Appearance of floating resting eggs in the rotifers *Brachionus plicatilis* and *B. rotundiformis*

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Resting eggs of the rotifers *Brachionus plicatilis* and *B. rotundiformis* are generally found in the bottom sediments in the nature. Here, environmental conditions tend to inhibit the hatching of resting eggs because oxygen concentrations and light levels are low. However, several workers have also reported the appearance of floating resting eggs in these species. These are characterized by a black ring inside of the extra-embryonic space. If this floating behavior can be utilized to avoid the inhibitory environmental effects which reduce the hatching rate of these eggs, then this mechanism clearly has important ecological significance.

In this study, the factors which induce flotation in resting eggs were investigated in the laboratory. Changes in temperature, salinity, photoperiod, dissolved oxygen and ammonium sulfate concentration did not increase the frequency of floating eggs in either species, irrespective of the developmental stage of the egg. Floating eggs appeared only when resting eggs were exposed to air. These results suggested that flotation of resting eggs may not be due to a biological process in the normal life cycle of the rotifer. It seems more likely that it occurs as an artifact derived from the methods used in handling mud samples or managing of rotifer cultures.

Key words: Rotifera, *Brachinonus plicatilis, Brachinonus rotundiformis,* resting egg, flotation

Monogonont rotifers reproduce both asexually and sexually and, in the sexual reproductive phase, resting eggs are formed through mating between males and young mictic females. The resting eggs of *Brachionus* are covered by a thick shell and they remain dormant until external conditions are suitable for hatching. These resting eggs play an important part in maintaining the species and may be responsible for their population biomass and geological distribution. They could also be used in aquaculture in a similar way to cladoceran ephippia and brine shrimp cysts[1)].

Resting eggs generally sink to the sediments, but Ito[2)], working on the sediments of eel culture pond, found that 13−16% of the resting eggs of *Brachionus* floated just before hatching. He noted that all of the floating eggs had a black ring around the extra-embryonic space. The same phenomenon was observed in resting eggs in rotifer mass culture tanks[3)]. So far, the factors which lead to the appearance of these floating eggs have not been identified. This study investigates the effect of external conditions on the appearance of floating eggs in a laboratory.

Materials and Methods

The marine monogonont rotifer *Brachionus plicatilis* formerly included L-and S-type groups. In the workshop of VIIth International Rotifer Symposium, held in Mikolajki, Poland, it was decided that L- and S-types should be classified as two different species. This decision was based on an accumulation of fundamental information[4-11)]. The former L- and S-type *Brachionus plicatilis* were renamed *B. plicatilis* and *B. rotundiformis,* respectively[12)].

In this study, I used both *B. plicatilis* (University of Tokyo strain, originally sampled at an eel culture pond in Mie prefecture) and *B. rotundiformis* (Lake Hamana strain) resting eggs. *B. rotundiformis* can produce two types of resting egg, one type which is released by the female and another which remains inside the maternal lorica[13,14)]. Only the former type was used in the experiments.

Floating eggs have a black ring inside the extra-embryonic space. This could easily be explained if a gas bubble had formed in the extra-embryonic space. The eggs would then float, due to the buoyancy of the gas bubble, and the outline of the bubble would appear black, due to the total reflection of light. Two hypotheses were set up to explain the mechanism leading to the formation of such a gas bubble. These were 1) metabolic activity within the resting egg which produces gas outside the cytoplasm (i. e. an internal factor), and 2) the intrusion of air from outside resting egg shell (i. e. an external factor). I conducted two series of experiments to test these hypotheses.

Experiment 1. Internal factors

B. plicatilis Newly released resting eggs of *B. plicatilis* were collected daily between April, 1981 to October, 1984 from 500 or 1000 ml cultures which had been fed on *Chlamydomonas* sp. The eggs were observed under a compound microscope from just after the release by the fertilized mictic female until they had hatched[15]. The eggs were incubated under various external conditions which might induce gas production from inside the embryo. Temperatures were set at 5, 10, 15, 20, 25, 30 and 35℃, salinity was adjusted to 7.2, 14.4 and 28.9 ppt and photoperiod of OL: 24D, 12L: 12D and 24L: 0D were used. Salinity was regulated by the dilution of natural seawater with distilled water. Light intensity was set at 2,000 lux by using white fluorescence bulbs.

B. rotundiformis Resting eggs of *B. rotundiformis* were collected from an outdoor pond at the Fisheries Laboratory, University of Tokyo in October, 1980. The eggs were stored at 5℃ in total darkness for 3 months before the experiments began. A range of temperatures, salinities and photoperiods were used for the experiment, as described for *B. plicatilis.* In addition, concentrations of dissolved oxygen (DO) and ammonium sulfate (0, 1, 10, 100 and 1,000ppm) were also controlled in these experiments as these factors also vary in the sediments in the natural environment. The dissolved oxygen concentration of the experimental sea water was regulated at three levels by supplying oxygen and nitrogen gas. The sea water was then transferred to 50ml glass DO bottles together with 100 resting eggs. The bottles were then capped tightly by lids which had been coated with silicon grease. The bottles were opened after five days and percentage of floating eggs and of hatched eggs were determined under a stereomicroscope. Dissolved oxygen concentration was determined at the beginning and end of the incubation period using the Winkler method. For the experiments in which dissolved oxygen and ammonium sulfate concentrations were varied, other external conditions were held constant, as follows: temperature, 25℃, salinity 28.9ppt; photoperiod 24L: OD.

Exp. Ⅱ. External factors

It was assumed that any intrusion of air into the extra-embryonic space of resting eggs would probably occur when the eggs were exposed to desiccation, or the rotifer cultures were being aerated. So, the following experiments were conducted to determine whether these factors influenced the production of floating eggs. Resting eggs of *B. plicatilis* and *B. rotundiformis* were used in the experiments.

1) Influence of desiccation

Approximately, 100−200 resting eggs were air dried in incubators (Fujimoto Rika Co.) at 5℃ and 25℃, and at photoperiods of 0L: 24D and 24L: 0D (see Table 3 for details). Initially, the eggs were desiccated in either 0.5ml of seawater (28.9 ppt in salinity) or deionized water. After one hour of desiccation, all of the eggs were transferred to 28.9 ppt seawater and the percentage of floating eggs was monitored until the eggs hatched.

2) Influence of aeration

Between 1,000 and 8,000 resting eggs were placed in glass beakers containing 1 liter of seawater (28.9 ppt salinity) and aerated at 500−1,000 ml per a minute for 24 hours. The beakers were incubated at temperatures of 10℃ and 25℃, and photoperiods of 0L: 24D or 24L: 0D (see Table 3 for details). Only those eggs which remained beneath the surface of the water were included in the results. Those which became attached to the glass beaker above the surface of the water were removed from the experiment as these may have become desiccated during the course of the experiment.

Results and Discussion

Variations in temperature, salinity and photoperiod did not induce floating egg production in either *B. plicatilis* or *B. rotundiformis.* In *B. plicatilis,* the production of gas bubble inside the embryo could not be induced at all in the maturation and postmaturation stages of egg development. Regulation of ammonium sulfate and dissolved oxygen concentration did not cause flotation in *B. rotundiformis* (Table 1, 2).

Only desiccation or aeration caused appearance of floating eggs in both species (Fig. 1). Table 3 presents data on the appearance of floating resting eggs of *B. rotundiformis.* The proportion of floating eggs among eggs which had been desiccated in deionized water was more than 90%, while the proportion of floating eggs among those eggs which had been desiccated in seawater was only 2−4%. The reason for this seemed to be that eggs which had been dried in seawater were surrounded by salt crystals after desiccation and this decreased the likelihood of contact with air. Resting eggs of *B. plicatilis* showed a similar trend for all stages from formation to post-maturation. Aeration of the culture medium cause 10% floating eggs.

Ito[1] reported that there was no difference in hatching rate between floating eggs and the ordinary "sinking" eggs. My results suggested that the hatching rate was lower in floating eggs, over a range of incubation temperatures and salinities

Table 1. Effect of dissolved oxygen (%) on the flotation and hatching of *B. rotundiformis* resting eggs.

DO at the beginning	DO at the end	Percent flotation	Percent hatching
10.5	5.1	0	0
27.7	4.1	0	46.1
96.2	66.7	0	70.6

Table 2. Effect of ammonium sulfate concentration on the flotation and hatching of *B. rotundiformis* resting eggs.

Ammonium sulfate (ppm)	Percent flotation	Percent hatching
0	0	79.5
1	0	74.0
10	0	0
100	0	0
1,000	0	0

Table 3. Effect of contact with air on the appearance of floating resting eggs in *B. rotundiformis.*

Treatment	Temperature (℃)	Photoperiod	Percent floating eggs (ratio)
Desiccation	25	24L:0D	88.0 (73/83)
in deionized	25	0L:24D	95.1 (173/182)
water	5	24L:0D	95.4 (104/109)
	5	0L:24D	98.7 (157/159)
Desiccation	25	24L:0D	2.0 (2/100)
in sea water*	5	24L:0D	4.0 (4/100)
Aeration	25	24L:0D	14.2 (209/1470)
	25	0L:24D	1.4 (110/7825)
	10	24L:0D	11.4 (450/3950)
	10	0L:24D	11.8 (158/1337)

* 28.9 ppt salinity

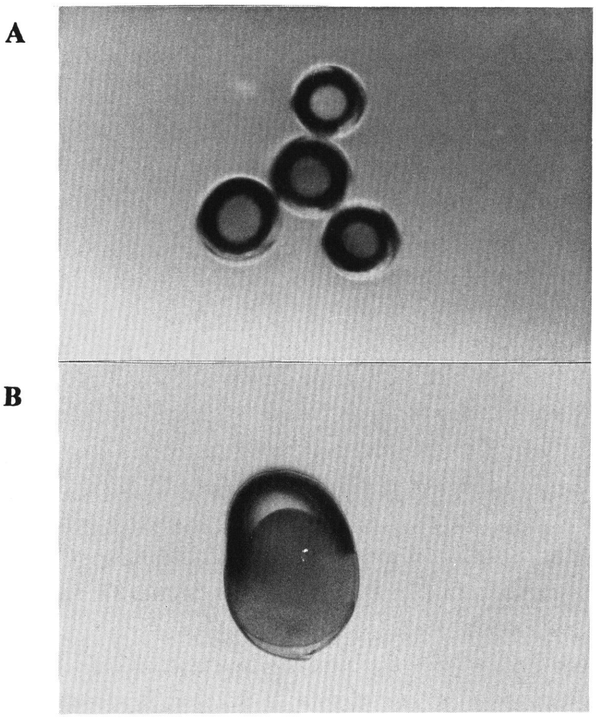

Fig. 1. Floating resting eggs appeared after the contact with air. A— Top view from the water surface. B—Lateral view.

(Fig. 2). Since the eggs had already been exposed to light, it was probably the effect of desiccation on the resting eggs during their development which had reduced their hatchability.

In my experiments, floating eggs appeared only when the eggs made contact with air. It is still possible, however, that the appearance of floating eggs is actually caused by factors that were not examined in this study. There is some published information concerning the hatching of resting eggs in *B. plicatilis* and *B. rotundiformis*[2, 15–18]. This suggests that the resting eggs of both species require light for hatching. However, most resting eggs in their natural environment are distributed on the sediments where they probably receive little or no irradiation. Both *B. plicatlis* and *B. rotundiformis* naturally occur in an eutrophic estuary whose bottom environment tend to be reductional due to the low oxygen concentration, ecially in the summer when the water is warmer. Thus, the floating of resting eggs could be an adaptation to enable these eggs to avoid an environment which inhibits hatching.

Very recently, however, Hagiwara *et al.*[19] reported that the photo-chemical reaction, rather than the direct irradia-

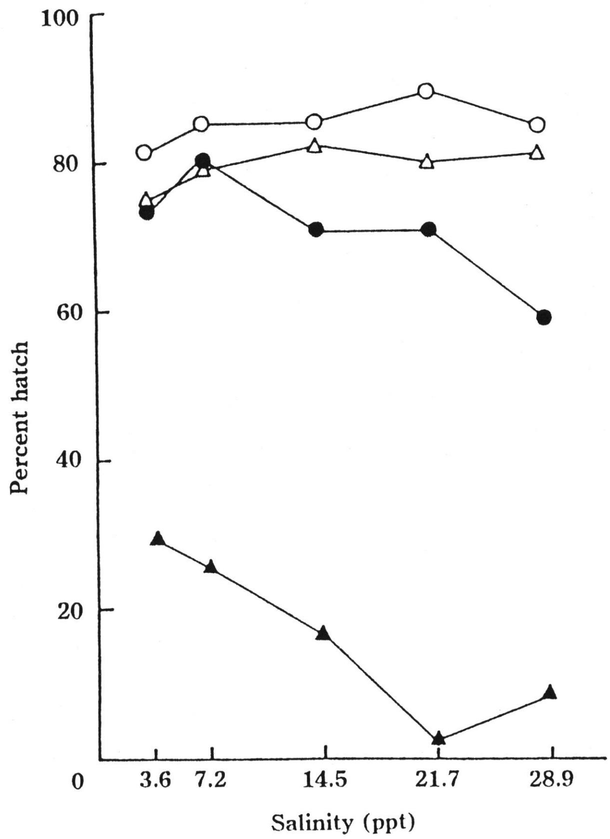

Fig. 2. Percent hatch of floating and demersal resting eggs under the effect of temperature and salinity.
○— demersal egg (25℃), ●— floating egg (25℃), △—demersal egg (20℃), ▲—floating egg (20℃)

esption may have a significant role on resting egg hatching in nature. They observed the highest hatching of resting eggs at 350−400nm wavelength, whose high energy irradiation poduces active oxygen in the water environment through photolysis on organic matter[20]. Hagiwara *et al.*[19] also showed that the additions of hydrogen peroxide, prostaglandins, and seawater (stored in the light) caused resting eggs to hatch even when incubated in the dark. Prostaglandins are produced through oxidation from n–3 and n–6 unsaturated fatty acids that are contained in resting eggs [21]. The photolysis at the sea surface produces hydroxyl radical as well as its reactive daughter products that may persist long to reach the shallow sea bottom[20]. From these, Hagiwara *et al.*[19] assumed that the UV photolysis could be a cause to produce prostaglandins in resting egg embryo, followed by the induction of resting egg hatching. Ito[2] reports that the eel ponds that he examined dry out during the winter. He also dried the sediments he collected before isolating the resting eggs. So, the floating eggs he observed probably resulted from exposure to air. In mass cultures at aquaculture centers, rotifer resting eggs have a greater chance of being exposed to the air because these cultures are often aerated to increase their oxygen supply. In contrast, there have been no reports of floating eggs from researchers who have observed rotifers at an individual level in laboratory cultures. This seems to support the hypothesis that floating eggs are the product of exposure to the air.

Floating eggs observed in this study were probably produced by the replacement of fluid in the extra–embryonic space with air from the surrounding environment. The surface tension would then cause the formation of an air bubble.

From their electron microscope observations, Wurdak *et al.*[22] revealed that outer shell of the resting egg of *B. calyciflorus* is porous. This was also found to be the case in *B. plicatilis* and *B. rotundiformis*[23]. Little is known about the transport of dissolved substances across the egg membrane, but it seems likely that research on this topic could provide information which would improve our understanding of the factors which control dormancy in resting eggs. By using information obtained in this research, resting eggs in the sediments could be isolated easily by desiccation. This would enable large quantities of resting eggs to be obtained from the natural environment[15] or from artificial ponds[24] for use as seeds for starting rotifer mass cultures.

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References

1) A. Hagiwara: Practical use of rotifer cysts. *The Israel. J. Aquac. Bamidgeh,* **46,** 13−21. (1994)

2) T. Ito: Studies on the "mizukawari" in eel–culture ponds. XI The hatching activity of dormant eggs of *Brachionus plicatilis* O. F. Müller. *Rep. Fac. Fish. Pref. Univ. Mie,* **3,** 178−192. (1958) (In Japanese with English abstract)

3) M. Furusawa: Biological features on neonates of *Brachionus plicatilis* hatched from resting eggs. *Annual report for 1977 fiscal year,* Ishikawa Pref. Fish. Res. Inst. and Ishikawa Pref. Sea Farming Center, 91−97. (1979) (In Japanese)

4) Y. Fu, K. Hirayama, and Y. Natsukari: Morphological differences between two types of the rotifer *Brachionus plicatilis* O. F. Müller. *J. Exp. Mar. Biol. Ecol.* **151,** 29 −41. (1991)

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

6) Y. Fu, A. Hagiwara, and K. Hirayama: Cross–mating among S and L type *Brachionus plicatilis* strains. Nippon Suisan Gakkaishi **59,** 2009−2016. (1993)

7) F. M. I. Rumengan, H. Kayano, and K. Hirayama: Karyotypes of S and L type rotifers *Brachionus plicatilis* O. F. Müller. *J. Exp. Mar. Biol. Ecol.,* **154,** 171−176. (1991).

8) F. M. I. Rumengan, and K. Hirayama: Growth responses of genetically distinct S and L type rotifer (*Brachionus plicatilis)* strains to different temperatures. "The Second Asian Fisheries Forum", Asian Fisheries Society, Tokyo, Japan (R. Hirano and I. Hanyu, ed.) pp. 37−40. (1990)

9) A. Hagiwara, and K. Hirayama: Preservation of rotifers and its application in the finfish hatchery. *TML Conference Proceeding* **3,** 61−71. (1993).

10) A. Gomez, and M. Serra: Behavioral reproductive isolation among sympatric strains of *Brachionus plicatilis* Müller 1786: Insights in to the status of this taxonomic species. *Hydrobiol.,* **313/314,** 114−119. (1995).

11) R. Rico–Martinez, and T. W. Snell: Mating behavior and mate recognition pheromone blocking of male receptors in *Brachionus plicatilis* Müller (Rotifera). *Hydrobiol.,* **313/314,** 105−110. (1995).

12) H. Segers: Nomenclatural consequences of some recent studies on *Brachionus plicatilis* (Rotifera, Brachionidae). *Hydrobiol.,* **313/314,** 121−127. (1995).

13) M. Okauchi, and K. Fukusho: Different modes in car–

rying resting eggs between two wild types of the rotifer *Brachionus plicatilis* from Matsusaka. *Bull. Japan. Soc. Sci. Fish.* **51,** 1907. (1985)

14) M. J. Carmona, A. Gomez, and M. Serra: Mictic patterns of *Brachionus plicatilis* Müller in small ponds. *Hydrobiol.,* **313/314,** 365－371. (1995).

15) A. Hagiwara, A. Hino, and R. Hirano: Combined effects of environmental conditions on the hatching of fertilized eggs of the rotifer *Brachinus plicatilis* collected from an outdoor pond. *Nippon Suisan Gakkaishi,* **51,** 755－758. (1985).

16) E. Lubzens, R. Fishler, and V. Berdugo-White: Induction of sexual reproduction and resting egg production in *Brachionus plicatilis* reared in sea water. *Hydrobiol.,* **73,** 55－58. (1980).

17) G. Minkoff, E. Lubzens, and D. Kahan: Environmental factors affecting hatching of rotifer (*Brachionus plicatilis*) resting eggs. *Hydrobiol.,* **104,** 61－69. (1983).

18) A. Hagiwara, and A. Hino: Effect of incubation and preservation on resting egg hatching and mixis in the derived clones of the rotifer *Brachionus plicatilis. Hydrobiol.,* **186/187,** 415－421. (1989).

19) A. Hagiwara, N. Hoshi, F. Kawahara, K. Tominaga, and K. Hirayama: Resting eggs of the marine rotifer *Brachionus plicatilis* Müller: development, and effect of irradiation on hatching. *Hydrobiol.,* **313/314,** 223－229. (1995).

20) K. Mopper, and X. Zhou: Hydroxyl radical photoproduction in the sea and its potential impact on marine processes. *Science,* **250,** 661－664. (1990).

21) A. Hagiwara, K. Hamada, A. Nishi, K. Imaizumi, and K. Hirayama: Dietary value of neonates from rotifer *Brachionus plicatilis* resting eggs for red sea bream larvae. *Nippon Suisan Gakkaishi,* **59,** 99－104. (1993).

22) E. S. Wurdak, J. J. Gilbert, and R. Jales: Fine structure of resting eggs of the rotifers *Brachionus calyciflorus* and *Asplanchna sieboldi. Trans. Amer. Micros. Soc.* **97,** 49－72. (1978).

23) N. Munuswamy, A. Hagiwara, G. Murugan, K. Hirayama, and H. J. Dumont: Structural differences between the resting eggs of *Brachionus plicatilis* and *Brachionus rotundiformis* (Rotifera, Brachionidae): an electron microscopic study. *Hydrobiol.* (in press).

24) A. Hagiwara, K. Hamada, A. Nishi, K. Imaizumi, and K. Hirayama: Mass production of rotifer *Brachionus plicatilis* resting eggs in $50\,m^3$ tanks. *Nippon Suisan Gakkaishi,* **59,** 93－98. (1993).

海産ツボワムシ類の浮遊性休眠卵の出現

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海産ツボワムシの休眠卵はふつう沈降性を示し，天然では汽水域の底泥中に分布するが，孵化直前の休眠卵の中には，卵空室部にガスがたまって浮遊性を示す例が報告されている。休眠卵の孵化には光照射が必要なので，前述の現象を孵化に阻害的な底質環境を回避する手段と捉えることができるかもしれない。その形成機構を2種の海産ツボワムシ（*Brachionus plicatilis, B. rotundiformis*）の休眠卵を材料として調べた。環境因子（水温，塩分，光条件，溶存酸素量，硫化アンモニウム濃度）を様々に調節して，卵の保存とインキュベートを行ったが，浮遊卵の出現は全く観察されなかった。一方，乾燥処理や培養槽の瀑気など，休眠卵と空気の接触の影響をみたところ，卵空室部内にガスが取り込まれ，浮遊卵が高率で出現した。この現象は2種のワムシに共通にみられた。したがって，この現象を，孵化に必要な光照射の機会を増大するための手段として考えることは難しい。過去の浮遊卵の観察例は，休眠卵を分離する際に採取した底泥を乾燥したり，培養槽に通気を施すなどの人工的な操作に基づくものと考える。既往の知見と併せると，底泥に分布する休眠卵は光の直接照射よりも光化学的な作用機構によって孵化する可能性が高い。