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Ecotoxicity of veterinary enrofloxacin and ciprofloxacin antibiotics on anuran amphibian larvae

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ABSTRACT

The ecological risks posed by two β-diketone antibiotics (DKAs, enrofloxacin, ENR and ciprofloxacin, CPX), characterized by their long persistence in aqueous environments and known deleterious effect on model organisms such as zebrafish were analysed using *Rhinella arenarum* larvae. Sublethal tests were conducted using environmentally relevant concentrations of both ENR and CPX (1–1000 $\mu g\,L^{-1}$) under standard laboratory conditions for 96 h. Biological endpoints and biomarkers evaluated were body size, shape, development and growth rates, and antioxidant enzymes (glutathione-S-transferase, GST; Catalase, CAT). Risk assessment was analysed based on ration quotients (RQ). The size and shape measurements of the larvae exposed to concentrations greater than 10 $\mu g\,L^{-1}$ of CPX were lower compared to controls (Dunnett post hoc $p < 0.05$) and presented signs of emaciation. Concentrations of 1000 $\mu g\,L^{-1}$ of CPX induced GST activity, in contrast with inhibited GST and CAT of larvae exposed to ENR. Risk assessments indicated that concentrations greater than or equal to10 $\mu g\,L^{-1}$ of CPX and ENR are ecotoxic for development, growth, detoxifying, and oxidative stress enzymes. It is suggested that additional risk assessments may provide evidence of bioaccumulation of CPX and ENR in tissues or organs of amphibian larvae by mesocosm sediment test conditions. Finally, intestinal microbiome studies should be considered to establish the mechanisms of action of both antibiotics.

1. Introduction

Over the past decades, the scientific community has been alerted by the increasing levels of veterinary pharmaceuticals recorded in freshwaters, including groundwater and sources of drinking water (Farré et al., 2008; Oggier et al., 2010; Rico et al., 2013). Pharmaceuticals reach the aquatic systems through several pathways, such as direct disposal of domestic surplus drugs, inadequate processing in the effluent treatment plants (Li and Randak, 2009) or incomplete removal mechanisms (Van Doorslaer et al., 2014). In general, 10–90% of pharmaceuticals and their metabolites are excreted via urine and feces (Kümmerer et al., 2000; Zhang et al., 2014, 2015). Besides their applications to livestock, poultry or swine feedlots, pharmaceuticals are widely used in aquaculture (Rico et al., 2014a). The continuous entry of pharmaceuticals into the aquatic environment from wastewater effluents or leaching and runoff of agricultural soils amended with manure, even at low concentrations, may pose long-term risks to aquatic and terrestrial organisms (Klavarioti et al., 2009; Martini et al., 2012; Rico et al., 2014b). In surface waters, pharmaceuticals and their metabolites generally include several pharmaco-therapeutic classes, such as antibiotics, antipyretics, anti-inflammatory drugs, β-blockers, lipid regulators, hormones, antidepressants, and anesthetics (Kümmerer, 2009).

In the '70s, the first evidence on pharmacologically active compounds (clorphibric acid) in aquatic systems was reported (Hignite and Azarnoff, 1977), but the occurrence of pharmaceuticals in the environment became an emerging concern only in the mid-1990s, when new analytical technologies were accessible (Heberer and Stan, 1997; Zuccato and Castiglioni, 2009). There are severe concerns over this bioactive compound role in enhancing antibiotic resistance among pathogenic bacteria (Homem and Santos, 2011). Fluoroquinolones, classes of commonly used β-diketone antibiotics (DKAs) persist for long time in the aquatic environment (Qin and

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Liu, 2013; Wang et al., 2014) due to their massive dose and frequent application of DKAs in human and livestock (Zhang et al., 2016). This environmental problem can exacerbate bacterial resistance in considerable proportion (Redgrave et al., 2014). Enrofloxacin (ENR) and ciprofloxacin (CPX) are the most used drugs in human (since the 1980s) and veterinary (since the 1990s) medicine to treatment of urinary tract, enteric, low respiratory tract, and bone infections, being mainly active against Gram-negative bacteria (López-Cadenas et al., 2013).

During the last century, different technologies were developed to remove fluoroquinolones from different aquatic systems (Van Doorslaer et al., 2014; Alcaráz et al., 2015). However, their removal is complex and they can be found in numerous water matrices worldwide at concentrations that range among ng L^{-1} to mg L^{-1} (Reinstorf et al., 2008). For instance, Watkinson et al. (2009) found 1.30 μg L^{-1} CPX concentration in water bodies of Australia, whereas Gibs et al. (2013) reported CPX residues (0.077 μg L^{-1}) in aquatic systems located downstream to the wastewater treatment plant discharges of New Jersey. Higher concentrations at concentrations above 14 mg L^{-1} of CPX were detected in areas with no or poor wastewater treatment (Larsson et al., 2007, Fick et al., 2009).

Therefore, in an ecotoxicological analysis regarding to emerging pollutants it is important to include acute effects and sub-lethal effects on non-target organisms (Rico et al., 2014a). ENR and CPX, affect growth and reproduction of cyanobacterium *Microcystis aeruginosa*, green algae species *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata* and duckweed *Lemna minor* at concentrations ranging from 1.96 to 53 μg L^{-1} (Robinson et al., 2005). Moreover, in crustacean *Daphnia magna*, zebrafish *Danio rerio* and fathead minnow *Pimephales promelas* have limited toxicity with no-observed-effect concentrations (NOEC) at 10 mg L^{-1} (Robinson et al., 2005; Iannacone and Alvariño, 2009; Plhalova et al., 2014; Dalla Bona et al., 2015). In addition, Wang et al. (2014) and Zhang et al. (2016) observed changes in both creatine kinase activity and creatine concentration, swimming behaviour, and severe histopathological changes of zebrafish heart tissue. To date, knowledge about mode of action of fluoroquinolones in amphibians is limited (Howard et al., 2010). ENR and CPX, pose risks to sensitive aquatic organisms that have identical and/or similar target molecules as antibiotics are developed to target-specific molecular pathways (Martins et al., 2012; Santos et al., 2013), and generally the model organisms use for investigate their ecotoxicity were performed on exotic species (Krull and Barros, 2012).

The present study approached the risk of ENR and its main metabolite CPX at relevant environmental concentrations on biological endpoints of amphibian larvae based on exposition (ecotoxicity assays at sublethal concentrations), and effects (morphological development and growth rates; GST and CAT activities).

2. Materials and methods

2.1. Stock and test solutions

ENR and CPX were purchased from Sigma–Aldrich (Steinheim, Germany). All standards were of analytical grade (purity >95%). Standard stock solutions of ENR and CPX at 1000 mg L^{-1} were prepared in methanol and stored at −20 °C. Based on the reported environmental concentrations of both antibiotics in lentic aquatic systems (MEC, 8.77 μg $L^{-1}{}_{ENR}$; 7.49 μg $L^{-1}{}_{CPX}$; Wei et al., 2012), where amphibians usually use to reproduce, several test solutions were prepared by taking appropriate aliquots of the standard stock solutions, evaporating the methanol with N_2, and re-suspending in the corresponding volume of water having a final concentrations of 1, 10 μg L^{-1} (MEC solutions), 100 and 1000 μg L^{-1}. Test solutions were prepared in 200 ml of dechlorinated tap water (DTW), whereas reference or control assay consisted in 200 ml of DTW (Alcaráz et al., 2015). Record of fluoroquinolones in aquatic environments in Argentina are possible due to their area the most used veterinary medicine (García et al., 2006), and traces were found in preliminary water sampling at 1 μg L^{-1} concentration. Despite the widely used in veterinary (feedlots of swine, poultry and livestock) no regulation and no treatment plants exists for washed off and poultry manures in Argentina.

2.2. Study species and Acute Toxicity Test Design

Eggs and embryos of the South American common toad *R. arenarum* (Anura: Bufonidae) were selected as model test organisms. Three surface egg strings of *R. arenarum* were collected from temporary ponds (31°40′29″ S–60°20′13″ W, protected reserve, Entre Ríos Province, Argentina) in November 2014; these sites are considered as unpolluted. This anuran species has a Neotropical distribution from southern Brazil, Argentina (south of Chubut Province), Uruguay and Bolivia (Kwet et al., 2004). It is frequently occur in different habitats comprising vegetated areas, ponds, wetlands, monocultives, and urban lands. This toad species is listed as "Least Concern" IUCN (2010), and it is used as sentinel species in numerous studies in Argentina (Junges et al., 2013; Lajmanovich et al., 2014). In addition, the quite fast development of aquatic life-stages (1–3 weeks until metamorphosis) is beneficial to laboratory work.

Egg strings of various cohorts (50 cm of each string) were randomly mixed, to genetic homogenization before adding to experimental microcosms, and they were maintained under lab conditions (24 ± 2 °C, 12/12-h photoperiod cycle) until they reached the development stages St 23–25 (Gosner stage, 1960, larvae with complete opercula) to perform toxicity test.

Due to lack of information in the literature about the effects of those antibiotics on sentinel and native anuran species, the first step was to elucidate the direct toxicity on *R. arenarum* larvae. Short-term static tests (96 h) were conducted using 200 ml sterile plastic recipients (85 mm diameter, 110 mm height).

Larvae (Stage 26) were exposed to 96 h to concentrations of ENR and CPX (1 μg L^{-1} and 10 μg L^{-1} –measured environmental concentrations – and 100, and 1000 μg L^{-1}) and a negative control (with dechlorinated tap water, DTW).

ENR and CPX treatments were made in triplicate with ten tadpoles per aquarium ($n = 30$). Average tadpole size (snout-tail tip) was 26 ± 1.3 mm and weight was 0.02 ± 0.05 g. The water temperature and pH were 24 °C and 6.5, respectively. A photoperiod consisted of 16 h light (>100 Lx)/8 h dark segments for each day, to simulate the photoperiod expected in environment and prevent photolysis of antibiotics (Babić et al., 2013). Survival, presence of faeces in the bottom of each aquarium (qualitative scale, low enough faeces < 40% of the aquarium bottom; medium: covering = 50% of the aquarium bottom, high: faeces cover more than 60%), temperature, pH, and dissolved oxygen were recorded daily with standard digital instruments and Aquamerk® kits, respectively. Larvae were fed 0.2 g of boiled lettuce and a similar amount was added at 48 h if all food were ingested. No differences in larvae mortality were found within replicates during the study period (Fisher's exact test $p > 0.05$); and therefore, we pooled data ($N = 30$) from replicates of each antibiotic and control treatments for all analyses. The pooled data based on mortality rather than development and growth rates were used because mortality was follow each day and is the most common parameter to pooling data in replicate treatments (Peltzer et al., 2013).

2.3. Biological endpoints

Larvae were treated according to ASIH et al. (2004) guidelines and with approval of Facultad de Bioquímica y Ciencias Biológicas

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(FBCB) bioethics committee at the end of the experiment (96 h). Each larva was weighed using an electronic field balance to the nearest 0.01 g, and then was gently blotted to remove excess water. Ten larvae per antibiotic concentration and control were placed individually in Eppendorf tubes and preserved on −20 °C freezer until to determine enzyme biomarkers. The remnant larvae were euthanized (MS-222) and preserved into vials with 10% formalin to measure development and growth endpoints. The remnant specimens were deposited in the Herpetological Collection (FBCB-UNL).

2.3.1. Development and growth

Body size and weight standard measures of larvae were biological endpoints while on lateral views of bodies was used to morphometric geometry analysis. The first ones were used to development and growth rates at the end of the experiments. Photographs of larvae were digitally recorded with a Moticam 10.0 camera mounted on an Arcano® stereoscopic microscope. Larvae were staged (Gosner, 1960) and morphological parameters were recorded using ImageJ software which is available free over the Internet (at http://rsb.info.nih.gov/ij/index.html).

Total length, body length, body width, tail fin length, tail muscle depth, and tail fin depth (Teplitsky et al., 2003) were standard measurements.

The shape of tadpoles ($N = 100$) was captured by digitizing 21 landmarks on lateral photos of each individual (Fig. 1, and full details in supplementary information) based on Katzenberger et al. (2014) using tpsDig2 software (Rohlf, 2010).

Growth and development rates were estimated according to equations from Teplitsky et al. (2003) per antibiotic treatments and controls. Indeed, developmental inhibition was also calculated according to Stancova et al. (2014).

2.3.2. Oxidative stress biomarkers and detoxifying enzymes

Ten larvae (Stages 28–29) from each treatment were randomly selected for glutathione-S-transferase (GST) and catalase (CAT) enzymes activities (see Section 2.3, see details in supplementary information). M tris buffer (hydroxymethyl) aminomethane hydrochloride (pH 7.6), 1 M ethylenediaminetetraacetic acid (EDTA), and 1 mM sacarose using a polytron. The homogenates were centrifuged at 10,000 r.p.m. at 4 °C for 15 min and the supernatant was collected. Protein concentrations were determined according to the Biuret method (Kingbley, 1942). GST activity was quantified following the method described by Habig et al. (1974) and adapted by Habdous et al. (2002), using a spectrophotometer, and expressing the results as $\mu mol\ min^{-1}\ mg^{-1}$ of protein using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}\ cm^{-1}$. CAT activity was measured by the method described by Aebi (1984), expressed as $\mu mol\ min^{-1}\ mg^{-1}$ of protein using a molar extinction coefficient of H_2O_2 40·10^{-3} L mol cm^{-1}.

2.4. Analytical procedures

To know the stability (permanency of analytes, antibiotics) at different solution concentrations with larvae and food (boiled lettuce) and without organisms and food at the final period studied (96 h), were determined by high performance liquid chromatography (HPLC). The chromatographic studies were performed on an Agilent 1100 LC instrument (Agilent Technologies, Germany). It is equipped with quaternary pump, degasser, oven column compartment, autosampler, UV–visible diode array detector (DAD), fluorescence detector (FLD), and ChemStation software package to control the instrument, data acquisition and data analysis. The chromatographic column was a Zorbax Eclipse XDB-C18, 75 mm × 4.6 mm, 3.5 μm particle size (Agilent Technology, Germany). The column temperature was measured by setting the oven temperature at 40 °C. The mobile phase consisted in a mixture of 20 mmol L^{-1} sodium acetate buffer (pH 4.0), acetonitrile and methanol (79:10:11). Samples were analysed in isocratic mode. The flow rate was maintained at 1.00 mL min^{-1}. The excitation and emission wavelengths were set at 277 nm and 450 nm, respectively. Samples were filtered through 0.45 μm nylon membrane filters before injection.

2.5. Statistical analysis

Standard morphological measures were analyzed by applying a principal component analysis (PCA), which allowed the reduction of the six measured morphological variables (referred to overall size and shape) to two principal components (PCs) (Peltzer et al., 2013). The resulting PC factors scores were then used in a MANOVA (Wilks' lambda multivariate test statistic) to determine if there were significant overall differences among treatments in the response vector control and mean sizes and shapes of tadpoles treated with the antibiotics (see result PCI and PCII). Univariate analysis of variance (ANOVA) test followed by post hoc-tests were also used for treatment significance for biological endpoints, indeed for enzymes activities. Unpaired Welch t test as also performed to compared development and growth rates between antibiotics treatments. Similarly, MANOVA and canonical variation analysis (CVA) were used to estimate the overall shape differences based on morphometric geometrics. Mahalanobis/Procrustes distances were considered as estimates of group differences. To test normality and homogeneity of variance of data, Shapiro–Wilk test and the Levene median test were used (Zar, 1999). IBM software was used to statistical analyses. For all tests, significance at 0.05 was used.

2.6. Risk estimations

Risk estimations were performed to characterize the adverse effects of these antibiotics in real aquatic environments, using the risk quotient (RQ). The RQ is the ratio between the highest measured environmental concentration (MEC) in potential breeding sites of amphibian, such as ponds, in the literature (8.77 $\mu g\ L^{-1}{}_{ENR}$; 7.49 $\mu g\ L^{-1}{}_{CPX}$; Wei et al., 2012), and the concentration at which no effect is expected (PNEC) for each one (VICH, 2004). The PNECs were derived by using the toxicity values calculated in this study as NOEC (no observed effect concentration), the highest concentration of each antibiotic at which statistically significant differences with controls were not detected (Plhalova et al ., 2014). An assessment factor (AF) (AF = 100) indicated for acute exposure and NOEC was used (VICH, 2004). A RQ value of less than 1 when the AF was applied indicates an insignificant risk for the aquatic environment, and the mitigation or further assessments are not needed (Svartz et al., 2016). Ecological Structure Activity Relationships (ECOSAR-U.S. EPA) software was used to simulate the toxicity of both antibiotics under aqueous conditions based on log of octanol/water partition coefficient, log K_{ow} (Gros et al., 2010), a common 'indicator' of bioaccumulation potential derived from structure (OECD, 1992). In general, very high log K_{ow} compounds (above 5) deserve very careful treatment as these have very low estimated solubility (<0.5) (Thomaidi et al., 2015). Tiered framework phases and conceptual site model (CSM) were considered (U.S. EPA, 1992).

3. Results

During the experiments of CPX and ENR with without larvae and lettuce, concentrations of each initial nominal concentration (1:10:100, and 1000 $\mu g\ L^{-1}$) of both antibiotics did not fall below 80–100% during the bioassay (96 h), becoming more stable. In contrasts, in the trials with larvae and lettuce, CPX and ENR were

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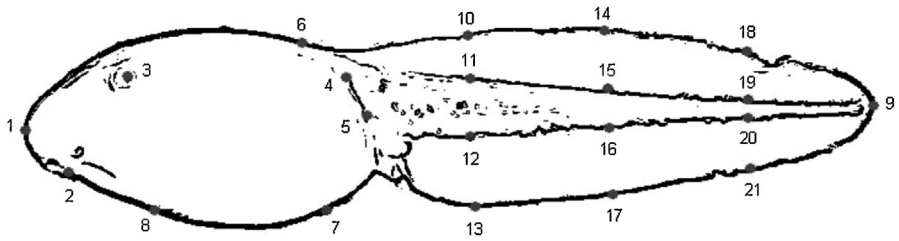

Fig. 1. Description of position of the 21 landmarks used for geometric morphometric analyses in *R. arenarum* tadpoles.

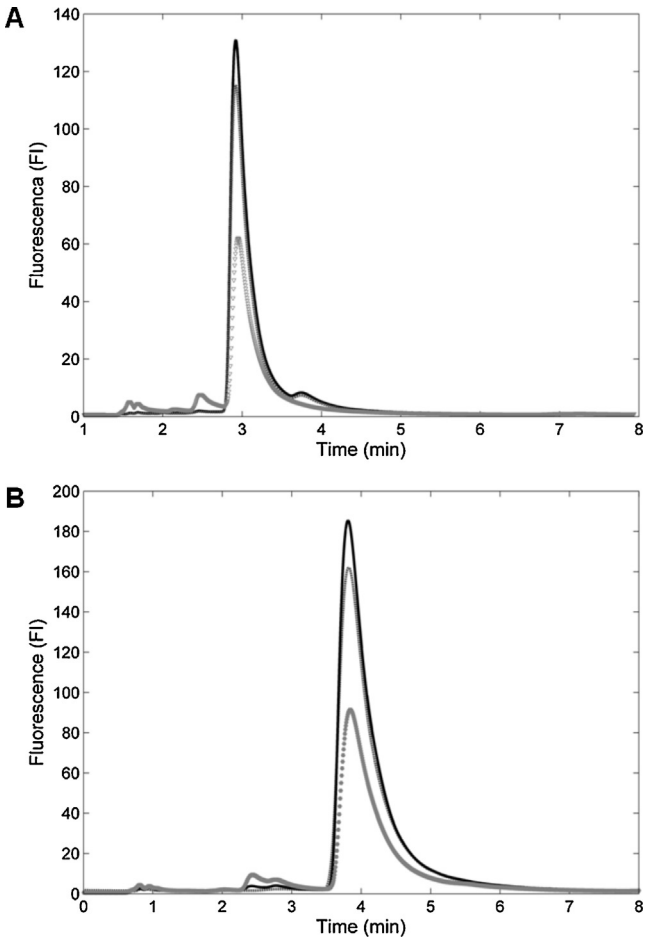

Fig. 2. CPX (A) and ENR (B) stabilities and absorptions without (grey bar lines) and with organisms and food (grey point lines), and initial concentration (black continuous lines) of a sample concentration (1000 μg L^{-1}) tested at 96 h. Fluorescence intensity (FI), Chromatographic screening time (min).

absorbed in ranges of 60–90% (Fig. 2). No degradation products of antibiotics were observed in any treatment.

3.1. Biological endpoints

In all CPX or ENR-exposed larvae, mortality did not exceed 2% during the 96 h experimental period. No mortality was detected in the control group. CPX-exposed larvae released more faeces (high, 60% of the aquarium bottom) than ENR-exposed larvae (low, <40%), and had loss of appetite (not eat, all boiled lettuce remained in the bottom) since 72 h.

3.2. Standard measures

Both antibiotic treatments correlated positively with size (PCI) and shape (PCII) of larvae (Table 1). MANOVA performed on larval size and shape standard measurements found significant effects on the interaction among concentrations and type of antibiotic exposures (Wilks' Lambda λ 0.68; $F = 1.91$; DF = 240; $p < 0.001$, Table 1) and per antibiotic treatments (Wilks' Lambda λ_{CPX} 0.41; $F = 1.77$; DF = 140; $p < 0.05$; Wilks' Lambda λ_{ENR}0.30; $F = 2.57$; DF = 140; $p < 0.001$). As a result, the effect of the antibiotics on each measure of size and shape in separate ANOVAs and Duncan post hoc comparison test can be seen in Table 1. Among the two antibiotic treatments, CPX produced significant (Dunnett post hoc test $p < 0.05$) shorter and emaciated animals ($mean_{totallength10\,\mu gL^{-1}} = 28.13$ mm, SE = 1.5; $mean_{totallength100\,\mu gL^{-1}} = 26.08$ mm, SE = 2; $mean_{totallength1000\,\mu gL^{-1}} = 26.69$ mm, SE = 1.5) than control individuals ($mean_{totallenght} = 31$ mm, SE = 1.5; $mean_{bodywidth} = 6.71$ mm; SE = 0.1). In addition, larvae exposed at ENR are similar in size and shape to those of controls, differing (Dunnett post hoc test $p < 0.05$) slighter in total length at concentrations > 10 μg L^{-1} ($mean_{totallength10\,\mu gL^{-1}} = 27.59$ mm; SE = 0.6; $mean_{totallength100\,\mu gL^{-1}} = 28.06$ mm; SE = 0.1; $mean_{totallength1000\,\mu gL^{-1}} = 28.01$ mm; SE = 0.3).

3.3. Development and growth rates

Antibiotic treatments had significant effects on larval development ($F = 6.81$; DF = 2; $p < 0.05$) and growth rates ($F = 7.29$; DF = 2; $p < 0.05$) of *R. arenarum* larvae, respect to controls (Dunnet test < 0.05, in both cases). Development and growth rates were significantly slighter in ENR than in CPX treatments (unpaired Welch t $test_{developmentrates} = 0.27$; DF = 6; $p < 0.001$; t $test_{growthrates} = 0.34$; DF = 6; $p < 0.05$), being inhibited at percentages higher than 13% at 1, 10 and 1000 μg L^{-1}. Thus, development inhibition percentages higher than 10% were accounted for 100 and 1000 μg L^{-1} in CPX treatments respect to the controls.

3.4. Morphometrical geometry

Average body shape changes in tadpoles exposed to ENR and CPX treatments varied significantly with respect to those of control larvae (Wilks' λ = 0.001, $F = 2.138$, $p < 0.001$). The Procrustes distance between control and CPX exposed larvae, control and ENR, and the two antibiotics were 0.04, 0.02 and 0.03, respectively ($p < 0.0001$ for permutation tests for all three pairwise comparisons). The body shape differences from the overall mean showed characteristic features for each of the three treatments (Fig. 3a–c). The canonical variation analysis (CVA) of the pooled samples also showed similar differences among treatments. Although larvae average shapes were clearly distinct among treatments, the 90% equal frequency ellipses indicated considerable overlap in the scatter of data (Fig. 3). The CV component I (75.87%) established larvae CPX exposed apart from the other two treatments, and there were

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Table 1
Results of the PCA, MANOVA and ANOVA on *Rhinella arenarum* morphological larvae responses to the different antibiotic treatments.

Measure traits	PCI	PCII	Univariate morphological response								
	Size	Shape	Ciprofloxacin ($\mu g\,L^{-1}$)					Enrofloxacin ($\mu g\,L^{-1}$)			
			1	10	100	1000		1	10	100	1000
Total length	0.43	−0.12	***F*=5.93 *p*<0.001**	NS	S	S	S	*F*=1.32 ***p*<0.001**	NS	S	S	S
Body length	0.42	0.38	***F*=4.83 *p*<0.05**	NS	NS	S	S	*F*=1.34 *p*>0.05	NS	NS	NS	NS
Body width	0.39	0.64	***F*=3.31 *p*<0.05**	NS	NS	S	S	*F*=0.97 *p*>0.05	NS	NS	NS	NS
Tail fin length	0.41	−0.42	***F*=4.32 *p*<0.05**	NS	S	S	S	*F*=1.22 *p*>0.05	NS	NS	NS	NS
Tail muscle depth	0.40	−0.52	*F*=1.34 *p*>0.05	NS	NS	NS	NS	0.47 *p*>0.05	NS	NS	NS	NS
Tail fin depth	0.41	0.03	***F*=3.52 *p*<0.05**	NS	NS	S	S	**2.61** *p*>0.05	NS	NS	NS	NS
Explained percentage	90%	5%	**MANOVA Wilks' Lambda λ 0.41; *F*=1.77; DF=140; *p*<0.05**					**MANOVA Wilks' Lambda λ 0.30; *F*=2.57; DF=140; *p*<0.001**				
Antibiotic Treatment Effect	***F*=47.48; DF=2; *p*<0.05**	***F*=4.93; DF=2; *p*<0.05**	**MANOVA Wilks' Lambda λ 0.68; *F*=1.91; DF=240; *p*<0.001**									

Bold letter indicate significant values.
Significant, S ($p<0.05$; Dunnett post hoc test); non-significant, NS.

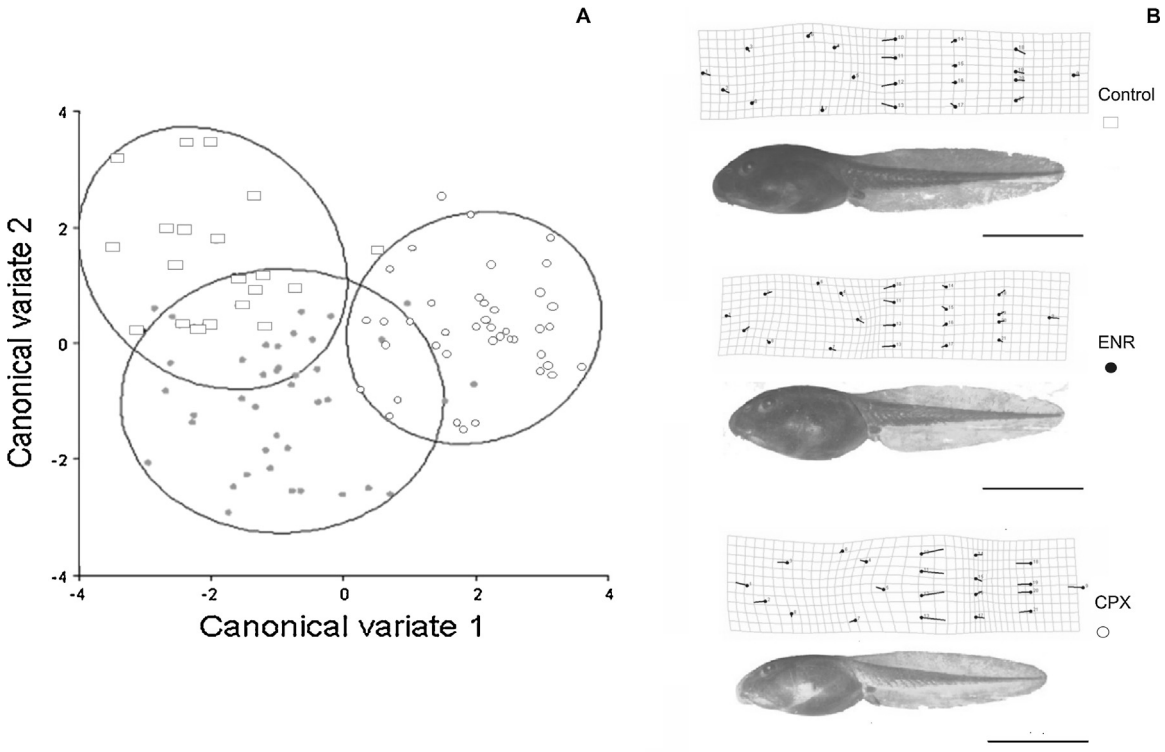

Fig. 3. Morphometric geometry variation in shape of *R. arenarum* larvae exposed to antibiotics. (A) Scatterplot from CVA on individuals of each antibiotic treatments (ENR-light grey dots, CPX-unfilled dots) and (controls-square), (B) Thin plate spline deformation grids describe shape variation of each antibiotic treatments (ENR and CPX) and control, with landmarks (black dots) and vectors (indicate direction of variation). Transformation grids magnified ×5. Example of typical larvae or each treatment, bar 1.45 mm.

associated with the relative length and height of the body, tail muscle and fin. The CV component II (24.12%) revealed contrast between larvae ENR exposed and controls, corresponding to differences in the relative body length.

Ciprofloxacin exposed larvae had smaller body than those exposed to the other treatments, and its transformation grid was therefore characterized by a relative lateral anterior–posterior contraction. In contrast, larvae exposed to the other treatments (ENR and control individuals) showed various degrees of lateral anterior–posterior elongation. The elongation of ENR exposed larvae was most pronounced in the tail, whereas the body was relatively shorter.

3.5. Oxidative stress enzymes

The effects of acute exposure to CPX and ENR on GST and CAT of *R. arenarum* larvae are shown in Fig. 4. Overall, oxidative stress and detoxification enzyme activities (GST and CAT) of toad lar-

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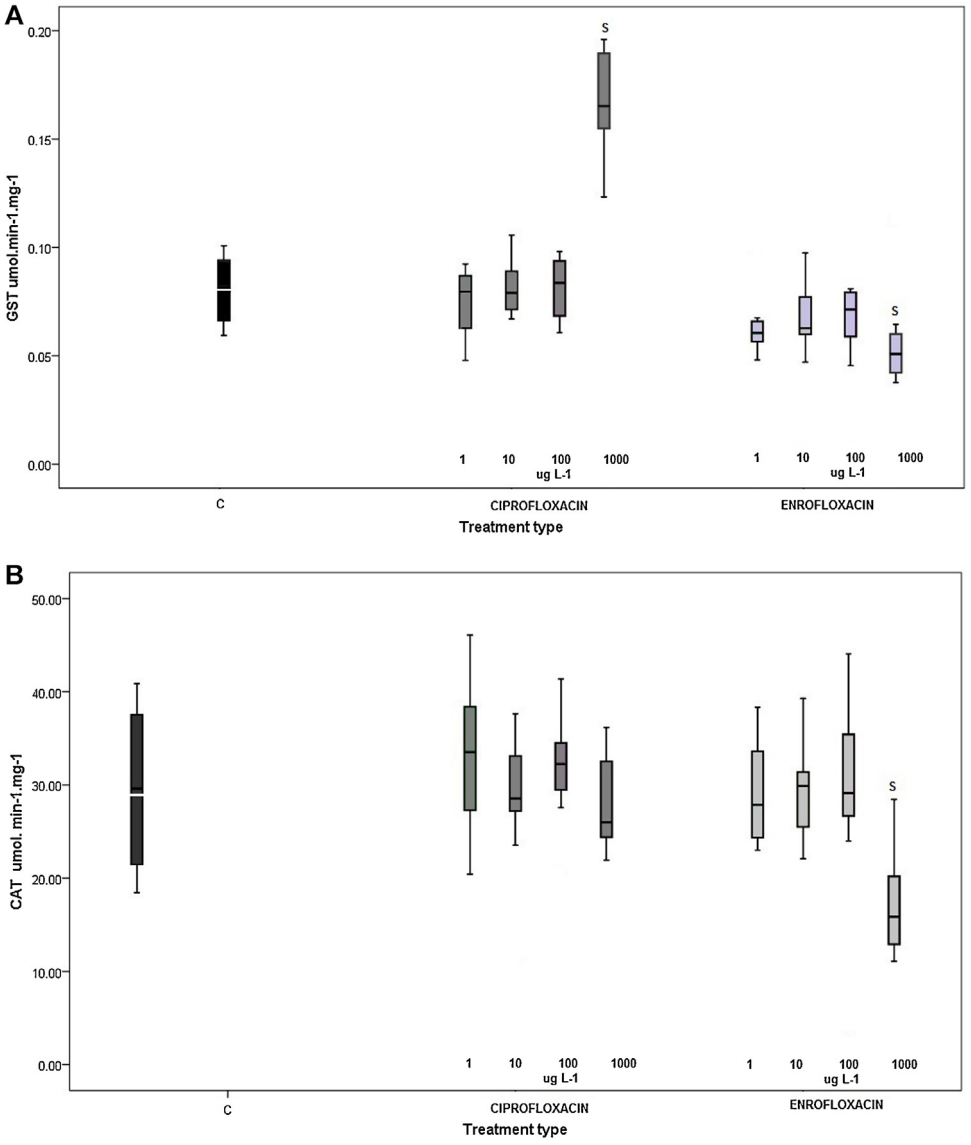

Fig. 4. GST (A) and CAT (B) activities on *R. arenarum* larvae exposed to CPX and ENR. Control, black box; CPX, dark grey; ENR; light grey. Statistical significance respect to the control group (S).

vae were affected by concentrations higher than 10 μg L^{-1} of both antibiotics treatments (MANOVA Wilks' lambda $\lambda = 0.34$, $F = 20.86$, DF = 89; $p < 0.0001$).

GST exhibited significantly higher activity ($F = 61.87$, DF = 4) at 1000 μg L^{-1} of CPX (GST $_{CPX1000\ \mu gL^{-1}} = 0.16 \pm 0.1$ μmol min^{-1} mg^{-1}). This induction of GST was statistical higher than the control group (GST $_{CPXcontrol} = 0.074 \pm 0.14$ μmol min^{-1} mg^{-1}; Dunnett post hoc test t $p < 0.0001$). In contrast, the GST activities of larvae exposed to ENR was significantly lower ($F = 6.27$, DF = 4; $p < 0.001$) at 1000 μg L^{-1} ($GST_{ENR1000\ \mu gL}-1 = 0.058 \pm 0.04$ μmol min^{-1} mg^{-1}) than in the controls, being these differences significant at $p > 0.05$).

The mean values of CAT activity was significantly reduced ($F = 5.80$, DF = 3; Dunnett post hoc test t $p < 0.05$; Fig. 4) in larvae exposed to 1000 μg L^{-1} of ENR (CAT $_{ENR1000\ \mu gL^{-1}} = 16.89 \pm 5.21$ μmol min^{-1} mg^{-1}) in comparison to control (CAT $_{ENRcontrol} = 30 \pm 0.1$ μmol min^{-1} mg^{-1}). Mean CAT activities in CPX treatments of larvae are similar to values of control ($F = 0.98$, DF = 3; Dunnett post hoc test t $p > 0.05$, Fig. 4).

3.6. Ecotoxicological risk

No observed effect concentration (NOEC) for both antibiotics was 1 μg L^{-1} for all biological endpoints at 96 h. Accordingly, an assessment factor (AF) of 100 was used, and a predicted no effect concentration (PNEC) of 1 μg L^{-1} was obtained based on the NOEC. RQs for both antibiotic were higher than 1 (Table 2), indicating significant ecotoxicological impact on short-term exposure. Both antibiotics were hydrophilic, obtaining log K_{ow} lower than 1 on ECOSAR simulations (Table 2).

4. Discussion

In aquatic systems, there are no studies indicating the deleterious effects of fluoroquinolone antibiotics on amphibian larvae, probably because these antibiotics are not yet described as toxic for amphibian or they are recently included in environmental screening as emerging contaminants and in the emergence of antibiotic resistance-bacteria (Redgrave et al., 2014; Van Boeckel et al., 2015). In general, orally administered antibiotics in veterinary medicine are excreted with faeces and litter manure, which are then usually

Table 2
Ration quotient risk (RQ) and Ecological Structure Activity Relationship (ECOSAR) of ENR and CPX. Chemical abstract service (CAS number), AF (assessment factor), no observed effect concentration (NOEC), Maximum measured environmental concentration (MEC), predicted no effect concentration (PNEC), and Quantitative structure–activity relationship models (QSAR).

Anuran species (stages 26-32)	Antibiotic	Study	NOEC	AF	MEC	PNEC	Log K_{wo}	Solubility (mg L^{-1})	Baseline QSAR Limitation Log K_{wo} fish (96 h)	RQs
Rhinella arenarum	Enrofloxacin CAS 093106-60-6 ENR	NOEC(96 h)	1 μg L^{-1}	100	8.77	0.1	0.70	2007	5	**RQ > 1**
Rhinella arenarum	Ciprofloxacin CAS 085721-33-1 CPX	NOEC(96 h)	1 μg L^{-1}	100	7.49	0.1	0.28	0.0003	5	**RQ > 1**

used in crop and vegetable field manure or fertilization, or derive via pluvial runoff of upland to ponds becoming a threat to aquatic organisms (Migliore et al., 1996). While effects of many pharmaceuticals are known in aquatic organisms (Załęska-Radziwiłł et al., 2011; Martins et al., 2012), there is little information about the impacts of fluoroquinolone antibiotics on amphibian. Using an amphibian experimental model *Xenopus laevis*, Richards and Cole (2006) determined CPX to be nontoxic and non-teratogenic.

The experiment with *R. arenarum* larvae evidenced that at 96 h exposures at 10, 100 and 1000 μg L^{-1} concentrations of ENR and CPX antibiotics induced impairments on its biological endpoints, mainly on development, growth and antioxidant enzyme activities. Those outcomes might exert potential damages on long-term maintenance of *R. arenarum* natural larvae populations continuously exposed to the input of antibiotics.

CPX and ENR did not caused significant mortality (<3%) on *R. arenarum* larvae. Similarly, Robinson et al. (2005) found that these fluoroquinolones caused less than 8% mortality at a concentration of 10 mg L^{-1} of fathead minnow's larvae (*Pimephales promelas*). Exposure of amphibian in early life stage to ENR resulted in a significant decrease in development and growth at concentrations higher than 10 μg L^{-1}. Generally, stress conditions such as polluted aquatic environment result of amphibian in early larvae stages (Peltzer et al., 2013). It seems that early life stages of amphibians are particularly vulnerable to damage caused by ENR. Moreover, *R. arenarum* larvae exposed to CPX reduce its body shape (results from morphogeometry) and size (results from standard measures) at short term of exposure. Despite these sublethal effects being non-specific for this antibiotic, as previously reported for other contaminants (Aronson et al., 2012), the reduced body size can be explained in three ways; firstly, fluoroquinolones induce neurotoxicity (Kamath, 2013) and the larvae may present non-feeding behaviour, reducing food intake in part due to a reduction of swimming (Peltzer et al., 2013), and secondly, these antibiotics are condro-bone toxic (Li et al., 2004), and may affect condrogenesis mainly in the larval skull that occur during metamorphosis. In contrast, Załęska-Radziwiłł et al. (2011) found that CPX stimulated of growth in juvenile zebra fish *D. rerio*. Similarly, Robinson et al. (2005) also described an increase in weight in *P. promelas* after seven days of exposure to CPX (10 mg kg^{-1}). Histological studies at bone and cartilaginous levels are necessary to elucidate ENR-CPX growth impairments in larvae, considering that amphibian metabolizes ENR to CPX (Howard et al., 2010). Thirdly, normal microbiome of amphibian larval gut (Lajmanovich et al., 2001) could be altered by antibiotics, as demonstrated for fathead minnow *P. promelas*'s guts after seven days of exposure at environmental relevant antimicrobial triclosan concentrations (Narrowe et al., 2015). The latter study suggests that even low-level environmental exposure to triclosan can induce significant short-term changes to the gut microbiome and host health. In this context, energetic gains from a fermentative microbial digestion within of guts of amphibian larvae and their direct relation to body size (Pryor and Bjorndal, 2013; Colombo et al., 2015) could change in the presence of antibiotics in water. More research is needed to focus on the potential effects of emerging aquatic contaminants on the intestinal microbiota, and hence, the effect on digestive function of amphibians. This suggestion is in accordance with symptoms of diarrhoea and loss of appetite observed in CPX-exposed larvae. Sometimes an imbalance in the commensal gut microbiota due to antibiotics can result in intestinal problems, decreasing 20% of the tadpole's daily energy intake by fermentation (Colombo et al., 2015), and other visible symptoms such as antibiotic-associated diarrhoea and poor growth rates (McFarland, 1998). In addition, microbiome gut starvation (Narrowe et al., 2015), gut immune systems and pathogens resistance (Redgrave et al., 2014, Colombo et al., 2015) in herbivorous amphibian larvae exposed to relevant concentrations of CPX and ENX could be included in future ecotoxicological risk assessment.

Oxidative stress biomarkers are used for evaluation of toxic effect of emerging contaminants such as pharmaceuticals and other pollutants on fish larvae (Plhalova et al., 2014) and anuran larvae (Lajmanovich et al., 2013). In this sense, defensive mechanisms of amphibian larvae are capable of counteracting the influence of reactive oxygen species (ROS) resulting from the metabolism of various pollutants (Sparling et al., 2001). Negative impact on biochemical processes linked with the presence of ROS and free radicals are observed in *R. arenarum* larvae exposed to CPX and ENR. The activity of GST of *R. arenarum* larvae exposed to CPX showed a significant increase at relevant concentration of 1000 μg L^{-1}. Similarly, a significant increase of GST was also observed in *D. rerio* exposed at 0.7 and 100 μg L^{-1} of CPX concentrations (Plhalova et al., 2014). Indeed, Bartoskova et al. (2014) found an increase in GST activity of *D. rerio* in the presence of another fluoroquinolone, norfloxacin (NFX) at 0.1 μg L^{-1} concentration. This might indicate that 1000 μg L^{-1} of CPX led to the activation of the detoxifying system and preventing the oxidative damage in *R. arenarum* larvae at short term exposure (96 h). In addition, the same concentrations of ENR produced the depletion of biotransform toxicants within larvae.

Catalase acts against reactive oxygen molecules (ROS) and converts hydrogen peroxide into water and oxygen (Li et al., 2010). The CAT activities of *R. arenarum* larvae exposed to CPX were similar to those found in the controls. Bartoskova et al. (2014) reported a significant increase of CAT activity in fish exposed to NFX, while Andrieu et al. (2015) found CAT inhibition at a higher concentration (50,000 μg L^{-1} CPX) in nile Tilapia *Oreochromis niloticus*. However, a concentration of 1000 μg L^{-1} of ENR inhibited CAT of toad larvae below the CAT activities of control samples, similarly to findings of Wang et al. (2009) who reported alteration in CAT activities in the gills and brain of fish after ENR treatments. Although, it has been recently demonstrated that the structural and functional changes of CAT are closely associated with increased risk of oxidative stress

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induced by fluoroquinolones (Qin and Liu, 2013) the interaction mechanisms between ENR and CAT are not yet fully understood (Stancova et al., 2014). Thus, these results reinforces the idea of the presence of oxidants that could lead to the inactivation of the enzymatic activity (Bagnyukova et al., 2006; Lushchak et al., 2009) and may be a sign of interference of this antioxidant defence pathway being the hydrogen peroxide the responsible for this inhibition (Modesto and Martinez, 2010). In addition, this may be possible that a deficiency of CAT activity due to its inhibition was compensated by enhanced GST activity. In this sense, the complex pathway of interaction between GST-CAT and the activity of one enzyme influences the activity of other enzymes should be investigated in larvae exposed to short and long period at ENR and CPX.

Furthermore, the risk quotient (RQ) could be a useful measure that characterizes potential ecological risk of a stressor (VICH, 2004). The ecotoxicity values obtained at 10 $\mu g\,L^{-1}$ were similar to the maximum concentration found in lentic environments (Wei et al., 2012). In this sense, the results of the risk assessment performed in our study indicate that the environmental release of ENR and CPX pose risks to *R. arenarum* tadpoles poses. Therefore, according to the results, the cut-off value used in the risk assessment of both antibiotics (1 $\mu g\,L^{-1}$) provides a sufficient protection level for amphibian larvae. Martins et al. (2012) pointed that the producers were more sensitive trophic level than consumers, being the overall decreasing order of sensitivity *Lemna minor* > *Pseudokirchneriella subcapitata* > *Vibrio fischeri* > *Daphnia magna* > *Gambusia holbrooki* (Martins et al., 2012). Similarly, a microcosm study investigating the effects of ENR on tropical freshwater communities could not identify significant effects of ENR on cyanobacteria species (Rico et al., 2014a). However, the integration of exposure and effect data in the PEC/PNEC ratios showed that CPX may pose a risk to the most sensitive aquatic species, particularly when aquatic samples with high concentrations of this antibiotic are considered, e.g., in hospital effluents. Such worst-case situation pre-empts a possible hazard for ecosystem integrity and functioning (Martins et al., 2012). Nevertheless, our evaluation is only focused on the toxicity of two antibiotics on amphibian larvae at environmental relevant concentration under lab condition, but in the aquatic systems pharmaceuticals are present as mixtures of an unlimited diversity of natural characteristics and exogenous inputs, which should be taken into account when evaluating ecotoxicological effects (Pomati et al., 2008). However, some studies, like those performed by Cleuvers (2004) revealed that a mixture of pharmaceuticals were toxic at concentrations at which a single drug showed either no or only little effect. In this context, more research should be needed to better understand the toxicity of antibiotics on anuran larvae at real environments and to assess potential side-effects on ecosystem structure and environments contaminated by other emerging contaminant (such as pesticides) and in relation environments.

The present study on a native anuran species helps setting priorities for further testing, considering that there is limited and controversial experimental data available regarding fate or toxicity. Since, recent investigation (Andrieu et al., 2015) has indicated that fluoroquinolone antibiotics accumulate in sediments downstream effluent discharges, further assessments should be carried out by testing potential toxic effects of contaminated sediments (ERA, Phase III "further assessments" or Tier C) (VICH, 2004) or conceptual site model (CSM, describing the complete exposure pathways that will be evaluated in the ERA including the assessment biological endpoints, U.S. EPA, 1992).

5. Conclusions

Our results lead us to conclude that environmental concentrations greater than or equal to 10 $\mu g\,L^{-1}$ of CPX and ENR have sublethal effect on *R. arenarum*, mainly affecting larval development, size, shape and growth, indeed altering enzyme activities related to oxidative stress. Although this is the first study pointing out that CPX and ENR impair amphibian larvae, explanation of those deleterious effects is complex and the ecological context should be considered. In another words, antibiotic contaminations and physiological factors (growth, development, oxidative stress enzymes) may alter ecological death of larvae (larvae may be unable to function in an ecological context due to alterations in their normal behaviour, Scott and Sloman, 2004). In this context, further assessments such bioaccumulation studies may provide evidences of CPX and ENX accumulation in amphibian larvae and consequently ecological impairments both in water and aquatic system sediments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etap.2017.01.021.

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