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Dietary effect of selenium-fortified *Chlorella vulgaris* on reproduction of *Brachionus plicatilis* species complex (Rotifera: Monogononta)

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We investigated the effects of fortifying a diet of *Chlorella vulgaris* with Selenium (Se) on sexual and asexual reproduction of rotifers in the *Brachionus plicatilis* species complex: i.e., two strains of *B. plicatilis* sensu stricto and one of *B. rotundiformis*. These rotifers were cultured for 8-10 days on one of three different diets that were adjusted to provide the same dry weight of food: non-fortified *Chlorella*, Se-fortified *Chlorella*, and *Nannochloropsis oculata*. *B. plicatilits* (Makishima strain), which is obligatorily asexual, showed no difference in population growth rate among the three different diets (r = 0.55-0.61). On the other hand, *B. plicatilis* (NH17L strain), which reproduces by cyclical parthenogenesis, showed higher population growth (r = 0.25) and also higher rates of fertilization (35.9%) and absolute resting egg production (2803.9 eggs/g food) with the Se-fortified *Chlorella* diet than with other foods. Although *B. rotundiformis* (Kochi strain), which also exhibits cyclical parthenogenesis, showed no differences in population growth among the three different diets (r = 0.42-0.48), sexual reproduction parameters were different depending on the feeding regime. The highest mixis (26.2%), fertilization (72.6%), and resting egg production (3489.9 eggs/g food) were observed with the Se-fortified *Chlorella* diet. We posit that the effect of Se-fortified diet was greater on the resting egg production by enhancing male fertility than on population growth.

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1 Introduction

Selenium (Se) is an unusual, essential trace element in aquatic environments. Too little leads to dietary deficiency, which can limit growth, but in high amounts it has toxic effects (Watanabe et al. 1997; Baines et al. 2002; Hamilton 2004). For example, we know that Se-deficient females are infertile and their offspring suffer from various symptoms including muscular weakness (Bedwal & Bahuguna 1994). Se deficiency also causes serious nutritional and developmental problems in mammals (e.g. Oldfield et al. 1960). On the other hand, organisms fed Se-fortified foods show enhanced growth and development (Poston et al. 1976; Gatlin & Wilson 1986; Price et al. 1987). In the case of fish, 0.3-0.7 mg of Se per kg of dry food enhanced growth of juvenile rainbow trout (*Oncorhynchus mykiss*) (Hilton et al. 1980) and grouper (*Epinephelus malabaricus*) (Lin & Shiau 2007). Thus, in aquaculture providing optimal level of Se in the diet of the live feed organisms is critical to their optimal production.

Rotifers of the genus *Brachionus* are widely used as the first food of marine larvae in aquaculture. However, rotifers are known to possess lower levels of micronutrients than in copepods, which also are an important prey for many marine larvae in the wild (Hamre et al. 2008; Srivastava et al. 2011). Se concentrations in rotifers are in the range of 0.08-0.09 mg/kg DW, a level that is about 30x lower than in copepods (3-5 mg/kg DW; Hamre et al. 2008), and 3 to 8x lower than the Se requirements for juvenile fish (Penglase et al. 2011). As a result, Se enrichment of rotifers is needed for effective larviculture. This study investigated whether a diet of Se fortified *Chlorella* affects reproduction of members of the *Brachionus plicatilis* species complex: i.e., two strains of *B. plicatilis* sensu stricto and one of *Brachionus rotundiformis*. One strain of *B. plicatilis* is obligatorily asexual (Makishima), while the other reproduces by cyclical parthenogenesis (NH17L); *B. rotundiformis* (Kochi strain) also reproduces by cyclical parthenogenesis.

2 Materials and Methods

2.1 General culture protocol and asexual culture of the *B. plicatilis* (Makishima strain)

The original source of the stock culture of *B. plicatilis* (Makishima strain) was collected at Makishima (Japan) and stock cultures have been maintained under laboratory condition for more than 10 years (Fu et al. 1991; Ruttanapornvareesakul et al. 2007). From this stock, several rotifers were transferred to 100 mL of culture medium and were cultured at 25°C under complete darkness (preliminary culture). The culture medium used was prepared by diluting natural seawater with Milli-Q water (Millipore 0.22 μm) to 22 ppt followed by GF/C filtration and sterilization (at 121°C, for 20 minutes). The rotifers were fed daily with *Nannochloropsis oculata* that had been cultured in Erd-Schreider medium under the following conditions: 22°C, 22 ppt, constant illumination, with gentle aeration. To prepare the food, we centrifugation a small amount of the algal culture at 5000 rpm, for 10 min, re-suspended it in culture medium, and fed the rotifers at 7×10^6 cells/ml. From the preliminary culture, 500 female rotifers were randomly selected and were placed into 100 mL of stock medium. We cultured those rotifers with one of three different algae: (1) non-fortified *Chlorella* (Super Fresh Chlorella-V12 containing 0.0 μg Se/g DW offered from Chlorella Industry Company, Fukuoka, Japan), (2) Se-fortified *Chlorella* (that containing 3.3 μg Se/g DW) by adding sodium selenite (Na_2SeO_3) to culture medium, and (3) *N. oculata* cultured in laboratory. We fed the same dry weight of algae to the rotifers every 12 h. Both *Chlorella* feeding regimes were provided to the rotifers at 2.5×10^6 cells/ml, but *N. oculata* was provided at 7×10^6 cells/ml. Triplicate cultures of three different diets were cultured for 8-10 days under complete darkness with aeration at 10 mL/min. We monitored the rotifer cultures daily and determined the number of animals per 1 mL. The mean of triplicate counts was used to calculate population growth rate (r) by the following equation.

$$r = \ln(N_t/N_0)/t,$$

where t is the culture days, and N_0 and N_t are the number of all female rotifers on days 0 and t, respectively.

2.2 Culture protocols for *B. plicatilis* (NH17L strain) and *B. rotundiformis* (Kochi

strain)

NH17L originated from Tokyo University and the Kochi strain was collected from Kochi (Japan). Both have been cultured in our laboratory for more than 15 years. These strains were cultured under the same conditions as described above for either 8 days (Kochi) or 10 days (NH17L). The population levels of females was recorded daily according to their reproductive status: i.e., females without eggs (?F), female-producing amictic females (FF), male-producing mictic females (MF), and resting egg producing mictic females (RF). Females without eggs (?F) included immature females, post-reproductive females, and non-spawning adult females (Sudzuki 1964; Hagiwara et al. 1988). The population growth was calculated as noted above, with two additional parameters (Hagiwara et al. 1988).

$$\text{Mixis (\%)} = [(MF + RF) / (FF + MF + RF)] \times 100$$

$$\text{Fertilization (\%)} = [(RF) / (MF + RF)] \times 100$$

Resting eggs were harvested on the last day of culture and the number of resting eggs per gram of dry weight of each food was calculated to evaluate the efficiency of food treatment on their production.

2. 3 Statistical analysis

Effects of Se-fortified *Chlorella* on the sexual and asexual reproduction of rotifer were evaluated by ANOVA followed by Tukey-Kramer *post hoc* test. All of the statistical analysis was carried out using Statview version 5.0 software (SAS Institute, Inc., USA).

3 Results

3.1 Parthenogenetic reproduction

Brachionus plicatilis (Makishima strain) fed on *N. oculata* showed higher population growth on day 3 (117.8 ± 16.2 ind./mL) compared to Se-fortified *C. vulgaris* diet (54.9 ± 14.1 ind./mL, Tukey-Kramer, $p < 0.05$, Fig. 1). Rotifers fed with *N. oculata* and *C. vulgaris* grew until day 5 after which growth ceased. However, the Se-fortified rotifer population grew continuously until day 7, but decreased on the last day of culture. On day 7, the Se-fortified diet led to the highest population density (753.7 ± 218.0 ind./mL) and this value was the highest among those values with three different feeding regimes (Tukey-Kramer, $p < 0.05$, Fig. 1). There were no significant differences in r or in the highest population density during culture among three different feeding regimes, but a tendency of active population growth was observed in the Se-enriched group (Table 1).

3.2 Cyclical parthenogenesis (sexual and asexual reproduction)

The highest density of *B. plicatilis* (NH17L strain) was seen with the Se-fortified *C. vulgaris* on day 9 (161.1 ± 23.4 ind./mL; Fig. 2a). Moreover, r was also higher with Se-fortified diet than other two diets (Tukey-Kramer, $p < 0.05$, Table 2). The sexual reproduction parameters showed different patterns depending on diet. Mixis was higher with both *Chlorella* diets than *Nannochloropsis*, while fertilization rate was highest with Se-fortified *Chlorella* (Tukey-Kramer, $p < 0.05$, Table 2). Number of resting eggs produced per gram of dry food was also the highest with Se-fortified *Chlorella*.

Brachionus rotundiformis (Kochi strain) showed a slightly different pattern from *B. plicatilis* (NH17L). There were no differences in r (0.42-0.48) during culture days (8 d), but the highest density of individuals was observed with Se-fortified *Chlorella* (Fig. 2b). Mixis with Se-fortified *Chlorella* (26.2%) and fertilization rate with Se-fortified *Chlorella* (72.6%) and *Nannochloropsis* (58.5%) were higher than the other diets. Moreover, the highest production of resting eggs per a gram of dry food was also observed with Se-fortified diet (3489.9 eggs/g of food; Tukey-Kramer, $p < 0.05$, Table 3).

4 Discussion

We found that the Se-fortified diet strongly influenced sexual reproduction of both *Brachionus* species thereby enhancing resting egg formation of rotifers (Table 2, 3). According to previous studies, fertilized female rotifers actively produce resting eggs with a diet of *N. oculata* compared to one of *C. vulgaris* (Hamada et al. 1993). Moreover, male fertility has been shown to be limited a diet of *Chlorella* (Snell & Hoff 1987). In our study, the pattern of mixis induction was different in *B. plicatilis* (NH17L strain) and *B. rotundiformis* (Kochi strain), while the same pattern of fertilization was observed among treatments, as the highest activity was with Se fortification.

Selenoprotein, phospholipid hydroperoxide glutathione peroxidase (PHGPx) is the family of glutathione peroxidase (GPx) and an element of cell membrane (Ursini et al. 1997). PHGPx existing in the mitochondria on the midpiece of mature spermatozoa maintains the active state of spermatozoa in humans. Thus, Se deficiency induces mechanical instability of spermatozoa (Ursini et al. 1999). Spermatozoa production in rotifers may be influenced in a similar manner, thus leading to successful fertilization with Se fortification. Due to the fact that males do not feed (King & Miracle 1980), the quality of their sperm depends on the nourishment provided to their mothers. This may account for the observation that male-producing females fed a diet of Se-fortified algae resulted in higher resting egg production (Table 2, 3).

Nannochloropsis oculata also has been shown to be an efficient food for the population growth of *Brachionus* species (Yúfera & Navarro 1995; Viayeh et al. 2010). However, *C. vulgaris* fortified with vitamin B_{12} greatly improved rotifer population growth to the same level as that with *N. oculata* (Hirayama et al. 1989). Commercial *C. vulgaris* used in this study was fortified with vitamin B_{12} and the same level of population growth was observed with both *C. vulgaris* diets (Table 1, 2, 3). Although there were no significant differences in population growth rate, the highest density of rotifers was observed with the Se-fortified diet (Fig. 1, 2a, b). Se is an integral ingredient of GPx that protects cells and membranes from oxidative damage by destroying hydrogen peroxide (Bauersachs et al. 1993; Awad et al. 1994). Therefore, the dietary effect of Se may have been to reduce oxidative stress. In a study of grouper and African catfish (Abdel-Tawwab et al. 2007; Lin & Shiau 2007) showed a positive influence of dietary Se. We propose that Se-enriched rotifers showed improved reproductive abilities based on the antioxidant abilities of GPx (Fig. 1, 2ab). Thus, dietary fortification of rotifers with Se is recommended for high-density mass culture of rotifers.

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Tables

Table 1. Effect of different diets on the population growth (*r*) and the highest density of the rotifer *Brachionus plicatilis* sensu stricto (Makishima strain) during 8 days of batch culture. Value indicates mean ± standard deviation.

Diet	Population growth rate (*r*)	Highest density (ind./mL)
C. vulgaris	0.58±0.06	532.8±213.3
C. vulgaris + Se	0.61±0.01	753.7±218.0
N. oculata	0.55±0.01	415.8±118.8

Table 2. Effect of different diets on the population growth rate (*r*) and sexual reproduction parameters of *Brachionus plicatilis* sensu stricto (NH17L strain) during 10 days of batch culture. Value indicates mean ± standard deviation.

Diet	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Resting eggs /g of feed
C. vulgaris	$0.19±0.02^{b}$	$20.2±2.7^{a}$	$2.6±3.1^{b}$	$79.3±36.8^{b}$
C. vulgaris + Se	$0.25±0.01^{a}$	$12.9±4.7^{a}$	$35.9±11.4^{a}$	$2803.9±748.6^{a}$
N. oculata	$0.20±0.02^{b}$	$3.9±1.0^{b}$	$0±0^{b}$	$108.3±67.2^{b}$

a > b, Tukey-Kramer, $p < 0.05$, n = 3

Table 3. Effect of different diets on the population growth rate (*r*) and sexual reproduction parameters of *Brachionus rotundiformis* (Kochi strain) during 8 days of batch culture. Value indicates mean ± standard deviation.

Diet	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Resting eggs /g of feed
C. vulgaris	0.42±0.03	18.1±3.1^{b}	1.1±1.1^{b}	96.9±59.6^{b}
C. vulgaris + Se	0.48±0.04	26.2±3.4^{a}	72.6±4.8^{a}	3489.9±1421.7^{a}
N. oculata	0.46±0.03	6.2±3.4^{c}	58.5±17.6^{a}	859.0±734.9^{b}

a > b > c, Tukey-Kramer, $p < 0.05$, n = 3

Figures

Figure 1. Population growth of *B. plicatilis* sensu stricto (Makishima strain) cultured with three different diets. Each plot and bar indicates the mean and standard deviation. Letters on the lines represent significant differences (a > b, Tukey-Kramer, $p < 0.05$, n = 3).

Figure 2. Population growth of two different rotifer species (NH17L strain of *B. plicatilis* sensu stricto (a) and Kochi strain of *B. rotundiformis* (b)). Each plot and bar indicates the mean and standard deviation of three replicates. Letters on the lines represent significant differences (a > b, Tukey-Kramer, $p < 0.05$, n = 3).

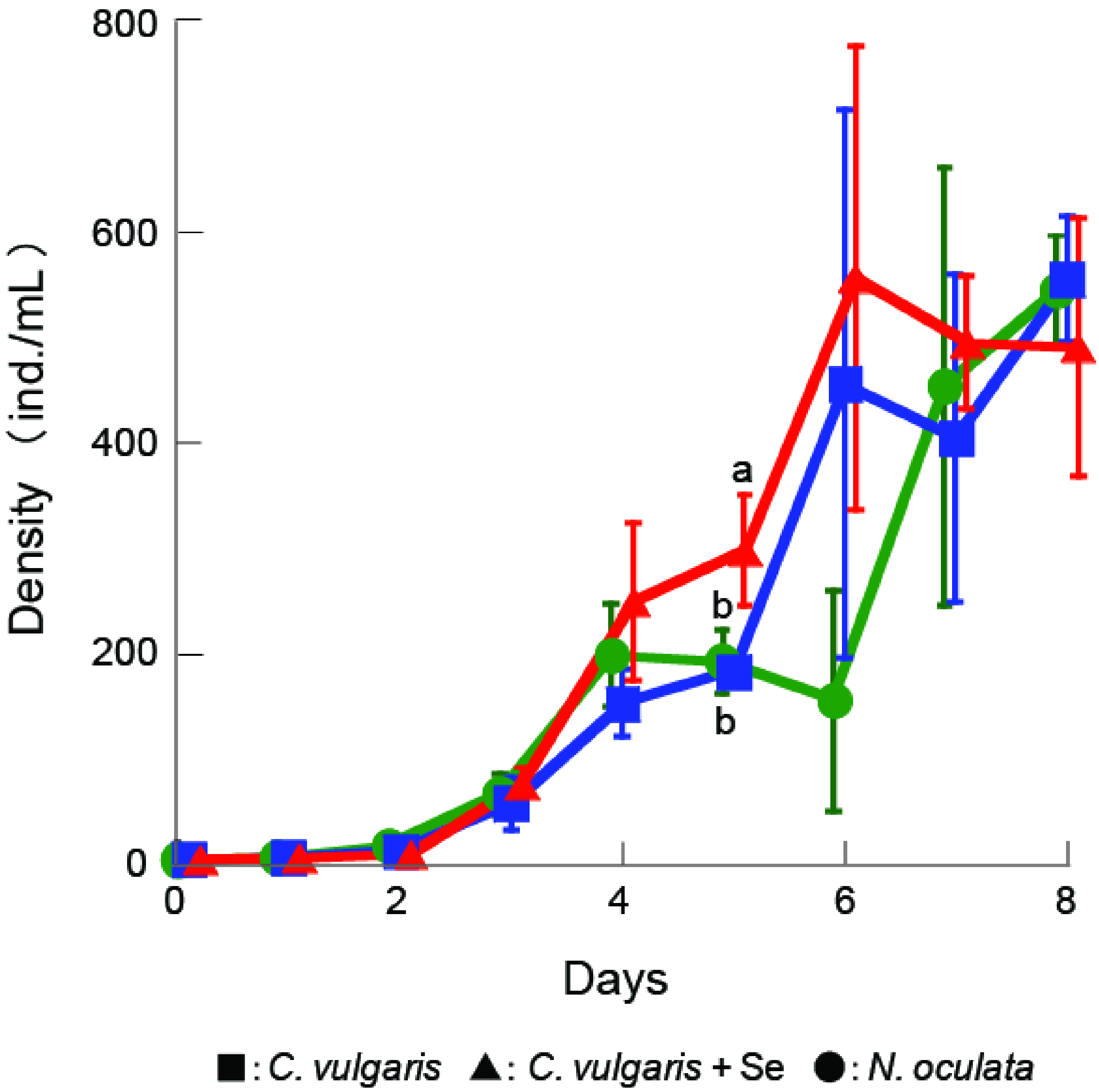
Density （ind./mL）
800
600
400
200
0
0
2
4
6
8
Days
a
b
b
■: C. vulgaris ▲: C. vulgaris + Se ●: N. oculata

Fig. 1

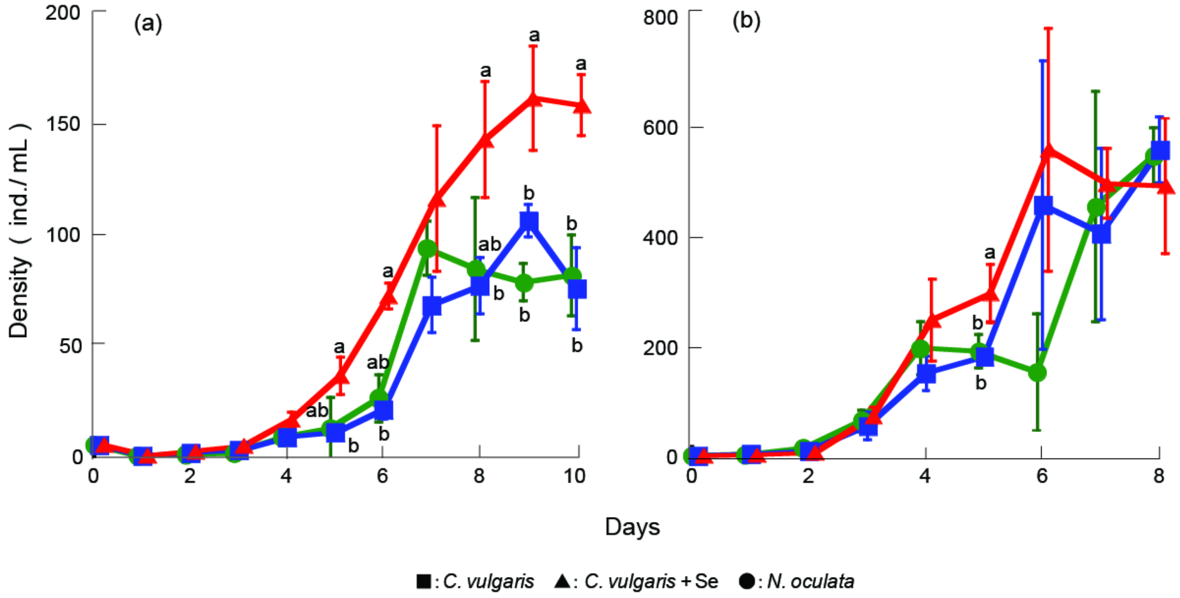
(a)
(b)
Density (ind./mL)
200
150
100
50
0
800
600
400
200
0
2
4
6
8
10
Days
■: C. vulgaris ▲: C. vulgaris + Se ●: N. oculata

Fig. 2