



Did decades of glyphosate use have selected for resistant amphibians in agricultural habitats? [☆]

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ABSTRACT

Glyphosate-based herbicides are used worldwide, and glyphosate's primary metabolite (aminomethylphosphonic acid: AMPA), is globally retrieved in surface waters. AMPA induces various adverse effects on aquatic wildlife, including selective mortality, which suggests that glyphosate exposure may have selected for AMPA-resistant individuals. We tested this hypothesis using spined toads (*Bufo spinosus*), an amphibian found in a variety of habitats, from AMPA-exposed agricultural lands to AMPA-free forested areas. We predicted that the offspring of individuals originating from agricultural habitats would develop AMPA-resistance - and be less prone to develop adverse effects from AMPA exposure. To investigate this question, we performed a common garden brood-rearing experiment. The embryos and larvae of 40 spined toad pairs captured in agricultural and forest ponds were exposed either to an environmental relevant concentration of AMPA ($0.4 \mu\text{g L}^{-1}$) or to control conditions ($n = 8160$ embryos, $n = 240$ tadpoles). We monitored development durations, developmental abnormalities and morphology, measured across key developmental stages. Although we observed significant effects of AMPA on fitness parameters in each group, these effects were not exacerbated in individuals from AMPA-free habitats. We suggest that temporal and/or spatial dynamics of contamination, as well as gene flow between exposed and preserved populations, may hinder adaptive divergence between populations. Yet, we show strong adverse effects of AMPA exposure at early developmental stages. AMPA could therefore be one of the numerous causes of declining wild amphibian populations.

1. Introduction

Habitat heterogeneity across space and time is a strong driver of phenotypic variability (Stearns, 1989), and such heterogeneity is expected to favour genotypes able to produce different phenotypes in response to environmental variations (Stearns, 1992). Originally thought to unfold over long periods of time, it has now been demonstrated that adaptive evolution, which is influenced by phenotypic plasticity (Wund, 2012), can occur during very short timescales (Boag and Grant, 1981; Hairston Jr et al., 2005; Whitehead et al., 2017). Human activities are nowadays considered as the dominant pressures affecting the fitness of many species, and have been shown to act as potent selective pressures (Palumbi, 2001; Stearns, 1992; Whitehead et al., 2017). Among anthropogenic activities affecting biodiversity, land-use change and especially intensive agriculture is a significant

global pressure, converting natural habitats to intensely managed systems (Dou et al., 2021; Dudley and Alexander, 2017).

The responses of wildlife to agricultural land-use are numerous. For instance, agricultural lands have been associated with decreased hatching success in American kestrels *Falco sparverius* (Touihri et al., 2019), modified dispersion and exploratory movements in Iberian lynx *Lynx pardinus* (Gastón et al., 2016) or increased chemical defence in adult common toads *Bufo bufo* (Bókonyi et al., 2019). With regard to the specific effects of pest control agrochemicals, they can act at various levels from genetics to phenotypic plasticity, resulting in physiological, behavioural and fitness alterations (Saaristo et al., 2018). Considering wild population can develop resistance to agrochemicals (Almeida et al., 2021; Cothran et al., 2013), those substances could thus impact the capacity of populations to persist into the future by altering the strength and targets of evolutionary selection.

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Among the numerous agrochemicals used in agricultural landscapes, glyphosate [N-(phosphonomethyl)glycine] has been considered as a virtually ideal herbicide (Duke and Powles, 2008). Glyphosate is a low-cost highly effective broad-spectrum herbicide, and is the only molecule that is highly effective at inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase of the shikimate pathway. The shikimate pathway is found specifically in microorganisms and plants, therefore glyphosate was considered to be one of the least toxic pesticides to animals (Herrmann and Weaver, 1999; Williams et al., 2000). Yet, its widespread use in agricultural lands has contaminated soils and waters. In the wild, glyphosate is rapidly and primarily metabolized to 2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA). AMPA has a longer half-life than its parent compound, 76–240 days and 2–142 days, respectively (Annett et al., 2014; Giesy et al., 2000; Grunewald et al., 2001) and is more frequently detected in surface waters worldwide (Silva et al., 2018). In addition, it is now recognized that glyphosate and AMPA can cause severe acute and chronic toxicological effects such as cytotoxicity, genotoxicity, increased oxidative stress, disrupted estrogen pathway, impaired cerebral functions, increased mortality and embryonic developmental failure (Cheron et al., 2022; Cheron and Brischoux, 2020; Gill et al., 2018; Howe et al., 2004; Lancôt et al., 2014, 2013; Mann and Bidwell, 1999; Matozzo et al., 2020, 2018; Navarro-Martín et al., 2014; Peillex and Pelletier, 2020; Suppa et al., 2020; Trudeau et al., 2020; Tsui and Chu, 2003); with higher teratogenic effects of AMPA in comparison to glyphosate (Zhang et al., 2021).

Importantly, it has recently been shown that AMPA, at concentrations similar to those measured in the wild, could alter embryonic development, induce selective mortality and affect hatchling phenotype in an amphibian species, the spined toad *Bufo spinosus* (Cheron et al., 2022; Cheron and Brischoux, 2020). Individuals that survived being more resistant to both AMPA and AMPA-mediated effects, these findings suggest that long-term use of glyphosate could act as a selective pressure (Cheron et al., 2022). Spined toads should be particularly well-suited to investigate the selective effects of AMPA, as they are widespread in Western Europe, they occur in a variety of habitats and persist in agricultural areas (Guillot et al., 2016). Finally, the aquatic embryonic and larval phases of the spined toad, coincide with the timing of glyphosate application in agroecosystems (Berger et al., 2013; Lenhardt et al., 2015). Individuals could therefore be exposed to agrochemicals across their entire life. Earlier studies found no glyphosate resistance in agricultural habitats in different anuran species (Bókonyi et al., 2017; Cothran et al., 2013), but those tadpoles were exposed to high concentrations of glyphosate and not to AMPA, which has a longer half-life and higher teratogenic effects (Zhang et al., 2021).

The aim of the present study was to investigate whether offspring of individuals originating from glyphosate-exposed habitats, such as agricultural areas, would be resistant to AMPA, in comparison to offspring originating from glyphosate-free habitats. To do so we captured breeding spined toads from forested (preserved) and agricultural (altered) habitats, and reared their offspring under common-garden conditions. To test whether individuals from agricultural habitats were resistant to AMPA, we exposed half of their offspring to an environmentally relevant concentration of AMPA, and the other half to control (without AMPA) conditions, during the embryonic and larval development until metamorphosis. The same was done with individuals from preserved habitats. Then, we quantified fitness parameters in both groups and predicted that historically exposed populations (agricultural origin) would be less impacted by AMPA exposure than preserved populations (forest origin). More specifically, because selective mortality has been shown to occur during embryonic development, we predicted that embryos from forest sites should be more sensitive to AMPA than their agricultural counterparts. Because of this AMPA-mediated selective mortality of susceptible embryos, we predicted that surviving larvae from forest sites should respond similarly than those from agricultural areas.

2. Materials and methods

2.1. Study sites and capture

We selected four breeding ponds in contrasted habitats (Fig. S1), two of which were located in highly forested areas and the two others in agricultural landscapes; all situated in the southern part of the Deux-Sèvres department (France). Such site selection allowed making simple habitat classifications (“Forest” vs “Agricultural”). Pond distances were considered large enough to avoid sampling individuals from a same population (i.e., ~18 km between the forest sites; ~20 km between the agricultural sites, shortest distance between ponds: 4 km) (Kovar et al., 2009; Sinsch, 1988). To ascertain the classification of our habitats, in 2020, AMPA analyses were conducted monthly (February to June) in water from the two monitored forest ponds. AMPA concentrations were below limits of detection (<0.030 µg L⁻¹). In contrast, in one of the monitored agricultural ponds and in another one not included in the present study, AMPA concentrations in June ranged between 0.17 and 2.8 µg L⁻¹. Between 28/01/21 and 22/02/21, we captured 10 spined toad amplexed pairs per sites (40 pairs in total). Pairs were spotted at night by patrolling along the breeding sites with head lights. In each pond, the 10 first couples were collected to guarantee comparable individual quality between the different sites.

2.2. Housing, treatment and measurements

Each pair was housed in a 20 L aquarium containing a branch for the female to wrap her egg strings around, after laying (2–17 days after capture) males and females were released in their habitat. Within 6 h post laying, the egg strings (hence “clutch”) from both forest and agricultural origin were removed from the aquarium, and six segments of 34 eggs per clutch were placed in individual aquariums (n = 240 aquariums) with 2 L of dechlorinated tap water in common-garden conditions. Among the six segments of a same clutch, three were exposed to 0.4 µg L⁻¹ (±0.01 µg L⁻¹) of AMPA (AMPA group) and the remaining three segments, non-exposed to AMPA, constituted the control group. The AMPA solution was obtained by dissolving commercial crystalline powder (Aminomethylphosphonic acid, 99% purity, ACROS ORGANICS™) with dechlorinated tap water. The selected AMPA concentration (0.4 µg L⁻¹) was representative of actual concentrations found in surface water in our study area and based on a previous study showing sublethal effects at environmental concentrations (Cheron and Brischoux, 2020). We monitored the duration of embryonic development (between egg-laying and hatching), once approximately 90% of the 34 eggs of a segment reached Gosner stage 25 (Gosner, 1960), we calculated three metrics: the hatching success (percentage of hatched tadpoles), the rate of deformed tadpoles (percentage of individuals with oedema, crooked spines, or other deformities, see Fig. S2) and the rate of late tadpoles (percentage of individuals which Gosner stage was at least 5 stages later than Gosner stage 25, see Fig. S2). Those metrics were calculated for n = 8160 individuals (240 segments of 34 eggs). From the 34 hatchlings originating from each segment, one healthy looking tadpole was selected, this resulted in 240 experimental individuals (n = 120 in each experimental group), the remaining tadpoles were released in the breeding pond of their parents. Each tadpole was individually kept in a 2 L aquarium either with dechlorinated tap water or AMPA according to the treatment experienced during embryonic development. As a consequence, each individual was exposed during both embryonic and larval development to the same treatment. The larval development was monitored through six key Gosner stages (25, 30, 37, 41, 42 and 46). Tadpoles’ development was checked daily and when a tadpole reached one of those stages it was photographed on graph paper for body, tail, and total length (snout to tail tip) measurements using the free software ImageJ (<https://imagej.nih.gov/ij/>), as these traits are known to exhibit plasticity in response to AMPA contamination (Cheron and Brischoux, 2020). Upon metamorphosis we calculated the scaled mass index (SMI)

developed by Peig and Green (2009) with the following formula: $SMI = \text{body mass} \times (0.996/SVL)^{0.398}$ and mortality was monitored from hatching to metamorphosis.

From the egg stage until metamorphosis, the 240 tadpoles were kept under simulated 12:12 h day and night period, in a room at 17 °C to avoid any variation of the basal metabolism and therefore development. Water was changed weekly, with an addition of AMPA at 0.4 µg L⁻¹ for the contaminated tanks. Upon hatching, the tadpoles were fed with organic ground spinach *ad libitum*.

2.3. Statistical analysis

Our data was tested for homogeneity of variance and normality, we also checked normality of the residuals using diagnostics plots. Some variables slightly diverged from normality (see violin plots Figure S3). Yet, because the F-statistic is extremely robust to violation of the normality assumption when sample sizes are equivalent among groups and degrees of freedom are large (both conditions were met in our analyses), we also used parametric test to analyse those variables. We used linear and generalized linear mixed-effect models (LMMs and GLMMs) to test the effects of AMPA treatment according to habitat on fitness parameters. It has to be noticed that in the forest group, there was one clutch where the deformity rate was very high (ranging from 50 to 100% for all segments), this clutch was therefore discarded from further analyses, resulting in $n = 234$.

The response variables were: embryonic phase duration, hatching success, deformity rate and late rate calculated for $n = 7956$ eggs, then during the larval phase, for the 234 tadpoles followed until metamorphosis the response variables were: duration to reach the next monitored stage, body length, tail length, snout to tail tip length (STL) and SMI (Peig and Green, 2009) at stage 46. Mortality was analysed with a binomial model (GLMM) and all other response variables were analysed with Gaussian models (LMMs). In each model, fixed variables were: treatment (AMPA versus Control), habitat (agricultural vs forest), their interaction and the clutch identity was set as a random variable. Mixed-effects models were fitted using the 'lmer' or 'glmer' functions of the 'lme4' package (Bates et al., 2015) in R v.3.6.3 (R Core Team, 2019). Kenward-Roger approximation was used to calculate degrees of freedom. For calculating linear contrasts, we used the 'lsmeans' package (Lenth, 2016). From each model, we report the treatment effect (difference between control and AMPA-exposed animals) for each habitat type as least-squares means with standard errors (SEs) and with 84% confidence intervals (CIs). Comparing two 84% CIs will give an approximate $\alpha = 0.05$ test for the difference (Payton et al., 2003); thus, in our case, lack of overlap between the two 84% CIs indicates a significant difference in the AMPA effect between forest and agricultural habitats. Finally, time-dependent survival according to treatment and habitat was tested using a Kaplan-Meier analysis with the 'survival' package (Therneau, 2022).

3. Results

Embryonic development duration (i.e. from egg-laying to hatching) was not related to AMPA exposure, in neither habitat, nor were hatching success, late rate or morphological measures ($n = 7956$, Fig. 1, Table 1). However, the proportion of deformed hatchlings ($n = 7956$) was higher in eggs exposed to AMPA in forest individuals only (Fig. 1, Table 1). During the embryonic phase, we did not observe differential responses to AMPA exposure according to the habitat (Fig. 1).

During the larval phase ($n = 234$), in the agricultural group only, the overall duration to reach metamorphosis was longer in AMPA exposed individuals in comparison to controls (Table 1). This result was the consequence of a ~2-day delay to reach Gosner stage 30, added to a ~4-day delay to reach Gosner stage 42 (Fig. 2, Table 1). In the Forest group, AMPA-exposed individuals reached Gosner stage 30 approximately 2-days after control individuals (Fig. 2, Table 1). Yet in the forest group,

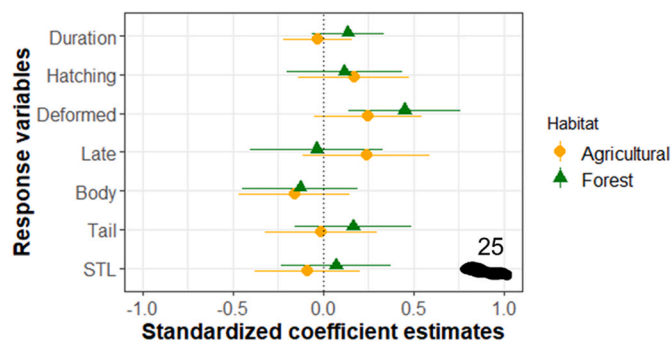


Fig. 1. Effects size of fitness parameters according to habitat in response to AMPA exposure during the embryonic stage of spined toads. The variables are the duration of the embryonic stage (egg-laying to hatching), hatching success, deformity rate, late rate and morphological measures (body length, tail length and snout to tail tip length = STL). Embryonic stage duration was obtained from $n = 234$ egg segments. Hatching success, deformity rate and late rate were calculated from $n = 7956$ eggs, and morphological measures on $n = 234$ hatchlings. The figure illustrates scaled estimates of model outputs and 84% confidence interval from mixed effect models. An effect is considered as significant when its confidence interval does not cross zero, the effects between habitats are considered significant when their confidence intervals do not overlap. Yellow circles represent agricultural originating individuals and green triangles forest individuals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

this delay was cleared after stage 30 and until metamorphosis (Fig. 2, Table 1).

The strongest effects of AMPA on morphological measures were observed at Gosner stage 30, where AMPA-exposed tadpoles from both habitats had shorter tails than controls, resulting in shorter total length (STL, Table 1). In contrast, at Gosner stage 37, AMPA-exposed tadpoles had longer bodies than controls in the Agricultural group (Table 1). At metamorphosis (Gosner stage 46), SVL and body condition (SMI) were not related to AMPA exposure in either habitat (Fig. 2, Table 1), nor was overall mortality (Table 1). During the larval phase and until metamorphosis, the effects of AMPA on morphology and body condition were not habitat-dependent (Fig. 2, Fig. S4, Table 1). Time-dependent survival was not different in exposed or control tadpoles in either habitat ($p > 0.160$ for all tests, Fig. S4, Table S1).

4. Discussion

Contrarily to our prediction, several decades of glyphosate exposure in agricultural lands did not confer an adaptive response to spined toad tadpoles. Although AMPA exposure did increase the proportion of deformed hatchlings in the forest populations, which are known to be non-viable (Beattie et al., 1992; Chinathamby et al., 2006; F. Brischox personal observation), the effects of AMPA were not stronger than in the agricultural group. In addition, the effects of AMPA exposure on morphology were not habitat-dependent, although in the agricultural group, AMPA-induced morphological differences persisted over a longer time (Agricultural: stage 30 and 37; Forest: stages 30). Yet, they were cleared at metamorphosis and among the toadlets that survived until metamorphosis (47.5% and 59.2% in the agricultural and forest groups, respectively), AMPA exposure was not related to SVL neither to body condition or mortality. AMPA exposure increased the total developmental length in tadpoles from the agricultural group, yet the development duration was not significantly different from that of forest individuals. Thus, individuals from both habitats did not respond differently to AMPA exposure, we have therefore no evidence of AMPA-resistance in spined toads originating from agricultural habitats, and we could explain this result by the several complementary and non-mutually exclusive hypotheses that follow.

A previous study conducted by Cheron and Brischox (2020),

Table 1

Effect of AMPA on fitness parameters according to habitat in developing spined toads. Values are estimates, standard errors (SE), degrees of freedom (df), upper and lower limits (CL) of the 84% confidence intervals obtained from the contrasts of the treatment (control vs AMPA) × habitat (agricultural vs forest) interaction of LMMs and GLMMs. Embryonic stage duration, hatching success, deformity rate and late rate were calculated out of 234 segments of 34 eggs (n = 7956), from stage 25 metrics are obtained for n = 234 individuals (sub-sample sizes are given for each stage). Total development duration spans from egg-laying to metamorphosis (Gosner stage 46), toadlet's body condition was calculated using the scaled mass index of Peig and Green (2009). Mortality was categorized as 0 = alive, 1 = dead. STL = snout to tail length, SVL = snout to vent length. Values in bold are significant at the 0.05 level. Reference level is "control".

Response variables		Agricultural					Forest					
		Estimate	SE	df	lower CL	upper CL	estimate	SE	df	lower CL	upper CL	
Embryonic stages		<i>Control Agricultural (n = 2040) - AMPA Agricultural (n = 2040)</i>					<i>Control Forest (n = 1938) - AMPA Forest (n = 1938)</i>					
Laying → 25	n											
	Duration	-0.064	0.18	188	-0.44	0.32	0.27	0.189	188.2	-0.12	0.67	
	Hatching success	5.60	4.82	188.2	-4.480	15.7	3.91	5.03	188.7	-6.6	14.4	
	Deformity rate	2.92	1.69	188.1	-0.61	6.45	5.34	1.76	188.6	1.66	9.01	
25	Late rate	3.13	2.20	188.4	-1.47	7.73	-0.46	2.29	189.5	-5.25	4.33	
	n	<i>Control Agricultural (n = 60) - AMPA Agricultural (n = 59)</i>					<i>Control Forest (n = 54) - AMPA Forest (n = 56)</i>					
	STL	-0.007	0.012	188.2	-0.032	0.017	0.006	0.0123	188.6	-0.020	0.032	
	Body length	-0.006	0.006	188.2	-0.018	0.006	-0.005	0.006	188.8	-0.018	0.008	
Larval stages	Tail length	-0.001	0.008	188.2	-0.018	0.017	0.009	0.009	188.7	-0.009	0.028	
	n	<i>Control Agricultural (n = 59) - AMPA Agricultural (n = 53)</i>					<i>Control Forest (n = 53) - AMPA Forest (n = 55)</i>					
	25 → 30	Duration	2.11	0.91	181.9	4.02	0.20	2.22	0.93	180.3	0.28	4.16
	30	STL	-0.061	0.023	179.5	-0.013	-0.108	-0.055	0.023	179.2	-0.103	-0.007
30 → 37	Body length	-0.019	0.010	179.8	0.002	-0.040	-0.014	0.010	179.3	-0.035	0.008	
	Tail length	-0.041	0.014	179.5	-0.011	-0.071	-0.041	0.015	179.2	-0.071	-0.010	
	n	<i>Control Agricultural (n = 49) - AMPA Agricultural (n = 46)</i>					<i>Control Forest (n = 47) - AMPA Forest (n = 52)</i>					
	37	Duration	0.52	2.60	159.5	-4.92	5.96	1.84	2.54	157.3	-3.48	7.15
37 → 41	STL	0.048	0.031	159.8	-0.017	0.113	0.047	0.030	157.5	-0.017	0.110	
	Body length	0.037	0.014	160.1	0.008	0.067	0.023	0.014	157.8	-0.006	0.052	
	Tail length	0.006	0.022	158.8	-0.040	0.052	0.027	0.022	156.7	-0.018	0.072	
	n	<i>Control Agricultural (n = 43) - AMPA Agricultural (n = 43)</i>					<i>Control Forest (n = 42) - AMPA Forest (n = 50)</i>					
41 → 42	Duration	-3.22	1.75	147.7	-6.89	0.45	-2.70	1.69	142.4	-6.23	0.84	
	STL	0.002	0.037	146	-0.074	0.078	-0.052	0.035	141.3	-0.125	0.022	
	Body length	0.007	0.019	150	-0.032	0.046	-0.007	0.018	144.2	-0.044	0.031	
	Tail length	-0.003	0.026	145.1	-0.058	0.052	-0.048	0.025	140.7	-0.101	0.005	
42 → 46	n	<i>Control Agricultural (n = 23) - AMPA Agricultural (n = 31)</i>					<i>Control Forest (n = 32) - AMPA Forest (n = 39)</i>					
	42	Duration	4.27	2.03	115	0.003	8.530	-0.053	1.72	99	-3.66	3.56
	STL	-0.076	0.080	114.4	-0.243	0.091	0.020	0.067	98.6	-0.122	0.161	
	Body length	0.015	0.020	107.8	-0.028	0.057	0.009	0.017	94.1	-0.026	0.045	
42 → 46	Tail length	-0.096	0.079	116.9	-0.262	0.069	-0.003	0.067	100.9	-0.144	0.139	
	n	<i>Control Agricultural (n = 23) - AMPA Agricultural (n = 31)</i>					<i>Control Forest (n = 32) - AMPA Forest (n = 39)</i>					
	46	Duration	0.80	0.85	119.1	-0.98	2.58	0.04	0.72	103.3	-1.48	1.56
	SVL	0.005	0.024	117.8	-0.046	0.055	0.006	0.020	101.7	-0.036	0.049	
Laying → 46	Toadlet SMI	-0.011	0.009	116.2	-0.030	0.008	0.004	0.008	99.9	-0.012	0.020	
	n	<i>Control Agricultural (n = 23) - AMPA Agricultural (n = 31)</i>					<i>Control Forest (n = 32) - AMPA Forest (n = 39)</i>					
	Total duration	7.52	3.58	107.2	0.01	15.03	1.70	2.95	95	-4.50	7.90	
	n	<i>Control Agricultural (n = 60) - AMPA Agricultural (n = 60)</i>					<i>Control Forest (n = 57) - AMPA Forest (n = 57)</i>					
25 → 46	Mortality	-0.62	0.37	∞	-1.39	0.16	-0.36	0.38	∞	-1.16	0.43	

reported non-monotonic effects of AMPA on embryonic mortality. Embryonic mortality was higher than for controls at concentrations which were around $0.07 \mu\text{g L}^{-1}$ and $0.32 \mu\text{g L}^{-1}$, whereas AMPA did not influence embryonic mortality at higher concentrations ($3.6 \mu\text{g L}^{-1}$). In the present study we monitored a higher proportion of non-viable hatchlings in AMPA-exposed individuals ($0.4 \mu\text{g L}^{-1}$) in forest individuals only. In France, environmental concentrations of AMPA in aquatic environments range from $0.1 \mu\text{g L}^{-1}$ to $6.6 \mu\text{g L}^{-1}$ (data from Water Agencies "Agence de l'eau Loire Bretagne"). In the area where we conducted our study, maybe AMPA concentrations in water bodies were too high to lead to high enough selective embryonic mortality in spined toads (e.g. $>3.6 \mu\text{g L}^{-1}$ as observed in Cheron and Brisoux (2020)), to represent a strong selective pressure for this species. In addition, these concentrations in water bodies are likely to vary within a year, according to temperature, precipitation, and between years, according to the nature of the crop, i.e. cultivated or fallow land (Edwards, 1975; Grandcoin et al., 2017; Medalie et al., 2020). Therefore, within a same pond, some years could select towards AMPA-resistant individuals, whereas in other years there would be no selection. Given the longevity of spined toads (ca. 10 years in the wild) it is likely that a homogenization of genotypes would occur between generations and mask AMPA-resistant individuals.

Although exposure to non-persistent chemicals may last for only a

short period of time, it is important to examine their long-term effects and the existence of any sensitive life stage. Any delay in metamorphosis or morphological changes could impact demographic processes of the population, potentially leading to declines or local extinction. Another explanation could therefore be that AMPA-exposed tadpoles, that present an upregulated oxidative stress response (Cheron et al., 2022), will be selected against later in their life as observed in other taxa. For example, in three-spined sticklebacks *Gasterosteus aculeatus*, a temperature-related growth rate increase induced oxidative damage during adulthood (Kim et al., 2019). In European staling *Sturnus vulgaris*, oxidative damage in early-life affected inflammatory response in adults (Nettle et al., 2017). Male Soay sheep *Ovis aries* lambs with lower protein carbonyls, a marker of oxidative damage, were less likely to survive their first-winter (Christensen et al., 2016), and this relationship was not observed in female lambs. AMPA-driven selection could thus occur in adult spined toads, it would therefore be interesting to conduct trans-generational studies to verify this hypothesis. Interestingly, during the field season of 2020 conducted in our study area, females did not migrate to breed in sites surrounded by agricultural areas (Renouit et al., 2021), this could result from a sex-specific AMPA-related selection towards males rather than females on sexually mature individuals (Renouit et al., 2021). Yet we cannot exclude that some biotic or abiotic factors, other than AMPA exposure, would have rendered the monitored ponds

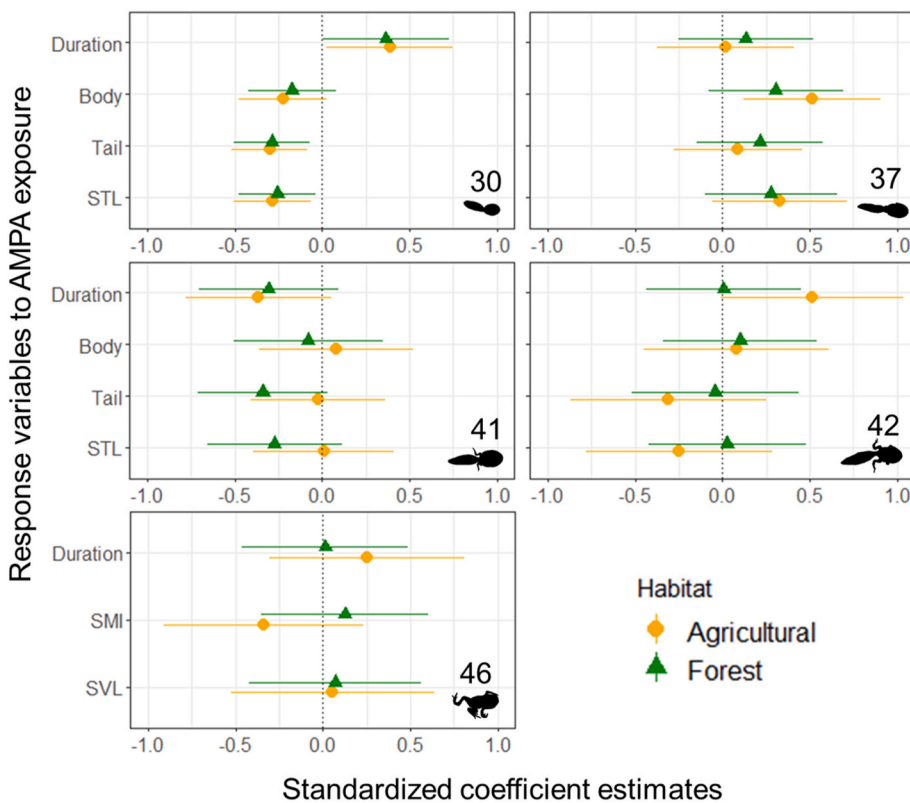


Fig. 2. Effects size of fitness parameters according to habitat in response to AMPA exposure during the larval stage of spined toads. The variables represent the duration of the larval stage and morphological measures (body length, tail length, snout to tail tip length = STL). For Gosner stage 46, we used the measure snout to vent length (SVL) as tail resorption was complete in most individuals, and calculated their scaled mass index as a measure of body condition. Sample sizes for each stage are given in Table 1. The figure illustrates scaled estimates of model outputs and 84% confidence interval from mixed effect models. An effect is considered as significant when its confidence interval does not cross zero, the effects between habitats are considered significant when their confidence intervals do not overlap. Yellow circles represent agricultural originating individuals and green triangles forest individuals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unattractive to female spined toads during that given year, as, at least during the following year, breeding females were observed in other agricultural sites.

Finally, we could explain this result by the fact that spined toad migration distance could be larger than expected, and instead of having habitat-related populations, we could be studying one unique population on our entire study area covering 76 km². Although we followed individuals from distant ponds (>4 km), smaller unmonitored ponds comprised between our study sites could act as breeding zones, where AMPA-resistant individuals would potentially reproduce with forest non-resistant individuals, resulting in a homogenised spined toad population with mixed genotypes. Adult spined toads' foraging area is not delimited by habitat structure (Indermaur et al., 2009) and from one pond to the next, there could be genetic mixture leading to a meta-population inhabiting a large territory. In other *Bufo* species such as *Bufo calamita*, the connectivity between neighbouring breeding ponds can be maintained up to a distance of 12 km according to the nature of the soil (Sinsch et al., 2012). The absence of adaptive response to AMPA exposure in agricultural toads in comparison to forest toads could therefore result from the absence of habitat-dependent genetic pools. Our results contrast with that of Almeida et al. (2021), which report differential genetic adaptation in *Daphnia magna* populations according to pesticides most commonly used in a 200 m radius around the studied pond. Considering the classic pattern of isolation by distance, which is determined by a positive correlation between genetic differentiation among populations and geographic distances (Wright, 1943), an organism's dispersal capacities, but also longevity is likely to affect gene flow between populations and therefore local adaptations. Further studies using genetics to identify segregated populations in areas more or less sprayed by glyphosate would be needed, in order to better address the question of AMPA as a selection pressure.

Still, we show strong effects of AMPA on deformity occurrence at hatching, morphology and developmental duration regardless the parents' habitat. These results suggest teratogenic effects of AMPA and a potential disruption of hormones involved in developmental plasticity.

AMPA and glyphosate exposure have been reported to induce deformities in several aquatic vertebrates such as amphibians and fish (Babalola et al., 2019; Bach et al., 2018; Smith et al., 2019; Zhang et al., 2021), and as previously mentioned, deformed hatchlings are not viable (Beattie et al., 1992; Chinathamby et al., 2006). Metamorphosis in amphibians is principally orchestrated by thyroid hormones (THs) and glucocorticoids (GCs), which in turn act on a number of other hormones (Lorenz et al., 2009; Navarro-Martín et al., 2012; Sachs and Buchholz, 2019). Exposure to glyphosate and metabolites are likely to disrupt these hormonal axes and the expression of targeted genes. For instance, exposure to Roundup WeatherMax® in natural wetlands altered the mRNA levels of thyroid- and stress-related genes of wood frogs (*Lithobates sylvaticus*) tadpoles (Lanctôt et al., 2013). The effects of AMPA on tadpole morphology and development may threaten wild populations. Shorter tails and larger bodies are maladaptive traits to cope with predators. For example, tadpoles reared in predator-exposed ponds had shorter bodies, deeper tail fins and longer tails (Buskirk and Relyea, 1998). A shorter body and a longer tail shall confer increased swimming speed, acceleration, and manoeuvrability, which are adaptive traits to predation avoidance. A longer time to develop may also be non-adaptive in the wild, increasing the risk of pond desiccation before emergence. AMPA exposed tadpoles may therefore be easier prey to catch and may have a higher mortality rate in the wild.

5. Conclusion

Despite strong evidences obtained in laboratory conditions from previous studies (i.e. increased embryonic mortality in the AMPA-exposed group), the results of this study give little evidence that glyphosate's primary degradation product, AMPA, induces a selective pressure in the wild. Still, we have to remain cautious on our conclusions, since housing conditions were optimal and do not reflect reality. Moreover, it has to be noticed that our monitoring of larval stages was conducted on healthy looking individuals, which may be better able to cope with AMPA exposure. Within and between years temporal and

spatial dynamics of contamination, as well as gene flow between exposed and preserved populations may hinder adaptive divergence between populations. In addition, wild organisms are exposed to a multitude of biotic (e.g. density, food scarcity, predation) and abiotic (e.g. temperature, desiccation probability, other contaminants) stressors, which may act synergistically with AMPA exposure. Further studies taking into account the presence of multiple stressors could help to better pinpoint the habitat-related effects of AMPA on wildlife. Yet, we show strong evidence of adverse effects of AMPA exposure at early developmental stages. AMPA exposure over a very short period of time (embryonic development: ~17 days) leads to deformities, which are lethal. This result is alarming, although glyphosate's primary metabolite increases teratogenicity, exposed spined toad populations have not developed resistance to AMPA. Glyphosate application in agricultural lands could therefore be one of the numerous causes of declining wild amphibian populations.

Author statement

Sabrina Tartu: Fieldwork; Lab monitoring; Formal analysis; Investigation; Visualization; Writing - original draft, review & editing; **Mathias Renoirt:** Fieldwork; Lab monitoring; **Marion Cheron:** Fieldwork; **Léa-Lise Gisselman:** Preliminary analyses; **Solenn Catoire:** Preliminary analyses; **François Brischoux:** Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Writing - review & editing.

Ethics statement

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de L'Enseignement Supérieur, de la Recherche et de L'innovation) under permits APAFIS#13477-2018,032,614,077,834 v7, APAFIS#23728-2020011613221913 v4 and DREAL/2020D/8041.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2022.119823>.

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