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Effect of Cadmium on the Developmental Instability of *Anabas testudineus* through Evaluation of Fluctuating Asymmetry of Its Bilateral Traits

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Abstract

Biomonitoring using fish has always been a popular method of assessing the toxic effects of cadmium on an organism. Increasing concentrations of cadmium in water bodies has led to bioaccumulation in the fish contributing to various morphological alterations. Among the known effects of these alterations is developmental instability via fluctuating asymmetry. Fluctuating asymmetry is increasingly being utilized as a simple yet effective method to evaluate the toxic effects of cadmium on the stability of development. This study used climbing perch (*Anabas testudieneus*) to assess the developmental instability caused by low level cadmium exposure by determining the fluctuating asymmetry in four bilateral traits; length of paired fins (pectoral and pelvic fins) diameter of eye and weight of gills. The left and right sides of the traits were measured once every four weeks for 16 weeks. The average measurements from both sides of the traits were used to calculate the FA value. Results found that the highest mean FA value in all traits except for pelvic fins were obtained in the highest treatment group, 0.015 mg/L. The increasing concentrations of cadmium exposure had a significant effect on the FA value of length of pectoral fins and diameter of eye but did not significantly influence the FA of weight of gills and FA of pelvic fin length. There was also a significant interaction between the duration of exposure and treatment groups in FA value of length of pectoral fin and diameter of eye, $p < 0.05$. The results from the study indicate that prolonged exposure to sub lethal concentrations of cadmium is able to cause significant effects on the normal development of length of pectoral fin and diameter of eye.

Keywords

Cadmium, *A. testudineus*, Fluctuating Asymmetry, Eye, Gills, Pelvic Fin, Pectoral Fin

1. Introduction

Heavy metal contamination of rivers teaming with aquatic life is a global issue. Among these heavy metals, cadmium is considered to be one of the most toxic heavy metals [1]. Cadmium is a nonessential heavy metal which exists in the environment in very small concentrations and often as impurities in non-ferrous metals like zinc, lead and copper [2]. Until recently, the reported release of cadmium into the atmosphere is approximately 30,000 tons annually of which between 4,000 tons and 13,000 tons are of anthropogenic sources [3]. Mining activities of smelting zinc bearing ores, fossil fuel combustion, agricultural fertilizer and municipal waste discharge are the primary culprits for this predicament [4]. Classified as a Group 1 Confirmed Human Carcinogen [5], cadmium concentration in the environment has increased to levels elevated than the background concentration to subsequently contaminate the water. The highly toxic effects of cadmium on all mammals and fish can be explained by the metal's inherent ability to rapidly accumulate in these living organisms [6]. It's persistence in water is equally troubling, particularly to the fish inhabiting these waters that are constantly in contact with the increasingly elevated levels of pollutants [7], The danger posed by increased cadmium concentrations in water is recognized by authoritative bodies and concentration limits have been set for monitoring purposes as stated in **Table 1**. However, very few bodies have set concentration limits for monitoring of cadmium concentration in fish. This factor coupled with the lack of standardized limits globally warrants further research into the toxic effects of cadmium on fish.

Table 1. Concentration limits of cadmium in freshwater [8] [9] [10].

Standard limits of cadmium in water (mg/L)		Source
Freshwater ecosystem	0.001	National Water Quality Standards, Malaysia
	0.0018	Environmental Protection Agency
	0.00072	National Recommended Ambient Water Quality Criteria (AWQC)
Drinking water	0.003	National Drinking Water Quality Standards, Malaysia
	0.003	World Health Organization
	0.005	Environmental Protection Agency

Human consumption of fish has long been practiced and is ubiquitous in the diet of the general population due to its nutritive values [11]. However, with the increasing cadmium pollution of water bodies, humans are at risk of exposure through consumption of cadmium contaminated fish [12]. It has been stated previously that cadmium possess bio-accumulative properties which poses a hazard to human health through prolonged consumption of contaminated fish. Fish are often higher in the aquatic food chain and, therefore, will accumulate substantial amount of heavy metal [13].

Cadmium exerts its effect differently in different organisms, but studies have determined that it is highly toxic to all mammals and fish [14]. [15] found that cadmium is able to cause developmental delay as early on in the larval stage of fish. Studies posits the disturbance in development is due to changes in the cell and production of excess reactive oxygen species (ROS) causing cell death or apoptosis [16]. Therefore, cadmium has been studied to cause detrimental effects to target organs by inducing stress in cells that regulates development. The disturbances in the development of fish are referred to as "developmental instability" which is the inability of an organism to express a certain trait due to genetic or environmental stress [17]. In ecotoxicology studies, alterations to an organism's development have been quantified and used to analyse the extent of environmental pollution. Variations in traits are associated with the organisms' response to environmental stress [18]. This is due to alterations in the organisms' homeostasis following perturbations in optimum living conditions [19]. This goes to show how susceptible an organisms' development is to the introduction of contaminants as it acts upon the homeostatic mechanisms.

There is a myriad of biomonitoring tools used in research to assess the effects of cadmium on fish. However, the most accurate and used quantitative tool to measure developmental instability is fluctuating asymmetry [20]. By definition, fluctuating asymmetry is random deviations between the right and left side of bilaterally symmetrical organisms [21] [22]. Poor homeostatic performance is able to act upon the development of an organisms which is manifested in structural shifts in bilaterally symmetrical traits [22]. Fluctuating asymmetry studies often use morphometric (measured) characteristics to assess these minute structural shifts. Morphometric measurements compare the sum average difference in measurement between selected bilateral traits of the left and right side of an organism [23]. In comparison to other biomonitoring tools that is time and energy consuming with more complex methods, FA is regarded as a simple yet accurate tool to measure developmental effects that does not require a large sample size.

Anabas testudineus or Climbing Perch is a Teleost fish that is native to Asia [24]. It favours brackish waters of canals, lakes, ponds, swamps and estuaries and can withstand unfavourable conditions. Ease of reproduction and rearing makes it a species of economic importance [25]. The species' omnivorous nature means that they accumulate large amounts of cadmium following ingestion of biotic elements that have also accumulated cadmium [26]. The Climbing Perch is hardy and able to withstand harsh environments [27]. This adaptability makes it an appropriate biomonitoring species to study the prolonged effects of cadmium on living organisms, as they are resilient enough to withstand the introduction of a toxicant but able to exhibit the effects in alterations to physiological and morphological function. This study assessed the effects of cadmium on the developmental instability of *Anabas testudineus* utilizing fluctuating asymmetry on the length of paired fins (pectoral fins and pelvic fins), weight of gills and diameter of eye. The study also measured the FA value on the aforementioned parameters and compare the FA values between different concentrations as well as to compare the FA values between treatment groups and duration of exposure.

2. Materials and Methods

This study is an experimental study utilizing a random and experimental study design. An animal ethics approval was received prior to conducting the experiment (UKMAEC APPROVAL No: FSK/2020/MOHD SHAM/25-N0V./1137-DEC.-2020-DEC.-2021). Four hundred individuals of *A. testudineus* were bought and reared in the laboratory. Water changes with corresponding cadmium concentration were done every two weeks to ensure cadmium concentrations were maintained throughout the research duration. The fish fry was divided into four different treatment groups exposed to varying concentrations of cadmium. To ensure the survival of the fish fry were maintained at 89% according to a study by [28], the stocking density of the fish fry were at 4 fish per litre of water [29].

Approximately 100 fish fries were placed in different treatment groups exposed to different concentrations of cadmium nitrate: control group exposed to 0.000 mg/L cadmium nitrate, low treatment group exposed to 0.005 mg/L cadmium nitrate, medium treatment group exposed to 0.010 mg/L cadmium nitrate and high treatment group exposed to 0.015 mg/L cadmium nitrate.

The study duration was 16 weeks and sampling, measurement and analysis were conducted every four weeks. In each sampling, about 20 fish were sampled, measured and analysed to obtain the fluctuating asymmetry value.

The length of both the left and right sides of the paired fins (pectoral and pelvic fins) and diameter of both sides of the eye were measured while the gills from both sides were dissected and weighed separately. To account for measurement error, all measurements were taken by the same person for a frequency of three times. To obtain the FA value, the difference in the average measurement of the trait on the left and right sides were divided by the sum total of the average measurements of the left and right sides of the trait that was divided by two.

Statistical analysis on the parameters was conducted using SPSS 2.0 version 25. Descriptive analysis was used to measure the FA value on the length of pectoral and pelvic fins, diameter of eye and weight of gills for each week. Normality tests will be run prior to analysis to compare the FA value between different concentrations using One Way ANOVA. To compare the FA values between different treatment groups and duration of exposure, a Two Way ANOVA was used.

3. Results and Discussion

Four bilateral traits were used to measure the effects of cadmium on the developmental instability in *A. testudineus*. The average fluctuating asymmetry value for length of pectoral fins ranged from 5.27% to 10.02%. The highest average FA value was obtained in samples in treatment group 0.015 mg/L 10.02 % ±11.50. There is no observable increasing trend as the concentration increases as shown in Figure 1. Analysis using One-Way ANOVA revealed that the FA value for length of pectoral fin is significantly influenced by different concentration of exposure, $p < 0.05$. Comparison of the FA value between treatment groups found a significant difference between treatment group 0.005 mg/L and 0.015 mg/L at $p < 0.05$. However, there was no significant difference in the FA value between control group and all three treatment groups, $p > 0.05$.

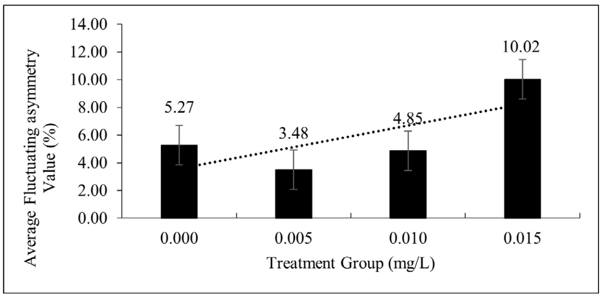

Figure 1. Average Fluctuating Asymmetry (FA) value of length of pectoral fin across treatment group.

The mean fluctuating asymmetry value for FA length of pelvic fin ranged from 2.86% to 4.29%, with the highest mean FA value found in treatment group 0.010 mg/L with 4.29% ± 2.97. Tests to determine whether different exposure concentration significantly affected the FA value using One Way ANOVA revealed no statistical significance between the two variables, $p > 0.05$. Comparison of the FA value between control treatment group and all three treatment groups found no significant difference. Similarly, there was no significant difference in FA value between all three treatment groups, $p>0.05$. Observation into the trend of mean FA value of pelvic fin does not show the value increasing consistently as concentration increases as shown in Figure 2.

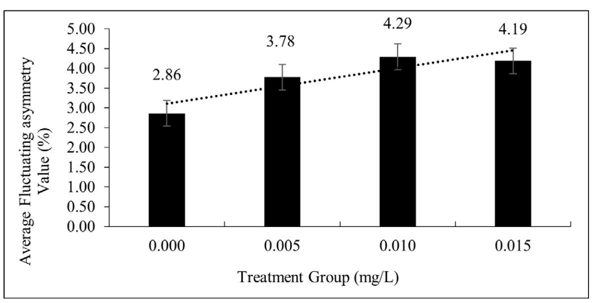

Figure 2. Average Fluctuating Asymmetry (FA) value of length of pelvic fin across treatment groups.

The parameter of gill weight produced FA values greater than other traits assessed. Analysis into the average FA value for weight of gills ranged from 24.75% to 29.04%. Treatment group 0.015 mg/L obtained the highest FA value with a mean FA value of 29.04% ±23.56. A One-Way ANOVA test found that the increase in FA value for weight of gills across treatment groups was not statistically significant, $p > 0.05$. Comparison of the mean FA value between treatment groups found no significant difference between the control group and treatment groups nor were there any observed between the treatment groups, $p > 0.05$. There is a fluctuating trend in the FA value for weight of gills across treatment group, with no observable increasing or decreasing FA value with increasing concentration as shown in **Figure 3**. Inconsistencies in FA value of weight of gills were also reported by [30] in a study that found no statistically significant difference between the left and right gill rakers and gill lamellae of the left and right sides of *Lebeo oguenensis*. Despite this, the FA value obtained for gills in this study is greater than the values of other traits measured. [31] reported that experimental studies on fish exposure to cadmium found that gills store a large quantity of cadmium subsequently causing morphological alteration. Therefore, it is expected that for an organ continuously in contact with cadmium-contaminated water, the gills would have a greater FA value than other bilateral traits. This is supported by [12] that found histopathological alterations in gills of *Oreochromis niloticus* which manifested in necrosis and atrophy of gill lamellae. We believed that the high standard deviation found in the 0.015 mg/L treatment may stem from histopathological alterations in a small number of the fish studied.

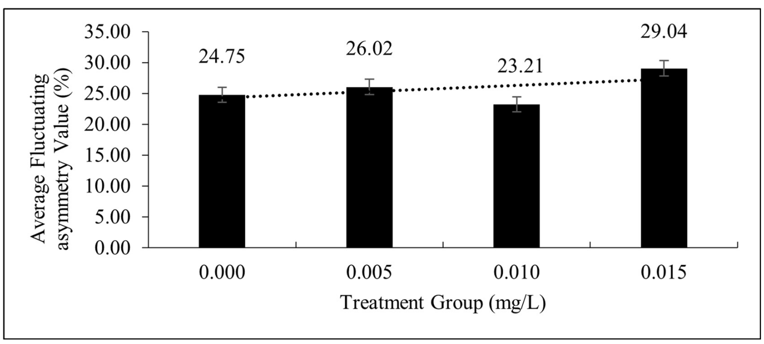

Figure 3. Average Fluctuating Asymmetry (FA) value of weight of gills across treatment group.

The diameter of the eye was also assessed by measuring the FA value after prolonged exposure to cadmium. The average FA value for diameter of eye ranged from 2.00% to 5.20%, with the greatest mean FA value found in treatment group 0.015 mg/L at 5.20% ±2.80. Analysis using One Way ANOVA determined that the increase in FA value of diameter of eye was significantly influenced by exposure to different concentrations of cadmium, $p < 0.05$. Comparison of the FA value between the treatment groups found that the control group had significantly lower FA value than treatment group 0.010 mg/L and 0.015 mg/L, $p < 0.05$ (**Figure 4**).

There was also a significant difference in the FA value between treatment group 0.005 mg/L and 0.015 mg/L, $p < 0.05$. The results found the range of mean FA values of diameter of eye to be lower than mean FA value of the pectoral fins. This is contrary to a previous study by [22] that reported the average FA value for diameter of eye in *Menidia beryllina* was greater than the value measured in the pectoral and pelvic fins.

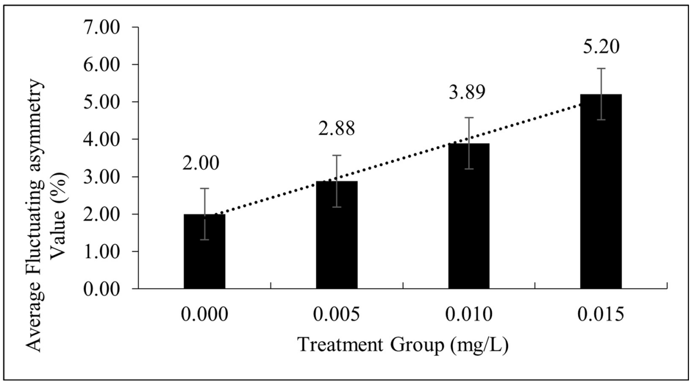

Figure 4. Average Fluctuating Asymmetry (FA) value of diameter of eye across treatment group.

Overall trend of FA value among the three traits measured indicates an increase in FA value with increasing concentration. Exposure to environmental stress will result in energy being spent to control and adapt to non-optimum conditions. This is supported by [32] who discussed that exposure of A. testudineus to cadmium resulted in excess energy being diverted to respond to oxidative stress subsequently reducing the energy reserves for growth and development. Some research suggests that fin lengths is a less sensitive traits to measure developmental instability than eye due to its functional importance. In his study, [22] found that fin asymmetry of *Menidia beryllina* following exposure to environmental stress was less pronounced than diameter of eye. He discusses that due to the fin's functional importance, the FA value is much lower because of its strong stabilizing selection. This is because the energy needed to produce asymmetry is costly, developmental stability will act to reduce FA in the fins as opposed to less expensive asymmetries in the eye. Despite this theory, the *Anabas testudineus* used in this study were reared in aquariums under laboratory conditions therefore it is possible that due to less space for locomotion, the fins have less functional importance as opposed to the gills and eyes. [33] extensively reviewed FA studies and determined that habitat factors in functional importance; pectoral fins of fish in sluggish waters will not likely be used for stabilization and, therefore, be able to demonstrate FA. This theory is supported by [34] and his study on Greenfinch that found traits of less functional importance to exhibit significantly higher levels fluctuating asymmetry than traits of more functional importance.

To compare the FA value of *Anabas testudineus* between treatment group and duration of exposure, a Two-Way ANOVA was utilized. The first parameter measured was length of pectoral fin. No increasing trend can be observed in the FA value of length of pectoral fin in different treatment groups as the duration of exposure increases as shown in **Figure 5**. However, an analysis to compare the FA value for length of pectoral fin between different exposure concentrations and duration of exposure using Two Way ANOVA found a significant interaction effect between treatment group and duration of exposure, $p < 0.05$. Analysis of the main effect found that there was a significant mean difference in FA value of length of pectoral fin between duration of exposure, $p < 0.05$. Similarly, there was a significant main effect for different exposure concentration, $p < 0.05$. What these results indicate is that the mean FA value for length of pectoral fin is significantly different between treatment groups and between duration of exposure. These results are congruent to the findings by [35] which found a significant difference in the FA value between treatment and exposure duration.

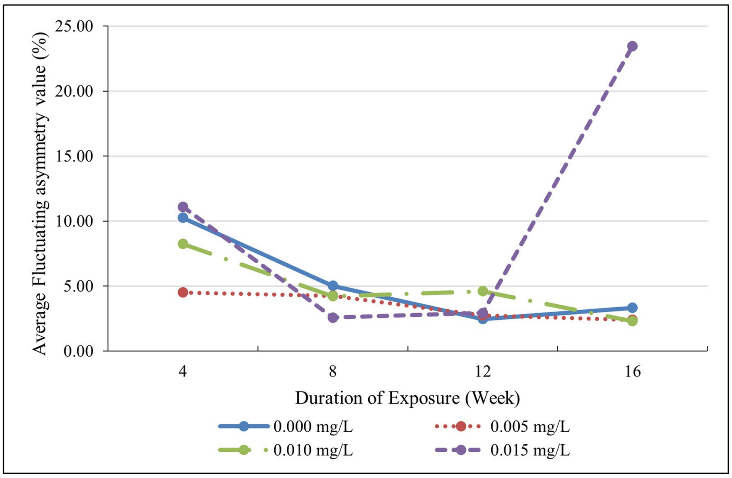

Figure 5. Average Fluctuating Asymmetry (FA) value (%) length of pectoral fin across duration of exposure.

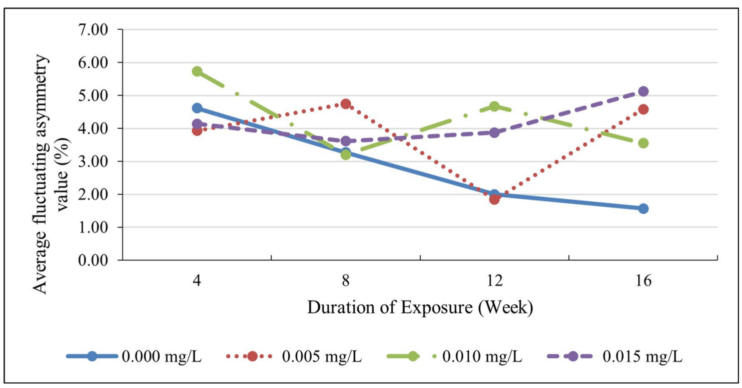

Figure 6. Average Fluctuating Asymmetry (FA) value (%) of length of pelvic fin across duration of exposure.

A Two-Way ANOVA was used to determine whether duration of exposure or different exposure concentration have an effect on FA value of pelvic fin and whether a significant interaction exists between the two variables. The analysis found that there was no significant interaction effect on the FA value of pelvic fin between different exposure concentration and duration of exposure, $p>0.05$. Main effect analysis revealed that there was no significant main effect for different treatment groups and similarly no significant mean difference of FA value of length of pelvic fin between duration of exposure, $p > 0.05$. **Figure 6** shows the trend in FA values across duration of exposure which indicates a decreasing trend in the FA value for length of pelvic fin in control group. However, trend in mean values in the other treatment groups show fluctuation across duration of exposure.

Analysis into the FA value of diameter of eye found that there was a significant interaction between treatment group and duration of exposure, $p < 0.05$. The main effect analysis revealed that there was a significant effect in FA value between treatment group, $p < 0.05$. The FA value of diameter of eye is therefore significantly affected by different exposure concentrations. There was, however, no significant main effect for duration of exposure, $p > 0.05$. As shown in **Figure 7**, the trend in FA values for diameter of eye does not indicate an increase in FA values according to treatment group as duration of exposure increases. In saying this however, the significant difference in FA values of diameter of eye between treatment groups does indicate dose-dependency. This is supported by [33] who reported that eye asymmetry was more significantly observed in response to environmental stress than in paired fins. These results are however contrary to a study by [36] that found no significant relationship between environmental contamination and high FA values of diameter of eye in freshwater three-spine stickleback fish. The variations in FA value of morphometric traits will be discussed further on.

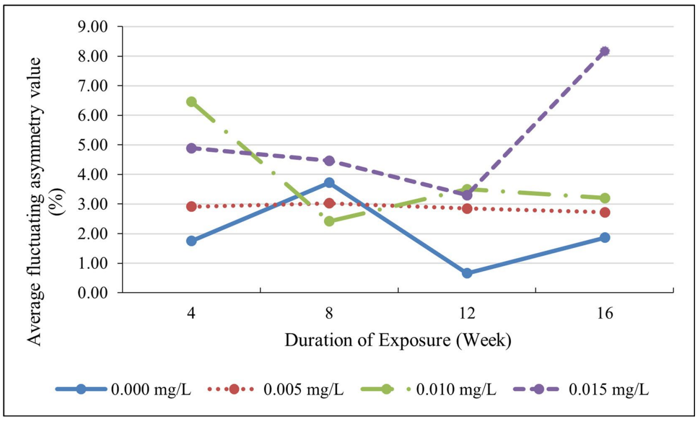

Figure 7. Average Fluctuating Asymmetry (FA) value of diameter of eye across duration of exposure.

The trend in FA values of weight of gills according to treatment group do not consistently show an increasing pattern (**Figure 8**). This is supported by the Two-Way ANOVA analysis which found no significant interaction in FA value of weight of gills between different treatment group and duration of exposure, p >

0.05.The main effects analysis did not find any significant difference in the FA value of weight of gills between duration of exposure and between treatment group, $p > 0.05$ While the gills obtained higher FA values as compared to other bilateral traits measured, there was no significant interaction between duration of exposure and treatment group, $p > 0.05$. The gills are regarded as one of the most sensitive indicators of environmental stress and yet studies have shown its ability to acclimatize to environmental disturbances [37]. It is likely that the varying FA values in gill weight as concentration and duration increases is attributed to developmental interference. Studies have reported that some parts of the gills are controlled by the same genetic mechanism which induces varied susceptibility to a stressor therefore making gill asymmetry a function of genetics [30]. Although no significant effect was found between FA of weight of gills and treatment group, treatment groups 0.005 mg/L and 0.015 mg/L obtained higher mean FA value than control treatment group. This indicates that exposure to cadmium does induce developmental instability and that exogenous stressor factors in the increase in FA values rather than merely genetic or developmental interference.

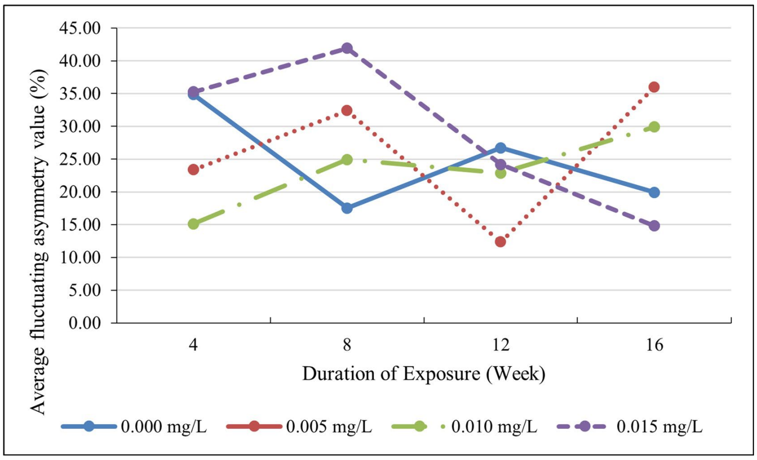

Figure 8. Average Fluctuating Asymmetry (FA) value (%) of weight of gills across duration of exposure.

While an observable increase in FA value was observed in the diameter of eye, no consistent negative or positive trend were observed for length of pectoral fin and weight of gills. Inconsistencies in results of FA value are not uncommon in literature. Varying degrees of FA value obtained in this study in all parameters were also observed in previous fish ecotoxicology studies in response to environmental stress [30]. A factor that contributes to varying FA values for a bilateral trait is body size. While the factor of size had been taken into consideration in this study, small deviations in the body size of *A. testudineus* may greatly influence the average FA value. [38] found a significant relationship between diameter of eye and standard length of fish. Lucentini [39] also reported that larger fish exhibit greater asymmetry.

The ability of cadmium to cause adverse effects on the development suggests that DNA damage due to cadmium exposure is significantly greater at highest concentration. DNA damage as stated is linked to the generation of reactive oxygen species (ROS) in excess [16]. The formation of ROS due to cadmium exposure is related to the disruption in biochemical parameters like the inhibition of important enzymes [40]. Among these enzymes important to development, estrogen is affected by exposure to high concentrations of cadmium. This is supported by a study by [41] that reported a significant interaction between developmental instability and estrogenic activity of *Trichomysterus aerolatus* subsequently increasing the FA value. Additionally, these alterations in the biochemical parameters contributes to the disruption of homeostatic mechanisms for growth which causes small deviations from the symmetry of bilateral organs, like the traits used in this study [22].

The choice of *Anabas testudineus* as the sentinel species in this study was due to its hardiness and adaptability in harsh environments. The significant FA values observed for length of pectoral fin and diameter of eye with increasing concentrations proves that while it is strong enough to survive cadmium-contaminated conditions, FA can still be detected in significant values. This statement can be supported by a study by [42] who observed histopathological changes in *Anabas testudineus* following exposure to cadmium.

4. Conclusion

Results from the study showed that the highest mean FA value for all the traits except for length of pelvic fin was obtained in the highest treatment group, 0.015 mg/L. While there was no observable consistent increase in FA value of pectoral fin with increasing duration of exposure and increasing concentrations, there was a significant main effect in FA value according to treatment group and duration of exposure on the FA pectoral fin, $p < 0.05$. Unlike pectoral fins, there was no significant increase in FA pelvic fin length with increasing concentration or increasing duration of exposure, as evident in the non-significant results of the interaction effect between treatment group and duration of exposure. The results also show that the FA value for diameter eye significantly increases as the concentration increases with a significant mean difference in the FA values between different treatment groups. While the mean FA value of weight of gills is greater than the mean FA value of the other morphometric traits, concentration and duration of exposure did not significantly impact the FA value. Overall, it can be reported that exposure of *A. testudineus* to cadmium causes developmental instability in the length of pectoral and pelvic fins, diameter of the eye and gill weight as the highest mean FA value was obtained by the highest treatment groups. Limitations of the study extends to precision of measurement, as fluctuating asymmetry is known to be highly sensitive. Careful consideration and control of the size of the fish sampled should be exercised in future studies. Conclusively, future studies can be conducted using fluctuating asymmetry as a measurement tool to assess the effects of anthropogenic stressors on the developmental instability in fish on other bilateral traits.

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Conflict of Interest Statements

The authors whose names are listed clarify that they have no participation or affiliation in any organizations that involve financial or non-financial interests in the subject or matter discussed in this manuscript.

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