International Journal on Advanced Science Engineering Information Technology

Vol.12 (2022) No. 5
ISSN: 2088-5334

Survival Rates of *Oryzias Celebensis* Embryo Reared in Different Media in an Attempt to Provide Embryos for Ecotoxicological Studies

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***Abstract*— Fish embryos from the genus *Oryzias* have long been used as animal models in ecotoxicological research. Research on the survival rate of *Oryzias celebensis* embryos in different rearing media has been carried out. In this study, the media used were embryo rearing medium (ERM), bottled water (BW), Pattunuang river water (PRW), and well water (WW). The data obtained is analyzed with a one-way ANOVA statistical test and descriptive analysis in the form of tables and figures. The results of this study indicated that the embryogenesis processes of *O. celebensis* in all media were faster than the development of *O. latipes,* which was used as a reference for embryogenesis observations. The yolk volume in each medium decreased in size along with the development of the embryogenesis phases. The results of the one-way ANOVA statistical test showed that the rearing medium was significantly different ($P<0.05$) concerning the hatching time parameter and not significantly different ($P>0.05$) on the parameters of survival rate of the embryo (SRe) and total larval length. The water quality of the rearing medium was still in a condition that the O. celebensis embryos could tolerate except for the concentration of $CaCO_3$, which could affect the hatching time. This study concludes that embryo rearing medium (ERM) solution is the best medium for rearing *O. celebensis* embryos for ecotoxicological studies. The hatching time parameter of the *O. celebensis* embryo has the potential to be used as a biomarker in ecotoxicological studies.**

***Keywords*— Embryogenesis; embryo survival rate; embryo rearing medium; hatching time; *Oryzias celebensis*; ecotoxicology.**

Manuscript received 17 Sep. 2021; revised 15 Dec. 2021; accepted 15 Feb. 2022. Date of publication 31 Oct. 2022.

I. INTRODUCTION

Oryzias celebensis is a paddy or medaka fish species in South Sulawesi [1], [2]. For a long time, paddy fish have been used as the best test animals for various fields, one of which is ecotoxicology [3]–[8]. This is because paddy fish have a fast growth rate, short lifespan, and life cycle, are easy to identify and cultivate and have a wide geographic distribution [9]–[12]. Not only in fish but embryos from paddy fish can also be used as ecotoxicological test biota [13]–[22]. One of the requirements as a test biota is that this paddy fish embryo has a sensitivity to various important contaminants such as xenobiotics[18], [23]–[25]. In addition to having sensitivity to various contaminants, this paddy fish also has a transparent embryo and chorion, making it easier to observe under a microscope [23], [26], [27].

Oryzias latipes, the paddy fish, distributed in Japan, Korea, and China, have long been used as model organisms in ecotoxicological studies [28], [29]. Currently, *O. melastigma* living in seawater is getting attention for use as a model organism in ecotoxicological research [30]–[34]. *Oryzias celebensis* is a paddy fish that can be easily found in the fresh and brackish waters of South Sulawesi. Unfortunately, this species has not been popularly used as a test animal in ecotoxicological research [8]. Likewise, *O. celebensis* embryos have not been used as test animals in ecotoxicological research. Therefore, it is very important to study *O celebensis* embryos as the basis for using them as test animals in the field of ecotoxicology.

Observations on fish embryos certainly require proper maintenance, including placement in the right media. Embryo rearing medium (ERM) solution is a medium that is generally used in laboratory-based embryo rearing. This solution has the function of assisting in protection from bacteria that can damage eggs [12]. Although the ERM solution is good to use, it requires several chemical compounds which are expensive to make. So, it is necessary to find alternative media to be used

in the maintenance of fish embryos *of O. celebensis*. Therefore, it is necessary to conduct a study to compare ERM and other incubation media that are easy to obtain and cheap, such as bottled water, river water where *O celebensis* lives, and well water that is available in the laboratory.

II. Materials and Method

A. Chemical

The chemicals that were used to make ERM were 10.0 g NaCl, 0.3 g KCl, 0.4 g CaCl 2 H_2O, 1.63 g $MgSO_4$, 1 ml of $NaHCO_3$ (0.25 g/20 ml H_2O). The chemicals were purchased from Merck, Germany.

B. Eggs production

Several pairs of *O. celebensis* brood stocks were placed in an aquarium with 20 liters of water. Fish are incubated until they produce eggs. The broodstocks were fed *Artemia* sp., nauplii, and Feng Li commercial feed during incubation.

C. The experiment

The study was conducted using a completely randomized design (CRD) with four treatments (maintenance media) and five replications. In this study, the media used were embryo rearing medium (ERM) as media A, bottled water (BW) as media B, Pattunuang river water (PRW) as media C, and well water (WW) as media D. Each experimental unit consisted of one egg. The eggs used in this study were fertilized eggs which were characterized by the formation of the perivitelline space. The eggs that have been selected were then put into a microplate containing 2 ml of rearing media according to the microplate wells that have been determined. The total number of eggs used was 20 eggs, and eggs originated from the same female broodstock.

D. Research variable

Six research variables were observed and analyzed in the study. The variables were embryogenesis, the survival rate of an embryo (SRe), yolk volume, hatching time, larvae's total body length, and incubation media's water quality.

1) Embryogenesis: embryogenesis development was observed under a trinocular microscope at 40x magnification. The development of the *O. celebensis* embryo was identified concerning the study of González-Doncel *et al.* [35], that observed the embryogenesis of *O. latipes.*

2) Survival Rate of Embryo (SRe): The survival rate of the embryo can be calculated by dividing the number of live embryos by the number of embryos fertilized before hatching using the following equation (1)[36]

$$\text{SRe} = \frac{\text{Number of live embryos}}{\text{Total of live embryos}} \text{ x } 100 \qquad (1)$$

3) Yolk volume: The yolk volume was calculated by the following equation (2)[37], [38].

$$\text{YV} = \frac{\pi}{6} \text{ x } (l^2 \text{x h}) \qquad (2)$$

YV= Yolk volume (mm^3), l = yolk length (mm), h= tolk high (mm), π= 3.1416

4) Hatching Time: Observation of hatching time was performed by observing if the chorionic membrane of the egg had ruptured and the larvae were actively moving. Only embryos capable of fully expelling the chorion are considered hatch [15]. *Oryzias celebensis* larvae were observed under a binocular stereo microscope with 20x magnification.

5) The total body length of larva: The total body length was measured from the lower jaw's tip to the caudal fin's tip [15]. All newly hatched larvae were measured for total body length using the Image Raster 3.0 application.

6) Water Quality of incubation media: Water quality parameters measured were pH, temperature, NaCl (Sodium Chloride), NO_3 (Nitrate), $CaCO_3$, and SO_4 (Sulfate).

E. Data Analysis

Data analysis was carried out descriptively and statistically. Descriptive analysis was carried out on embryogenesis parameters, egg yolk volume, and water quality of incubation media. Furthermore, statistical analysis was carried out using the one-way ANOVA test to compare embryo survival, hatching time, and total body length on different incubation media. Before the ANOVA test was performed, using the GraphPad Prism 5 software, the data were first tested for normality and homogeneity.

III. Results and Discussion

A. Embryogenesis

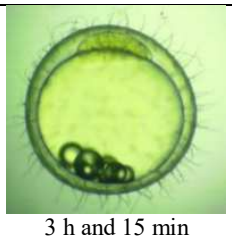
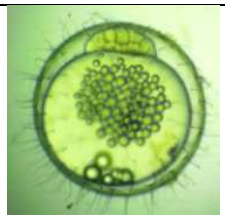
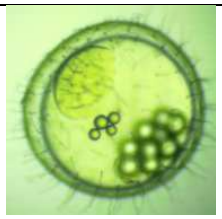
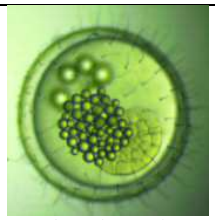
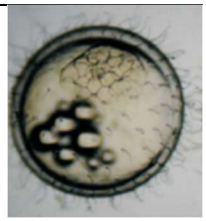
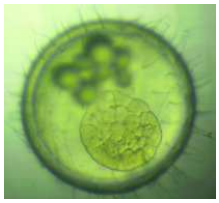
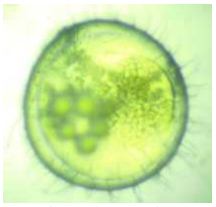
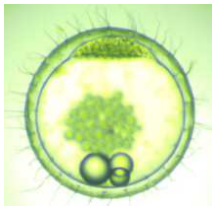
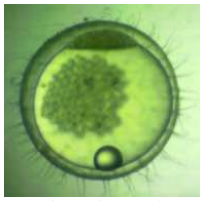
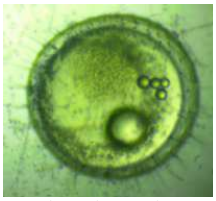
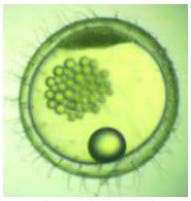
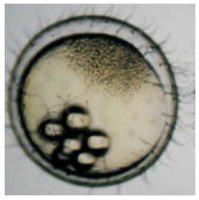
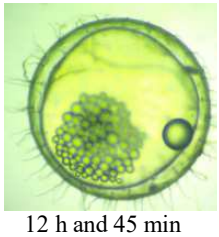
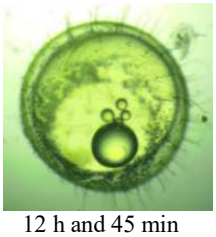
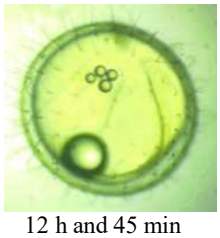
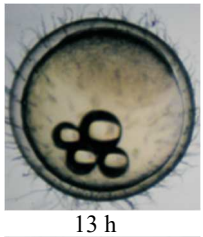
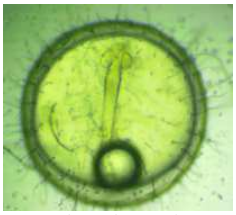
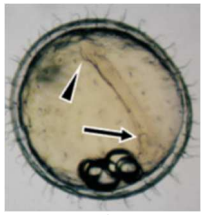
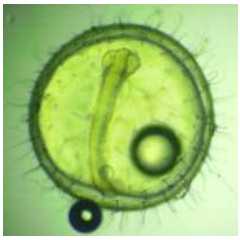
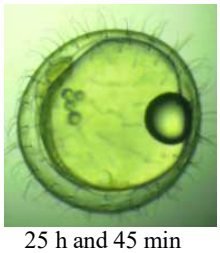
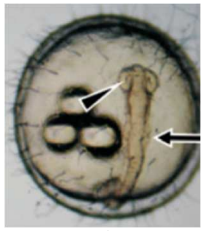
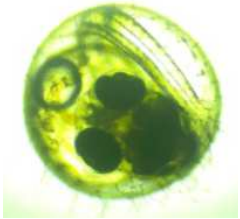
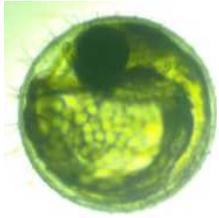
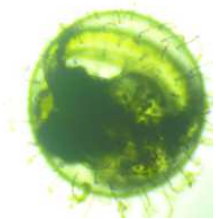
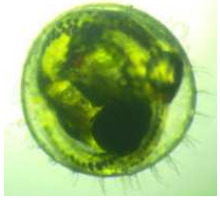
The developmental stages of *O. celebensis* embryogenesis can be summarized into six phases: cell division (cleavage), morula, blastula, gastrula, neurula, organogenesis, and hatching. The development of *O. celebensis* embryos on different media can be seen in Table I. From the observations, it is known that the development of *O. celebensis* embryos in each medium is not much different from the embryogenesis material of *O. latipes* that is used as a reference González-Doncel *et al.* [35].

In terms of time, there were differences in the developmental period between *O. celebensis* and *O. latipes* embryos. *Oryzias celebensis* embryo development was faster than *O. latipes* where the final organogenesis phase in *O. celebensis* ended on day 8 (164 h and 45 min days post-fertilization/dpf) while *O. latipes* ended on day 9 (200 dpf). Klüver [39] stated that hatching of *O. celebensis* embryos occurred on the eighth day after fertilization.

B. The Survival Rate of the Embryo (SRe)

Survival can be defined as the chance of survival of an individual or organism within a certain time [24]. The results of the statistical analysis of the embryo survival rate (SRe) can be seen in (Fig. 1) showed that the SRe was not significantly different ($P>0.05$) against the rearing media. In embryo rearing medium (A), bottled water (B), and Pattunuang river water (C), the highest SRe level was 100%, while well water (D) had the lowest SRe level at 60%. This was because, in media Pattunuang river water (C), two of the embryos died due to the presence of microorganisms and fungi on the chorion surface.

TABLE I
ORYZIAS CELEBENSIS DEVELOPMENT IN THE DIFFERENT INCUBATION MEDIUMS

Stage	A (ERM)	B (BW)	C (PRW)	D (WW)	[12].
Cleavage	 3 h and 15 min	 3 h and 15 min	 3 h and 15 min	 3 h and 15 min	 3 h 30 m
Morula	 3 h and 45 min	 3 h and 45 min	 3 h and 45 min	 3 h and 45 min	 4 h
Blastula	 6 h and 15 min	 6 h and 15 min	 6 h and 15 min	 6 h and 15 min	 6 h 30 m
Gastrula	 12 h and 45 min	 12 h and 45 min	 12 h and 45 min	 12 h and 45 min	 13 h
Neurula	 23 h and 45 min	 23 h and 45 min	 23 h and 45 min	 23 h and 45 min	 24 h
Organogenesis*	 25 h and 45 min	 25 h and 45 min	 25 h and 45 min	 25 h and 45 min	 29 h
Organogenesis**	 164 h and 45 min	 164 h and 45 min	 164 h and 45 min	 164 h and 45 min	 174 h

NOTE: *Early; ** Late

According to Elameen *et al.* [40], if fungi and bacteria attack the embryo development process, the embryo's ability to hatch will be reduced and even cause the death of the embryo.

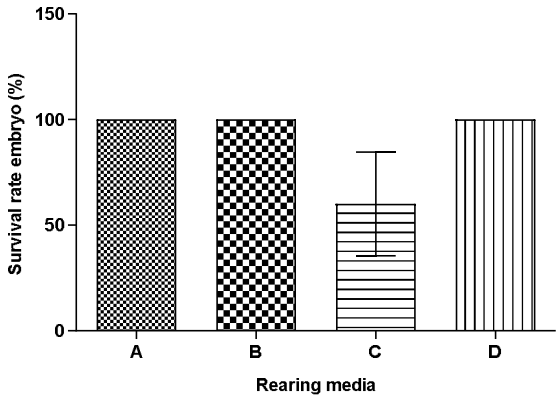


Fig. 1 Graph of survival rate embryo (SRe) *O. celebensis* on each rearing media. Embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D).

C. Yolk Volume

Yolk volume measurement was used to evaluate embryo growth and metabolic rate [15]. The results of the measurement of yolk volume in media Embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D) in each phase decreased along with the development of the embryo in a more advanced phase (Fig. 2). Bik *et al.* [41] stated that the yolk continues to shrink in line with the development of the embryo, the energy contained in the yolk moves to the embryo's organs. One of the functions of egg yolk during the early stages of fish embryo development is to provide nutrients for organogenesis, while its role at a later stage is to provide energy through metabolic processes [42], [43].

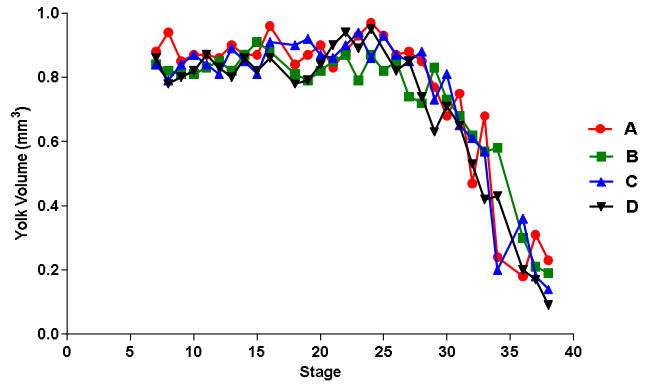


Fig. 2 The pattern of decreasing yolk volume of *O. celebensis* embryos during the study was on embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D) [44].

During the observation, it was also found that there were oil droplets that were not perfectly integrated with the oil globules located at the vegetal pole in the embryos of media bottled water (B), Pattunuang river water (C), and well water (D) (Table I). The reason why the oil droplets do not integrate with the oil globules is unknown. Therefore, there is a need for an in-depth study of it to enrich the interpretation of *Oryzias* embryo development, especially for ecotoxicological purposes. In *O. latipes* oil globule does not affect the development and growth of the embryo. Embryos that have their oil globules removed were hatched in a similar length to those that are still intact or normal [42].

D. Hatching time

The release of the embryo from the egg envelope is the result of several processes commonly referred to as hatching [45], [46]. The results of statistical analysis showed that the hatching time of the embryos on the rearing media was significantly different ($P<0.05$). The differences were found in media bottled water (B) and well water (D) (Fig. 3). The difference in hatching time was related to the $CaCO_3$ level in the rearing medium. The $CaCO_3$ content of media Embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D) was 28.03, 38.04, 8.08, 4.04 mg/l, respectively.

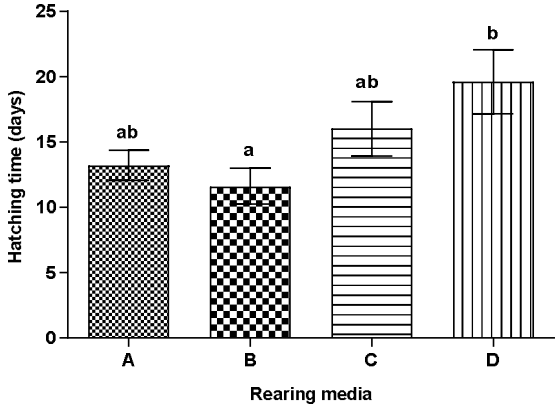


Fig. 3 Graph of hatching time embryo *O. celebensis* on each rearing medium. Different superscripts indicate a significant difference ($P>0.05$). Embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D).

Some freshwater fish embryos such as *Poecilia reticulata*, *Betta splendens*, *Danio rerio*, *O Latipes* the hatching enzyme productions were influenced by Ca^{2+} ions [47]–[50]. The low $CaCO_3$ levels in media Pattunuang river water (C) and well water (D) were suspected to be the cause of the slow hatching of embryos. In medaka eggs, the chorion consists of two layers, namely a thin outer and a thick inner layer. The hatching enzymes in medaka only digest the inner layer of the chorion in which the process consists of two steps, namely choriolytic swelling caused by HCE (High choriolytic enzyme) and dissolution of the swollen structure by LCE (low choriolytic enzyme) [46], [49], [50]. Besides these two enzymes, one more enzyme that works in the hatching process has been found, namely the pactacin enzyme [51].

The production of these three enzymes, HCE (High choriolytic enzyme), LCE (low choriolytic enzyme) and pactacin together with hatching time and hatching success of *O. celebensis* embryos has the potential to be used as biomarkers for ecotoxicological studies. Several studies have shown that contaminants can affect hatching time and success of embryos of the genus *Oryzias* [15][25][52]. To ensure in more detail the mechanism of the effect of pollutants on hatching time and success, research at the biochemical and molecular levels in *O. celebensis* embryos is necessary.

E. The Total Body Length of Larvae

Based on the statistical test results, the total body length of larvae at hatching in each rearing medium showed no significant difference (P>0.05). The measurement results showed that the average total body length of larvae in embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D) had almost the same size (Fig. 4). The total body length of *O. celebensis* larvae at hatching is not much different from the total body length of larvae of other species such as *O. latipes* 4.2 mm [45], and *Oryzias wolasi* 4.48 mm [6].

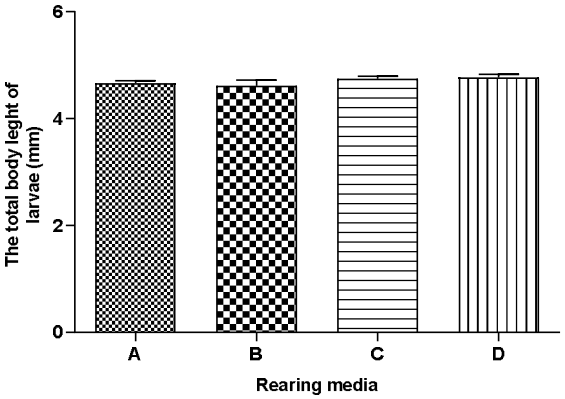


Fig. 4 Graph of the total body length of larvae *O. celebensis* on each rearing medium. Embryo rearing medium (A), bottled water (B), Pattunuang river water (C), and well water (D).

F. Water quality of incubation media

One factor that supports the success of the fish embryo incubation process is the water quality of the rearing media. The rearing media temperature is assumed to be the same as the temperature measured in the broodstock rearing aquarium, which is 26-27ºC. Then the results of pH measurements in each rearing medium can be seen in
(Table II). The temperature in this study was still in the normal range for *Oryzias* embryos [53], [54], and pH 6-9 was the range good in rearing medaka fish eggs [54].

TABLE II
WATER QUALITY OF REARING MEDIA

Water quality	A	B	C	D
pH	6.44	7.67	8.03	7.62
Parameters	Mineral concentrations (mg/l)			
	B	C	D	Threshold values
Na	20	-	-	<50 [55]
Mg	1.39	-	-	>1 [56]
SO_4	1.91	2.27	<0.001	2-80 [55]
NO_3	0.26	1.137	0.698	<10 [57]
$CaCO_3$	38.04	8.07	4,04	35.5 [58]
NaCl	-	2.92	4.67	0.3-10 [59]

- Parameters not measured

The mineral content in each rearing media can be seen in (Table II). Embryo rearing medium (A) was the control medium in this study. In general, Embryo rearing medium (A) is a rearing medium used to incubate fish embryos. The main components of ERM are NaCl, KCl, CaCl, and $MgSO_4$. Many previous studies that have used ERM as rearing media include Lee et al. [54] and Myla et al. [55] in the rearing of *O. latipes* embryos and Souders II *et a*l. [56] and Maharaj *et al.* [57] in *D. rerio* embryos. The mineral components in media bottled water (B), Pattunuang river water (C), and well water (D) can be seen in (Table II). The low levels of SO_4 in media D (well water) were probably due to the lack of mineralization processes in the water [64]. The measurement results show that $CaCO_3$ in media Pattunuang river water (C) and well water (D) has a lower value than the threshold value. Chanu *et al.* [47] have reported that an incubation medium with 5 mg/l $CaCO_3$ causes a very low survival rate of *D. rerio* embryos and larvae. However, the results of this study were not in line with the current study, where the embryos of media Pattunuang river water (C) and well water (D) developed well, and the larvae did not experience any abnormalities. It was just that the hatching time was slower than media embryo rearing medium (A) and bottled water (B).

IV. CONCLUSION

Based on the results of the study, it could be concluded that the most suitable medium for the rearing of *O. celebensis* embryos was the embryo rearing medium (ERM). Embryo rearing medium solution is a rearing medium generally used to incubate *Oryzias* embryos in the laboratory. Bottled water, Pattunuang river water, and well water are not optimal when used as an alternative rearing media for *O. celebensis* embryos, since the embryos reared in these three media still resulted in the non-fusion of all the oil droplets, making it difficult to observe the embryos.

Therefore, there is a need for further studies of the mechanism underlying the non-integration of oil droplets into the larger oil globules in *O. celebensis* embryos. The studies will enrich the way of interpreting *Oryzias* embryonic data, especially in the field of ecotoxicology. If we order from best to worst, then the order of medium that can be used as an incubation medium for *O. celebensis* embryos is embryo rearing media, bottled water, Pattunuang river water, and well water.

ACKNOWLEDGMENT

We gratefully acknowledge Prof. Dr. Ir. Joeharnani Tresnati, DEA for providing a laboratory for the experiment. We also express our deepest gratitude LPPM Universitas Hasanuddin for funding this work (contract number: 3469/UN4.1/KEP/2022) and the Ministry of Education, Culture, Research, and Technology of Indonesia for funding this work (contract number: 752/UN4.22./PT.02.00/2021).

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