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Stellenbosch University

ABSTRACT

Many anthropogenic chemicals, especially from agricultural and environmental practices are generating global ecological concerns. The fact that many of these substances have been linked to endocrine system modulation, particularly the thyroid homeostasis is becoming a topical issue. Several studies support the hypothesis that the thyroid system represents major target of endocrine disruption. Using a Xenopus Metamorphosis Assay, this study assessed the comparative thyroidal impact of three glyphosate-based formulations including Roundup, Kilo Max and Enviro glyphosate. The result showed that Roundup at 0.6 mg/L significantly increased whole-body length (WBL), and reduced the whole-body mass (WBM) alongside a significant increase in the thyroid gland, but without impacting stage development. The Kilo Max significantly reduced both WBL and snout-vent length (SVL) at 190 and 280 mg/L, while it increased the full-limb length (FLL) at 90 mg/L only. Kilo Max also significantly reduced the stage development at 190 mg/L and 280 mg/L, without impacting the thyroid gland. Although, Enviro glyphosate, reduced WBL, SVL and WBM of the tadpoles at 28 mg/L, without impacts the stage development and thyroid histology. This result confirms that Roundup formulation is thyroid active as it affect the thyroid gland, while Kilo Max and Enviro glyphosate formulations only showed extra-thyroidal effects, since it has no effect on thyroid gland.

Keywords: thyroid, herbicide, amphibian, Roundup, Kilo Max, Enviro glyphosate.

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ABSTRACT

Many anthropogenic chemicals, especially from agricultural and environmental practices are generating global ecological concerns. The fact that many of these substances have been linked to endocrine system modulation, particularly the thyroid homeostasis is becoming a topical issue. Several studies support the hypothesis that the thyroid system represents a major target of endocrine disruption. Using a Xenopus Metamorphosis Assay, this study assessed the comparative thyroidal impact of three glyphosate-based formulations including Roundup, Kilo Max and Enviro glyphosate. The result showed that Roundup at 0.6 mg/L significantly increased whole-body length (WBL),and reduced the whole-body mass (WBM) alongside a significant increase in the thyroid gland, but without impacting stage development. The Kilo Max significantly reduced both WBL and snout-vent length (SVL) at 190 and 280 mg/L, while it increased the full-limb length (FLL) at 90 mg/L only. Kilo Max also significantly reduced the stage development at 190 mg/L and 280 mg/L, without impacting the thyroid gland. Although, Enviro glyphosate, reduced WBL, SVL and WBM of the tadpoles at 28 mg/L, without impacting the stage development and thyroid histology. This result confirms that Roundup formulation is thyroid active as it affects the thyroid gland, while Kilo Max and Enviro glyphosate formulations only showed extra-thyroidal effects, with no effect on thyroid gland. This result points to the underlying role of surfactants in the thyroid disruption and not the main glyphosate. Regulatory agencies must do the needful, to subject all aquatic formulations to relevant physiological endpoints to characterize their impacts particularly on amphibians, given their global declining status.

Keywords: thyroid, herbicide, amphibian, Roundup, Kilo Max, Enviro glyphosate.

Author α σ ρ Ɯ: Department of Botany & Zoology, Stellenbosch University, Stellenbosch, 7600, South Africa.
σ: Department of Zoology & Environmental Biology, Lagos State University, Lagos, Nigeria.
ρ: Department of Microbiology, Stellenbosch University.
σ: Department of Paraclinical Sciences, University of Pretoria, South Africa.

Highlights

- Roundup significantly increased thyroid gland at 0.6 mg L, increased the WBL and reduced the WBM, without impacting the developmental stage of the tadpoles.
- Kilo Max significantly reduced the developmental stage, WBL and SVL at 190 and 280 mg/L, while increasing the FLL at 90 mg/L, without impacting the thyroid glands.
- Enviro glyphosate reduced WBL, SVL and WBM at 28 mg/L without impacting the stage development.
- Roundup is thyroid active while Kilo Max and Enviro glyphosate only showed extra-thyroidal actions.

I. INTRODUCTION

There are no longer any pristine areas without environmental pollutants (Bergman *et al.*, 2013). The environment today harbors numerous anthropogenic substances emanating from

agricultural, industrial, and environmental management (WHO/UNEP, 1997; Bergman *et al.,* 2013; EPA, 2016). A particular concern is the intensive use of pesticides such as insecticides, herbicides, fungicides and acaricides among others, which has led to ubiquitous contamination in many environmental media (Oritz-Dalgado *et al*., 2019) that raises serious concern over their non-target impacts through contamination of soil and water matrices (Carvalho, 2017**;** Babalola *et al*., **2**019). These chemicals, apart from their toxic effects, also possess differential physiological effects that may include thyroidal, reproductive, genotoxicity and teratogenicity (Babalola *et al*., 2020). Even though there are about 800 chemicals known or suspected to interfere with the endocrine system in one way or the other (mostly *in-vitro*), only small fractions of these chemicals have been investigated in intact organisms (Bergman *et al.,* 2013).

Pesticides are regularly introduced into the environment, through a variety of anthropogenic practices to combat pests that mainly influences agricultural yield and sustainability. However, several of these pesticides alter the delicate balance between species that characterize a functioning ecosystem and produce many physiological and biochemical changes in freshwater organisms by influencing the activities of several enzymes (Khan and Law, 2005)**.** Many of these pesticides have also been shown with the capacity to decrease the total protein content in amphibians, suggesting changes in the biochemical system of the non-target organisms (Khan *et al*., 2003). Some of these pesticides have also been shown to increase the toxic nature of the non-target organisms, through the alteration of their biochemical system (Bokony *et al.,* 2017). There are increasing negative impacts on the ability of non-target organisms to develop and reproduce properly given the level of these harmful substances that are continually introduced into the environment (Kortenkamps *et al.* 2011). Today, despite the intensive ecotoxicological research, there are still gaps about the ecological impacts of many environmental contaminants on non-target organisms (Bokony *et al.,* 2020).

Amphibians are an important component of the aquatic habitat, especially in tropical regions of the world (McDarmid, 1992), they are decreasing at an alarming rate around the world (Stuarts *et al*., 2004; Khan and Law, 2005; Munoz *et al.,* 2014). Although several factors have been suggested as possible causes of this decline, chemical pollutants (such as pesticides) have been highlighted as a major contributing factor (Sparling *et al*, 2001; Egea Serano *et al*., 2012; Munoz *et al*., 2014). In most amphibian species, their active reproductive cycles and early onset of development coincide both in time and places with pesticides application, which may therefore impact a critical window of amphibian fecundity and survival (Carlsson, 2019)**.** This global extinction of species and population declines among the amphibians is considered a critical threat to the global biodiversity (Whittaker *et al*., 2013). Even though Egea-Seranno *et al.* (2012)have noted that there is lack of deep understanding of how different types of pollutants affect amphibians, but evidence have shown that low-dose effects and non-monotonic response in amphibian species suggests that many pathways might be affected differentially (Vanderbing *et al.,* 2012). This is particularly true for the thyroid system.

Due to the complex nature of the thyroid system, numerous possible mechanisms of agonistic and/or antagonistic actions may influence amphibian metamorphic processes at different molecular levels (Fort *et al*., 2011; Gilbert *et al.,* 2012)**.** Optiz *et al.* (2005) summarized various possible thyroid system-disrupting effects of chemicals that strongly support the hypothesis that thyroid system represents another important target of endocrine disruption activities**.** The central effects of many anthropogenic chemicals on the thyroid system can be seen in many metamorphic organisms like amphibians, where they disrupt, arrest or lead to abnormal growth. The fact that the rising thyroid hormones from the thyroid gland of a growing tadpoles orchestrate the sequential changes of metamorphosis in the tadpole organs (Schreiber *et al.* 2001), any insult on the thyroid gland by these anthropogenic chemicals results in disruption of metamorphic

processes. The alteration of the thyroid system is of serious concern because the thyroid hormones mediate major physiological processes, including growth regulation, general metabolism and metamorphosis (Denver, 1997; Hermelink *et al.* 2010). Today, because of the considerable number of chemicals that have been shown to alter the thyroid system in vertebrates, the disruption of the thyroid axis has been identified as an important consideration for the regulation of chemicals (Miyata and Ose, 2012).

The Xenopus metamorphosis assay (XEMA) is used globally as an *in vivo* assessment protocol to identify agonistic and/or antagonistic effects of chemical toxicants on the thyroid system of the African clawed frog, *Xenopus laevis*. This assay is based on the fact that the metamorphosis phase in amphibians development is generally dependent on proper synthesis and regulation of thyroid hormones, which when modulated can lead to measurable developmental effects in the test organisms (Grim *et al.*, 2009). These measurable endpoints of this assay include hind limb length, thyroid gland histopathology and assessment of metamorphic stage development compared to control exposure group, and the identification of growth abnormalities and morphological features of the exposed organisms (OECD, 2007). The uniqueness of this protocol is the fact that the entire process occurs in aquatic environments, the ecological niche of these anuran larva, where the effects of thyroid disruption are greater (Helbing *et al.* 2010).

Glyphosate, for example, is the current leading herbicide globally (Sihtmae *et al.,* 2013; Turhan *et al.*, 2020). They are used for plant control, in no-till farming practises, and in the agricultural production of "glyphosate-ready" genetically modified crops (Gomez-Oritz *et al.,* 2017). The herbicide is also one of the leading formulations approved for aquatic weeds management. For example, a Working for Water initiative by the Western Cape Government of South Africa project to eradicate invasive alien vegetation, aimed to promote freshwater security, relied on the use of glyphosate formulations as one of the herbicides of choice. There are several commercial formulations of glyphosate herbicide including Roundup, Kilo Max, Environ glyphosate, Touchdown, Glyfos Bio and Roundup Bioactive etc. These different glyphosate formulations contain various surfactants, some of which are already of global concern. Despite substantial advances in Glyphosate toxicological studies, several uncertainties and gaps still exist, particularly on amphibians (Munoz *et al.*, 2014; Turhan *et al.*, 2020). For example, studies have pointed at the polyethoxylated tallow amine (POEA) surfactant in Roundup for the adverse role, leading to the emergence and development of several new POEA-free formulations (Howe *et al.*, 2004; Turhan *et al.*, 2020).

For the purpose of extending the knowledge regarding the potential adverse health effects of glyphosate formulations on amphibian development, this study evaluated the thyroid-disrupting potential of three aquatic glyphosate-based formulations using a Xenopus Metamorphosis Assay protocol. The selected glyphosate formulations were selected due to their regular use in managing aquatic weeds in the South African Working for Water Project and hence, may be closely associated with the pollution of freshwater ecosystems and exposure to aquatic wildlife.

II. MATERIALS AND METHODS

2.1. Test chemicals

The three glyphosate formulations selected for the study include Roundup (360 g acid equivalent (a.e.)/L) by Monsanto Ltd, South Africa, Enviro glyphosate (360 g a.e /L) by Enviro Industries Ltd, South Africa and Kilo Max (700 g a.e /kg) Volcano Agro-science Ltd, South Africa. Roundup contains polyethoxylated tallow amine (POEA) surfactant. The Kilo Max formulation contains an undisclosed POEA-free surfactant. Enviro glyphosate contains polyethylene alkylamine surfactant.

2.2. Africa clawed frog (Xenopus laevis) husbandry and breeding of Tadpoles

From the healthy in-house *X. laevis* breeding stock, two sexually mature males and females were selected. The males and females were

separately maintained in 15 L glass tanks, filled with charcoal filtered water buffered with 2.5 g iodated sea salt and 0.8 g $NaHCO_3$ /10 L (Kloas *et al.* 1999). The frogs were fed with fish pellets (Aqua-Nutro, South Africa) three times weekly, and their holding tanks were washed and refilled with fresh charcoal filtered water immediately after their feeding. The breeding induction was performed according to ASTM, (1998) protocol. In brief, the adult males and females were first primed with 100 IU human chorionic gonadotropin (hCG) (Merck Ltd, Germany), which was injected into their dorsal lymph sac, four days before the mating. The males and females were given a second round of 100 IU and 300 IU hCG respectively, just prior to the mating. Male and female breeding pairs were placed together in a 15 L glass tank that was lined with plastic netting to isolate and protect the eggs from the adults. After the eggs deposition, the breeding pair were removed from the chamber, and an oxygen pipe was dropped to the water tank to increase the oxygen volume available to the eggs. After the emergence of the tadpoles, the newly hatched tadpoles were spread into several new 15 L tanks at a density of 40 tadpoles per tank, to avoid the overcrowding effects. The resultant tadpoles were staged using developmental atlas by Nieuwkoop and Faber (1994). Starting from NF-stage 47-48, the tadpoles were fed with Sera Micron (Sera Heinsberg, Germany), at 30 mg/animal/day twice daily until they reached NF-stage 51. All the husbandry, breeding and maintenance of tadpoles and exposure protocols were approved by the Animal Research Ethical Committee of the Stellenbosch University (Approval no- SU-ACUM 12-00015).

2.3 Test procedure

2.3.1. Exposure set-up

At the attainment of NF-stage 51, a total of twenty (20) healthy tadpoles were selected from the holding tanks and allocated to new 15 L exposure tanks. The exposure tanks were replicated twice at each of the exposure concentrations. Following the XEMA experimental protocol, the exposures were done under controlled climate conditions including water temperature at 23 ± 1 ^{0}C, pH range of 7.5 - 8.5, ensured dissolved oxygen of >6.5 mg/L and a 12 hour of light and dark photoperiod ($L_{12}D_{12}$) regime (Organization for Economic Co-operation and Development, 2008). The tadpoles food ration was increased from the initial 30 mg/animal/day, to 50 mg/animal/day to compensate for their increased growth. While exposure mortality was monitored twice daily, the exposure medium was totally renewed every third day. The whole experiment was repeated twice independently. Only mortality incidence below 10% in the control group was accepted as basis for the acceptance of the experiment.

2.3.2. Exposure Concentrations

Arising from the initial 96-hour LC_{50} value derived at NF-stage 48 of *X laevis tadpoles* (Babalola and Wyk, 2017), three exposure concentrations for each of the herbicide formulations were selected (Table I).

2.3.3. Analytical assessment of experimental concentration

Random samples from each of the exposure medium concentrations were taken and analyzed to confirm the experimental concentrations. In brief, 100 ml of water sample was taken from each of the selected exposure tanks into 150ml glass bottles. In the case of replicates, 100ml was taken from each and pooled together, from where a single 100ml was obtained. The water samples were then frozen in an iced pack and transported to the Synexa Life Sciences certified laboratory, (Cape Town, South Africa), for analysis within two hours after collection. The glyphosate analyses and quantification were performed using liquid chromatography coupled with mass spectrometry. The results of the exposure water sample analysis (Table I addendum) showed that there was no major difference to the predicted nominal concentrations during the exposure study.

2.4. Autopsy procedure and morphometric measurements

At the termination of the 21-day exposure, all the survived tadpoles were collected and gently euthanized in 0.1 % benzocaine (Sigma). The tadpoles were then blotted dry, individually

weighed (to the nearest 0.01 g), and measured for their snout–vent length (SVL) (to the nearest 0.1 mm) prior to preservation in Davidson's solution **(**OECD, 2007**)** for 72 hours. The tadpoles were then transferred to 4 % neutral buffered formalin for preservation (OECD, 2007**;** Shi *et al.*, 2012). The fore-limb length (FLL) and hind-limb length (HLL) were measured using Leica EZ4D stereo microscope (Leica Microscope Ltd, Germany) (to nearest 0.1 mm). with metric trace ruler that has the capacity to measure both straight and curved lines using traced lines of the limbs. The tadpole's heads just posterior to the eye, (containing the thyroid gland) were carefully severed transversely using a sharp blade and subjected to routine (paraffin wax imbedding) for histological procedures (Bancroft and Steven, 1977), followed by sectioning, mounting and staining.

2.5. Developmental Stage (NF-stage) determination

To establish the NF developmental stages of the tadpoles (according to Nieuwkoop and Faber, 1994), five median developmental stage of the tadpoles per tank and per concentration were haphazardly selected and compared to the median developmental stage of the control group for histopathological studies of the thyroid gland.

2.6. Histological Procedures

The lower jaws housing the thyroid glands were removed from the formalin, and thoroughly washed in running tap water for 10 minutes before they were processed for routine paraffin wax-based histology. Following the routine paraffin wax embedding and staining (Bancroft and Steven, 1977), the jaw samples were first dehydrated in series of graded concentrations of alcohol before been embedded (in frontal plane to facilitate the caudal surface of the tissue first) in histowax (Histolab Product, Sweden). The embedded lower jaw tissues were sectioned at 8 µm using Reichert-Jung microtome (Cambridge Instrument, Germany). The sections were mounted on clean, albumin coated glass slides, before being oven-dried at 40 °C overnight. The sections were subsequently dewaxed, then stained with haematoxylin and eosin (Bancroft and Stevens, 1977). The stained slides were cleared in xylene and mounted with glass cover using resin-based mounting medium (DPX, Sigma Ltd)

2.6.1. Histological measurement of the thyroid

Using the image of the right-side of the thyroid from each of the tadpoles, Leica DMLB light microscope equipped with a digital camera (Leica Microscope Ltd, Germany) was used for all measurements of the thyroid follicle epithelium cell heights. This was done by determining the length of the base to the apical edge of the cell. For each tadpole specimen, 15 epithelial cell height measurements were taken for four thyroid gland follicles, resulting in 60 epithelial cell height measurements per individual. A mean value was then calculated with other individual group members for follicle cell height. Follicular cross sectional area (follicle lumen area), and the thyroid cross sectional area were also measured and calculated (using image analysis software (Sigmascan, Systat Software Inc.). This was done by measuring the cross sectional area of all the serial sections and then summed together. Ten follicles were measured in each section, making ten thyroid follicles in each tadpole. The data was then combined for all the tadpoles per exposure concentration group.

2.7. Data analysis

A non-parametric Kruskal-Wallis test was used to assess variance in median NF-stage among concentration (since developmental stage constitutes ordinal data), followed by Dunn's multiple comparison test (DMCt) to identify significant pairwise differences in stages (Shi *et al.*, 2012). Normality and homogeneity of variance in WBM, WBL and SVL data were analyzed with Shapiro-Wilk's and Levene's tests respectively. One-way ANOVA or Kruskal-Wallis ANOVA test (K-W ANOVA) for non-parametric data was subsequently used to analyse for variations among concentration groups. Front limb length (FLL) and hind limb length (HLL) were normalized to snout-vent length in order to correct for the growth effect (or size related NF stages) (Coady *et al.*, 2010). Normality of the normalized FLL and HLL was evaluated using residuals' normal

probability plots and the Shapiro-Wilks test, whereas Levene's test was applied to test for homogeneity of variance. The treatment's effect (i.e. specific pesticide concentration), developmental stage and the treatment stage interaction on FLL and HLL was tested using mixed model ANOVA, using individual tadpoles as random factor. Pairwise differences in WBM, WBL, SVL and the normalized FLL and HLL between pesticide treatments and the control groups were assessed using the Tukey HSD test with Spjotfoll/Stoline correction for parametric data or the Dunn's test for non-parametric data. Significant differences were taken at $P<0.05$. All statistical analyses were done using Sigma Statistica V12 (Statsoft Inc., USA).

III. RESULTS

3.1 - Mortality

No mortality incidence was observed in all the experimental tanks throughout the 21-day exposure period.

3.2. -Kilo max

3.2.1.-Variation in Developmental Stages

Following the 21-day treatment with the Kilo Max formulation, the frequency distribution of developmental stages ranged from NF-stage 54 to 64 (Fig 1).

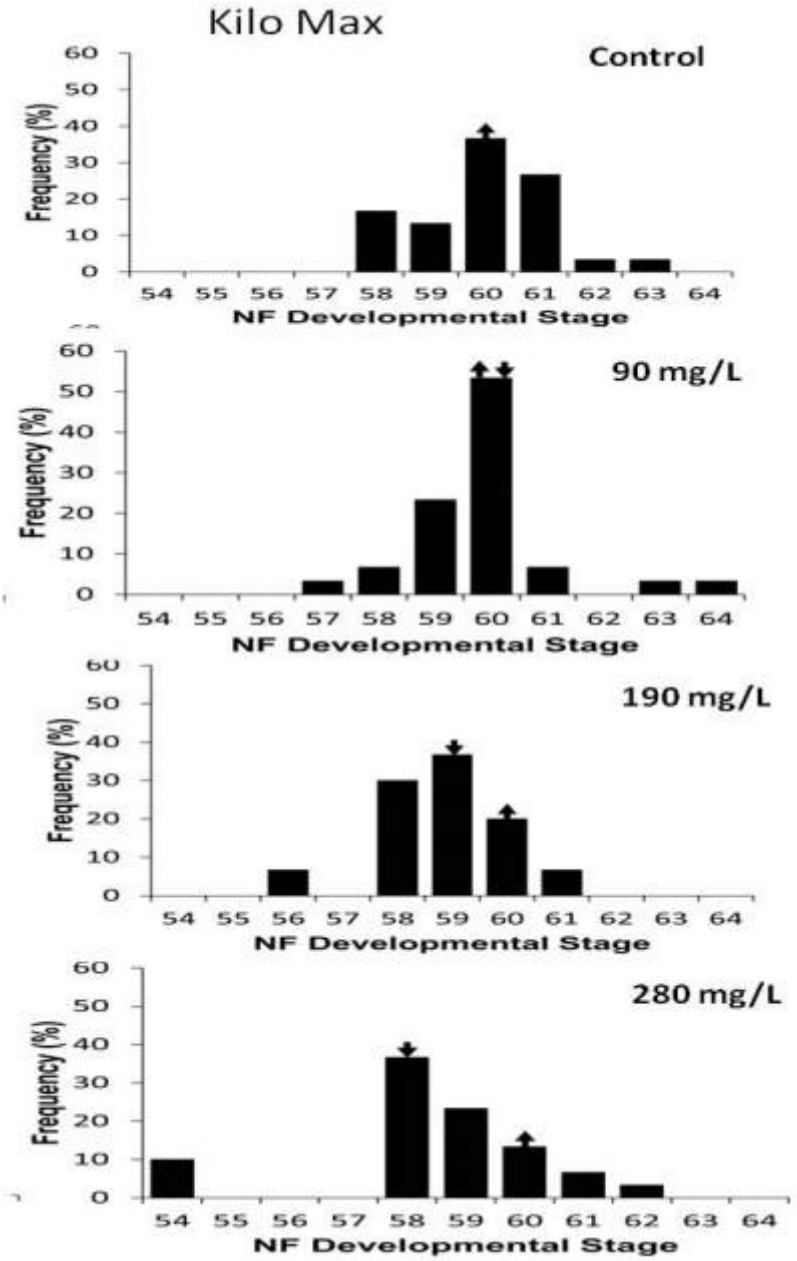

Fig. 1: The frequency distributions (n= 20) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* larval exposed to concentrations series of Kilo Max formulation (a) control, (b) 90 mg/L, (c) 190 mg/L and (d) 280 mg/L. The asterisk indicates significant difference (Post -HOC; P< 0.05) relative to the median stage in the control. The upward arrow showed the median at the control relative to the downward arrow that showed the median distribution of developmental stage at the various concentrations.

At both the control group and concentration of 90 mg/L, the median was NF-stage 60. The exposure at concentrations of 190 mg/L and 280 mg/L showed a significant delay in development with a median NF-stage 58. The exposure impact of the Kilo Max formulation delayed the NF developmental stages of the exposure in a dose dependent manner across all the exposure concentrations.

The exposure impact of the Roundup formulation on the treated tadpoles produced a developmental stage that ranged from NF-stage 57 to NF- stage 64 (Fig. 2).

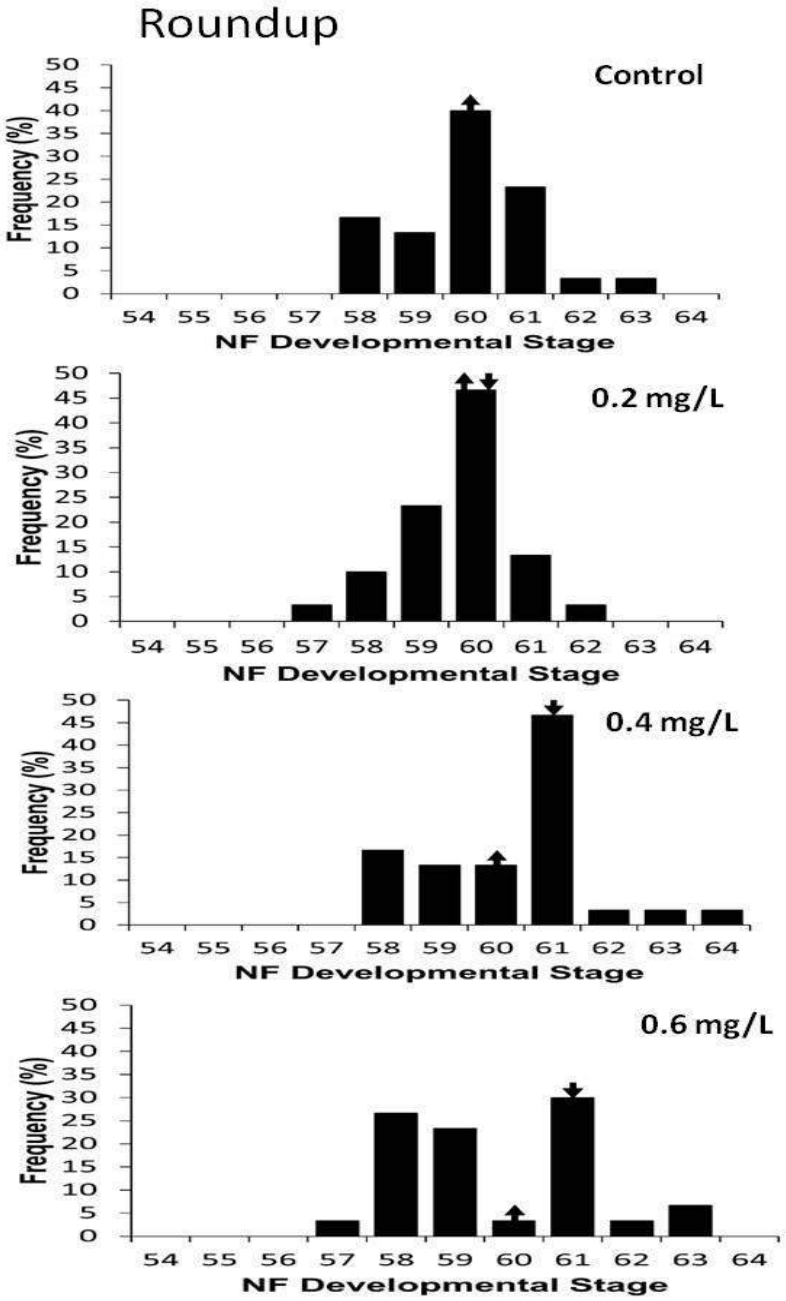

Fig. 2: The frequency distributions (n=20) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* larval exposed for 21-day to graduated concentrations of Roundup (b) 0.2 mg/L, (c) 0.4 mg/L (d) 0.6 mg/L relative to the control (a). The upward arrow showed the median at the control relative to the downward arrow that showed the median of developmental stages at the various concentrations.

The median of developmental stages shifted from NF-stage 60 at 0.2 mg/L to NF-stage 61 at concentrations of 0.4 mg/L and 0.6 mg/L (Fig 2). However, Kruskal-Wallis ANOVA test showed that these observed shifts were not significant compared to the control ($P > 0.05$).

For Enviro glyphosate treated tadpoles, the rate of stage development varied between NF-stage 57 through NF-stage 62. Using the Kruskal-Wallis ANOVA test and Dunn's multi-comparison test there was no significant variation in developmental stage distribution of the treated

tadpoles compared to the control ($P > 0.05$). (Fig 3).

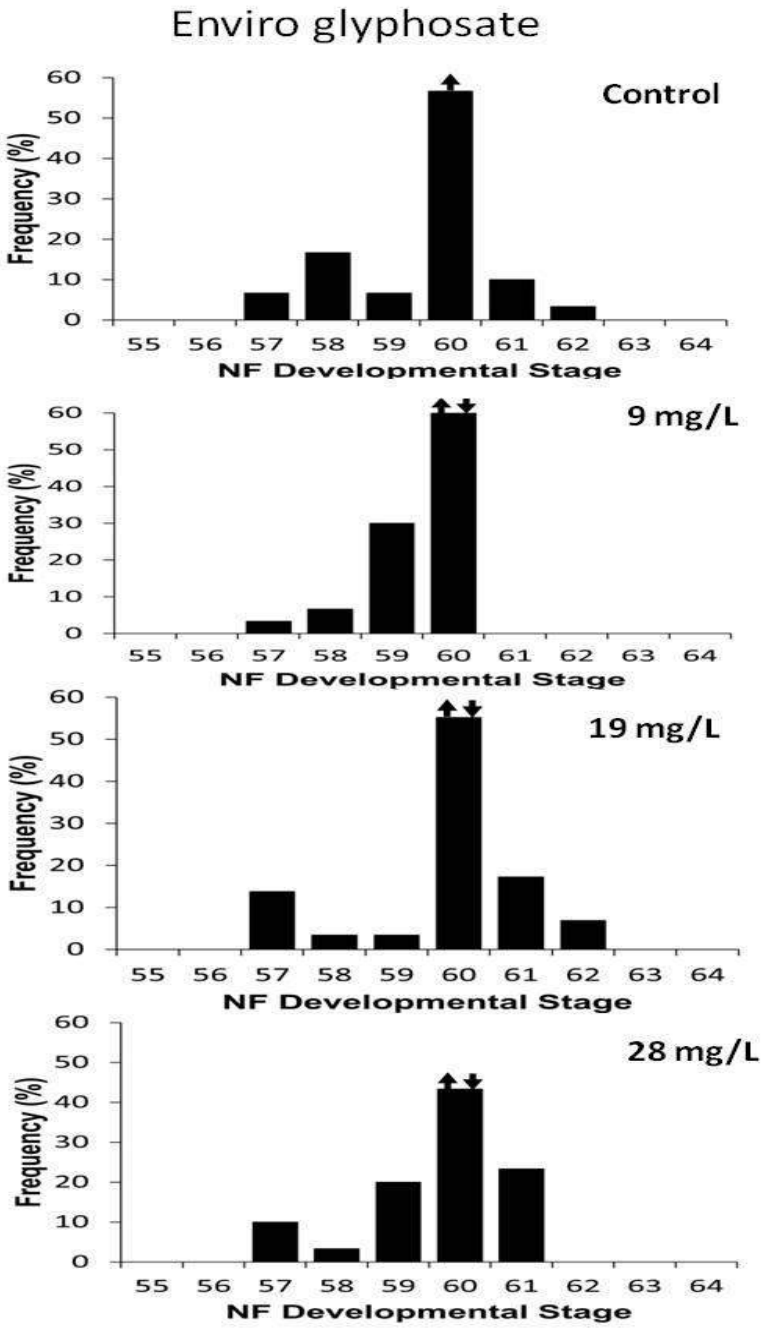

Fig. 3: The frequency distributions (n=20) of developmental stages (Nieuwkoop and Faber, 1958) attained by *X. laevis* larvae are exposed for 21-day to graduated concentrations of Enviro glyphosate (b) 9 mg/L, (c) 19 mg/L (d) 28 mg/L relative to the control (a). The upward arrow showed the median at the control relative to downward arrow that showed the median of developmental stage at the various concentrations

For developmental stages, comparing the NF developmental stages medians between the Kilo Max treated tadpoles and that of the control group, the Kruskal-Wallis ANOVA test showed significant variation in the median of developmental stages, this was further confirmed with multi-comparison analysis, which showed a significant delay in developmental medians at 190 mg/L and 280 mg/L relative to the control tadpoles (Fig. 4).

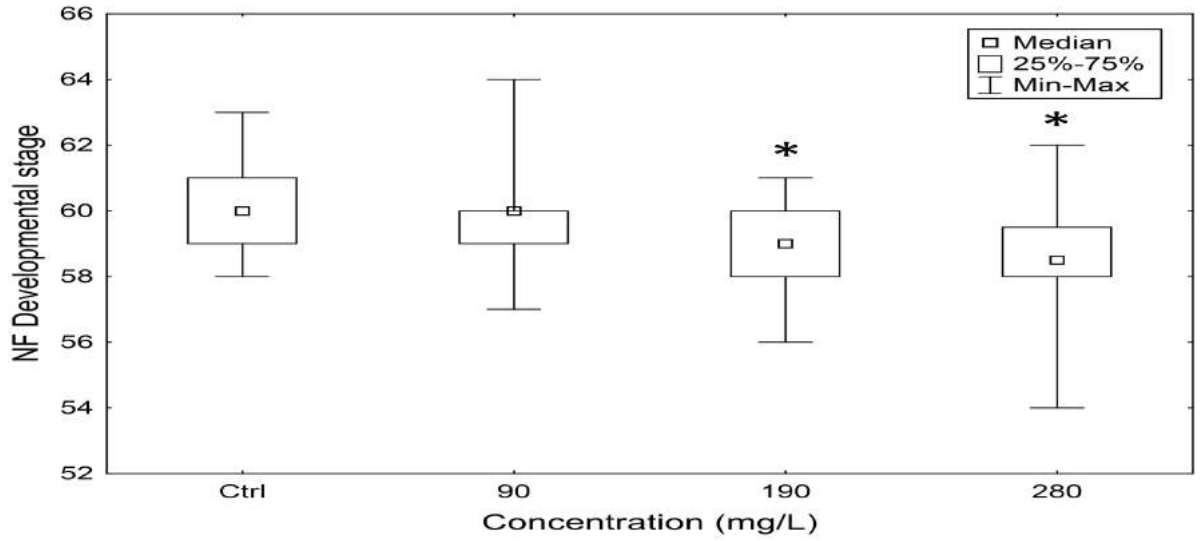

Fig. 4: Stage differentiation of *X. laevis* after 21-day exposure to graded concentrations of Kilo Max compared to the control. Asterisks indicate significant difference (DMCt; P< 0.05) from the control.

For Roundup formulation, the exposure showed no variation in the rate of the tadpoles development, as there was no variation in the median of developmental stages at all the exposure concentrations compared to the control exposure (Fig 5).

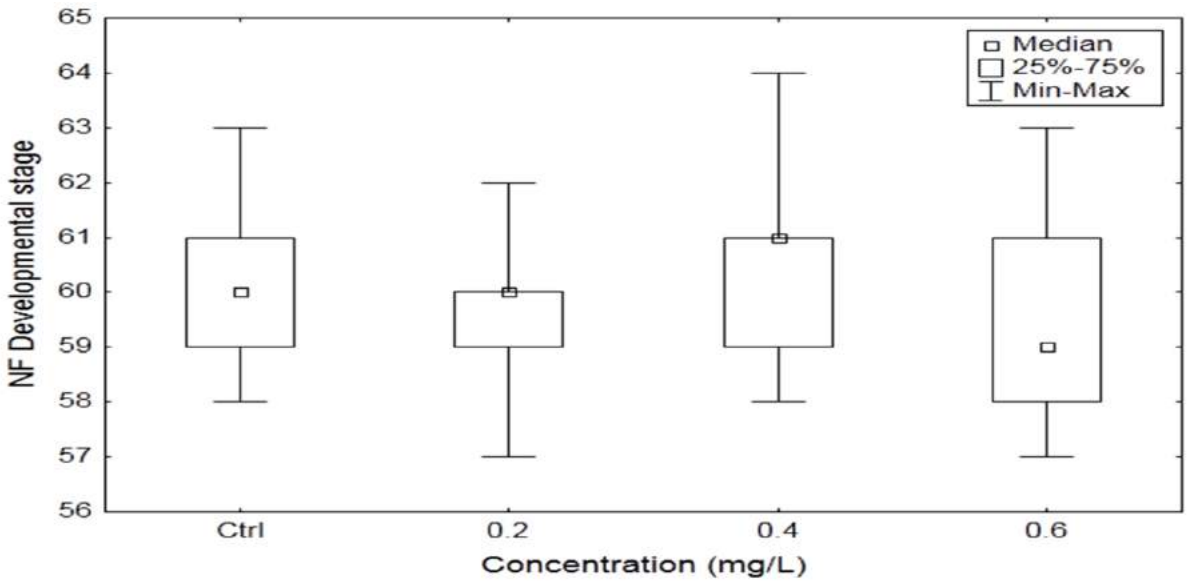

Fig. 5: Stage differentiation of *Xenopus laevis* larval following 21-day exposure to graded concentrations of 0.2, 0.4 and 0.6 mg/L of Roundup formulation compared to the control (Ctrl).

In the case of Enviro glyphosate, the rate of the development of the treated tadpoles showed no variation as the median of developmental stages remained constant at all the exposure concentrations as well as the control (Fig 6).

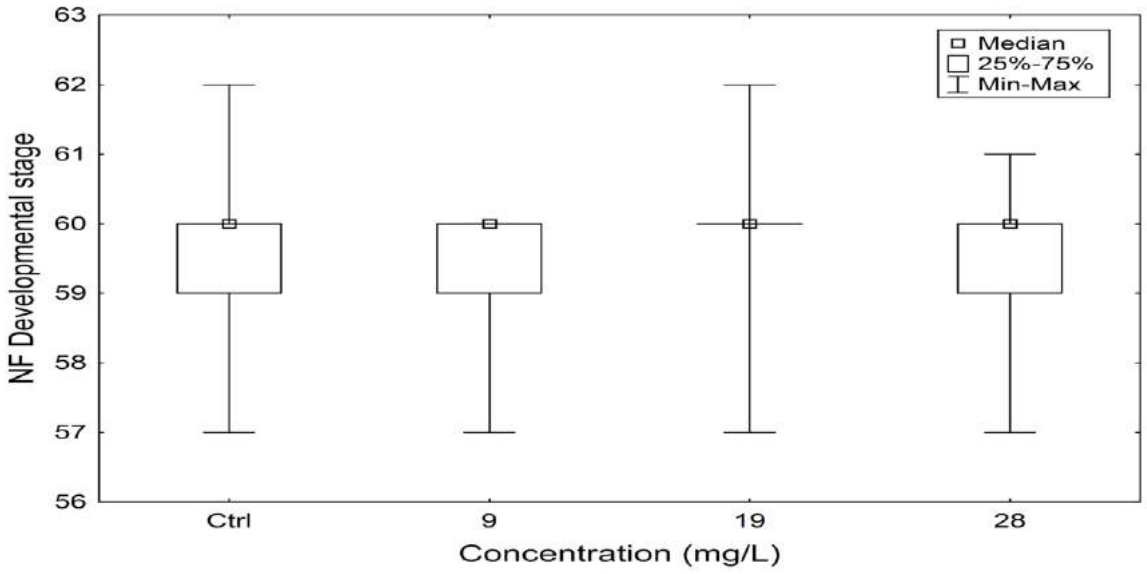

Fig. 6: Stage differentiation following 21-day exposure to graded concentrations of 9, 19, and 28 mg/L of Enviro glyphosate formulation compared to the control (Ctrl).

3.1.3. Morphometric Analyses in Kilo max Treated Tadpoles

The exposure of tadpoles to Kilo Max formulation significantly reduced the mean WBL (Fig. 7a) and SVL (Fig. 7b) at the two highest concentrations of 190 mg/L and 280 mg/L compared to the control (Tukey HSD test; $P < 0.05$). The Kilo Max formulation at all the exposure concentrations also showed a significant concentration dependent reduction in WBM compared to the control (Fig. 7c). The mean FLL (normalized) (Fig. 7d) also significantly increased at 90 mg/L compared to the control (Tukey HSD Test; $P < 0.05$).

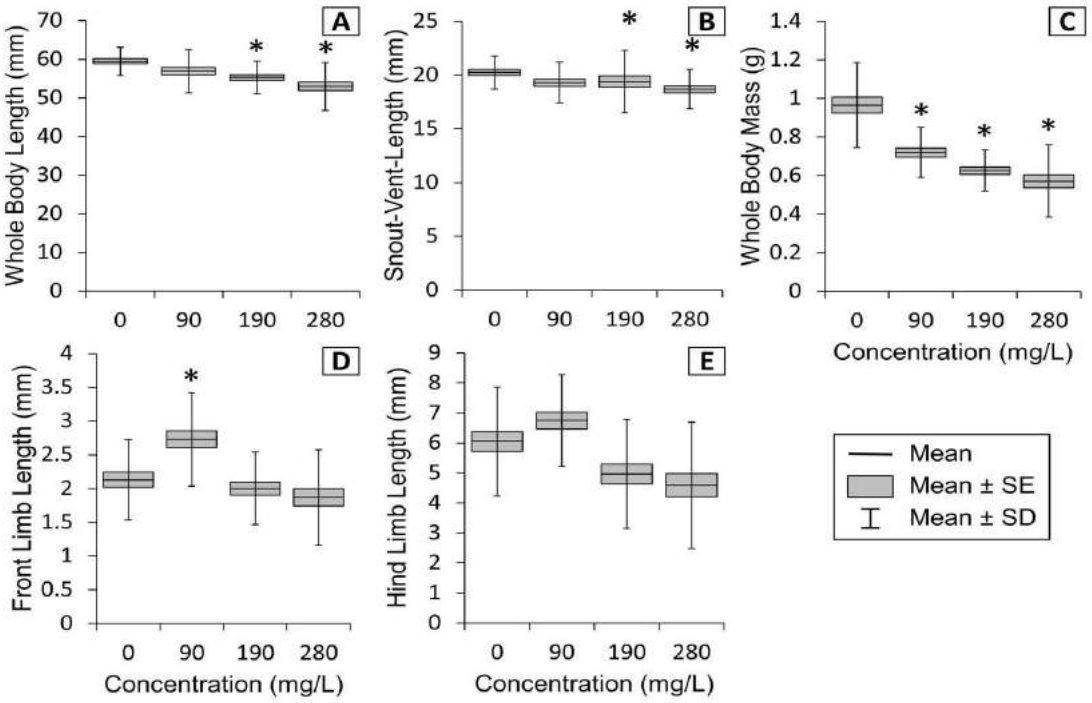

Fig. 7: Exposure impacts of graded concentrations of Kilo Max formulation on treated *Xenopus laevis larval* (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Front Limb Length, (e) Hind Limb Length. Asterisks indicate significant difference ($P< 0.05$) from the control.

The exposure of the tadpoles to Roundup formulation resulted in a higher mean WBL (Fig.8a) and SVL (Fig. 8b). But using the Kruskal-Wallis ANOVA test, followed by Tukey HSD multiple comparison test, only the mean WBL was significantly different (P< 0.05) at the lowest exposure concentration of 0.2 mg/L compared to the control. The Roundup treated tadpoles showed concentration dependent reduction in mean WBM (Fig. 8c), which was confirmed with the Kruskal-Wallis ANOVA test followed by Tukey HSD multiple comparison test at concentrations of 0.4 and 0.6 mg/L (P < 0.05) compared to the control.

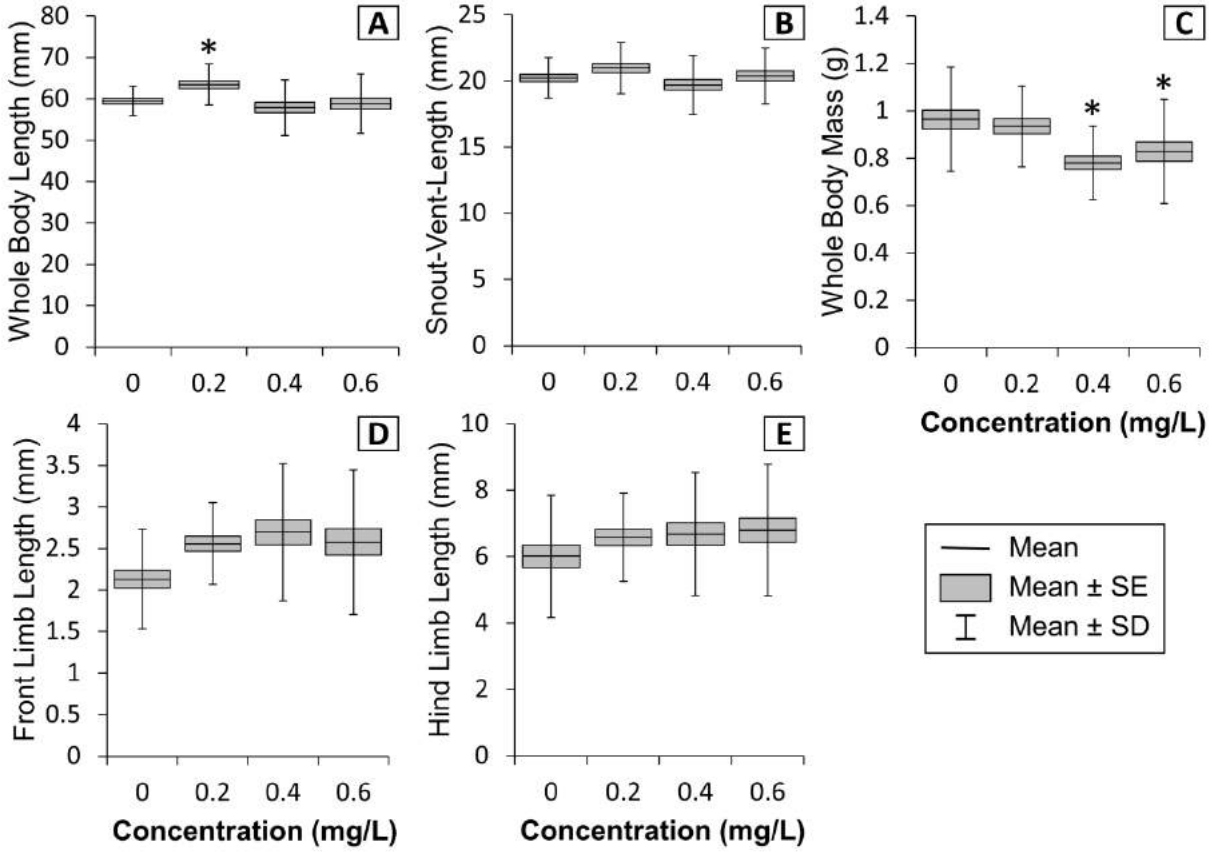

Fig 8: Exposure impacts of graded concentrations of Roundup formulation on treated *Xenopus laevis tadpoles* on (a) Whole Body Length, (b) Snout-Vent Length (c) Whole body Mass, (d) Front Limb Length, (e) Hind Limb Length. Asterisks indicate significant difference (P< 0.05) from the control.

The mean WBL (Fig 9a), WBM (Fig 9c) and SVL (Fig 9b) of the Enviro glyphosate treated tadpoles were lower compared to the control. This significant reduction was confirmed by the Kruskal-Wallis ANOVA test, followed by Tukey HSD multiple comparison tests at only 28 mg/L compared to the control (P< 0.05).

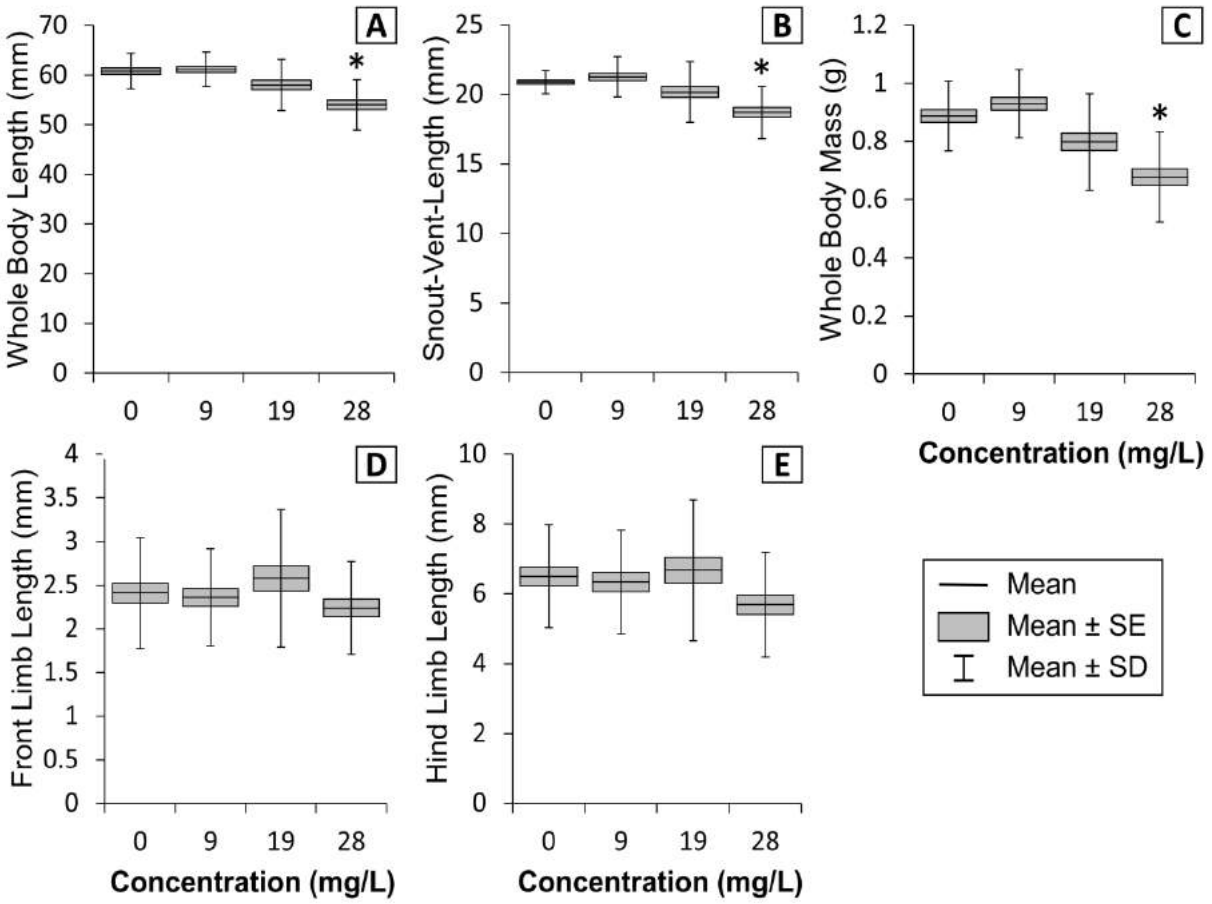

Fig 9: Exposure impacts of graded concentrations of Enviro glyphosate on treated *Xenopus laevis* tadpoles (a) Whole Body Length, (b) Snout-vent Length (c) Whole body Mass, (d) Front Limb Length, (e) Hind Limb Length. Asterisks indicate significant difference (P< 0.05) from the control.

3.1.4. Histopathological Endpoints

The exposure of developing tadpoles to Kilo Max formulation showed slight atrophy of both the gland area and the Colloidal area (Fig 10 b,c,d; Table 2)., which were not significantly different compared to the control (Fig 10 a). But the follicle epithelium showed evidence of hypertrophy, with a significant increase at all exposure concentrations compared to the control (K-W ANOVA Test; P< 0.05). Following the exposure to the Roundup formulation, the gland area (Fig 10e) and colloidal (luminal) area of the thyroid gland of the treated tadpoles showed significant increase in hypertrophy (P< 0.05) at the concentration of 0.6 mg/L compared to the control (Fig 10 a). The height of the follicle epithelium also showed hypertrophy with a significant increase (P< 0.05) at all exposure concentrations compared to the control (Table II). The exposure of tadpoles to Enviro glyphosate formulation showed evidence of atrophy of the colloidal area, with a significant reduction at concentrations of 9 and 19 mg/L (Fig 10 f and g) compared to the control (Fig 10 a) (K-W ANOVA Test; P< 0.05) (Table 2). The follicle epithelium also showed evidence of hypertrophy with a significant increase (K-W ANOVA Test; P< 0.05) at the two exposure concentrations of 19 and 28 mg/L (Fig 10 g and h) compared to the control (Fig 10a).. For the gland area, there was no significant difference in the treated tadpoles (P> 0.05) relative to the control (Fig 10 a).

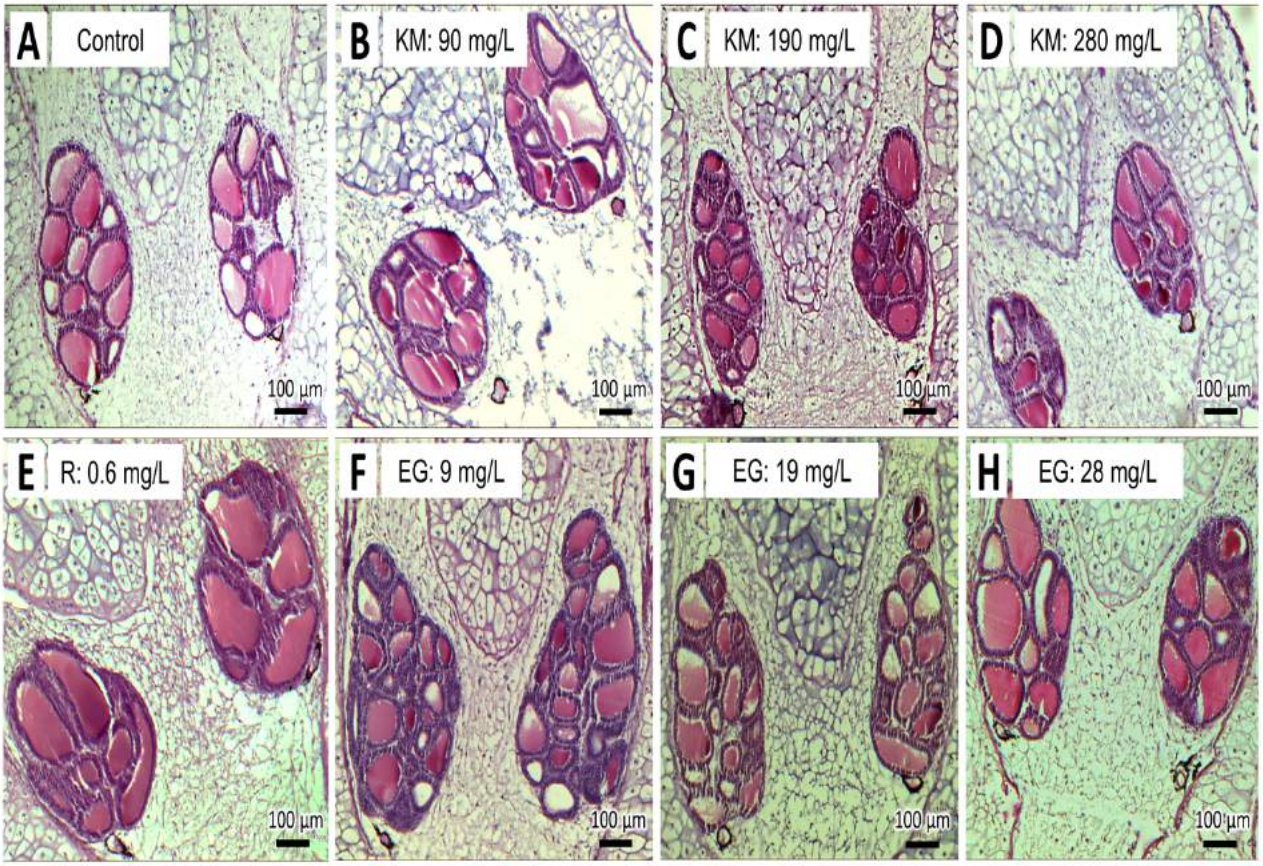

Fig 10: Histological cross-sections of the *X. laevis* thyroid glands exposed to graded concentrations (90-280mg/L) of the Kilo Max (10 b, c, d), and concentration (0.6 mg/L) of Roundup (10 e) as well as graded concentrations (9-28 mg/L) of Enviro glyphosate (EG) (10 f, g, h) relative to the control (10 a) (Mag 100X).

IV. DISCUSSION

All the tadpoles selected for this study were healthy. The control tadpoles passed through the metamorphosis at the expected international standard. The treated tadpoles at all the exposure concentrations of the three formulations showed no acute toxicity effect on the survival of the *X. laevis,* as no single tadpole died during the studies. The overall mean body mass and snout-vent length (± SD) for the control tadpoles after 21-day were 0.96 ± 0.23 and 20.23 ± 1.54 for Roundup, 0.96 ± 0.22 and 20.22 ± 1.56 for Kilo Max, and 0.99 ± 0.23 and 20.24 ± 1.78 for Enviro Glyphosate. These size and length values were even slightly higher than the recommended international standard as spelt out in OECD's phase 1 pre-validation study with NF stage 51 tadpoles, which has 0.94g and 19.5 mm as the mean WBM and mean SVL respectively (OECD, 2007). The control tadpoles were distributed between NF-stages 58-63 for Roundup and Kilo Max, and between NF 57-62 for Enviro glyphosate, with all their median developmental stage at NF-stage 60 at exposure termination. These stages are consistent with OECD, 2007 pre-validation guidelines recommendations that control tadpoles should reach a minimum median stage of NF 57 at the termination of the study (OECD, 2007).

The Kilo Max formulation significantly inhibited developmental stages at 190 and 280 mg/L but without impacting on the histopathology of the thyroid gland. The inhibition of developmental stages in this current study supported the earlier result of Babalola *et al.* (2019), where they showed concentration dependent growth inhibition of Kilo Max formulation on early larval stages of *X. laevis* at concentration of 190- 280 mg/L. This no impact on the thyroid gland, coupled with reduction in the tadpoles' sizes,

body mass and whole-body length suggests a non-thyroidal action, and extra-thyroidal toxic activities (Coady *et al.*, 2014). As noted by Fort *et al.* (2011), non-thyroidal action occurs when a toxicant affects whole body length, snout-to-vent length and whole-body mass, but not hind limb length and thyroid gland histology. As also pointed out by Optiz *et al.* (2005), total blockage of thyroid hormone (TH) synthesis and thus complete inhibition of metamorphosis does not necessarily inhibit growth of the tadpoles. This means that the concentration dependent growth inhibition observed in tadpoles treated with the Kilo Max is non-thyroidal at the current exposure concentrations. These non-thyroidal effects supported the findings in Babalola *et al.* (2019), where they noted that Kilo Max formulation is not a growth disruptor as its minimum concentration inhibiting growth (MCIG) ratio of 0.82 exceeded that of 0.32 benchmark ratios for growth inhibiting potential (Bantle *et al.,* 1999).

The tadpoles exposed to Roundup formulation showed a significant reduction in MBM at concentrations of 0.4 and 0.6 mg/L. This finding is consistent with the result of Howe *et al.* (2004), who noted that Northern leopard frog (*R. pipiens)* tadpoles were significantly smaller compared to the control tadpoles when exposed to Roundup formulation. This reduction in body mass in both *R. pipiens* and *X. laevis* species is possibly a physiological response to toxic effects of the Roundup formulation, as the same effects were observed in tadpoles exposed to the POEA surfactant alone (Howe *et al.*, 2004). This current reduction in body mass, particularly at concentration below the expected environmental concentration of Roundup at 1.43 mg/L (Govandarajulu, 2008) could have serious implications on the growth and reproduction of the amphibians in the wild. Several researchers have shown numerous implications of the size reduction, including increased chance of predation, and possible influence for lower survival rate and later reproductive fitness (Howe *et al.*, 2004; Gupta, 2012). The size reduction may also have strong downstream effects on adult phenotype and fitness (Dmitriew and Rowe, 2011). This means that the reduction in *X. laevis* body mass in tadpoles treated with Roundup formulation will have negative impacts on reproductive fitness and subsequently wider effects on the wild population.

The developmental stages of tadpoles treated with Roundup formulation was not significantly affected when compared to the control group. This result is consistent with the previous findings from our research group (Babalola *et al.*, 2019), where the Roundup formulation at concentrations of 0.5 -1.3 mg/L showed no significant inhibitory effects on the growth of embryo and early larval stages of *X. laevis*. The current results are also consistent with the findings of Lanctot *et al.* (2013), where they reported that Roundup formulation did not affect the growth of wood frog (*Lithobates sylvaticus)* tadpoles. However, the histopathological evidence in the thyroid gland showed inhibiting activities. The hypertrophy of the thyroid gland with a significant increase at concentration of 0.6 mg/L, coupled with hypertrophy of the follicle epithelium height at all exposure concentrations, as well as hypertrophy of colloidal area (goitre phenotype), at a concentration of 0.6 mg/L, showed the interaction of the Roundup formulation with the thyroid pathway. This occurrence of goitre phenotype may be an indication of thyroid hormone synthesis, either by modulating iodine uptake or biosynthesis of THs through hyperthyroidism (Mirata and Ose, 2012). That there is inhibitory effect on thyroid at the current highest exposure concentration of 0.6 mg/L suggests the initiation of inhibitory processes which normally commences with alteration in thyroid gland at concentrations below the onset of reduction in tadpoles developmental stages, majorly due to the compensatory growth responses (Carlsson *et al.,* 2019). This is consistent with the result of Howe *et al.* (2004), who noted the inhibitory impacts of Roundup formulation on the *R. pipiens* tadpoles. As noted by several researchers, low level of thyroid hormones (THs) usually activates the HPT for increased secretion of TSH from the pituitary gland, resulting in excessive stimulation, and an increase in thyroid gland size (OECD, 2008).

For Enviro glyphosate formulation, both the developmental stages and histological evidence

showed no significant difference between the treated tadpoles and the control. However, the reduction in body mass, SVL and WBL at concentration of 28 mg/L, also suggested the involvement of toxic properties of the formulation rather than the thyroidal activities (OECD, 2008; Fort *et al*., 2011). The reduction in body mass, SVL and WBL is consistent with the results of Babalola *et al*. (2019), where it was noted that exposure of embryo-larval to the Enviro glyphosate formulation resulted in a significant growth inhibition at concentration of 440 mg a.e. /L compared to the control. According to them, the Enviro glyphosate MCIG of 0.94 as against the benchmark of 0.30 makes the formulation a non-growth disruptor. Therefore, Enviro glyphosate formulation does not have thyroidal effects on the growing *X. laevis* at the current exposure concentrations.

From the exposure impacts of these three glyphosate formulations, it is clear that the exposure produced two varieties of actions. The first is the total growth inhibition that occurred in the Kilo Max and Enviro Glyphosate formulation, where the reduction in tadpoles' size, body mass, SVL and WBL, without gland alteration compared to the selective reduction in body mass and alteration in gland histology as shown in Roundup treated tadpoles. Several deductibles of interest can be obtained from this result including concentrations at exposure impacts, the potential contributions of active glyphosate and the potential role of surfactants .

The total growth inhibition observed in both Kilo Max and Enviro glyphosate formulations occurred at higher concentrations compared to the observed effects in Roundup formulation. This means that higher concentrations of active glyphosate are involved in the observed impacts of Kilo Max and Enviro glyphosate than in the Roundup formulation. This fact effectively rules out the active glyphosate as the direct cause of thyroidal activities as observed in Roundup formulation. This support the findings of Howe *et al*., 2004 and Turhan *et al*., 2020 that the toxic effects in glyphosate formulations is not a function of the active glyphosate, but rather the effects of the surfactants

On the role of added surfactants, relying on the two partitioning of the results again, the total growth inhibition as occurred in Kilo Max and Enviro glyphosate formulations without gland alteration compared to the selective body mass reduction and gland alteration in Roundup clearly indicates the role of added surfactants. That the Kilo Max (with an unknown surfactant and Enviro glyphosate with polyethylene alkalamine surfactant only caused general toxic action, without impacting the thyroid gland compared to the POEA in Roundup, which caused specific thyroid-driven body mass and gland alteration. This shows that the surfactants in Kilo Max and Enviro glyphosate are not thyroid active compared to the POEA in the Roundup formulation

It is evident from the current exposure study using the three formulations that the active glyphosate ingredient is not mediating the thyroid-disrupting activity. In addition, the surfactants in both Kilo Max and Enviro glyphosate only produced toxic impacts but are not thyroid active, unlike the POEA in Roundup that disrupted the thyroid-driven activities in the treated tadpoles. The issue of surfactants have always been very controversial for several reasons. First, the identity of the surfactants are usually shrouded in secrecy by the manufacturers of the pesticides under the guise of trademark protection. This makes it generally difficult to identify the surfactant, and access their exposure impacts as well as environmental effects. As noted by Mesnage *et al*., 2019, the issues of surfactant could be very confusing. According to them, hiding the identity of the surfactant by the producers usually makes it difficult to identify the toxicity of the co-formulas, and makes it even harder to specify their health effects both on human beings and the environment.

V. CONCLUSION AND RECOMMENDATIONS

In this study, it is very obvious that the three glyphosate formulations showed two opposing reactions on the growth and development of the treated tadpoles. The Roundup formulation, even at a low environmental relevant concentration of 0.6 mg/L, significantly increased the thyroid

gland, hypertrophy of the follicle epithelium height at all exposure concentrations, and hypertrophy of colloidal area at concentration of 0.6 mg/L, showing the inhibitory potential of the Roundup formulation on thyroid homeostasis. The Kilo Max formulation, even though significantly inhibited the developmental stages of treated tadpoles, shows similar no effects, just like Enviro glyphosate formulation on the histopathology of the thyroid gland, suggesting a no thyroid-disrupting activities, but rather other toxic impacts.

These observed impacts from these formulations clearly showed three important points, the active glyphosate play no role in thyroid activities and, therefore, is not thyroid active, the surfactants in Kilo Max and Enviro glyphosate formulations are not thyroid active, unlike the POEA surfactant in Roundup, which is thyroid active and not biologically inert as already pointed out by many studies This result confirms that the new emerging surfactants have differential characteristics compared to the old POEA surfactant. This study also showed that the activities of these new glyphosate formulations are likely the mirror of their surfactant, particularly in regard to the thyroid activities. Therefore, assessment of the thyroidal activities of aquatic herbicides should be mandatory before herbicides are approved for application.

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Data Accessibility

The data are available online in the doctoral thesis of the lead author.

Ethical care Statement

Xenopus laevis used for this study were collected, cared for, and treated under strict compliance with all ethical practices and law.

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