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Hermaphroditism induction of Sub-Lethal Dose of Atrazine and Atrazine-Nitrate on the Egyptian Toad, *Sclerophrys Regularis*

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Abstract: In this study, atrazine has been shown to act as a potent endocrine disruptor in amphibians either alone or in combination with nitrate under stable laboratory conditions, causing altered gonadal development at the sub-lethal concentrations. A control group and three treatments were tested; atrazine, nitrate, atrazine- nitrate treatments with doses of 300 µg/L, 200 mg/L and their combination, respectively. Atrazine exhibited increase the presence of testicular oocytes TOs in most treated specimens; furthermore, the sizes of seminiferous tubules were decreased compared to control group. Conversely no intersex individuals were detected in the nitrate treatment alone. The combined effects of nitrate and atrazine were not additive or synergistic but were similar to the effects of atrazine in raising the percentage of intersex but with increasing the size of testicular oocyte compared to atrazine alone. On the other hand, the control group in the present study did not contain the rudimentary testicular oocytes and showed normal structures. These gonadal abnormalities may reduce reproductive success and could be a major factor behind amphibian declines. Also, these negative impacts may be a bioindicator alarming the ecosystem disrupting caused by the uncontrolled apply of these chemicals in agriculture.

Keywords: Atrazine, Nitrate, Intersex, Amphibians, *Sclerophrys Regularis*

1. Introduction

Amphibians are important ecological components of both wet and dry lands [1, 2]. Among vertebrates they are distinctive in many ways and contribute in many ecosystem services, so they have an important ecological and human role. Amphibians, a unique group of vertebrates containing over 6,300 known species, are threatened worldwide [3] and the number of extinct and threatened species will probably continue to rise [4]. There is a little evidence for a single factor causing this decline but multiple factors as increased ultraviolet radiation, fungal and bacterial epidemics, droughts, climate changes, habitat destruction and fragmentation, exotic species, heavy metals, acid rain, pesticides can act together to cause mortality or sublethal effects.

The present study will intensely focus on the atrazine and nitrate as the most common herbicide and fertilizer, respectively addressing their role in amphibian decline in general and their effect on *Amietophrynus regularis* in particular which is a common Egyptian toad using agricultural fields and other disturbed areas and thus could be exposed to contaminants through several different routes and across all life stages. Atrazine, one of the most widely used pesticides in the world, can be transported more than 1,000 km from the point of application via rainfall [5] even in

remote areas where it is not used. Although nitrogen enters ecosystems from sewage effluents, industrial waste, atmospheric deposition and other sources, agricultural application of nitrogen-based fertilizers remains the major source of nitrates in the environment [6]. Safe levels of nitrate in groundwater for humans are 10 mg/L nitrate [7], exceeding this level may result in severe disorders [8]. The current work aims to evaluate the possible occurrence of hermaphrodites in amphibian as a biotoxicity of such chemicals using histopathological techniques and to assess the impact of human activities on amphibian and environment.

2. Materials and Methods

2.1. Collection of Test Organism

2.1.1. Studied Species

In this study the Egyptian toad *Amietophrynus regularis*, recently *Sclerophrys regularis* [9], was the tested species. This is an African species that is very widely distributed and ranges from Senegal to Egypt and to many African countries, it is classified as Least Concern according to the International Union for Conservation of Nature [10] Red List of Threatened Species. It is the common toad species found in Egypt.

2.1.2. Sampling

Tadpoles of the amphibian species were collected from Al-wahat region in Egypt, this region is a natural habitat with a minimum level of contamination to obtain less interfering of other negative impacts, tadpoles are collected at their earliest stage that is characterized by their bodies look like small pin head, larvae of Gosner stage (24-27), nearly 2-3 weeks post-hatching [11]. Collection was performed at the initiation of mating season at the end of March. Rearing and testing were done in the postgraduate ecotoxicological research laboratory at the Department of Zoology, University of Al-Azhar, Cairo, Egypt. Four hundreds of larval tadpoles were collected in aerated plastic bags immersing into their ground water and transferred to the laboratory. Tadpoles are equally distributed into four plastic containers in a width of (37×40 cm) and a height of (25 cm), each containing 8 Liter of dechlorinated tap water. They were allowed to acclimatize for seven days in the holding containers prior to the bioassay [12]. Tadpoles were fed on dried algae and fish feeding minute grains available in the market. Larvae were reared on natural conditions of a 12:12 hrs light: dark cycle (dark from 4:50 p.m. to 4:50 a.m.) and room temperature was at 30-35°C throughout the duration of the experiment, the water in each container was changed every 3 days [13].

2.2. Test Chemicals

The chemicals used for the toxicity tests, were the herbicide organochlorine, Atrazine (C8H14ClN5; 6-chloro-4-N-ethyl-2-N-propan-2-yl-1, 3, 5-triazine-2, 4-diamine) and sodium nitrate NaNo3 fertilizers, The chemicals are commonly used on farms in Egypt and worldwide for controlling weeds or as fertilizer.

2.3. Test Water

Water used for toxicity testes was dechlorinated tap water. The water was dechlorinated as stock by adding calcium thiosulphate crystals (few crystals per 50 liters were added) which have no effect on pH or oxygen dissolved [14] and air pumping for 1 hr). This water was used for acclimatization, control tests, and for making the various concentrations of the test chemicals.

2.4. Test Solutions

Stock solutions of 300 µg/L from pure commercially available Atrazine and 200 mg/L for toxicity test [14].

2.5. Experimental Design

Toxicity tests were conducted by preparing 4 containers. Each container contains 8 liters of dechlorinated tap water and constantly inserted air pump in each with concentrations of; 300 µg/l of Atrazine, 200 mg/l of sodium nitrate, combination of 300 µg/l of Atrazine and 200 mg/l of sodium nitrate and non of both as control. These concentrations are chosen as sublethal doses according to pre-study test with some concentrations which resulted in a sub-lethal dose of atrazine at 300 µg/l and of nitrate at 200 mg/l. Larval tadpoles were distributed into the containers as 100 larvae per container to make four groups which were labeled A , N , AN and C groups, respectively. New solution of water and chemicals was prepared at each container every 3 days since atrazine has a minimum half-life of 48 hours in water [15], and this work had been done until metamorphosis (complete tail reabsorption—Niewkwoop–Faber Stage 66) was reached.

2.6. Histopathological Study

Histological study was applied on gonads of the metamorphed toads, individuals of the same stage and size at each group were sorted at the end of the experiment after most individuals have been metamorphed (after 12 weeks of care) to be examined. Animals were anesthetized and dissected tissues were fixed with 10% neutral formalin for 24 hours. Afterwards, tissues were preserved in 70% ethyl alcohol. Tissues were dehydrated in ascending ethanol series, cleared with methyl benzoate, and then embedded in paraffin. Tissues were sectioned at 7 µ and stained with haematoxyline and eosin (H&E) for general histological examination. In addition, tissues were stained by Masson's trichrome stain for investigation of collagen fibers (green) [16]. Slides were examined by light microscope (Zeiss) model 25 and photographed using microscope-computerized camera, this examination was done to evaluate the impact of pollution on toad's gonads.

2.7. Statistical Analysis

LDS test on SPSS software package program (Version 17)

was done to test the testes size and the oocytes diameter and number. Probability values ≤0.05 and ≤0.01 were defined as significant throughout the current work. However, the values > 0.05 were considered non–significant. Statistically non-significant, significant and highly significant outputs were accompanied by symbols NS, * and ** respectively.

3. Results

On studying the effects of sub-lethal concentrations of nitrate, atrazine and their combination on gonadal differentiation of the toad population, the gonadal development and gonadal morphology were observed, whereas gonads of control group showed normal structure and collagen content.

3.1. Atrazine and Histopathological Alteration of Gonads

Testicular oocytes were observed in most individuals treated with atrazine (Figures 1 d&1 e), also atrazine showed decrease in the size of seminiferous tubules (Figure 1 e) compared to with control group (Figure 1 c).

3.2. Nitrate and Histopathological Alteration of Gonads

No intersex individuals were found at nitrate treatment alone (Figures 2 a & 2 b), only great reduction in size of testes and seminiferous tubules and increase of collagen fibers were observed.

3.3. Atrazine-Nitrate Combination and Histopathological Alteration of Gonads

The combined effect of nitrate and atrazine combination (Figure 2c) was not additive or synergistic, it was similar to the effect of atrazine in raising the percentage of intersex but with increasing the size of testicular oocytes and decreasing in their numbers compared to atrazine alone (Tables 3 & 4 and Figures 4 & 5).

3.4. Effect of Tested Chemicals on Testes Size

In the present study, the length and width of testes were good measurements to evaluate the effect of chemical treatments on testes size; overall, treatments affected size of testes by decreasing or increasing them through changing their length and/or width. Atrazine treatments showed an increase in size of testes by increasing their length (185.25±3.12 μm) and width (134.93±2.86 μm) compared to control group (length137.28±4.75 μm, width 115.66±2.85 μm). Atrazine-nitrate treatment showed increase in size of testes by increasing only their width (131.96±11.71 μm) compared to control group. Nitrate treatment showed reduction in size of testes compared to control by decreasing their length (105.53±3.06 μm) and width (103.98±4.16 μm). The higher reduction in testes size was exhibited in nitrate-treated specimens among all treatments. The higher increase in testes size was showed in atrazine-treated specimens among all treatments (Table 1 and Figure 3).

- Statistical analysis

To compare the different sizes of the testes, LSD was done (Table 2) and demonstrated that:

1. Testes length and width showed highly significant difference between C and both A and N ($p \leq 0.01$).
2. Testes width showed highly significant difference between C and AN ($p \leq 0.01$).
3. There was non-significant differences in testes length between C and AN ($p < 0.05$).
4. Testes length and width showed highly significant difference between N and both A and AN ($p \leq 0.01$).
5. Testes length showed highly significant difference between A and both N and AN ($p \leq 0.01$).

3.5. Testicular Oocytes (TOs) Diameter and Number Between A and AN Treated Groups

The occurrence of TOs exhibited difference between atrazine and atrazine-nitrate treated animals. Such difference in the present study was evaluated by the average diameter and numbers of TOs occurrence. The mean diameter of testicular oocytes of specimens exposed to atrazine-nitrate treatment (146.95±7.84 μm) was larger than that of atrazine treatment (135.82±8.15 μm). Conversely, the mean number of testicular oocytes occurred in gonads of animals exposed to atrazine-nitrate treatment was lower (14.33±0.51 oocytes) than that of atrazine treatment (23.50±2.38 oocytes) (Table 3 and Figures 4&5).

Table 1. *Mean testes length and width of Sclerophrys regularis treated with nitrate (N) and atrazine-nitrate (AN) and control (C).*

Treatment		Testes length	Testes width
C	Mean	137.2805	115.6628
	Std. Deviation	4.7508	2.8511
A	Mean	185.2595	134.9385
	Std. Deviation	3.1251	2.8662
N	Mean	105.5338	103.9806
	Std. Deviation	3.0693	4.1638
AN	Mean	137.0603	131.9642
	Std. Deviation	16.9846	11.7101
Total	Mean	139.5245	121.1044
	Std. Deviation	29.3174	14.1914

Table 2. *LDS multiple comparisons of Sclerophrys regularis testes length and width of different treatments.*

Dependent Variable	(I) ex	(J) ex	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Testis length	C	A	-47.97901-**	3.79305	.000	-55.6140-	-40.3440-
		N	31.74669**	3.63157	.000	24.4367	39.0567
		AN	.22023 NS	3.63157	.952	-7.0897-	7.5302
	A	C	47.97901**	3.79305	.000	40.3440	55.6140
		N	79.72570**	3.79305	.000	72.0907	87.3607
		AN	48.19924**	3.79305	.000	40.5642	55.8343

Dependent Variable	(I) ex	(J) ex	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Testis width	N	C	-31.74669-**	3.63157	.000	-39.0567-	-24.4367-
		A	-79.72570-**	3.79305	.000	-87.3607-	-72.0907-
		AN	-31.52646-**	3.63157	.000	-38.8364-	-24.2165-
	AN	C	-.22023- NS	3.63157	.952	-7.5302-	7.0897
		A	-48.19924-**	3.79305	.000	-55.8343-	-40.5642-
		N	31.52646**	3.63157	.000	24.2165	38.8364
	C	A	-19.27561-**	2.72366	.000	-24.7581-	-13.7932-
		N	11.68223**	2.60771	.000	6.4332	16.9313
		AN	-16.30131-**	2.60771	.000	-21.5504-	-11.0523-
	A	C	19.27561**	2.72366	.000	13.7932	24.7581
		N	30.95784**	2.72366	.000	25.4754	36.4403
		AN	2.97430 NS	2.72366	.281	-2.5082-	8.4568
	N	C	-11.68223-**	2.60771	.000	-16.9313-	-6.4332-
		A	-30.95784-**	2.72366	.000	-36.4403-	-25.4754-
		AN	-27.98354-**	2.60771	.000	-33.2326-	-22.7345-
	AN	C	16.30131**	2.60771	.000	11.0523	21.5504
		A	-2.97430- NS	2.72366	.281	-8.4568-	2.5082
		N	27.98354**	2.60771	.000	22.7345	33.2326

*: The mean difference is significant at the 0.05 levels
**: The mean difference is significant at the 0.01 levels
NS: The mean difference is not significant

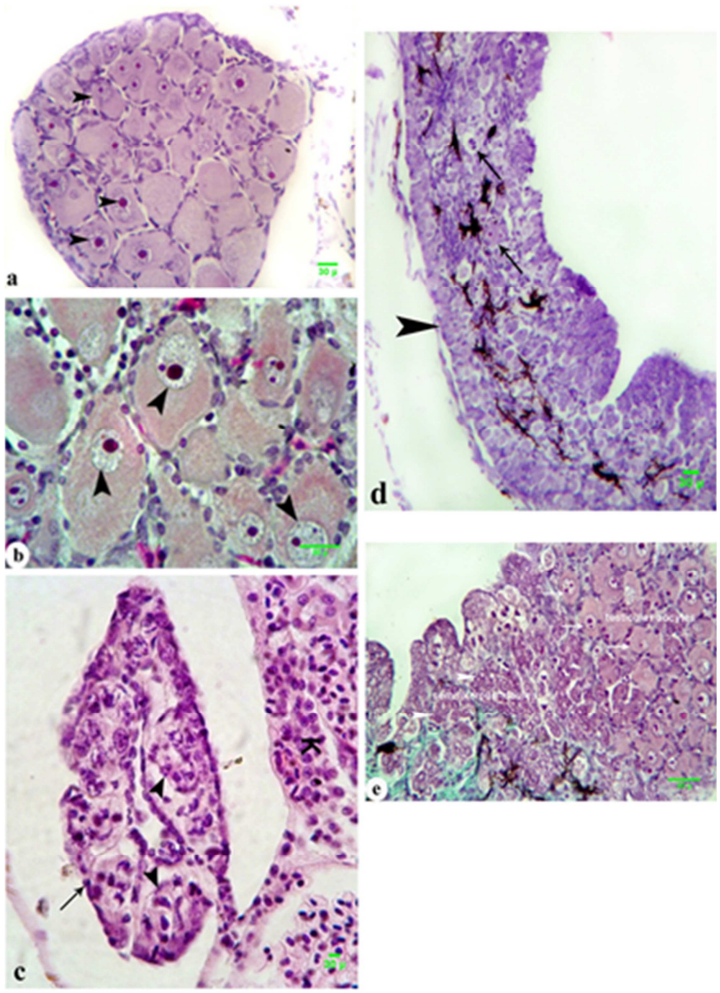

Figure 1. *Gonads of control and atrazine treated tadpole of Sclerophrys regularis.*

(a) ovary of the control group showing normal oocytes (Arrowhead). (H&E) (b) normal oocytes (Arrowhead). (Masson's trichrome) (c) normal testis (Arrow) attached to the kidney (K); with normal seminiferous tubules (Arrowhead). (H&E) (d) Hermaphroditic gonad of group exposed to 300 µg/l Atrazine; showing testis (arrowhead) with testicular oocytes (arrow). (H&E) (e) Hermaphroditic gonad of group exposed to 300 µg/l Atrazine; showing seminiferous tubules (arrowhead) with large number of testicular oocytes (arrow) and increase of collagen fibres (green). (Masson's trichrome).

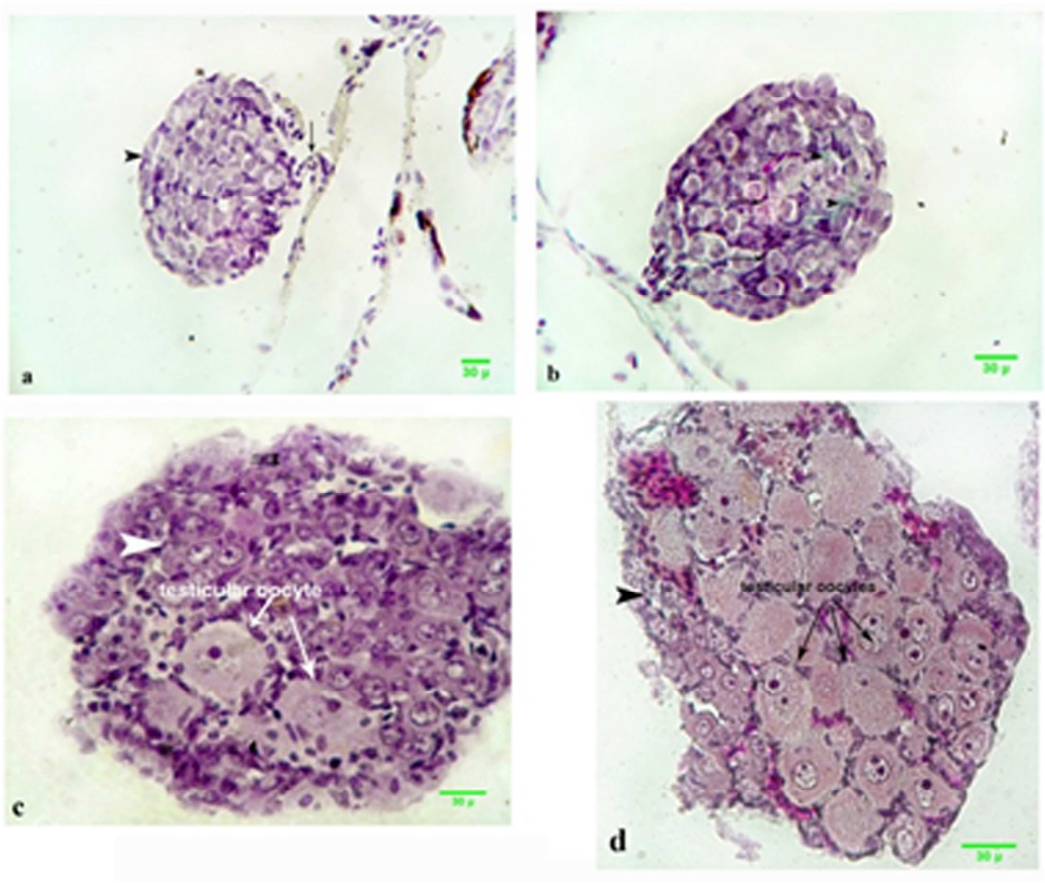

Figure 2. *Gonads of nitrate and atrazine-nitrate treatment tadpole of Sclerophrys regularis.*

(a) testis of group exposed to 200 mg/l nitrate showing great reduction in the size of testis (arrowhead) attached to the kidney (arrow) with degeneration and reduction of the seminiferous tubules. (H&E) (b) testis of group exposed to 200 mg/l nitrate showing; great reduction in the size of testis and the seminiferous tubules (arrow) with the appearance of fibrosis of collagen fibers (green) (arrowhead). (Masson's trichrome) (c) gonad of group exposed to 300 µg/l atrazine&200 mg/l nitrate showing testicular oocytes (arrow) and seminiferous tubules (arrowhead) and undifferentiated tissue (double arrows). (H&E) (d) gonad of group exposed to 300 µg/l atrazine & 200 mg/l nitrate showing testicular oocytes (arrow) and seminiferous tubules (arrowhead).(Masson's trichrome).

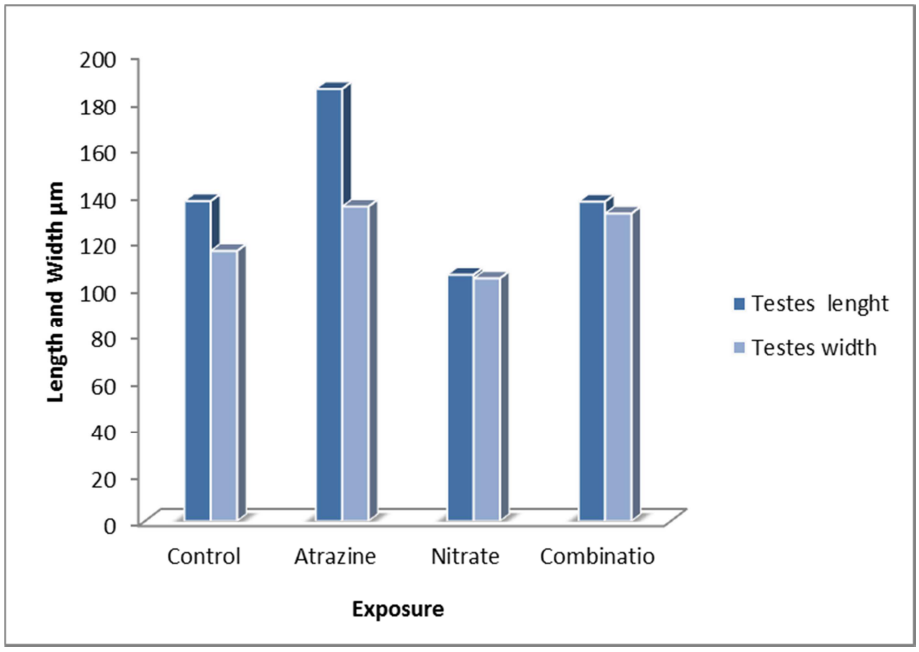

Figure 3. *Testes length and width of Sclerophrys regularis exposed to Atrazine and Nitrate.*

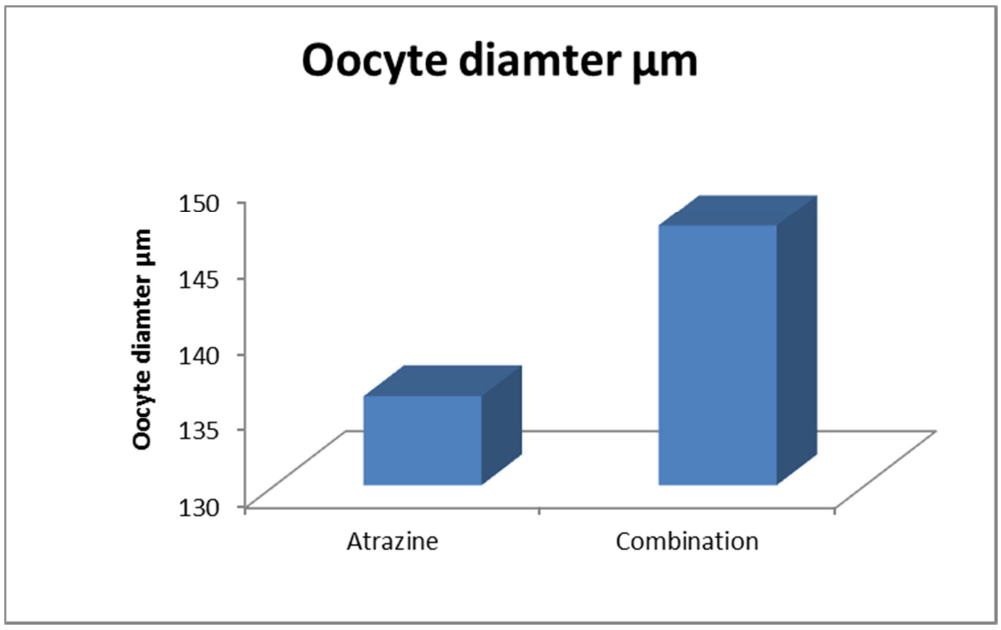

Figure 4. *Mean testicular oocytes diameter of Sclerophrys regularis exposed to atrazine and combination treatment.*

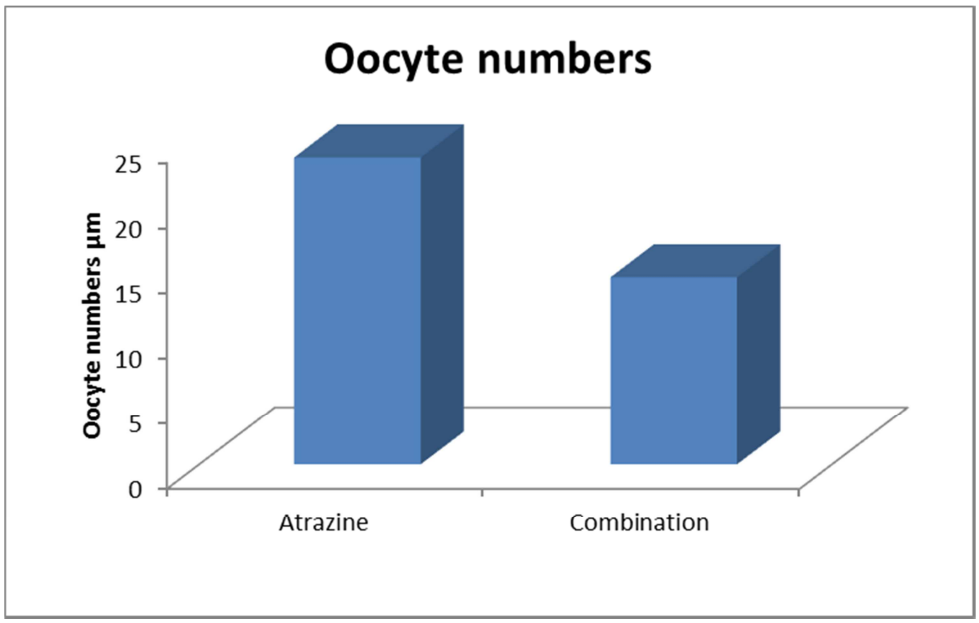

Figure 5. *Mean testicular oocyte numbers of Sclerophrys regularis exposed to atrazine and combination treatment.*

Table 3. *Means of testicular oocytes diameter and numbers at atrazine and atrazine-nitrate treated Sclerophrys regularis.*

Exposure	Oocyte numbers		Oocyte diameter	
	Mean	Std. Deviation	Mean	Std. Deviation
Atrazine	23.5000	2.3805	135.8256	8.1506
Combination	14 .3333	0.51640	146.9552	7.8415

Table 4. *LDS multiple comparisons of testicular oocytes diameter and numbers at atrazine and atrazine-nitrate treated Sclerophrys regularis.*

Exposure	Significance between atrazine and atrazine nitrate treatments
Oocyte numbers	Highly significant (.01**)
Oocyte diameter	Significant (.05*)

*: The mean difference is significant at the 0.05 levels
**: The mean difference is significant at the 0.01 levels
NS: The mean difference is not significant

4. Discussion

This study assessed the potential influence of atrazine and nitrate exposure on *Sclerophrys regularis* toads reared under controlled laboratory conditions, by investing possible effects of such contaminants on gonadal development on exposure to sublethal doses, that seemed to be relevant to environmental concentrations at many areas worldwide. 300 µg/l atrazine, 200 mg/l nitrate and combined dose treated animals experienced significantly development of testicular oocytes (TOs) in gonads and decreased the size of testes.

In the present study gonadal abnormalities were observed in the experimental animals following atrazine and/or nitrate exposure, however, the forms of abnormalities were not consistent between individuals and the frequency of abnormalities in treated groups differ from the complete absence of abnormalities observed in the control group. Nonetheless, all of the abnormalities occurred in atrazine exposed tadpoles were concurred with other studies that have indicated that atrazine increases the presence of testicular oocytes (TOs) [17], causes testicular dysgenesis [18] and reduces testis size [19].

All those previous studies operated different ranges of concentrations, so future meta-analysis of all previous data sets may increase the sensitivity of detecting significance in the frequency of gonads abnormalities observed in tadpoles exposed to atrazine. The controls of this study did not contain the rudimentary testicular oocytes occasionally observed in the normal unexposed development of *Rana pipiens* that believed to degenerate once the testis further develops and starts to produce androgens [20], these rudimentary testicular oocytes were completely lacked at the control group of this study addressing *Sclerophrys regularis*, so TOs from the different treatments conducted on this study were compared.

A significant difference in size of testicular oocytes was observed between atrazine and atrazine-nitrate combination treated tadpoles. The TOs in atrazine-nitrate treated tadpoles were significantly ($p \leq 0.05$) larger (mean diameter =146.95 µm) than the TOs of atrazine treated tadpoles (mean diameter =135.82 µm) which were measured quantitatively in this study. Conversely, nitrate reduced the number of TOs induced when combined to atrazine with difference showed to be highly significant ($p > 0.01$). The increased proportions of intersex in atrazine and combined treatment compared to the control were synergistic with the records of previous studies [21 and 22] concerned with high percentage of sex ratio increasing of female. The potential sex ratio could not be detected because most individuals examined were shown to be intersex and due to the lacking signs of sex determination in the so early metamorphed toads. The mechanism responsible for increasing proportions of intersex compared to control is likely to be a local endocrine effect in the gonad, as the hypothalamo–pituitary–gonadal axis becomes functional only shortly before the metamorphic climax, so direct feminizing effects of either atrazine or nitrate-combined cannot be excluded.

The presence of amphibian TOs has been negatively related with a decreased number of spermatogonia in seminiferous tubules [23]. Furthermore, it has been observed that small amounts of dehydrotestosterone (DHT) produced within the amphibian testes inhibit the production of TOs [24], and hence, the decrease of seminiferous tubules in the atrazine exposed testes observed histologically in this study, may be a result of decreased DHT production, and this may also result in less inhibition of oocyte growth [24, 20]. These interpretations were synergetic with the hypothesis that atrazine induces aromatase and promotes the conversion of testosterone to estrogen and that the production of hermaphrodites may be referred to this disruption in steroidogenesis [25]. Since the frequency of testicular oocytes may reflect endocrine disruption, therefore, the observed significant increase in size of TOs in the gonads between treatments of this study suggest TOs diameter and number to be a potential sensitive endocrine disrupting endpoint reflecting altered steroidogenesis in the testes. Future work with this data set should assess the influence of atrazine on gonad maturity, as well as female oocyte diameter and number.

Although, no intersex individuals were detected in the nitrate alone, great reduction in size of testes and seminiferous tubules were observed. On contrast, intersex was detected in nitrate-treated individuals in rare studies [26]. In atrazine treated animals, the highly significant increase in size of testes may be due to the increased number of TOs indicating that endocrine disruption tends toward feminizing action. Conversely, the highly significant reduction in size of testes in nitrate treated animals indicate that endocrine alteration tend toward decreasing androgens without increasing hormones that induce feminization. These suggestions are in consequence with the highly significant reduction in testes size at atrazine treatment when combined to nitrate compared to that in atrazine alone and the highly significant decrease in TOs number of the animal suggesting the nitrate may be contrary to or reduce the feminizing action of atrazine.

It remains unclear why nitrate caused an increase in follicle size of TOs when combined to atrazine and in contrast why it alone showed no mechanism to develop TOs. The endocrine control of these processes is not well understood, and the molecular mechanisms of action of the contaminants investigated are not known; however, both compounds nitrate and atrazine have been implicated in alteration of steroidogenesis in amphibians. The endocrine control of testicular oocyte growth and their environmental relevance requires further investigation.

This study has demonstrated changes in sex type driven toward feminization. Though the alteration of growth and development induced in this study and since the steroidogenesis effects occurred in other studies [26] without major changes in animal growth or general morphological development, they are likely to reflect specific endocrine or reproductive mechanisms. Since steroid hormone

concentrations and secondary sexual traits correlate with reproductive activity and success, affected toads likely have reduced reproductive success. These reproductive abnormalities could certainly contribute to amphibian population decline [27] occurring in areas exposed to highly concentrated agricultural contaminants.

Moreover, fibrosis was clearly seen in atrazine and nitrate treatments, indicating the severe negative action of both chemicals on gonadal tissues of amphibians.

In the present study has explored the effects of atrazine and nitrate on amphibian; From the chemical point of view, atrazine is capable of interacting synergistically with other agricultural chemicals to decrease survival, growth or metamorphosis of amphibian larvae [28] and it is possible that an interaction between atrazine and nitrate could increase the impairment rather than a single action [29]. Some authors discussed the possible mechanism of interaction between atrazine and nitrate as combined together, this mechanism involves the oxygen-carrying capacity of larval blood because nitrite can cause methemoglobinemia and atrazine is known to reduce circulating erythrocytes, also nitrate and atrazine may increase the risk due to nitrosamine formation [30], many nitrosamines are known to be carcinogens [31]. During digestion nitrate is reduced to nitrite and many secondary amines are nitrosated in the presence of nitrite, atrazine is a secondary amine that nitrosates to form N-nitrosoatrazine (NNAT) which has been shown to significantly increase chromosomal abnormalities in lymphocytes at low concentrations. These interactions were clearly seen to cause severe negative impacts rather than atrazine or nitrate can cause alone, these impacts were supported by our results which statistically seemed to have the higher increase in gonadal abnormalities in combined treatment compared to control and all other treatments regarding the presented data, indicating that double impact represented by atrazine, synergistically with nitrate have ability to increase impairment of development in *Sclerophrys regularis*. These results also were in accordance with results from several studies as mentioned previously. The problem that both chemicals mostly showed to be found in agriculture areas together, the problem also extend to include all contaminants when many chemicals interact together in agriculture. There is mounting evidence that some amphibian species living in regions of intensive agrochemical contaminants use suffer great developmental and survival impacts [27]. Considering this possibility and the fact that atrazine and nitrate co-occur ecologically, these results suggest the interaction between atrazine and nitrate to increase developmental instability of amphibian populations.

Rather than the observed effect on gonads, atrazine and/or nitrate had a wide range of sever effects concerning growth and body size at metamorphosis [32], genotoxicity [33] or malformations [34], indicating the disrupting action of such chemicals on amphibian development and sexual maturity. Over the years, human activities in agrochemical use have increased causing disturbances to all organisms in the ecosystem either in direct or indirect route, while the high concentrations were detected as well as low concentrations [35]; by this approach the present study can be a good assessment for relevant chronic high exposure.

The obtained results also indicate amphibians can act as bioindicator at high level execution alarming increased toxins at the environment and their effects. The adverse effects yielded indicate their sensitivity to environment change and pesticide toxicity, their life between land and water and having them a permeable skin allows toxins to move relatively freely and concentrate into their bodies. The results also were good bioindication that atrazine behaves as an endocrine disruptor and organogenesis fluctuation impact.

Organogenesis fluctuation, gonadal abnormalities and other impacts may act as indirect contributor in amphibian decline [36] in which these abnormalities impair reproductive success, activity, behavior and fitness of the animals and make them more vulnerable to be attacked by enemies and lower their population by generations.

Environmental Protection Agency (EPA) has considered atrazine as well as nitrate to be an ecological risk to the aquatic community-population level due to the off-target impacts on aquatic animals and plants in neighboring watersheds, where the concentrations used may exceed their levels of concern. Therefore, contamination can be found in nearly all surface and ground waters, in both agriculture and non-agriculture areas.

5. Conclusions

It could be suggested that atrazine and nitrate may alter reproductive axes in amphibians. Atrazine increases the presence of testicular oocytes TOs, causes testicular dysgenesis and reduces testis size Atrazine has a demascunilization/feminization effect that can be partial or complete what depends on the dose and time of exposure. Nitrate reduced the number of TOs induced when combined to atrazine. No intersex individuals were detected in the nitrate alone with great reduction in size of testes and seminiferous tubules were observed. These reproductive abnormalities could certainly contribute to amphibian population decline occurring in areas exposed to highly concentrate agricultural contaminants. Accordingly, we recommend not using herbicides and soil fertilizers in excess quantities recommended by the World Health Organization because of their harmful impact on living organisms and their impact on human health.

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