PN-ABT-368

Mass culture of the rotifer
Brachionus plicatilis
Isolation of clones and
high yield production
AID-CDR Project No.
DHR-5544-G-SS-9067-00
Years 1989-1992
H21/94

Principal investigators:

1. Dr. E. Lubzens
National Institute of Oceanography
Israel Oceanographic & Limnological Research
P.O. Box 8030, Haifa 31080, Israel

2. Dr. Liana McManus
Marine Science Institute
University of the Philippines
U.P.P.O. Box 1
Diliman, Quezon City 1101
Philippines

General Introduction

The enclosed includes all the reports prepared during the course of the research project which was approved in August 1989 and lasted until December 31, 1992.

During the research project, Dr. Liana McManus visited Israel with her assistant in the spring of 1990. She studied here basic methods for culturing rotifers and algae. Their training here resulted in the establishment of research facilities at the field station of the Marine Science Institute in Bolinao, Philippines. These research facilities are shown in photographs enclosed in a separate envelope taken at Bolinao in December 1992 during the visit of Dr. E. Lubzens to the Philippines.

The research project resulted in identifying rotifer strains with potentially extremely high reproductive rates (DUM, SAP, OTON) and the best conditions for obtaining these high rates. These include relatively high temperatures (30°C or higher) and low salinities (10 or 20 ‰). The nutritional quality of rotifers depended on the food they were fed. Rotifers that contain the polyunsaturated fatty acids required by marine fish larvae could be obtained by either feeding them with algae or with commercially available enrichment foods that contain fish oil. It was found that frozen algal paste could replace fresh algae (*Nannochloropsis* sp.) in both stabilizing mass cultures and in enrichment of rotifers.

Development of a simple method using restriction endonuclease analysis of mt DNA provided evidence for accidental contamination of cultures during the course of the project. This method has been used for obtaining profiles of all rotifer strains kept at IOLR, Haifa, Israel in the "live strain library" which is available to all fish growers. Several countries (including those of the Mediterranean area; Turkey, Greece, Spain, Italy and others - Norway, U.K., Japan and Singapore) take advantage of this library for obtaining strains that are most suitable to their needs and culture conditions.

A

Table of Contents:

1. Research Report - June 1991 (Israel and Philippines)
2. Research Report - September 1991 (Philippines)
3. Research Report - April 1992 (Philippines)
4. Research Report - July 1992 (Israel)
5. Research Report - October 1992 (Philippines)
6. Research Report - December 1992 (Israel)

Mass culture of the rotifer *Brachionus plicatilis*
Isolation of clones and high yield production
Research Report, June 1991

Introduction

The rotifer *Brachionus plicatilis* (O.F. Muller) is used as the first and only food source of marine fish larvae raised in intensive mariculture facilities. About 40,000 - 100,000 rotifers are required for raising one marine fish larva during the first 20 days of its life in captivity. This imposes the setting up of very large mass cultures of this rotifer in hatcheries raising marine fish. Today, the supply of rotifers in these facilities is totally dependent on these mass cultures. The reproductive rates of the rotifers will dictate the volumes and facilities required for raising them in the numbers needed daily, as food for the growing larvae. Determining the optimal reproductive rates, in the location of the hathcery, will result in a more efficient and dependable supply of rotifers.

In the first stages of this research project, we set out to develop a routine small-scale experimental setup for determining optimal reproductive rates of *B. plicatilis*. We also tested the effect of food concentration, salinity and temperature on the reproductive rates of three strains (populations?) provided to us from the Philippines. Following a short visit by Dr. L. McManus and her assistant, the same routine method is being used by her, on location, to check the same strains. The facilities in our laboratory in Israel enable us to carry out experiments under controlled temperature conditions. These are under construction in the Marine Science Laboratory in the Philippines. In parallel, a simple method was developed for the extraction of mt DNA. The extracted mt DNA was subjected to restriction enzyme digestions, and the differences between strains/clones is under investigation.

Israel:

Materials and Methods

Rotifers are routinely maintained in small batch cultures at three different temperatures (20, 25 and 30 °C) and a salinity of 30 ‰. They are fed on algae (*Nannochloropsis* sp.) every other day. The algae are raised in 3 liter or 10 liter batch culture under constant illumination in a 25°C temperature controlled room. Algae are centrifuged, and the resulting suspended pellet is used for feeding the rotifers in the experiments described below. The concentration of algae in the pellet was usually 10^{10} cells ml^{-1} which is equivalent to 10 mg ml^{-1}.

Experimental Setup

Rotifers were pre-adapted to the conditions of temperature and salinity for 10-20 days, prior to performing the experiments. The routine setup consisted of suspending, in 20 ml of sea water, about 20 or 5 rotifers per ml (Experiments 1-9 and Experiments 10-13, respectively) on the first day of the experiment. Each replicate was prepared in a 50 ml Ehrlenmeyer flask, and three replicates were used for each treatment. In each experiment, 12-16 flasks were prepared, which were divided into 3-4 different treatments (combinations of temperatures and salinities). Rotifers were fed daily on algae (*Nannochloropsis* sp.). Each experiment lasted for 96 hours, and flasks were not illuminated. The culture media was changed 48 hours after the beginning of the experiment, by sieving the contents of each flask on a 27 µm mesh nylon sieve and resuspending the rotifers in freshly prepared sieved sea water. The reproductive rate and percent of eggs for each replicate were determined by counting rotifers on the first and last day of the experiment. Counts were made by withdrawing samples in a glass pipette and counting the number of females and the eggs they carry, under a binocular microscope (X 12). Each sample was replaced in the flask before a new one was withdrawn. At least three samples were counted for each replicate, and the average number was used in the calculations. The reproductive rate (G) for each replicate was calculated by:

$$G = \frac{1}{T} \ln \frac{N_t}{N_o}$$

where T = days of the experiment (4 days)

N_o = the initial number of rotifers and the eggs they carry in each flask

N_t = the final number of rotifers and the eggs they carry in each flask

The percent of eggs was calculated by:

$$\%\ of\ eggs = \frac{No.\ of\ eggs}{Total\ no.\ of\ females\ in\ sample} X\ 100$$

Results and Conclusions

The three strains (OTON, SAP, DUM) received from the Philippines are relatively small size strains. Initial culture attempts in our laboratory revealed that they are adapted to high temperatures. Attempts to maintain these strains at 20°C failed, and even at 25°C their reproductive rates were very low. They are routinely maintained in our laboratory at 30°C. In the experiments shown in Tables 1-3, their reproductive rates were examined at two temperatures (30 and 36°C) and four salinities (10, 20, 30 and 40 ‰). In evaluating the conditions for promoting the highest reproductive rates, we also paid attention to the percent of eggs carried by females. This value indicates the physiological condition of the culture and its potential for further reproduction. A low percent of eggs indicates starvation or the effect of other adverse conditions that will eventually result in the loss or collapse of the culture. The first experiments (Experiments 1-4) are not reported here, since the percent of eggs carried by females was very low or eggs were completely absent at the end of the experiment. This was, in most cases, a result of starvation. We therefore carried out preliminary observations on the amount of food that should be provided to ensure the presence of excess food in the tested cultures. These observations revealed that not only the total amount of food provided each day to each culture was important, but also that the amount of food (algae) should be divided into several feeding periods throughout the day. This is especially important in the highly reproductive cultures, where the total amount of food required per day

is relatively high, and if it is provided once a day, it results in the death of the rotifers.

For strain OTON, which was the first strain examined, we found that reproductive rates (values of G) varied between a mean of 0.77 to 0.93, and the percent of eggs changed between 21.8 to 68.5, when cultured at 30°C in a salinity of 30 ‰ (30 S). Reducing the salinity or increasing the temperature resulted in higher reproductive rates. However, the effect of reducing salinity was more pronounced than that of the increase in temperature. By reducing the salinity to 10 S, mean reproductive rates reached 1.31 or 1.35. Increasing the culture temperatures to 36°C resulted in G values of 0.90-0.97.

The second strain that was examined (SAP) showed even higher reproductive rates than those of OTON. At 30°C and a salinity of 30 S, the mean G values ranged between 1.27-1.56. Reducing the salinity to 20 S resulted in mean G values ranging between 1.45-1.61. Lower salinities (10 S) did not increase dramatically the G values (1.56). Increasing the culture temperature to 36°C did not increase reproductive rates. However, decreasing the culture temperature to 25°C reduced significantly the mean G values, which reached 0.53-0.62 and 0.72-0.76 for cultures maintained in 20 and 30 S salinity, respectively. Our attempts to maintain cultures at 10 S at 36°C were not very successful, and therefore this possibility was not examined. The full statistical analysis of these results awaits the completion of similar experiments currently under way for strain DUM. The full analysis will compare all three strains and will provide a basis for choosing the strain with the highest reproductive potential and define the culture conditions that promote its maximal reproductive rates.

The most striking result about the three strains examined here is their potential for very high rates of reproduction. Most of the strains reported until now, and those that we have in our laboratory, show maximal reproductive rates of 0.45-1.00, when fed on *Nannochloropsis* sp. also used in the present series of experiments. It is also quite clear that the two strains require relatively high temperatures (30°C)

for their cultures. In evaluating the reproductive potential of the two strains examined so far, SAP shows a higher potential for reproduction than OTON. However, SAP cultures were found to be unstable at low salinities.

A simple method was developed for the extraction of mt DNA from rotifers that requires relatively small numbers (about 2-3 x 10^6 individuals). Preliminary results on restriction enzyme digestions show differences between strains. These will be presented in our next report. The method was developed using strains available in our laboratory, while waiting for those from the Philippines. We have now extracted mt DNA from the OTON and SAP strains, and they will be examined in the next few weeks.

Future work

1. Continue with experiments testing the DUM strain.
2. Perform full statistical analysis of the results comparing the three strains.
3. Produce restriction enzyme profiles of clones and strains from the Philippines.
4. Initiate upscale experiments using yeast and algae for Philippine strains, at the salinity and temperature promoting high reproductive rates.

Table 1. The reproductive rates (G) and the percent of eggs carried by females of rotifers (strain OTON) raised at four salinities (10, 20, 30 and 40 ‰) and two temperatures (30 and 36 °C). The total amount of food provided during the experiments is shown in the last column.

Expt. no.	Temp. °C	Salinity ‰	Reproductive rate (G)	Eggs (%)	Algae (x 10^7 cells)
5	30	30	0.77 ± 0.05 (1)	21.8 ± 1.6	224
			0.82 ± 0.05 (2)	7.0 ± 4.9	320
	36	30	0.80 ± 0.06 (3)	20.4 ± 4.7	224
			0.93 ± 0.09 (4)	11.9 ± 8.8	320
6	30	10	1.19 ± 0.02	0	272
		20	0.98 ± 0.01	18.5 ± 12.3	224
		30	0.85 ± 0.06	26.4 ± 8.0	208
7	30	10	1.31 ± 0.02	6.3 ± 0.6	368
		20	--	--	272
		30	--	--	256
		40	0.42 ± 0.67	39.8 ± 6.1	144
8	30	10	1.35 ± 0.04	31.7 ± 2.1	528
		20	1.26 ± 0.03	51.5 ± 1.4	416
		30	0.91 ± 0.05	68.5 ± 9.8	256
9	30	20	1.15 ± 0.06	9.6 ± 6.1	352
		30	0.93 ± 0.05	33.7 ± 11.4	240
	36	20	1.23 ± 0.04	8.63 ± 0.07	417
		30	0.90 ± 0.08	35.8 ± 10.0	256
10	30	20	1.17 ± 0.07	43.7 ± 5.4	84
		30	0.78 ± 0.11	25.8 ± 3.2	76
	36	20	1.22 ± 0.04	24.3 ± 12.2	96
		30	0.97 ± 0.01	19.7 ± 5.0	80

Table 2a. The reproductive rates (G) and percent of eggs carried by females of rotifers (strain OTON) raised at 30°C and salinity 30 ‰

Experiment no.	Reproductive rate (G)	Eggs (%)
5	0.77 ± 0.05	21.8 ± 1.6
	0.82 ± 0.05	7.0 ± 4.9
6	0.85 ± 0.06	26.4 ± 8.0
8	0.91 ± 0.05	68.5 ± 9.8
9	0.93 ± 0.05	33.7 ± 11.4
10	0.78 ± 0.11	25.8 ± 3.2

Table 2b. The reproductive rates (G) and percent of eggs carried by females of rotifers (strain OTON) raised at 30°C and salinity 20 ‰

Experiment no.	Reproductive rate (G)	Eggs (%)
6	0.98 ± 0.01	18.5 ± 12.3
8	1.26 ± 0.03	51.5 ± 1.4
9	1.15 ± 0.06	9.6 ± 6.1
10	1.17 ± 0.07	43.7 ± 5.4

Table 2c. The reproductive rates (G) and percent of eggs carried by females of rotifers (strain OTON) raised at 30°C and salinity 10 ‰

Experiment no.	Reproductive rate (G)	Eggs (%)
6	1.19 ± 0.02	0
7	1.31 ± 0.02	6.3 ± 0.6
8	1.35 ± 0.04	31.7 ± 2.1

Table 2d. The reproductive rates (G) and percent of eggs carried by females of rotifers (strain OTON) raised at 36°C and salinity 30 ‰

Experiment no.	Reproductive rate (G)	Eggs (%)
5	0.80 ± 0.06	20.4 ± 4.7
	0.93 ± 0.09	11.9 ± 8.0
9	0.90 ± 0.08	35.8 ± 10.0
10	0.97 ± 0.01	19.7 ± 5.0

Table 2e. The reproductive rates (G) and percent of eggs carried by females of rotifers (strain OTON) raised at 36°C and salinity 20 ‰

Experiment no.	Reproductive rate (G)	Eggs (%)
9	1.23 ± 0.04	8.63 ± 0.07
10	1.22 ± 0.04	24.3 ± 12.2

Table 3. The reproductive rates (G) and the percent of eggs carried by females of rotifers (strain SAP) raised at four salinities (10, 20, 30 and 40 ‰) and two temperatures (30 and 36 °C). The total amount of food provided for each treatment is shown in the last column.

Expt. no.	Temp. °C	Salinity ‰	Reproductive rate (G)	Eggs (%)	Algae (x 10^7 cells)
11	30	20	1.61 ± 0.01	--	176
		30	1.56 ± 0.01	26.5 ± 5.9	176
	36	20	1.57 ± 0.04	--	176
		30	1.56 ± 0.02	--	176
12	25	20	0.76 ± 0.04	81.9 ± 8.9	104
		30	0.56 ± 0.06	61.1 ± 15.4	104
	30	20	1.55 ± 0.18	53.9 ± 28.9	152
		30	1.38 ± 0.06	51.5 ± 20.7	152
13	25	20	0.72 ± 0.10	65.1 ± 8.5	-
		30	0.62 ± 0.04	67.9 ± 3.6	-
14	30	10	1.56 ± 0.01	31.4 ± 15.3	128
		20	1.45 ± 0.01	44.2 ± 27.0	136
		30	1.27 ± 0.05	72.6 ± 11.2	120
		40	0.93 ± 0.14	90.3 ± 5.9	88

MASS CULTURE OF THE ROTIFER *BRACHIONUS PLICATILIS* - ISOLATION OF CLONES AND HIGH YIELD PRODUCTION

Progress Report, Marine Science Institute, Philippines

Activities

Major activities undertaken for the period April - August 1990 were as follows:

April 16-18. Dr. Liana T. McManus and her research assistant, Ms. Ma. Catalina Ranola, visited the Southeast Asia Fisheries Development Center (SEAFDEC) to obtain live rotifer samples from Panay (central Philippines) strains. They also established informal collaborative ties such that SEAFDEC will maintain a reference culture for Central Philippines while Dr. McManus sets one up for Northern and Southern Philippines to be based at the Marine Science Institute. Cultures obtained from three sites and one from Kochi, Japan, were obtained from Ms. Milaoros de la Pena.

May 21 - June 5. Dr. McManus and Ms. Catalina Ranola were at IOLR, Israel, in the laboratory of Dr. Lubzens, for standardization of culture methods to be used in the project. These included media preparation, rotifer culture maintenance, and experimental protocol for determining the effects of environmental parameters (temperature, salinity, food levels) on reproductive potential of rotifers. For the administrative mechanics of the project, Dr. McManus talked with Mr. Arieli, IOLR's accountant, regarding financial matters. The Philippine visit also included a trip to the Eilat rotifer mass production facility.

June 21-22. Ms. Ranola went to SEAFDEC to obtain live samples of rotifers and algae. Samples were sent to Dr. Lubzens for experiments on reproductive potential.

July. Maintenance of experimental cultures from Central Philippines.

Aug. 8. Sampling in Tambac Bay for isolation of rotifers. A transect sampling was done in stations with salinities of 15, 18, 20, 25 and 30 ppt. Rotifers were isolated from 18, 20 and 25 ppt and are currently in culture.

Aug. 23. Dr. McManus and two assistants went to the Binangonan Freshwater Station of SEAFDEC in Laguna de Bay, which seasonally receives seawater intrusion from Manila Bay. Sampling from both Laguna de Bay and Manila Bay will be done subsequently.

Plans

Sampling in Laguna de Bay, Manila Bay and in brackishwater areas in Mindanao are planned for the period September 1990 to February 1991. Other activities will include isolation of geographical strains and maintenance of cultures. Experiments which require the purchase of necessary equipment, such as a centrifuge and the construction of constant temperature rooms, await the release of funds.

MARINE SCIENCE INSTITUTE

US AID/IOLR GRANT NO. DHR-5544-G-SS-9067-00

MASS CULTURE OF THE ROTIFER *BRACHIONUS PLICATILIS*

Progress Report, Sept. 1, 1990 to Feb. 28, 1991

Activities

Major activities undertaken for the period Sept. 1, 1990 to Feb. 28, 1991 were as follows:

1. Upscaling of new strains and purification of algal cultures (October-November 1990.

   In September, we lost 4 strains from SEAFDEC and one new isolate from Tambac Bay because of contaminated algal cultures. We obtained new rotifer samples from SEAFDEC (strains SAPIAN, AKLAN and OTON) in late September and purified algal cultures at the same time. Ciliate infestation was observed to have originated from cultures of *Isochrysis galbana*.

2. Comparison of IOLR media and Walne's media (November 1990).

   In order to hasten the upscaling of algae (after the ciliate contamination), we compared the growth of *Tetraselmis tetrahele* and *I. galbana* in IOLR media and in Walne's media, the latter being the growth media used previously. Results showed that both species grew better in the IOLR "improved" recipe. Close examination of the two media indicated that concentrations of major components differed significantly. The IOLR media was markedly enriched in terms of nitrates, phosphates, trace metals and vitamins.

3. Preliminary experiments on food preference by rotifers (January-February 1990).

   Initial experiments on food preference were done to determine which algal species will be used for maintenance of rotifer cultures. *I. galbana* (about 1.5 M/mil), *T. tetrahele* (about 1.0 M/mil), and a mixture of *T. tetrahele* and *Chlorella virginica* (perhaps this is the same as the IOLR *Nannochloropsis*) (at approximately 1.0 M/mil and 0.1 M/mil, respectively) were given to all three

strains (SAPIAN, AKLAN and OTON). Using G (reproductive potential) as the parameter, *T. tetrahele* gave the highest estimates of reproductive potential. Although this experiment was preliminary, it was the basis for our choice of the latter as the maintenance food species. Later experiments, which will be designed to account for differences in cell size and perhaps CHN content, need to be done to validate this initial result.

4. Initial screening for asexually reproducing clones (January - February 1991). Following the procedure for isolation of rotifer clones for mass cultivation and production of resting eggs as described in Appendix A of the project document, we tested the three SEAFDEC strains for mixis. For this particular experiment, cultures were initiated with amictic females, each bearing a single egg. Culture conditions for the assay were 16 ppt at 24-28°C, and food at 5 x 10^6 cells ml^{-1} of *T. tetrathele*. Every three days, the assay was repeated over a period of 15 days. Following are the results:

Strain	SAPIAN	AKLAN	OTON
Ave. % eggs	49.9 ± 5.5	49.1 ± 4.0	49.8 ± 4.7
Ave. G	1.42 ± 0.08	1.44 ± 0.16	1.58 ± 0.16
Ave. % mixis	3.5 ± 2.8	1.7 ± 1.4	2.7 ± 1.5

13

Mass culture of the rotifer *Brachionus plicatilis*

Isolation of clones and high yield production

Research Report, September 1991

Introduction

The rotifer Brachionus plicatilis is an important food species for marine fish and invertebrate larvae in culture. Propagation techniques for this species, especially those intended to promote mass production, have to be developed to support intensive mariculture efforts. A first step is to determine culture conditions which favor high reproductive rates of rotifers.

In collaboration with research at the IOLR under Dr. E. Lubzens, the Philippine team has obtained rotifer samples from different geographical locations in the country to study their respective reproductive potentials at various levels of salinity, temperature and food. This report summarizes the results of initial experiments conducted at room temperature (24-30°C). Upon construction of a temperature-controlled room, the effects of various temperature levels will be studied as well.

Materials and Methods

Collection of samples. Rotifers were obtained from existing cultures in SEAFDEC and in the Mindanao State University (Naawan Campus). Those from SEAFDEC were originally collected from wild populations in 3 provinces (SAPIAN from Sapian, Capiz; DUM from Dumaguet, Aklan (DUM is also coded as AKL for Aklan); OTON from Oton, Iloilo; and NAAWAN from Naawan, Misamis Oriental). Samples obtained from Naawan

were originally taken from fishponds fringing Panguil Bay.

Maintenance. Cultures are maintained in 500 ml flasks in an airconditioned room with temperature ranging from 24 to 30°C. They are fed with the algae Tetraselmis tetrahele at 0.5 X 10^6 cells per rot ml daily offered in three doses. The algae are raised in 8 liter carboys under constant illumination and the same temperature range as that for rotifers. They are centrifuged, the resulting concentrate subsampled to determine density of cells ml^{-1}, and then fed to the rotifers.

Experimental set-up. Three kinds of experiments were done to determine the level of mixis at two salinity levels (Series #1), the optimal food concentration at 32 ‰ and room temperature (Series #3), and the optimal salinity level at optimal food concentration and room temperature (Series #4). Three to four geographically distinct rotifer populations were used in these 3 series (SAPIAN, AKLAN, OTON and NAAWAN).

Before the experiments were started, resting eggs from each population were isolated. A pure culture, i.e. culture propagated from a single resting egg, was maintained for each of the four populations, and from which rotifers for the three series of experiments were obtained. Because a low level of mixis occurs, the cultures, over time, are no longer pure.

Prior to an experiment, rotifers are acclimatized to experimental salinity levels for at least a week. The general procedure used is based on the routine methods established by Dr. Lubzens at IOLR. A number of rotifers are suspended in 20 ml seawater contained in 50 ml flasks, the number of which are divided into 3 replicate flasks per treatment. Rotifers are given Tetraselmis tetrahele daily in 3 doses. Water is changed every other

day by sieving rotifers using a 25 υm mesh nylon sieve and resuspending the animals in freshly filtered (0.45 υm filter) sea water. Rotifers are counted on the first and fourth (last) day of an experiment by pipetting a given volume from each flask unto counting wells which are observed under an M3C Wild stereomicroscope. The number of males, females and eggs are counted to determine percent mixis (%males/females), percent eggs (%eggs/total where total counts = females + males + eggs), and reproductive potential G ($G = \ln (N_t/N_o)/T$ in days). Three replicate counts are made for each flask without replacement, the average of which are used in statistical comparisons.

Preliminary statistical tests were limited to determining trends within a population. Comparisons of rotifer populations will be done after the completion of replicate experiments where systematic errors will have been minimized.

Results

The results of experiments reported here are preliminary in that we are still completing replicate experiments. Trends we have thus far detected may still change. In comparing our results with those obtained from parallel experiments at the IOLR, two major points should be considered. First, our cultures are routinely maintained using the algal species *Tetraselmis tetrahele*. Presumably, rotifers in ponds bloom following a *Tetraselmis* bloom. In the absence of *Nannochloropsis* and because our source laboratories used this species, we opted to do the same. We did preliminary experiments using various concentrations of *Isochrysis galbana* where we obtained extremely low reproductive rates ranging from 0.11 to 0.28. Second, our algal and rotifer cultures are maintained in airconditioned rooms only so that temperature is not as controlled as that

in temperature-controlled walk-in incubators. How this biases the reproductive rates and other parameters such as mixis levels will be ascertained only by experiments where temperature will be controlled.

Table 1 summarizes the results of eight experiments done to determine primarily the level of mixis of our 4 rotifer populations at 16 and 32‰. Pairwise comparison of means for each population show that mixis levels are the same at the 2 salinities for SAPIAN and NAAWAN rotifers. AKLAN animals significantly decreased sexual reproduction at 32‰ while OTON rotifers increased mixis with a salinity increase. Reproductive rates remained the same with an increase in salinity for SAPIAN and NAAWAN populations. FOR AKLAN and OTON rotifers, their reproductive rates decreased at 32‰.

The responses of 3 rotifer populations to different concentrations of food are shown in Table 2. ANOVA and Duncan's Multiple Range Test (DMRT) of the reproductive rates (G's) of AKLAN rotifers indicated that they were highest at 4 X 10^7 cells. They decreased below and above this optimal food level. For SAPIAN and OTON rotifers, reproductive rates did not respond in a significant manner to increasing food concentration. Whether this means that they saturate or remain unsatiated within the tested range of concentrations will have to be determined further. In the next series of experiments, the optimal food level obtained for AKLAN was used for the other populations in determining the effect of 3 salinities.

At the optimal food level, AKLAN rotifers decreased their reproductive rates with increasing salinity (Table 3). For SAPIAN, OTON and NAAWAN populations, their

reproductive rates remain statistically unchanged.

Discussion

The small-sized rotifers we have so far studied seem to have high reproductive potentials as observed by Dr. Lubzens. SAPIAN, AKLAN (= DUM) and NAAWAN populations all showed reproductive rates between 1.09 and 1.34 in Series #3 and #4 experiments. We also corroborate the observation of the Israel Team that SAPIAN rotifers consistently showed higher reproductive rates that OTON rotifers (Tables 2 and 3). The percent of eggs never reached below 30% for all populations including OTON.

Except for the AKLAN (=DUM) population, which has so far shown clear responses to salinity gradients and food levels at room temperature range, SAPIAN and OTON rotifers seems unresponsive to similar changes in salinity and food. The biases introduced by fluctuating temperature between 22 to 30°C and other systematic errors introduced during the experiments need to be further minimized before more conclusive trends can be observed.

Future work

1. Replicate all experiments at specific temperature levels.
2. Collect rotifers from the wild, e.g. from Tambac or Laguna de Bay.
3. Perform statistical analyses to determine which population exhibits the highest reproductive rates at optimal levels of salinity, temperature and food.

Table 1. Mixis levels (%males/females), the percent of eggs carried by females, and the reproductive rates (G) of 4 geographically distinct rotifer populations raised at 2 salinities (16 and 32‰) at room temperature range (24.5-29.5°C). Food (Tetraselmis tetrahele) was provided daily at a concentration of 3 X 10^7 cells rot ml^{-1}.

PARAMETER	SALINITY (‰)	SAPIAN	AKLAN	OTON	NAAWAN
%Mixis	16	1.4±1.0	10.7±4.3	1.1±0.8	3.8±1.3
	32	2.0±0.6	1.2±0.9	8.0±0.9	3.5±1.4
%Eggs	16	45.3±1.6	45.0±1.9	49.2±2.4	50.2±2.3
	32	45.9±1.3	41.4±4.0	39.2±3.5	44.8±3.6
Reproductive rate (G)	16	1.62±0.03	1.48±0.03	1.73±0.02	1.54±0.01
	32	1.50±0.07	1.17±0.03	1.17±0.01	1.45±0.12
Statistics					
%Mixis		16‰=32‰	16‰>32‰	16‰<32‰	16‰=32‰
G		16‰=32‰	16‰>32‰	16‰>32‰	16‰=32‰

Notes:

1. This summary includes results of eight experiments.
2. Pairwise comparisons of means for each population were done at the 95% significance level.
3. A two-way ANOVA will be done to compare responses between populations after completion of replicate experiments.

Table 2. Mixis levels, the percent of eggs carried by females, and the reproductive rates (G) of 4 geographically distinct rotifer populations raised at 5 concentrations of Tetraselmis tetrahele. Experimental cultures were at 32‰ and at room temperature range (21.5-30.0°C).

ALGAE ($X 10^7$)	PARAMETER	SAPIAN	AKLAN	OTON
1	%Mixis	42.7±17.4	0.0	19.9±1.4
	%Eggs	34.8±6.8	42.7±2.8	40.5±11.3
	G	1.09±0.04	0.99±0.08	1.22±0.17
2	%Mixis	33.8±14.9	0.0	20.2±5.8
	%Eggs	41.9±1.3	38.0±4.6	52.9±4.6
	G	1.18±0.07	1.14±0.05	1.10±0.06
4	%Mixis	14.7±1.0	0.0	20.5±3.8
	%Eggs	44.2±2.7	41.7±5.8	54.5±2.1
	G	1.24±0.06	1.21±0.12	1.12±0.02
8	%Mixis	12.9±5.3	0.0	13.5±2.2
	%Eggs	44.2±1.9	41.5±3.8	52.5±3.1
	G	1.26±0.14	1.16±0.10	1.13±0.08
16	%Mixis	4.5±0.6	0.0	4.5±3.3
	%Eggs	43.8±1.1	35.2±1.1	53.2±1.4
	G	1.18±0.08	1.02±0.01	0.93±0.10

Notes:

1. This table includes results of three experiments, 1 experiment for each population.

2. One-way analysis of variance for each population was done at 95% significance level. They indicated significantly different treatment means, using reproductive rate as the parameter, only for the AKLAN population. Duncan's multiple range test for multiple comparison of means showed that the reproductive rate of AKLAN rotifers was significantly highest at $4 X 10^7$ cells. G's at 2 and 8 million cells were 2nd highest; those for 1 and 16 million cells were lowest.

   The reproductive rates of SAPIAN and OTON rotifers apparently did not respond in some significant manner to increasing food concentration.

3. A two-way ANOVA will be done to compare responses of different rotifer populations after completion of replicate experiments.

Table 3. Mixis levels, the percent of eggs carried by females, and the reproductive rates of 4 rotifer populations raised at 3 salinity levels. Cultures were maintained at 4 X 10^7 cells of Tetraselmis tetrahele and at room temperature range.

SALINITY (‰)	PARAMETER	SAPIAN	AKLAN	OTON	NAAWAN
16	%Mixis	5.8±3.9	6.4±3.5	14.5±1.9	6.6±1.3
	%Eggs	43.7±5.4	45.6±1.7	33.3±6.7	49.4±7.3
	G	1.34±0.03	1.33±0.05	1.11±0.22	1.32±0.07
24	%Mixis	16.2±4.0	33.1±15.1	20.9±5.8	7.8±2.3
	%Eggs	44.4±1.5	40.7±8.8	42.1±5.0	42.9±2.9
	G	1.21±0.21	1.27±0.05	1.19±0.08	1.32±0.03
32	%Mixis	37.3±2.1	19.9±6.6	10.7±2.1	8.8±1.4
	%Eggs	42.6±4.2	36.9±5.3	39.3±2.5	48.0±1.4
	G	1.08±0.06	1.15±0.01	1.00±0.05	1.28±0.03

Notes:

1. This table summarizes results of the 2nd trial run for each population or a total of 4 experiments.

2. Four one-way analysis of variance were done and indicated that except for AKLAN, the reproductive rates remained significantly the same with increasing salinity for SAPIAN, OTON and NAAWAN rotifers. For AKLAN rotifers, their reproductive potential significantly decreased with increasing salinity as shown by the Duncan's Multiple Range Test.

3. A two-way ANOVA to compare the responses of different rotifer populations will be done after the completion of replicate experiments.

Mass culture of the rotifer *Brachionus plicatilis*
Isolation of clones and high yield production
Research Report, April 1992

Introduction

The rotifer Brachionus plicatilis is an important food species for marine fish and invertebrate larvae in culture. Propagation techniques for this species, especially those intended for mass production, have to be developed to support intensive mariculture efforts. A first step is to determine culture conditions which favor high reproductive rates of rotifers.

In collaboration with research at the IOLR under Dr. E. Lubzens, the Philippine team has obtained rotifer samples from different geographical locations in the country to study their respective reproductive potentials at various levels of salinity, temperature and food. We have one strain from northern Philippines (BANI), three from the central area (AKLAN, OTON, and SAPIAN), and one from the south (NAAWAN). This report summarizes the results of 23 out of 26 experiments conducted on all five strains. Experiments on mixis previously reported were not included here. Subsequent experiments using a water bath with temperature control, will focus on the effects of temperature (25, 30 and 35°C) on reproductive potential.

Materials and Methods

Collection of samples. Rotifers were obtained from existing cultures in SEAFDEC and in the Mindanao State University (Naawan Campus). Those from SEAFDEC were originally collected from wild populations in 3 provinces (SAPIAN from Sapian, Capiz; DUM from Dumaguet, Aklan (DUM is also coded as AKL for AKLAN); OTON from Oton, Iloilo; and NAAWAN from Naawan, Misamis Oriental). Samples obtained from Naawan were originally taken from fishponds fringing Panguil Bay.

Maintenance. Cultures are maintained in 500 ml flasks in an airconditioned room with

temperature ranging from 23.0 to 31°C. They are fed with the algae Tetraselmis tetrahele at 4.0 X 10^6 cells per rot ml daily, and offered in three doses. The algae are raised in 8 liter carboys under constant illumination and the same temperature range as that for rotifers. They are centrifuged, the resulting concentrate subsampled to determine density of cells ml^{-1}, and then fed to the rotifers.

Experimental set-up. Before the experiments were started, resting eggs from each population were isolated. A pure culture, i.e. culture propagated from a single resting egg, was maintained for each of the four populations, and from which rotifers for the three series of experiments were obtained. Because a low level of mixis occurs, the cultures, over time, are no longer pure. Rotifers are acclimatized to experimental salinity levels for at least a week, if they are different from maintenance conditions.

The general experimental procedure followed is based on the methods established by Dr. Lubzens of IOLR. A number of rotifers (1-10 per ml) are suspended in 20 ml seawater contained in 50 ml flasks, the number of which are subdivided into 3 replicate flasks per treatment. Rotifers are given algae daily in three doses. Water is changed every other day by sieving rotifers using a 25 υm mesh nylon sieve and resuspending the animals in freshly filtered (0.45 υm filter) sea water. Rotifers are counted on the first and fourth (last) day of an experiment by pipetting a given volume from each flask and directly counting rotifers under an M8 Wild dissecting scope while inside the pipette. The number of males, females and eggs are counted to determine percent mixis (%males/females), percent eggs (% eggs/total where total = females + males + eggs), and reproductive potential G ($G = \ln (N_t/N_o)/T$ in days). Three replicate counts are made for each flask with replacement, the average and standard deviation of which are used in statistical comparisons.

Preliminary statistical tests were limited to determining trends within a population.

Comparisons of rotifer populations will be done after the completion of replicate experiments where systematic errors will have been minimized, and temperature fully controlled.

Results

In comparing our results with those obtained from parallel experiments at the IOLR, two major points should be considered. First, our cultures are routinely maintained using the algal species Tetraselmis tetrahele. Presumably, rotifers in ponds bloom following a Tetraselmis bloom. In the absence of Nannochloropsis and because our source laboratories used this species, we opted to do the same. We did preliminary experiments using various concentrations of Isochrysis galbana where we obtained extremely low reproductive rates ranging from 0.11 to 0.28. Second, our algal and rotifer cultures are maintained in airconditioned rooms only so that temperature is not as controlled as that in walk-in incubators. How this biases the reproductive rates and other parameters will be ascertained only by temperature-controlled experiments.

Luzon Strain. BANI, a strain from northwestern Luzon, exhibited the highest reproductive potential of 1.55 $\pm$ 0.01 at 16 ppt, 4 X 10^6 algal cells, 27.0 - 28.5°C (Table 1), compared to all 4 other strains tested under various experimental conditions. At 32 ppt and temperature range of 27.0 - 30.0°C, optimal food level was 4 X 10^6 T. tetrahele cells. At food concentrations lower and higher than this level, reproductive potentials were significantly reduced.

Visayan Strains. SAPIAN, OTON, and AKLAN strains generally preferred 16 ppt of salinity like BANI (Tables 2, 3 and 4). Except for OTON, SAPIAN and AKLAN both showed their highest reproductive potentials at 4 X 10^6 food cells and at room temperature range.

Mindanao Strain. NAAWAN from central Mindanao, tolerates 16 and 24 ppt with a slightly higher reproductive potential at the former (Table 5). Food saturation was reached at 2 X 10^6 food cells and continued at 8 X 10^6 cells.

General comments. We are monitoring the size changes among the 5 rotifer populations over time. So far, BANI is the biggest; the three Visayan strains are intermediate; and NAAWAN is the smallest. The latter, however, have increased in length after 5 months and achieving size ranges similar to the Visayan populations.

Future Work

1. Replicate experiments at 25, 30 and 35°C focusing on BANI, SAPIAN and NAAWAN populations.
2. Isolation of small and big morphs for each population.
3. Monitor size changes for each population. Low percent of mixis occurs so that monitoring of a single strain from a single resting egg is not yet possible.
4. Perform statistical analyses to determine which population exhibits the highest reproductive rates at optimal levels of salinity, temperature and food.

Table 1. Reproductive potentials, G, and % eggs of BANI at three salinity (a) and five food levels (b).

(a)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	27.0 - 28.5	32	4	1.36 ± 0.01	30.0 ± 3.30
		24		1.48 ± 0.02	24.1 ± 4.51
		16		1.55 ± 0.01	12.1 ± 0.81

(b)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
2	27.0 - 30.0	32	1	1.15 ± 0.01	9.1 ± 3.81
			2	1.25 ± 0.04	16.1 ± 5.67
			4	1.40 ± 0.01	31.0 ± 3.69
			8	1.41 ± 0.03	40.2 ± 8.35
			16	0.97 ± 0.01	41.8 ± 1.37
3	27.0 - 28.5	32	1	1.03 ± 0.00	16.9 ± 2.41
			2	1.19 ± 0.01	21.0 ± 2.33
			4	1.27 ± 0.02	26.4 ± 1.82
			8	1.21 ± 0.03	36.4 ± 3.82
			16	0.60 ± 0.01	20.2 ± 10.60

Table 2. Reproductive potentials, G, and percent eggs of SAPIAN at three salinity (a) and five food levels (b).

(a)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	24.5 - 27.5	32	4	1.08 ± 0.06	42.6 ± 4.22
		24		1.21 ± 0.21	44.4 ± 1.48
		16		1.34 ± 0.03	43.7 ± 5.44
3	25.0 - 27.5	32	4	1.21 ± 0.03	36.2 ± 1.88
		24		1.25 ± 0.09	23.3 ± 4.51
		16		1.28 ± 0.06	25.2 ± 9.28
4	25.0 - 29.5	32	4	1.25 ± 0.02	42.8 ± 2.20
		24		1.33 ± 0.01	46.1 ± 2.69
		16		1.43 ± 0.04	40.1 ± 1.51
5	25.5 - 30.0	32	4	1.19 ± 0.04	37.1 ± 1.49
		24		1.34 ± 0.03	33.7 ± 1.78
		16		1.39 ± 0.01	31.6 ± 7.80

(b)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	23.0 - 31.0	32	1	1.09 ± 0.04	34.8 ± 6.75
			2	1.18 ± 0.07	41.9 ± 1.33
			4	1.24 ± 0.06	44.2 ± 2.68
			8	1.26 ± 0.14	44.2 ± 1.86
			16	1.18 ± 0.08	43.8 ± 1.07
2	25.5 - 30.0	32	1	1.00 ± 0.02	25.0 ± 3.60
			2	1.14 ± 0.02	32.6 ± 3.66
			4	1.21 ± 0.01	39.7 ± 2.58
			8	1.19 ± 0.01	43.0 ± 2.48
			16	1.14 ± 0.00	47.6 ± 2.91

Table 3. Reproductive potentials, G, and percent eggs of OTON at three salinity (a) and five food (b) levels.

(a)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	26.0 -28.0	32	4	1.19 ± 0.08	50.5 ± 4.53
		24		1.22 ± 0.17	57.7 ± 4.60
		16		1.33 ± 0.02	39.3 ± 0.67
2	25.0 - 28.5	32	4	1.00 ± 0.05	39.3 ± 2.48
		24		1.19 ± 0.08	42.1 ± 4.96
		16		1.11 ± 0.22	33.3 ± 6.65
3	25.0 - 28.0	32	4	1.18 ± 0.03	45.4 ± 1.82
		24		1.34 ± 0.02	47.3 ± 2.39
		16		1.46 ± 0.03	48.2 ± 3.21

(b)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	21.5 - 30.0	32	1	1.22 ± 0.17	40.5 ± 11.32
			2	1.10 ± 0.06	52.9 ± 4.60
			4	1.12 ± 0.02	54.5 ± 2.06
			8	1.13 ± 0.08	52.5 ± 3.10
			16	0.93 ± 0.10	53.2 ± 1.35

Table 4. Reproductive potentials, G, and percent eggs of AKLAN at three salinity (a) and five to six food (b) levels.

(a)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
3	24.5 - 27.0	32	4	1.15 ± 0.01	36.9 ± 5.33
		24		1.27 ± 0.05	40.7 ± 8.80
		16		1.33 ± 0.05	45.6 ± 1.72
4	25.0 - 28.0	32	4	1.17 ± 0.01	37.2 ± 0.65
		24		1.33 ± 0.02	37.8 ± 1.84
		16		1.35 ± 0.02	28.5 ± 8.03

(b)

Trial	Temp (°)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	23.0 - 26.0	32	1	ND	ND
			2	1.09 ± 0.06	8.1 ± 2.30
			4	1.13 ± 0.04	8.3 ± 2.92
			8	0.87 ± 0.09	10.1 ± 4.70
			16	0.60 ± 0.09	19.1 ± 3.65
			32	0.46 ± 0.13	40.3 ± 14.52
2	23.0 - 26.0	32	1	0.99 ± 0.08	42.7 ± 2.82
			2	1.14 ± 0.05	38.0 ± 4.65
			4	1.21 ± 0.12	41.7 ± 5.83
			8	1.16 ± 0.10	41.5 ± 3.76
			16	1.02 ± 0.01	35.2 ± 1.11
3	25.0 - 27.5	32	1	0.97 ± 0.03	25.0 ± 8.77
			2	1.16 ± 0.04	21.8 ± 0.55
			4	1.24 ± 0.06	29.9 ± 5.43
			8	1.15 ± 0.03	25.5 ± 5.20
			16	0.97 ± 0.07	45.0 ± 3.97

Table 5. Reproductive potentials, G, and percent eggs of NAAWAN at three salinity (a) and five to six food (b) levels.

(a)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	24.5 - 27.0	32	4	1.28 ± 0.14	46.2 ± 3.03
		24		1.31 ± 0.09	44.6 ± 3.77
		16		1.34 ± 0.02	42.1 ± 3.86
2	25.0 - 27.0	32	4	1.28 ± 0.03	48.0 ± 1.40
		24		1.32 ± 0.03	42.9 ± 3.59
		16		1.32 ± 0.07	47.4 ± 3.83
3	24.5 - 27.0	32	4	1.17 ± 0.03	40.0 ± 4.22
		24		1.31 ± 0.05	40.2 ± 1.34
		16		1.40 ± 0.03	46.2 ± 2.27

(b)

Trial	Temp (°C)	Salinity (‰)	Food Level (X 10^6)	G	% Eggs
1	24.5 - 27.0	32	1	0.79 ± 0.13	44.2 ± 9.47
			2	0.92 ± 0.11	45.1 ± 4.33
			4	0.92 ± 0.00	47.9 ± 3.72
			8	0.91 ± 0.08	48.7 ± 2.99
			16	1.00 ± 0.04	47.5 ± 7.79
2	25.0 - 28.0	32	1	1.07 ± 0.05	19.6 ± 13.21
			2	1.21 ± 0.04	40.6 ± 1.19
			4	1.24 ± 0.04	41.6 ± 0.70
			8	1.10 ± 0.08	46.9 ± 5.03
			16	0.88 ± 0.06	46.8 ± 5.88
			32	0.72 ± 0.04	56.4 ± 4.17

Mass culture of the rotifer *Brachionus plicatilis*

Isolation of clones and high yield production

Research Report, October 1992

Introduction

The rotifer *Brachionus plicatilis* is an important food species for marine fish and invertebrate larvae in culture. Propagation techniques for this species, especially those intended for mass production, have to be developed to support intensive mariculture efforts. A first step is to determine culture conditions which favor high reproductive rates of rotifers.

In collaboration with research at the IOLR under Dr. E. Lubzens, the Philippine team has obtained rotifer samples from different geographical locations in the country to study their respective reproductive potentials at various levels of salinity, temperature and food. To date, we have studied the responses of 1 strain from northern Philippines (BANI), 3 from the central area (SAPIAN, AKLAN and OTON), and 1 from the southern part (NAAWAN). This report summarizes the results of 25 experiments, 18 of which were done using a temperature-controlled water bath. Three strains (BANI, SAPIAN and NAAWAN) were used in all 25 experiments. The first set of experiments was designed to determine the effects of 3 temperature levels and 3 salinities on the reproductive rates of the rotifers which were maintained at 4 x 10^6 cells of *Tetraselmis tetrathele* rot ml^{-1}. The second set was conducted to determine the effects of 5 density levels of *T. tetrathele* on rotifers maintained at 2 salinity levels and at the optimal temperature of 30°C. The third and fourth sets consisted of experiments using *Isochrysis galbana* as the food species. Experiments with 5 levels of *I. galbana* fed to rotifers which were maintained at 32‰ and at room temperature (25 - 30°C) made up the third set. The fourth set consisted of experiments using 5 food levels of *I. galbana* fed to rotifers which were maintained at 2 salinity levels and at 30°C. To date, 3

more experiments for the fourth set have to be completed.

Materials and Methods

Collection of samples. Rotifers were obtained from existing cultures in SEAFDEC and in the Mindanao State University (Naawan Campus). Those from SEAFDEC were originally collected from wild populations in 3 provinces (SAPIAN from Sapian, Capiz; DUM from Dumaguet, Aklan (DUM is also coded as AKL for AKLAN); OTON from Oton, Iloilo; and NAAWAN from Naawan, Misamis Oriental). Samples obtained from Naawan were originally taken from fishponds fringing Panguil Bay.

Maintenance. Cultures were maintained in 500 ml flasks in an airconditioned room with temperature ranging from 23.0 to 31°C. They were fed with the algae *Tetraselmis tetrathele* at 4.0 X 10^6 cells per rot ml daily, and offered in three doses. The algae were raised in 8 liter carboys under constant illumination and the same temperature range as that for rotifers. They were centrifuged, the resulting concentrate subsampled to determine density of cells ml^{-1}, and then fed to the rotifers.

Experimental set-up. Before the experiments were started, resting eggs from each population were isolated. A pure culture, i.e. culture propagated from a single resting egg, was maintained for each of the 3 populations, and from which rotifers for the 4 sets of experiments were obtained. Because a low level of mixis occurs, the cultures, over time, were no longer pure. Rotifers were acclimatized to experimental salinity and temperature levels, and to the experimental food species for at least a week to a month, if they differed from maintenance conditions.

The general experimental procedure followed was based on the methods established by Dr. Lubzens of IOLR. A number of rotifers (1-10 per ml) were suspended in 20 ml

Table 1. The effects of 3 temperature levels, and 3 salinity levels on the reproductive rates of 3 geographically distinct populations of *Brachionus plicatilis*. The rotifers were fed with 4 x 10^6 cells of *Tetraselmis tetrathele* rot ml^{-1}.

Strain	Temperature (°C)	16‰	24‰	32‰
Bani	25	1.17 ± 0.02	1.12 ± 0.03	0.60 ± 0.19
	30	1.56 ± 0.03	1.52 ± 0.01	1.46 ± 0.01
	35	1.36 ± 0.00	1.35 ± 0.01	1.03 ± 0.29
Sapian	25	1.21 ± 0.00	0.91 ± 0.00	1.00 ± 0.00
	30	1.53 ± 0.01	1.46 ± 0.00	1.39 ± 0.03
	35	0.86 ± 0.04	1.36 ± 0.04	1.25 ± 0.01
Naawan	25	1.32 ± 0.02	1.18 ± 0.00	0.97 ± 0.01
	30	1.48 ± 0.01	1.45 ± 0.04	1.37 ± 0.01
	35	0.85 ± 0.00	1.42 ± 0.03	1.33 ± 0.02

seawater contained in 50 ml flasks, the number of which were subdivided into 3 replicate flasks per treatment. Rotifers were given algae daily in three doses. Water was changed every other day by sieving rotifers using a 25 µm mesh nylon sieve and resuspending the animals in freshly filtered (0.45 µm filter) sea water. Rotifers were counted on the first and fourth (last) day of an experiment by pipetting a given volume from each flask and directly counting rotifers under an M8 Wild dissecting scope while inside the pipette. The number of males, females and eggs were counted to determine percent mixis (%males/females), percent eggs (% eggs/total where total = females + males + eggs), and reproductive potential G ($G = \ln (N_t/N_o)/T$ in days). Three replicate counts were made for each flask with replacement, the average and standard deviation of which are used in statistical comparisons.

Statistical tests using one- or two-way analyses of variance were limited to determining trends within a population. Significance level was set at 0.05 and Tukey's test was used in multiple mean comparisons.

Results

Table 1 and Fig. 1 summarize the reproductive rates of the 3 rotifer strains obtained from the first set of experiments. A two-way ANOVA done for each strain gave significant variance ratios for temperature, salinity, and the interaction between these 2 factors. For BANI strain, reproductive rates obtained at 25, 30 and 35°C were higher at 16‰ and 24‰ than at 32‰. At all salinity levels, 30°C was optimal for BANI rotifers compared to 25 and 25°C.

For SAPIAN rotifers, animals maintained at 16‰ gave the highest reproductive rates at both 25 and 30°C compared to those placed at other salinity levels. However, at 35°C, rotifers reared at 24‰ reproduced the fastest. At all salinity levels, animals reared at 30°C

exhibited the highest reproductive rates. In the case of the NAAWAN strain, individuals reared at 25 and 30°C reproduced the fastest at 16‰; at 35°C, animals maintained at 24‰ gave the highest rates. At 16 and 32‰, 30°C was optimal; at 24‰, organisms maintained at 30 and 35°C gave rates higher than those reared at 25°C. Thus, the interaction between temperature and salinity did not allow for unequivocal conclusions to be made. Furthermore, it was possible that food may have limited reproductive rates at 35°C, as validated in the next set of experiments. The F values due to factor interaction was highest among NAAWAN rotifers, intermediate among SAPIAN rotifers, and least among BANI rotifers.

The effects of 5 density levels of *Tetraselmis tetrathele* and 2 salinity levels on the 3 rotifer strains maintained at 30°C are summarized in Table 2 and Fig. 2. A 2-way ANOVA for BANI rotifers indicated no significant interaction between food and salinity. Optimal reproductive rates were obtained at 8 x 10^6 *T. tetrathele* cells rot ml^{-1} at both 16 and 32‰. Statistical analyses for SAPIAN and NAAWAN populations showed significant factor interaction, specially in the former. For the SAPIAN population, 4 x 10^6 cells rot ml^{-1} was the optimal food level at 16‰. At 32‰, reproductive rates of the rotifers increased with increasing food concentration and did not exhibit a satiety level. For NAAWAN rotifers, they required 8 x 10^6 cells rot ml^{-1} at 16‰; and half of this concentration at 32‰. While the 3 rotifer populations indicated food-limited reproductive rates at 1 or both salinity levels, rates obtained at 4 x 10^6 *T. tetrathele* cells in the previous set (30°C, 16‰) were significantly higher than those obtained at similar or even higher food levels in the second set.

Rotifers belonging to the 3 strains were used in testing the effect of *Isochrysis galbana* as food species (Table 3 and Fig. 3). They were maintained at room temperature which ranged from 25 to 30°C and at 32‰. One-way ANOVA for each strain indicated significant F values except for NAAWAN rotifers. For the BANI population, reproductive rates at food

levels ranging from 4 to 32 x 10^6 cells rot ml^{-1} did not differ significantly from each other. Similarly, rates obtained at food levels ranging from 16 to 64 x 10^6 cells rot ml^{-1} were not statistically different from one another, but were significantly lower than the first statistical group. In the case of SAPIAN rotifers, reproductive rates were statistically similar at food ranging from 4 to 32 x 10^6 cells rot ml^{-1}, all of which were significantly higher than rates obtained at 64 x 10^6 cells rot ml^{-1}. For the NAAWAN population, the 5 food levels of *I. galbana* did not have any significant effect on reproductive rate.

The effect of various densities of *I. galbana* on reproductive rates of 3 rotifer populations was further tested under a more controlled temperature level, i.e. 30°C. The rotifers were maintained at 16 and 32‰ (Table 4). A two-way ANOVA for BANI rotifers showed a significant factor interaction; thus, the need to qualify under what conditions a food level was optimal. At 16 and 32‰, reproductive rates were highest at 16 x 10^6 cells rot ml^{-1}. For SAPIAN at 32‰, reproductive rates were highest at the same food level. In all experiments using *I. galbana* as food species, reproductive rates were significantly lower than those obtained when *Tetraselmis tetrathele* was used.

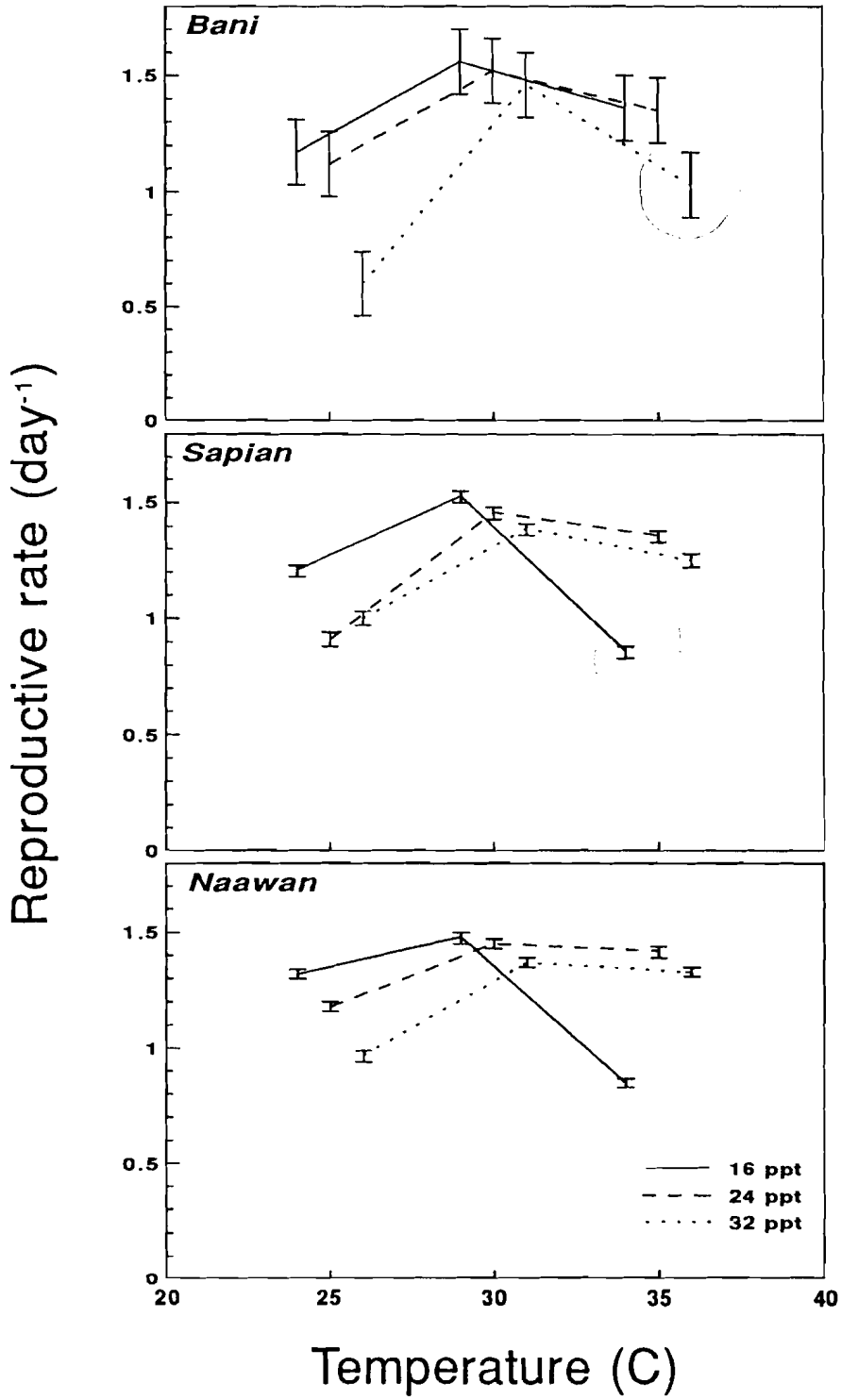


Figure 1. Reproductive rates of *BANI, SAPIAN* and *NAAWAN* rotifers at 3 temperature (25, 30 and 35°C) and 3 salinity (16, 24 and 32‰) levels. Entries were offset at each temperature level to avoid graphic overlap.

Table 4. The effects of 5 food levels of *Isochrysis galbana* on the reproductive rates of 3 *Brachionus plicatilis* strains maintained at 2 salinity levels and at 30°C.

Strain	Food Level (x 10^6)	16‰	32‰
Bani	4	0.75 ± 0.03	0.79 ± 0.03
	8	0.83 ± 0.00	0.89 ± 0.01
	16	1.02 ± 0.03	0.98 ± 0.00
	32	0.77 ± 0.00	0.85 ± 0.01
	64	0.00 ± 0.00	0.41 ± 0.02
Sapian	4	0.80 ± 0.01	0.78 ± 0.01
	8	0.94 ± 0.01	0.91 ± 0.01
	16	0.99 ± 0.02	0.96 ± 0.01
	32	0.86 ± 0.03	0.83 ± 0.02
	64	0.00 ± 0.00	0.00 ± 0.00
Naawan	4	0.91 ± 0.02	0.66 ± 0.02
	8	1.02 ± 0.04	0.66 ± 0.08
	16	1.09 ± 0.00	0.85 ± 0.01
	32	1.13 ± 0.00	0.78 ± 0.02
	64	0.00 ± 0.00	0.00 ± 0.00

Table 4. The effects of 5 food levels of *Isochrysis galbana* on the reproductive rates of 3 *Brachionus plicatilis* strains maintained at 2 salinity levels and at 30°C.

Strain	Food Level (x 10^6)	16‰	32‰
Bani	4	0.75 ± 0.03	0.79 ± 0.03
	8	0.83 ± 0.00	0.89 ± 0.01
	16	1.02 ± 0.03	0.98 ± 0.00
	32	0.77 ± 0.00	0.85 ± 0.01
	64	0.00 ± 0.00	0.41 ± 0.02
Sapian	4	0.80 ± 0.01	0.78 ± 0.01
	8	0.94 ± 0.01	0.91 ± 0.01
	16	0.99 ± 0.02	0.96 ± 0.01
	32	0.86 ± 0.03	0.83 ± 0.02
	64	0.00 ± 0.00	0.00 ± 0.00
Naawan	4	0.91 ± 0.02	0.66 ± 0.02
	8	1.02 ± 0.04	0.66 ± 0.08
	16	1.09 ± 0.00	0.85 ± 0.01
	32	1.13 ± 0.00	0.78 ± 0.02
	64	0.00 ± 0.00	0.00 ± 0.00

Table 2. The effects of 5 density levels of *Tetraselmis tetrathele* and 2 salinity levels on *Brachionus plicatilis* maintained at 30°C.

Strain	Food Level (x 10^6)	16‰	32‰
Bani	1	1.08 ± 0.00	0.92 ± 0.12
	2	1.21 ± 0.01	1.18 ± 0.01
	4	1.37 ± 0.01	1.22 ± 0.10
	8	1.43 ± 0.02	1.37 ± 0.09
	16	1.35 ± 0.01	1.21 ± 0.03
Sapian	1	1.19 ± 0.01	1.11 ± 0.00
	2	1.37 ± 0.03	1.18 ± 0.02
	4	1.47 ± 0.00	1.34 ± 0.00
	8	1.47 ± 0.02	1.54 ± 0.01
	16	1.21 ± 0.02	1.62 ± 0.01
Naawan	1	1.10 ± 0.01	1.09 ± 0.01
	2	1.22 ± 0.01	1.19 ± 0.02
	4	1.39 ± 0.01	1.38 ±0.01
	8	1.43 ± 0.01	1.25 ± 0.03
	16	0.86 ± 0.02	0.93 ± 0.03

Table 3. The effects of 5 food levels of *Isochrysis galbana* on the reproductive rates of *Brachionus plicatilis* strains (Bani, Sapian and Naawan) maintained at 32‰ and at room temperature range (25 - 30°C).

Strain	Trial	Food Level (x 10^6)				
		4	8	16	32	64
Bani	1	0.81 ± 0.03	0.86 ± 0.02	0.87 ± 0.04	0.99 ± 0.01	0.43 ± 0.39
	2	0.76 ± 0.01	0.80 ± 0.01	0.78 ± 0.00	0.77 ± 0.01	0.72 ± 0.00
Sapian	1	0.82 ± 0.02	0.91 ± 0.01	0.91 ± 0.01	0.87 ± 0.14	0.49 ± 0.40
	2	0.81 ± 0.03	0.86 ± 0.02	0.87 ± 0.04	0.99 ± 0.01	0.43 ± 0.39
Naawan	1	0.69 ± 0.02	0.84 ± 0.02	0.99 ± 0.00	1.08 ± 0.00	1.18 ± 0.01
	2	0.68 ± 0.11	0.87 ± 0.01	0.97 ± 0.03	0.76 ± 0.02	0.00 ± 0.43
	3	0.87 ± 0.00	0.80 ± 0.01	0.74 ± 0.01	0.69 ± 0.05	0.59 ± 0.39

Table 4. The effects of 5 food levels of *Isochrysis galbana* on the reproductive rates of 3 *Brachionus plicatilis* strains maintained at 2 salinity levels and at 30°C.

Strain	Food Level (x 10^6)	16‰	32‰
Bani	4	0.75 ± 0.03	0.79 ± 0.03
	8	0.83 ± 0.00	0.89 ± 0.01
	16	1.02 ± 0.03	0.98 ± 0.00
	32	0.77 ± 0.00	0.85 ± 0.01
	64	0.00 ± 0.00	0.41 ± 0.02
Sapian	4	Ongoing	0.78 ± 0.01
	8		0.91 ± 0.01
	16		0.96 ± 0.01
	32		0.83 ± 0.02
	64		0.00 ± 0.00
Naawan	4	Ongoing	Ongoing
	8		
	16		
	32		
	64		

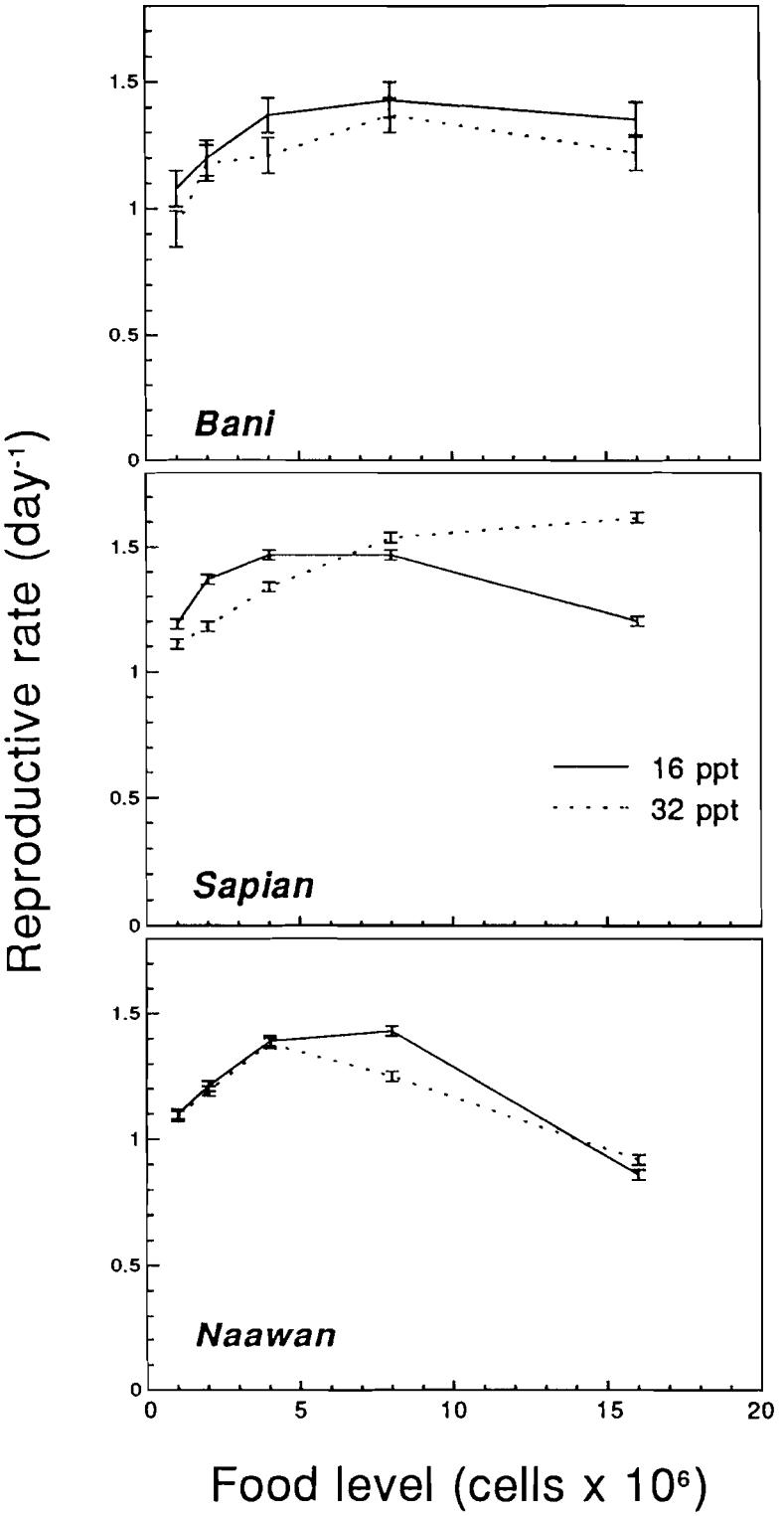


Figure 2. Reproductive rates of 3 rotifer strains fed with *Tetraselmis tetrathele* at 5 densities (cells x 10^6 rot ml^{-1}) and maintained at 30°C, and at 16 and 32‰.

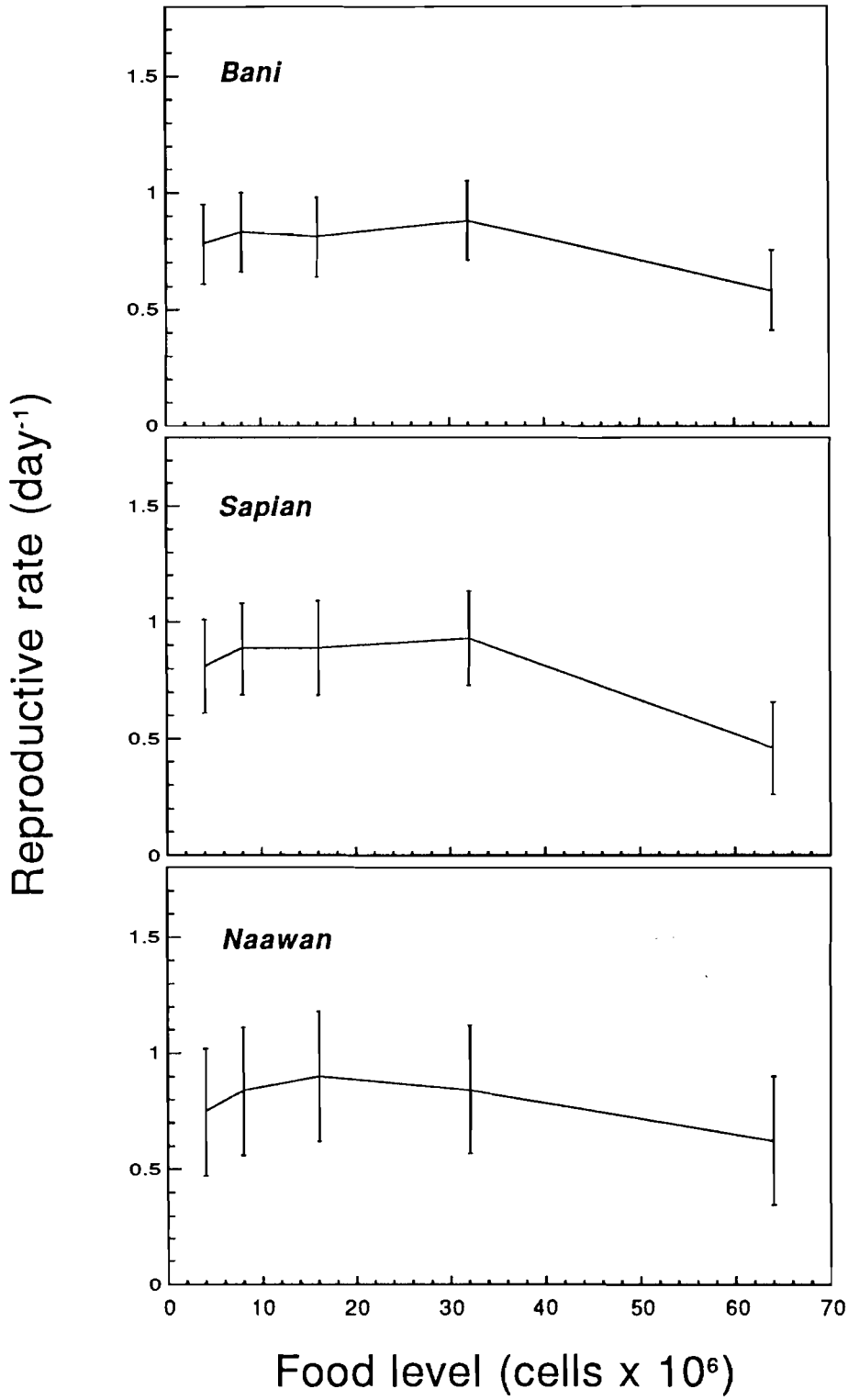


Figure 3. Reproductive rates of 3 rotifer strains fed with *Isochrysis galbana* at 5 food densities (cells x 10^6 rot ml^{-1}) and maintained at room temperature range (25-30°C) and at 32‰.

Mass culture of the rotifer *Brachionus plicatilis*

Isolation of clones and high yield production

Research Report, July 199~~1~~2

X

Introduction

In the previous report we showed results of reproductive rates (G) mainly for two rotifer strains (OTON, SAP) obtained from the Philippines, which were cultured on algae (*Nannochloropsis* sp.). In the present report, we show results for the third strain (DUM) obtained from the Philippines. We completed some experiments with strains OTON and SAP at high temperatures which were not tested previously.

From our results and culture experience, the rotifers of strain SAP seem to be the best candidates for mass cultures at the high temperature conditions (30°C) prevailing in the Philippines. The highest reproductive rates with this strain were obtained when it was cultured at a salinity of 10 ‰. However, at this salinity a high production of males occurred, which should be avoided in mass cultures of rotifers, that are intended as food for fish larvae. Due to the higher cost of algae, yeast is used as food instead of algae, in large scale cultures. Yeast fed rotifers lack essential long chained unsaturated fatty acids, which are essential for the survival of marine fish larvae. Our aims were:

1) to determine the reproductive rates of SAP rotifers fed on yeast and compare it to the reproductive rates obtained with rotifers fed on algae.
2) to determine the fatty acid composition of rotifers fed on yeast or algae.
3) to determine the appropriate combination of yeast and algae that should be provided to rotifers in order to obtain optimal reproductive rates and the best combination of fatty acid composition.

Materials and Methods

The reproductive rates for rotifers were determined as described in the previous report.

Rotifers from the strain SAP were used in experiments relating the reproductive rate (G) to the amount of food (algae or yeast) fed to rotifers (Fig. 1 in

the results section). Rotifers in this series of experiments were cultured at 30°C and in sea water (salinity 20 ‰). On the first day of the experiment, about 30,000 rotifers were suspended in 500 ml sea water in 1 litre Ehrlenmeyer flasks. Rotifers were fed daily for three consecutive days, with known amounts of algae or yeast. The food was provided in amounts ranging from 0.1 g (dry weight) up to 3.0 g (dry weight) per 10^6 rotifers per day. Three replicate flasks were used for each of the tested food concentrations. The number of rotifers and the number of eggs carried by females were counted in three replicate samples removed from each flask. The volume of samples taken for counting ranged from 1 ml to 0.1 ml, according to the density of the rotifers. At least 50 rotifers were counted in each sample, and the sample was returned to the flask before the next one was taken. The reproductive rates (G) were calculated (as shown in the previous report) from counts made on the first and last day of the experiment (3 days). After the last counts were made on day 3, rotifers were starved for 1 hour and taken for fatty acid analysis.

For determining the dry weight of algae (*Nannochloropsis* sp.), algae were taken from batch cultures during the log phase of their growth (culture density: 6×10^9 cells ml^{-1}). Algae were centrifuged at 5,000 g in 250 ml cups, the pellet was resuspended in a small volume of membrane filtered sea water, and the number of cells ml^{-1} was counted in a hemocytometer. Aliquots of 0.5-6 ml from the algal concentrate were sieved through pre-weighed GF/C filters (4.7 cm in diameter). After drying at 60°C for 24 hours, the filters were weighed and the weight was related to the number of cells per sample (Fig. 2). Dry weight of baker's yeast was determined by drying 10 samples weighing 1 g (wet weight) each at 60°C for 24 hours.

Results and Conclusions

1. Reproductive rates of rotifers fed on algae

The reproductive rates (G) for rotifers of strain DUM are shown in Table 1. This strain is clearly thermophilic and can be cultured at temperatures reaching 36 or 38°C. The best reproductive rates were obtained at the lowest salinities (10‰),

where mixis also occurred. Increasing the salinity from 20‰ to 30‰ had a small effect on the reproductive rates, but reduced dramatically the occurrence of mixis. Therefore, for mass cultures, this strain should be cultured at 30‰ and at temperatures of 30-36°C.

The results on SAP and OTON (Table 2) reveal also that the highest reproductive rates were obtained with rotifers cultured at a salinity of 10‰. However, again, at this salinity, high mixis occurred, which was reduced in cultures maintained at 20 or 30‰. Due to the smaller size of SAP rotifers, we decided to use this strain for future consideration in mass cultures.

2. The effect of yeast on the reproductive rate of SAP strain rotifers

The accepted practice today in mass cultivation of rotifers is to feed rotifers with baker's yeast, in order to reduce the cost of production. Raising algae as the sole food of rotifers is extremely costly and also leads to dependence on a live culture, which may fail periodically. Yeast, however, lacks essential long chain highly unsaturated fatty acids (HUFA), which are essential to marine fish larvae. The accepted practice today is to enrich rotifers with algae containing HUFA for 8-24 hours before they are transferred to the fish tanks as food. From our results (Fig. 1), it is clear that the reproductive rate of rotifers is dramatically reduced, when they are fed on yeast. While the plateau is reached at similar amounts of food supplied (1.255-2.00 g dry weight per 10^6 rotifers), the reproductive rate of algae fed rotifers is 1.4 per day compared to 0.6 per day for yeast fed rotifers.

The total amount of fatty acids of rotifers was related to the amount of food they received. There were only slight differences between rotifers fed yeast and those fed on algae (Table 3). However, rotifers fed on algae contained considerable amounts of EPA (20:5 ω3), while those fed on yeast lacked these essential fatty acids. Rotifers fed on algae contained also considerable amounts of 16:0, and those fed on yeast were relatively enriched with 18:1 n9. The content of rotifers fed with yeast in combination with increasing amounts of algae is currently being investigated.

It is possible that including some algae in the diet of yeast fed rotifers could increase their reproductive rates and their HUFA content. In terms of management, this would mean that instead of providing a large amount of algae at the time of enrichment, the same amount of algae could be provided during the culture period of the rotifer. This would contribute to higher production of rotifers. Also, these rotifers would contain HUFA that are required by fish larvae and reduce the necessity of the enrichment period. The optimal combinations of yeast and algae that should be provided in order to obtain higher production rates of rotifers and optimal amounts of HUFA are currently being determined in our laboratory.

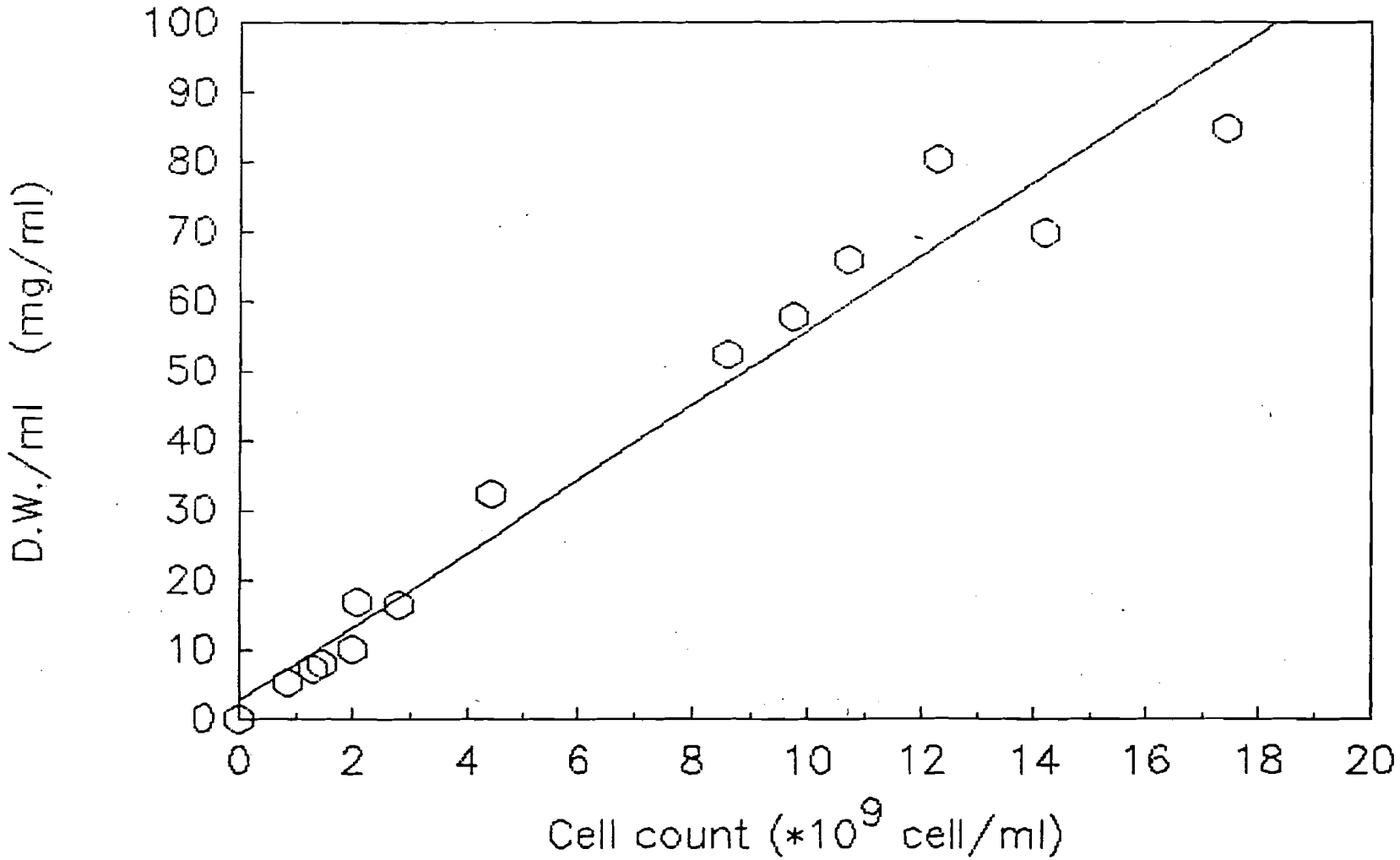
Table 1. The reproductive rates (G) of rotifers (strain DUM) raised at three salinities (10, 20 and 30 ‰) and three temperatures (30, 36 and 38°C)

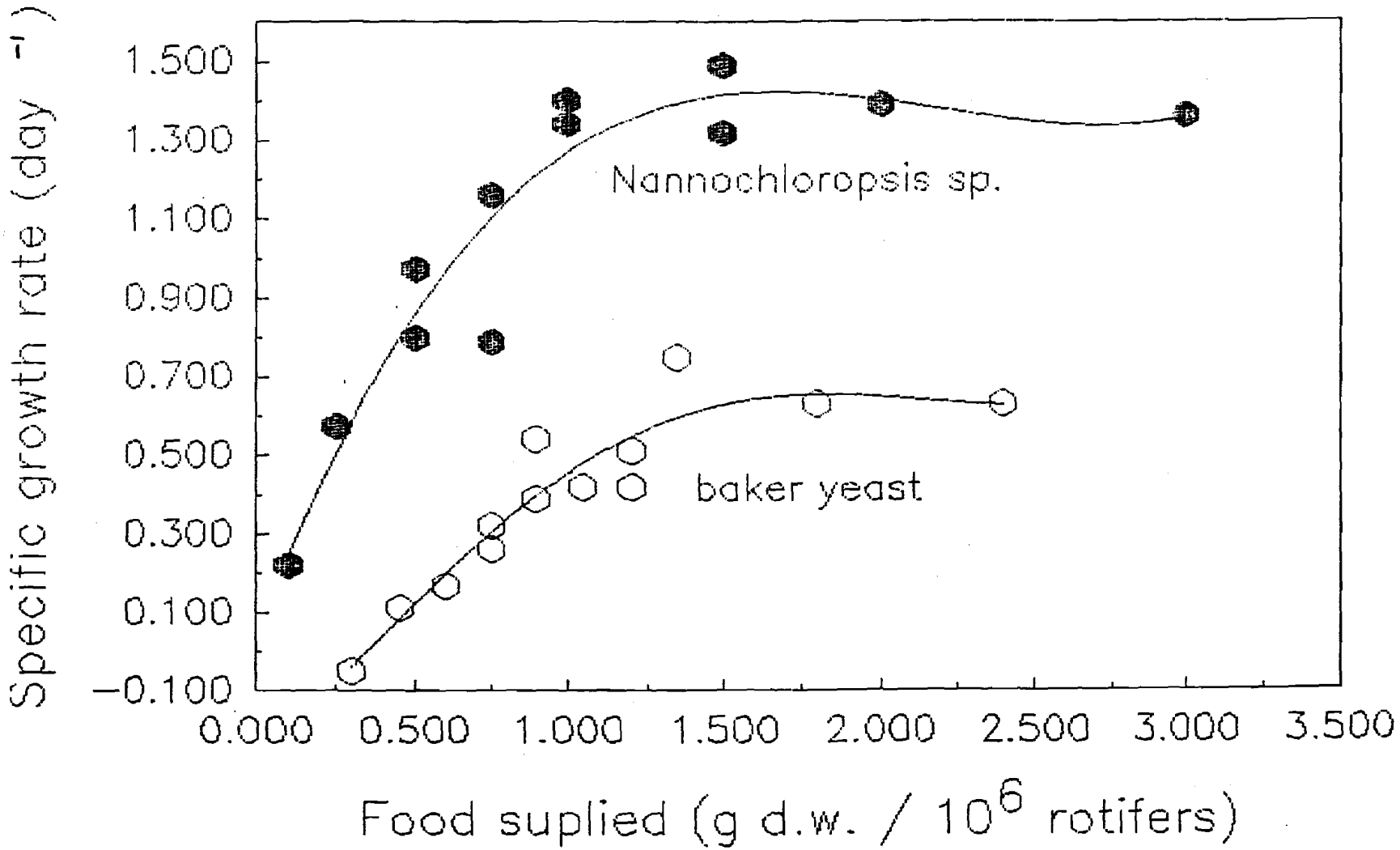
Experiment no.	Temp °C	Salinity ‰	Reproductive rate (G)
14	30	20	1.70 ± 0.04
		30	1.72 ± 0.02
	30	20	1.69 ± 0.04
		30	1.63 ± 0.03
15	30	20	1.64 ± 0.04
		30	1.57 ± 0.06
	36	20	1.75 ± 0.05
		30	1.72 ± 0.06
16	30	10	2.21 ± 0.04
		20	2.05 ± 0.04
	38	10	2.24 ± 0.05
		20	2.34 ± 0.03

Table 2. The reproductive rates (G) of two rotifer strains (SAP and OTON) raised at four salinities (10, 20, 30 and 40 ‰) and at 30 or 35 °C

Strain	Temp °C	Salinity ‰	Reproductive rate (G)
SAP	30	10	2.10 ± 0.01
		20	1.71 ± 0.10
		30	1.62 ± 0.06
		40	1.12 ± 0.16
	35	10	2.13 ± 0.01
		20	2.09 ± 0.02
		30	1.77 ± 0.09
		40	1.52 ± 0.04
OTON	35	10	1.91 ± 0.06
		20	1.83 ± 0.02
		30	1.36 ± 0.05
		40	dead

FIG 2


D.W./ml (mg/ml)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Cell count (*10^9 cell/ml)


Specific growth rate (day⁻¹)
1.500
1.300
1.100
0.900
0.700
0.500
0.300
0.100
−0.100
0.000
0.500
1.000
1.500
2.000
2.500
3.000
3.500
Food suplied (g d.w. / 10^6 rotifers)
Nannochloropsis sp.
baker yeast

Mass culture of the rotifer *Brachionus plicatilis*
Isolation of clones and high yield production
(Third Year - Israel)

INTRODUCTION

The third year of the project was devoted to: (a) nutritional improvement of cultured rotifers; (b) identification of rotifer strains obtained from the Philippines by using restriction endonuclease analysis of mt DNA.

Nutritional improvement of cultured rotifers

The rotifers provide the only nutritional source of cultured marine larvae during the initial period of their life which lasts 15-20 days after hatching from their eggs. Lipids are an essential compnent of these nutritional requirements. While yeast-fed rotifers synthesize lipids *de novo* (Lubzens et al., 1985), including polyunsaturated fatty acids (PUFA), the total amount that is accumulated in them is insufficient. The common practice in marine hatcheries includes mass cultivation of rotifers using yeast as a cheap source of food, and enrichment of rotifers with lipids for a short period (8-24 h) before they are supplied to the marine fish larvae. Rotifers are enriched with algae containing the essential fatty acids (EFA) or with formulated diets fortified with fish oil. These enrichment foods are significantly more expensive than yeast.

We tested the reproductive rates and lipid content of rotifers fed on *Nannochloropsis* sp. and compared them to yeast-fed rotifers. *Nannochloropsis* sp. is rich in eicosapentaenoic acid (EPA, 20:5 ω3), while fish oils contain both EPA and docosahexaenoic acid (DHA, 22:6 ω3). Several species of marine fish were found to require both EPA and DHA; for freshwater species, DHA is not essential (Watanabe, 1993). The euryhaline rotifers of the species *Brachionus plicatilis* were also found to be suitable for freshwater carp larvae (Lubzens et al., 1987).

In addition to serving for EPA enrichment of rotifers, *Nannochloropsis* is important in supporting rotifer biomass production. It is well known that reproductive rates of rotifers fed on algae are higher than those fed yeast. Furthermore, algal-raised rotifer cultures are more stable and less prone to collapse than yeast-fed mass cultures. However, supplying live algae is expensive and results in dependence of rotifer cultures on a direct supply from algal cultures. Replacement of live algae with frozen ones could be one solution. We tested the possible replacement of live algae with frozen concentrated mass for both biomass production and lipid enrichment of rotifers.

Restriction enzyme analysis of mt DNA

The large quantities required daily in these fish hatcheries depend on daily production from continuous or semi-continuous cultures. Rotifer strains vary considerably in their reproductive potentials, with r values from 0.55 to 1.15 (Snell and Carrillo, 1984; Lubzens, 1987; Lubzens et al., 1989). Larger stocks of rotifers with low reproduction rates must be kept than of those with fast reproduction rates. Since in several cases fish larvae showed a clear age effect on their feeding preference of different size rotifers (Fukusho, 1983; Korunuma and Fukusho, 1987; Lubzens et al., 1989), obtaining higher survival rates of fish larvae involves culturing more than one rotifer strain on location. Furthermore, keeping the various live strains available may result in accidental contamination. Means for identifying a specific rotifer strain or clone are essential in maintaining such a live collection. In addition to morphological differences, allozyme patterns (Fu et al., 1991) and restriction mapping of the mt DNA genome (King, 1989) have been proposed for distinguishing among clones of different populations. The use of mt DNA for population studies is widespread and well known (Avise et al., 1987; Moritz et al., 1987; Caccone et al., 1988).

We report here on a simplified and rapid method for the isolation of mt DNA from various strains of *B. plicatilis* and show that restriction enzyme analysis can be used to distinguish among rotifer strains of importance in mariculture.

The profiles from restriction endonuclease analysis of mt DNA obtained for three rotifer strains received from the Philippines at the initiation of the program were compared with the cultured strains brought back from the Philippines at the end of the program.

MATERIALS AND METHODS

Nutritional improvement of cultured rotifers

Algae

Fresh algae (*Nannochloropsis* sp.) were cultured as described in previous reports. Frozen biomass was prepared after centrifugation of mass cultured algae and obtained from Dr. Assaf Sukenik, IOLR, Haifa, Israel.

Yeast

Fresh baker's yeast was obtained from local bakeries and stored for up to one week at 4°C.

Rotifers

Rotifers from the SAP strain (size 171.7 × 116.6 µm) were used in the reproductive rate and nutritional quality experiments. In restriction endonuclease analysis of mt DNA, six rotifer cultures were used. Rotifers of the strains SAP, DUM and OTON were obtained in 1990 from Dr. L. McManus. Strains SAPIAN, BANI and OTONI were brought back from the Philippines in 1992.

For extraction of mt DNA, rotifers were batch cultured in 3-4 litres of membrane filtered (0.45 µm) natural sea water at 25 or 30 °C and at a salinity of 20 or 30 ‰, depending on the strain. They were fed on live or frozen *Nannochloropsis* sp. Twenty-four hours before extraction of mt DNA, rotifers were sieved (60 µm mesh) and transferred to freshly sieved sea water (at the appropriate salinity) and starved overnight.

Extraction of mt DNA

The extraction of mt DNA was carried out by a modification of the method reported for *Drosophila* (Tamura and Aotsuka, 1988) and is given in detail:

Procedure:

Stages 1-7 are performed on ice and in a precooled centrifuge (a Sorvall RC-5C or a microfuge can be used throughout the preparation).

1. Sieve rotifers through a 60 mesh sieve and resuspend at a density of 100,000 per ml, in precooled homogenizing buffer (solution STE).
2. Homogenize the rotifers in a glass (15 ml) homogenizer (Dounce tissue grinder). Use at least 15 strokes. Check whether rotifers are homogenized by looking at a small drop under a microscope.
3. Transfer the homogenate to Eppendorf vials (about 1.5 ml per vial) and centrifuge at 1,000 g for 2 min. Repeat this stage twice, by transferring the supernatant after the first centrifugation to new vials and centrifuging them at 1,000 g.
4. Transfer the supernatant to new vials and centrifuge at 2,000 g for 11 min.
5. Discard the supernatant and suspend the precipitate in solution #1, so that the final volume is 50 µl. Use a vortex to achieve full suspension.
6. Prepare a fresh solution of solution #2 and add 100 µl to each test tube. Mix (vortex) and incubate on ice for 5 min.
7. Add 75 µl of precooled solution #3 to each test tube. Mix (vortex) and incubate again on ice for 5 min.
8. Centrifuge test tubes at 12,000 g for 6 min (4°C).
9. Remove the supernatant from each test tube. Combine the supernatant of every three tubes into one. Now add 1/2 volume of a chloroform-isoamyl solution and another 1/2 volume of phenol. Mix (vortex) the contents of each test tube and centrifuge at room temperature for 3 min at 12,000 rpm.

10. Transfer carefully the upper liquid phase into clean test tubes. If a significant white precipitate between the two phases is found, repeat step 9 again. Add two volumes of precooled absolute alcohol (-20°C). Mix and leave overnight at -20°C.

    Perform the following steps on the next day:

11. Centrifuge tubes at R.T. for 15 min at 12,000 rpm. Discard the supernatant and wash the precipitate by adding 1 ml of cold (-20°C) 70% ethanol. Mix gently and centrifuge again at 12,000 g for 15 min.
12. Discard supernatant and dry the precipitate in vacuum for 30 min or until the precipitate is transparent and dry.
13. Suspend the precipitate in 10 µl of water, mix (vortex) and leave in the refrigerator (4°C) at least overnight. Between 50-70 µg of DNA are collected from each extraction.
14. Store the tubes at 4°C. Take aliquots for digestion with endonuclease enzymes.

Solutions:

1. Solution STE (homogenizing buffer): Mix 15 ml Tris-Cl (1 M, pH=7.5), 10 ml EDTA (0.5 M, pH=8.0) and 475 ml distilled water in a 500 ml bottle and autoclave. Keep refrigerated until use. Weigh into sterile 50 ml conical vials (Coster, U.S.A.) 4.27 g of sucrose. Add 50 ml of the sterile Tris-Cl and EDTA solution prepared before, mix thoroughly and keep on ice for Step 1 in the Procedure.
2. Solution #1: Mix 1 ml of Tris-Cl(1 M, pH=8.0), 2 ml of EDTA (0.5 M, pH=8.0) and 3 ml NaCl (5 M) in 1 liter of distilled water. Autoclave the solution and keep refrigerated until use (Step 5 in the Procedure).
3. Solution #2: Prepare this solution immediately before use (Step 6 in the Procedure) by mixing 200 µl of NaOH (from 1 M stock solution), 700 µl of sterile H_2O (sterile; double distilled) and 100 µl SDS (from 10% stock solution).

4. Solution #3: Dissolve 58.89 g of potassium acetate (KAc) in a final volume of 120 ml H_2O. Add 23 ml of glacial acetic acid and 57 ml of H_2O (final volume - 200 ml; pH ranging between 4.8-5.4).
5. Preparation of chloroform-isoamyl solution: Add to 96 ml chloroform 4 ml of isoamyl.
6. Preparation of phenol solution: Dissolve the crystals (at 60°C) and add an equal volume of H_2O (double distilled and sterile). Keep in a refrigerator in a dark bottle covered with aluminum foil. Prior to use (Step 9 in the Procedure), add to the required volume of phenol 1/2 to 1/3 volumes of TE buffer. Mix and evaluate the pH by placing a drop on pH paper. Titrate with NaOH to obtain pH = 8.0.
7. TE buffer: Prepare an X10 sterile stock solution by mixing 10 ml Tris-Cl (1 M, pH = 8.0) and 2 ml EDTA (0.5 M) in a final volume of 100 ml. Dilute the TE buffer solution before use in the phenol solution step (see above).

A sample containing about 5 µg of DNA is incubated with 2 µl of a restriction enzyme. A buffer specific to each enzyme (2 µl) and 2 µl of RNAse are added. The total volume is made up to 22 µl with distilled sterile H_2O. After incubation (overnight at 37°C), 2 µl of gel ladding buffer is added, and each sample is run on a 1% agarose gel. Agarose gels (1%) were prepared and run in a horizontal minigel apparatus (Hoefer Scientific Instruments, USA) for 2 h (95 V) as described by Ausubel et al. (1989). Gels were visualized in U.V. by ethidium bromide and photographed with a polaroid camera. Fragment analysis was carried out by comparing the mobility of rotifer DNA samples to that of DNA molecular weight markers (digested with Hind III).

RESULTS

Nutritional improvement of cultured rotifers

The effect of various food sources (fresh or dried yeast; live, frozen or dried *Nannochloropsis*) on the reproductive rate, total fatty acid content and fatty acid

distribution in rotifers is shown in Table 1. Results show that live *Nannochloropsis* supported the highest rates of reproduction, followed by frozen algae or fresh yeast. The relatively high rates obtained with yeast ($r = 0.84$) were due to the fact that the daily portion (6 gm fresh weight or 2 gm dry weight per 10^6 rotifers) was supplied to the rotifers in 4-6 meals, at equal intervals over a 24 h period. The reproductive rates were greatly reduced when rotifers were fed on equivalent amounts of dried algae or dried yeast. Almost no differences were found between rotifers fed fresh or frozen algae in their total fatty acids (TFA) and fatty acid distribution. Yeast-fed rotifers showed reduced amounts of 16:0 and lacked PUFA such as arachidonic acid (20:4 ω6), EPA (20:5 ω3), docosapentaenoic acid (DPA; 22:5 ω3) and DHA (22:6 ω3). On the other hand, the rotifers contained relatively more palmitoleic acid (16:1 ω7) and oleic acid (18:1 ω9). Rotifers fed on dried yeast lacked stearic acid (18:0), oleic acid (18:1 ω9) and linoleic acid (18:2 ω6), and contained significantly smaller amounts of TFA. Rotifers fed on dried algae contained DHA, which was not found in the other treatment groups in this experiment.

The fatty acid composition in rotifers enriched with formulated food (Superselco, Artemia Systems, Belgium) for 6 or 12 h is given in Table 2. Results show that this food source provides significant amounts of DHA in addition to EPA.

Variable reproductive rates were found in rotifers fed a combination of yeast and *Nannochloropsis* at various combinations (Table 3a,b). No clear trend could be demonstrated. However, the EPA content clearly depended on the presence and proportion of algae in the diet. All rotifers in these experiments lacked 20:4 ω6.

Identification of rotifer strains obtained from the Philippines by restriction endonuclease analysis of mt DNA

Fragment sizes produced by restriction enzyme digestion of 6 clones are shown in Table 4a,b. From comparisons between strains (Table 5), it is clear that the initial strains we received in 1990, namely SAP, DUM and OTON, were different from each other. Two of the strains received in 1992 (OTONI and SAPIAN) were found to be

completely identical, while BANI was completely different from both OTONI and SAPIAN. Also, SAPIAN or OTONI and SAP were not fully identical. Similarly, BANI and DUM differed from each other.

DISCUSSION

The results of the last year of research clearly showed that the nutritional quality of rotifers mainly depends on the food they receive. No clear effect was found in the reproductive rates of rotifers fed yeast with increasing amounts of *Nannochloropsis* in their diet. The small differences found between yeast-fed and algae-fed rotifers was mainly due to the exceptionally high reproductive rates found in yeast-fed rotifers (> 0.76 day^{-1}). This could be attributed to the practice of distributing the total food dose over 4-6 meals during a period of 24 h. However, large-scale experiments in the National Center for Mariculture (part of our institute) demonstrated the stabilizing effects of incorporation of *Nannochloropsis* (fresh or frozen) into the rotifer diets. The accepted practice today is to include 20% algae in the daily food supplied to mass cultivated rotifers (in 10 m^3 tanks) or to feed the rotifer cultures with algae once a week. Frozen algae mass was found adequate as a nutritional source of important lipids such as EPA and was also found suitalbe in replacing fresh algae in stabilization of rotifer cultures.

The method developed for identifying rotifer clones or strains through digestive endonuclease analysis of mt DNA was useful in providing evidence on the full identity between OTONI and SAPIAN cultures. This shows that occasional unintentional contaminations between rotifer cultures occur in laboratories, and until now it was not possible to discriminate or identify these events.

REFERENCES

Avise, J.C., J. Arnold, R.M. Ball, E. Bermingham, T. Lamb, J.E. Neigel, C.A. Reeb and N.C. Saunders. 1987. Intraspecific phylogeography: The mitochondrial bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18: 489-522.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (eds.). 1989. Current Protocols in Molecular Biology. Vols. 1 & 2. Wiley-Interscience, New York.

Caccone, A., G.D. Amato and J.R. Powell. 1988. Rates and patterns of scnDNA and mtDNA divergence within the *Drosophila melanogaster* subgroup. Genetics 118: 671-683.

Fu, Y., K. Hirayama and Y. Natsukari. 1991. Genetic divergence between S and L. type strains of the rotifer *Brachionus plicatilis* O.F. Müller. J. Exp. Mar. Biol. Ecol. 151: 43-56.

Fukusho, K. 1983. Present status and problems in culture of the rotifer *Brachionus plicatilis* for fry production of marine fishes in Japan. Symp. Int. Aquacult., Coquimbo, Chile, Sept. 1983: 361-374.

King, C.E. 1989. Molecular genetics of rotifers: Preliminary restriction mapping of the mitochondrial genome of *Brachionus plicatilis*. Hydrobiologia 186/187: 375-380.

Korunuma, K. and K. Fukusho. 1987. Rearing of Marine Fish Larvae in Japan. IDRC, Ottawa, Canada: 109 pp.

Lubzens, E. 1987. Raising rotifers for use in aquaculture. Hydrobiologia 147: 245-255.

Lubzens, E., A. Marko and A. Tietz. 1985. *De novo* synthesis of fatty acids in the rotifer *Brachionus plicatilis*. Aquaculture 47: 27-37.

Lubzens, E., S. Rothbard, A. Blumenthal, G. Kolodny, B. Perry, B. Olund, Y. Wax and H. Farbstein. 1987. Possible use of *Brachionus plicatilis* (O.F. Müller) as food for freshwater cyprinid larvae. Aquaculture 60: 143-155.

Lubzens, E., A. Tandler and G. Minkoff. 1989. Rotifers as food in aquaculture. Hydrobiologia 186/187: 387-400.

Moritz, C., T.E. Dowling and W.M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18: 269-292.

Snell, T.W. and K. Carrillo. 1984. Body size variation among strains of the rotifer *Brachionus plicatilis*. Aquaculture 37: 359-367.

Tamura, K. and T. Aotsuka. 1988. Rapid isolation method of animal mitochondrial DNA by the alkaline lysis procedure. Biochem. Genet. 26: 815-820.

Watanabe, T. 1993. Importance of docosahexanoic acid in marine larval fish. J. World Aquacult. Soc. 24: 152-161.

Table 1. The effect of various food sources on the specific reproductive rate, total fatty acid (TFA) content and fatty acid distribution in rotifers. Values show mean (standard deviations in parentheses) of three or more replicates.

	Live *Nanno-chloropsis* sp	Frozen *Nanno-chloropsis* sp	Dried *Nanno-chloropsis* sp	Yeast	Dried Yeast
Reproductive Rate (day^{-1})	1.1 [a] (0.13)	0.89 [b] (0.16)	0.64 [c] (0.09)	0.84 [bc] (0.19)	0.42 [d] (0.10)
TFA $\mu g/10^6$ rotifers	15.5 [a] (3.8)	12.0 [ab] (1.2)	11.3 [b] (1.9)	8.7 [bc] (0.9)	7.1 [c] (0.4)
FA distrib. % weight					
14:0	9.4 (1.5)	5.7 (0.2)	5.6 (0.4)	2.8 (0.3)	4.9 (0.3)
16:0	20.7 (4.2)	21.3 (1.7)	24.7 (1.2)	8.2 (2.0)	8.7 (1.0)
16:1	20.3 (3.4)	26.1 (3.6)	16.8 (0.5)	36.0 (6.7)	60.6 (1.1)
18:0	5.1 (2.0)	5.9 (2.3)	7.9 (0.6)	9.9 (4.7)	-
18:1 ω9	6.0 (1.3)	3.4 (0.2)	4.9 (0.3)	27.9 (4.2)	25.7 (0.3)
18:1 ω7	5.0 (0.7)	6.7 (2.5)	6.6 (0.6)	11.7 (0.9)	
18:2	5.9 (1.0)	3.6 (1.0)	5.8 (0.4)	3.6 (1.7)	
20:4 ω6	5.4 (0.8)	5.0 (1.2)	5.1 (0.1)		
20:5 ω3	14.2 (2.8)	17.1 (3.7)	12.4 (1.0)		
22:5 ω3	4.2 (1.4)	5.2 (2.3)	3.9 (0.8)		
22:6 ω3			6.2 (0.4)		

In all cases (except dried algae), 2 gm dried weight (dw) of specified food was supplied to 10^6 rotifers per day, divided into four feeding portions. This amount of food yielded maximal reproductive rates in rotifers. An amount of 4.0 gm/10^6 rotifers per day of dried yeast was needed to obtain maximal reproductive rates. Duncan's multiple range test was performed on the reproductive rate values ($p<0.0001$) and TFA ($p<0.0001$). Means with the same superscript letter along each line are not significantly different from one another.

Table 2. Fatty acid composition in rotifers enriched with various foods for 6 or 12 hrs (weight % of total fatty acids). Rotifers were cultured in 20 ‰ sea water at 30°C.

	Control yeast, no enrichment		Super Selco	
	6 hrs	12 hrs	6 hrs	12 hrs
14:0	3.3 (0.3)	5.6 (0.5)	2.4 (0.2)	2.8 (0.3)
16:0	6.7 (1.0)	5.1 (1.1)	5.2 (0.1)	8.8 (0.4)
16:1	47.9 (3.0)	45.1 (3.0)	26.1 (1.0)	23.5 (1.0)
18:0	5.7 (0.9)	12.7 (1.1)	4.1 (0.8)	6.0 (0.7)
18:1 ω9	29.9 (2.4)	24.0 (0.9)	18.5 (0.5)	18.3 (0.5)
18:1 ω7	--	--	7.9 (0.3)	--
18:2 ω6	--	4.6 (0.8)	3.4 (0.1)	3.3 (0.5)
20:4 ω6	--	--	--	--
20:5 ω3	--	--	19.1 (0.6)	20.0 (1.0)
22:5 ω3	--	--	5.1 (0.4)	--
22:6 ω3	--	2.9 (0.5)	12.2 (0.4)	17.1 (1.3)

Table 3a. The effect of food source on the reproductive rate, total fatty acid content and fatty acid distribution of rotifers. Rotifers were fed on a combination of yeast and *Nannochloropsis*.

	Exp. 1		Exp. 2				Exp. 3
Yeast (gm)	1.2	1.0	1.5	1.5	1.5	1.0	1.5
Algae (gm)	0.3	0.5	0.1	0.3	0.5	0.5	0.3
Total (gm)	1.5	1.5	1.6	1.8	2.0	1.5	1.8
Reproductive rate (day^{-1})	0.79 ± 0.14	1.01 ± 0.04	0.89 ± 0.09	0.99 ± 0.02	1.19 ± 0.08	0.98 ± 0.07	1.04 ± 0.10
TFA, $\mu g/10^6$ rotifers	8.91 ± 2.62	12.23 ± 1.71	5.88 ± 1.36	9.62 ± 1.16	10.22 ± 3.09	12.82 ± 1.76	6.90 ± 1.04
Fatty acid distribution:							
14:0	3.59 ± 0.54	5.09 ± 0.39	3.29 ± 0.56	4.12 ± 0.30	4.75 ± 0.47	5.07 ± 0.37	3.98 ± 0.50
16:0	19.03 ± 0.27	16.06 ± 2.01	10.88 ± 1.73	12.37 ± 0.38	14.83 ± 0.75	14.94 ± 0.38	13.90 ± 0.71
16:1	22.33 ± 2.12	19.85 ± 0.87	27.17 ± 2.24	26.06 ± 1.09	26.17 ± 1.88	23.12 ± 0.84	22.68 ± 2.98
18:0	4.03 ± 0.22	3.56 ± 0.36	4.87 ± 0.60	3.40 ± 0.59	4.15 ± 0.64	3.36 ± 0.15	3.84 ± 0.48
18:1 ω-9	16.85 ± 0.17	10.97 ± 0.56	19.72 ± 2.23	16.24 ± 1.11	13.14 ± 0.52	12.19 ± 0.90	13.69 ± 1.90
18:1 ω-7	5.78 ± 0.48	4.91 ± 0.39	6.59 ± 0.88	6.10 ± 0.20	5.91 ± 0.50	5.90 ± 0.12	6.91 ± 0.69
20:3 ω-6	3.58 ± 0.37	4.21 ± 0.27	3.40 ± 0.31	3.50 ± 0.17	4.07 ± 0.56	4.29 ± 0.19	4.19 ± 0.59
20:5 ω-3	0.64 ± 0.10	0.29 ± 0.25	0	0	0	0	0
22:5 ω-3	7.04 ± 1.20	9.36 ± 0.69	4.43 ± 0.94	6.58 ± 0.56	8.59 ± 1.32	8.83 ± 0.69	7.35 ± 0.92
22:6 ω-3	2.48 ± 2.48	4.16 ± 0.27	0.67 ± 0.94	3.95 ± 0.17	4.83 ± 0.57	5.38 ± 0.34	4.64 ± 0.50

Table 3b. The effect of food source on the reproductive rate, total fatty acid content and fatty acid distribution of rotifers. Rotifers were fed on a combination of yeast and *Nannochloropsis*.

	Exp. 3		Exp. 4			
Yeast (gm)	1.5	1.0	1.75	1.75	2.25	1.85
Algae (gm)	0.5	0.5	0.10	0.5	-	-
Total (gm)	2.0	1.5	1.85	1.80	2.25	1.85
Reproductive rate (day^{-1})	1.04 ± 0.26	0.93 ± 0.20	0.85 ± 0.15	0.93 ± 0.25	0.91 ± 0.13	0.60 ± 0.09
TFA, $\mu g/10^6$ rotifers	9.24 ± 2.22	7.80 ± 1.61	8.56 ± 2.00	15.33 ± 4.36	14.62 ± 4.40	11.39 ± 0.75
Fatty acid distribution:						
14:0	5.73 ± 1.04	4.66 ± 0.85	1.53 ± 0.33	3.35 ± 0.06	1.63 ± 0.18	1.51 ± 0.21
16:0	16.05 ± 0.87	15,76 ± 1.50	9.30 ± 0.53	13.23 ± 0.54	6.28 ± 1.10	6.52 ± 0.81
16:1	24.05 ± 1.67	19.56 ± 3.28	17.30 ± 3.11	20.02 ± 0.93	24.85 ± 2.89	22.76 ± 3.45
18:0	3.75 ± 0.51	3.53 ± 0.43	3.75 ± 0.60	3.27 ± 0.29	3.32 ± 0.50	3.46 ± 0.27
18:1 ω-9	9.64 ± 3.0	10.23 ± 1.08	17.15 ± 3.26	13.06 ± 0.6	24.36 ± 1.89	22.56 ± 3.0
18:1 ω-7	5.81 ± 0.36	5.78 ± 0.46	6.60 ± 1.16	6.21 ± 0.36	8.86 ± 0.77	8.28 ± 0.96
18:2	4.68 ± 0.40	4.99 ± 0.62	1.95 ± 0.38	3.23 ± 0.23	1.78 ± 0.69	1.29 ± 0.31
20:3 ω-6	0	0	0	0	0	0
20:5 ω-3	9.17 ± 1.19	8.20 ± 1.61	3.28 ± 0.53	8.80 ± 0.19	0	0
22:5 ω-3	4.68 ± 0.40	4.55 ± 1.32	1.91 ± 0.57	5.10 ± 0.29	0.15 ± 0.30	0
22:6 ω-3	-	-	-	0.63 ± 0.38	1.25 ± 0.29	1.17 ± 0.18

Table 4a. Fragment sizes produced by restriction enzyme digestion of *B. plicatilis* Philippine strains

Enzyme	Strain					
	OTONI	OTON	SAPIAN	SAP	BANI	DUM
Hind III	5.0	not	5.0	not	not	not
	3.2	tested	3.2	tested	digested	tested
	3.0		3.0			
	2.0		2.0			
	1.4		1.4			
	1.25		1.25			
	0.75		0.75			
	0.47		0.47			
Total	17.07		17.07			
Eco RV	10.0	13.0	10	11	4.3	9.4
	6.3	4.2	6.3	6.1	3.6	6.0
	4.3		4.3	4.2		4.5
Total	20.6	17.2	20.6	21.3	7.9	19.7
Xba I	10.0	6.9	10.0	11.0	not	not
	(double)	3.9	(double)	(double)	digested	digested
		2.4				
		1.7				
		1.3				
Total	20.0	16.2	20.0	22.0		

Table 4b. Fragment sizes produced by restriction enzyme digestion of *B. plicatilis* Philippine strains.

Enzyme	Strain					
	OTONI	OTON	SAPIAN	SAP	BANI	DUM
Eco RI	4.4	not	4.4	12.0	4.5	not
	3.3	digested	3.3	3.0	3.8	digested
	1.2		1.2	3.2	3.4	
	0.95		0.95	1.2		
	0.27		0.27	0.95		
				0.28		
Total	10.12		10.12	21.53	11.7	
Ava I	10.5	not	10.5	11.0	not	11.0
	(double)	digested	(double)	(double)	digested	(double)
Total	21.0		21.0	22.0		22.0
Bgl II	5.5	7.4	5.5	6.4	5.5	6.5
	4.4	3.8	4.0	3.9	3.5	4.1
	3.3	2.9	3.3	2.8	2.4	3.0
	1.8	2.5	1.8	1.8	2.2	1.8
	1.1	1.4	1.1	1.1		1.0
	0.78	0.79	0.78	0.74		0.7
	0.68	0.34	0.68	0.60		0.6
	0.40		0.40	0.38		0.33
Total	17.96	19.13	17.56	17.72	13.6	18.03

Table 5. Comparisons in fragment sizes produced by restriction enzyme digestion of *B. plicatilis* Philippine strains.

Comparison between strains	Enzyme					
	Hind III	Eco RV	Xba I	Eco RI	Ava I	Bgl II
OTON-SAP	*	-	-	-	-	-
OTON-DUM	*	-	-	+	+	-
SAP-DUM	*	+	-	-	+	+
OTONI-SAPIAN	+	+	+	+	+	+
OTONI-BANI	-	-	-	-	-	-
SAPIAN-BANI	-	-	-	-	-	-
OTONI-OTON	-	-	-	-	-	-
OTONI-SAP	*	+	+	-	+	-
OTONI-DUM	+	-	-	-	+	-
SAPIAN-SAP	*	+	+	-	+	+
SAPIAN-OTON	*	-	-	-	-	-
SAPIAN-DUM	*	+	-	-	+	-
BANI-OTON	*	-	-	-	+	-
BANI-SAP	*	-	-	-	-	-
BANI-DUM	*	-	+	-	-	-

* not tested
\+ similar
\- not similar