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Circadian Variation of the RNA/DNA Ratios in Marbled Flounder *Pleuronectes yokohamae*

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ABSTRACT **Laboratory-reared marbled flounder (*Pleuronectes yokohamae*) juveniles (23.2±0.2 mm total length; age 89 day) were sampled over a 48-h period to determine any diel patterns in RNA/DNA ratios. RNA/DNA ratios were highest during daytime periods (0800, 1100, 1400, 1700 h) and significantly reduced at night (2000, 2300, 0200, 0500 h). Findings from this study indicate a diel variation in biochemical condition and suggest that special care should be taken in designing sampling plans, including sampling time and data analysis to account for this source of variability.**

Key words : Marbled flounder, *Pleuronectes yokohamae*, RNA/DNA ratios, circadian variation

INTRODUCTION

The Marbled flounder *Pleuronectes yokohamae* is commercially important species in Korea, but its resources has been decreased recently. For a restocking of this species, artificial propagation has been initiated and a part of them was released into coastal waters of Korea. To maximize the likelihood of post-release survival rate of hatchery-reared fish, more fundamental approaches examining whether the hatchery-reared fish is suited for a life in the sea, and the comparison of the differences between the reared and indigenous fish in a variety of respects should be carried out prior to consideration of the relation with biotic and abiotic factors in its habitats. A variety of techniques such as morphometric, histological, and biochemical analyses for diagnosing the nutritional condition of fish larvae and juveniles have been developed and applied to both laboratory-reared and wild fishes (Buckley, 1979; Theilacker and Watanabe, 1989; Gwak *et al*., 1999). One biochemical analysis, the ratio of RNA to DNA, has been proven to be a useful and reliable indicator of nutritional condition (Buckley, 1980) and growth of larval and juvenile fish (Gwak and Tanaka, 2002). The quantity of deoxyribonucleic acid (DNA) is believed to be normally stable under the changeable environment. The quantity of ribonucleic acid (RNA, primarily associated with ribosomes) is closely related to the rate of protein synthesis. Since both larval and juvenile growth is dependent upon protein synthesis, the RNA/DNA ratio has been shown to have high sensitivity to feeding levels, and can be used as an index of fish growth.

Some studies have revealed that there are several sources of environmental variability which influence biochemical condition in fish, these include biotic sources such as food availability (Clemmesen, 1994; Gwak and Tanaka, 2001), and maternal origin (Heyer *et al*., 2001). Some abiotic sources of variability such as water temperature (Buckley, 1982; Clemmesen, 1996), and salinity (Juerss *et al*., 1987) have also been identified. However, variable results in diel variations in RNA/DNA ratios have been reported. Rooker and Holt (1996) reported a characteristic diel pattern in RNA/DNA ratio of laboratory-reared larval *Sciaenops ocellatus*. Gronkjaer *et al*. (1997) also documented the influence of sampling time on RNA/DNA ratio of *Gadus morhua* larvae sampled in the Baltic. However, Bailey *et al*. (1995) was unable to demonstrate a diel effect on RNA content using SL as covariate with *Theragra chalcogramma* larvae. Diel periodicity in RNA/DNA ratio could possibly produce strong confounding effects on the interpretation of the results, if such variations can be demonstrated. Therefore, this study was designed to examine diel variations in RNA/DNA ratios of juvenile marbled flounder *P. yokohamae*. Information from this study will assist in designing sampling plans, including sampling time for future condition studies on both laboratory-reared and wild-caught *P. yokohamae* juveniles.

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MATERIALS AND METHODS

1. Experimental fish and sampling

Artificially fertilized eggs were obtained from mature male (35.1 cm TL) and female (40.3 cm TL) o*f P. yokohamae* caught by the set net. Artificially hatched larvae were reared at the Gyeongsangnamdo Fisheries Resources and Research Institute. Larval rearing was conducted in an indoor transparent polycarbonate tanks (500 L) at 13.0 ±0.5˚C under natural photoperiod conditions. The larvae were fed rotifers (*Brachionus plicatilis*) cultured with *Nannochloropsis oculata*, for 18 days after first feeding, and then supplemented with *Artemia* nauplii enriched with squid liver oil. Before start of the experiment, the juveniles were transferred to 300 L polycarbonate tanks after size selection and fed to satiation three times per day (0900, 1300 and 1700 hours) on *Artemia*. Experiment was carried out under the light/dark regimen (0700 ~1800, day; 1800~0700, night). Average light intensity was about 258 lux.

To determine any diel patterns in RNA/DNA ratios, 89 day old laboratory-reared juveniles were sampled at 3 h intervals over a 48 h period beginning at 0800 hours. To determine survival, dead fish were counted everyday during the experiment. At each sampling interval, ten juvenile fish were randomly sampled from two replicate 300 L tanks, anesthetized with tricane methanesulfonate (MS222) and measured (total length) to the nearest 0.1 mm on ice. Gut contents were removed to avoid the effects of food organisms on RNA/DNA ratios. Samples were stored at −80˚C until later analysis.

2. Quantification of nucleic acids

All chemicals used in the RNA/DNA analyses were analytical grade: DNA from salmon sperm (Wako Chemicals, Osaka, Japan), RNA from yeast (Kanto Chemicals, Tokyo, Japan), RNase from bovine pancreas (Nacalai Tesque, Inc., Kyoto, Japan), and Ethidium Bromide (Nacalai Tesque, Inc., Kyoto, Japan). The methodology described in Clemmesen (1988) was used with slight modifications. Tris-EDTA buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, adjusted to pH 8.0 with HCl) was added to 10 mL glass homogenizer placed in 4˚C ice-cold water, and the whole body was completely homogenized. The aliquot for RNA and DNA contents was centrifuged at 6,000 rpm for 10 min at 4˚C after Phenol treatment. The quantity of RNA and DNA in the whole body was determined individually by using a nucleic acid specific fluorescent dye method developed by Clemmesen (1993), and slightly modified by Sato *et al.* (1995). Total nucleic acid contents (RNA+DNA) were determined fluorometrically with Hitachi 650-10LC (excitation, 360 nm; emission, 590 nm) by adding 50 μL EB-buffer solution. DNA contents were determined in the same way after another 150 μL aliquot was enzymatically digested with 20 μL RNase for 30 min at 37˚C. Salmon sperm DNA (Wako pure Chem. Co. Ltd.) and yeast RNA (Kanto Chem. Co. Ltd.) were used as standards.

3. Statistical analysis

The effects of day and night (0700~1800, day; 1800~0700, night) and time-of-day on RNA/DNA ratios were examined by two-way ANOVA. A one-way ANOVA was used to examine diel variations in RNA/DNA ratios. Significant ($P<0.001$) results were examined further using Tukey's HSD multiple-comparison test to determine which levels of the main effect(s) differed from other levels. Student's *t*-test was used to compare the value in RNA/DNA ratios and total length between daytime and nighttime. Significance was accepted for $P<0.05$. All statistical analyses and data presentations were carried out using STATISTIX 7.0 for Windows (Analytical Software, Tallahassee, Florida, USA).

RESULTS

Highly significant differences in RNA/DNA ratios were observed over a 48 h period ($P<0.001$) (Fig. 1). The effects of day and night hours and time-of-day on RNA/DNA ratios examined by a two-way ANOVA indicated that the RNA/DNA ratios were significantly influenced by both of them ($P<0.001$) (Table 1). The one-way ANOVA followed by the Tukey test ($P<0.001$), revealed that RNA/DNA ratios remained relatively high during daytime (0800, 1100, 1400, 1700 hours). Peak values were observed at 1400 hour on the first day and 1100 hour on the second. Significant declines in RNA/DNA ratios were observed at night (2000 hour) on the first day and remained relatively low during nighttime (2300, 0200 hours). Similar fluctuations in RNA/DNA ratios were observed on the second day. Comparisons of mean RNA/DNA ratios shows that the values were significantly higher ($P<0.05$) during daytime (3.95 ± 0.49, n=80) than those of nighttime (2.44 ± 0.28, n=80). There was no significant difference ($P>0.05$) in total length between daytime (23.1 ± 0.2 mm, n=80) and nighttime (23.2 ± 0.3 mm, n=80). No dead fish were observed during the experiment.

DISCUSSION

The aim of this study was to evaluate the diel variation of RNA/DNA ratios in *P. yokohamae*. These data demonstrate that a circadian rhythm in RNA/DNA ratios do occur in laboratory-reared juvenile *P. yokohamae*. A similar positive relationship between sampling time and RNA/DNA ratios has been reported for immature rainbow

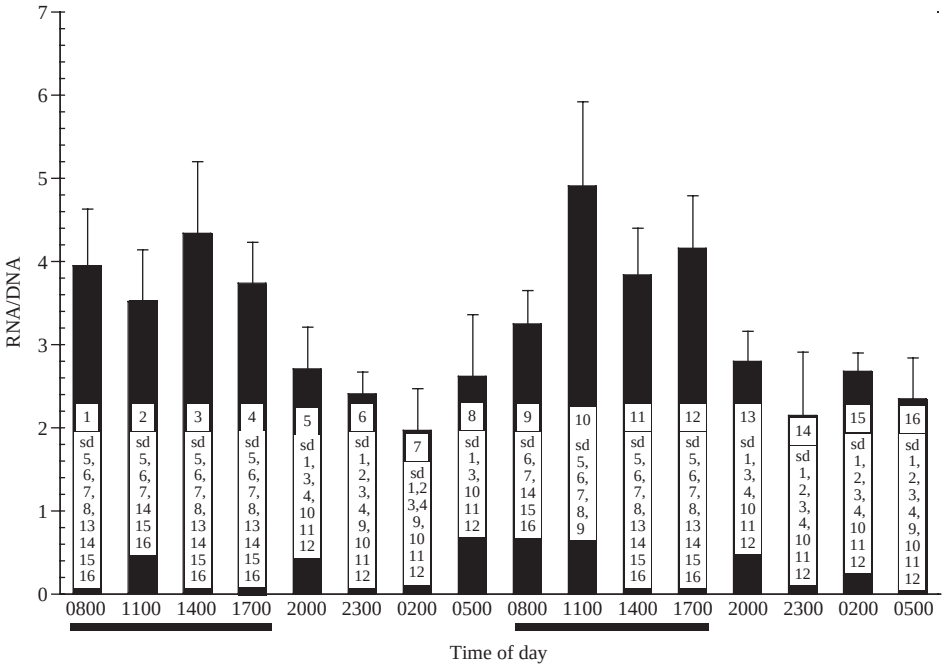

Fig. 1. Diel variations in RNA/DNA ratios of *Pleuronectes yokohamae* juveniles over the 48 h period. Values are means (10 individuals) ± standard deviation. Numbers in each bar show the significant differences (sd) between the 16 samples (Tukey's test, $P < 0.001$). The dark bar indicates the light period.

Table 1. Summary of a two-way ANOVA for combined data, fixed effect of RNA/DNA

Ind. var.	*df* effect	MS effect	*df* error	MS error	*F*	*P*-Level
1	1	45.289	109	15.029	223.19	0.001*
2	5	7.497	109	15.029	9.16	0.001*
1 × 2	5	0.92	109	15.029	0.18	0.35

1=day and night (0700 ~ 1800, day; 1800 ~ 0700, night), 2=time (h). Ind. var.: independent variable; df: degrees of freedom; MS: mean square.
*$P < 0.001$

trout *Oncorhynchus mykiss* (Mugiya and Oka, 1991), and larval red drum *S. ocellatus* (Rooker and Holt, 1996). They reported that high ratios during daytime periods were followed by a steep decrease at night. The present study on juvenile *P. yokohamae* also indicated that high RNA/DNA ratios were present during daytime sampling periods (0800, 1100, 1400, 1700 hours) and significant reductions occurred at night (2000, 2300, 0200, 0500 hours). In *Sardina pilchardus* Chicharo *et al*. (1998) also suggested that in twilight and early hours of the night the values of RNA : DNA were significantly higher. However, with *Clupea harengus* larvae, Clemmesen (1996) found no diel pattern in RNA/DNA ratios. Thus, this difference may be a specific characteristic of this particular species. The presence of a strong diel pattern in RNA/DNA ratios of warm water species compared to those of cold water species might be caused by differences in metabolic rates, food requirements and digestion times between them (Buckley *et al*., 1999).

Two possibilities could explain the results from the present study: (1) the effect of temperature on RNA/DNA ratios, (2) circadian periodicities in endocrine activity (mainly growth-regulating hormone). Temperature influences physiological processes and has been shown to effect RNA/DNA ratios (Buckley, 1982; Mathers *et al*., 1992). However, Gwak (1999) reported that Japanese flounder larvae and juveniles reared under temperatures (14 ~ 22°C) with enough food showed no significant difference in RNA/DNA ratios. Therefore, temperature effects caused by daily differences (1°C) between day and night can be disregarded. Circadian periodicities in cell division rate or growth-regulating hormone has been reported in plasma and pituitary levels in salmonids (Leatherland *et al*., 1974; Bates *et al*., 1989), insects (Lee *et al*., 1996), and rats and sheep (Coon *et al*., 1995). A primary role of growth hormone is the stimulation of somatic growth. This function is apparently achieved by active protein synthesis which is closely related to increased amino acid incorporation into protein, and increased number of rRNA in the muscle and their activity (Lied *et al*., 1982; Loughna and Goldspink, 1984). In fact the same diel patterns were observed for the bivalves *Ruditapes decussatus* and *Cerastoderma edule* (Chicharo *et al*., 1998). Therefore, the second explanation on diel variations in RNA/DNA ratios appears more plausible.

Moreover, Gutierrez *et al*. (1984) reported that these rhythms are influenced by and vary depending on photoperiod. The present results also show significant differences in RNA/DNA ratios between daytime and nighttime. Accordingly, it could be postulated that photoperiod probably play some role in producing diel variations in RNA/DNA ratios. The effects of feeding on diel variations in RNA/DNA ratios during this experiment could be disregarded because the RNA/DNA ratios do not reflect increase in prey availability over a period of hours (Clemmesen, 1994). The influence of juvenile length on these results should also be avoided, because some authors (Suthers, 1992; Clemmesen, 1994; Rooker and Holt, 1996; Suthers *et al*., 1996) argue that the RNA/DNA ratio increases with body length. In any event, the present study selected juvenile fish of similar lengths.

Considering the findings of the present study, care should be taken when using RNA/DNA ratio as a condition measure of juvenile marbled flounder in the field of fisheries recruitment and aquaculture. Further studies should be undertaken to confirm this characteristic diel pattern with field-collected fish.

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문치가자미 RNA/DNA의 일주기적 변화

곽우석

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요 약 : 문치가자미 *Pleuronectes yokohamae*의 RNA/DNA비 일주기 변화를 조사하기 위하여 부화후 89일, 전장 23.2±0.2 mm의 치어를 48시간 동안 일정한 시간 간격으로 채집하였다. RNA/DNA비는 주간(0800, 1100, 1400, 1700)에 높았고 야간(2000, 2300, 0200, 0500 hours)에는 낮게 나타났다. 이번 연구결과에서 문치가자미의 생화학적 일주기 변화를 확인하였고, 이와 같은 RNA/DNA비의 일주기적 변화를 고려하여 시료 채집시간 결정을 포함한 시료채집 계획작성과 data분석이 필요할 것으로 생각된다.

찾아보기 낱말 : 문치가자미, *Pleuronectes yokohamae*, RNA/DNA 비, circadian variation