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Reproductive endpoints of *Rhinella arenarum* (Anura, Bufonidae): Populations that persist in agroecosystems and their use for the environmental health assessment

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A R T I C L E I N F O

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A B S T R A C T

Degradation of the environment by agriculture affects the persistence and health of the amphibian populations. Characteristics related to reproduction of anuran can be used to evaluate the status of populations and as endpoints in environmental health assessment. In this *in situ* study the aspects related to the amplexus and ovipositions of the bioindicator species *Rhinella arenarum* that inhabits agroecosystems were analyzed. The hypothesis of this study is that perturbations of agroecosystems have a negative impact on the size of reproductive adults, on the size of ovipositions and eggs, and on the survival of eggs and embryos. Study area is located in the rural landscape of central Argentina. Four sampling sites were selected: C1, C2 and C3 are ponds on agroecosystems; and SM is a reference site that is not affected by agriculture or livestock. Abundance of amplexus pairs, oviposition and tadpoles per site was recorded. Individuals´ snout–vent length (SVL) in amplexus was measured. The fecundity was calculated like number of eggs per oviposition. The eggs' Gosner stage, the diameter eggs and the frequency of dead and abnormal eggs were recorded by oviposition. Killing-power between egg-embryo and egg-tadpole was calculated. The higher phosphate concentration was detected in all agroecosystems and nitrate was detected in C1 and C2. Conductivity, salinity and SDT were higher in C1 site Male SVL from the SM site was lower than the other sites while the largest SVL was of female from the C3 site. The higher frequencies of sprouted eggs and of dead eggs were recorded in the C2 site. Egg diameter was associated with SM and correlated negatively to SVL of the male and female. No correlation between female SVL and oviposition size was recorded. Killing-power in the passage from egg to tadpole classes was higher in the three agroecosystems. The hypothesis of this study was corroborated in part. Reproductive adults in agroecosystems did not have smaller body size. However, in the agroecosystem ponds, the eggs with smaller diameter were registered, the oviposition had higher frequency of abnormal eggs and the higher mortality was registered. This confirms the high sensitivity of the early stages to environmental disturbances and sustains their use as endpoints for the environmental health assessment.

1. Introduction

Expansion of the agricultural frontier is the main factor associated with the degradation of habitat quality (GAA, 2004) that affects 89% of the species of threatened anurans (Young et al., 2004). The degradation of ecosystems is mainly caused by the presence of livestock, the tillage of cropping lands and the incorporation of excessive nutrients and toxic elements through the application of biocides and fertilizers (Pengue, 2004). Many chemicals used in agriculture can contaminate the ponds causing serious damage to aquatic ecosystems (Knutson et al., 2004). In addition, agriculture alters the geomorphology of aquatic and terrestrial environments, modifying the hydroperiod, the vegetation cover, the environmental productivity and the trophic network. All this causes that persistence and health of amphibian populations are to be compromised (Beja and Alcazar, 2003; Gray and Smith, 2005; Mann et al., 2009; Gonçalves et al., 2015; Hegde and Krishnamurthy, 2014; Peltzer

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et al., 2017; Zhelev et al., 2017).

Analysis of endpoints allows the assessment and monitoring of environmental health (de Acevedo and da Mata, 2003; Attademo et al., 2014; Api et al., 2015; Babini et al., 2016; Pollo et al., 2016; Zhelev et al., 2017). Their advantage is that they allow detecting the biological effects of pollutants and other environmental stressors on the health and integrity of organisms (Livingstone, 1993). Endpoints are specific characteristics of the bioindicator and any change registered on these characters is an indicator that one or more physical or chemical requirements of the bioindicator are outside their required limit as a consequence of a change in the environmental condition (Hawkins, 2007).

Characteristics related to reproduction, such as the size of the organisms in the amplexus, fecundity, survival and size of the eggs are part of the life history of a species. These characteristics are not immutable. The life history of an organism is set within the limits given by the genotype of the individual, but in different environments the phenotypic expression of the genotype can vary, known as phenotypic plasticity (Sorci et al., 1996). The trend of these characters can be used to evaluate the status of populations in different environments (Morrison and Hero, 2003) and also as endpoints in environmental health assessment (Johnson, 2005). Amphibians are excellent bioindicators of environmental change (Rowe and Beebee, 2003; Hopkins, 2007) and they have been extensively used in toxicological tests to assess the toxic effects of contaminants on their growth, development, or reproduction (Feng et al., 2004; Lajmanovich et al., 2003). Their aquatic-terrestrial life cycle places them in doublejeopardy because a disturbance to the quality or availability of either habitat can disrupt their life cycle and affect populations (Dunson et al., 1992).

In Argentina, the percentage of territory under agricultural practice is increasing (Nori et al., 2013; Kopta et al., 2016) and anuran populations are being strongly affected (Duré et al., 2008; Attademo et al., 2014; Peltzer et al., 2013; Pérez-Iglesias et al., 2018). However, some anuran species are able to survive as meta-populations in altered landscapes (Knutson et al., 2004). Having knowledge about the intraspecific variation in the life history traits of amphibians in unfathomable environments for their development and survival is important for the conservation of the amphibian population (Morrison and Hero, 2003). In the agroecosystems of the central area of Argentina, populations of the native specie *Rhinella arenarum* (Hensel, 1867) are recorded. This specie has several characteristics that constitute it as good bioindicator of the environmental health (Hawkins, 2007). Several lab studies have reported the lethal and sublethal effect of agrochemicals on *R. arenarum* (Howe et al., 1998; Venturino et al., 2003; Ferrari et al., 2005; Vera Candioti et al., 2010; Bosch et al., 2011; Brodeur et al., 2013; Lajmanovich et al., 2013, 2014; Babini et al., 2016) however the *in situ* studies are few.

In situ tests are of great importance because they can incorporate the effects, mechanisms and dynamics of the system that are difficult to incorporate into laboratory tests, which can be the case with multiple or unidentified stressors, such as during pulse exposures from storm water or toxicant application (Relyea and Hoverman, 2006; Crane et al., 2007). *In situ* studies about *R. arenarum* that inhabit these agroecosystems have reported on their population abundance (Bionda et al., 2013) and diverse sublethal effects in adult organisms and tadpoles (Bionda et al., 2011a; Caraffa et al., 2014; Babini et al., 2015a, 2017; Pollo et al., 2015). Then, taking into account the need and value of *in situ* tests and that reproductive endpoints of anurans are indicators of environmental health, in this paper the aspects related to the amplexus and ovipositions of the bioindicator species *Rhinella arenarum* that inhabits agroecosystems were analyzed. The hypothesis of this study is that perturbations of agroecosystems have a negative impact on the size of reproductive adults, on the size of ovipositions and eggs, and on the survival of eggs and embryos.

2. Material and method

2.1. Study area and sampling sites

Study area is located in the rural landscape of central Argentina. Sampling was carried out in ponds from the south of Córdoba province, near the Río Cuarto city (33° 10 'S −64° 20' O; 420 m.a.s.l). The main socio-economic activity of the area is agricultural-livestock production. The predominant climate of this region is temperate, with dry winters and hot summers. The summers present temperatures up to 43 °C, with an average temperature of 23 °C. In winter, temperatures can drop to −7.5 °C, presenting a winter of few rains and with frequent frosts from April until August (Capitanelli, 1979a, 1979b).

Four sampling sites with different degree of human disturbance were selected: **C1** (coordinates 33°05′51″ S, 64°26′02″ W): rural landscape with a pond near to the crop (about 50 m) used by cattle to drink water (livestock load: 60 cows / 9 ha); **C2** (coordinates 33°06′09″ S, 64°25′32″ W): rural landscape with pond close to the crop (about 60 m) and not used by cattle; **C3** (coordinates 33°05′39″ S, 64°25′58″ W): rural environment with pond away from the crop (about 300 m) and used by cattle discontinuously, every three days (livestock load: 140 cows / 43 ha); **SM** (coordinates 33°06′42 S, 64°18′12 W): semi-modified landscape. This site was used as a reference site (Hawkins, 2007) not affected by agriculture or cattle. Pond is near to a protected natural area, the native forest "El Espinal", located within the National University of Río Cuarto.

Ponds of all study sites are temporary and have no fishes. The area of the ponds per site is: C1: 196 m^2; C2: 251 m^2; C3: 265 m^2; SM: 227 m^2. During sampling season the sites were surrounded by soybean and corn crops. The vegetation cover percentage in the ponds margins per site is as follows: C1: 60%; C2: 90%, C3: 70%, SM: 100%. The C1 and C3 sites are sparsely vegetated with plants with heights up to 0.3 m and are affected by livestock grazing. Gramineae predominate in the margins were *Cynodon dactylon, Trichloris* sp.*, Digitaria* sp. *and Eulisine* sp. In C2 site the predominant vegetation is composed of coastal aquatic plants with heights greater than 0.5 m typical of marsh as *Typha angustifolia*, *T. latifolia*, *Cortaderia selloana*, *Sorgum halepense* and floating plants as *Azolla* sp., *Lemna* sp., *Salvinia minima* and *Centella* sp. The SM site has pond with aquatic vegetation of marsh plants higher than 1.5 m as *Typha angustifolia*, *T. latifolia*, *Sorgum halepense* and also floating plants as *Centella* sp. and *Rorippa* sp. In addition, a small strip of native forest (biogeographic Pampean province) alternating with grasslands is surrounding the pond (Doffo, 1989).

2.2. Rhinella arenarum

Native species *R. arenarum* (Hensel, 1867) has several characteristics that constitute it good bioindicator of the environmental health (EPA, 1998; Hawkins, 2007). Its sensitivity to varied xenobiotics was demonstrated in several studies (Howe et al., 1998; Venturino et al., 2003; Bosch et al., 2011; Lajmanovich et al., 2014). This anuran species has a wide Neotropical distribution (Frost, 2017) and it is commonly found in forests, wetlands, riversides, urban and agricultural lands. Also, it is easy to acclimate under laboratory conditions (Kwet et al., 2004). According to Duellman and Trueb (1994) *R. arenarum* has the reproductive mode M I.1.; the eggs are aquatic, small and black in gelatinous strings deposited in the water (Cei, 1980; Babini et al., 2015b). Tadpoles of *R. arenarum* are black, the labial tooth row formula is 2(2)/3 or 2(2)/(1)3 and have benthic habits, remaining in the margins of bodies of water (Cei, 1980; Gallardo, 1987).

2.3. Field work

Sampling season was carried out between 2012 and 2013, during specie's breeding season. Breeding season began in September, when the first amplexus was recorded, and ended in February, when there were no more tadpoles in ponds from sampling sites. During these months, all study sites were sampled on the same way, with a frequency of 2 and 3 times a week.

Physical-chemical parameters were relieve from environment: water and air temperature (at 100 cm from the ground), pH, conductivity, total dissolved solids (TDS) and salinity of water bodies using a digital multiparameter 35-series 35425–10 test (Oakton Instruments 625 E Bunker Court Vernon Hills, IL 60061, USA). Dissolved oxygen was measured using a meter HD3030. These parameters allow the characterization of water quality (Gatica et al., 2012). In addition, it is known that pH and dissolved oxygen values influence the development and survival of anurans (Gauthier et al., 2004; Saenz et al., 2013; Arenas Rodriguez, 2014).

Besides, water samples from each pond were taken to determine the levels of nitrate and phosphorus, because they are indicators of contamination by agricultural-livestock activity and are used as indicators of water quality (Camargo and Alonso, 2006, 2007; Herrero and Gil, 2008). Levels of nitrate and phosphorus were determined through the colorimetric and visible spectrophotometric methods of analysis, respectively. These analyses were performed by the area of Hydrology, Geology Department, Río Cuarto National University.

Visual Encounter Surveys (Heyer et al., 1994) was used for sampling of pairs in amplexus, ovipositions (clutches) and tadpoles from the ponds. In the field the individuals´ snout–vent length (SVL) in amplexus was measured with a Vernier caliper (0.01-mm precision) and they were released immediately after. Abundance of ovipositions was recorded at each site. The fecundity or number of eggs per oviposition was calculated considering only the oviposition that still maintained the shape of string, because 24 h after oviposition, the gelatinous substance of the string begins to degrade. Ovipositions were collected with a net and they are weighed with a digital balance of 0.01 g accuracy. Approximately 5% of the total mass of each oviposition was then removed and stored in Formol Buffer to be transferred to the laboratory. Subsequently the ovipositions were returned to the water.

Transects of one meter by the margin of the ponds were made with a net (0.5 m wide) and the number of captured tadpoles was counted. By sampling date, 10 transects were performed being the distance between transects was greater than 30 m. These sampling was repeated 1 time a week, beginning when tadpoles were recorded and ending when the tadpoles completed their metamorphosis. The mean number of tadpoles caught in one transect (surface of one transect: 0.5 m^2) was calculated. This allowed knowing the density of tadpoles per site. Approximately 5% of tadpoles captured in a sampling were anesthetized with a solution at 0.5% of Methanesulfonate Salt (3-Aminobenzoic Acid Ethyl Ester Sigma-Aldrich™) and preserved in Formol Buffer for laboratory analysis.

2.4. Laboratory work

The egg can be defined as the ovule or gamete through gastrulation (1–12 Gosner stages) and its vitelline membrane of ovarian origin, immersed in the jelly produced in the oviduct (Altig and McDiarmid, 2007). In the laboratory, stage (Gosner, 1960) of eggs and tadpoles, number of eggs and diameter of the eggs was recorded using a binocular loupe Zeiss West Germany 47 50 52–9901. In addition, number of dead eggs and eggs with anomalies were counted.

Number of eggs counted was extrapolated to the total mass of the oviposition to estimate the number of eggs per oviposition. Density of eggs per site was estimated as follows: (F*A) / S; being; F: fecundity (mean number of eggs per oviposition); A: number of amplexus registered per site (C1: 26, C2: 27, C3: 26, SM: 23); S: surface of a pond from site (C1: 196 m^2; C2: 251 m^2; C3: 265 m^2; SM: 227 m^2).

Dead eggs and abnormal eggs were considered as non-viable eggs, and this data was used to calculate the proportion of viable eggs that became embryos.

Killing-power indicates how strongly the mortality acts in each stage or class of age. This parameter is calculated as follows: k = Log_{10} (N_X / N_{X+1}); being N_X: number of individuals of the age class X; N_{X+1}: number of individuals at a later time (Begon et al., 2006). Killing power was calculated between the egg and embryo classes, and between the egg and tadpole classes.

2.5. Statistic analysis

Physical-chemical parameters, male and female SVL and oviposition size (mass of the oviposition and number of eggs per oviposition) were analyzed by ANOVA. The assumptions of normal distribution and homogeneity of variances were tested with the Kolmogorov-Smirnov and Levene tests, respectively. When these assumptions were met, the parametric ANOVA analysis was performed, and the post-hoc DGC test (Test of DiRienzo, Guzmán and Casanoves) was used to test differences between means (Di Rienzo et al., 2002). This test uses the multivariate cluster analysis technique, mean chain or UPGMA (unweighted pair-group method using an arithmetic average) in a distance matrix obtained from the sampling means (Balzarini et al., 2008).

Linear Mixed Models (LMMs) was using for analyzed egg diameter. We included Site (S) as fixed factor and Gosner Stage (GS) and Oviposition (O) as a random factor. The null model with the intercept only was also evaluated. The best model was selected using the Akaike information criterion (AIC) and Bayesian information criterion (BIC) methods (AIC and BIC minors implies a better fit of the data to the model).

Data of frequency of sprouted eggs and of dead eggs were adjusted to a generalized linear model (GLM). These response variables were adjusted to a binomial distribution and *logit* link function (Nelder and Wendderburn, 1972; Myers et al., 2002).

Principal Component Analysis (PCA) with parameters: male and female SVL, oviposition size (oviposition mass and number of eggs), egg diameter, frequency of sprouted eggs and of dead eggs per site was performed. We standardized the data set before plotting the PCA because all variables have different units. The Biplot was performed with Principal Component 1 and 2 (PC1 and PC2).

InfoStat 2017 (Di Rienzo et al., 2017) and R 3.3.2 (R Core Team, 2016) were used for all analyses.

3. Results

3.1. Environmental parameters

No statistically significant differences in temperatures of air and water between sites were recorded (Table 1). Values of conductivity, salinity and SDT were higher in C1 site, having significant difference to the values of the other sites. Significant differences between the sites for pH were recorded. The most basic pH values were recorded in C1 and C3 sites, whereas the highest acid values were recorded in C2 (minimum value: 6.5). Respect to the laboratory analysis of ions, the higher phosphate concentration was detected in the three agroecosystems, whereas nitrate was detected only in C1 and C2 (Table 1).

3.2. Reproductive endpoints

The analysis of the variance indicated a significant difference for the male SVL and the female SVL between sites (ANOVA male SVL: $F_{3, 43}$: 3.74, α: 0.05; ANOVA female SVL: $F_{3, 43}$: 4.42, α: 0.05) (Table 2). Male SVL from the SM site was lower than the other sites. Female SVL from the C3 site was larger than of the other sites.

Regard to the oviposition size, analysis of the variance was significant for the mass of the oviposition, registering at C2 site the highest weight (ANOVA: $F_{3, 38}$: 9.24, α: 0.05) (Table 2). Significant difference was not detected for the number of eggs per oviposition between sites (ANOVA: $F_{3, 38}$: 1.4, α: 0.05).

Model that best fit the data of egg diameter was: Site (S) + Gosner

Table 1
Environmental parameters (mean ± standard deviation) per sites. Statistical value F, degrees of freedom of the model and error and p-value of analysis of the variance and DGC *post-hoc* test.

	Sites				F_{df} P-value	DGC post-hoc test
	C1	C2	C3	SM		
Water Temp. (°C)	21.3 ± 4.9	21.7 ± 5.5	21.3 ± 4.5	23.8 ± 3.3	$F_{3,\ 84}$: 0.82P: 0.4893	
Air Temp. (°C)	21.8 ± 4.6	22.32 ± 5.6	23.98 ± 6.2	24.87 ± 3.9	$F_{3,\ 84}$: 1.44P: 0.2366	
Conductivity (μS cm^{-1})	1175.5 ± 403.8	173.8 ± 40.23	524.6 ± 393.2	334.2 ± 179	$F_{3,\ 84}$: 50.55P < 0.0001	C1 > C3 > C2,SM
Salinity (mg L^{-1})	578.4 ± 204.6	143.5 ± 207.6	263.9 ± 190.8	160.5 ± 87.3	$F_{3,\ 84}$: 27.77P < 0.0001	C1 > C3,C2,SM
TDS (mg L^{-1})	806.4 ± 286.2	123.1 ± 28.8	386 ± 274.3	237.3 ± 127.5	$F_{3,\ 84}$: 46.9P < 0.0001	C1 > C3 > C2,SM
pH	8.8 ± 0.5	7.5 ± 0.8	9.2 ± 0.7	8.3 ± 0.6	$F_{3,\ 84}$: 25.99P < 0.0001	C3,C1 > SM > C2
Dissolved Oxygen (%; ppm)	79.9% (6.96 ppm)	77.2% (6 ppm)	76% (6.61 ppm)	70.2% (5.14 ppm)		
Phosphate (mg. PO_4^{3-} L^{-1})	9	9	9	5.7		
Nitrate (mg. NO_3^- L^{-1})	1	1	ND	ND		

Analytical error of the Phosphate and Nitrate measurements is 0.5%; the detection limit of the determination of NO^{3-} is 0.2 mg L^{-1}. ND: not detected or observed. TDS: total dissolved solids.

Stage (GS) + Oviposition (O), AIC: −691, BIC: −663 (Model S: AIC = -¬524, BIC = −505. Model S + GS: AIC = −545, BIC = −522. Model S + O: AIC = −578, BIC = −556). Egg diameter analysis revealed significant difference between sites (LMM: $F_{3,\ 302}$: 10.47) (Table 2). Eggs of the ovipositions from SM site had larger diameter, while those eggs of smaller diameter were those of the C3 site ovipositions.

Dead eggs and abnormal eggs were recorded. Abnormal eggs were called by their appearance as sprouted eggs (Fig. 1). Frequency of sprouted eggs and of dead eggs was statistically different between sites (GLM Sprouted eggs: $F_{3,\ 39}$: 301.6; GLM Dead eggs: $F_{3,\ 39}$: 86.3) (Table 2). The higher frequency of sprouted eggs and the higher frequency of dead eggs were recorded in ovipositions of C2 site. The SM ovipositions recorded the lowest frequencies of sprouted eggs and dead eggs.

Principal components, PC1 and PC2, of the ACP accounted for 94.6% of the total variability of the data (Fig. 2). Eigenvectors show the coefficients with which each original variable was weighted to form the PC1 and PC2 (Table 3). PC1 emphasizes the variability introduced by egg diameter, being the only variable with negative weight. Parameters related to oviposture size, oviposition mass and number of eggs, had a positive correlation with frequency of sprouted eggs and dead eggs. These variables were associated with C2 site. Egg diameter was associated with SM and correlated negatively to SVL of the male and female. No correlation between female SVL and oviposture size was recorded.

3.3. Mortality

Killing-power in the egg-to-embryo passage was larger at the C2 site, and lower at the SM site (Table 4). Killing-power in the passage from egg to tadpole classes was higher in the three agroecosystems, and lower in the SM site (Table 3).

4. Discussion

The need to determine the effects of environmental changes on the first stages of amphibian development, due to its role in population regulation, has been noted since 1980 (Wilbur, 1980). In this study, different reproductive endpoints of *R. arenarum* were analyzed in order to evaluate environmental health and its effect on populations of the species.

4.1. Environmental parameters

In site C1, high conductivity values were recorded. Salinity and total dissolved solids also had the highest values, compared to the other sites. In aqueous solutions, the salinity and total dissolved solids are directly proportional to the conductivity, which is a function of the ionic concentration (calcium, magnesium, sodium, sulfate and carbonate). The high conductivity values indicate mineralization processes, which could be caused by the anthropogenic activities on these sites (Gatica et al., 2012). In all sites the dissolved oxygen concentration was within the normal range for amphibians, greater than 60% (Gauthier et al., 2004).

At site C2 acidic pH values were recorded. In fresh water, pH values in a range of 6.5–8 are optimal for survival and physiology in most large aquatic organisms. Out of this pH range, as the high values that were registered mainly in C1 and C3, direct toxic effects can occur and stress levels are high (García and Fontúrbel, 2003; Ohrel and Register, 2006). In amphibians, the early stages would be the most sensitive to both acid and alkaline stress, with genetic disorders occurring from the egg stage (Pough and Wilson, 1977). In tadpoles were recorded sublethal effects as late embryonic development, smaller body size and reduction in locomotor performance with far pH to neutrality values (Arenas Rodriguez, 2014).

High concentrations of nitrate and phosphate are indicators of contamination by agricultural-livestock activity and are used as indicators of water quality in numerous studies (Sánchez de Fuentes, 2000; Perdomo et al., 2001; Blanco et al., 2004; Arumi et al., 2007;

Table 2
Reproductive endpoints per sites. Mean, standard deviation, p-value of analysis and DGC *post-hoc* test.

	Site				P-value	DGC post-hoc test
	C1	C2	C3	SM		
Male SVL (mm)	101.1 ± 4.5	103.2 ± 4.5	107.8 ± 2.6	90.7 ± 4.5	0.0178	C3,C2,C1 > SM
Female SVL (mm)	109.4 ± 3.9	112.9 ± 3.9	120.3 ± 2.3	105.4 ± 3.9	0.0085	C3 > C2,C1,SM
Oviposition mass (g)	317 ± 42	612 ± 46	351 ± 39	345 ± 46	0.0001	C2 > C3,SM,C1
Number of eggs	24,721 ± 3249	34,535 ± 3592	29,469 ± 2989	28,029 ± 3592	0.257	
Egg diameter (mm)	1.67 ± 0.02	1.65 ± 0.02	1.60 ± 0.02	1.72 ± 0.02	< 0.0001	SM > C2,C1 > C3
Sprouted eggs (°/$_{00}$)	0.66 ± 0.2	100 ± 2.6	1.9 ± 0.35	0.36 ± 0.18	< 0.0001	C2 > C3 > C1,SM
Dead eggs (°/$_{00}$)	40 ± 1.6	70 ± 2.1	40 ± 1.6	20 ± 1.4	< 0.0001	C2 > C1,C3 > SM

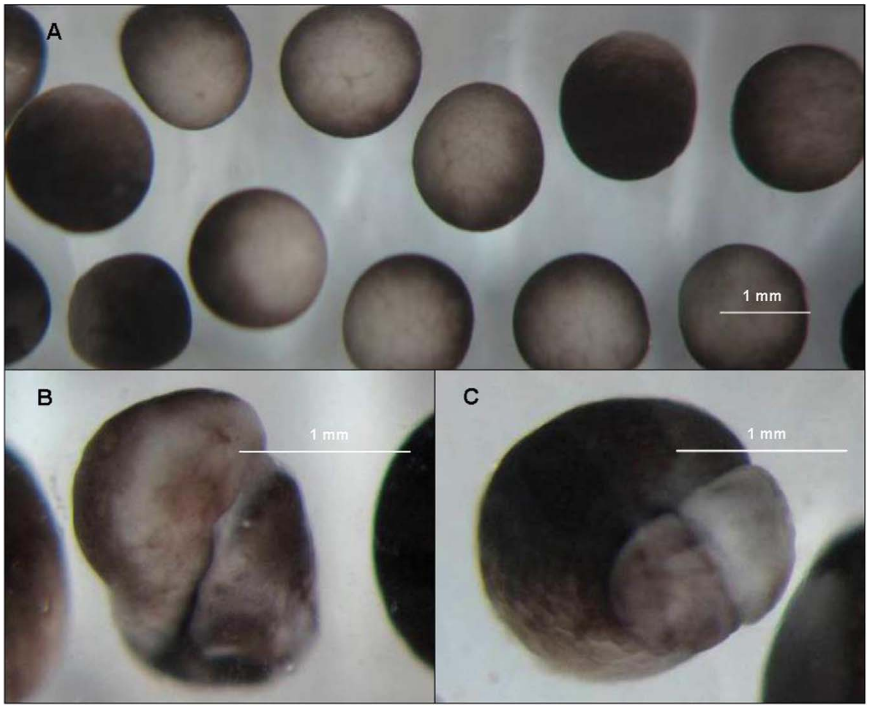

Fig. 1. Photographs of eggs: A) normal eggs in 9 Gosner stage, B) dead egg and C) egg sprouted.

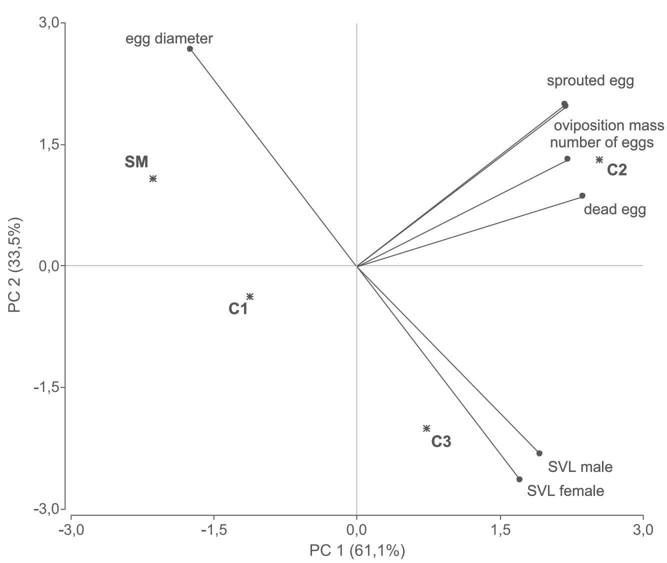

Fig. 2. Biplot of reproductive endpoints obtained by PCA.

Table 3
Eigenvectors of PCA of the reproductive endpoints.

Endpoints	Eigenvectors	
	PC1 (61.1%)	PC2 (33.5%)
Male SVL	0.35	−0.43
Female SVL	0.31	−0.48
Oviposition mass	0.4	0.36
Number of eggs	0.41	0.24
Egg diameter	−0.32	0.49
Sprouted eggs	0.4	0.37
Dead eggs	0.43	0.16

Table 4
Density of eggs, embryos and tadpoles (organism per square meter; org/m2) and frequency of non-viable eggs (total amount of dead and sprouted eggs; $^{\circ}/_{00}$) per site. Killing power (k) between egg to embryo, between embryo to tadpole and between egg to tadpole classes.

	C1	C2	C3	SM
Eggs (org/m^2)	3279	3715	2891	2840
Non-viable eggs ($^{\circ}/_{00}$)	44	161	42	24
Embryos (org/m^2)	3135	3117	2771	2773
Tadpoles (org/m^2)	220	248	184	246
k egg-embryo	0.019	0.076	0.018	0.010
k embryo-tadpole	1.154	1.099	1.178	1.052
k egg-tadpole	1.173	1.175	1.196	1.062

Camargo and Alonso, 2007; Herrero and Gil, 2008). Phosphate was found in high concentrations in all three agroecosystems. It is known that phosphate concentrations higher than 0.1 mg L^{-1} favours eutrophication processes and decreases dissolved oxygen in water bodies (DOF, 1989; Macleod and Haygarth, 2003), so in sites C1, C2 and C3 this nutrient could be harming all the aquatic aerobic organisms of these communities, including the aquatic stages of amphibians (Mitsch and Gosselink, 2000). Nitrogen was registered in the nitrate form in C1 and C2. There is a linear relationship between agricultural-livestock activity and the increase of this element (Ongley, 1997; Langan, 2003). Nitrate and phosphate favours eutrophication processes in water bodies (EEA, 2005).

4.2. Reproductive endpoints

Female body size is important for amplexus reproduction in any kind of mating pattern, because a larger female could offer higher fecundity (Gutiérrez and Luddecke, 2002; Fan et al., 2013; Bionda et al., 2011a). This relationship was not recorded in this study, since in C3 the females with larger SVL were found, however the ovipositions of these sites did not have a greater number of eggs than the other sites. On the other hand, the fact that reproductive adults did not have smaller size in agroecosystems could be because that parameters such as body size and body condition might be misleading indicators of health condition, particularly when they are used to estimate health status of populations from habitats with different levels of alteration (Pólo-Cavia et al., 2010).

The characteristics of ovipositions, such as the number of eggs can indicate the conservation status of amphibians (Lavilla et al., 2000). The weight per cord varied according to the sites in which they were registered, being in C2 where the ovipositions of greater weight were registered. However, there were no differences in the number of eggs per cord between sites. This would indicate that ovipositions of C2 have a greater amount of jelly between the eggs, which would confer greater weight but equal number of eggs than the ovipositions of the other sites. Jelly provides mechanical support to the ovule, fixes the eggs to each other or to a structure of the environment, increases the entry of congenital sperm and prevents heterospecific sperm (Barbieri and Del Pino, 1975). In addition, it has been reported that jelly protects eggs from contaminants from the environment and from predators or pathogens (Marquis et al., 2006; Altig and McDiarmid, 2007). Therefore, ovipositions with more amount of jelly in C2 could be associated with these environmental stressors than being present in this site, ovipositions with more jelly would be favoured.

Larger diameter eggs were registered at the SM site. The optimal size of the eggs can be correlated to environmental factors and it is associated with the duration of the larval period (Berven and Chadra, 1988). Egg size affects offspring survival by influencing size, shape, growth and development during embryonic development (Kaplan and King, 1997). Ovipostures with larger average egg sizes tend to have shorter development times and the tadpoles have larger sizes and longer survival (Hutchings, 1991). The size of the egg is affected by the amount of energy available for reproduction (Morrison and Hero, 2003), so the SM site organisms, being a healthier environment, could have more energy available to invest in ovipositions with larger diameter eggs.

In agroecosystem C2, a high percentage of anomalous eggs was recorded, which were named as sprouted eggs. No bibliography was found that reported this particular type of egg anomaly. However, at the beginning of the 20th century, some studies have already reported abnormalities in eggs and anuran embryos associated with variations in salinity, oxygen and temperature (Morgan, 1902). It is known that the proliferation of morphological deformities is a sublethal response of the early stages of anurans when exposed to contaminants (Cooke, 1981). Different abnormalities in the cleavage process have been associated with chemical substances capable of damaging the mitotic beams of the cells, which generates a deficient differentiation in the development of eggs and can lead to the death of the embryo (Witschi, 1952). In addition C2 also recorded the highest frequency of dead eggs. When eggs or early embryos are exposed to adverse environmental conditions, such as poisons, extreme temperatures or lack of oxygen, a blastemic degradation process is triggered, known as blastophthoria that leads to the death and disintegration of the egg or embryo (Witschi, 1952). These adverse conditions could be affecting the early stages of development and generating the highest value of killing-power in the egg-embryo passage in C2 and the highest values of total killing-power (from egg to tadpole) in the three agroecosystems.

4.3. Persistence in unfavourable aquatic ecosystem

Therefore, the smaller diameter of the eggs and the greater frequency of abnormal and dead eggs in the ovipositions of agroecosystems are indicating an unfavourable aquatic ecosystem for anuran development. This could have a negative impact on the survival of the populations. However, the monitoring of these populations since 2006 indicates that they persist (Bionda et al., 2011b). This could be due to the high number of eggs per oviposition of the species, which confers a high intrinsic growth rate and persistence capacity (Duellman and Trueb, 1994).

In addition, it is necessary to bear in mind that frequently amphibian populations are organized as interconnected subpopulations, which behave as source or sink populations, forming a meta-population (Petranka, 2007). Study area physiography favours the creation of small temporary ponds in rainy periods, usually on the sides of roads (Bridarolli and di Tada, 1996). These ephemeral habitats could be used by *R. arenarum*, thanks to the great phenotypic plasticity in the time of their metamorphosis (Acosta, 2009) and to be acting as source populations when the populations of the agroecosystems ponds diminish.

4.4. Conclusion

Aspects related to the amplexus and ovipositions of the bioindicator species *Rhinella arenarum* that inhabits agroecosystems were analyzed. The hypothesis was that perturbations of agroecosystems have a negative impact on the size of reproductive adults, on the size of ovipositions and eggs, and on the survival of eggs and embryos. This hypothesis was corroborated in part. Amplexed adults in agroecosystems were not smaller. However, the oviposition eggs had smaller size, higher frequency of abnormal eggs and higher mortality in agroecosystems. This confirms the high sensitivity of the early stages to environmental disturbances and sustains their use as endpoints for the environmental health assessment. Therefore, we believe that it would be important to carry out future studies on gene flow between populations and we recommend the conservation sites of small temporary ponds, such as those that can be formed on road side, for the persistence of anuran populations in agroecosystems.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.02.050.

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