Biosaintifika 14 (3) (2022): 383-390
Journal of Biology & Biology Education
p-ISSN 2085-191X | e-ISSN 2338-7610
https://journal.unnes.ac.id/nju/index.php/biosaintifika

Effect of Chlorpyrifos Insecticide On The Ossification of Wader Pari Fish Vertebrae (*Rasbora lateristriata*)

Dian Respati Ayu, Bambang Retnoaji*

Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Sel., Sendowo, Sinduadi, Kec. Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta 55281, Indonesia.

*Corresponding Author: bambang.retnoaji@ugm.ac.id

Submitted: 2022-06-08. Revised: 2022-08-15. Accepted: 2022-11-23

Abstract. Due to high growth, chlorpyrifos was one of the solutions to increase food production. However, this compound is dangerous because it belongs to the organophosphate group, which blocks the activity of the acetylcholinesterase (AChE) enzyme and causes nervous failure in an animal. Due to heavy rain, this insecticide will run off and contaminate the river's body and affect aquatic life. *Rasbora lateristriata* pari was a non-target animal of chlorpyrifos. This study aimed to determine the effect of chlorpyrifos exposure on the behavior of the R. lateristriata's feeding and swimming response, as well as the morphology and ossification process of the vertebrae. The study was conducted for seven days. Four groups of fish were exposed to chlorpyrifos with concentrations of 0, 0.001, 0.005, and 0.01 ppm. Observation of the ossification and morphological process of vertebrae is carried out using AR-Ab bone staining. Observation of *R. lateristriata's* behaviors is carried out using descriptive analysis. It was found that the fish exposed to chlorpyrifos showed some behavioral abnormalities in swimming and feeding responses. Fish exposed to 0.005 ppm chlorpyrifos showed vertebral abnormalities, which is haemal kyphosis. Kyphosis refers to Λ-shaped vertebral curvature. However, the ossification process represented by Ar-Ab staining is going well. The novelty in this study relates to the research results obtained. This study provides an initial overview of the vertebrae of *R. lateristriata* exposed to the insecticide chlorpyrifos. The benefits of this findings are to become a source of initial information related to *R. lateristriata* for future research

Key words: Alizarine red-Alcian blue, Chlorpyrifos, vertebrae, *R. lateristriata*.

How to Cite: Ayu, D. R., Retnoaji, B. (2022). Effect of Chlorpyrifos Insecticide On The Ossification of Wader Pari Fish Vertebrae (*Rasbora lateristriata* Bleeker, 1854). *Biosaintifika: Journal of Biology & Biology Education*, *14* (3), 383-390.

DOI: http://dx.doi.org/ 10.15294/biosaintifika.v14i3.30657

INTRODUCTION

Indonesia is an agrarian country with a high growth rate, causing food demand to increase. Therefore, many Indonesian farmers are using additional insecticides and pesticides to maintain the quality and quantity of agricultural products. One commonly used type of insecticide is chlorpyrifos. Chlorpyrifos belongs to the organophosphate group. This insecticide's principal mechanism is blocking the activity of the acetylcholinesterase (AChE) enzyme, which regulates stimuli signals Ventura et al., (2015). Thus resulting in nervous system failure in exposed animals. According to Harsanti et al. (2015), chlorpyrifos insecticide is used intensively by shallot farmers in Bandung, Yogyakarta, and Denpasar, Bali. This insecticide is carried away by rainwater so that it flows from the farmland to the waters leading to the river. Almost all types of insecticides have a wide spectrum of toxicity and are not selective. Thus the overuse of chlorpyrifos in the environment can cause damage to the target and non-target organisms. Therefore, this insecticide is very dangerous for aquatic and terrestrial ecosystems (Ali, 2020).

One of the non-target aquatic organisms is a *R. lateristriata*. *Rasbora lateristriata* is a freshwater fish native to Indonesia. According to Retnoaji et al. (2016), This fish is very popular for consumption because of its high nutritional and economic value. Unfortunately, R. lateristriata pari conservation status by IUCN (International Union for Conservation of Nature) Red List are included in vulnerable endangered (VU) situations, and this indicates that *R. lateristriata* will be at risk of becoming extinct in the future. Thus, the contamination of chlorpyrifos in water bodies can increase the risk of extinction of *R. lateristriata* in the wild as well as poisoning non-target organisms in the water. Richendrfer et al. (2012), in their research, reported that one of the parameters of the successful growth of R. lateristriata fish is a well-underway osteogenesis process.

If the fish bones are deformed during development, then the success rate of fish life will decrease. In addition, abnormalities in fish bones are able to affect the physiological, morphological, and behavioral aspects of *R. lateristriata* Sharbidre et al., (2011). Research on *R. lateristriata* has not been done much, especially on bones and osteogenesis processes. Therefore, it is necessary to conduct research related to the effect of chlorpyrifos on the behavior of eating and swimming responses of *R. lateristriata* and the morphology and osteogenesis process of its vertebrae.

The purpose of this study was to determine the behavioral changes of *R. lateristriata* Bleeker, 1854) which were treated with chlorpyrifos insecticide. In addition, to determine the effect of chlorpyrifos insecticide on the morphology of the vertebrae of the *R. lateristriata* and to determine the ossification of the vertebrae of the *R. lateristriata* exposed to chlorpyrifos insecticide. This research is expected to be able to provide benefits for increasing knowledge and insight related to the dangers of excessive use of insecticides for the environment, improving laboratory skills by conducting various research methods for histological observations, as well as providing information related to R. lateristriata fish farming processes that are beneficial for *R. lateristriata* breeders so that prevention processes can be carried out for river contamination to produce high-quality *R. lateristriata* fish.

METHODS

Experimental design

This research was conducted in the Laboratory of Histology and Animal Embryology, Faculty of Biology, Gadjah Mada University. The research started on weekdays from August 2020 - March 2021. The process of observation and data retrieval is conducted from August to January. The method of processing and analyzing data is carried out from February to April. The tested factor is the different concentrations of chlorpyrifos. Preliminary tests were conducted to determine the concentration of chlorpyrifos to be used. The data obtained indicates the range of concentrations to be used is 0, 0.001, 0.005, and 0.01 ppm.

Fish culture

One month-old fish were selected, and five normal fish (active swimming and not defective) were put into a jam bottle filled with chlorpyrifos. Fish were exposed to chlorpyrifos with a concentration of 0, 0.001, 0.005, and 0.01 ppm by four repetitions for 168 hours. Feed is given two times a day, and water is replaced daily. If any fish die, a fixation process is carried out immediately.

Measured parameters

Survival Rate

Survival rate measurement was calculated using this formula as follows SR= (No-Nt)/No, where SR= survival rate (%), Nt= total fish died during the experiment, No= total fish at the start of the experiment.

Water quality

During the study, water temperature (^{0}C), pH, and dissolved oxygen (mg L-1) were measured.

Data analysis

All measured data were analyzed to one-way ANOVA and followed by the Duncan multi-range test at a 95% confidence level using IBM SPSS Statistic ver. 21.0.

Behavior observation

Behavioral observations were carried out using qualitative analysis methods every day for one week. Observations include the response of fish to feed and swimming movements.

Bone staining

After seven days of treatment, the fish were fixed using 96% alcohol for ± four days. Furthermore, the fish were stained with AR-AB bone staining for ± 4-5 days. Then the fish were rinsed with distilled water twice. After that, the fish were cleared using 0.5% KOH until the fish were clear and the bones could be seen with the naked eye. Then cleaned with a MOLL solution containing 20 ml of glycerin, 1 ml, 1% KOH, and 79% distilled water for several minutes. Finally, the fish should be preserved in 100% Glycerin solution.

RESULTS AND DISCUSSION

Survival rate data of *R. lateristriata* (Table 1) showed a decrease in survival from a concentration of 0.01 ppm to 93.3% on the 7th day. While in the control treatment, 0.001 ppm

Table 1. The survival rate, heart rate and operculum opening of (*R. lateristriata*) exposed to chlorpyrifos (0 ppm, 0.001 ppm, 0.005 ppm, 0.01 ppm) for one week

Chlorpyrifos concentration (ppm)	Survival rate (%)	Heart rate (mean±SD)	Operculum opening (mean±SD)
0 ppm	100	254.2±23[a*]	134.66±5.03[a]
0.001 ppm	100	245.6±27[a*]	188.66±9.01[b]
0.005 ppm	100	213.2±14[b*]	196.0±25.05[b]
0.01 ppm	93.3	217±6[b*]	126±27.05[a]

The mean value (±SD) at the same column with similar superscript are not significantly different (P<0.05)

and 0.005 ppm survival rate was 100%. Viant et al., (2006) reported in their research that exposure to chlorpyrifos during the development and growth of fish caused disturbances in development and growth. So it can reduce the survival rate of fish. Richendrfer et al., (2012) state that, exposure to insecticides in fish can cause chronic stress which results in decreased survival rates. Survival rate data were analyzed using linear regression to determine LC50 168 hours. LC50 was identified in 0.078 ppm concentration. This proves that the concentration used in the chlorpyrifos treatment test gave a sublethal effect on.

In addition, the measurement of 24 hpf R. lateristriata larvae heart rate (Table 1) showed a decrease in heart rate as the concentration of chlorpyrifos increased. The average heart rate was 254.2 bpm (control), 245.6 bpm (0.001), 213.2 bpm (0.005) and 217 bpm (0.01 ppm). The data were analyzed using a one-way ANOVA test and showed that exposure to chlorpyrifos with concentrations of 0.001, 0.005 and 0.01 ppm gave significantly different results from the control treatment (p> 0.005). This is because organophosphate insecticides are inhibitors of the acetylcholinesterase (AChE) enzyme. In zebrafish, the M2 Ach muscarinic receptor plays an important role in controlling heart rate. Watson et al., (2014) reported a decrease in the heart rate of zebrafish larvae exposed to chlorpyrifos for 5 days. This is caused by the accumulation of acetylcholine in the synaptic gap which results in excessive stimulation of the M2 Ach receptor, thus triggering bradycardia (slower heartbeat).

The results showed that one-month-old fish with control treatment had an average operculum opening of 134.6 bpm, while in the treatment 0.001 ppm (188.6 bpm), 0.005 ppm (196 bpm) and 0.01 ppm (126 bpm). Duncan's test results showed that fish operculum openings at concentrations of 0.005 ppm and 0.001 ppm were not significantly different. However, it was significantly different from the control treatment and 0.01 ppm. Sharbidre et al., (2011) in their research showed that exposure to chlorpyrifos in fish caused toxic stress, some of the symptoms of which were increased operculum opening, irregular swimming movements and changes in the body becoming weaker after several hours of exposure (subsequent lethargy).

Table 2 shows the behavioral analysis of 1-month-old fish exposed to chlorpyrifos for 1 week. The response to the feed was observed shortly after being fed. While the response of

Table 2. The behavior of *R. lateristriata* exposed to chlorpyrifos (0 ppm, 0.001 ppm, 0.005 ppm, 0.01 ppm) for one weeks

Chlorphyrifos concentration (ppm)	Response to feed*	Locomotor Activity
0 ppm	Active	Normal and active movement
0.001 ppm	Passive	Moves irregularly, body tilts to the side, looks weak, convulsions
0.005 ppm	Passive	Moves irregularly, body tilts to the side, looks weak, convulsions
0.01 ppm	Passive	Undirected defecation, the body tilted to the side, sometimes the fish's body position is upside down, looks weak, convulsing

*The fish's active response to food is characterized by moving to the surface immediately after being fed, the passive response is indicated by the opposite swimming movements was observed every 10 minutes after the water was replaced. The first parameter that was observed was the reaction of the fish to the feed. Only fish in the control treatment (0 ppm) were seen to be active, i.e. They immediately went to the surface to get feed while the feed was given. Meanwhile, fish at 0.001, 0.005 and 0.01 ppm treatments showed a passive response to feed. This passive response is indicated by the fish being seen waiting for the feed to sink (not rushing to the surface). The abnormal movements of the fish observed were the fish moving undirected, convulsing and the body tilted to the side. After a while, the fish began to look normal and tended to be weak so many fish were found under the bottom of the treatment container.

Chlorpyrifos plays a role in inactivating the acetylcholinesterase enzyme which causes the accumulation of acetylcholine it interferes with the neurotransmission process. In more detail, it causes hyperstimulation of ach receptors, namely nicotinic and muscarin receptors which are found in various body tissues such as the brain (central nervous system) and muscles (locomotion system). In the peripheral nervous system ach, acts as a neurotransmitter at the neuromuscular junction between motor nerves and skeletal muscles. So that the induction of chlorpyrifos causes movement abnormalities in fish (Colović et al., 2013). That is what causes movement abnormalities in fish exposed to chlorpyrifos. Moreover, another possibility that can cause swimming behavioral abnormalities in fish is vitamin deficiency. In zebrafish, vitamin C deficiency can exacerbate vitamin E, promoting muscle abnormalities called myopathy (Lebold et al., 2013). Myopathies are an inflammation in skeletal muscle that causes muscles to weaken, which can interfere with their contraction activity (Cassandrini et al., 2017). Based on research (Barohn et al., 2014), it is known that several causes of myopathies are Drug-induced myopathies, Endocrine myopathies, Inflammatory/immune myopathies, and Myopathies associated with other systemic illnesses.

Research conducted by (Greer et al., 2019) on juvenile rainbow trout exposed to chlorpyrifos (10, 20, and 40 g/l) for seven days showed a decrease in olfactory response. In addition, fish exposed to chlorpyrifos at a concentration of 1.38 g/l significantly reduced serine lipase enzyme activity and altered lipid metabolites in the trout brain. Therefore, chlorpyrifos can interfere with neuronal signaling and impact neurobehavioral responses in aquatic animals. In the same journal, it is also stated that this occurs through inhibition of acetylcholinesterase (AChE), which leads to accumulation of acetylcholine and prolonged stimulation of cholinergic receptors throughout the central and peripheral nervous systems.

The morphological structure of the *R. lateristriata* refers to the morphological and anatomical structure of the zebrafish (D. *Rerio*) (Bryson-Richardson et.al., 2007). According to (Hadi et.al., 2021) the fish's body is divided into three parts: head, body and caudal. Figure 1 shows the structure of the exoskeleton which fish consist of the head, upper and lower jaws, radial cartilage, dorsal fin, anal fin and caudal fin. Observation of the morphology of the exoskeleton of R. lateristriata pari did not show any abnormalities. The dorsal, anal and caudal fins are intact and undamaged. Likewise, the skeleton of the head is observed to be normal.

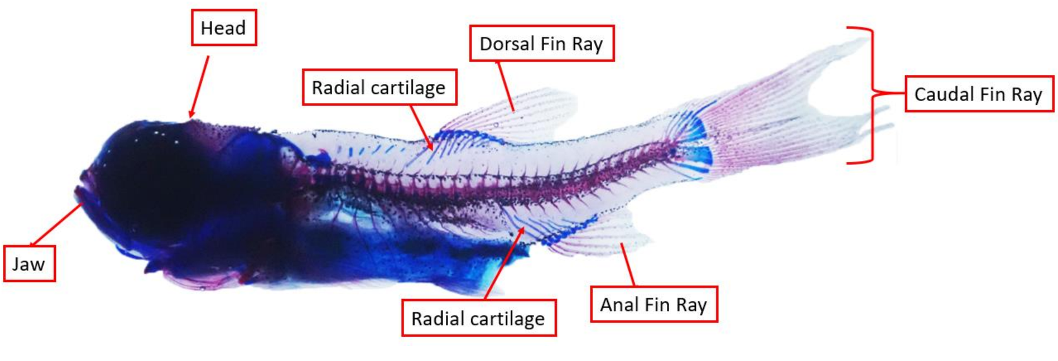


Figure 1. Morphological structure of *R. lateristriata*

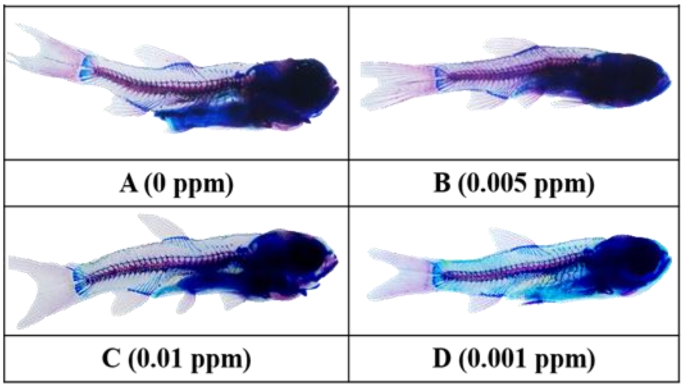


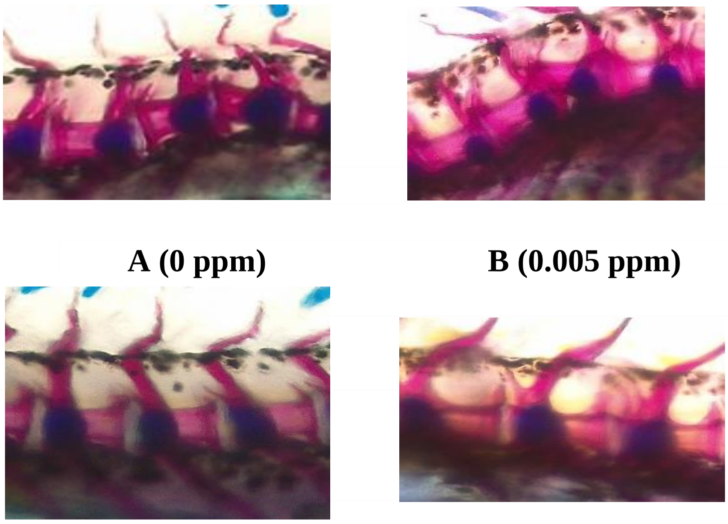
Figure 2. The results of staining *R. lateristriata* fish skeleton using Ar-Ab staining on chlorpyrifos treatment A (0 ppm), B (0.005 ppm), C (0.01 ppm) and D (0.001 ppm)

Observation of bone damage was carried out qualitatively by looking at the intensity of the color formed after the staining process. Ar-Ab (Alcian Blue/Alizarin Red) is a type of bone stain that is commonly used. This type of bone dye can visualize and differentiate the types of cartilage and true bone. The cartilage will be stained in blue (Alcian Blue), while true bone will be stained in red (Alizarin Red) (Ovchinnikov, 2016). The working principle of this dye is Alizarin red is sensitive to bind BCP-crystal deposition (Basic Calcium-Phosphate) which is known to be deposited a lot in true bone (osteum). This is what causes real bones to be stained red by Alizarin red (Terkeltaub, 2017). Meanwhile, Alcian blue dye works by binding acidic carbohydrates compounds that accumulate in the cartilage matrix, so that the cartilage will be colored blue (Eggerschwiler et al., 2019).

Colors that are visualized more clearly or densely indicate that the osteogenesis process is going well. This is because alizarin red binds to the minerals phosphate and calcium in the true bone matrix. This mineralization process is in line with the process of osteogenesis. Therefore, it can be seen if the color is not clearly visualized or even not colored indicating a disturbance in the mineralization and osteogenesis process. Based on Figure 2. Ar-Ab staining in each treatment is known to have almost the same color intensity. This shows that the calcification process in R. lateristriata fish bones is going well. Cartilage is stained blue and true bone is stained red. In addition, the curvature of the vertebral column at 0, 0.001, 0.005 and 0.01 ppm treatment looks normal. In (Figure 2) the vertebral arch looks normal, and the outer morphology of the R. lateristriata fish looks normal. Morphological observations consisted of the shape of the skeleton of the head, upper and lower jaws, the amount of radial cartilage, the shape of the dorsal, anal and tail fins.

Based on Figure 3, it is known that there are abnormalities in the control treatment and 0.005 ppm which is indicated by a green arrow. The damage is a vertebral dislocation. While the treatment of 0.01 ppm and 0.001 vertebrae tend to look normal. (Figure 3.b) the dislocation occurred in the 11th rib bearing, where the vertebra was fractured so that it was higher and overlapped compared to the adjacent rib bearing. The same thing was also found in the control treatment (Figure 3. A) which occurred on the 6th rib bearing, the rib bearings looked misaligned but did not overlap or were broken. This deformation of the vertebrae was found in only one of the six fish tested. Therefore, it is necessary to carry out further testing regarding the effect of chlorpyrifos on the vertebral morpho-anatomy of the R. lateristriata fish. Further testing is in the form of biochemical tests to determine the sublethal effect of insecticides and to determine the physiological status of fish. In addition, it is necessary to do X-rays and radiography tests to observe the morpho-anatomy of the vertebrae of *R. lateristriata* exposed to chlorpyrifos.

Based on research (Bernabò et al., 2011), various pesticides are endocrine disruptors (EDCs) for fish, such as chlorpyrifos. Chlorpyrifos can affect thyroid hormone signaling by blocking, mimicking, or synergizing with endogenous hormones through direct interaction of thyroid receptors (TRs). Disturbances in the thyroid hormone signaling process can trigger changes in fish body



A (0 ppm) **B (0.005 ppm)**

C (0.01 ppm) **D (0.001 ppm)**

Figure 3. The results of staining the vertebrae of R. lateristriata fish using Ar-Ab staining on chlorpyrifos treatment A (0 ppm), B (0.005 ppm), C (0.01 ppm) and D (0.001 ppm)

structure, viability, ossification process, vision, behavior, metabolism, growth, pigmentation, digestive and muscular systems, and fish survival rates. In addition, the thyroid hormone plays an essential role in normal bone development, especially in osteoblast differentiation (Darras et al., 2015).

In addition, abnormalities in the vertebrae and bone development can be influenced by various factors. The main factor that causes abnormalities in the skeleton of fish is inadequate or excessive nutrition. Some of them, such as vitamin C deficiency, can cause vertebral bone abnormalities, namely lordosis, kyphosis, and scoliosis (Sato et al., 1982). Overdose of vitamin A can induce deformities in the spine, namely vertebral curvatures, compression and fusion (Fernández, et al., 2012). Vitamin K deficiency can cause irregular vertebral rows. Lack of food phosphorus levels causes skeletal anomalies in Zebra Fish (Costa et al., 2018).

According to (Berillis, 2015) some chemicals are able to induce neuromuscular damage that results in abnormalities in the spine of fish. Lordosis and kyphosis are the most common vertebral abnormalities in fish. D. *Labrax* fish have vertebral abnormalities in the form of haemal kyphosis, which is a condition where the vertebrae are curved to form an Λ-shaped (Fragkoulis et.al, 2019). Haemal kyphosis is believed to be the same vertebral abnormality as R. lateristriata fish exposed to chlorpyrifos at a concentration of 0.005 ppm (Figure 3. B). This condition is believed to be the result of extreme muscle contraction due to inhibition of the cholinesterase enzyme. Several factors that cause bone damage due to other insecticides are damage to the embryonic phase, nutritional deficits, heredity, environmental factors, chemical exposure and so on (Berillis, 2015).

One of the obstacles faced in this research is the lack of a clear standardization of classification to determine the type of abnormality in the vertebrae of fish of the genus Rasbora. As far as is known, many terms are used to describe a vertebral abnormality without a clear explanation, and different words are used to denote the same abnormality. Instead, the same word denotes a much different bone disorder. These obstacles are also found in (Boglione et al., 2013) research on Skeletal anomalies in reared European fish larvae and juveniles.

Therefore, the novelty in this study relates to the research results obtained, especially the effect of the insecticide chlorpyrifos on the vertebral ossification process and the behavior of *R. lateristriata*. *R. lateristriata* is a freshwater fish native to Indonesia. Although it is a popular consumption fish, research related to this fish has not been comprehensive, so a lot of research and further review is needed. This study provides an initial overview of the vertebrae of *R.*

lateristriata exposed to the insecticide chlorpyrifos. The benefits of this findings are to become a source of initial information related to *R. lateristriata* for further research. The benefits that are expected for social and environmental interests are awareness of the survival and sustainability of the *R. lateristriata* so that it can reduce the things that cause it to become prone to extinction. In addition, this research is useful for showing the negative impact of excessive use of insecticides, so it is hoped that insecticide users will use insecticides wisely and according to the rules so as to minimize the negative impact on the environment.

CONCLUSION

This study identified that the chlorpyrifos insecticide at concentrations of 0.001, 0.005 and 0.01 ppm can have a negative effect on food responses and cause abnormalities in swimming movements. The vertebrae morphology of the R. lateristriata fish in the 0.005 ppm treatment showed an abnormal structure which is vertebral haemal kyphosis, particularly at 11th vertebrae. The process of vertebrae ossification of *R. lateristriata* that represented by the colour intensity of AR-AB in each treatment showed normal results. The findings of this research are expected to be basic information and a source of initial knowledge for further scientific research related to effect of chlorpyrifos insecticied to *R. lateristriata*. It is expected to conducted further testing regarding biochemical tests to determine the sublethal effect of insecticides, X-rays and radiography tests to observe the morpho-anatomy of the vertebrae of *R. lateristriata* exposed to chlorpyrifos

ACKNOWLEDGEMENT

The authors would like to thank GAMA WADER team for their assistance during experimental period in the Laboratory of Histology and Animal Embryology, Faculty of Biology, Gadjah Mada University. Our gratitude to the Gadjah Mada University that facilitated the authors to conduct this research.

REFERENCES

Ali S. J. (2020). Monocrotophos, an organophosphorus insecticide, induces cortical and trabecular bone loss in Swiss albino mice. Chemico-biological interactions, 329, 109112. https://doi.org/10.1016/j.cbi.2020.109112

Barohn, R. J., Dimachkie, M. M., & Jackson, C. E. (2014). A pattern recognition approach to patients with a suspected myopathy. Neurologic clinics, 32(3), 569–vii. https://doi.org/10.1016/j.ncl.2014.04.008

Berillis, P. (2015). Factors That Can Lead To The Development Of Skeletal. Journal of Fisheries Sciences, 9(3), 017-023.

Boglione, C., Gisbert, E., Gavaia, P., E. Witten, P., Moren, M., Fontagné, S. and Koumoundouros, G. (2013), Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. Rev Aquacult, 5: S121-S167. https://doi.org/10.1111/raq.12016

Bryson-Richardson, R. J., Silke, B., Thomas, F. S., Thomas, E. H., Nicholas, J. C., Abigail, J. G., James, S., and Peter, D. C. (2007). FishNet: an online database of zebrafish anatomy. BioMed Central Biology 5(34), 1-8.

Cassandrini, D., Trovato, R., Rubegni, A. (2017), Congenital myopathies: clinical phenotypes and new diagnostic tools. Ital J Pediatr 43, 101. https://doi.org/10.1186/s13052-017-0419-z.

Colović, M. B., Krstić, D. Z., Lazarević-Pašti, T. D., Bondžić, A. M., & Vasić, V. M. (2013). Acetylcholinesterase inhibitors: pharmacology and toxicology. Current neuropharmacology, 11(3), 315–335.

Costa, J. M., Sartori, M., Nascimento, N., Kadri, S. M., Ribolla, P., Pinhal, D., & Pezzato, L. E. (2018). Inadequate Dietary Phosphorus Levels Cause Skeletal Anomalies and Alter Osteocalcin Gene Expression in Zebrafish. International journal of molecular sciences, 19(2), 364. https://doi.org/10.3390/ijms19020364

Eggerschwiler, B., Daisy, D. C., Hans-Christoph, P., Elisa, A. C. and Paolo, C. (2019). Automated digital image quantification of histological staining for the analysis of the trilineage differentiation potential of mesenchymal stem cells. Journal of Stem Cell Research & Therapy, 10(69),1-10.

Fragkoulis, S., A. Printzi, G., Geladakis, N., Katribouzas and G. Koumoundouros. (2019). Recovery of haemal lordosis in Gilthead seabream (Sparus aurata L.). Scientific Report 9(9832), 1-11.

Hadi, R. A. P., Winarni, E. T., Bhagawati, D., & Nuryanto, A. (2021). Morphological Differences between Chaetodon auriga and Chaetodon vagabundus based on Truss Morphometric Characters. Biosaintifika:

Journal of Biology & Biology Education, 13(3), 297-304.

Harsanti, E. S., E. Martono, H.A., Sudibyakto dan E. Sugiharto. (2015). Residu Insektisida Klorpirifos Dalam Tanah Dan Produk Bawang Merah Allium Ascalonicum L, di Sentra Produksi Bawang Merah di Kabupaten Bantul, Yogyakarta. Jurnal Ecolab, 9(1), 01 - 46.

Lebold, K. M., Löhr, C. V., Barton, C. L., Miller, G. W., Labut, E. M., Tanguay, R. L., & Traber, M. G. (2013). Chronic vitamin E deficiency promotes vitamin C deficiency in zebrafish leading to degenerative myopathy and impaired swimming behavior. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP, 157(4), 382–389. https://doi.org/10.1016/j.cbpc.2013.03.007

Ovchinnikov, D. (2009). Alcian Blue/Alizarin Red Staining of Cartilage and Bone in Mouse. Cold Spring Harbor Protocol, 4(3), 1-2.

Retnoaji, B., Nanda, F., Sartika, D., Eunike, N., Oktaviani, D. D., Afriani, D. (2016). The effect of volcanic dust on histological structure of wader pari (Rasbora lateristriata Bleeker, 1854) organs. AIP Conference Proceedings, 1744(1).

Richendrfer, H., Pelkowski, S. D., Colwill, R. M., and Creton, R. (2012). Developmental sub-chronic exposure to chlorpyrifos reduces anxiety-related behavior in zebrafish larvae. Neurotoxicol. Teratol. 34, 458–465. doi: 10.1016/j.ntt.2012.04.010

Sharbidre, A. A., Metkari, V., and Patode, P. (2011). Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, Poecilia reticulata. Pesticide Biochemistry and Physiology, 10, 132–141.

Terkeltaub, R. (2017). Kelley and Firestein's Textbook of Rheumatology (Tenth Edition). Chapter 96 - Calcium Crystal Disease: Calcium Pyrophosphate Dihydrate and Basic Calcium Phosphate Vol.2. Elsevier, 1645-1665.

Ventura, C., Núñez, M., Randi, A., Venturino, A., and Cocca, C. (2015). The Organophosphorus Chlorpyrifos As A Breast Cancer Risk Factor. Nova Science Publishers, Inc, New York. P-16.

Watson, F. L., Schmidt, H., Turman, Z. K., Hole, N., Garcia, H., Gregg, J., Tilghman, J., Fradinger, E. A. (2014). Organophosphate pesticides induce morphological abnormalities and decrease locomotor activity and heart rate in Danio rerio and Xenopus laevis. Environ Toxicol Chem, 33(6),1337-45.