Alterations in pigmentation and morphology of goldfish (*Carassius auratus*) exposed to sublethal treatment with mercury

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Abstract. Even in low concentrations, organic trace pollutants, including Mercury (Hg), threatens early fish developmental stages. In this study, the pigmentation and morphology changes of goldfish were determined for fish subjected to various concentrations of mercury ($HgCL_2$). Eight-day-old comet fish (*Carassius auratus*) larvae were kept in a 3-liter basin, with a stocking density of 10 individuals/L. Fish samples were collected after 64 days of exposure to sublethal concentrations of Hg. The concentrations were 0.01, 0.03 and 0.06 ppm (3–20% of LC_{50}-96h). Digital microscopy was used to observe goldfish morphology, while pigmentation was observed using Modified Toca Color Finder (M-TCF) to determine color changes. The results showed that changes occurred in fish exposed to Hg in all concentrations. The color changes of goldfish slowly became pale. The fish presented abnormal shapes, especially in the caudal peduncle area, for a Hg concentration of 0.06 ppm. This study concluded that the exposure to Hg can change fish color and morphology. Mercury exposure, even at lower concentrations, led to the same effect on fish larvae, resulting in defects or morphological and pigmentation abnormalities.
Key Words: deformity, morphology, skin coloration, trace pollutants.

Introduction. Currently, water pollution is occurring everywhere, particularly in developing countries, as a result of human activities that change the physical, chemical and biological properties of ecosystems, such as mining or agriculture. Pollution is caused by the entry of foreign substances in the natural environment. Water pollution generally occurs in the watershed or other areas where waste is disposed into bodies of water due to the concentration of population and industrialization. The influence of industrial waste pollutants containing heavy metals such as arsenic (As), lead (Pb) cadmium (Cd), mercury (Hg), and other heavy metals can lead to biota disturbances and even death in most organisms (Azaman et al 2015; Suyatna et al 2017; Junianto et al 2017).

Heavy metals in the water can be absorbed and accumulated by all body tissues of aquatic biota directly by contact with water or indirectly from the food chain (Jezierska & Witeska 2006). Toxic pollutants can cause death (lethal) or harm (sublethal), disrupting the growth, behavior and morphological characteristics of various aquatic organisms that try to cope through biochemical, haematological or physiological responses (Handy 1994). Environmental changes and habitat niches can directly cause abnormalities in the physiology and morphology of fish organs (Devi & Mishra 2013; Seebacher et al 2016). Body malformation occurrences increase and the survival rate is reduced, especially in the early developmental stages of fish (Jezierska et al 2009; Sfakianakis et al 2015).

Over the past three decades, many aspects of heavy metal contamination in the aquatic environment with regard to distribution, toxicity and bioaccumulation in organisms have been investigated. Heavy metal pollution has dreadful effects on fish reproduction. Heavy metals disrupt reproductive hormone secretion (Ebrahimi & Taherianfard 2011) and accumulate in the fish gonads (Annabi et al 2013). Spermatozoa motility of sea bass (*Dicentrarchus labrax*) and zebra fish (*Danio rerio*) were reduced due to mercury and cadmium toxicity (Abascal et al 2007; Acosta et al 2016). In the end, the metals may reduce fish fecundity, leading to a long-term decline and eventual extinction of fish species (Ebrahimi & Taherianfard 2011; Irianto et al 2017).

Recently, there have been a few studies regarding the effect of mercury exposure on the development of fish embryos. Early life exposure of experimental fish toward inorganic mercury and MeHg have resulted in deformities such as eye, tail and fin alterations (Samson & Shenker 2000). Organic (MeHg) and inorganic mercury (HgCl2) both induced metallothionein (MT) production and produced embryo developmental toxicity in medaka fish (*Oryzias latipes*) (Dong et al 2016). They altered fish phenotypes, producing pericardial edemas with elongated or "tube" heart, failure of swim bladder inflation and reduced eye pigmentation.

In high-value ornamental species such as goldfish (*Carassius auratus*), emphasis should be placed on achieving high levels of skin pigmentation, which together with body shape, fin shape and body size are the most important quality criteria for their market value (Gouveia et al 2003; Eşanu et al 2015). The coloration of skin is determined by pigment cells (Sugimoto 2002). The epidermal layer can adjust depending on environment and sexual activity, since the number and location of the chromatophores affect the color brightness in fish. The changes are caused by stress due to the environment, lack of sunlight, disease or feed (Almeida et al 2008; Yedier et al 2014; Kurnia et al 2019).

Our environment is increasingly contaminated with heavy metals as a result of mining, deforestation, waste disposal and fuel combustion. It is suspected that the environment is one of the major factors that affect the development and pigmentation of embryos and larvae in fish. Sub lethal environmental changes within fish have been demonstrated by many sensitive histopathological and biochemical techniques. Unfortunately, pigmentation and morphological growth at early developmental stages of fish often remain obscure regarding these effects. The morphological alteration of Hg exposed comet fish larvae has also been less studied. Therefore, the objective of the present study was to evaluate the effects of mercury (Hg) on the pigmentation and changes in the morphology of comet fish (*C. auratus*) larvae. The results may assist in the definition of future coastal and river management measures, specifically targeted at monitoring heavy metal contamination.

Material and Method

Experimental fish. Research was conducted in a hatchery station at the Fisheries and Marine Sciences Faculty from Halu Oleo University. Comet fish (*C. auratus*) were reared until they reached the reproduction stage. They were kept in a 1000 L fibertank with a continuously aerated water. The brood fish were fed with a commercial pelleted diet (30% crude protein) at ratio of 3% body weight per day for eight weeks. The brood fish were regularly monitored to determine gonad maturation prior to spawning. Natural spawning occured in an aquarium with hapa, in a ratio of females to males of 1:1. After hatching, larvae were fed with a chicken egg yolk formula starting with the third day to the 7^{th} day post hatching (7 dph).

Study design and treatments. The experiment started at 8 dph. Larvae were collected and separated into 4 groups based on different treatments. The completely randomized design was utilized in this study. Three different doses of $HgCl_2$ were tested in 3 replications. The control group was exposed only to fresh water media. Experiments were performed each in a 3 L plastic container, containing 25 fish larvae. Water was continuously aerated. The test animals were each exposed to one of the following nominal Hg concentrations: 0 (control - K), 0.01 ppm (A), 0.03 ppm (B) and 0.06 ppm (C). Skin color, morphology and abnormal tail shape were recorded every 4 days, during a 56 day period. During the experiment, fish with the age 8-11 days were fed with *Artemia* spp., and afterwards the larvae were fed with pellet feed (PF-800) until the end of the experiment.

Mercury dosage determination. The determination of Hg concentrations in this experiment was based on LC_{50}-96 hours, 0.396 ppm (Hidayat et al 2016). The maximum concentration used in this study (0.06 ppm) corresponds to 20% of the LC_{50}-96. The type of mercury used in this study was mercury chloride ($HgCl_2$, Merck, Germany). Water stocking was prepared for each treatment dosage group. The water was changed every two weeks with Hg stock with the same concentration as the maintenance media. Water quality maintenance was carried out every day using the siphon technique to clean uneaten feed and feces from the bottom of the basin.

Pigment brightness and morphological development. There are two main variables measured in this research, pigment brightness and morphological development.

The brightness of fish pigmentation was measured and recorded using color benchmarks, M-TCF (Modified Toca Color Finder) color chart (Siebeck et al 2008), color standards being set first (Figure 1). Parameters set through M-TCF were quantified visually by a ranking system consisting in the colors of comet fish. The determination of the level of brightness pigmentation changes in comet ornamental fish ranked from the lowest, 1, to the highest, 7, with color gradations from very light to more concentrated. Observations were performed every 4 days for 64 days. The color measurement of the fish samples required three independent observers to obtain precise results.

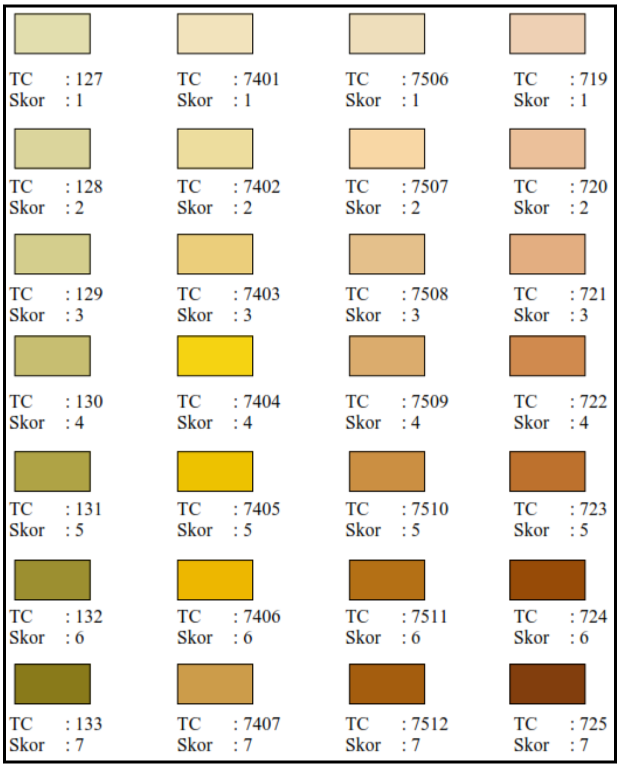

Figure 1. Modified Toca Color Finder color chart used in this study.

***Morphological development* (*fins and tail*)**. Under a microscope, Hg exposed larvae were observed to analyze the development of larval fin and tail morphology. Observations were made every 4 days, for 64 days. Observations were made by comparing the level of development of the comet fish larvae from the control lot with that of fish from other treatments, until a definitive shape was formed and completed.

Data analysis. Recorded observational data, including comet fish color brightness and development of fins and tail morphology were analyzed descriptively using Excel 2016.

Results and Discussion

Pigmentation in comet fish larvae. Based on the results of the assessment of the panelists regarding the brightness of comet fish larvae pigmentation, it was observed that fish presented pigments in the Hg exposed treatments (0.01-0.06 ppm) (Figure 2). Figure 2 presents the average values of comet fish pigmentation brightness score for 64 days. During observations, larvae presented the highest average scores from 5.33 to 3.44, at a concentration of 0 ppm, from the 4th to the 64th day. In treatment A, at a concentration of 0.01 ppm from the 4th to the 64th day, the average score was from 4.56 to 3.22. Even in the 12th day the score was at the lowest value, 2. The brightness score of fish in treatment B in the study period was from 3.89 to 2.89, the lowest score bring observed in the 12th day, 1.89. In treatment C, the brightness score of fish ranged from 3.33 to 2.89, while the lowest scores were observed in the 12th and 24th days, 1.56. This low scores indicate a poor coloration quality of the fish, because goldfish should be pigmented with an orange - red color in order to achieve consumer acceptance.

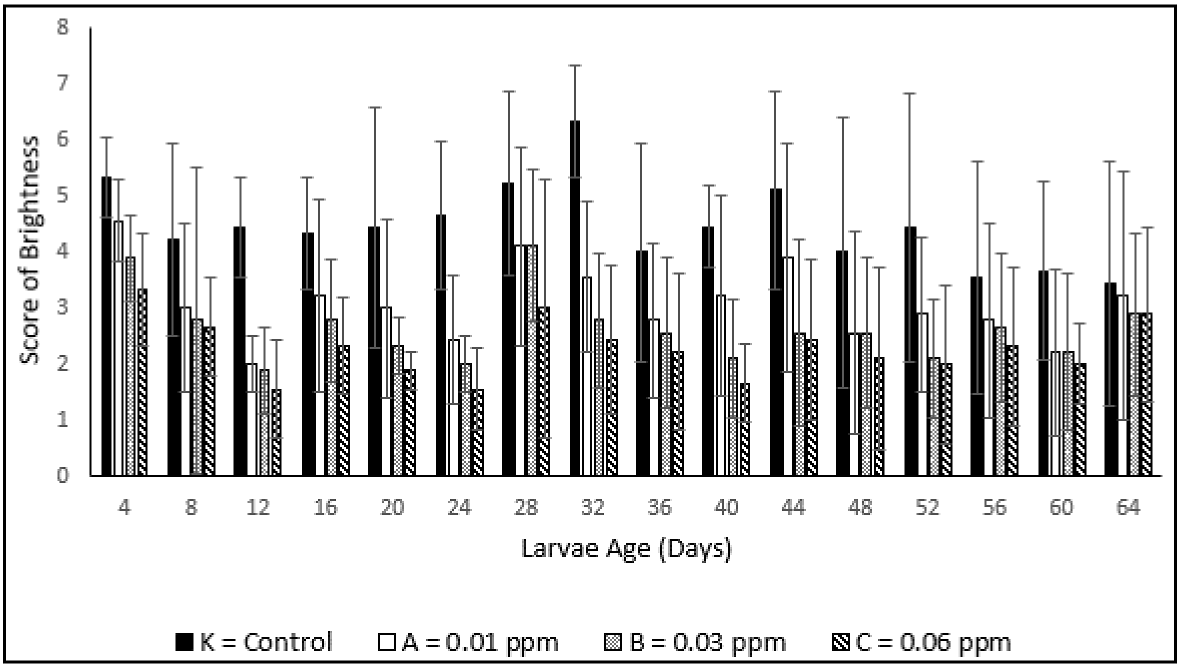

Figure 2. The score of brightness of Hg exposed comet fish (*Carassius auratus*) at different ages, according to Modified Toca Colour Finder.

The effect of sublethal mercury administration can be seen in the brightness of the fish color (Figure 2). From the figure, it can be seen that fish in the control were brighter (not pale), while in the mercury treatments at sub lethal concentrations (0.01, 0.03 and 0.06 ppm), their color was pale in the 64 days of rearing. These results showed that the pigment was damaged by the mercury treatment. These results showed that the pigment in the fish epidermis was damaged by the mercury treatment, even at the low concentrations. The first observation on the 4th day showed that there was a difference in the average score of the pigment brightness in the control treatment, A, B and C. This was maintained throughout the experiment (64th day). These results showed the difference in the percentage of bright colored fish compared to pale colored fish due to Hg exposure.

Development of larval morphology. Different abnormal aspects were observed at the base of the tail of the comet fish (Figure 4). At 35 days of exposure to 0.06 ppm Hg (treatment C), the comet fish showed a bending of the tail at the base, while there was no visible bending of the fish tail in treatment A and B. The bending of the tail at the base in the 0.06 ppm Hg treatment rises to 12% at the age of the 51 days. In treatment

B (0.03 Hg ppm) and A (0.01 Hg ppm), the bending at the base of the tail was observable at the age of 43 days. In treatment B, 7% of the fish presented bending of the tail at the age of 51 days, while in treatment A 5% of the fish showed the bending of the tail at the age of 51 days. Hg exposure resulted in negative and abnormal effects on the base of the tail, while the control treatment showed normal development at the base of the fish tail. It can be stated that even in lower concentrations, the exposure of mercury still has a toxic effect on aquatic organisms.

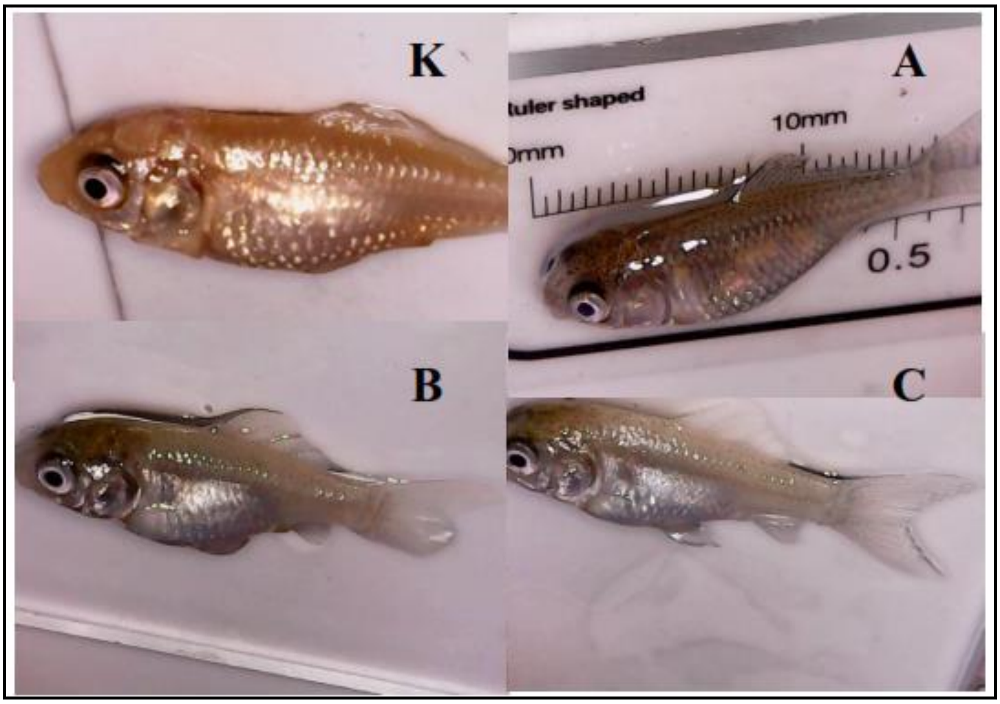

Figure 3. Difference in the pigmentation brightness of Hg exposed comet fish (*Carassius auratus*) at 62 days of age; K – control with 0 Hg ppm; A – treatment with 0.01 Hg ppm; B – treatment with 0.03 Hg ppm; C - 0.06 Hg ppm.

Metal ions are usually absorbed through passive diffusion or transportation through the gills, while metals associated with organic materials are ingested and absorbed through the intestine by endocytosis (Olsson et al 1998). The results of this study showed that low levels of exposure to mercury had an impact on goldfish morphology. Based on the observation in this study, fish pigmentation shows the appearance of heavy masses of yellow pigment spots (xanthophores) along and over the dorsal muscles at 2-4 days of age for goldfish larvae, while the Pacific red snapper (*Lutjanus peru*) was the first to complete pigmentation in the eyes (Estrada-Godínez et al 2015). Pigmentation in goldfish has been reported to be concentrated above the lateral line at 35 days of age (Rahaman et al 2011). Fish color generally showed maximum development after 3 weeks and subsequently no change in color (stable color) (Solihah et al 2015). In this research, the brightness reduced steadily since first day of observation until the 12th day, increasing slightly only on the 28th day. Pollutants have an effect on the skin by causing oxidative stress. The increase in the brightness of the fish on the 28th day might be due to the fact that the fish had begun to withstand the pressure that occurs in their environment. The extended stress period, however, might have lead to fatigue, which was characterized by a decrease in brightness in the following days. However, in all treatment, Hg exposed fish showed low brightness. This showed that Hg is very influential regarding the brightness of the color of ornamental fish. It is suspected that the color change was due to physiological changes in the larvae. The mechanism of melanocortin may occur in this case. Melanocortin is a complex neuroendocrine signaling mechanism involved in numerous vertebrate physiological processes, including pigmentation, steroidogenesis and metabolic control (Cal et al 2017).

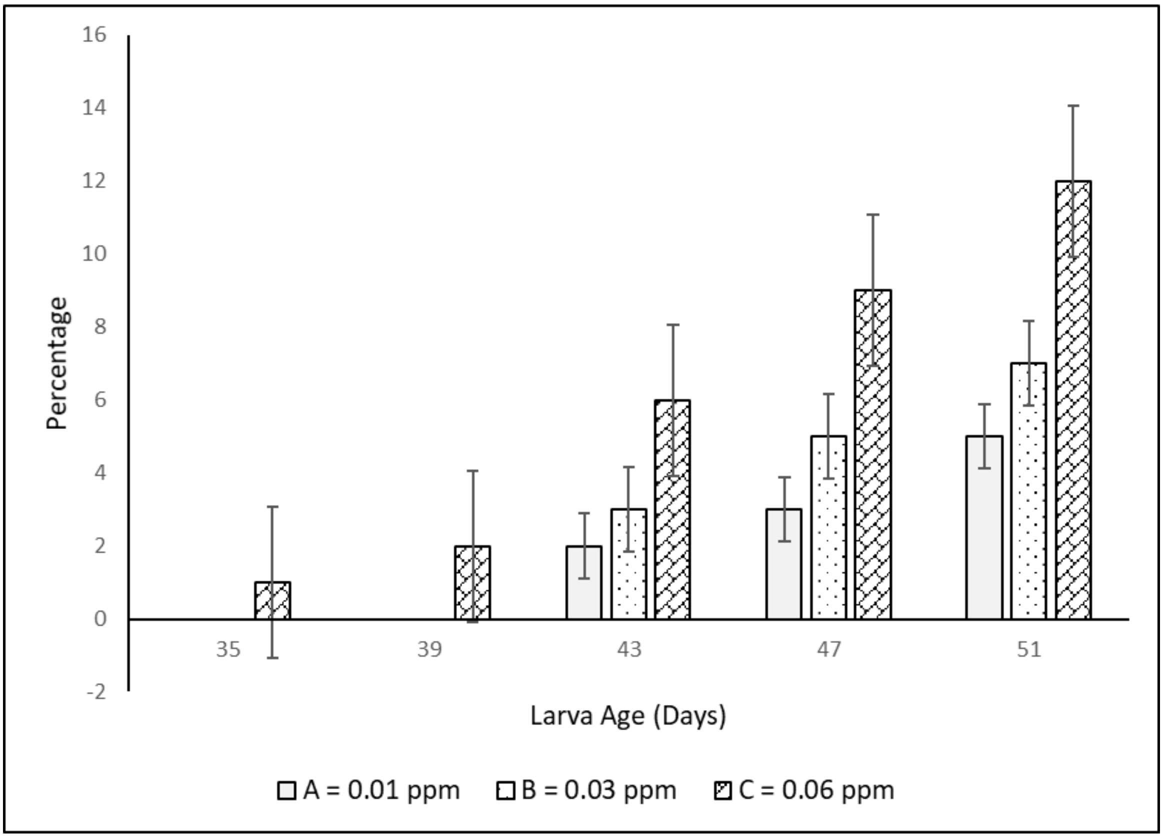

Figure 4. Percentage of comet fish (*Carassius auratus*) showing symptoms of tail bending in mercury treatments.

Morphology, appearance and other gross characteristics can be biomarkers for assessing water quality (Kroon et al 2017). Water pollution can damage the surface barrier mechanism of fish skin and cause changes in organizing cellular components. These changes can affect the normal physiology of the epidermis. The Hg from contaminated water will be accumulated in the body of the fish and damage or stimulate the enzymatic system (Gill et al 1990). Heavy metals will stay in the skin of the fish and fish scales and enter into the body of the fish through the gills, in the bloodstream and will then spread to all organs. Heavy metal in the body of fish will react with proteins and can damage melanin (McGraw 2003), which plays a role in producing the coloration in fish. Hg exposed fish look pale or the color brightness decreases because Hg has damaged the skin, and consequently melanin. Organisms inhabiting aquatic environments may be vulnerable to changes in the environment. Changes in the environment can distort the transmission and interpretation of pigment, based on signals between species (Price et al 2008).

The morphological changes observed in the present work prove that mercury can cause gross malformations. Based on the observations on morphological developments, comet fish larvae showed that the morphological development was not much different in each treatment in the early days of the experiment. When the fish exposure to Hg continued, they start to present differences in morphological development in the treatments at the age of 11 days to 35 and 39 days.

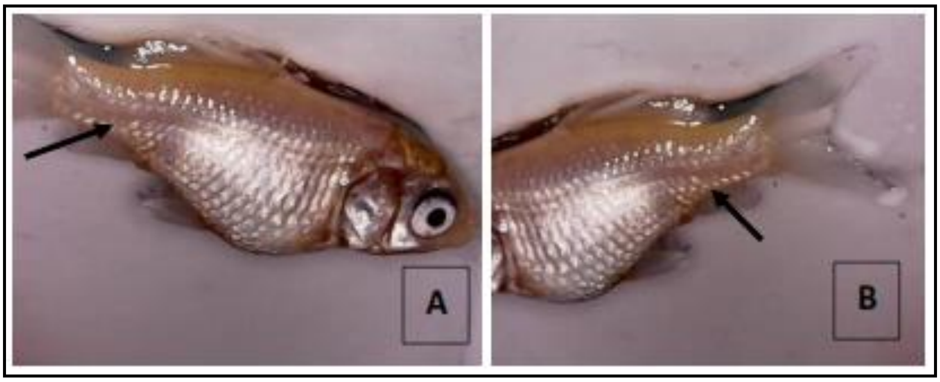

Figure 5. Bending at the tail base of Hg exposed fish at treatment C (0.06 Hg ppm) at age of 35 days (A) and 43 days (B).

The exposure to Hg in both low and high concentrations in aquatic environments resulted in changes in comet fish larva morphology, especially in the development of fins and tails. The highest concentration treatment in this research (0.06 ppm) resulted in the bending of the tail base (Figure 5). This was also observed by Devlin (2006), who observed that Hg can produce abnormal developments in fish. Growth inhibition is one of the most obvious symptoms of the influence of metal toxicity in fish larvae (Heydarnejad et al 2013; Rani et al 2015). These observations are in line with research that found that there was a negative correlation between heavy metal accumulation in fish muscle and their body weight (Łuczyńska & Brucka-Jastrzębska 2005). These toxic elements may interfere metabolically with nutritionally essential metals such as iron, calcium, copper and zinc (Tchounwou et al 2012). This causes the growth of abnormal fin bone tissue. This is consistent with the statement that heavy metals can cause malformations and mortality, which are associated with the metal concentration (Kennedy 2011).

The comet fish larvae reach a definitive form after 35 to 39 days from hatching, when the post larval stage has ended. The caudal fin, pelvic fin, pectoral fin and dorsal fin of the offspring were undamaged. However, in Hg exposed larvae, abnormal development was observed after 35 days, in the form of tail base bending, especially in treatment C (0.06 Hg ppm). Organisms exposed to low concentrations of heavy metals for long periods of time will not result in death, but can have sublethal influences. This can affect reproductive behavior or morphological traits (Kazlauskienė & Stasiūnaitė 1999). It is suspected that heavy metals in polluted waters, even in low concentrations, have the same effect, but the percentage of affected individuals is lower. Changes in the environment and habitat can directly cause abnormalities in the morphology of fish organs (Ebbesson & Braithwaite 2012; Shuai et al 2018).

Conclusions. In summary, the present study shows that exposure to Hg at the tested sublethal concentrations resulted in a decrease in the brightness level of pigmentation in goldfish. Treatments exposing fish to mercury have damaged the pigment in the epidermis of the larvae, even in the low concentrations. Hg exposure also cause slow morphological development and abnormalities in the morphology of fish larvae (mainly at the base of the tail), particularly at a concentration of 0.06 ppm, the highest concentration in this study.

Acknowledgements. The author would like to thank The Ministry of Research, Technology, and Higher Education of The Republic of Indonesia for funding this research through Competitive Based Research (PBK).

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Received: 28 April 2019. Accepted: 16 July 2019. Published online: 9 December 2019.
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How to cite this article:
Nur I., Erni W. A., Idris M., Yusnaini, 2019 Alterations in pigmentation and morphology of goldfish (*Carassius auratus*) exposed to sublethal treatment with mercury. AACL Bioflux 12(6):2147-2156.