Offprint from "Marine Biology"
International Journal on Life in Oceans and Coastal Waters, Vol. 10, No. 2, July 1971, Pages 183—188

Mass culture of the rotifer *Brachionus plicatilis* and its evaluation as a food for larval anchovies

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Abstract

Growth rates of anchovy larvae, *Engraulis mordax*, reared for 19 days under constant environmental conditions on a diet of laboratory-cultured organisms, exceeded the growth rates of anchovies fed on a diet of wild plankton. The rotifer *Brachionus plicatilis* was found to be a nutritous food source when fed to the larvae in concentrations of 10 to 20/ml and in combination with the dinoflagellate *Gymnodinium splendens* (100/ml). Optimum conditions were determined for mass culture of the rotifer. A high food concentration was the most important parameter needed to assure a high yield of rotifers. Large volumes (464 l) of the unicellular flagellate *Dunaliella* sp. were cultured for feeding the rotifers. The rotifer culture technique described produces approximately 2.5×10^6 organisms/day, providing a reliable food source for rearing studies. The lengths of *B. plicatilis* (without eggs) ranged between 99 and 281 μ, most rotifers being larger than 164 μ and less than 231 μ. Individuals weighed 0.16 μg and contained 8×10^{-4} cal.

Introduction

As part of a larger program on the biology of the northern anchovy *Engraulis mordax*, a study was initiated at the Fishery-Oceanography Center, La Jolla, California, USA, to rear larval anchovies using a variety of cultured food organisms. In an earlier report (Lasker et al., 1970) we showed that first-feeding anchovy larvae grew well initially on the cultured dinoflagellate *Gymnodinium splendens* (53μ diameter) but soon required a larger food organism. Veligers of the bubble snail *Bulla gouldiana* were of the proper size (140μ) and could be cultured in the laboratory, but growth rates of larval anchovies fed on them never reached those of larvae fed on a diet of wild plankton.

Because Japanese workers have used the mixohaline rotifer *Brachionus plicatilis* in fish culture, particularly for feeding larvae of the yellowtail *Seriola dorsalis* (Harada, 1970), we investigated the possibility of using this rotifer as food for larval anchovies. Although Ito (1960) published a study on factors affecting *B. plicatilis* reproduction in small cultures, he did not study reproduction in mass cultures which are needed to produce large numbers of rotifers to feed fish larvae.

Vasil'eva and Okuneva (1961) reared the rotifer *Brachionus rubens* in outdoor concrete tanks, but the environmental conditions were not controlled. In contrast to *B. plicatilis*, fecund female *B. rubens* attach to a substrate, therefore, only a portion of the latter's population is available as a forage organism.

In any rotifer culturing procedure, where the maximum number of individuals is desired, parthenogenetic (amictic) reproduction must be optimized and conditions avoided which cause sexual (mictic) reproduction, which produces smaller males and resting eggs. A number of factors are known to alter the ratio of mictic to amictic females; these are species composition and crowding (Gilbert, 1963), temperature, and the quantity and quality of algal food (Edmondson, 1965; King, 1966) and salinity (Ito, 1960). Quantitative studies are not yet available on the mass culture of rotifers, nor of a rotifer's nutritional adequacy with respect to larval fish growth, therefore, in this study we define laboratory conditions which can be used for mass culturing *Brachionus plicatilis* with the maximum production of amictic females. We compare reproduction of *B. plicatilis* on different algal diets, and assess the rotifer as a food for the larval anchovy *Engraulis mordax*.

Algal cultures

The algae we tried as rotifer food were *Monochrysis lutheri*, *Dunaliella* sp., *Nannochloris* sp. and *Exuviella* sp., obtained from Dr. W. H. Thomas of the Scripps Institution of Oceanography. The algae were unialgal, but not bacteria-free, and were cultured in 75% seawater (100% seawater = 33‰ S) containing soil extract (Lasker et al., 1970).

When large quantities of algae were desired, we chose to culture *Dunaliella* which we found to be compatible with the nutritional requirements of *Brachionus plicatilis* (p. 184), and the easiest of the 4 species to culture because it did not require vitamins or soil extract in the medium. *Dunaliella* medium was essentially the same as described by Thomas (1964). We prepared the seawater by filtering ultraviolet (UV)

light-treated aquarium seawater through two Cuno Aqua-pure filters (pore size = 5 μ), a large capacity Millipore cartridge pre-filter and filter (pore size = 0.45 μ), and finally the water was re-irradiated with UV. EDTA (ethylenediamine tetracetate, disodium salt) 0.001%, was added with the nutrients. The medium was not autoclaved.

Twenty-liter carboys filled with 16 l of medium were inoculated with *Dunaliella* sp. The medium was bubbled with 5% CO_2 in air, stirred with a magnetic stirrer, and continuously illuminated between 500 and 700 ft-c at 23° to 25 °C. Fig. 1 illustrates the growth curve for a *Dunaliella* culture as measured by cell

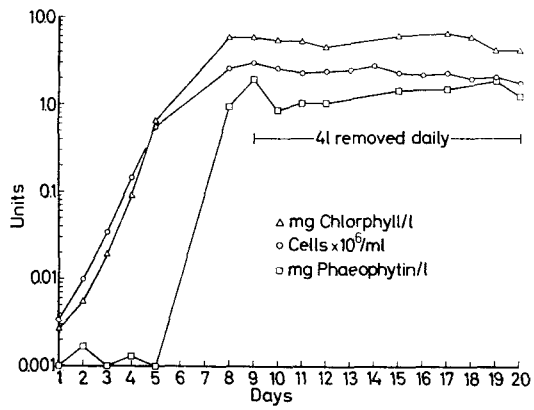

Fig. 1. Growth rate of *Dunaliella* sp. in a 16 l culture. The cell numbers increased from 3.4×10^3 to 3.0×10^6/ml in 9 days. At peak growth, $^1/_4$ of the culture is removed daily and replaced with fresh media. Using this procedure, a healthy culture with a sustained yield can be maintained for 10 more days

count, chlorophyll and phaeophytin production (HOLM-HANSEN et al., 1965). The cells increased from 3.4×10^3 to 3.0×10^6/ml in 9 days. The increase in the rate of phaeophytin production with a concomitant decrease in the rate of chlorophyll production indicates that the culture has reached its peak. At peak growth, the culture is used as an inoculum (p. 185) or $\frac{1}{4}$ of the culture can be withdrawn for rotifer feeding and replaced by fresh medium each day. Under the latter conditions the culture maintains a high cell density for 10 more days. The time to peak growth can be decreased from 9 to 4 or 5 days if the *Dunaliella* inoculum is increased to give a concentration of approximately 3×10^4 cells/ml.

Culture conditions for *Brachionus plicatilis*

Temperature influences the fecundity of rotifers. Maximum reproduction of *Brachionus plicatilis* occurs between 30° to 34 °C. However, since the algae we used do not tolerate high temperatures, all experiments and culturing were performed between 21° and 25 °C.

Brachionus plicatilis doubling times at 24 °C were used to compare the effectiveness of 4 algae as food. KING (1966) found that he required about 50 μg dry weight of algae/ml to maintain a maximum population size of the rotifer *Euchlanis dilatata*. In our experiments, therefore, we always fed *B. plicatilis* (similar in size to *E. dilatata*) an algal dry weight in excess of that used by KING. Cell counts equivalent to 50 μg dry weight for the algae we used are: (1) *Dunaliella* sp. 0.3×10^6 cells/ml; (2) *Exuviella* sp. 0.2×10^6 cells/ml; (3) *Monochrysis lutheri* 1.5×10^6 cells/ml (PARSONS et al., 1961). There is no weight data available for *Nannochloris*, therefore, because it is smaller than *Monochrysis*, higher cell concentrations were used. All algal cells were counted with a Coulter Counter, Model A.

In the initial experiments (Table 1), 10 or 100 female rotifers with eggs were inoculated into cultures of each of the algae listed above, when algal cell growth was in the logarithmic phase and the number of cells was 0.6 to 4×10^6/ml, depending on the species. Algae were added as necessary to maintain a constant cell density. *Brachionus plicatilis* individuals were counted at different time intervals from 4 to 17 days.

In a second series of experiments, 2,000 rotifers of all sizes were added to algal flasks and counted after 4 days.

Doubling times were calculated by dividing $\log_e 2$ by the instantaneous growth rate (K)

$$K = \frac{\log_e N_t - \log_e N_o}{t} \quad (1)$$

where N_o is the number of rotifers in the inoculum, N_t is the final number after time t in days.

Doubling times ranged from 0.8 to 1.1 days at 24 °C. There were no obvious differences in reproductive rates between rotifers feeding on different algal diets of similar dry weights (Table 1).

Dunaliella sp. was used as the main diet to determine the relationship between the doubling time and the concentration of algae. Only female rotifers with eggs were used for the original inoculum. Algal cell concentration was varied from 1.0×10^5 cells/ml to 1.0×10^6 cells/ml (Table 2). Doubling times decreased from almost 3 days to about 1 day, with a tenfold increase in algal cells.

The reproductive rate of *Brachionus plicatilis* was the same in 25 and 33‰ S seawater, provided food concentrations were comparable. Consequently, we used the latter salinity for mass culturing the rotifers. When cultures became dense with *B. plicatilis* we noted no decrease in doubling times as long as food was in excess, and we concluded that crowding, up to 200 rotifers/ml (Tables 1 and 2), did not inhibit reproduction in this species.

Table 1. *Brachionus plicatilis. Reproductive rates of rotifers fed on 4 algal diets, at 24 °C*

Diet	Cell concentration (No/ml)	Initial number of rotifers (N_o)	Final number of rotifers (N_t)	Time in days (t)	Instantaneous growth rate (K)	Doubling time (days)	Final concentration of rotifers (No/ml)	Container size (ml)
Monochrysis lutheri	3×10^6	10	36×10^3	12	0.68	1.0	18	2000
		10	130	4	0.64	1.1	1	100
		2000	42×10^3	4	0.76	0.9	200	2000
Nannochloris sp.	4×10^6	10	16×10^3	12	0.62	1.1	8	2000
		10	270	4	0.82	0.8	3	100
		2000	30×10^3	4	0.68	1.0	152	2000
Exuviella sp.	6×10^6	10	190	4	0.74	0.9	2	100
		35	12×10^4	17	0.61	1.1	60	2000
Dunaliella sp.	1×10^6	10	54×10^3	13	0.66	1.1	27	2000
		10	230	4	0.78	0.9	2	100
		2000	45×10^3	4	0.78	0.9	220	2000

Table 2. *Brachionus plicatilis. Reproductive rates of rotifers fed on several concentrations of Dunaliella sp. at 24 °C*

Diet	Cell concentration (No/ml)	Initial number of rotifers (N_o)	Final number of rotifers (N_t)	Time in days (t)	Instantaneous growth rate (K)	Doubling time (days)	Final concentration of rotifers (No/ml)	Container size (ml)
Dunaliella sp.	1×10^5	80	900	10	0.24	2.9	0.5	2000
	1×10^5	80	850	8	0.30	2.3	0.4	2000
	1×10^5	7	90	10	0.26	2.7	0.9	100
	2×10^5	20	3×10^4	20	0.37	1.9	15	2000
	2×10^5	20	6×10^4	20	0.40	1.8	30	2000
	1×10^6[a]	100	93×10^3	9	0.76	0.9	46	2000

[a] See Table 1 for additional data.

Mass culture of *Brachionus plicatilis*

To mass culture rotifers, a large volume of *Dunaliella* sp. was needed. The fiberglass water tables, available in the Fishery-Oceanography Center aquarium (LASKER and VLYMEN, 1969), were filled to a depth of 13 cm (464 l) with *Dunaliella* medium and inoculated with 32 l of a culture of *Dunaliella* which gave a final concentration of 2×10^5 cells/ml. Water-table cultures were aerated and kept under constant illumination (140 to 170 ft-c). Temperature was not controlled, but aquarium temperatures were relatively constant and only varied between 21° and 25 °C. When the number of *Dunaliella* cells reached 1×10^6/ml (usually in 2 to 4 days) rotifers were added. With such heavy concentrations of *Dunaliella* it was rare to find any other species of algae contaminating the culture during the first few weeks.

An inoculum of 2×10^4 rotifers yielded 2.5×10^6 rotifers/day after 4 or 5 days. The rotifers were harvested daily for about a month, but the production rate decreased, as the alga was consumed faster than it reproduced; therefore, 4 l of *Dunaliella*, from the 16 l cultures, were added as necessary.

Alternatively, rotifers could be kept at 13° to 17 °C in a 500 l holding tank for months provided they were fed *Nannochloris* at a minimum concentration of 1×10^6 cells/ml. Aeration, 200 ft-c illumination for 14 h/day, and addition of enough nutrients for 500 l of medium monthly were required. *Brachionus plicatilis* reproduces slowly at this temperature, maintaining a constant density, thus assuring a continuous source.

Rotifers were collected for feeding fish larvae by slowly circulating the water with a submersible pump through a plastic tube, one end of which was covered with 64 μ mesh netting (Nitex).

Size, weight and caloric content of *Brachionus plicatilis*

Our clone of *Brachionus plicatilis* was isolated from the Salton Sea by Dr. I. HAYDOCK of the Southern California Water Research Project. The size of the

individuals (without eggs) resulting from this clone ranged between 99 to 281 μ long and 66 to 182 μ wide, with about 80% of the population between 164 to 231 μ long and 99 to 149 μ wide. Attached eggs add about 90 μ to a rotifer's length.

Dry weights averaged 0.16 ± 0.01 μg/individual in 4 determinations. Weights were determined by collecting a known number of rotifers on a washed and tared Nitex (nylon 35 μ mesh) circle which had been cut to fit a 25 mm Millipore filter holder. The rotifers were washed with distilled water, dried to a constant weight at 60 °C, and weighed with a Cahn electrobalance to ±2 μg. The samples were scraped off the Nitex, pelleted and subsequently used for calorie and ash measurements.

The caloric content was measured with a Parr 1411 non-adiabatic calorimeter. Benzoic acid was added to the samples weighing less than 40 mg. The average of 4 determinations, converted to ash-free values, was 5335 ± 139 cal/g dry weight organic substance.

The amount of ash was estimated by incinerating samples in a muffle furnace at 500° to 525 °C. The mean for 7 measurements was 7.8 ± 2.0% ash on a dry weight basis.

Rearing anchovy larvae on a rotifer diet

The rearing methods we used were the same as described by LASKER et al. (1970). Anchovy eggs were collected at sea and sorted into the experimental containers at 17 °C. Both small, 10 l containers, with 100 eggs in each and large, 510 l tanks containing 2,000 to 4,000 eggs were used for assessing the feeding conditions necessary for maximum growth. The large tanks were initially filled half full to reduce the quantity of food organisms required, and gradually filled to capacity when the rotifer production exceeded the daily demand.

The growth rates of the rotifer-fed larvae were compared with larvae fed veligers and wild plankton (LASKER et al., 1970; KRAMER and ZWEIFEL, 1970). The mean larval standard length after 19 days on each experiment diet was the criterion for determining the adequacy of the diet.

Growth of anchovy larvae in small containers

Data on the length achieved by *Engraulis mordax* in rearing experiments on each of 4 feeding regimes using rotifers as the basic food and the previous results where veliger and wild plankton diets were used (LASKER et al., 1970) are summarized in Table 3. *Gymnodinium splendens* (100/ml) was added as the first food (except in Group II) followed by the test organism on the 4th day after hatching (hatching = day 0). *Dunaliella* sp. at a density of 1×10^6 cells/ml was added to the larval fish rearing containers to maintain the rotifers in a well-fed condition. Anchovy larvae do not feed on *Dunaliella* sp.

In the first experiment (Group II) rotifers were used as the only food for *Engraulis mordax*. About 7% of the individuals in a *Brachionus plicatilis* culture are 80 μ or less in width, which is an acceptable size for first feeding anchovy larvae (BERNER, 1959). Thus, at a rotifer concentration of 15 to 20/ml, at least 1 rotifer/ml was of a satisfactory size for larval anchovy initial feeding. There was a high mortality of the larvae in the first 9 days which correlated with the mortality curves for starving larvae (LASKER et al., 1970), unlike experiments where *Gymnodinium splendens* was added. However, the final mean length, 7.8 mm, of the few larvae which succeeded in capturing enough prey during the first days of feeding was significantly larger ($p = < 0.05$) than the mean length, 7.0 mm, for veliger-fed larvae (Group I).

In the next series of tests, rotifers were fed to the larvae in progressively increasing concentrations and in combination with *Gymnodinium splendens*. We tried to maintain food densities which could be compared with the earlier wild plankton and veliger experiments (LASKER et al., 1970) and also included a high rotifer density experiment. With each increase in the concentration of rotifers offered as food for the larvae, there was a significant increase in the resulting size of the larval anchovies (see probabilities, groups III, IV, V, Table 3).

In Group III, the rotifer concentration varied between 2 to 6/ml, averaging about 3 to 4/ml. Survival at this food density was very low, similar to the *Brachionus plicatilis* only diet (Group II); however, the larvae were significantly larger than veliger-fed larvae ($p = < 0.01$), averaging 8.2 mm standard length.

In Group IV, the concentration of food offered to the larvae and the percent survival of the larvae were comparable to the veliger experiments (Group I). The rotifer densities varied between 2 to 10/ml and averaged about 7/ml. The combined larval mean length, 8.6 mm, was greater than the mean length achieved with a veliger food ($p = < 0.01$) and also greater than the length attained on the lower density rotifer diet (Table 3).

The best larval anchovy growth rates were attained in the high food density experiments, Group V, when 10 to 20 rotifers/ml were fed. Although there was a difference between two treatments within this group — Table 3 (Va) 8.9 mm differed from (Vc) 9.5 mm ($p = < 0.01$) — it is quite clear that a high food density should be maintained for larvae feeding on rotifers. The Group V mean larval length, 9.1 mm, is the greatest we have obtained in small containers on cultured food. It is significantly larger ($p = < 0.01$) than all other experimental diets except for a diet of wild plankton (Table 3). The average anchovy growth rate for 19 days in 10 l containers on the high density rotifer diet is 0.31 mm/day as compared to 0.38 mm/day for wild-plankton-fed larvae.

Table 3. *Growth and survival of anchovy larvae (Engraulis mordax) after 19 days on experimental diets, in small containers, at 17 °C*

Group	Diet	Food density (No/ml)		Number of larvae	Mean larval length (mm)	Standard deviation (mm)	Range in individual lengths (mm)	Group mean length (mm)	Survival (%)	Probabilities for equal lengths between groups
I[a]	*Gymnodinium splendens* Veligers	100—200 6—8		121	7.0	1.1	4.9 – 9.6		12—47	
										II vs I – p = < 0.05
II	*Brachionus plicatilis*	15—20		12	7.8	1.0	6.1— 9.8		14	
										III vs II – p = < 0.05
III	*Gymnodinium splendens*	100								
	Brachionus plicatilis	2—6	a)	18	8.5	0.7	6.7— 9.7		18	
			b)	9	8.3	0.8	7.3— 9.7	8.2	9	
			c)	48	8.1	1.1	6.0—10.3		—	
										IV vs III – p = < 0.05
IV	*Gymnodinium splendens*	100								
	Brachionus plicatilis	2—10	a)	18	8.0	1.4	5.9— 9.8		19	
			b)	44	8.7	1.5	5.8—11.7	8.6	52	
			c)	40	8.8	1.8	6.1—13.3		41	
										V vs IV – p = < 0.01
V	*Gymnodinium splendens*	100								
	Brachionus plicatilis	10—20	a)	37	8.9	1.1	6.6—11.0		39	
			b)	29	9.0	1.5	5.9—11.6	9.1	—	
			c)	40	9.5	1.0	6.9—11.8		43	
										VI vs V – p = < 0.01
VI[a]	Wild plankton	2		12	10.5	1.4	8.1—13.0			

[a] Data from LASKER et al. (1970).

Table 4. *Length of anchovy larvae (Engraulis mordax) after 15 and 19 days on experimental diets in large tanks*

Group	Diet	Food density (No/ml)	Age (days)	Number of larvae	Mean larval length (mm)	Standard deviation (mm)	Range in individual lengths (mm)	Container size (l)	Temperature (°C)
I	*Gymnodinium splendens*	50—100							
	Brachionus plicatilis	4—8	15	20	8.4	1.3	5.9—10.7	510	17.0
	Artemia, day 15	<1	19	10	10.1	0.8	8.9—11.1		17.0
II	*Gymnodinium splendens*	50—100							
	Brachionus plicatilis	10—20	15	20	10.5	0.8	9.0—11.8	510	17.0—18.0
	Artemia, day 15	<1	19	20	12.0	1.2	9.4—13.9		17.0—18.1
III	Wild plankton[a]	<1—>2	15	51	8.8	1.2	5.8—11.1	380	17.0
	Artemia, day 17	<1	19	35	10.6	2.1	5.5—13.7		17.0

[a] Data from KRAMER and ZWEIFEL (1970). The 15 and 19 day mean lengths of the larval anchovies reared on a wild-plankton diet were obtained by combining KRAMER and ZWEIFEL's data for days 14, 15 and 16, and for days 18 and 20.

Growth of anchovy larvae in large containers

Two thousand *Engraulis mordax* eggs were used in the first experiment, and the larvae were reared at 17 °C on a diet of *Gymnodinium splendens* and rotifers at 4 to 8/ml. *Dunaliella* sp. was added to supply the rotifers with food (1×10^5 cells/ml), and the diet was supplemented with newly hatched *Artemia* nauplii from the 15th day. The mean lengths achieved by larvae on the 15th and 19th day of this diet were compared with KRAMER and ZWEIFEL's (1970) results on anchovies reared at 17 °C on a diet of wild plankton (1 to 2 organisms/ml), and supplemented with *Artemia* nauplii from the 17th day. The growth rates on both diets were the same. The growth attained on days 15 and 19 for rotifer-fed larvae, 8.4 and 10.1 mm, did not differ significantly from the wild-plankton-fed larvae, 8.8 and 10.6 mm (Table 4).

In a second experiment, 4,000 laboratory-spawned and fertilized anchovy eggs (LEONG, 1971) were added to the large tank. The feeding regime was the same

as in the first trial except that the rotifer concentration was doubled, to test the results obtained from the high density small container experiment. The temperature held at 17 °C for 10 days and, due to a failure in the temperature control, the tank warmed to 18 °C on the 15th day and was 18.1 °C on day 19. The mean length of the larvae on this high-density rotifer diet (10 to 20/ml) was significantly ($p = < 0.01$) greater than the wild plankton control (Kramer and Zweifel, 1970) on both the 15th and the 19th day (Table 4). The average growth rate of the rotifer-fed larvae for 19 days was 0.46 mm/day, or 0.07 mm/day more than the wild plankton-fed larvae. The success of this trial can be attributed to the increased food ration, although there may have been some effect from the higher temperature.

In conclusion, laboratory cultured rotifers, fed in high concentrations, appear to be a nutritious food source for anchovy larvae. For the first time in our experience, the larval anchovy early growth rate on a diet other than that of wild plankton equalled and exceeded that of larvae fed on wild plankton.

Summary

1. Optimum conditions for mass culturing the rotifer *Brachionus plicatilis* are described. The time for the rotifer population to double in size was determined at different temperatures, salinities, food concentrations, and on various algal diets. A high food concentration was the most important parameter needed to assure a high yield of rotifers. Under the conditions given, 2.5×10^6 rotifers can be harvested daily.

2. A technique is described for culturing large volumes of the marine flagellate *Dunaliella* sp. Dense, 16 l cultures with 3×10^6 cells/ml were used as the inoculum for a 464 l tank in which the rotifers were cultured. Cell concentrations in the large tanks increased to 1×10^6/ml in 2 to 4 days.

3. *B. plicatilis* is evaluated as a food for larval anchovies. The mean lengths of anchovies (*Engraulis mordax*) after feeding for 19 days on rotifer diets are compared with previous experiments in which either gastropod veligers or wild plankton were fed. Even at a very low density of 3 to 4/ml, rotifers are a more valuable food source for the larvae than veligers at a concentration of 6 to 8/ml. Larval growth rates obtained on the highest density diet (10 to 20 rotifers/ml) exceeded the wild plankton controls.

4. The size of *B. plicatilis* (without eggs) ranged between 99 to 281 μ long and 66 to 182 μ wide. The dry weight averaged 0.16 μg/individual, ash was 7.8% of the dry weight, and caloric content 5335 cal/g dry organic substance.

Acknowledgements. We thank Dr. R. Lasker for constructive criticism during the preparation of the manuscript, B. May for the use of his data on ash content of *Brachionus plicatilis*, and Mr. J. Zweifel for statistical analysis of the data. This work was supported in part by Sea Grant Program No. GH-52 to the University of California, San Diego, California, USA.

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Date of final manuscript acceptance: April 16, 1971. Communicated by J. Bunt, Miami