Water Air Soil Pollut (2015) 226:427
DOI 10.1007/s11270-015-2695-9

Harmful Effects of the Dermal Intake of Commercial Formulations Containing Chlorpyrifos, 2,4-D, and Glyphosate on the Common Toad *Rhinella arenarum* (Anura: Bufonidae)

Rafael C. Lajmanovich · Andrés M. Attademo · María F. Simoniello · Gisela L. Poletta · Celina M. Junges · Paola M. Peltzer · Paula Grenón · Mariana C. Cabagna-Zenklusen

Received: 4 August 2015 / Accepted: 13 November 2015
© Springer International Publishing Switzerland 2015

Abstract Amphibians have complex life cycles with aquatic and terrestrial life and uncovered skins; therefore, they are exposed to chemical contamination, where dermal exposure is a significant route for pesticide uptake in both habitats. In this study, measurements in blood samples such as levels of butyrylcholinesterase (BChE), carboxylesterase (CbE), glutathione S-transferases (GST), thiobarbituric acid reactive substances (TBARS), modified alkaline comet assay (ACA) for detection of oxidized bases (FPG and Endo III sites), as well as the ratio of heterophils and lymphocytes (H/L), were evaluated as non-destructive biomarkers to monitor dermal pesticide exposure in male toads of *Rhinella arenarum*. Toads were exposed to a solution containing a nominal concentration of commercial formulations of the insecticide chlorpyrifos (CPF, 10 mg/L), and herbicides 2,4-D and glyphosate (GLY) (20 mg/L, respectively). After 48 h of exposure, the levels of plasma B-sterases (BChE and CbE) were inhibited (55 and 43 %, respectively) in toads exposed to CPF. Also, the activity of GST was inducted for dermal exposure to 2,4-D, as well as the levels of TBARS due to CPF exposure. Besides this, CPF and 2,4-D exposure induced oxidative DNA damage, and the H/L ratio decreased for the both herbicide exposures. Our results showed that exposure via dermal uptake to CPF, 2,4-D, and GLY in the common toad *R. arenarum* induced neurotoxicity, oxidative stress, and immunological depression. Thus, some blood biomarkers employed in our study (B-esterases, GST, levels of TBARS, ACA, and H/L ratio) might be used as predictors in health and ecological risk assessment of amphibian populations exposed to OP insecticides and herbicides.

Keywords Amphibians · Dermal exposure · Chlorpyrifos · 2,4-D · Glyphosate · Blood non-destructive parameters

R. C. Lajmanovich · A. M. Attademo · G. L. Poletta · C. M. Junges · P. M. Peltzer
National Council for Scientific and Technical Research (CONICET), Buenos Aires, Argentina

R. C. Lajmanovich (✉) · A. M. Attademo · M. F. Simoniello · G. L. Poletta · P. Grenón · M. C. Cabagna-Zenklusen
Faculty of Biochemistry and Biological Sciences, National University of Littoral (FBCB-UNL), Paraje el Pozo s/n, 3000 Santa Fe, Argentina
e-mail: lajmanovich@hotmail.com

1 Introduction

The integrated use of measurable indicators, such as cholinesterases (ChEs) and others, may be necessary for biomonitoring programs for assessing the impact of pesticides in amphibians living in agroecosystems (Lajmanovich et al. 2008; Attademo et al. 2011). The use of several biomarkers (i.e., pollutant-induced biological responses at the sub-individual level that are measured in nonlethal methodologies) is one of the first ecotoxicity phases in risk characterization of pollutants. A pollutant stress normally triggers a cascade of biological responses, each of which may, in theory, serve as biological endpoints that could be described with a

Published online: 20 November 2015

biomarker (Hook et al. 2014). Therefore, the measurement of blood butyrylcholinesterase (BChE) activity, such as and carboxylesterase (CbE) (for neurotoxicity), glutathione S-transferases (GST) and thiobarbituric acid reactive substances (TBARS) (for oxidative stress) is used as a biomarker to monitor pesticide exposures in wild species (Falfushinska et al. 2008; Poletta et al. 2012). The alkaline comet assay (ACA) is sufficiently sensitive for detecting DNA damage in frogs (Dhawan et al. 2009). Certainly, oxidative DNA damage is a valuable tool for the measurement of oxidative stress and may represent an optimal index for measurement of the potential risk of amphibian pesticide exposures (Ismail et al. 2014; de Lapuente et al. 2015). Moreover, the use of the bacterial enzymes Endonuclease III (Endo III) (to recognize oxidized pyrimidines) and formamidepyrimidine-DNA glycosylase (FPG) (to recognize oxidized purines, including 7,8-dihydro-8-oxo-guanine) were first described by Collins et al. (1993). Furthermore, the ratio of two leukocyte types, heterophils and lymphocytes (H/L ratio), has been increasingly used by to analyze immune function in amphibians exposed to pesticides (Davis et al. 2008).

In this sense, in vivo and in vitro research with the terrestrial life stages of amphibians have shown that the uptake of pesticides occurs rapidly through the permeable skin and it is much higher if compared to mammals (Quaranta et al. 2009). Dermal exposure presents a potentially significant but insufficiently studied route for pesticide uptake in terrestrial amphibians (Van Meter et al. 2014). Furthermore, studies using terrestrial life-stages of amphibians are really important because dermal exposure is seen as main absorption way of pesticides for adult and juvenile amphibians (Brühl et al. 2013).

In Argentina, after the prohibition of organochlorine (OC) and organophosphate (OP) pesticides (endosulfan and malathion), the use of alternative insecticides like chlorpyrifos (CPF) rapidly increased. CPF is widely used in grain cultivation and in numerous non-agricultural situations. CPF is one of the most frequently detected insecticides in our country, found in sediments, suspended particles, and in water (Jergentz et al. 2005). In addition, Loewy et al. (2011) detected CPF in surface water and sub-surface drains in the pome-fruit-growing region of Neuquén River Valley in 73 % of the samples. CPF caused severe birth defects in children exposed in utero (Sherman, 1996) and some effects on wild fauna through several diverse mechanisms (Chaturvedi et al. 2013). Mainly, CPF inhibits the ChEs enzymes, affects the nervous system of organisms (Sun and Chen 2008), and increases DNA damage in toads (Yin et al. 2009; Li et al. 2015). In some cases, the CPF formulated was found to be more toxic than the active ingredient, particularly to aquatic organisms (Ali et al. 2009). Several studies have documented an apparent connection between the presence of CPF residues and reductions in amphibian populations, at both local (Fellers et al. 2004) and landscape scales (Davidson et al. 2001).While some organophosphates are readily absorbed through the skin, studies in humans suggest that skin absorption of CPF is more limited (Hayes and Laws 1991).

The 2,4-dichlorophenoxyacetic acid (2,4-D) is a phenoxy herbicide that is related to the growth hormone indoleacetic acid. 2,4-D was developed during World War II and is composed of 50 % Agent Orange (EXTOXNET, 1996). In Argentina, in the main agricultural region, Humid Pampa, about 2200 tons of 2,4-D are annually applied in different crops (particularly, corn and soybean 2,4-D tolerant), comprising a total area of 3.4×10^6 ha (Merini et al. 2008). Despite the fact that this herbicide has a negative ecotoxicological profile and is a potent clastogen (Bukowska 2006), the effects of 2,4-D exposure on adult amphibians are poorly understood (Ryan et al. 2006).

Glyphosate (GLY) is the world's most widely used herbicide. It is used in horticulture, parks, and home gardens, but the largest use is in agriculture on the genetically modified glyphosate-resistant crop varieties (GMOs) (James 2008). In March 2015, GLY was classified as probably carcinogenic to humans for the International Agency for Research on Cancer (IARC) (Guyton et al. 2015). Although this news is relatively recent, several studies were alerting on the adverse consequences of DNA damage effects of GLY formulations on wildlife (i.e., in earthworms, snails, spiders, fish, amphibians, and reptiles) and mammals, including humans (Poletta et al. 2009; Benamú et al. 2010; Guilherme et al. 2012; Wagner et al. 2013; Braz-Mota et al. 2015; Gress et al. 2015).

The main part of ecotoxicological research for amphibians was done on aquatic life stages. As part of a continuing study to assess the adverse effect of pesticides on populations of anurans in Argentina, the purpose of this research was to evaluate some non-destructive biomarkers (BChE, GST, TBARS, ACA, and H/L ratio) under dermal exposure in male of *R. arenarum* toads to estimate the risk of CPF, 2,4-D, and GLY under laboratory conditions.

2 Materials and Methods

2.1 Reagents

2-Thiobarbituric acid (TBA), and Trichloroacetic acid (TCA), and Butylhydroxytoluene (BHT) were obtained from Merck® (USA) and Sigma-Aldrich® (Germany). Sodium dodecyl sulfate (SDS) was purchased from Calbiochem® (Canada). Butyrylthiocholine iodide (BuSCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), α-naphthyl acetate (α-NA), and Fast Red ITR salt were obtained from Sigma-Aldrich® (Germany). Reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) were obtained from Acros Organics (USA). All the other chemicals used in this study were acquired from Biopack® (Argentina). Dimethyl sulphoxide (DMSO) was purchased from Fluka. RPMI-1640 medium was purchased from HyClone. Low melting point agarose (LMPA), normal melting point agarose (NMPA) and the rest of reagents for ACA and general laboratory chemicals were provided by Sigma.

2.2 Pesticides Selection

The commercial formulations of pesticides used in experiments were: CPF (48 % active ingredient [a.i.], O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate; log K_{OW}=4.7; NUFARM®, Nufarm S.A. Argentina), 2,4-D (60.2 % a.i., 2,4-Dichlorophenoxy acetic acid dimethyl amine salt; log K_{OW}=0.65; ASI MAX 50®; CHEMOTECNICA S.A, Argentina), and GLY (74.7 % a.i., *N* (phosphonomethyl) glycine; log K_{OW}=−3.4; Roundup Ultra-Max®, Monsanto Co., Argentina). As the active ingredients are not expected to have similar mechanisms of action, metabolites, or toxicokinetic behavior as the commercial formulated products (Sparling et al. 2010), the pesticides were tested in this form, they were applied in cultivated fields and introduced into the environment. Likewise, Brühl et al. (2013) exposed terrestrial amphibians to pesticide formulations that contain additives.

2.3 Study Animals

Twenty adult male of *R. arenarum* were collected by hand from temporary ponds in artificial wetlands (31°39'52.90" S, 60°42'50.20" W, South Park Lake, Santa Fe, Santa Fe province, Argentina) in November 2014; these sites had not been treated with chemical pesticides, as determined by the laws to protect human and wildlife health. *R. arenarum* is used as model in ecotoxicology (Cabagna et al. 2005). This toad is frequently found in forests, wetlands, agricultural land and urban territories (Peltzer et al. 2006) and it has an extensive Neotropical distribution (IUCN 2010). These toads feed mainly on a variety of arthropods, and they play, therefore, an important role as biological controls, particularly in soybean crops (Attademo et al. 2005).

After capture, toads were quickly transported to the laboratory in darkened buckets containing water to minimize stress. Snout-vent length (SVL) (mm) and body weight (g) were recorded with digital caliper (precision, 0.01 mm). Toads were acclimated for 24 h before initiation of the experiment (individually in semi-transparent plastic water buckets; size: Ø 23 cm×28 cm) under laboratory conditions with a photoperiod 12–12 h (light 07:00–19:00 h), humidity (60±10 %), and temperature 24±2 °C.

2.4 Risk Exposure

A control group (CO, *N*=5) with 500 ml of dechlorinated tap water (DTW; pH 7.4±0.05; conductivity 165±12.5 μmhos/cm; dissolved oxygen concentration 6.5±1.5 mg/L; and hardness 50.6 mg/L $CaCO_3$) and the three treatments with pesticide formulations (nominal concentration) were employed: CPF (*N*=5, 10 mg/L), 2,4-D (*N*=5, 20 mg/L), and GLY (*N*=5, 20 mg/L). Doses were chosen in ranges usually lacking overt toxicity, mimicking a scenario with no alerting clinical signals that may erroneously lead to the assumption of absence of danger (Muller et al. 2014). Toads were randomly placed individually into a sterile bucket with 500 ml of tests solution (DTW or pesticides) equivalent to 3 cm deep for 48 h, and under the same laboratory condition as described previously.

2.5 Biomarkers

Blood samples (approximately 0.5 ml) were collected by a minimally cardiac puncture using a heparinized syringe (i.e., Attademo et al. 2011) after 48 h of dermal exposure. Although, this procedure is invasive, the use of anesthesia was avoided because of interferences in the interpretation of biomarker responses (Vernadakis and Routledge 1973). Busk et al. (2000) also reported that anesthesia might be more stressful to amphibians and reptiles than cardiac puncture. Likewise, Tyler

(1999) suggested that cardiac puncture is a reliable method. Therefore, we considered the practical experience of our personnel with amphibians, the health of animals after blood sampling, and the minimum blood volume required for experimental purposes as the criteria for selecting cardiac puncture as the most appropriate blood sampling technique instead of animal sacrifice (Gabor et al. 2013). Whole blood was used for lipid peroxidation and comet assay in erythrocytes and hematological indicators of stress, while the plasma was separated from the collect whole blood by centrifugation (at 10,000 rpm for 15 min) for enzymatic determinations.

Toads were maintained in the laboratory of ecotoxicology during a period of recovery, and after a general revision of body condition, they were released to the same sites where they had been captured. To do this, we had the approval of the Animal Ethics Committee of the Faculty of Biochemistry and Biological Sciences, National University of Littoral, and followed the guidelines of ASIH and SSAR (2001).

2.5.1 B-Esterases Determination

Plasma BChE activity was determined colorimetrically by the Ellman et al. (1961). The reaction medium included 930 μl 25 mM Tris–HCl, 1 mM $CaCl_2$ (pH=7.6), 50 μl 5,5′-dithiobis-2-nitrobenzoic acid (3 9 10^{-4} M, final concentration), 10 μl butyrylthiocholine iodide (2×10^{-3} M, final concentration) and 10 μl of plasma. The variation in optical density was recorded at 410 nm for 1 min at 25 °C using a Jenway 6405 UV–VIS spectrophotometer. Kinetic was carried out in duplicate. Plasma BChE activity was expressed as μmol of substrate hydrolyzed min^{-1} ml^{-1} of plasma using a molar extinction coefficient of 13.6×10^{-3} M^{-1} cm^{-1}. We did not determine the plasma.

Acetylcholinesterase (AChE) activity because the BChE is the enzyme that primarily contributes to total plasma cholinesterase activity in many vertebrate species (Sanchez-Hernandez and Moreno-Sanchez 2002).

Plasma CbE activity was measured by the Gomori method (1953) as adapted by Bunyan et al. (1968). The assay was carried out with 25 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} $CaCl_2$ (pH=7.6), and 10 μL plasma at 25 °C. The reaction was initiated by adding 50 μL *a*-naphthyl acetate (1.04 mg mL^{-1} in acetone) as substrate, and stopped after 10 min by addition of 500 μL of 2.5 % SDS and subsequently 500 μL of 0.1 % Fast Red ITR in 2.5 % Triton X-100 in water (freshly prepared). Samples were left in darkness for 30 min to develop, and the absorbance of the complex was read at 530 nm. Hydrolysis of α-NA was expressed as nmol of substrate hydrolyzed min^{-1} $ml^{-1}$of plasma using a molar extinction coefficient of 33.225×10^{3} M^{-1} cm^{-1}.

2.5.2 GST Activity

Plasma GST activity was determined as described by Habig et al. (1974) and adapted by Habdous et al. (2002) for serum GST activity in mammals. The reaction solution contained 100 mM Na-phosphate buffer (pH=6.5), 2 mM 1-chloro-2, 4 dinitrobenzene, 5 mM reduced glutathione, and the sample as well as the kinetics of reaction were monitored at 340 nm. GST was corrected for no enzymatic activity by subtracting lanks (buffer and GSH only) and the results were converted to specific activity in units of nmol of substrate hydrolyzed min^{-1} ml^{-1} of plasma using a molar extinction coefficient of 9.6×10^{3} M^{-1} cm^{-1}.

2.5.3 Lipid Peroxidation in Erythrocytes

Lipid peroxidation in erythrocytes was determined by measuring the production of color generated during the reaction of TBA with malondialdehyde (TBARS assay) according to the method of Buege and Aust (1978) with some modifications (Simoniello et al. 2010). An aliquot of washed erythrocytes were hemolyzed by adding demineralized water (Milli Q plus reagent grade) and mixed thoroughly with four volumes of reaction solution (15 %*w*/*v* TCA, 0.375 %*w*/*v* TBA, 0.25 mol l^{-1} HCl acid) and 4 % BHT to inhibit peroxidation stimulated by Fe^{3+} without affecting the formation of the MDA-TBA chromogen. The mixture was then heated at 92 °C for 45 min. After cooling, the flocculent precipitate was removed by centrifugation at 12000*g* for 10 min at 4 °C. The sample absorbance at 535 nm was determined, and the TBARS concentration was calculated using the extinction coefficient 1.56×10^{5} M^{-1} cm^{-1}. The MDA concentration in erythrocytes was expressed as mmol mg^{-1} protein.

2.5.4 Comet Assay Modified for Detection of Oxidized Bases (FPG and Endo III)

The ACA (pH>13) was then performed according to the method described by Singh et al. (1988), with the

following modifications (Poletta et al. 2008): blood samples were diluted 1:19 (*v*/*v*) with RPMI-1640 medium and used immediately. Then, 4.5 μL of each diluted blood sample (approximately 4.0×10^3 erythrocytes) was added to 300 μL of 1 % low melting point agarose (LMA) and three slides were prepared. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were immediately immersed in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM trizma base, 1 % Triton X-100 and DMSO 10 %; pH 10) and left at 4 °C overnight. After lysis slides were washed and excess liquid dabbed off with tissue; 50 μl of enzyme solution or buffer alone as control was placed on the gel and covered with a cover slip. Slides were put into moist box (prevents desiccation) and incubated at 37 °C for 30 min. At the end of the incubation period, coverslips were removed and slides were placed in an electrophoresis tank to continue with the comet assay (Poletta et al. 2012). The slides were then immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA; pH>13), first for unwinding (10 min) and then for electrophoresis (0.7–1 V cm^{-1}, 300 mAmp, 10 min at 4 °C). All of the steps were carried out under conditions of minimal illumination and low temperature (on ice). Once electrophoresis was completed, the slides were neutralized and dehydrated with ethanol. Slides were stained with acridine orange at the moment of analysis and one hundred randomly selected comets from each animal were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (damage index, $DI=n1+2.n2+3.n3+4.n4$), where $n1$, $n2$, $n3$ and $n4$ are the number of cells in each class of damage, respectively. The frequency of FPG or Endo III sites were estimated by subtracting the values obtained without enzymes from the values obtained with the enzyme.

2.5.5 Hematological Indicators of Stress

Two blood smears for each toad were prepared on clean slides, fixed, and stained by the May–Grunwald–Giemsa method (Dacie and Lewis 1984). To determine the counts of heterophil and lymphocyte, 100 cells per film were examined by light microscopy. All blood counts, including granulocytes (heterophil, basophil, and eosinophil) and non-granulocytes (lymphocyte and monocyte), were examined by the same investigator. The results are presented as the percentage of each cell occurring in each film. The heterophil/lymphocyte (H/L) ratio was examined as a response estimator of stress (Davis et al. 2008).

2.6 Data Analyses

All biomarkers data were expressed as the mean±SEM. The influence of pesticide treatments on each variable (B-esterases, GST enzyme, TBARS, ACA [FPG and Endo III sites], and H/L ratio) were analyzed with Kruskal–Wallis test and Dunn's test for post hoc comparisons (Lajmanovich et al. 2013). These statistical analyses were performed using BioEstat software 5.0 (Ayres et al. 2008). A value of $p<0.05$ was considered significant.

3 Results

Mean (±SD) length and body mass of male toads were 90.15±2.43 mm and 70.92±0.93 g, respectively. No signs of general behavioral disorders (e.g., hyperactivity, loss of coordination in both front and hind limbs, erratic swimming, etc.) were observed in exposed toads to CPF, 2,4 D, and GLY as a response to neurotoxicity.

3.1 B-Esterases Activities

The mean value of BChE activity in the non-pesticide exposed toads was 6.14±0.48 μmol min^1 mg^{-1} plasma at 48 h. BChE activity varied among groups exposed to different types of commercial pesticides, differing significantly in toads exposed to CPF (percentage of inhibition whit a 55.86 %) respect to BChE activity of the control group ($p<0.01$) (Fig. 1).

The CbE activity (mean±SEM) in the control group was 167.94±9.96 nmol min^{-1} mg^{-1} plasma at 48 h. Only CPF formulation inhibited CbE enzyme activity significantly ($p<0.01$) with respect to control in toads exposed. The percentage of inhibition is 43.11 % (Fig. 2).

3.2 Oxidative Stress

The mean value of GST activity in control toads was 82.21±4.52 nmol min^{-1} mg^{-1} plasma at 48 h. The induction of GST enzymatic activity with respect to

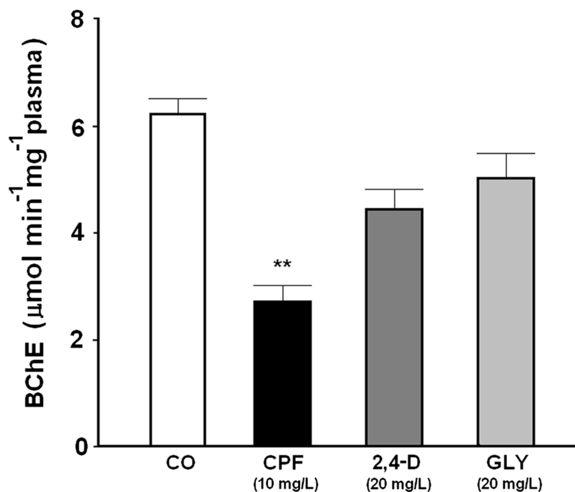

Fig. 1 Effects of commercial pesticide exposure (48 h) on the butyrylcholinesterase (BChE) activity in *R. arenarum* toads. *CO* control, *CPF* chlorpyrifos, *2,4-D* 2,4-dichlorophenoxy acetic acid, and *GLY* glyphosate. Data are expressed as mean±SEM, $N=5$. Significantly different form control (** $p<0.01$ Kruskal–Wallis test followed Dunn's post-test)

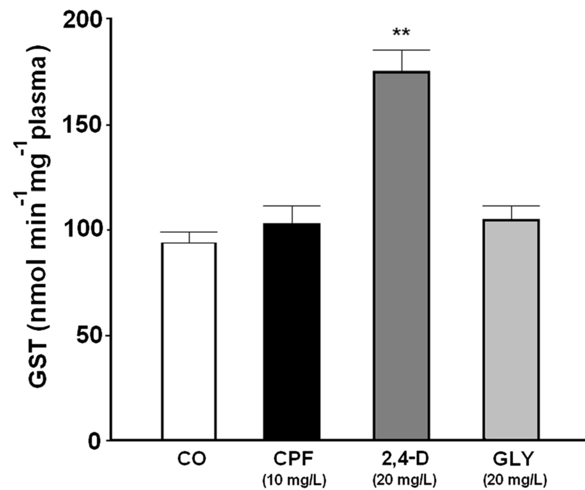

Fig. 3 Effects of commercial pesticide exposure (48 h) on the glutathione *S*-transferase (GST) activity in *R. arenarum* toads. *CO* control, *CPF* chlorpyrifos, *2,4-D* 2,4-dichlorophenoxy acetic acid, and *GLY* glyphosate. Data are expressed as mean±SEM, $N=5$. Significantly different form control (** $p<0.01$ Kruskal–Wallis test followed Dunn's post-test)

the controls was significant ($p<0.01$) for 2,4-D formulations across dermal exposure (Fig. 3).

The TBARS (mean±SEM) in the control group was 26.15±2.29 mmol/mg^{-1} protein at 48 h. TBARS levels were significantly increased compared with control groups in CPF exposure ($p<0.05$) (Fig. 4).

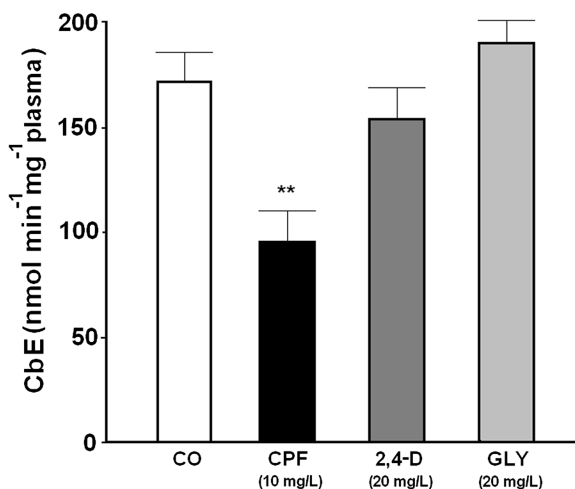

Fig. 2 Effects of commercial pesticide exposure (48 h) on the carboxylesterase (CbE) activity in *R. arenarum* toads. *CO* control, *CPF* chlorpyrifos, *2,4-D* 2,4-dichlorophenoxy acetic acid, and *GLY* glyphosate. Data are expressed as mean±SEM, $N=5$. Significantly different form control (** $p<0.01$ Kruskal–Wallis test followed Dunn's post-test)

3.3 DNA Damage

No differences were found in ACA (ID and FPG sites) between toads exposed to the different pesticides and the control group ($p>0.05$), although results demonstrated a significantly higher DNA damage in Endo III sites in CPF and 2,4-D exposed ($p<0.01$ by Kruskal–Wallis followed by Dunn's tests) (Fig. 5).

3.4 Blood Stress Index

Mean H/L ratio in control groups was 0.34±0.09. Toads exposed at 2,4-D and GLY formulations decreased the H/L ratio at 48 h ($p<0.05$) (Fig. 6).

4 Discussion

Amphibians may be particularly susceptible to anthropogenic chemicals for a multiplicity of reasons. Fundamentally, their complex life cycles (aquatic and terrestrial life stages) expose them to potential chemical contamination in both habitats. Certainly, offset evaporative skin losses, terrestrial anurans absorb water, mainly through hyper-vascularized skin in the ventral pelvic region, explain the vulnerability to pesticides uptakes from contaminated sediments, water, and soil (Sparling et al. 2001). Indeed, anurans moving across agricultural

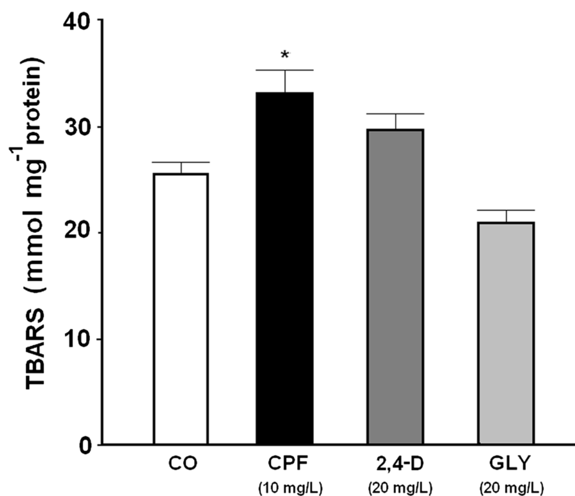

Fig. 4 Effects of commercial pesticide exposure (48 h) on the thiobarbituric acid reactive substance (TBARS) activity in *R. arenarum* toads. *CO* control, *CPF* chlorpyrifos, *2,4-D* 2,4-dichlorophenoxy acetic acid, and *GLY* glyphosate. Data are expressed as mean±SEM, $N=5$. Significantly different form control (* $p<0.05$ Kruskal–Wallis test followed Dunn's post-test)

fields may be at risk of pesticide skin and aerial exposure (Brühl et al. 2011). Besides, agricultural chemicals present on the vegetation or in soils can leach or diffuse by precipitation into a potential aquatic breeding system and subsequently affect amphibians (Storrs Méndez et al. 2009).

The use of some biomarkers is clearly needed in the evaluation of pesticide assessments. Esterase inhibition is the classical approach to monitoring environments probably polluted by OP pesticides (Robles-Mendoza et al. 2011). In our study, BChE and CbE activities were significantly inhibited (nearest of 50 %) in the plasma of *R. arenarum* toads after 48 h of dermal exposure to CPF. In addition, previous experiments showed similar results in B-esterases in another native species adults toads (*Rhinella schneideri,* Attademo et al. 2007), fish (*Synbranchus marmoratus*; Junges et al. 2010), and lizards (*Gallotia galloti*, Sanchez et al. 1997) exposed to OP. BChE and CbEs are important in reducing OP toxicity (Wheelock et al. 2004; Laguerre et al. 2009) and these isozymes may contribute to pesticide tolerance due to their capability to bind to OPs. In the *R. arenarum* individuals used in this study, BChE and CbEs may be decreasing the effective concentration of the pesticide (Wheelock et al. 2008) before they reach the blood–brain barrier in order to protect AChE from inhibition (Walker 1998).

After a 48-h exposure, the activity of the antioxidant enzyme (GST) showed an increase in toads exposed to all pesticides in relation to the control group; however, this effect was only significant for toads exposed to 2,4-D. Oxidative stress may occur if the equilibrium between oxidants and antioxidants is interrupted either by the reduction of antioxidant defenses or by the excessive increase of reactive oxygen species (ROS) (Valavanidis et al. 2006). Certainly, the toxic action of 2,4-D has been evolving from decades and now it is considered that 2,4-D also induces free radical reactions that lead to numerous unbeneficial changes in tissues. The increases of free radical levels can cause DNA damage and thus cell death (in apoptotic process) (Bukowska 2006). Increased activity of GST can reveal disorders that could be indicative of redox alterations

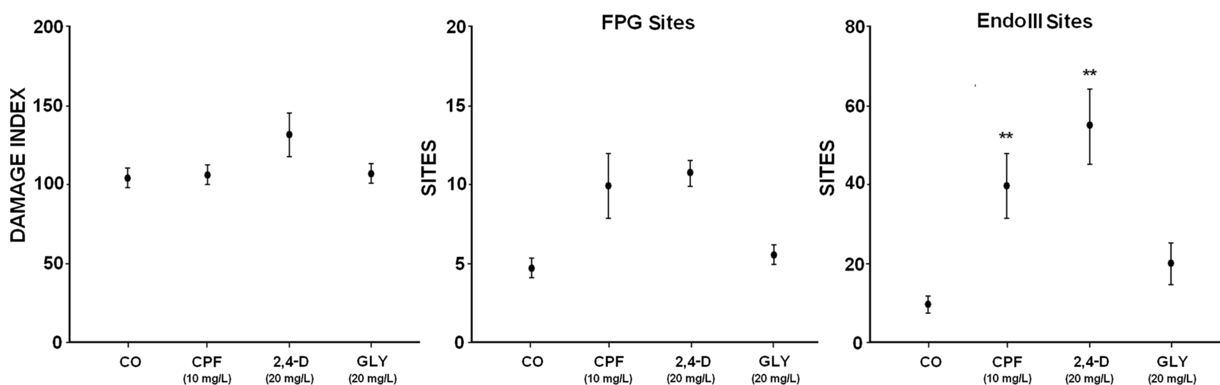

Fig. 5 Effects of commercial pesticide exposure (48 h) on the DNA damage quantified by comet assays in *R. arenarum* toads. The damage index (DI) and levels of formamidepyrimidine-DNA-glycosilase (FPG) and endonuclease III (Endo III) sites were calculated. *CO* control, *CPF* chlorpyrifos, *2,4-D* 2,4-dichlorophenoxy acetic acid, and *GLY* glyphosate. Data are expressed as mean±SEM, $N=5$. Significantly different form control (** $p<0.01$ Kruskal–Wallis test followed Dunn's post-test)

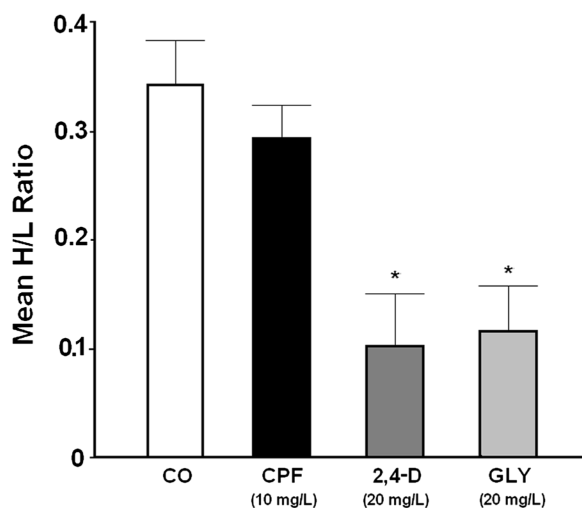

Fig. 6 Effects of commercial pesticide exposure (48 h) on mean heterophil/lymphocyte (H/L) ratio in peripheral blood from *R. arenarum* toads. *CO* control, *CPF* chlorpyrifos, *2,4-D* 2,4-dichlorophenoxy acetic acid, and *GLY* glyphosate. Data are expressed as mean±SEM, $N=5$. Significantly different form control (* $p<0.05$ Kruskal–Wallis test followed Dunn's post-test)

related to a possible oxidative stress situation (Oruç et al. 2004) as demonstrated in fish (Oruç and Uner 2002). In addition, the increased TBARS levels found in toad exposed to CPF may have resulted from an increase of free radicals as a consequence of stress condition. These findings are consistent with results of several other investigations in human cell lines where the administration of CPF caused oxidative damage and it was evidenced by an increase in TBARS (e.g., Qiao et al. 2005; Gultekin et al. 2006). Augmented levels of oxidative stress would be expected because of the increased intake and utilization of oxygen. Indeed, the relation between the increase in oxidative stress of wildlife after pesticide sprayed by concentration of TBARS in plasma and red blood cells is demonstrated for reptiles by Poletta et al. (2012).

In this study, the genotoxicity of three chemicals, CPF, 2,4-D, and GLY, was tested individually in dermal sub-lethal exposure. Following exposure to DNA, glycosylases (FPG and Endo III) were used to convert oxidized purines and pyrimidines, respectively, into DNA single-strand breaks. Indeed, the insecticide CPF and the herbicide 2,4-D demonstrated a significantly higher DNA damage (incubated with Endo III prior to analysis) in specimens treated, but no differences in FPG sites were observed. Muller et al. (2014) described a similar result in mice exposed to CPF. According to Collins et al. (1996) the use of these two enzymes has a substance effect on the measurement of ID multiplying the rate of damage observed four times. It has to be pointed out that malondialdehyde, a naturally occurring product of lipid peroxidation, is genotoxic and is capable of inducing DNA damage (Marnett 1999). Induction of oxidative DNA damage in erythrocytes of toad in CPF exposure, as observed in our study, further complements the enhanced lipid peroxidation, but it did not show a significant enhancement in 2,4-D. This could be due to some compensatory consequences, as GST increase. In contrast, notwithstanding GLY presents a substantial genotoxic risk, it did not cause a statistically significant increase in the DNA damage. In fact, either dermal penetration studies with Roundup® showed very low absorption (Williams et al. 2000), or our exposure was not enough in time to prove this effect. However, Roundup Ultra-Max® contains ethoxylate adjuvants responsible for many observed adverse effects in the exposed toads (e.g., cell toxicity) (Mesnage et al. 2013).

On the other hand, H/L ratios provide one measure of immune function (Norris and Evans 2000). Furthermore, granulocytic leukocytes of the amphibians are relatively poorly studied compared to those of other vertebrates (Shutler and Marcogliese 2011). However, we found significant relations between leukocytes and the herbicide exposure (2,4-D and GLY), where the H/L proportions were lower than control may reflect a general stress response (Davis et al. 2008). The two herbicides affected hematological and immunological parameters in aquatic vertebrates exposed to sub-lethal concentrations of both herbicides (e.g., Safahieh et al. 2012). Severe stress may decrease heterophils (heteropenia) and increase lymphocytes in the periphery (lymphocytosis), resulting in a low H/L ratio (Müller et al. 2011). Certainly, Attademo et al. (2011) found in the frog *Leptodactylus chaquensis* a differential leukocyte count and H/L ratio in two agroecosystem fields in contrast with a pristine forest.

Furthermore, in the risk evaluation of a pesticide, the Log K_{OW} is an important parameter when predicting the uptake across the dermis for amphibians (Quaranta et al. 2009). In this context, chlorpyrifos exceeds the Stockholm Convention criteria for bioaccumulation with most reported

values of log K_{OW} meeting or exceeding 5.0 (Gebremariam et al. 2012). CPF undergoes long-range derive and has been measured in the Arctic, in ice, snow, fog, air, seawater, lake sediment, fish and vegetation. Accordingly, the determination of the Bioconcentration Factor (BCF) values is needed to provide other lines of body-burden toad by CPF. In contrast, Log K_{OW} of the two herbicides studied has low mobility and only a slight tendency to leach in soil, but they are highly hydrophilic. Thus, taking into account the dermal characteristic of amphibians, pure herbicides and surfactants also represent risks for dermal absorption, as demonstrated by Willens et al. (2006). For example, inert ingredients and solvents can also alter the dermal absorption of herbicides, with effects being dependent on solvent specificity and concentration (Baynes and Riviere 1998).

5 Conclusions

In general, exposure and toxicity studies for adult amphibians are scarce, and the reported data indicate the need for further research, especially in light of the global amphibian decline and pesticide bioaccumulation. We studied a “realistic scenario” exposition with commercial formulations of three common pesticides and direct dermal toads exposure. Effects were not restricted to a specific class of pesticides and seem to be influenced not only by the active ingredient but also the formulation adjuvants. A decreasing ecotoxicity sequence in terms of dermal uptake at 48 h is the following CPF>2,4-D>GLY, producing neurotoxicity, oxidative stress, DNA damage, and immunological suppress in adults of the common toad *R. arenarum*. In fact, the blood parameters selected are good biomarkers to characterize the risk exposures of native amphibians exposed to OP insecticides and herbicides.

Acknowledgments This study was supported in part by the National Council for Scientific and Technical Research (CONICET), National Agency for Promotion of Science and Technology (ANPCyT), and the Course of Action for Research and Science Promotion (CAI + D-UNL). Authors are thankful to Dr. Andrew Collins (University of Oslo, Oslo, Norway) for providing the enzyme FPG and ENDO III. We also thank the anonymous reviewer for many valuable comments and suggestions.

Compliance with Ethical Standards Animals used in this research have been treated according to ASIH and SSAR (2001) criteria and with approval from the Animal Ethics Committee of the Faculty of Biochemistry and Biological Sciences. http://www.fbcb.unl.edu.ar/pages/investigacion/comite-de-etica.php

Conflict of Interest The authors declare that they have no competing interests.

References

Ali, D., Nagpure, N. S., Kumar, S., Kumar, R., Kushwaha, B., & Lakra, W. S. (2009). Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Food and Chemical Toxicology, 47*(3), 650–656. doi:10.1016/j.fct.2008.12.021.

ASIH, HL, & SSAR (2001). Guidelines for use of live amphibians and reptiles in field research. http://www.utexas.edu/depts/asih/herpcoll.htlm. Accessed 13 June 2014.

Attademo, A. M., Cabagna-Zenklusen, M., Lajmanovich, R. C., Peltzer, P. M., Junges, C. M., & Bassó, A. (2011). B-esterase activities and blood cell morphology in the frog *Leptodactylus chaquensis* (Amphibia: Leptodactylidae) on rice agroecosystems from Santa Fe Province (Argentina). *Ecotoxicology, 20*, 274–282. doi:10.1007/s10646-010-0579-8.

Attademo, A. M., Peltzer, P. M., & Lajmanovich, R. C. (2005). Amphibians occurring in soybean and implications for biological control in Argentina. *Agriculture, Ecosystems and Environment*, *106*(1), 389–394. doi:10.1016/j.agee.2004.08.012 DOI:10.1016/j.agee.2004.08.012#doilink

Attademo, A. M., Peltzer, P. M., Lajmanovich, R. C., Cabagna, M., & Fiorenza, G. (2007). Plasma B-esterases and glutathione S-transferase activities in the toad *Chaunus schneideri* (Amphibia, Anura) inhabiting rice agroecosystems of Argentina. *Ecotoxicology, 16*, 533–539. doi:10.1007/s10646-007-0154-0.

Ayres, M., Jr., Ayres, D., & Santos, A. (2008). *BioEstat*. Versão5.0. Belém, Pará, Brazil: Sociedade Civil Mamirauá, MCT-CNPq.

Baynes, R. E., & Riviere, J. E. (1998). Influence of inert ingredients in pesticide formulations on dermal absorption of carbaryl. *American Journal of Veterinary Research, 59*(2), 168–175.

Benamú, M. A., Schneider, M. I., & Sánchez, N. E. (2010). Effects of the herbicide glyphosate on biological attributes of *Alpaida veniliae* (Araneae, Araneidae), in laboratory. *Chemosphere, 78*(7), 871–876. doi:10.1016/j.chemosphere.2009.11.027.

Braz-Mota, S., Sadauskas-Henrique, H., Duarte, R. M., Val, A. L., & Almeida-Val, V. M. (2015). Roundup® exposure promotes gills and liver impairments, DNA damage and inhibition of brain cholinergic activity in the Amazon teleost fish *Colossoma macropomum. Chemosphere, 135*, 53–60. doi:10.1016/j. chemosphere .2015.03.042.

Brühl, C. A., Pieper, S., & Weber, B. (2011). Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environmental Toxicology and Chemistry, 30*(11), 2465–2472. doi:10.1002/etc.650.

Brühl, C. A., Schmidt, T., Pieper, S., & Alscher, A. (2013). Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Scientific Reports, 3*, 1135. doi:10.1038/srep01135.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. In S. Fleischer & L. Packer (Eds.), *Methods in Enzymology (Vol. 52, pp. 302–310)*. New York: Academic Press.

Bukowska, B. (2006). Toxicity of 2,4-dichlorophenoxyacetic acid—molecular mechanisms. *Polish Journal of Environmental Studies, 15*(3), 365–374.

Bunyan, P. J., Jennings, D. M., & Taylor, A. (1968). Organophosphorus poisoning. Properties of avian esterases. *Journal of Agricultural and Food Chemistry, 16*(2), 326–331. doi:10.1021/jf60156a028.

Busk, M., Jensen, F. B., & Wang, T. (2000). Effects of feeding on metabolism, gas transport, and acid–base balance in the bullfrog *Rana catesbeiana. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 278*(1), 85–95.

Cabagna, M. C., Lajmanovich, R. C., Stringhini, G. A., Sanchez-Hernandez, J. C., & Peltzer, P. M. (2005). Hematological parameters of health status in common toad *Bufo arenarum* in agroecosystems of Santa Fe Province, Argentina. *Applied Herpetology, 2*(4), 373–380. doi:10.1163/157075405774483085.

Chaturvedi, M., Sharma, C., & Tiwari, M. (2013). Effects of pesticides on human beings and farm animals: a case study. *Research Journal of Chemical and Environmental Sciences, 1*(3), 14–19.

Collins, A. R., Dusinská, M., Gedik, C. M., & Stětina, R. (1996). Oxidative damage to DNA: do we have a reliable biomarker? *Environmental Health Perspectives, 104*, 465–469. doi:10.1289/ehp.96104s3465.

Collins, A. R., Duthie, S. J., & Dobson, V. L. (1993). Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis, 14*, 1733–1735. doi:10.1093/carcin/14.9.1733.

Dacie, J. V., & Lewis, S. M. (1984). *Practical hematology.* New York: Churchill Livingstone.

Davidson, C., Shaffer, H. B., & Jennings, M. R. (2001). Declines of the California red-legged frog: climate, UV-B, habitat, and pesticides hypotheses. *Ecological Applications, 11*(2), 464–479. doi:10.1890/1051-0761(2001)011[0464:DOTCRL]2.0.CO;2.

Davis, A. K., Maney, D. L., & Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology, 22*(5), 760–772. doi:10.1111/j.1365-2435.2008.01467.x.

de Lapuente, J., Lourenço, J., Mendo, S. A., Borràs, M., Martins, M. G., Costa, P. M., & Pacheco, M. (2015). The Comet Assay and its applications in the field of ecotoxicology: a mature tool that continues to expand its perspectives. *Frontiers in Genetic, 6*, 180. doi:10.3389/fgene.2015.00180.

Dhawan, A., Bajpayee, M., & Parmar, D. (2009). Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biology and Toxicology, 25*, 5–32. doi:10.1007/s10565-008-9072-z.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology, 7*(2), 88–95. doi:10.1016/0006-2952(61)90145-9.

Extension Toxicology Network (EXTOXNET). (1996). *Pesticide Information Profiles (PIP) - 2,4-D.*

Falfushinska, H. I., Romanchuk, L. D., & Stolyar, O. B. (2008). Different responses of biochemical markers in frogs (*Rana ridibunda*) from urban and rural wetlands to the effect of carbamate fungicide. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 148*(3), 223–229. doi:10.1016/j.cbpc.2008.05.018.

Fellers, G. M., McConnell, L. L., Pratt, D., & Datta, S. (2004). Pesticides in mountain yellow-legged frogs (*Rana muscosa*) from the Sierra Nevada Mountains of California, USA. *Environmental Toxicology and Chemistry/SETAC, 23*(9), 2170–2177. doi:10.1897/03-491.

Gabor, C. R., Bosch, J., Fries, J. N., & Davis, D. R. (2013). A non-invasive water-borne hormone assay for amphibians. *Amphibia-Reptilia, 34*(2), 151–162.

Gebremariam, S. Y., Beutel, M. W., Yonge, D. R., Flury, M., & Harsh, J. B. (2012). Adsorption and desorption of chlorpyrifos to soils and sediments. *Reviews of Environmental Contamination and Toxicology, 215*, 123–175. doi:10.1007/978-1-4614-1463-6_3.

Gress, S., Lemoine, S., Séralini, G. E., & Puddu, P. E. (2015). Glyphosate-based herbicides potently affect cardiovascular system in mammals: review of the literature. *Cardiovascular Toxicology, 15*(2), 117–126. doi:10.1007/s12012-014-9282-y.

Gomori, G. (1953). Human esterases. *Journal of Laboratory and Clinical Medicine, 42*, 445–453.

Guilherme, S., Gaivão, I., Santos, M. A., & Pacheco, M. (2012). DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide-elucidation of organ-specificity and the role of oxidative stress. *Mutation Research, 743*(1–2), 1–9. doi:10.1016/j.mrgentox.2011.10.017.

Gultekin, F., Patat, S., Akca, H., Akdogan, M., & Altuntas, I. (2006). Melatonin can suppress the cytotoxic effects of chlorpyrifos on human hepG2 cell lines. *Human & Experimental Toxicology, 25*(2), 47–55. doi:10.1191/0960327106ht584oa.

Guyton, K. Z., Loomis, D., Grosse, Y., El Ghissassi, F., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Mattock, H., & Straif, K. (2015). Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. *The Lancet Oncology, 16*, 490–491. doi:10.1016/S1470-2045(15)70134-8.

Habdous, M., Vincent-Viry, M., Visvikis, S., & Siest, G. (2002). Rapid spectrophotometric method for serum glutathione S-transferases activity. *Clinica Chimica Acta, 326*(1–2), 131–142. doi:10.1016/S0009-8981(02)00329-7.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry, 249*(22), 7130–7139.

Hayes, W. J., & Laws, E. R. (1991*).* Handbook of pesticide toxicology: classes of pesticides. New York: Academic Press Inc.

Hook, S. E., Gallagher, E. P., & Batley, G. E. (2014). The role of biomarkers in the assessment of aquatic ecosystem health.

Integrated Environmental Assessment and Management, 10(3), 327–341. doi:10.1002/ieam.1530.

IUCN: The International Union for Conservation of Nature and Natural Resources. An analysis of amphibians on the 2010, IUCN Red List, 2010. www.iucnredlist.org/amphibians (Accessed 22 July 2014).

Ismail, M., Khan, Q. M., Ali, R., Ali, T., & Mobeen, A. (2014). Evaluation of the genotoxicity of chlorpyrifos in common indus valley toad, *Bufo stomaticus* using alkaline single-cell gel electrophoresis (comet) assay. *Agricultural Sciences*, *5*, 376–382. doi:10.4236/as.2014.54039 DOI:10.4236/as.2014.54039#_blank

James, C. (2008). ISAAA Briefs brief 39 Global status of commercialized biotech/GM crops: 2008. Ithaca: ISAAA.

Jergentz, S., Mugni, H., Bonetto, C., Schulz, R., & Mugnu, H. (2005). Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. *Chemosphere, 61*, 817–826. doi:10.1016/j.chemosphere.2005.04.036.

Junges, C. M., Lajmanovich, R. C., Peltzer, P. M., Attademo, A. M., & Bassó, A. (2010). Predator–prey interactions between *Synbranchus marmoratus* (Teleostei: Synbranchidae) and *Hypsiboas pulchellus* tadpoles (Amphibia: Hylidae): Importance of lateral line in nocturnal predation and effects of fenitrothion exposure. *Chemosphere, 81*(10), 1233–1238. doi:10.1016/j.chemosphere.2010.09.035.

Laguerre, C., Sánchez-Hernández, J. C., Kohler, H. R., Triebskorn, R., Capowiez, Y., Rault, M., & Mazzia, C. (2009). B-type esterases in the snail *Xeropicta derbentina*: an enzymological analysis to evaluate their use as biomarkers of pesticide exposure. *Environmental Pollution, 157*, 199–207. doi:10.1016/j.envpol.2008.07.003.

Lajmanovich, R. C., Junges, C. M., Attademo, A. M., Peltzer, P. M., Cabagna-Zenklusen, M. C., & Basso, A. (2013). Individual and mixture toxicity of commercial formulations containing glyphosate, metsulfuron-methyl, bispyribac-sodium, and picloram on *Rhinella arenarum* tadpoles. *Water, Air, & Soil Pollution, 224*, 1404. doi:10.1007/s11270-012-1404-1.

Lajmanovich, R. C., Sánchez-Hernández, J. C., Peltzer, P. M., Attademo, A. M., Fiorenza, G. S., Cabagna-Zenklusen, M. C., & Bassó, A. (2008). Levels of plasma B-esterases and glutathione-S-transferase activities in three South American toad species. *Toxicological & Environmental Chemistry, 90*(6), 1145–1161. doi:10.1080/02772240801923107.

Li, D., Huang, Q., Lu, M., Zhang, L., Yang, Z., Zong, M., & Tao, L. (2015). The organophosphate insecticide chlorpyrifos confers its genotoxic effects by inducing DNA damage and cell apoptosis. *Chemosphere, 135*, 387–393. doi:10.1016/j.chemosphere.2015.05.024.

Loewy, R. M., Monza, L. B., Kirs, V. E., & Savini, M. C. (2011). Pesticide distribution in an agricultural environment in Argentina. *Journal of Environmental Science and Health. Part. B, 46*(8), 662–670. doi:10.1080/03601234.2012.592051.

Marnett, L. J. (1999). Lipid peroxidation-DNA damage by malondialdehyde. *Mutation Research, 424*, 83–95. doi:10.1016/S0027-5107(99)00010-X.

Merini, L. J., Cuadrado, V., & Giulietti, A. M. (2008). Spiking solvent, humidity and their impact on 2,4-D and 2,4-DCP extractability from high humic matter content soils. *Chemosphere, 71*(11), 2168–2172. doi:10.1016/j.chemosphere.2007.12.025.

Mesnage, R., Bernay, B., & Séralini, G. E. (2013). Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. *Toxicology, 313*, 122–128. doi:10.1016/j.tox.2012.09.006.

Müller, C., Jenni-Eiermann, S., & Jenni, L. (2011). Heterophils/Lymphocytes-ratio and circulating corticosterone do not indicate the same stress imposed on Eurasian kestrel nestlings. *Functional Ecology, 25*(3), 566–576. doi:10.1111/j.1365-2435.2010.01816.x.

Muller, M., Hess, L., Tardivo, A., Lajmanovich, R., Attademo, A., Poletta, G., Simoniello, M., Yodice, A., Lavarello, S., Chialvo, D., & Scremin, O. (2014). Neurologic dysfunction and genotoxicity induced by low levels of chlorpyrifos. *Neurotoxicology, 45*, 22–30. doi:10.1016/j.neuro.2014.08.01.

Norris, K., & Evans, M. R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology, 11*(1), 19–26. doi:10.1093/beheco/11.1.19.

Oruç, E. O., & Uner, N. (2002). Marker enzyme assessment in the liver of *Cyprinus carpio* (L.) exposed to 2,4-D and azinphosmethyl. *Journal of Biochemical and Molecular Toxicology, 16*(4), 182–188. doi:10.1002/jbt.10040.

Oruç, E. O., Sevgiler, Y., & Uner, N. (2004). Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 137*(1), 43–51. doi:10.1016/j.cca.2003.11.006.

Peltzer, P. M., Lajmanovich, R. C., Attademo, A. M., & Beltzer, A. H. (2006). Diversity of anurans across agricultural ponds in Argentina. *Biodiversity and Conservation, 15*, 3499–3513. doi:10.1007/s10531-004-2940-9.

Poletta, G. L., Larriera, A., Kleinsorge, E., & Mudry, M. D. (2008). *Caiman latirostris* (broad-snouted caiman) as a sentinel organism for genotoxic monitoring: basal values determination of micronucleus and comet assay. *Mutation Research, 650*, 202–209. doi:10.1016/j.mrgentox.2007.12.001.

Poletta, G. L., Larriera, A., Kleinsorge, E., & Mudry, M. D. (2009). Genotoxicity of the herbicide formulation Roundup®(glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test. *Mutation Research, 672*, 95–102. doi:10.1016/j.mrgentox.2008.10.007.

Poletta, G., Simoniello, M. F., Porcel de Peralta, M., Kleinsorge, E., Siroski, P., & Mudry, M. (2012). Evaluation of pesticide-induced DNA damage and oxidative stress on human and wildlife populations in Santa Fe province (Argentina). In A. J. A. Gomez & E. M. L. de Ortega (Eds.), *Pesticides: Characteristics, Uses and Health Implications* (pp. 1–23). Nova Science Publishers.

Qiao, D., Seidler, F. J., & Slotkin, T. A. (2005). Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos. *Toxicology and Applied Pharmacology, 206*, 7–26. doi:10.1016/j.taap.2004.11.003.

Quaranta, A., Bellantuono, V., Cassano, G., & Lippe, C. (2009). Why amphibians are more sensitive than mammals to xenobiotics. *PLoS ONE, 4*(11), e7699.

Robles-Mendoza, C., Zúñiga-Lagunes, S. R., Ponce de León-Hill, C. A., Hernández-Soto, J., & Vanegas-Pérez, C. (2011).

Esterases activity in the axolotl *Ambystoma mexicanum* exposed to chlorpyrifos and its implication to motor activity. *Aquatic Toxicology, 105*(3–4), 728–734. doi:10.1371/journal.pone.0007699.

Ryan, T., Scott, C., & Douthitt, B. (2006). Sub-lethal effects of 2,4-D exposure on golf course amphibians. *USGA Turfgrass and Environmental Research Online, 5*(16), 1–14.

Safahieh, I., Jaddi, Y., Yavari, V., & Reza Saligheh, Z. (2012). Sub-lethal effects of herbicide Paraquat on hematological parameters of benny fish *Mesopotamichthys sharpeyi*—international proceedings of conference full-text database. In *Proceedings of 2012 2nd International Conference on Biotechnology and Environment Management* (pp. 141–145). Phuket, Thailand: IACSIT Press, Singapore.

Sanchez, J. C., Fossi, M. C., & Focardi, S. (1997). Serum B esterases as an nondestructive biomarker for monitoring the exposure of reptiles to organophosphorus insecticides. *Ecotoxicology and Environmental Safety, 38*, 45–52. doi:10.1006/eesa.1997.1560.

Sanchez-Hernandez, J. C., & Moreno-Sanchez, B. M. (2002). Lizard cholinesterases as biomarkers of pesticide exposure: enzymological characterization. *Environmental Toxicology and Chemistry / SETAC, 21*(11), 2319–2325.

Sherman, J. D. (1996). Chlorpyrifos (Dursban)-associated birth defects: report of four cases. *Archives of Environmental Health, 51*(1), 5–8. doi:10.1002/etc.5620211109.

Shutler, D., & Marcogliese, D. J. (2011). Leukocyte profiles of northern leopard frogs, *Lithobates pipiens*, exposed to pesticides and hematozoa in agricultural wetlands. *Copeia, 2011*(2), 301–307. doi:10.1643/CP-10-065.

Simoniello, M. F., Kleinsorge, E. C., Scagnetti, J. A., Mastandrea, C., Grigolato, R. A., Paonessa, A. M., & Carballo, M. A. (2010). Biomarkers of cellular reaction to pesticide exposure in a rural population. *Biomarkers, 15*, 52–60. doi:10.3109/13547500903276378.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*, 184–191. doi:10.1016/0014-4827(88)90265-0.

Sparling, D., Fellers, G., & McConnell, L. (2001). Pesticides and amphibian population declines in California, USA. *Environmental Toxicology and Chemistry, 20*, 1591–1595. doi:10.1002/etc.5620200725.

Sparling, D. W., Linder, G., Bishop, C. A., & Krest, S. (2010). Ecotoxicology of amphibians and reptiles, (Sparling, D. W., Linder, G., Bishop, C. A. & Krest, S. Eds.) (Second Edi.). CRC Press.

Storrs Méndez, S. I., Tillitt, D. E., Rittenhouse, T. A. G., & Semlitsch, R. D. (2009). Behavioral response and kinetics of terrestrial atrazine exposure in American toads (*Bufo americanus*). *Archives of Environmental Contamination and Toxicology, 57*(3), 590–597. doi:10.1007/s00244-009-9292-0.

Sun, F., & Chen, H. S. (2008). Monitoring of pesticide chlorpyrifos residue in farmed fish: investigation of possible sources. *Chemosphere, 71*(10), 1866–1869. doi:10.1016/j.chemosphere.2008.01.034.

Tyler, M. J. (1999). Frogs and toads as experimental animals. *Australian and New Zealand Council for the Care of Animals in Research and Teaching Ltd (ANZCCART) News, 12*, 1–4.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety, 64*(2), 178–189. doi:10.1016/j.ecoenv.2005.03.013.

Van Meter, R. J., Glinski, D. A., Hong, T., Cyterski, M., Henderson, W. M., & Purucker, S. T. (2014). Estimating terrestrial amphibian pesticide body burden through dermal exposure. *Environmental Pollution, 193*, 262–268. doi:10.1016/j.envpol.2014.07.003.

Vernadakis, A., & Rutledge, C. O. (1973). Effects of ether and pentobarbital anaesthesia on the activities of brain acetylcholinesterase and butyrylcholinesterase in young adult rats. *Journal of Neurochemistry, 20*(5), 1503–1504.

Wagner, N., Reichenbecher, W., Teichmann, H., Tappeser, B., & Lötters, S. (2013). Questions concerning the potential impact of glyphosate based herbicides on amphibians. *Environmental Toxicology and Chemistry, 32*(8), 1688–1700. doi:10.1002/etc.2268.

Walker, C. H. (1998). The use of biomarkers to measure the interactive effects of chemicals. *Ecotoxicology and Environmental Safety, 40*, 65–70. doi:10.1006/eesa.1998.1643.

Wheelock, C. E., Miller, J. L., Miller, M. G., Shan, G., Gee, S. J., & Hammock, B. D. (2004). Development of toxicity identification evaluation (TIE) procedures for pyrethroid detection using esterase activity. *Environmental Toxicology and Chemistry, 11*, 2699–2708. doi:10.1897/03-544.

Wheelock, C. E., Phillips, B. M., Anderson, B. S., Miller, J. L., Miller, M. J., & Hammock, B. D. (2008). Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). *Reviews of Environmental Contamination and Toxicology, 195*, 117–178. doi:10.1007/978-0-387-77030-7_5.

Williams, G. M., Kroes, R., & Munro, I. C. (2000). Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology, 31*(2), 117–165. doi:10.1006/rtph.1999.1371.

Willens, S., Stoskopf, M. K., Baynes, R. E., Lewbart, G. A., Taylor, S. K., & Kennedy-Stoskopf, S. (2006). Percutaneous malathion absorption in the harvested perfused anuran pelvic limb. *Environmental Toxicology and Pharmacology, 22*(3), 263–267. doi:10.1016/j.etap.2006.04.009.

Yin, X., Zhu, G., Li, X. B., & Liu, S. (2009). Genotoxicity evaluation of chlorpyrifos to amphibian Chinese toad (Amphibian: Anura) by Comet assay and Micronucleus test. *Mutation Research, 680*(1–2), 2–6. doi:10.1016/j.mrgentox.2009.05.018.