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BARD

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Rotifer Resting Eggs and Their Application to Marine Aquaculture

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Section a.

ABSTRACT

Our research program was aimed at elucidating the possibility of producing rotifer resting eggs for use in mariculture.

During the course of our research, we found that the major factors determining resting egg production are genetic. Production of resting eggs varied not only between different zoogeographical strains but also between clones isolated within each strain. It was possible to obtain clones capable only of asexual reproduction; other isolated clones produced relatively large numbers of males, yet others were able to produce relatively large numbers of resting eggs. Environmental conditions such as salinity or food (quality and quantity) modulated these genetic tendencies. The highest rates of resting egg production were obtained at intermediate temperatures, salinities and food levels. By manipulating these culture conditions, it was possible to synchronize the occurrence of mixis and resting egg production. The main problem today is not the production of mictic females, which may reach levels exceeding 70% of the total population, but to increase the proportion of fertilized females producing resting eggs. Production of resting eggs in 10 litre volumes was not significantly different from that in 10 ml experimental flasks.

Resting eggs produced and isolated in our laboratory show an obligatory dormant period which lasts about one month. Following this period, eggs were found to hatch best following exposure to light, at a salinity of 16 ‰ and low temperature (10°C). These eggs can be preserved for extended periods of time (at least for two years) without significant loss of viability if preserved

in the dark in 16 ‰ salinity sea water. Some attempts were made to preserve these eggs in dry form, resulting in some loss in viability.

Our research efforts resulted in two immediate applications in using rotifers as food for fish larvae. The rotifer Brachionus plicatilis was found to improve and accelerate development of fresh water fish larvae (carp). Selective behaviour of Sparus aurata larvae suggests using small body size rotifer strains as food for these larvae during the first 2-4 days of their life, followed by large body size rotifer strains.

A SUMMARY OF BARD GRANT RESEARCH

1983

University of Tampa

I. Introduction

During 1983 our research at the University of Tampa concerned three primary areas of investigation: rotifer body size, resting egg production, and genetic variation among rotifer strains. The experiments reported here were planned and executed in collaboration with Dr. Esther Lubzens in Haifa and Dr. Amos Tandler in Elat. A summary of our results follows.

II. Project Report

A) Rotifer Body Size

We completed our examination of rotifer body size this year and the resulting manuscript has been published in the journal *Aquaculture* (see attached). Briefly, our results showed that rotifer body size could be altered to only a small degree by culture conditions such as temperature, salinity and diet. However, a factorial ANOVA demonstrated that the type of rotifer strain utilized had a large effect on body size. Our conclusion, therefore, is that rotifer body size is determined primarily by genetic as opposed to environmental factors.

Experiments complimentary to those described above were carried out in Elat by Dr. Amos Tandler and outlined in last year's report. Their results showed that larval Sparus aurata indeed show selectivity of rotifers based on body size. It appears that young Sparus larvae ingest smaller rotifers, then switch to larger rotifers as the larvae increase in size. This switching of prey size with larval growth underscores the need for rotifers of various sizes to satisfy the changing larval demands. We have been able to identify and supply the hatchery in Elat with a variety of B. plicatilis strains that meet the larval Sparus aurata prey size requirements.

B) Resting Egg Production

1) Female susceptibility to fertilization

As reported last year, our experimental results and those of Dr. Lubzens suggested that mixis could be induced in a large proportion of rotifer females by manipulating the culture conditions. Environments that promote sexual reproduction are ones with lower salinities, moderate temperatures, and intermediate food levels of particular types of algae. By manipulating these

culture variables, we have been able to induce mixis in up to 90% of females in certain B. plicatilis strains.

Although we have been successful at inducing mixis, the rate of fertilization has been consistently low. It is not unusual to record only 2-3% of the females in a population fertilized and carrying resting eggs. The highest percentage of fertilization we have ever recorded is 15% and this on only one occasion. From these data it was obvious that a large proportion of females can be mictic and yet only a small fraction of them would actually be fertilized. These observations led us to suspect that all mictic females are not fertilizable, consequently, several of this year's experiments were designed to investigate this possibility.

An example of data that supports the notion that female susceptibility to fertilization fluctuates temporally is presented in Figure 1. Mixis was induced in the Russian strain of B. plicatilis by transferring an amictic culture to growth conditions. Growth conditions include abundant food, fresh medium and moderate salinities and temperatures as described in last year's report. After 3 days, mictic females began to appear in the population. Neonate females were collected within 15 minutes of their births and placed in 50 ul of medium with 3 males. Male copulation with all females was observed soon after introduction of the males. The fertility of males from this strain has been verified on several occasions as evidenced by their resting egg production in other populations prior to and after the completion of this experiment.

A total of 64 mictic females were isolated and, despite the fact that copulation was observed in each case, none of these mictic females was fertilized as determined by their failure to produce resting eggs. It should also be noted that females from this strain have engaged in extensive resting egg production both before and after this experiment. Furthermore, while resting egg formation failed during this period, a strong mictic response was recorded as evidenced by high male density in this population. This experiment has been repeated three times and the results are summarized in Table 1. Over the five months of these experiments, all 127 of the mictic females examined failed to become fertilized after observed copulations. Since this strain is capable of prolific resting egg production, the failure to become fertilized must have been a temporary block. This variability in the susceptibility to fertilization is presumably under the control of as yet unidentified environmental parameters.

If the susceptibility of mictic females to fertilization does indeed fluctuate temporally, our experiments have implicated two parameters as having a possible role. The data thus far suggest that algal diets play a determining role, while other observations implicate conditioning of the medium by the rotifers.

Last year we reported that algal diet seemed to have a role in resting egg production (Figure 2). Diets containing yeast along with the green algae Chlorella or Dunaliella, or the blue-green Schizothrix, promoted the greatest resting egg production. This observation was reinforced this year in additional diet experiments using the Russian strain of B. plicatilis. We have continued to work primarily with this rotifer strain because our results have indicated that this strain has the greatest resting egg production capacity of any tested.

The results presented in Table 2 summarize the resting egg production after 10 days in 3 ml cultures inoculated with 5 females. The populations were assayed daily for females, males and resting egg-carrying females. The four Russian clones listed in Table 2 all were isolated from the same resting egg population.

Resting eggs were produced on all four diets, but the largest number was produced on either a Chlorella-Tetraselmis or a mixed algal diet. This mixed diet consisted of equal concentrations of Chlorella, Dunaliella, Tetraselmis, and Synechococcus. We are now in the process of repeating this experiment with more replication to determine the generality of our results. If repeatable, these results clearly suggest that mixed algal diets composed of particular algal species promote the greatest resting egg production.

We now also have experiments in progress to test the ability of rotifer-conditioned medium to induce resting egg production in the Russian strain. Hino and Hirano (1976) and Dr. Lubzens and her colleagues have shown that frequent medium changes can lower the level of mixis in a population. This effect is presumably the result of a conditioning factor which is excreted into the medium by the rotifers and is required for mixis and perhaps resting egg production. The experiments we have underway attempt to determine if the addition of conditioned medium to a rotifer population alters the timing or level of mixis or enhances resting egg production.

2) Rates of Asexual vs. Sexual Reproduction

In last year's report, it was shown that temperature, salinity, and food level all modulate the mictic response in B. plicatilis. Maximum sexual

reproduction occurred at moderate temperatures, salinities, and food levels. Corroborating results for salinity and food levels were reported by Dr. Lubzens for her Dor strain of B. plicatilis. This year we completed experiments which allowed us to directly compare the effect of the above three factors on both asexual and sexual reproduction.

Preliminary observations suggested that asexual and sexual reproduction respond differently to temperature, salinity and food level changes. We examined asexual and sexual reproductive rates of the Tampa Bay B. plicatilis strain (McK8) at several temperatures, salinities and food levels (Figs. 3,4,5). These data were collected from laboratory populations cultured in 50 ml of Instant Ocean medium (Snell, Bierberich, and Fuerst, 1983) on a diet of Chlorella vulgaris. The mean population growth rate per individual (R) was calculated as $R = \ln N_t - \ln N_0 / T$, where $T = 1$ day.

It can be seen in all three of these figures that male production (sexual reproduction) was more sensitive to temperature, salinity and food level effects than asexual reproduction. The reproductive response curves of mictic and amictic females are not coincident, rather the amictic females maintain higher reproductive rates over a broader range of environments than mictic females. Furthermore, the cessation of sex at environmental extremes appears to be a general response since it occurs in all treatments. In a generalized model of the reproductive response (Figure 6), it can be seen that a consequence of the differential response of asexual females to physiological stress is to produce zones of exclusively asexual reproduction at environmental extremes. In these zones asexual reproduction continues in physiological conditions where sexuality ceases.

These data have important implications concerning our current understanding of sexual reproduction and its integration into the rotifer life cycle. Rotifer sexuality appears to be regulated by both inducible and repressible elements. Population density plays an inducing role, probably through the conditioning of the medium by some as yet unidentified factor. However, even in the presence of this inducing factor, sex can be repressed by physiological stress such as high temperatures or salinities or low food levels. We are now drafting a manuscript that reports these results and develops a model to include both the inducible and repressible aspects of sex in the rotifer life cycle.

3) The Effects of Resting Egg Production on Population Dynamics

If we are to develop the means to mass produce resting eggs, we will need to understand the relationship between sex and population growth in rotifers. This relationship was examined using computer simulation of both exponential and logistic population growth models. The simulation demonstrated that both the frequency of sex and the proportion of a female's daughters reproducing sexually had effects on population growth and resting egg production. As the frequency of sex decreased, the proportion of sexual daughters required for maximum resting egg production increased. Adding density-dependence to the model also shifted this optimum towards a higher proportion of sexual daughters. When population growth was unconstrained by density, the optimum proportion of sexual daughters varied between 10-50%, depending on the frequency of sex. This compares to 25-64% when the greatest density restraints were applied at $K=500$ females. Increased population growth rate (r) increases the optimum proportion of sexual daughters nonlinearly and these effects are exaggerated as sex becomes less frequent.

An examination of data from laboratory populations of four Brachionus plicatilis strains reveals that the average proportion of sexual daughters is 21% and in close agreement with that predicted by the simulation. The level of sex in rotifer populations, therefore, appears to be a tradeoff, with females producing parthenogenetic daughters that contribute to population growth and sexual daughters that contribute to resting egg production. A complete explanation of the simulation and a discussion of the results is presented in the attached manuscript.

C) Genetic Similarities Among Brachionus plicatilis strains

Selective breeding may make it possible to improve the characteristics of rotifers that make them useful to aquaculture. Traits like body size (Snell and Carillo, 1984) and resting egg production capacity are to some extent under genetic control and therefore susceptible to manipulation through selection. Since crosses are not easy to accomplish between rotifer strains, it would be useful to know a priori which pairings would be likely to succeed. A first step towards acquiring this knowledge is a determination of the genetic relatedness among the B. plicatilis strains currently in culture.

Using polyacrylamide gel electrophoresis, we have estimated the level of genetic similarity in several enzymes among 13 strains. A cluster analysis

has indicated that several of the strains have quite similar isozyme patterns, while other strains appear to have no affinities. A full description of these results can be found in the attached pre-print.

While ours is only a preliminary survey, it is hoped that this technique will be useful in resolving some of the systematic problems that still plague B. plicatilis and other rotifers.

III. Other Activities

During 1983 Dr. Lubzens and I met in Israel for three weeks in June to discuss the results of our research and plan the experiments to be completed during the final phase of the grant. While in Israel, I visited IOLR in Haifa, conferred with Dr. Lubzen's research team, and presented a seminar to the general scientific staff summarizing my work on rotifers over the past two years. I also visited the IOLR hatchery in Elat where I met with Dr. Amos Tandler, discussed results of our research, and talked over larval rearing problems that the hatchery staff were having in the Sparus aurata project. I also presented a seminar in Elat concerning rotifer body size and its genetic and environmental determinants.

IV. Summary and Conclusions

Over the past three years we have determined the major environmental factors regulating resting egg production in the rotifer Brachionus plicatilis. The highest rates of resting egg production occur at intermediate temperatures, salinities, and food levels and on a mixed algal diet. The influence of genetic factors on resting egg production also has been investigated. After surveying 17 B. plicatilis strains for resting egg production capacity, the Russian strain stood out above all others. This strain has been shown to yield nearly twice the resting egg output as any other strain tested, a characteristic that could make a critical difference in a mass production operation.

Body size variation among several strains has also been examined. Both large and small strains have been identified and placed in permanent culture which will serve as a repository of different rotifer body sizes available to all aquaculturists. This collection is sure to be useful as the rearing of fish larvae continues to expand. It also has been demonstrated that culture conditions have only a small influence on rotifer body size and that genetic factors are its primary determinant.

At the present time, we are quite successful at manipulating rotifer strain and culture environment to produce a high proportion of mictic females. We have recorded instances where mictic females have comprised up to 90% of a population. The current bottleneck in resting egg production is not in producing the mictic females, but in getting these females fertilized. It is not uncommon to obtain only 2-15% fertilization among mictic females in a population. Our data suggest that all mictic females are not fertilizable and that some as yet unidentified environmental factors regulate this susceptibility to fertilization. If we are to be successful in the mass production of resting eggs, I believe that the problem of female susceptibility to fertilization must be solved and it is here that I feel future research must focus.

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- Snell, T.W. and K. Carillo. 1984. Body size variation among strains of the rotifer Brachionus plicatilis. Aquaculture 37: 354-367.

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1. Snell, T.W., C.J. Bieberich and R. Fuerst. 1983. The effects of green and blue-green algal diets on the reproductive rate of the rotifer Brachionus plicatilis. Aquaculture. 31: 21-30.
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6. Snell, T.W. and B.C. Winkler. 1984. Isozyme analysis of rotifer proteins. Biochem. Syst. and Ecology, in press. APPENDIX ONE.
7. Snell, T.W. and E. Lubzens. Sex, Population dynamics and resting egg production in rotifers. In preparation. APPENDIX TWO.
8. Snell, T.W. and E. Lubzens. Comparative reproductive response of sexual and asexual female rotifers to physiological stress. In preparation.

Table 1. A summary of four fertilization experiments. Each Neonate female was exposed to 3 males and copulation was observed in every case.

Neonates isolated.	444
Number surviving to reproduce. .	404
Number amictic	277 (69%)
Number mictic	127 (31%)
Number fertilized	0

Table 2. The effects of various diets on resting egg production: C-Chlorella vulgaris; C sp.-Chlorella sp.; Sch-Schizothrix calicola; E. coli-Escherchia coli; Yea-Baker's yeast; Dun-Dunaliella salina; Tetra-Tetraselmis sp.; Syn-Synechococcus cedrorum; mixed-C, Tetra, Syn, Dun. Resting eggs represent the total number of resting eggs produced after 10 days from an original inoculum of 20 females into a 100 ml culture.

<u>Rotifer Strain</u>	<u>Diet</u>	<u>Experiment 1</u> <u>Resting eggs produced</u>	<u>Experiment 2</u> <u>Resting eggs produced</u>
RUS	C-Sch	-	0
	C-E. Coli	-	0
	C-Yea	0	-
	C sp.	-	0
	C-Dun	-	0
	C-Tetra	1	4
	C-Syn	0	-
	mixed	20	0
RUS' 1	C-Sch	-	2
	C-E. coli	-	0
	C-Yea	0	-
	C sp.	-	0
	C-Dun	-	0
	C-Tetra	0	0
	C-Syn	1	-
	mixed	14	5
RUS' 2	C-Sch	-	0
	C-E. coli	-	2
	C-Yea	0	-
	C sp.	-	0
	C-Dun	-	0
	C-Tetra	50	10
	C-Syn	0	-
	mixed	0	0
RUS' 3	C-Sch	-	0
	C-E. coli	-	0
	C-Yea	0	-
	C sp.	-	0
	C-Dun	-	0
	C-Tetra	0	0
	C-Syn	0	-
	mixed	3	0

Figure Captions

- Figure 1. Population dynamics and mixis. The female and male curves plot density per ml. The histogram bars represent the percentage of mictic females in the total female population. The numbers in the bars are the number of mictic neonates isolated on each day.
- Figure 2. The effect of diet on mixis. The unmarked columns are percent males, and the diagonal strips are percent resting egg-carrying females in the population. SCH - Schizothrix calcicola, CHL - Chlorella sp., YEA - baker's yeast.
- Figure 3. The effect of temperature on the reproductive rate of sexual and asexual females. Population growth rate is r - the change in the number of males or females per individual per day. Vertical lines indicate standard error.
- Figure 4. The effect of salinity on the reproductive rate of sexual and asexual females. Population growth rate is r - the change in the number of males or females per individual per day. Vertical lines indicate standard error.
- Figure 5. The effect of food quantity on the reproductive rate of sexual and asexual females. Population growth rate is r - the change in the number of males or females per individual per day. Vertical lines indicate standard error.
- Figure 6. Comparative reproductive response curves of sexual and asexual females. The environmental gradient refers to temperature, salinity or food quantity.

ROTIFER DENSITY

Fig

DAYS

PERCENT OF POPULATION

POPULATION GROWTH RATE

TEMPERATURE

POPULATION GROWTH RATE

0.4

0.2

0

ASEXUAL

SEXUAL

0.25

2.5

5

10

15

20

ug CHLORELLA PER ML

REPRODUCTIVE RATE

ENVIRONMENTAL GRADIENT

Section c.

REPORT BY DR. E. LUBZENS, IOLR, HAIFA

I. Introduction

During the last phase of our BARD project we report on four projects:

- A. The effect of vitamin B12 on asexual and sexual reproduction in Brachionus plicatilis.
- B. Isolation of rotifer clones for mass cultivation and production of resting eggs.
- C. Production of resting eggs, preservation and hatching.
- D. Rotifers (Brachionus plicatilis) improve growth rate of carp (Cyprinus carpio) larvae - APPENDIX THREE.

In part (C) above we attempted to upscale resting egg production to 10 litre volumes in order to simulate possible problems that may be encountered in mass production. Part (D) was carried out during the course of the year when a new hatchery was being built in Eilat and experiments using Sparus aurata larvae had to be postponed. We also include in this report a paper entitled "Salinity dependence of sexual and asexual reproduction in the rotifer Brachionus plicatilis" - APPENDIX FOUR.

II. Project Report

A. The effect of vitamin B12 on asexual and sexual reproduction in Brachionus plicatilis

1) Introduction

It has been shown that adding vitamin B12 (VB12) to aseptic cultures of Brachionus plicatilis is essential for maintenance of adequate reproduction of this rotifer (Scott, 1981). Our previous work (Lubzens et al., 1985) showed that sexual reproduction appears under conditions favoring relatively high reproduction rates. We therefore decided to examine the possible role of VB12 in inducing and maintaining sexual reproduction and production of resting eggs in this rotifer.

Our experiments were carried out first in Clone B12 (CB12) which was isolated from resting eggs of a culture originating from rotifers collected at Dor, south of Haifa. This clone produces males and resting eggs when cultured at salinities lower than 30 ‰ as described previously (Lubzens et al., 1985). Appearance of sexual reproduction may obliterate the possible effect of VB12, due to the production of males and resting eggs which do not contribute to the increase in size of population. Therefore, the effect of VB12 was also tested in another clone, Clone N.R.E. (CNRE) which was also isolated in our laboratory from resting eggs, but was incapable of producing males or resting eggs under all tested culture conditions.

2) Materials and Methods

Cultures. Chlorella stigmatophora and Brachionus plicatilis were cultured and maintained in membrane filtered sea water (salinity 40 ‰) as described previously (Lubzens et al., 1980; Minkoff et al., 1983).

Vitamin B12 was purchased from Sigma Chemical Co., U.S.A., freshly prepared every 2-3 days in distilled water and stored at 4°C.

3) Experimental set-up

i. Experiments with clone B12 (CB12)

The effect of four concentrations of VB12 (100, 200, 400 and 800 pg ml⁻¹) was studied in rotifers maintained at two salinities (20 ‰ and 40 ‰ S) and compared to control groups not receiving any externally added VB12. Since cultures were maintained in natural sea water in nonsterile conditions, the possible presence of small amounts of VB12 was not ruled out.

Rotifers used in the two experimental series described below were removed from exponentially growing cultures maintained in a medium of 40 ‰ salinity. In the first experimental series, where the effect of VB12 was tested in rotifers cultured in a medium of 40 ‰ salinity, each of the five treatment groups consisted of five replicates. Each replicate group consisted of ten rotifers which were introduced at the onset of the experiment into 10 ml of sea water containing 3×10^6 cells ml⁻¹ of Chlorella stigmatophora in 50 ml flasks. VB12 was added to each treatment at one of the concentrations stated above (0, 100, 200, 400 or 800 pg ml⁻¹). All flasks were kept on a rotatory shaker in a temperature controlled room (25 ± 1°C) under constant illumination. The number of rotifers and the number of eggs they carried were counted every

2-3 days. Following the count, rotifers were sieved and transferred to fresh medium. In the second series of experiments, an identical set-up was used, except that rotifers were transferred at the onset of the experiment from a culture maintained at 40 % S into a medium of 20 % salinity. In this series, the number of mictic females, males and resting eggs was also counted during the course of the experiment.

ii. Experiments with clone N.R.E. (CNRE)

The effect of VB12 (400 pg ml^{-1}) was tested in two experimental groups maintained at two salinities (20 % and 40 % S) and compared to controls not receiving any externally added source of this vitamin. Rotifers cultured during the course of the experiment in a 20 % S medium were transferred to this salinity at the onset of the experiment. Each of the four experimental groups consisted of three replicate 100 ml flasks, into which 25 rotifers were initially introduced in 25 ml medium, containing $3 \times 10^6 \text{ cells ml}^{-1}$ of C. stigmatophora. The number of rotifers was counted every 2 days in 1 ml samples, which were returned to the flasks after the count was made. Following the count, rotifers were sieved and transferred to fresh medium.

The reproductive potential of each experimental group was tested four days after the onset of the experiment. Eight females, each carrying one egg, were removed from each of the replicate flasks and transferred for further incubation into culture plates. Each female was incubated in a single 3 ml well containing 1 ml of freshly prepared medium to which the female was previously exposed. The number of females in each well was counted after 24 or 48 hours in these cultures at 20 % S or 40 % S, respectively.

4) Results and discussion

While the addition of VB12 to the culture medium improves rotifer rate of reproduction in those cultures maintained at 40 ‰ S (Fig. A1), no effect was observed in rotifers cultured in a medium of 20 ‰ salinity (Fig. A2). A positive response to VB12 was already achieved by adding 100 pg ml⁻¹ medium. Increasing the amount of VB12 to 200, 400 or 800 pg ml⁻¹ resulted in small but statistically insignificant changes in rotifer numbers. Vitamin B12 had no effect on the appearance of mixis (Fig. A3), its level or the number of resting eggs produced in the course of the experiment using clone B12 (Table A1).

The response to VB12 by clone NRE rotifers was also found to depend on the salinity in which they were maintained. In this clone, which reproduces only asexually, results clearly show (Fig. A4) that the number of rotifers was about 5-fold higher in those cultures receiving VB12 and maintained at 40 ‰ S than in controls receiving none (307 rotifers ml⁻¹ vs. 57 in controls). Furthermore, the reproductive potential of rotifers exposed to VB12 was double that of controls (Fig. A5). These differences were not observed among rotifers cultured in 20 ‰ S medium. It is not possible to compare our results with those of Scott (1981), as the salinity used in culturing rotifers in his experiments is not mentioned.

The exact ways in which VB12 functions are yet unknown. It probably involves the synthesis of methyl groups of choline, methionine and nucleic acids. The experimental results shown here suggest that at relatively high salinities, higher amounts of VB12 than those present in natural sea water are required for rotifers fed on algae. We also observed (unpublished results) that yeast-fed rotifers do not require additional VB12 in their media. The exact mode of

action of this vitamin in rotifers is yet unknown.

In conclusion, the increased reproductive rate observed in rotifers cultured with VB12 at the higher salinities is not associated with the appearance of sexual reproduction and therefore has no effect on the production of resting eggs.

5) References

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Table A1. Number of resting eggs produced by rotifers exposed to increasing concentrations of vitamin B12 in their growth media.

Concentration of vitamin B12 pg ml ⁻¹	Number of resting eggs produced (Mean $\pm$ S.D. - 5 replicate groups)
0	406 $\pm$ 184
100	473 $\pm$ 177
200	280 $\pm$ 51
400	275 $\pm$ 178
800	321 $\pm$ 167

Legend to Figures

Figure A1. The effect of increasing vitamin B12 concentrations on the number of rotifers (clone B12) cultured in sea water (salinity 40 ‰).

Figure A2. The effect of increasing vitamin B12 concentrations on the number of rotifers (clone B12) cultured in diluted sea water (salinity 20 ‰).

Figure A3. The effect of increasing vitamin B12 concentrations on mixis in rotifers (clone B12) cultured in diluted sea water (salinity 20 ‰). Mixis is expressed either as the percent of mictic to amictic females (top) or males to total number of females (bottom).

Figure A4. The effect of vitamin B12 (400 pg ml^{-1}) on rotifers (clone N.R.E.) cultured at two salinities (20 ‰ or 40 ‰).

Figure A5. The effect of vitamin B12 on the reproductive potential of rotifers (clone N.R.E.) cultured at two salinities (20 ‰ or 40 ‰).

Fig. A1

Fig. A2

Fig. A3

Fig. A4

Fig. A5

B. Isolation of rotifer clones for mass cultivation and production of resting eggs

1) Introduction

During the course of research carried out in our laboratory in recent years, we observed that some clones originating from resting eggs produced by a previously isolated clone did not show any ability to undergo mixis and were therefore unable to produce resting eggs. One of these clones, clone N.R.E., has been used in our laboratory for the past two years in routine mass cultures of B. plicatilis as food for marine or fresh water fish larvae (Lubzens et al., 1984). The advantage of this clone is that it permits relatively reliable predictions of the number of rotifers produced in the course of time. Collapse of cultures due to appearance of males and resting eggs is thus avoided. The daily increase in 200 litre volume containers ranged between 10-25%, depending on food quality and quantity and on salinity.

Following these results, we decided to explore the possibility of isolating a clone which could be used for high production of resting eggs.

2) Materials and methods

Resting eggs produced in our laboratory by clone B12 and stored for one year at 4°C (in the dark) were used in the experiments described below. One hundred eggs were each placed in a well containing 1 ml sea water (16 ‰ S) and algae (10^6 cells ml^{-1} C. stigmatophora), and incubated at 20°C under constant illumination (Step 1). Of these, 70 eggs hatched after 2-4 days and rotifers of each clone were cultured at $25 \pm 1^\circ\text{C}$ under constant illumination and fed on C. stigmatophora until several rotifers were produced. These rotifers were then

transferred (Step 2) into a 25 ml flask containing 10 ml sea water (salinity 16 ‰) and algae, and placed on a rotatory shaker in a temperature controlled room ($25 \pm 1^{\circ}\text{C}$) under constant illumination. After one week of incubation, rotifers of each clone were transferred to fresh medium containing sea water at a higher salinity (30 ‰ S) and algae (Step 3). All cultures were renewed once a week. Following one week incubation at Step 3, rotifers were transferred to fresh medium at a salinity of 40 ‰ S (Step 4) and were cultured for 2-3 weeks at this salinity. At this stage only 36 clones managed to produce cultures that could be used in an assay testing their ability to undergo mixis and the production of resting eggs.

The assay was based on following the appearance of males in cultures of each clone when rotifers were transferred from high salinity media (40 ‰ S) to lower salinity media (16 ‰ S). Fifteen females, each carrying one amictic egg, were transferred from 40 ‰ S cultures to 50 ml flasks containing 20 ml culture medium for each one of the 36 clones (salinity 16 ‰ and 3×10^6 cells ml^{-1} of C. stigmatophora). Two replicates were made for each clone and these were compared to clone B12 (the parent clone). All flasks were placed on a rotatory shaker under constant illumination in a temperature controlled room ($25 \pm 1^{\circ}\text{C}$). The number of amictic and mictic females was counted every 2-3 days in 3×1 ml samples removed from each flask and returned to it after the count was made. Following the count, 100 rotifers of each flask were transferred to 20 ml of fresh medium. The experiment lasted for 16 days.

3) Results and discussion

Out of the 37 tested clones (36 new clones and the parent clone B12), ten died out during the course of the experiment, six of them before their reproductive rate (G) was determined. The isolated clones could be divided into three categories:

- (1) amictic clones
- (2) poor mictic clones with no resting egg production
- (3) good to excellent mictic clones producing resting eggs.

Out of the 37 clones, 11 belonged to category (1), 16 to (2) and 10 to (3). Mixis was determined as the relative number of males per female in the counted samples and this was averaged for 7 observations made. Clones belonging to category (3) were tested in two separate experiments, showing similar results (Table B1). No correlation was found between the rate of reproduction (G) and mictic levels of each clone. Furthermore, the relative number of males was not always correlated with high resting egg production, although more experiments in this line are required.

Results presented here show clearly that rotifers descended from resting eggs produced by one clone parent have different tendencies towards asexual or sexual reproduction. Similar conclusions were made by Buchner (1977) and Hino and Hirano (1977). Our results are in accord with those of Hino and Hirano (1977) which also showed that about 70% of their clones exhibited mixis early in the line of the clone (until the 8th generation after isolation) and that the level of mixis varied greatly between strains. Hino and Hirano (1977) made their observations on rotifers continuously cultured in sea water at 16‰ chlorinity fed on Chlamydomonas sp. Our method of assay synchronizes the appearance of

sexual reproduction and the production of resting eggs. These and our results are of great importance not only in understanding the survival of rotifers in nature but also for the rotifer breeder. The preliminary results presented here show that it may be possible to isolate clones with relatively high asexual reproductive rates suitable for mass production, similar to the clone we use today for this purpose (clone N.R.E.). Also, clones more suitable than the parent clone (clone B12) can be isolated for the production of resting eggs. These results show that in the process of resting egg production, a process of genetic segregation occurs which requires further attention. Genetic improvement through cross breeding should also be investigated in the future.

4) References

- Buchner, H. 1977. Physiological basis of the reproduction of heterogeneous Rotatoria. Arch. Hydrobiol. Beih. Ergebn. Limnol. 8: 167-168.
- Hino, A. and R. Hirano. 1977. Ecological studies on the mechanism of bisexual reproduction in the rotifer Brachionus plicatilis. II. Effect of cumulative parthenogenetic generation on the frequency of bisexual reproduction. Bull. Jpn. Soc. Sci. Fish. 43: 1147-1155.
- Lubzens, E., G. Sagie, G. Minkoff, E. Meragelman and A. Schneller. 1984. Rotifers (Brachionus plicatilis) improve growth rate of carp (Cyprinus carpio) larvae. Bamidgeh 36: 41-46.

Table B1. Assessment of clones for mixis and rate of reproduction

a. Category 1: clones showing only asexual reproduction

Clone no.	Average rate of reproduction (G)
6	0.82
7	0.78
8	0.80
9	0.83
17 (died)	0.93
23	1.04
41	0.92

Clones 21, 26, 30, 45 died before G was determined but showed only amictic reproduction.

b. Category 2: clones showing low mictic levels and almost no production of resting eggs

Clone no.	Average rate of reproduction (G)	Average mictic level (% of males/female)
13	0.48	12
14	0.41	2
16	0.61	22
19	0.76	6
22	0.92	0.5
31	0.65	5
35 (died)	-	0.5
36	0.72	2
37 (died)	0.54	17
39	0.75	9
42	0.86	2
44	0.74	6

Clones 12, 24, 33, 41 showed mixis but died before the above parameters were determined.

c. Category 3: clones showing medium to high mictic levels and
producing resting eggs

Clone no.	Average rate of reproduction (G)	Average mictic level (% of males/female)	Relative no. of resting eggs
B12	0.81	29	+++
3	0.68	16	+++
4	0.72	70	+++
5	0.67	74	++
10	0.87	26	+++++
20	0.81	39	++++
25	0.72	20	+++++
27	0.75	19	+++
34	0.73	42	+
40	0.57	55	+++++
43	0.99	32	+++

C. Production of resting eggs, preservation and hatching

1) Introduction

Preliminary attempts were made to produce resting eggs in 25 litre conical containers in order to simulate possible problems that could be encountered in mass production. In the reported experiments, rotifers were fed on either yeast or yeast with algae (Chlorella stigmatophora). Resting eggs produced were used to test two methods of isolation and several methods for their preservation.

2) Materials and methods

i. Rotifers

Rotifers of clone B12 were used in the course of the experiments. Before the onset of the experiments, cultures were maintained on Chlorella stigmatophora at a salinity of 40 ‰ and a temperature of $25 \pm 1^{\circ}\text{C}$ under constant illumination. These rotifers were sieved and transferred to three 25 litre conical containers, each containing 10 litres of sea water at a salinity of 16 ‰. Rotifers were suspended at a density of 1 rotifer ml^{-1} . Temperature was maintained at $24 \pm 2^{\circ}\text{C}$ through a control box attached to a thermoregulated thermometer. Cultures were aerated by airstones, and rotifers were fed on algae in combination with yeast (Experiment 1) or on yeast alone (Experiment 2). The number of rotifers and the appearance of males and resting eggs were followed every 1-3 days.

ii. Food

Algae. Chlorella stigmatophora was cultured in 20 litre flasks and used as described previously (Minkoff et al., 1982). Algae were supplied daily to rotifer cultures at a density of 3×10^6 cells ml^{-1} .

Yeast. Caked baker's yeast was purchased from local grocery shops and stored for up to 5 days at 4°C. The amount of yeast fed to rotifers was 10^6 g rotifer⁻¹. The same amount of yeast was added to algal-fed rotifers, when density of cultures exceeded 5 rotifers ml⁻¹.

iii. Isolation of resting eggs

Resting eggs produced in the experimental containers were collected at the end of the experiment. Some of these eggs were suspended in distilled water, sonified, transferred to separatory glass funnels and collected by gravitation. Eggs were preserved, dried, in distilled water or sea water of two salinities (20 ‰ or 40 ‰) at 3 different temperatures (4°C, 20°C or -18°C) in scintillation vials wrapped with aluminum foil. Other eggs were washed in distilled water and placed on sucrose gradients made of four sucrose concentrations (25, 50, 75 or 100 gm in 100 ml distilled water) in 5 ml centrifuge tubes. Tubes were centrifuged at a velocity of 3000 RPM (1075 g) for 10 minutes using a Sorvall RC-5 centrifuge (DuPont, U.S.A.). Following centrifugation, eggs were recovered from the border between sucrose densities of 25 and 50%. These eggs were preserved either in sucrose solutions (25 or 50%) or sea water at two salinities (20 and 40 ‰).

iv. Hatching of preserved resting eggs

In order to test methods of isolation and preservation, resting eggs were hatched, at least one month after their collection.

Eggs to be hatched were suspended for 2 hrs in distilled water on a rotatory shaker table at $20 \pm 1^\circ\text{C}$, under direct illumination. Following this period of incubation, undamaged eggs were transferred to petri dishes containing sea water

(salinity 16 ‰) and algae (C. stigmatophora at a concentration of 0.5×10^6 cells ml^{-1}) and further incubated at the same conditions of temperature and light for 72 hours. For each treatment combination, between 80-200 eggs were tested. Every 24 hours, rotifers hatched from the resting eggs were counted and removed. The total percent of hatched eggs was calculated as the number of eggs hatched after 72 hours of the total incubated eggs. Under these conditions of incubation, no reproduction of rotifers occurred during the 24 hour interval period between observations.

3) Results and discussion

i. Production of resting eggs

The number of rotifers increased from 1 rotifer ml^{-1} to 300 rotifers ml^{-1} in a similar pattern in all 3 replicate containers (each containing 10 litres) in Experiment 1 (Fig. C1), where rotifers were fed only on algae. Mictic levels reached 60-75% on days 6-8 after the onset of the experiment, similar to our previous results obtained in small (10 ml) experimental cultures. Small variations were observed in the number of resting eggs, which were collected at the termination of the experiments (Table C1), considering a large error introduced by methods of isolation and counting. Similarly, the relative number of resting eggs produced per female (r/f) varied little between replicate containers and was relatively higher (0.16-0.17) than levels obtained by us previously on algal-fed rotifers, which ranged between 0.09-0.12 (unpublished results). In total, about 10^6 resting eggs were produced in 30 litres by 6×10^6 rotifers fed on algae and yeast during the course of the experiment.

Rotifers fed only on yeast hardly produced any resting eggs (Experiment 2) (Table C2), although mictic levels in these cultures were similar to those observed in Experiment 1 (Fig. C2). The total number of rotifers found in each of the culture containers was similar to that observed in cultures of Experiment 1. Yeast used as food maintained rotifer cultures and did not affect the production of males, but eliminated the production of resting eggs. It is not known whether the effect of yeast is direct, a lack in energy required for the production of resting eggs (as suggested by Gilbert, 1977, 1980), or indirect, affecting water quality (mainly pH being lower in these cultures). The exact combination of yeast and algae should be further investigated, in order to obtain a feeding regime containing minimum amounts of algae and permitting resting egg production. Reducing the amount of algae used could reduce significantly the cost of resting egg production.

ii. Hatching of preserved resting eggs

Effect of food

Although a relatively small number of eggs was produced by females fed only on yeast, yeast had no effect on the hatching of resting eggs (Table C3) when preserved for one month at 4°C in 16 ‰ salinity sea water. The relatively low hatching levels and large individual variations observed may be due to the short period of storage. In previous work (Minkoff et al., 1983), we observed an obligatory dormant period lasting for about one month.

Effect of isolation and preservation method on hatching

No hatching was observed in resting eggs preserved in distilled water, sucrose solutions (25 or 50%) or in sea water at a salinity of 40 ‰ (Table C4). Best results were obtained in hatching eggs preserved in diluted sea water (salinity 16 ‰) at 5° or 20°C. It was possible to preserve dried eggs, and best results were obtained if they were rinsed in sea water (salinity of 16 or 40 ‰) prior to drying at room temperature. Resting eggs isolated on sucrose gradients could be preserved in diluted sea water (salinity 16 ‰) without significant loss in their viability.

4) Summary and conclusions

Production of resting eggs in 10 litre volumes was found to be similar to that obtained with rotifers cultured in small experimental flasks containing 10 ml. Feeding rotifers on yeast produced relatively large numbers of females, did not affect mictic levels, but resulted in almost no production of resting eggs. In order to decrease the requirements for algae, which are costly to produce and maintain, more experiments are needed to elucidate the optimum combination of algae and yeast that should be provided for the production of resting eggs.

Resting eggs were best preserved in diluted sea water of 16 ‰ salinity at 5 or 20°C in the dark. Under these conditions, eggs produced by yeast-fed females hatched in a similar way to those produced by females which were fed also on algae. Eggs could be isolated by sucrose gradients but survived preservation only when stored in diluted sea water of 16 ‰ salinity. In order to obtain viable dry stored rotifer resting eggs, they must be washed in sea water (16 ‰ or 40 ‰ salinity) prior to drying.

5) References

- Gilbert, J.J. 1977. Mictic female production in monogonant rotifers. Arch. Hydrobiol. Beih. Ergebn. Limnol. 8: 142-155.
- Gilbert, J.J. 1980. Female polymorphism and sexual reproduction in the rotifer Asplanchna: evolution of their relationship and control of dietary tocopherol. Am. Nat. 116: 409-431.
- Minkoff, G., E. Lubzens and D. Kahan. 1983. Environmental factors affecting hatching of rotifer (Brachionus plicatilis) resting eggs. Hydrobiologia 104: 61-69.

Table C1. Production of resting eggs by rotifers fed on algae and yeast.

Resting eggs were collected at the termination of Experiment 1 and their number estimated from 5 x 1 ml samples withdrawn and returned to collection vessel.

Replicate container (10 litres)	Number of resting eggs (r)	No. of rotifers at termination of experiment (f)	Ratio r/f
(1)	369.2×10^3	215.3×10^4	0.17
(2)	318.0×10^3	204.7×10^4	0.16
(3)	311.0×10^3	181.3×10^4	0.17
Total	998.2×10^3	601.3×10^4	0.17 ± 0.01

Table C2. Production of resting eggs by rotifers fed on yeast.

Resting eggs were collected at the termination of Experiment 2 and their number estimated from 5 x 1 ml samples withdrawn and returned to collection vessel.

Replicate container (10 litres)	Number of resting eggs (r)	No. of rotifers at termination of experiment (f)	Ratio r/f
(1)	4×10^3	206×10^4	1.94×10^{-3}
(2)	6×10^3	238×10^4	2.52×10^{-3}
(3)	negligible	214×10^4	-
Total	10×10^3	658×10^4	1.52×10^{-3}

Table C3. Comparison in hatching of rotifer resting eggs produced by females fed on yeast or on yeast in combination with algae. Eggs were preserved for one month at 5°C in dilute sea water (salinity 16 ‰) covered in aluminum foil. Each experiment consisted of 80 eggs divided into 4 groups.

Food fed to rotifers	% of hatching (Av. $\pm$ S.D.)		
	Exp. 1	Exp. 2	Exp. 3
Yeast	20 $\pm$ 19	28 $\pm$ 12	26 $\pm$ 10
Algae + yeast	23 $\pm$ 21	30 $\pm$ 12	22 $\pm$ 12

Table C4. Hatching of rotifer resting eggs preserved for 4-6 months at -18° , 5° or 20°C in sea water (16 ‰ or 40 ‰ salinity) or dried form. Eggs were pre-incubated in distilled water (2 hrs) at 20°C under illumination and transferred to petri dishes (4 replicates, each containing 20 eggs) containing sea water (salinity 16 ‰) and algae (0.5×10^6 cells ml^{-1} of *Chlorella stigmatophora*). The number of eggs hatched during 72 hrs of incubation at 20°C under constant illumination is expressed in percent of total eggs incubated.

	Salinity during preservation period (‰)	Temperature of preservation ($^{\circ}\text{C}$)	Method of preservation	% of hatching (Av. $\pm$ S.D.)
1	16	5	in solution	43 ± 12
2			dried	21 ± 7
3		20	in solution	53 ± 9
4			dried	0
5	40	-18	in solution	0
6			dried	15 ± 0
7		5	dried	27 ± 9
8			dried	28 ± 12
9	distilled water	-18	dried	0
10		5	dried	14 ± 11
11		20	dried	0
12		-18	dried	0
13	16	5	in solution (after sucrose gradient separation)	40 ± 7.6

Legends to Figures

Figure C1. The increase in number of rotifers (clone B12) cultured in diluted sea water (salinity 16 ‰) in 25 litre conical containers (three replicates, A, B and C) fed on algae and yeast (Experiment 1).

Figure C2. The relative number of males to females (% mixis) in rotifer cultures fed on algae with yeast (o) or on yeast alone (●).

Fig. C1

Fig. C2

○ fed algae
● fed yeast

III. Summary and Conclusions

The aim of our project was to explore the possible use of rotifer (Brachionus plicatilis) resting eggs for aquaculture. These resting eggs could be used either as a reserve stock for initiation of mass cultures or directly as a source of rotifers in mariculture hatcheries.

Establishing this system involved research into the factors affecting sexual reproduction and production of resting eggs, their hatching under controlled conditions and long-term preservation.

During the course of our work we have determined the major environmental factors involved in the induction of resting egg production. Intermediate temperatures, salinities and food levels of mixed algal diets support the production of resting eggs.

Production of resting eggs was found to differ in 17 zoogeographical strains examined. The Russian strain was the most productive, its output being twice that of any other tested strains. Variation was also found among clones isolated from single resting eggs produced by a parent clone, suggesting segregation during sexual reproduction. Occurrence of high mictic levels among clones did not assure high production of resting eggs.

Although we were quite successful in manipulating rotifer strain and culture environment to produce high proportions of mictic females (above 90%), fertilization rates resulting in the production of resting eggs were relatively low (2-15%). Further research into the factors involved in fertilization are required for feasible economic mass production of these resting eggs.

Rotifer resting eggs were found to hatch under controlled conditions. Their hatching rate depended on the method of preservation, exposure to light, appropriate salinity and temperature. These factors could be manipulated to obtain 70-80% hatching rate. This rate is sufficient for routine use of resting eggs, considering the high rate of rotifer reproduction.

Resting eggs were best preserved in sea water at a salinity of 16 ‰, in the dark and at temperatures ranging from 4-20°C. Although dry preserved eggs could be more convenient for commercial use, these show some loss in viability.

Rotifer body size was found to be genetically determined. Small size rotifers were found to be consumed in preference to larger ones by Sparus aurata larvae during the first 2-4 days of their life. Use of these small sized rotifers may increase survival of fish larvae during their critical period of development.

Further exploitation of B. plicatilis rotifers and their resting eggs was suggested by using them to feed fresh water fish (carp) larvae. Accelerated development of these larvae as a result of rotifers may be of economic importance in freshwater hatcheries, either by increasing production using the same facilities or by the introduction of larger larvae, capable of competing with predators, into outdoor pools.

In conclusion, our collaborative research efforts resulted in covering almost all the research program proposed to BARD. Commercial application of the production of rotifer resting eggs awaits further research into increasing the relative number of resting eggs produced by cultured rotifers.

IV. Publications

- Helps, S. 1982. An examination of prey size selection and its subsequent effect on survival and growth of larval gilthead sea bream (Sparus aurata). M.Sc. Thesis, Plymouth Polytechnic, U.K.
- Lubzens, E., G. Minkoff and S. Marom. 1985. Salinity dependence of sexual and asexual reproduction in the rotifer Brachionus plicatilis. Mar. Biol. (in press)
- Lubzens, E., G. Sagie, G. Minkoff, E. Meragelman and A. Schneller. 1984. Rotifers (Brachionus plicatilis) improve growth of carp (Cyprinus carpio) larvae. Bamidgeh 36: 41-46.
- Meragelman, E., E. Lubzens and G. Minkoff. 1985. A modular system for small scale mass production of the rotifer Brachionus plicatilis (submitted for publication).
- Minkoff, G. 1983. Environmental factors affecting mictic female production and hatching of Brachionus plicatilis (O.F. Muller) resting eggs (Rotifera). M.Sc. Thesis, Hebrew University, Israel.
- Minkoff, G., E. Lubzens and D. Kahan. 1983. Environmental factors affecting hatching of rotifer (Brachionus plicatilis) resting eggs. Hydrobiologia 104: 61-69.
- Minkoff, G., E. Lubzens and E. Meragelman. 1985. Improving rotifer (Brachionus plicatilis) asexual reproduction rates by salinity manipulations (submitted for publication).

Section d.

Description of Cooperation

Dr. T. Snell visited Israel on two occasions (1981 and 1983). During his stay, exchange and discussions led to coordination in experimental work. Furthermore, during his first visit we initiated experiments on following the time sequence of events leading to the appearance of mixis in rotifers transferred from high salinity to low salinity culture media. Discussions with Dr. A. Tandler in Eilat led to the experiments using small body size rotifer strains as food for fish larvae during the first few days of their life. The collaborative program enabled us to cover almost all the planned experiments in the research proposal submitted to BARD.

Section e.

Benefit to Agriculture - Aquaculture

1. Use of small body size rotifers to feed Sparus aurata larvae during the first few days of their life may lead to higher survival of these economically important larvae.
2. Use of Brachionus plicatilis in improving growth and development of freshwater carp larvae may permit the use of facilities and techniques developed for rotifer mass cultivation and preservation for freshwater aquaculture.
3. Controlled production, preservation and hatching of rotifer resting eggs may permit their use in aquaculture. Immediate practical application awaits increased efficiency of resting egg production in order to reduce the cost of the product.