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Bioaccumulation of Chromium, Iron, and the Expression of TNF-α and Caspase-3 in Mudskipper (*Periophthalmus* spp.) from Ambon Island Waters, Indonesia

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ABSTRACT

TNF-α protein and caspase-3 in mudskipper muscle can be used as a biomarker to evaluate the environmental conditions. Therefore, this study aimed to analyze the heavy metal contents, specifically Cr and Fe, in the sediments from the mangrove area, as well as the expression of TNF-α and caspase-3 in mudskipper muscle. The samples used were the sediments and mudskippers from the waters of Poka, Waai, and Rutong Villages. The heavy metals were analyzed using Atomic Absorption Spectrophotometry (AAS), while the TNF-α and caspase-3 expressions were assessed with immunohistochemical staining. Observations were carried out using an optical microscope with 1000x magnification. The results showed that the proportion of Fe accumulated in mudskipper at the three locations was greater than Cr, while Cr was higher in the sediment compared to Fe. Moreover, the TNF-α and caspase-3 expressions appeared in muscle organs in varying amounts. This indicates that Cr and Fe under excessive conditions can be toxic to mudskipper causing muscle inflammation and cell death, which leads to the expression of TNF-α and caspase-3.

Keywords: heavy metals Cr and Fe; TNF-α; caspase-3; mudskipper.

INTRODUCTION

Chromium (Cr) and Iron (Fe) are essential heavy metal groups in the periodic table. They are needed in low amounts by living organisms to enhance metabolism (Witkowska et al., 2021; Yousif et al., 2021). Cr functions in the metabolism of carbohydrates, lipids, proteins, and amino acids (Pechova and Pavlata, 2007). In general, Cr(III) is classified as an essential heavy metal, while Cr(VI) is toxic to humans and animals (Abbas et al., 2016; Govind et al., 2014). Bielicka et al. (2005) explained that Cr is a bio element often found as an active component of GTF (Glucose Tolerance Factor) and plays a role in glucose metabolism. Meanwhile, Fe is an essential component in the formation of hemoglobin, oxidative metabolism, cofactors for proteins and enzymes, DNA synthesis, as well as electron transport (Al-Fartusie and Mohssan, 2017; Speich et al., 2001). Zhao et al. (2014) reported that Fe is an important element in zebrafish metabolism, because it acts as a modulator of hepcidin expression in skeletal muscle, liver, and notochord. Although heavy metals, such as Cr and Fe have physiological functions in fish, they can also pollute the environment and become toxic due to excessive accumulation in aquatic organisms (Jaber et al., 2021; Javed and Usmani, 2017; Shaaban et al., 2021).

The entry of Cr and Fe in marine waters can reduce the water quality because their accumulation into the ecosystems leads to pollution (Azratul et al., 2017; Farombi et al., 2007; Pantopoulos et al., 2012). In addition, Afsan et al. (2014) revealed that Fe, Cu, Zn, Mn, and Cr are the heavy metals that pollute waters and accumulate more dominantly in fish tissues. Nisha et al. (2016) showed that the Cr toxicity in Danio rerio fish causes body

discoloration, frequent opening of the mouth, abnormal swimming movements, and excessive mucus secretion, while Mamta & Trivedi (2016) reported that the exposure to Cr of 2.6 mg/L for 60 days caused an increase in lactic acid in muscles and blood. Aslam and Yousafzai (2017) also concluded that the exposure to this heavy metal can alter various activities of the enzymes succinate, pyruvate, and lactate dehydrogenase in the kidneys, brain, and liver. Furthermore, the high accumulation of Fe in the fish body causes degeneration of muscle fibers and atrophy of the bundles (Aris and Tamrin, 2020). Jaishankar et al. (2014) reported that iron toxicity leads to a cellular reduction in mitochondria and lysosomes.

Biomonitoring programs are used to measure contaminants in a marine organism; however, biomarkers which are measurable parameters at various levels of biological organization including molecular, cellular, or physiological have recently been developed (Hamza-Chaffai, 2014). Biomarkers are defined as substances associated with observable biochemical, physiological, or other changes in the tissues or body fluids of an organism (Dalzochio et al., 2016; Leomanni et al., 2016). In addition, Dey et al. (2016) stated that they are causal intermediate effects of xenobiotic exposure, thereby providing an early warning signal about the potential damage at the cellular and sub-cellular levels in certain organs and/or tissues under contaminated aquatic environments.

Hamdy et al. (2016) showed that the caspase-12 protein is a useful biomarker for water pollution, while Falfushynska et al. (2014) stated that the caspase-3 activity can be used for the determination of toxic effects. In addition, Rumahlatu et al. (2019) argued that the TNF-α protein is applicable generally as a biomarker for marine organisms, and more specifically in the *D. setosum* species as a bioindicator. Yildirim and Danabas (2014) revealed that the immunomodulating factor TNF-α, can be used as a bioindicator in fish health and water quality assessment. Sandri et al. (2001) & Silva et al. (2015) found the caspase-3 expression in dystrophic muscles, but not in normal ones. Meanwhile, the TNF-α expression can be found in skeletal muscle fibers due to injury and muscular dystrophy. It also functions in the regeneration of these muscles (Alvares, et al. 2020; Li, 2003; Li and Reid, 2001; Wang et al., 2019). To determine the expression of caspase-3 and TNF-α in an organism used for biomonitoring, one type of fish that can be utilized is Mudskipper.

Mudskipper can accumulate heavy metals through sediments; hence, it is used as a biomarker of oxidative stress to evaluate the side effects of heavy metals (Santoso et al., 2021; Zaccon et al., 2017). The habitat is in the mangrove area with a muddy substrate in which various nutrients and heavy metals accumulate. Furthermore, this fish is important for biological and ecological-toxicological studies and also known as potential bio-indicators in environmental monitoring and assessment of coastal waters as well as tropical or subtropical soft-bottom intertidal systems. The relationship with the mangrove environment culminates in adaptability and increases the sensitivity to pollution; therefore, this can be the basis for bio ecotoxicological studies (Ansari et al., 2014; You et al., 2018).

Several studies related to the accumulation of Cr and Fe were conducted in the Maluku seas and various marine waters. Siahaya et al. (2013) reported that the accumulation of Cr in the marine waters of Poka-Rumah Tiga was greater in sponge tissue (*Callispongia* sp) compared to those in seawater and sediments. Manullang et al. (2017) showed that the accumulation rate of Fe (27,598–51,716 mg/kg) was higher than Cu (13.7–44.8 mg/kg) in the Poka coastal sediments, while El-Batrawy (2018) found that the degree of heavy metals accumulation in *O. niloticus* muscle was Fe>Zn>Mn>Ni>Pb>Cu. This shows that Cr and Fe are more easily accumulated in tissues due to their function in metabolism. Furthermore, Arifin (2001) reported that the concentration of Cr is high in the eastern and western parts of Indonesia, where the elevated concentration in the waters of the eastern part is influenced by the high mineral content in the highlands.

Sediment is a container submerged by seawater and a colloid suspended by organic and mineral material; hence, it can mediate the transfer of heavy metals for living things such as macroinverts, fish, and amphibians (Greenfield, 2012; Yunus et al., 2020). Martinez-Guijarro et al. (2019) explained that the presence of heavy metals in sediments is influenced by human activities, and it accumulates in living tissue, causing short to long-term toxic effects. Furthermore, the sediments in the mangrove areas of Rutong, Waai, and Poka Villages function as a medium for the transfer of heavy metals such as Cr and Fe for mudskipper.

The risk of Cr and Fe accumulation is high in mudskipper muscles, and this fish actively moves in the mud; hence, they need healthy muscles. Santoso et al. (2020) argued that this species can be used for biomonitoring in aquatic ecosystems as well as biomarkers to detect oxidative stress, genotoxicity, and immunotoxicity. Several studies also reported the accumulation of Cr and Fe in other species, including heavy accumulation of Cr in *Periophthalmus modestus* (Liu et al., 2019), Cr and Fe in *Periophthalmus argentilineatus* (Kruitwagen et al., 2008), and Fe in *Gobius boddarti* species (Ahmed et al., 2011). Adhihetty and Hood (2003) explained that the expression of caspase-3 in skeletal muscle is caused by apoptosis as evidenced by myonuclear decay, while Guan et al. (2021) reported that the expression of IL-10 as a pro-inflammatory cytokine in the intestinal organ of *Boleophthalmus pectinirostris* increased significantly in an underwater environment. Presently, there are no previous studies on the expression of caspase-3 as an executor of apoptosis and TNF-α as a pro-inflammatory cytokine in mudskipper muscles due to the Cr and Fe exposure, specifically in those that live in the mangrove forest area of Ambon Island, Indonesia. Therefore, this study aimed to examine the accumulation of heavy metals, namely Cr and Fe, in sediments and mudskippers as well as to analyze the expression of caspase-3 and TNF-α in the muscles.

MATERIALS AND METHODS

Study area

This study was conducted in the mangrove ecosystem of Ambon Island, Indonesia (Figure 1). The locations include Ferry-Poka Port (Poka-I station), Poka-Diesel Power Plant Area (Poka-II station), Waai Beach I (Waai-I station), Waai Beach 2 (Waai-II station), Waai Beach Rutong I (station Rutong-I), and Rutong Beach II (station Rutong-II).

Sample collection

The sediment and mudskipper samples were collected from the mangrove areas of Poka, Waai, and Rutong Villages. The samples were placed in different plastic bags, labeled, and stored in a box filled with ice. Subsequently, all were brought to the laboratory for further analysis of heavy metals and immunohistochemistry.

Sample preparation and analysis of the Cr and Fe heavy metals

The heavy metal contents, namely Cr and Fe, were analyzed at the Research and Industrial Standardization Center of Maluku Province, the Environmental Health Center, and the Center for Disease Control Engineering – Ambon,

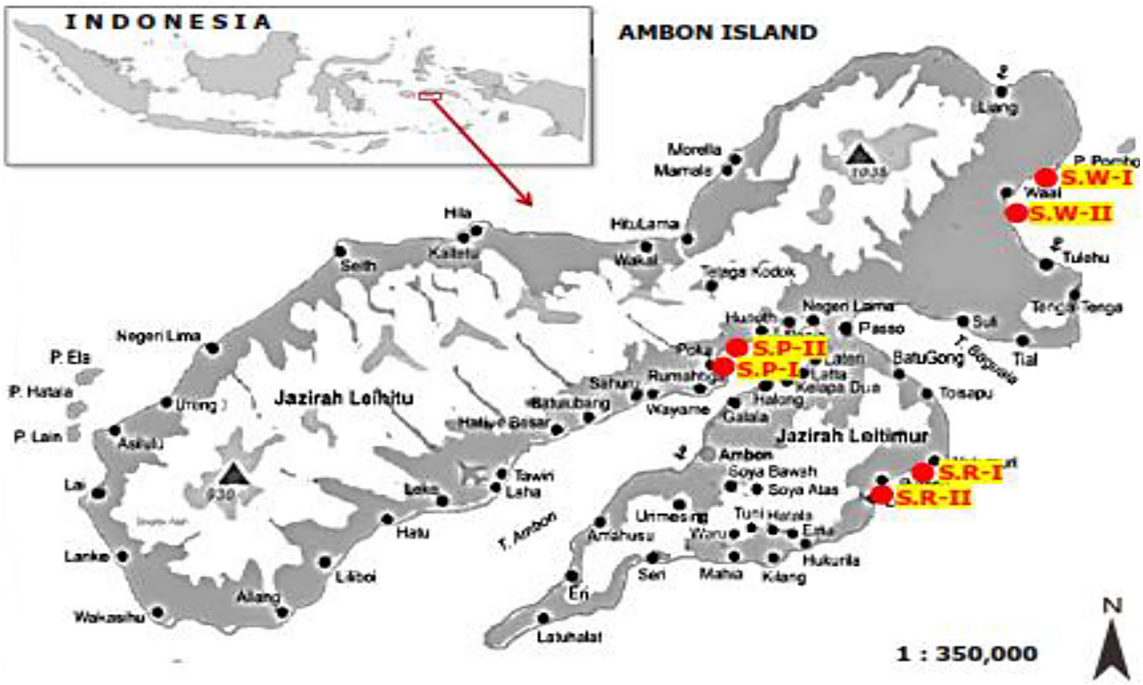

Figure 1. Sampling locations (SP-1: Station Poka-I; SP-2: Station Poka-II; SW-I: Station Waai-I; SW-II: Station Waai-II; SR-I: Station Rutong- I; SR-II: Rutong-II Station)

Indonesia. The samples were initially prepared; then, calibration curves were made and analyzed using an Atomic Absorption Spectrophotometer (AAS) to determine individual heavy metals. Furthermore, the absorbance of the sample solution was entered into the calibration curve, while the concentration of heavy metals in the sample (wet weight) was calculated using an equation by Baranowska et al. (2015) as follows:

$$\text{Content, ppm} = a/b \quad (1)$$

where: a – the amount of metal µg from the measurement results with AAS,
b – sample weight (5.0 g).

Caspase-3 immunohistochemical staining

Immunohistochemical staining of caspase-3 was carried out at the Zoology Laboratory, Faculty of Mathematics and Natural Sciences, Pattimura University. The stages were modified based on the procedure of Leite et al. (2016), (1) each piece of muscle tissue was subjected to antigen retrieval for 10 minutes in citrate buffer (pH 6.0), allowed to stand at room temperature for 30 minutes, washed using phosphate buffer saline (PBS), and then incubated with Protein block (Blocking agent sniper) for 30 minutes. (2) Incubation was further carried out using primary antibody caspase-3 with a dilution of 150 times dissolved in serum at room temperature; then, the preparation was washed using PBS. (3) Universal Link secondary antibody was incubated, and then washed using PBS followed by Trecavidin-HRP at room temperature. (4) Re-washing was performed with PBS 2x, while Chromogen diamino benzidine (DAB) was dripped for 20–30 seconds, and immersed in Lilie Mayer's Hematoxylin solution as a counterstain for 1–2 minutes. (5) Immersion was carried out in lithium carbonate for 1 minute, followed by washing with running water. In addition, dehydration was carried out using ethanol and washing with xylol; then, the sample was covered with aqueous mounting media. (6) The slide was mounted with entelan, and lastly, (7) observations were made on an optilab microscope with 1000x magnification.

TNF-α immunohistochemical staining

TNF-α immunohistochemical staining was carried out at the Zoology Laboratory, Faculty of Mathematics and Natural Sciences, Pattimura University. It was performed by modifying the stages of Neves et al. (2015). (1) The slide was immersed in 0.3% of H_2O_2 for 30 minutes, then rinsed with water followed by 1x PBS. (2) Incubation was carried out with 1% normal serum/PBS [Mix 1x 3.5 ml PBS, pH 7.4 and 1 drop containing approximately 35 l/drop of normal serum in a tube for 30 min at room temperature and (3) normal serum was dropped from the slide. (4) Incubation of the first sample was diluted using PBS sections by optimizing antibody titer before starting Immunohistochemistry in a humid chamber for 1h at room temperature. (5) The slides were then rinsed with 1x PBS 3 times each for 5 minutes and (6) they were incubated with diluted PBS Biotin-labeled secondary antibody of 1,4 1x PBS, pH 7.4 ml, and 1 drop containing approximately 35 l/drop of biotinylated anti-mouse and TNF-α in a tube for 30 min at room temperature. (7) The slides were rinsed with 1x PBS 3 times, each for 5 minutes. (8) Preparation of the detection solution includes Mix 1xPBS 1.33 ml of 1 drop with 35 l/drop of solution A and 1 drop of solution B in a tube. The mixture was incubated at room temperature for 30 minutes before use, (9) the detection solution was added to the tissue portion, and then incubated at room temperature for 30 minutes. (10) Rinsing was carried out with 1x PBS for 3 times, each for 5 minutes. (11) A fresh expansion solution was prepared with Mix 1.6 ml of DAB buffer and 1 drop of liquid DAB in a tube. (12) Solution development was added to cover the network for 5–30 minutes. (13) The reaction was stopped by immersing the tissue in water. (14) Counter stain slide was used when necessary, while Harris Hematoxylin was used for core staining. (14) Slide installation with entelan was conducted, and (15) observations were performed on an optical microscope with 1000x magnification.

Data analysis

The data were analyzed descriptively to explain the average value of heavy metal concentrations of Cr and Fe, as well as the expression of TNF-α. In addition, visualization in the form of images was carried out to show the condition of mudskipper body tissue expressing caspase-3 and TNF-α.

RESULTS AND DISCUSSION

Heavy metal concentration in sediment and mudskipper

The heavy metals accumulated in the mangrove areas of Poka, Waai, and Rutong Villages were in the order Fe>Cr (Table 1), while those in sediments were Cr>Fe (Table 1).

The results presented in Table 1 are in line with several studies which reported that the accumulation of Fe in fish is higher than Cr in the Bay of Bengal (Rakib et al., 2021). The order of heavy metals accumulation in Karachi Pakistan and the coral ecosystem of Krakatau Island is Fe>Zn>Cu>Mn and Fe>Zn>Cr, respectively (Yousif et al., 2021; Murwani et al., 2019). Furthermore, Fe was reportedly higher than Cr in the body of *Rasbora tornieri* fish (Intamat et al., 2016) and shrimp (Ezemonye et al., 2019). The accumulation of Fe is also greater than other essential heavy metals in various weights of *Oreochromis mossambicus* (Shinde et al., 2020).

These explanations indicate that Fe has a high affinity for fish bodies, Fytianos and Lourantou (2004), stated that its high affinity is bound to organic matter, while Rosli et al. (2018) explained that it tends to accumulate more in some species of fish compared to other essential heavy metals, and plays a role in the formation of red blood cells. According to Galbraith et al. (2019), Fe is one of the essential metals that can accumulate in red muscle organs containing myoglobin. Meanwhile, Mansour et al. (2019) stated that the location in fish is on the side of the body under the skin and serves to perform muscle contractions for movement. The accumulation of heavy metals in the body of mudskipper fish is also influenced by habitat and bioaccumulation through the food chain. The fish that live in mud accumulate more heavy metals than pelagic species (Jiang et al., 2018; Mirghaed et al., 2018).

The characteristics of Fe are inversely proportional to Cr which has a high bond with the sediment fraction, amounting to 89% (Morillo et al., 2004). Several studies reported that the accumulation of Cr in sediments was higher than in seawater (Li et al., 2013; Rifkin et al., 2004; Ferrans et al., 2021). Morillo et al. (2004) explained that the marine sediments with high amounts of Cr were associated with the residual fraction of 80% and 10% organic-sulfide matter. This study is also in line with that by Soulivongsa et al. (2020) which reported that the Cr in sediments was higher than in fish with values of 20.71 mg/kg and 4.72 mg/kg, respectively.

Cr and Fe pollute the environment and aquatic organisms. On the basis of the location of the mangrove area, the analysis showed that the order of Cr and Fe concentrations, in the sediment was Waai>Rutong>Poka and Rutong>Poka>Waai, while in mudskipper was Poka>Waai>Rutong and Poka>Rutong>Waai, respectively (Table 1). Differences in the accumulation of both are influenced by the source of pollution in the particular location. The high Cr in the Waai mangrove area is due to a large number of fishing activities in the forms of painting and coating on speedboats or paint peeling off the walls of the boat and dissolving in seawater and then accumulating in the sediment. Castritsi-Catharios et al. (2014) reported that heavy metals pollute the aquatic environment through human activities such as agriculture, transportation, pharmaceutical products,

Table 1. Heavy metal concentrations in sediment and mudskipper

Location	Station	Mudskipper (ppm)		Sediment (ppm)	
		Cr	Fe	Cr	Fe
Poka	S.P-I	0.0445	0.0521	0.2193	0.0984
	S.P-II	0.0387	0.0884	0.1446	0.0768
Mean±SD		0.0416 ± 0.0041	0.0703 ± 0.0257	0.1820 ± 0.0528	0.0876 ± 0.0153
Waai	S.W-I	0.0405	0.1207	0.1100	0.1117
	S.W-II	0.0432	0.1120	0.1347	0.1009
Mean±SD		0.0419 ± 0.0019	0.1164 ± 0.0062	0.1224 ± 0.0175	0.1063 ± 0.0076
Rutong	S.R-1	0.0669	0.076	0.1839	0.0733
	S.R-II	0.0429	0.0917	0.1761	0.0814
Mean±SD		0.0549 ± 0.0170	0.0839 ± 0.0111	0.1800 ± 0.0055	0.0774 ± 0.0057

Note: S.P-1: Station Poka-I; S.P-2: Station Poka-II; S.W-I: Station Waai-I; S.W-II: Station Waai-II; S.R-I: Station Rutong-I; S.R-II: Station Rutong-II.

or naturally through biogeochemical processes in the waters. The sources of Cr pollution in marine waters are through anthropogenic means, namely electroplating and the paint industry, as well as naturally through geoaccumulation, rain, and innate particles that accumulate heavy metals from rivers (Afshan et al., 2014; Harikumar et al., 2010; Tabari et al., 2010). According to Yunus et al. (2020), Cr is used in wood preservatives, cement protective coatings, paints, paper, rubber, carpets, and teals. This topic was also examined by Praveena and Lin (2015) who stated that Cr is sourced from the shipping activity at the Dickson port of Malaysia. Meanwhile, the highest source of Fe pollution in the mangrove area of the Rutong village is due to a large number of household waste activities. A similar result was also reported by Shu et al. (2020) and Li et al. (2020) which found that the source of Fe pollution is anthropogenic activity in the form of household waste.

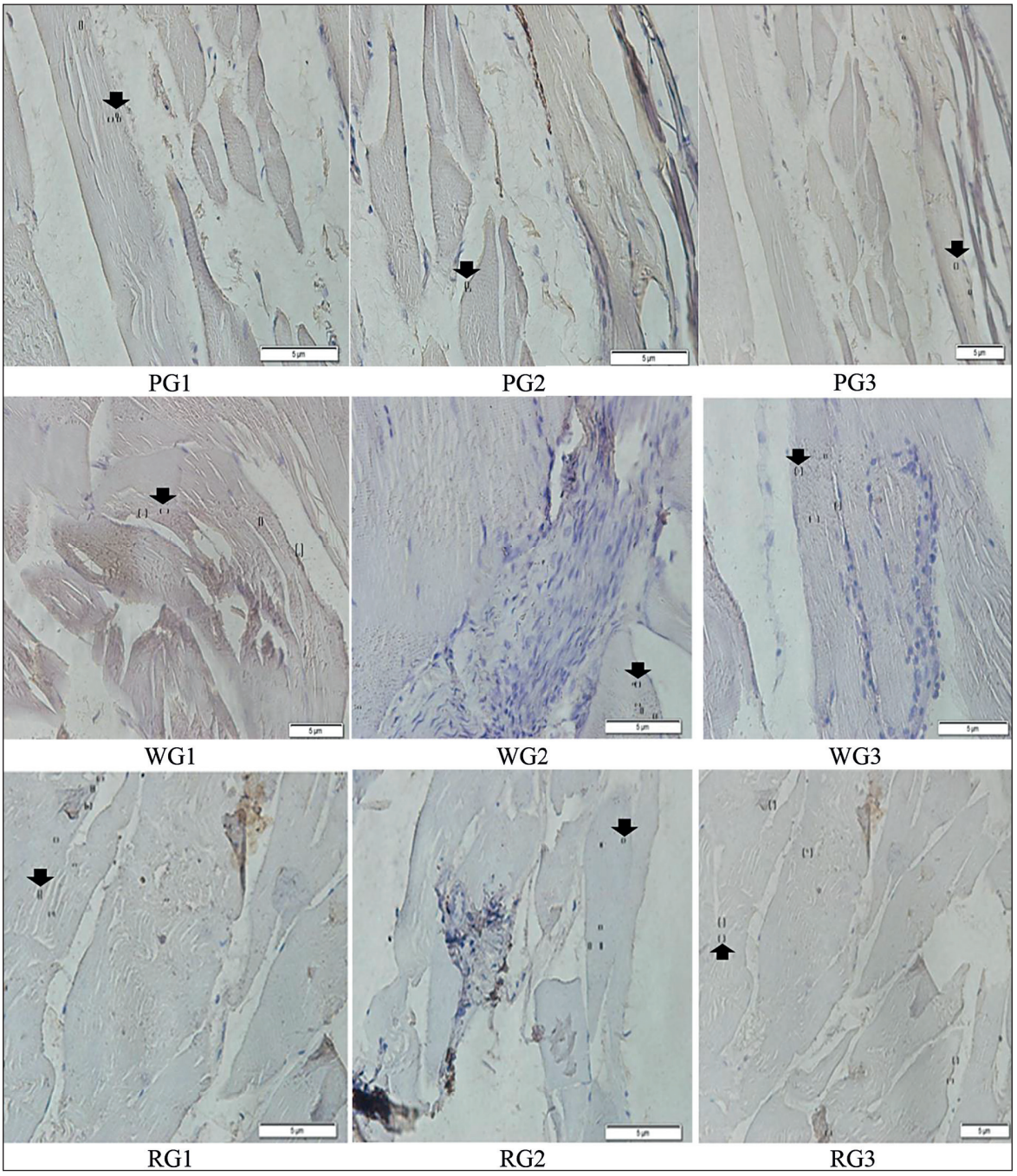

Figure 2. The TNF-α expression in mudskipper muscle cells. Black arrows indicate the TNF-α expression. Staining using immunohistochemistry at 1000x magnification. Captions: PG1, PG2, PG3 (Poka Figure 1,2,3); WG1, WG2, WG3 (Waai Figure 1,2,3); RG1, RG2, RG3 (Rutong Figure 1,2,3)

TNF-α expression in mudskipper

The immunohistochemistry test results for the TNF-α expression in mudskipper are shown in Figure 2. The calculation of the total TNF-α expressed in mudskipper muscle tissue can be shown in Table 2.

The results show that the accumulation of heavy metals in mudskipper causes muscle damage; hence, it can be a biomarker in the aquatic environment. Steinhagen et al., (2004) explained that Cr is toxic to *Cyprinus carpio* fish, causing a decrease in the immune cells. Meanwhile, Kaur et al. (2018) reported that the Zn, Pb, Mn, Cu, and Cr contained in *Labeo rohita* muscle tissue cause damages such as shortening and elongation of muscle bundles, edema, and necrosis. Shah et al. (2020) also revealed that the Cr in *Ctenopharyngodon idella* induced inflammation, necrosis, degeneration, edema, muscle fiber zigzags, and lesions. Khan et al. (2020) showed that Fe accumulation was higher than Cr, while Abdel-Khalek et al. (2020) explained that the Fe bound to fish muscles is higher than Al. Mudskipper muscle is not a metabolic tissue, but an organ of heavy metal accumulation, because it is covered by skin that is constantly in water and mud. This is consistent with Bibak et al. (2021) and Santoso et al. (2021) who stated that mudskipper muscle is a skin-wrapped tissue that is in contact with pollutants dissolved in seawater and mangrove sediments.

Furthermore, Sharma et al. (2014) argued that heavy metals are one of the causes of reactive oxygen species (ROS) production in tissue through oxidative stress conditions. Powers et al. (2011) explained that high levels of ROS cause contractile dysfunction of skeletal muscles, leading to fatigue. Excessive Fe in cells leads to increased production of reactive oxygen species (ROS) such as hydroxyl radicals (H_2O), superoxide radicals (O_2-), or hydrogen peroxide (H_2O_2). These activities can trigger mitochondrial respiratory dysfunction (Galaris et al., 2019; Chen et al., 2018). The increase in TNF-α is in line with the rise in ROS production (Suematsu et al., 2003; Jiang et al., 2020). Valenzuela et al. (2017) reported that skeletal muscle in *Paralichthys adpersus* responds to pathogens by expressing the pro-inflammatory cytokine TNF-α. The results in Figure 2 show that the mudskipper muscles can express TNF-α when heavy metal accumulation causes inflammation and edema. The expression in muscle tissue marked with brown color indicates a mechanism against oxidative stress by the accumulation of Fe and Cr. According to Rumahlatu et al. (2019), it stated that the higher the Cd level, the higher the TNF-α expression in the liver of *Diedema setosum*. In addition, Yin et al. (2018) reported that TNF-α expression also appears due to the exposure to Cu, Cr, Cd, and Pb.

The calculation results show that the amount of TNF-α expression in mudskipper muscle tissue varies (Table 2). The mudskipper muscle cells that express the most TNF-α originate from the Rutong

Table 2. Total TNF-α expression in mudskipper muscle

Location	Figure-	Total TNF-α expression in each observation				
		I	II	III	IV	V
Poka	1	1	0	3	0	0
	2	0	2	0	0	1
	3	1	0	0	1	1
	Mean ± SD	0.6667 ± 0.5774	0.6667 ± 1.1547	1 ± 1.7321	0.3333 ± 0.5774	0.6667 ± 0.5774 3.4
Waai	1	2	0	1	1	0
	2	0	0	0	2	2
	3	0	2	2	0	0
	Mean ± SD	0.6667 ± 1.1545	0.6667 ± 1.1545	1 ± 1	1 ± 1	0.6667 ± 1.1545 4.1
Rutong	1	4	1	1	1	2
	2	2	0	1	2	0
	3	2	0	0	0	0
	Mean ± SD	2.6667 ± 1.1547	0.3333 ± 0.5774	0.6667 ± 0.5774	1±1	0.6667 ± 1.1545 5.4

coast. In the case of this study, it can be explained that the household waste and human activities in the Rutong mangrove waters are able to accumulate heavy metals in the mudskipper's body. This accumulation can induce the TNF-α expression in mudskipper muscle cells. Muscle cells, although not metabolizing tissue, can be damaged like any other tissue. Trovato et al. (2016) explained that muscle is one of the tissues that can be damaged due to various factors. The large amount of TNF-α expression is the activity of muscle cells to fight oxidative stress due to the accumulation of Cr and Fe. The same opinion that TNF-α expression does appear under the conditions of muscle damage and muscle inflammation is also expressed by Renström et al. (2017). Tidbal et al. (2010) explained that the TNF-α expression that appears in muscle cells can regenerate damaged muscles.

Caspase-3 expression in mudskipper muscle cells

The immunohistochemical test results on the expression of caspase-3 in mudskipper muscle tissue are shown in Figure 3, while the amount expressed is shown in Table 3.

The results show that the accumulation of heavy metals also activates the protein caspase-3 which is involved in the cell apoptotic pathway. Due to the continuous accumulation of oxidative stress, the cell will enter a death phase through the caspase-mediated apoptotic pathway (Ott et al., 2007; Alarifi et al., 2014; Lushchak, 2014). Furthermore, the high increase in ROS in muscle cells can affect the mitochondrial metabolic processes. This induces an increase in Ca^{2+} which then mediates the apoptotic pathway through caspase-3. The consequences include atrophy, which leads to muscle weakness, as well as loss of mass and function, sarcopenia, and inflammation (Barbieri and Sestili, 2012).

Several previous studies reported that heavy metals affect cell apoptosis mediated by caspase-3. Anvarifar et al. (2018) found that Cr causes apoptosis in fish cells, while Rumahlatu et al. (2014) explained that high concentrations of Cd increase the caspase-3 expression and activate apoptosis in *D. setosum* liver cells. Abdel-Emam and Ali (2021) also reported that Pb causes oxidative stress in rat liver cells which stimulates the caspase-3 expression as an indicator of inflammation and liver cell apoptosis. According to Jiaxin et al. (2020), Cd also activates the expression in carp neutrophil cells. In addition, Shaw et al. (2022) reported that low levels of Cr(VI) can be toxic and cause apoptosis in zebrafish liver. Renu et al. (2021) revealed that the exposure to Cr can activate caspase-3 in the liver of living organisms, while Jiang et al. (2015) reported that the accumulation of Cu causes oxidative damage to carp muscle while activating caspase-3 signaling. Furthermore, da Silva et al. (2014) showed that the high accumulation of iron (Fe) in rat brain cells affects mitochondrial metabolism and leads to the loss of synaptic signaling processes

Table 3. Muscle cells expressing the protein caspase-3

Location	Figure-	The number of caspase-3 expressions in each observation				
		I	II	III	IV	V
Poka	1	1	1	0	0	0
	2	2	1	3	1	4
	3	1	2	0	0	1
	Mean ± SD	1.3333 ± 0.5774	1.3333 ± 0.5774	1 ± 1.7321	0.3333 ± 0.5774	1.6667 ± 2.0817 1.12
Waai	1	0	0	3	1	0
	2	2	1	0	2	8
	3	18	16	5	0	5
	Mean ± SD	6.6667 ± 9.8658	5.6667 ± 8.9629	2.6667 ± 2.5166	1 ± 1	4.3333 ± 4.0415 4.08
Rutong	1	12	8	3	1	2
	2	5	8	2	0	0
	3	3	4	2	4	2
	Mean ± SD	6.6667 ± 4.7258	6.6667 ± 2.3094	2.3333 ± 0.5774	1.6667 ± 2.0817	1.3333 ± 1.1547 3.74

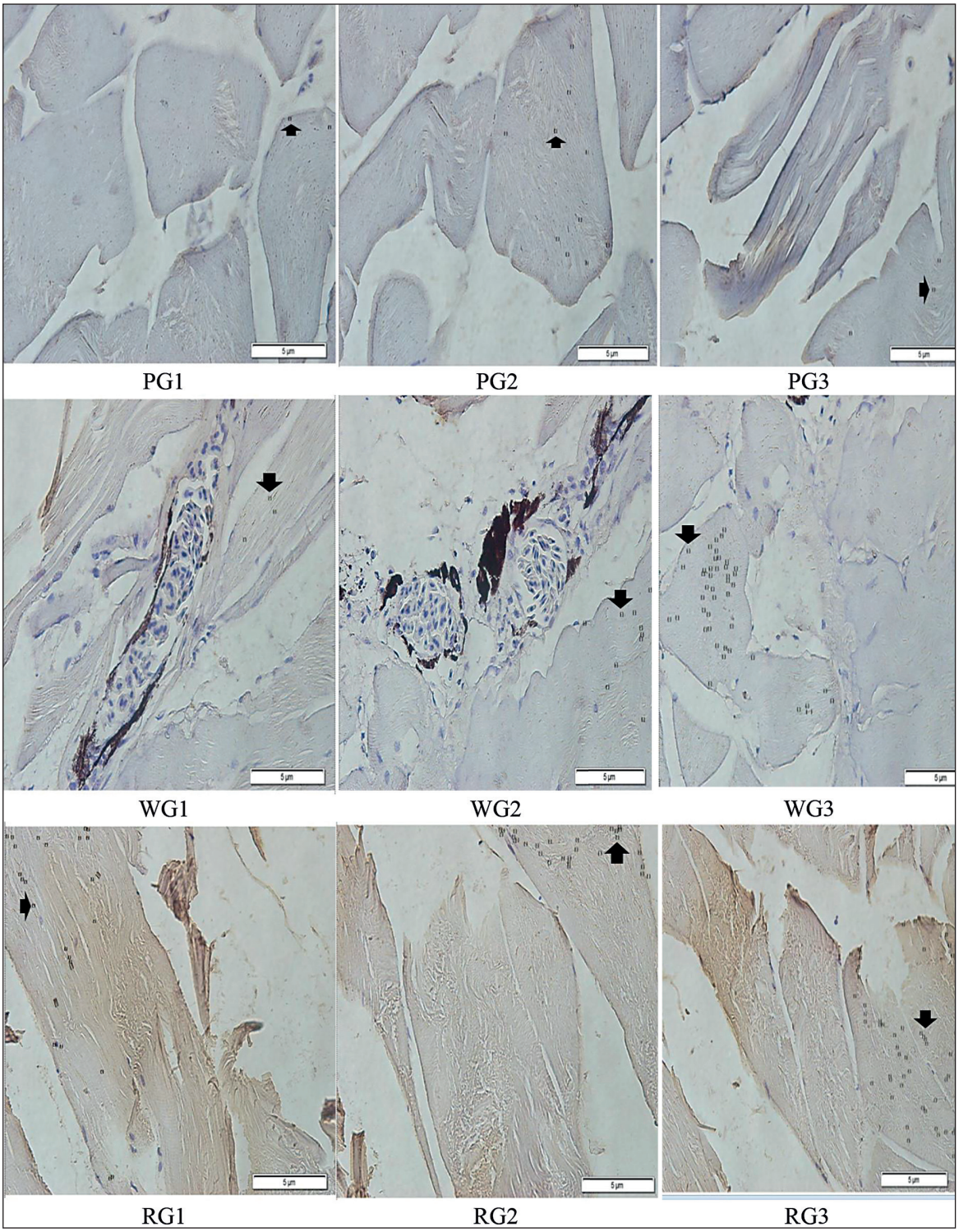

Figure 3. The caspase-3 expression in mudskipper muscle cells. Brown arrows indicate the caspase-3 expression. Staining using immunohistochemistry at 1000x magnification. Captions: PG1, PG2, PG3 (Poka Figure 1,2,3); WG1, WG2, WG3 (Waai Figure 1,2,3); RG1, RG2, RG3 (Rutong Figure 1,2,3)

in brain neurons, which ultimately triggers neuronal cell apoptosis. On the basis of these studies, the caspase-3 expression was caused by the accumulation of Fe and Cr. It causes muscular dystrophy and the death of cells that make up mudskipper muscle tissue.

The calculation results showed that the amount of caspase-3 expression in mudskipper muscle tissue varied (Table 3). The mudskipper muscle cells that express the most caspase-3 originate from the Waai coast. In the case of this study, it can be explained that human activities and ship

activity waste in the Waai mangrove waters can affect the accumulation of heavy metals in the mudskipper body. This accumulation can induce the caspase-3 expression in mudskipper muscle cells. The research on the expression of caspase-3 due to accumulation of Fe and Cr metals in fish muscle has not been studied, but has been reported by other researchers on other heavy metals, Guo et al. (2020) reported that the Cu accumulation in chicken muscle further induces caspase-3 to perform apoptosis through endoplasmic reticulum stress; Wang et al. (2018) reported a similar case that Cu and As accumulated in chicken muscle induce caspase-3 for apoptosis. Smuder et al. (2010) continued that oxidative stress in cells is closely related to myofibril proteolysis in muscle cells and further accelerates apoptosis by caspase-3.

CONCLUSIONS

On the basis of the results, Cr and Fe are toxic to mudskipper, the order of heavy metals accumulation in the three mangrove ecosystems namely Poka, Waai, and Rutong was Fe>Cr, while the order in sediments was Cr>Fe. In addition, the TNF-α and caspase-3 expressions appeared in mudskipper muscle organ in varying amounts. The results also showed that muscle as a non-metabolic tissue can accumulate Cr and Fe, leading to inflammation and apoptosis characterized by the appearance of the TNF-α and caspase-3 expressions under conditions of heavy metal accumulation. This indicates that the accumulation of Cr and Fe can be used by mudskipper as a biomarker of mangrove water conditions in Ambon Island.

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