水温および塩素量がシオミズツボワムシの受精卵形成に与える影響

誌名	日本水産學會誌
ISSN	00215392
著者	萩原, 篤志 日野, 明徳 平野, 礼次郎
巻/号	54巻4号
掲載ページ	p. 569-575
発行年月	1988年4月

Nippon Suisan Gakkaishi 54(4), 569–575 (1988)

Effects of Temperature and Chlorinity on Resting Egg Formation in the Rotifer *Brachionus plicatilis*[*1,2]

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(Received September 30, 1987)

The effect of temperature (15–30°C) and chlorinity (4–16‰) on resting egg production of the rotifer Brachionus plicatilis was investigated. The rate of resting egg formation was found to increase at lower temperatures and chlorinities. The number of resting eggs produced by 10,000 rotifers at 15°C and 4‰ Cl′ was 8,300, as opposed to 6.1 at 30°C and 16‰ Cl′. Further, the steps which precede egg formation, i.e., appearance of mictic females, fecundity of male-producing mictic females, mating and fecundity of resting egg-producing females, were activated at lower temperatures and chlorinities. The fecundity of each female type and the lifetime swimming distance (life span×swimming speed) of males increased at lower temperatures and chlorinities. These factors were used as an index of biological activity.

The data demonstrate a concurrence of optimum conditions for resting egg production with rotifer biological viability. This suggests that greater numbers of mictic females occur and more resting eggs are formed when the individual rotifers are physiologically vigorous.

In recent years, the monogonont rotifer *Brachionus plicatilis* has become an essential food supply in the production of larval fish and crustaceans. The primary constraint to widespread use of rotifers for larval rearing is difficulty in producing sufficient biomass. Monogonot rotifers are heterogonous, alternating parthenogenesis with bisexual reproduction. In parthenogenesis, amictic females produce offspring without male intervention, yielding a large population in a matter of days. In bisexual reproduction, resting eggs are the final product. Resting eggs remain dormant unless environmental conditions are suitable for hatching. Even under optimum conditions, hatching of resting eggs occurs one week later which delays their contribution to population growth. These factors may enable culturists to maintain large quantities of resting eggs. The eggs can be used as seed for rotifer mass culture or to supply neonates directly to fish larvae, in the manner of *Artemia* resting eggs.

In *Brachionus plicatilis*, no amphoteric nor mictic females which produce both males and resting eggs are found. Therefore, the five stages of bisexual reproduction which result in the production of resting eggs in this species are: mictic female production by amictic females; male production by unfertilized mictic females; mating of male with young mictic females; fertilization; and, resting egg production by fertilized mictic females.

Hino and Hirano[1–5] and Snell[6] examined the mechanism involved in the appearance of mictic females, the first stage of bisexual reproduction. Imamura *et al.*[7] succeeded in producing a larger number of resting eggs by increasing temperatures, Lubzens *et al.*[8] and Minkoff *et al.*[9] reported that resting eggs production was increased by maintaining rotifers at reduced salinities.

In this study, the effect of temperature and chlorinity on the rate of resting egg formation is examined in both large (500 m*l*) and small (5–10 m*l*) rotifer cultures.

Materials and Methods

B. plicatilis typicus (one subspecies of the L-type or large type rotifer) from the stock maintained at the University of Tokyo was employed in this

*1 Studies on the Formation and Hatching of Fertilized Eggs of the Rotifer *Brachionus plicatilis*—II. (シオミズツボワムシ受精卵の形成および孵化生態に関する研究—II).

*2 An outline of this report was presented at the spring meeting of the Japanese Society of Scientific Fisheries, Tokyo, April, 1983.

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study. This stock originated in an eel pond in Mie Prefecture, Japan and has been cultured in the laboratory since 1969.

In this study, one strain of rotifer, produced from a single resting egg, was employed. This egg was produced two years earlier at 25°C, 16‰ Cl′ and under a OL: 24D photoperiod. The egg was kept at 5°C, 16‰ Cl′ and OL: 24D.

Rotifers were cultured in 500-m*l* beakers at 4 different temperatures (15, 20, 25 and 30°C±1°C) at 16‰ and in 3 different chlorinities (4, 8 and 16‰ Cl′) at 25°C, plus one culture of low temperature and chlorinity (15°C and 4‰). The chlorinity of the culture medium was adjusted by dilution of natural seawater with deionized water and the medium was then sterilized by boiling.

Rotifers were fed from a live culture of a marine *Chlamydomonas* sp. Centrifuged algae was fed daily to restored density to 3 to 6×10^5 cells/m*l*. The *Chlamydomonas* was grown on Miquel-Nelson-Matsue's medium in which the chlorinity had been adjusted using the method detailed above, under 2000 lux continuous lighting.

Prior to the experiment, rotifers were acclimated to the seven culture conditions for 10 to 20 days. From each culture, juvenile rotifers were stocked at a density of 10/m*l* in glass bottles. Total female density is expressed as rotifer density. The bottle bottoms were funnel-shaped for ease of resting egg collection and removal of bottom deposits. Samples containing at least 200 females were collected from each culture every 24 hours and examined microscopically. Female rotifers were classified by modification of Sudzuki's[10] criteria and evaluated on the basis of the types of eggs carried, thus:

?♀··young females before laying eggs, old females which have finished spawning or females which lack spawning ability.

♀♀··amictic females which parthenogenetically produce diploid eggs that hatch into amictic or mictic females. The egg is oval and grayish in color.

♂♀··mictic females which meiotically produce haploid eggs that parthenogenetically hatch into males. The egg is round, grayish, and about half the size of amictic eggs.

D♀··mictic females which produce diploid fertilized eggs (resting eggs) after meiosis. These hatch into amictic females. The eggs is oval, comparable in size to amictic eggs, with brown or orange-colored cytoplasm and a hyaline cavity on one side of the egg.

In addition, the number of males in the samples were counted. Then ♀♀, ♂♀, D♀ and ♂ were returned to the culture medium. ?♀ were cultured separately in a 5-cm petri dish until eggs were found and the number of each female type was counted. After separation from bottom deposits, the number of resting eggs was counted.

Data were obtained from day 1 and continued until the population exceeded 100/m*l* (Nth day). The percentage of mictic females (M) in the total sample, the mating efficiency (ME) (number of D♀/1,000 males) and the number of resting eggs (R) (produced by 10,000 female rotifers) were calculated using the following formulas:

$$M=[\{n(♂♀)+n(D♀)\}/n(♀)]\times100$$

where

n (♂♀)=the number of ♂♀ which appeared on the Nth day, including the individuals found in ?♀

n (D♀)=the number of D♀ obtained in the same way as n (♂♀),

n (♀) =total number of ?♀, ♀♀, ♂♀ and D♀ which appeared on the Nth day

and

$$ME=[n(D♀)/n(♂)]\times1{,}000$$

where

n (♂)=total number of ♂ which appeared on the 1st to Nth day, calculated approximately by the following formula:

$$n(♂)=\sum Di/L_M$$

where

D_i=number of males which appeared on the *i* th day,

L_M=life span of males cultured in petri dishes

and

$$R=[D+D'\times(V/V')]\times10{,}000/100V$$

where

D=number of resting eggs collected from the culture bottom until density of rotifers reached 100/m*l*

D′=number of eggs produced by separately cultured D♀ including individuals found in ?♀, which appeared on the Nth day of observation

V =volume of culture medium (500 m*l*)

V′=volume sampled for Nth day observation.

The life span of females exceeds the number of days in this experiment and no female mortality was expected.

Following an evaluation of the ?♀ in the mass-culture experiment, 5 to 20 individuals each of ♀♀, ♂♀ and D♀ were identified and cultured in separate petri dishes (5–10 m*l* in volume) at a

density of 1 to 2/m*l*. Daily observations were made to assess survivorship of each female type and to count offspring from ♀♀ and ♂♀, and the number of resting eggs produced by D♀.

From 4 to 8 males produced by ♂♀ were transferred within 6 hours of birth to another petri dish to continue culture. In a preliminary experiment, mortality of males occurred on the second day at 25°C and on the fifth day at 15°C. Males were observed, therefore, every 2 h at 30 25°C, every 6 h at 20°C and every 12 h at 15°C to determine life span. Five to ten males were observed within 6 h after birth to estimate swimming velocity. Each male was put under a stereomicroscope and the pattern of swimming was recorded with a drawing mirror. After magnification, the path length was measured with a curvimeter. The life-time swimming distance of males was arrived at by multiplying the life span by swimming speed.

Hourly samples of ?♀, produced by 100 ♀♀ at 25°C and 8‰ Cl′ were separated into petri dishes. Fifteen hours after sampling began, 20 one-hour-old males were stocked into each petri dish. Approximately 24 h later, eggs were observed and female types determined.

Results

Although the culture period and maximum density obtained varied among experimental conditions, a general trend in the appearance of ♀♀, ♂♀, ♂, D♀ and resting eggs was apparent. A summary of the appearance of each rotifer type and resting eggs that occurred in the mid-range of temperatures and salinities tested (20°C and 16‰ Cl′) is presented in Fig. 1. Note that resting eggs are the product of four steps during bisexual reproduction.

The rate of resting egg production, of mictic females and the index of mating efficiency in each culture are shown in Table 1. The number of resting eggs produced by 10,000 rotifers increased in proportion to decreases in temperatures at 16‰ Cl′. At 30°C, 6.1 resting eggs were produced, 102.0 at 25°C and 232.3 at 20°C. At 25°C, the number of resting eggs produced increased as chlorinities decreased and numbered 102.0 at 16‰ Cl′, 500.5 at 8‰ Cl′ and 2007.4 at 4‰ Cl′. The combination of low temperature and low chlorinity (15°C and 4‰ Cl′) resulted in more mictic females and higher mating efficiency. In this culture, 10,000 rotifers produced 8,300 resting eggs (Table 1). It should be noted that the maximum density

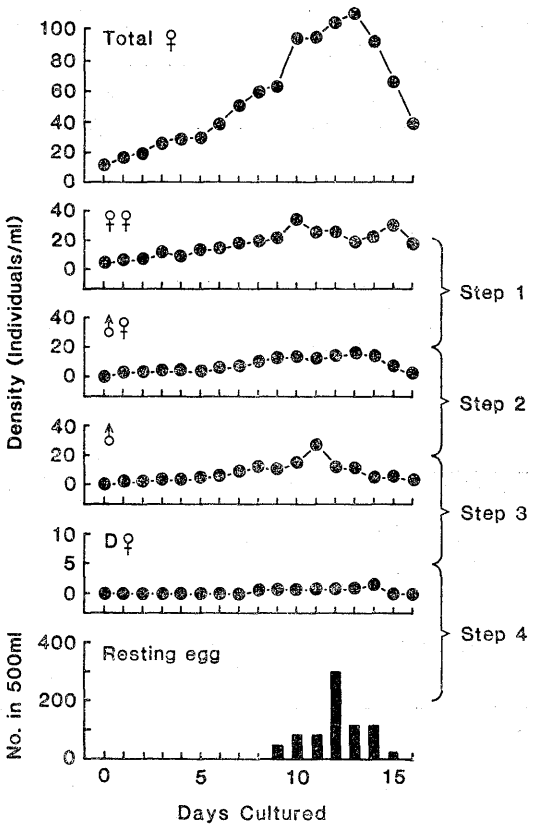

Fig. 1. Four steps of bisexual reproduction at 20°C and 16‰ Cl′. See text for explanation of symbols.

Table 1. Effect of temperature and chlorinity on bisexual reproduction in a 500-m*l* beaker culture of rotifers

Culture Condition		Rate of Mic. F. Appearance (%)	Mating Efficiency	No. of Resting Eggs Produced by 10,000 Rotifers
30°C	16‰ Cl′	1.9	3.1	6.1
	8‰ Cl′	—	—	—
	4‰ Cl′	—	—	—
25°C	16‰ Cl′	10.8	98.6	102.0
	8‰ Cl′	14.3	182.5	500.5
	4‰ Cl′	23.0	458.6	2007.4
20°C	16‰ Cl′	28.9	61.9	232.3
	8‰ Cl′	—	—	—
	4‰ Cl′	—	—	—
15°C	16‰ Cl′	47.3	1484.8	7221.0
	8‰ Cl′	—	—	—
	4+ Cl′	33.9	829.1	8300.0

The data above were obtained from a culture ranging in density from 10 to 100 individuals/m*l* except at 15°C and 16 ‰Cl′, where the maximum density was 21.0 individuals/m*l*.

Table 2. Effect of temperature and chlorinity on the life span of each female type

Culture Condition		Life Span ♀♀ (days)	Life Span ♂♀ (days)	Life Span D♀ (days)
30°C	16‰ Cl′	—	4.0±1.6 (5)	4.6±1.5 (5)
	8‰ Cl′	—	—	—
	4‰ Cl′	—	—	—
25°C	16‰ Cl′	8.6±1.5 (17)	7.4±1.3 (5)	6.5±1.8 (6)
	8‰ Cl′	12.7±4.8 (17)	7.7±3.9 (7)	6.7±1.6 (6)
	4‰ Cl′	10.9±4.3 (20)	—	7.6±1.6 (13)
20°C	16‰ Cl′	—	9.7±4.4 (20)	12.9±1.5 (10)
	8‰ Cl′	—	—	—
	4‰ Cl′	—	—	—
15°C	16‰ Cl′	14.1±2.2 (8)	12.3±2.3 (12)	12.9±3.6 (11)
	8‰ Cl′	—	—	—
	4‰ Cl′	14.8±3.4 (9)	16.2±1.0 (9)	13.3±3.6 (10)

Numbers in parentheses indicates number of individuals tested.

Table 3. Effect of temperature and chlorinity on the fecundity of each female type

Culture Condition		Fecundity ♀♀ (Offspring/ female)	Fecundity ♂♀ (Offspring/ female)	Fecundity D♀ (Eggs/ female)
30°C	16‰ Cl′	—	3.0	1.0
	8‰ Cl′	—	—	—
	4‰ Cl′	—	—	—
25°C	16‰ Cl′	7.0	6.8	1.3
	8‰ Cl′	18.9	19.9	1.7
	4‰ Cl′	16.3	—	1.9
20°C	16‰ Cl′	—	8.5	1.8
	8‰ Cl′	—	—	—
	4‰ Cl′	—	—	—
15°C	16‰ Cl′	15.0	13.4	2.8
	8‰ Cl′	—	—	—
	4‰ Cl′	21.2	21.8	2.8

Figures under column ♀♀ and ♂♀ indicate number of offspring produced. Figures under column D♀ indicate number of resting eggs produced. Each is the mean value of 5–20 individuals.

of rotifers obtained at 15°C and 16‰ Cl′ was only 21/m*l*. This makes it difficult to compare with data from other cultures. Life span and numbers of offspring varied inversely with temperature and chlorinity (Tables 2 and 3). There was, however, no difference between 4 and 8‰ Cl′ at 25°C. The numbers of offspring produced by ♀♀ and ♂♀ were about the same despite the culture condition, e.g. 7.0 and 6.8 at 25°C and 16‰ Cl′. Low fecundity was observed in D♀, varying from 1.0 to 2.8 among the conditions.

Male life span is longer at lower temperatures (Table 4). At 15°C, the life span was 96.0 h or 4 times the life span at 30°C. There was, however, no significant difference in life spans among

Table 4. Effect of temperature and chlorinity on the life span and swimming speed of male rotifers

Culture Condition		Life Span (hours)	Swimming Speed (meters/hour)
30°C	16‰ Cl′	24.0± 0.0 (5)	3.60 (5)
	8‰ Cl′	—	—
	4‰ Cl′	—	—
25°C	16‰ Cl′	47.2± 2.3 (5)	2.52 (5)
	8‰ Cl′	46.0±10.1 (4)	2.76 (6)
	4‰ Cl′	48.7± 1.0 (6)	3.24 (4)
20°C	16‰ Cl′	80.8± 6.3 (8)	2.40 (6)
	8‰ Cl′	—	—
	4‰ Cl′	—	—
15°C	16‰ Cl′	96.0±29.4 (6)	1.80 (5)
	8‰ Cl′	—	—
	4‰ Cl′	92.4±16.6 (5)	2.76 (5)

Numbers in parentheses indicate number of individuals tested.

Table 5. Number of female types and percentage of mictic females sampled at various times after birth

Time (hr) after birth	Number of each female type ♀♀	♂♀	D♀	Mic. F. (%)
1	11	1	1	15.4
2	13	0	0	0
3	—	—	—	—
4	8	0	1	11.1
5	10	0	0	0
6	16	0	1	5.9
7	19	0	0	0
8	25	2	1	10.7
9	13	3	2	27.8
10	6	4	0	40.0
11	6	2	0	25.0
12	8	8	0	50.0
13	8	9	0	52.9
14	3	6	0	66.7
15	8	7	0	46.7

— indicates no data available.

chlorinities at 25°C. Also, males swam faster at higher temperatures and in lower chlorinities; the velocity at 30°C is twice that at 15°C.

In culture with males, mictic females become D♀ only during the first 9 h after birth (Table 5). Beyond this age, they became ♂♀.

Discussion

Resting eggs are the product of a series of processes under the influence of a variety of factors. Study of these factors has produced several indices by which to evaluate bisexual reproduction. Hino and Hirano[4] suggested that environmental conditions during resting egg formation affect the bisexual reproductive pattern in the derivative strain. To account for this, only one strain of rotifers with an identical gene type and known previous history was employed in this study.

Rotifer populations contain several generations of mictic and amictic females, as well as males, though their ratios change both within the population and among populations. Mictic females are produced parthenogenetically, therefore, bisexual reproduction depends on the progress of parthenogenesis. Table 5 shows that 9 h after birth, no further D♀ appear. Mictic females do not produce resting eggs unless mated shortly after birth.

Hino and Hirano[2,3,5] reported several factors which increase the appearance rate of mictic females using the same stock of *B. plicatilis* used in the current experiments. They employed a successive individual culture method which can distinguish between the influence that rotifer density, temperature or salinity exert on the appearance of mictic females. They found that more mictic females were produced at higher densities (10/m*l*), lower temperatures (15°C) and lower chlorinities (4‰). In the current study, the production of mictic females increased during the exponential growth phase (Fig. 1). This is consistent with the observation that the appearance of mictic females is coincident with higher densities. The reason for this relationship on the physiological level is currently unknown.[2] In individual culture, Hino and Hirano[3,5] found that the number of mictic females increased at lower temperatures and chlorinities. A similar observation was found in the mass culture experiments of Lubzens[11] and Lubzens *et al.*[12] Our results show that not only the appearance rate of mictic females, but also the mating efficiency and fecundity of ♂♀ and D♀ appeared to increase at lower temperatures and chlorinities (Table 1). The effect of temperature and chlorinity on resting egg formation was observed at each of the four steps involved in bisexual reproduction. This results in a geometric increase in resting egg production.

The life span of each female type increased at lower temperatures probably because of a decrease in metabolic rate (Table 2). The total number of offspring produced by one female (termed net production rate) is used as a measure of viability and reproductive potential (Table 3). The ability of each female type to spawn in the environmental ranges tested, increased both at lower temperatures and at lower chlorinities. The longest life spans and greatest fecundity occurred at 15°C and 4‰ Cl′. These two external factors exert a combined influence on female fecundity. Results of our study differ from those reported by Snell.[6] He found that the reproductive responses of ♀♀ and ♂♀ differed when salinity and temperature were changed. We observed that all three female types responded similarly to the same changes. He also observed that the fecundity of ♀♀ in a sub-tropical rotifer strain increased at higher temperatures. Hirayama *et al.*[13] reported that the fecundity of ♀♀ was 3.0 at 30°C, 12.9–16.4 at 16–27°C and 4.7 at 10°C. This variation in observations probably results from differences in rotifer stock (Ito *et al.*,[14] Snell[6]) or differences in algal food species (Hirayama[15]).

Swimming velocity increased at higher temperatures and in lower chlorinities. In this experiment, males were found to change swimming direction more often at 4 and 8‰ Cl′ than at 16‰ Cl′, but traces of male swimming paths were recorded in only two dimensions. Therefore, the difference in swimming speeds among chlorinities should be greater than that indicated by path measurements.

According to Gilbert,[16] mating of *Brachionus* is triggered by contact between males and females. Therefore, males swimming faster should come in contact with females more often, resulting in an increase in mating. The mating frequency of a male is therefore dependent on the total distance it swims. Male swimming distance can be used as a measure of viability in the same way that offspring production is used with females. Table 6 shows that males swim greater distances at lower chlorinities. Also, males swim for a

Table 6. Index of lifetime swimming distance of male rotifers

Culture Condition		Distance (meters)
30°C	16‰ Cl′	86.4
	8‰ Cl′	—
	4‰ Cl′	—
25°C	16‰ Cl′	118.9
	8‰ Cl′	127.0
	4‰ Cl′	157.8
20°C	16‰ Cl′	193.8
	8‰ Cl′	—
	4‰ Cl′	—
15°C	16‰ Cl′	172.8
	8‰ Cl′	—
	4‰ Cl′	255.0

Distance swum = life span of male rotifer × swimming speed (Table 4).

longer period at lower temperatures despite the fact that they swim more slowly. The greatest lifetime swimming distance was observed at 15°C and 4‰ Cl′. Theoretically, this should result in an increase in male/female encounters.

Using the criteria of egg production in the female and swimming distance in the male, it appears that the reproductive potential of both sexes increases at lower temperatures and lower chlorinities, within the ranges tested.

The formation of resting eggs, the four reproductive steps which precede it, and the viability of males and females all increased at lower temperatures or chlorinities. This concurrence suggests that mictic females are produced when the physiological state of individual rotifers is favorable. This is consistent with the hypothesis stated by Lubzens *et al.*[12] and Snell[6] that mictic females appear when conditions are moderate.

Bisexual reproduction does not contribute immediately to the standing crop and occurs infrequently at higher temperatures and chlorinities, e.g. 30°C and 16‰ Cl′. Since the population growth rate increases at higher temperatures, it appears that higher temperatures and chlorinities are better suited to mass culture of rotifers for larval feeds. Comparing 25°C and 4‰ Cl′ to 15°C and 4‰ Cl′ shows that the latter is better suited to resting egg formation because a greater number of mictic females appear and active resting egg production is maintained for a longer period. The former temperature and chlorinity, however, are adequate for producing resting eggs over a shorter period.

Imamura *et al.*[7] reported the production of 3.2×10^7 resting eggs in a 4 m^3 tank in 11 days. They improved resting egg production by increasing temperatures from between 4 and 15°C to between 23 and 30°C. One possible explanation for this result is that initial low temperatures induce mictic female appearance and the subsequent increase in temperature speeds succeeding steps of resting egg production.

Studies on red sea bream (*Pagrus major*) (Kitajima *et al.*[17]) and black porgy (*Acanthopagrus schlegeli*) (Okauchi *et al.*[18]) indicated that the neonates of 2.0×10^9 resting eggs can provide feed for 1.0×10^5 fish larvae, assuming 100% hatching.

To employ rotifer resting eggs in the same manner as *Artemia* cysts, rotifers must be cultured in large tanks. In larger tanks, however, such factors as contamination by protozoans and a decline of water quality may inhibit resting egg formation. Solving these problems is crucial to the control of resting egg production in large quantities and is an area of future investigation. It is also important to select or breed a strain which has a high bisexual reproductive potential and is resistant to both protozoan contamination and decline in water quality. Studies on this aspect of rotifer culture are proceeding.

Acknowledgments

Portions of this study were supported by a Grant in Aid of Scientific Research from the Ministry of Education, Japan and by the U.S. Agency for International Development (DAN-4161-A-00-4055-00). The authors wish to thank Cheng-Sheng Lee, Clyde Tamaru and Vernon Sato for their review and comments and Anita Belanger for preparation of the manuscript.

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