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# Usability of selenium fortified *Chlorella* diet for rotifer resting egg production and hatching

Chengyan HAN[1,2,3], Hee-Jin KIM[4], Mingyou LI[1,2,3] and Atsushi HAGIWARA[4,5,*]

**Abstract:** Our previous study elucidated that commercial *Chlorella vulgaris* induces the resting egg production of the euryhaline rotifer *Brachionus plicatilis* sensu stricto caused by selenium (Se) fortification. This study confirmed production efficiency and hatchability of resting eggs with the commercial Se-fortified *C. vulgaris*. The aforementioned parameters were compared to those with *Tetraselmis tetrathele* (control) which is an optimal diet for resting egg production and hatching. The rotifers pre-cultured with *Nannochloropsis oculata*, and the produced resting eggs were employed to test diet effects. The hatching rate of collected resting eggs was estimated and then hatchlings were used for next generation culture. For the resting egg production with two different diets, *T. tetrathele* induced significantly higher productivity in the first generation, while no differences were observed in the second generations between two diets *T. tetrathele* and *C. vulgaris*. The resting eggs with *N. oculata* showed 6.0% of hatching rate, whereas it notably increased with two tested diets. The highest hatching rate was observed with *C. vulgaris* feeding (74.0−81.0%) through the all tested generations. The obtained results exhibit the possibility of *C. vulgaris* feeding for the mass production of rotifer resting eggs.

**Key words:** Rotifera; Selenium; *Chlorella vulgaris*; Resting egg; Hatchability

The rotifer *Brachionus plicatilis* sensu stricto exhibits heterogenic life cycle that includes sexual (mictic) and asexual (amictic) reproduction phases (Hagiwara and Hino 1989). Sexual reproduction of rotifers is regulated by various external factors such as population density (Snell and Boyer 1988; Hino and Hirano 1976), temperature (Hino and Hirano 1985), salinity (Hino and Hirano 1988) and food (Snell et al. 1983; Hotos 2002; Hagiwara et al. 2005). Resting eggs are the final product of sexual reproduction and can be used like *Artemia* cyst (Hagiwara et al. 1997). Among the various external factors, resting egg production and its hatchability strongly depend on maternal diet and feeding history (Snell and Hoff 1985; Hagiwara and Hino 1990). The microalgae, *Tetraselmis tetrathele* is so far considered as an optimal diet for the induction of sexual reproduction (resting egg production) and higher hatching rate of resting eggs, although the nutritional mechanism of its action is unknown. It was also reported that repeated generation (via resting eggs) by feeding *Tetraselmis* gradually enhanced resting egg hatchability (from 30.5 to 90% of hatching rate) (Hagiwara and Hino 1989). For the mass production of rotifer resting eggs, however, the cost of commercial

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[1] Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China.
[2] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Shanghai Ocean University, Ministry of Education, Shanghai 201306, China.
[3] National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai 201306, China.
[4] Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan.
[5] Organizaion for Marine Science and Technology, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan.
[*] Corresponding author: Tel/Fax, (+81) 95-819-2830; E-mail, hagiwara@nagasaki-u.ac.jp (A. Hagiwara).

*Tetraselmis* spp. is not proper in terms of economic cost-effectiveness (Brown 2002). Hence, a cheaper substitute is required for profitable resting eggs production.

Initial commercial microalgae *Chlorella* was nutritionally deficient for rotifer growth, although, now it can yield equivalent rotifer production to *Nannochloropsis oculata*, due to possibility of nutrient fortification using various elements such as vitamin $B_{12}$ (Hirayama et al. 1989) and selenium (Se) (Kim et al. 2014). The commercial *C. vulgaris* is now recognized as one of the best diet for rotifer population growth, and aquaculture facilities are generally using commercial *C. vulgaris* for intensive mass culture of rotifers (Hagiwara et al. 2001). Previously, we observed that *Chlorella* fortified with Se enhanced not only asexual (population growth) but also sexual reproduction of rotifers; induced the highest mixis induction, fertilization, and resting egg production compared to non Se-fortified *Chlorella* and *N. oculata* (Kim et al. 2014), while the hatchability of the resting eggs produced with the commercial *Chlorella* fortified with Se was not reported. This study evaluated the efficiency of the commercial *Chlorella* diet for the mass production of resting eggs by comparing production and hatching rate of resting eggs with *T. tetrathele* which is known as the optimal diet for the rotifer sexual reproduction.

## Materials and Methods

This study employed L-type rotifer, *B. plicatilis* s. s. (Japanese NH1L strain). Rotifer culture medium (at 17 ppt) was prepared by dilution of natural seawater with Milli-Q water (Millipore 0.22 mm) followed by GF/C filtration and autoclaving (121℃, 15 min). Two rotifer diets *N. oculata* and *T. tetrathele* were independently cultured using the modified Erd-Schreiber medium (Hagiwara et al. 1994) at 22℃, and 22 ppt under continuous illumination with gentle aeration. For the *Chlorella* diet, live liquid paste of commercial Super Fresh Chlorella-V12 (*C. vulgaris*; Se-fortified at lower than 3.3 μg Se/g by Chlorella Industrial Company Ltd. in Japan) was supplied to rotifers.

One generation is designated as a cycle of resting egg formation, diapauses and hatching followed by Hagiwara and Hino (1989). The rotifers were pre-cultured with 30 amictic female rotifers, which were randomly selected from laboratory stock cultures. The rotifers were cultured in 30 m*l* of sterilized medium (17 ppt of diluted natural seawater), and fed daily with *N. oculata* ($7.0 \times 10^6$ cells/m*l*) at 25℃ under complete darkness for 14 days in triplicate. Resting eggs were harvested on the last day of culture and kept at 25℃ under total darkness for two weeks. To calculate hatching rate of produced resting eggs, 20 resting eggs were transferred into a well of 6-well microplate containing 5 m*l* of 17 ppt seawater. Five replicates were incubated at 25℃ and 24-h illumination (at 2,000 lx) during four days. The number of hatchlings was daily counted and pipetted out from each well. At the end of incubation, the hatching rate (%) was calculated using the cumulative number of hatchlings.

For the first generation, 30 neonates were randomly selected from the resting eggs formed by the pre-generation. The neonates were inoculated into 30 m*l* of *T. tetrathele* (at $2.5 \times 10^5$ cells/m*l*) or commercial *Chlorella* (at $2.5 \times 10^6$ cells/m*l*) suspension. These cultures were maintained at 25℃ under complete darkness for 14 days in triplicate. During the culture period, the numbers of four-type females: females without eggs (?F), female producing amictic females (FF), male producing mictic females (MF), and resting egg producing mictic females (RF) were recorded daily. Based on the obtained data, the following three reproductive parameters were estimated.

Population growth rate ($r$): $\ln (N_t / N_0) / t$

Mixis (%): $[(MF+RF) / (FF+MF+RF)] \times 100$

Fertilization (%): $[RF / (MF+RF)] \times 100$,

where $t$ is culture days, and $N_0$ and $N_t$ are the number of all the types of female rotifers on Days 0 and $t$, respectively. Resting eggs were harvested on the last day of culture and the total number of produced resting eggs was

**Table 1.** Effects of different diets on the sexual and asexual reproductions of 1st and 2nd generations of the rotifer *Brachionus plicatilis* s. s. in 30 m*l* cultures

| Generation | Diet | Population growth rate (*r*) | Mixis (%) | Fertilization (%) | Resting eggs/m*l* |
|---|---|---|---|---|---|
| 1st | *T. tetrathele* | 0.30 ± 0.01[a] | 4.1 ± 0.1[a] | 56.9 ± 1.8[a] | 243.3 ± 62.7[a] |
| | *C. vulgaris* | 0.31 ± 0.01[a] | 3.8 ± 0.4[a] | 51.7 ± 3.3[a] | 54.3 ± 14.0[b] |
| 2nd | *T. tetrathele* | 0.26 ± 0.01[b] | 1.4 ± 1.2[b] | 4.4 ± 7.7[b] | 25.7 ± 7.5[b] |
| | *C. vulgaris* | 0.28 ± 0.02[b] | 0.6 ± 0.6[b] | 3.6 ± 3.9[b] | 30.0 ± 7.0[b] |

Data are means ± standard deviation of triplicates. The alphabets denote significant differences in each column through generations (a > b, Tukey HSD test, $p < 0.05$, $n = 3$).

determined. After harvesting, the eggs were kept at 25℃ for two weeks under total darkness (Hagiwara and Hino 1989), and then their hatching rate was estimated with the same methods as the pre-culture. The aforementioned methods for first generation are applied for the second generation.

Reproductive parameters and hatching rates of resting egg produced were statically analyzed with two-way ANOVA followed by Tukey HSD test. The statistical analysis was conducted with R version 3.1.3 (R Development Core Team, 2015).

## Results

The population growth rate (*r*) showed no significant differences between two diets, *T. tetrathele* and *C. vulgaris* in each generation (Table 1). The sexual reproduction parameters, mixis and fertilization also showed no significant differences related to diet species in each generations (Table 1). These reproductive parameters had significant differences between two tested generations (Two-way ANOVA, $p < 0.01$ for population growth rate, $p < 0.0001$ for mixis and fertilization). The resting egg production of first generation showed differences related to the diet species and significantly higher production was observed with *T. tetrathele* (243.3 ± 62.7, Tukey HSD test, $p < 0.05$, $n = 3$). However, no significant differences were observed between *T. tetrathele* and *C. vulgaris* in the second generation (Table 1).

The hatching rate of resting eggs formed by the pre-cultured rotifers fed on *N. oculata* was 6.0% (Fig. 1). Through the hatchling cultures with the two different diets, the produced resting eggs exhibited the higher hatching rate with *C. vulgaris* diet within two generations (Tukey HSD test, $p < 0.05$, $n = 5$). The hatching rates for *C. vulgaris* and *T. tetrathele* were 81.0 ± 6.5% and 64.0 ± 11.0% in the first generation, and 74.0 ± 14.9% and 65.0 ± 11.0% in the second generation, respectively.

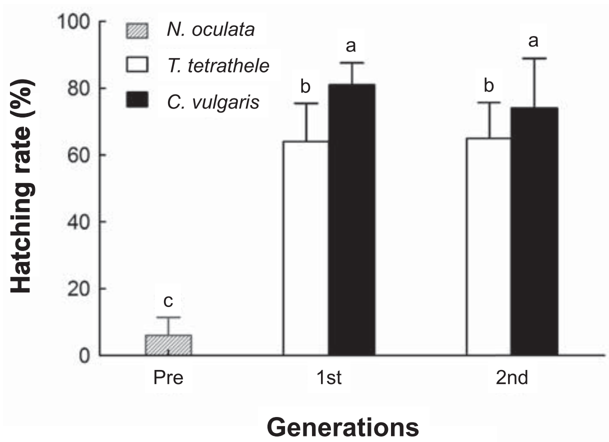


**Fig. 1.** Hatching rates (%) of resting eggs produced by three different diets: *Nannochloropsis oculata*, *Tetraselmis tetrathele*, commercial Se-fortified *Chlorella vulgaris* through three generations (pre, 1st and 2nd) of *Brachionus plicatilis* s. s. Each column and error bar indicates mean and standard deviation of five replicates. The alphabets denote significant differences among the three tested diets (a > b > c, Tukey HSD test, $p < 0.05$, $n = 5$).

## Discussion

The condensed live paste of freshwater *Chlorella* has been used as a commercial diet for the rotifer *Brachionus* spp. since 1982 (Maruyama et al. 1997). Generally, *Chlorophytes* (*Dunaliella* spp. and *Chlorella* spp.) have low nutritional value and are not suitable as a single species diet for rotifers (Hirayama and Funamoto 1983). To supply the nutritional deficiencies, the commercial *Chlorella* diet has been enriched with essential nutrients i.e., vitamins and essential fatty acids making it suitable for the intensive mass culture of rotifers as well as for feeding larval stage of fish (Maruyama and Hirayama 1993; Hayashi et al. 2001). Moreover, commercial *Chlorella* is cheaper than other algal diets; 15,000 Japanese Yen (US$ 140–150) for 18 *l* of volume with 20 billion cells/m*l* of density (Hagiwara et al. 2001). Currently, the commercial *Chlorella* is further fortified with selenium (Se) and significantly improves population growth as well as sexual reproduction of the rotifer *B. plicatilis* s. s. compared to those fed with non Se-fortified *Chlorella* and *N. oculata* (Kim et al. 2014). In this study, the employed rotifers showed the same level of sexual and asexual reproductive parameters between two tested diets *C. vulgaris* and *T. tetrathele* within a generation, while the higher resting egg production was shown with *T. tetrathele* in the first generation (Table 1). The employed *Chlorella* is a freshwater species and difficult to maintain their condition in saline water, and thus aeration is needed to maintain culture condition of euryhaline rotifers (Kim et al. 2014). For the commercial *Chlorella*, the employed rotifers were cultured without aeration, and it may cause of low resting egg production. In the observation between first and second generations, the reproductive parameters showed significant differences. The sexual reproduction of rotifers is a clone specific characteristics. The clone individuals derived from resting eggs which are produced by a single fertilized female showed mixis rate ranging 0–12% (Hagiwara and Hino 1989; Lubzens 1989). Moreover, large variations of mixis and fertilization were observed among trials because of the characteristics of stem females which is hatchlings from resting eggs (Hagiwara et al. 1997; Hagiwara and Lee 1991; Hagiwara et al. 1993a). The stem females are vulnerable to unfavorable conditions and the obtained characters can be inherited by their offspring through the maternal cytoplasm (Hagiwara et al. 2005). Therefore, these features should affect the variation of reproductive parameters between two generations.

The final products of sexual reproduction, resting eggs are one of preservation method of rotifers and can be used as seeds of mass cultures for the following season (Hagiwara and Lee 1991; Hagiwara et al. 1993a; Hagiwara et al. 1993b). In addition, resting eggs can be hatched and used to feed fish larvae directly similar to *Artemia* cyst (Hagiwara et al. 1997). Thus, mass production of qualitative resting eggs with *Chlorella* diet has synergetic economic effects on aquaculture facilities in terms of labor reduction. The hatching rate of resting eggs is used as a criterion to evaluate the quality of produced eggs. The diapause period determines the hatching ability of resting eggs (Hagiwara and Hino 1989), and it depends on water temperature; the optimal period is extended with temperature decreases in *B. plicatilis* s. s. Therefore, this study optimized diapause period (for simultaneous hatching) at 25℃ for 15 days to calculate the exact hatchability of the resting eggs produced during the limited period of observation. After the diapause phase, the diet effects on resting egg hatchability were observed through two serial generations. Although the obtained results showed increases of hatching rates of resting eggs with both diets, *T. tetrathele* and the commercial *Chlorella* compared to *N. oculata* which is diet for pre-generation, the higher hatching rate was observed with the commercial *Chlorella* through the generations observed (Fig. 1). The commercial *Chlorella* is fortified with Se by supplying sodium selenite ($Na_2SeO_3$) to culture medium. It was figured out that the fortified Se induced higher population growth and resting egg production by comparing to those with non-fortified *Chlorella* in the rotifer *B. plicatilis* s. s. (Kim et al. 2014). The fortified Se also has a significant function in an oxidative metabolism (Ursini et al. 1997). The dissolved oxygen level can influence the hatchability of resting eggs (Hagiwara et al. 1985). The oxidation of fatty acid in resting eggs is an expected hatching mechanism related to peroxide production by light irradiation (Hagiwara et al. 1995). Therefore, the higher hatchability of resting eggs with the commercial *Chlorella* might be induced by the function of Se. The obtained results support that the efficiency of the commercial *Chlorella* fortified with Se for the mass production of qualitative resting eggs in the rotifer *B. plicatilis* s. s.

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## References

Brown, M. R. (2002) Nutritional value and use of microalgae in aquaculture. Avances en Nutrición Acuícola VI. Memorias del VI Simposium Internacional de Nutrición Acuícola 3: pp. 281–292.

Hagiwara, A., M. D. Balompapueng, N. Munuswamy and K. Hirayama (1997) Mass production and preservation of the resting eggs of the euryhaline rotifer *Brachionus plicatilis* and *B. rotundiformis*. *Aquaculture*, **155**, 223–230.

Hagiwara, A., W. G. Gallardo, M. Assavaaree, T. Korani and A. B. de Aranjo (2001) Live food production in Japan; recent progress and future aspects. *Aquaculture*, **200**, 111–127.

Hagiwara, A., K. Hamada, S. Hori and K. Hirayama (1994) Increased sexual reproduction in *Brachionus plicatilis* (rotifera) with the addition of bacteria and rotifer extracts. *J. Exp. Mar. Biol. Ecol.*, **181**, 1-8.

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

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

Hagiwara, A., A. Hino and R. Hirano (1985) Studies on the appearance of floating fertilized eggs in the rotifer *Brachionus plicatilis*. *Aquaculture Science*, **32**, 207–212.

Hagiwara, A. and A. Hino (1989) Effect of incubation and preservation on resting egg hatching and mixis in the derived clones of the rotifer *Brachionus plicatilis*. *Hydrobiologia*, **186**, 415–421.

Hagiwara, A. and A. Hino (1990) Feeding history and hatching of resting eggs in the marine rotifer *Brachionus plicatilis*. *Nippon Suisan Gakkaishi*, **56**, 1965–1971.

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

Hagiwara, A., Y. Kadota and A. Hino (2005) Material effect by stem females in *Brachionus plicatilis*: effect of starvation on mixis induction in offspring. *Hydrobiologia*, **546**, 275–279.

Hagiwara, A. and C-S Lee (1991) Resting egg formation of the L- and S-type rotifer *Brachionus plicatilis* under different water temperature. *Nippon Suisan Gakkaishi*, **75**, 1645–1650.

Hayashi, M., T. Yukino, I. Maruyama, S. Kido and S. Kitaoka (2001) Uptake and accumulation of exogenous docosahexaenoic acid by *Chlorella*. *Biosci. Biotechnol. Bichem.*, **65**, 202–204.

Hino, A. and R. Hirano (1976) Ecological studies on the mechanism of bisexual reproduction in the rotifer *Brachionus plicatilis*–I. General aspects of bisexual reproduction. *Nippon Suisan Gakkaishi*, **43**, 1147–1155.

Hino, A. and R. Hirano (1985) Relationship between the temperature given at the time of fertilized egg formation and bisexual reproduction pattern in the deriving strain of the rotifer *Brachionus plicatilis*. *Nippon Suisan Gakkaishi*, **51**, 511–514.

Hino, A. and R. Hirano (1988) Relationship between water chlorinity and bisexual reproduction rate in the rotifer *Brachionus plicatilis*. *Nippon Suisan Gakkaishi*, **54**, 1329–1332.

Hirayama, K. and H. Funamoto (1983) Supplementary effect of several nutrients on nutritive deficiency of baker's yeast for population growth of the rotifer *Brachionus plicatilis*. *Nippon Suisan Gakkaishi*, **49**, 505–510.

Hirayama, K., I. Maruyama and T. Maeda (1989) Nutritional effect of freshwater *Chlorella* on growth of the rotifer *Brachionus plicatilis*. *Hydrobiologia*, **186/187**, 39–42.

Hotos, G. N. (2002) Selectivity of the rotifer *Brachionus plicatilis* fed mixtures of algal species with various cell volumes and cell densities. *Aquac. Res.*, **33**, 949–957.

Kim, H.-J., K. Nakamura and A. Hagiwara (2014) Dietary effect of selenium-fortified *Chlorella vulgaris* on reproduction of *Brachionus plicatilis* species complex (Rotifera: Monogononta). *Int. Rev. Hydrobiol.*, **99**, 161–165.

Lubzens, E. (1989) Possible use of rotifers resting eggs and preserved live rotifers (*Brachionus plicatilis*) in aquaculture. In: De Pauw, N. et al. (Ed.) Aquaculture: a biotechnology in progress, **1**, pp. 741–750.

Maruyama, I., T. Nakao, I., Shigeno, Y. Ando and K. Hirayama (1997) Application of unicellular algae *Chlorella vulgaris* for the mass-culture of marine rotifer *Brachionus*. *Hydrobiologia*, **358**, 133–138.

Maruyama, I. and K. Hirayama (1993) The culture of the rotifer *Brachionus plicatilis* with *Chlorella vulgaris* containing Vitamin $B_{12}$ in its cells. *J. World Aquacult. Soc.*, **24**, 194–198.

R Development Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Snell, T. W., C. J. Bieberich and R. Fuerst (1983) The effects of green and blue-green diets on the reproductive rate of the rotifer *Brachionus plicatilis*. *Aquaculture*, **31**, 21-30.

Snell, T. W. and E. M. Boyer (1988) Thresholds for mictic female production in the rotifer *Brachionus plicatilis* (Muller). *J. Exp. Mar. Biol.*, **124**, 157-162.

Snell, T. W. and F. H. Hoff (1985) The effect of environmental factors on resting egg production in the rotifer *Brachionus plicatilis. J. World Aquac. Soc.*, **16**, 484-497.

Ursini, F., M. Maiorino and A. Roveri (1997) Phospholipid hydroperoxide glutathione eroxidase (PHGPx): More than an antioxidant enzyme. *Biomed. Environ. Sci.*, **10**, 327-332.

# シオミズツボワムシの耐久卵形成に対する セレン強化クロレラの有効性

韓程燕・金禧珍・李名友・萩原篤志

シオミズツボワムシ *Brachionus plicatilis* sensu stricto（L 型，NH1L 株）の耐久卵量産のため，セレン強化クロレラ（*Chlorella vulgaris*，スーパー生クロレラ，クロレラ工業）の有効性が確認されているが，形成された耐久卵の孵化については不明のままである。本研究ではセレン強化クロレラの耐久卵孵化に対する有効性を確認するため，親世代（*Nannochloropsis oculata* 給餌）から次の 2 世代（クロレラ又は *Tetraselmis tetrathele* 給餌）を通じ，耐久卵形成率と孵化率を比較した。クロレラを給餌したワムシは，耐久卵を経由した世代において，従来最も良いと報告された *T. tetrathele* を給餌したワムシより，安定的に孵化率が高い耐久卵を形成することが確かめられた。