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Effect of Cadmium on the Developmental Instability of *Anabas testudineus* through Evaluation of Fluctuating Asymmetry of Eye Diameter

Fazlin Hazirah Mohd, Sharifah Nadrah Syed Idrus, Mohd Riduan Abdullah, Mohd Sham Othman*

Center for Toxicology & Health Risk Studies (CORE), Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia
Email: *mso@ukm.edu.my

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Abstract

Biomonitoring using fish has been a popular method of assessing the toxic effects of cadmium concentrations of cadmium in water bodies and has led to bioaccumulation in fish contributing to various morphological alterations. Among the known effects of these alterations is developmental instability via fluctuating asymmetry (FA). The utilization of FA data is 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 FA in the diameter of the eye because of low-level cadmium exposure. 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 for the diameter of the eye was obtained in the highest treatment group, 0.015 mg/L. The results also indicate that increasing concentrations of cadmium exposure had a statistically significant effect on the FA of eye diameter. There was also a statistically significant interaction between the duration of exposure and treatment groups in FA of the diameter of the eye. The results from the study indicate that prolonged exposure to sub-lethal concentrations of cadmium can cause significant effects on the normal development of eyes in *Anabas testudineus*. This study can add to the body of research regarding fluctuating asymmetry as a tool to study developmental toxicants and assess other bilateral organs.

Keywords

Biomonitoring, Cadmium, Eye, Fluctuating Asymmetry, *Anabas testudineus*

1. Introduction

Heavy metal contamination of rivers teaming with aquatic life is a global issue. Among these heavy metals, cadmium is one of the most toxic heavy metals [1]. Cadmium is a nonessential heavy metal that 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 4000 tons and 13,000 tons are from anthropogenic sources [3]. Mining activities, fossil fuel combustion, use of agricultural fertilizer and municipal waste discharge have been cited to be the culprits of this predicament [4]. Classified as a Group 1 Confirmed Human Carcinogen [5], the concentration of cadmium in the aquatic system has increased to elevated levels [6]. 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 [7]. Its 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 [8]. 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, few bodies have set concentration limits for monitoring cadmium pollution through fish. This factor coupled with the lack of standardized limits globally warrants further research into the toxic effects of cadmium on fish.

Human consumption of fish has long been practiced and is ubiquitous in the diet of the general population due to its nutritive value [12]. However, with the increasing cadmium pollution of water bodies, humans are at risk of exposure through the consumption of cadmium-contaminated fish [13]. It has been stated previously that cadmium possesses bio-accumulative properties, posing a hazard to human health through prolonged consumption of contaminated fish. Fish, which are often higher in the aquatic food chain, will accumulate a substantial amount of heavy metal through the indirect uptake of organisms from lower trophic levels [14].

Cadmium exerts its effect differently in different organisms, but studies can definitively state that it is highly toxic to all mammals and fish [15]. Studies have found that cadmium can cause developmental delay as early as the larval stage of

Table 1. Concentration limits of Cadmium in freshwater [9] [10] [11].

Type of Aquatic Bodies	Standard Limits of Cadmium in Aquatic Bodies (mg/L)	Source
Freshwater Ecosystem	0.001	National Water Quality Standards, Malaysia
	0.00072	National Recommended Ambient Water Quality Criteria (AWQC), US. Environmental Protection Agency

fish [16]. Researchers in this study cited that oxidative stress was among the causes of developmental disturbance induced by cadmium exposure [17]. Cadmium has been studied to cause detrimental effects to target organs by inducing stress in cells that regulate 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 [18]. In ecotoxicology studies, alterations to an organism's development have been measured and used to analyze the extent of environmental pollution. Variations in traits are associated with the organisms' response to environmental stress [19]. This is due to alterations in the organisms' metabolism following perturbations of optimum living conditions [20]. The findings put forth by previous studies indicate the susceptibility of an organism's development to the introduction of contaminants by acting upon the homeostatic mechanisms.

There is a myriad of biomonitoring tools used in research to assess the effects of cadmium on fish. However, fluctuating asymmetry is the most accurate and used quantitative tool to measure developmental instability [21]. Fluctuating asymmetry (FA) are random deviation between the left and right sides of bilaterally symmetrical organisms [22] [23]. The same reference also stated that poor homeostatic performance can act upon the development of an organism 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 sides of an organism [24]. In comparison to other biomonitoring tools that are time and energy-consuming with more complex methods, FA is regarded as a simple yet accurate tool to measure developmental effects without requiring a large sample size.

Anabas testudineus or Climbing Perch is a Teleost fish that is native to Asia [25]. **Figure 1** shows an image of a Climbing Perch. It favors brackish waters of canals, lakes, ponds, swamps, and estuaries and can withstand unfavorable conditions [26]. This species is given particular attention in ecotoxicology studies due to its' omnivorous nature. [27]. Being omnivorous allows for the accumulation of large amounts of pollutants following the ingestion of contaminated biotic elements [18]. Furthermore, Climbing Perch is hardy and able to withstand harsh environments [28]. In the context of laboratory studies, 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 of morphological function. This study will assess the effects of cadmium on the developmental instability of *Anabas testudineus* utilizing fluctuating asymmetry on the diameter of the eye. The study will also measure the FA value on the parameter and compare the FA values between different concentrations as well as compare the FA values between treatment groups and duration of exposure.

Figure 1. Climbing perch.

2. Materials and Method

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 under controlled conditions. The fish fry were divided into four different treatment groups exposed to varying concentrations of cadmium. To ensure the survival of the fish fry was maintained at 89% [29] the stocking density of the fish fry was kept at 4 fish per liter of water [30].

Approximately 100 fries were placed in different treatment groups exposed to different concentrations of cadmium nitrate: control group exposed to 0.000 mg/L cadmium nitrate solution, low treatment group exposed to 0.005 mg/L cadmium nitrate solution, medium treatment group exposed to 0.010 mg/L cadmium nitrate solution and high treatment group exposed to 0.015 mg/L cadmium nitrate solution. 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 analyzed to obtain the fluctuating asymmetry value.

The diameter of both sides of the eye was measured using Vernier calipers. 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 was divided by the sum of the average measurements of the left and right sides of the trait and divided by two.

Statistical analysis was conducted using SPSS 2.0 version 25. Descriptive analysis was used to measure the FA value on the diameter of the eye for each week (**Figure 2**). Normality tests were 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 the duration of exposure, a Two-Way ANOVA was used.

3. Results & Discussion

The diameter of the eye was assessed by measuring the FA value after prolonged

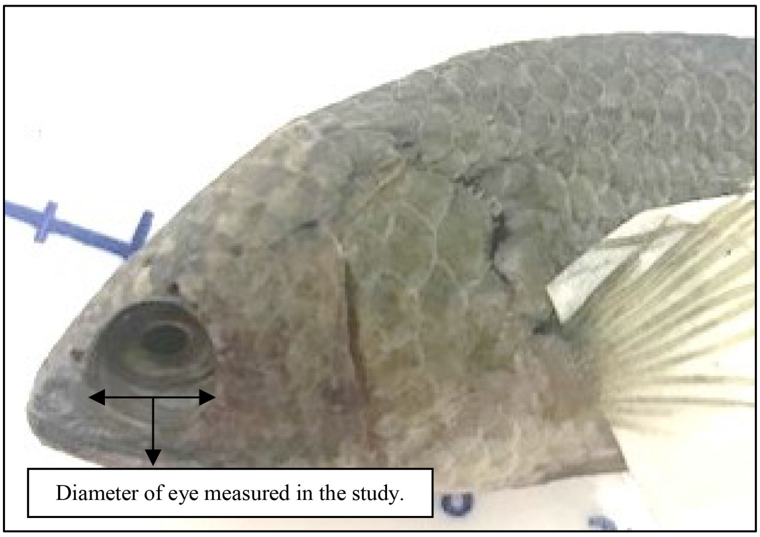

Figure 2. Diameter of eye taken to measure the FA value.

exposure to cadmium. The average FA value for the diameter of the eye ranged from 2.00% to 5.20%, with the greatest mean FA value found in the treatment group 0.015 mg/L at 5.20% ± 2.80. Analysis using One Way ANOVA determined that the exposure to different concentrations of cadmium had a statistically significant influence on the increase in FA value of the diameter of the eye, $p < 0.05$. A comparison of the FA value between the treatment groups found that the control group had a significantly lower FA value than the treatment group 0.010 mg/L and 0.015 mg/L, $p < 0.05$. Statistically, there was also a significant difference in the FA value between the treatment group of 0.005 mg/L and 0.015 mg/L, $p < 0.05$. These results are congruent with a study by [22] which found the FA value for eye diameter in *Menidia beryllina* was greater following exposure to environmental contaminants.

To compare the FA value of *Anabas testudineus* between the treatment group and duration of exposure and to investigate further the effects of concentration and exposure duration on FA, a Two-Way ANOVA was utilized. Analysis into the FA value of the diameter of the eye found the interaction between the treatment group and duration of exposure to be statistically significant, $p < 0.05$. The main effect analysis revealed that there was a statistically significant effect in the FA value of the diameter of the eye between treatment groups, $p < 0.05$. The FA value of the diameter of the eye is therefore significantly affected by different exposure concentrations, $p < 0.05$. There was however no statistically significant main effect for the duration of exposure, $p > 0.05$. As shown in **Figure 3**, the trend in FA values for the diameter of the eye does not indicate a consistent increase in FA values according to the treatment group as the duration of exposure increases therefore not in a time-dependent manner. In saying this, however, the significant difference in FA values of the diameter of the eye between treatment groups does indicate dose dependency. This is supported by a previous study that reported that cadmium-induced damage to the retinal structure of adult

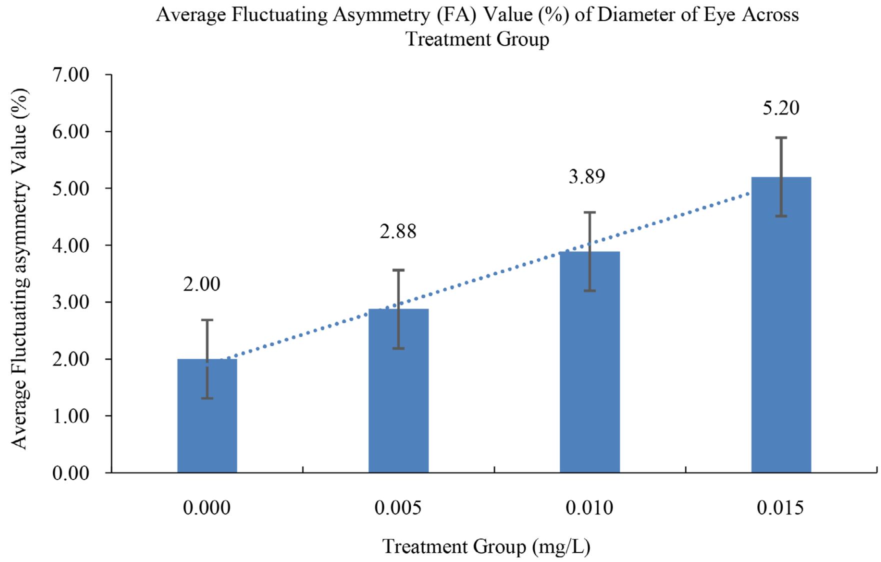

Figure 3. Average Fluctuating Asymmetry (FA) value of diameter of eye across treatment group.

Danio rerio increased with dose [31]. These results are however contrary to a study by Kenny & von Hippel (2014) that found no significant relationship between environmental contamination and high FA values of the diameter of the eye in freshwater Threespine stickleback fish [32].

Cadmium-induced developmental instability can be attributed to simultaneous effects on biochemical parameters brought upon by DNA damage. The adverse effects on the development of *A. testudineus* are proven in a previous study which found that DNA damage due to cadmium exposure is significantly greater at the highest concentration [33]. The previous study cited that damage to DNA is linked to the excessive generation of reactive oxygen species (ROS). The formation of ROS due to cadmium exposure is related to the disruption in biochemical parameters like the inhibition of important enzymes [34]. Among these enzymes important to development, estrogen is affected by exposure to high concentrations of cadmium. This is supported by a study that reported a significant interaction between developmental instability and estrogenic activity of *Trichomysterus aerolatus* subsequently increasing the FA value [35]. In summary, the alterations in these biochemical parameters contribute to the disruption of developmental homeostasis which causes small deviations from the symmetry of bilateral organs, like the traits used in this study. The significant effects on the stable development of the eye caused by prolonged exposure to cadmium can therefore be attributed to adverse effects on biochemical parameters.

The increase in duration and exposure to cadmium burdens the metabolic system subsequently disrupting the growth process [20]. One study discusses the reduction of energy in the context of the fish's biochemical system; cadmium-induced oxidative stress in *A. testudineus* consumes the energy otherwise used for growth and development to respond to cellular stress [36]. The significant difference in the FA value between the control group and the highest treatment group found in this study may be due to the saturation of energy for stable development as the exposure concentration increases. This developmental instability can be evaluated through FA. This can be supported by a study that cited chronic exposure to environmental pollutants may disrupt developmental homeostasis in which the morphological disturbances can be evaluated through FA [37]. The significant interaction between the increasing concentration of cadmium and the duration of exposure found for eye diameter may be due to disturbances to developmental homeostasis whereby the energy required to maintain developmental stability is used for survival and adaptation.

The developmental instability observed in this study can also be caused by dysregulation of antioxidant mechanisms. As discussed, exposure of fish to cadmium induces a stress response involving oxidative damage following accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in their bodies [38]. When the fish's antioxidant defence system is saturated as a result of increase in free radicals, adverse cellular responses occur leading to cell damage [39]. This theory is further compounded by studies that indicate cadmium possess an ability to cause DNA damage, cell cycle arrest and apoptosis alongside oxidative damage [40]. Increase in ROS has also been shown to induce the production of malondialdehyde (MDA) through lipid peroxidation of unsaturated fatty acids which results in similar disturbances to cell membrane structure [41]. These perturbations of cellular response against oxidative damage may affect enzymatic function relating to cell proliferation and differentiation [38]. Studies on the development of *Drosopohilia* conclude that cell proliferation is an integral part of eye development which in and of itself is a precisely regulated process [42] [43]. Any such attack on this process may induce morphological disturbances of the eyes, an organ that grows simultaneously as the body grows in fish [44]. Therefore, a marked increase in ROS due to Cd exposure disrupts important enzymatic functions for cell proliferation and differentiation subsequently affecting the development of organs.

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 the diameter of the eye with increasing concentrations prove that while it is strong enough to survive cadmium-contaminated conditions, FA can still be detected in significant values (**Figure 4**). This statement can be supported by a previous study that observed histopathological changes in *Anabas testudineus* following exposure to cadmium [45].

While significant findings were found to prove that developmental instability of the eye increases as the concentration of cadmium exposure increases, the FA values did not significantly increase with time. The study duration of 16 weeks may be insufficient time for pronounced asymmetry to be measured and significant interactions to be formed. A factor that contributes to fluctuating FA values

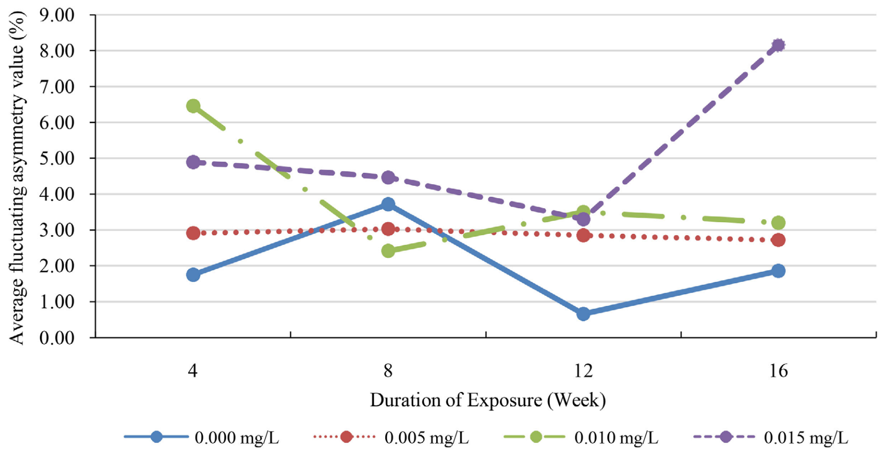

Figure 4. Average Fluctuating Asymmetry (FA) value of diameter of eye across duration of exposure.

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. Previous studies found a significant relationship between the diameter of the eye and the standard length of fish [46]. Larger fish have also been studied to exhibit greater asymmetry [47].

4. Conclusion

Results from the study showed that the highest mean FA value for the diameter of the eye was obtained in the highest treatment group, 0.015 mg/L. The results also show that the FA value for the diameter eye significantly increases as the concentration increases with a significant mean difference in the FA values between different treatment groups. A comparison of the mean FA value between different exposure concentrations and duration of exposure found a statistically significant interaction for eye diameter. Overall, it can be reported that exposure of *A. testudineus* to cadmium causes developmental instability (DI) of the eye as the highest mean FA value was obtained by the highest treatment groups. Limitations of the study extend to the 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 DI in fish on other bilateral traits.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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