Dietary value

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Dietary Value of Waste-Fed Rotifer *Brachionus rotundiformis* on the Larval Rearing of Japanese Whiting *Sillago japonica*

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Abstract. Live food resources are useful for larval fish rearing. However, production of sufficient live food resources is expensive. This study employed a cost-effective rotifer culture technique using fish waste diet (FWD) and investigate the effect of the FWD-fed rotifer on larval rearing of the Japanese whiting, *Sillago japonica*. Fertilized eggs of *S. japonica* were hatched in polycarbonate tanks containing 100 l of artificial seawater at 10 eggs l^{-1} with 50 ml min^{-1} of aeration at room temperature. Two diets (i.e. FWD-fed rotifers and rotifers fed with super fresh Chlorella-V12 as control) were used with 10 rotifers ml^{-1} for 10 days. Fish were sampled every two days for morphometric and gut content analysis. Fatty acid analysis was done for both rotifers and fish larvae. The fish larvae fed with FWD-rotifers had higher total length than those given control diet. There was no significant difference in survival rate, viability, dry weight, gut content, head length, eye diameter, and body depth between the two diets. The DHA recorded of total lipid for the fish given FWD-rotifer and control fish are3 5.2% and 18.2% respectively. The use of waste-fed rotifers is cost-effective method to enhance the production of larval fish rearing in hatcheries.

1 Introduction

At the close of endogenous feeding phase, most marine larval fishes still have small mouth gap and rudimentary digestive track and therefore, cannot ingest and digest inert feed [1, 2]. At this critical phase, the larval fishes require timely and adequate supply of appropriate live feed that are readily ingested, efficiently digested and with essential nutrition for their healthy growth and development [3]. Depending on the rotifer culture method and condition, essential nutrients may be deficient, sometimes lower than the required threshold for healthy fish development [4]. Therefore, due to the possibility of nutritional deficiencies, rotifers are often provided with supplements to improve their nutritional value for larviculture. The freshly cultured microalgae, sometimes enriched with essential nutrients such as EPA, DHA and vitamin B_{12}, have been commonly preferred as first choice diet for the rotifers [5].

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However, year round cultivation of sufficient and nutritious microalgae is a costly and laborious task for many hatcheries all over the world, thus limits the continuous production of sufficient live foods. Lack of sufficient live food sometimes disrupts the fish seedling production programs in the microalgae-based hatcheries [6]. In our previous studies where the FWD was used to culture different rotifer species, we obtained about 1,200 individuals ml^{-1} of the rotifer, *Brachionus rotundiformis* [7], and about 1,600 individuals ml^{-1} of *Proales similis* [8] within 10 days of culture. In addition, the rotifers had relatively higher EPA and DHA contents than those rotifers cultured using normal (non-enriched) *C. vulgaris* diet. This finding demonstrates a possibility of obtaining nutrient-rich rotifers without necessarily feeding them on enriched microalgae or supplementing them with the expensive commercial emulsions. FWD therefore presents an opportunity to lower production cost in aquaculture hatcheries. However, the suitability of the FWD-fed rotifers for larval fish rearing still remains unclear. To explore on this aspect, the present study was conducted to determine the effects of the rotifer, *B. rotundiformis* (SS-type) fed with FWD, on the larval growth and development of the Japanese whiting *Sillago japonica*. *S. japonica* is an ecologically valuable fish resource distributed along the coasts of Japan, South Korea, Taiwan and the Philippines [9].

2 Materials and methods

2.1 Rotifer preparation

The inoculant rotifer, *B. rotundiformis* (SS-type; Thai strain) was obtained from Nagasaki Prefectural Institute of Fisheries, Japan. The SS-type rotifer is small in size (i.e. 70 – 160 μm), and is considered appropriate for the small-mouth fish larvae such as of *S. japonica* [10]. The rotifers employed in the test experiment were cultured using the FWD technology that has been extensively described in our patent registered under number: P00201609066 in Indonesia. In this article, we have only presented a flow chart of the rotifer production unit using FWD in Figure 1. Meanwhile, the rotifers used for the control experiment were batch cultured in 50 l of artificial seawater (22 ppt) and fed with HUFA enriched *Chlorella vulgaris* diet (Super Fresh Chlorella-V12, Chlorella Industry Co. Ltd., Fukuoka, Japan) at 25°C with aeration (Figure 2). The *C. vulgaris* diet was daily maintained at 7.0×10^6 cells ml^{-1} in the rotifer cultures [11].

2.2 Rotifer swimming speed

To determine the viability of the rotifer, swimming speed was recorded for 30 seconds under a stereomicroscope at 10× (SteREO Discovery V8, Zeiss, Germany) equipped with a digital camera (AxioCam, HSm) and image-analysis software (AxioVision 4.8). Five rotifers were sampled from each treatment and placed in 20 μl water drop, in which the swimming speed was measured using Dipp Motion Pro version 8.0 (DITECT Co. Ltd., Japan) in millimeters per second (mm s^{-1}).

2.3 Larviculture experiment

Fertilized eggs of *S. japonica* were obtained from the same institute as rotifers. The eggs were carefully transferred into six polycarbonate tanks each containing 100 l of artificial sea water (33 ppt) at 10 eggs l^{-1} with aeration fixed at 50 ml min^{-1} [12]. After hatching, larval fish development was monitored every 6 hours until mouth opening and then, the feeding process began (Fig. 3). Two feeding regimes i.e. the test diet (FWD-fed rotifers) and control diet

(rotifers fed with enriched *C. vulgaris*) were each triplicated in the culture tanks. The fish larvae were reared at 25°C with 12-h diurnal photoperiod (900–2100), for 10 days. The density of rotifers (diet) in each larval fish rearing tank was maintained at 10 ind. ml $^{-1}$ throughout the rearing period from 2 days post hatching (dph), which was the time of mouth opening. Ten larval fish were randomly sampled every 2 days from 2 dph for morphometric and gut content analysis. The fish samples were kept in small screw-cap bottles anesthetized with 2-3 drops of MS 222 followed by 5% formalin fixation [12].

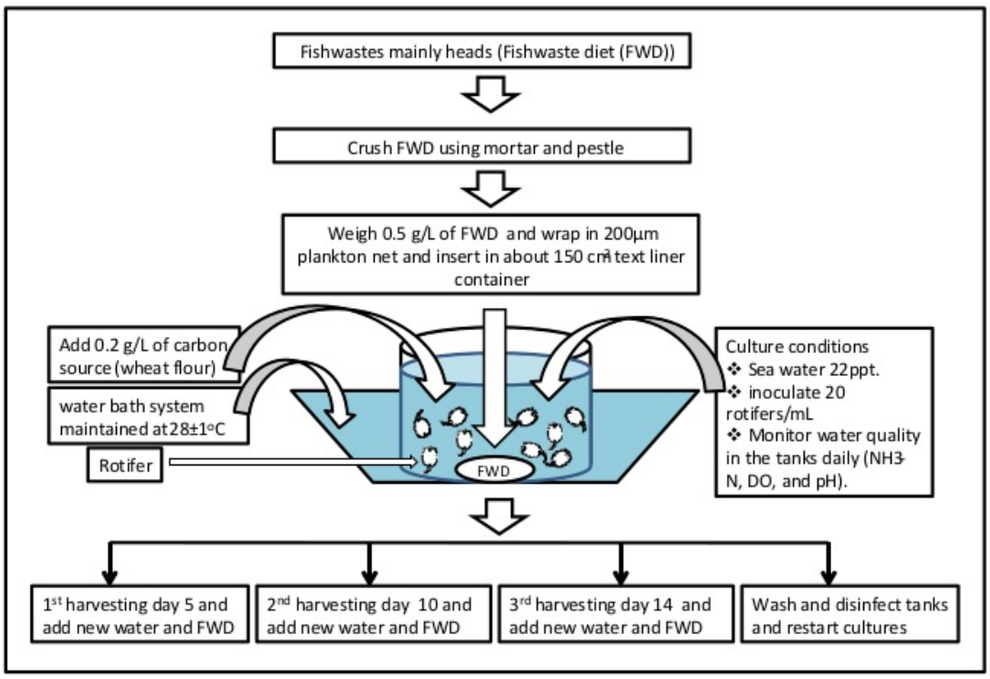

Figure 1. Flowchart for rotifer production using the low-cost FWD technology

Figure 2. Rotifer batch cultures with HUFA enriched *Chlorella vulgaris* diet (Super Fresh Chlorella V12, Chlorella Industry Co. Ltd., Fukuoka, Japan

The hatching rate and survival activity index (SAI) experiments were performed to determine the quality of the fish eggs and fish larval survivability during starvation, respectively [13]. Here, 20 eggs were placed in a 500 ml beaker containing 100 ml of artificial sea water (33 ppt) at 25°C in total darkness, without aeration and feeding. Dead larvae were counted and removed every 24 h until total larval mortality was reached. Triplicate

observations were used to calculate the hatching rate and SAI. The percentage of eggs that hatched normally was calculated after 24 h by the formula: [Normally hatched larvae / total number of eggs] × 100%, while SAI was calculated using the equation of [13],

$$SAI = \frac{1}{N}\sum_{i=1}^{K}(N - hi)\times i \quad (1)$$

where N = total number of examined larvae, hi = cumulated mortality by i-th day and K = number of days elapsed until all larvae died due to starvation. Morphological characteristics of the larval fish samples i.e. total and standard length, eye diameter, body depth and head length (Fig. 3), were measured using a microscopic measurement system that included a stereomicroscope (Discovery V8, Zeiss, Germany) equipped with a digital camera (AxioCam, HSm) and image-analysis software (AxioVision 4.8). At 10 dph, the percent survival of the fish larvae was calculated from the average number of surviving fish larvae in each culture tank. Also, the viability test of the fish larvae was determined at 10 dph by subjecting 10 fish larvae from each replicate tank to 30 s of air exposure before determining the rate of their survival thereafter. The dry weight of the fish larvae was measured in pre-weighed aluminum boats dried at 60°C for 24 h and weighed using ultra-micro chemical balance (Mettler Toledo, USA).

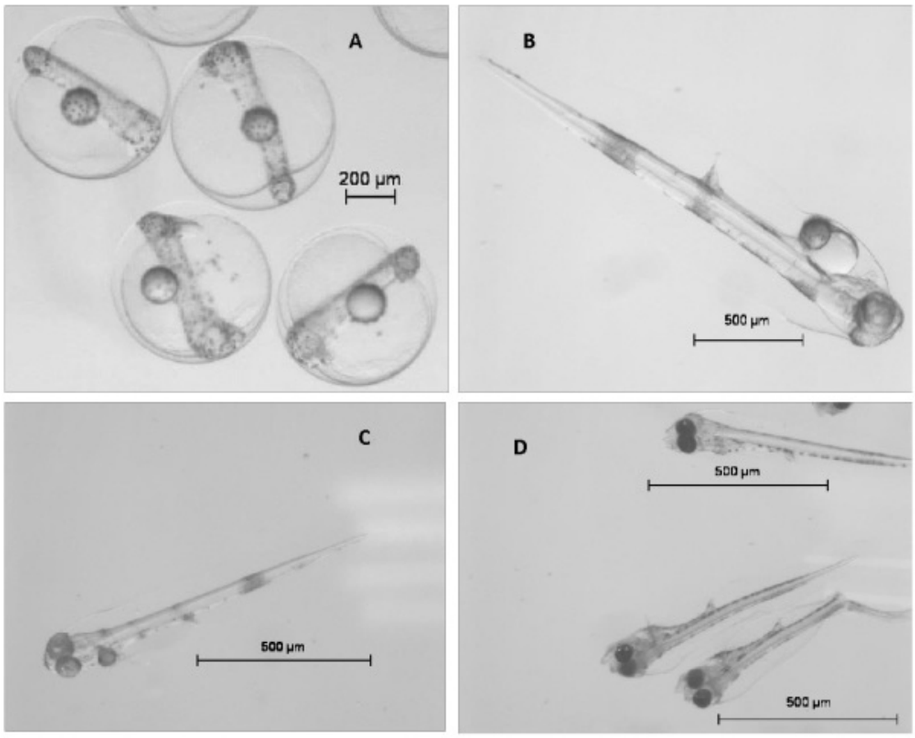

Figure 3. Larval developmental stages of *Sillago japonica*; A) egg stage, B) newly hatched larvae with a bigger egg yolk, C) 1 dph showing reduced egg yolk size, D) 2 dph showing mouth opening (end of exogenous feeding)

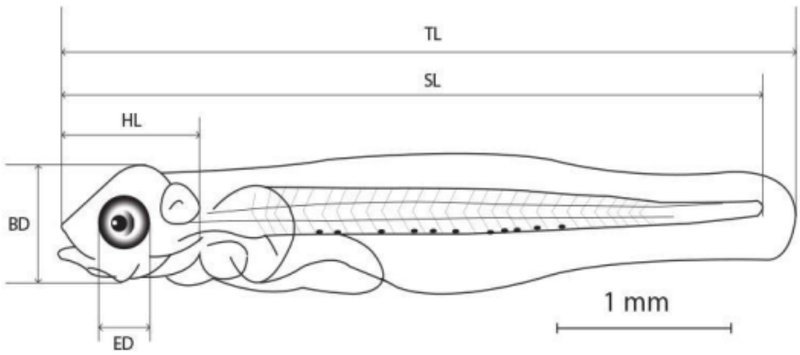

Figure 4. The five morphological characteristics to estimate larval growth and development of *S. japonica*. TL, total length; SL, standard length; HL, head length; ED, eye diameter; BD, body depth

2.4 Fatty acid analysis

At 10 dph, all the fish larvae were harvested from each treatment tank, dried using filter paper from beneath the harvesting mesh and kept at -80°C until biochemical analysis. The total lipid and fatty acid composition of the fish larvae and rotifer samples were performed by Chlorella Industry Co., Fukuoka, Japan. The sample methanol lysates were prepared at 100 °C for 2 h after the addition of 2 M hydrogen chloride methanol. Fatty acid methyl esters (FAME) were extracted by n-hexane. Gas chromatography analysis was performed using a GC-2010 (Shimadzu Scientific Instruments, Inc.) equipped with a HR-SS-10 column (Shinwa Chemical Industries, Ltd.). The column temperature was regulated at 150 to 220 °C. Individual fatty acids were quantified by means of the response factor to 15:0 fatty acids as the internal standard [14].

2.5 Statistical analysis

Statistical analysis was carried out using the R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test for homogeneity was used to test the equality of variances. Repeated ANOVA measures were used to test the effects of the diets on fish larval morphological parameters, gut content and swimming speed. The larval fish survival and viability tests were analyzed using Kruskal-Wallis test and Log-Rank test for groups, respectively. Where significant differences were detected, the Tukey HSD post hoc test was performed to locate them at $p<0.05$.

3 Results

The hatching rate of *S. japonica* eggs was 93.3±2.9%. The hatched fish larvae survived up to 5 days of starvation, with a SAI of 5.8±1.1. The rotifer swimming speed was affected by the diets (One way ANOVA, $F=14.51$, $p=0.01$), where the control-rotifers had significantly higher speed (0.83 mm s^{-1}) than FWD-rotifers (0.65 mm s^{-1}) (Tukey HSD, $p=0.01$). The number of ingested rotifers by individual fish larvae was significantly affected by age of fish larvae (Two-way ANOVA, $F=128.04$, $p=0.00$), but not the diets ($F=0.24$, $p=0.62$). The fish larvae from the two treatments ingested similar amounts of rotifers each day and, at 10 dph, about 10.5±3.1 and 11.6±2.7 rotifers were ingested by the FWD-fish and control-fish,

respectively. At 10 dph, there was no significant differences in morphological parameters e.g. head length (One-way ANOVA, F=2.46, p=0.13), eye diameter (One-way ANOVA, F=2.46, p=0.12) and body depth (One-way ANOVA, F=0.16, p=0.69) between FWD-fish and control-fish. However, there was a significantly higher total and standard length for the FWD-fish than the control-fish (p=0.00) (Table 1). There was no significant differences in fish larval survival rate (Kruskal-Wallis test, χ^2=0.19, df=1, p=0.66), viability (Log-rank test for groups, χ^2 = 0.4, df=1, p=0.55) and dry weight (One-way ANOVA, F=6.41, p=0.06) between the two diets (Table 2). The nutritional value of rotifers and fish larvae is summarized in Table 3. Due to high fish mortality experienced during the experiment, the quantities of fish sample were insufficient for total lipid analysis. Nonetheless, percent compositions of DHA were 5.2 and 18.2 for FWD- and control- fish, respectively. The EPA was under detectable limit in both samples. The ratio of DHA: ARA was 2.4 and 8.3 for the FWD- and control- fish, respectively (Table 4).

Table 1: Morphological parameters of the fish larvae as at 10 dph, the values are mean length (mm) ± SD, with different numbers of fish larvae per replicate. One-way ANOVA, Tukey HSD, different superscripts in each column denote significant differences at p<0.05 between the diets, a>b

Diet	Total length (n=30)	Standard length (n=30)	Head length (n=12)	Eye diameter (n=30)	Body depth (n=12)
FWD	3.65 ± 0.30^a	3.33 ± 0.33^a	0.76 ± 0.03	0.25 ± 0.02	0.69 ± 0.11
Control	3.42 ± 0.22^b	2.94 ± 0.33^b	0.74 ± 0.05	0.24 ± 0.02	0.68 ± 0.11

Table 2: Means ± SD of fish larval characteristics at 10 dph. The fish dry weight at 2 dph was 0.08 ± 0.01mg/ind in both diets, n=3

Diets	Dry weight (mg/ind)	Survival rate (%)	Viability (%)
FWD	0.26 ± 0.03	9.70 ± 9.06	58.8 ± 9.3
Control	0.21 ± 0.01	4.93 ± 0.83	64.4 ±7.9

4 Discussion

The results of this study show that fish larval rearing is possible with cheaply produced rotifers. This is important for improving fish larviculture especially in the developing countries, where aquaculture is a significant pillar for food and nutrition security. The fish eggs employed in the experiment were of high quality (93.3±2.9% hatching rate) and, the fish larvae showed high survivability during starvation (SAI: 5.8±1.1). These results are comparable to those of [15] who reported 95% and 4.1 – 8.4 hatching rate and SAI, respectively for *S. japonica* larvae. There was unprecedented high fish mortality (i.e. survival rates of 9.7±9.1 % and 4.9 ±0.8 % for FWD treatment and control, respectively), and the reason was not clearly determined. However, it was suspected that aeration rate employed in the current study was probably not optimal for this fish species. It could have also been possible that the fish larvae suffered from unknown microbial influence. In our previous study, FWD generated huge density of bacterial communities, of which some could possess lethal characteristics to cultured animals [7,8]. Further studies are recommended to determine the optimum aeration rate for *S. japonica* larvae, and also to screen the possible microbial communities contained in the culture facilities.

The viability of the FWD-fed rotifers as determined by the swimming speed may have been weaker than control-rotifers perhaps due to high bacterial turbidity in the FWD culture medium. More active rotifers are normally preferred in larviculture because they improve larval fish development due to the ability to stimulate fish raptorial behaviour [16].

Table 3: Total lipids (Total, mg/g dry weight) and fatty acid composition (% of total lipids) of the rotifers fed with FWD and control diet, and of the fish (*S. japonica*) larvae cultured with the FWD- and control-rotifers for 10 days. * = total lipids not measured due to limited sample quantity

	Rotifers fed with		*S. japonica* larvae fed with	
	FWD	Control diet	FWD-fed rotifers	Control-rotifers
Total lipids	20.5	23.3	*	*
Fatty acids				
14:0	5.4	3.6	1.1	2.8
14:1	2.3	0.2	0.4	0.1
16:0	15.2	18.7	14.5	17.4
16:1	3.0	2.1	1.1	5.1
16:2	0.6	1.2	1.3	0.5
18:0	4.3	7.0	10.0	5.6
18:1	4.8	14.7	4.9	13.6
18:2*n*-6	4.1	1.3	21.8	9.5
18:2*n*-3	0.5	0.7	2.4	1.0
20:0	0.3	0.8	0.0	0.4
20:1	0.6	0.3	0.3	0.1
20:4*n*-6	0.0	2.5	2.2	2.2
20:5*n*-3	1.9	6.6	0.0	0.0
22:0	0.0	0.3	1.0	0.3
22:1	15.1	0.4	2.7	5.0
24:0	1.4	1.3	0.4	0.6
24:1	0.8	0.5	1.1	0.3
22:5*n*-3	0.5	2.2	3.0	2.1
22:6*n*-3	1.7	18.8	5.2	18.2
Others	37.4	16.8	26.6	15.3
DHA/EPA	0.9	2.8	0.0	0.0
DHA/ARA	0.0	7.5	2.4	8.3
Total	100	100	100	100

The results showed that diet did not determine the number of rotifers ingested by the larval fish, thus suggesting equal palatability between the FWD-fed rotifers and those fed on conventional enriched algal diet. Due to same ingestion rate of rotifers, the fish morphometric measurements (head length, eye diameter and body depth), survival, viability and dry weight. However, a significantly higher total and standard length for the fish given FWD-rotifers than those given control diet was realized. These results indicate the superiority of FWD as rotifer diet for fish larviculture production. The fact that there was no significant difference in survival rate, viability, dry weight, gut content, head length, eye diameter, and body depth between the two diets indicate that waste-fed rotifers are as good as established larviculture technologies.

Previous larval fish rearing data confirm that survivability of fish larvae strongly depends on the amount of DHA and ARA present in the diet [17,18,12]. Since most marine fish larvae cannot synthesize DHA from precursor molecules e.g. EPA or α-linolenic acid (ALA), supplying DHA-rich feeds to fish larvae is important [16]. The DHA/ARA ratio of 2.4

obtained in this study is less than the optimal ratio of 10.0 required for proper larval fish development [18]. Nonetheless, the similarity of larval fish development parameters between the test and control diet suggests a possibility of FWD for the larviculture of *S. japonica*. The technology for FWD-fed rotifers promises appears a major leap toward developing a cheap and nutritionally rich diet for the rotifers, which are valuable food sources in larviculture. Based on the food value of the bioflocs [7, 8]. The FWD culture fluid can be considered a cheaper enrichment medium for low quality larval fish foods e.g. bakers' yeast and, may reduce the need for the expensive enrichment emulsions. However, further studies are needed for this suggestion. Further experiments are also recommended to test the FWD in other fish species for larviculture. The advantage of FWD is that it is significantly cheaper because fishwaste, which is the main ingredient in the diet, can be obtained for free. Therefore, the FWD-rotifers provides the means to develop more economic ways of improving aquaculture of commercially important fish species in small-scale tropical hatcheries.

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