

Temperature Dependent of Mitotic Interval for Grass Puffer, *Takifugu niphobles*

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ABSTRACT : The objective of this study was to determine the mitotic intervals (τ_0) of two consecutive cell divisions and synchronous embryonic cleavage in grass puffer, *Takifugu niphobles* at different water temperatures (18, 20, 22, and 24°C). The color of the fertilized egg was light yellowish. The egg type was demersal and unadhesive. Egg weight was 0.09 ± 0.002 mg. The sizes of unfertilized eggs were smaller than fertilized eggs in major axis and minor axis at 20°C ($p < 0.05$). The size of the fertilized egg of 18°C water temperature group at the blastodisc stage was the smallest ($p < 0.05$), but no significant differences were observed in the other water temperatures group except 18°C water temperature group ($p > 0.05$). The first cleavage stages at 18, 20, 22, and 24°C were at 75, 90, 105, and 120 mins, respectively. As water temperature was increased, embryonic development and formation time of the first cleavage furrow were accelerated. There were negative correlation between τ_0 and water temperature for grass puffer ($Y = -1.225X + 70.05$, $R^2 = 0.988$, $n = 10$, where Y was τ_0 and X was temperature). This study confirmed that successful hatching of grass puffer was related to water temperature. Chromosome manipulation will be helpful for this species using cleavage frequency and τ_0 .

Key words : Mitotic intervals (τ_0), Water temperature, Grass puffer

INTRODUCTION

The grass puffer, *Takifugu niphobles*, is the most commonly found fish in adjacent seas of Korea. Grass puffer inhabits in coastal and brackish waters where sea water and fresh water are mixed with low salinity. They usually eat small crustaceans, shellfish, and cobwebbs (Honma et

al., 1980; Oh et al., 2000). Grass puffer exhibits unique spawning behaviors. It spawns in semilunar cycles from late spring (May) to summer (August). It will spawn to the intertidal zone and lay eggs in rock slits (Honma et al., 1980; Oh et al., 2000; Masaomi et al., 2013; Goo et al., 2015; Gil et al., 2017b). The grass puffer can grow up to 20 cm. It is the smallest adult fish among Tetraodontidae

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fish (Nozaki et al., 1976; Oh et al., 2000). Because of its large population and small size, it is widely used as an ornamental fish. It is also possible to replace the expensive puffer fish dishes.

For grass puffer, anesthetic and physiological effects of clove oil and lidocaine-HCl, effect of starvation, sexual dimorphism were previously studied (Gil et al., 2017a; Kim et al., 2017; Park, 2017). However, unlike previous studies, the objective of this study was to determine cleavage frequency and mitotic intervals of cell divisions in grass puffer at different water temperatures. Effective controls of the first cleavage and second ootid for chromosome manipulation in fish are dependent on the type and duration time of treatment. It is well known that the second meiosis is affected by water temperature, hydraulic pressure, and chemical treatment (Thorgaard et al., 1981; Thorgaard & Allen, 1987; Ahn, 1995). Therefore, among different methods of fish breeding techniques, producing polyploidy, gynogenetic, and androgenetic diploid fish through chromosome manipulation is very important for the aquaculture industry (Thorgaard, 1983; Thorgaard & Allen, 1987; Mims et al., 1997). Recently, Masaomi et al. (2013) have induced triploid grass puffer. Yoo et al. (2018) have also produced hybrids and hybrid triploids between river puffer and tiger puffer.

Among different techniques for chromosome manipulation, inhibiting the first cleavage and first or second polar body emission has been adapted to induce tetraploid or mitotic gynogenetic and androgenetic diploid (Mims et al., 1997; Masaomi et al., 2013). An appropriate Dettlaff unit is determined by the duration of one mitotic cycle during the time of synchronous cleavage divisions under different water temperatures. The Dettlaff unit is the duration in minutes of one mitotic cycle during early simultaneously embryonic beginning or the interval between two continuous cell divisions separation (Shelton, 1993; Saat & Veersalu, 1996; Shelton et al., 1997). When measured over a range of temperatures, the relationship between mitotic

intervals (τ_0) and temperature as determined by regression analysis can be used to predict developmental events that are influenced by temperature within a single species or between species with similar spawning biology (Dettlaff, 1986). Previous studies have been limited to egg development of grass puffer (Oh et al., 2000). Grass puffer is a very useful sample for fishery laboratory animals in physiological, hematological, and other experiments. Therefore, the objective of this study is to determine the mitotic intervals (τ_0) of two consecutive cell divisions and synchronous embryonic cleavage in grass puffer at different water temperatures (18, 20, 22, and 24°C).

MATERIALS AND METHODS

1. Rearing condition and sampling

Grass puffer, *Takifugu niphobles*, were collected from the harbor at Korea Maritime and Ocean University (KMOU), Busan, Korea, from May to August, 2016. Samples were transported to and reared in Fishery Genetics and Breeding Sciences Laboratory of the KMOU, Busan, Korea. Initial measurements of body weight (BW) and total length (TL) were made to the nearest 0.1 g and 0.1 cm using an electric balance (AX 200, Shimadzu Corp., Japan) and digital vernier calipers (CD-20 CP; Mitutoyo, Japan), respectively. The TL and BW of each species were 13.6±2.15 cm and 29.7±4.26 g in females and 12.7±2.53 cm and 26.5±3.29 g in males, respectively. Experimental samples were acclimated for one week in aquariums (200 L) with different water temperatures (18, 20, 22, and 24°C) to have sufficient adaptation.

2. Egg collection and measurements

We injected into the grass puffer 1,000 IU/g BW human chorionic gonadotropin (HCG, Sigma-Aldrich St. Louis, MO, USA). Twenty-four hours after the injection, eggs and sperm were extracted by pressing the abdomens. They were mixed in sufficient seawater by drying method. Ferti-

lized eggs were adequately aerated and filtered in plastic sea water tank (20 L). Comparing the size of unfertilized eggs and fertilized eggs, unfertilized eggs were measured at 20°C while the fertilized eggs were divided into four different groups based on water temperature (18, 20, 22, and 24°C). Color, type, weight, and size (major axis, minor axis, and extracellular matrix) of unfertilized and fertilized eggs (when blastodisc stage was developed) of grass puffers were compared. The weights of eggs were measured by weighing 10 eggs and dividing the number by 10. The diameters of eggs were measured using the eyepiece micrometer of a microscope (Axioskop, Zeiss, Germany) under 50 × magnification. Egg development was confirmed by sampling at intervals of 5 mins from 1 hr after fertilization.

3. Mitotic interval and first cleavage

To investigate the period of the first cleavage of fertilized eggs depending on different temperatures also known as the Dettlaff unit (mitotic interval, τ_0) of different cleavage frequencies and water temperatures, eggs were fertilized at 18, 20, 22, and 24°C and sampled every 5 mins after fertilization. Each sample was fixed in 10% neutral formalin (100 mL formalin, 6.5 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4 g KH_2PO_4 , fresh water 900 mL) and kept at 4°C until observation. τ_0 was examined under microscope with 50 × magnification. For τ_0 , the time it took for about 10% of the entire eggs to reach 2-cell stage (τ_I) and 8-cell stage (τ_{III}) at each hatching temperature were measured according to the method of Ignat'eva (1975). Average τ_0 was calculated by the formula $\tau_0 = (\tau_{III} - \tau_I) / 2$. Linear regression analysis was performed to find the relationship between the average τ_0 and water temperature. This experiment was performed in triplicates.

4. Statistical analysis

The relationship between the mean mitotic interval and water temperature was examined by simple linear regres-

sion using SPSS Software (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

As shown in Table 1 and Fig. 1, egg color of grass puffer, *Takifugu niphobles*, is light yellowish. Egg type is unadhesive and demersal. Egg weight was measured at 0.09 ± 0.002 mg at water temperature of 20°C. Among various puffer fish species, the eggs of grass puffer are light yellowish, unadhesive, and demersal with lots of oil globules (Oh et al., 2000). Regarding other puffer fish species, the eggs of tiger puffer (*T. rubripes*), river puffer (*T. obscurus*), and panther puffer (*T. pardalis*) are opaque and milky-white in color (Jang et al., 1996; Han, 1999; Han et al., 2001). The major axis of unfertilized egg was 1.05 ± 0.041 mm. The minor axis of unfertilized egg was 1.01 ± 0.038 mm. The extracellular matrix of unfertilized egg was 0.09 ± 0.005 mm. The sizes of fertilized eggs at four different water temperatures were significantly smaller than that of the other three groups in the long axis and short axis at 18°C and no significant difference was found in the other three groups ($p < 0.05$). The sizes of extracellular matrix showed no significant ($p > 0.05$) difference among four water temperature groups. When fertilized eggs were compared to unfertilized eggs at the same temperature of 20°C, the major axis of fertilized egg was 1.36 times larger and the minor axis was 1.39 times larger ($p < 0.05$) than those of unfertilized eggs.

As shown in Fig. 2, egg development was accelerated when water temperature was increased from 18°C to 24°C. Egg developmental rates of the four water temperature groups were similar to each other. However, the time that eggs reached the first cleavage was 30 mins at 18°C, 45 mins at 20°C, 60 mins at 22°C, and 75 mins at 24°C. After 90 mins, the first cleavage was finished in all water temperature groups. Oh et al. (2000) have reported the embryogenesis and early ontogenesis of a grass puffer. As mentioned by Oh et al. (2000), two-cell stage of grass puffer

Table 1. Characteristics of unfertilized and fertilized eggs of grass puffer, *Takifugu niphobles* at different water temperatures*

Characteristics of egg	Color		Type		
	Light yellowish		Unadhesive and demersal		
Egg weight (mg)	0.09±0.002 ^a				
Egg size (mm)	Water temperature for egg development (°C)				
		18	20	22	24
Unfertilized egg	Major axis	1.05±0.041 ^a			
	Minor axis	1.01±0.038 ^a			
	Extracellular matrix	0.09±0.005 ^a			
Fertilized egg	Major axis	1.39±0.032 ^a	1.43±0.039 ^b	1.45±0.064 ^b	1.46±0.043 ^b
	Minor axis	1.36±0.035 ^a	1.41±0.048 ^b	1.42±0.053 ^b	1.43±0.052 ^b
	Extracellular matrix	0.09±0.009 ^a	0.09±0.005 ^a	0.11±0.009 ^a	0.09±0.008 ^a

*Each value is presented as mean±SE of triplicated groups (n=30). Means in rows with the different superscript letter are significantly different (p<0.05). Fertilized egg sizes were measured for blastodisc stage at each water temperature.

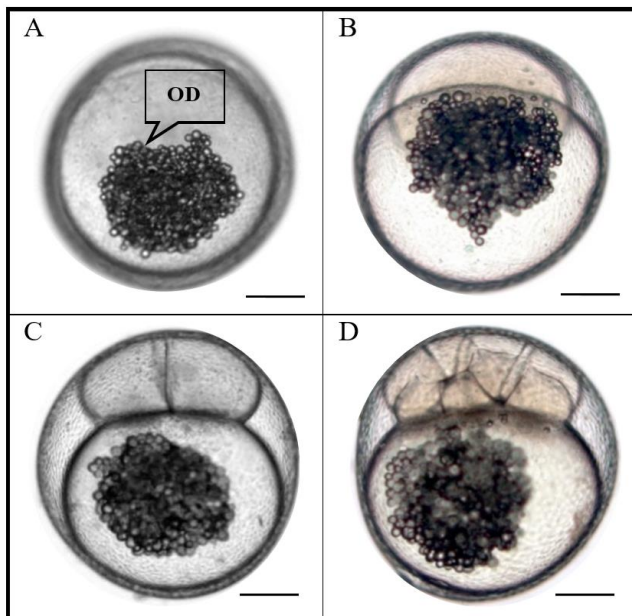


Fig. 1. Egg development of grass puffer, *Takifugu niphobles*. (A) Fertilized egg, (B) Blastodisc stage, (C) 2-cell stage, and (D) 8-cell stage. Bars: 25 μm. OD: oil droplet.

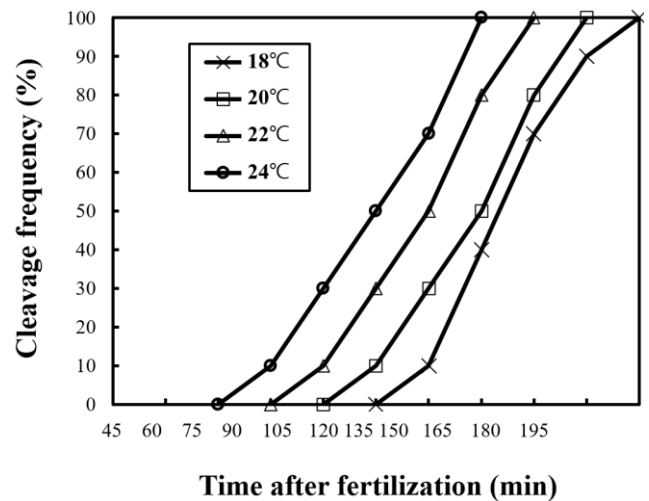


Fig. 2. The percentages of grass puffer, *Takifugu niphobles*, eggs developing to anaphase of the first cleavage at four different temperatures after fertilization.

egg can be reached within two hours after fertilization at 22°C and 8-cell stage of egg can be reached at 4 hours

after fertilization. Although our experimental water temperatures were different from those of Oh et al. (2000), the time needed to reach the 2-cell stage and the 8-cell stage at 18, 20, 22, and 24°C was similar to that of Oh et al. (2000) at 20°C. Egg development stage of tiger puffer is different from that of grass puffer. For example, the time needed to reach 2-cell stage and 8-cell stage has been reported to be 3:30 and 5:50 hours after fertilization, respectively (Han, 1999). Therefore, there is a time difference in egg development stage among different fish species or the same fish species at different temperatures. This could be used to identify the taxonomic ranges of different fish species. The eggs of grass puffer showed faster development at higher temperature (Fig. 2). As the water temperature was increased, the slope of the first cleavage frequency with elapsed time after fertilization was increased. Approximately 10% of fertilized eggs reached first cleavage frequency every 15 min. These results are similar to those reported by Ignat'eva (1975), Saat & Veersalu (1996), Park & Im (2001), and Kim et al. (2011) showing that faster development has occurred at higher water temperature.

As shown in Fig. 3, the time of mitotic intervals (τ_0) was measured according to water temperature (18, 20, 22, and 24°C). Unlike the tendency of cleavage frequency, τ_0 was negatively correlated with water temperature ($Y = -1.225X + 70.05$, $R^2 = 0.988$ where Y was τ_0 and X was temperature). Of the four water temperature groups, τ_0 was 53 ± 1.9 mins at 18°C, 49 ± 2.3 mins at 20°C, 45 ± 1.7 mins at 22°C, and 40 ± 1.8 mins at 24°C. The duration of one mitotic cycle during the period of identical cell divisions (τ_0) has proven to be an appropriate unit for comparing the duration of development processes at different temperatures in poikilothermic animals undergoing identical cell divisions during their early development (Shelton, 1993, 1997; Park & Im, 2001; Kim et al., 2011). According to results shown in Fig. 3, we found that grass puffer showed faster development with decreased mitotic intervals when water temperature was increased, indicating a strong negative correlation

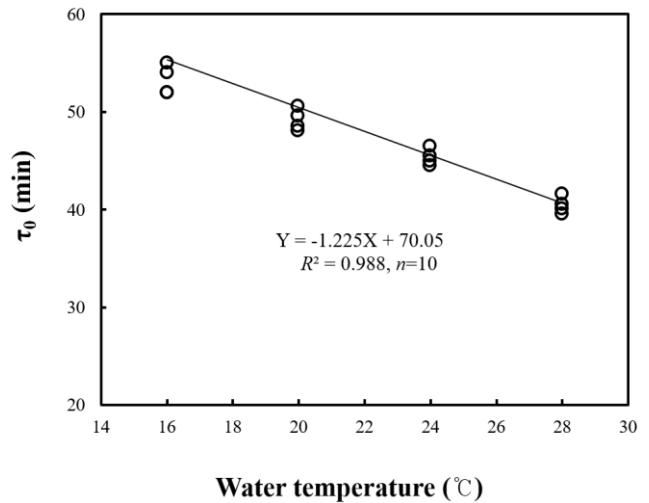


Fig. 3. Mitotic interval (τ_0 , Y) of grass puffer, *Takifugu niphobles*, as function of temperature (X). Temperatures used are within normal range for spawning and early development for this species.

between τ_0 and water temperature. Besides this study, similar results on the reduction of mitotic interval according to rising water temperature have been reported in far eastern catfish (*Silurus asotus*), black plaice (*Pleuronectes obscurus*), and Korean rose bitterling, *Rhodeus uyekii* (Park & Im, 2001; Park and Im, 2010; Kim et al., 2011).

In summary, this study demonstrated obvious specific and clear differences in the time of the first cleavage and mitotic intervals at different temperatures in grass puffer. Data obtained from this study will add value to biological *Takifugu* species aquaculture database. This study will aid future investigations on seeding production by artificial fertilization, induction of triploid for producing sterile organisms, and induction of tetraploid, mitotic gynogenetic diploid, and androgenetic diploid grass puffer.

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eries, No. 1, Regarding Experimental Animals, No. 9,932) and the ethic guideline of KMOU.

REFERENCES

- Ahn CM (1995) Effects of photoperiod and water temperature on the reproductive cycle of the spring-spawning bitterling, *Rhodeus uyekii* (Pisces: Cyprinidae). Korean J Ichthyol 7:33-42.
- Dettlaff TA (1986) The rate of development in poikilothermic animals calculated in astronomical and relative time units. J Therm Biol 11:1-7.
- Gil HW, Lee TH, Choi CY, Kang CB, Park I-S (2017a) Anesthetic and physiological effects of clove oil and lidocaine-HCl on the grass puffer, *Takifugu niphobles*. Ocean Polar Res 39:1-11.
- Gil HW, Lee TH, Choi CY, Kang CB, Park I-S (2017b) Induction of artificial spawning by human chorionic gonadotropin in the grass puffer, *Takifugu niphobles*. J Fish Mar Sci Edu 29:409-414.
- Goo IB, Park I-S, Gil HW, Im JH (2015) Stimulation of spermiation by human chorionic gonadotropin and carp pituitary extract in grass puffer, *Takifugu niphobles*. Dev Reprod 19:253-258.
- Han KH, Cho JK, Lee SH, Hwang DS, Yoo DJ (2001) Spawning behavior and early life history of *Takifugu pardalis* (Teleostei: Tetraodontidae) in Korea. Kor J Ichthyol 3:181-189.
- Han KN (1999) Development of eggs, larvae and juveniles of the puffer, *Takifugu rubripes* reared in the laboratory. Japan J Ichthyol 33:186-194.
- Honma Y, Ozawa T, Chiba A (1980) Maturation and spawning behavior of the puffer, *Fugu niphobles*, occurring on the coast of Sado Island in the Sea of Japan (a preliminary report). Jpn J Ichthyol 27:129-138.
- Ignat'eva GM (1975) Temperature dependence of cleavage rates in carp, pike, and whitefish. Sov J Dev Biol 5:24-28.
- Jang SI, Kang HW, Han HG (1996) Embryonic, larval and juvenile stages in yellow puffer, *Takifugu obscurus*. J Aquacult 1:11-18.
- Kim B-S, Lim SG, Gil HW, Park I-S (2011) Temperature dependent index of mitotic interval (τ_0) for chromosome manipulation in Korean rose bitterling, *Rhodeus uyekii*. Fish Aquat Sci 14:429-433.
- Kim DS, Gil HW, Lee TH, Park I-S (2017) Comparative analysis of sexual dimorphism between spawning season and non-spawning season in the grass puffer, *Takifugu niphobles*. J Fish Mar Sci Edu 29:447-452.
- Masaomi H, Yutaka T, Kadoo M (2013) Gonadal development and fertility of triploid grass puffer, *Takifugu niphobles* induced by cold shock treatment. Mar Biotechnol 15:133-144.
- Mims SD, Shelton WL, Linhart O, Wang C (1997) Induced meiotic gynogenesis of paddlefish, *Polyodon spathula*. J World Aquacult Soc 28:334-343.
- Nozaki M, Tsutsumi T, Kobayashi H, Takei Y, Ichikawa T, Tsuneki K, Miyagawa K, Uemura H, Tatsumi Y (1976) Spawning habit of the puffer, *Fugu niphobles* (Jordan et Snyder). Zool Mag 85:156-168.
- Oh SH, Han KH, Kim YM, Joung HH, Shin SS, Kim YU (2000) Spawning behavior and early life history of grass puffer, *Takifugu niphobles*. Kor J Ichthyol 4:236-243.
- Park I-S, Im S-Y (2010) Egg development and mitotic interval (τ_0) in black plaice, *Pleuronectes obscurus* (Herzenstein). Fish Aquat Sci 13:278-283.
- Park I-S (2017) Effect of starvation on the just hatched grass puffer, *Takifugu niphobles* larvae. JMLS 2:27-33.
- Park I-S, Im JH (2001) Determination of the temperature dependent index of mitotic interval (τ_0) for chromosome manipulation in far eastern catfish, *Silurus asotus*. Korean J Ichthyol 13:85-88.
- Saat T, Veersalu A (1996) Duration of synchronous cleavage cycles and rate of development at different temperatures in the Baltic herring. J Fish Biol 48:658-663.
- Shelton WL (1993) Determination of the developmental

- duration (τ_0) for ploidy manipulation in carps. *Isr J Aquacult* 45:73-81.
- Shelton WL, Mims SD, Clark JA, Hiott AE, Wang C (1997) A temperature-dependent index of mitotic interval (τ_0) for chromosome manipulation in paddlefish and shovelnose sturgeon. *Prog Fish-Cult* 59:229-234.
- Thorgaard GH (1983) Chromosome set manipulation and sex control in fish. In: *Fish Physiology*. Vol. 9B. Hoar WS, Randall DJ and Donaldson EM, eds. Academic Press, New York, NY, pp 405-434.
- Thorgaard GH, Allen SK Jr (1987) Chromosome manipulation and markers in fishery management. In: *Population Genetics and Fishery Management*. Ryman N and Utter F, eds. University of Washington Press, Seattle, WA, US, pp 319-331.
- Thorgaard GH, Jazwin ME, Stier AR (1981) Polyploidy induced by heat shock in rainbow trout. *Trans Amer Fish Soc* 110:546-550.
- Yoo GY, Lee TH, Gil HW, Lim SG, Park IS (2018) Cytogenetic analysis of hybrid and hybrid triploid between river puffer, *Takifugu obscurus* and tiger puffer, *T. rubripes*. *Aquacult Res* 49:637-650.