Environmental factors affecting hatching of rotifer (*Brachionus plicatilis*) resting eggs

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Abstract

Hatching experiments were carried out on a population of *Brachionus plicatilis* (Dor strain) resting eggs produced in batch laboratory cultures under controlled conditions and then stored for at least one month at 4 °C in the dark. Light was found to be obligatory for termination of dormancy. Over the temperature range of 10–30 °C (at 9.0‰ salinity), hatching was optimal (40–70%) at 10–15 °C and decreased linearly with the rise in incubation temperature. Resting eggs incubated over a salinity range of 9–40‰ (at 15 °C) showed optimal hatching at 16‰. Incubation of resting eggs in distilled water permitted normal embryonic development, but neonates died at eclosion. Presence of algae, *Chlorella stigmatophora* (0.5×10^6 cell ml^{-1}), was found to aid hatching.

Introduction

Dormancy ensures the survival of a species during adverse environmental conditions and is broken in response to conditions that favour continued development. The effect of some external factors (i.e. temperature, salinity, light and oxygen levels) on hatching has been studied in several aquatic invertebrates such as copepods (see review by Grice & Marcus 1981), *Daphnia* (Pancella & Stross 1963; Stross 1966 & 1971), *Artemia* (Jennings & Whittaker 1941; Clegg 1964; Sorgeloos 1980), as well as two species of rotifers (Ito 1960; Pourriot *et al.* 1980 & 1981; Lubzens 1981).

The rotifer resting egg is an embryo in an arrested stage of development surrounded by three protective shell layers (Wurdak *et al.* 1978). Apart from being dependent on environmental conditions, hatching is probably modified by as yet undetermined physiological and genetical factors (see reviews by Ruttner-Kolisko 1972; Gilbert 1974).

In the present paper, the effect of temperature, salinity, light and algae on hatching of resting eggs in a clone of *Brachionus plicatilis* was studied. Some preliminary results on hatching of resting eggs from a mixed population of *B. plicatilis* have been previously reported (Lubzens *et al.* 1980; Lubzens 1981).

Material and methods

Rotifers

A single clone of *Brachionus plicatilis* was collected from the Dor fish farms (about 40 km south of Haifa). Rotifers were batch cultured in Mediterranean sea water (M.S.W.) under constant illumination at 25 °C. They were fed *Chlorella stigmatophora* (3×10^6 cells ml^{-1}) supplemented with fresh bakers' yeast (1×10^{-6} gr rot^{-1}). Rotifers reproduced mainly asexually when cultured in M.S.W. of 40‰ salinity.

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Sea water

Mediterranean sea water of 40‰ salinity (as determined by a refractometer, Bio-Marine) and pH of 7.5–8.0 was collected 200 m offshore from the Israel Oceanographic & Limnological Research station, Haifa, and stored for 1–4 weeks in 20 l plastic containers. Prior to use, M.S.W. was membrane filtered (Millipore 0.45 μm). In cases where lower salinities were required, M.S.W. was diluted with deionized fresh water.

Algae

Chlorella stigmatophora (No. 211/20, Culture Centre of Algae and Protozoa, Cambridge, England) was cultured in 2–3 l batches at 20–22 °C under constant illumination. Culture medium was prepared from M.S.W. enriched with a modification of Guillards f/2. medium (Lubzens 1981). Algae in log phase of growth were harvested by 15 min centrifugation at 4200 g at room temperature. The algae pellet was resuspended in M.S.W. to desired densities as determined by a Coulter Counter Model ZB.

Production of resting eggs

Resting eggs were produced as described previously (Lubzens 1981) by transferring rotifers to lower salinities (9‰ or 20‰). One to two weeks following the appearance of the first mictic females, resting eggs were collected by sieving through an 85 μm net and then separating them from remaining debris. Resting eggs were resuspended in diluted M.S.W. (9‰ or 20‰ according to the salinity in which they were produced) and stored for at least one month at 4 °C in 15 ml glass vials covered with aluminium foil. Preliminary results showed that resting eggs had to be stored for at least one month before hatching.

Resting egg hatching

Undamaged resting eggs, translucent, brown and vacuolated in appearance, were individually collected after storage and hatched in either test tubes or petri dishes. Hatching conditions, unless otherwise stated, were in a medium containing $0.5 - 1.0 \times 10^6$ algal cells ml^{-1} and constant illumination. Ten resting eggs were distributed in each test tube containing 3 ml medium. Forty to four hundred resting eggs were incubated in each petri dish (10 cm diameter) containing 16 ml medium. These resting eggs were transferred in groups of 10–40 onto 1 cm diameter sieves (65 μm) which facilitated observations and handling. Preliminary experiments showed that hatching was not affected by resting egg crowding. The incubation media were changed every 2–3 days during the course of the experiments.

During the process of hatching, three distinct stages of embryonic development were observed using a stereoscopic light microscope (×10–40):
(1) Embryos in the diapause stage were characterized by their light brown colour.
(2) Embryos in the early to late developmental stages were characterized by their yellow colour. Occasionally, the eye pigment and movement of cilia and mastax could be noticed.
(3) Embryos that reinitiated their development but failed to hatch were characterized by their grey colour.

Experimental procedure

Temperature effect

Two sets of experiments were carried out at six different temperatures (5, 10, 15, 20, 25 and 30, each ± 1 °C) at 9.0‰ salinity. Resting eggs for each experiment originated from a different batch.

Experiment 1 consisted of two-month-old resting eggs which were distributed in groups of 10 per test tube and incubated in water baths, 10 test tubes for each temperature.

Experiment 2 consisted of one-month-old resting eggs which were distributed 20 per sieve. Ten sieves in petri dishes were incubated at each temperature. Resting eggs that did not hatch after incubations at 10, 20, 25 and 30 °C were transferred on day 12 for additional incubation at 15 °C.

Salinity effect

Resting eggs stored for 2 months at 9.0‰ salinity were tested at six different salinities (0, 9, 16, 24, 32 and 40‰). Two hundred resting eggs were used for each salinity; they were divided equally onto 10 sieves and incubated at 15 °C. Every 1–3 days the developmental stages of the resting eggs were examined. Hatched rotifers were removed and unhatched resting eggs transferred to fresh medium.

Algal effect

The effect of different algal concentrations (0.5, 1.0, 3.0 and 6×10^6 cells ml^{-1}) on hatching was investigated using eggs stored for about three months at 9.0‰ salinity. At each algal concentration, 100 resting eggs were distributed on four sieves and incubated at 9.0‰ salinity at 15 °C under constant illumination. In order to elucidate the beneficial factors within the algae, an experiment was carried out in which the following treatments were tested:

Treatment 1 and 2: medium with (1×10^6 cell ml^{-1}) or without algae.

Treatment 3: medium containing heat-killed algae (3 min at 80 °C).

Treatment 4: the filtrate (Millipore 0.45 μm) from an algal suspension (1×10^6 cells ml^{-1}) incubated at 15 °C for 24 h.

All the above-mentioned experiments were carried out in 9.0‰ salinity at 15 °C under light and dark conditions, except for treatment 3 which was carried out only in the light.

Illumination effect

Resting eggs produced at 20‰ salinity were sorted (see section on hatching of resting eggs, above) into test tubes in groups of 50 and then stored in the above salinity at 4 °C in the dark for two months. Prior to experiments, resting eggs were warmed up for 2 h to 15 °C. An experimental group consisted of at least 100 eggs distributed in groups of 10 per sieve in a 20‰ salinity medium which did not contain algae. Each group was exposed to one of the following durations of illumination (40 W cool white fluorescent, 6.3 W m^{-2}): 0.2, 4, 8, 24, 48, 72 and 144 h. After light exposure, petri dishes were sealed with aluminium foil and returned to a 15 °C incubator. After a total of 7 days incubation, hatching percentage and the developmental stages of the resting eggs were recorded (see above section – Resting egg hatching).

Analysis of results

Statistical comparison of results among treatments in the various experiments was carried out using Student's t-test.

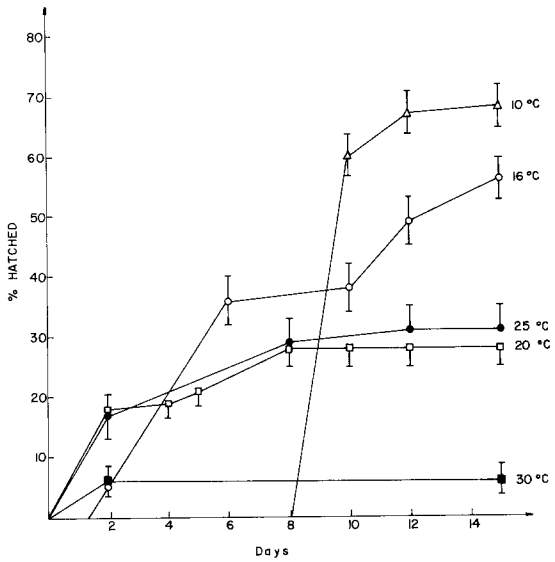

Fig. 1. Temperature effect on hatching of *B. plicatilis* resting eggs. Resting eggs were incubated in the light in a medium of 9.0‰ salinity containing 1×10^6 cells ml^{-1} of *Chlorella stigmatophora*. (Mean ± S.E.M.)

Results

Temperature effect

Figures 1 & 2 show that hatching of resting eggs occurred in the range of temperatures 10–30 °C, with a maximal percentage at 10 °C (68%) and lowest at 30 °C (6%). Over this range, an inverse relationship between temperature and hatching percentage was observed. Resting eggs at high temperatures hatched after a shorter (2 days) incubation period than those at lower temperatures (4–10 days). The time required for 50% of the maximal number of resting eggs to hatch ($E_{50\%}$) at each temperature was interpolated from Fig. 1 and plotted in Fig. 4. $E_{50\%}$ decreases exponentially with the increase in incubation temperature.

In another set of experiments (Fig. 3), resting eggs that did not hatch at incubation temperatures ranging from 20 to 30 °C were induced to hatch after being transferred to a lower temperature of 15 °C (see arrow in Fig. 3). The final hatching percent of these eggs was similar to that obtained for eggs incubated at 15 °C only (38–44%). However, resting eggs first incubated at 30 °C showed a significantly lower level of hatching (36%) after the four additional incubation days at 15 °C ($p < 0.025$).

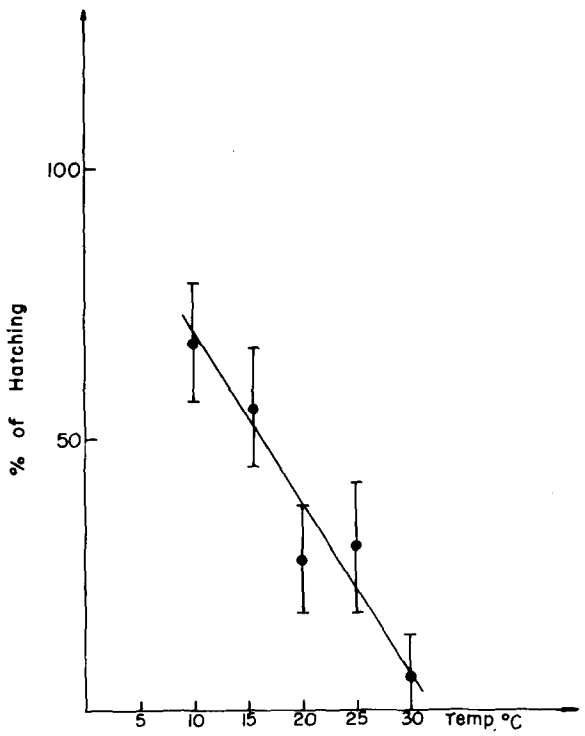

Fig. 2. Hatching after 15 days of *B. plicatilis* resting eggs as a function of incubation temperature (conditions as in Fig. 1). (Mean ± S.E.M.)

Salinity effect

Figure 5 shows that hatching at 40‰ salinity started after four days incubation and reached 50% hatching between days 6–7 (Table 1), while at the lower ranges (9–32‰ salinity) hatching started on day 3 and reached 50% hatching between days 4–5.

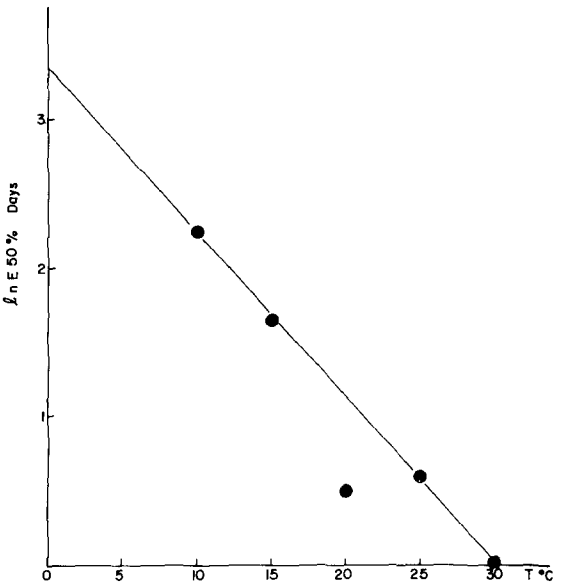

Fig. 4. The relationship between $E_{50\%}$ (the time required for 50% of the maximal number of resting eggs to hatch) and the temperature of incubation for *B. plicatilis* (interpolation from Fig. 1). $E_{50\%} = e^{3.32-.11\,T\,^{\circ}C}$.

Table 1. The relationship between $E_{50\%}$ (time required for 50% of final hatching) and the incubation media salinity for *B. plicatilis* resting eggs. (Interpolation from Fig. 5).

Salinity (‰)	$E_{50\%}$ (days)
0	0
8	4.7
16	4.3
24	4.4
32	4.5
40	6.7

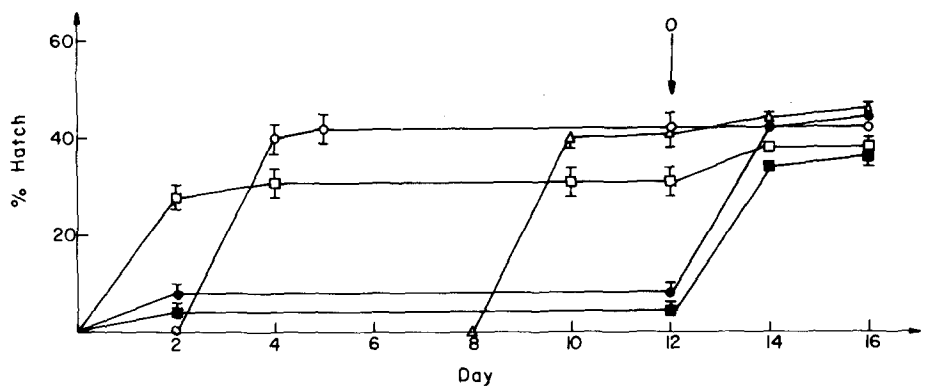

Fig. 3. Effect of different temperatures on hatching of *B. plicatilis* resting eggs. Resting eggs that did not hatch at the temperature range of 20–30 °C were transferred on day 12 to the lower temperature of 15 °C, for an additional 4 days of incubation (see arrow). Hatching conditions as in Fig. 1. △ 10 °C, ○ 15 °C, □ 20 °C, ● 25 °C, ▪ 30 °C. (Mean ± S.E.M.)

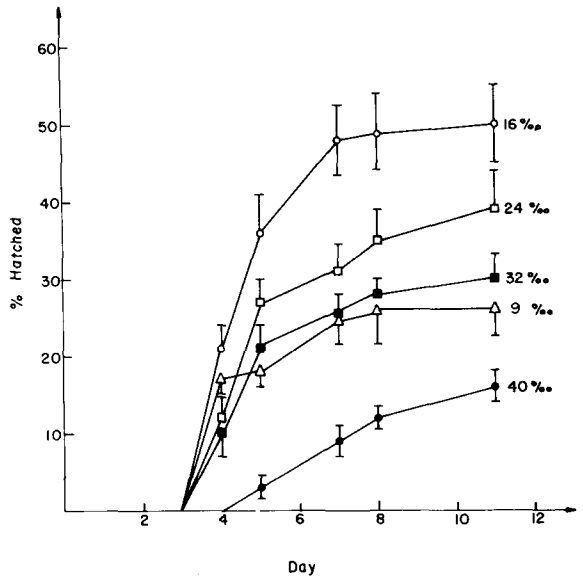

Fig. 5. Effect of different salinities on hatching of *B. plicatilis* resting eggs. Eggs were incubated in the light at 15 °C in a medium containing 10^6 cells ml^{-1} of *Chlorella stigmatophora*. (Mean ± S.E.M.)

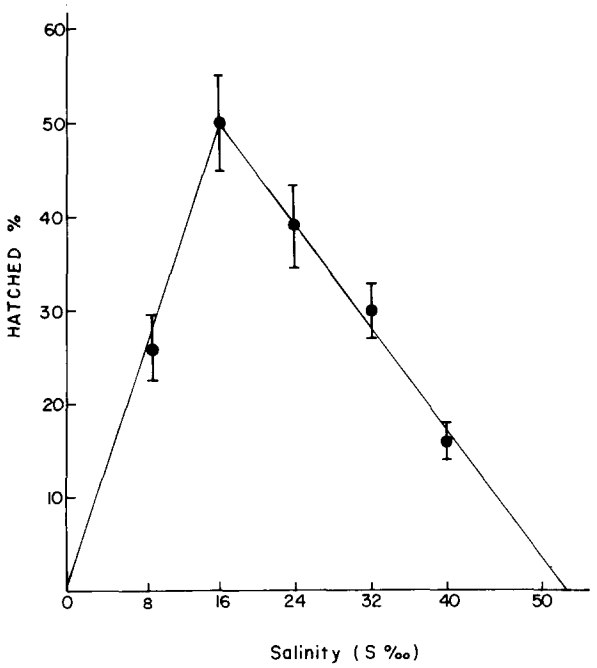

Fig. 6. Effect of different salinities on hatching of *B. plicatilis* resting eggs after 11 days of incubation (conditions as in Fig. 5). (Mean ± S.E.M.)

Plotting the final hatching percent at each salinity (Fig. 6) showed hatching to be optimal at 16‰ salinity. It was reduced significantly at salinities much higher or lower than the optimal salinity of 16‰ ($p < 0.005$ at 9 and 32‰ salinity; but $p < 0.1$ at 24‰ salinity).

The stages of embryonic development observed on days 5 and 11 showed two patterns which were most pronounced at the two extreme salinities (Fig. 7A, B). In distilled water, most of the embryos had resumed development (became yellow) by day 5 but died (became grey) within the egg shell by day 11. At 40‰ salinity, a large proportion of resting eggs (about 50%) remained dormant (brown) throughout the experiment and relatively few of those resuming development died within the egg. In the intermediary range of 9–32‰ salinity the increase in salinity caused a progressively higher number of eggs to remain dormant. However, there was no pronounced difference in the number of embryos dying without hatching within the 9–40‰ salinity range.

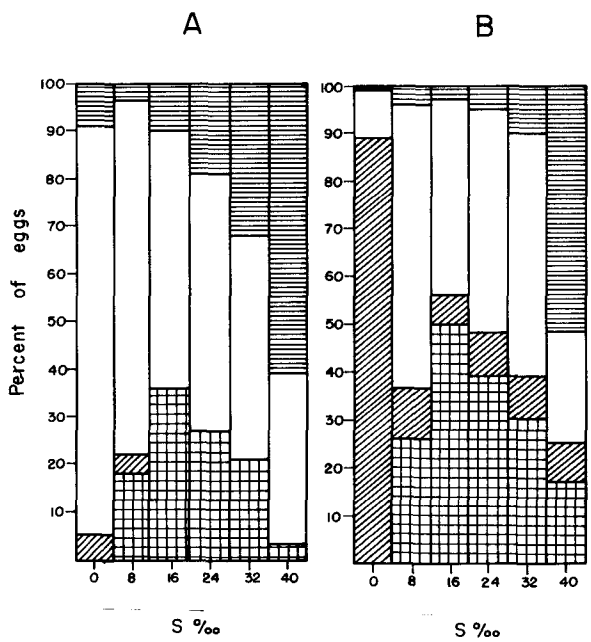

Fig. 7. Effect of salinity on the developmental state of *B. plicatilis* resting eggs after (A) 5 days, (B) 11 days of incubation (incubation conditions as in Fig. 5). ▤ Dormant embryos, □ Developing embryos, ▦ Hatched rotifers, ▨ Dead embryos.

Table 2. Effect of *Chlorella stigmatophora* concentrations on hatching of *B. plicatilis* resting eggs. Resting eggs were incubated at 15 °C and 9‰ S.

Algae concentrations $\times 10^6$ cells ml^{-1}	0	0.5	1	3	6
Hatcing % (Mean ± S.E.M.)	36 ± 6	60 ± 10	55 ± 8	53 ± 15	51 ± 7

Significance level between 0 and 0.5×10^6 cells ml^{-1}: $0.05 < \alpha < 1$

Significance level between 0 cells ml^{-1} and all the rest: $0.05 < \alpha < 0.1$

Table 3. Effect of different fractions of algal suspension (1×10^6 cells ml^{-1}) on hatching of *B. plicatilis* resting eggs in dark and in light. Incubation conditions were 15 °C and 9‰ S.

Group	Illumination	Treatment	Hatching % (Mean ± S.E.M.)
1	+	— Algae	48 ± 3.2
2	+	Dead Algae	42 ± 5.5
3	+	Filtrate	45 ± 3.4
4	+	+ Algae	54 ± 4.9
5	-	— Algae	18 ± 2.9
6	-	Filtrate	13 ± 2.9
7	-	+ Algae	15 ± 2.9

Significance level between groups 4 and 1: $0.1 < \alpha < 0.2$
Significance level between groups 4 and 2: $0.05 < \alpha < 0.1$
Significance level between groups 4 and 1 + 2 + 3: α significant at all levels.

Algae effect

The effect of various algal concentrations in the incubation medium on resting egg hatching is shown in Table 2. Although the presence of algae increased hatching percent from 36% to 60% from zero and 0.5×10^6 cells ml^{-1}, respectively, the effect was of marginal statistical value ($0.05 < p < 0.1$). A further increase in the algal concentration did not increase the hatching percent.

In the second experiment (Table 3), dark-incubated resting eggs exhibited a very low hatching percent (13–18%) in comparison with resting eggs incubated in the light (42–54%). The highest percentage (54 ± 4.9%) was obtained for resting eggs incubated with live algae, whereas the other experimental groups (dead algae, filtrate and lack of algae) in light gave lower hatching percentages (42–48%). The above differences were statistically significant.

Illumination effect

Table 4 and Figure 8 show that after 7 days of incubation there was a difference between resting eggs incubated under light or dark conditions. In complete darkness, the development of resting eggs and their hatching was very low (Table 4). On the other hand, light exposures had a pronounced effect on the initiation of development and hatching of resting eggs. Increasing the illumination exposure from 10 min to 24 h caused a gradual increase in the percentage of activated eggs from 48% to 79% and of hatching from 19% to 41%. Maximal hatching (50%) was obtained for resting eggs illuminated for 48 h. The percentage of resting eggs found with

Table 4. Effect of illumination on hatching percentage of *B. plicatilis* resting eggs. After exposure to increasing periods of light, resting eggs were further incubated in the dark. Resting eggs were incubated in a 20‰ S medium not containing algae at 15 °C for a total period of 7 days.

Duration of light exposure (h)	Dormant resting eggs % (Mean ± S.E.M.)	Activated resting eggs % (Mean ± S.E.M.)	Hatched resting eggs % (Mean ± S.E.M.)	N* (replicates)
0	85	13	3	1
	68	30	4	1
0.2	50 ± 4.3	48 ± 4.2	19 ± 3.2	12
4	57 ± 5.7	44 ± 5.0	16 ± 3.5	10
8	33 ± 4.9	67 ± 5.0	31 ± 3.5	12
24	22 ± 4.1	79 ± 5.0	41 ± 5.1	10
48	15 ± 3.8	84 ± 3.0	50 ± 4.1	10
72	18 ± 5.7	82 ± 5.0	47 ± 5.4	10
144	20 ± 4.0	80 ± 3.8	43 ± 5.8	12

* Control groups (0 h duration of exposure) had one replicate each of 50 eggs. All other groups had 10 eggs per replicate.

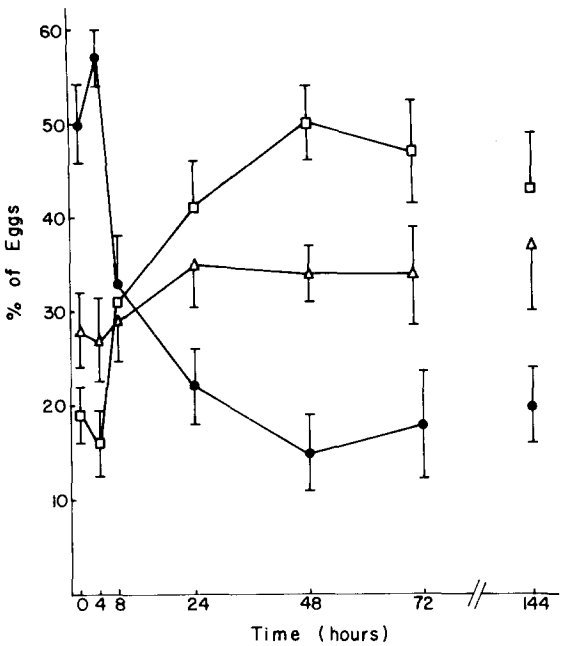

Fig. 8. Effect of illumination on the developmental state of *B. plicatilis* resting eggs. After exposure to increasing periods of light, resting eggs were transferred to dark for further incubation at 15 °C for a total period of 7 days. Incubation medium of 20‰ salinity did not contain algae. ● Dormant embryos, △ Developing embryos, □ Hatched rotifers. (Mean ± S.E.M.)

various embryonic developmental stages (yellow colour) was hardly affected by the duration of light exposure.

Discussion

In the present study, it was found that resting eggs of *Brachionus plicatilis* hatched over a wide range of temperatures (10–30 °C) and salinities (9–40‰), and after only a short (10 min) or continuous light exposure. Complete darkness, low temperatures and, possibly, salinities above 40‰ interfere with the process.

Hatching levels of *B. plicatilis* resting eggs decreased linearly with the rise in temperature (Fig. 2). This inverse relationship could be attributed to lowering of dissolved oxygen levels with temperature increase. Preliminary tests (Winkler's method, Strickland & Parsons 1960) showed linear increase in oxygen levels from 0.44 O_2 mg ml^{-1} at 30 °C to 0.69 O_2 mg ml^{-1} at 10 °C in the incubation medium. Oxygen was found to be required in hatching dormant stages in other species of *Brachionus* (*B. bakeri*; Lite & Whitney 1925) as well as in other invertebrates: *Artemia* cysts (Sorgeloos & Persoone 1975) and copepod eggs (Kasahara *et al.* 1975; Uye & Fleminger 1976).

Developmental rate of *B. plicatilis* resting eggs was exponentially dependent on the incubation temperature (Fig. 4). A similar relationship was reported for subitaneous (McLaren 1966) and diapause (Kasahara & Uye 1979) copepod eggs.

In all salinities tested, re-initiation of embryonic development was observed. However, 16‰ salinity, which was optimal for hatching, was also optimal for *B. plicatilis* growth (Ruttner-Kolisko 1972; Walker 1981). The pronounced decrease in hatching that occurred at 40‰ salinity coincided with a greater proportion of eggs remaining dormant in *Artemia* (Clegg 1964). Osmotic stress at 9‰ or below might have been the cause for reduced hatching also at this range, although embryonic development rate was unaffected. A similar phenomenon has been observed for dormant eggs of the estuarine copepod *Acartia californiensis* (Johnson 1980). Death that occurred in *B. plicatilis* resting eggs placed in distilled water before hatching was probably due to lysis of neonates after rupturing the inner layer, prior to excystment. The inner layer has been assumed to act as an osmotic barrier (Wurdak *et al.* 1978).

In some instances we observed neonates trapped between operculum and outer shell layer before complete excystment but after rupturing of hatching membrane. These neonates suffer from depletion of energy resources, which could be fatal unless food is available up to full excystment. This could explain the higher hatching levels (Table 2) obtained when live algae were added to the incubation media. The higher hatching levels of resting eggs incubated in the presence of live algae may also be due to removal of waste products (i.e. CO_2) by these algae.

Of all the environmental factors tested here, light seems to be the only obligatory stimulus for hatching of *B. plicatilis* resting eggs. This conclusion could be further supported by preliminary results in which incubation of resting eggs for one month in the dark under hatching conditions (15 °C and 20‰ S) showed that hatching occurred only upon light exposure. Light was also found to be obligato-

ry for hatching of *B. rubens* resting eggs, provided other environmental conditions (i.e. temperature) were favourable. This necessity disappeared after extended periods of storage at 4 °C (Pourriot *et al.* 1981). In *Daphnia pulex* it was found that light was essential for diapause release but prolonged storage in constant dark eliminated this requirement (Stross 1966). A second beneficial effect of light was shown in our experiments in which prolonged light exposure (over 24 h) increased the number of activated and hatched resting eggs to optimal levels (Table 4).

In the experiments reported here, successful hatching never exceeded 70%. This could be due to various internal and external factors. Gilbert (1974) has already mentioned that diversity of genotype may be the primary basis for sporadic hatching of resting eggs. This adaptive mechanism ensures the survival of a species in fluctuating environments. Levels of hatching may also be under genetic regulation (Ruttner-Kolisko 1969). Furthermore, the physiological conditions of the mictic females and the food they consume may affect hatching of the resting eggs they produce (Gilbert 1974).

It is possible that in our experiments, higher hatching levels were prevented by structural resistance of the outer egg shell, enabling only a fraction of the activated embryos to excyst. Decrease in hatching may also have resulted from suboptimal storage conditions of the resting eggs. Moreover, hatching levels might have been raised had resting eggs been incubated under simultaneously optimal conditions of light, salinity, temperature and algal concentration.

Acknowledgement

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