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Limbs Asymmetry as Biomarker in the Egyptian Toad *Amietophrynus regularis* Exposed to Atrazine and Nitrates

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Abstract: Many amphibians occupy appositional roles between different animal taxa; despite this, many species are threatened with decline and extinction. Several and different contributors and factors were previously reviewed to contribute amphibian disappearance. Between those factors are chemical pollution, heavy metals, climate change, disease, etc. The most commonly used index of developmental instability is fluctuating asymmetry (FA), as a feature of environmental impact was also reviewed. F.A is the variance in right minus left values of bilaterally symmetrical structures. The results indicate that individuals exposed to atrazine or that exposed to both atrazine and nitrate exhibit a significantly higher degree of FA ($p<0.01$) while FA measured in individuals treated only with nitrate was not significant ($p<0.05$) These results constitute the first assessment of FA in this species in Egypt and suggest that the degree of FA in skeletal traits cane be a useful indicator of the degree of environmental stress experienced by amphibian populations.

Keywords: *Amietophrynus regularis*, Atrazine, Nitrates, Fluctuating Asymmetry

1. Introduction

In recent years, the biomonitoring became an integral part of the general environmental monitoring. Amphibians as inhabitants of two environments, aquatic and terrestrial, are successfully used as indicators of the damage of biotopes caused by anthropogenic pollution [1, 2, 3]. One of the simplest, easiest and cheapest methods of bioindication is the estimation of the fluctuating asymmetry (FA) as an integral indicator of developmental stability of amphibian populations. Recently, the concept of amphibian decline has gained a worldwide focus. To manage populations and prevent declines, it is important to understand how different types of stresses and their timing affect populations. Some stresses are initiated gradually and occur over long periods of time; others are relatively sudden and short term. The second type of stress is more dynamic, and its effects are more difficult to predict. Some chemicals are persistent, whereas others, such as nutrients, are very dynamic, exhibiting changes in concentration on various timescales [4]. One example is the natural influx (or pulse) of nutrients, such as nitrate, as a result of runoff during a storm event [5, 6]. Most toxicology studies examine the effects of constant concentrations of various chemical compounds [7, 8], but pulses may affect organisms differently than static levels do. Fluctuating asymmetry is a measure of the degree to which structures that are bilaterally symmetrical depart from perfect symmetry [9]. A failure of developmental regulation was suggested to increase with levels of environmental stress. In fact, measures like size, weight and condition have been applied, but also, fluctuating asymmetry has been put forward as a biomarker of stress [10]. Accordingly, fluctuating asymmetry will be used as a useful tool for monitoring stress levels in amphibian populations [11, 12]. Nowadays our environments are facing many and varied sources of hazards and toxic substances such as heavy metals, herbicides, pesticides, insecticides, radioactive materials, polychlorinated biphenyls, sewages, etc.

Amphibians are especially at risk from agricultural contaminants because they have permeable skin and eggs that readily absorb chemicals from the environment. Moreover, many species complete their life cycles in ponds and streams adjacent to agricultural fields where agrichemicals are applied, and these applications often coincide with breeding and larval development [13] Atrazine is an herbicide used predominantly in corn and sorghum production, and is one of the most prevalent herbicides found in the environment [14, 15]. Atrazine exposure has produced effects on the endocrine system of amphibians, fish, reptiles and human cell lines in the µg/L range within laboratory studies [16]. Nitrogenous compounds are known to be lethal to some species of amphibian larvae at or near ecologically relevant concentrations [17, 18]. In fact, fewer studies have examined the sublethal effects of nitrogenous compounds on larval amphibians [19]. He [19] exposed *Bufo americanus* and *Hyla chrysoscelis* tadpoles to concentrations of nitrate, a pervasive stressor in the environment, up to 5 mg/L of $NO3^{-}$in constant concentrations and in pulses at three different points during development. Reactive nitrogen includes ammonium, nitrite, and nitrate, but because of nitrification, ammonium and nitrite are converted to nitrate in the presence of oxygen [20]. For this reason, nitrates are the most concentrated form of inorganic nitrogen in aquatic systems [20] and should be a focus when examining the effects of increased nitrogen in the environment. One important effect of nitrates is toxicity to aquatic organisms, such as amphibians [21]. Although less toxic to amphibians compared with ammonium or nitrite [22]. Nitrates still have lethal effects at relatively low concentrations and many nitrate toxicity tests have examined lethal impacts of nitrate [23]. *Amietophrynus regularis* is of conservation concern for several reasons; it is an important indicator to the health of the environment. It acts as active insect biocontrol in most Egyptian agricultural fields in addition it is considered as an important part of the food items of many animals. The purpose of this work is to study the levels of fluctuating asymmetry in the limbs of Amietophrynus regularis exposed to atrazine and nitrates as a bioindicator to health of the environment.

2. Materials and Methods

2.1. Sampling

The Egyptian toad (*Amietophrynus regularis*), is a species of toad in the family Bufonidae. It is found widely in the Subsaharan Africa, with its range extending to the oases in Algeria and Libya as well as to northern Nilotic Egypt. In Egypt this species is largely restricted to the Nile Valley and Delta, parts of the western Mediterranean coast, the Suez Canal Zone and Kharga and Bahariya oases [24]. During the breeding season, tadpoles of *Amietophrynus regularis* were collected by nets from clean water body. After that, animals transported to laboratory in air- provided tank. Tadpoles are collected at their earliest stage, nearly three weeks post - hatching [23]. Larval tadpoles are equally distributed into four plastic containers in a width of (37×40 cm) and a height of (25 Cm), each containing 4 Liter of dechlorinated tap water. Animals were acclimatized and fed for seven days as recommended [25].

2.2. Test Chemicals Preparation

Pure atrazine and sodium nitrate were dissolved in water to make 1 liter from each.

2.3. Experiment Design

Four containers of 8 liter were prepared for chemical treatments.

Solutions were diluted from stocks as follow: 300 µg/L of Atrazine 200 mg/L of sodium nitrate. 300 µg/L of atrazine + 200 mg/L of nitrate were mixed to make combination. 0/L atrazine and nitrate as control. larval tadpoles are distributed into the containers as 100 larvae in each to make four groups which were labeled respectively, atrazine (A), nitrate (N), combined (AN) and control (C) groups. The solution in each container was changed every 3 days and the food was added. A new stock solution for atrazine was made up every 3 days immediately before each water change since atrazine has a minimum half-life of 48hours in water [23] and that had been done until metamorphosis is reached.

2.4. Asymmetry Study

This technique was applied on the metamorphic toads. To obtain accurate asymmetry measurements, forty toads from each group were tested. In addition, each bone was measured 3 times to minimize errors. The measured bones were hind limb (femur and tibio-fibula mm) and forelimb (humerus and radio-ulna mm). The measurements of bones were based on [26, 27].

2.5. Skeletal Asymmetry

All muscles and connective tissues from the forelimbs and hind limbs were removed. The humerus and radio-ulna in forelimbs, and femur and tibio-fibula in the hind limbs was measured using caliper. General distribution for right minus left hind limb and forelimb were done separately. Fluctuating asymmetry was calculated according to [28], as:

$$FA = \frac{|R-L|}{Size} \quad \text{where Size} = \frac{(R+L)}{2}$$

R = length of the right limb and L = length of left limb. To test the total limb asymmetries, the asymmetry traits for each limb were added together, where hind limb represented by (Femur + Tibio-Fibula) and forelimb (Humerus +Radio-ulna).

2.6. Statistical Analysis

To test the current data, SPSS version 17 program was applied. Normal distribution curve was graphed to test normality as well as equality of tested means. Analysis of variance ANOVA and least significant differences LSD were used to compare the effectiveness of chemicals in relevant to animal growth. Levene's Test was also applied to verify the different significances between limbs in the term of fluctuating asymmetry.

3. Results

In the present study, the curve of normality was applied to evaluate the deviation between measured means. The total mean between right minus left forelimbs as well as hind limbs were centered on zero in normally treated animals (Fig. 2).

Slight measurements were recorded as fluctuating asymmetry after the exposure of experimental chemicals. All means (R-L) recorded were positively biased either in nitrate or atrazine exposed toads. On the hand, recorded values around zero were right biased, regardless of the type of chemical exposure. The combination of atrazine and nitrate potentially affected the normal distribution of both limbs. The range of recorded FA means ranged between (0.05±0.004 mm) in nitrate exposed animals to (0.40±0.29 mm) in combination exposed animals. Generalized ANOVA test has revealed the presence of significant differences of the means of FA between hind ($F=36.722$, $p<0.01$) and forelimbs ($F=34.494$, $p<0.01$) (Table 1 and Fig. 1). Consequently, the LSD statistical analysis was performed to check and categorize the induction of FA in relevant to chemical action (potential). This least significant differences LSD test has shown that:

- From the statistical point of view, there was no significant differences between the means of FA recorded from both control and NO_3 exposed animals ($p<0.05$). Despite this, from the lateral point of view the scientists of laterality consider this case as a deviation from normal limb distribution as long as it deviated from zero. Consequently, the negative values (-0.060, FAHL) and (-0.041, FAFL) between the means of fluctuating asymmetry in NO_3 exposed group minus control group is good scientific evidence of the induction of FA (Table 2).
- The means of difference between control & atrazine FA (Table 2) gave the highest levels of significance ($p<0.01$), whether in hind or forelimb (-0.332^{aa} and -0.373^{aa}) respectively.
- Also the means of difference between NO_3 & atrazine FA (Table 2) gave the highest levels of significance ($p<0.01$) whether in hind or forelimb (-0.272^{aa} and -0.33^{aa}) respectively. Considering the recorded values, atrazine potentially exceeded nitrate in recorded means of FA in both limbs.
- In order to verify the potential of atrazine and nitrate to affect the development and growth of *Amietophrynus regularis* in the term of limb asymmetry, Levene's Test (Tables 3-5) was applied. The results of Levene's Test have summarized that nitrate could affect limb than more forelimb. Also FA in animals exposed to atrazine was higher than those exposed to nitrate or control groups (Table 4).

Table 1. *General ANOVA test of FA in hind and forelimbs of Amietophrynus regularis.*

Asymmetry trait		Sum of Squares	df	Mean Square	F	Sig.
FAHL[1]	Between Groups	1.883	2	.942	36.722	.000
	Within Groups	2.375	107	.027		
	Total	4.258	109			
FAFL[2]	Between Groups	2.488	2	1.244		
	Within Groups	2.947	107	.034		
	Total	5.435	109		34.494	.000

[1] fluctuating asymmetry in hind limb, [2] fluctuating asymmetry in forelimb

Table 2. *LSD test of FA in hind and forelimb of Amietophrynus regularis exposed to nitrate and atrazine in relevant to control groups.*

Limb FA	Control & Nitrate	Control & Atrazine	Nitrate & Atrazine
FAHL	-0.060^{NS}	-0.332^{aa}	-0.272^{aa}
FAFL	-0.041^{NS}	-0.373^{aa}	-0.33^{aa}

a: The mean difference is significant at the 0.05 levels, aa: The mean difference is significant at the 0.01 levels, NS: The mean difference is not significant

Table 3. *Two group test of hind and forelimbs of Amietophrynus regularis exposed to nitrate compared to control groups.*

Levene's Test for EV[6]				t-test for Equality of Means						
		F	Sig.	t	df	Sig	MD[3]	SED[4]	95% CID[5]	
									Lower	Upper
FAHL	EVA[1]	9.854	.002	-3.455	78	.001	-.07100	.02055	-.11191	-.03009
	EVNA[2]			-3.455	70.30	.001	-.07100	.02055	-.11198	-.03002
FAFL	EVA	1.080	.302	-3.022	78	.003	-.04525	.01497	-.07506	-.01544
	EVNA			-3.022	70.80	.003	-.04525	.01497	-.07510	-.01540

[1]Equal variances not assumed, [2]Equal variances not assumed, [3]Mean Difference,
[4]Std. Error Difference, [5]Confidence Interval of the Difference, [6]Equality of Variances

Table 4. *Two group test of hind and forelimbs of Amietophrynus regularis exposed to Atrazine compared to control groups.*

Levene's Test for EV[6]				t-test for Equality of Means						
		F	Sig.	t	df	Sig	MD[3]	SED[4]	95% CID[5]	
									Lower	Upper
FAHL	EVA[1]	60.430	.000	-8.970	78	.000	-.38425	.04284	-.38425	.04284
	EVNA[2]			-8.970	45.457	.000	-.38425	.04284	-.38425	.04284
FAFL	EVA	92.587	.000	-8.462	78	.000	-.42650	.05040	-.42650	.05040
	EVNA			-8.462	41.411	.000	-.42650	.05040	-.42650	.05040

[1]Equal variances not assumed, [2]Equal variances not assumed, [3]Mean Difference,
[4]Std. Error Difference, [5]Confidence Interval of the Difference, [6]Equality of Variances

Table 5. *Two group test of hind and forelimbs of Amietophrynus regularis exposed to nitrate and Atrazine.*

Levene's Test for EV[6]				t-test for Equality of Means						
		F	Sig.	t	df	Sig	MD[3]	SED[4]	95% CID[5]	
									Lower	Upper
FAHL	EVA[1]	37.839	.000	-7.049	78	.000	-.31325	.04444	-.40173	-.22477
	EVNA[2]			-7.049	51.594	.000	-.31325	.04444	-.40245	-.22405
FAFL	EVA	78.655	.000	-7.460	78	.000	-.38125	.05111	-.48300	-.27950
	EVNA			-7.460	43.664	.000	-.38125	.05111	-.48428	-.27822

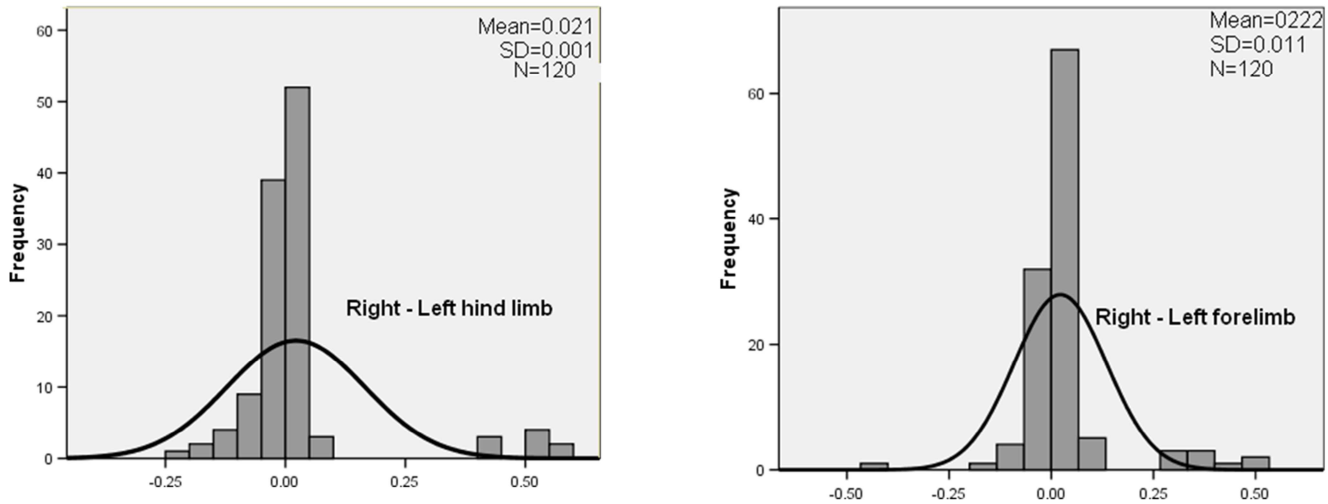

Figure 1. *General singed different means of FA in hind and forelimbs of metamorphed Amietophrynus regularis*

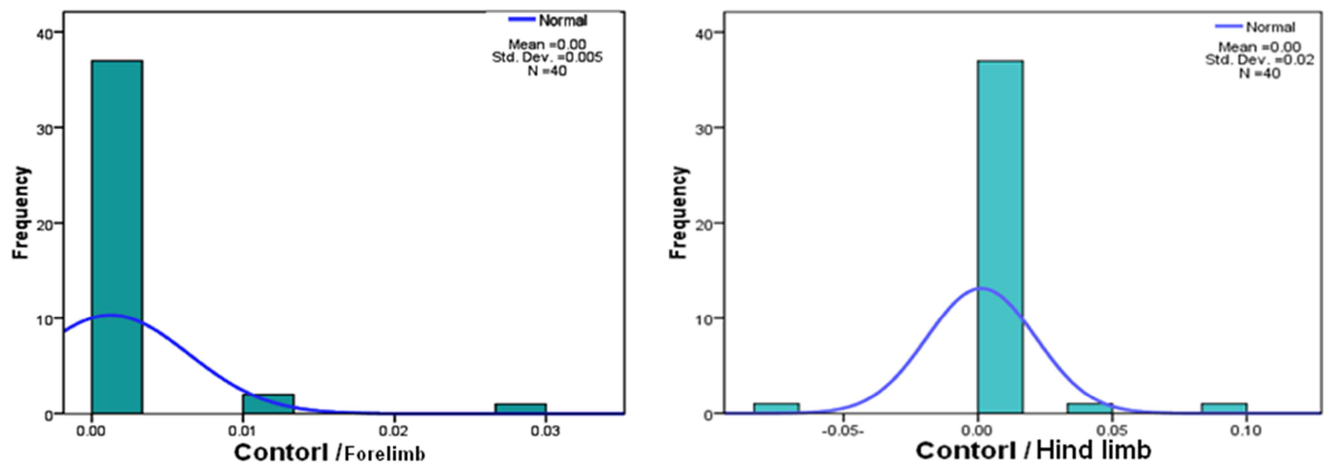

Figure 2. *Right – left limbs of Amietophrynus regularis control groups.*

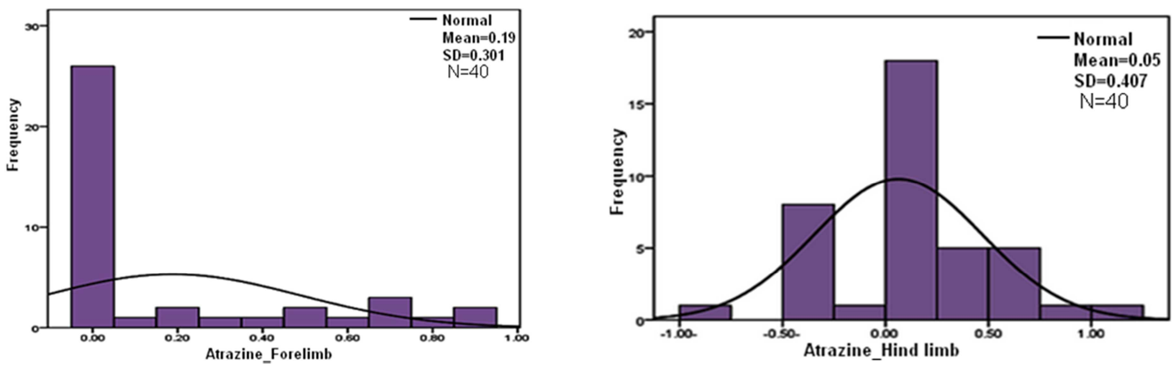

Figure 3. *Right – left limbs of Amietophrynus regularis exposed to atrazine.*

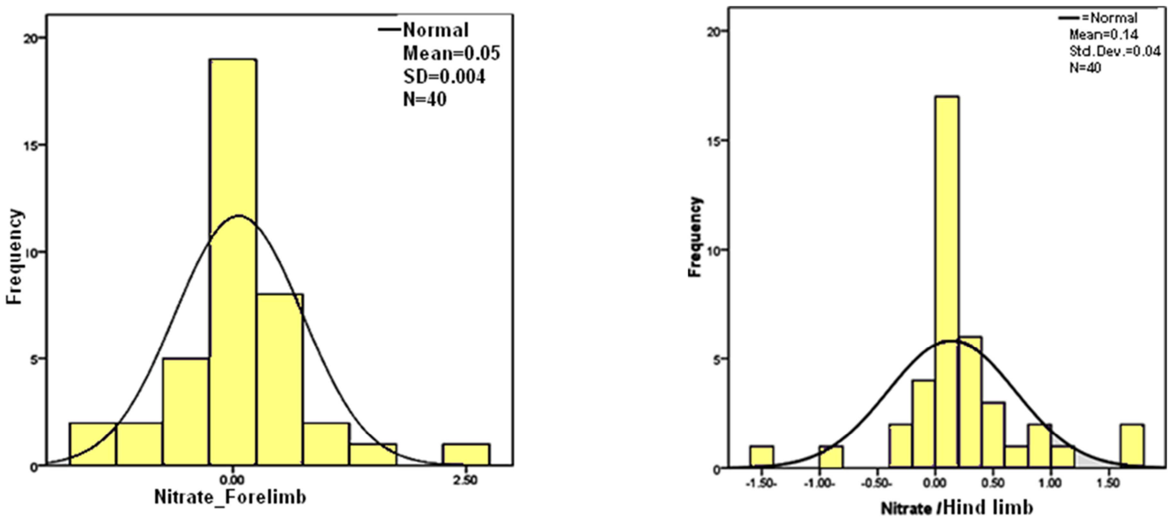

Figure 4. *Right – left limbs of Amietophrynus regularis exposed to nitrate.*

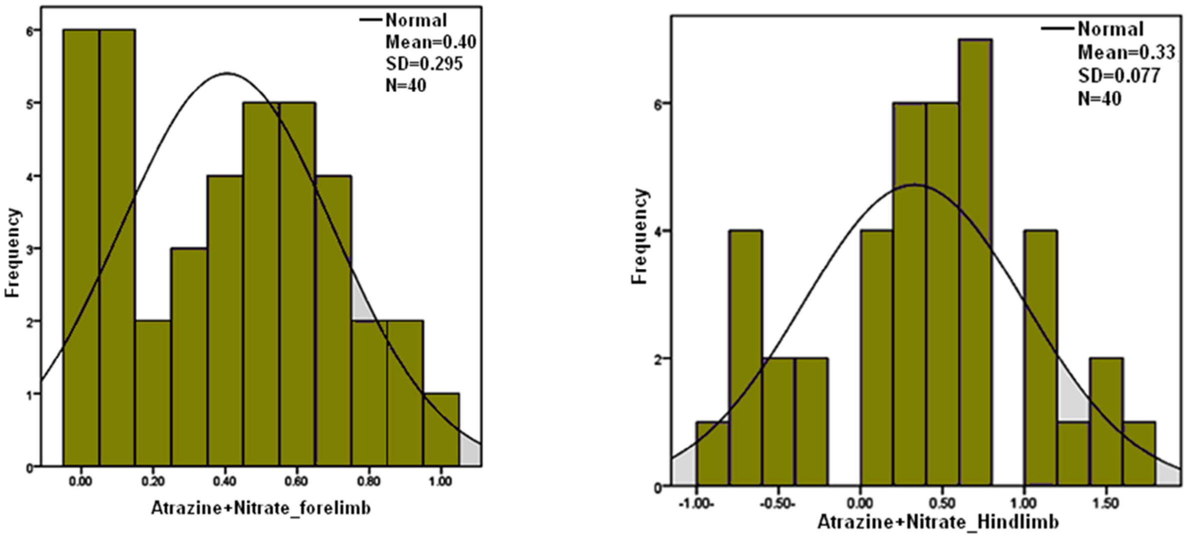

Figure 5. *Right – left limbs of Amietophrynus regularis exposed to combined atrazine + nitrate*

4. Discussion

This study has explored the effects of atrazine and nitrate to the development and limb asymmetry of the amphibian *Amietophrynus regularis*.

From the chemical point of view, atrazine is capable of interacting synergistically with other agricultural chemicals to decrease survival of amphibian larvae [39] and it is possible that an interaction between atrazine and nitrate could impair survival, growth, or metamorphosis of larvae. [40] Discussed the possible mechanism of interaction between atrazine and nitrate as combined together. This mechanism involves the oxygen-carrying capacity of larval blood. This study has explored the effects of atrazine and nitrate to the development and limb asymmetry of the amphibian *Amietophrynus regularis*. From the chemical point of view, atrazine is capable of interacting synergistically with other agricultural chemicals to decrease survival of amphibian larvae [39] and it is possible that an interaction between atrazine and nitrate could impair survival, growth, or metamorphosis of larvae. [40] 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 (from reduced nitrate) can cause methemoglobinemia and atrazine is known to reduce circulating erythrocytes. A comprehensive review of published literature on the developmental effects of atrazine on amphibians, reptiles, and fish has just been completed [41]. Nitrate can have significant effects on anuran larvae, both through direct toxicological effects on survivorship, but also through behavioral effects [19]. Nitrate has been shown to reduce activity or feeding rate in a variety of other amphibian larvae [31, 32]. The decrease in activity level of tadpoles or larvae in the presence of nitrate may actually decrease predation risk since activity in tadpoles frequently increases predation [33]. However, reduced activity may also affect the competitive abilities of tadpoles because activity level has sometimes been correlated with competitive ability in tadpoles [34, 35]. Regarding natural habitat, adult toads are likely to be exposed to nitrate as while crossing agricultural fields that fertilized with nitrates to reach breeding sites, and embryos and larvae are at risk from runoff into surface waters [36]. Sodium nitrate (40, 100 mg NO_3-/L) were found to cause retarded growth and increased mortality of common toad (*Bufo bufo*) [36] and larval stages of Australian tree frog (*Litoria caerulea*) [37]. Furthermore, several amphibian species were observed to suffer disequilibrium, developmental abnormalities, and increased mortality in acute and chronic studies at levels comparable to those found in the environment [31, 32]. The toxic effect of nitrates can be attribute to the fact that, nitrate can be converted to nitrite through bacterial reduction in the gut of the amphibians consuming plant material containing nitrate. Nitrite is reported to induce methemoglobinemia in larvae of *Rana catesbeiana* by oxidizing the Fe^{2+} to Fe^{3+}, thus preventing oxygen transport [38]. Tolerance exposure of anuran tadpoles for nitrate and other toxic substances was found to be time dependent [28, 29, 30]. The fluctuating asymmetry aspect of this study was unique regarding the method of measurement and can be debated as an effective indicator of stress. The presence of FA was found to be right biased in general. The means of difference between control & atrazine FA gave the highest levels ($p<0.01$) of significance (Table 2), whether in hind (-0.332^{aa}) or forelimb (-0.373^{aa}). Also, the combined atrazine and nitrate were potentially affect metamorphed *Amietophrynus regularis* targeting its skeletal behavior, With mean record ranged from (33±0.08) in hind limb to (0.40±0.30) in forelimb. Any left -right asymmetry observed at two months post metamorphosis may have originated during the larval stage due to developmental noise or other unexplained geometrical variance [42]. The results corroborate the earlier studies indicating that environmental stressors that influences the development and growth of amphibian embryos and tadpoles (e.g. [43, 44, 45, 46]. Our results indicate that the stress effects induced by atrazine and nitrate are also visible at later (adult) life stages once the individuals have changed from an aquatic (larvae) to a mostly terrestrial (adults) life. These effects were very clear limps FA, suggesting that FA can be a useful indicator of the degree of environmental stress experienced by amphibians. Increased FA among individuals exposed to acid stress has been observed in fishes [47]. But our results provide the first test in amphibians giving the alarming evidence for global decline of amphibian giving the alarming evidence for global decline of amphibian populations [12, 48]. FA could provide a useful metric for identifying populations under stress when direct population size estimates are difficult or impossible to obtain. Alternate measuring technique s and markers may prove more effective at detecting smaller variations. Perhaps more sensitive software would also prove more advantageous.

5. Conclusion

This study explored the effects of environmentally relevant concentrations of nitrate and atrazine on the development instability of the toad *A. regularis.* The presence of no accordance between hind and forelimb FA, not only affect animal's movement but also biological and physiological behaviors including feeding and breeding. Intensive agrochemicals activities can lead to environmental contamination and affect non-target organisms including amphibians. Toads with asymmetric limbs will be easy to preyed, can't reach food sources, lose breeding pools, have gradual immune system disorders and consequently may die. The present investigation reaffirms the opportunities for assessing the developmental stability of *A. regularis* populations that live in conditions of anthropogenic pollution through the method of FA. The results provide better opportunities to use FA in *A. regularis* populations for bioindication and biomonitoring, and for parallel and independent analyses on the physicochemical analyses assessment of the environmental condition. Development of FA analyses may thus provide researchers with a robust biological indicator of environmental health, which can potentially be used to monitor areas sensitive to ecological disturbance or where there are human health concerns. Proper screening of individuals with a stress indicator may have warned biologists about environmental problems and led to changes in human behavior before the stressor dramatically affected sensitive biota. Amphibian biologists thus need an early-warning system that could identify stressed animals before the stressor causes population or regional harm. Such an indicator should be able to measure stress-induced effects before drastic changes in morphology take place which would subsequently decrease the organism's survival and reproduction. One such indicator is obtained by measuring developmental stability.

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