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Toxicity Detection of Pollutants in the Tallo River Using Simple Biomarkers of *Oryzias celebensis* Embryo

Khusnul Yaqin [a,*], Sri Wahyuni Rahim [b], Basse Siang Parawansa [b], Hadiratul Kudsiah [b], Rahmadi Tambaru [c], Dwi Kesuma Sari [d], Etty Riani [e], Andi Dina Hardiana [a], Meimulya [a], Yulia Indasari Lalombo [a]

[a] *Postgraduate School of Fisheries, Universitas Hasanuddin, Tamalanrea, Makassar, 90425, Indonesia*
[b] *Study Program of Aquatic Resources Management, Department of Fisheries, Universitas Hasanuddin, Tamalanrea,Makassar, Indonesia*
[c] *Department of Marine Science, Faculty of Marine Science and Fisheries, Universitas Hasanuddin, Tamalanrea, Makassar, Indonesia*
[d] *Department of Veterinary Medicine, Faculty of Medicine, Universitas Hasanuddin, Tamalanrea,Makassar, Indonesia*
[e] *Faculty of Fisheries and Marine Science, Bogor Agricultural University, IPB Darmaga Campus, Bogor 16680, West Java, Indonesia*
Corresponding author: [*]khusnul@unhas.ac.id

***Abstract*— The current research demonstrated the use of simple biomarkers from *Oryzias celebensis* embryos to detect the effects of pollutants from the Tallo River. The biomarkers used in this research were somite number, egg biometrics, heart rate, survival rate, and hatching time. Pollutants were sampled from three hot spots in the Tallo River (Station 1 (S1), Station 2 (S2) and Station 3 (S3)). Two exposure models were used in this study: *in sit*u and *semi-in situ* exposure. *In situ* exposure was carried out by placing ten embryos (stage 17) at each station for six days. *Semi-in situ* embryos (10 animals) were exposed to polluted water from each station and embryo rearing media (ERM) as a control at stage 17 until hatching in the laboratory. Biomarker data were analyzed using ANOVA (Analysis of variance) to determine the sensitivity of each biomarker used. Almost all the embryos used as model animals in the *in situ* experiment died, except for two embryos placed in stations 2 and 3. The embryos from station 2 hatched after 18 days. The embryos from station 3 died after 18 days. The *semi in situ* experiment showed that the number of somites and heart rate showed statistically significant differences between embryos exposed to water from S1 and control embryos ($p < 0.05$). Other endpoints did not show potential as biomarkers. This study suggested that the number of somites and heart rate of *O. celebensis* embryos could potentially be used as biomarkers in effect-based monitoring.**

***Keywords*—Bet hedging; embryo; in situ; *Oryzias celebensis*; river pollutants; simple biomarkers; Tallo river.**

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I. INTRODUCTION

Fish of the genus *Oryzias* have long been used as animal models in toxicological research. These fish have advantages over other animal models that make them ideal for toxicological testing [1]–[7]. *Oryzias* have a relatively small size, which reduces space requirements and can minimise toxicological test waste. The life cycle of *Oryzias* is relatively short [4], [8]–[11] so it can be used to determine the effects of contaminants at all stages of its life cycle in a short period. These fish are also easily adapted to laboratory environments and are sensitive to contaminants [2], [8].

In addition to *Oryzias* adult fish, *Oryzias* embryos can also be used as animal models or sentinel organisms for ecotoxicological studies and their derivatives. The use of fish embryos will facilitate the work of researchers or practitioners in environmental studies, as the use of embryos does not require an ethical clearance. Fish embryos are not protected organisms, because the developmental stages of fish that do not yet have feeding activity are not classified as protected according to the new European Directive 2010/63/EU on the protection of animals used for scientific purposes[12], [13].

The hard and clear chorion makes *Oryzias* embryos easy to observe under a microscope [6], [14], so biomarkers associated with the use of embryos are called non-destructive biomarkers (NDB). The chorion of *Oryzias* is thicker than that of zebrafish [9], [15]. Using *Oryzias* embryos, observable biomarkers extend from the early stages of embryogenesis to hatching. If the adult *Oryzias* is considered a model animal that can reduce space, cost and waste due to its small size, then the *Oryzias* embryo is much smaller, about 1.5 mm [6].

Therefore, in terms of size, embryos are more worthwhile than adult fish. It's just that the hard chorion creates some obstacles in the exposure process [16]. This problem can be overcome by dechlorination.

Research on the use of embryos as animal models in ecotoxicological studies has been widely conducted using *in vivo* approaches [17]–[25]. The embryos of three species, namely *Oryzias latipes, O. melastigma* (the name of this species has been changed to *O. dancena* [26]) and *O. javanicus* showed the same response to organotin contaminants [27]. Exposure of *O. javanicus* embryos to benzo[a]pyrene BaP resulted in fin damage, spinal curvature and cardiac oedema [28]. *In vivo*, exposure of *O. dancena* embryos to the synthetic estrogen 17α-ethinylestradiol (EE2) resulted in growth retardation, including decreased embryonic heart rate, delayed appearance of eye pigmentation, decreased hatching rate and impaired larval movement [17]. Although the Organisation for Economic Co-operation and Development (OECD) has established test guidelines (OECD TGs) using *O. latipes*, which are often used in the ecological risk assessment of environmental chemicals, there have been few studies using *Oryzias* embryos with an *in situ* approach, particularly using embryos of *Oryzias celebensis*, a fish native to Sulawesi, Indonesia.

This paper discusses the results of using *O. celebensis* embryos as animal models in ecotoxicological studies using *in situ* and *semi-in situ* approaches to pollutants in the Tallo River. The Tallo River is one of the rivers in Makassar City. This river is polluted by industrial and domestic waste disposal. Tragically, several tributaries of this river have become dumping grounds. It is hoped that the results of this research can be used to develop the use of *O. celeb*ensis embryos as sentinel organisms to monitor water quality in the Tallo River and other rivers using simple biomarkers.

II. Materials and Method

A. Materials

The materials used in this study were NaCl, KCl, CaCl, $MgSO_4$ and $NaHCO_3$. The chemicals were purchased from Merck, Germany

B. Broodstock Sampling

The broodstocks of *O. celebensis* were collected from the Patunuangase River, Maros Regency. A total of 150 pairs of *O. celebensis* were collected from the Patunuangase River, Maros. The broodstocks were brought to the laboratory and acclimatised and reared until fertilisation occurred. During rearing, the broodstocks were fed with *Artemia* sp nauplii, Fengli 0 and Othohime B. Feeding was performed in the morning, afternoon and evening.

C. River Water Sampling

After the broodstock produced eggs, water was sampled from two tributaries and the Tallo River. The sampling stations are shown in Figure 1. Station 1 (5° 7'17.64"S, 119°28'51.39"E) is a tributary of the Biringromang, station 2 (5° 7'9.90"S, 119°28'1.94"E) is the mainstream of the Tallo River in front of the Lakkang jetty, and station 3 (5° 6'39.44"S, 119°28'5.98"E) is a tributary of the Bontoa. The water was sampled seven times. During four days of sampling at station 1 and station 3, the river water was black. The mainstream of Tallo River water was brown. This indicates that the Tallo River was heavily polluted.

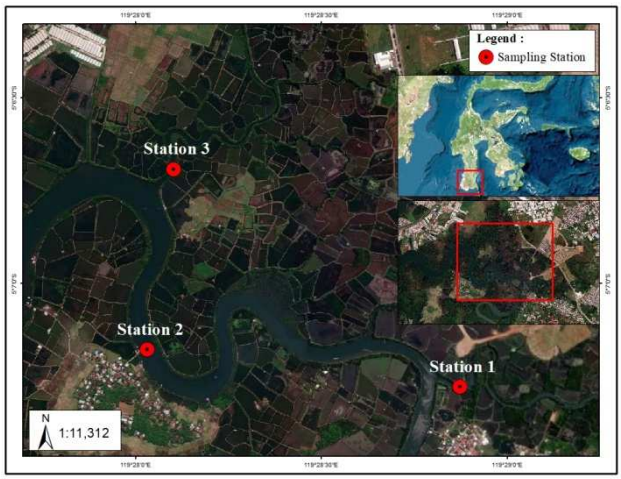

Fig. 1 Sampling stations at the River of Tallo

D. Exposure to Contaminants

Two exposure methods were used in this study, *semi-in situ* and *in situ*. The *semi-in situ* method was carried out by taking water samples at three stations (Figure 1) and as material to contaminate the embryos maintained in a 24-well microplate. As a control, 10 embryos were incubated with ERM (embryo-rearing media). This medium consists of 10.0 g NaCl, 0.3 g KCl, 0.4 g CaCl 2 H_2O, 1.63 g $MgSO_4$ mixed with 1 ml $NaHCO_3$ (0.25 g/20 ml H_2O). It was then dissolved with Aquabidest 1:10.

Ten embryos were used in each microplate representing each station and control. The amount of river water pollutants from each station and the ERM was approximately 2.5 ml. The pollutants and ERM were replaced every day until the embryos hatched, i.e. seven days. Exposure was performed on embryos at stage 17. Embryos were incubated at 26 °C with a 12:12 photoperiod during the exposure period. The embryos were then observed, videotaped and photographed to analyse all the embryogenic parameters used in this study. This is because *O. celebensis* embryos have optically clear chorions.

In situ exposure was carried out by placing a microplate filled with *O.celebensis* embryos in a plastic container on the bottom of the Tallo River at the research station for seven days. Each microplate contained 10 embryos. After seven days, the microplate was harvested and the embryo was observed.

E. Parameters Observed in the Study

Embryo parameters measured were the number of somites, heart rate, egg diameter, yolk diameter, yolk absorption rate, survival rate, the total body length of the newly hatched embryo and hatching time. Embryos with abnormal yolk sac enlargement were classified as having yolk sac oedema. The number of dead embryos was also scored at each replicate.

1) Number of Somites: The number of somites was counted directly from the images taken with a microscope at 40x magnification. The number of somites was counted at stages 19-23.

2) Egg Diameter: Egg diameter was measured horizontally and vertically. The egg diameter was calculated using the following formula [29]:

$$Ds = \sqrt{Dh \times Dv}$$

where Ds is the actual diameter (mm); Dh is the horizontal diameter (mm); Dv is the vertical diameter (mm)

3) Yolk Sac Volume: The yolk sac volume can be calculated using the following equation [30], [31]:

$$V = \frac{\pi}{6} \times LH^2$$

where V is the yolk sac volume (mm^3); L is the length of the yolk sac diameter (mm); H is the height yolk sac diameter (mm).

4) Yolk Sac Absorption Rate: The yolk sac absorption rate was calculated using the formula [32]:

$$YS_{AR} = \frac{Vo-Vt}{Tt-To}$$

where YS_{AR} is the yolk sac absorption rate (mm^3/hour); Vo is the initial yolk sac volume (mm^3); Vt is the final yolk sac volume (mm^3); To is the initial time (hours), Tt is the final time.

5) Heart Rate: The heart rate was measured by recording the time taken to reach 30 beats. The time to reach 30 beats for each embryo was converted to the number of heartbeats per minute using the appropriate formula [33], [34]:

$$N = 30/T \times 60$$

where N is the number of embryonic heartbeats per minute (beat/minute); T: the time to reach 30 beats

6) The Total Body Length of the Newly Hatched Larvae: The larvae were observed using a binocular microscope with 20x magnification. Larval images were then documented using the Optilab application. All newly hatched larvae were measured for the total body length using Image Raster 3.0.

7) Survival Rate: Embryo survival rate was calculated using the formula [35], [36] as follows:

$$SR = \frac{Nt}{No} x100\%$$

where SR is Survival rate; Nt is the number of live embryos at the end of the study; No is the number of embryos at the start of the study

8) Hatching Time: Observation of hatching time was conducted by checking whether the chorionic membrane of the eggs had broken and the larvae were actively moving. Hatched eggs were then recorded daily from the first day of hatching until all embryos in each rearing medium had hatched. Only embryos that were able to fully emerge from the chorion were considered hatched, the others were considered unhatched [37].

F. Data Analysis

Non-parametric Kruskal-Wallis analysis was used to determine differences in each of the parameters studied, and post-hoc Dunn's test was used to determine differences between stations. Data that could not be analysed statistically inferentially were analysed statistically descriptively.

III. Results and Discussion

The *semi-in situ* approach showed that 97,5% of the embryos hatched successfully. Meanwhile, the *in situ* approach showed that almost all embryos died, except for two embryos that were placed at stations 2 and 3. The live embryos were found at stage 33, when normally developing embryos should have reached stage 37. The embryos at station 2 hatched after 18 days of fertilisation. The embryos at station 3 did not hatch and died on day 18. The results of observing the parameters used as biomarkers or endpoints in studies of *semi-in situ* exposed embryos are explained in the following section.

A. Somite

Somites are temporary structures that appear only briefly during early development. They then differentiate into vital structures, including the axial skeleton, the dorsal dermis and all the striated muscles of the adult body, under the influence of various growth factors secreted by the surrounding tissues. The segmental organisation of the somites along the posterior-anterior axis of the embryo is then reflected in the segmental properties of the spinal cord and dorsal root ganglia [38], [39]. Somites are very important in the development of the *Oryzias* embryo as they provide the basis for the formation of skeletal muscles, vertebrae and some skin tissues. Their sequential formation and differentiation are tightly regulated by genetic mechanisms that ensure proper embryonic development [40].

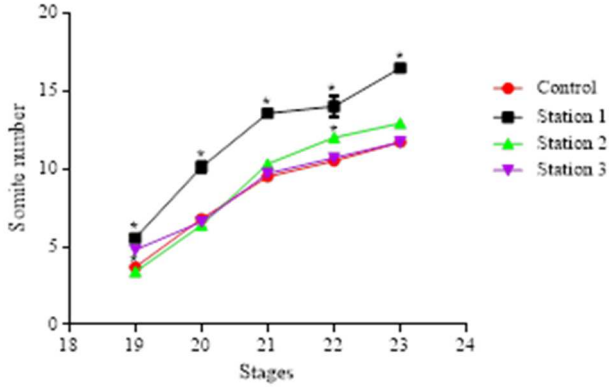

Fig. 2 The number of *O. celebensis* embryo somites incubated in the control treatment, water from stations 1, 2 and 3. The asterisks indicate a statistically significant difference from the control treatment ($p<0.05$).

After the embryo of *O. celebensis* was exposed to water from the Tallo River, the embryo underwent a stimulation of somite development. The most significant stimulation occurred in the water treatment from station 1. From phase 19 to phase 23, somite development was faster in embryos exposed to water from station 1 than in the control. Exposure to water from station 2 showed that there was more growth than the control at stage 22 only. Meanwhile, exposure to water from station 3 showed a more significant number of somites at stage 19 (Figure 2).

Oryzias embryos have long been used as model organisms in ecotoxicology research with various endpoints or biomarkers. In this study, biomarkers that are easy to observe

and measure in *O. celebensis* embryos were used. One of the biomarkers used was the number of somites in the early stages of *O. celebensis* embryogenesis.

In vertebrates, including *Oryzias*, the somites play an important role in the development of the musculoskeletal system. The somites consist of the dermomyotome, myotome and sclerotome, which form the basis of the axial organisation of the musculoskeletal system. The sclerotome gives rise to the vertebrae and ribs, while the dorsal tendon originates from the syndetome. The skeletal muscles of the trunk are derived from the dermyotome and myotome [43]. Somitogenesis is essential for the development and controls the differentiation of the peripheral spinal cord and skeletal muscle [44]. Zebrafish, *Danio rerio*, exposed to river polluted sediment showed a reduction in the number of somites [41]. Other research showed that *D. rerio* that exposed to ionizing radiation from radionuclides was experienced somite apoptosis [42]. Pb has been reported to cause twisted somite in zebrafish embryos [43].

The malformation of the somites leads to changes in axial curvature [44]. A damaged axial curvature can lead to a damaged spine [45]. The damage to the spine can be predicted to affect the swimming ability of adult fish, which will eventually have the ecological consequence that the fish may become extinct because the ability to avoid predators and the ability to predate will also be reduced. Both of these things can reduce their survival in the wild. The damage to the fish's ability to move becomes worse if the somite development disorder is projected onto the fish's muscle formation. Furthermore, if we consider that somites differentiate into other organs such as kidneys and blood vessels, embryos that experience somite development disorders will have consequences for the fish's ability to excrete waste and disrupt growth as a whole. This will reduce the Darwinian fitness of an aquatic biota, with ecological consequences. It was suggested that failure of somite formation was a core biomarker associated with mortality [46]–[55].

The current research showed that contaminants in the Tallo River water could cause an increase in the number of somites. Zhao et al. [56] reported that both diazepam and 2-methylamino-5-chlorobenzophenone (MACB) increased the number of somites in zebrafish embryos. In the view of Zhao et al. [56], the triggering of somite development showed that the two nerve inhibitors trigger the development of fish embryos early in their development. This suggested that most of the pollutants in the Tallo River water, particularly at S1, were neuroinhibitors.

Understanding the consequences of abnormal somite development in fish embryos is not only essential for developmental biology but also for fisheries management and conservation efforts through the use of somite development as a biomarker. By using somite development in *O. celebensis* embryos as a biomarker, we obtain a non-destructive biomarker as the sentinel organism does not need to be killed.

B. Egg Diameter

The effects of pollutants on the egg diameter of *Oryzias* embryos may vary depending on the specific pollutant and its concentration, as well as the duration of exposure. Such pollutants can cause swelling in *O. melastigma* embryonic eggs [57], [58]. The current study illustrated that swelling occurred in *O. celebensis* eggs exposed to water from stations 1 and 3 compared to the control statistically ($p<0.05$). The egg diameters of station 2 were not significantly different from those of the control.

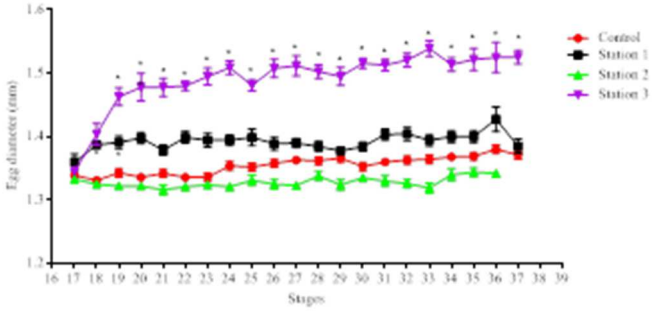

Fig. 3 Diameter of *O. celebensis* eggs exposed to water from stations 1, 2, and 3 compared to the control. The asterisks indicate a statistically significant difference from the control treatment ($p<0.05$).

Certain pollutants, such as metals e.g. mercury [59], Cu, Cd, Pb [60] and organochlorine compounds [61], may interfere with normal egg development. Exposure to metal substances results in reduced egg size diameter [59], [60] due to impaired cell division or impaired yolk formation. On the other hand, some contaminants, such as endocrine disruption chemicals [61] can disrupt the normal hormonal balance in fish embryos. This can lead to an abnormal build-up of lipids or other substances in the egg, which may increase the size of the egg [57]. The diameter of fish eggs may increase due to hydration caused by high molecular weight substances in the perivitelline fluid [62].

C. The volume of Yolk Sac

The yolk sac volume of *O. celebensis* embryos measured from stage 17 to hatching showed that there was a decrease in yolk sac volume from stage 17 to 37. After stages 31 to 35, the volume of the yolk sac decreases sharply also observed by Iwamatsu et al. [63] in *O. latipes*. This means that the yolk, as an energy source for the embryo during its development, was used by the embryo in the growth processes. From stations 1 and 3, we observed that the yolk sac volume was statistically larger than the control ($p < 0.05$). In other words, there was yolk sac oedema at stations 1 and 3, whereas there was yolk sac shrinkage at station 2 descriptively.

This did not happen at all stages. However, at stages 36 and 37, oedema was still present in embryos incubated with water from stations 1 and 3. The yolk sac volume data were used to calculate the yolk absorption rate. In the embryo of *O. latipes*, the volume of the yolk sac decreased sharply between 2 and 8 days after fertilisation [63].

When the yolk sac volume of an *O. celebensis* embryo was measured from stage 17 to hatching, it was found that the yolk sac volume decreased from stage 17 to stage 37. Iwamatsu et al. [63] also reported that the volume of the yolk sac drastically declines from stages 31 to 35 in *O. latipes*.

Mu et al. [64] found that *O. melastigma* embryos exposed to phenanthrene developed oedema, while those exposed to retene suffered heart tissue damage. In contrast, *O. latipes* embryos injected with tributyltin (TBT) and polychlorinated biphenyls (PCBs) *in* ovo experienced yolk sac volume shrinkage [65]. These current results suggested that changes in yolk sac volume had the potential to be used as a biomarker,

particularly in the final stages of embryogenesis such as stages 36 and 37 (Figure 4). However, further studies are needed to investigate changes in yolk sac volume as a biomarker of the contaminants.

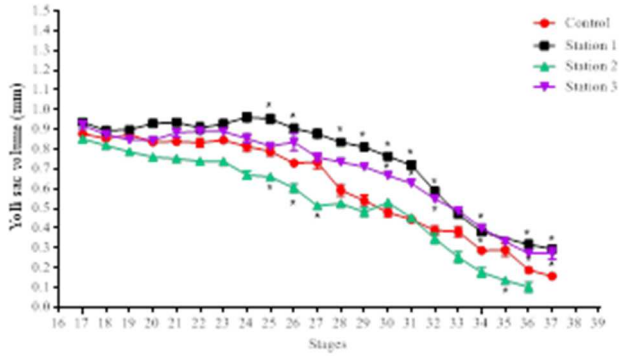

Fig. 4 Yolk sac volume of *O. celebensis* embryos exposed to water from the Tello River. The asterisks indicate a statistically significant difference from the control treatment ($p<0.05$).

D. Yolk Sac Absorption in Oryzias celebensis Embryo

In vertebrates, yolk sac absorption is an essential developmental process that occurs during the early stages of embryonic development [67]. The yolk sac is a membranous structure that contains nutrients, including proteins, lipids and carbohydrates, that are necessary for the nutrition of the developing embryo [68]. As the embryo develops, it goes through a series of developmental stages during which it relies on the yolk sac for nutrition [68], [69]. In *Oryzias* embryos, the yolk sac begins to be absorbed around 24 hours after fertilisation [66]. The yolk sac is still relatively large and occupies a significant proportion of the embryo's body cavity. Over time, the yolk sac becomes smaller as it is absorbed by the fish embryo.

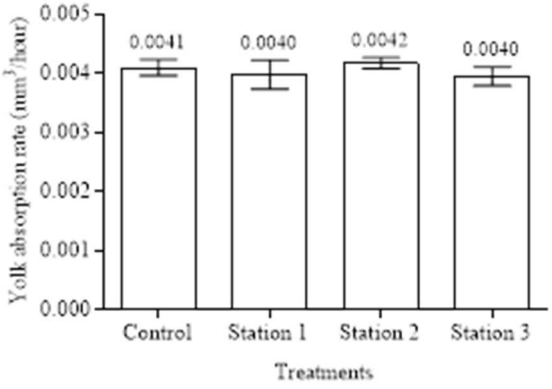

Fig. 5 Yolk sac absorption of *O. celebensis* embryos exposed to Tallo River water.

The nutrients in the yolk sac are metabolised, absorbed and used by the embryo or other extra-embryonic tissues [70]. The yolk sac helps the embryo to grow and develop at different stages of embryogenesis. Therefore, understanding the activity of yolk sac absorption in *O. celebensis* embryos concerning exposure to pollutants may help efforts to use the potential yolk sac absorption rate as a simple biomarker.

Statistical tests of the yolk sac absorption rate depicted that there was no significant difference between all treatments and controls (Figure 5). The absorption rate of *O. celebensis* embryos ranged from 0.004-0.0042 mm^3/hour.

These results indicated that the embryos at all stations used the yolk sac as energy for embryonic development properly. The yolk nutrients were metabolised and absorbed by the yolk sac membrane and then used by the embryo or other extraembryonic tissues. The pollutants present at all stations did not affect the use of energy stored in the yolk sac, which is an important activity in the process of embryo development until hatching. However, Mu et al. [64] found that the yolk sac of *O. melastigma* embryos experienced swelling after exposure to phenanthrene. If the findings of Mu et al., [64] are viewed from the perspective of yolk sac absorption, then exposure to phenanthrene may cause the embryo to lack the ability to absorb the yolk sac as an energy source.

E. Heart Rate

The heart rate of an *Oryzias* embryo is indeed frequently employed in ecotoxicology as a biomarker to evaluate the potentially harmful impacts of environmental pollutants or chemicals [8], [21], [58], [71]–[74]. This approach is utilized to assess the toxicity of various substances on aquatic organisms, particularly *Oryzias* species like *O. melastigma* [8], [34]. Changes in the heart rate of *Oryzias* embryos serve as a valuable indicator of embryonic toxicity and provide insight into the adverse effects of contaminants in *O. celebensis*, which is still rare, especially for *in situ* studies.

In this study, there were signs of an increase in the heart rate of embryos exposed to river water from Station 2, but by the end of the stage, stages 35 (189.10 ±32.62 bpm) and 36 (211.00 ± 20.43 bpm), the heart rate of *O. celebensis* embryos returned to normal (stage 35: 198.85 ± 19.30 bpm; stage 36: 190.5 ± 22.92 bpm).

Embryos exposed to water from the Tallo River at station 3 experienced the same thing (Figure 6). In the early stages of embryogenesis, the heart rate of the embryos increases, but in the last stages, 35 (184.40 ± 11.84 bpm), 36 (207.50 ± 12.08 bpm), 37 (191.00 ± 44.41 bpm), the heart rate returns to normal (Figure 6). In contrast, embryos exposed to Tallo River water at station 1 showed a decrease in heart rate at stages 30 (116.89 ± 11.52 bpm), 31 (126.22 ± 7.33 bpm), 32 (125.22 ± 6.02 bpm), 35 (165.02 ±13.86 bpm), 36 (142,88 ± 8,17 bpm), and 37 (142,88 8,17 bpm) (Figure 6). However, at stages 33 (145.44 ± 12.36 bpm) and 34 (68.00 ± 12.61 bpm), the heart rate returns to the control or normal. Stages 35, 36 and 37 are important signs of irreversible heart rate abnormalities. In the last three stages, the decrease in heart rate did not return to normal until hatching.

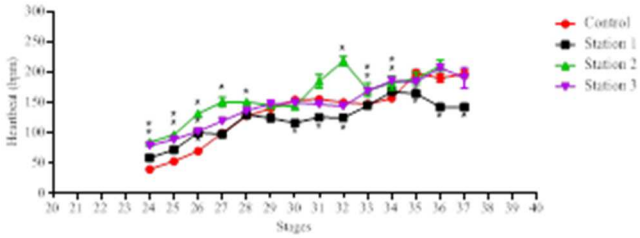

Fig. 6 Heart rate (bpm = beats per minute) of *O. celebensis* embryos exposed to water from the Tallo River. The asterisks indicate a statistically significant difference from the control treatment ($p<0.05$).

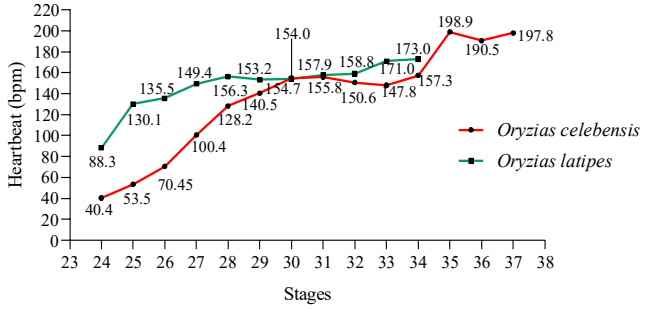

Fig. 7 Heart rate comparison (bpm = beat per minute) of embryo *O. celebensis* and *O. latipes*. The data on the heart rate of *O. latipes* are modified from Gierten et al., [75].

Compared to the heart rate of *O. latipes* [75], the heart rate of *O. celebensis* is slower than that of *O. latipes*. However, after stage 29, both heart rates are almost the same until the hatching stage (Figure 7).

The current study revealed that *O. celebensis* embryos exposed to water from stations considered polluted in several areas of the Tallo River showed the presence of tachycardia or bradycardia in *O. celebensis* embryos. Embryos exposed to water from S2 and S3 exhibited tachycardia in early and mid-stages and then the heart rate became normal in the late stages (stages 35-37). However, embryos exposed to water from S1 experienced bradycardia in the mid to late stages (stages 35-37). These results illustrated that the heart rate in the last stages needs to be taken into account when heart rate is used as a biomarker to detect pollutants. Liu et al., [57] found that *O. melastigma* embryos exposed to $MnCl_2$ experienced an increase in heart rate or tachycardia. Liu et al., [8] also observed tachycardia of the *O. melastigma* embryos exposed to Ni. Chen et al. [33], in addition to finding the phenomenon of tachycardia in *O. melastigma* embryos, also found bradycardia in *O. melastigma* embryos. Tachycardia occurred when embryos were exposed to microplastics at a concentration of 1 x 10^3 particles/ml microplastics and bradycardia occurred when embryos were exposed to 1 x 10^6 particles/ml microplastics [33]. From the research by Chen et al., [33], it can be concluded that when *O. melastigma* embryos were exposed to relatively low concentrations of microplastics, the embryos experienced tachycardia, but when the embryos were exposed to relatively high concentrations of microplastics, bradycardia occurred. Eventually, if the heart rate is to be used as a biomarker, two response models must be taken into account, tachycardia and bradycardia, and the heart rate in the last stage, stage 35-37, is the most appropriate heart rate state in using the heart rate as a biomarker. This makes sense, of course, because late-stage heart rate damage is an accumulation of early and middle-stage damage.

F. The Total Body Length of The Newly Hatched Embryo

From the results of statistical analysis, embryos exposed to Tallo River water at stations 1 and 2 had shorter total newly hatched body lengths compared to those of controls (5.53 ± 0.21). The mean total body length of newly hatched embryos at stations 1 and 2 was 5.22 ± 0.23 and 5.16 ± 0.13 mm, respectively. However, in embryos exposed to Tallo River water at station 3, the total body lengths of newly hatched (5.44 ± 0.41) were not significantly different from those of the control (Figure 8).

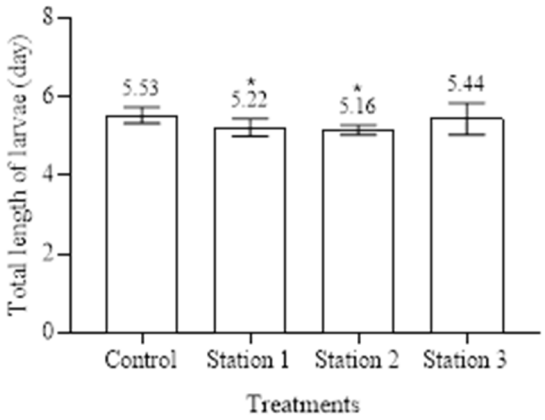

Fig. 8 Total body length of the newly hatched embryos of *O. celebensis* exposed to Tallo River water. The asterisks indicate a statistically significant difference from the control treatment ($p<0.05$).

In fish, the total body length of the newly hatched larvae is theoretically a projection of the metabolic rate (yolk sac absorption) and heart rate [27]. The higher the metabolic rate and heart rate in the embryo, the longer the total body length of the newly hatched larvae will be. Wang et al., [76] found that in *O. melastigma* embryos exposed to Cu, the total body length of the newly hatched larvae was longer, the yolk sac volume was smaller and the heart rate was faster compared to the control embryos. What was found by Wang et al., [76] was consistent with the above theory that the total body length of the newly hatched larvae is the result of yolk sac absorption, which is consistent with a higher heart rate. However, the results of the current study did not seem to be consistent with the above theory; the low heart rate in embryos exposed to Tallo River water did not have implications for the low yolk sac absorption but did have implications for shortening the total body length of the newly hatched larvae. The difference between the results of the current study and those of Wang et al., [76] was due to the contaminants used in the research. Wang et al., [76] used a single pollutant, Cu, while the current study used Tallo River water, which naturally contains a variety of pollutants that were discharged into the Tallo River. The variety of pollutants in the Tallo River caused different effects on the embryos. Embryos contaminated by Tallo River water could not fully use the energy absorbed from the yolk sac for growth but were used to repair other damage caused by the mixture of pollutants from Tallo River water. The energy required to cope with stress from pollutants may divert some energy away from growth [77]. This resulted in a non-linear consistency between heart rate, yolk sac absorption and total body length of newly hatched larvae.

G. Survival Rate

The survival rate of *O. celebensis* embryos exposed to Tallo River water at station 1 was 90%. Furthermore, the survival rate of *O. celebensis* embryos incubated with control media (ERM) and Tallo River water at stations 2 and 3 was 100% (Figure 9). However, the hatching process did not occur simultaneously.

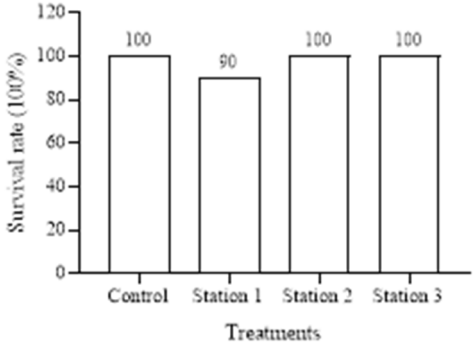

Fig. 9 Survival rate of *Oryzias celebensis* embryos exposed to Tallo River water.

After the embryo has successfully passed through the stages of embryogenesis, it hatches. In the research, the data between hatching ability and survival rate became one data because during the experiment no embryos were found that hatched and died simultaneously. Embryonic death was indicated by the absence of a heartbeat. The results showed that embryonic death occurred in embryos from S1 (10%). This reinforces the somite and heart rate data indicating the effect of contaminants on embryos exposed to Tallo River water from S1.

H. Hatching Time

Hatching time indicates the time required for *O. celebensis* embryos to hatch. Statistical tests showed that there was a significant difference between the control and Tallo River water-exposed embryos ($p<0.05$). The difference occurred between embryos incubated in Tallo River water station 3 and those incubated in control media.

Embryos incubated in Tallo River water at station 3 hatched faster (8.40 ± 1.35 days) than those incubated in control media (11.30 ± 4.11 days). However, one embryo in the control treatment showed a bet-hedging phenomenon [78]–[80], i.e. the embryo slowed down the hatching time by hatching on day 23. Bet-hedging is a life history strategy that allows populations to survive under unpredictably variable conditions [78]–[80].

In ecotoxicological studies, the phenomenon of bet-hedging needs to be carefully considered and should be taken into account. In contrast, exposure of the *Oryzias* embryos to only TBT increased the hatching times of embryos, whereas exposure to only PCBs decreased the hatching times[65].

The parameter of hatching time was used as an endpoint or biomarker by several researchers on *Oryzias* embryos [57], [58], [81], [82]. The current study showed that *O celebensis* embryos exposed to Tallo River water, namely from S3, had an accelerated hatching time compared to controls. Iwamatsu [66] found that the normal hatching time for *O. latipes* at a temperature of 26 ° C was 10 days after fertilisation. The current study showed that the control hatching time was 11.3 days, but there was one embryo in the control that experienced bed-hedging with a hatching time of 23 days. If this bet-hedging data is considered an outlier and eliminated, then the average hatch time in the control treatment was 10 days. It can therefore be concluded that the control hatching time treatment was consistent with the normal hatching time of *O. latipes* [66].

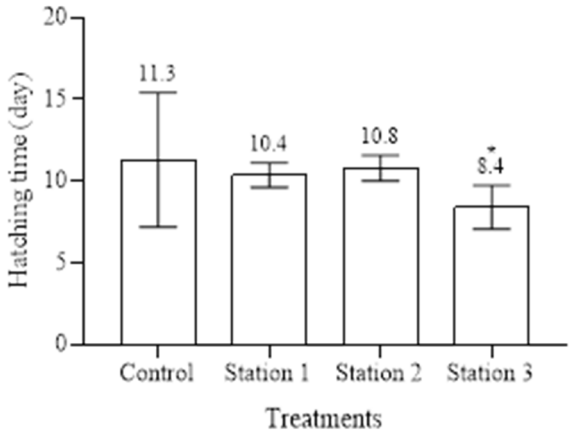

Fig. 10 Hatching time of *O. celebensis* embryos exposed to Tallo River water. Hatching time of *O. celebensis* embryos exposed to Tallo River water. The asterisks indicate a statistically significant difference from the control treatment ($p<0.05$).

In the context of the phenomenon of bet-hedging on the hatching time of *Oryzias* embryos, the use of hatching time as a biomarker needs serious and careful consideration. Bet-hedging could be a typical adaptive strategy used in response to rapid environmental changes [83-90]. Typically, in a group of embryos produced by broodstock, a small proportion are prepared as bet-hedging embryos [83]. However, it is not yet clear what the characteristics of these bet-hedging embryos are. It is therefore necessary to consider bet-hedging data as outlier data so that they do not interfere with statistical analysis.

IV. Conclusion

The current research suggested that the number of somites and heart rate of *Oryzias celebensis* embryos have the potential to be used to detect contaminants in polluted river water, such as the Tallo River. Other endpoints such as egg biometrics, survival rate and hatching time using a statistical approach did not show the ability to detect contaminants from the Tallo River. However, more in-depth research needs to be carried out to determine the mechanisms underlying the changes in somite number and heart rate, particularly using a genetic approach. In this way, as research progresses, simple biomarkers, somite number and heart rate, can be used as ecotoxicological test tools both in the laboratory and in the field studies.

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