



Genotoxic and melanic alterations in *Lithobates catesbeianus* (anura) tadpoles exposed to fipronil insecticide

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Abstract

The aim of this study was to evaluate the genotoxic and morphological systemic effects of both an acute and a chronic exposure of bullfrog tadpoles to fipronil. *Lithobates catesbeianus* tadpoles had morphological biomarkers (skin, liver, and blood) analyzed at Gosner stages 36–38, when exposed to four different concentrations of Regent® 800 WG (80% fipronil): 0.00 (control), 0.04, 0.08, 0.4 mg/L, and four experimental times: 4, 8, 12, and 16 days. Body darkness responded directly to the treatment and exposure time. There was a treatment-dependent decrease in darkness of heads and tails. In relation to the biometric analysis, fipronil induced a decrease in the individual weight and liver mass at the end of the experiments, whereas the hepatosomatic index did not vary according to the treatment. For the exposed animals and for the control group, the area of hepatic melanin increased as exposure time increased. Fipronil has genotoxic effects on *L. catesbeianus* tadpoles even after short exposure times (e.g., 4 and 8 days), and the main nuclear abnormality is in the anucleate cells. A relevant correlation was observed between genotoxic biomarkers and cutaneous and internal melanin. The frequency of nuclear abnormalities is inversely correlated both with the hepatic melanin area and with the cutaneous melanin of animals. Fipronil has distinct systemic effects on tadpoles based on its concentration, as well as on its exposure time. Such alterations (pigmentation level and rate of erythrocyte abnormality) result in morphological and physiological effects, which may compromise the behavior and survival of the anurans.

Keywords Biomarkers · Body darkness · Genotoxicity · Melanin · Melanomacrophages · Nuclear abnormalities

Introduction

In the last 10 years, Brazil has become the biggest toxic agrochemical market in the world, corresponding to a 190% increase in the national market (Lopes and Albuquerque 2018; Anvisa 2019; MMA 2018). The increase in the use of such chemical substances results in water resource contamination

(Thompson et al. 2007; Ribeiro et al. 2013) and may affect natural ecosystems (Konstantinou et al. 2006; Hildenbrandt et al. 2008; Foit et al. 2010) along with the non-target species which live in these habitats (Mann et al. 2009).

Fipronil (C₁₂H₄Cl₂F₆N₄OS) is a new-generation insecticide known worldwide (Roberts and Reigart 2013); besides being used in agriculture, it is a veterinary medication for the control of pests, parasites, and larvae (Zhao and Salgado 2010; Amaral 2012). Fipronil is responsible for causing endocrine disruption (Silva et al. 2011); thus, it is classified as carcinogenic for humans (Fent 2014). It also causes genotoxicity (Tomiazzi et al. 2018), hepatotoxicity (Ferreira et al. 2012), reproductive toxicity (Josende et al. 2015), behavioral, immunological, and mortality alterations (Pochini and Hoverman 2017). Among the products in which Fipronil is the active ingredient, one of which, Regent 800 WG, is classed as a highly toxic—red label (Anvisa 2019). It acts by means of contact or ingestion and is delivered by land or air (Agrofit 2020). Thus, it may be transported and found in aquatic environments where it causes great damage to the local biota (Albuquerque et al. 2016; Bernabò et al. 2016). Simon-

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Delso et al. (2015) summarized some reasons for the success of systemic insecticides (fipronil and neonicotinoids) based on the similarities of their properties, e.g., toxicity and physico-chemical profiles, and also reviewed their trends, uses, mode of action, and their metabolites, in addition to documenting their share of the global market in the last 25 years. In amphibians, fipronil may spread once or twice faster than in mammals (Wagner et al. 2016) and its effects in anurans are related to enzymatic alterations (Reynaud et al. 2012; Gripp et al. 2017) and metamorphosis time (Boscolo et al. 2017).

Experiments regarding the concentrations of fipronil in aquatic environments are lacking, mainly in the ponds that serve as a transient environment for amphibians that live close to the cultivation areas, especially during application periods. These concentrations in a realistic condition may be higher than those that have been described for freshwater rivers. However, according to de Toffoli et al. (2015), in the soil close to agricultural areas of Northwest Brazil, environmentally relevant concentrations of fipronil, fipronil sulfone, and fipronil sulfide are present, ranging from 35 to 180 µg/kg, which is very close to the concentration in the water used in our experimental protocols.

The acute toxicity for pesticides on aquatic organisms can be measured by establishing the LC50 or the concentration in water (e.g., mg/L) which is toxic to the test organisms. According to a review of the effects of fipronil on vertebrate wildlife (Gibbons et al. 2015), there is a marked variation among vertebrate's taxa and different systemic insecticides in acute toxicity. In that study, neonicotinoid and fipronil insecticides could exert their impact on vertebrates either directly, through their overt toxicity, or indirectly, for example by reducing their food supply. For amphibians, no fipronil toxicity experiments have been reported; however, for some fish, the toxicity of fipronil varies according to species, with lethal concentrations (LC50) ranging from 0.042 mg/L in *Oreochromis niloticus* (African tilapia) to 0.43 mg/L in *Cyprinus carpio* (European carp; Qureshi et al. 2016). The LC50 in *Rhamdia quelen* (silver catfish) is 0.81 mg/L at 48 h of study (Fredianelli et al. 2019).

Anurans are biomarkers of the environment quality, since they have a quick response to environmental stress; therefore, they are vulnerable to terrestrial and aquatic contamination (Brodeur and Candioti 2017). Their organs and internal membranes contain melanin (Franco-Belussi et al. 2016)—an endogenously extracted polymer (Césarini 1996) which absorbs and neutralizes free radicals and other potentially toxic agents derived from the cellular catabolism (Zuasti et al. 1989; Agius and Roberts 2003). In the skin, melanin is the first barrier against UV radiation (Montagna and Carlisle 1991) and also enables the adaptive interaction of animals and the environment (e.g., camouflage, cryptic coloration) (Aspengren et al. 2009; Sköld et al. 2016). Researchers have found evidence of internal melanin alterations and skin coloration due to

environmental contaminants (Franco-Belussi et al. 2018; Pérez-Iglesias et al. 2019; Scaia et al. 2019); these are correlated to genotoxic effects (Franco-Belussi et al. 2018), which show that aquatic contaminants may cause damage to the adaptive capacity of animals to their environment.

Melanomacrophages (MMs) are cells which are present in the hematopoietic organs of fish and anurans. Such cells, with a phagocytic activity similar to that of macrophages (Agius 1980), contain melanin and substances of the cellular catabolism, such as hemosiderin and lipofuscin (Agius and Roberts 2003). MMs are morphological biomarkers of effects and are used as tools to evaluate the effects of contaminants, e.g., glyphosate (Pérez-Iglesias et al. 2016; Bach et al. 2018; Pérez-Iglesias et al. 2019), benzo[α]pyrene (Fanali et al. 2017, 2018), flutamide (Gregorio et al. 2016), pyrethroid (Oliveira et al. 2016), carbaryl (Çakici 2015), and sexual steroid hormones (Zieri et al. 2015). Hence, these cells are important in order to detect tissue alterations as a result of possible xenobiotics present in the environment. However, there are few studies that evaluate the role of these cells in tadpoles and there are no reports on the possible effects that fipronil may have on them.

Therefore, the objective of this study was to evaluate the systemic effects of both acute and chronic exposure of bullfrog tadpoles to fipronil. In order to do so, morphological biomarkers such as morphological nuclear alterations in erythrocytes were examined, in addition to histological analyses of the liver and animal coloration. Moreover, a comparative approach was used regarding genotoxic responses, by assaying the melanin present both in the liver and the skin of the animal, in order to test the hypothesis that it plays a role in protecting tissues (De Oliveira et al. 2017).

Materials and methods

Animal sampling and experimental design

In total, 128 *Lithobates catesbeianus* (Shaw 1802) tadpoles were studied, at Gosner stages 36–38; they were obtained from UNESP's Centro de Aquicultura (CAUNESP) in Jaboticabal, São Paulo, Brazil. Animals were kept in aquaria containing 1 L water/animal, with constant aeration, controlled temperature (27.0 ± 1 °C), and natural photoperiod (~12-h light:12-h dark). Animals were fed with a protein meal every day for 7 days before experiments began.

After that acclimatation period, animals were randomly rearranged in 32 aquaria with 4 specimens in each tank; the proposed population density was 1 animal per liter of water. The specimens were exposed to four different concentrations of Regent® 800 WG (80% fipronil): 0.00 (control), 0.04, 0.08, and 0.4 mg/L, and four periods of exposure: 4, 8, 12, and 16 days. These concentrations are in accordance with Toffoli et al.

(2015) agricultural soil of Northwest of Brazil (i.e., 35 to 180 $\mu\text{g}/\text{kg}$). Each group was analyzed in duplicate, with a total of $N = 8/\text{concentration}/\text{time}$. The water was totally renewed every 48 h with the replacement of compound concentrations in the treated groups. These animals were also fed every 48 h.

After the experimental periods, animals were photographed and then euthanized with benzocaine (10 g/L); their blood was sampled through cardiac puncture with a heparinized syringe and needles for the obtention of blood extensions. Subsequently, animals were desiccated and had their livers removed. All manipulation and care of animals followed the NIH Guide for Care and Use of Laboratory Animals; all procedures were previously approved by the Ethical Committee and Animal Experimentation from IBILCE/UNESP (CEUA #199/2018).

Biometric analyses

Following the 7-day acclimatization period, an exploratory analysis of the biomass that composed each replicate was conducted, immediately before the beginning of the experiments. The biomass behavior was evaluated for each subgroup (control and treated), both before and after the experiments, aiming to observe possible sub-lethal alterations of anatomic or developmental character. Animals were weighed on an analytical balance with 0.001-g precision and measured with a pachymeter (0.01 mm); the livers were also weighed. Based on this data, it was possible to calculate the hepatosomatic index ($\text{HSI} = \text{liver weight (g)}/\text{animal weight (g)} \times 100$).

Body darkness analyses

In order to quantify body darkness, we used Photoshop® to standardize the area measured. Landmarks were used to standardize measurements in each animal (Franco-Belussi et al. 2018). All image analyses were performed in accordance with Svensson et al. (2005). The digital images were converted to CIE $L^*a^*b^*$, which consisted of three parameters: L^* (lightness) represents the relative lightness ranging from total black to total white; a^* (redness) represents the balance between red and green; and b^* (yellowness) represents the balance between yellow and blue. Afterwards, the following formula was used: $100 - (100 \times L^*/255)$, considering the relative area of L^* (Svensson et al. 2005; Franco-Belussi et al. 2018).

Hepatic melanin

The liver was fixed in Methacarn (60% methanol, 30% chloroform, and 10% acetic acid) for 3 h, then dehydrated and immersed in historesin (Leica). Cuts of 2 μm were stained in hematoxylin-eosin and photographed in a microscope coupled to the image capturing system (Leica DM4B). The melanin area present in the liver was measured through the ImagePro-plus 6.0 software by using a differential staining technique

(Santos et al. 2014). In order to do so, 25 photomicrographs were used for each animal.

Nuclear abnormalities in erythrocytes

Blood smears were stained with Giemsa 7.5% for 15 min and subsequently washed in running water. Following this, 1000 erythrocytes were analyzed for each animal through a light microscope (Leica DM4000). The nuclear abnormalities were evaluated according to Franco-Belussi et al. (2016).

Statistical analysis

In order to model the ratio of nuclear abnormalities, a generalized linear model (GLM) was built with a binomial distribution and a *log* link function, including treatment and time of exposure; each one containing a categorical predictor with 4 levels, in addition to their interactions. We tested our model assumptions by using diagnostic plots through the R (R Core Team 2017) package *splot* (Lüdtke 2016). Residuals had homogeneity of variance and normal distribution.

In order to model the darkness (response variables), a generalized linear mixed-effects models (GLMM) with beta distribution was fitted, including treatment and time as categorical predictor variables with four levels each, along with their interactions (Franco-Belussi et al. 2018). In order to model the area of hepatic melanin, a GLMM was used, but instead with a Gaussian distribution.

Pearson correlation was used in order to test for a correlation between the ratio of nuclear abnormalities and body darkness. In addition, nuclear abnormalities were tested with hepatic melanin. All analyses were conducted through R v. 3.4.0 (R Core Team 2017).

Results

Biometrics

Table 1 contains the assayed biometric variables. No anatomical alteration was verified qualitatively. The 16-day group had an increase in biomass, compared to their biomass at the beginning of the experiment ($P < 0.001$; Table S1). By assaying the individual's variables, fipronil was observed to cause a decrease in the individual weight in 4-days of 0.04 mg/L ($P = 0.03$; Table S2) and liver mass in 4 days of 0.04-mg/L and 0.08-mg/L treatments ($P < 0.05$; Table S3). Although the treatments decreased liver weight, the hepatosomatic index (HSI) did not vary according to the treatment (Table S4). Similarly, total length did not vary among control and treatment groups (Table S5). All biometric variables result in significant differences in at least one experimental time (Table 1; S1-S5) showing the effects of time of exposure.

Table 1 Biometric variables

Time	Treatment	Biomass initial	Biomass final	Individual weight (g)	Total length (cm)	Weight liver (g)	HSI
4 days	0.00 mg/L	6.65 ± 0.30	6.19 ± 0.40	1.55 ± 0.26	5.74 ± 0.39	0.050 ± 0.01	3.34 ± 0.94
	0.04 mg/L	4.48 ± 0.001	4.21 ± 0.02	1.05 ± 0.21*	5.29 ± 0.60 ^a	0.028 ± 0.006 ^{*,a}	2.68 ± 0.72 ^{a,b}
	0.08 mg/L	5.04 ± 0.37	4.71 ± 0.39	1.18 ± 0.49	5.32 ± 0.94	0.033 ± 0.02*	2.88 ± 0.93
	0.40 mg/L	5.67 ± 0.61	5.17 ± 0.62	1.29 ± 0.23 ^{a,c}	5.56 ± 0.21 ^a	0.050 ± 0.01	3.87 ± 0.91 ^a
8 days	0.00 mg/L	5.56 ± 0.09	5.72 ± 0.11	1.43 ± 0.25	5.70 ± 0.40	0.046 ± 0.01	3.25 ± 0.56
	0.04 mg/L	5.02 ± 0.02	5.23 ± 0.03	1.31 ± 0.27	5.44 ± 0.40 ^a	0.045 ± 0.006 ^b	3.54 ± 0.87 ^a
	0.08 mg/L	5.94 ± 0.42	5.99 ± 0.41	1.50 ± 0.22	5.68 ± 0.35	0.048 ± 0.006	3.25 ± 0.53
	0.40 mg/L	6.51 ± 0.62	6.65 ± 0.34	1.66 ± 0.38 ^a	5.95 ± 0.48 ^a	0.049 ± 0.01	2.95 ± 0.64 ^b
12 days	0.00 mg/L	6.65 ± 0.74	6.71 ± 0.59	1.68 ± 0.35	6.04 ± 0.68	0.052 ± 0.02	3.09 ± 0.75
	0.04 mg/L	6.12 ± 0.77	6.12 ± 0.66	1.50 ± 0.61	5.90 ± 0.93 ^a	0.04 ± 0.02 ^a	2.49 ± 0.56 ^b
	0.08 mg/L	6.08 ± 0.19	6.33 ± 0.21	1.60 ± 0.33	5.88 ± 0.70	0.042 ± 0.01	2.63 ± 0.51
	0.40 mg/L	7.87 ± 0.35	7.53 ± 1.12	2.15 ± 0.22 ^b	6.67 ± 0.46 ^b	0.066 ± 0.007	3.09 ± 0.42 ^a
16 days	0.00 mg/L	6.90 ± 0.20	7.43 ± 0.39 [#]	1.86 ± 0.44	6.41 ± 0.60	0.051 ± 0.02	2.68 ± 0.40
	0.04 mg/L	5.73 ± 0.36	6.87 ± 0.32 [#]	1.72 ± 0.52	6.34 ± 0.79 ^b	0.044 ± 0.01 ^b	2.64 ± 0.53 ^b
	0.08 mg/L	6.12 ± 0.77	6.54 ± 0.88 [#]	1.64 ± 0.27	6.04 ± 0.30	0.044 ± 0.007	2.79 ± 0.68
	0.40 mg/L	5.41 ± 0.72	6.21 ± 0.70 [#]	1.55 ± 0.24 ^c	6.02 ± 0.21 ^a	0.040 ± 0.006	2.62 ± 0.26 ^b

Different letters showing differences between distinct experimental times at the same treatment. Mean ± SE. ($p < 0.05$)

SD, standard deviation; HSI, hepatosomatic index

*Show differences between control and treatment in the same time

Show differences between initial and final biomass in same time and treatment

Body darkness

The color of *L. catesbeianus* tadpoles responds directly to the treatment and exposure time. There was a decrease in the darkness of heads and tails, thus indicating it is both time- and treatment-dependent (Fig. 1).

The head pigmentation at initial times (i.e., 4 and 8 days) decreased at the lowest concentration (0.04 mg/L; Fig. 2; Table S6); such response persisted during the other times. The tail darkness decreased in 8 days, only at the 0.08-mg/L concentration, whereas for 12 and 16 days, there was a decrease in pigmentation at the lowest concentration (0.04 mg/L; Figs. 1 and 2; Table S7).

Hepatic melanin

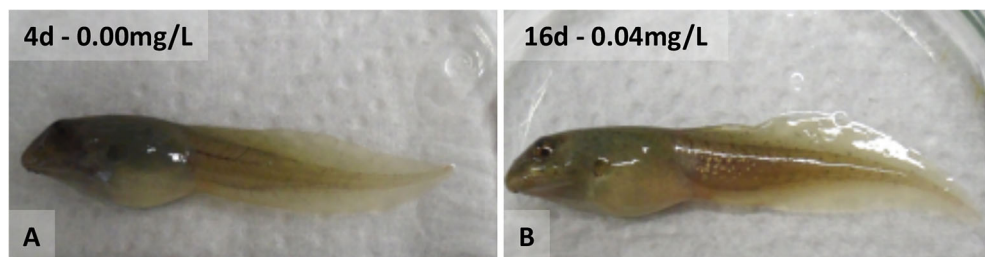
The area of hepatic melanin increased as exposure time increased in all treatments, including in the control group (Figs.

3 and 4; Table S8). For the exposed animals, hepatic melanin decreased at a 0.04-mg/L concentration of fipronil after 4 days of exposure ($P = 0.0001$; Table S8); however, after 8 days at the same concentration, there was a substantial increase ($P = 0.0009$; Table S8), which indicates an acute response to the treatment. For the other exposure times, only 12 days had a treatment effect, i.e., there was an increase in hepatic pigmentation at 0.08-mg/L concentration, thus suggesting a chronic effect of such concentration ($P < 0.0001$; Fig. 4; Table S8). By considering the effects of time and treatment together, it is noteworthy that after 16 days of exposure, all concentrations led to the same response (Fig. 4; Table S8).

Nuclear abnormalities

Fipronil presents genotoxic effects on *L. catesbeianus* tadpoles in short exposure times (e.g., 4 and 8 days) ($P < 0.05$; Figs. 5 and 6; Table S9). That may be due to the

Fig. 1 a *L. catesbeianus* tadpoles exposed to fipronil at a concentration of 0.00 (control group) after 4 days of exposure. b *L. catesbeianus* tadpoles exposed to fipronil at a concentration of 0.04 mg/L after 16 days of exposure



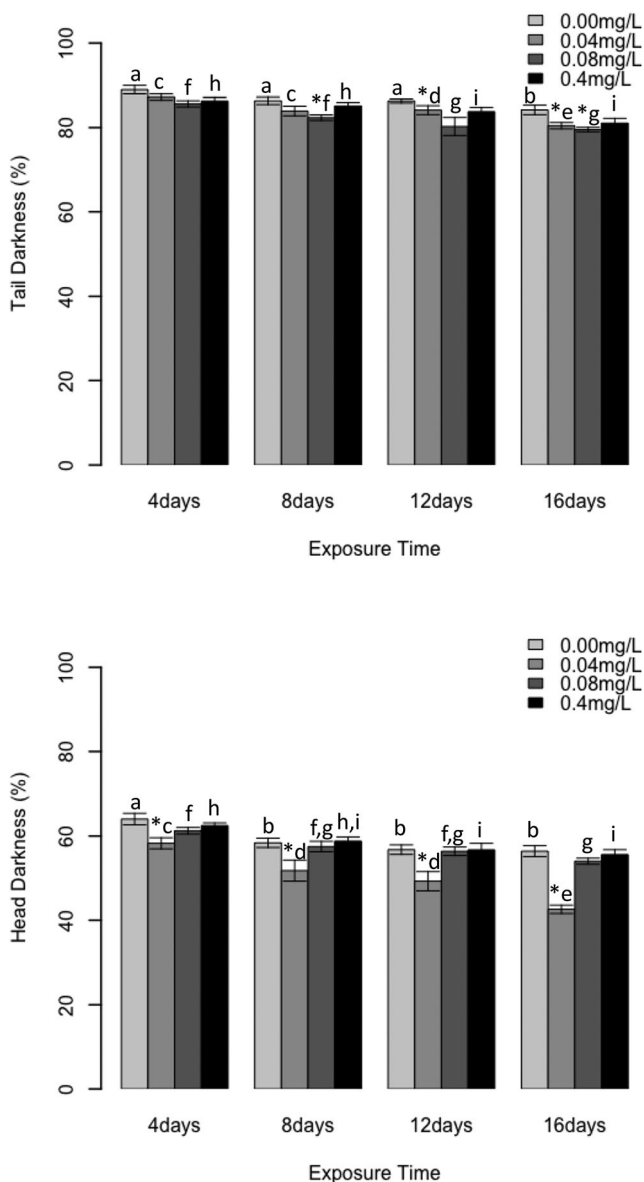


Fig. 2 Head and tail darkening percentage. Different letters indicate differences between the same treatment at different exposure times. Asterisk (*) represents differences between control and treatment at the same exposure time ($P < 0.05$). Data is provided as the mean \pm SE

fact that in tadpoles, the erythrocyte replacement rate tends to be higher at initial developmental stages. The main nuclear abnormality observed was that of anucleate cells (Fig. 5). At the 4-day exposure time, it was possible to observe an increase in the nuclear abnormalities in animals exposed to 0.04 and 0.08 mg/L fipronil ($P < 0.0001$; Table S9); when the time was 8 days, the increase was observed in all the exposed animals ($P < 0.005$; Fig. 6; Table S9). For 16-day exposure, the increase in nuclear abnormality frequency was observed at the concentrations of 0.08 and 0.4 mg/L ($P < 0.01$; Fig. 6; Table S9) showing a dependency of time and treatment.

Compared analysis between nuclear abnormalities and melanin

Although significant, the frequency of nuclear abnormalities is weakly correlated with both hepatic melanin area ($r = -0.23$; $P = 0.03$), and cutaneous melanin ($r = -0.22$; $P = 0.04$).

Discussion

Exposure to the commercial insecticide Regent® 800 WG caused a decrease in the individual's weight (4 days–0.04 mg/L) and liver mass (4 days–0.04, 0.08 mg/L), whereas the total length and hepatosomatic index did not suffer significant effects when comparing control and treatment groups. *Physalaemus nattereri* tadpoles exposed to the same insecticide (0.5–1.5 mg/L) had an accelerated development (Boscolo et al. 2017), which may result in individuals with a lower mass (Cauble and Wagner 2005) and size (Denver and Crespi 2006). Similar results have been observed for the insecticide endosulfan, which caused a weight decrease in *Bufo bufo* (Brunelli et al. 2009). In turn, the insecticides carbaryl, malathion, and permethrin have led to an increase in the mass of *Bufo americanus* and *Rana clamitans*; moreover, there was a developmental delay (Boone 2008), which was also observed for endosulfan (Brunelli et al. 2009).

Even though there were effects regarding the biometric parameters of *L. catesbeianus*, those responses were occasional and nonlinear and they may be related to the intrinsic properties of each animal, which have then culminated in body weight and liver mass reduction. Considering that there was no mortality and that animals after 16 days had biomass gain (Table 1), we assume that a normal development of the tadpoles occurred. However, other effects at morphological and cellular levels were observed in this study.

Fipronil caused the agglomeration of melanin granules, which decreased the skin pigmentation of the head and tail of *L. catesbeianus*. The dispersion of granules, which led to a darkening of the body, was observed for tadpoles exposed to different contaminants, such as insecticides (Pandey and Tomar 1985), herbicides (Pérez-Iglesias et al. 2019), and endocrine disruptors (Scaia et al. 2019). Fish exposed to nitrate/phenol/hexachlorocyclohexane had decreased pigmentation (Daiwile et al. 2015). This difference may be explained due to the mechanism of action of the compounds and the physiological responses of each species. Another determining factor in the response of chromatophores is the exposure time. Fish treated with arsenic suffered an initial reduction in their pigmentation (i.e., 7–60 days of exposure), followed by an increase during longer periods (i.e., 90 days), suggesting a physiological adaptation mechanism of melanophores (Allen et al. 2004). Therefore, pigmentation is an important biomarker and, once conserved within the various animal groups, can provide an intrinsic characteristic

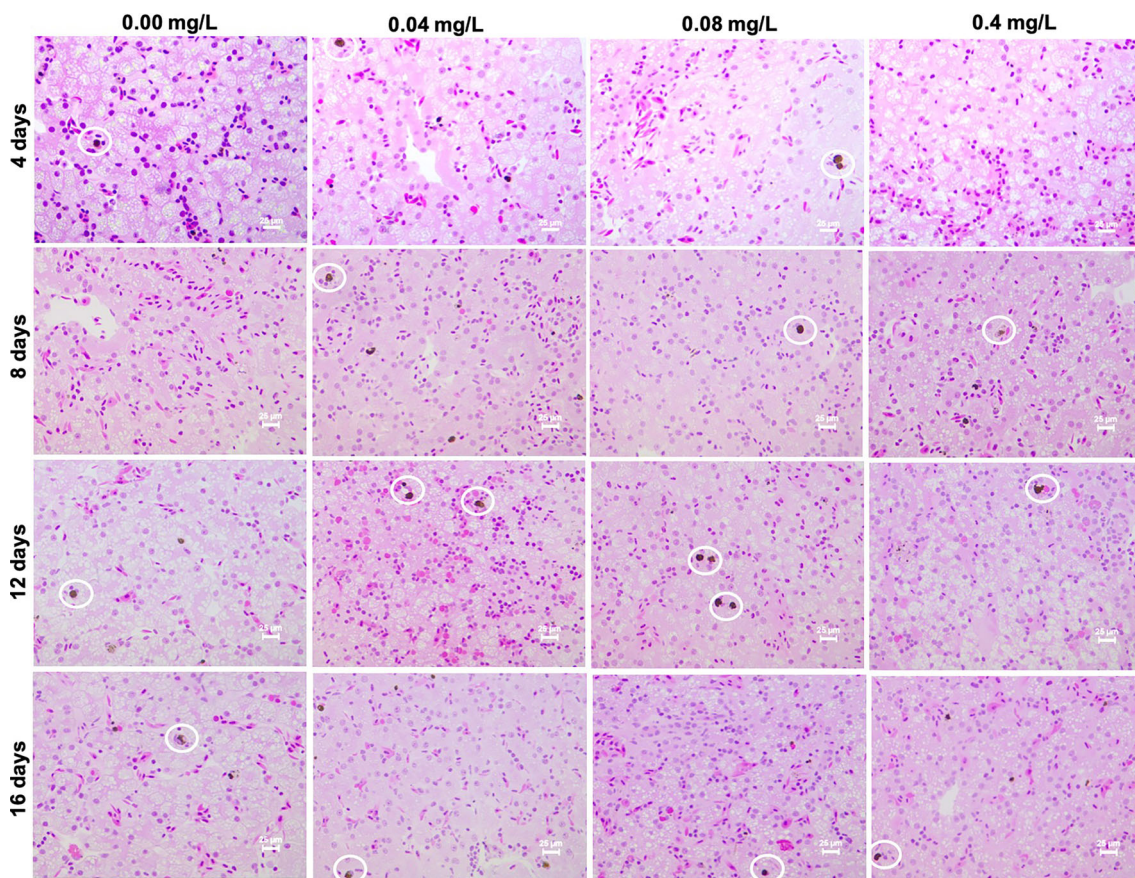


Fig. 3 Histology of the hepatic tissue showing hepatic melanin (circles). HE—25 μm

to their cells and/or melanin. The conservation of pigmentation has been reported for some amphibian taxa (Provete et al. 2012).

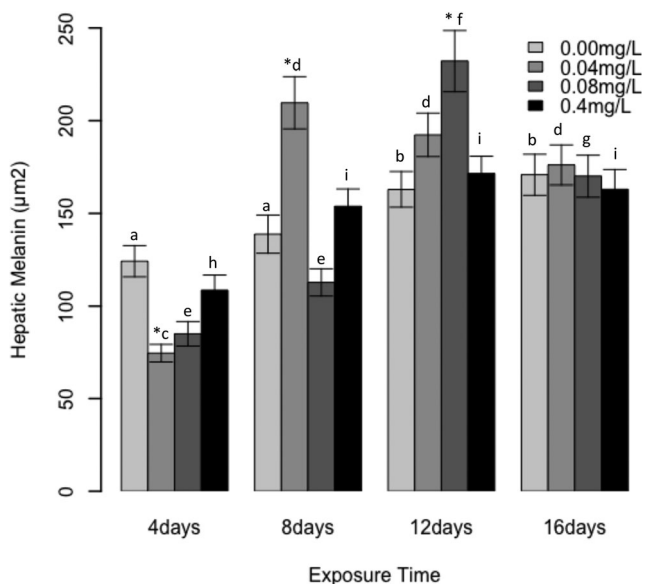


Fig. 4 Hepatic melanin area. Different letters indicate differences between the same treatment at different exposure times. Asterisk (*) represents differences between control and treatment at the same exposure time ($P < 0.05$). Mean \pm SE

Hepatic melanin was also responsive to fipronil exposure, with a decrease after 4 days and subsequent increase after 8 and 12 days. Hence, time is a relevant factor, because the same concentration (0.04 mg/L) resulted in an inverse response as days advanced. Alterations in this hepatic pigment occur as a response to different chemical compounds and to contrasting land use patterns, i.e., agriculture activities (Franco-Belussi et al. 2020), in which there may be an increase (Pérez-Iglesias et al. 2016; De Oliveira et al. 2017; Pérez-Iglesias et al. 2019) or a decrease (Regnault et al. 2014; Fanali et al. 2017, 2018). MMs are associated with detoxifying pollutants (Pérez-Iglesias et al. 2016) and neutralizing free radicals (Agius and Roberts 2003; Barni et al. 1999); hence, the decrease in melanin may be an initial stress caused by fipronil, whereas the increase may be a physiological adaptation of the cell—trying to reestablish the normal condition of the organ. This fact is corroborated by the 16-day group, which did not present any significant difference in the melanin area.

Blood cells reflect all the physical and chemical alterations in organisms which are exposed to insecticides and pesticides (Ambali et al. 2011). The *L. catenulatus* tadpoles exposed to fipronil had an increase in the total rate of nuclear abnormalities after 4 (0.04–0.08 mg/L), 8 (0.04–0.08–0.4 mg/L), and 16 days (0.08–0.4 mg/L). Some studies also evaluated the systemic effects of both an acute and a chronic exposure of aquatic

Fig. 5 Nuclear abnormalities. **a** Normal erythrocyte (arrow) and anucleate erythrocyte. **b** Micronucleus. **c** Segmented nucleus. **d** Binucleated erythrocyte

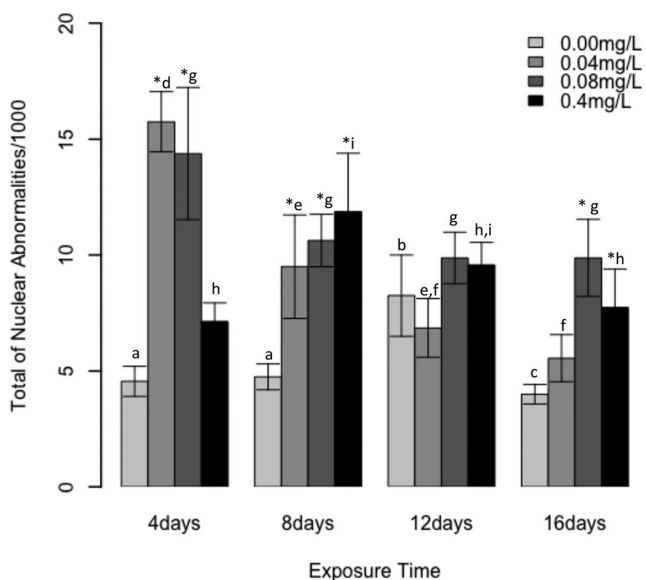
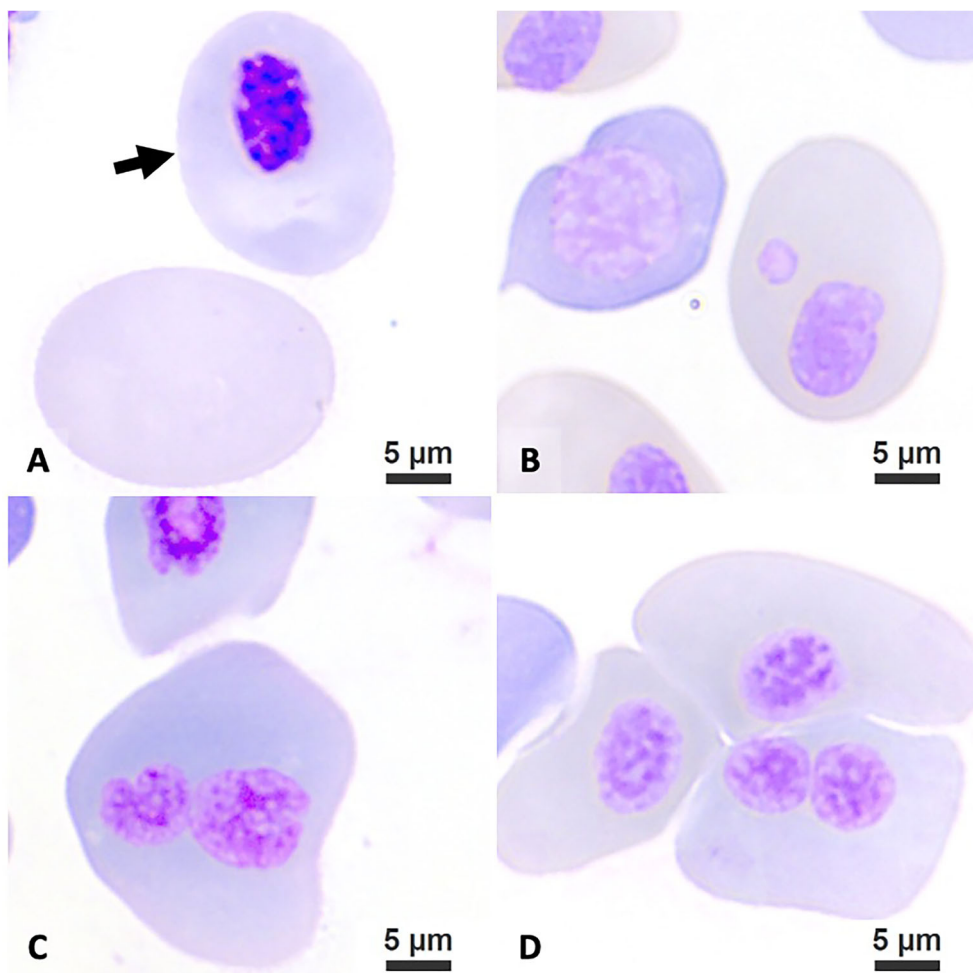


Fig. 6 Total nuclear abnormalities. Different letters indicate differences between the same treatment at different exposure times. Asterisk (*) represents differences between control and treatment at the same exposure time ($P < 0.05$). Mean \pm SE

vertebrates to fipronil. In the experiment carried out by Ghisi et al. (2011) in the catfish (*Rhamdia quelen*), the results suggest that only the highest concentrations of fipronil cause damage in erythrocytes, but none of the concentrations (0.05–0.23 $\mu\text{g/L}$) were sufficient to alter the DNA or the morphology of the gill cells. The authors suggest the need for studies using higher concentrations, other exposure times and other tissues. Further studies in carp fish (*Cyprinus carpio*) exposed for 96 h to sub-lethal concentrations of fipronil (400 $\mu\text{g/L}$) reported morphological injuries (Qureshi et al. 2016). These injuries in carp fish included erythrocyte abnormalities which included necrosis, micronucleus, and hyperchromatosis; histopathological changes in the gills, liver, and kidney. Particularly, in the liver, these effects occur as karyorrhexis, hepatocellular hypertrophy, nuclear hypertrophy, melanomacrophage aggregates, and central vein contraction. The toxicity in fish (concentrations of 0.3–0.4 mg/L) was also reported by Fredianelli et al. (2019), which included a clinical condition of anemia, alterations in liver enzyme levels, nuclear erythrocyte changes and liver, gill, and kidney damage. In this study, the rate of abnormalities was higher for the 4-day group and decreased as the experiment advanced. Such results highlight the recycling dynamics of erythrocytes with alterations at short

time intervals, which makes it difficult to observe the genotoxic effects during prolonged times at low concentrations of fipronil.

Based on our data, we demonstrated that the higher the melanin quantity, both in the skin and the liver, the lower the rate of nuclear abnormalities observed in erythrocytes. This comparative approach between genotoxic responses and morphological properties of visceral (hepatic) and somatic (cutaneous) melanin is in accordance with the assumptions by De Oliveira et al. (2017), who identified melanin as having a protective role for tissues, due to the toxicity of environmental chemical contaminants.

Our experimental design, particularly the concentrations of the compound, was based on the most recent studies of these aquatic vertebrates. In addition to the LC50 for these aquatic vertebrates, was to obtain systemic effects comparable with the data in the literature on amphibians. There are some studies with tadpoles of Neotropical anurans having been exposed to higher concentrations of fipronil (0.5 and 1.5 mg/L); these are related to biochemical biomarkers in *Scinax fuscovarius* (Margarido et al. 2013) and related to antioxidant enzyme responses and the metamorphosis in *Physalaemus nattereri* (Boscolo et al. 2017). Some studies are very close to our contamination levels of fipronil (0.04, 0.08, 0.4 mg/L): showing oxidative stress and neurotoxic effects (at concentrations 0.026, 0.05, 0.1, 0.5, 1.5 mg/L) in *Physalaemus gracilis* tadpoles (Rutkoski et al. 2020) and lipid peroxidation and enzymatic antioxidant defense in *Eupemphix nattereri* tadpoles exposed to intermediary concentrations in water and sediment (0.035, 0.12, 0.18 mg/kg) (Gripp et al. 2017).

For Neotropical amphibians, evidence showed that the higher concentrations of fipronil (0.5 and 1.5 mg/L) was responsible for alterations in antioxidant enzymes of both *Scinax fuscovarius* tadpoles (Margarido et al. 2013) and *Physalaemus nattereri* tadpoles (Boscolo et al. 2017). As both direct and indirect effects, there are increases in the levels of oxidative damage of the cellular components due to an increase of the susceptibility of the animals to oxidative stress. Gripp et al. (2017) reported similar conclusions about fipronil and its degradation products promoting oxidative stress in *Eupemphix nattereri* tadpoles exposed to environmentally relevant concentrations (0.035 to 0.18 mg/kg), which decreased physiological performance in the long-term. Rutkoski et al. (2020) observed changes that suggest the occurrence of oxidative damage and alterations in the central nervous system at concentrations ranging from 0.026 to 1.5 mg/L in *Physalaemus gracilis* tadpoles. As a result, we hypothesize that the sum of each specific effect can trigger systemic alterations, leading to detrimental results at the population level.

Conclusion

Fipronil has systemic effects based on its concentration (0.04, 0.08, and 0.4 mg/L) and exposure time (4, 8, 12, and 16 days). Such alterations are related to the pigment alterations and rate

of abnormality in erythrocytes, which altogether, result in morphological and physiological effects which may compromise the behavior and survival of the organism.

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Authors' contributions ATS: investigation, methodology. BSLV: conceptualization, investigation, methodology, writing original draft, and review. LFB: conceptualization, methodology, formal analysis, writing original draft, and review and editing. CO: conceptualization, resources, writing original draft, and review and editing. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethical approval All manipulation and care of animal's procedures were previously approved by the Ethical Committee and Animal Experimentation from IBILCE/UNESP (CEUA #199/2018).

Competing interests The authors declare that they have no competing interests.

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