J. Fish. Sci. Tech. 5(3), 191~199, 2002

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Endogenous Rhythm in Oxygen Consumption by the Pacific Oyster *Crassostrea gigas* (Thunberg)

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(Received April 2002, Accepted August 2002)

Pacific oysters *Crassostrea gigas* (Thunberg) were collected on April, 1999 and March~September, 2000 from Goseung Bay along the southern coast of Korea. The oysters tested collected from a depth of 0.5~2 m in which they cultured by a long line hanging method. The oxygen consumption rates (OCR) of oysters held under constant temperature and darkness (CC), were determined using an automatic intermittent-flow-respirometer (AIFR). Depending on holding periods after oyster collection, the experiments were divided into two groups: Group 7-d (held to ambient temperature for ca. 7 days) and Group 21-d (held to ambient temperature for ca. 21 days). The OCR for Group 7-d single oyster displayed two peaks every day under CC, while Group 21-d single oyster showed one peak every day. It is likely that the rhythmic patterns (12.6~12.8 hours) of the OCR in the Group 7-d single oyster may have been influenced by tidal currents at the sampling site. The rhythmic patterns (24.3~24.7 hours) in the Group 21-d single oyster may have been shifted from two peaks to one peak each day under CC. The present study concludes that the OCR rhythm of wild oysters in nature is governed by two lunar-day clocks (24.8 hours); one driving one peak and the other driving the second peak. When oysters are subjected to the long-term CC conditions, one of the two-clock systems is depressed or only intermittently becomes active. However, the OCR rhythms by two to three oysters occurred arrhythmic patterns during the experiments and exhibited some evidence of weak rhythmicity of compared to those of a single oyster. It could be partly due to differences group effects.

Key words: Pacific oyster, *Crassostrea gigas*, Oxygen consumption, Endogenous rhythm, Circalunidian rhythm

Introduction

Many marine organisms possess endogenous rhythms which enable them to synchronize their behaviour and physiology with cyclical changes in the environment. Most of the early studies of bivalve rhythms have focused on the coincidental events of the behaviour and physiology of freshly-collected specimens with natural environmental conditions (Zann, 1973a; 1973b; Beentjes and Williams, 1986; Ameyaw-Akumfi and Naylor, 1987; Palmer and Williams, 1993). However, most of these species are found in the intertidal zone.

Tidal rhythms are entrained by cues associated with tides, salinity, temperature, hydrostatic pressure and mechanical agitation (Morgan, 1996; Palmer, 1996). The tidal rhythms of bivalves are mainly controlled not by exogenous factors but by intrinsic or endogenous clocks with the known exception of the New Zealand cockle *Chitone stutchburyi* (Beentjes and Williams, 1986). Naylor (1976) and Ameyaw-Akumfi and Naylor (1987) stated that sessile intertidal animals are less likely than motile species to have evolved endogenous control of tidal rhythmicity; there would be less adaptive advantages for sessile species to develop anticipatory responses to tidal rises and falls. Manila clam, *Ruditapes philippinarum,* removed from their natural environ-

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ment and maintained for nine weeks in continuously immersed conditions exhibited a clear endogenous circatidal rhythm in oxygen consumption (Kim et al., 1999).

Many researchers have attempted to demonstrate the existence of diurnal and tidal rhythms in the metabolic activity (filtration, digestion, etc.) of oyster species as reviewed by Morton (1971) and Higgins (1980). The digestive processes of the oyster, *Crassostrea gigas*, which lives on intertidal mud flats, are discontinuous and related to the tidal cycle as reported by Morton (1977). However, subsequent attempts by several researchers to replicate these results have been unsuccessful. Palmer (1980) working with the American oyster, *C. virginica*, found no evidence for endogenous control of filtration rate. Higgins (1980) also reported that a time series analysis of valve movements showed that oysters exhibited a 24-h periodicity of valve activity when subjected to a 24-h feeding schedule but exhibited no consistent periodicity under continuous feeding or unfed conditions. Therefore, it appears that the evidence for endogenous activity rhythms in oysters is inconclusive. Although several studies have been conducted to determine oxygen consumption rhythms in oysters (Shumway, 1982; Shumway and Koehn, 1982; Bougrier et al., 1998), data on the rhythmic patterns of oxygen consumption by oysters in the subtidal zone are still rare.

The purpose of the present study was to test the occurrence of an endogenous circalunidian rhythm in oxygen consumption of *C. gigas* collected in subtidal zone, using an automatic intermittent-flow-respirometer (AIFR) which allows for long-term measurements.

Materials and Methods

Experimental animals

Pacific oysters, *C. gigas*, were collected on April, 1999 and March~September 2000 from Goseung Bay (tidal currents of 40~130 cm/sec at the ebb and 20~120 cm/sec at the flood occur each day) (MMAF, 2000) along the southern coast of Korea. Mean water depth of the sampling area was 5 m, and the area was not exposed during low tide. The oysters used in the present study were reared using a long line hanging culture system and continuously submerged during the growing period. The oysters collected from a depth of 0.5~2 m were transported to the laboratory immediately (within 6-h) by plane. All periphytes (including polychaetes, barnacles etc.) attached to the oysters were removed using a brush. Oysters were then kept continuously immersed for 7 to 21-d in a holding tank (50 cm deep and 500-L) under the laboratory conditions of 12-h (L) light and 12-h dark (D) cycle.

Depending on holding time after collection, the experimental animals were divided into two groups: Group 7-d and Group 21-d. Group 7-d oysters were held to laboratory conditions for ca. 7-d before the experiment. Group 21-d oysters were held to laboratory conditions for ca. 21-d before the experiment. Seawaters in the holding tank were changed twice a week. Oysters were not fed during holding and throughout the experiments in order to exclude effects on oxygen consumption due to feeding and digestion. Measurements of oxygen consumption rates (OCR) were conducted in 22 replicated experiments for 37 individuals (Table 1). One to three oysters were placed in a respirometer at one time. After each trial, these oysters were removed and the shell lengths measured. Shell length (SL) of the experimental oysters, defined as the longest distance between the anterior and posterior margin (Quayle, 1988), were measured with Vernier-calipers to 0.1 mm. Oyster meat was dried in an oven at 80℃ until a constant weight was reached and weighed to the nearest 0.01 g.

Table 1. Experimental parameters, mean shell length (cm) and somatic-tissue weight (g DW) of the oysters, *Crassostrea gigas*, under laboratory conditions (mean ± SD, where given)

	Group 7-d	Group 21-d
Mean shell length (cm)	7.9 ± 1.2	7.4 ± 1.0
Somatic-tissue weight (g DW)	2.1 ± 0.9	1.6 ± 0.3
Temperature (℃)	19.2 ± 0.4	18.8 ± 0.3
Salinity (PSU)	32.4 ± 0.8	32.5 ± 0.1
Duration (h) of experiments	90.1~425.6	120.3~170.4
Number of individuals (n)	16	21
Number of experiments (N)	10	12

Experimental design

OCRs were measured for 90.1 to 425.6 hours, using an automatic intermittent-flow-respirometer (AIFR, one system with two chambers). Bacteria

were filtered from the water used in experiments by sterile membrane filters (with two Sartorius Capsule Filters, input-pore diameter 0.2 μm and output-pore diameter 0.07 μm). Background oxygen consumption of bacteria was measured by running blanks (i.e., without oyster) throughout the experimental period. Oxygen levels in the 0.3-L (for a single oyster) and 1.4-L (for two to three oysters) experimental chamber were maintained between 85% and 95% of saturation to minimize any physiologic stress on the oysters due to hypoxia. When the oxygen level dropped below the predetermined limit, the magnetic drive gear pump and 3-way magnetic valve (332F, Nortec, Germany) automatically flushed the chamber with oxygen-saturated seawater from a 20-L storage tank until the selected oxygen level was reached. No measurements were taken during this flushing. After each experiment, the chamber was rinsed with oxygen-saturated water and the probe voltage was examined to ascertain whether it had deviated from the pre-experiment gauge voltage. After calibrating the oxygen probe (15 μ PO_2, Eschweiler, Germany), data collection began and was controlled throughout the experiment automatically by a computer. Measurements were taken in an incubator (RI-50-1060, REVCO, USA) under constant darkness and temperature (Tables 1, 2). The magnetic drive gear pump (MS-Z, Ismatec Sa, Switzerland) supplied horizontal water at 690 mℓ min^{-1}. The digital controlling unit recorded the oxygen level through a picoammeter every second. Mean OCR (mOCR) values for the test oysters were calculated and displayed graphically at 90-second intervals. All data, including local and experimental time (seconds), temperature (℃), air pressure (hPa), oxygen consumption (mℓ O_2 h^{-1}), and oxygen levels (%), were stored directly on a hard disk for future analysis. Oyster OCR was calculated from the changes in oxygen saturation level in the test chambers with time. Saturation concentration, KO_2 (mℓ l^{-1}), was calculated for standard conditions (atmospheric pressure P_{atm} = 1 atm = 1,013 mbar) as a function of temperature and salinity according to Weiss (1970). More detailed descriptions of calculations methods and schematic illustration of the apparatus are given in Kim et al. (1996, 1997, 2001).

Table 2. Experimental parameters and oxygen consumption rate of *Crassostrea gigas* under laboratory conditions (1~3 specimens of the oysters were measured for about 144.0~169.9-h). Statistical values were calculated for each batch from the 4,154 to 5,593 data points (mean ± SD, where given)

	Group 7-d	Group 21-d	
	A	A	B
Individual number (n)	1	1	1
Mean shell length (cm)	8.3 ± 0.5	9.8	8.6
Somatic-tissue weight (g DW)	1.1 ± 0.3	1.5	1.2
Duration (h) of the experiment	144.0	169.7	169.9
Number of points measured	4,154	4,955	5,593
Temperature (℃)	23.2 ± 0.8	21.8 ± 0.3	21.9 ± 0.3
Salinity (PSU)	30.4	29.0	29.0
Oxygen saturation level (%)	85.2~94.7	85.3~94.8	85.3~94.7
Mean oxygen consumption (mℓ O_2 g^{-1} DW h^{-1})	0.87 ± 0.62	1.02 ± 0.58	0.70 ± 0.36

*Oysters of Group 7-d and Group 21-d were held to laboratory conditions for ca. 7-d and ca. 21-d, respectively, following the collection and before subjecting to experiments.

Analysis of oxygen consumption records

Rhythmicity of OCRs was determined using a maximum entropy spectral analysis (MESA) of raw data transformed into 10-minute lag intervals. The time series were analyzed for periodicity using MESA spectra following the procedures and algorithms described by Dowse and Ringo (1989). Analysis of the OCR rhythms was performed using a 2% weighted smooth curve procedure. We used the locally weighted least squares error method (KaleidaGraphy custom program for Macintosh, Synergy Software) to plot a best-fit smooth curve through the center of the data. The value of 2% yielded the best-fit curve in repeated tests. Statistical values were computed for each batch of data points.

Results

Group 7-d (Oysters held to laboratory conditions for ca. 7-d after collection before subjecting to experiments)

The OCR for Group 7-d oysters exhibited rhythmicity throughout the single-oyster experiment. The observed frequency of rhythmicity was 12 cycles in 6-d (144.0-h) for a single oyster, indicating an approximately 12-h semidiurnal rhythmicity of oxygen consumption. The mean oxygen consumption rate

(mOCR) for the oysters appeared to gradually increase with time and temperature (Fig. 1, A). The single oyster mOCR averaged over the duration of the experiment and over the range of oxygen levels (between 94.4 and 85.4%) was 0.87 ± 0.62 mℓ O_2 g^{-1} DW h^{-1} (Table 2). MESA spectra of the data set presented in Fig. 1 indicated that the OCR peaks mainly occurred at 12.7-h intervals, which corresponds to a circatidal rhythm (Fig. 2, A). The OCR also showed minor peaks in shorter periods of 7.9-h intervals. The OCR by two oysters exhibited the arrhythmicity during the first few days (Fig. 1, B). There was some evidence of weak circatidal rhythm (Fig. 2, B).

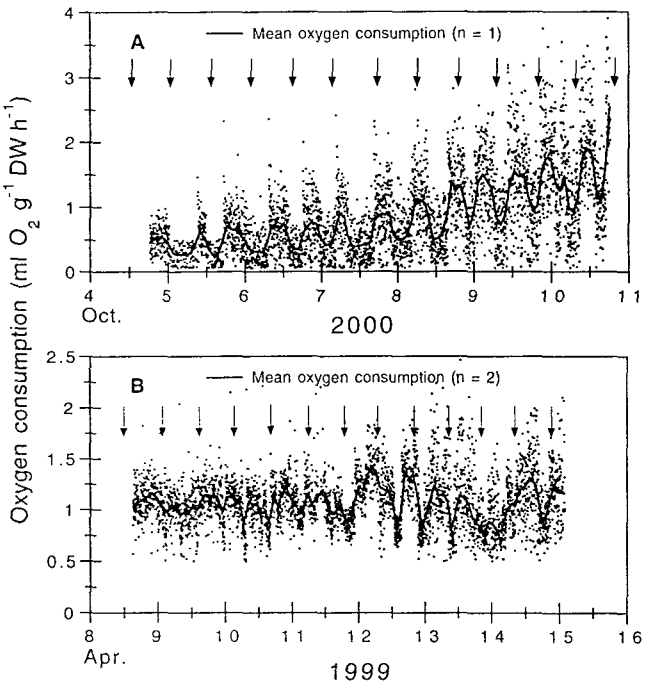

Fig. 1. Time series of the oxygen consumption rate (mℓ O_2 g^{-1} DW h^{-1}) for 144.0-h (A) and for 148.0-h (B) for oysters, *Crassostrea gigas*, held for approximately 7-d after collection before subjecting to experiments. Oysters were held at 23.2 ± 0.8℃ (A) and 20.4 ± 0.6℃ (B), and under constant darkness. The data represent semidiurnal patterns displayed by a single oyster (A) and by two oysters (B) during the experiment. The curve for the mean oxygen consumption rate through the centre of the data represent a weighted smooth curve of 2%. Dots represent the mean oxygen consumption rate during 90-s intervals. Arrows indicate scheduled times of high tide at the collection site.

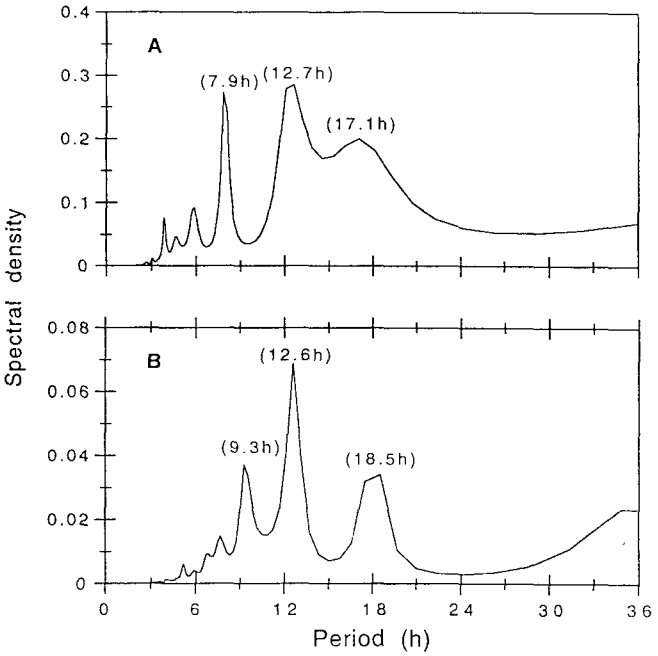

Fig. 2. Maximum Entropy Spectral Analysis (MESA) spectra for the data presented in Figs. 1A and 1B. Time periods (h) corresponding to the dominant peaks in the MESA plots are given in the parentheses.

Group 21-d (Oysters held to laboratory conditions for ca. 21-d after collection before subjecting to experiments)

The OCR of Group 21-d oyster exhibited rhythmicity throughout the experiments conducted under constant darkness and at 21.4 ± 0.5℃ (constant condition: CC). The oyster mOCR values measured from 17 October to 27 October 2000 were fitted to a weighted smooth curve of 2% (Fig. 3). The OCR was highly variable, ranging from 0.10 to 2.06 mℓ O_2 g^{-1} DW h^{-1}. The single oyster mOCR averaged over the duration of the experiment and the range of oxygen levels (between 94.4 and 85.4%) was 1.02 ± 0.58 (Group 21-d, A) and 0.70 ± 0.36 (Group 21-d, B) mℓ O_2 g^{-1} DW h^{-1} (Table 2). The results of two experiments covering 169.7-h (Fig. 3A) and 169.9-h (Fig. 3B) showed a relatively stronger pattern and longer time span in the 24 hours cycle. The OCR rhythm patterns of two data were somewhat differ for about 2-d during the experiments, even though the oysters were kept in the same laboratory. The

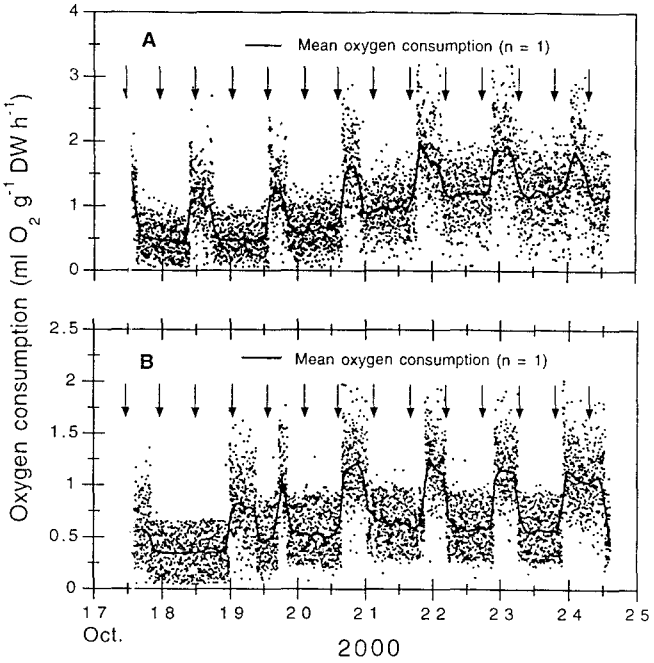

Fig. 3. Time series of the oxygen consumption rate (mℓ O_2 g^{-1} DW h^{-1}) during 169.7-h (A) and 169.9-h experimental period (B) by a single oyster, *Crassostrea gigas*, held for 21-d after collection before subjecting to experiments. Oysters were kept at 21.8 ± 0.3℃ (A) and 21.9 ± 0.3℃ (B), and they were kept under constant darkness. (A) and (B) represent diurnal patterns displayed by a single oyster during the experiments. Arrows indicate scheduled times of high tide at the collection site.

rhythm observed showed about 7 peaks during the experimental period of 169.7-h (7.1-d) (Fig. 3A) and a slightly delayed peak on the 2nd day of the replicate experiment (Fig. 3B). The widths of these peaks were about 7- to 9-h (mean 8-h) during the experiments. The low mean value OCR of the experimental oysters ranged from 0.52 to 1.22 mℓ O_2 g^{-1} DW h^{-1} during 16-h experiment, and the high mean values were estimated as 1.15 to 1.90 mℓ O_2 g^{-1} DW h^{-1} during 8-h experiment (Table 3). The MESA plots for the data presented in Figs. 3A and 3B indicated that the OCR peaks mainly occurred at 24.7- and 24.3-h intervals, which correspond to a cirlunidian rhythm (Figs. 4A, 4B). The OCR also showed minor peaks in the short period of 16.6-h interval. The OCR by three oysters exhibited the arrhythmicity (Fig. 5). There was some evidence of weak 24 hours rhythm (Fig. 6), although they exhibited small peaks (11.6-h and 17.4-h).

Table 3. Mean oxygen consumption rates (mOCR) of the oyster, *Crassostrea gigas*, were calculated by the difference of high value for 8-h and low value for 16-h of the OCR measured during the experimental periods (± SD)

	Mean oxygen consumption rate (mOCR) (mℓ O_2 g^{-1} DW h^{-1})			
	Fig. 3A (X)		Fig. 3B (Y)	
	Low value	High value	Low value	High value
I	0.52 ± 0.30	1.15 ± 0.43	0.65 ± 0.22	1.19 ± 0.28
II	0.61 ± 0.27	1.28 ± 0.42	0.60 ± 0.19	1.22 ± 0.28
III	0.86 ± 0.34	1.63 ± 0.48	0.58 ± 0.20	1.19 ± 0.26
IV	1.14 ± 0.43	1.87 ± 0.53	0.61 ± 0.27	1.10 ± 0.28
V	1.20 ± 0.30	1.90 ± 0.56		
VI	1.22 ± 0.45	1.81 ± 0.54		

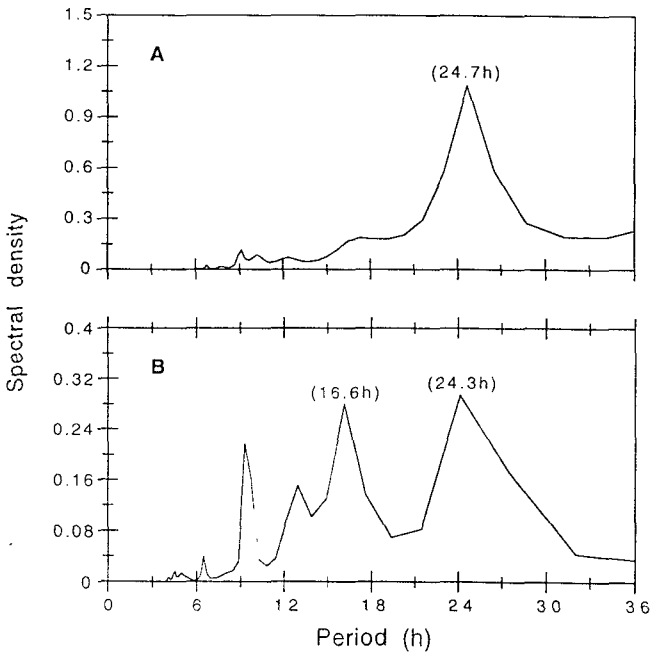

Fig. 4. Maximum Entropy Spectral Analysis (MESA) spectra for the data presented in Figs. 3A and 3B. Time period (h) corresponding to the dominant peaks in the MESA plots are given in the parentheses.

Discussion

The mOCR (0.7~1.02 mℓ O_2 g^{-1} DW h^{-1}) of the oyster *C. gigas* in this study differs from some other oxygen uptake protocols of *C. virginica* (reviewed by Willson and Burnett, 2000) in that OCR was used as the closed or open flow system instead of AIFR system. It could be partly due to differences

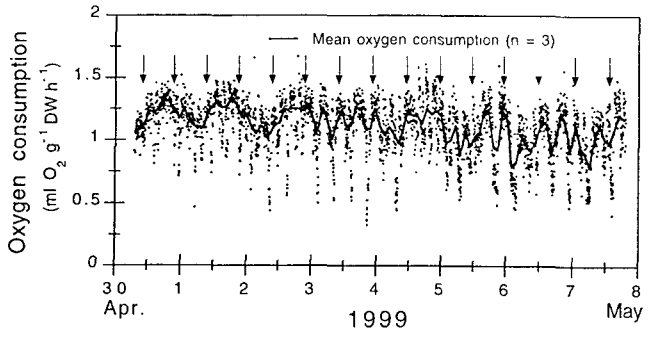

Fig. 5. Time series of oxygen consumption rate (mℓ O_2 g^{-1} DW h^{-1}) during 207.1-h by three oysters, *Crassostrea gigas*, held for 21-d after collection before subjecting to experiments. Oysters were kept at 21.2 ± 0.7℃ and they were kept under constant darkness. Arrows indicate scheduled times of high tide at the collection site.

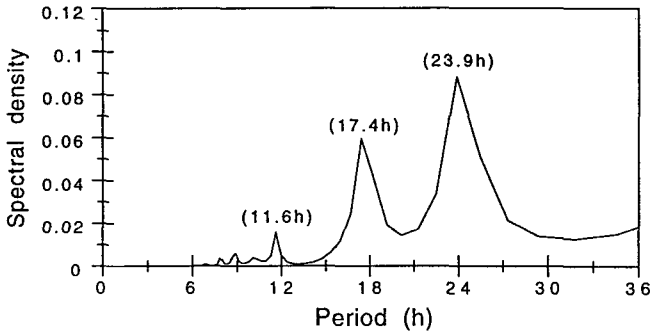

Fig. 6. Maximum Entropy Spectral Analysis (MESA) spectra for the data presented in Fig. 5. Time period (h) corresponding to the dominant peaks in the MESA plots are given in the parentheses.

in metabolic activity among species and experimental methods. Most respirometers (closed and flow-through) are incapable of correlating oxygen uptake with defined activity patterns, which limits their usefulness in bioenergetic studies (Wrona and Davis, 1984).

In the present study, *C. gigas* were protected from extrinsic factors such as light, food, temperature, tide, which could affect their rhythmic activities. Nevertheless, the OCR of the Group 7-d oysters (held for 7-d) exhibited a strong 12.4 hours cycle mode during the experiments under CC. It is likely that semi-diurnal patterns of the OCR in the Group 7-d oysters may have been influenced by tidal currents at the sampling site where tidal currents of 40~130 cm/sec at the ebb and 20~120 cm/sec at the flood occur each day (MMAF, 2000). However, the OCR of the Group 21-d oysters (held for 21-d) exhibited a strong 24.8 hours cycle mode during the experiments under CC. This may indicate that the "oscillator" was shifted from two peaks to one peak each day in the same physiological process.

In this study, the OCR of two to three oysters exhibited highly variable and arrhythmicity or minor peaks compared to those of a single oyster, although there are a weak 12.4 and 24 hours rhythms. However, the OCR of three Manila clams, *Ruditapes philippinarum*, intertidal-dwelling species, exhibited a circatidal rhythmicity under CC, which corresponds with a periodicity of 12.1 to 12.6-h (Kim et al., 1999). It could be partly due to differences among individuals, group effects, or the habitat of species. Ameyaw-Akumfi and Naylor (1987) stated that the rhythmicity of intertidal organisms, especially sessile species, is likely to be an exogenous rather than endogenous response to tidal fluctuations because there would be little adaptative advantage for sessile species to develop anticipatory responses to rise and fall of tides.

The periodic points of the OCR by a single oyster apparently moved or changed with time as shown in Figures 3A and 3B. However, periodic and regular peaks were recorded for the Washington clams, *Saxidomus purpuratus*, collected from sublittoral zones during about 15 days of observation suggesting that the Washington clams have different physiology from oysters. The observed OCR rhythmic periods of Washington clam did not shift the predicted 50 minutes each day, and the periodic point was relatively consistent (Kim et al., in press). The difference may have risen from the dissimilar environmental conditions of collection sites for those shellfish, i.e., Washington clams were collected from the seabed of 7 m water depth.

The clock hypotheses in marine organisms have been intensively reviewed by Palmer (1995a). For example, Barnwell (1968) reported that when two species of Caribbean crabs, *Uca mordax* and *U. minax*, were transported overland to the Pacific, they displayed two activity peaks per day in the laboratory. Also, three patterns in the swimming activity of the sand-beach isopod, *Excirolana chiltoni*, were observed; diurnal activity, semi-diurnal equal activity, and semi-diurnal unequal activity (Klapow, 1972). Several hypotheses have been raised to ex-

plain these phenomena (Enright, 1975; Webb, 1976; Bolt and Naylor, 1985; Palmer, 1996). However, there are disagreements as to whether a circadian and a circatidal rhythm are present separately in an organism (Webb, 1976), or whether circatidal or combined circatidal and circadian rhythmicities are governed by a single bimodal circadian clock with two peaks per cycle (Enright, 1975).

Another hypothesis (circalunidian rhythm) is that lunar rhythms, which consist of two peaks every day, are governed by two lunar-day clocks; one driving one peak and the other clock driving the second peak (Palmer, 1995a; 1995b; 1996; 1997). Morgan and Iwama (1990) stated that changes in metabolic rate in relation to an endogenous rhythm would be very difficult to measure accurately. So far, therefore, there is controversy related to the theories of circadian, circatidal and circalunidian rhythms (Palmer, 1995b; 1997; Naylor, 1996; 1997; Aldrich, 1997; Williams, 1998). Aldrich (1997) stated that as the cases rest without disproof of those theories, it is necessary to compile more data gathered in consistent manner, and carry out more analyses using those data for conclusive descriptions.

Endogenous rhythm of freshly collected wild oysters subjected to CC environment for 7 days, in the present study, was 24.8 hours with two physiological clocks. That is to say a two-clock system, X-clock and Y-clock governed the oysters' respiration rhythm (Fig. 7, upper panel). Moreover, when both physiological clocks, X-clock and Y-clock, were in active coupling mode, then, the period between two periodic rhythmic points were 12.4 hours. However, when the oysters were held in the laboratory conditions for more than 21 days before their respiration rhythms were measured, apparently X-clock of a 24.8 hour-period was depressed or uncoupled in oyster physiology. It seemed that only Y-clock of 24.8 hours was in active mode (Fig. 7, middle panel). Further observation indicated that X-clock of oysters did not stay depressed but intermittently became active during the experimental period (Fig. 7, bottom panel). These observations were similar to a long-term observation study conducted on respiration activities of Washington clams (Kim et al., in press). Therefore, the results of this study suggest that respiration of the oysters have a circalunidian rhythm governed by two-clock endogenous rhythm

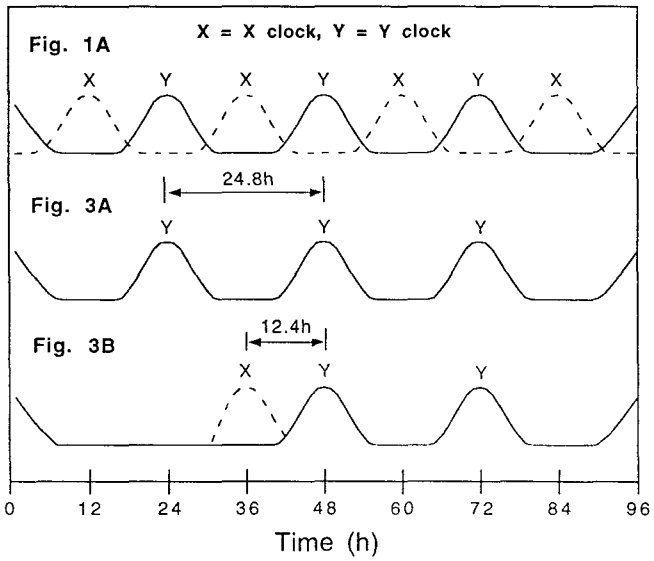

Fig. 7. The oysters, *Crassostrea gigas*, have a circalunidian rhythm governed by two-clock endogenous rhythm each having a 24.8 hour period (X-clock and Y-clock); one driving one peak and the other clock driving the second peak. The rhythm of respiration in oyster governed by two clocks (Fig. 1A). The rhythm of respiration in oyster governed by one clocks and one of the two-clock system depressed (Fig. 3A) or only intermittently becams active (Fig. 3B) under constant temperature and darkness.

each having a 24.8 hour period (X-clock and Y-clock); one driving one peak and the other clock driving the second peak.

The question is raised as to what are the possible advantages in changing rhythmic clocks from two peaks to one peak each day under CC ? It is generally accepted that the main function of a physiological clock is to alert an organism in advance of some periodic environmental event (Palmer, 1996). Therefore, we can postulate that when marine organisms are exposed to a long-term CC environment, one of the main functions of the clock mechanism may be to alter the rhythmicity of their physiological processes. The starved *C. gigas* adopted one peak OCR cycle from the previous two peaks each day to minimize energy loss under CC.

Acknowledgements

This study was supported by the Ministry of Science and Technology (MOST) of Korea under

the National Research Laboratory (2000-N-NL-01-16) project, and also supported the Ministry of Maritime Affairs and Fisheries' Special Grants for Korea Fisheries Research and Development Project.

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