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1 **Agrochemicals disrupt multiple endocrine axes in**  
2 **amphibians**

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35 **Abstract**

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

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

## 67 **Introduction**

68  
69 There are countless benefits to intensive agricultural practices, including high  
70 productivity, labour concentration, and efficiencies, and thus, major health and  
71 economic benefits to people. Loss of natural habitat due to agricultural development is  
72 one important contribution to global amphibian declines. This is accompanied by the  
73 use of a range of agrochemicals for crop and farm animal management. There is  
74 accumulating and controversial evidence that some are endocrine disrupting chemicals  
75 (EDCs), or have the potential to disrupt other key physiological control processes that  
76 may contribute to reduced amphibian health and significant population declines. The  
77 most obvious agrochemicals are the various herbicides, insecticides, fungicides, and  
78 fertilizers used (Table 1). However, animal growth promoters, manure and sewage run-  
79 off, natural and synthetic steroids, pharmaceuticals and metals can also be considered

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

82

83 **TABLE 1 NEAR HERE**

84

85 The class of Amphibia is comprised of three orders: Anura (frogs and toads,~  
86 6,500 species), Caudata (newts and salamanders, ~680 species), and the  
87 Gymnophiona (caecilians, ~205 species). Of these, most research on basic endocrine  
88 control of development and reproduction, as well as the processes underlying endocrine  
89 disruption are largely undertaken with a few anuran species. For the most part, we limit  
90 the scope of this review to frogs and toads because data are missing in the other  
91 groups. Amphibians appear to be particularly susceptible to disruption of development,  
92 because metamorphosis is dependent on the thyroid hormones (THs), and that THs and  
93 their receptors are often altered by environmental contaminants. Moreover, sexual  
94 development is highly plastic and sex reversal is relatively easy to induce with  
95 exogenous sex steroid treatments, among others. Crosstalk between the thyroid,  
96 metabolic, and gonadal axes gives rise to additional processes that can be affected by  
97 EDCs. Given that tadpole and adult amphibian skin is permeable, many species can  
98 take up and respond to pollutants at both life-history stages.

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

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

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

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

118 The control of the synthesis, secretion, and metabolism of THs has been described for  
119 anurans in several elegant reviews and will not be covered in detail here (Denver et al.,  
120 2002; Buchholz et al., 2006; Denver, 2013). Briefly, metamorphic progression is  
121 accompanied by a gradual increase in THs and developmental- and tissue-dependent  
122 expression patterns of the TRs and deiodinase enzymes. In particular, the type 2  
123 deiodinase (DIO2) is required for local conversion of T4 to the more active T3, and  
124 metabolism of these to less active forms by type 3 deiodinase (DIO3). Contaminants  
125 have the potential to cause adverse effects through interference at many levels:

126 synthesis of THs (sodium iodide symporter (NIS), thyroid peroxidase (TPO), thyroid  
127 stimulating hormone (TSH), thyroid stimulating hormone receptor (TSHR)), plasma  
128 transport proteins, cellular uptake mechanisms, TRs, and TH metabolism (e.g.,  
129 deiodinase enzymes). Interference with feedback loops and hormonal crosstalk  
130 represent other potential mechanisms of endocrine disruption. Several agricultural  
131 contaminants have been identified as disruptors of the HPT axis in vertebrates  
132 (Leemans et al., 2019). Because TH-signaling is highly conserved across vertebrate  
133 species, and anuran metamorphosis is TH-dependent (Heimeier and Shi, 2010),  
134 amphibians represent ideal animal models to assess endocrine disruption.  
135 Contaminants that affect TH-dependent processes have been associated with  
136 alterations in the rate of metamorphosis (Heimeier and Shi, 2010; Miyata and Ose,  
137 2012). On the cellular and molecular levels, thyroid gland histology or disruption of the  
138 actions of exogenous THs or TH-dependent processes are parameters frequently  
139 studied to assess thyroid disruption. Studies on pituitary TSH and the direct  
140 measurement of T4, T3 and metabolites in anurans are lacking in general and more  
141 specifically as related to endocrine disruption. Measuring and interpreting circulating  
142 hormone levels, particularly in small tadpoles, presents a significant technical challenge.  
143 Therefore, many studies have used highly sensitive molecular endpoints, such as gene  
144 expression of TH-responsive genes, to examine the effects of agrochemicals on the  
145 HPT of amphibians (Crump et al., 2002; Lancôt et al., 2013; Navarro-Martín et al.,  
146 2014).

147

148 **FIGURE 1 NEAR HERE**

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

158

## 159 **2.1 Fertilizers and Metals**

### 160 **2.1.1 Fertilizers**

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

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



172 *gargarizans*) tadpoles exposed to a relatively high concentration (100 mg/L) of nitrate  
173 exhibited delayed metamorphosis, reduced circulating T4 and T3 levels, down-  
174 regulation of *dio2* and up-regulation of *dio3* mRNA, and partial colloid depletion in  
175 thyroid gland follicles (Wang et al., 2015). Interestingly, *B. gargarizans* exposed to 5 -  
176 200 mg/L nitrate at embryonic stages (when maternally-transferred HPT molecular  
177 components are present), exhibited an increased rate of development, down-regulation  
178 of transporter genes (solute carrier family 16 member 2 (*slc16a2*) and (solute carrier  
179 organic anion transporter family, member 1C) *slco1c*) and of *dio2* and *dio3*, and an up-  
180 regulation of *tra* (Xie et al., 2019). In this study, T4 levels and *trβ* expression were not  
181 affected by nitrate exposure. Similarly, exposure 5 and 50 mg/L nitrate did not report  
182 transcriptional alterations of TH-responsive genes (*trβ* and Rana larval keratin type I) in  
183 *Lithobates catesbeianus* (American bullfrog) tadpole tail fin tissue (Hinter et al., 2012).  
184 Therefore, it is suggested that nitrate does not interact with T4, but disrupts gene  
185 expression following conversion to reactive nitric oxide; thereby, disrupting essential  
186 proteins, and affecting transcription and mRNA stability (Poulsen et al., 2018). The  
187 species-specific sensitivities and the molecular mechanisms by which nitrate disrupts  
188 the HPT axis in amphibians remains to be elucidated.

### 189 **2.1.2 Metals**

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

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

202         Copper occurs naturally in the environment; however, deposits of anthropogenic  
203 Cu reach surface waters from agricultural runoff. Several studies have demonstrated  
204 that Cu exposure can delay the rate of metamorphosis in *L. pipiens* (Chen et al., 2007),  
205 *L. sphenoccephalus* (Southern leopard frog; Lance et al. 2012), *L. sylvatica* (Wood frog;  
206 Peles, 2013), *B. gargarizans* (Chai et al., 2014; Wang et al., 2016; Chai et al., 2017),  
207 and *B. arabicus* (Arabian toad, Barry, 2011). At the histological level, Cu causes  
208 follicular hyperplasia in the thyroid gland of *B. gargarizans* (Wang et al., 2016).  
209 Exposure to 32 and 64 µg/L Cu induced an up-regulation of *dio3* expression, but down-  
210 regulations of both *tra* and *trβ* (Wang et al., 2016). Similarly, exposure to 64 µg/L Cu  
211 induced down-regulation of *dio2*, *dio3*, *tra*, and *trβ* mRNA levels in a separate study of  
212 *B. gargarizans* (Chai et al., 2017).

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

218 2003). In contrast, Cd exposure at 855 µg/L Cd arrested metamorphosis in *Xenopus*  
219 *laevis* (African clawed frog; Sharma and Patiño, 2008), and interestingly, these effects  
220 were exacerbated in male *X. laevis* tadpoles when estradiol-17β (E2) was present  
221 (Sharma and Patiño, 2010). Sharma and Patiño (2008) also observed a reduction in  
222 follicle cell height in *X. laevis* tadpoles. Chronic Cd exposure (50, 100, and 500 µg/L) in  
223 *B. gargarizans* tadpoles caused retarded metamorphosis, follicular hyperplasia of the  
224 thyroid, and downregulated *dio2*, *tra*, and *trβ* transcripts (Sun et al., 2018). Similarly,  
225 metamorphosis was arrested at a pro-metamorphic stage in *Pleurodeles waltl* (Iberian  
226 ribbed newt) exposed to 1.2 mg/L Cd (Flament et al., 2003). Several studies reported  
227 that Cd decreases plasma T4 levels, without altering T3 levels (reviewed in Buha et al.,  
228 2018). The direct effect on the thyroid gland is suggested to be due to oxidative stress  
229 in mitochondria of follicular cells; thereby, altering production and/or secretion of T4  
230 (Buha et al., 2018).

## 231 **2.2. Fungicides**

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

241 Li et al., 2016). In *P. nigromaculatus*, 28-day larval exposure affected the rate of  
242 metamorphosis and significantly altered transcriptional regulation (Zhang W et al.,  
243 2020). In the brain, transcripts of *tsh $\beta$*  and *dio2* were upregulated, while *tr $\beta$* , *dio2*, *matrix*  
244 *metalloproteinase 2*, *TH-responsive basic leucine zipper transcription factor (TH/bZIP)*,  
245 *and sonic hedgehog* genes were up-regulated in the intestine (Zhang W et al., 2020).  
246 Interestingly, despite the observed inhibitory effects of triadimefon on development, TH-  
247 responsive genes were induced, suggesting a compensatory transcriptional response.  
248 Pre-metamorphic (Nieuwkoop and Faber (NF) stage 51) *X. laevis* tadpoles exposed to  
249 triadimefon for 21 days exhibited similar adverse effects (Zhang W et al., 2020). Anti-  
250 thyroidogenic effects were observed, including reduced rate of metamorphosis,  
251 decreased levels of T3 and T4, and an alteration of HPT-related gene expression. The  
252 authors conducted *in silico* ligand–TR docking computational analyses, which  
253 suggested that triadimefon could interact with TRs; however, direct evidence is lacking.

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

263 The imidazole prochloraz, is widely used in Asia, Australia, Europe, and South  
264 America. It has been shown to delay metamorphosis, elevate T3 levels, and cause  
265 thyroid hyperplasia in *R. temporaria* (European common frog) (Brande-Lavridsen et al.  
266 2008). Similarly, *X. laevis* tadpoles exposed to 20, 60 and 180 µg/L prochloraz exhibited  
267 elevated T4 level and mild follicular cell hypertrophy in thyroid gland histology of the 180  
268 µg/L treatment group (Haselman et al., 2018). These effects were observed without  
269 concurrent alteration of T3 level or disruption of metamorphic rate. Therefore, it was  
270 suggested that prochloraz exposure interfered with the HPT-axis by altering T4  
271 metabolism in the liver and kidney or through affecting the negative-feedback  
272 mechanisms. Interestingly, prochloraz has also been implicated in the disruption of  
273 other endocrine pathways through aromatase inhibition. Exposure to 115 and 251 µg/L  
274 prochloraz lowered plasma testosterone levels, disrupted gonadal differentiation, and  
275 caused a male-biased sex ratio in *R. temporaria* (Brande-Lavridsen et al., 2008). It was  
276 suggested that prochloraz may act through an anti-androgen mode of action (Haselman  
277 et al., 2018), but this is far from clear. Given the potential for prochloraz to disrupt both  
278 thyroid and gonadal functions, it will be important to determine the mechanisms of  
279 action within the context of hormonal crosstalk (Figure 1).

### 280 **2.3 Insecticides and acaricides**

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

286 regulation of *trβ* in testis and liver of adult male *R. temporaria* exposed to DDE.  
287 Similarly, in larval *R. temporaria*, DDE caused a decrease in *trβ* expression in brain and  
288 tail tissue that was negatively correlated with tail length and width (Mortensen et al.,  
289 2006).

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

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

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

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

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

336         Since their introduction in the early 1990s, neonicotinoids have become the most  
337 commonly used type of insecticide in the world (Jeschke et al., 2011). Although  
338 neonicotinoids have been implicated in the disruption of HPT function in lizards (Wang,  
339 Xu et al., 2019), birds (Pandey and Mohanty, 2017; 2015), and rats (Ibrahim et al.,  
340 2015; Şekeroğlu et al., 2014), there is a relative lack of data concerning their effects in  
341 amphibians. In *Eremias argus* (Mongolian racerunner lizard), three neonicotinoids  
342 (dinotefuran, thiamethoxam, and imidacloprid) affected the plasma concentrations of T4,  
343 and the transformation of T4 to T3 (Wang, Xu et al., 2019). In larval *L. sylvaticus*,  
344 exposure to imidacloprid at 10 and 100 µg/L (above current environmental levels),  
345 increased survival and delayed completion of metamorphosis compared with controls  
346 (Robinson et al., 2017), suggesting that the amphibian HPT axis may be altered by  
347 neonicotinoids.

348

## 349 **2.4 Herbicides**

### 350 **2.4.1 Atrazine**

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



355 consistent effects of atrazine on the thyroid function in amphibians (Solomon et al.,  
356 2008). It is particularly challenging to interpret the current data due to species-specific  
357 sensitivities and non-monotonic effects of atrazine. For example, atrazine reduced *in*  
358 *vitro* corticosterone production in *L. catesbeianus*, but not *X. laevis* (Goulet and Hontela,  
359 2003). In one study, atrazine exposure delayed metamorphosis at 100 µg/L in *X. laevis*  
360 (Freeman and Rayburn, 2005). In contrast, other authors report that atrazine exposure  
361 in larval *X. laevis* at similar concentrations (40 - 160 µg/L) had no effect on the rate of  
362 metamorphosis; whereas, 20 and 320 µg/L of atrazine accelerated or delayed  
363 metamorphosis, respectively (Sullivan and Spence, 2003). Similarly, in *L. clamitans*, 10  
364 µg/L, but not 25 µg/L atrazine, delayed metamorphosis (Coady et al., 2005). In larval  
365 *Ambystoma tigrinum* (tiger salamander), atrazine exposure delayed and accelerated  
366 metamorphosis at 75 µg/L and 250 µg/L, respectively (Larson et al., 1998). Both 75 and  
367 250 µg/L treatments caused an elevation of plasma T4 in pro-metamorphic (stage-IV),  
368 but not at pre-metamorphic (stage-II) larvae (Larson et al., 1998).

369         Several other studies have reported no effects of atrazine on metamorphic  
370 endpoints (rate, forelimb emergence, tail resorption) in *X. laevis* (Carr et al., 2003;  
371 Coady et al., 2005; Kloas et al., 2009), *Limnodynastes tasmaniensis* (spotted grass  
372 frog; Spolyarich et al., 2010), *A. americanus* (Freeman et al., 2005), or *L. pipiens* (Allran  
373 and Karasov, 2000; Orton et al., 2006). In an investigation using sensitive gene  
374 expression endpoints, low levels of atrazine (1.8 µg/L) significantly reduced  
375 metamorphic success (number of animals reaching metamorphosis) without affecting  
376 body weight, snout-vent length, or age at metamorphosis in *L. pipiens* (Langlois et al.,  
377 2010). In the same study, animals that did not metamorphose, exhibited a decreased

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

#### 384 **2.4.3 Phenylurea herbicides**

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

400

### 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 *trα* 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.*

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

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

436

#### 437 **2.4.4 Glyphosate**

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

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

447         Laboratory exposures to the glyphosate-based herbicide VisionMax® had direct  
448 effects on the metamorphosis of *L. sylvaticus* tadpoles by decreasing their development  
449 rates when exposed to 2.9 mg of acid equivalents (a.e.) per L (Navarro-Martín et al.,  
450 2014). These glyphosate-exposed animals took an additional 7 days to reach  
451 metamorphic climax when compared to controls. Tadpoles exposed to VisionMax®  
452 were larger in size and in weight with respect to the control group, but no effects on the  
453 condition factor were observed. At the molecular level, VisionMax® reduced normal  
454 developmental changes in tail *trβ* or *dio3*, glucocorticoid receptor (*gr11*) mRNA levels in  
455 *L. sylvaticus* tadpoles, suggesting a partial inhibition of the stress axis. The authors  
456 hypothesized that the up-regulation of transcripts triggering metamorphosis, such as *trβ*  
457 or *dio3*, could be the result of a compensatory response to prevent developmental delay  
458 (Navarro-Martín et al., 2014). These data also suggest that VisionMax® could shift the  
459 energy expenditure from development to growth.

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

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

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

## 491 **2.5 Agrochemical mixtures:**

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

513

### 514 **3. Disruption of growth and metabolism**

515 Life-history theory states that animals have to partition limited resources between  
516 growth and reproduction, and hormones control these processes (Figure 1). Since  
517 resources are limited there are physiological constraints at the larval and the adult  
518 stages that cannot be modified without causing deleterious effects on growth and  
519 reproduction (Lardner and Loman, 2003). At the larval stage, disruption of metabolism  
520 can have a profound and lasting effect across the lifespan of an organism (Mueller et  
521 al., 2015). Indeed, larval development rates determine the period of water availability in  
522 temporary ponds and the size and weight at metamorphosis which are all directly linked  
523 with mortality. At the adult stage, disruption of metabolism can mimic reduction of  
524 resources which is known to increase the risk of mortality associated with reproduction.  
525 In such events, skipping reproduction for allocating resources to growth might postpone  
526 reproductive event at the following year (Lardner and Loman, 2003) and maintaining  
527 reproduction might lead to progeny with reduced growth capacities (Regnault et al.,  
528 2018). Amphibians are particularly exposed to agrochemicals through water during  
529 periods critical for optimal metabolism, growth or reproduction, (Fan et al., 2007a,  
530 2007b; Hayes et al., 2011; Duarte-Guterman et al., 2014).

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



537 Whitaker, Ide, 2011; Zaya, Amini, Whitaker, Kohler, Ide, 2011), higher concentrations of  
538 atrazine have been shown to decrease snout vent length, body weight (Diana et al.,  
539 2000; Sullivan and Spence, 2003), and survival at metamorphosis (Hoskins et al.,  
540 2018). Discrepancy between low doses and high doses atrazine have been associated  
541 with non-monotonous effects (Diana et al., 2000; Freeman et al., 2005; Coady et al.,  
542 2005). For example, exposure to atrazine resulted in a significant acceleration of  
543 metamorphosis at 1 mg/L and a significant increase in the duration of metamorphosis at  
544 5 mg/L, whereas no significant difference was observed with 0.1 mg/L (Brodeur et al.,  
545 2009). Many studies have shown that exposures to glyphosate and formulations with  
546 POEA and POEA alone increased mortality and decreases size and developmental  
547 rates of tadpoles (Edginton et al., 2004; Howe et al., 2004; Relyea, 2004, 2005, 2012;  
548 Relyea and Jones, 2009; Jones et al., 2010, 2011; Williams and Semlitsch, 2010;  
549 Moore et al., 2012; Lanctôt et al., 2014). Concerning insecticides and fungicides, fewer  
550 data are available, but a direct link between exposure to these classes of agrochemicals  
551 and growth alterations has been established for the insecticides carbaryl (Boone et al.,  
552 2013),  $\alpha$ -cypermethrin alone (Xu and Huang, 2017) or in combination with the herbicide  
553 oxadiazon (Mesleard et al., 2016), the carbamate methomyl (Trachantong et al., 2017)  
554 and the fungicides copper (Peles, 2013), triphenyltin (Higley et al., 2013) and  
555 cyproconazole (Zhang W et al., 2019).

556 In several studies, alterations of tadpole growth under pesticide exposure have  
557 been associated with metabolic disruption. Tadpoles are required to balance their  
558 limited energy reserves for maintenance, growth, metamorphosis, and locomotion, while  
559 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 ( $\geq 250$  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

583 quinclorac exposure induced a significant decrease in levels of glycogen, proteins,  
584 cholesterol, and total lipids in gill, liver, and muscle in *L. catesbeianus* (Dornelles and  
585 Oliveira, 2014), suggesting that several classes of herbicides might be associated to  
586 metabolic disorders. *Microhyla fissipes* tadpoles (farmland frog) exposed to 60–  
587 120 mg/L glyphosate-based herbicides showed preference for warm temperatures,  
588 exhibited a suppressed growth, and a reduced tail development, together with the  
589 down-regulation of carbohydrate and lipid catabolism and the up-regulation of the amino  
590 acid catabolism. These results suggested a trade-off between protein synthesis and  
591 energy production with respect to amino acid allocation, and provide a metabolic  
592 explanation as to why growth was suppressed tadpoles exposed to a glyphosate-based  
593 herbicide (Wang, Chang et al., 2019).

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

606 metabolism (Zaya, Amini, Whitaker, Ide, 2011; Zaya, Amini, Whitaker, Kohler, Ide,  
607 2011). These results suggest that tadpoles exposed to pesticides undergo a global  
608 stress response that might involve the glucocorticoid axis, and increased metabolism  
609 may be a compensatory mechanism to promote survival (Higley et al., 2013; Strong et  
610 al., 2017).

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

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

631         Despite the absence of clear correlations between disruption of metabolism by  
632 agrochemical in adults and decreased growth capacities of progeny, impacts of  
633 pesticide exposure and metabolism impairments in post-metamorphic amphibians have  
634 been clearly demonstrated (Van Meter et al., 2018). In adults, disruption of lipid,  
635 carbohydrate, and protein metabolism following insecticide exposure has been shown.  
636 For example, a decrease in the total lipid content of liver, muscle, testes, and ovaries  
637 were observed in *Minervarya andamanensis* (Andaman frog) with lethal concentration of  
638 malathion, whereas exposure to sub-lethal concentrations were associated to an  
639 increase in total lipids, free fatty acids, glycerol, and lipase activity (Gurushankara et al.,  
640 2007). A negative relationship between p,p-DDE exposure and protein and  
641 carbohydrate storage associated with increased energy consumed has been shown in  
642 *Xenopus muelleri* (Müller's clawed frog) (Wolmarans et al., 2018). *L. sphenoccephala*  
643 exposed to single, double, or triple pesticide mixtures of bifenthrin (insecticide),  
644 metolachlor (herbicide) and triadimefon (fungicide) presented an increase in amino  
645 acids and other key metabolites concentrations in liver, suggesting depletion of energy  
646 resources (Glinski et al., 2018). *Bufo regularis* (African toad) exposed to endosulfan  
647 showed an increase in serum glucose levels with a concomitant reduction in liver  
648 glycogen, indicating disorders in carbohydrate metabolism (Isioma and Lawrence,  
649 2013). In addition, an inhibition of normal oxidative metabolism associated to lactic acid  
650 accumulation in blood has been shown following exposure of *Hoplobatrachus tigerinus*

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

#### 653 **4. Disruption of sexual differentiation**

654 As in many other vertebrate species, both female and male heteromorphic sex  
655 determining systems occur in amphibians. However, in the majority of species, sex is  
656 genetically determined by homomorphic sex chromosomes (Bachtrog et al 2014),  
657 suggesting that a variety of sex-determining genes exist (Ito 2018). Sexual  
658 differentiation in amphibians is driven by sex steroid hormones. In this regard, sexually  
659 dimorphic expression of steroidogenic-related genes, for example the cytochrome P450  
660 19 (*cyp19a1*), that converts androgens to estrogens, and the cytochrome 17 $\alpha$ -  
661 hydroxylase/17,20 lyase (*cyp17a1*), responsible for the conversion of progestogens to  
662 androgens, have been observed in different frog species, including *S. tropicalis* and *L.*  
663 *sylvaticus*. These two genes can be used as biomarkers of phenotypic sex (Navarro-  
664 Martin et al., 2012). As observed in birds, reptiles, and fish, frog gonadal development is  
665 sensitive to sex steroids (Hayes, 1998). In frogs, this process takes place mostly during  
666 the aquatic larval period making these organisms very sensitive to environmental EDCs.  
667 Moreover, many agrochemical applications occur in Spring, which coincides with the  
668 seasonal period of frog spawning and sensitive phases of tadpole development. Sex  
669 steroids are key players in gonadal differentiation and their action can be disrupted by  
670 estrogenic, androgenic, anti-androgenic, and progestagenic chemicals used in animal  
671 agriculture. Up to now, literature has focused on gonadal morphology, sex ratios  
672 (presence of intersex, as well as sex reversal as endpoints for studying endocrine  
673 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.

697 Many pesticides alone or combined in mixtures have the ability to act as  
698 androgen receptor (AR) antagonists *in vitro* and *in vivo* (Orton et al., 2011; 2012).  
699 Feminizing effects has been observed in *S. tropicalis* metamorphs exposed to 9 µg/L of  
700 linuron (Orton et al., 2018). The authors conclude that a disruption in the *ar/foxl2*  
701 signaling during gonadal differentiation could be responsible of the observed feminized  
702 sex ratio. A comparative toxicogenomics database analysis using transcriptomic  
703 datasets suggested that phenylurea herbicides, such as linuron and diuron, exert similar  
704 transcriptomic responses as the ones produced by reproductive hormones (Marlatt and  
705 Martyniuk 2017). In particular, linuron was found to affect transcripts related to steroid  
706 hormone biosynthesis and metabolism of xenobiotics by cytochrome P450, among  
707 others. Numerous studies have revealed effects leading to gonadal feminization caused  
708 by atrazine in many vertebrate species (Hayes et al. 2011); however, several  
709 researchers have failed to demonstrate anuran feminization (reviewed by Orton and  
710 Tyler, 2015). In addition to this controversy, the mode of action of atrazine remains  
711 unclear and needs to be elucidated. From one side, it was shown that it can perturb  
712 steroidogenesis *in vitro* in amphibian tissues (Goulet and Hontela, 2003; Orton et al.,  
713 2009), overstimulate endogenous estrogen production (Crain et al., 1997; Sanderson et  
714 al., 2001), up-regulate of brain *era* mRNA levels as well as disrupting of 5β-reductase  
715 activity which regulates androgen bioavailability by catalyzing the conversion of  
716 testosterone to 5β-dihydrotestosterone (Langlois et al., 2010). On the other hand, *in*  
717 *vitro* receptor binding assays failed to demonstrate that atrazine directly interact with  
718 either the estrogen or androgen receptors (Kojima et al., 2004; Orton et al., 2009) and  
719 does not alter aromatase activity *in vivo* (Rohr & McCoy, 2010).



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

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

743            Masculinizing effects can be induced not only directly by interacting with ARs, but  
744 also indirectly by blockade of estrogen synthesis through aromatase inhibition. For  
745 example, when the irreversible aromatase inhibitor 4-hydroxyandrostenedione (4-OHA)  
746 was implanted intraperitoneally into *L. catesbeianus* tadpoles, gonadal abnormalities at  
747 the histological level were observed (Yu et al., 1993). These included degenerating  
748 ovaries with regressed oocytes (4 %), intersex (17 %), and full differentiation into testes  
749 (79 %). Moreover, these gonadal alterations were accompanied by decreased E2 and  
750 increased T secretions (Yu et al., 1993). Agrochemicals may exert adverse effects in  
751 amphibians by acting through similar mechanisms. For example, the acaricide  
752 azocyclotin may have both androgenic and anti-estrogenic action in *X. laevis* (Li et al.,  
753 2017). Tadpoles exposed chronically (4 months) to 0.05 - 0.5 µg/L exhibited a male-  
754 biased sex-ratio, increased T and pregnenolone levels, hermaphroditic gonadal  
755 development, and altered gene expression of steroidogenic enzymes (cyp17 and  
756 aromatase). Another example can be found in *R. temporaria* in which waterborne  
757 exposures from hatch to metamorphosis to the fungicide and aromatase inhibitor  
758 prochloraz (115 and 251 µg/L) significantly increased the proportion of histological  
759 males (80-85.7 %) and hermaphrodites in the final population (Brande-Lavridsen et al.,  
760 2008).

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

766 However, effects on ovarian and oviductal development were observed in adults,  
767 demonstrating that developmental exposures can have long-term effects (Kvarnryd et al  
768 2011). These long-term effects seem to be mediated by perturbations in the anti-  
769 mullerian hormone and progesterone signalling since LNG exposures altered *amh*,  
770 *amhr2*, *ipgr* and *mpgr beta* mRNA levels in a concentration-, sex- and developmental  
771 stage-specific manner (Säfholm et al 2016). Given that synthetic proestrogens can affect  
772 development, it will be important to screen agrichemicals for progesterone-like activities  
773 in amphibians.

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

789 gonadal histology were assessed in three frog species (*H. arborea*, *B. viridis* and *X.*  
790 *laevis*) exposed to bisphenol A (BPA), the intensity of these effects varied across the  
791 three families (Hylidae, Bufonidae and Pipidae) (Tamschick, 2016; Tamschick et al.,  
792 2016). There may be some relevance of BPA to agriculture, as in some cases, human  
793 sewage is being applied to fields as fertilizer, and thus BPA has been detected in  
794 agricultural soils (Zhang Z et al., 2015). Another pollutant of concern are polychlorinated  
795 biphenyls (PCBs) that have been associated with the use of organic waste as fertilizers  
796 in agricultural soils in some countries (Antolín-Rodríguez et al., 2016). A study that  
797 assessed effects of PCBs on *X. laevis* found that there was no effect of PCB3 and  
798 PCB5 on the proportion of females, but they observed a reduction of males with  
799 morphologically normal testes (Qin et al., 2003). Compared to *L. clamitans*, *L. pipiens*  
800 tadpoles were found to have lower hatching success and survival with higher incidences  
801 of edema when exposed to PCBs (Rosenshield et al., 1999). These results suggest that  
802 different species sensitivity related to sex differentiation might exist for this compound.  
803 Further evidence in *X. laevis* supports variations among population responsiveness  
804 when sex ratios were evaluated following E2 exposure (Luong, 2016). In this regard,  
805 Orton and Routledge (2011) studied differences in growth, metamorphic development,  
806 and sexual differentiation in eggs from *Bufo bufo* (common toad) collected from sites  
807 with different agricultural intensity activities. Their results suggested that maternal  
808 exposures and/or *in ovo* events could have more influence on this developmental  
809 process than the tadpole rearing environment. For that reason, they concluded that  
810 traditional exposure scenarios (starting at the larval stage) could be missing crucial  
811 developmental windows to assess amphibian sensitivity to pollutants.

812 One of the challenges to assess the effects of agrochemicals in aquatic  
813 organisms is that in most cases, the manufacturers do not provide the information on  
814 the full composition of formulations and that the same active compound can be  
815 manufactured under different names and by different companies. A clear example is the  
816 glyphosate-based herbicides. For example, a study carried out using glyphosate-based  
817 herbicides containing POEA as the surfactant (Roundup Original® and Roundup  
818 Transorb® formulations) revealed the presence of gonadal abnormalities in *L. pipiens*,  
819 but no effects were observed when glyphosate was tested alone (Howe et al., 2004).  
820 However, no observed effects in sex ratios or gonadal abnormalities were observed in  
821 *L. sylvaticus* exposed to VisionMax® under chronic laboratory conditions or to Roundup  
822 WeatherMax® under environmental pulse laboratory and field conditions (Lanctôt et al.,  
823 2013, 2014, Navarro-Martín et al., 2014).

## 824 **5. Disruption of anuran vocalization**

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

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

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

841         Atrazine has been associated with feminization in frogs. Male *X. laevis* exposed  
842 to atrazine from embryo to adult had lower plasma testosterone levels than controls,  
843 and were out-competed by controls when attempting to mate with females. They also  
844 displayed female-like morphology, including changes in larynx structure, vocalization  
845 quality was not assessed (Hayes, Khoury et al., 2010). Vinclozolin, a commonly used  
846 fungicide with anti-androgenic effects, affected songs of *X. laevis*. Exposed males  
847 experienced a decrease in advertisement calls and an increase in types of call  
848 indicating sexual non-receptivity (Hoffmann and Kloas 2010). A recent metabolomic  
849 study found that *Limnodynastes peronei* (striped marsh frog) larvae exposed to atrazine  
850 presented alterations in steroidogenesis and cholesterol metabolism and suggested that  
851 detected changes in formic acid could be related to alterations in the steroidogenic  
852 conversion of androgens into estrogens (Melvin et al., 2018). Exposure to flutamide, a  
853 model compound for anti-androgenicity, also reduced advertisement calls (Behrends et  
854 al. 2010). As expected, opposite effects were produced with the potent androgen  
855 agonist methyl-dihydrotestosterone (MDTH; Hoffmann and Kloas 2012a). This  
856 androgen is used in fish aquaculture, so there is a possibility for amphibian exposures  
857 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

881 17 $\alpha$ -ethinylestradiol (EE2), total calling duration was not affected, but a lower proportion  
882 of the calls were advertisement calls, which also had fewer clicks. They also produced  
883 more rasping calls, indicating a sexually unreceptive state (Hoffmann and Kloas 2012b).  
884 The effects of EE2 were reversed by co-exposure to either tamoxifen or fluvastatin,  
885 both pharmaceutical anti-estrogens (Hoffmann and Kloas 2012c). Estrogens had  
886 contrasting effects in the Western clawed frog (*S. tropicalis*); exposure to estradiol and  
887 4-tert octylphenol increased the number of calls from males (Schwendiman and Propper  
888 2012). This may be due to species differences, or how data was collected. Behaviors  
889 were noted by an observer rather than recorded with microphones, which is difficult to  
890 do so accurately as calls are underwater. Duration and type of call was also not  
891 evaluated in this study.

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

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



904 2012a). Indirect endocrine disruption was tested with *X. laevis* using playbacks of males  
905 exposed to EE2 and females preferred calls from control males over exposed  
906 (Hoffmann and Kloas 2012b). In both these cases, phonotaxis was measured as time  
907 spent near the speaker, which may or may not be due to attraction to the sound, and  
908 the effect sizes were small. Since E2 is able to induce phonotaxis, effects of other (anti)-  
909 estrogenic compounds on phonotaxis should be examined. While estrogenic  
910 compounds may increase phonotactic response, preference and selectivity may be  
911 affected. Using established attractive and unattractive playbacks for *E. pustulosus*  
912 female choosiness was found to decrease as receptivity (latency to approach)  
913 increased. The less selective frogs were also closer to egg-laying (Lynch et al. 2005).  
914 Mate choice is conditional, and a large body of evidence shows that higher quality  
915 females show a stronger preference for attractive traits in males (Cotton et al., 2006).  
916 For example, larger female *Hyperolius marmoratus* (African reed frogs) show a stronger  
917 preference for low-pitched male calls (Jennions et al., 1995). Female crickets on a high-  
918 quality diet show stronger preference for high call rate and responded faster to  
919 playbacks with typically preferred features (Hunt et al., 2005). Therefore, negative  
920 effects of EDCs on stress and overall health may further disrupt mate choice.

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

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

931

## 932 **6. Conclusions and future directions**

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

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

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

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

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

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

996 In the broader context, several authors have attempted to link exposure  
997 to pollutants, including agrochemicals, to amphibian population declines (Hayes, Falso  
998 et al., 2010; Orton and Tyler 2015). On the global scale, population declines are unlikely  
999 to be linked to a single agrochemical, or even a single class of compounds. On the  
1000 other hand, it is clear that numerous agrochemicals, alone and in mixtures, have  
1001 multiple actions to disrupt development, metabolism, stress, and reproduction. Some  
1002 agrochemicals result in detrimental effects in the descendant generation. There is thus  
1003 the potential for major effects on the physiology of amphibian species. That many  
1004 populations of amphibians are exposed to agrochemicals in addition to being subjected  
1005 to other ecosystem threats, such as habitat loss and climate change, indicates that  
1006 future research must attempt to address both cellular and molecular mechanisms of  
1007 endocrine disruption under multiple stressor scenarios in this class of vertebrates.

1008

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1020 **Captions for table and figures**

1021

1022 **TABLE 1.** Summary of major endocrine-disrupting effects of agricultural fungicide,  
1023 herbicides, fertilizers and metals in select amphibian species/. Please note that. This  
1024 table serves to illustrate the range of effects and species. Studies and other key  
1025 examples can be found in the relevant sections of the main text.

1026

1027 **FIGURE 1. Hormonal control system in anurans.**

1028 Corticotropin-releasing factor (CRF) is a dual function neuropeptide (thick black arrows  
1029 and associated pathways) in anurans. The CRF is secreted from hypothalamic nerve  
1030 terminals in the median eminence and transported to the pituitary to trigger synthesis  
1031 and release of thyroid stimulating hormone (TSH) from the pituitary gland. In turn, TSH  
1032 stimulates the development of the thyroid, leading to initial synthesis and release of  
1033 THs. It is principally T3 that drives the positive autoregulation of the TH receptors to  
1034 induce metamorphosis-related genes and to reduce the expression of growth-related  
1035 genes. The CRF system is better known for the neuroendocrine regulation of the stress  
1036 axis. It stimulated the synthesis and release of adrenocorticotrophic hormone (ACTH)  
1037 from the pituitary, which in turn, stimulates corticosterone (CORT) from the  
1038 steroidogenic cells of the interrenal (amphibian equivalent to mammalian adrenal cortex  
1039 embedded within the kidney complex). Corticosteroids negatively feedback at the level  
1040 of CRF and ACTH to attenuate glucocorticoid synthesis. Corticosterone is the main  
1041 adrenal steroid in amphibians. While CORT does not drive metamorphosis alone, it can  
1042 potentiate the actions of THs to accelerate the process. The tripeptide thyrotropin-  
1043 releasing hormone (TRH) was originally named for that action because of its role in  
1044 mammals. In anurans, it does not effectively stimulate TSH. On the other hand, it  
1045 stimulates prolactin (PRL) and growth hormone (GH; grey arrows and associated  
1046 pathways) to promote tadpole growth. Both PRL and GH can negatively regulating  
1047 several metamorphic processes. Growth hormone-regulated hepatic insulin-like growth  
1048 factor (IGF) production and IGFs (thin dashed arrow) regulate numerous growth-related  
1049 processes, and potentially aspects of metamorphosis. Pancreatic hormones, such as  
1050 insulin (INS), are important for many aspects of growth, metabolism, and  
1051 metamorphosis (thin black arrows and associated pathways). Recent data indicate that  
1052 the pancreas is also a target for endocrine disruption. Metabolic disruption can also lead  
1053 to reduced reproduction. While often not considered along with the thyroid axis, there is  
1054 accumulating evidence indicating important  
1055 regulation of the thyroid and reproductive axes (shown by the X; see also Flood et al.  
1056 2013 and Duarte-Guterman et al., 2014). The principal stimulatory neuropeptide  
1057 gonadotropin-releasing hormone (GnRH) stimulates the pituitary synthesize the  
1058 gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (hatched  
1059 arrows and associated pathways). The gonadotropins stimulate development of the  
1060 ovaries and testes, and drive steroidogenesis. These include progesterone (P4),  
1061 testosterone (T), 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT), and estradiol (E2) (see Vu and

1062 Trudeau, 2016). The THs can regulate numerous systems within the hypothalamo-  
1063 pituitary-gonadal axis to affect sexual development in tadpoles, or reproductive  
1064 processes in adults. Similarly, the sex steroids can impact the thyroid- and growth  
1065 systems to modulate metamorphosis. Note that feedback regulation at the  
1066 hypothalamus and pituitary by THs, CORT, and sex steroids exists, but is not depicted  
1067 on the figure. For further details, refer to documented actions of endocrine disrupting  
1068 chemicals in the main text.  
1069  
1070

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Chemical	Species	Exposure	Effects	Reference
<b>Fungicides</b>				
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
<b>Herbicides</b>				
	<i>X. laevis</i>	NF 47-62 at 400 µg/L	Disrupted energy metabolism.	Zaya et al., 2011
Atrazine	<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

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### 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

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### 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

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