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Histopathological Changes and Metallothionein Levels in *Filopaludina javanica* During Depuration

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Abstract. The heavy metal lead has been reported to pollute waters along the Glagah River, Lamongan Regency, Indonesia. Lead contamination in all organs of the Tutut snail *(Filopaludina javanica)* was detected at 2.92 ± 0.5 ppm. Therefore, lead contamination can cause severe problems because lead can accidentally accumulate in the human body through the food chain. One effort to reduce risks related to food safety is Depuration. The research aimed to analyze the effect of Depuration using a flushing system on histopathological changes and metallothionein levels in all organs of the spotted snail *(Filopaludina javanica)*. The research used a Completely Randomized Design with four treatments and three replications. Treatment includes flushing time (6 hours, 12 hours, 18 and 24 hours). The MT response was tested statistically using One Way Anova, while the histopathological analysis used a descriptive method by comparing before treatment and after flushing treatment for 24 hours, to determine metallothionein levels, the ELISA (Enzyme-Linked Immunosorbent Assay) method was used. The results showed tissue damage in edema, hyperplasia, and vacuolation. Tissue damage before and after Depuration was relatively the same and included in the low category, with damage ranging from (0-6.1%). Meanwhile, metallothionein analysis has not shown a significant decrease in levels. Metallothionein levels increased with the duration of flushing. Further checks showed that the media water used in flushing during Depuration contained Fe and Zn, which resulted in increased MT levels.

Keywords: *Filopaludina javanica*, Metallothionein Levels, Histopathology, Lead Reduction

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1. Introduction

District is a Lamongan Regency, East Java Province, Indonesia sub-district. In several sub-districts in Lamongan Regency, there are Tutut Snails *(Filopaludina javanica)*. The presence of spotted snails in rivers and ponds ranges from 10 – 100 individuals/m2. Most of these snails are used for animal feed, and a small part are used for human consumption. The research results show that Glagah District, Lamongan Regency rice fields contain Pb: 0.67 ppm [1]. The Pb levels are the highest compared to several sub-districts in Lamongan Regency. According to [2] and [3], the heavy metal content in the flesh of aquatic organisms is Glagah usually higher than the heavy metal content in waters because these heavy metals will accumulate in the flesh. If snails contain Pb, it is feared that it will enter the human body as the leading consumer.

The heavy metal Pb that enters the flesh of the spotted snail *(Filopaludina javanica*) can cause poisoning for people who consume it because Pb is a heavy metal that has high toxicity and is accumulated in the human body [4]. According to [5] and [6], the heavy metal lead will cause lung damage and nerve damage. Therefore, efforts are needed to reduce the heavy metal content in shells so that their negative impact on the health of people who consume them can be prevented.

Efforts to maintain the quality and safety of food products can be made by providing special treatment by reducing Pb levels in the spotted snail (*Filopaludina javanika*). According to [7] and [8], Pb release can be done by rinsing or by soaking and draining freshwater snails *(Filopaludina javanica)* continuously with clean water before being given to livestock and consumed by humans.

Metallothionein (MT) is a biological compound that interacts with metals naturally. Thus, metallothionein plays an essential role in managing the metabolic mechanisms and cellular transition of metallic materials in the body, especially heavy metal ions [9], [10], [11]. MT has been reported in vertebrates, including many species of fish and aquatic invertebrates, especially mollusks. The MT test can detect the presence of certain heavy metals in aquatic organisms exposed to heavy metals, including the Tutut snail *(Filopaludian javanika)* [12], [13]. Metallothionein (MT) is also essential for the defense and detoxification of non-essential metals such as lead (Pb), Cadmium (Cd), and Mercury (Hg) [14].

Depuration using the flushing method can repair specific organ tissues in shellfish. Histological observations determine and compare tissue conditions before and after treatment. The advantages of this observation include disease diagnosis, such as the cause of infection and disease classification, as well as being able to provide an overview of the condition of spotted snail tissue that is contaminated with spotted snails that have received treatment so that it can be seen whether the tissue is healthy or not in a sample. Meanwhile, observations of metallothionein levels were carried out to determine the response to decreasing lead levels after the depuration process. The MT test can precisely detect the presence of lead metal. Based on the explanation above, it is necessary to conduct research regarding Histopathological Changes and Metallothionein Levels in *Filopaludina javanica* During Depuration.

2. Methods

2.1 Research Location

The research was conducted in a Gayam Hamlet, Soko Village, Glagah District, Lamongan Regency field laboratory. Testing for lead metal Pb in the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, while metallothionein (MT) level testing in the Laboratory (FAAL) of the Faculty of Medicine, Brawijaya University, Malang.

2.2 Freshwater Snail Sampling

Freshwater snails *(Filopaludina javanica)* measuring 25-27 (twenty-five to twenty-seven) mm and weighing 3-4 grams were taken around the Glagah River area, Lamongan, Indonesia. Next, the freshwater snails are stored in a container and left alive for depuration processing. The container storing spotted snails *(Filopaludina javanica)* is dry without using water [15].

2.3 Analysis of Metallothionein Levels
Freshwater snail tissue was homogenized using a mortar, and 3 ml of buffer (0.5 M sucrose, 20 mM Tris-HCL buffer, pH 8.6, containing 0.01% β-mercaptoethanol) was added and placed in a tissue homogenizer. The homogenate was centrifuged at 30,000 rpm for 20 minutes to obtain the supernatant. 1.05 ml of ethanol and 80 ml of chloroform/1 ml of supernatant were added and centrifuged at 6000 rpm for 10 minutes. Next, 3 ml of ethanol was added, and the supernatant was stored at 20oC for 1 hour. Metallothionein was purified by centrifugation with 300 ml 5 mM Tris-HCL, one mM EDTA, and pH 7. Metallothionein levels were analyzed using an ELISA reader with a wavelength of 450 nm. The absorption results were then converted using a standard curve.

2.4 Analisis Histopatologi
Tutut snail tissue was preserved using 10% formalin. Next, the tissue was blocked with paraffin and cut using a microtome with a thickness of 3-5 microns. Deparaffinization was done in the oven for 30 minutes at 70-80 and xylol immersion for 15 and 5 minutes. Rehydrate using graded alcohol for 3 minutes (100%, 96%, 80%, 70%, 50%, and 30%) and washing water for 15 minutes. Tissue staining using hematoxylin and eosin. Next, observations were carried out using a compound microscope.

2.5 Data Analysis
The research used the experimental method with experimental design, which was a completely randomized design (CRD) using four treatments with three replications, while the control used was Tutut snails (*Filopaludina javanica)* without Depuration. Treatment includes flushing time (6 hours, 12 hours, 18 and 24 hours). The MT response was tested using One Way Anova, while histopathological analysis used a descriptive method by comparing before and after treatment (flushing for 24 hours). All statistical analyses were done using the software program SPSS 16.0 (Statistics Product and Service Solution) and Microsoft Exel 2010.

3. Results and Discussion

3.1. Metallothionein
The results of the analysis of metallothionein levels in "whole organs" are presented in Table 1. Based on the results of the ANOVA test, the average metallothionein levels after flushing treatment showed no significant difference ($P>0.05$). The heavy metals influence metallothionein levels in the bodies of freshwater snails. The decrease in metallothionein levels in the 6-hour rinse is likely due to decreased Pb levels from before treatment, in contrast to the flushing treatment for 12, 18, and 24 hours, which showed an increase in metallothionein levels.

Metallothionein levels during treatment experienced increases and decreases thought to be influenced by the depuration process. The decrease in metallothionein levels is thought to be due to the excretion process by the leopard snail, and the Pb levels in the body are shed during the flushing process. The longer the flushing time, the more Pb content is released or separated from the protein structure bonds of the body of the spotted snail *(Filopaludina javanica)* along with the mucus contained in the body. According to [16], the Depuration of toxic materials in green mussels is the most quickly released from the digestive organs, at around 50%. In contrast, the slowest is released from the gill organs, which is around 5%. The digestive organs contain metallothionein, a binder for heavy metals. Metallothionein molecules are light and easily soluble (Cytosolid), and there is a relationship between metallothionein (MT) levels and

Pb, Cd, and Hg levels in oyster (Crassostrea iredalei) gills. Metallothionein levels in the gills of oysters (C. Iredale) are related to Pb, Cd, and Hg levels, so these factors influence metallothionein production in the oyster body [17]

Table 1. Analysis of Metallothionein Levels

Sample Code	Depuration Time (hours)	Metallothionein Levels (ng/ml)
A	0	0.873 ± 0.135
B	6	0.826 ± 0.147
C	12	1.035 ± 0.387
D	18	0.950 ± 0.365
E	24	1.376 ± 0.497

Metallothionein in all organs of freshwater snails *(Filopaludina javanica)* in several treatments showed an increase, thought to be caused by the media water used, which was drilled well water. The laboratory analysis results show that the well water used in flushing contains other metals, such as Zn at 0.05 ppm and Fe at 0.02 ppm. The snails accumulate the metal during flushing. Iron (Fe) is an essential metal required by living organisms, but it can have a toxic impact at certain levels. with levels[18]. The research results by [19] showed that metallothionein levels in gills increased, increasing Pb levels with a coefficient (r) of 0.89.

3.2. Histopathology

The results of tissue analysis of the snail's "whole organ" can be observed in several tissues, such as the outer stomach wall called digestive diverticula (Figure 1A), gills (Figure 1B), and stomach (Figure 1C). Digestive diverticula tissue experiences cell swelling (edema) of 4%. The gills (Figure 1B) experienced hyperplasia of 6.1%, and the stomach (Figure 1C) identified several abnormalities, such as edema of 2.1% and vacuoles of 0.2%. The erosion of epithelial cells usually characterizes gills experiencing hyperplasia, widened lacunae accompanied by loose red blood cells, and pillar cells. According to [20], hyperplasia is the process of excessive tissue formation due to an increase in cell volume. Meanwhile, the image (1Cv) includes a type of damage, usually called vacuolation, or cavities in cells filled with fluid that appear white.

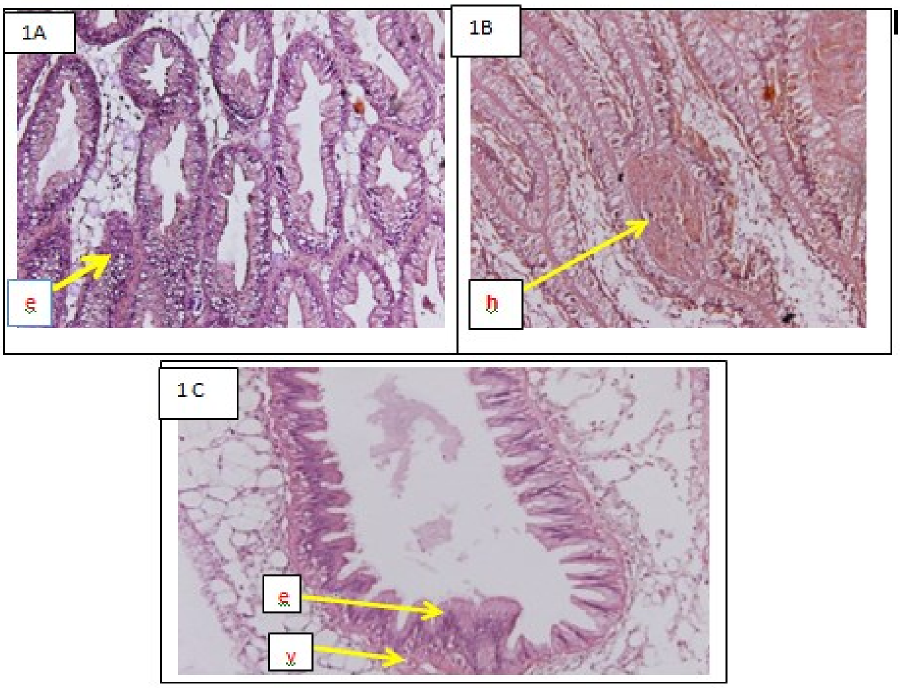

Figure 1. Results of Histological Observation of "Whole organ" *(Filopaludina Javanica)* before washing with some damage to the digestive tract tissue (A), gills (B), and stomach (C). Information: (e) Edema or swelling of cells or excessive accumulation of fluid in body tissue, (h) Hyperplasia or the process of excessive tissue formation due to an increase in the number of cells, (v) Voids or cavities in cells filled with fluid, (200x magnification)

Tissue damage to the "Overall Organs" of freshwater snails (*Filopaludina Javanica)* in controls ranged from 2.3 - to 6.1%; this value is relatively low. According to [21], the above damage (Figure 1) is classified as low because the percentage of tissue damage ranges from 0 – 30%. This shows that the levels of the heavy metal Pb in the control "whole organ" (Figure 1) damage do not affect the reproduction and life of the spotted snail (Filopaludina javanica). They are adding to research [22] that low histological damage does not result in the death of living organisms. Disrupt specific organ systems.

Rinsing for 24 hours did not significantly affect "whole organ" damage changes. Damage to "whole organs" was still visible in the digestive tract tissue, with a percentage of 1.6%. The total damage to gill tissue was 3.9% from two damages, Hyperplasia 3.3% and Vaccination 0.6%. In contrast to the histopathological condition of the stomach (Figure 2C), no abnormalities were found in edema, hyperplasia, etc. The stomach can be recognized because it has a folded structure forming folds with cylindrical ciliated epithelium, eosinophilic cytoplasm, and oval basophilic nuclei. A round pyknotic nucleus is visible at the basal part of the gastric epithelium. A pink mass was found in the lumen, thought to come from mucous secretions mixed with food. In the lamina propria, pink fibers with basophilic nuclei.

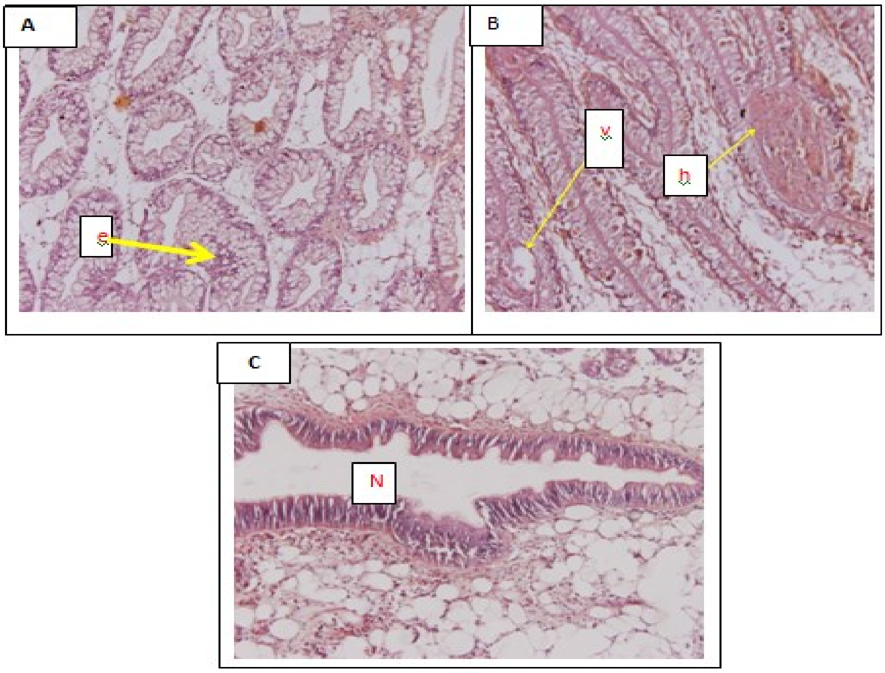

Figure 2. Results of histological observations of the "Whole Organ" of the Tutut Snail after rinsing for 24 hours on the digestive tissue/gastrointestinal tract (2A), gills (2B), and stomach (2C). Information: (e) Edema or swelling of cells or excessive accumulation of fluid in body tissue, (h) Hyperplasia or the process of excessive tissue formation due to an increase in the number of cells, (v) Voids or cavities in cells filled with fluid, (N) Normal, (200x magnification).

Damage to freshwater snails' "Whole Organ" tissue in the 24-hour rinsing treatment ranged from 1.6 - 3.9%. The highest damage occurred to the gills at 3.9% and the lowest to the digestive diverticula tissue at 1.6%. [21] damage to snails (Figure 2) is in the low category. Tissue damage is divided into 3: low category with a percentage of tissue damage of 0-30%, medium category with a percentage of tissue damage between 30-70%, and heavy category with a percentage of tissue damage between 70 – 100%. Thus, depuration treatment shows changes in tissue structure that are better than before, although not yet significant.

4. Conclusion

Based on the existing findings, it can be concluded as follows: First, Tissue damage before and after treatment shows a low level of damage with a tissue damage value of (0-6.1%). Tissue damage does not cause death; it only disrupts specific organ systems. Second, an analysis of metallothionein levels has not shown a significant decrease. MT levels experienced an increasing trend during the flushing treatment, which was thought to be due to the water media used containing Fe and Zn. Third: Depuration activities using a flushing system should use media water that is cheap and free of heavy metals, and the technique should use a biofiltration system.

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