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Nutritive Value of 'Tahiti *Isochrysis*' *Isochrysis* sp. for Larval Greasy Back Shrimp, *Metapenaeus ensis*

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The diatom, *Chaetoceros gracilis*, is extensively used as a nutritive food organism in the rearing of shrimp larvae in Japan. The large-scale outdoor culture of this species is, however, difficult during the summer season. To access the suitability of 'Tahiti *Isochrysis*' *Isochrysis* sp. (T-*Iso*) as a substitute for *C. gracilis*, we investigated the comparative nutritive value of these algae for larvae of greasy back shrimp, *Metapenaeus ensis*, by feeding experiments and fatty acids analyses. Further, T-*Iso* was cultured in large-scale outdoor tanks to determine its suitability for culture during the spawning season of *M. ensis* in summer.

Larvae fed on T-*Iso* only were considerably delayed in development as compared to those fed on *C. gracilis*. On the eicosapentaenoic acid, which is known as an essential fatty acid of Kuruma prawn *Penaeus japonicus* larvae, the content of T-*Iso* was less than that of *C. gracilis*. Thus, the nutritive value of T-*Iso* seemed to be lower than that of *C. gracilis*. However, the survival rate and the development of larvae fed on T-*Iso* and *C. gracilis* mixture were better than those of larvae fed on *C. gracilis* or T-*Iso* in single use. Further, T-*Iso* showed a steady and constant increase in density in both 100-*l* and 500-*l* outdoor tanks under high temperature conditions like *Tetraselmis tetrathele* and *Nannochloropsis oculata*. Therefore, T-*Iso* is useful as a supplemental food alga to *C. gracilis*, particularly during summer periods of high temperature and achieve a high survival rate and good growth of the shrimp larvae.

Key words: *Chaetoceros gracilis*, food organism, *Isochrysis* sp., larval rearing, *Metapenaeus ensis*

Introduction

Some species of the planktonic diatoms which are cultured using outdoor ponds are fed to shrimp larvae at the protozoeal (PZ) and mysis (M) stages. *Chaetoceros gracilis*, which is popular food diatom (Chu 1989), is generally used for rearing shrimp larvae. Many species of shrimp extensively spawn during the hot summer in Japan. However, it is difficult to prepare the enough diatoms being used as food of larvae under bad weather condition. Sometimes it leads to an insufficient food supply and starvation of the larvae. As a counter-measure against such a food deficiency, *Nannochloropsis oculata*, fresh water *Chlorella* and baker's yeast are commonly fed instead of diatoms despite their low nutritive value.

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Isochrysis sp., commonly referred to as 'Tahiti *Isochrysis*' (the alga is hereafter abbreviated to T-*Iso*), is a tropical flagellate and is used as food for bivalve larvae (Helm and Laing 1987). However, T-*Iso* has never been used as an food for shrimp larvae in Japan. T-*Iso* is nearly equal to other food diatom species in cell size and can be readily cultivated under high temperature (Kaplan *et al.* 1986, Boussiba *et al.* 1988). Therefore, we expected that the alga may be useful as a substitute food alga for shrimp larvae.

Sanchez (1986) confirmed that the survival rate and development of *Penaeus vannamei* larvae fed on T-*Iso* were superior to those of larvae fed on *Bacteriastrum hyalinum* and *Prorocentrum micans*. However, *B. hyalinum* and *P. micans* are not generally used as a larval food in Japan. In this study, we compared the survival and development of greasy back shrimp, *Metapenaeus ensis*, larvae fed on T-*Iso* or *C. gracilis* and investigated the growth of T-*Iso* by outdoor batch style culture in order to evaluate the feasibility of using T-*Iso* as a substitute food for shrimp larvae at the protozoeal and mysis stages.

Materials and Methods

The food value of T-*Iso* for the shrimp larvae was evaluated by two feeding experiments and by analyzing the fatty acid composition of the total lipids from cells of T-*Iso*, *C. gracilis* and from the bodies of *M. ensis* larvae. Further, T-*Iso* was cultured in 100-*l* and 500-*l* polycarbonate tanks to evaluate the possibility of mass-culture during the spawning season of the shrimp.

Feeding Experiment 1 *M. ensis* nauplii used in this experiment were hatched from eggs obtained from a single female. Six hours after hatching, vigorous nauplii were collected using pipettes and black plastic Petri dishes, and were randomly divided into six groups of 5,000 larvae each. Each group was held in a 30-*l* polycarbonate tank containing 25-*l* of filtered sea water with the food algae. Three groups were fed on T-*Iso* (test groups; I-1～I-3) and the other three groups were fed on *C. gracilis* (control groups; C-1～C-3). This experiment was terminated after seven days.

During the experiment, the cell densities of algae in all tanks were measured twice daily with a Coulter Counter (ZM Type) and adjusted to the fixed densities by adding food algae or by draining and adding filtered sea water. The rearing water temperature was kept at 25°C and air was continuously supplied at a rate of 400～500 m*l*/min per tank.

Both species of algae were cultured using 10-*l* glass carboys in a temperature and illumination controlled room (about 22°C and 80 $\mu Em^{-2}s^{-1}$ continuously). They were harvested during the stationary growth phase, and fed to the larvae. Before the experiment, diameters of both algae (about 14,000 cells) were measured using a Coulter Counter to compare the cell size.

At the end of the experiment, all living larvae in each tank were counted and the survival rates were calculated. Further, 100 larvae were randomly collected from each tank and examined under a microscope to identify their development following the morphological classification of Fudinaga (1942).

Feeding Experiment 2 The experiment was done using eggs obtained from a different female to experiment 1. Vigorous nauplii were randomly divided into six groups of 5,000 larvae. Two groups were

fed on a combination of T-*Iso* and *C. gracilis* (test groups; IC-1, -2) and the other groups were fed on either *C. gracilis* or T-*Iso* (control groups; C-1, -2, I-1, -2). The densities of the algae in each tank were adjusted to the fixed densities. Other methods are the same as the feeding experiment 1.

Analytical Methods of Fatty Acids T-*Iso* and *C. gracilis* were collected and centrifuged at 4,000 rpm. The larvae fed on each algal species or combined species were sampled by plankton nets after the experiment 1 and 2, and were washed twice with fresh water. These samples were stored at −80°C until analysis. Total lipids were extracted by chloroform-methanol according to Folch *et al.* (1957). The fatty acids of the total lipids were methyl-esterified and analyzed by gas-liquid chromatography (GLC; Shimadzu GC-9A). GLC operating conditions for determination of fatty acids are summarized in Table 1.

Table 1. GLC operating conditions for the determination of fatty acid composition

	Operating condition
Apparatus	Shimadzu gas-liquid chromatography GC-9A with a hydrogen flame ionization detector
Column	Thermon 3000 Ã, 50 m×0.25 mmØ
Temperature	Column: 200°C
	Injection port: 230°C
Carrier gas	Nitrogen
Flow rate	Nitrogen: 1.5 kg/cm^2
	Hydrogen: 0.6 kg/cm^2
	Air: 0.6 kg/cm^2

Outdoor Culture Experiments Outdoor culture experiments using 100-*l* tanks were conducted to compare the growth rate of T-*Iso* with that of *N. oculata* and *T. tetrathele*. Batch style culture was adopted for this trial. Twelve 100-*l* polycarbonate tanks containing 80-*l* of modified Guillard F medium (Guillard and Ryther 1962) were set in a sunny location. Each tank was covered with a polycarbonate plate and was continuously supplied with air at a rate of 8～10-*l*/min per tank. T-*Iso*, *N. oculata* and *T. tetrathele* which grew exponentially in a culture room were inoculated 4 replicates per species and were cultured for seven days. All cultures were unialgal but non-axenic. The cell densities of T-*Iso*, *N. oculata* and *T. tetrathele* at the beginning of the experiment were adjusted to about 20×10^4, 160×10^4, 5×10^4 cells/m*l*, respectively, so that the inoculated total cell volume of each alga was approximately equal. Estimates of the cell densities of all cultures were made daily using a Coulter Counter, and the growth rate was calculated from the following expression (Guillard 1973):

$$\text{Growth rate (divisions/day)} = \log_2 (N_t/N_0)/t$$

where N_t = final cell count, N_0 = initial cell count, and t = time (days).

In the 500-*l* scale trial, T-*Iso* was cultured in a polycarbonate tank with 500-*l* of modified Guillard F medium. The tank was set in a sunny location and air was continuously supplied by four aerators at a

rate of 8~10-*l*/min. Inocula were provided from a 10-*l* stock culture of T-*Iso* which had already grown to the maximum cell density of about 8×10^6 cells/m*l* in the culture room. The cell density immediately after inoculation in each trial was adjusted to about 3×10^5 cells/m*l*. Estimates of cell density and water temperature were monitored daily at 10 a.m. The trial proceeded for 7 days and was repeated four times during July to August.

Results

Survival and Development of Larvae Results of feeding experiments 1 and 2 are shown in Tables 2 and 3, respectively. The average survival rates and their standard deviations of larvae at the end of experiment in the T-*Iso* and control, *C. gracilis* groups were 73.7% (S.D.=6.4) and 64.4% (S.D.=7.0) in experiment 1, respectively. Results of the analysis of variance showed that the differences in survival rates between both groups were significant ($P<0.05$). The average survival rates were 67.1% (IC-1, 2), 54.7% (C-1, 2) and 49.7% (I-1, 2) in experiment 2. With regard to larval development, clear differences were observed between groups. The larval development in T-*Iso* groups was considerably delayed compared with that in *C. gracilis* groups. In experiment 1 (Table 2), 88% to 100% of larvae in *C. gracilis* groups developed to M3 stage at the end of the experiment, while 70% to 99% of the T-*Iso* groups were at M2 stage and 0% to 30% only had developed to M3 stage. The average survival rates of larvae in experiment 2 (Table 3) were 67.1% (IC-1 and IC-2), 54.7% (C-1 and C-2), and 49.7% (I-1 and I-2). Although almost all the larvae in the combined and *C. gracilis* tanks developed to M3 stage at the end of the experiment, the larvae in T-*Iso* tanks remained at the M1 or M2 stages.

Table 2. Survival rate and metamorphic stage of *Metapenaeus ensis* larvae fed on *Isochrysis* sp. [T-*Iso*; (I)] or *Chaetoceros gracilis* (C) at the end of the feeding experiment 1

Tank	Cell density of algae*1 ($\times10^4$ cells/m*l*)	Number of nauplii*2 (N/25-*l*)	Number of larvae*3 (N/25-*l*)	Survival rate (%)	Larval metamorphic stage*4		
					M1	M2	M3
I-1	13–15	5,000	3,450	69.0	0	70	30
I-2	13–15	5,000	3,558	71.2	1	73	26
I-3	13–15	5,000	4,052	81.0	1	99	0
C-1	10–12	5,000	3,550	71.0	0	0	100
C-2	10–12	5,000	2,854	57.1	0	0	100
C-3	10–12	5,000	3,257	65.1	0	12	88

*1 The densities of T-*Iso* (Tank I-1, I-2, I-3) or *C. gracilis* (Tank C-1, C-2, C-3) maintained during the experiment

*2 The number of *M. ensis* nauplii introduced to a tank at the start of the experiment

*3 The number of living larvae in a tank at the ending of the experiment

*4 The metamorphic stage distribution of 100 larvae collected from each tank at the end of the experiment (M1: Mysis 1, M2: Mysis 2, M3: Mysis 3)

Fatty Acid Compositions of Larvae and Food Algae The main fatty acid compositions of the total lipids of larvae and the food algae in feeding experiment 1 and experiment 2 are shown in Table 4. The

Table 3. Survival rate and metamorphic stage of *M. ensis* larvae fed on the combination of T-*Iso* and *C. gracilis* (IC), T-*Iso* (I) and *C. gracilis* (C) at the end of the feeding experiment 2

Tank	Feeding density ($\times 10^4$ cells/ml)*1		Number of nauplii*2 (N/25-*l*)	Number of larvae*3 (N/25-*l*)	Survival rate (%)	Larval stage*4		
	T-*Iso*	*C. gracilis*				M1	M2	M3
IC-1	5–7	8–10	5,000	3,050	61.0	0	0	100
IC-2	5–7	8–10	5,000	3,658	73.2	0	0	100
C-1	0	16–18	5,000	2,355	47.1	0	0	100
C-2	0	16–18	5,000	3,109	62.2	0	0	100
I-1	10–12	0	5,000	2,312	46.2	62	38	0
I-2	10–12	0	5,000	2,655	53.1	80	20	0

*1 The densities of T-*Iso* (Tank IC-1, IC-2, I-1, I-2) or *C. gracilis* (Tank IC-1, IC-2, C-1, C-2) maintained during the experiment

*2 The number of *M. ensis* nauplii introduced to a tank at the start of the experiment

*3 The number of living larvae in a tank at the ending of the experiment

*4 The metamorphic stage distribution of 100 larvae collected from each tank at the end of the experiment (M1: Mysis 1, M2: Mysis 2, M3: Mysis 3)

Table 4. Fatty acid composition of the total lipids from the whole bodies of *M. ensis* larvae fed on T-*Iso*, *C. gracilis* and a combination of T-*Iso* and *C. gracilis*, and from the food algae in the feeding experiment 1 and 2 (Area %)

Fatty acid	Larvae fed on T-*Iso*	Larvae fed on *C. gracilis*	Larvae fed on T-*Iso* and *C. gracilis*	T-*Iso*	*C. gracilis*
16:0	24.0	9.9	18.2	6.0	16.0
16:1	11.9	15.5	8.1	6.8	0.2
18:0	4.2	3.9	4.0	—	1.2
18:1(n-9)	15.2	4.7	7.3	5.2	1.7
18:2(n-6)	3.9	3.1	2.2	3.6	1.8
18:3(n-3)	2.1	0.7	—	5.8	1.5
18:4(n-3)	2.0	0.4	3.0	28.9	0.5
20:1	3.4	—	3.7	0.5	—
20:4(n-6)	0.9	0.8	4.5	—	3.0
20:5(n-3)	3.6	6.7	5.4	0.5	22.0
22:6(n-3)	3.4	1.3	3.1	2.8	0.9
ΣPUFA	15.9	13.0	18.2	42.1	29.7
Σ(n-3)HUFA	7.0	8.0	8.5	3.3	22.9

total lipid content in T-*Iso* and *C. gracilis* were 4.7% and 5.0% on a wet matter basis, respectively. A high percentage of 18:4(n-3) was recognized in T-*Iso*, but the percentage of 20:5(n-3) was low. On the other hand, *C. gracilis* contained a high level of 20:5(n-3). Further, T-*Iso* contained 22:6(n-3) about three times more than *C. gracilis*. The larvae fed on T-*Iso* contained more 18:4(n-3), 22:6(n-3) but less 20:5(n-3) compared with those fed on *C. gracilis*, and the relative contents of fatty acids of the larvae fed

on a combination of both T-*Iso* and *C. gracilis* in feeding experiment 2 were intermediate among larvae fed on either T-*Iso* or *C. gracilis*.

Shape and Size of T-Iso The shape of T-*Iso* and *C. gracilis* are shown in Fig. 1, and the frequency distributions of cell diameters in Fig. 2. The mode diameter of T-*Iso* and *C. gracilis* are about 3.9 to 4.1 μm and 4.9 to 5.1 μm, respectively. Although T-*Iso* showed obvious phototaxis and sometimes attached to the culture vessels, it was scattered easily in vessels by the aeration and seldomly made flocks or linkages.

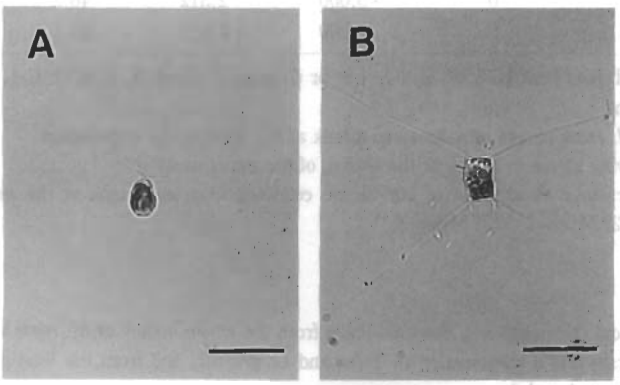

Fig. 1. *Isochrysis* sp. (T-*Iso*) (A) and *Chaetoceros gracilis* (B) used in this study. Scale bars represent 10 μm.

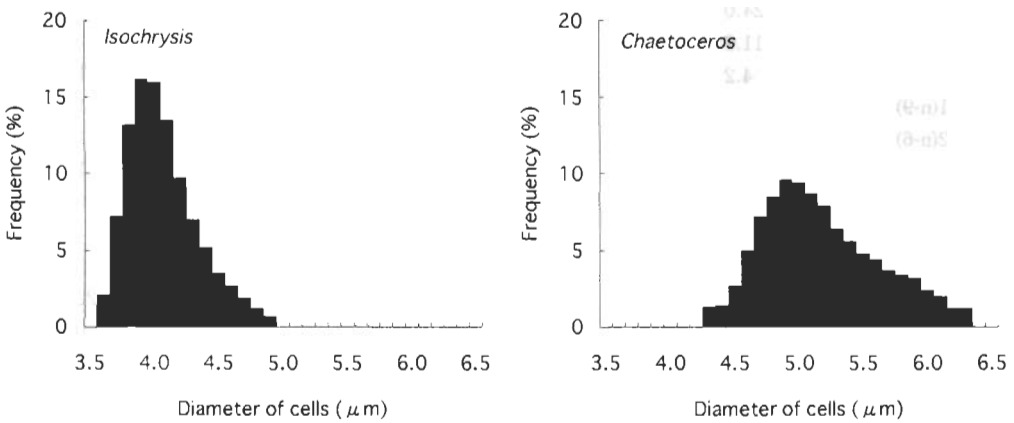

Fig. 2. Cell diameter distributions of T-*Iso* and *C. gracilis* collected at the stationary growth phase for each species.

Growth of T-Iso in Outdoor Culture Tanks The average growth rates and standard deviations of T-*Iso*, *N. oculata*, and *T. tetrathele* cultured in 100-*l* tanks were 0.52 (S.D.=0.03), 0.50 (S.D.=0.05) and 0.56 (S.D.=0.05), respectively (Table 5). Results of the analysis of variance showed that differences in growth rates between them were no significant ($P<0.05$). The growth phases of T-*Iso* in the four 500-*l*

scale trials are shown in Fig. 3. During the experiments, clear weather continued and the water temperature in tanks ranged between 23°C and 30°C. Although the inoculated cell populations had already reached the stationary phase, lag phases were scarcely observed or continued for only one day in these trials. T-*Iso* grew exponentially at a rate of 0.61 to 0.77 divisions/day after inoculation for 6 days. On *N. oculata*, the most serious problem in the large-scale outdoor culture is the sudden culture collapse during the hot and rainy season. However, such phenomena were not observed during our four trials.

Table 5. Growth rates of T-*Iso*, *Nannochloropsis oculata* and *Tetraselmis tetrathele* in 100-*l* outdoor culture tanks set in a sunny location for seven days

Algae	Cell densities of algae after inoculation ($\times 10^4$ cells/m*l*)	Cell densities of algae at the end of the experiment ($\times 10^4$ cells/m*l*)	Growth rate* (divisions/day)
T-*Iso*	20.35	466.68	0.50
	15.40	523.05	0.56
	22.55	533.23	0.51
	23.65	496.93	0.49
N. oculata	174.00	4215.75	0.51
	123.50	4056.25	0.56
	189.00	3151.50	0.45
	157.10	3192.75	0.48
T. tetrathele	5.50	168.30	0.55
	5.50	190.58	0.56
	3.85	208.45	0.64
	6.60	171.88	0.52

* Growth rate $=\log_2 (N_t/N_0)/7$ (N_0: Initial cell count, N_t: Final cell count)

Discussion

In general, the nutritive value of algae to shrimp larvae should be evaluated by larval rearing experiments and quantitative analysis of the essential nutrients for the larval growth. Therefore, we evaluated the nutritive value of T-*Iso* to *M. ensis* larvae on the basis of the results of the feeding experiments, fatty acid components of both T-*Iso* and larvae fed on the alga, and also the growth of the alga in large-scale outdoor culture tanks in this study.

Nutritive Value of T-Iso Evaluated from Survival Rates and Development of Larvae The average survival rate of the test groups in feeding experiment 1 seemed to be higher than that of control groups. However, if the experiment continued until the most larvae in the test groups developed to M3 stage, the survival rate would have to approximated to that of the control groups. Generally, the larval development is delayed by feeding of a low nutritive diets. From the results of experiments 1 and 2, the development of larvae fed on T-*Iso* only was clearly delayed compared with that of larvae fed on *C. gracilis*. Thus, the nutritive value of T-*Iso* only seems to be inferior to that of *C. gracilis*.

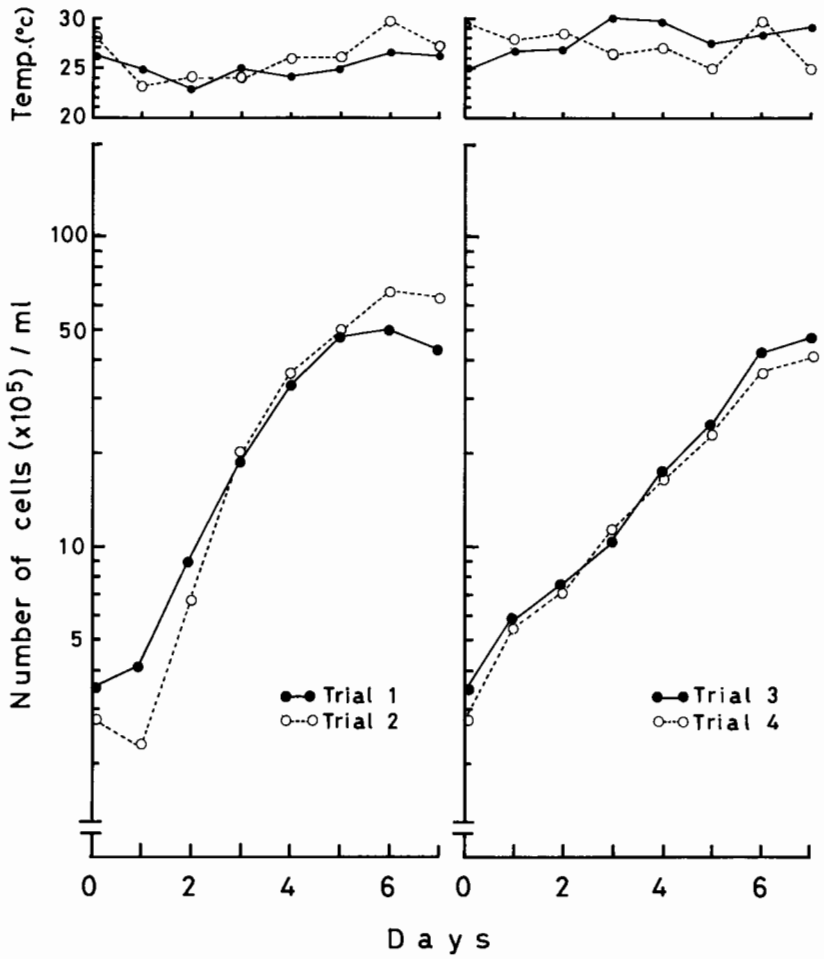

Fig. 3. Cell density of T-*Iso* and water temperature changes in the outdoor culture experiments (Trial 1~4) with 500-*l* tanks in summer. Trials 1, 2, 3 and 4 were carried out from July 1 to 8, July 10 to 17, July 20 to 27 and August 1 to 8, respectively.

***Effect of Combination Feeding both T-*Iso *and* C. gracilis** The diameter of T-*Iso* was about 1 μm smaller than that of *C. gracilis*. The PZ1 stage of *Penaeus monodon* is initially capable of ingesting diet particles of a size range of 4~5 μm (Tobias-Qunitio and Villegas 1982). As *M. ensis* larvae are almost equal in body length to *P. monodon* larvae, T-*Iso* seems to be taken readily like *C. gracilis*. The survival rate and development of the larvae fed on T-*Iso* in combination with *C. gracilis* in feeding experiment 2 were higher than those of the larvae fed on T-*Iso* or *C. gracilis* only. These results indicate that the nutritive defects of T-*Iso* seem to be compensated by the combination feeding. Therefore, T-*Iso* can be useful as an auxiliary food. Moreover, the feeding both algae in combination is more suitable for larval rearing.

Nutritive Value of T-Iso Evaluated from Fatty Acids Compositions Although the fatty acid compositions of T-*Iso* and *C. gracilis* usually changes slightly under various culture conditions (Su *et al.* 1988, Brown *et al.* 1993), the results of the analyses in this study were in close agreement with data from previous reports (Sukenik and Wahnon 1991, Fernandez-Reiriz *et al.* 1989). It was interesting that T-*Iso* contained much 18:4(n-3), which is contained a little in other food algae. The nutritive value of various fatty acids for shrimp larvae have been investigated previously, and 18:2(n-6), 18:3(n-3), 20:5(n-3) and 22:6(n-3) have been reported to be essential fatty acids (EFA) for *Penaeus japonicus* (Kanazawa and Teshima 1977, Kanazawa *et al.* 1979). Further, 20:5(n-3) and 22:6(n-3) appeared to be highly essential (Kanazawa *et al.* 1979). However, the percentage of 20:5(n-3) in T-*Iso* is only 0.02% of wet sample [we multiplied total lipid content(%) by the fatty acid content(%)]. The percentage of 22:6(n-3), 18:3(n-3) and 18:2(n-6) in T-*Iso* were estimated at 0.08%, 0.17% and 0.11%, respectively. Therefore, the total percentage of essential fatty acids in T-*Iso* was 0.38%. The nutritive value of 18:4(n-3) for shrimp larvae has been little investigated, but its nutritive value appears to be lower than that of 20:5(n-3) or 22:6(n-3). On the other hand, the percentage of 20:5(n-3), 22:6(n-3), 18:3(n-3) and 18:2(n-6) in *C. gracilis* was estimated at 1.1%, 0.05%, 0.08% and 0.09% of wet sample, respectively. The total percentage of these fatty acids in *C. gracilis* was 1.32%. Thus, *C. gracilis* contains essential fatty acids about three times more than T-*Iso*, and its nutritive value was higher than that of T-*Iso* judging from the fatty acid composition. The low 20:5(n-3) content in T-*Iso* might be one of the reasons why the development of larvae fed on only T-*Iso* was delayed in the feeding experiments 1 and 2. The larvae fed on T-*Iso* contained high percentages of 18:1(n-9), 18:3(n-3), 18:4(n-3) and 22:6(n-3), whereas they showed low 20:5(n-3) contents as compared with larvae fed on *C. gracilis*. Such a tendency closely correlates with the fatty acid composition of T-*Iso*. However, the contents of n-3 highly unsaturated fatty acids (n-3 HUFA) showed only slight differences between them. Therefore, the larvae fed on T-*Iso* seemed to be able to live. The high survival rate of the larvae fed on combination food in experiment 2 may be due to the enough supply of 20:5(n-3) and 22:6(n-3) from both algae.

Productivity in Large-scale Outdoor Tanks From the results of 100-*l* outdoor culture experiment, T-*Iso* was approximately equal to *N. oculata* and *T. tetrathele* with regards to growth rate. *N. oculata* and *T. tetrathele* are known as easily cultivatable unicellular algae (Maruyama *et al.* 1986, Okauchi and Fukusho 1984), and are mass produced as food for the rotifer *Brachionus plicatilis* and shrimp larvae. Moreover, T-*Iso* can be readily produced in 500-*l* outdoor culture tanks during the spawning season of the shrimp in summer.

Conclusion *C. gracilis* is a nutritious food alga, but its large scale culture is relatively difficult in summer compared with *N. oculata* and *T. tetrathele* in Japan. As shown in this study, the nutritive value of T-*Iso* is lower than that of *C. gracilis*, however, T-*Iso* can be readily cultured in outdoor culture tanks in summer. Although the uni-species feeding of T-*Iso* is inadequate for larval rearing, the use of T-*Iso* in combination with *C. gracilis*, or temporally as a substitute food alga seem advantageous. Moreover, the good development and high survival rates of the larvae can be obtained by the combination feeding of T-*Iso* and *C. gracilis*, so that we can reduce the quantity of *C. gracilis* used. The most suitable combi-

nation ratio of the algae should be investigated to develop a more efficient feeding methods of T-*Iso* as a food alga for *Metapenaeus* larvae.

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イソクリシス（タヒチ株）のヨシエビ幼生に対する餌料価値

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キートセロス *Chaetoceros gracilis* はクルマエビ類幼生の餌料として広く利用されているが，屋外での安定した大量培養は困難である。そこで，屋外での大量培養が比較的容易なイソクリシス（タヒチ株）*Isochrysis* sp. を餌料として利用することを目的に，ヨシエビ *Metapenaeus ensis* 幼生への餌料価値を調べた。飼育試験及び含有高度不飽和脂肪酸組成から，タヒチ株の餌料価値はキートセロスに比べて劣るが，キートセロスとタヒチ株の併用給餌により幼生の成長及び生残率は，各藻類を単独に給餌した場合と比べ，向上することがわかった。また，タヒチ株は屋外培養が容易であることも確認できた。タヒチ株を併用することにより，キートセロスの給餌量を約 1/2 に軽減することができ，クルマエビ類種苗への安定した給餌が可能になると期待される。