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Genotoxicity and limbs asymmetry in the Egyptian toad (*Sclerophrys regularis)* as biomarkers for heavy metals toxicity

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ABSTRACT

Many species of amphibians are facing remarkable decline and extinction throughout the world. Chemical contamination, such as heavy metals, is one of the factors contributing to the extinction of amphibians. Wetland biota, particularly amphibians, may be harmed by heavy metal contamination. The emergence of micronucleated erythrocytes (MN) and nuclear lesions (NL), collectively known as MN-Test, in nucleated erythrocytes is an excellent tool for detecting genotoxicity or chromosomal damage. Furthermore, developmental instability, also known as fluctuating asymmetry (FA), is broadly applied to assess changes in the health of the environment under natural conditions when it is subjected to long-range pollution. Some heavy metals (MN, Fe, Pb, Cd, and Cr) were measured in water – soil from two separate environments of Egyptian toad "*Sclerophrys regularis*" (EL Giza and El Wahat regions, Egypt). MN and FA traits were also assessed to compare and evaluate the impact of heavy metals pollution. The current results have revealed that EL Giza is more polluted than EL Wahat, and toads inhabiting EL Giza showed higher micronucleated MN and NL of RBCs than that from EL Wahat. Two patterns of fluctuating asymmetry; the right hindlimb was larger than the left limb and the right forelimb was larger than the left forelimb, were recorded from EL Giza. On the other hand, opposite results were obtained for toad processed from El Wahat. Statistical analysis indicated the presence of strong relation between heavy metals distribution in the environment and the investigated biomarkers ($0.05 \geq P \leq 0.01$).

INTRODUCTION

The recent dramatic declines in amphibian populations and the higher rate threat of extinction, which exceeds one third of the current species, have received attention of conservationists **(Gower *et al*., 2013)**. The biodiversity characteristics that amphibians exhibit are indicated through their ability to coexist in two different ecosystems, whether it is aquatic or terrestrial, given the fact that each ecosystem imposes a number of constraints that amphibians have to biologically abide by **Lion *et al*. (2019)**.

Consequently, it is safe to say that amphibians' anatomical structure makes it easier for both liquids and gases to be absorbed by their unique permeable skin. This biological trait allows them to be utilized as indicators for environmental pollution. Unfortunately, the same naturally distinct trait makes amphibians more exposed to toxic component **(Ives, 2021)**. **Saber *et al.* (2019)** demonstrated that habitat destruction one of the main reason of amphibians decline. Also, chemical contaminations consider one of the primary causes for that amphibian's vulnerability to extinction in natural habitats **(Egea-Serrano *et al*., 2012)**. Heavy metals are a part of the chemical pollutants, which can accumulate in the amphibian body and have the potential to disturb their development/growth and cannot be degraded **(Spolyarich *et al*. 2011)**. In general, trace metals can be assorted as their biologically function to essential and non-essential and their toxicity altitude with high concentration or metabolic deficiencies **(Mustafa, 2019)**. Heavy metals on the blood parameters of many a biota including amphibian have extradite global attention (**Sievers *et al.*, 2018 and Khattab *et al.*, 2021**). So this study used fluctuating asymmetry in measuring developmental instability and genotoxicity in diagnosing the structural status of chromosomes of red blood cell of Egyptian toad (*Sclerophrys regularis)* as biomarkers for toxicity of heavy metals impact to evaluate the harmful effect of certain heavy metals.

MATERIALS AND METHODS

1. Study area

Two areas (**Fig. 1**) were selected for the current study: Abu Rawash area, located about 8 kilometres north of EL Giza (site 1), Egypt. It is located at the main stream with global position at ٣٠° ٠١` ٥٦″ N, 31° ٠٤` ٢٩″ E. It has an industrial area with a large number of factories so it is receives a variety of pollutants. El Bawiti area, northern Bahariya Oases, western desert, Egypt. The Bahariya Oases (El Wahat) (site 2) located in the mid of the western desert at about 370 km to the southwest of Cairo. It located between latitudes 28^{o} 00′, 28^{o} 50′ N and longitudes 28^{o} 20′, 29^{o} 10′ E. This is an arid area with total rainfall between 3 to 6 mm/year. On the other hand, springs and wells are the main two-groundwater resources of water there.

Fig. 1. A map of the two studied regions, site 1 (Abu Rawash) and site 2 (El-Bawiti).

2. Sampling

Soil and water (5 replicates for each site) were sampled in acid washed plastic bags and bottles. Toads and samples collected were transported according to the recommended technique for Ecological studies. Some heavy metals (MN, Fe, Pb, Cd, and Cr) were determined in water and soil of the investigated environments. After acid-digestion, heavy metals in water (µg/l) and soil (µg/g) samples were determined by Graphite Furnace AA (GFAA) spectroscopy according to **Jackwerth and Würfels (1994).**

3. Genotoxicity using MN-test.

Blood samples were withdrawn by cardiac puncture **(Mgbenka *et al.*, 2003 and Parasuraman *et al.*, 2010)** and smeared on cleaned glass microscope slides. After 20 minutes of fixation in pure methanol, the slides were air–dried and stained with a 10% Giemsa solution. A total of 2000 erythrocytes were counted per toad, and five randomly selected animals from each group were screened and scored under 1000-fold magnification. A computerised camera was used to picture the scored RBCs with (MN/NL). Nuclear abnormalities of the erythrocytes were classified and identified according to **Bosch *et al.* (2011) and Osman *et al.* (2012).** The frequencies of micronucleated RBCs and RBCs abnormalities (nuclear lesions) were calculated as follows:

$$\textbf{RBCs (MN/NL) ‰} = \frac{\text{Number of cells containing micronuclei}}{\text{Total number of cells counted}} \times 1000$$

4. Fluctuating asymmetry in limbs

Random numbers of 60 toads from each site were examined to get accurate asymmetry measurements. The measured bones were hindlimb (femur and tibio-fibula mm) and forelimb (humerus and radio-ulnamm). Each bone was measured three times with a Calliper to avoid errors. The measurements of bones were based on **Sherman *et***

***al.* (2009)**. Fluctuating asymmetry was calculated according to **Ortiz-Santaliestra *et al.* (2006)** 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

5. Statistical analysis

The current data was processed using the statistical tool SPSS (V20). Throughout the current study, significant prospect values of 0.05 and 0.01 were used. Significant values were defined as those between 0.05 and 0.01 (both included).

RESULTS

1. Heavy metals in the investigated habitats (water – soil)

Based on the current data **(Table 1)**, metals concentrations in water were found in the following order: Fe > Mn > Cd > Pb > Cr in the water stampeded from the two regions. Regarding the mean concentrations of heavy metals in water, Iron (Fe) exhibited tremendous concentration in water sampled from El Wahat (515.14±120.43) when compared to El Giza (314.05±101.75). Also, the mean value of manganese of Abu Rawash (11.23±1.86) was twice that of El Bawiti (5.86±2.14). The mean concentration of cadmium fluctuated as 0.784±0.569 µg/l at El Giza and 0.170±0.163 µg/l at El Wahat. Lead concentration increased from 0.284±0.379µg/l at site 1 then declined to 0.150±0.255 µg/l at site 2. The mean concentrations of chromium increased from 0.0120±0.227 µg/l at site 2 to maximum value 0.274±0.004 µg/l at site 1. The means of the concentration of the five heavy metals in soil are presented in **Table (1)**. As well as water, heavy metals concentration in soil was higher at site 1 in comparison to the second site. So, the mean concentration of manganese in soil analysed from site 1 was 93.014±34.866 µg/g, followed by 12.624±2.838 µg /g in site 2. The measured lead in site 2 was 0.120±1.283 µg /g, but it was 7.676±1.283µg/g in site 1. Chromium peaked in site 1 (6.110±0.8050 µg/g), then exhibited a closed values (0.596±0.311µg/g) in site 2 . The high concentration of iron was observed in site 1 with mean 6984.234±2524.747 µg/g, but exhibited 2189.888±3804.536µg/g in site 2. Also, cadmium peaked at site 1 (1.020±0.496µg/g) but dropped in site 2 (0.270±0.352µg/g).

Table 1. Means ± SD of heavy metals concentrations of water (µg/l) and soil (µg/g) sampled from the two investigated sites.

Parameters	El Giza		El Wahat	
	Water	Soil	Water	Soil
Fe	515.140±120.43	6984.234±252447	314.050±101.75	2189.888±38536
Mn	11.238±1.86	93.014±34.866	5.868±2.14	12.624±2.838
Pb	0.284±0.37	7.676±1.283	0.150±0.25	0.120±0.083
Cd	0.784±0.56	1.020±0.496	0.170±0.16	0.270±0.352
Cr	0.274±0.22	6.110±0.8050	0.0120±0.00	0.596±0.311

2. ***Genotoxicity of erythrocytes***

All assessed micronucleated (MN) and nuclear lesions (NL) of RBCs were higher in El Giza specimens than in EL Wahat specimens (**Table 2** and **Fig. 2**). For example, the mean micronucleated RBCs (‰) of *Sclerophrys regularis* was 19.600±1.516‰ in site 1 and 9.000±1.581 in site 2. Binucleated RBCs were higher in animals collected from El Giza (12.400±1.816‰) compared to El Wahat (7.800±0.836‰). Among the biomarkers studied, MN and BN erythrocytes had the highest mean values. Strikingly, kidney shaped nuclei (8.000±.836‰) from site 2 were lower than those from site 1, namely El Giza (10.400±1.140‰). Irregular shaped nuclei scored from El Wahat animals (13.000±0.707 ‰) were four times those of El Giza (3.800±1.303‰). Oppositely, lobed from El Giza ware (6.000±1.224) twice the mean values from El Wahat (3.600±1.673‰). As well as lobed, heart shaped nuclei from El Giza (6.400±1.673‰) were several folds those from El Wahat (2.600±1.140‰). Vacuolated nuclei of site 1 (12.200±1.303 ‰) were more than five times of those of site 2 (2.800±1.483‰). Regarding the results of the T-test all parameters differed significantly between the two assessed regions ($p \leq 0.01$).

3. ***Correlation between MN & NL and heavy metals distribution in habitat***

Regarding **Table (3)**, MN and BN positively correlated with Mn, Fe, and Cr found in water ($0.05>p<0.01$), but MN and BN positively correlated with the five heavy metals in soil ($0.05>p\leq0.01$). Heart shaped positively correlated with Mn and Pb in water habitat ($0.05>p<0.01$), and correlated also with all metals in soil ($0.05>p\leq0.01$). Irregular shaped negative correlated with all metal ($0.05>p\leq0.01$) except Pb gave no significant ($p>0.05$) in water, but in soil irregular shaped negatively correlated with all except Cd ($p<0.01$), lobed nuclei were positively correlated with Mn, Fe, and Cr in water ($0.05>p<0.01$), and also correlated with metal in soil except Cd ($p\leq0.01$). Kidney shape and vacuolated nuclei were positively correlated with Fe and Mn in water ($p<0.05$) and kidney shaped positively correlated with all ($0.05\geq p\leq0.01$). Notched give significant with studded metal in soil and water ($p\leq0.01$)) except Pb in water, but vacuolated nuclei showed significant correlation with all heavy metals in soil ($p<0.01$) except Cd.

Table 2. Means ±SD of micro nucleated and nuclear lesions (‰) of *Sclerophrys regularis* RBCs collected from the two investigated sites.

Parameters	El Giza	El Wahat
MN‰	19.600±1.516	9.000±1.581
BN‰	12.400±1.816	7.800±0.836
Irregular shaped‰	3.800±1.303	13.000±0.707
Heart- shaped‰	6.400±1.673	2.600±1.140
Kidney- shaped‰	10.400±1.140	8.000±0.836
Vacuolated nuclei‰	12.200±1.303	2.800±1.483
Lobed shape‰	6.000±1.224	3.600±1.673
Notched nuclei‰	4.666±2.081	0.666±1.154

Table 3. Correlation coefficients between MN& NL and heavy metals concentrations in water and soil from the two sites.

Parameters		**Heavy metals in water**					**Heavy metals in soil**				
		Cd	**Pb**	**Cr**	**Fe**	**Mn**	**Cd**	**Pb**	**Cr**	**Fe**	**Mn**
MN	r	0.59^{NS}	0.472^{NS}	0.699*	0.787**	0.857**	0.702*	0.924**	0.955**	0.969**	0.860**
	sig	0.072	0.168	0.025	0.007	0.002	0.024	0.000	0.000	0.000	0.001
BN	r	0.456^{NS}	0.352^{NS}	0.770**	0.770**	0.857**	0.739**	0.825**	0.879**	0.883**	0.925**
	sig	0.186	0.319	.009	0.009	.002	0.015	0.003	0.001	0.001	0.000
Irreg. Sh.	r	0.680*	0.508^{NS}	.665*	0.638*	0.871**	0.595^{NS}	0.953**	0.969**	.983**	0.826**
	sig	0.030	0.134	0.036	0.047	0.001	0.069	0.000	0.000	0.000	0.003
Heart Sh.	r	0.512^{NS}	0.652*	0.522^{NS}	0.580^{NS}	0.750**	0.662*	0.948**	0.905**	0.868**	0.709*
	sig	0.130	0.041	0.122	0.079	0.012	0.037	0.000	0.000	0.001	0.022
Kid. Sh.	r	0.607^{NS}	0.438^{NS}	0.558^{NS}	0.652*	0.690*	0.623*	0.934**	0.903**	0.884**	0.811**
	sig	0.063	0.205	0.094	0.041	0.027	0.054	0.000	0.000	0.001	0.004
Vacuol.	r	0.541^{NS}	0.598^{NS}	0.614^{NS}	0.745**	0.755**	0.613^{NS}	0.931**	0.922**	0.941**	0.780**
	sig	0.106	0.068	0.059	0.013	0.012	0.060	0.000	0.000	0.000	0.008
Lobed Sh.	r	0.395^{NS}	0.560^{NS}	0.705*	0.798**	0.713*	0.550^{NS}	0.855**	0.834**	0.862**	0.797**
	sig	0.259	0.092	0.023	0.006	0.021	0.099	0.002	0.003	0.001	0.006
Notched	r	0.684*	0.365^{NS}	0.657*	0.697*	0.726**	0.737**	0.962**	0.932**	0.881**	0.828**
	sig	0.029	0.299	0.039	0.025	0.017	0.015	0.000	0.000	0.001	0.003

*: The mean difference is significant at the 0.05levels

**: The mean difference is significant at the 0.0levels

NS: The mean difference is not significant

Lobed Sh.= lobed shape, Heart Sh.= heart shape, Kid. Sh.= kidney shape, Irreg. Sh.= Irregular shape, Vacuol.= vacuolated

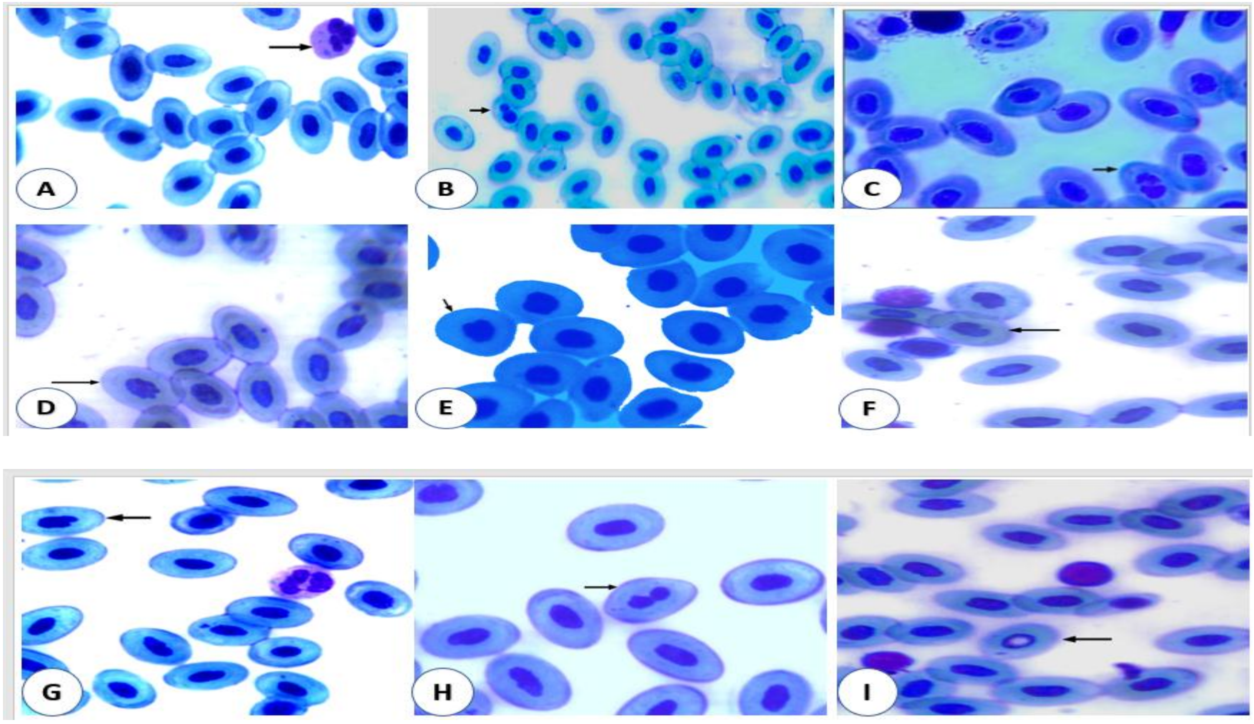

Fig. 2. Photomicrographs of erythrocytes of *Sclerophrys regularis* collected from the two investigated sites. Normal RBCs with WBCs (A), micronucleated MN RBC (B), binucleated RBC (C), lobed shape nucleus (D), heart – shaped nucleus(E), kidney–shaped nucleus (F), irregular – shaped nucleus (G), notched nucleus (H) and vacuolated nucleus (I), arrows, ×= 1000, Giemsa stain.

4. ***Fluctuating asymmetry (Right-Left limb distributions)***

The right minus left forelimb showed a negative value (–0.0016 mm) in the tabulated data **(Tables 4, 5)** and plotted data **(Figs. 3-6),** but the right minus left hindlimbs gave a positive difference (0.0059 mm). This means that fluctuating asymmetry in *Sclerophrys regularis* inhabiting El Giza exhibited two patterns of fluctuating asymmetry. The right hindlimb was larger than the left limb, while the right forelimb was larger than the left forelimb. **Table (5)** and **Figs. (5,6),** the opposite results were obtained for toads processed from EL Wahat. The fluctuating asymmetry was right biased since the mean value of right – left forelimb was positive (0.0005 mm). Furthermore, the fluctuating asymmetry in the hindlimb was negative, with a mean value of (–0.0020 mm).

Table 4. The statistics of FA of *Sclerophrys regularis* collected from site 1 (El Giza).

		FAFL	FAHL
N	Valid	58	58
	Missing	60	60
Mean		-.0016	.0059
Median		.0000	.0002

FAFL= Fluctuating asymmetry in forelimb, FAHL = Fluctuating asymmetry in hindlimb

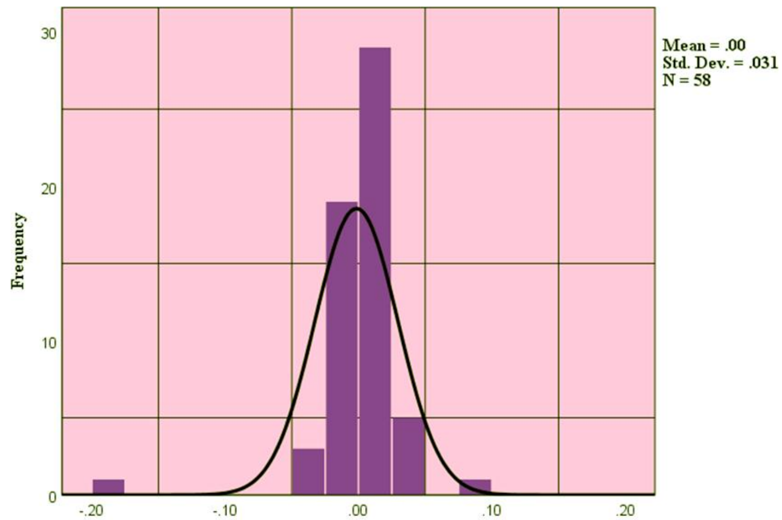

Fig. 3. Distribution of FA in forelimb of *Sclerophrys regularis* collected from site 1 (El Giza).

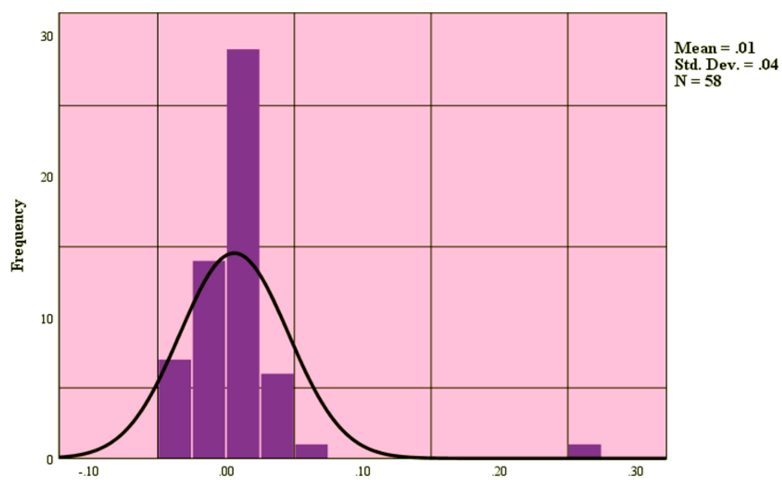

Fig. 4. Distribution of FA in hindlimb of *Sclerophrys regularis* collected from site 1 (El Giza).

Table 5. The statistics of FA of *Sclerophrys regularis* collected from site 2 (El Wahat).

		FAFL	FAHL
N	Valid	60	60
	Missing	58	58
Mean		0.0005	–0.0020
Std. Deviation		.00865	0.01346

FAFL= Fluctuating asymmetry in forelimb, FAHL = Fluctuating asymmetry in hind limb

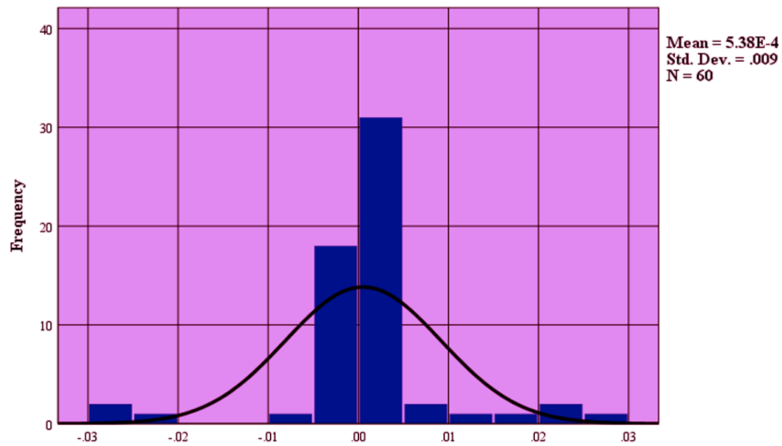

Fig. 5. Distribution of FA in forelimb of *Sclerophrys regularis* collected from site 2 (El Wahat).

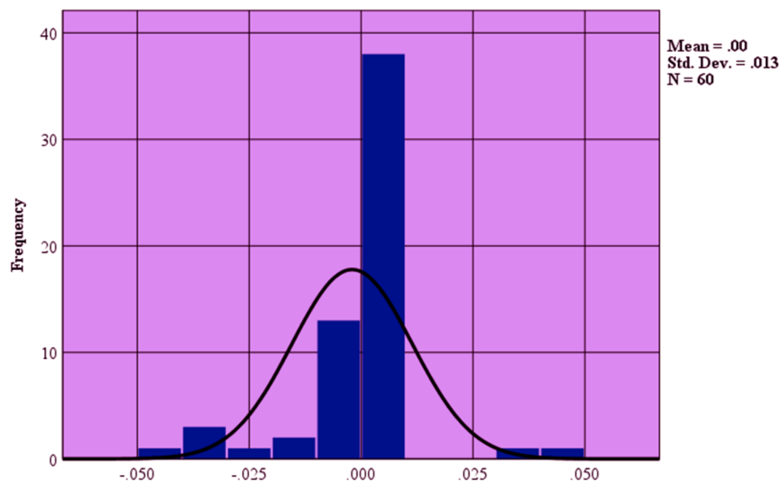

Fig. 6. Distribution of FA in hind limb of *Sclerophrys regularis* collected from site 2 (El Wahat).

DISCUSSION

Bio-monitoring is a crucial evaluation tool used around the world to offer critical up-to-date information on the state and health of amphibians **(Brodeur *et al.*, 2020)**. As a sequence of increment anthropogenic chemical pollution boosted and adversely affected terrestrial and aquatic environments. It is well known that many heavy metals discompose toxic effects in humans and animals **(Sharma *et al.*, 2014 ; Campbell Grant *et al.*, 2016)**. After ingesting environmental toxins from natural ecosystems, heavy metals for instance travel through the bloodstream to organs and tissues, where they are deposited **(Fazio *et al.*, 2014; Dupuy *et al.*, 2015).** They also stacked more in response to their proportion in the environment **(Said *et al.*, 2017; Osman *et al.*, 2018).** Several heavy metals lead to a variety of disorders *via* DNA breakage, morphological alterations and finally cell damage or death **(Doherty *et al.*, 2010 ; Güner and Muranli, 2011).** Damage to DNA may be repaired, induce mutation, or lead to necrosis, or apoptosis **(Leonard *et***

***al*., 2004; Pereira *et al*., 2006 ; Jadhav *et al*., 2007).** After cell division, micronuclei are chromosomal fragments or entire chromosomes that were not integrated into the nucleus **(Lidiyam *et al*., 2013).** The MN has been frequently used to detect clastogenic events or eugenic consequences in a variety of animals **(Udroiu, 2008)**. The MN test was used to evaluate DNA damage as a biomarker for genotoxic effects at the subcellular level, as well as an early response to chromosomal breakage and loss **(Garaj-Vrhovac *et al.*, 2008; Güner and Muranli, 2011).** A rise in the production of micronuclei, according to the present findings. El Giza had higher mean values for all MN, BN, and NL of *Sclerophrys regularis* erythrocytes than El Wahat. The present data showed accordance with several studies deal with MN and LN induction resulted from metal exposure from environment. Aside from the pollutant's genotoxic effects, as determined by the MN test and nuclear lesions, various morphological abnormalities of *Sclerophrys regularis* RBCs were discovered **(Ercal *et al.*, 2001)**. Heavy metals may directly interfere with DNA synthesis, as indicated visually in RBCs morphological abnormalities include unusual forms of RBCs (poikilocytosis) and large cells than the normal (megaloblastic/macrocytic anaemia), in addition to micronucleated RBCs and nuclear lesions **(Florence *et al*., 2006)**. In addition, FA of bilaterally symmetrical characteristics is a valuable sign of developmental instability that might increase in response to external environmental stress **(Breno *et al*. 2013 and Ahmed *et al*,. 2020)**. Animals exposed to environmental contaminants have shown increased levels of FA **(Guo *et al*., 2017).** According to **Güner and Muranli (2011), t**here is also naturally-occurring asymmetry, which is a small variation that arises in an organism independent of stress, increased FA among individuals exposed to pesticides stress has been spotted in fishes **(Allenbach *et al* ., 1999).** Egyptian toad exposed to atrazine and nitrates at tadpole stages exhibited fluctuating asymmetry when reached metamorphic stages **(Saber *et al*., 2016).** Alternate measuring techniques and markers may demonstrate more effective at detecting minimal variations. So instability arises as a result of organisms' inability to tolerate surrounding habitat pressure throughout development. Individuals who are better able to buffer themselves against the negative impacts of environmental stress have a distinct edge.

CONCLUSION

Based essure throughout development. Individuals who are better able to buffer themselves against the negative impacts of environmental stress have a distinct edge. In the current findings, the patterns of alteration including genotoxicity and fluctuating asymmetry can be used as potential biomarkers for environmental bio-monitoring. This approach allows us to assess the degrees of environmental influence on associate fauna from one extreme to the other, while also providing a database for decision-makers concerned with natural resources and their long-term use.

REFERENCES

Ahmed, S.S.; Saber, S.A.; Osman, A. G., El-Sayed, A.M. and Fouda, M.A. (2020). Detection of pollution effect using biomarker responses Fluctuating asymmetry in *Brachidontus rostratus* at Alexandria, Egypt.

Allenbach, D. M.; Sullivan, K. B. and Lydy, M. J. (1999). Higher fluctuating asymmetry as a measure of susceptibility to pesticides in fishes. *Environmental Toxicology and Chemistry: An International Journal*., 18(5): 899-905.

Bosch, B.; Mañas, F.; Gorla, N. and Aiassa, D. (2011). Micronucleus test in post metamorphic Odontophrynus cordobae and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. *Journal of Toxicology and Environmental Health Sciences*., 3:155-163.

B**reno, M.; Bots, J.; Schaepdrijver, L. D. and Dongen, S. V. (2013).** Fluctuating asymmetry as risk marker for stress and structural defects in a toxicologic experiment. Birth Defects Research, *Developmental and Reproductive Toxicology*., 98(4): 310–317.

Brodeur, J.C.; Bahl, M.F.; Natale, G.S. and Poliserpi, M.B. (2020). Biomarker and hematological fieldwork with amphibians: is it necessary to sample all night? *Environmental Science and Pollution Research*., 27:17152-17161.

Campbell- Grant, E.; Miller, D.; Schmidt, B.; Adams, M.; Amburgey, S.; Chambert, T.; Cruickshank, S.; Fisher, R.; Green, D. and Hossack, B. (2016). Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *International Journal of Scientific Reports.*

Doherty, V. F.; Ogunkuade , O. O. and Kanife, U. C. (2010). Biomarkers of oxidative stress and heavy metal levels as Indicators of environmental pollution in some selected fishes in Lagos, Nigeria. *American-Eurasian Journal of Agricultural and Environmental Sciences.*, 7 (3): 359-365.

Dupuy, C.; Galland, C.; Pichereau, V.; Sanchez, W.; Riso, R.; Labonne, M.; Amara, R.; Charrier, G.; Fournier, M.and Laroche, J. (2015). Assessment of the European flounder responses to chemical stress in the English Channel, considering biomarkers and life history traits. *Mar Pollut Bull.*, 95:634-645.

Ercal, N.; Gurer-Orhan, H. and Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. *Current Topics in Medicinal Chemistry.*, 1(6): 529-539.

Egea-Serrano, A.; Relyea, R. A.; Tejedo, M. and Torralva, M. (2012). Understanding of the impact of chemicals on amphibians: a meta-analytic review. *Ecology and evolution.*, 2(7): 1382-1397.

Fazio, F.; Piccione, G.; Tribulato, K.; Ferrantelli, V.; Giangrosso, G.; Arfuso, F. and Faggio, C. (2014). Bioaccumulation of heavy metals in blood and tissue of striped mullet in two Italian lakes. *Journal of aquatic animal health.*, 26:278-284.

Florence, A.; Joseph, J. and Steven, H. (2006). Megaloblastic Anemia and Other Causes of Macrocytosis, *Department of Internal medicin*., 4(3): 236–241.

Garaj-Vrhovac, V.; Gajski, G. and Ravlić, S. (2008). Efficacy of HUMN criteria for scoring the micronucleus assay in human lymphocytes exposed to a low concentration of p, p'-DDT. *Brazilian Journal of Medical and Biological Research.*, 41: 473-476.

Gower, D.J.; Kassahun, R.; Schwaller, S.; Largen, M.J.; Collen, B.; Spawls, S.; Menegon, M.; Zimkus, B.M.; De Sá, R.; Mengistu, A.A.; Gebresenbet, F.; Moore, R.D.; Saber, S.A. and Loader, S.P. (2013). Longterm data for endemic frog genera reveal potential conservation crisis in the Bale Mountains, *Ethiopia. Oryx*., 47 (1): 59-69.

Güner, U. and Muranli, F. D. G. (2011). Micronucleus test, nuclear abnormalities and accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853). Tur*kish Journal of Fisheries and Aquatic Sciences*, 11(4), 615-622.

Guo, R.; Zhang, W.; Ai, S.; Ren, L. and Zhang, Y. (2017). Fluctuating asymmetry rather than oxidative stress in *(Bufo raddei)* can be an accurate indicator of environmental pollution induced by heavy metals. *Environmental monitoring and assessment*, 189(6): 1-10.

Ives, A. (2021). Thyroid gland development in the larval Northwestern salamander *(Ambystoma gracile)*. *In SFU Undergraduate Research Symposium Journal*., 2, 1.

Jackwerth, E. and Würfels, M. (1994). Der Druckaufschluß—apparative Möglichkeiten, Probleme und Anwendungen. Pages 121-138 Probennahme und Aufschluß. Springer.

Jadhav, S. H.; Sarkar, S. N.; Patil, R. D. and Tripathi, H. C. (2007). Effects of subchronic exposure via drinking water to a mixture of eight water-contaminating metals: a biochemical and histopathological study in male rats. *Archives of Environmental Contamination and Toxicology*., 53:667–677.

Leonard, S. S.; Harris, G. K. and Shi, X. (2004). Metal-induced oxidative stress and signal transduction Free Radical Biology and Medicine., 37(12): 1921–1942.

Lidiyam, I.; Palak, K. and Olga, K. (2013). Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond, Department of Biological Sciences, University of Lethbridge, Lethbridge, AB, Canada., https://doi.org/10.3389/fgene.2013.00131

Lion, M. B.; Mazzochini, G. G.; Garda, A. A.; Lee, T. M.; Bickford, D.; Costa, G. C. and Fonseca, C. R. (2019). Global patterns of terrestriality in amphibian reproduction. *Global Ecology and Biogeography.*, 28(6): 744–756.

Mgbenka, B. O.; Oluah, N. S. and Umeike, I. (2003). Effect ofgammalin 20 (lindane) on differential white blood cell counts of the African catfish, (*Clarias albopunctatus)*. *Bulletin of Environmental Contamination and Toxicology.*, 71(2): 0248–0254.

Mustafa, M. A. (2019). Elemental Analysis of Scalp Hair Samples from Workers in the Industrial Area of Omdurman by using Spectroscopic Techniques (Doctoral dissertation, Sudan University of Science and Technology).

Khattab, N.; Saber, S.; El-Salkh, B. and Said, R. (2021). The Efficiency of *(Sclerophrys regularis)* as a Bioindicator. *Egyptian Academic Journal of Biological Sciences, Zoology*., 13(1): 91-101.

Osman, A. G; Abuel-Fadl, K. Y. and Kloas, W. (2012). In situ evaluation of the genotoxic potential of the river Nile: II. Detection of DNA strand-breakage and apoptosis in *Oreochromis niloticus niloticus* (Linnaeus, 1758) and *Clarias gariepinus* (Burchell, 1822). *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*., 747:14-21.

Osman, A.G.; AbouelFadl, K.Y.; Abd El Baset, M.; Mahmoud, U.M.; Kloas, W. and Moustafa, M.A. (2018). Blood biomarkers in Nile tilapia *Oreochromis niloticus niloticus* and African catfish *Clarias gariepinus* to evaluate water quality of the river Nile. *Journal of Fisheries Sciences*., 12:1-15.

Ortiz-Santaliestra, M. E.; Marco, A.; Fernández, M. J. and Lizana, M. (2006). Influence of developmental stage on sensitivity to ammonium nitrate of aquatic stages of amphibians," *EnvironmentalToxicology Chemistry*., 25:105–111.

Parasuraman, S.; Raveendran, R. and Kesavan, R. (2010). Blood sample collection in small laboratory animals. *Journal of Pharmacology and Pharmacotherapeutics*., 1(2): 87.

Pereira, R.; Pereira, M. L.; Ribeiro, R. and Gonçalves, F. (2006). Tissues and hair residues and histopathology in wild rats *(Rattus rattus L)* and Algerian mice (*Mus spretus* Lataste) from an abandoned mine area (Southeast Portugal). *Environmental Pollution.*, 139(3): 561–575.

Saber, S. A.; El Salkh, B. A.; Said, A. S.; Said, R. E. and Gadel-Rab, A. G. (2016). Limbs asymmetry as biomarker in the Egyptian toad Amietophrynus regularis Exposed to Atrazine and Nitrates. *Interational Journal Ecotoxicology Ecobiology*., 1(3): 103–110.

Saber, A. S.; Kassahun, R.; Loader, P .S. and El Kafrawy, B. S. (2019). Amphibian diversity in relation to environmental change in Harenna Forest, Bale Mountains National Park, Ethiopia: A remote sensing and GIS approach. *Egyptian Journal of Aquatic Biology and Fisheries.*, 23(3): 139-149.

Said, R.E.; AbdAllah, A.T.; Mostafa, M.A. and El-Shimy, N.A. (2017). Efficiency of *Polychaete Nereis* (Neanthes) Succinea as Biomonitor for Heavy Metals Pollution in the Red Sea, *Egypt. Advances in Bioscience and Bioengineering.*, 5:86-91.

Sharma, B.; Singh, S. and Siddiqi, N.J. (2014). Biomedical Implications of Heavy Metals Induced Imbalances in Redox Systems. *BioMed Research International*.,640754.

Sherman, E.; Tock, K. and Clarke, C. (2009). Fluctuating asymmetry in *(Ichthyophonus sp).* infected newts, (*Notophthalmus viridescens)*, from Vermont. *Applied Herpetology*., 6(4): 369-378.

Sievers, M.; Hale, R.; Swearer, S. E. and Parris, K. M. (2018). Contaminant mixtures interact to impair predator-avoidance behaviours and survival in a larval amphibian. *Ecotoxicology and Environmental Safety.*, 161:482-488.

Spolyarich, N.; Hyne, R. V.; Wilson, S. P.; Palmer, C. G. and Byrne, M. (2011). Morphological abnormalities in frogs from a rice-growing region in NSW, Australia, with investigations into pesticide exposure. *Environmental Monitoring and Assessment*., 173(1–4): 397–407.

Udroiu, I. (2008). The micronucleus test for aquatic toxicology. Aquatic Toxicology Research Focus. Elías Svensson, *NovaScience Publishers*.,pp. 145-160.