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Effects of Glyphosate-Based Herbicide Sub-Lethal Concentrations on Fish Feeding Behavior

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Abstract Glyphosate-based herbicides are widely used in agricultural systems. Although the target organism are particularly plant organisms, there are numerous studies showing adverse effects in aquatic animals, such as inhibition of acetyl cholinesterase, effects on kidney, liver, and gill and stressors effects. This study analyzed the effects of commercial formulation of glyphosate on feeding behavior in Pacu (*Piaractus mesopotamicus*). Fish were exposed to three glyphosate concentrations (0.2, 0.6, and 1.8 ppm) for 15 days. At concentrations of 0.2 and 0.6 ppm, food intake decreased on day 13 and then returned to normal on day 15. At the highest glyphosate-based herbicide concentration, 1.8 ppm, food consumption decreased dramatically and did not recover on day 15. This study showed that glyphosate-based herbicide at sub-lethal concentrations can affect feed intake in pacu and consequently inhibits its growth.

Keywords Roundup · Herbicide · Feeding behavior · *Piaractus mesopotamicus*

Glyphosate is a potent organophosphorous substance, widely used as an herbicide in agricultural production to control plant pests such as weeds (Timmermann et al. 2003). Glyphosate is systemic herbicide acting in plant organisms by inhibiting the synthesis of some essential amino acids (as tyrosine, tryptophan and phenylalanine), thus leading to death of growing plants (Cole 1985; Franz et al. 1997). However, concerns about the exposure of animals and human to this substance persist, as several studies showed negative effects on animals (Giesyet al. 2000), such as inhibiting acetyl cholinesterase, effects on kidney, liver, and gill (in aquatic animals) and various stressors effects (Cattaneo et al. 2011; Glusczak et al. 2006; Menéndez-Helman et al. 2012; Lajmanovich et al. 2011; Langiano and Martinez 2008; Modesto and Martinez 2010; Salbegoet al. 2010; Sandrini et al. 2013).

Herbicides can reach aquatic environments via agricultural runoff and leaching processes, as well as by direct applications to control invasive aquatic weeds (Annett et al. 2014). Once in the aquatic ecosystems, herbicides may reduce environmental quality and influence essential ecosystem webs by reducing species diversity and community structures, modifying food chains, changing patterns of energy flow, nutrient cycling and changing the stability and resilience of ecosystems.

The laws in USA, European countries and Brazil allow the use of this herbicide and determine maximum concentrations in water bodies (7 ppm) in the United States, 0.001 ppm in European countries and 0.65 ppm glyphosate in Brazil (Amarante-Junior and Santos 2002). Despite establishing limits in the application of the herbicide and maximum concentration in water bodies, the increased and indiscriminate use of these products results in contamination of aquatic environments with concentrations well above those permitted by law (Annett et al. 2014).

Fish growth is a consequence of a cascade of behavioral and physiological events, and it is assumed that the stress have a negative impact on organisms due to prolonged activation of the hypothalamic-pituitary-interrenal (HPI) axis (Bernier et al. 2004). Environmental, social and physical

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stressors, mediated by components of the HPI axis, can affect fish growth by inhibiting food intake, absorption or conversion efficiency of food into body (Benoit et al. 2000; Bernier et al. 2004; Heinrichs and Richard 1999; Smagin et al. 1998).

Despite the identified harmful physiological effects of exposure to glyphosate, especially at high concentrations, there has been no attempt to evaluate the long-term impact of low concentrations of glyphosate in the water, especially concerning possible effects on key behaviors for survival. Thus, the objective of this study was to determine the effect of glyphosate-based herbicide on fish feeding behavior.

Materials and Methods

Pacu (*Piaractus mesopotamicus*) was chosen as animal model, since it is a native species in Brazil, with a high ecological and economic relevance. Fish were obtained from an aquaculture farm and acclimatized in a vivarium for a month before the experiments. The fish (length 7.35 ± 0.35 cm and weight $12.53\text{g} \pm 1.64$) were maintained in 300 L tanks with a constant aeration, at a density of 1 fish/L. Water temperature ranged between 24 and 26°C, pH averaged 7, ammonia was maintained below 0.04 mg/L and a 12 h light phase from 06:00 am to 06:00 pm was applied. The fish were fed once daily (until satiety) with tropical fish food (36% of protein). Ethics committee of animal use (CEUA) of UNESP, Botucatu, SP, Brazil (protocol number 484), previously approved all procedures.

The commercial glyphosate formulation used was Isopropylamine salt of N-phosphonomethyl glycine 480 g/L, 3-iodo 2-propynyl butyl carbamate at 0.017%. We calculate the lethal concentration (LC50) to establish sublethal concentrations for this study. For that, we randomly distributed 50 juvenile pacu in five groups of 10 fish, at different glyphosate concentrations (0, 1, 10, 50 and 100 ppm). There was no difference in length ($p = 0.69$) and weight ($p = 0.78$) between groups. The LC50 for 48 h was calculated through trimmed Spearman–Karber method (Hamilton et al. 1977) with TSK package on R software. The LC50 found was 22.36 ppm. The concentrations used in this work were sub-lethal; 0.2 (0.9% of LC50), 0.6 (2.68% of LC50) and 1.8 ppm (8% of LC50).

The experimental design consisted of measuring the latency to feed and the amount of food ingestion before exposure and measuring it again on 10, 11, 13,14 and 15 days after exposure to different sub-lethal concentrations of glyphosate-based herbicide (0.2, 0.6 and 1.8 ppm).

We placed 18 fish in 20 L glass aquariums individually and allowed them to acclimate for 48 h before the beginning of experiments. At the first day, prior to adding the glyphosate-based herbicide, we gave 10 fish-feed pellets for each fish and measured the latency to feed and number of pellets ingested after 20 min. Twenty-four hours after that we added the glyphosate-based herbicide to the aquarium water at the concentrations 0.2, 0.6 and 1.8 ppm. To assess the rate of glyphosate degradation in the water at initial concentrations of 0.2, 0.6 and 1.8 ppm, glyphosate concentrations were monitored during 8 days, according to Hidalgo et al. (2004). The method showed a limit of detection (LD) of glyphosate in water of 0.05 µg/L, a limit of quantification (LQ) of 0.15 µg/L, a maximum detectable concentration of 5 µg/L and a mean recovery rate of 101%. For quantification, a four-point calibration curve (0.075, 0.2, 0.75 and 5 µg/L) was constructed with two replicates each. An analytical quality control solution (0.5 µg/L) was also used. All samples were analyzed for duplicate and those with glyphosate concentrations above the calibration curve were diluted until concentration could be reliably estimated. During 8 days, Glyphosate concentrations decreased by 3.95%, 3.18% and 4.96%, respectively (Table 1), with a clear linear trend (linear regression, $R^2 = 0.89$, 0.97 and 0.99, respectively). Accordingly, we could conservatively estimate that by day 15 (the last experimental day) glyphosate decrease would not exceed 10% of the initial concentration in all treatments. Thus it seemed reasonable to assume that possible effects of glyphosate and differential treatment effects would be maintained throughout the experimental period and would not be biased by differential degradation rates.

For the following 15 days, the fish were daily fed as in the first day. On each of days 10, 11, 13, 14 and 15 we measured the latency to feed and number of pellets ingested 20 min after feeding.

For assessment of the effect of glyphosate-based herbicide on feeding behavior we compared the latency to feed and number of eaten pellets among the different concentration treatments using repeated measures ANOVA, with day as random effect. Tukey–Kramer HSD test was used for *aposteriori* analysis. Normality and homoscedasticity were verified through Kolmogorov–Smirnovand Levene tests, respectively. To meet ANOVA assumptions, data of the number of ingested pellets was $\log(x + 1)$ transformed. Significance level was set at $\alpha = 0.05$.

Table 1 Glyphosate concentrations (ppm) in the aquaria water during 8 days and percentage of initial concentration (in parentheses)

Day	Glyphosate concentration (ppm)		
1	0.2 (100%)	0.6 (100%)	1.8 (100%)
2	0.197 (98.62%)	0. 594 (99.02%)	1.780 (98.87%)
4	0.197 (98.66%)	0. 590 (98.32%)	1.756 (97.54%)
6	0.196 (97.78%)	0.585 (97.55%)	1.733 (96.27%)
8	0.192 (96.05%)	0.581 (96.82%)	1.711 (95.04%)

Results and Discussion

The exposure of *P. mesopotamicus* to 1.8 ppm of glyphosate-based herbicide decreased the number of pellets ingested by fish ($p = 0.00001$; Fig. 1), as compared to control (before glyphosate-based herbicide exposure). At 1.8 ppm, the pellets consumption was low since day 10 and kept lower until the last day of the experiment (15th day) (Fig. 1). However, the exposure of 0.2 ppm led to lower ingestion of pellets just on day 13 ($p = 0.0003$) and 14 ($p = 0.038$), going back to a normal consumption at 15 days of exposure ($p = 0.73$). A similar pattern was observed at 0.6 ppm concentration with lower ingestion on day 13 ($p = 0.01$), but the recovery was on day 14. Glyphosate-based herbicide exposure also significantly increased the latency to feed relative to 'before' at 1.8 ppm, but didn't increase significantly at 0.6 and 0.2 ppm (Fig. 2).

These negative effects on the fish feeding behavior are perturbing in light of the fact that the highest tested concentration (1.8 ppm) can also be found in the field (Thompson et al. 2004). This effect can be intensified, considering that fishes do not seem to avoid glyphosate and there is no scientific consensus that they avoid commercial formulations as used in this study (Tierney et al. 2010). Kasumyan (2001) has reviewed effects of pollutants on feeding behavior of fishes. Here we show for the first time the effects of long-term exposure (10–15 days) to glyphosate-based herbicides on feeding behavior of fish, specifically on *P. mesopotamicus*, an important species from the economic and conservation point of view.

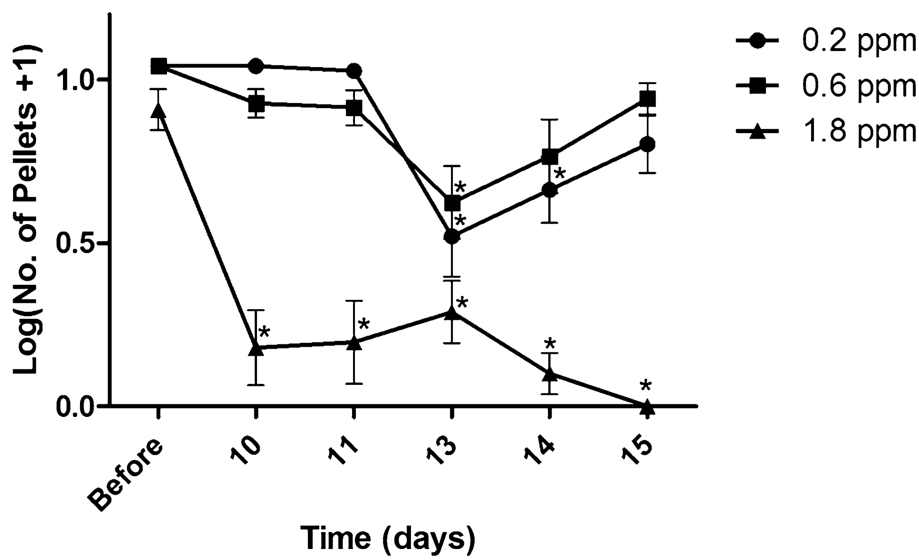

Fig. 1 Number of pellets ingested by pacu (*Piaractus mesopotamicus*) before and on days 10, 11, 13, 14 and 15 after exposure to different concentrations of glyphosate-based herbicide. *Indicates statistical significance of each time-point from before exposure time. *Symbols* (*circle, square* and *triangle*) represent the mean of each treatment and bars represent the standard error of the mean

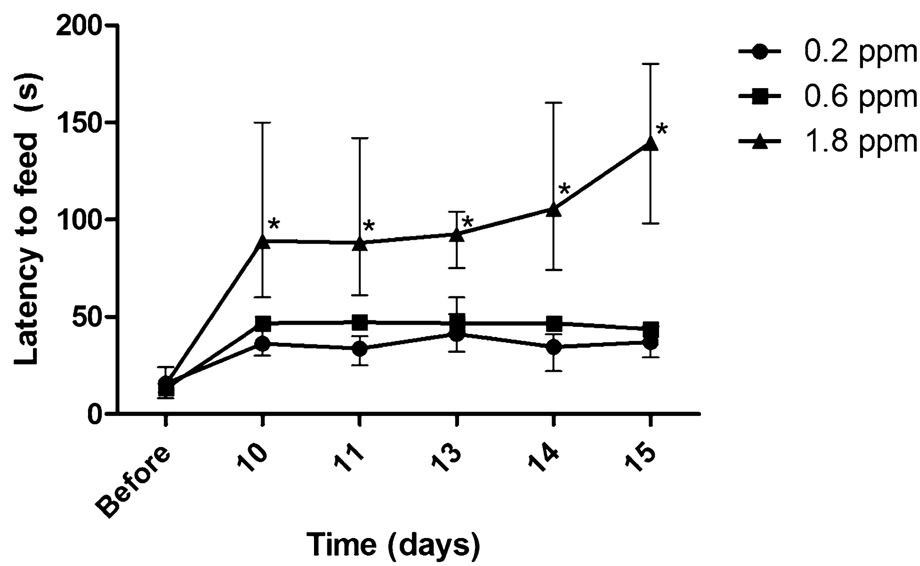

Fig. 2 Latency in seconds of Pacu (*Piaractus mesopotamicus*) to start feeding before exposure and days 10, 11, 13, 14 and 15 after exposure to different concentrations of glyphosate-based herbicide. *Indicates statistical significance of each time-point from before exposure time. *Symbols* (*circle, square* and *triangle*) represent the mean of each treatment and bars represent the standard error of the mean

There are some explanations for why glyphosate-based herbicides might lead to reduced food intake and higher latency to feed. One of them is that the presence of the herbicide in the water can modify the taste of food, leading to reduced attractiveness of food to fishes. Another explanation is disruption of the olfactory system. Tierneyet al. (2006) showed that acute exposure of *Onchorynchus kisutch* to 1 ppm or higher of glyphosate (just the active substance) leads to reduced Electro-Olfactogramactivity when it detects Lserine in the environment. Their argument is that glyphosate resemble the aminoacid glycine and there is some overlapping for the same active site for Lserine substance. As a result, fish cannot detect Lserine and it does not respond to its presence. However, when the active site is available again (during recovering time), the fish restore their initial capability to detect Lserine.

For commercial formulations, as used in this study, Tierneyet al. (2007a) showed that Roundup® is considerable more toxic than the active ingredient (glyphosate only), probably due to negative effects of surfactants on the olfactory system (Sutterlin and Sutterlin 1971).

Another possible cause for olfactory system disruption is through Acetyl cholinesterase (ACHE) impairment. It is well known that glyphosate-based herbicides leads to disruption of ACHE activity, resulting in malfunction of neuro-transmission (Glusczak et al. 2006; Menéndez-Helman et al. 2012; Modesto and Martinez 2010; Salbego et al. 2010; Sandrini et al. 2013). Considering that production of mucous on olfactory epithelium is up regulated by the secretion of ACHE, the lack of this enzyme can result in over-production of mucous, increasing the thickness of mucous layer and the distance between odoriferous molecules and active sites of neuroreceptors, leading to lack of olfactory sensitivity (Inglis et al. 1997; Jarrard et al. 2004; Tierney et al. 2007b).

One factor that we should consider is the stress response of the fish. Experimental manipulations, new environment and social isolation usually lead to stress response in fish, manifested in increased cortisol levels. However, sub-lethal concentrations of agrichemicals were reported to decrease plasma cortisol levels and disrupt stress response in fish (Cerciato et al. 2008). It is well known that cortisol leads to increased food intake (Bernier et al. 2004). Thus, we suggest that the reducing effect of glyphosate on food ingestion was mediated by cortisol; the manipulation at the beginning of the experiment could have caused some stress response on those fish, resulting in cortisol release and stimulating food intake. As we submitted them to glyphosate treatments, depending on concentration (as in Bernier et al. 2004; Cerciato et al. 2008), cortisol release was disrupted, resulting in reduced feed ingestion in the highest tested concentration (1.8 ppm). Glyphosate also have impacts on fish growth that can be related to food intake or food conversion efficiency (Bernier et al. 2004; Salbego et al. 2010).

In an economic perspective, *P. mesopotamicus* is highly appreciated for human consumption and aquaculture. Glyphosate-based herbicides, especially Roundup®, is broadly used in farms for weed control and can reach (through runoff and leaching) aquaculture ponds and reduce algal food resource in the water. There by, it might reduce the growth rate of herbivore fishes in these aquaculture systems, resulting in losses to fish growers.

In a conservation perspective, considering that concentrations used in this study is compatible with reality (from 0.00001 to 1.9 ppm; Annett et al. 2014), and considering that that fishes cannot avoid and possibly do not have an avoidance behavior with glyphosate, they can be directly affected by glyphosate-based herbicides that impose reduced consumption of food. In common situations where natural food is scarce, this can be very dangerous, because animals must get all food that they need to survive in unfavorable times and places.

There are regulations for using glyphosate-based herbicides and governments around the world determine limits to glyphosate concentrations in natural water bodies. EPA (USA) established 7 ppm, while Brazilian government stated 0.65 ppm (CONAMA 357) and European governments only 0.001 ppm (Amarante-Junior and Santos 2002). According to our results, EPA and Brazilian government need to review their laws, looking not just on survival of native species in natural environments, but also on their well-being.

In conclusion, the present study shows for the first time that long-term exposure to sub-lethal concentrations of glyphosate-based herbicides can decrease feeding activity and appetite in *P. mesopotamicus*, a native species in Brazil with great ecological and economical relevance. Such interference obviously has deleterious fitness consequences for individual fish, and in the long run it may adversely affect whole fish populations. This fact has a great impact in terms of conservation, considering that glyphosate-based herbicides are widespread at sub-lethal concentration in natural environments. It is therefore advisable for scientists to look for seemingly subtle effects such as those related to feeding behavior without any evident damage, and furthermore, for decision maker to consider revision of permitted herbicides levels in the aquatic environment.

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