Xu *et al*. 2019). These lethal concentrations were more than 100 times higher than TBBC and DTBP. For *L. catesbeianus*, the perfluoroalkyl substance mixtures PFOS and PFOA had LC_{50}s (144 and 1004 mg/l, respectively) almost 1000 times higher (Flynn *et al*. 2019) compared to TBBC and DTBP acute toxicity for *S. tropicalis* and *L. pipiens* in this study. Toxicity comparisons to these SPAs should be taken with caution, different species, sensitivity and time of exposure might affect response.

Table 5. Lethal concentration (LC_{50}) comparison between TBBC and DTBP to other potential endocrine disruptors in amphibians and /or fish.

Compound	96 h[a] LC_{50} (mg/l)	Species and stage of exposure	Authors
TBBC	0.07	*S. tropicalis* NF9	This study
	0.17	*L. pipiens* G25	
DTBP	0.52	*L. pipiens* G25	This study
BPA	7.7	*S. tropicalis* NF9	Trudeau lab 2017
BPA	4.7	*X. laevis*	Iwamuro *et al*. 2003
BPA	19.2 [b]	*R. arenarum* G4	Wolkowicz *et al*. 2014
	7.1 [d]		
BPA	2.4	*X. laevis* NF2	Arancio *et al*. 2019
BPAF	0.004		
BPA	6.67	*D. rerio* 3dpf	Mai *et al*. 2018
BPA	8.04	*D. rerio*	Chow *et al*. 2013
4-tOP	0.26 [e]	*L. pipiens* G26	Hogan *et al*. 2006
	0.57 [e]	*L. pipiens* G36	
	0.15 [e]	*L. sylvaticus* G26	
4-tOP	0.20	*D. rerio* 5hpf	Saputra *et al*. 2016
AO2246	1.7	*D. rerio*	Yang *et al.* 2018
TBHQ	9.2		
BHA	17.9		
BHT	44.0		
NP	59.14	*X. laevis* NF7-9	Xu *et al*. 2019
4-NP	10.13		
NP12	14.60		
NP	2.0	*X. laevis* NF45	
4-NP	2.0		
NP12	10.57		
PFOS	144	*R. catesbeiana* G25	Flynn *et al*. 2019
PFOA	1004		
NA	4.10 [c]	*L. pipiens* G5	Melvin and Trudeau 2012b
	2.95 [c]	*S. tropicalis* NF4	
NA (S1M)	10.4	*S. tropicalis* NF9-10	Gutierrez-Villagomez *et al*. 2019
NA (S2M)	11.7		
NA (AEO)	52.3		

[a] 96h or otherwise specified, [b] 48h, [c] 72h, [d] 168h, [e] 360h

The lethal concentration of TBBC was two times lower for *S. tropicalis* compared to *L. pipiens*. Different species may show a difference in survival sensitivity to chemicals (Moore *et al*. 2012), in this case, it seems like the lab model species *S. tropicalis* is more sensitive than the local species *L. pipiens* to TBBC. Though, it is important to note that *L. pipiens* was not exposed to TBBC during the same early developmental stage (i.e., blastula stage) as *S. tropicalis*, which may influence developmental effects (Wagner *et al*. 2017; Xu *et al*. 2019). For example, *X. laevis* has shown more sensitivity to BPA when embryos are exposed at stage NF2 (Arancio *et al*. 2019) than when exposed at NF7 (Iwamuro *et al*. 2003). On the other hand, this same species, *X. laevis,* showed more sensitivity at later stages (larvae) when exposed to three different nonylphenol compounds (Xu *et al*. 2019) and to a glyphosate-based herbicide (Wagner *et al*. 2017) than when embryos were exposed (Xu *et al*. 2019; Wagner *et al*. 2017). To better understand sensitivity in survival for both species with the TBBC compound, it will be necessary to determine the lethal concentration for *S. tropicalis* at later developmental stages, and for *L. pipiens* at the blastula stage.

Acute exposure to TBBC on *L. pipiens* resulted in body size variation between the different concentrations (at 0.025 and 0.05 mg/l tadpole body was larger and at concentrations higher than 0.1 mg, body size was smaller). One possible reason could be that initial body size variability at day 0 was reflected at the end of the 96h, although visually they were similar in size when selected. Photographing tadpoles before exposure would allow for potential initial size differences to be accounted for or controlled between treatments at the beginning of an exposure (see Young *et al*. 2019).

The malformations found in this study are considered to be common developmental effects consistent with other chemicals commonly found in commercial products. For example, BPA exposure at 4.6 and/or 5.7 mg/l, showed short body length, head and tail abnormalities, edema and abnormal gut coiling (Iwamuro *et al*. 2003; Oka *et al*. 2003; Sone *et al*. 2004; Imakoa *et al*. 2007; Baba *et al*. 2009) and loss of pigment (Baba *et al*. 2009) in *X. laevis* tadpoles. In the present study, TBBC developmental effects were similar to the effects found in

the positive control treatment (BPA at 5 mg/l), however, BPA exposed tadpoles showed more frequency in abnormalities. Other commercial compounds that affect embryonic development and produce similar abnormalities in amphibians are herbicides (Wagner *et al*. 2017), nonylphenols (Xu *et al*. 2019), and naphthenic acids (Melvin and Trudeau 2012b; Gutierrez-Villagomez *et al*. 2019). In other aquatic organisms, such as, *D. rerio*, BHA, TBHQ, and AO2246 also decreased body size in a dose-response manner (Yang *et al.* 2018).

The present study shows the two SPAs tested affected amphibian embryonic development at concentrations as low as 0.025 mg/l, impacting tadpole size and causing malformations at 0.05 mg/l. The TBBC and DTBP are mass produced compounds, used in many common products, hence depending on their environmental release, they could pose a risk to aquatic life. Further research is needed to determine if exposure to TBBC will continue to affect growth and development after chronic exposure using sublethal concentrations, which may be more relevant to environmental exposures. Such information would help to understand the consequences of exposure to this chemical throughout amphibian development and using local species would help understanding whether local populations could be affected. Therefore, it is necessary to investigate the mechanisms in which TBBC and DTBP are affecting embryonic development, especially the decrease of body size in the two amphibian species studied and the several abnormalities on *S. tropicalis*.

Therefore, the purpose of the next chapter was to investigate whether TBBC at lower and more environmentally relevant concentrations would still affect amphibian growth in a chronic exposure, and if it would also affect metamorphosis. The calculated lethal concentration for TBBC and DTBP and their developmental toxicity risks on amphibians will be added to the ecotoxicological information used by the CMP to support risk assessments for these compounds.

Chapter 3

Chronic exposure to 4, 4-thiobis (6-t-butyl-m-cresol; TBBC) affects development of *Silurana tropicalis tadpoles*

3.1 Introduction

Many chemicals continue to be released into the environment at concentrations well below lethal levels, but little is known about their long-term impact on living organisms. Understanding the effect of sublethal, ecologically relevant concentrations of exposure to chemicals on survival and development is essential to determine the sensitivity species might have to chemical pollution (Egea-Serrano *et al*. 2012). Endocrine disruptors alter development in many species including amphibians, which are sensitive to these compounds. The use of environmentally relevant concentrations during chronic exposure might reveal effects otherwise not shown when animals are exposed for a short period of time (Eggen *et al*. 2004).

Amphibians are particularly sensitive to environmental contamination (Hayes *et al*. 2010; Egea-Serrano *et al.* 2012). Contaminants can be absorbed through their semi-permeable skin and affect development, reproduction, growth and metabolism, which are regulated by hormones (Heimer and Shi 2010; Carr and Patiño 2011; Mengeling *et al*. 2017; Xu *et al*. 2019). Amphibian metamorphosis represents an excellent model to evaluate thyroid hormone (TH) signaling disruption during development, especially because the TH action and mechanisms are similar to those of other vertebrates and may have effects on development, growth and metabolism (Berg *et al*. 2009; Heimer and Shi 2010; Mitsui *et al*. 2006). Previous studies have revealed that chronic exposure to chemicals at environmentally relevant concentrations have measurable effects on amphibians. For example, sodium perchlorate (SP), used in propellants and explosives, delayed development and increased growth in *Lithobates sylvaticus* (wood frogs) when exposed for two weeks to a concentration of 14 mg/l (Bulaeva *et al*. 2015). The same compound, SP, at a concentration of 250 µg/l resulted in comparable effects on *X. laevis* when exposed for 3 weeks (Ruthsatz *et al* 2018). Exposure to 0.8 µg/l of TCS (Triclosan, a

widely used antibacterial) increased total length and weight but did not affect metamorphosis in *X. laevis* when exposed at premetamorphosis (Fort *et al*. 2011). However, at 30 µg/l, TCS delayed development and increased growth on *Pseudacris regilla* (Pacific tree frog; Marlatt *et al*. 2013). In a one-year chronic exposure at 0.05 µg/l, TCS and benzopyrene delayed metamorphosis and induced a pre-diabetic state on *S. tropicalis*, these effects were also observed in their progeny which experienced a decrease in reproduction and viability (Regnault *et al*. 2018).

Bisphenol A (BPA), a highly produced and widely used plasticizer, was used in this study as a positive control. It has moderate toxicity with a chemical structure similar to TH and has been found to affect metamorphosis in chronically exposed amphibians (Heimer and Shi 2010). For example, at 2.28 and 5.7 mg/l, BPA delayed development and suppressed the T_3 action on tail resorption in *X. laevis* (Iwamuro *et al*. 2003). At 0.22 mg/l, BPA exposure suppressed metamorphosis, tail reduction and hind limb elongation in *S. tropicalis* (Goto *et al*. 2006). In T_3 induced *R. rugosa*, BPA suppressed the tail reduction induced by T_3; and downregulated TR on T_3 treated *X. laevis* tadpoles (Goto *et al*. 2006). Chronic exposure to BPA at concentrations ranging from 2.28 to 228 µg/l has increased growth in males of *X. laevis* (Oehlmann *et al*. 2009). At 0.023 µg/l BPA reduced weight of *X. laevis* and at 228 µg/l reduced weight and body size of *H. arborea* (Tamschick *et al*. 2016). Hence, BPA has been proposed to have TH suppressing effects on aquatic organisms (Canesi and Fabri 2015).

The chemical of interest in this study is the 4,4-thiobis (6-t-butyl-m-cresol; TBBC), which is a synthetic phenolic antioxidant used in many plastic and rubber products. A few studies have analyzed TBBC toxicity effects to lab mammal species, in which TBBC irritated the stomach and accumulated in adipose tissues and kidneys of mice and rats (Birnbaum *et al*. 1983; NTP 1994; Munson *et al.* 1988; Takahashi and Oishi 2006), and TBBC was also found to have androgen and estrogen receptor antagonist activity (Satoh *et al* 2008). The presence of this chemical in the environment has now been confirmed, from non-detected to a maximum of 8.8 ng/l in

influent and 1.6 ng/l in effluent of wastewater treatment plants in Canada; thus, it has the potential to affect aquatic organisms (Lu *et al*. 2019).

To date, there is no known information on chronic exposure to TBBC on amphibian development. In chapter 2, TBBC affected early embryonic development producing abnormalities and reducing growth in *S. tropicalis* at concentrations as low as 0.025 mg/l. For the present chapter 3, gross morphological endpoints were investigated to evaluate TBBC chronic effects on the TH mediated metamorphosis in *S. tropicalis* during chronic exposure at sublethal concentrations. The objective was to determine the chronic effects the TBBC compound on the metamorphic development of *S. tropicalis* tadpoles.

3.2 Methods

3.2.1 TBBC chronic exposure

To analyze the effects on survival, growth and development after a chronic exposure (from 48 to 52 days) to TBBC on *S. tropicalis*, 28 to 37 tadpoles at stage NF47-48 from three clutches were placed in glass tanks with 3L of treatment solution (treatments outlined below) for a total of 759 tadpoles. After the third day of exposure, an additional litre of solution was added to each tank to compensate for the density of the growing tadpoles. The solution temperature was maintained between 24.3 to 25.5ºC and a pH range from 6.9 to 7.6 throughout the whole experiment. There were five replicates per treatment and five treatments: solvent control (dimethyl sulfoxide, DMSO), positive control (0.5 mg/l BPA), and low 0.002 µg/l, medium 0.1µg/l, and high 5 µg/l TBBC concentrations. The lowest TBBC concentration (0.002 µg/l) was chosen to capture the concentration found in the environment (Lu *et al*. 2019). For this experiment, there was no water control, only a solvent control (DMSO) at 0.002% was used as the negative control and is referred to in this chapter as control. The highest concentration tested was 20% of the lowest concentration used in the acute exposure, where TBBC at 0.025 mg/l significantly reduced body size in *S. tropicalis* tadpoles. Treatment solution was replaced two times a week with 50% renewal of treatment solution and survival was analyzed every 24 h

with dead tadpoles removed. Tadpoles that reached stage NF66 before week 7 were removed, anesthetized, weighed and photographed for body measurements using ImageJ 1.52a software. Tadpoles not completing metamorphosis by the end of the exposure period were anaesthetized and processed to collect mass and size, as described above and developmental stage recorded according to Nieuwkoop-Faber (1994). The experiments were performed at the Aquatic facility at the University of Ottawa following the Canadian Council on Animal Care (CCAC) guidelines.

3.2.2 Chemical analysis

Water samples were analyzed with an ultra-performance liquid chromatography tandem mass spectrometer (UPLC-MS-MS), which consisted of a Waters Acquity LC coupled to Water Xevo TQS triple quadrupole mass spectrometer (Lu *et al*. 2019). The method detection limit (MDL) corresponded to 0.006 ng/ml. The average ± standard deviation recovery on spiked samples was 101 ± 0.87%. The relative standard deviation corresponding to the analysis of three replicates was 11%. Sample analysis was conducted by the Environment and Climate Change laboratory of Dr. Amila De Silva at the Canadian Centre for Inland Waters (CCIW), in Burlington, Ontario.

3.2.3 Statistical analysis

Statistical analyses were calculated using the R Statistical Software (R Core Team 2019) version 3.6.1. Survival was assessed using a General Linear Model with binomial distribution. To determine if there were any differences in time (days) to complete metamorphosis, only stage NF66 metamorphs were analyzed using a Generalized linear mixed model with lmer function from lme4 package (Zuur *et al*. 2009). Tadpoles were separated into five developmental stage groups (pre-metamorphosis (NF47-55), pro-metamorphosis (NF56-57), metamorphic climax (NF58-62), completing metamorphosis (NF63-65), and metamorphs (NF66)) to analyze proportion of tadpoles at each developmental stage category between treatments using General Linear Models with binomial distribution. Generalized Linear Mixed

Models with lmer function were used to analyze the effects of TBBC treatments on tadpole body size (SVL) and body mass in two developmental groups only: metamorphic climax (NF58-62) and tadpoles that completed metamorphosis (i.e., metamorphs; NF66). The TH levels peak during metamorphic climax with tadpoles going through dramatic developmental changes, therefore, disruption in the TH pathway would potentially show changes in gross morphometric endpoints during this time. The second developmental group evaluated, that is, tadpoles that completed metamorphosis, provided information on time to metamorphosis. The TBBC treatments were included as fixed effects and each replicate (tank) as a random effect to compensate for non-independence of tadpoles within the same tank. A significant *p* value was set as 0.05. Model fit was analyzed by plotting residuals vs fitted values to verify homogeneity, histograms of residuals were used to assess normality and residual vs Treatment was plotted to check independence (Zuur *et al*. 2009).

3.3 Results

Tadpole survival was not significantly different between controls and most treatments, where survival was 93.9% for controls, 92% for 0.1 µg/l TBBC and 92% for 5 µg/l TBBC, however, survival was significantly reduced to 83.4% at 0.002 µg/l TBBC (Table 6). There was no difference in survival between control and the 0.5 mg/l BPA treatment (t= 1.114, df= 8, p= 0. 2976), where survival was 87.2%.

Considering only metamorphs (i.e. NF66), it took on average 46.3 (± 2.7, n= 17) days for tadpoles in control treatments to complete metamorphosis, followed by the BPA treatment at 47.2 (±2.4, n= 9) days. For TBBC treatments, days to complete metamorphosis was 47.7 (±1.9, n= 18) days at 0.002 ug/L, 48 (±2.3, n= 13) days at 0.1 µg/l and 48.3 (±2.4, n= 9) days at 5 µg/l. The time to metamorphosis was not significantly different between control and TBBC treatments (Table 7). There was also no difference in time to metamorphosis between control and BPA treatments (W= 64.5, p= 0.522). There was a significantly higher proportion of tadpoles in the premetamorphosis stage (NF47-55) at the highest TBBC treatment (5 µg/l) compared to

Table 6. Effects of chronic TBBC exposure on tadpole survival in *Silurana tropicalis*.

	Estimate	Std Error	t value	Pr (> | t |)
glm (Alive ~ Treatment)				
Intercept	2.77	0.44	6.31	< 0.001
0.002 µg/l	-1.15	0.52	-2.22	0.041
0.1 µg/l	-0.31	0.58	-0.53	0.601
5 µg/l	-0.38	0.57	-0.67	0.513
Null deviance:	1.43	On 19 degrees of freedom		
Residual deviance:	1.08	On 16 degrees of freedom		

Table 7. Effects on chronic TBBC exposure on time (days) to metamorphosis in *Silurana tropicalis* NF66 metamorphs.

	Estimate	Std Error	z value	Pr (> | z|)
Days ~ Treatment				
Intercept	3.84	0.03	107.69	> 0.001
0.002 µg/l	0.03	0.05	0.59	0.555
0.1 µg/l	0.03	0.05	0.65	0.515
5 µg/l	0.04	0.06	0.70	0.484

controls (X_2= 23.42, df= 12, p = 0.024). However, there was no differences between controls and TBBC treatments in the proportion of tadpoles in the other developmental groups (p > 0.05): prometamorphosis (NF56-57), metamorphic climax (NF58-62), tadpoles reaching metamorphosis (NF63-65) and metamorphs (NF66; Fig 9).

In the metamorphic climax developmental group, tadpoles had a 14.5% lower body mass at 0.1 and 5 µg/l TBBC compared to controls. Body size (SVL) was not different between TBBC treatments and controls (Table 8, Fig 10a, b). Tadpoles exposed to 0.5 mg/l of BPA had a 6.7% lower body mass than controls (W= 1230, p= 0.048) but did not differ in SVL between these two groups (t= 0.5475, df= 88, p= 0.57). Growth was decreased for tadpoles that

completed metamorphosis (NF66), where SVL significantly decreased at 5 µg/l TBBC, while body mass was reduced at the lower TBBC concentration of 0.002 µg/l (Table 9, Fig 11a, b). There were no significant differences in growth between metamorphs exposed to BPA and controls (body mass: t= 0.3896, df= 24, p= 0.7002; SVL: t= 1.3627, df= 24, p= 0.1856).

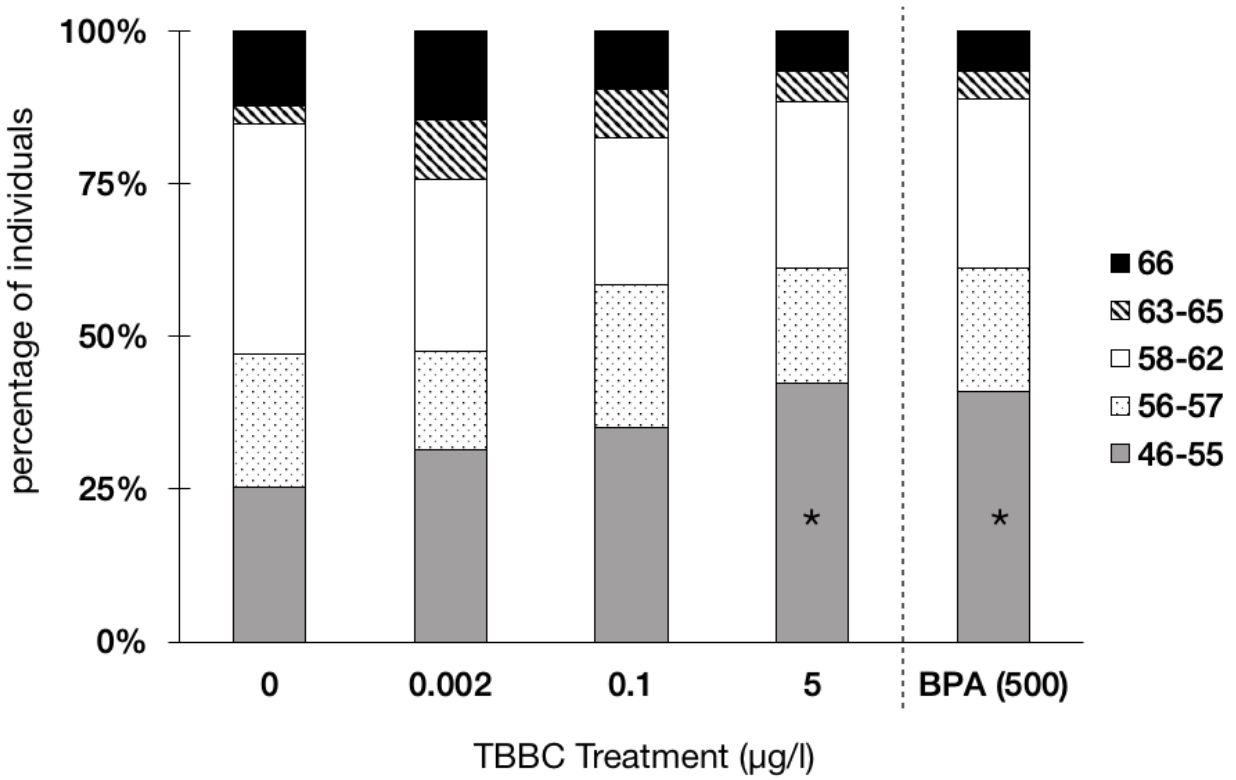


Figure 9. Effects of chronic TBBC exposure on the proportion of tadpoles in each developmental group (premetamorhosis NF46-55, prometamorphosis NF56-57, metamorphic climax NF58-62, reaching metamorphosis NF63-65, end of metamorphosis NF66) after 7 weeks. The BPA treatment (positive control) is separated by a dashed line for clarity. Asterisk (*) indicate significance at $p < 0.05$.

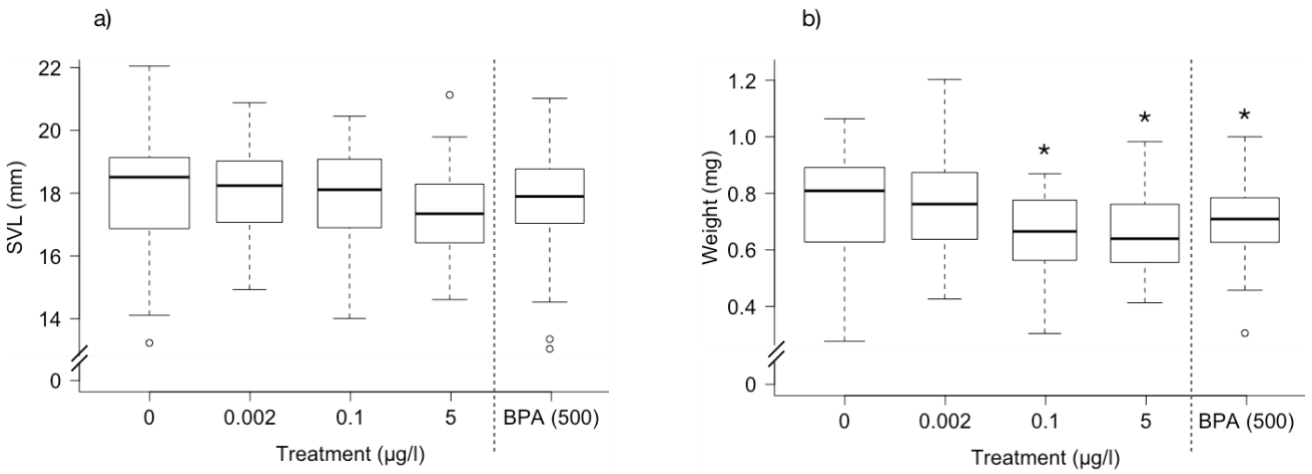


Figure 10. Effects of chronic TBBC exposure on a) snout to vent length (SVL) and b) body mass, at metamorphic climax (i.e., NF58-62) in *Silurana tropicalis* tadpoles. The BPA treatment group (positive control) is separated by a dashed line for clarity. Bolded bar represents the mean and standard error in box, circles are outliers. Asterisk (*) indicate significance at $p < 0.05$.

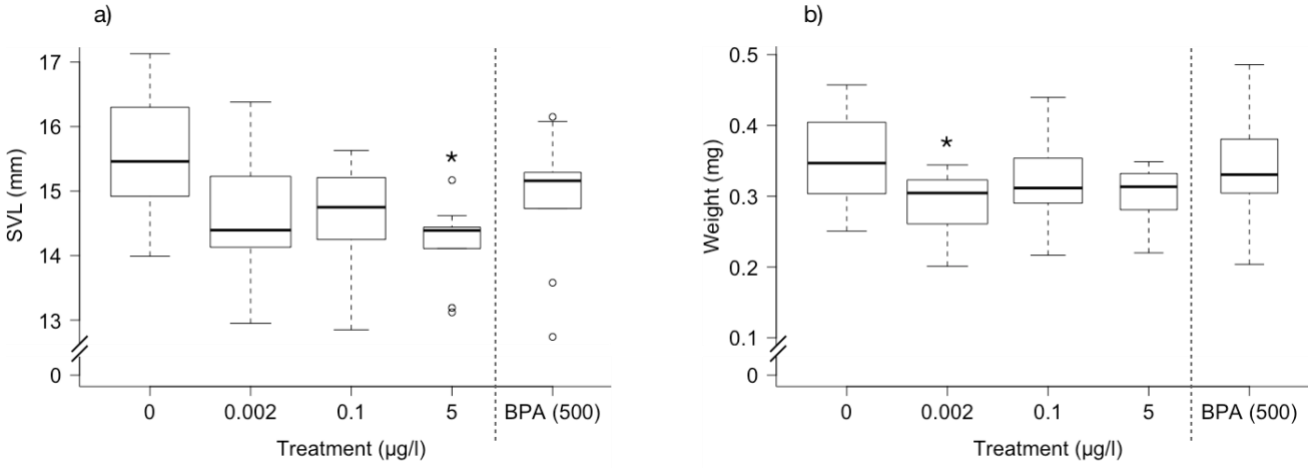


Figure 11. Effects of chronic TBBC exposure on a) snout to vent length (SVL) and b) body mass, in *Silurana tropicalis* metamorphs (i.e., NF66). The BPA treatment group (positive control) is separated by a dashed line for clarity. Bolded bar represents the mean and standard error in box, circles are outliers. Asterisk (*) indicate significance at $p < 0.05$.

Table 8. Effects on chronic TBBC exposure on body size (snout to vent: SVL) and body mass at metamorphic climax (NF stages 58-62) in *Silurana tropicalis*.

	Estimate	Std Error	df	t value	Pr (> | t |)
SVL ~ Treatment + Stage + (1 | Tank))					
Intercept	52.02	5.66	146.98	9.20	> 0.001
0.002 µg/l	-0.002	0.59	15.64	-0.004	0.997
0.1 µg/l	-0.30	0.59	16.04	-0.50	0.623
5 µg/l	-0.84	0.59	15.54	-1.43	0.172
Stage	-0.57	0.09	145.99	-6.00	> 0.001
Body mass ~ Treatment + Stage + (1 | Tank))					
Intercept	5.31	0.63	149.17	8.47	> 0.001
0.002 µg/l	0.005	0.05	15.48	0.09	0.928
0.1 µg/l	-0.12	0.05	15.87	-2.13	0.049
5 µg/l	-0.13	0.05	15.13	-2.32	0.034
Stage	-0.08	0.01	148.66	-7.25	> 0.001

Table 9 Effects on chronic TBBC exposure on body size (snout to vent: SVL) and body mass at the end of metamorphosis (NF66) in *Silurana tropicalis*.

	Estimate	Std Error	df	t value	Pr (> | t|)
SVL ~ Treatment + (1 | Tank))					
Intercept	15.32	0.29	12.01	52.81	> 0.001
0.002 µg/l	-0.64	0.40	11.64	-1.58	0.140
0.1 µg/l	-0.67	0.43	12.14	-1.54	0.148
5 µg/l	-1.08	0.45	15.05	-2.40	0.030
Body mass ~ Treatment + (1 | Tank))					
Intercept	0.34	0.01	10.20	22.94	> 0.001
0.002 µg/l	-0.05	0.02	10.15	-2.51	0.031
0.1 µg/l	-0.02	0.02	10.84	-0.99	0.341
5 µg/l	-0.04	0.02	16.43	-1.63	0.122

3.4 Discussion

The TBBC compound is present in many products of daily use including construction materials, laboratory chemicals, furniture, computers, cameras, lamps, refrigerators, footwear, paper, cardboard, toys, and food packaging. However, knowledge of its presence in the environment is limited to one recent study (Lu *et al*. 2019) in which TBBC was found up to 1.6 ng/l in effluents of wastewater treatment plants in Canada. As such, the implications of wildlife exposure to TBBC are not yet known. Also, of note is the similarity in chemical structure of TBBC to endogenous THs, which might be indicative of potential effects on thyroid pathways in the body. These combined observations led to the investigation of the toxic effects of TBBC on *S. tropicalis*, and to determine its potential as an endocrine disruptor.

In this study, *S. tropicalis* tadpoles were chronically exposed to the synthetic antioxidant TBBC at sublethal environmentally relevant concentrations for seven weeks. After chronic TBBC exposure to sublethal concentrations, survival was significantly reduced to 83% at the lower TBBC concentration (0.002 µg/l), but still within the acceptable mortality rate for the test (i.e., < 20% mortality). Specific reason for this mortality was not found as the conditions in the lab chamber were similar for all exposure vessels.

Chronic exposure to TBBC reduced tadpole growth at metamorphic climax and at the end of metamorphosis. In the case of tadpoles at metamorphic climax, TBBC exposure did not affect SVL; however, it reduced body mass by 14.5% at 0.1 and 5 µg/l concentrations. Moreover, tadpoles that completed metamorphosis saw a decrease of 17% in body mass at the lower TBBC concentration of 0.002 µg/l and a decrease of 8% of SVL at 5 µg/l of TBBC. Similar to this study, other chemicals have been shown to disrupt hormonal pathways leading to decreases in amphibian growth, for example, PFAS have been found to decrease growth in American bullfrogs (*Rana catesbeiana*; Flynn *et al*. 2019), and nonylphenols have been found to reduce growth in *X. laevis* (Xu *et al*. 2019). It is not known however, if the tadpoles tested in this

study would have been able to compensate for the lower body mass after metamorphosis if exposure had continued.

Currently, it is recognized that body size at metamorphosis is positively associated with various fitness components (Semlitsch *et al*. 1988; Cabrera-Guzman *et al*. 2013; Jennette *et al*. 2019). For example, larger size at metamorphosis has been correlated to a larger size at adulthood. And this larger size also increased their survival, especially during the first weeks after metamorphosis (Altwegg and Reyer 2003; Berven 1990; Chelgren *et al*. 2006). Furthermore, larger metamorphs had longer legs (Johansson *et al*. 2010), and larger metamorphs can jump farther and for longer periods than smaller metamorphs, increasing their ability to find better habitats (John-Alder and Morin 1990; Johansson *et al*. 2010) and increasing their dispersion distance as well (Chelgren *et al*. 2006). Another fitness factor affected by size at metamorphosis is reproduction, since larger female juveniles would start their first reproduction earlier than smaller ones, they would also have bigger clutches (Berven 1990; Tejedo 1992) and higher fecundity than the smaller counterparts (Awkerman and Raimondo 2018). In this context of larger size at metamorphosis associated with higher fitness, I would predict that TBBC exposure in *S. tropicalis* would probably adversely affect life fitness as a result of reduced body size. In this regard, it would be interesting to conduct a longer TBBC exposure study to investigate whether this decrease in growth would affect maturation and reproductive fitness.

In this study, TBBC exposure reduced tadpoles' body mass at metamorphic climax and metamorphs, while metamorphosis was not delayed or accelerated. Body size at metamorphosis is a plastic response to environmental factors (Laurila and Kujasalo 1999; Cabrera-Guzman *et al*. 2013). In general, amphibians under environmental stress are often found to accelerate metamorphosis with a cost of a reduction in adult body size (Crespi and Denver 2005; Davidson *et al*. 2011). The present study suggests that TBBC exposure was not inducing significant stress in *S. tropicalis* to accelerate metamorphosis. Future studies on the mechanisms that affect growth including TH and the growth hormone/insulin like factor pathways would help to understand why metamorphosis was not affected.

Time to metamorphosis took two days longer for metamorphs exposed to 5 µg/l of TBBC compared to controls, although this delay was not statistically significant and likely not biologically significant for a fully aquatic amphibian. Other chemicals have delayed time to metamorphosis for longer periods of time. For example, *L. sylvaticus* tadpoles exposed to sodium perchlorate, took 11 days longer to reach metamorphic climax (Bulaeva *et al.* 2015), and up to 5 days longer in the case of *X. laevis* (Ruthsatz *et al*. 2018). The glyphosate-based herbicide Vision Max® formulation exposure to *L. sylvaticus* was found to delay metamorphic climax by 7 days (Navarro-Martin *et al*. 2014). The different results with our study suggest that exposure to TBBC at the concentrations tested was not interfering with the thyroid pathway with respect to time to metamorphosis.

Chronic exposure at 5 µg/l TBBC and BPA exposure at 0.5 mg/l maintained a higher proportion of individuals in premetamorphic stages. A higher proportion of tadpoles developing at a slower rate implies that more tadpoles remain exposed to aquatic contaminants for longer periods of time which could further exacerbate the harmful effects of these contaminants.

In the present study, the positive control, BPA, at a concentration of 0.5 mg/l decreased body mass in tadpoles at metamorphic climax, however, BPA did not affect SVL in tadpoles at metamorphic climax nor did it affect growth when metamorphosis was completed. Furthermore, exposure to BPA did not affect time to metamorphosis. In previous studies, BPA decreased growth on *X. laevis* (at 0.023 µg/l) and on *Hyla arborea* (at 228 µg/l; Tamschick *et al.* 2016) but was found to increase tadpole size in *X. laevis* males (at 2.28 to 228 µg/l; Oehlman *et al.* 2009). One potential explanation for the different findings across these studies is that BPA might have different effects on metabolic rate across species (Lindholst *et al*. 2003). Another potential explanation is that the developmental stage at which tadpoles are exposed to BPA can impact the study findings. For example, Arancio *et al*. (2019) found that *X. laevis* was more sensitive to BPA when exposed at stage NF2. Chronic exposure to BPA did not affect development in this study, which is consistent with previous studies exposing *X. laevis* to BPA to concentrations below 0.5 mg/l (Pickford *et al*. 2003; Levi *et al*. 2004). However, when *X. laevis* was exposed to

BPA at a concentration of 2.28 mg/l, metamorphosis was delayed (Iwamuro *et al*. 2003). I would therefore recommend using a higher concentration of BPA as the positive control when evaluating body size, body mass and metamorphic development.

Results from this study suggest that environmentally relevant concentrations of TBBC exposure reduce tadpole growth at metamorphic climax and at the end of metamorphosis in *S. tropicalis*. There were, however, no effects of TBBC exposure on metamorphic development. Further research is necessary to understand the mechanisms underlying the decrease in growth, including the growth hormone/insulin like factor considering that time to metamorphosis was not affected. Moreover, to confirm that TBBC does not affect the TH pathway, it will be necessary to evaluate histology and molecular endpoints in addition to morphometrics. For example, thyroid histology could provide information of chemical effects on the thyroid gland size and tissue such as thyroid hypertrophy and hyperplasia (Fort *et al*. 2007). For molecular endpoints, during metamorphic climax, the TR-mediated genes, TRβ and dio3, increase expression in brain and tail, and if their expression is decreased, then the TH levels would decrease as well, leading to slower or inhibition of metamorphic changes (Navarro-Martin *et al*. 2012; Fort *et al*. 2007). Together, the use of histology, molecular and gross morphometric endpoints would help to understand the mechanisms of TBBC effects on the endocrine systems in amphibians.

Metabolism of TBBC and/or DTBP in amphibians has not yet been studied. However, in lab mice and rats, TBBC has been found to accumulate in liver and adipose tissue after rapid distribution throughout the body when exposed at high doses (oral: 5, 50 and 500 mg/kg body weight; intravenous: 5 mg/kg body weight), and excreted in feces (Birnbaum *et al*. 1983). Repeated exposure to TBBC would also result in some accumulation of unmetabolized compound in liver and lipid-rich tissues (Birnbaum *et al*. 1983). Birnbaum *et al*. (1983) indicate that TBBC is readily metabolized and excreted. In this regard, metabolic information on SPAs is needed to understand the relationship between how much is actually entering the body, its

distribution, accumulation and excretion, and how the kinetics are related to the effects found. The present study did not evaluate absorption and/or dietary uptake, burden, metabolism and excretion of SPAs by the tadpoles and/or froglets, therefore, information about the concentration inside the body and whether it was the parent compound or any metabolite that affected growth remains unknown. Further investigations in this regard would be needed to understand the relation between TBBC exposure and the effects in these amphibian species.

Chapter 4

General conclusions

This study reveals for the first time the lethal concentration of two commonly used synthetic antioxidants on amphibians, TBBC and DTBP. Both chemicals affected embryonic development after acute exposure on the lab model species, *S. tropicalis*, and the native species *L. pipiens*. Specifically, TBBC reduced tadpole size at 0.025 mg/l and produced malformations at 0.05 mg/l in *S. tropicalis* tadpoles. Sublethal concentrations of chronic TBBC exposure affected growth during metamorphosis in *S. tropicalis*, however, TBBC did not affect time to metamorphosis. Exposure to TBBC specifically affected body mass at the lower, more environmentally relevant concentration (0.002 µg/l), but also affected size at 0.1 and 5 µg/l. These results call for further investigations on the mechanisms behind TBBC exposure affecting growth, e.g., including regulation of genes and binding affinities with hormone receptors and transporters on the growth hormone/insulin like factor. Furthermore, investigations regarding how a decrease in body size at metamorphosis would affect future fitness such as survival and reproduction are necessary especially when finding that TBBC affected this endpoint even at nominal concentrations as low as 0.002 µg/l, which is also an environmental relevant concentration.

Future studies should also address the LC_{50} for DTBP with *S. tropicalis* to compare its toxicity with TBBC with the same species. Acute exposures with TBBC and *L. pipiens* at earlier developmental stages would also be important to understand developmental sensitivity of this species. Chronic exposures to TBBC and DTBP with *L. pipiens* would give a better view of how these SPAs might affect growth and development in both species.

There is little known about the risks of exposure to these two SPAs for wildlife and humans. For example, environmental exposure or migration of TBBC from relevant consumer products (e.g., food containers) has not been documented, but effects from the usage of nitrile

gloves have found skin sensitivity in people (Rich *et al*. 1991). Therefore, it is important to investigate how these, and similar chemicals may affect other biological systems, such as neurological or reproductive systems. Developing this basic ecotoxicity knowledge will provide decision makers with more information and allow better guidelines on the use, management and disposal of the products containing these chemicals, while minimizing the risk of exposure to wildlife and humans.

Increasing numbers of chemicals are being released into the environment, and for many of these compounds there is little or no ecotoxicological information. It is essential to have at least basic ecotoxicity information for highly produced and widely used chemicals that have a risk of being released into the environment, not only because environmental contamination is an important factor in amphibian population decline (Hayes *et al*. 2010), but also for the risk it poses to wildlife in general. Therefore, it is necessary to increase efforts for environmental monitoring of SPAs, targeting TBBC and DTBP especially close to production areas, landfill sites and effluents of wastewater plants that may have higher risks for SPAs release into the environment to be able to evaluate the risk of exposure from these SPAs on aquatic organisms.

Overall, the results from this thesis should be considered as a base for future studies to elucidate the potential risk TBBC exposure might have on the environment. The compounds TBBC and DTBP are only two of more than 1500 chemicals that are being analyzed by the CMP3. Addressing more chemicals for environmental monitoring and evaluating their endocrine disruption is necessary. This thesis contributes to fill the gaps on basic ecotoxicity information of these SPAs within CMP, especially regarding embryonic and larval developmental toxicity of amphibians.

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