Agrochemicals disrupt multiple endocrine axes in amphibians

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Abstract

Concern over global amphibian declines and possible links to agrochemical use has led to research on the endocrine disrupting actions of agrochemicals, such as fertilizers, fungicides, insecticides, acaricides, herbicides, metals, and mixtures. Amphibians, like other species, have to partition resources for body maintenance, growth, and reproduction. Recent studies suggest that metabolic impairments induced by endocrine disrupting chemicals, and more particularly agrichemicals, may disrupt physiological constraints associated with these limited resources and could cause deleterious effects on growth and reproduction. Metabolic disruption has hardly been considered for amphibian species following agrichemical exposure. As for metamorphosis, the key thyroid hormone-dependent developmental phase for amphibians, it can either be advanced or delayed by agrichemicals with consequences for juvenile and adult health and survival. While numerous agrichemicals affect anuran sexual development, including sex reversal and intersex in several species, little is known about the mechanisms involved in dysregulation of the sex differentiation processes. Adult anurans display stereotypical male mating calls and female phonotaxis responses leading to successful amplexus and spawning. These are hormone-dependent behaviours at the foundation of reproductive success. Therefore, male vocalizations are highly ecologically-relevant and may be a non-invasive low-cost method for the assessment of endocrine disruption at the population level. While it is clear that agrichemicals disrupt multiple endocrine systems in frogs, very little has been uncovered regarding the molecular and cellular mechanisms at the basis of these actions. This is surprising, given the importance of the frog models to our deep

understanding of developmental biology and thyroid hormone action to understand human health. Several agrichemicals were found to have multiple endocrine effects at once (e.g., targeting both the thyroid and gonadal axes); therefore, the assessment of agrichemicals that alter cross-talk between hormonal systems must be further addressed. Given the diversity of life-history traits in Anura, Caudata, and the Gymnophiona, it is essential that studies on endocrine disruption expand to include the lesser known taxa. Research under ecologically-relevant conditions will also be paramount. Closer collaboration between molecular and cellular endocrinologists and ecotoxicologists and ecologists is thus recommended.

Introduction

There are countless benefits to intensive agricultural practices, including high productivity, labour concentration, and efficiencies, and thus, major health and economic benefits to people. Loss of natural habitat due to agricultural development is one important contribution to global amphibian declines. This is accompanied by the use of a range of agrochemicals for crop and farm animal management. There is accumulating and controversial evidence that some are endocrine disrupting chemicals (EDCs), or have the potential to disrupt other key physiological control processes that may contribute to reduced amphibian health and significant population declines. The most obvious agrochemicals are the various herbicides, insecticides, fungicides, and fertilizers used (Table 1). However, animal growth promoters, manure and sewage runoff, natural and synthetic steroids, pharmaceuticals and metals can also be considered

as agrochemical pollutants as they are used and measured near-by agricultural activities.

TABLE 1 NEAR HERE

The class of Amphibia is comprised of three orders: Anura (frogs and toads, ~6,500 species), Caudata (newts and salamanders, ~680 species), and the Gymnophiona (caecilians, ~205 species). Of these, most research on basic endocrine control of development and reproduction, as well as the processes underlying endocrine disruption are largely undertaken with a few anuran species. For the most part, we limit the scope of this review to frogs and toads because data are missing in the other groups. Amphibians appear to be particularly susceptible to disruption of development, because metamorphosis is dependent on the thyroid hormones (THs), and that THs and their receptors are often altered by environmental contaminants. Moreover, sexual development is highly plastic and sex reversal is relatively easy to induce with exogenous sex steroid treatments, among others. Crosstalk between the thyroid, metabolic, and gonadal axes gives rise to additional processes that can be affected by EDCs. Given that tadpole and adult amphibian skin is permeable, many species can take up and respond to pollutants at both life-history stages.

Our main objective was to critically review amphibian data on the disruption of metamorphosis, growth, metabolism, and aspects of sexual differentiation and behaviour by agrochemicals. Information on the molecular and cellular mechanisms of action of a given agrochemical may be available, but more often it is not. In this

instance, we review the effects of model endocrine disruptors when there is a paucity of information on agrochemicals. The second objective was to identify areas requiring collaborative research concerning disruption of the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-gonad (HPG) axis, and the resultant impacts on amphibian population health.

2. Metamorphosis and disruption of the hypothalamic-pituitary-thyroid axis

The HPT axis (Figure 1) is critically important in regulating amphibian development, metamorphosis, and physiology and has been reviewed extensively (Denver et al., 2002; Shi, 2000; Denver, 2009; 2013). The action of THs, thyroxine (T4) and triiodothyronine (T3), are mediated through the TH receptors (TR α and TR β) (Yaoita and Brown, 1990; Eliceiri and Brown, 1994). The TRs are liganded transcription factors that dimerize with the retinoic acid receptor (RXR) to induce tissue-specific actions by binding to thyroid response elements (TREs) in promoter regions of TH-responsive genes, thus, controlling most aspects of the metamorphic progression (Helbing and Atkinson, 1994; Shi, 2000; Buchholz et al., 2004, 2005, 2017).

The control of the synthesis, secretion, and metabolism of THs has been described for anurans in several elegant reviews and will not be covered in detail here (Denver et al., 2002; Buchholz et al., 2006; Denver, 2013). Briefly, metamorphic progression is accompanied by a gradual increase in THs and developmental- and tissue-dependent expression patterns of the TRs and deiodinase enzymes. In particular, the type 2 deiodinase (DIO2) is required for local conversion of T4 to the more active T3, and metabolism of these to less active forms by type 3 deiodinase (DIO3). Contaminants have the potential to cause adverse effects through interference at many levels:

synthesis of THs (sodium iodide symporter (NIS), thyroid peroxidase (TPO), thyroid stimulating hormone (TSH), thyroid stimulating hormone receptor (TSHR)), plasma transport proteins, cellular uptake mechanisms, TRs, and TH metabolism (e.g., deiodinase enzymes). Interference with feedback loops and hormonal crosstalk represent other potential mechanisms of endocrine disruption. Several agricultural contaminants have been identified as disruptors of the HPT axis in vertebrates (Leemans et al., 2019). Because TH-signaling is highly conserved across vertebrate species, and anuran metamorphosis is TH-dependent (Heimeier and Shi, 2010), amphibians represent ideal animal models to assess endocrine disruption. Contaminants that affect TH-dependent processes have been associated with alterations in the rate of metamorphosis (Heimeier and Shi, 2010; Miyata and Ose, 2012). On the cellular and molecular levels, thyroid gland histology or disruption of the actions of exogenous THs or TH-dependent processes are parameters frequently studied to assess thyroid disruption. Studies on pituitary TSH and the direct measurement of T4, T3 and metabolites in anurans are lacking in general and more specifically as related to endocrine disruption. Measuring and interpreting circulating hormone levels, particularly in small tadpoles, presents a significant technical challenge. Therefore, many studies have used highly sensitive molecular endpoints, such as gene expression of TH-responsive genes, to examine the effects of agrochemicals on the HPT of amphibians (Crump et al., 2002; Lancôt et al., 2013; Navarro-Martín et al., 2014).

FIGURE 1 NEAR HERE

Relevant here is that the developmentally changing levels of T4 and T3, along with tissue-dependent developmental variations in the TRs in anurans drive metamorphosis. Tadpoles are an excellent model system to unravel the complexities of disruption of hormone-dependent developmental processes. Variations in hormone levels superimposed on changing TRs may lead to major differences in sensitivity and response to a given agrochemical (Rosenfeld et al., 2017). Therefore, it is essential that effects be tested at different developmental stages. While this is far from clear, we provide examples of numerous agrochemicals that impact TH-dependent processes in amphibians.

2.1 Fertilizers and Metals

2.1.1 Fertilizers

Nitrogenous fertilizers are commonly applied in agricultural operations and are present in agricultural runoff (Lawniczak et al., 2016). Nitrate and nitrite have been shown to affect the HPT axis in several vertebrate species (Edwards et al., 2006) by competing with uptake of iodide by the thyroid gland, thereby inhibiting TH synthesis (Tonacchera et al., 2004; De Groef et al., 2006; Ward et al., 2010). Therefore, nitrogenous fertilizers represent a class of agrochemicals that may potentially interfere with the HPT axis of wild amphibians.

Several studies indicate that exposure to nitrate altered rates of amphibian metamorphic development with apparent species-dependent sensitivities (Wyngaarden et al., 1953; Xu and Oldham, 1997; Edwards et al., 2006; Ortiz-Santaliestra and Sparling, 2007; Allran and Karasov, 2009; Wang et al., 2015). Chinese toad (*Bufo*

gargarizans) tadpoles exposed to a relatively high concentration (100 mg/L) of nitrate exhibited delayed metamorphosis, reduced circulating T4 and T3 levels, down-regulation of *dio2* and up-regulation of *dio3* mRNA, and partial colloid depletion in thyroid gland follicles (Wang et al., 2015). Interestingly, *B. gargarizans* exposed to 5 - 200 mg/L nitrate at embryonic stages (when maternally-transferred HPT molecular components are present), exhibited an increased rate of development, down-regulation of transporter genes (solute carrier family 16 member 2 (*slc16a2*) and (solute carrier organic anion transporter family, member 1C) *slco1c*) and of *dio2* and *dio3*, and an up-regulation of *trα* (Xie et al., 2019). In this study, T4 levels and *trβ* expression were not affected by nitrate exposure. Similarly, exposure 5 and 50 mg/L nitrate did not report transcriptional alterations of TH-responsive genes (*trβ* and Rana larval keratin type I) in *Lithobates catesbeianus* (American bullfrog) tadpole tail fin tissue (Hinther et al., 2012). Therefore, it is suggested that nitrate does not interact with T4, but disrupts gene expression following conversion to reactive nitric oxide; thereby, disrupting essential proteins, and affecting transcription and mRNA stability (Poulsen et al., 2018). The species-specific sensitivities and the molecular mechanisms by which nitrate disrupts the HPT axis in amphibians remains to be elucidated.

2.1.2 Metals

Metals, such as lead (Pb), copper (Cu), and cadmium (Cd), are aquatic contaminants associated with agricultural operations (Naveedullah et al., 2013). A major source of metal contamination from agriculture is from the application of fertilizer that is manufactured from phosphate rock, which includes trace levels of metals (Sabiha-Javied et al., 2009). Some metals are anti-thyroidogenic and decrease metamorphic

rate, thereby posing a threat to wild amphibians (Thambirajah et al., 2019). Sublethal concentrations of Pb has been shown to inhibit metamorphosis in *Bufo arenarum* (Pérez-Coll et al., 1988, 1990), *L. pipiens* (Northern leopard frog; Chen et al., 2006), *Pelophylax nigromaculatus* (Black spotted frog; Huang et al., 2014) and *B. gargarizans* (Yang et al., 2019). In *B. gargarizans*, Pb exposure at 1000 µg/L caused follicular cell hyperplasia and colloid depletion in thyroid glands, increased the transcript levels of *dio2*, *tra*, and *trβ*, while decreasing *dio3* mRNA levels (Yang et al., 2019).

Copper occurs naturally in the environment; however, deposits of anthropogenic Cu reach surface waters from agricultural runoff. Several studies have demonstrated that Cu exposure can delay the rate of metamorphosis in *L. pipiens* (Chen et al., 2007), *L. sphenoccephalus* (Southern leopard frog; Lance et al. 2012), *L. sylvatica* (Wood frog; Peles, 2013), *B. gargarizans* (Chai et al., 2014; Wang et al., 2016; Chai et al., 2017), and *B. arabicus* (Arabian toad, Barry, 2011). At the histological level, Cu causes follicular hyperplasia in the thyroid gland of *B. gargarizans* (Wang et al., 2016).

Exposure to 32 and 64 µg/L Cu induced an up-regulation of *dio3* expression, but down-regulations of both *tra* and *trβ* (Wang et al., 2016). Similarly, exposure to 64 µg/L Cu induced down-regulation of *dio2*, *dio3*, *tra*, and *trβ* mRNA levels in a separate study of *B. gargarizans* (Chai et al., 2017).

Phosphate-based fertilizers contain varying amounts of Cd, and agricultural interventions have been shown to enhance leaching of geogenic Cd from soil (Hariprasad and Dayanada, 2013). Sub-lethal Cd exposure may have HPT-disrupting effects in amphibians. Cd exposure at 5 and 54 µg/L was found to accelerate metamorphosis in *Anaxyrus americanus* tadpoles (American toad; James and Little,

2003). In contrast, Cd exposure at 855 µg/L Cd arrested metamorphosis in *Xenopus laevis* (African clawed frog; Sharma and Patiño, 2008), and interestingly, these effects were exacerbated in male *X. laevis* tadpoles when estradiol-17β (E2) was present (Sharma and Patiño, 2010). Sharma and Patiño (2008) also observed a reduction in follicle cell height in *X. laevis* tadpoles. Chronic Cd exposure (50, 100, and 500 µg/L) in *B. gargarizans* tadpoles caused retarded metamorphosis, follicular hyperplasia of the thyroid, and downregulated *dio2*, *tra*, and *trβ* transcripts (Sun et al., 2018). Similarly, metamorphosis was arrested at a pro-metamorphic stage in *Pleurodeles waltl* (Iberian ribbed newt) exposed to 1.2 mg/L Cd (Flament et al., 2003). Several studies reported that Cd decreases plasma T4 levels, without altering T3 levels (reviewed in Buha et al., 2018). The direct effect on the thyroid gland is suggested to be due to oxidative stress in mitochondria of follicular cells; thereby, altering production and/or secretion of T4 (Buha et al., 2018).

2.2. Fungicides

The azole fungicides are broad-spectrum fungicides used widely in agriculture. The imidazole fungicide, cyproconazole, adversely affected both the HPT and HPG axes in *P. nigromaculata* (Zhang W et al., 2019). Chronic cyproconazole exposure caused a decreased rate of metamorphic development and exposure to 1 mg/L cyproconazole reduced colloid and follicles of the thyroid gland. Moreover, T3 and T4, and thyroid-related gene transcript levels were significantly affected in a stage- and concentration-dependent manner (Zhang W et al., 2019). Similarly, the triazole fungicide, triadimefon is the most commonly used agricultural fungicide and has been implicated as having anti-thyroidogenic action in amphibians (Zhang W et al., 2020; Li,

Li et al., 2016). In *P. nigromaculatus*, 28-day larval exposure affected the rate of metamorphosis and significantly altered transcriptional regulation (Zhang W et al., 2020). In the brain, transcripts of *tsh β* and *dio2* were upregulated, while *tr β* , *dio2*, *matrix metalloproteinase 2*, *TH-responsive basic leucine zipper transcription factor (TH/bZIP)*, and *sonic hedgehog* genes were up-regulated in the intestine (Zhang W et al., 2020). Interestingly, despite the observed inhibitory effects of triadimefon on development, TH-responsive genes were induced, suggesting a compensatory transcriptional response. Pre-metamorphic (Nieuwkoop and Faber (NF) stage 51) *X. laevis* tadpoles exposed to triadimefon for 21 days exhibited similar adverse effects (Zhang W et al., 2020). Anti-thyroidogenic effects were observed, including reduced rate of metamorphosis, decreased levels of T3 and T4, and an alteration of HPT-related gene expression. The authors conducted *in silico* ligand–TR docking computational analyses, which suggested that triadimefon could interact with TRs; however, direct evidence is lacking.

Mancozeb is a dithiocarbamate that is extensively used in orchard crops and has been shown to induce adverse effects on TH-related developmental processes in frogs. Mancozeb (16, 80, and 400 $\mu\text{g/L}$) reduced growth in a concentration-dependent manner in *L. pipiens* (Shenoy et al., 2009) and induced physical deformities in eye and limb development in *A. americanus* tadpoles (Harris et al., 2000). TH-disruptive action of mancozeb (e.g., decreasing circulating T4 levels) has been characterized in other vertebrates (Nordby et al., 2005; Axelstad et al., 2011; Pandey and Mohanty, 2015), however, the underlying molecular pathways involved in these effects of fungicides in amphibians have not yet been assessed using more sensitive analytical tools.

The imidazole prochloraz, is widely used in Asia, Australia, Europe, and South America. It has been shown to delay metamorphosis, elevate T3 levels, and cause thyroid hyperplasia in *R. temporaria* (European common frog) (Brande-Lavridsen et al. 2008). Similarly, *X. laevis* tadpoles exposed to 20, 60 and 180 µg/L prochloraz exhibited elevated T4 level and mild follicular cell hypertrophy in thyroid gland histology of the 180 µg/L treatment group (Haselman et al., 2018). These effects were observed without concurrent alteration of T3 level or disruption of metamorphic rate. Therefore, it was suggested that prochloraz exposure interfered with the HPT-axis by altering T4 metabolism in the liver and kidney or through affecting the negative-feedback mechanisms. Interestingly, prochloraz has also been implicated in the disruption of other endocrine pathways through aromatase inhibition. Exposure to 115 and 251 µg/L prochloraz lowered plasma testosterone levels, disrupted gonadal differentiation, and caused a male-biased sex ratio in *R. temporaria* (Brande-Lavridsen et al., 2008). It was suggested that prochloraz may act through an anti-androgen mode of action (Haselman et al., 2018), but this is far from clear. Given the potential for prochloraz to disrupt both thyroid and gonadal functions, it will be important to determine the mechanisms of action within the context of hormonal crosstalk (Figure 1).

2.3 Insecticides and acaricides

Organochlorine compounds are established TH disruptors that likely act through competition for binding to TH transport proteins in serum (Cheek et al., 1999b). Dichlorodiphenyltrichloroethane (DDT) is a legacy organochlorine insecticide that persists in the environment with its metabolites, such as dichlorodiphenyldichloroethylene (DDE). Arukwe and Jenssen (2005) reported a down-

regulation of *trβ* in testis and liver of adult male *R. temporaria* exposed to DDE. Similarly, in larval *R. temporaria*, DDE caused a decrease in *trβ* expression in brain and tail tissue that was negatively correlated with tail length and width (Mortensen et al., 2006).

The organochlorine miticide dicofol is structurally related to DDT. In binding affinity assays using [¹²⁵I]T₃ and bullfrog transthyretin (TTR), dicofol was found to have a biphasic effect. T₃ binding to TTR was stimulated at low concentrations (0.4 – 400 nM) and inhibited at a high concentration (40,000 nM) (Ishihara et al., 2003). Similarly, the organochlorine pesticide, methoxychlor, was originally introduced as a replacement for DDT, but was subsequently banned due, in part, to its endocrine disrupting properties (USEPA, 2004). Fort et al. (2004) explored the sensitivity of *X. laevis* to this compound with endpoints related to the HPT and HPG axes. In tadpoles, methoxychlor exposure delayed metamorphosis, reduced circulating T₃ levels, and induced follicular hyperplasia of the thyroid glands at a concentration of 0.1 mg/L. Reproductive assays in adult *X. laevis* indicated that methoxychlor exposure adversely affected fecundity, and reproductive behaviors in both males and females. Both sexes exhibited a concentration-dependent reduction in gonad mass and gamete quantity. In breeding studies where exposed animals were paired with controls, the frequency of amplexus, fertilization, and embryo viability was affected by adult female methoxychlor exposure, and to a lesser extent by male exposure (Fort et al., 2004). Therefore, methoxychlor is an EDC affecting both the HPT and HPG axes in frogs.

Organotin compounds have a variety of applications in agriculture as biocidal agents and have also been implicated in disruption of both HPG and HPT axes (Santos-

Silva et al., 2018). The acaricide azocyclotin is an organotin compound extensively used in many agricultural practices due to its wide mode of action of disrupting ATP formation by inhibiting oxidative phosphorylation (Van Leeuwen et al., 2010). A 21-day exposure of azocyclotin to *X. laevis* tadpoles caused delayed metamorphosis, decreased circulating T3 concentration, and down-regulated expression of thyroid-responsive genes. More specifically, 0.1 and 0.5 µg/L azocyclotin down-regulated the expression of *trβ*, *dio2*, basic transcription element binding protein (*bteb*), matrix metalloproteinase 2, and stromelysin-3 (Li, Cao et al., 2016). Similarly, other organotin pesticides such as fentin hydroxide and fenbutatin oxide, were shown to induce TH disruption by decreasing T3 level in *X. laevis* tadpoles (Li et al., 2019). Both compounds inhibited metamorphic rate and decreased *trβ*, *bteb*, and *dio2* expression; thereby, decreasing T3 level. Transcripts of genes associated with promoting TH production (*tshβ* and *slc5a5*) (Wang et al., 2013) were up-regulated, suggesting a compensatory transcriptional response to decreased T3 levels. It was also found that fentin hydroxide, but not fenbutatin oxide antagonizes T3-TRβ complex binding, which affects the TH-dependent transcriptional regulation (Li et al., 2019). The present literature highlights the need for further investigations into the molecular mechanisms of action of specific organotin compounds.

Carbaryl is a carbamate insecticide that inactivates acetylcholinesterases. Bridges (2000) demonstrated that carbaryl exposure retarded metamorphosis in *L. sphenoccephalus*. In contrast, Boone (2008) reported that carbaryl also accelerated metamorphosis in *L. clamitans* (green frog) and in *A. americanus*. In a subsequent study, Boone et al. (2013) revealed that 3 day exposure to 1 mg/L carbaryl did not affect

L. clamitans metamorphosis, but did induce an up-regulation of *tra* and *trβ* transcripts in the brain, which persisted for several months following metamorphosis. These studies highlight the importance of exposure timing and life-cycle testing, as well as the potential legacy impact of a transient exposure to EDCs.

Since their introduction in the early 1990s, neonicotinoids have become the most commonly used type of insecticide in the world (Jeschke et al., 2011). Although neonicotinoids have been implicated in the disruption of HPT function in lizards (Wang, Xu et al., 2019), birds (Pandey and Mohanty, 2017; 2015), and rats (Ibrahim et al., 2015; Şekeroğlu et al., 2014), there is a relative lack of data concerning their effects in amphibians. In *Eremias argus* (Mongolian racerunner lizard), three neonicotinoids (dinotefuran, thiamethoxam, and imidacloprid) affected the plasma concentrations of T4, and the transformation of T4 to T3 (Wang, Xu et al., 2019). In larval *L. sylvaticus*, exposure to imidacloprid at 10 and 100 µg/L (above current environmental levels), increased survival and delayed completion of metamorphosis compared with controls (Robinson et al., 2017), suggesting that the amphibian HPT axis may be altered by neonicotinoids.

2.4 Herbicides

2.4.1 Atrazine

The triazine herbicide atrazine is used in ~80 countries and has been shown to alter TH production in vertebrates (Ghinea et al., 1979; Kornilovskaya et al., 1996; Stoker et al., 2000). The effects of atrazine on amphibians have been well studied, primarily with endpoints related to reproduction. Early studies did not demonstrate

consistent effects of atrazine on the thyroid function in amphibians (Solomon et al., 2008). It is particularly challenging to interpret the current data due to species-specific sensitivities and non-monotonic effects of atrazine. For example, atrazine reduced *in vitro* corticosterone production in *L. catesbeianus*, but not *X. laevis* (Goulet and Hontela, 2003). In one study, atrazine exposure delayed metamorphosis at 100 µg/L in *X. laevis* (Freeman and Rayburn, 2005). In contrast, other authors report that atrazine exposure in larval *X. laevis* at similar concentrations (40 - 160 µg/L) had no effect on the rate of metamorphosis; whereas, 20 and 320 µg/L of atrazine accelerated or delayed metamorphosis, respectively (Sullivan and Spence, 2003). Similarly, in *L. clamitans*, 10 µg/L, but not 25 µg/L atrazine, delayed metamorphosis (Coady et al., 2005). In larval *Ambystoma tigrinum* (tiger salamander), atrazine exposure delayed and accelerated metamorphosis at 75 µg/L and 250 µg/L, respectively (Larson et al., 1998). Both 75 and 250 µg/L treatments caused an elevation of plasma T4 in pro-metamorphic (stage-IV), but not at pre-metamorphic (stage-II) larvae (Larson et al., 1998).

Several other studies have reported no effects of atrazine on metamorphic endpoints (rate, forelimb emergence, tail resorption) in *X. laevis* (Carr et al., 2003; Coady et al., 2005; Kloas et al., 2009), *Limnodynastes tasmaniensis* (spotted grass frog; Spolyarich et al., 2010), *A. americanus* (Freeman et al., 2005), or *L. pipiens* (Allran and Karasov, 2000; Orton et al., 2006). In an investigation using sensitive gene expression endpoints, low levels of atrazine (1.8 µg/L) significantly reduced metamorphic success (number of animals reaching metamorphosis) without affecting body weight, snout-vent length, or age at metamorphosis in *L. pipiens* (Langlois et al., 2010). In the same study, animals that did not metamorphose, exhibited a decreased

expression of *dio3* in the tail, suggesting that these animals were attempting to compensate for the anti-metamorphic effects of atrazine through a reduction in TH metabolism. Given the potential for disruption of multiple hormonal axes in some amphibian species, targeted investigations on the cellular and molecular basis of atrazine and other triazines (active ingredients and additives to commercial formulations) is clearly warranted.

2.4.3 Phenylurea herbicides

Urea-based herbicides are widely used in agriculture to control grasses and broad-leaf weeds and have been associated with chronic toxicity in vertebrates (Marlatt and Martyniuk, 2017). While there is evidence that these compounds may disrupt the HPT axis in other vertebrates, there is a relative lack of data for these compounds, especially in amphibians. Diuron is a widely used herbicide of the arylurea class that acts by inhibiting photosynthesis. Diuron and its metabolite, 3,4-dichloroanilin are commonly detected in freshwater ecosystems. Both compounds were shown to disrupt TH-related gene expression and plasma T3 level in *L. catesbeianus* tadpoles (Freitas et al., 2016). In transgenic *X. laevis* tadpoles expressing the TH/bZIP-gfp reporter gene, linuron exposure significantly increased fluorescence in the head region without T3 co-treatment (Spirhanzlova et al., 2017), inducing TH-like actions. Additionally, linuron has anti-androgenic (Marlatt and Martyniuk, 2017) and anti-estrogenic effects (Spirhanzlova et al., 2017). Therefore, phenylurea herbicides having multiple hormonal activities, may represent a particular threat to wild amphibian populations and should be further investigated.

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401 **2.4.3 Chloroacetanilide herbicides**

402 Butachlor is a chloroacetanilide herbicide used extensively in rice agriculture that
403 has been identified as a HPT disruptor in vertebrates (Zhu et al. 2014; Li, Wang et al.
404 2016; Shuman-Goodier et al., 2017). Exposure of *Rhinella marina* (cane toad) tadpoles
405 to butachlor increased expression of *trβ*, but did not affect expression of another TH-
406 responsive gene, *Krüppel-like factor 9 (klf9)* (Shuman-Goodier et al., 2017), and did not
407 affect the rate of metamorphosis. In *X. laevis*, butachlor promoted metamorphosis,
408 increased T3 and T4 levels and up-regulated *trβ* expression (Li, Wang et al., 2016).

409 Acetochlor is another chloroacetanilide type herbicide and the third-most
410 commonly detected herbicide in surface waters in the USA (Foley et al., 2008).
411 Acetochlor has been implicated in altering the HPT in other vertebrates (*Gobiocypris*
412 *rarus*, rare minnow; Li et al., 2009), *Rattus norvegicus* (rat; Rollerová et al., 2011),
413 *Danio rerio* (zebrafish; Yang et al., 2015) and accelerating the rate of metamorphosis in
414 *L. pipiens* (Cheek et al., 1999a) and *X. laevis* larvae (Crump et al., 2002). Several
415 studies reported no effect of acetochlor in the absence of exogenous T3 (Cheek et al.,
416 1999a; Crump et al., 2002; Turque et al., 2005). In *L. catesbeianus* and *L. pipiens*,
417 exposure to acetochlor during T3-induced metamorphosis resulted in reduced time to
418 forelimb emergence (Cheek et al. 1999a; Velhoen and Helbing, 2001). Acetochlor
419 induced an up-regulation of *tra* and *trβ* in the brains of premetamorphic *L. catesbeianus*
420 tadpoles and this up-regulation was enhanced by exogenous T3 treatment (Helbing et
421 al., 2006). Moreover, co-exposure of *L. catesbeianus* to acetochlor and T3 induced a
422 synergistic up-regulation of *trβ* (Veldhoen and Helbing, 2001). In T3 pre-treated *X.*

laevis tadpoles, acetochlor amplified the transcriptional response of a TH/bZIP promoter-driven eGFP reporter gene in the head region of transgenic tadpoles (Turque et al., 2005). That acetochlor magnifies the T3-induced response for many up-regulated genes and attenuates the response for down-regulated ones, suggests a mechanism of disruption that involves TH-dependent transcriptional regulation. Because acetochlor does not appear to bind directly to TR β (Cheek et al. 1999b), it has been suggested that epigenetic mechanisms may be involved (Crump et al., 2002; Thambirajah et al., 2019). While plausible, this hypothesis remains to be rigorously tested.

While acetochlor modulates the HPT axis in amphibians, no studies to date have reported alterations of the HPG axis or other reproductive-related endpoints in acetochlor-treated tadpoles. Acetochlor exposure adversely affects reproduction in rats by interacting with high-affinity to uterine ER (Rollerová et al., 2011, 2000). Thus, the effects of acetochlor on amphibian reproduction should be investigated.

2.4.4 Glyphosate

The broad-spectrum organophosphate herbicide glyphosate is used worldwide to kill weeds and grasses in crop fields. The effects of glyphosate on development and growth in several frog species has been reported (e.g., Edge et al., 2011, Edge, Gahl et al, 2014, Edge, Thompson et al., 2014; Bach et al., 2016), but few studies have examined molecular effects to elucidate its mode of action. In the first studies to investigate the molecular effects of glyphosate exposure, Roundup® formulations were shown to disrupt steroidogenesis in mouse Leydig tumour cell line through disruption of

steroid acute regulatory protein (StAR) expression (Walsh et al., 2000). In several frog species, Roundup® increased *trβ* mRNA (Howe et al., 2004).

Laboratory exposures to the glyphosate-based herbicide VisionMax® had direct effects on the metamorphosis of *L. sylvaticus* tadpoles by decreasing their development rates when exposed to 2.9 mg of acid equivalents (a.e.) per L (Navarro-Martín et al., 2014). These glyphosate-exposed animals took an additional 7 days to reach metamorphic climax when compared to controls. Tadpoles exposed to VisionMax® were larger in size and in weight with respect to the control group, but no effects on the condition factor were observed. At the molecular level, VisionMax® reduced normal developmental changes in tail *trβ* or *dio3*, glucocorticoid receptor (*gr11*) mRNA levels in *L. sylvaticus* tadpoles, suggesting a partial inhibition of the stress axis. The authors hypothesized that the up-regulation of transcripts triggering metamorphosis, such as *trβ* or *dio3*, could be the result of a compensatory response to prevent developmental delay (Navarro-Martín et al., 2014). These data also suggest that VisionMax® could shift the energy expenditure from development to growth.

In an attempt to mimic a agriculturally-relevant scenario, a parallel study was conducted to evaluate the effect of the Roundup WeatherMax®, Roundup Vision® as well as isopropylamine salt of glyphosate (IPA) and the polyethoxylated tallowamine (POEA) surfactant of Vision® by exposing *L. sylvaticus* tadpoles to two pulse applications (Lanctôt et al. 2014). In this case, developmental rates were not affected and only differences in the condition factor of animals exposed to IPA and POEA were observed, demonstrating that milder effects resulted from environmentally realistic exposures. Nevertheless, this study demonstrated that exposures were able to alter the

developmentally-regulated increases of mRNA levels of key genes controlling development, such as *trb*, *grll*, *dio2* and *dio3*, in tail and/or brain. These results reinforce the idea that a compensatory mechanism to regulate TH levels may occur in tadpoles exposed to glyphosate-based herbicides, but the authors did not measure TH levels. Although no significant effects were found related to growth and development, these results pointed out the need to elucidate the molecular mechanisms of action that could translate to long-term effects on fitness in juveniles or adults exposed to glyphosate-based herbicides during early development.

Another study assessed the effects of agriculturally-relevant concentrations of glyphosate-based herbicides on whole ecosystems using *in situ* split pond design of wetland exposures to Roundup WeatherMax® (Lancôt et al., 2013). Although small significant differences in growth and development were found in exposed *L. sylvaticus* tadpoles compared to controlled wetlands, trends were not consistent between years or ponds. Based on this, the authors concluded that pulse exposures of Roundup WeatherMax® may pose minimal risk to larval amphibian development. Interestingly and in line with the laboratory studies, however, exposed tadpoles from some wetlands presented altered mRNA levels of thyroid and stress related genes, supporting the idea that glyphosate-based herbicides have the potential to alter hormonal pathways during tadpole development. These results raised several questions that remain to be answered: 1) would these alterations at the molecular level be translated into functionally biological changes in juvenile or adult later life? 2) Are the observed changes due to direct exposure to herbicides or an indirect consequence of alteration of food resources by these chemicals?

2.5 Agrochemical mixtures:

Considering that agrochemicals are present in the environment in complex mixtures, there is a lack of data examining the effects of environmentally relevant mixtures of agrochemicals on the amphibian HPT axis. Mosconi et al. (2005) documented elevated plasma T3 and T4 frogs (*Pelophylax lessonae* and *P. esculenta*) captured in Italy at agricultural sites compared to those from pristine areas. However, this study did not characterize or quantify the agrochemicals at each site, nor did they examine other TH-related cellular or molecular endpoints. Hayes et al. (2006) were amongst the first to specifically address the effects of pesticide mixtures in multiple endocrine systems. In a complex study of *L. pipiens* and *X. laevis* tadpoles, the effects of low doses four herbicides, two fungicides, and three insecticides used on american cornfields were assessed. The important conclusion was that study of single pesticide exposures is underestimating likely effects on growth, metamorphosis, and stress hormone production. In some cases, mixtures can negate effects, and in other cases mixtures can synergize. Recently, *A. americanus* tadpoles were exposed to water from a retention pond receiving runoff from a pesticide-treated corn operation. Results indicated that the complex mixture of agrochemicals accelerated metamorphic timing and induced a significant up-regulation of *trβ* and *dio3* mRNA in liver at metamorphic climax (Thomson and Langlois, unpublished) indicating the importance of conducting ecotoxicological work in real-world settings. It is clear that future studies must consider relevant mixtures to determine the potential endocrine disruption actions of agrochemicals.

3. Disruption of growth and metabolism

Life-history theory states that animals have to partition limited resources between growth and reproduction, and hormones control these processes (Figure 1). Since resources are limited there are physiological constraints at the larval and the adult stages that cannot be modified without causing deleterious effects on growth and reproduction (Lardner and Loman, 2003). At the larval stage, disruption of metabolism can have a profound and lasting effect across the lifespan of an organism (Mueller et al., 2015). Indeed, larval development rates determine the period of water availability in temporary ponds and the size and weight at metamorphosis which are all directly linked with mortality. At the adult stage, disruption of metabolism can mimic reduction of resources which is known to increase the risk of mortality associated with reproduction. In such events, skipping reproduction for allocating resources to growth might postpone reproductive event at the following year (Lardner and Loman, 2003) and maintaining reproduction might lead to progeny with reduced growth capacities (Regnault et al., 2018). Amphibians are particularly exposed to agrochemicals through water during periods critical for optimal metabolism, growth or reproduction, (Fan et al., 2007a, 2007b; Hayes et al., 2011; Duarte-Guterman et al., 2014).

Exposure to agrochemicals during tadpole development has been widely studied and among effects reported alteration of growth has been associated to all types of pesticides. Concerning herbicides, atrazine, and glyphosate have been the most studied for their impacts on tadpole development. Whereas exposures to low environmentally relevant concentrations have not been associated with significant effects on growth (Carr et al., 2003; Coady et al., 2005; Orton et al., 2006) or minor effects (Zaya, Amini,

Whitaker, Ide, 2011; Zaya, Amini, Whitaker, Kohler, Ide, 2011), higher concentrations of atrazine have been shown to decrease snout vent length, body weight (Diana et al., 2000; Sullivan and Spence, 2003), and survival at metamorphosis (Hoskins et al., 2018). Discrepancy between low doses and high doses atrazine have been associated with non-monotonous effects (Diana et al., 2000; Freeman et al., 2005; Coady et al., 2005). For example, exposure to atrazine resulted in a significant acceleration of metamorphosis at 1 mg/L and a significant increase in the duration of metamorphosis at 5 mg/L, whereas no significant difference was observed with 0.1 mg/L (Brodeur et al., 2009). Many studies have shown that exposures to glyphosate and formulations with POEA and POEA alone increased mortality and decreases size and developmental rates of tadpoles (Edginton et al., 2004; Howe et al., 2004; Relyea, 2004, 2005, 2012; Relyea and Jones, 2009; Jones et al., 2010, 2011; Williams and Semlitsch, 2010; Moore et al., 2012; Lanctôt et al., 2014). Concerning insecticides and fungicides, fewer data are available, but a direct link between exposure to these classes of agrochemicals and growth alterations has been established for the insecticides carbaryl (Boone et al., 2013), α -cypermethrin alone (Xu and Huang, 2017) or in combination with the herbicide oxadiazon (Mesleard et al., 2016), the carbamate methomyl (Trachantong et al., 2017) and the fungicides copper (Peles, 2013), triphenyltin (Higley et al., 2013) and cyproconazole (Zhang W et al., 2019).

In several studies, alterations of tadpole growth under pesticide exposure have been associated with metabolic disruption. Tadpoles are required to balance their limited energy reserves for maintenance, growth, metamorphosis, and locomotion, while maintaining adequate energy stores for basal metabolism (Melvin et al., 2016).

560 Chemical exposure (e.g., naphthenic acids) during sensitive developmental stages can
561 disrupt energy balance in tadpoles (Melvin et al., 2013) by requiring an animal to
562 allocate more energy towards detoxification pathways or altering various metabolic
563 processes that may ultimately influence energy homeostasis (Melvin et al., 2016).
564 Tadpoles exposed pesticide-contaminated water presented decreased levels of
565 biochemical constituents, such as glycogen, suggesting compensatory metabolic
566 mechanisms (Strong et al., 2016, 2017). The relation between atrazine exposure and
567 modification of tadpole metabolism and growth was first studied by Langerveld et al.
568 (2009) in *X. laevis*. In this study, exposure to atrazine at 400 µg/L was associated to a
569 reduction in fat body size, lipid storage organ, suggesting an energy deficit in these
570 tadpoles (Langerveld et al., 2009). In addition, *X. laevis* tadpoles exposed throughout
571 development to 200 or 400 µg/L atrazine, were smaller and had smaller fat bodies.
572 These morphological parameters were associated with an increased transcription of
573 genes involved in the conversion of lipids and proteins into energy and an increase in
574 ADP:ATP ratios at the end of exposure suggesting tadpoles may have had difficulty in
575 maintaining energy homeostasis (Zaya, Amini, Whitaker, Ide, 2011; Zaya, Amini,
576 Whitaker, Kohler, Ide, 2011). These metabolic effects of atrazine were confirmed in *A.*
577 *americanus* and *Hyla versicolor* (grey tree frog), where exposed tadpoles displayed
578 perturbations in a number of classes of biological macromolecules, including fatty acids,
579 amino acids, purine nucleosides, pyrimidines, and mono- and di-saccharides. These
580 results suggested that higher concentrations of atrazine (≥ 250 µg/L) impact energy
581 metabolism of larval amphibians by diverting energy to detoxification, tissue repair and
582 restoring homeostasis (Snyder et al., 2017). Moreover, atrazine, glyphosate, and

quinclorac exposure induced a significant decrease in levels of glycogen, proteins, cholesterol, and total lipids in gill, liver, and muscle in *L. catesbeianus* (Dornelles and Oliveira, 2014), suggesting that several classes of herbicides might be associated to metabolic disorders. *Microhyla fissipes* tadpoles (farmland frog) exposed to 60–120 mg/L glyphosate-based herbicides showed preference for warm temperatures, exhibited a suppressed growth, and a reduced tail development, together with the down-regulation of carbohydrate and lipid catabolism and the up-regulation of the amino acid catabolism. These results suggested a trade-off between protein synthesis and energy production with respect to amino acid allocation, and provide a metabolic explanation as to why growth was suppressed tadpoles exposed to a glyphosate-based herbicide (Wang, Chang et al., 2019).

Molecular mechanisms and hormonal disruption associated with metabolic disorders have been poorly studied in tadpoles. Insight into comprehensive mechanism of pesticide impacts have been pinpointed with the fungicide triphenyltin in *L. sylvaticus* and atrazine in *X. laevis*. In *L. sylvaticus* larvae that survived to metamorphic climax during exposure to triphenyltin for as long as 100 days, abundance of transcripts of peroxisome proliferator-activated receptors (*ppar* α and *ppar* γ), cytochrome p4504B1 (*cyp4b1*), fatty acid synthase (*fas*), and lipoprotein lipase (*lpl*) were greater than in controls, suggesting that an up-regulation of lipid metabolism might have been important for survival and development of these animals (Higley et al., 2013). In *X. laevis* tadpoles exposed to atrazine, a decrease in glucocorticoid receptor protein and an increase in *ppar* β mRNA were observed, suggesting receptor down-regulation in response to a glucocorticoid surge associated with changes in energy status and lipid

metabolism (Zaya, Amini, Whitaker, Ide, 2011; Zaya, Amini, Whitaker, Kohler, Ide, 2011). These results suggest that tadpoles exposed to pesticides undergo a global stress response that might involve the glucocorticoid axis, and increased metabolism may be a compensatory mechanism to promote survival (Higley et al., 2013; Strong et al., 2017).

Alteration of tadpole growth has been also associated with parental disruption of metabolism following exposure to endocrine disruptors. Although not yet documented for agrochemical exposure, this pattern has been clearly demonstrated with the polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP) and the organochlorine, triclosan (TCS). Both acute and chronic exposure to BaP and TCS can induce marked metabolic disorders in *Silurana tropicalis* (Western clawed frog) associated with impaired lipid and carbohydrate metabolisms (Regnault et al., 2014, 2016, 2018; Usal et al., 2019). Molecular mechanisms clearly demonstrated that the insulin-regulated processes were affected by EDC exposure. Indeed, female *S. tropicalis* exposed from tadpole stage to BaP or TCS at concentrations of 50 ng·L⁻¹ displayed glucose intolerance syndrome, liver steatosis, liver mitochondrial dysfunction, liver transcriptomic signature, and pancreatic insulin hypersecretion, all typical of a prediabetes state. Moreover, after 1 year of depuration, *S. tropicalis* that had been exposed to BaP still displayed hepatic disorders and a marked insulin secretory defect resulting in glucose intolerance, suggesting a diabetes-like state (Regnault et al., 2018). The exposed animals produced progeny that metamorphosed later, were smaller and lighter at metamorphosis, and had reduced reproductive success, thus linking metabolic disorders in genitors to tadpole development impairments. These multigenerational

effects have been associated to decreased-lipid levels in eggs, but epigenetic DNA modifications could also occur (Regnault et al., 2018; Usal et al., 2019).

Despite the absence of clear correlations between disruption of metabolism by agrochemical in adults and decreased growth capacities of progeny, impacts of pesticide exposure and metabolism impairments in post-metamorphic amphibians have been clearly demonstrated (Van Meter et al., 2018). In adults, disruption of lipid, carbohydrate, and protein metabolism following insecticide exposure has been shown. For example, a decrease in the total lipid content of liver, muscle, testes, and ovaries were observed in *Minervarya andamanensis* (Andaman frog) with lethal concentration of malathion, whereas exposure to sub-lethal concentrations were associated to an increase in total lipids, free fatty acids, glycerol, and lipase activity (Gurushankara et al., 2007). A negative relationship between p,p-DDE exposure and protein and carbohydrate storage associated with increased energy consumed has been shown in *Xenopus muelleri* (Müller's clawed frog) (Wolmarans et al., 2018). *L. sphenoccephala* exposed to single, double, or triple pesticide mixtures of bifenthrin (insecticide), metolachlor (herbicide) and triadimefon (fungicide) presented an increase in amino acids and other key metabolites concentrations in liver, suggesting depletion of energy resources (Glinski et al., 2018). *Bufo regularis* (African toad) exposed to endosulfan showed an increase in serum glucose levels with a concomitant reduction in liver glycogen, indicating disorders in carbohydrate metabolism (Isioma and Lawrence, 2013). In addition, an inhibition of normal oxidative metabolism associated to lactic acid accumulation in blood has been shown following exposure of *Hoplobatrachus tigerinus*

(Indus Valley bullfrog) to the organophosphate phosalone (Ramalingam and Kasinathadurai, 1989).

4. Disruption of sexual differentiation

As in many other vertebrate species, both female and male heteromorphic sex determining systems occur in amphibians. However, in the majority of species, sex is genetically determined by homomorphic sex chromosomes (Bachtrog et al 2014), suggesting that a variety of sex-determining genes exist (Ito 2018). Sexual differentiation in amphibians is driven by sex steroid hormones. In this regard, sexually dimorphic expression of steroidogenic-related genes, for example the cytochrome P450 19 (*cyp19a1*), that converts androgens to estrogens, and the cytochrome 17 α -hydroxylase/17,20 lyase (*cyp17a1*), responsible for the conversion of progestogens to androgens, have been observed in different frog species, including *S. tropicalis* and *L. sylvaticus*. These two genes can be used as biomarkers of phenotypic sex (Navarro-Martin et al., 2012). As observed in birds, reptiles, and fish, frog gonadal development is sensitive to sex steroids (Hayes, 1998). In frogs, this process takes place mostly during the aquatic larval period making these organisms very sensitive to environmental EDCs. Moreover, many agrochemical applications occur in Spring, which coincides with the seasonal period of frog spawning and sensitive phases of tadpole development. Sex steroids are key players in gonadal differentiation and their action can be disrupted by estrogenic, androgenic, anti-androgenic, and progestagenic chemicals used in animal agriculture. Up to now, literature has focused on gonadal morphology, sex ratios (presence of intersex, as well as sex reversal as endpoints for studying endocrine disruption). The presence of intersex gonads in frogs have been observed in agricultural

674 areas and after exposure to atrazine (Hayes, Collins et al., 2002, Hayes, Haston et al.,
675 2002; Hayes et al., 2003; McCoy et al., 2008). In contrast, recent studies (Lambert et
676 al., 2019). showed that frequency of intersex and sexual genotype-phenotype
677 discordance were not correlated in *L. clamitans* collected from ponds found in
678 underdeveloped landscapes, forested landscapes, or suburban neighborhoods,
679 suggesting that the mechanism by which they occur may be different. Lambert et al.
680 (2019) concluded that the presence of intersex or sex reversal was not associated with
681 land use, suggesting that sex reversal in natural scenarios could be related to multi-
682 stressor responses (including temperature). Clearly, many questions remain
683 unanswered. Directed research must be conducted to link early agrochemical exposure
684 during sexual development with adult frog gonadal status months or years later.

685 Nevertheless, compelling evidence from laboratory and mesocosms studies of
686 feminizing or masculinizing effects in frogs by many environmentally relevant chemicals
687 (such as pharmaceuticals, organic persistent pollutants (PCBs, PBDEs, BPA, PFOS)
688 pesticides and chemical mixtures among others) have been elegantly summarized
689 (Orton and Tyler 2015; Slaby et al., 2019). Many of these compounds are not directly
690 relevant to agricultural practices but serve to demonstrate that sex differentiation
691 processes are sensitive to a range of EDCs. For example, finasteride (5-alpha
692 reductase inhibitor, the enzyme that converts T to DHT) was found to induce feminizing
693 effects, while the aromatase inhibitors flavone (MacKenzie et al., 2003) and fadrozole
694 (Duarte-Guterman et al., 2009) induce masculinization. Therefore, it has been
695 demonstrated that exposure to synthetic steroids or chemicals can affect steroid
696 biosynthesis and disrupt gonadal differentiation in anurans.

Many pesticides alone or combined in mixtures have the ability to act as androgen receptor (AR) antagonists *in vitro* and *in vivo* (Orton et al., 2011; 2012). Feminizing effects has been observed in *S. tropicalis* metamorphs exposed to 9 µg/L of linuron (Orton et al., 2018). The authors conclude that a disruption in the *ar/foxl2* signaling during gonadal differentiation could be responsible of the observed feminized sex ratio. A comparative toxicogenomics database analysis using transcriptomic datasets suggested that phenylurea herbicides, such as linuron and diuron, exert similar transcriptomic responses as the ones produced by reproductive hormones (Marlatt and Martyniuk 2017). In particular, linuron was found to affect transcripts related to steroid hormone biosynthesis and metabolism of xenobiotics by cytochrome P450, among others. Numerous studies have revealed effects leading to gonadal feminization caused by atrazine in many vertebrate species (Hayes et al. 2011); however, several researchers have failed to demonstrate anuran feminization (reviewed by Orton and Tyler, 2015). In addition to this controversy, the mode of action of atrazine remains unclear and needs to be elucidated. From one side, it was shown that it can perturb steroidogenesis *in vitro* in amphibian tissues (Goulet and Hontela, 2003; Orton et al., 2009), overstimulate endogenous estrogen production (Crain et al., 1997; Sanderson et al., 2001), up-regulate of brain *era* mRNA levels as well as disrupting of 5β-reductase activity which regulates androgen bioavailability by catalyzing the conversion of testosterone to 5β-dihydrotestosterone (Langlois et al., 2010). On the other hand, *in vitro* receptor binding assays failed to demonstrate that atrazine directly interact with either the estrogen or androgen receptors (Kojima et al., 2004; Orton et al., 2009) and does not alter aromatase activity *in vivo* (Rohr & McCoy, 2010).

Masculinizing effects in amphibians have also been observed by exposure to androgens and progestogens. Testosterone and trenbolone acetate (a synthetic androgen) are used as growth promotants (to increase muscle growth and appetite) in animal agriculture. Exposure to trenbolone acetate has been shown to masculinize *P. nigromaculatus* (Li et al., 2014) and *S. tropicalis* (Olmstead et al., 2012), accelerate Müllerian duct regression in *X. laevis* (Haselman et al., 2016), induce adverse effects on gonadal morphology and differentiation in *X. laevis*, *Bufo viridis*, and *Hyla arborea* (Rozenblut-Kościsty et al., 2019). Although the reproductive effects of trenbolone acetate have been reported, cellular and molecular mechanism of action remains unknown.

A very recent study has shown alterations of gonadal development and the synthesis of endogenous hormones in adolescent mice. This study demonstrates a decrease of serum testosterone levels in mice after trenbolone exposure together with the downregulation of the expression of three genes (*3b-HSD*, *P450scc* and *Cyp17a1*) involved in steroidogenesis (Zhang S et al., 2019). Progesterone (P4) and its synthetic analogue, melengestrol acetate, a veterinary pharmaceutical used to suppress estrus and promote growth in cattle (Orlando and Ellerstad, 2014), were shown to alter expression of sex-steroid-related genes in *S. tropicalis* embryos (Thomson and Langlois, 2018). Relatively high concentrations of P4 have been implicated in male-biased sex ratios in amphibians (reviewed in Hayes, 1998). The mechanism of androgenic action is possibly through the AR, following biotransformation of P4 to an androgenic steroid (Lutz et al., 2001); however, further investigation is required to confirm this hypothesis.

Masculinizing effects can be induced not only directly by interacting with ARs, but also indirectly by blockade of estrogen synthesis through aromatase inhibition. For example, when the irreversible aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) was implanted intraperitoneally into *L. catesbeianus* tadpoles, gonadal abnormalities at the histological level were observed (Yu et al., 1993). These included degenerating ovaries with regressed oocytes (4 %), intersex (17 %), and full differentiation into testes (79 %). Moreover, these gonadal alterations were accompanied by decreased E2 and increased T secretions (Yu et al., 1993). Agrochemicals may exert adverse effects in amphibians by acting through similar mechanisms. For example, the acaricide azocyclotin may have both androgenic and anti-estrogenic action in *X. laevis* (Li et al., 2017). Tadpoles exposed chronically (4 months) to 0.05 - 0.5 µg/L exhibited a male-biased sex-ratio, increased T and pregnenolone levels, hermaphroditic gonadal development, and altered gene expression of steroidogenic enzymes (cyp17 and aromatase). Another example can be found in *R. temporaria* in which waterborne exposures from hatch to metamorphosis to the fungicide and aromatase inhibitor prochloraz (115 and 251 µg/L) significantly increased the proportion of histological males (80-85.7 %) and hermaphrodites in the final population (Brande-Lavridsen et al., 2008).

Levonorgestrel (LNG) is a contraceptive progestagen not usually associated with agriculture, but may be applied to fields through human sewage applications (Tang et al., 2012), and can serve here as a model progestagen. So far, no effects on sex ratios have been observed by exposures to LNG during tadpole development in *X. laevis* and *S. tropicalis* (Kvarnryd et al 2011, Lorenz et al 2011, Säfholm et al 2016).

However, effects on ovarian and oviductal development were observed in adults, demonstrating that developmental exposures can have long-term effects (Kvarnryd et al 2011). These long-term effects seem to be mediated by perturbations in the anti-mullerian hormone and progesterone signalling since LNG exposures altered *amh*, *amhr2*, *ipgr* and *mpgr beta* mRNA levels in a concentration-, sex- and developmental stage-specific manner (Säfholm et al 2016). Given that synthetic proestrogens can affect development, it will be important to screen agrichemicals for progesterone-like activities in amphibians.

Amphibians have divergent patterns of sex determination, sexual differentiation, and development, and therefore, variable responsiveness to hormones and EDCs. The degree of sensitivity a species has to a particular contaminant must be considered when assessing the toxicity of a compound. Piprek et al. (2012) classified amphibians into two categories: highly sensitive and less sensitive species. Rank sensitivities depends not only on the species being studied, but also on the type of EDC being tested (Bridges and Semlitsch, 2000). Another important factor to be considered is the different sensitivities of tadpole life stages (Hogan et al., 2008), indicating that prior to any comparative study, windows of exposure sensitivity assessments are required. Comparative studies have examined the spectrum of amphibian sensitivities to sex steroids and EDCs at both the family and population level. *L. pipiens* has been reported to be more susceptible to sex reversal and to develop intersex gonads than *L. sylvaticus* (Mackenzie et al., 2003). However, *L. sylvaticus* appeared to be more sensitive than *L. pipiens* when developmental endpoints, such as mortality and body size were examined following similar estrogenic exposures (Hogan et al., 2006). When sex ratios and

gonadal histology were assessed in three frog species (*H. arborea*, *B. viridis* and *X. laevis*) exposed to bisphenol A (BPA), the intensity of these effects varied across the three families (Hylidae, Bufonidae and Pipidae) (Tamschick, 2016; Tamschick et al., 2016). There may be some relevance of BPA to agriculture, as in some cases, human sewage is being applied to fields as fertilizer, and thus BPA has been detected in agricultural soils (Zhang Z et al., 2015). Another pollutant of concern are polychlorinated biphenyls (PCBs) that have been associated with the use of organic waste as fertilizers in agricultural soils in some countries (Antolín-Rodríguez et al., 2016). A study that assessed effects of PCBs on *X. laevis* found that there was no effect of PCB3 and PCB5 on the proportion of females, but they observed a reduction of males with morphologically normal testes (Qin et al., 2003). Compared to *L. clamitans*, *L. pipiens* tadpoles were found to have lower hatching success and survival with higher incidences of edema when exposed to PCBs (Rosenshield et al., 1999). These results suggest that different species sensitivity related to sex differentiation might exist for this compound. Further evidence in *X. laevis* supports variations among population responsiveness when sex ratios were evaluated following E2 exposure (Luong, 2016). In this regard, Orton and Routledge (2011) studied differences in growth, metamorphic development, and sexual differentiation in eggs from *Bufo bufo* (common toad) collected from sites with different agricultural intensity activities. Their results suggested that maternal exposures and/or *in ovo* events could have more influence on this developmental process than the tadpole rearing environment. For that reason, they concluded that traditional exposure scenarios (starting at the larval stage) could be missing crucial developmental windows to assess amphibian sensitivity to pollutants.

One of the challenges to assess the effects of agrochemicals in aquatic organisms is that in most cases, the manufacturers do not provide the information on the full composition of formulations and that the same active compound can be manufactured under different names and by different companies. A clear example is the glyphosate-based herbicides. For example, a study carried out using glyphosate-based herbicides containing POEA as the surfactant (Roundup Original® and Roundup Transorb® formulations) revealed the presence of gonadal abnormalities in *L. pipiens*, but no effects were observed when glyphosate was tested alone (Howe et al., 2004). However, no observed effects in sex ratios or gonadal abnormalities were observed in *L. sylvaticus* exposed to VisionMax® under chronic laboratory conditions or to Roundup WeatherMax® under environmental pulse laboratory and field conditions (Lanctôt et al., 2013, 2014, Navarro-Martín et al., 2014).

5. Disruption of anuran vocalization

Vocalization is the main form of communication in anurans, conveying information on identity, location, and quality. Vocalization is sexually dimorphic, with males producing advertisement calls to attract females for mating. As such, vocalization is influenced by both inter- and intra-sexual selection.

Androgens play an important role in male courtship and reproduction (Figure 1). Amplexus, the mating position of anurans, occurs when a male clasps a female to externally fertilize the eggs. In *X. laevis*, amplexus and vocalization can both be induced by human chorionic gonadotropin (hCG), an analog of luteinizing hormone which triggers gonadal steroid production. In castrated adult males, hCG no longer promotes calling. However, exogenous testosterone and dihydrotestosterone both restore calling,

and laryngeal muscles are dense in AR, suggesting that the behavior is dependent on testicular androgens (Wetzel and Kelley 1983). Calling was also inhibited by castration and restored with exogenous androgens in several other species such as *Hyla cinerea* (green tree frog; Burmeister and Wilczynski 2001). In *L. pipiens*, amplexus and vocalization were induced in intact males with pituitary implants, inhibited in castrates, and restored with testicular implants (Palka and Gorbman 1973).

Atrazine has been associated with feminization in frogs. Male *X. laevis* exposed to atrazine from embryo to adult had lower plasma testosterone levels than controls, and were out-competed by controls when attempting to mate with females. They also displayed female-like morphology, including changes in larynx structure, vocalization quality was not assessed (Hayes, Khoury et al., 2010). Vinclozolin, a commonly used fungicide with anti-androgenic effects, affected songs of *X. laevis*. Exposed males experienced a decrease in advertisement calls and an increase in types of call indicating sexual non-receptivity (Hoffmann and Kloas 2010). A recent metabolomic study found that *Limnodynastes peronei* (striped marsh frog) larvae exposed to atrazine presented alterations in steroidogenesis and cholesterol metabolism and suggested that detected changes in formic acid could be related to alterations in the steroidogenic conversion of androgens into estrogens (Melvin et al., 2018). Exposure to flutamide, a model compound for anti-androgenicity, also reduced advertisement calls (Behrends et al. 2010). As expected, opposite effects were produced with the potent androgen agonist methyl-dihydrotestosterone (MDTH; Hoffmann and Kloas 2012a). This androgen is used in fish aquaculture, so there is a possibility for amphibian exposures to occur because of. water waste disposal. The DDT metabolite DDE was found to

858 provoke both anti-androgenic-like effects, such as a decrease production of
859 advertisement calls, and estrogenic-like effects, denoted by an increased amount of
860 rasping and a slightly higher bandwidth (Hoffmann and Kloas 2016). This duality was
861 found to be concentration-dependent with lower DDE concentrations (10^{-11} M) resulting
862 in estrogen-specific responses, while higher concentrations (10^{-9} M) affected producing
863 anti-androgenic responses.

864 Androgens also contribute to the development of sexually dimorphic calls.
865 Gonadectomized juvenile *X. laevis* of both sexes given testicular implants produce male
866 advertisement calls as adults, but only if the frogs received the implant at <6 months
867 old, suggesting a critical period. This is likely related to sexually dimorphic vocal organs,
868 which has been extensively described in *X. laevis*. Nerves in male laryngeal muscles
869 are able to contract and relax more quickly than in females, allowing the production of
870 rapid trills that make up the advertisement calls. Males also have more fast-twitch fibers,
871 while females have more slow-twitch. The surge in THs during metamorphosis is
872 required for the larynx to become androgen-sensitive (Cohen and Kelley 1996;
873 Robertson and Kelley, 1996). As thyroid disruption has been well-studied in tadpoles
874 and juvenile frogs, effects on adult laryngeal biomechanisms and ability to vocalize as
875 adults could be a future area of research. Since atrazine can feminize larynx
876 morphology in adult male frogs (Hayes, Khoury et al. 2010), sound quality is likely to be
877 disrupted.

878 Estrogens can also affect vocal communication. Pharmaceutical estrogens enter
879 water through use of contraceptives, but livestock waste is also a major source of
880 estradiol, estrone, and estriol (Hanselman et al., 2003). In male *X. laevis* exposed to

17 α -ethinylestradiol (EE2), total calling duration was not affected, but a lower proportion of the calls were advertisement calls, which also had fewer clicks. They also produced more rasping calls, indicating a sexually unreceptive state (Hoffmann and Kloas 2012b). The effects of EE2 were reversed by co-exposure to either tamoxifen or fluvastatin, both pharmaceutical anti-estrogens (Hoffmann and Kloas 2012c). Estrogens had contrasting effects in the Western clawed frog (*S. tropicalis*); exposure to estradiol and 4-tert octylphenol increased the number of calls from males (Schwendiman and Propper 2012). This may be due to species differences, or how data was collected. Behaviors were noted by an observer rather than recorded with microphones, which is difficult to do so accurately as calls are underwater. Duration and type of call was also not evaluated in this study.

As males produce mating calls, females respond with phonotaxis, or movement relative to acoustic stimuli. Playback experiments have demonstrated hormone-dependent phonotaxis towards conspecific calls. In *Engystomops pustulosus* (túngara frogs), injections of hCG led to a dose-dependent increase in plasma E2, as well as increasing phonotactic response to male calls. Phonotaxis could also be induced with exogenous E2 alone, and co-injection of E2 and P4 (Chakraborty and Burmeister 2009). Exposure to recordings of male choruses also increased plasma E2, demonstrating the physiological importance of E2 (Lynch and Wilczynski 2006).

Phonotaxis has been poorly studied in the context of endocrine disruption. Direct disruption was tested with female *X. laevis* exposed to the androgen agonist MDHT. Exposed female frogs showed a slightly stronger phonotactic response, although the effect was only significant at one dose on two of four days tested (Hoffmann and Kloas

2012a). Indirect endocrine disruption was tested with *X. laevis* using playbacks of males exposed to EE2 and females preferred calls from control males over exposed (Hoffmann and Kloas 2012b). In both these cases, phonotaxis was measured as time spent near the speaker, which may or may not be due to attraction to the sound, and the effect sizes were small. Since E2 is able to induce phonotaxis, effects of other (anti)-estrogenic compounds on phonotaxis should be examined. While estrogenic compounds may increase phonotactic response, preference and selectivity may be affected. Using established attractive and unattractive playbacks for *E. pustulosus* female choosiness was found to decrease as receptivity (latency to approach) increased. The less selective frogs were also closer to egg-laying (Lynch et al. 2005). Mate choice is conditional, and a large body of evidence shows that higher quality females show a stronger preference for attractive traits in males (Cotton et al., 2006). For example, larger female *Hyperolius marmoratus* (African reed frogs) show a stronger preference for low-pitched male calls (Jennions et al., 1995). Female crickets on a high-quality diet show stronger preference for high call rate and responded faster to playbacks with typically preferred features (Hunt et al., 2005). Therefore, negative effects of EDCs on stress and overall health may further disrupt mate choice.

Behavioural effects of endocrine disruption have not been as well-studied compared to other endpoints. While underused, behaviour can be an effective indicator as it is the output of integrating neural, endocrine, anatomical, and physiological responses to EDCs. Measurement of behavioural endpoints have the practical advantage of being non-lethal, allowing for repeated measures and long-term studies. Methods should be standardized, especially for more cryptic and subjective behaviors

like phonotaxis. However, vocalization is much more obvious and can be a particularly easy and cost-effective endpoint to measure. Previous studies have largely analyzed acoustic waveforms manually, however the process could be automated for higher throughput and accuracy.

6. Conclusions and future directions

Evidence suggests that many classes of agrochemicals, including fertilizers, fungicides, insecticides, acaricides, herbicides, metals, and mixtures negatively affect endocrine signaling in amphibians. In most cases, there is very little known about the mechanisms of actions of these chemicals to lead to adverse outcomes in development, growth, metabolism and reproduction. In numerous cases, investigators observe effects on gene expression endpoints targeting specific endocrine pathways, but it is unclear if these are linked to the apical responses, or the result of the downstream effect on metamorphosis, sexual development, or some other phenotype observed.

Thyroidal physiology is essential to development and metamorphosis in anurans, however, the underlying molecular pathways involved in disruption of growth and metamorphosis in amphibians have rarely been assessed using sensitive analytical tools. Given that the general plan of hypothalamo-pituitary control of endocrine physiology is known (Fig. 1), is it surprising that few studies have attempted to quantify effects at the brain and pituitary. Future investigations should further categorize compounds implicated in the alterations of metamorphic rate through analysis of molecular endpoints (e.g., gene expression of TH-responsive genes). A confounding factor when assessing EDCs of the HPT axis is that compounds may induce

developmental stage-dependent differential effects, so experimental designs that employ exposures at multiple life history stages are of paramount importance. Moreover, there are few examples of studies assessing the effects of agrochemicals on TH levels. There are numerous reasons for this. Firstly, synthesis, interconversion and metabolism of THs by the DIOs is complex and difficult both to assess and interpret. The lack of specificity, bias and imprecision are analytical problems associated with many extraction methods and assays previously utilized for the determination THs. Emerging methods such as ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry that are optimized for amphibians (Luna et al., 2013; Hornung et al., 2015; Hansen et al., 2016) will be essential to comprehensive analysis of thyroid disruption.

Many of the agrochemicals lead to disruption of sexual development, including sex reversal. Additionally, more recent data indicate that metabolic disruption may impact reproductive success in subsequent generations. Maternal transfer of chemicals and epigenetic inheritance of potential effects should be investigated in amphibian models. Emerging evidence indicates significant cross-talk between the thyroid and reproductive axes, yet few studies have attempted to determine the consequential effects of disruption of one on the other. Certain chemicals (e.g., atrazine) have been implicated as EDCs of the thyroid, reproductive, and interrenal axes. In contrast, the apparent lack of pleiotropic effects by others (e.g., acetochlor), represents an important gap in our knowledge of cross-talk. Therefore, targeted investigations to characterize the cellular and molecular basis of interactions between endocrine axes are required and would contribute significantly to our understanding of neuroendocrine disruption

processes (Rosenfeld et al., 2017). Future studies must target the molecular pathways underlying sex reversal in tadpoles, and additionally, the consequences for reproductive success. Although sexual differentiation in amphibians is determined genetically, it is highly plastic where sex steroids, environmental factors, or contaminants may interact to affect developmental outcomes. Cause-and-effect studies are clearly lacking. For example, that an estrogenic or antiandrogenic agrochemical can affect sex ratios is suggestive of steroid-mediated disruption, but if that effect was blocked by a known receptor antagonist, it would be strong evidence that indeed the agrochemical was acting via nuclear estrogen or androgen receptor signaling. Given that there are also important differences in the responses of the various taxa and species, it is therefore critical that future studies be designed to determine the mechanisms of action of agrochemicals in Amphibia.

Many investigations to date have focused on single-agrochemical exposures. Considering that agrochemicals are present in the environment in complex mixtures, there is a relative lack of data examining the effects of environmentally relevant mixtures. It is therefore pertinent to evaluate the potential interactive effects of agrochemicals on amphibian health at the cellular and molecular levels. Field studies are very informative of the state of amphibian populations; however, it is challenging to establish causality between the exposure scenarios and the observed phenotypes in agricultural field settings, since there are many confounding variables. Therefore, we suggest that laboratory studies be designed in collaboration with field biologists, thus that mechanisms of action of agrochemicals can be established under more relevant scenarios.

In the broader context, several authors have attempted to link exposure to pollutants, including agrochemicals, to amphibian population declines (Hayes, Falso et al., 2010; Orton and Tyler 2015). On the global scale, population declines are unlikely to be linked to a single agrochemical, or even a single class of compounds. On the other hand, it is clear that numerous agrochemicals, alone and in mixtures, have multiple actions to disrupt development, metabolism, stress, and reproduction. Some agrochemicals result in detrimental effects in the descendant generation. There is thus the potential for major effects on the physiology of amphibian species. That many populations of amphibians are exposed to agrochemicals in addition to being subjected to other ecosystem threats, such as habitat loss and climate change, indicates that future research must attempt to address both cellular and molecular mechanisms of endocrine disruption under multiple stressor scenarios in this class of vertebrates.

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Captions for table and figures

TABLE 1. Summary of major endocrine-disrupting effects of agricultural fungicide, herbicides, fertilizers and metals in select amphibian species/. Please note that. This table serves to illustrate the range of effects and species. Studies and other key examples can be found in the relevant sections of the main text.

FIGURE 1. Hormonal control system in anurans.

Corticotropin-releasing factor (CRF) is a dual function neuropeptide (thick black arrows and associated pathways) in anurans. The CRF is secreted from hypothalamic nerve terminals in the median eminence and transported to the pituitary to trigger synthesis and release of thyroid stimulating hormone (TSH) from the pituitary gland. In turn, TSH stimulates the development of the thyroid, leading to initial synthesis and release of THs. It is principally T3 that drives the positive autoregulation of the TH receptors to induce metamorphosis-related genes and to reduce the expression of growth-related genes. The CRF system is better known for the neuroendocrine regulation of the stress axis. It stimulates the synthesis and release of adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn, stimulates corticosterone (CORT) from the steroidogenic cells of the interrenal (amphibian equivalent to mammalian adrenal cortex embedded within the kidney complex). Corticosteroids negatively feedback at the level of CRF and ACTH to attenuate glucocorticoid synthesis. Corticosterone is the main adrenal steroid in amphibians. While CORT does not drive metamorphosis alone, it can potentiate the actions of THs to accelerate the process. The tripeptide thyrotropin-releasing hormone (TRH) was originally named for that action because of its role in mammals. In anurans, it does not effectively stimulate TSH. On the other hand, it stimulates prolactin (PRL) and growth hormone (GH; grey arrows and associated pathways) to promote tadpole growth. Both PRL and GH can negatively regulating several metamorphic processes. Growth hormone-regulated hepatic insulin-like growth factor (IGF) production and IGFs (thin dashed arrow) regulate numerous growth-related processes, and potentially aspects of metamorphosis. Pancreatic hormones, such as insulin (INS), are important for many aspects of growth, metabolism, and metamorphosis (thin black arrows and associated pathways). Recent data indicate that the pancreas is also a target for endocrine disruption. Metabolic disruption can also lead to reduced reproduction. While often not considered along with the thyroid axis, there is accumulating evidence indicating important regulation of the thyroid and reproductive axes (shown by the X; see also Flood et al. 2013 and Duarte-Guterman et al., 2014). The principal stimulatory neuropeptide gonadotropin-releasing hormone (GnRH) stimulates the pituitary synthesize the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (hatched arrows and associated pathways). The gonadotropins stimulate development of the ovaries and testes, and drive steroidogenesis. These include progesterone (P4), testosterone (T), 5 α -dihydrotestosterone (5 α -DHT), and estradiol (E2) (see Vu and

Trudeau, 2016). The THs can regulate numerous systems within the hypothalamo-pituitary-gonadal axis to affect sexual development in tadpoles, or reproductive processes in adults. Similarly, the sex steroids can impact the thyroid- and growth systems to modulate metamorphosis. Note that feedback regulation at the hypothalamus and pituitary by THs, CORT, and sex steroids exists, but is not depicted on the figure. For further details, refer to documented actions of endocrine disrupting chemicals in the main text.

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Chemical	Species	Exposure	Effects	Reference
Fungicides				
Triphenyltin	<i>L. sylvaticus</i>	100 days at 0.1 – 1 µg/L	Upregulated lipid metabolism.	Higley et al., 2013
Triclosan	<i>S. tropicalis</i>	1 yr at 200 ng/L	Disrupted energy metabolism.	Regnault et al., 2018
Triadimefon	<i>P. nigromaculatus</i>	28 days at 26 mg/L	Reduced metamorphic rate. Upregulated tshβ, dio2, trβ, dio2, matrix metalloproteinase 2, TH/bZIP, and sonic hedgehog.	Zhang W et al., 2018
Vinclozolin	<i>X. laevis</i>	4 days at 28.6 ng/L – 286 µg/L	Reduced advertisement calling. Increased rasping (unreceptive call). Altered acoustic properties.	Hoffmann & Kloas, 2010
Herbicides				
Atrazine	<i>X. laevis</i>	NF 47-62 at 400 µg/L	Disrupted energy metabolism.	Zaya et al., 2011
	<i>L. pipiens</i>	GS 25-42 at 1.6–3.7 µg/L	Downregulated dio3. Reduced metamorphic success.	Langlois et al., 2010
	<i>L. pipiens</i>	GS 27-42 at 1.6–3.7 µg/L	Female-biased sex ratio.	Langlois et al., 2010
	<i>L. pipiens</i>	GS 23-metamorphic climax at 0.1-25 ppb	Female-biased sex ratio, gonadal dysgenesis.	Hayes et al., 2003
Glyphosate-based (VisionMax®)	<i>L. sylvaticus</i>	GS 25-42 at 2.9 mg a.e./L	Reduced metamorphic rate. Downregulated dio2. Upregulated trβ dio2, dio3, trβ, and grll.	Navarro-Martin et al., 2014
Glyphosate-based (WeatherMax®)	<i>L. sylvaticus</i>	GS 6-37 at 2.9 mg a.e./L	Downregulated trβ.	Lanctot et al., 2013
Linuron	<i>S. tropicalis</i> ,	Embryo to NF stage 40 at 9 µg/L	Female-biased sex ratio.	Orton et al., 2018

Fertilizers and Metals

Nitrate	<i>B. gargarizans</i>	GS 26- metamorphic climax at 100 mg/L	Reduced metamorphic rate. Partial colloid depletion. Reduced T3 and T4 levels. Downregulated dio2. Upregulated dio3.	Wang et al., 2015
Lead	<i>B. gargarizans</i>	GS 26-42 at 50 - 1000 µg/L	Follicular hyperplasia and colloid depletion. 50 µg/L: Increased metamorphic rate. Upregulated dio2, trα, and trβ. Downregulated dio3. 1000 µg/L: Decreased metamorphic rate. Downregulated ↓ dio2, trα and trβ. Upregulated dio3.	Yang et al., 2019

Other pesticides

Azocyclotin	<i>X. laevis</i>	21 d at 0.1- 0.5 µg/L	Reduced metamorphic rate and T3 level. Downregulated dio2, trβ, bteb, matrix metalloproteinase 2, stromelysin-3.	Li et al., 2016
DDE (metabolite of DDT)	<i>X. laevis</i>	4 days at 3.18-318 ng/L	Reduced advertisement calling, increased rasping (unreceptive call).	Hoffmann & Kloas, 2016