Imazapyr herbicideformulation induced multiple abnormalities in gonadal development of Xenopus laevis at environmentally relevant concentrations

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Research Article

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Abstract

Mankind is now in the era of environmental contamination and pollution, where the environment has become a warehouse of its several toxicants. In fact, there are no longer any pristine area without these anthropogenic toxicants, with serious consequences on wildlife's physiological processes including growth and reproduction. The widespread occurrence of pesticides for example, is being linked to numerous reproductive malformations in wildlife organisms, but the degree of association has not been characterised. Using the extended Xenopus Metamorphosis Assay (XEMA) protocol, the exposure impacts of imazapyr herbicide formulation (Arsenal), approved for aquatic environment was assessed on gonadal development of *Xenopus laevis* at environmentally relevant concentrations of 0.5, 2.0, and 3.5 mg/L. The formulation significantly reduced the mean body mass at premetamorphosis (NF-stage 55) at 3.5 mg/L concentration compared to the control. In sex ratio, the exposure only showed marginal fluctuations at all the exposure concentrations. For gonadal malformations, an abnormality index of 17.5%, 25%, and 35% was derived at 0.5 mg/L, 2.0, and 3.5 mg/L concentrations respectively, with malformations including tissues separation, segmented aplasia, aplasia, mixed sex, narrow hypoplasia, and angular deformity. This study showed that at relevant environmental concentrations, this formulation induced concentration dependent complex gonadal malformations, suggesting its potential capacities to induced serious reproductive disruptions that can negatively impacts fecundity, fertility, and general reproductive fitness of amphibians. In order to protect the wildlife from reproductive impacts, there is a serious need for cautions in application of this herbicide formulation

1. Introduction

The environment today has now become a warehouse for several anthropogenic chemicals, which has penetrated to all environmental compartments. There are no longer any pristine areas without environmental toxicants globally (WHO, 2013). The intensive use of many pesticides including herbicides, insecticide, fungicides and acaricides, has led to ubiquitous contamination being present in many environmental media (Egea-Serano *et al*., 2012; Munoz *et al*., 2015). Though the effects of these contaminants may vary among the different taxa (Hoffman *et al*., 2003), the widespread nature of various contaminants play a role in general deterioration of health in the wildlife (WHO, 2013), and assessing the risk of these contaminants on reproductive system is now an imperative task (Bokony *et al*., 2020).

Global declines of many wildlife, particularly in amphibians, have been linked with a wide range of environmental pressures and stressors, including diseases, climate change, habitat loss, and pollution (Orton and Tyler, 2015). In the area of pollution, a wide range of toxicants have shown negative impacts on reproductive health of wildlife including Fish (Jobling *et al*., 2002; Jobling *et al*., 2008; Scholz and Kluver, 2009; Khan *et al*., 2015), amphibian (McDaniel *et al*., 2008; Mann *et al*., 2009), reptiles (Crain, 1997; Crain *et al*., 1998) and bird (Giesy *et al*., 2003).

Despite the current documentation of amphibian decline and malformations, there are still only few reports on the use of amphibian as models for abnormalities of reproductive system and processes due to effects of pesticides pollution (Khan and Law, 2005; Bokony *et al*., 2020). In spite of diverse evidence from numerous field studies showing that the reproductive and development of wildlife organisms may have been impacted by anthropogenic chemicals (Khan and Law, 2005; WHO, 2013; Bokony *et al*., 2020), very little is still known about the ecological impacts of many of the environmental toxicants affecting the reproductive system. Of particular importance are the aquatic herbicides that are approved for the treatment and management of aquatic weeds by various international bodies, including UNEP, USEPA, and European Union. These herbicides include Imazapyr, Glufosinate ammonium, Diquat dibromide, some glyphosates, Endothall, Fluridone, Copper sulfate etc

Imazapyr is one of the globally used herbicides. It is mobile and persistent, with half-life of about 50 months on soil surface (Vizantinopoulon and Lolos, 1994). Imazapyr is one of the aquatic herbicides approved for the management of weeds in aquatic environment (Yahnke *et al*., 2013). It has several formulations including Arsenal, Chopper and Format. The Arsenal formulation consists of 25 g/L active Imazapyr, 186 g/L of ammonium hydroxide, 18 g/L of nonyl phenol ethoxylate (9 ethoxylated rings) and water (Grisolia *et al*., 2004). Concentrations of up to 5.7 mg/L have been measured in surface water and treated sediment with no overlying canopy, according to the Washington State Department of Agriculture (WSDA, 2003). . This current study therefore aimed to assess the exposure impacts of Imazapyr herbicide (Arsenal formulation) on sexual development and

reproductive health, using *X. laevis* as sentinel species. This study intends to add to the emerging body of knowledge on the impact of pesticides on wildlife reproductive system.

2. Materials And Methods

2.1. **Test chemical**

The herbicide formulation: Arsenal (250 g/L, Imazapyr) (BASF Chemical Ltd., South Africa).

2.2. **Test organisms**.

Care of *Xenopus laevis* and Breeding of Tadpoles

Two sexually mature males and females frogs, were selected from the University in-house healthy breeding stock of *X. laevis*. The male and female were maintained in separate 15 L glass tanks containing buffered reverse osmosis (RO) water (2.5 g iodated sea and 0.8 g Na HCO_3 salt/ 10 L) (Kloas *et al*., 1999). They were fed three times per week with fish pellets (Aqua-Nutro, South Africa). Breeding induction was performed following the ASTM, (1998) protocol. Briefly, the males and females were primed, four days prior to the commencement of mating, 100 IU of human chorionic gonadotropin (hCG) (Merck Ltd, Germany), was injected into their dorsal lymph sac. The males and females received further 100 and 300 IU respectively just prior to mating. The breeding pair of male and female was then placed together in a new 15 L tanks lined with plastic netting (to separate the adults from the eggs). The breeding glass tanks were placed at a well-ventilated but quiet corner, away from human disturbances. The eggs were well aerated to protect them from inadequate oxygen supply. The emerging tadpoles were distributed into several 15 L tanks containing 10 L buffered RO water at a density of 40 tadpoles per tank. They were fed with Sera Micron (Sera, Heinsberg, DE) containing 50.2% protein, 8.1% fat, 4.2 % fibre, and 11.0 % ash (Lutz *et al.,* 2008) 30 mg/animal/day initially, which was later increased to 50 mg/animal/day in order to account for the increased in sizes. The feeding was done until they attained the desired NF-stage 51. All the breeding and husbandry procedures were approved by the Animal Research Ethics Committee of the Stellenbosch University, Stellenbosch, South Africa (Approval no- SU-ACUM 12-00015**).**

2.3. **Test procedure**

2.3.1. Exposure set-up

At NF stage 51, a total of twenty randomly selected tadpoles were allocated from holding tanks and transferred into 15 L exposure tanks containing 10 L of reversed osmosis water, and replicated twice at each of the selected concentrations (n=40). Following the experimental protocol by Organization for Economic Co-operation and Development (OECD), 2008), the exposure experiment was done in a controlled climate room with the following physical conditions; water temperature of 24 ± 1 ° C, pH ranging between 7.5 - 8.5, dissolve oxygen concentration of >6.5 mg/L and a 12 hours of light and dark photoperiod ($L_{12}D_{12}$). The exposure concentrations were centred on 15, 30, and 45 % of 96 hour LC_{50} of the herbicide at NF 48 of *X. laevis* (Babalola and Wyk, 2018*).* The exposure medium was completely changed every three days, after each feeding regime of Sera Micron. The mixture of this Sera Micron feed and water dilution have been tested for both estrogenicity and androgenicity using yeast estrogen screen (YES) and yeast androgen screen (YAS) (De Boever *et al*., 2001). At the end of the exposure, the metamorphs were collected, weight, dissected, and assessed for various reproductive malformations and the carcasses were preserved for future references.

2.3.2. Exposure Concentrations

Three exposure concentrations were selected which was centred around environmental relevant concentrations of 5.77 mg/L (based on the 15, 30, and 45%).

2.3.3. Nominal Concentration test

Samples of the exposure water were taken one hour after the introduction of the herbicide, to confirm the exposure concentrations of the formulation. The exposure water samples were stored in icebox at temperature of -5 ^{O}C and sent to Envirotech Laboratory, Lagos Nigeria for analysis using the gas chromatography, at detection limit of 0.05 µg /L. The concentrations detected showed low variations relative to the predicted nominal concentrations (Addendum Table 1).

2.4. Survival and Developmental Disruption

The embryonic survival rate was assessed at 96 hour post hatching of the tadpoles. Growth (size) disruption was also assessed using body mass (to the nearest gram) at prometamorphic (NF-stage 55) and at metamorphosis (NF-stage 66), compared to the control. Gonadal sex ratio was assessed using Leica DMLB light microscope and image analysing software, which were then compared between the exposure concentrations and to that of the control. Histological analysis was performed to distinguish the sex tissues of the males and the females.

2.5. Assessment of Gonadal Morphology

Gonadal morphological malformations were assessed by dissecting the young metamorphs at the gastrointestinal cavity, to expose the gonads. Using a dissecting stereo microscope, the sex tissues were categorised as testes, ovaries or as improper formation of part(s) of the organs and the gonads were assessed for gross morphology abnormalities using the following characteristics by Lutz *et al*. (2008):

2.6. Histological Sex Validation

Microscopic views of testes and ovaries were validated by histological examination of 10 selected males and females. The males were identified using the absence of ovarian cavity and the growth of germ cells in the medullar region. For the females, they were identified using the presence of ovarian cavity and the growth of germ cell in the cortical region (Fig. 1)

2.7. Data Analysis

Normality and homogeneity of variance was evaluated using the respective Shapiro-wilk's and Levene's tests. Mean body mass at pro-metamorphosis and at the metamorphosis were compared to the respective untreated controls using one way ANOVA, followed by DUNN'S multiple comparison of mean rank test for non-parametric data. Differences in gonadal sex ratio at each of the concentrations and control were analysed with Chi (X^2) square test using Graphpad online software (Graphpad Software Inc., USA).

3. Results

3.1. Survival

The 96 hour post-hatching survival rate of tadpoles was 96 %.

3.2. Body Mass Effects

The mean body mass (MBM) of the *X. laevis* juveniles exposed to this formulation at prometamorphic (NF-stage 55) fluctuated in concentrations dependent manner. The mean body mass increased from 0.9 g at a concentration of 0.5 mg/L to 0.97 g at a concentration of 2.0 mg/L, and to 0.91 g at 3.5 mg/L compared to the 0.8 g of the Control (Table 3). The increased body mass was statistically significant ($P < 0.05$) only at 3.5 mg/L concentration compared to the Control. At metamorphosis (NF-stage 66), the mean body mass (MBM) increased in concentrations dependent manner, which were not statistically significant at all the exposed concentrations relative to the Control (Table 3).

3.3. Sex Ratio Effects

The percentage sex ratio of the *X. laevis* metamorphs following exposure to this formulation only showed marginal effects. The ratio moved from 47.5:52.5 (F: M) at a concentrations of 0.5 and 2.0 mg/L down to ratio of 45:55 (F: M) at a concentration of

3.5 mg/L compared to ratio of 47.5:52.5 (F: M) in the Control. None of the ratios were significantly different compared to the Control (Fig. 3).

3.4. Gonadal Malformation Effects

The Arsenal formulation induced numerous gonadal morphological malformations in the treated juveniles. At 0.5 mg/L concentration, the recorded abnormality index was 17.5 %. The observed abnormalities included tissue separation (the most widespread), segmented aplasia, aplasia, partly thin segmented hypoplasia, and angular deformity (Fig. 4). At a concentration of 2.0 mg/L, the abnormality index recorded increased to 25 % with abnormalities that included tissue separation, segmented aplasia, aplasia, hypoplasia, and angular deformity. The most widespread abnormalities were tissue separation (Table 4). At 3.5 mg/L concentration, the abnormality index observed was 35 %. The induced abnormalities included mixed sex, being the most widespread, aplasia, segmented hypoplasia, segmented aplasia, and narrow hypoplasia respectively (Fig. 4).

4. Discussion

Pesticides application (including herbicides, insecticides, fungicides etc) has continued to generate serious health and ecological concerns on non-target organisms (Diamanti-Kandarakis *et al*., 2009; Babalola *et al*., 2018). The reprotoxicity impacts of these pesticides for example are particularly worrisome giving the direct impacts of reproduction on recruitment and biodiversity. The exposure impacts of many aquatic herbicide formulations on reproductive organs and system of the non-target organisms like amphibians remains unclear (Taylor *et al*., 2005; Oka *et al*., 2008). This study examined the exposure impacts of imazapyr aquatic herbicide formulation on growth and development of gonadal/reproductive system of amphibian using *X. laevis*. The results showed that the imazapyr herbicide formulation impacts negatively on both the body mass of *X. laevis* at prometamorphosis stage and on their gonadal development through widespread gonad abnormalities.

Impacts on body mass.

The Arsenal formulation produced increased body mass at prometamorphic stage which was significantly different at the highest exposure concentration relative to the Control. At metamorphosis stage, the body mass also showed similar concentration dependent increased, but which was not significantly different to that of the control. Increased body mass at prometamorphic and metamorphic stage of development have being linked to exposure to estrogenic activities (Phuge and Gramapurohit, 2015). This suggests that this Arsenal formulation might be anti-androgenic or estrogenic. Therefore, the current increased in body mass could be a signal to anti-androgenic or estrogenic property of the Arsenal herbicide formulation. The impacts of such increased in body mass on the reproductive physiology, particularly fecundity and fertility could be huge, since the exposed frogs would have to divert many physiological processes and resources that could have been used for growth and reproduction to counter the exposure impacts of this herbicide formulation. This means that the exposed frogs will be impacted negatively on the growth and reproductive development.

Additionally, since concentrations of 5.78 mg/L have been measured in surface water and treated sediment with no overlying canopy (WSDA, 2003), suggesting that the frog could be facing higher concentration effects from the Arsenal formulation in the environment, compared to the current exposure concentration range of 0.5 mg/L to 3.5 mg/L. This mean that further studies at environmentally relevant concentration of 5.7 mg/L will help to clear all doubt on the impacts of this formulation on the body mass.

Sex Ratio

Although the sex ratios of the treated metamorphs showed no significant shift at the current highest exposure concentration of 3.5 mg /L, similar further study at expected environmental concentration (EEC) of 5.7 mg/L will be very important in clearing doubt of the potential effects and enrich the scientific and ecological understanding of this herbicide formulation on reproduction

Gonadal Malformations

Gonadal malformations, even though its various kinds has been noted to occur naturally in wild amphibian populations (Hanken, 1983; Read and Tyler, 1994), the severities of malformations only become worrisome when it show a dose dependent pattern as found in this current study. The occurrence of 18-35 % malformations in this formulation shows the high potential reproductive toxicity. As noted by several authors, the occurrence of gonadal abnormalities could lead directly to reproductive dysfunction, in a way that could negatively impair reproductive success of the exposed individuals (Quellet *et al.*, 1997; Howe *et al.*, 2004; Qin *et al.*, 2005; McCoy *et al.*, 2008; McDaniel *et al.*, 2008). The widespread nature of these pesticide induced amphibian gonadal malformations could also be a contributing factor to the global decline that is currently being witnessed (Qin *et al.,* 2005). Of particular interest is the occurrence of up to 35 % at concentration of 3.5 mg/L, which is still below the current environmental relevant concentration of 5.78 mg/L. This suggests that this formulation is a potential reproductive threat to amphibians in aquatic environment.

Conclusions

The evidence from this study have shown the capacities of Arsenal formulation (imazapyr) to negatively impair the reproductive fitness of *X. laevis* though increased body mass as well as induced gonadal malformations. That these observed effects are all at environmentally relevant concentrations of this formulation makes it more worrisome. Even though studies are still required to highlight the impacts of these effects on the reproductive fitness of the amphibians, especially the gonadal malformations effects, this study has laid a foundation moving forward. This study has also added to the growing body of evidence pointing to the needs for further review and assessment of the role of pesticides, particularly aquatic herbicides that are approved in managing aquatic weeds, in the etiology of numerous gonadal malformations as witness in this study. It is also important for continuous assessment of exposure impacts of many pesticides as they affect reproductive system in aquatic vertebrates and to develop early warning biomarkers of reproductive toxicant in aquatic system, which will help safeguard the aquatic biodiversity.

Declarations

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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 practises and law.

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Tables

Table 1: The selected exposure concentrations (based on the 15, 30 and 45% of 96 hour LC_{50} at NF stage 48) (Babalola & Wyk, 2018) for the Arsenal herbicide formulation.

Formulation	Concentration (mg a.e./L)
Arsenal (ERC)	0, 0.5, 2.0, and 3.5

Table 2: Gonad abnormality index characteristics as reported by Lutz *et al*. (2008)

Identifier	Description
Adhesion	Gonads that join to other abdominal tissues
Aplasia (agenesis)	Total lack of gonads development
Segmented aplasia	Longitudinal discontinuous gonads (e.g. tissue separation, extraneous gonadal tissue)
Bifurcation	Division of gonads oriented longitudinally
Angular deformity	Gonads that bends to an excessive degree (e.g. gonad folded)
Displaced gonads	Gonads or section of it not typically located (e.g. lateral, or medially)
Fused	The fusion of left and right gonads to a varying degree
Hypertrophy	Enlargement of gonads (e.g. wider, thick or enlarged)
Segmented hypertrophy	Excessive enlargement of one or more areas of the gonads (e.g. mass enlargement, pearling and partly thick)
Hypoplasia	Decrease in gonad's size (e.g. narrow, slightly narrow, and truncated, slightly truncated, thin and margin entire)
Segmented hypoplasia	Gonad where one or more parts are excessively reduced, attenuated, or poorly developed but not separated (including partly narrow, partly thin, margin slightly, margin partially entire and pearling)
Intersex	Gonads where ovary and testicular tissues are present in separate structures
Mixed sex	Gonads where ovary and testicular tissues are present in the same structure
Translucent	Gonads that appear not so dense (e.g. slightly translucent)
Segmental translucent	Section(s) of the gonads appears not so dense
Malformation index	The percentage of the observed abnormality

Table 3: The impacts of the Arsenal herbicide formulation on mean body mass (MBM) at both NF-stage 55 (prometamorphic) and NF-stage 66 (metamorphic stages) of *X. laevis*. Asterisks indicate significant difference from the control treatment

Herbicide Formulation	Conc. (mg/L)	MBM ± SD @ Prometamorphic (NF 55) (g)	MBM ± SD @ Metamorphosis (NF 66) (g)
Arsenal	0	0.8 ± 0.3	1.03 ± 0.15
	0.5	0.9 ± 0.18	1.05 ± 0.17
	2.0	0.97 ± 0.14	1.07 ± 0.22
	3.5	*0.91 ± 0.22**	1.13 ± 0.18

Table 4- Types of gonadal morphological malformations, Abnormalities Index, and % Sex ratio at various graded concentrations of the Arsenal formulation

Herbicide	Conc. (mg/L)	Types and no of morphological malformation	Abnormality Index (%)	% Sex ratio F:M
Arsenal	0	Segmented aplasia (1), narrow hypoplasia (1)	7.5	47.5:52.5
	0.5	Aplasia (1), angular deformity (1), segmental aplasia (1), tissue separation (2), partly narrow segmented (1), partly thin segmented hypoplasia (1)	17.5	47.5:52.5
	2.0	Aplasia (2), angular deformity (1), segmental aplasia (1), tissue separation (3), hypoplasia (2), slightly translucent (1)	25	47.5:52.5
	3.5	Mixed sex (5). Segmented hypoplasia (2), aplasia (4), segmental aplasia (1), narrow hypoplasia (1).	35	45:55

Addendum Table 1: The gas chromatography analytical result for Arsenal formulation (Imazapyr). The limit of detection was 0.05 µg/L.

Nominal (mg/L)	Detected A (mg/L)	Detected B (mg/L)
0	0	0
0.5	0.46	0.48
2.0	1.94	1.96
3.5	3.40	3.45

Figures

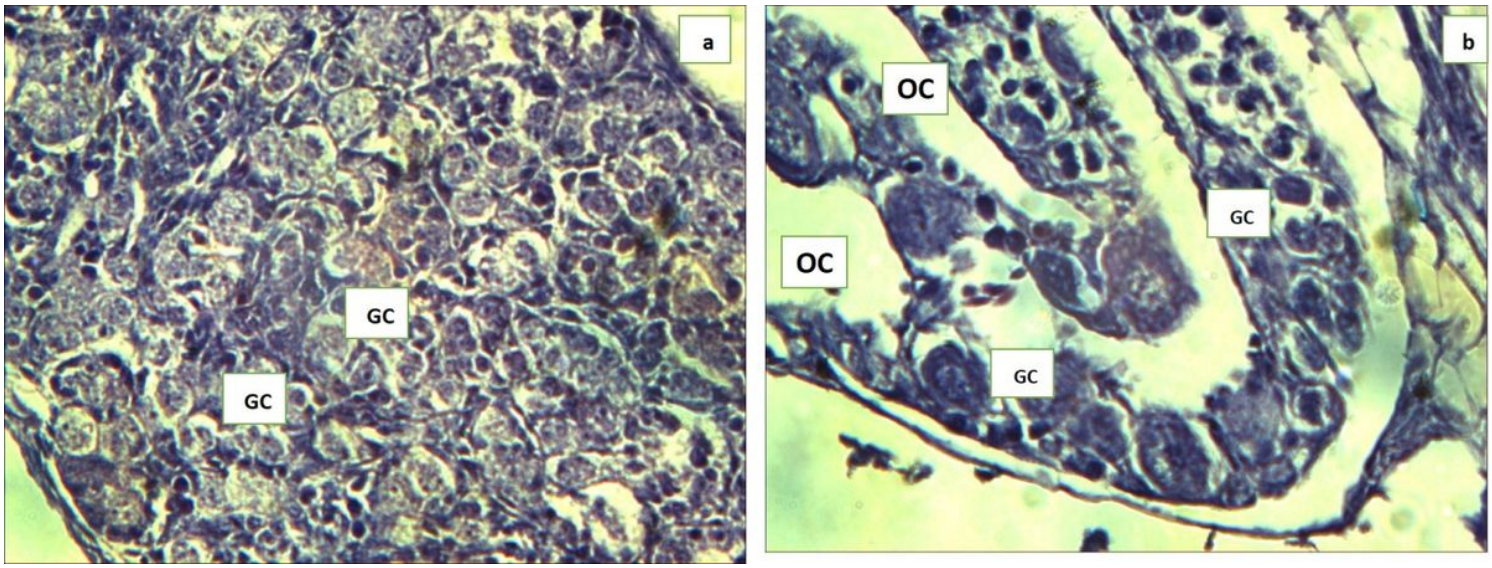

Figure 1

Histology (400X) for sex ratio determination of X. laevis (a) testis and (b) ovary

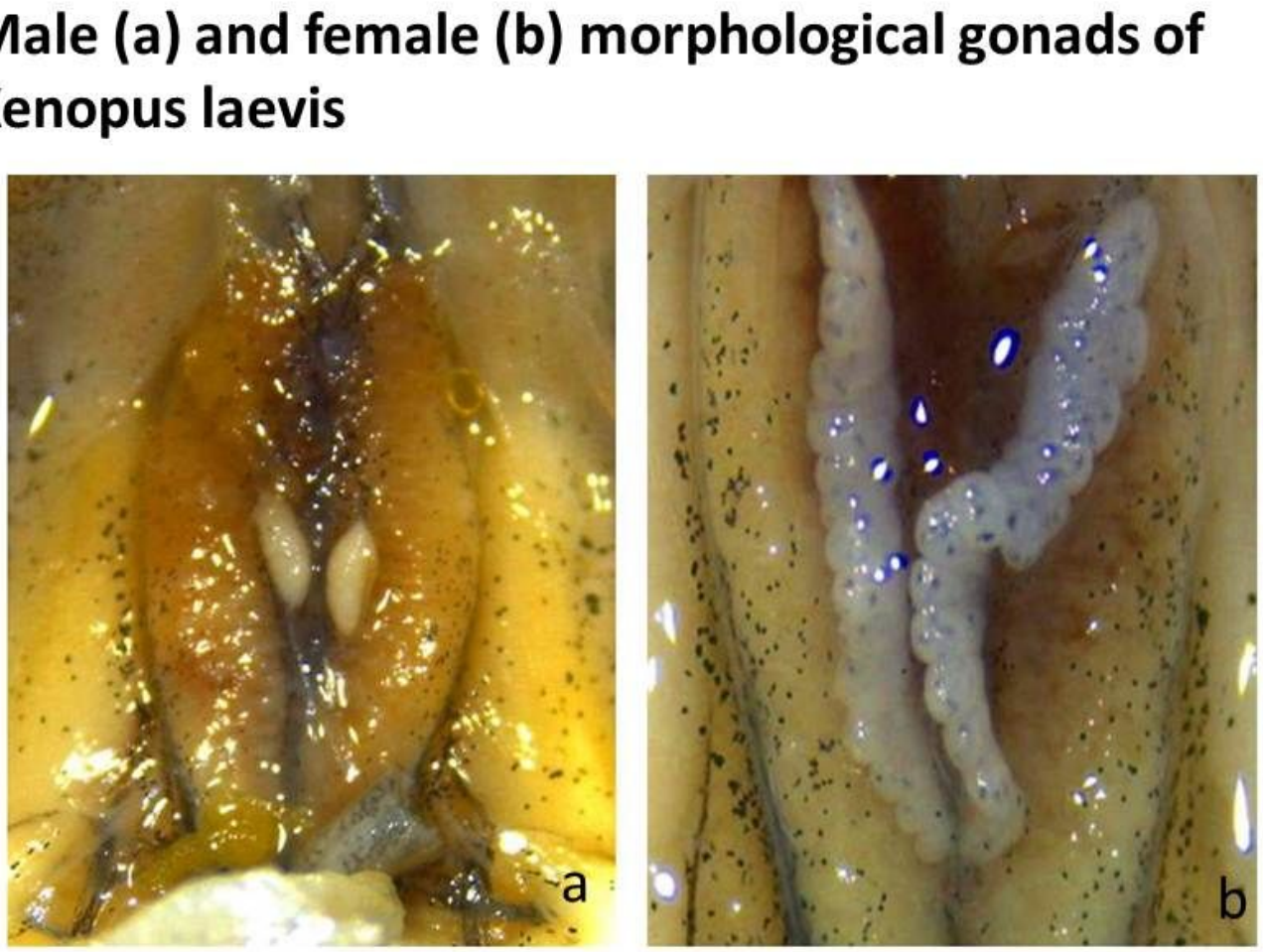

Figure 2

sample of male (a) and female (b) morphological gonads (100X) of X. laevis showing the testis (a) and the ovary (b).

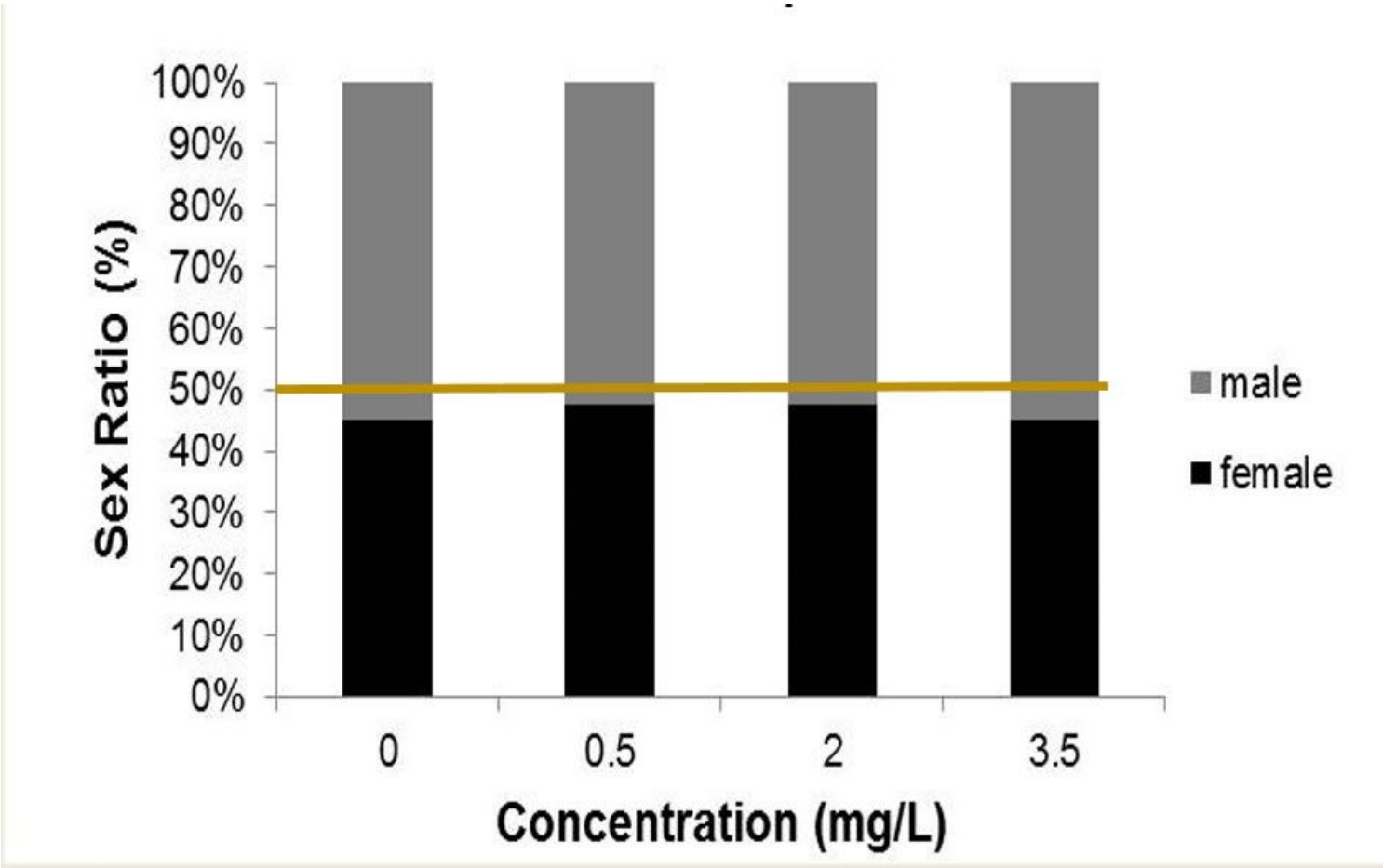

Figure 3

Percentages of sex ratios of Arsenal formulation exposed X. laevis compared to the Control. The horizontal line indicates the 50% average mark point.

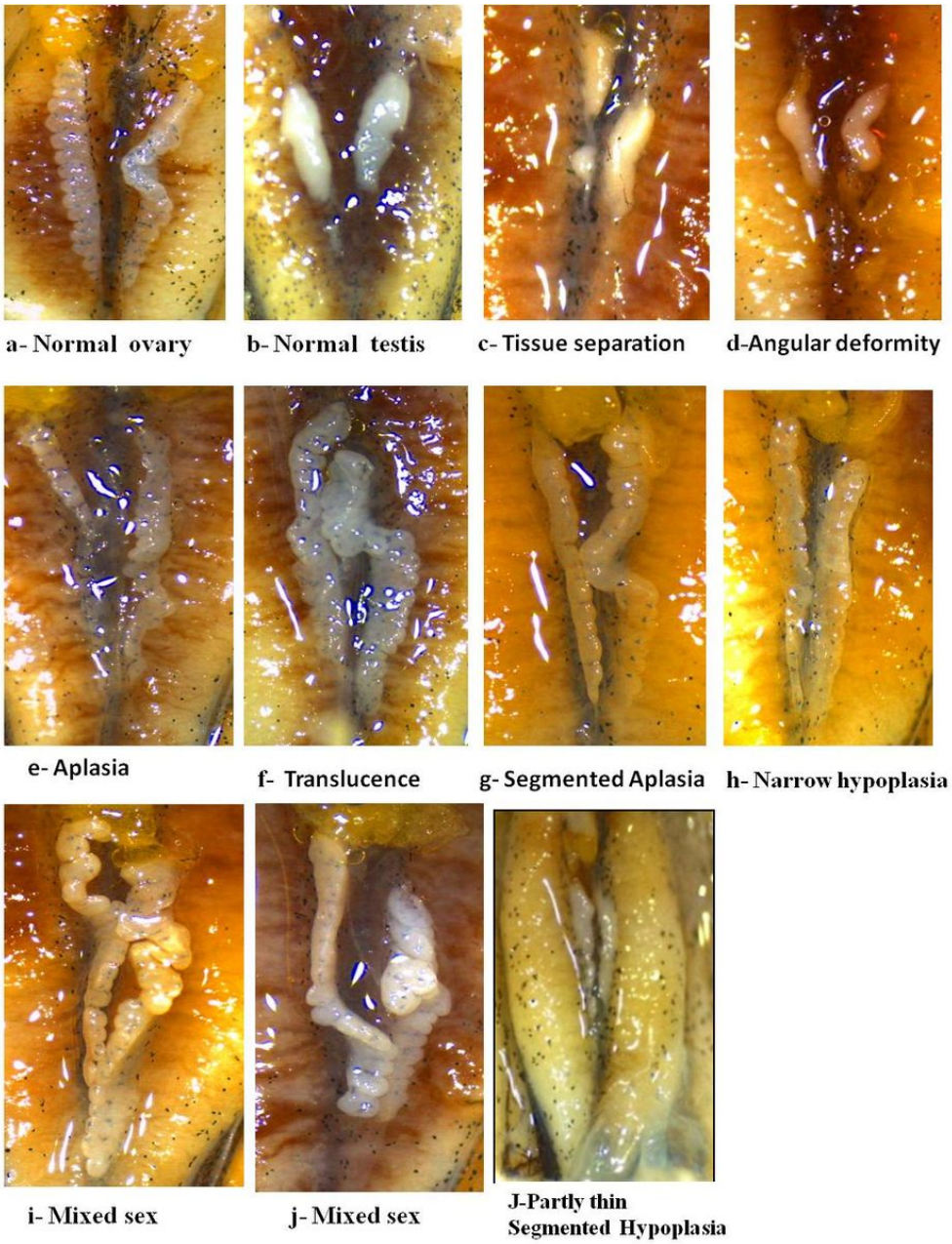

Figure 4

Various gonadal morphological abnormalities in X. laevis juveniles treated with relevant environmental concentrations of Arsenal herbicide formulation.