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Amphibian malformations and body condition across an agricultural landscape of northwest Argentina

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ABSTRACT: Agricultural landscapes support large amphibian populations because they provide habitat for many species, although agriculture affects amphibians through various mechanisms. Pollution with agrochemicals is the major threat to amphibian populations after habitat loss, as chemicals alter the ecophysiology of amphibians, putting their health and survival at risk. We aimed to assess the effect of different environments, sites, width of forest buffers and sampling years on the health of amphibians, which was estimated through the prevalence of malformations and body condition. During 3 yr of pitfall trapping, we captured 4491 amphibians. The prevalence of malformations was higher in the croplands than in the forests, while the body condition was better within forests. The prevalence of malformations was higher in the narrower forest site than in the wider forest site. The prevalence of malformations and the body condition were higher in the third year. The prevalence of malformations differed by species. We found 11 types of malformation, which mainly affected limbs and were unilateral or bilaterally asymmetrical. Our results showed that the prevalence of malformations and body condition reflect different aspects of the health of amphibians and that forest individuals are healthier than those from croplands. The results also highlight the importance of spatial configuration besides the conservation of natural habitats to preserve healthy amphibians in agricultural landscapes. The types of malformation that we found suggest that agrochemicals could be an important cause of malformations.

KEY WORDS: Anurans · Abnormalities · Agrochemicals · Amphibian disease · Spatial configuration

INTRODUCTION

Agricultural landscapes support large amphibian populations because they provide habitat for many species (e.g. Attademo et al. 2005, Prado & Rossa-Feres 2014, Guerra & Aráoz 2015). However, in the long term, declining lowland amphibian populations are mostly associated with habitat loss resulting from agricultural expansion (Mann et al. 2009), because agriculture affects amphibians through different mechanisms (e.g. Hazell et al. 2001, Johansson et al. 2005). The mechanical disturbances generated by agricultural practices alter the soil surface and water bodies, affecting or removing the habitat where most of the life cycle of amphibians occurs (Bishop et al. 1999, Knutson et al. 2004). After habitat loss, pollution with agrochemicals is the major threat to amphibians (Collins & Storfer 2003, Boone et al.

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2007, Mann et al. 2009), as chemicals alter the ecophysiology of amphibians, putting their health and survival at risk (e.g. Agostini et al. 2010, Attademo et al. 2015, Lajmanovich et al. 2015). For example, the larvae of many species that breed in aquatic habitats in agricultural lands at the time of the application of agrochemicals are exposed to these compounds during their development (Bridges & Boone 2003, Mann et al. 2009). Several field studies have shown that agrochemicals induce malformations in tadpoles and metamorphs (e.g. Taylor et al. 2005, Agostini et al. 2013) or reduce growth during metamorphosis, affecting health in adulthood (e.g. Brodeur et al. 2011, Hegde & Krishnamurthy 2014, Wagner et al. 2014).

Malformations are permanent structural defects resulting from the abnormal development of organisms (Meteyer et al. 2000). The etiology of amphibian malformations occurring in the wild remains unclear, but agrochemicals are suspected to be one of their major causes since pesticides and fertilizers could directly affect the normal development of tadpoles or increase their susceptibility to infectious diseases and to UV-B radiation (Taylor et al. 2005, Reeves et al. 2010, Lunde & Johnson 2012). Laboratory studies have shown the association between the concentration of single or combined agrochemicals and the development of amphibian malformations (e.g. Lajmanovich et al. 2003, Dimitrie & Sparling 2014). Some pesticides alter the enzymatic activity of amphibians (e.g. Attademo et al. 2014, 2015), reducing their immune functions and increasing their infection by parasites (e.g. Attademo et al. 2011), which could result in malformations (e.g. Kiesecker 2002, Johnson & Chase 2004, Rohr et al. 2008). Therefore, the prevalence of amphibian malformations is an indicator of the health of amphibians (Ouellet et al. 1997). However, the interpretation of this indicator is not straightforward. Amphibian species have different population phenologies, body sizes, larval development periods and habitat preferences, which affect their sensitivity to teratogenic factors. This differential sensitivity influences the prevalence and the type of malformations of each species (e.g. Johnson et al. 2001b, Agostini et al. 2013). Moreover, the causal agent of malformations may fluctuate through time (e.g. Ouellet et al. 1997), producing variations in the prevalence of malformations between years.

Different types of malformation result from exposure to different teratogenic factors (Lannoo 2009), so the nature of malformations could give some information about their origin. For example, limb malformations of amphibians have been attributed to parasites, UV-B radiation, chemical contaminants and their interactions (Stocum 2000, Blaustein & Johnson 2003, Piha et al. 2006). The agents causing forelimb malformations differ from those causing hindlimb malformations (Gardiner & Hoppe 1999), which could be related to the level of exposure to environmental toxins during development (Stocum 2000). Several studies have reported higher prevalence of hindlimb than forelimb malformations in agroecosystems (e.g. Ouellet et al. 1997, Kiesecker 2002, Eaton et al. 2004, Taylor et al. 2005, Piha et al. 2006, Gurushankara et al. 2007, Spolyarich et al. 2011). Other studies have linked polydactyly, polymelia and cutaneous fusion to infection with parasites (e.g. Johnson et al. 2001a,b, 2002, Kiesecker 2002, Johnson & Sutherland 2003) and associated bilaterally symmetrical limb malformations to UV-B radiation (e.g. Ankley et al. 1998, 2002, 2004, Gardiner & Hoppe 1999).

Although the body length of amphibians depends mainly on the time since metamorphosis, the body mass depends on the resource uptake, which is related to the health of the individual (e.g. Relyea 2004, Langerveld et al. 2009, Attademo et al. 2014, Dimitrie & Sparling 2014). Thus, body condition, which is estimated from the relationship between body mass and snout-to-vent length (SVL), is an indicator of the individual health of amphibians (Schulte-Hostedde et al. 2005). A relatively reduced mass in relation to the animal length is a sign of energy deficits (Zaya et al. 2011a). Agriculture affects the body mass of amphibians by different means. On the one hand, agrochemicals and their combination with other factors may affect individuals through changes in the expression of genes (e.g. those responsible for digestive enzymes, growth or energetic homeostasis) or through the disruption of their endocrine and immune systems. On the other hand, agricultural practices may affect the environment by reducing habitat quality or food availability. These changes can limit the resource uptake of amphibians, reducing their body mass, which is reflected in a decrease of body condition (Brodeur et al. 2011, Zaya et al. 2011a,b, Wagner et al. 2014). So it is expected that the average body condition of adult amphibians will worsen in stressful environments, such as croplands.

Most field studies that assess the effect of agriculture on the prevalence of malformations are not able to discriminate the background prevalence from that induced by agricultural practices because they compare the prevalence of malformations at different locations, which differ in other aspects (e.g. environmental factors, genetic pools). Besides, most field studies assessing the prevalence of amphibian malformations have taken place in the Northern Hemisphere and in developed countries (Ouellet 2000, Lannoo 2009), which have particular environmental pollution histories. Furthermore, although other studies have demonstrated that the width of forest buffers influences the behavior and abundance of amphibians (e.g. Harper et al. 2008, Freidenfelds et al. 2011, Silva et al. 2012a,b), none of them have evaluated the effect of these buffers on their health. To our knowledge, this is the first systematic and intensive survey of the prevalence of malformations in wild frogs of South America that compares the effect of adjacent agricultural and natural habitats, the spatial configuration and the width of forest buffers on the health of amphibians in agricultural landscapes.

During a 3 yr survey in a heterogeneous agricultural landscape of Tucumán Province, Argentina, we obtained a large sample of 4491 specimens to assess the differential effects of environment on amphibian health. Our specific aims were to (1) estimate a baseline of malformation prevalence for our study site and compare it with the generally accepted baseline (0 to 5%); (2) assess the potential effect of the species and the SVL of the specimen on its probability of presenting a malformation; (3) assess the effect of the environments, sites, width of forest buffers and the sampling years on the prevalence of malformations and the body condition; (4) compare the frequency between different types of malformation, and the frequency of hindlimb and forelimb malformations between environments and species; and (5) assess the relation between the prevalence of malformations and the body condition. We aimed to accurately estimate the background malformation prevalence, which was expected to be within the range of reported baselines. We predicted that the prevalence of malformations would differ by species and decrease with SVL. We also expected that malformation prevalence would be higher in the croplands than in the forests and higher in the narrower than in the wider forest site, and that it would differ between years. Being within an agroecosystem, we expected that agrochemical-related malformations would be more frequent than other types of malformation. Finally, we expected that the patterns of body condition would be inversely related to the patterns of malformation prevalence.

MATERIALS AND METHODS

Study area

The survey was carried out in Lules Farm, Lules Department, Tucumán Province, in the Yungas piedmont between 450 and 750 m above sea level (Sesma et al. 2010). This area is characterized by a subtropical climate with an average annual temperature of 19°C, marked seasonality (Brown & Malizia 2004) and a monzonic rainfall regime with dry winters and rainy summers (Grau et al. 2010). Some streams of the Lules River basin, the most important hydrological system in the area, are channeled for irrigation and flow along the farm from northwest to southeast. The farm is mainly covered with sugarcane and lemon plantations, but keeps remnants of secondary Yungas forest connected with the natural vegetation of the Sierra de San Javier.

We selected 6 sites on Lules Farm for the survey: 2 within the piedmont forest (26° 53′ 29″ S, 65° 21′ 26″ W and 26° 52′ 49″ S, 65° 20′ 40″ W), 2 within the sugarcane crops (26° 54′ 02″ S, 65° 19′ 44″ W and 26° 52′ 57″ S, 65° 20′ 12″ W) and 2 within the lemon plantations (26° 53′ 47″ S, 65° 20′ 39″ W and 26° 53′ 13″ S, 65° 20′ 09″ W). The 6 sites were located 20 m from a water course, which constitutes an attractor for amphibians, and at least 450 m from each other. The distance between sites with the same cover type was at least 1200 m. The average distance between all sampling sites was 1541 m (Fig. 1). All the sites had similar topographies and characteristics, but the forest 1 site was wider than the forest 2 site; the distance from the center of the water course to the edge of the forest was 190 m at the first site and 80 m at the second site. The analyzed landscape is contiguous as no hard edges separate the different types of environments, so amphibians had the possibility of moving between environments and sites. However, this movement is not very likely, due to the limited home ranges of amphibians (Wells 2007). Moreover, the movement of amphibians between environments makes differences more difficult to detect, so we consider that our analyses are conservative.

Sampling methods and design

We used the pitfall trapping method (Corn 2001). Traps consisted of cylindrical plastic buckets 33 cm diameter and 39 cm high, with side perforations to prevent flooding. At each site, we placed 20 pitfall traps arranged in 5 rows separated by 40 m. In each

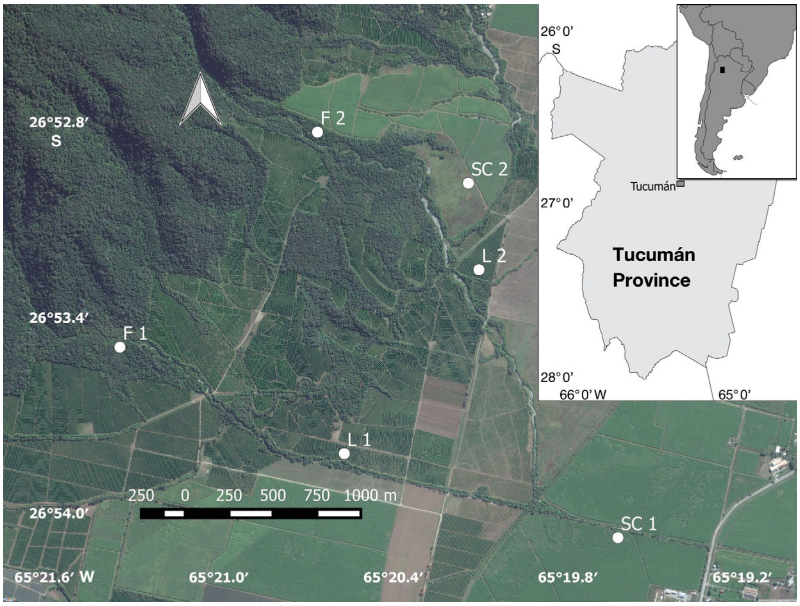

Fig. 1. Location of the 6 sampling sites in Lules Farm, Tucumán Province, Argentina. The inset shows the location of the study area. F1: forest 1; F2: forest 2; L1: lemon 1; L2: lemon 2; SC1: sugarcane 1; SC2: sugarcane 2. Image: Google Earth

row, we placed 4 traps separated by 10 m. It was not possible to install the fences suggested by Corn (2001), which increases the efficacy of the capture method, because they would have interfered with agricultural tasks. We conducted 114 surveys from February 2007 to April 2010, which totaled 1131 trapping days. Traps were checked about every week during spring and summer, and every fortnight in autumn and winter when the activity of amphibians decreases almost completely. This interval between surveys was established through a preliminary analysis and it was considered to be a good comproomise between sampling effort in a long-lasting study and the maintenance of health and survival of amphibians. Although some amphibians may have lost mass between surveys, which affects their body condition, it is likely that this loss was not biased between environments, so the results were not affected. Lules Farm provided information on the herbicides, insecticides, fungicides and fertilizers applied in the croplands under study.

Case definition

We identified the species of each captured specimen, measured its SVL using a Mitutoyo digital solar caliper (accuracy ±0.02 mm), weighed it using an electric weighing scale PRECision TH200 (accuracy ±0.1 g) and examined it for external malformations (e.g. reduced, incomplete or missing limbs or eyes). Finally, we released the individual near the capture area. Malformations are easily distinguished from traumatic deformities (e.g. amputations), which present characteristic awkward forms and healing tissue (Ouellet et al. 1997, Meteyer 2000, Taylor et al. 2005). We classified malformations according to Meteyer (2000) and Medina et al. (2013). All the classifications in this study were performed by a single researcher based on field observations, notes and pictures. During the first sampling year, 258 captured individuals were marked by means of toe clipping but, owing to the low number of general recaptures (11) and particularly those occurring in sites different from the first capture (1), we discontinued the procedure in the successive sampling years.

Body condition is an indicator of the health and fitness of an individual and can be estimated as the residual of a regression line of the body mass on the SVL (Schulte-Hostedde et al. 2005). The regression line estimates the expected body mass for a given SVL, so that the sign and magnitude of the residual indicate the condition of the individuals (Schulte-Hostedde et al. 2005). Some individuals that were not weighed in the field were omitted from this analysis. We separately estimated the body condition for the 5 most abundant species because it was not possible to get a reliable regression from the reduced number of individuals of the remaining species. As most of the species presented allometry (i.e. differential change in 2 biological variables in response to age) we log-transformed the mass and SVL of the individuals to linearize the association.

Data analyses

Malformations were only detected in the 4 most abundant species, so we estimated the prevalence of malformations for these species (*Leptodactylus latinasus*, *Physalaemus biligonigerus*, *Pleurodema borellii* and *Rhinella arenarum*). The rest of the individuals were clumped together in a single group (*L. chaquensis*, *L. mystacinus*, *Pleurodema tucumanum*, *Odontophrynus americanus*, *Oreobates discoidalis*, *R. schneideri*, *Phyllomedusa sauvagii*, *Scinax fuscovarius*). Malformations are rare events with low frequencies, so models with multiple explaining variables are difficult to parameterize due to a lack of convergence or high uncertainties in the estimates. As we considered that some attributes of the individuals may affect the likelihood of presenting a malformation, we used a 2-step method to statistically analyze the prevalence of malformations under different conditions taking into account these attributes. In the first step, we evaluated the effect of the species and the SVL of the specimen on its probability of presenting a malformation, and in the second step we used the estimated probabilities to assess the effect of the site, the environment and the sampling year on the prevalence of malformations through resampling methods.

As species differ in their size ranges, we standardized SVL within each species to make them comparable. To obtain the standardized SVL (SSVL) for every specimen, we estimated the difference between its SVL and the mean for its species and divided this difference in the standard deviation of the SVL of the species. To analyze the effect of individual features on the probability of presenting a malformation, we performed and compared different logistic regressions to predict the presence of malformations. For all of these regressions, we used a logit link function and we compared their Akaike's information criterion to select the best fitting model. We compared 4 models that considered different sets of predictive variables: (1) only the SSVL; (2) only the species; (3) the species and the SSVL; and (4) the species, the SSVL and their interaction. This analysis showed that the probability of presenting a malformation depends on the amphibian species and on the size of the individual. The size of the individuals and the abundance of the species varied between environments, but the reduced number of malformations did not permit the effect of the site, habitat type or sampling year to be included in the logistic model. So we performed a permutation analysis to evaluate the effect of the site, the environment and the sampling year on the prevalence of malformations controlling the size and the species effects (Gotelli 2000). For every individual, we used the best fitted regression (i.e. the one that considered the species and the SSVL but not their interaction) to estimate its probability of presenting a malformation and we used these probabilities to sample the observed number of malformed specimens in 100 000 simulations. In every simulation, we recorded the number of malformed individuals at each site, environment and year. To statistically evaluate the significance of the pattern, we computed the probability of randomly getting a greater (for croplands), lower (for forest) or greater and lower (for each sampling year) number of malformed individuals than the corresponding observed value in each site, each environment and each year.

To assess whether the frequency of hindlimb and forelimb malformations varied between environments, we performed a chi-squared test. As the expected number of malformations was sometimes below 5, we evaluated the statistical significance of the test through permutations because the results of Pearson's chi-squared test are inaccurate when expected values are small (Hope 1968).

To assess differences in body condition between environments, sites and years, we used separate linear models. To make a single analysis, we clumped the body conditions of the 5 most abundant species (*L. latinasus*, *O. discoidalis*, *P. biligonigerus*, *P. borellii* and *R. arenarum*), which were the only ones for which this metric was estimated. Although we acknowledge there may be some interactions between the factors (i.e. sites, environments and years), we analyzed their effect separately to facilitate the comparison with the analysis of prevalence of malformations. All the statistical analyses were performed with R software (R Core Team 2015).

RESULTS

We captured and measured 4491 individuals of 12 species (Table S1 in the Supplement at www.int-res.com/articles/suppl/d121p105_supp.xlsx): *Leptodactylus chaquensis*, *L. latinasus*, *L. mystacinus*, *Physalaemus biligonigerus*, *Pleurodema borellii*, *Pleurodema tucumanum* (Leptodactylidae), *Odontophrynus americanus* (Odontophrynidae), *Oreobates discoidalis* (Craugastoridae), *Rhinella arenarum*, *R. schneideri* (Bufonidae), *Phyllomedusa sauvagii, Scinax fuscovarius* (Hylidae). Overall, 79 specimens (1.8%) of 4 species (*L. latinasus*, *P. biligonigerus*, *P. borellii*, *R. arenarum*) showed evidence of malformation (Table S2 in the Supplement). We observed 126 cases of malformation, because 31 specimens had more than one type of malformation.

The prevalence of malformations differed by species ($\chi^2 = 15.4$, $p = 0.005$). The prevalence was highest in *P. borellii* (4%); was intermediate in *P. biligonigerus* (3%) and *L. latinasus* (2%); and was low in *R. arenarum* (1%). None of the 179 individuals that were clumped in a single group for the analyses presented malformations. The probability of presenting a malformation increased with size in the 4 species and the model that included the species identity as well as its SSVL was the one that best explained the occurrence of malformations. *P. borellii* was the species with the highest probability of presenting malformation across most of the range of SSVL, but this probability was even higher in specimens of *P. biligonigerus* with SSVL above 2. In contrast, this probability remained very low along the complete range of SSVL of *R. arenarum* (Fig. 2).

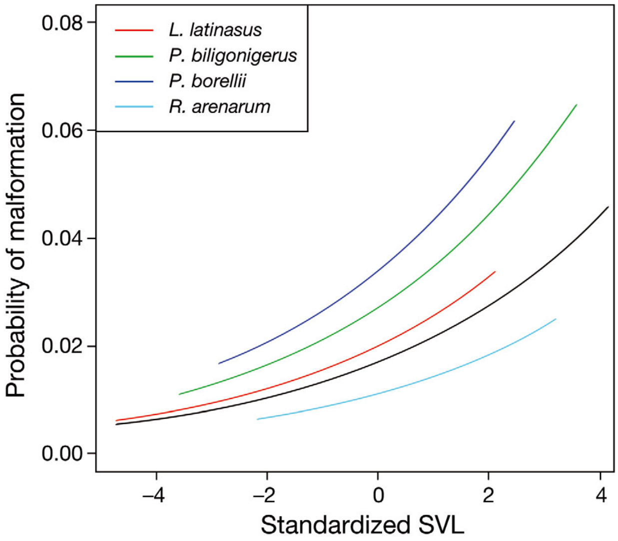

Fig. 2. Logistic regressions of the presence of malformations on the standardized snout-to-vent-length (SSVL) for the 4 most abundant species (*Leptodactylus latinasus*, *Physalaemus biligonigerus*, *Pleurodema borellii* and *Rhinella arenarum*) and for the complete assemblage (black line)

Malformed frogs were observed in all the environments, but prevalence was higher in the croplands than in the forests, though differences were not significant. Prevalence was 2.2 % in lemon, 1.6 % in sugarcane and 1.3 % in forests. The range of malformation rate was less variable between the cropland sites (1.4 to 2.3 %) than between both forest sites (0.2 to 2.0 %). Although these differences were not significant among environments, the prevalence of malformations in the wider forest (forest 1) was significantly below the random expectations (Table 1). The overall prevalence of malformations was well below average in the first year of sampling and it increased significantly in the third year of sampling ($p < 0.001$; Table 1, Fig. 3).

We observed 11 types of malformation; 3 of them affected the head of the amphibians and the other 8 affected limbs. Cephalic malformations were anophthalmia (missing eye), microphthalmia (small eye) and brachygnathia (abnormal shortness of lower jaw). Limb malformations were amelia (totally missing limb), ectromelia (incomplete limb with the lower portion of the leg missing), hemimelia (short bone but distal limb and foot are present), brachydactyly (reduced number of phalanges), ectrodactyly (completely missing digit including the metatarsal bone and phalanges), polydactyly (more than the normal number of metatarsal bones), polyphalangy (duplicate set of phalanges), according to Meteyer (2000), and digits expanded at the end (the skeletal elements are not affected), according to Medina et al. (2013). The malformations occurred on the right and/or left body side. Most of the malformations occurred in limbs (94 %), and individuals with hindlimb malformations (65 %) were more frequent than individuals with forelimb malformations. Although this propor-

Table 1. Variation of prevalence of malformations and body condition between sites, environments and sampling years. Malf.: malformed specimens; captures: obtained specimens; prevalence: percentage of malformed specimens; Year 1: February 2007 to March 2008; Year 2: April 2008 to March 2009; Year 3: April 2009 to April 2010. The p-values of malformations were estimated through 100 000 permutations and the estimates of body conditions and their p-values were obtained from a linear model

Factors		Malformation				Body condition		
Category	Class	Malf.	Captures	Prevalence (%)	p	Estimate	SE	p
Site	Forest 1	1	421	0.23	0.008	0.017	0.012	0.164
	Forest 2	12	576	2.08	0.871	0.014	0.010	0.143
	Lemon 1	22	979	2.25	0.280	0.013	0.008	0.088
	Lemon 2	18	860	2.09	0.345	−0.008	0.008	0.278
	Sugarcane 1	16	957	1.67	0.248	−0.013	0.008	0.113
	Sugarcane 2	10	698	1.43	0.767	−0.013	0.009	0.171
Environment	Forest	13	997	1.30	0.252	0.015	0.008	0.044
	Lemon	40	1839	2.18	0.204	0.002	0.005	0.657
	Sugarcane	26	1655	1.57	0.655	−0.013	0.006	0.036
Year	Year 1	11	1693	0.65	<0.001	<0.000	0.006	0.999
	Year 2	31	1650	1.88	0.581	−0.012	0.006	0.050
	Year 3	37	1148	3.22	<0.001	0.015	0.007	0.024

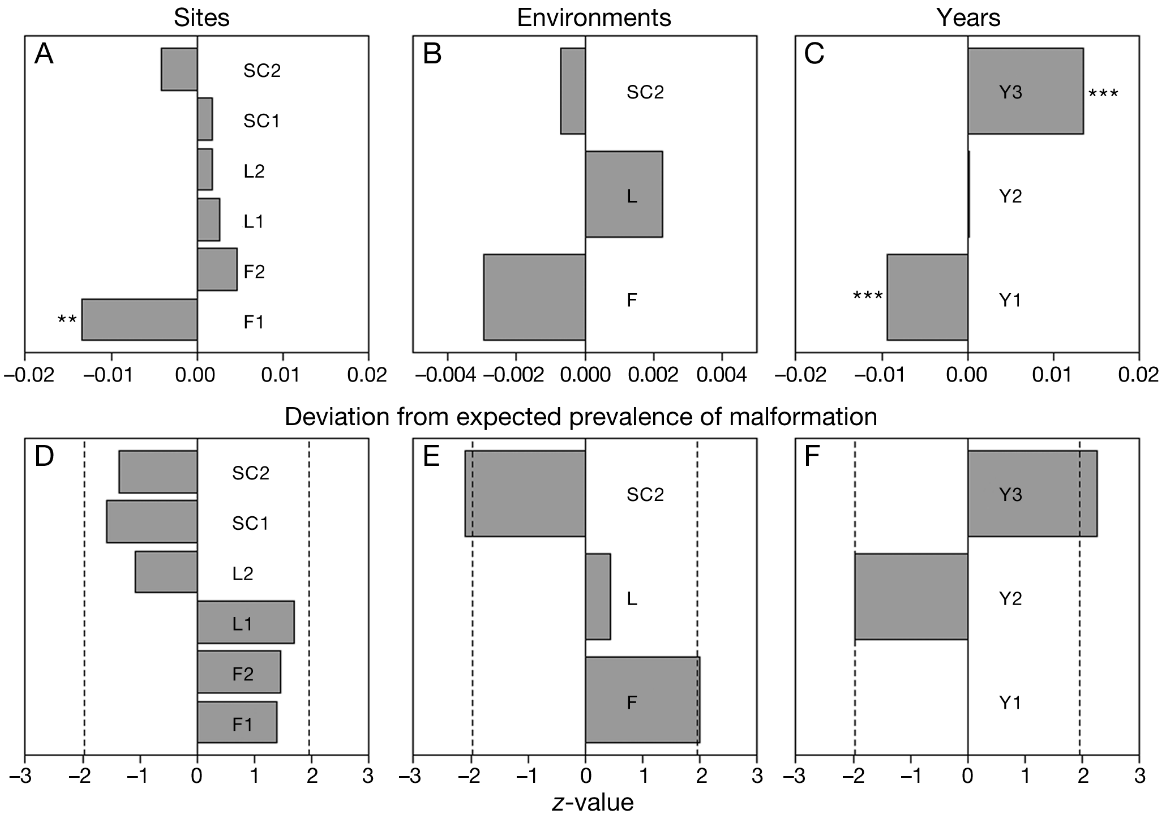

Fig. 3. Effect of different factors on the health of amphibians (site/environment abbreviations as in Fig. 1). (A–C) Differences between observed and expected prevalence of malformations. Expected prevalence was estimated taking into account the species identity and the standardized snout-to-vent length (SSVL) of the individuals captured under each situation. The statistical significance was assessed through 10 000 random simulations. Statistical assessment for sites and environments were evaluated with 1-tail tests. Asterisks indicate significance ($^{**}\alpha = 0.01$; $^{***}\alpha = 0.001$). (D–F) Normalized *z*-values of the effect of different situations on the body condition of individuals estimated through linear models. Dashed lines show the significance threshold of $\alpha = 0.05$

tion was higher in lemon crops (75%) and lower in sugarcane crops (53%), the difference was not significant (p = 0.265). Overall, the most abundant type of malformation was ectrodactyly (40%), and the rarest ones were brachygnathia, hemimelia and polyphalangy (one case of each). Some types of malformation were more frequent in the forests and lemon plantations than in the sugarcane crops. *R. arenarum* was the species with highest variety of types of malformation (9) and 3 of them were only observed in this species (microphthalmia, brachygnathia and polydactyly), while hemimelia and polyphalangy occurred only in *L. latinasus* (Table 2).

Forest environments had a significant positive effect on the body condition of individual amphibians while sugarcane affected it negatively. However, we did not find significant effects of sites on the body condition. In the second sampling year, body condition was significantly below zero (p = 0.050), while in the third year it was significantly high (p = 0.024; Table 1, Fig. 3).

DISCUSSION

The overall prevalence of malformed frogs (1.8%) observed in this study was within the expected background malformations frequency of 0 to 5% (Lunde & Johnson 2012), as found in several studies performed in agricultural landscapes (e.g. Cooke 1981, Gillilland et al. 2001, Eaton et al. 2004, Piha et al. 2006, Gurushankara et al. 2007, Mann et al. 2009, Böll et al. 2013). The low prevalence of malformations found in this study could be explained to some extent by the fact that most baselines of prevalence of malformations are based on surveys that were carried out in industrialized countries in the Northern Hemisphere with longer histories of environmental pollution, while this study was performed in a developing country. The contribution of a methodological issue cannot be discarded since in this survey traumatic deformities (which are sometimes erroneously computed as malformations) were omitted from our analysis (Taylor et al. 2005).

Table 2. Prevalence and type of malformations by environment and species

		Environment			Species				
		Forest	Lemon	Sugarcane	*Leptodactylus latinasus*	*Physalaemus biligonigerus*	*Pleurodema borellii*	*Rhinella arenarum*	Total
Forelimb malformation		7	12	21	16	5	11	8	40
Hindlimb malformation		10	51	16	47	6	7	17	77
Malformation with unrecorded location		0	1	1	1	0	0	1	2
Limb malformation	Amelia	1	1	0	0	0	1	1	2
	Ectromelia	2	7	9	12	2	0	4	18
	Hemimelia	0	1	0	1	0	0	0	1
	Digits expanded at the end	7	8	18	9	4	11	9	33
	Brachydactyly	1	9	2	8	2	0	2	12
	Ectrodactyly	4	37	9	33	3	6	8	50
	Polydactyly	2	0	0	0	0	0	2	2
	Polyphalangy	0	1	0	1	0	0	0	1
Cephalic malformation	Anophthalmia	1	3	0	1	0	1	2	4
	Microphthalmia	0	1	1	0	0	0	2	2
	Brachygnathia	1	0	0	0	0	0	1	1

Our results showed that the prevalence of amphibian malformations in agricultural landscapes depends to some extent on the vegetation cover of the site and that the spatial configuration of the surrounding sites may influence this prevalence. The higher prevalence of malformations observed in the narrower natural area (forest 2) than in the wider natural area (forest 1) reinforces the idea that the width of natural areas is important for buffering the effect of neighboring agriculture and for preserving these semi-natural systems. Several authors have demonstrated that the composition of land cover types and their spatial configuration affect amphibians in tropical agricultural landscapes and that the width of forest buffers influences the behavior and abundance of amphibians (e.g. Harper et al. 2008, Freidenfelds et al. 2011, Silva et al. 2012a,b). However, none of them evaluated the effect on the health of amphibians. Our study emphasizes the importance of preserving forest fragments of adequate size in agricultural areas to protect wildlife, as it suggests that malformations are mainly produced within croplands and that sites are permeable to the movement of amphibians only to some extent.

The variation of prevalence of malformations between years suggests that the causal agent fluctuates through time and it is not a permanent characteristic of the studied croplands or the studied amphibians (e.g. Ouellet et al. 1997). There are no evident differences in the nature or in the frequency of agrochemical applications between years (Table S3 in the Supplement). Moreover, malformation is developed mainly during tadpole stage and the age of individuals could not be accurately estimated, so no formal correlational analysis was performed. We acknowledge that the variation of malformation prevalence between years could be due to some environmental variables that were not taken into account in this study. For example, water quality, which varies between years depending on runoff sources, rainfall, temperature and other factors, could affect the development of tadpoles (Taylor et al. 2005). The fluctuating stress factor could also affect the abundance of amphibians in this area, which decreased with time according to Guerra & Aráoz (2015).

The types of malformations found in this survey suggest that agrochemicals are the main cause of malformations. Most of the chemicals used in the studied croplands have known teratogenic effects (see Table S3). The fact that lemon crops, in which the application of agrochemicals are more frequent (Table S3) have higher prevalence and a higher proportion of hindlimb to forelimb malformations (though differences were not significant) supports the idea that agrochemicals are responsible at least for an important proportion of the malformations. The agent causing hindlimb malformations seem to be related to the level of exposure to environmental toxins during development (Stocum 2000) and several studies have reported higher prevalence of hindlimb than forelimb malformations in agroecosystems (e.g. Ouellet et al. 1997, Kiesecker 2002, Eaton et al. 2004, Taylor et al. 2005, Piha et al. 2006, Gurushankara et al. 2007, Spolyarich et al. 2011). Our results do not permit us to discard the possibility that malformations could be the result of the combination or synergistic interactions between factors, such as agrochemicals, UV-B radiation and infections with parasites (Reeves et al. 2010, Lunde & Johnson 2012). However, several types of malformation linked to infection with parasites were absent (polymelia and cutaneous fusion) or scarce (polydactyly) in our survey (e.g. Johnson et al. 2001a,b, 2002, Kiesecker 2002, Johnson & Sutherland 2003). In the same way, there were no bilaterally symmetrical limb malformations typically associated with UV-B radiation (e.g. Ankley et al. 1998, 2002, 2004, Gardiner & Hoppe 1999). Besides, the types of malformation seem to be site specific (Burkhart et al. 2000) and the prevalence of malformations seems to be highly variable over time, suggesting that their causal agent may fluctuate from year to year (e.g. Ouellet et al. 1997).

All the species that exhibited malformations are abundant in areas affected by anthropogenic disturbances (e.g. Attademo et al. 2005, Guerra & Aráoz 2015). The variation of prevalence and types of malformation between them could result from differences in their natural history (Johnson et al. 2001a, Agostini et al. 2013) and consequently in their sensitivity (e.g. Relyea 2009, Attademo et al. 2014). For example, *Pleurodema borellii* and *Physalaemus biligonigerus* — the 2 species with the highest prevalence of malformations — lay eggs in foam nests that float on top of temporary or permanent water bodies and tadpole development occurs there (Lavilla et al. 2000). In contrast, the eggs and tadpoles of *Rhinella arenarum* and *Leptodactylus latinasus* — species with lower prevalence of malformations — would be less exposed to malformation factors. *R. arenarum* lays eggs in a continuous jelly tube at the bottom of temporary lentic or slowly lotic water bodies (Lavilla et al. 2000). For example, we observed their clutches in an irrigation channel, with flowing good quality water from the Lules River basin (Sesma et al. 2010). *L. latinasus* lay eggs in a foam nest in underground chambers where early larval stages also take place. After flooding, tadpoles complete their development in proximate water bodies (Lavilla et al. 2000). Agostini et al. (2013) also suggested a link between the prevalence and types of malformation and the levels of exposure to malformation factors during early stages and the habits of juveniles and adults.

This study also reinforces the idea that agricultural practices may negatively affect other indicators of amphibian health besides the prevalence of malformations. For example, body condition, which is an indicator of the health of individuals and which probably reflects the quality of the environment during adulthood, had higher scores within forest than in croplands. Since the body condition is an indicator of the quality of the environment, we expected it to inversely vary with the prevalence of malformations. We observed this pattern between environments, but not between sampling years. Both the prevalence of malformations and the body condition had their highest values during the third sampling year. It is possible that body condition is affected by density through intraspecific competition and it decreases when amphibians increase (Guerra & Aráoz 2015); but it is also possible that the prevalence of malformations and the body condition are reflecting different aspects of environmental quality. For example, malformations are generated during the larval stage and during metamorphosis (Meteyer et al. 2000), while body condition may reflect the environmental quality during adulthood (Schulte-Hostedde et al. 2005).

Through this study, we highlight the importance of accurately estimating an adequate baseline of the health of amphibians to correctly assess the effect of different types of land cover. However, we acknowledge that more studies should be carried out to define a general baseline of the prevalence of malformations in the Southern Hemisphere, where the history of environmental pollution is shorter and probably less intense than in the Northern Hemisphere. We emphasize the need for further research with more replications to confirm the effect of spatial configuration on the health of amphibians. We consider that different tools (e.g. ecological, epidemiological, environmental and public health) should be integrated to understand the environmental challenges involved in malformations and population declines of amphibians (Burkhart et al. 2000, Johnson & Bowerman 2010), especially taking into account the increase in amphibian malformations through time (Reeves et al. 2013).

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