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Egg Development and Mitotic Interval (τ_0) in Black Plaice, *Pleuronectes obscurus* (Herzenstein)

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Black plaice, *Pleuronectes obscurus* (Herzenstein), were collected and fertilized to observe egg development, temperature-related cleavage rates, and mitotic intervals (τ_0). Fertilized egg was demersal, adhesive, and did not contain oil globules. After 1.75 h, the blastodisc formed, and hatching took place 121 h after fertilization at 14°C. The hatched larvae were 3.5±0.16 mm in total length. At higher temperatures, eggs developed faster and underwent further identical developmental processes. Additionally, τ_0 and water temperature were strongly negatively correlated at all temperatures (Y=-2.6981X+98.767, R^2=0.9831, where Y is τ_0, and X is temperature) for black plaice.

Key words: Egg development, Mitotic interval (τ_0), Black plaice, *Pleuronectes obscurus* (Herzenstein)

Introduction

Black plaice, *Pleuronectes obscurus* (Herzenstein, 1890), in the order Pleuronectiformes, is widely distributed throughout the South, East, and West Seas and around Japan, in the East China Sea. This fish generally inhabits coastal areas but has also been introduced into estuaries. Its average total length is approximately 40 cm, and it spawns in the spring. Eggs are demersal and adhesive (Choi et al., 2002). Black plaice is an expensive food that is used in raw, sliced fish dishes and therefore has high commercial value.

To date, a cytogenetic study (Kim et al., 2007) has been the only reported study of black plaice in Korea. Thus far, the early life history and seeding production of Pleuronectidae have been examined in the stone flounder (*Kareius bicoloratus*), marbled sole (*Pleuronectes yokohamae*), spotted flounder (*Verasper variegatus*), and brown sole (*P. herzensteini*) (Kim, 1982; Kim et al., 1983, 1985; Cho et al., 1995; Han and Kim, 1997, 1999), but not the black plaice.

The ability to effectively manipulate ploidy through the application of suitable shocks (temperature, pressure, or chemical) early in egg development requires empirical determination of the magnitude, duration, and time of application of the shock (Thorgaard, 1983). The genetic material of a gynogenetic haploid can be doubled through a controlled second meiotic division and first cleavage. The second meiotic division can be controlled by temperature, hydrostatic pressure, and chemical treatment, which can also be used to induce triploidy. The first cleavage can be controlled by heat-shock and hypostatic pressure, which are used to induce tetraploidy and can be implemented together or separately (Thorgaard, 1986; Komen et al., 1991).

The effective controlled release of the second ootid and the first cleavage are dependent on the type, intensity, and duration of treatment (Thorgaard et al., 1981; Onozato and Yamaha, 1983; Thorgaard and Allen, 1987). Thus, the control of first cleavage, a means of chromosome engineering, can affect the short-term enhancement of aquaculture production (Thorgaard, 1983). Additionally, the control of first cleavage has applications for the induction of tetraploidy, mitotic gynogenetic diploidy, and androgenetic diploidy using chromosome engineering. Thus, to practice effective control of first cleavage, an understanding of the temperature-dependence of this process is important (Thorgaard, 1983; Mair, 1993).

The Dettlaff unit (τ_0) is the duration (min) of one mitotic cycle during early synchronous embryonic cleavage or the interval between two consecutive cell

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divisions (Saat and Veersalu, 1996a, 1996b; Shelton et al., 1997). When measured over a range of temperatures, the relationship between τ_0 and temperature, determined by regression analysis, can predict the developmental events affected by temperature both within species and between species with similar spawning biology (Dettlaff, 1986). Thus, we examined egg development using artificial fertilization to document patterns for future industrialization uses. We also assessed temperature-related τ_0 and cleavage rates to establish the most efficient procedures for chromosome manipulation in black plaice.

Materials and Methods

Broodstock and artificial fertilization

Fish were collected from the coastal areas of Hari, Young-do, and Busan, Korea, from February to March 2007. Fish were transported to and maintained at the Fishery Genetics and Breeding Science Laboratory, Korea Maritime University.

The external morphology of broodstock was recorded with a digital camera using a photocopy stand (Nikon D80, Japan). Fish were weighed to the nearest 0.01 g using an electronic balance (JW-1; Acom, Pocheon, Korea), and body lengths were measured to the nearest 0.01 cm using digital vernier calipers (CD-20CP; Mitutoyo, Kawasaki, Japan). Sperm and eggs were artificially fertilized using the dry process (Park et al., 2006).

Observations of reproductive characteristics

We estimated a gonadosomatic index (GSI) and a hepatosomatic index (HSI) using the following formulas: GSI (%)=gonad weight (g) × 10^2/body weight (g); HSI (%)=liver weight (g) × 10^2/body weight (g).

We also recorded the number of eggs brooded over for hatching per female weight unit as well as the diameter of fertilized eggs. The number of eggs brooded over for hatching per female weight unit was calculated using the following formula: number of eggs brooded over for hatching/100 g. The number of eggs brooded over for hatching was calculated as: the weight of all eggs for hatching/average weight of one egg for hatching. We also measured the diameter of 50 fertilized eggs at 50× under an optical microscope (Axiostar plus, Zeiss, Germany).

Egg development, hatching, and early rearing

Fertilized eggs were placed in an aerated, square plastic tank (36×51×31 cm). Natural seawater was filtered through a 100 μm closed-pore filter, and one-third of the incubated water was exchanged once per day. Water temperatures were maintained at 14± 0.5°C. Samples were generally taken at 5 min intervals immediately after fertilization and at 30 min and 1 h intervals after the start of development. Samples were fixed in a 5% neutral formalin solution and examined under an optical microscope. A developmental stage was considered to have been reached when approximately 80% of the developing embryos were found to be within that stage. At 2 days post-hatching, we began feeding and rearing the fish with 5 rotifers/mL twice per day.

Observations of first cleavage and mitotic interval (τ_0)

To assess the temperature dependence of the first cleavage and mitotic interval (τ_0), water temperatures were maintained using temperature-controlled water baths set at 6, 10, 14, 18, 22, and 26°C. Samples were generally taken at 5-min 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 sea water) at 4°C before observation. Sampled embryos were examined at 50× magnification.

Timing of the appearance of the first cleavage furrow was recorded and used as the start for the timing of subsequent cell divisions. The times at which approximately 10% of the developing embryos reached the two- (τ_I) and eight-cell (τ_{III}) stages were recorded. Value of 10% was selected based on the recommendation of Ignatyeva (1975). Mean mitotic cycle intervals (τ_0) were calculated as $\tau_0=(\tau_{III}-\tau_I)/2$. The relationship between the mean mitotic interval and water temperature was examined using simple linear regression.

Results

Fig. 1 presents the external morphology of a representative fish used in this experiment. The means (±SD) of body length and broodstock weight were 31.9±3.81 cm and 864.1±331.87 g (n=10) and 26.4±2.18 cm and 285.9±71.06 g (n=10) respectively (Table 1).

Reproductive characteristics

GSI and HSI values of broodstock are presented in Table 1. Average GSI of females was approximately 27% higher than average GSI of males. In contrast, average HSI of males were encompassed by those of females, average HSI of mates were 0.05% higher than those males. Average number of eggs brooded over for hatching per female weight unit was 92,861±5,002 eggs/100 g.

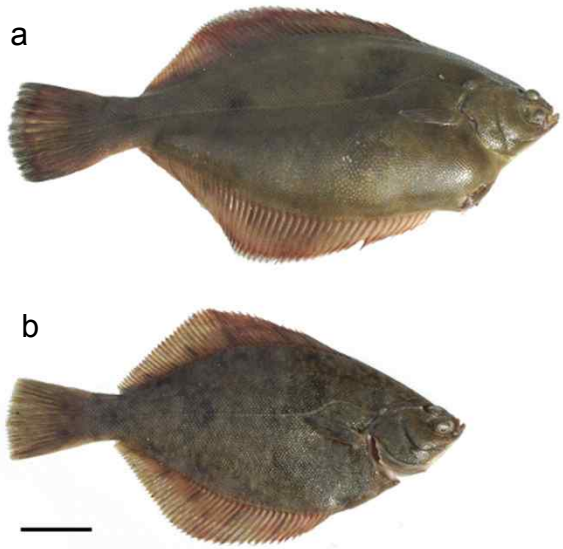

Fig. 1. External morphology of the broodstock black plaice, *Pleuronectes obscurus* used in this study. (a) female and (b) male. Bar is 6 cm.

Timing of first cleavage

Eggs of black plaice exhibited faster development at higher temperatures (Fig. 2). As the water temperature increased, the slope of the relationship between the frequency of the first cleavage and time elapsed since fertilization increased.

Mitotic interval (τ_0)

τ_0 and water temperature were strongly negatively correlated at all temperatures (Y=-2.6981X+98.767, R^2=0.9831, where Y is τ_0, and X is temperature; Fig. 3).

Egg development and hatching

Table 2 and Fig. 4 present the development of eggs that were artificially fertilized at 14°C. Fertilized eggs were demersal and adhesive and measured an average of 0.84±0.010 mm (n=50) in diameter (Fig. 4-A). The eggs did not contain oil globules. After 1.75 h, the blastodisc formed (Fig. 4-B). The 2-, 4-, 8-, 16-, 32-, and 64-cell stages were attained at 2.5, 3.5, 4.5, 5.5, 7.5, and 8.5 h, respectively (Figs. 4-C~H); the morula stage was reached in 10 h (Fig. 4-I), and the blastula stage was reached in 16 h (Fig. 4-J). The eggs began the gastrula stage at 20 h (Fig. 4-K), and the embryonic body began to form at 27 h (Fig. 4-M).

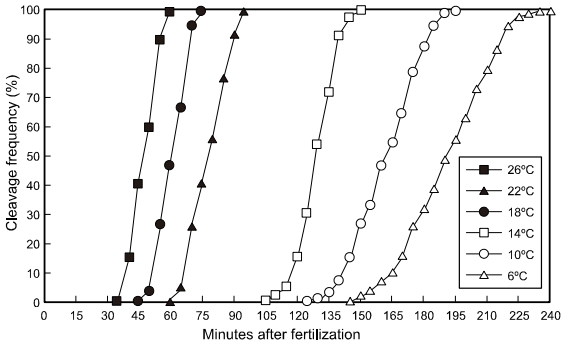

Fig. 2. The percentages of black plaice, *Pleuronectes obscurus* eggs developed to anaphase of first cleavage at six different temperatures overtime.

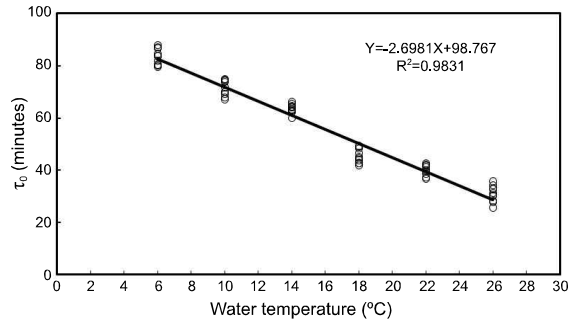

Fig. 3. Mitotic intervals (τ_0, Y) for black plaice, *Pleuronectes obscurus* as functions of temperature (X). Temperatures used are within the normal range for spawning and early, development in this species. Eggs from three females to were fertilized with pooled sperm from five males and were distributed among the temperature treatments.

Optic vesicles appeared in 44 h (Fig. 4-N), and the auditory vesicles and Kupffer's vesicle were apparent at 72 h (Fig. 4-O). At 90 h, Kupffer's vesicle had disappeared and movements of heart and tail were observed (Fig. 4-P). Hatching took place 121 h after fertilization at 14°C. Hatched larvae were an average of 3.5±0.16 mm in total length (Fig. 4-Q).

Discussion

Eggs of Pleuronectidae are spherical, inadhesive, and floating, whereas eggs of several other species are demersal and adhesive, including those of black

Table 1. Standard lenth, body weight, godadosomatic (GSI) and hepatosomatic indices (HSI) of broodstock black plaice, *Pleuronectes obscures*

	Body length (cm)	Standard weight (g)	GSI (%)	HSI (%)
Female (*n*=10)	31.9 ± 3.81	864.1 ± 231.8	30.2 ± 1.43	1.0 ± 0.49
Male (*n*=10)	26.4 ± 2.18	285.9 ± 71.0	2.3 ± 0.78	0.8 ± 0.19

Table 2. Egg development of the black plaice, *Pleuronectes obscurus* at 14°C

Time (hr : min)	Stages*	Descriptions
-	A	Fertilized egg
1 : 45	B	Elevation of blastodisc
2 : 30	C	2-celled stage
3 : 30	D	4-celled stage
4 : 30	E	8-celled stage
5 : 30	F	16-celled stage
7 : 30	G	32-celled stage
8 : 30	H	64-celled stage
10 : 00	I	Morula stage
16 : 00	J	Blastula stage
20 : 00	K	Beginning of gastrulation
23 : 00	L	Postgastrula stage
27 : 00	M	Formation of the embryonic body
44 : 00	N	Less than 10 mytomes stage, formation of optic vesicles
72 : 00	O	More than 14 mytomes stage, formation of auditory vesicles and Kupffer's vesicle
90 : 00	P	20 and above mytomes stage, disappearance of Kupffer's vesicle
121 : 00	Q	Hatching of embryo

*Stages (A-Q) reffering from Fig. 2.

plaice (*Pleuronectes obscurus*) and marbled sole (*P. yokohamae*). Black plaice eggs do not contain oil globules (Kim et al., 1983). In the present study, the mean diameter of black plaice eggs was 0.84± 0.010 mm, which is similar to values for marbled sole and smaller than those of brown sole (*P. herzensteini*) and stone flounder (*Kareius bicoloratus*) (Kim, 1982; Kim et al., 1983; Han and Kim, 1999).

Our results indicated that the time required to form the blastodiscs in the eggs of black plaice was similar to that observed for stone flounder, although the incubation temperature for black plaice was higher than those used for stone flounder and marbled sole (Kim, 1982; Kim et al., 1983). However, time until hatching for black plaice was longer than that observed for stone flounder and was similar to that of marbled sole, which also exhibited relatively slow formation of the blastodisc (Kim, 1982; Kim et al., 1983). In contrast, time until hatching for black plaice was longer than that for brown sole at the same temperature (Han and Kim, 1999).

During development of black plaice eggs, auditory vesicles and Kupffer's vesicle appeared at the same time. However, for eggs of stone flounder, marbled sole, and brown sole, development proceeded in the following order: optic vesicles appeared, Kupffer's vesicle disappeared, and movements of the heart were observed (Kim, 1982; Kim et al., 1983). Thus, within the same species, differences in egg development depend on incubation temperature, whereas across species at the same temperature, these differences depend on species-specific characteristics (Kim, 1982; Kim et al., 1983; Han and Kim, 1999).

In terms of temperature-related cleavage rates and mitotic intervals (τ_0), we found that black plaice eggs underwent cleavage within the temperature range of 6 to 26°C. Based on our results, we determined that black plaice eggs exhibited faster development and decreased mitotic intervals with increasing water temperature, indicating that τ_0 and water temperature were strongly negatively correlated. Temperature-dependence of τ_0 in black plaice is similar to observations for winter flounder (*Pseudopleuronectes americanus*; Park and Johnson, 2002), far eastern catfish (*Silurus asotus*; Park and Im, 2001), greenlings (*Hexagrammos otakii*; Park et al., 2006), baltic herring (*Clupea harengus membras*; Saat and Veersalu, 1996b), perch (*Perca fluviatilis*; Saat and Veersalu, 1996a), and ruffe (*Gymnocephalus cernuus*; Saat and Veersalu, 1996a).

Additionally, the relationships between τ_0 and water temperature in fish are typically curvilinear, providing that temperatures are within the range for which the species of fish naturally spawns and develops (Saat and Veersalu, 1996a, 1996b; Park and Im, 2001; Park and Johnson, 2002; Park et al., 2006). Observed linear relationship between τ_0 and temperature was consistent with data for the developmental rates of black carp and winter flounder (Shelton and Rothbard, 1993; Park and Johnson, 2002). However, additional observations are needed, as the available data suggest that the relationship between τ_0 and temperature is highly species-specific. Species-specificity of the development rate can be used to identify the taxonomic ranges of different fish species. Given the identity of mitotic events and the short time intervals (τ_0), chromosome manipulations in black plaice would be most efficient between

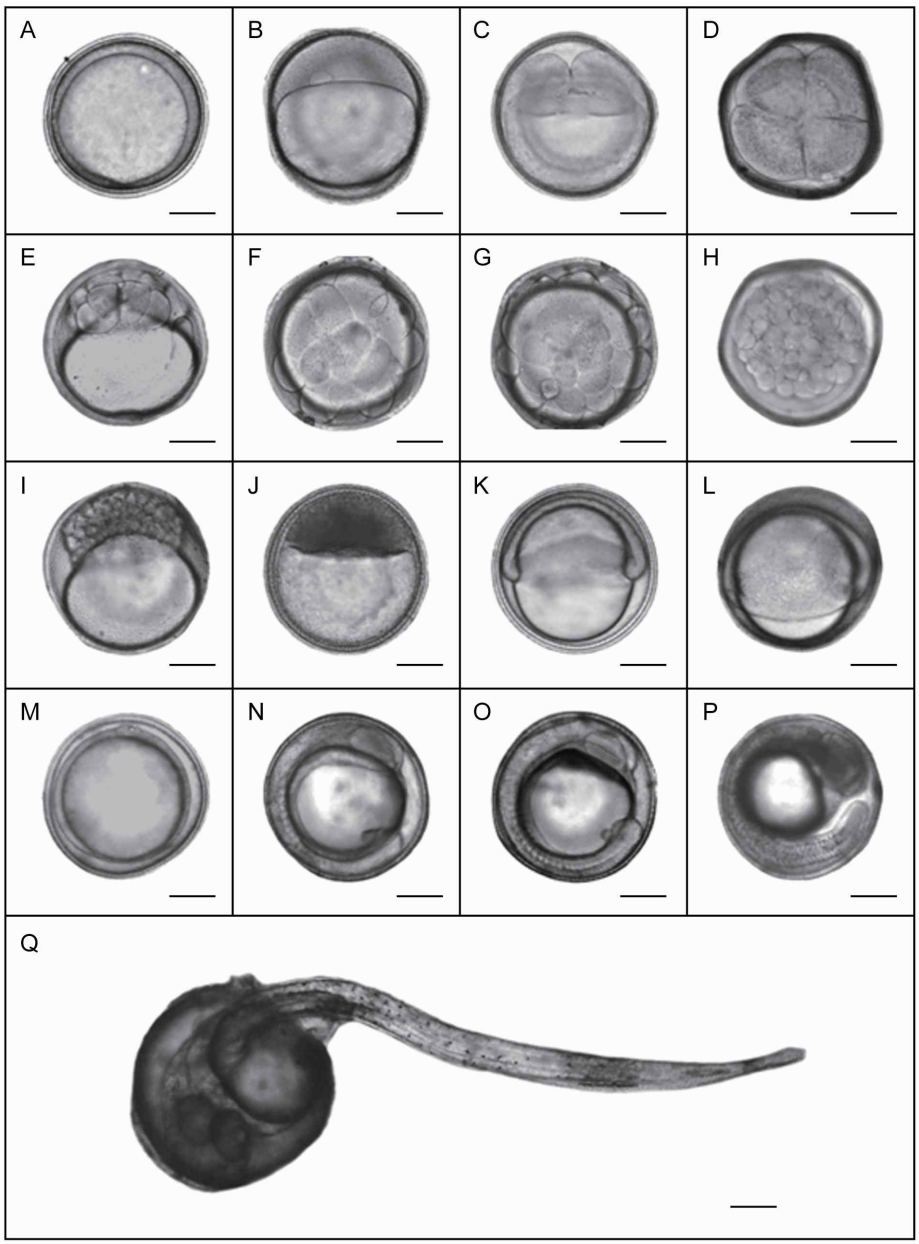

Fig. 4. The eggs development of the black plaice, *Pleuronectes obscurus*. Each developmental stages are detailed in Table 2. Bars are 200 μm.

6 and 10°C.

Overall, our study demonstrated clear differences in both the timing of the first cleavage and mitotic intervals at different temperatures in black plaice. The data provided here will be useful for the development of an optimal treatment protocol for chromosome manipulation. Furthermore, data on egg development will be valuable for use in a biological aquaculture database. Future studies should focus on seeding production techniques via artificial fertilization, early life history, and other characteristics of black plaice.

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current laws of Korea.

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