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Pesticides in the real world: The consequences of GMO-based intensive agriculture on native amphibians

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ABSTRACT

Pesticide use has been suggested as one of the major drivers of the global amphibian decline. Laboratory and mesocosm studies have addressed several questions to understand the mechanism by which pesticides cause detrimental effects on amphibians. However, the extrapolation of those results to natural populations may not be adequate to predict environmental impacts or to understand the role of pesticides in the amphibian decline. By using in situ enclosures, we evaluated the effects (survival and mobility) of common pesticides applied by farmers (cypermethrin, chlorpyrifos, endosulfan, glyphosate, and 2,4-Dichlorophenoxyacetic acid) on tadpoles. We assessed these effects in four common amphibian species from South America across 91 ponds located in the Pampas of central Argentina. We found that survival decreased in 13 out of 20 pesticides applications concomitantly with detection of pesticides in water ponds. 48 h after applications, mixtures containing endosulfan or chlorpyrifos reduced tadpole survival to < 1% while the cypermethrin mixtures reduced survival to 10%. In addition, we found impairment of mobility in all combination of pesticides, including glyphosate. The ecological context involved in our study represents the common exposure scenarios related to GMO-based agriculture practices in South America, with relevance at regional levels. We emphasize that multifaceted approaches developed to understand the role of pesticides in the amphibian decline need a conservation perspective. This will be achieved by work focusing on the integrated use of state-of-the-art techniques and resources for documenting pesticide effects over wild amphibians' populations, allowing conservation scientists to generate better management recommendations.

1. Introduction

The loss of biological diversity is one of the most severe human-caused global environmental problems. Hundreds of species and myriad populations are being driven to extinction every year (IPBES, 2019). Amphibians play an important role in the current biodiversity crisis, considering that many species have declined worldwide or simply gone extinct (Bishop et al., 2012). Nowadays, nearly 41% of amphibian species are threatened and 32 became extinct in the last century (IUCN, 2019). The causes of amphibian decline are complex; they may differ among species, populations, and life stages within a population; and are context-dependent with a multiplicity of stressor interaction driving declines (Blaustein et al., 2011). The exposure to chemical contaminants (including the 3.5 million tonnes of pesticides annually used, Pretty and Bharucha, 2015) is one of the most significant threats to amphibians, with nearly a fifth of all species affected (Chanson et al., 2008; Bishop et al., 2012).

The fact that large-scale pesticide use linked to agricultural practices has long been proposed as one of the major factors for amphibian population decline (Bishop et al., 2012) has promoted a great advance in the knowledge of amphibian ecotoxicology (Hopkins, 2007). A plethora of studies has reported adverse effects of pesticides which can

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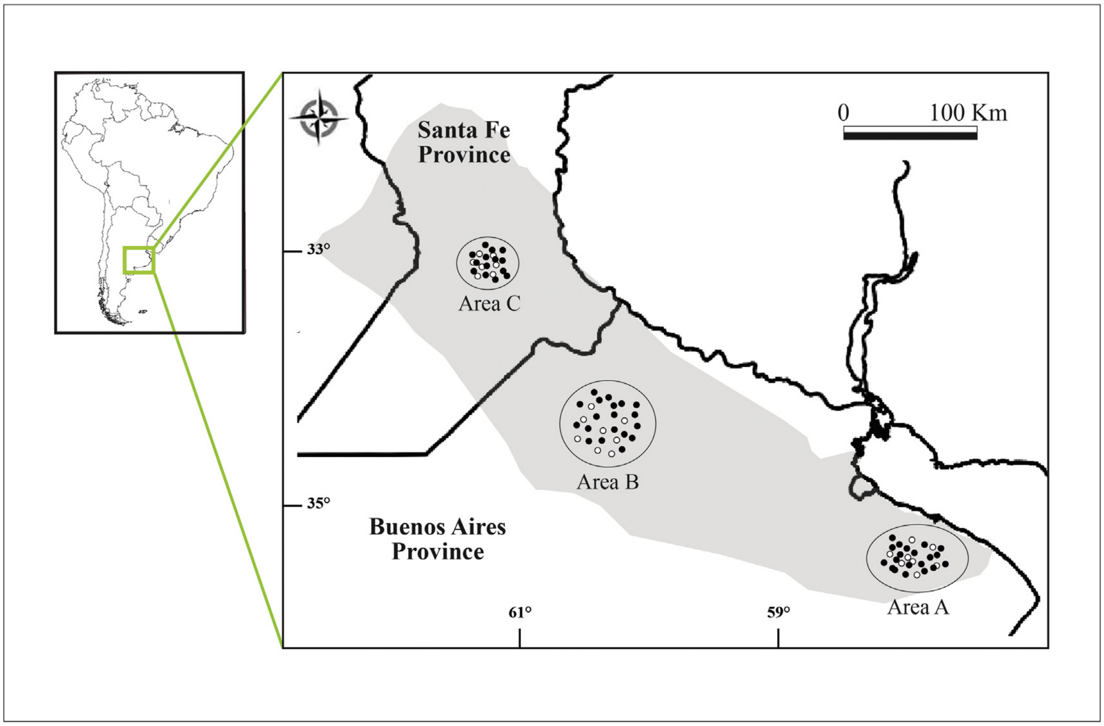

Fig. 1. Maps of the study site in Central Argentina. Gray surface indicates the Rolling Pampas where the three main areas were located. Black circles represent ponds adjacent to agricultural plots and white circles represent reference ponds.

affect tadpoles by decreasing survival, causing morphological abnormalities and impairing growth and development (Lehman and Williams, 2010). These studies used indirect methods (e.g. environmentally relevant concentrations exposures, biomarkers, laboratory and mesocosm experiments, and modeling efforts) to evaluate how pesticides may affect wild amphibian populations (Lehman and Williams, 2010; Shuman-Goodier and Propper, 2016). Nonetheless, the extrapolation of these effects to natural populations (or communities) may not be adequate to predict environmental impacts (Relyea and Hoverman, 2006; Köhler and Triebskorn, 2013), or to understand the role of pesticides in the amphibian decline (Chanson et al., 2008). Few studies have explored the effects of pesticides on amphibians under field conditions, and most of them have studied the effects of a single pesticide (Wojtaszek et al., 2004; Edge et al., 2012, 2014). Direct comparisons and extrapolation are still difficult owing to differences in test methodology, formulations tested, exposure regimes, environmental matrix, and species involved.

Agrochemicals have become an important component of worldwide agriculture systems and have enabled food production to more than double during the last century (Carvalho, 2017). In South America, the intensive agricultural model based on the GMO technological package (soy and corn seeds resistant to glyphosate, no-tillage and others agrochemical supplies) has applied since the '90s, mainly in grassland areas of Argentina, Brazil, Paraguay and Uruguay (Pengue, 2016). Soybean production in South America now covers over 57 million ha, more than on any other continent (USDA, 2015). In the past two decades, economic benefits have led to the expansion of the GMO crops into areas that had been considered marginal for this kind of production system, such as the fringes of the Amazon, Cerrado and Caatinga in Brazil, across Bolivia's and Argentinian Chaco forests, and parts of the Atlantic forests (Oliveira and Hecht, 2016). The system also builds on the 'post-frontier' areas where tenure regimes have been stabilized and consolidated, intensifying production over pastures and replacing less profitable crops (Brazilian Cerrado and the Pampas of Argentina and Uruguay) (Oliveira and Hecht, 2016). Further, it is expected that the South American countries will play a fundamental role in response to the growing worldwide demand for food and biofuels (Waroux et al., 2017). Therefore, it is anticipated that the expansion of the agricultural frontier and the intensification of current practices will continue, with the consequence being increased use of pesticides (Pretty and Bharucha, 2015). Since the beginning, pesticides have been massively used without critical evaluation, rigorous regulations and adequate information about its impacts on wildlife, leading to increasing public awareness and demanding for more scientific evidence about its consequences (López et al., 2012).

Many amphibian species have been able to persist in agricultural landscapes in South America, mainly restricted to small ponds and artificial irrigations channels and cutwaters (Attademo et al., 2005; Agostini et al., 2016; Suárez et al., 2016; Brodeur and Vera Candioti, 2017; Ribeiro et al., 2017). Although some species have been experiencing population declines and local extinctions (Carreira and Maneyro, 2015; Deutsch et al., 2017) there is a paucity of studies exploring whether native amphibian species are affected by pesticide exposure under natural scenarios. Some studies conducted in Argentina and Brazil have revealed that amphibian populations from agricultural landscapes exhibit abnormalities, alteration of enzymatic biomarkers and reduced body condition (Peltzer et al., 2008, Brodeur et al., 2011, Agostini et al., 2013, Sánchez-Domene et al., 2018). Nonetheless, the causative link between the effects observed in native populations and the pesticide inputs was not clearly demonstrated since a multiplicity of stress factors besides exposure to chemicals, could cause similar detrimental effects. Thus, the goal is to focus on the integrated use of state-of-the-art techniques and resources that will allow conservation scientists to assess the impacts of pesticides on native amphibians in relevant ecological contexts, and therefore being able to generate better management recommendations.

Here, we assessed whether chemical pest control management associated with GMO-based agriculture practices affects native amphibians, and we discuss the importance of those practices for their conservation. This was achieved by monitoring lethal and sublethal effects on amphibian larvae inhabiting ponds adjacent to agricultural farmlands and measured endpoints after farmers applied pesticides. First, we determined if pesticides applied in crops reached the ponds by measuring their concentrations. Then, by using in situ enclosures, we evaluated the effects of pesticide exposures on survival and mobility of tadpoles of four common amphibian species from South America (*Boana pulchella*, *Leptodactylus latrans*, *Rhinella fernandezae* and *R. arenarum*).

2. Materials and methods

2.1. Study area

The study was conducted in the Rolling Pampas (Fig. 1). This area was originally covered by grasslands, but nowadays supports the richest agricultural lands of South America (Soriano, 1991; Bilenca and Miñarro, 2004) and also hold an important diversity of amphibian species (≃23% of the amphibians from Argentina; Vaira et al., 2012). We studied a total of 91 temporary ponds grouped into three main areas (Fig. 1). Seventy-one ponds were located adjacent to agricultural plots (soybean crops) and therefore likely exposed to pesticide inputs. The 20 remaining ponds, without cropped plots in the surrounding basins, stand as reference ponds and were located in agro-ecosystems with grasslands for livestock use. The average of minimum distance from reference ponds to agricultural plots was 8.3 km (range: 6.8–11.5 km).

2.2. Amphibian species studied

In order to cover a representative portion of native amphibian diversity, we research pesticide effects using four species that belong to Hylidae (*B. pulchellus*), Leptodactylidae (*L. latrans*) and Bufonidae (*R. fernandezae* and *R. arenarum*) families (Frost, 2019). The species selected are well represented across the study area and previous studies showed that these species use ponds in agricultural landscapes as breeding sites (Agostini et al., 2013; Agostini and Burrowes, 2015; Brodeur and Vera Candioti, 2017).

2.3. Pesticides exposure and endpoints

The assessed applications were decided and conducted by farmers depending on the demands of each crop. All the applications of commercial formulations were dispersed with a self-propelled agricultural sprayer. To assess the effects of pesticide exposure at field conditions, pesticide application must be linked with the presence of tadpoles in the ponds. Therefore, the species used for the experiments as well as the number of ponds assessed depended on the reproductive events occurring before. We evaluated a total of 20 applications in 91 ponds (71 adjacent to agricultural plots and 20 reference ponds) during amphibian breeding seasons (October–March) from 2010 to 2012 and 2016 to 2017 (Table 1). Each pesticide evaluation involved ponds exposed to pesticides and a reference pond from the same area (see Fig. 1) allowing us to measure the endpoints on the same dates. The species assessed in each experiment and the pesticides applied are listed in Table 1. We studied the pesticide effects by using two endpoints: survival and mobility employing in situ enclosures.

To study the effects on tadpole survival and mobility, we performed five enclosures per pond (50 × 20 × 20 cm - 1 mm mesh). Each enclosure held 20 tadpoles (29–42 developmental stage, Gosner, 1960) obtained from the same ponds where the enclosures were placed. In order to ensure proper acclimatization of the tadpoles, we started the experiments 7 days before the pesticide applications and checked the tadpole conditions 48 h before the pesticide applications. We quantified live tadpoles and determined the impairment of motility at three times: T0 (24 h before pesticide applications), T1 (24 h after pesticide applications) and T2 (48 h after pesticide applications). To quantify the endpoints, we scooped out the tadpoles from the enclosures using small minnow nets and placed them in a plastic box (containing 1 l of water from the ponds) and waited 5 min to record the effect. Tadpoles were

Table 1
Pesticides applications assessed in ponds of central Pampas of Argentina. **AP**: number of pesticide application assessed. Area: (A: South of Rolling Pampas, B: Central of Rolling Pampas, C: North of Rolling Pampas). CY: cypermethrin. CP: chlorpyrifos. ENDO: endosulfan. GLY: glyphosate, 2,4-D: 2,4-Dichlorophenoxyacetic acid. **NP**: number of total ponds assessed in each pesticide application. **P ID**: identity of the ponds. C: ponds adjacent to agricultural plots. R: reference pond. *Bp*: *Boana pulchella*. *Ll*: *Leptodactylus latrans*. *Rf*: *Rhinella fernandezae*. *Ra*: *Rhinella arenarum*. **STD**: developmental stage (Gosner, 1960).

AP + area	Pesticides applied	NP	(P ID)	Species	STD
1A	CY-GLY	5	C1-4 + R1	*Bp*	40–42
2A	CY-GLY	3	C5-6 + R2	*Rf*	39–41
3A	CY-GLY	3	C7-8 + R3	*Ll*	32–36
4A	CY-GLY	3	C9-10 + R4	*Rf*	39–41
5B	CY-GLY	6	C11-15 + R5	*Ra*	31–34
6B	CY-GLY	5	C16-19 + R6	*Bp*	39–42
7C	CY-GLY	6	C20-24 + R7	*Bp*	37–39
8C	CY-GLY	5	C25-28 + R8	*Rf*	37–40
9C	CY-GLY	5	C29-32 + R9	*Bp*	39–41
10A	CP-GLY	3	C33-34 + R10	*Ll*	39–42
11B	CP-GLY	6	C35-39 + R11	*Bp*	32–36
12B	CP-GLY	5	C40-43 + R12	*Bp*	39–42
13A	END	3	C44-45 + R13	*Rf*	29–31
14A	CY-GLY-END	2	C46 + R14	*Ll*	30–37
15B	GLY – 2,4-D	6	C47-51 + R15	*Rf*	39–42
16B	GLY – 2,4-D	7	C52-57 + R16	*Rf*	40–41
17C	GLY – 2,4-D	5	C58-61 + R17	*Bp*	38–41
18A	GLY	5	C62-65 + R18	*Bp*	37–41
19A	GLY	3	C66-67 + R19	*Bp*	39–42
20C	GLY	5	C68-71 + R20	*Ra*	37–40

considered dead if they did not respond to repeated prodding or when showing any trace of decomposition. The impairment of mobility was assessed by gently prodding tadpoles and gauging their response as normal (larvae swims away immediately) or abnormal (delayed response, loss or impairment of swimming ability) (online Appendix A1-2). Effects on mobility were assessed in surviving tadpoles, therefore the evaluation on this effect was not conducted when the survival of tadpoles decreased under 10%. The quantification of the endpoints was made by the same person. We carried out this experimental design in ponds adjacent to agricultural plots simultaneously with the reference ponds.

2.4. Abiotic parameters and pesticide concentrations

Because pesticide toxicity may be modulated by abiotic factors (Laskowski et al., 2010), we measured abiotic parameters of the ponds (temperature, dissolved oxygen –DO- conductivity and pH) before the application (T0) by using a Lutron YK series 2000. For each application assessed, we measured the abiotic parameters in five different sites per ponds adjacent to agricultural plots as well as in the reference ponds.

We conducted a survey in order to study the occurrence and concentrations of pesticides in surface water before and after pesticide applications. We kept water samples in 250 ml plastic and glasses bottles (for glyphosate and insecticides/2,4-D analysis respectively) and we consecutively transported them to the laboratory facilities. Pesticides were identified and quantified by GC-μECD and HPLC-MS/UV after extraction and cleanup procedures (online Appendix C).

2.5. Statistical analyses

To identify the abiotic parameters that might affect the toxicity of pesticides, we evaluated differences between ponds adjacent to agricultural plots and reference ponds through the three main areas assessed by using general linear mixed models (Crawley, 2007). We performed separate analyses for each physiochemical factor (temperature, DO, conductivity, and pH) which was introduced to the models as response variables. The identity of the ponds (adjacent to agricultural plots and reference) was considered a fixed effect. Because we conducted sampling the same day for each application assessed, the application (1 – 20) and area (A, B and C) were introduced to the model as random effects to control the non-independence of the data.

We investigated the effect of pesticides on survival and mobility across time, employing generalized linear mixed models (GLMM) (Zuur et al., 2009) with binomial family distribution and *cbind* response terms (number of affected, number non-affected). We conducted these analyses separately for each pesticide application including time from the application (T0, T1 and T2) as fixed effects. We controlled the non-independence of data considering the pond identity and the enclosure as random effects. Since we were interested in assessing the effects of pesticides after reaching the water ponds, we did not include in the analysis data from those ponds where pesticides applied were not detected and effects were not registered. We used Tukey's honest significant difference (HSD) post hoc comparison tests to determine significant treatment differences. We used two-tailed test and Type I error rate (α) of 0.05. Statistical analyses were carried out using the lme4 and nlme packages (Bates et al., 2015; Pinheiro et al., 2019) implemented in R software, Version 3.6.0 (R Development Core Team, 2018).

3. Results

3.1. Abiotic parameters and pesticide concentrations

We did not find significant effects of the identity of the ponds on any abiotic parameters (temperature, DO, conductivity or pH) showing that these variables did not significantly ($p > 0.05$) vary among ponds adjacent to crops and those considered as reference ponds. Results of general linear mixed models including mean values and standard errors of abiotic parameters are shown in Table 2 (Online Supported Data A).

Before applications, we detected the insecticides endosulfan and chlorpyrifos in 10% ($n = 7$) of the samples from ponds adjacent to agricultural plots in concentrations ranging from 0.9 to 3.9 μg/l. After applications, we detected the pesticides assayed in 63 of the 71 ponds adjacent to crops. The ranges of total measured pesticide concentration in water were: 45.6–413.9 μg cypermethrin/l, 176.9–256.6 μg chlorpyrifos/l, 230.3–327.5 μg endosulfan/l, 70.4–209.6 μg 2,4-D/l and 31.2–330.3 μg glyphosate/l (Table 3, Online Supported Data B). We did not detect pesticides in any of the reference ponds. The 8 ponds adjacent to crops where we did not detect pesticides were C5 and C6 (corresponding to the A2), also C4, C15, C24, C32, C43 and C65. Concomitantly, the average of surviving tadpoles in those 8 ponds was 99.8% and we did not detect effects on mobility, so we removed those ponds from the analyses. Under these results, we assumed that pesticide concentrations detected after applications exceeding 10 μg/l, indicated that pesticides involved in each experiment effectively reached the ponds (Table 3, Online Supported Data B).

3.2. Tadpole survival

We found that survival significantly decreased ($p < 0.05$) after 13 of the 20 pesticide applications (Table 3). All pesticide applications involving endosulfan caused a significant decrease ($p < 0.05$) of survival at T1 and T2. After application of endosulfan (13A), only 0.5% (± 1.5) of tadpoles survived at T2, while no tadpoles survived after the application of cypermethrin + glyphosate + endosulfan (14A). All the applications of chlorpyrifos + glyphosate (10A, 11B and 12B) reduced survival to 4% (± 4.4) at T1 and 1.8% (± 3.4) at T2. Except for 2A where pesticides did not reach the pond, all the applications involving cypermethrin + glyphosate mixture (1A, 3-4A, 5-6B and 7-9C) caused a significant decrease ($p < 0.05$) on survival to 31.6% (± 11.2) and 10.5% (± 13.5) at T1 and T2 respectively. Only one application of glyphosate + 2,4-D mixture (15B) caused a significant decrease of tadpole survival ($p < 0.05$) reducing the survival to a 76.6% (± 11.5). None of the applications of glyphosate were associated with a significant decrease in survival ($p > 0.05$). The analyses also showed that survival at T2 was significantly lower (Tukey's post hoc analysis: $p < 0.05$) compared with those obtained at T1 in 8 of the 13 applications (1A, 3-4A, 5-6B and 7-9C) (Table 3). The total average of survival detected in control ponds was 99.95% (± 0.21), 99.91% (± 0.30), 99.8% (± 0.41) at T0, T1 and T2, respectively (Online Supported Data C).

3.3. Tadpole mobility

Results showed that the mobility of surviving tadpoles was negatively affected by all combination of pesticides (Table 3). The applications involving cypermethrin + glyphosate mixture (1A, 3-4A, 5-6B and 7-9C) caused a significant ($p < 0.05$) decrease of mobility in tadpoles exposed to 73.7% (± 11.6) at T1 and 95.3% (± 9.2) at T2. We also found significant effects ($p < 0.05$) on mobility in tadpoles exposed to the six applications involving glyphosate and glyphosate + 2,4-D mixture (15-16B, 17C, 18-19A and 20C). After application of glyphosate + 2,4-D mixture, 84.5% (± 11.6) of surviving tadpoles were negatively affected, while 79.4% (± 14.5) tadpoles were affected after glyphosate applications. The effects on mobility were significantly (Tukey's post hoc analysis: $p < 0.05$) higher at T2 than those observed at T1 in 7 of the 8 applications where we were able to conduct the analyses. The low number of surviving tadpoles exposed to endosulfan (13A), chlorpyrifos + glyphosate mixtures (10A, 11-12B) and cypermethrin + glyphosate + endosulfan mixture (14A) did not allow us to conduct the evaluation on mobility endpoint (Table 3). We did not detect tadpoles affected in any enclosures placed in the reference ponds (Online Supported Data C).

4. Discussion

This is the first study exploring the effects of pesticides on native amphibians involving a significant portion of high-intensity agricultural areas from South America. We found that chemical pest control can cause profound impacts on tadpoles of *B. pulchellus*, *L. latrans*, *R. fernandezae* and *R. arenarum* under real exposure scenarios. The pesticides involved in our study, as well as the way they were used, represent the usual practices carried out to control pests in soybean crops in South America (Argentina, South of Brazil and Paraguay; López et al., 2012).

Table 2
Abiotic parameters of the ponds assessed (data are given as mean values and standard errors). **T**: temperature. **DO**: dissolved oxygen. **C**: conductivity.

Pond identity	T (°C)[a]	DO (mg/L)[b]	C (mS/cm)[c]	pH[d]
Ponds adjents to crops ($n = 71$)	20.70 ± 0.96	9.23 ± 0.95	0.21 ± 0.06	7.05 ± 0.64
Reference ponds ($n = 20$)	20.80 ± 1.05	9.05 ± 0.80	0.19 ± 0.05	7.19 ± 0.78

[a] $F = 0.01$, $df = 1–51$, $p = 0.91$.
[b] $F = 0.56$, $df = 1–51$, $p = 0.46$.
[c] $F = 1.24$, $df = 1–51$, $p = 0.27$.
[d] $F = 0.06$, $df = 1–51$, $p = 0.79$.

Table 3
Percentages of survival and impairment of mobility across time in tadpoles from ponds receiving pesticides and ranges of pesticides concentration detected in water samples. **AP**: number of pesticide application assessed. **Area**: (A: South of Rolling Pampas, B: Central of Rolling Pampas, C: North of Rolling Pampas). CP: chlorpyrifos. ENDO: endosulfan. CY: cypermethrin. GLY: glyphosate, 2,4-D: 2,4-Dichlorophenoxyacetic acid. *Bp*: Boana pulchellus, *Ll*: *Leptodactylus latrans*, *Rf*: *Rhinella fernandezae*, *Ra*: *Rhinella arenarum*. T0: 24 h before application. T1: 24 h after application. T2: 48 h after application.

AP + Area	Pesticides applied	Pond ID	Species	Tadpole survival			Tadpole mobility		Range of pesticide detected μg/L
				T0	T1	T2	T1	T2	
1A	CY-GLY	C1–3	*Bp*	100	38.6 (±8.7)*	18 (±11.7)$^{\varphi}$	56 (±15.5)*	87.1 (±12.8)$^{\varphi}$	CY 195.3–365.35 - GLY 153.5–231.2
3A	CY-GLY	C7–8	*Ll*	100	27.5 (±10.6)*	1 (±2.1)$^{\varphi}$	85.4 (±2.0)*	–	CY 102.3–214.8 - GLY 67.3–137.2 **END 3.9**
4A	CY-GLY	C9–10	*Rf*	99	30.1 (±7.3)*	2.5 (±2.6)$^{\varphi}$	85 (±13.8)*	–	CY 149.5–184.5 - GLY 18.2–35.7
5B	CY-GLY	C11–14	*Ra*	100	29.5 (±5.5)*	1 (±2)$^{\varphi}$	71.1 (±0.9)*	–	CY 124.0–286.0 - GLY 89.2–320.7
6B	CY-GLY	C16–19	*Bp*	100	41.5 (±9.7)*	3.5 (±3.2)$^{\varphi}$	69.2 (±5.5)*	–	CY 239.3–354.9 - GLY 99.2–188.2
7C	CY-GLY	C20–23	*Bp*	100	20.2 (±1.7)*	9.2 (±5.5)$^{\varphi}$	85.1 (±13.2)*		CY 114.5–330.9 - GLY 73.4–156.8 **CP 1.5**
8C	CY-GLY	C25–28	*Rf*	100	29.6 (±8.3)*	6 (±3.8)$^{\varphi}$	78.6 (±9.2)*	–	CY 231–413.9 - GLY 105.4–211.8
9C	CY-GLY	C29–31	*Bp*	100	37.2 (±11.2)*	35.5 (±12.9)	59.3 (±11.8)*	75.3 (±13.7)$^{\varphi}$	CY 122.9–215.6 - GLY 121.2–196.9
10A	CP-GLY	C33–34	*Ll*	99	7.5 (±4.2)*	1.5 (±2.4)$^{\varphi}$	–	–	CP 245.3–256.6 - GLY 31.2–87.9
11B	CP-GLY	C35–39	*Bp*	100	1.6 (±2.2)*	0	–	–	CP 176.9–240.1 - GLY 104.2–155.3
12B	CP-GLY	C40–42	*Bp*	99	5.6 (±4.5)*	5 (±4.6)	–	–	CP 211.3–230.6 - GLY 98.8–105.7
13A	END	C44–45	*Rf*	99.9	4 (±4.5)*	0.5 (±1.6)	–	–	END 242.9–327.5
14A	CY-GLY-END	C46	*Ll*	100	2 (±4.4)*	0	–	–	CY 45.6 - GLY $<$ 0.5 - END 230.3
15B	GLY-2,4-D	C47–51	*Rf*	100	79.6 (±13.3)*	76.6 (±11.5)	76.8 (±6.1)*	77.2 (±4.7)	GLY 178.5–330.3 - 2,4-D 70.4–180.1 **END 1.4–1.6**
16B	GLY-2,4-D	C52–57	*Rf*	100	99.3 (±2.1)	98.8 (±2.8)	61.2 (±6.1)*	71.5 (±5.7)$^{\varphi}$	GLY 56.8–83.4 - 2,4-D 78.9–101.4
17C	GLY-2,4-D	C58–61	*Bp*	99	98.6 (±2.9)	96.9 (±4.5)	79.3 (±3.3)*	93.1 (±4.8)$^{\varphi}$	GLY 173.5–190.3 - 2,4-D 132.4–209.6
18A	GLY	C62–64	*Bp*	99	99.4 (±1.5)	98.9 (±2.1)	48.8 (±11.4)*	65.1 (±6.4)$^{\varphi}$	GLY 110.5–179.3
19A	GLY	C66–67	*Bp*	100	100	99.5 (±1.5)	67 (±5.3)*	79.8 (±3.9)$^{\varphi}$	GLY 54.5–92.5 **END 0.9**
20C	GLY	C68–71	*Bp*	100	99.2 (±1.8)	98.7 (±2.2)	74.1 (±4.3)*	94.1 (±4.1)$^{\varphi}$	GLY 214.5–315.5

– Effect not evaluated because the low number of tadpoles surviving.
Bold concentrations are indicating pesticides not applied but detected in water samples.
* Significant effects compared with T0 (Tukey's post hoc analysis: $p < 0.05$).
$^{\varphi}$ Significant effects compared with T1 (Tukey's post hoc analysis: $p < 0.05$).

Additionally, all assessed species have a wide distributional range and occur in a great variety of habitats (Cei, 1980; IUCN, 2019). Therefore, it is expected that the results obtained in this study are relevant at a regional level.

While studies about contaminant effects on aquatic wildlife have primarily focused on dose-response relationships to examine effective and lethal concentrations, the effects induced by pesticide exposures under real scenarios have been poorly demonstrated (Köhler and Triebskorn, 2013). The few studies carried out in agricultural landscapes from South America have revealed that amphibian populations occurring in areas exposed to pesticides, experienced anatomical and physiological disturbances (e.g. abnormalities, detrimental health parameters, and reduced the cholinesterase activity) (Peltzer et al., 2008; Brodeur et al., 2011; Agostini et al., 2013; Sánchez-Domene et al., 2018). Although these effects were first reported based on laboratory tests for the same species and pesticide families, evidence gathered in natural conditions were not enough to establish a definitive link with pesticides exposure (Brodeur et al., 2011; Agostini et al., 2013). By using in situ enclosures, we demonstrated that native amphibians exhibited decreases in tadpole survival and impaired mobility as a consequence of pesticide exposures involved in common practices carried out in soy crops from South America. This methodological approach allowed us to explore the effects of the most important pesticides families used worldwide through a large geographic scale.

For conservation outcomes, there is a need to increase the evidence about pesticide effects in relevant ecological contexts, which suppose assessing the impacts using not full-controlled experiments. Thus, we discuss some additional aspects regarding our experiments for assessing pesticides effect on native amphibians.

4.1. Pesticide interactions

Several authors have pointed out that pesticide toxicity might be altered by acting synergistically with other abiotic factors (Laskowski et al., 2010). For instance, high pH values increased the toxic effects of herbicides on aquatic taxa (Chen et al., 2004), while the temperature was a powerful modulator of sublethal toxicity impacting both uptake rates and metabolic rates of fishes (Camp and Buchwalter, 2016). It could be expected that the abiotic factors of the ponds varied among land use, modulating the toxicity of pesticides assessed. Nonetheless, our results showed no differences between exposed and reference ponds, suggesting that effects evidenced in this study were induced by pesticides.

4.2. Pesticide mixtures and commercial formulations

We evaluated different mixes of pesticides used in commercial formulations, which include the active ingredient and a cocktail of adjuvants and additives. Manufacturing companies often kept confidential (usually named 'inerts') the applied adjuvants and additives, making it difficult to monitor specific concentrations and effects (Mann et al., 2009). The toxicity of these compounds cannot be fully evaluated under laboratory conditions and policy regulation on pesticide use cannot be easily achieved. Additionally, it is already known that some commercial formulations are much more toxic for non-target species than their active ingredients acting alone (Tsui and Chu, 2003; Mesnage et al., 2019). Therefore, we can expect that observed effects in our study were also caused by adjuvants and additives, although it is virtually impossible to separate the detrimental effects caused by them from those induced by the active ingredients. Determining the source of toxicity in pesticides mixtures is undoubtedly necessary but these questions cannot be addressed under real scenarios of exposure since pesticides are globally applied as commercial formulations (Mesnage et al., 2013).

4.3. Pesticides in temporary ponds

It is generally recognized that pesticide concentrations found in natural habitats are often lower than the concentrations causing detrimental effects in experimental tests (Lehman and Williams, 2010). Our results indicate that after applications, all commercial formulations of insecticides reached high concentration in the water column causing lethal and sub lethal effects in tadpoles. For instance, all the application mixtures containing endosulfan or chlorpyrifos caused 99% of mortality under concentrations ranging 230.3–327.5 μg endosulfan/l and 176.9–256.6 μg cypermethrin/l which highly exceed the median lethal concentration (CL50) informed for several species exposed in laboratory toxicity tests (Agostini et al., 2010, Bernabò et al., 2011, Sparling and Fellers, 2007). The high pesticides concentration reported in our study are linked with the moment we surveyed the ponds (after the application) reflecting the difference from data usually reported in surveys that are not designed to detect peak concentrations (Relyea and Hoverman, 2006; Mann et al., 2009). Furthermore, as we conducted our study in small temporary ponds, it is expected that normal rates of pesticide applications would reach higher concentration comparing with those reported in literature obtained from rivers and lakes (Etchegoyen et al., 2017; Castro Berman et al., 2018).

4.4. Toxicity of pesticides

Considering the magnitude of the lethal effects caused by the pesticides assessed, our results are in agreement with previous studies conducted under laboratory conditions about the relative toxicity of them (Relyea, 2009; Agostini et al., 2010; Lehman and Williams, 2010; Bernabò et al., 2011; Edge et al., 2014). In this sense, our results showed that the relative toxicity for amphibian larvae follows the decreasing order: endosulfan, chlorpiryphos, cypermethrin, 2,4-D and glyphosate. Future researches are necessary to reach a more accurate understanding of the effective concentrations of each pesticide that are causing effects on tadpoles. These studies should include a representative monitoring of pesticide concentrations in water ponds.

Recently, the toxicity of glyphosate has been extensively discussed. Glyphosate (active ingredient) is considered as unlikely to be hazardous by WHO and slightly toxic by USEPA (Kegley et al., 2016). Many authors have concluded that under normal conditions of use this herbicide does not represent any hazard for aquatic wildlife due to the low persistence of the formulated compounds (Gill et al., 2018). Moreover, recent studies conducted in natural ponds have revealed no effects on *Lithobates sylvaticus* and *L. clamitans* tadpoles exposed to RoundUp WeatherMax™ and VisionMAXT™ (Edge et al., 2012, 2014). On the other hand, several studies have confirmed the relatively high toxicity of glyphosate-based products on amphibian larvae and questioned the risk of its worldwide use (Lajmanovich et al., 2003; Cauble and Wagner, 2005; Relyea and Jones, 2009). Our study showed that commercial formulations of glyphosate exposures caused adverse sublethal effects under concentrations ranging from 31.2 μg/l to 330.3 μg glyphosate/l.

Regarding sublethal effects, our results showed a higher impact registered at 48 h compared with 24 h after applications, suggesting that the effect on mobility could increase over time. On the other hand, we can expect that tadpoles have experienced recovery if the toxicity of pesticides decreases (mediated by breakdown process) (Narahashi et al., 2007). Studies assessing the recovery capabilities of aquatic biota after pesticide exposures have reported long recovered periods and irreversible injuries in central nervous systems (Bernabò et al., 2013; Bulaeva et al., 2015). The sublethal concentration inducing effects in our study may cause lethal effects in subsequent hours or days since a reduced of swimming may lead to reduce feeding and weakened predator avoidance (Weis et al., 2001)

4.5. Conservation implications

Documenting a definitive link between pesticides and the decline of any organism in nature has been a monumental task because it requires long-term monitoring data over large regions and, ideally, the ability to conduct controlled experiments at these same scales (Chanson et al., 2008). Indeed, there are very few publications showing strong evidence that pesticide exposure has directly caused amphibian populations decline (Davidson, 2004). In this regard, some authors have pointed that linking amphibian decline with contaminants in nature may be secondary if experimental evidence demonstrates convincingly the negative consequences of exposure to contaminants of individual amphibians and their populations (Boone et al., 2005). For a conservationist perspective, we argue that studies on pesticides effects under real scenarios of exposures are necessary to understand the real impact of pesticides over native amphibian populations. Furthermore, these kinds of studies need to be incorporated into a multifaceted approach in order to elucidate the causative link between pesticides as a driver of the worldwide decline. Additionally, since confirmed population decline is the focus of conservation initiatives, it is essential to gather evidence for causal links between pesticides effects on native amphibian populations in order to protect species occurring across agricultural landscapes. Finally, working closely with farmers, allowed scientists to obtain additional information about agricultural practices which is essential for studies evaluating pesticides impacts in real scenarios. By identifying products and information regarding wildlife impacts we enhance our ability to monitor and establish conservation efforts to help minimize impacts on amphibian populations.

5. Conclusion

Our work has triggered alarm about the detrimental impact of pesticides (insecticides and herbicides) on native amphibians inhabiting the shallow ponds of the richest agricultural lands of South America. We documented effects caused by pesticides on tadpoles which can compromise the viability of populations living in agricultural landscapes. The intensive agricultural model based on the GMO technological package currently applied in South America is expected to expand (and intensify) over the coming years. Therefore, it is also expected that native amphibian populations will continue being affected. We suggest that conservation priorities should be focused on developing a better policy legislation for pesticide use, including not only the protection of human settlements but also native terrestrial and wetland habitats. Additionally, there is an urgent need to build knowledge on the alternative management practices (e.g. borders management, crop rotation) to the extended and intensive use of pesticides for pest control. Finally, it is necessary to extend the knowledge on the effects of pesticide applications not only for other amphibian species but also for other taxa conforming aquatic communities with the purpose to achieve a more accurate understanding of the conflict involving pesticide use. This will enable us to predict and mitigate future pesticides impacts and establish conservation priorities for species, critical habitats, and ecosystems.

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Author statements

Agostini MG: Conceptualization, Formal analysis, Investigation, Writing, Writing - Review & Editing, Visualization, Funding acquisition. Project administration.

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Declaration of competing interests

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.

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Appendix A

Videos showing the determination of the impairment of mobility response on *R. arenarum* tadpoles. Captures were recording after a cypermethrin + glyphosate mixture exposure. A1: normal responses. A2: abnormal responses.

Appendix B

Analytical techniques for pesticides detection and quantification.

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