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Comercial insecticide fipronil alters antioxidant enzymes response and accelerates the metamorphosis in *Physalaemus nattereri* (Anura: Leiuperidae) tadpoles

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ABSTRACT

Fipronil is an insecticide widely used in sugarcane crops in several areas of the world. In tropical areas, the application of this and other pesticides is intensified during the rainy season due the soil moisture, which coincides with the period of reproduction for many amphibian species. Since this pesticide can be easily incorporated into ephemeral ponds by leaching, it is possible that many neotropical amphibians are being affected by exposure to fipronil, specially during the initial stages of life when they are exclusively aquatics. This study aimed to evaluate the effects of the commercial formulation of insecticide fipronil (Regent®800WG) on antioxidant enzymes and development stages of tadpoles of Physalaemus nattereri at nominal concentrations of 0.5 and 1.5 mg/L. Activity of antioxidant enzymes catalase (CAT), glutathione-S-transferase (GST) and glucose-6-phosphate dehydrogenase (G_6PDH) was reduced in animals exposed to two tested concentrations of fipronil (0.5 mg/L and 1.5 mg/L), with similar response after two and seven days of exposure. Glutathione reductase (GR) activity was not altered after treatments. After seven days of exposure, an evident acceleration on stages of development was observed in animals exposed to the highest concentration of fipronil. This study conclude that the commercial formulation of fipronil (Regent®800WG) impair the antioxidant system of P. nattereri and accelerates the metamorphosis of these tadpoles, which could result in more vulnerable adults, promoting adverse consequences at population level.

Keywords: *amphibians, antioxidant enzymes, stage of development, toxicity, pesticides.*

INTRODUCTION

Pesticides are among the most harmful substances released into the natural systems due to the high chemical stability, resistance to metabolism and rapid absorption [1]. In most seasonal tropical areas of the world, intensive agricultural activity and the large use of pesticides coincide with the rainy season [2]. During this period, the use of insecticides is also intensified because the favorable climatic conditions to insects reproduction, causing an increase in insect populations [3] including herbivores of crops [4,5]. Some studies have shown that the increase in rainfall intensity may results in a much bigger increase of surface runoff [6]. Leaching is also increased, because its absorption is intensified in the moist soil. Thus, through the direct release or by leaching during the rainy season, pesticides reach natural systems affecting non-target organisms, endangering especially the aquatic organisms [7,8]. Most neotropical anuran species occur and reproduce during the rainy season [9,10], and it is important to consider that most species have the first stage of life completely aquatic [9,11]. Regent®800WG is a commercial formulation of an insecticide that contains fipronil as its active ingredient (80%), and is one of the most used insecticides in sugarcane farming in Brazil [12], an agricultural activity with increasing demand due to the increased interest in renewable fuels. Fipronil (5-amino-1-[2, 6-dichloro-4(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile) acts in the insect central nervous system binding to insect gamma-aminobutyric acid (GABA) chloride channels and blocking the action of GABA, the normal passage of chloride ions and the transmission of normal neural impulse [12-15]. Fipronil has played an essential role in sugarcane pest control because of its high effectiveness against

insects that are resistant to other insecticides such as the pyrethroids, organophosphates, and carbamates [16]. In southeastern Brazil, São Paulo state contributes to approximately 20% of pesticide consumption in the country and 60% of sugarcane production. The northwestern area concentrates the main sugarcane farming activity of the São Paulo State. In this area 36 species of anurans have been recorded [10]. Considering the accelerated expansion of sugarcane cultivation in this region, there are strong reasons indicating that many of these species may be threatened by exposure to this insecticide.

Although several studies have pointed that pesticides can lead to many deleterious effects on aquatic organisms and the evidences that most of them are reaching amphibians' environment, few studies have been conducted to determine the potential negative effects of fipronil on tadpoles. Especially during the larval stage, amphibians have been shown to be more sensitive to contamination by pesticides than adults, since they are strictly aquatic and have higher skin permeability [17]. Some adverse effects of fipronil have been reported to other non-target organisms, such as the copepod *Amphiascus tenuiremis* which had their fertility, reproduction, and development delayed by fipronil at concentrations of 0.22 µg/L [18]. For amphibians, recent evidences also showed that the active ingredient fipronil can be responsible for alterations in antioxidant enzymes of *Scinax fuscovarius* tadpoles [2] increasing the susceptibility of the animals to oxidative stress that could lead to increases in the levels of oxidative damage cellular components [19,20]. Actually, it has been shown that numerous pesticides can impair the antioxidant defense system in tadpoles [2,21]. However, the sensitivity of species to these chemicals may be species-specific. Thus, the aim this study was to evaluate the effects of a formulation of the insecticide fipronil (Regent®800WG) on antioxidant enzymes and stages of development of *Physalaemus nattereri* tadpoles. This species occurs in open areas in central and southeastern Brazil, eastern Paraguay and Bolivia [22] and the major threat to this species is the spread of intensive agriculture. It is considered a generalist/wide-ranging species [10,22]. Generalist species have important ecological roles and, because their wide spatial distribution, their potential to influence the environmental conditions experienced by other species is great [23]. So, the effects of environmental contaminants in generalist species have to be carefully considered. We hypothesize that fipronil formulation may alter the antioxidant enzyme activity and also can change the normal development rates of tadpoles.

MATERIALS AND METHODS

Test Organisms

Tadpoles of *Physalaemus natterer* were collected in surrounding area of Macaubal, São Paulo State, Brazil in temporary ponds away from agriculture areas. After collected they were brought to the laboratory, where they were classified according the development stages described by Gosner [24]. Tadpoles in development stage were selected, when the legs are not present but only the limb buds are developed [24], since this is a period of intensive development. After identification of the development stages, the animals were acclimated and then exposed to the contaminant.

Tadpoles maintenance

Tadpoles were kept in 500 L indoor stock-tanks for acclimation before the beginning of the experiment. The tanks had dechlorinated water, temperature between 27°C and 28°C and were maintained under 12:12 h (light:dark) regimes. Commercial powdered food containing algal-based food (Sera Micron, Germany) was provided twice a day to satiation. External biological filters and constant aeration removed detritus and tadpole's feces, ensuring the water quality. Tanks were siphoned once a week to remove remaining leftovers.

Experimental design

The effect of commercial formulation of fipronil (Regent 800WG®) was tested at two concentrations: 0.5 mg/L and 1.5 mg/L. Concentrations selected in this study were based on previous studies that found about 0.2 mg/kg of soil for fipronil close to agricultural areas in the region of São José do Rio Preto, Brazil [25]. So we decided to test higher concentrations estimating a higher amount of fipronil very close to the crops, and during application periods, to simulate a worst scenario for the tadpoles. For this, tadpoles were weighed, measured (0.23g ± 0.04g and 2.84cm ± 0.11cm) and subjected to three treatments. Furthermore, the animals were subject to different exposure times (2 days and 7 days), based in previous studies evaluating effects of oxidative stress in tadpoles [2]. For this study, three replicates containing five tadpoles (n=15) each were used by each treatment. The animals were fed every two days with 0.5 g of food.

After the exposure period (2 days and 7 days) the animals were killed by lethal dose of benzocaine and the biometry was performed. This study was conducted in agreement with the precepts of National Council for the Control of

Animal Experimentation (CONCEA) and was approved by the Committee for Ethics on Using Animal (CEUA), UNESP, and São José do Rio Preto, SP, Brazil.

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich at the highest purity except the pesticide Regent 800WG®, which was obtained from in a local commerce supplier.

Sample Preparation and analysis

For the enzymatic measurement, tadpoles were entirely and individually homogeneized in a ratio of (1:4, w/v) in Tris buffer 20 mM (pH 7.4), sucrose 0.5 mM, KCl 0.15 mM and 1 mM protease inhibitor (PMSF). The samples were then centrifuged at 10000 g for 30 min at 4ºC. To obtain the cytosolic fraction, the supernatant portions were collected and re-centrifuged at 50 000 g for one additional hour at 4ºC. The supernatant obtained after this second centrifugation was used for the analysis of CAT, GST, G6PDH and GR assays.

The analysis of CAT was performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes. The activities of GR, GST and G6PDH were performed on a Victor TM X3 microplate reader (Perkin Elmer®). CAT activity was measured using the method described by Beutler, which monitors the rate of decomposition of hydrogen peroxide by the enzyme at 240 nm for 1 min. Specific activity was expressed as U/mg of protein-1 using a molar extinction coefficient of 0.071 mM^{-1} cm^{-1}. The assays were performed using Tris-HCl buffer (1 M, pH 8.0) with 5 mM EDTA and 10 mM H_2O_2 as the substrate.

GST activity was measured using the method described by Keen [26] which monitors the formation of the conjugate of 1-chloride-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) catalyzed by GST in the sample for 1 min at 340 nm. Specific activity was expressed as U/mg of protein-1 using a molar extinction coefficient of 9.6 mM/cm. The final volume of the assay was 110 µl, which contained a potassium phosphate buffer (0.2 M, pH 6.5), 1 mM CDNB (dissolved in 1.0 mL of absolute ethanol), 1 mM GSH and the sample.

GR activity was measured based on the Carlberg and Mannervik methodology [27], in which the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) is monitored for 2 min at 340 nm in the presence of the substrate glutathione disulfide (GSSG). The methodology consists of the reduction of GSSG to GSH by GR through the oxidation of NADPH. The final volume of the assay was 100 µL, which contained a potassium phosphate buffer (0.1 M, pH 7.5) with 2 mM GSSG (dissolved in buffer), 0.1 mM NADPH (dissolved in $NaHCO_3$ 0.1% (v/v)), 0.15 mM GSH and the sample. Specific activity was expressed as U/mg of $protein^{-1}$ using a molar extinction coefficient of 6.22 mM/cm.

The enzymatic assay of G_6PDH (EC 1.1.1.49) was based on the Glock and McLean methodology [28], which measures the formation of NADPH at 340 nm for 1 min. The assay consists of the reduction of $NADP^+$ to NADPH by the G6PDH using glucose-6-phosphate (G6P) as the substrate. The assay had a final volume of 205 µL, which contained Tris-HCl buffer (0.1 M, pH 7.4), $MgCl_2$, $NADP^+$, G6P and the sample. The reference blank did not contain G_6P. Specific activity was expressed as mU/mg of protein-1 using a molar extinction coefficient of 6.22 mM/cm. The quantification of proteins was performed using the Bradford assay [29] with Coomassie Brilliant Blue G-250 in an acidic solution. The absorbance values were determined at 595 nm, and the results were compared to the analytical curve prepared with bovine serum albumin (BSA) as the standard.

Statistical analysis

Data normality was evaluated by Shapiro Wilk's W and homocedasticity by Cochran's test. To assess whether there was a difference between the stages of development in animals in different treatments, the G test was performed with Yates' correction [30]. Biochemical parameters were compared using one-way ANOVA followed by Fisher's LSD. $P<0.05$ was established to infer statistical significance.

RESULTS

Development stage

After 2 days of exposure, tadpoles were classified at three different stages according to Gosner classification [24] (Gs30, Gs31, Gs32; Figure 1A). No differences were observed on stages of development after 2 days of exposure. However, there was acceleration on stages of development in tadpoles exposed to both concentrations of Regent®800WG ($P<0.0001$). After 7 days of exposure, tadpoles were classified at five different stages (Gs36, Gs37, Gs38, Gs39 and

Gs40; Figure 1B). We observed that the control group presented tadpoles distributed at younger stages of development, with 13% at Gs36, 27% at Gs37, 53% at Gs38 and 7% at Gs39. No animals reached stage Gs40 in the control group. For both concentrations, no tadpoles were classified at Gs36 and only 7% of tadpoles were in Gs37. After exposure to 0.5 mg/L of fipronil, 73% of the tadpoles reached stage Gs39 and 13% the stage Gs40. The treatment with higher concentration (1.5 mg/L) showed that 73% of the tadpoles reached stage Gs40.

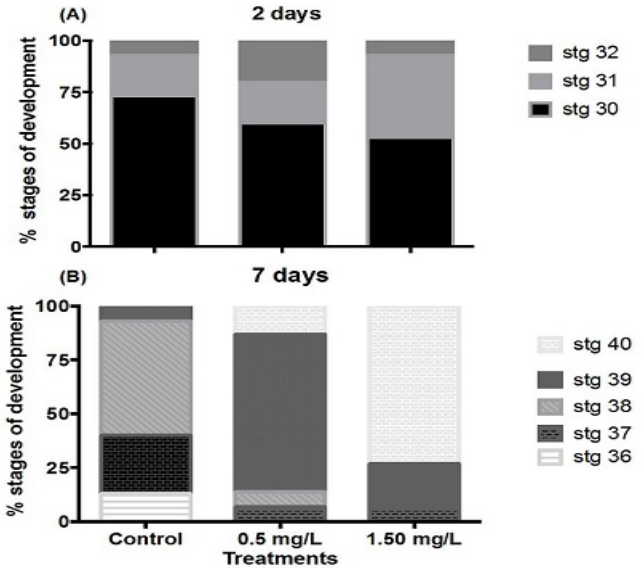

Figure 1: (A) Development stage of tadpoles of *Physalaemus nattereri* subjected to different concentrations of fipronil after 2 days of exposure and (B) after 7 days of exposure. Bars represent the development stage according to Gosner (1960).

Antioxidant enzymes

There was a decrease of CAT activity in tadpoles after 2 days of exposure in both treatments compared to the control group (P=0.003; Figure 2A). In addition, 7 days of exposure decreased CAT activity in the treatment with 0.5 mg/L compared to the control and the treatment with 1.5 mg/L (P=0.004; Figure 3A). A decrease on G_6PDH activity was also observed for both concentrations after 2 days (P=0.031; Figure 2B) and 7 days of exposure (P<221 0.003; Figure 3B). GST activity was decreased in tadpoles after 2 days of exposure in the treatment with 1.5 mg/L compared to the control and concentration 0.5 mg/L (P<0.0001; 223 Figure 2C). In addition, after 7 days of exposure, there was a decrease on GST activity at both concentrations compared to the control treatment (P<0.0009; Figure 3C). No differences were observed in enzyme activity of GR between treatments in both exposure periods (2 days: P=226 0.236; Figure 2D; 7 days: P=0.539; Figure 3D).

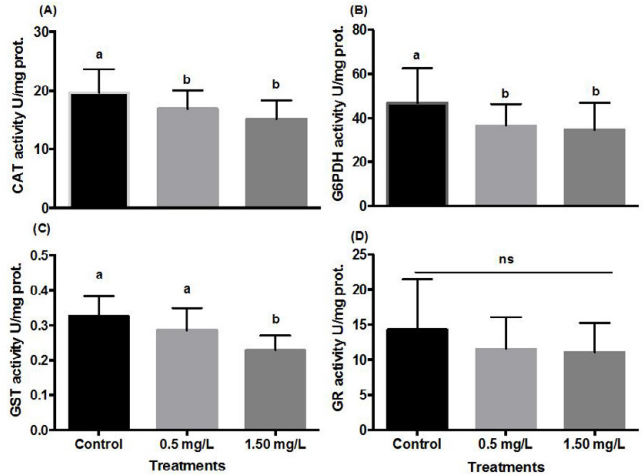

Figure 2: Data expressed as mean ± standard deviation. (A) Catalase (CAT), Glutathione *S*transferase, Glucose-6-phosphate-dehydrogenase (G6PDH) and Glutathione reductase (GR) activity in *Physalaemus nattereri* exposed to Control (without contaminant); 0.5 mg/L and 1.5 mg/L of Regent ® 800 WG after 2 days. Different letters indicate presence of statistical significance.

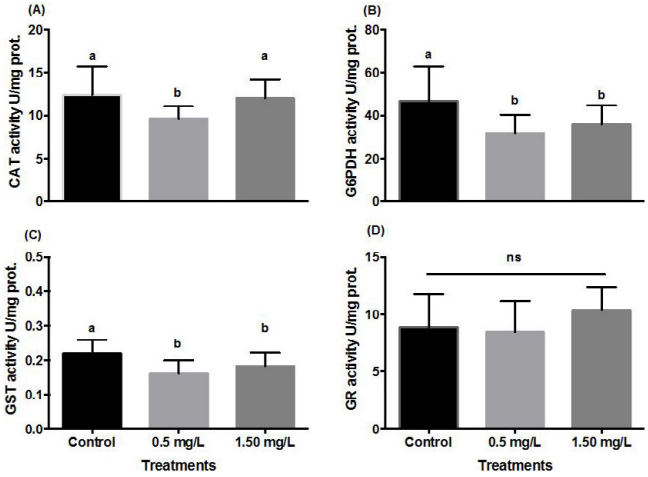

Figure 3: Data expressed as mean ± standard deviation. (A) Catalase (CAT), Glutathione *S*transferase, Glucose-6-phosphate-dehydrogenase (G6PDH) and Glutathione reductase (GR) activity in *Physalaemus nattereri* exposed to Control (without contaminant); 0.5 mg/L and 1.5 mg/L of Regent ® 800 WG after 7 days. Different letters indicate presence of statistical significance.

DISCUSSION

While much has been published about the ecotoxicological impacts caused by fipronil in fishes [31] little attention has been given the effects of fipronil in tadpoles, that often live in temporary ponds near sugarcane crops, culture in which fipronil is widely used. In this study we observed that antioxidant enzyme activity in tadpoles of P. nattereri was impaired by exposure to fipronil and acceleration in developmental stages of the tadpoles was observed after exposure to this insecticide.

Development of tadpoles was accelerated in both concentrations tested after 7 days exposed to fipronil. According to Hartman [32], the increased metamorphosis rate for tadpoles exposed to commercial pesticide Pyraclostrobin Headline® may be considered an indication of stress. Also, some pesticides have been known to promote a delay on metamorphosis in amphibians larvae, such as tadpoles of Rana temporaria exposed to the fungicide prochloraz and Ambystoma tigrinum exposed to herbicide atrazine [33,34]. Metamorphosis in amphibians can be mediated by different hormones, such as corticotrophic and thyroid hormones [35], and an environmental stress can induce higher levels of these hormones and start early development in anurans [36]. The time needed to complete the metamorphosis process has important and essential effects on physiological performance on adult amphibians. Acceleration on this process may result in smaller and more fragile adults, which are more vulnerable to predators and have the fecundity rates reduced [37,38]. Thus, environmental stressors such as pesticide exposure including the fipronil can cause disturbances in the developmental 11 rates of *Physalaemus nattereri*, which could indicate an endocrine deregulatory action of fipronil to tadpoles.

Activities of the enzymes CAT, GST and G6PDH were decreased after treatments with fipronil, showing that the antioxidant system and biotransformation reactions were also perturbed. The activity of antioxidant enzymes may be elevated or inhibited depending upon the type and concentration of the stressor [39]. We registered a decrease of CAT activity in animals exposed to the both concentrations of fipronil in both exposure periods (2 and 7 days). CAT is as heme-containing enzyme that facilitates the removal of H_2O_2 by metabolizing it into H_2O and O_2 [40]. This indicates that this enzyme could be affected in tadpoles at higher concentrations of fipronil, a fact that can result in a reduction of the capacity to scavenge H_2O_2. Several studies have demonstrated that CAT activity can be inhibited or have its activity decreased in different species of aquatic animals exposed to numerous pesticides [2,41,42]. These effects could impair the ability of these organisms to handle against reactive oxygen species generated as a consequence of exposure to these environmental contaminants. Clasen [43] showed that fipronil (Standak® – BASF) promoted inhibition of CAT in the fish C. carpio exposed to 0.65 mg. L−1, after 7, 30 and 90 days of exposure. Ballesteros [44] also observed a decrease in CAT activity in the fish Jenynsia multidentata exposed to endosulfan. The inhibition observed in this enzyme was also directly linked to the development of oxidative stress in common carp tissues after prolonged exposure to fipronil [43].

The enzyme Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP + oxidoreductase) catalyzes the first and rate-limiting step of the pentose-phosphate pathway and is an important celular source of NADPH, an

important co-factor for cytochrome P450 mediated biotransformation reactions, for antioxidant enzymes such as glutathione peroxidase, and for numerous other biosynthetic reactions [44]. Thus, the decrease in G6PDH activity of tadpoles from fipronil-exposed treatments compared to the control group can also contribute for a higher susceptibility to oxidative stress in tadpoles and an impairment of cellular metabolism. Nevertheless, the decrease in G6PDH activity did not affected the activity of GR, since GR was not affected by fipronil exposure in tadpoles. The enzyme GR is responsible for maintaining levels of GSH by reducing GSSG using electrons from NADPH [45]. Despite organophosphorus and carbamate pesticides have been related to decreases in GR activity in *Rana arenarum* tadpoles [46-48], fipronil has shown to present a puzzling effect in *S. fuscovarius* tadpoles [2] causing both increases, decreases or no alterations depending on the period of exposure and the fipronil concentration. This lack of changes in GR activity may be due to the short exposure time. Moreover, it could be suggested that even being not altered by fipronil GR activity could be limited by the lack of NADPH due to decreased G6PDH activity.

The enzyme GST is involved in the detoxification process and contributes to phase II of biotransformation, in which exogenous compounds are conjugated with endogenous macromolecules such as glucose, sulfate, and, in the case of GST, the tripeptide glutathione [39]. Our results showed that tadpoles of P. nattereri exposed to fipronil had a decrease on GST activity for both period of exposure, which agrees with previous studies on S. fuscovarius tadpoles exposed to the same commercial formulation of fipronil [2] and studies on fish (*J. multidentata*) exposed to endosulfan [43]. The inhibition of this enzyme should decrease the ability of tadpoles to metabolize environmental xenobiotics, including fipronil, turning the animals more vulnerable for intoxication.

The present study documented considerable impairment of antioxidants enzyme of *P.nattereri* tadpoles exposed to a commercial formulation of fipronil (Regent 800WG®), in different concentrations after short periods of exposure. There was a decrease in the activity of enzymes CAT, GST and G6PDH at both concentrations of fipronil tested. In addition, we also showed that fipronil accelerate metamorphosis process in tadpoles after 7 days of exposure, despite the mechanisms involved in this process remains to be further investigated. Despite the survival of tadpoles were not affected in the tested exposure periods, the increase in developmental rates could seriously impact anuran populations, i.e. by producing smaller adults. The size of the organisms is directly related to their survival, because affect their ability to escape from predators, and to get food [49]. Also, small females have low fertility [50]. Thus, there is a large ecological importance of these results for amphibian's conservation in current years, since many species are sharing areas of agriculture crops during the early stages of development, where the use of pesticides such as fipronil is constant and intensive.

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REFERENCES

[1]El-Amrani S; Pena-Abaurrea M; Sanz-Landaluze J; Ramos L; Guinea J; et al. *Sci Total Environ,* **2012**, 425, 184-190.

[2] Stefani Margarido TC; Felício AA; de Cerqueira Rossa-Feres D; Alves de Almeida E. *Mar Environ Res,* **2013**, 91, 61-67.

[3] Vasconcellos A, Andreazze R; Almeida AM; Araujo HFP; Oliveira ES; et al. *Ver Bras Entomol*, **2010**, 54, 471-476.

[4] Voraphab I, Hanboonsong Y; Kobori Y; Ikeda H; Osawa T; et al. *Ecol Res,* **2015,** 30, 415.

[5] Rosado JF, Picanço MC; Sarmento RA; Da Silva RS; Pedro-Neto M; et al. *Exp Appl Acarol,* **2015**, 66, 415-426.

[6] Schulz R. *J Environ Qual*, **2004**, 33, 419.

[7] Nebeker AV; Schuytema GS. *Arch Environ Contam Toxicol*, **1998**, 35, 441-446.

[8] Gooddy DC; Chilton J; Harrison I. *Sci Total Environ,* **2002**, 297, 67-83.

[9] Duellman WE; Trueb L. *The John Hopkins University Press*, **1994**, 670.

[10] Provete DB, Garey MV; Silva FR Da; Rossa-Feres DDC. *Biota Neotrop*, **2011**, 11, 377-391.

[11] Mcdiarmid, RW; Altig R. *McDiarmid Altig*, **1999.**

[12] Stark JD; Vargas RI. *Ecotoxicol Environ Saf Stehr*, **2005**, e62, 11-16.
[13] Stehr CM; Linbo TL; Incardona JP; Scholz NL. *Toxicol Sci,* **2006**, 92, 270-278.
[14] Bencic DC; Villeneuve DL; Biales AD; Blake L; Durhan EJ; et al. *Environ Toxicol Chem*, **2013**, 32, 1828-1834.
[15] Gosner K. *Herpetologica*, **1960,** 16, 183-190.
[16] Gunasekara AS; Troung T. *fipronilrev*, **2007,** 393, 1-27.
[17] Yan D; Jiang X; Xu S; Wang L; Bian Y; et al. *Chemosphere*, **2008,** 71, 1809-1815.
[18] Chandler TG; Cary TL; Volz DC; Walse SS; Ferry JL; et al. *Environ Toxicol Chem*, **2008**, 23, 117.
[19] Toyokuni S. *Pathol Int*, **1999**, 49, 91-102.
[20] Halliwell B. *Plant Physiol*, **2006**, 141, 312-22.
[21] Jones L; Gossett D; Banks S; McCallum M. *J Herpetol*, **2010**, 44, 222-228.
[22] Frost D. *American Museum of Natural History*, **2011**
[23] Gaston KJ. *Science,* **2010**, 327, 154-155.
[24] de Toffoli AL; da Mata K; Bisinoti MC; Moreira AB. *J Environ Sci Health B*, **2015**, 50, 753-759.
[25] Keen JH; Habig WH; Jakoby WB. *J Biol Chem*, **1976**, 251, 6183-6188.
[26] Carlberg I; Mannervik B. *Methods Enzymol*, **1985**, 113, 484-490.
[27] Glock GE; McLean P. *Biochem J*, 1953, 55, 400-4088.
[28] Bradford MM. *Anal Biochem*, **1976**, 72, 248-254.
[29] Sokal RR; Rohlf FJ. *Third Edition WH Freeman and Co*, **1995.**
[30] Wu H; Gao C; Guo Y; Zhang Y; Zhang J; et al. *Pestic Biochem Physiol*, **2014**, 115, 9-14.
[31] Hartman EAH; Belden JB; Smith LM; McMurry ST. *Ecotoxicology*, **2014**, 23, 396-403.
[32] D L Larson; S McDonald; J Fivizzani WE; Newton SJH. *Physiol Zool*, **2014**, 71, 671-679.
[33] Brande-lavridsen N; Christensen-dalsgaard J; Korsgaard B. *Open Zool*, **2010**, 3, 7-16.
[34] Denver RJ. *Netherlands J Zool*, **1995**, 45, 195-200.
[35] Denver RJ, *Horm Behav*, **1997**, 31, 169-179.
[36] Galloy V; Denoël M. *Acta Oecol*, **2010**, 36, 179-183.
[37] Katzenberger M; Tejedo M; Duarte H; Marangoni F; Beltrán JF; et al. *Ver da Biol*, **2012**, 8, 25-32.
[38] Fatima M; Usmani N; Firdaus F; Zafeer MF; Ahmad S; et al. *Comp Biochem Physiol Part C Toxicol Pharmacol*, **2015**, 176-177, 17-30.
[39] Van der Oost R; Beyer J; Vermeulen NP. *Environ Toxicol Pharmacol*, **2003**, 13, 57-149.
[40] Peltzer PM; Lajmanovich RC; Attademo AM; Junges CM; Cabagna-Zenklusen MC; et al. *Ecotoxicol Environ Saf*, **2013**, 98, 142-151.
[41] Sun L; Jin R; Peng Z; Zhou Q; Qian H; et al. *Ecotoxicology*, **2014,** 23, 1044-1054.
[42] Clasen B; Loro VL; Cattaneo R; Moraes B; Lópes T; et al. *Ecotoxicol Environ Saf*, **2012**, 77, 45-51.
[43] Ballesteros ML; Wunderlin DA; Bistoni MA. *Ecotoxicol Environ Saf*, **2009**, 72, 199-205.
[44] Kletzien RF, Harris PK, Foellmi LA. *FASEB J*, 1994,8, 174-181.
[45] Eriyamremu GE; Osagie VE; Omoregie SE; Omofoma CO. *Ecotoxicol Environ Saf*, **2008**, 71, 284-364.
[46] Ferrari A; Anguiano L; Lascano C; Sotomayor V; Rosenbaum E; et al. *J Biochem Mol Toxicol*, **2008**, 22, 259-267.
[47] Ferrari A; Lascano CI; Anguiano OL; D'Angelo AM; Venturino A; et al. *Aquat Toxicol*, **2009,** 93, 37-44.
[48] Ferrari A; Lascano C; Pechen de D'Angelo AM; Venturino A. *Comp Biochem Physiol C Toxicol Pharmacol*, **2011**, 153, 34-39.
[49] Cabrera-Guzmán E; Crossland MR; Brown GP; Shine R. *PLoS One*, **2013**, 8, e70121.
[50] Orton F; Baynes A; Clare F; Duffus ALJ; Larroze S; et al. *Ecotoxicology*, **2014**, 23, 1359-1365.