Short Communication

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Temperature–Salinity Dependence of the Mitotic Interval (τ_0) for Chromosomal Manipulation in Marine Medaka, *Oryzias dancena*

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ABSTRACT

Eggs of the marine medaka, *Oryzias dancena* were collected and fertilized to observe the temperature-salinity-related cleavage rates and mitotic intervals (τ_0). We investigated the relationship between τ_0 and five different water temperatures (18, 22, 26, 30, and 34°C) and four different salinities (0, 10, 20, and 30 ppt NaCl). As the water temperature increased, the slope of the first cleavage frequency increased with elapsed time after fertilization, and approximately 30% of fertilized eggs reached the first cleavage frequency at every 15-minute intervals. However, the slope of the first cleavage frequency did not differ significantly between 0 ppt and the other salinities (10, 20, or 30 ppt). At higher water temperatures, the eggs developed more rapidly, but no other developmental process was affected. At higher salinity, the hatching rates of the eggs decreased, and the hatching times were delayed. There was a strong negative correlation between τ_0 and water temperature as shown in this equation ($Y = -1.137X + 75.47$, $R^2 = 0.977$, where Y is τ_0 and X is water temperature). At a constant water temperature, τ_0 did not differ significantly in 0, 10, 20, and 30 ppt NaCl.

Keywords: Mitotic interval (τ_0); Temperature-Salinity Dependence; Marine Medaka; *Oryzias dancena*

Short Communication

The marine medaka, *Oryzias dancena* is a euryhaline teleost that can live in both freshwater and seawater [1]. It spawns 60 days after hatching, with a consequently short interval between generations [2,3]. Accordingly, the species has drawn attention as an experimental animal in aquaculture. The marine medaka is more tolerant of hyperosmotic environments than is the Japanese medaka, *O. latipes,* with higher survival rates in the adult fish and greater hatching rates in the oosperm [3-6]. That is, most of its physiological attributes are similar across a wide spectrum of salinities, ranging from fresh water to normal seawater [4-6]. Therefore, much attention has been directed at extending the utility of functional transgenic marine medaka strains for ornamental purposes, because they can be used at most naturally occurring salinities [7]. Recently, marine medaka has been in the spotlight as an experimental fish of transgenic fish research [7-10]. However, the practical application of transgenic fish has raised public and scientific concern about the ecological risks involved, especially those associated with the adverse consequences for natural gene pools, which can be genetically contaminated if unwanted transgenic animals are released [11]. For these reasons, much recent scientific research has focused on risk assessment in relation to transgenic fish, with particular emphasis on the reproductive confinement of transgenic stocks [12]. Triploidization involving blocking of the second meiotic division has been proposed as one approach to the generation of transgenic fish having depressed reproductive capacities [8-10,13].

The ability to effectively manipulate ploidy through the application of suitable shocks (temperature, pressure, or chemical) early in egg development requires the empirical determination of the magnitude, duration, and time that the shock should be

applied [8-10,14,15]. The genetic material of a gynogenetic haploid organism can be doubled by controlling the second meiotic division and the first cleavage. The second meiotic division can be controlled by water temperature, hydrostatic pressure, or chemical treatment, which can also be used to induce triploidy [15,16]. The first cleavage can be controlled by heat shock or hypostatic pressure, which are also used individually or sequentially to induce tetraploidy [17- 20].

The effectively controlled release of the second ootid and the first cleavage are dependent on the type, intensity, and duration of the treatment [21-23]. The method of controlling the first cleavage as a means of chromosomal engineering can also enhance short-term production in aquaculture [14]. It also has an application in the induction of tetraploidy, mitotic gynogenetic diploidy, or androgenetic diploidy using chromosomal engineering. To effectively control the first cleavage, an understanding of its temperature dependence is essential [14,24]. However, determination of mitotic interval is necessary for producing triploid and tetraploid. The Dettlaff unit (mitotic interval, τ_0) is the duration (in minute) of one mitotic cycle during the early synchronous embryonic cleavage or the interval between two consecutive cell divisions [18,20,25,26]. When measured over a range of water temperatures, the relationship between τ_0 and water temperature, as determined by regression analysis, can showthe developmental events that are influenced by temperature within a species and between species with a similar spawning biology [18,20,27].

Up to date, τ_0 has been used to estimate the optimal times for chromosomal manipulation in a variety of species, including the tench, Tinca tinca; common carp; *Cyprinus carpio*; paddlefish, *Polyodon spathula*; shovelnose sturgeon, *Scaphirhynchus platorynchus*; black crappie, *Pomoxis nigromaculatus*; far eastern catfish, *Silurus asotus*; winter flounder, *Pseudopleuronectes americanus*; greenling, *Hexagrammos otakii*; black plaice, *Pleuronectes obscurus*; and Korean rose bitterling, *Rhodeus uyekii* [26,28-36]. Because the marine medaka has a wide range of salinity tolerance, we identified the egg development achieved through artificial fertilization, and also assessed the temperature–salinity dependence of τ_0 and the embryo cleavage rates to establish the most efficient procedures for chromosome manipulation in the marine medaka.

Materials and Methods

The experimental group of diploid marine medaka, *Oryzias dancena* used in this study was reared according to the methods of the previous study [37]. Fish with a standard length of over 25 mm were used in this experiment; 35 males and 15 females were placed in each of two aquariums, and 2,000 fertilized eggs were collected immediately by net. The fertilized eggs of the experimental group (n = 100) were reared in a 100 L glass aquarium. To assess the temperature-salinity dependence of the first cleavage and τ_0, the water temperature was maintained in temperature-controlled water baths at 18, 22, 26, 30, or 34°C, and each experimental group was subjected to one of four salinities, 0, 10, 20, or 30 ppt NaCl. The water salinity of each group was regulated to remain constant throughout the experiment. Samples were generally taken at 5-minute intervals and fixed with 5% neutral formalin solution (50 mL formalin, 3.25 g $Na_2HPO_4 \cdot 12H_2O$, 2.25 g KH_2PO_4, 950 mL distilled water) at 4°C before observation. We measured the diameter of 10 fertilized eggs at a magnification of 50× under an optical microscope (Axiostar plus, Zeiss, Germany) equipped with a microscope camera (Axiocam MR, Zeiss). A specific developmental stage was considered to be reached when approximately 80% of the developing embryos were at that stage. In this study, all experiments were performed in triplicate.

The time of the appearance of the first cleavage furrow was recorded and was deemed to be the start of the subsequent cell division. The times at which approximately 10% of the developing embryos reached the two-cell (τ_{II}; Figure 1a) and eight-cell (τ_{III}; Figure 1b) stages were recorded. The value of 10% was selected according to the recommendation of the previous study [38]. The mean τ_0 value was calculated as $\tau_0 = (\tau_{III} - \tau_{II})/2$. The relationship between the mean τ_0 and the water temperature was examined by simple linear regression line. One-way and two-way ANOVA analysis were applied to determine water temperature and salinity effects. Duncun's multiple range test was applied when significant difference was observed.

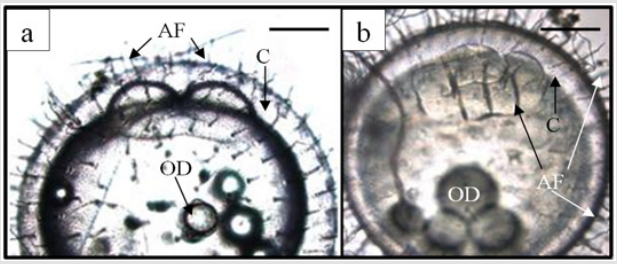

Figure 1: External morphology of egg development in marine medaka, *Oryzias dancena*. (a): 2-celled stage; (b): 8-celled stage. Scale bars are 100 μm. AF: attaching filament; C: chorion; OD: oil droplet.

Result

The fertilization, hatching rates, and hatching times of the marine medaka, *Oryzias dancena* eggs are shown in Table 1. The fertilization rates of the groups were not significantly different, and the hatching rates of each group did not differ significantly as the water temperature increased. With increasing water salinity, the hatching rates decreased at each specific water temperature, but the hatching rates of each group did not differ significantly at a specific salinity respectively. With increasing water salinity, the hatching time of each group was delayed in each specific water temperature and its time was delayed as the water temperature decreased in each specific salinity respectively. As water temperature increased, the two-cell stage and the eight-cell stage were reached earlier in a specific salinity. However, the times taken to reach the two-cell stage and eight-cell stage did not differ across different salinities at the same water temperature (Table 1).

Table 1: Fertilized and hatched rates, and 2 cell stage, 8 cell stage

and hatch-out time of each group in marine medaka, *Oryzias dancena*. 1)Each value (means of triplicate ± SD) having different superscript letters are significantly different (P<0.05). Fertilized rates of each group were analyzed at 24 hrs after fertilized. Hatched rates of each group were analyzed at 1 day after hatch-out. 2)Each time were analyzed when 80% of total eggs were reached.

Water Temperature	Salinity (ppt)	Rations (%) [1)]		Hatch-out (days)	Time After Fertilized (min)[2)]	
		Fertilized	Hatched		2-cell stage	8-cell stage
18	0	98±1.9^{a}	93±2.4^{a}	17	100	210
	10	97±3.8^{a}	80±3.4^{b}	17	101	212
	20	97±4.2^{a}	79±2.5^{b}	18	100	210
	30	98±2.1^{a}	77±3.0^{b}	18	102	209
22	0	98±1.8^{a}	94±2.9^{a}	16	95	200
	10	96±4.9^{a}	84±2.8^{b}	17	94	197
	20	98±1.7^{a}	78±3.2^{bc}	17	94	199
	30	97±4.0^{a}	77±3.0^{c}	17	95	201
26	0	97±4.5^{a}	95±3.0^{a}	14	90	180
	10	98±3.0^{a}	86±3.4^{b}	15	89	181
	20	97±3.5^{a}	79±3.6^{b}	16	91	180
	30	98±2.4^{a}	78±2.9^{b}	17	90	180
30	0	96±4.6^{a}	94±3.9^{a}	12	80	165
	10	97±3.9^{a}	86±3.4^{b}	13	81	164
	20	96±4.7^{a}	79±2.9^{b}	14	83	165
	30	98±2.5^{a}	78±3.1^{b}	15	80	166
34	0	98±3.2^{a}	93±3.7^{a}	11	75	150
	10	96±4.9^{a}	81±3.5^{b}	12	74	149
	20	96±5.0^{a}	79±3.6^{b}	13	76	150
	30	97±4.7^{a}	78±3.4^{b}	14	75	152

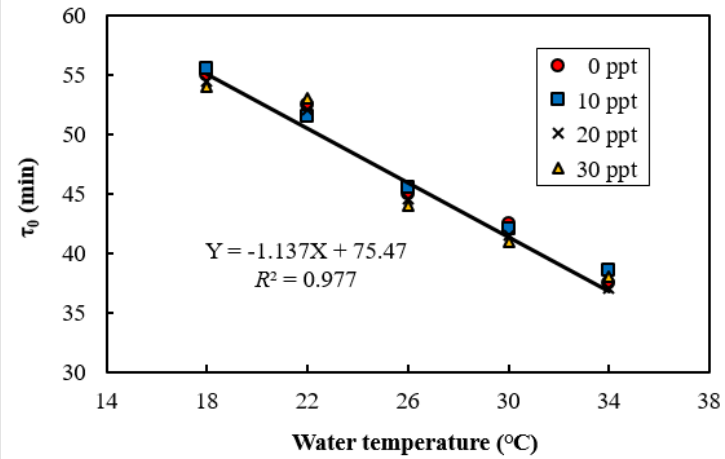

Figure 2: Mitotic intervals (τ0, Y) for marine medaka, *Oryzias dancena* as functions of temperature (X). Temperatures used are within the normal range for spawning and early, development in this species. Eggs from three females to were fertilized with pooled sperm from one male and were distributed between the temperature treatments and the salinity treatments. The experiments were executed three times.

As shown in Figure 2, the τ_0 values at 18, 22, 26, 30, and 34°C were 55, 52.5, 45, 42.5, and 37.5 minutes respectively, and there was a strong negative correlation between τ_0 and water temperature at all water temperatures (Y = –1.137X + 75.47, R^2 = 0.977, where Y is τ_0 and X is water temperature). Within the groups subjected to the same water temperature, τ_0 did not differ at salinities of 0, 10, 20, and 30 ppt NaCl. As shown in Figure 3, the eggs of the marine medaka at a salinity of 0 ppt showed the fastest development at higher water temperatures. The first cleavage began after 80, 75, 70, 60, and 55 minutes at the water temperatures of 18, 22, 20, 30,

and 34°C respectively. The embryos reached the two-cell stage after 100, 95, 90, 80, and 75 minutes at the water temperatures of 18, 22, 26, 30, and 34°C respectively. As the water temperature increased, the slope of the first cleavage frequency with elapsed time after fertilization increased, and approximately 30% of the fertilized eggs reached the first cleavage frequency at every 5-10 minutes intervals. However, the slope of the first cleavage frequency with time did not differ significantly between a salinity of 0 ppt and the other salinities (10, 20, and 30 ppt NaCl).

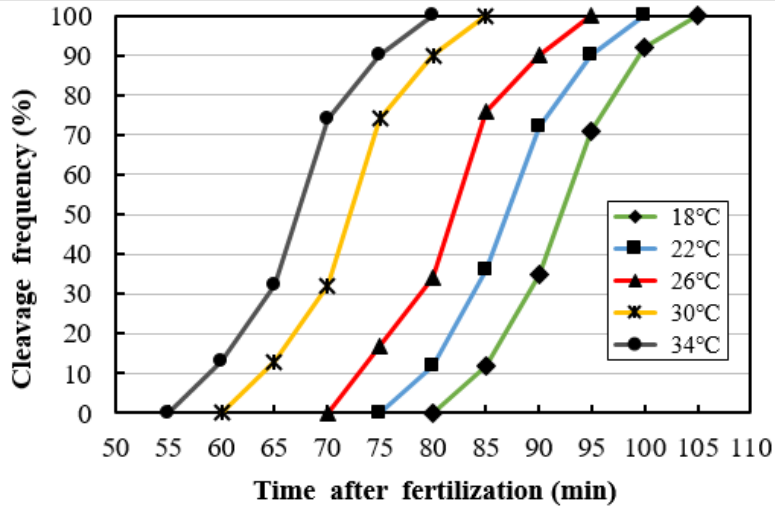

Figure 3: The percentages of marine medaka, *Oryzias dancena* eggs developed to anaphase of first cleavage at 0 ppt salinity and five different temperatures overtime.

Discussion

The previous study has reported the embryogenesis and early ontogenesis of the marine medaka, *Oryzias dancena* [2-3]. As they mentioned, the marine medaka eggs reaches the two-cell stage 90 minutes after fertilization at 26°C and 1 ppt NaCl and reaches the eight-cell stage 3 hrs after fertilization under these conditions. Although the experimental salinities used in our study differed from that of the previous study, the times required to reach the two-cell stage and eight-cell stage at 26°C and 0 ppt NaCl in our study were similar to the previous study at 26°C and 1 ppt NaCl [2]. Another previous study also reported the embryonic development and early viability of the marine medaka under different salinity conditions [38]. They reported that the hatching rates of their eggs decreased at higher salinities, and the hatching rate of the eggs was highest at 0 ppt NaCl among the salinities tested [38]. At higher salinities, the hatching times of the eggs were delayed, and its hatching time at 0 ppt NaCl was earliest among the experimental groups. In the salinity range of 0 ppt to 30 ppt NaCl, the hatching rates and hatching times in our study were similar to those of the previous study [38].

We also attempted to determine the water temperature-salinity dependence of the cleavage rates and τ_0 and found that the marine medaka eggs underwent cleavage within a temperature range of 18-34°C and a salinity range of 0-30 ppt NaCl. We found that the marine medaka eggs showed faster development with increasing water temperatures and water salinity, whereas τ_0 decreased with increasing water temperatures, which indicates a strong negative correlation between τ_0 and water temperature. Although τ_0 and hatching time after fertilization differed greatly from those of other species, the trend in the water temperature dependence of τ_0 for the marine medaka is similar to that in the black plaice, *Pleuronectes obscurus*; winter flounder, *Pseudopleuronectes americanus*; far eastern catfish, *Silurus asotus*; greenling, *Hexagrammos otakii*; Baltic herring, *Clupea harengus membras*; perch, *Perca fluviatilis*; ruffe, *Gymnocephalus cernuus*; Korean rose bitterling, *Rhodeus uyekii*; Korean bullhead, *Pseudobagrus fulvidraco*; grass puffer, Takifugu niphobles [18,20,25,32-36,39].

The relationship between τ_0 and water temperature in fish is typically curvilinear, when the temperatures are within the range in which the fish species naturally spawn and develop [18,20,25,32-35,39]. The linear response we observed in this study for τ_0 against water temperature is consistent with studies of developmental rates in the black carp, the winter flounder, and the black plaice [18,20,29,33,35]. However, additional observations are required. The available data suggest that the graphs of the dependence of τ_0 on the water temperature are highly species specific. Therefore, the species specificity of the developmental rate can be used to identify the taxonomic ranges of different fish species. We searched the previous study for determining the relationship between τ_0 and the water salinity. Unfortunately, no previous studies have reported such a relationship between τ_0 and the water salinity. That's because τ_0 of several species (having a wide spectrum of salinities) have not been researched so far. So, the future investigation of species having a wide spectrum of salinities needs to focus on the relationship between development and water salinity. The results of this study and an investigation of seeding production techniques

based on artificial fertilization, the induction of triploidy to produce sterile organisms, and the induction of tetraploidy, mitotic gynogenetic diploidy, and androgenetic diploidy in the marine medaka will facilitate such research in the future as well.

Conclusion

Considering the identity of the mitotic events and the short time intervals of mitotic intervals (τ_0), chromosomal manipulation in marine medaka, *Oryzias dancena* would be the most effective at 0 ppt salinity and water temperatures between 26 and 30°C. This study has demonstrated the specific and clear differences in the rates of hatching, the time of the first cleavage, τ0, and hatching time at different water temperatures and different salinities in the marine medaka. These data will be useful for the development of an optimal treatment protocol for its chromosomal manipulation. These data on eggs development will make a valuable contribution to a biological aquaculture database.

Declaration

Ethics Approval and Consent to Participate

The experiments performed in this study complied with the current laws of Korea (Ordinance of Agriculture, Food and Fisheries, No. 1 - the law regarding experimental animals, No. 9982) and the Ethical Guidelines of Korea Maritime & Ocean University, Korea.

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Conflict of Interest

The author has no financial or personal conflicts of interests.

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