The Mass Culture of the Freshwater Rotifers Brachionus Rubens Ehrenberg 1838 Using Different Algal Species Diets

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Abstract— The bottleneck of most inland freshwater aquaculture enterprises is in obtaining an adequate number of fingerlings, due to their high mortality at early life stages. Their successful production is hindered by many factors including an adequate supply of food at early larval stages. A study on the mass culture of freshwater rotifer species was conducted at the freshwater rotifers' laboratory in Cần Thơ University's College of Fisheries and Aquaculture. *Brachionus rubens* Ehrenberg 1838 were identified and inoculated in 30 identical Falcon cups of 50 ml at 5 female rotifers cup^{-1} in order to obtain cultures with sufficient rotifer density for the subsequent culture experiments. A feeding experiment to identify the algae diet that conferred the best culture performance was conducted. Four different feeding treatments involving monodiets of *Nannochloropsis oculata*, *Chaetoceros calcitrans* and *Chlorella vulgaris* algae species and additionally a mixture of the three algal species were run in a 7-day experiment period. 1.5 l of identical, transparent plastic bottles filled with 1 l of mineral bottle water were stocked at an initial density of 20 individual rotifers ml^{-1} were used in three triplicates. Water temperature and water pH were 26 ± 1 ˚C and 7.5 ± 1.5 respectively. The diet containing mixed algal species had significantly higher rotifer density and egg ratio on the 7th day. Blending microalgae species can enhance the culture performance of the freshwater rotifers *B. rubens* when compared to monospecific microalgae diets.

Keywords— Larviculture; Brachionus rubens; freshwater aquaculture; zooplankton; microalgae.

INTRODUCTION

With the pre-eminence of an exponential global human population growth, currently projected to hit a median of 10 billion by 2050 from the current 7.7 billion (FAO, 2017; The United Nations, 2017), there is a need to aim at higher production of food to confront the daunting challenge of feeding the ever-burgeoning human population. Aquaculture has been described as an important contributor to total global seafood production and is expected to solve the riddle behind food and nutrition security (Béné et al., 2015; Nadarajah and Flaaten, 2017; Foley, 2019). The United Nations Food and Agriculture Organization (FAO) and recent studies estimate that more than 50 % of the seafood consumed worldwide is coming from aquaculture and that production must double to meet the expected demand by 2030 (Bostock et al., 2010; FAO, 2016; 2017; 2018; Assefa and Abunna, 2018). Fortunately, aquaculture is the fastest-growing sector of agriculture in the world (Assefa and Abunna, 2018; Hossain and Shefat, 2018) and has grown at an annual rate of more than 5.8% since 2005 with an indication of continued growth (Yoshimatsu and Hossain, 2014; Bengtson, 2018; Bentoli, 2019) and with a potential to meet the needs and demands of the ever-expanding appetite of the human population (Rust et al., 2014; Kobayashi et al., 2015; Li and Robinson, 2015; FAO, 2016; Froehlich et al., 2017).

Fish larviculture has been described as a major bottleneck for the industrial upscaling of the aquaculture of many fish (Dhert et al., 2001; Ashraf et al., 2010b; Megaraja et al., 2016) because many aquaculture species cultured, especially marine species, are fixed on the scheme of motile prey organisms (Faruque et al., 2010) and encounter problems to accept inert or dry diets as starter food (Korstad et al., 1989). Moreover, feeding with dry diets has been demonstrated to delay gut development at that stage (Sargent et al., 1999; Sorgeloos et al., 2001; Hamre et al., 2008).

The success and profitability of any farming operation of fish and shellfish are highly contingent on the availability and ready supply of larvae for on-growing to market size among other factors (Suantika et al., 2003). Optimal fish and shellfish hatchery production is heavily reliant upon the development of a diet fulfilling the nutritional requirements of fish and shellfish larvae. Presently, in the larval and juvenile phases of many fish and crustacean species, the use of manufactured feed is not fully optimizable (Coutteau

and Sorgeloos, 1997; Støttrup and Lesley, 2003; Chesney, 2007; Faruque, 2010; Hyppolite and Laleye, 2012; Maehre et al., 2013). Even with much progress already made in defining larval digestive capabilities and nutritional requirements, there is still a wide knowledge lacuna on the specific nutritional requirements of many potential aquaculture species at their early stages (Hamre et al., 2013; Thépot et al., 2016). This is mainly attributed to factors such as their small mouth, usually with an opening of less than 0.1 mm, extremely fragile, rudimentary and/or undeveloped digestive system, and not physiologically fully developed, or simply lacking stomach. With much of the protein digestion taking place in hindgut epithelial cells and therefore susceptible to microbial or disease infections and poor acceptance of artificial diets witnessed fish species (Govoni et al., 1989; Schipp et al., 1999; Cahu and Zambonino-Infante, 2001; Tocher, 2010; Megaraja et al., 2016; Román Reyes, et al., 2017; Mondal et al., 2018). Distinctively, a good knowledge of larval nutritional requirements throughout the development cycle would contribute to optimizing diets and feeding protocols, and thereby improve larval and juvenile quality (Dabrowski et al., 1978; Rainuzzo et al., 1997; Hamre et al., 2013). Significant scientific research efforts have been dedicated to meeting the nutritional requirements of marine fish larvae using cultured live prey and inert replacement diets for larvae (Shields, 2001).

Live feeds remain essential inputs in hatcheries for many aquaculture species (Phillips and Matsuda, 2011), and this situation is projected to remain for the conceivable future (Dhont et al., 2013). Live food organisms include yeasts, bacteria, all plants (phytoplankton) and animals (zooplankton) which are used in finfish and shellfish larval rearing systems (Akhtar and Singh, 2012; Dhont et al., 2013; Ghosh et al., 2016).

The natural diet of larvae of most aquaculture fish and shellfish species consists of a wide diversity of phytoplankton species including diatoms, flagellates, green algae, and zooplankton organisms comprising of copepods, ciliates, cladocerans, decapod larvae, etc. found in great abundance in natural plankton, as elucidated by Turner (1984), Ekelemu (2015) and Mondal et al. (2018). Zooplanktons are a valuable source of protein, amino acids, lipids, fatty acids, carbohydrates, vitamins, minerals (New, 1999; Santhosh and Anil, 2013; Mondal et al., 2018) and enzymes (amylase, protease, exonuclease and esterase), which play an important role in larval nutrition (Flüchter, 1982; Munilla-Moran et al., 1990). They are easily digestible (Murugesan et al., 2010) and a study by Mims et al. (1991) revealed that the exoskeleton of the live organism (as roughage) is necessary for food digestion in fish fry. Being a source of carotene, they improve flavour, colour, and texture of fish fed on them (Spinelli, 1979).

Artemia nauplii, Artemia cysts, Daphnia spp., Tubifex and Moina are among commonly used and desirable zooplankton by farmers to raise fish larvae (Rónyai and Ruttkay, 1990; Subagja et al., 1998; Hung et al., 1999; Le et al., 2002; Rottmann et al., 2003). However, these live feed are generally bigger sizes and are only suitable for application after 4 hph but not within the first 24 hph (McGee, 2016). Moreover, Artemia, a marine organism dies within 2 h of introduction in freshwater Porticelli (1987) and Ovie (1997) cited in Ekelemu (2015), and therefore may not be effectively utilized as live feed for freshwater aquaculture species. Since larval sizes are dependent on the size of the egg they hatch from (Blaxter and Hempel, 1963), smaller eggs will hatch into tiny larvae that may not immediately accept bigger-sized prey.

In hatcheries, the growth and survival of larval fish depend on the availability of suitable food type (Mills et al., 1989; Fox and Flowers, 1990; Margulies, 1993; Duffy et al., 1996; Welker et al., 2004; Ma et al., 2013; Odo et al., 2015). Larval fish requires high protein food (42 % and 52 % for omnivorous and carnivorous fish respectively) for survival and growth (Tacon, 1990). Rotifers especially of the genus Brachionus are among the most commonly used zooplankton as live feed for fish larvae cultures (Howell, 1974; Halbach, 1984; Sugumar and Munuswamy, 2006) and are an ideal feed source for large quantity fish cultivation (Zhang et al., 2005; Arak and Mokashe, 2015). This genera is a proper live feed for the early larviculture because it guarantees faster growth rates, better health quality and higher survival rates in fish in the later stages of growth (Das et al., 2012). However, much of the studies have only been performed on B. calyciflorus with only a little information available on the culture and application of B. rubens as live feeds to larval fish. A study conducted by Schlüter in 1980 pointed out that B. rubens are suitable organisms for mass culture and highlighted some of the requirements for their successful culture. However, despite this discovery, only a few studies have focused on the culture techniques and utilization of this rotifer species. Lubzens (1987) highlighted three major factors to consider during the initiation of a mass culture of

rotifers. Firstly, it is critical to select rotifer strains that will be of appropriate size for the larvae. Moreover, culture conditions should be suitable and food quality and quantity should be maintained at adequate levels for rotifers. In addition, rotifer strains can vary in reproductive rate, individual size, optimum culture conditions, frequency of mixis and tolerance to environmental perturbation (Fukusho, 1989; James, 1989; Lubzens et al., 1989). Consequently, it is important to know the biology of rotifers, especially of the freshwater rotifers, whose application has not been widely exploited, despite their ease of culture (Oltra and Todolf, 1997), so as to be familiar with the manipulative strategies to stimulate their growth, multiplication, and application in larviculture technology, that will enable the optimization of their culture (Ashraf et al., 2010b).

Since prey selection by larval fish is dependent on prey quality, prey selection remains an important factor worth considering for successful larviculture as the food value of live food organisms for a particular fish species is primarily determined by its size and form (Hunter, 1980; Murugesan et al., 2010; Riley et al., 2010). According to Hunter (1980), small rotifers must also be supplied in greater numbers than larger ones, to meet the metabolic demands of the developing larvae (Fernandez-diaz et al., 1994). Evidently, while a small food organism is desirable for fish larvae in term of assimilation, the use of larger organisms confers better digestibility as long as the size of the food organisms does not interfere with the ingestion mechanisms of the fish larvae (Merchie, 1996), as fish would take a long time to attain satiation if fed with smaller live food organisms, and this would result in poor growth due to inefficient feeding and waste of energy (Riley et al., 2010).

Selection of a suitable algae species to feed rotifers must be considered as although there exist several algae species useful as rotifer feeds, research has found that some species are unsuitable in feeding B. rubens (Schlüter, 1980), or are simply of insufficient dietary components to meet the demand for live feeds (Cabrera et al., 2005), or may just be adequate for rotifer culture (Ben-Amotz et al., 1987; Tzovenis et al., 2003a, b). However, the quality coming out of large scale hatcheries could be unreliable (Birkou et al., 2012). Over 40 different species of microalgae, including Nannochloropsis spp., Chlorella spp. among others have been tried or used directly in larviculture or indirectly as food for zooplanktons including rotifers (Marin et al., 1994; Wikfors and Ohno, 2001; Cho et al., 2007; Ferreira et al., 2008; Mohr and Adrian, 2010; Ranjan et al., 2016).

Intensive production of rotifers is usually performed in batch cultures and continuous cultures within indoor or outdoor facilities. Indoor facilities are, however, preferred over outdoor facilities (Dhert, 1996). Basically, the production strategy is the same for indoor or outdoor facilities, but higher starting and harvesting densities enable the use of smaller production tanks (generally 1 to 2 m3) within intensive indoor facilities (Dhert, 2003). In batch culture, the rotifers are maintained at a constant volume with an increasing rotifer density by increasing the culture volume while in continuous culture, rotifer density is maintained constant by periodic harvesting. Today, high-density rotifer cultures have been developed producing more than 1500 rotifers.ml-1 within 4 days.

The objective of the present was to investigate the effects of three different microalgae species, namely N. oculata, C. calcitrans, and C. vulgaris, offered as monodiets and as a mixture of the three for the culture of freshwater rotifer, B. rubens on their culture performance; density, specific growth rates, ration of egg-carrying females and egg ratio.

MATERIALS AND METHODS

B. rubens were isolated under a microscope from a mixed population of zooplankton collected from the Hau River using the method described by Sharma (1983, 1996, 1998, 2005), Segers (1995) and Arimoro (2006). The species was then inoculated in 30 identical Falcon cups of 50 ml each at a density of 5 asexual female rotifers cup^{-}1 in order to obtain cultures with sufficient rotifer density for the subsequent culture experiment. They were fed on C. vulgaris and/or C. calcitrans for 7 days. They were then rinsed over a submerged filter of 50 µm mesh size that retained the rotifers. The culture media used for the experiment was bottled mineral water. Temperature and pH during the entire experimental period were 26 ± 1 °C and 7.5 ± 1.5 respectively.

To determine the most suitable algae diet that could provide the greatest culture performance to the rotifers, B. rubens, the rotifers were divided into four different treatment groups each in triplicate: (1) rotifers exclusively fed with C. vulgaris, (2) rotifers exclusively fed with C. calcitrans, (3) rotifers exclusively fed with N. oculata and (4) rotifers fed with a mixture of the three algal species (ratio 1:1:1 on a cell concentration basis). For treatments 1, 2 and 3 the individual algal cell concentration was maintained at a

density of 1.6 × 106 cell.ml⁻1 while for the mixed algae, each of the individual algae was mixed equally to give a final concentration of 1.6 × 106 cells.ml⁻1. In all the treatments, feeding was done at an interval of 2 h from 0700 h to 2100 h for 7 d. Every morning, before feeding, waste metabolites were removed by temporarily halting the aeration and allowing the medium to settle and decanting the first water layer containing rotifers. About 300 ml of culture water was exchanged this way in order to maintain the quality of the culture water while maintaining the culture volume at 1,000 ml for 7 d.

The experiment was conducted in an air-conditioned room where the temperature was 26 ± 1 °C with a constant illumination condition provided throughout, from four cool-white fluorescent light tubes (60 cm long, 210 V, Philips 60 W, which gave approximately a light intensity of 12 Klux at the culture surface) (Rapid Tables.com, 2019), placed 20 cm from the culture bottles. Gentle aeration was achieved using air tubes connected to the culture units. Prior to the commencement of any feeding trial, the up-scaled rotifers was starved for 24 h in order to minimize the effects of the diets they were fed before the experiment.

Three different algae species, (freshwater spp., C. vulgaris, marine spp., N. oculata (salinity 10 g l⁻1) and marine spp., C. calcitrans (salinity 20 g l⁻1)) were individually batch cultured in 5 l transparent bottles and fed on Walne's medium (Walne, 1970), under controlled indoor conditions at 25 ± 1 °C, continuous illumination (3,000 lux), and mild aeration to prevent algae from settling. Algae were harvested in the log phase of the growth period by centrifugation at a speed of 3,000 rpm, decanted, resuspended in a small quantity of distilled water and stored at 4 °C according to Graima et al. (2003) and Low and Toledo (2015). Fresh algal concentrate used was prepared after every 3 days.

The number of microalgae cells was determined in diluted aliquots of suspended concentrate by counting in a haemocytometer and further calculated using equation 1. Dry weights reported by Brown (1991) and Flores-Burgos et al. (2005) were used to calculate the dry weights of the algal diets to be fed. The dry weights for single cells of C. calcitrans, N. oculata and C. vulgaris were 11.3, 6.1 and 27.0 pg cell⁻1, respectively.

$$\boldsymbol{cells.ml^{-1}} = \frac{n_1 + n_2}{160} \; \boldsymbol{x\,10^6\,x\,d} \quad \text{.....Equation 1}$$

Where:

$n1$: total cells in the first counting chamber.
$n2$: total cells in the second counting chamber.
d: coefficient of dilution.

Each day, rotifer numbers, egg numbers – attached to the female rotifers and suspended in the water column (where all eggs were considered as amictic eggs), and egg-carrying female rotifers from each vessel of every feeding treatment were counted from a 1 ml sample using a Sedgewick-Rafter counting chamber and an Olympus CX 22 LED microscope at 10 × magnification. Three counts were made from the 1 ml sample and averages were collated. In the case of high densities, a volume of 500 µl was taken instead. Empty and transparent loricae belonging to dead rotifers were not counted. The average values of the three 1-ml samples were multiplied by the tank volume (1,000 ml) to estimate their totals in each treatment. Results of rotifer densities and the density of amictic eggs were expressed as numbers of individuals.ml⁻1, while the results of the ratio of egg-carrying females and egg ratio were reported as the proportion of the total rotifer population. All the results were presented in means ± S.E. The specific growth rate (SGR) expressed as µ was calculated using the formula in equation 2. The average SGR per treatment was calculated from the three replicates in each case.

$$SGR\ (\mu) = \left(\frac{\boldsymbol{ln\,N_t - ln\,N_0}}{\mathbf{t_1 - t_0}}\right) \quad \text{(Krebs, 1973)}$$

.....Equation 2

where: μ is the specific growth rate, N_t is rotifer density (individuals.ml^{-1}) at time t_1, N_0 is the initial rotifer density (individuals ml^{-1}), t_0 and t_1 are days of culture ($t_1 > t_0$).

The egg ratio (%) was calculated from the formula:

$$Egg\ ratio\ (\%) = \frac{Number\ of\ amictic\ eggs}{Total\ number\ of\ rotifers} \times 100$$

Equation 3: % egg ratio.

DATA ANALYSIS

All analyses were performed using the Real Statistics and Numerical Analysis for Excel (NumXL) tool packs version 2016, embedded on Excel and the results were reported as mean ± S.E. One-way Analysis of Variance (ANOVA) was used to determine any significant differences in the tested quantitative variables of culture performance in rotifers population: rotifer

densities, daily specific growth rates, ratio of the egg-carrying female rotifers, density of eggs and egg ratio.

RESULTS

The mean rotifer densities obtained from feeding rotifers on four different treatments of microalgae diets over the seven-day culture period showed a gradual increase (Table 1). Significant differences between mean rotifer densities were observed between some treatments from the second day onwards. The mixed diet was significantly better, about 15 % increment than all the others from day 2 until the end ($p < 0.05$). Except for day 5 rotifers fed on N. oculata and C. vulgaris did not show significant differences in densities from day 3 to the end. Rotifers fed on C. calcitrans showed significantly lower mean rotifer densities on the 6th and 7th day ($p < 0.05$) but had comparable densities to the other monospecific diets between day 3 and 5 ($p > 0.05$).

Table 1: The mean rotifer density (individuals.ml^{-}1) of rotifers fed with the four different diets over the 7-day culture period. Data are presented as mean ± S.E based on three replicates. Different superscript letters in the same row indicate a significant difference among treatments with Tukey's multiple comparison test; $p < 0.05$).

Feeding Treatment

Day	C. vulgaris	N. oculata	C. calcitrans	Mixed algae
1	20.0 ± 0.0a	20.0 ± 0.0a	20.0 ± 0.0a	20.0 ± 0.0a
2	57.6 ± 1.4a	79.9 ± 4.4b	50.3 ± 4.2a	80.2 ± 4.4b
3	79.7 ± 1.8a	92.1 ± 2.9a	74.9 ± 3.1a	156.7 ± 4.2b
4	114.2 ± 3.6a	136.8 ± 2.5a	119.4 ± 5.4a	241.7 ± 7.7b
5	153.3 ± 3.7a	217.7 ± 5.5b	163.1 ± 3.1a	276.3 ± 6.4c
6	244.9 ± 6.7a	258.0 ± 6.1a	215.0 ± 4.2b	288.6 ± 3.0c
7	277.0 ± 2.4a	285.4 ± 1.0a	234.6 ± 5.2b	313.8 ± 3.6c

Note: Mixed algae diet contains C. vulgaris, N. oculata and C. calcitrans in the ration 1:1:1 by cell concentration.

The highest daily specific growth rates were observed on the 2nd day for all the treatments but thereafter a decreasing trend was observed up to day 7 when the lowest SGRs were registered in all the treatments (Figure 4.1.1). The SGRs recorded on day 2 for both the mixed and N. oculata diets were significantly higher than those of C. calcitrans and C. vulgaris ($P < 0.05$) but were not significantly different between themselves ($p > 0.05$). The SGR observed between the 3rd and 7th days varied across the treatments and were significantly lower than those recorded on day 2 ($p < 0.05$). The SGR value recorded for the mixed diet on day 3 was significantly higher than the rest of the diets on that day ($p < 0.05$). N. oculata recorded the greatest drop in SGR value on the 3rd day but later picked up from the 4th and the 5th day before dropping again on days 6 and 7. On the 4th day, C. calcitrans and mixed diets had the highest SGR values which were non-significantly different between the two diets but were significantly higher than those recorded for N. oculata and C. vulgaris on the same day. On the 5th day, the SGR values were not significantly different between the mixed and N. oculata and were significantly higher in these two diets in comparison to the other two diets ($p < 0.05$). On the 6th day, the mixed and C. vulgaris diets had the highest SGR values which were not significantly different between themselves ($p > 0.05$) but were significantly higher than the other two monodiets ($p < 0.05$). On the last day, C. vulgaris and N. oculata had the lowest growth rate of all treatments (0.10 day^{-}1) and these rates were significantly lower than those recorded for rotifers fed on C. calcitrans and the mixed diets on the same day ($p < 0.05$). The mixed diet had the highest SGR on the final day (0.30) which was lower than 1.70 recorded for C. calcitrans on the same day.

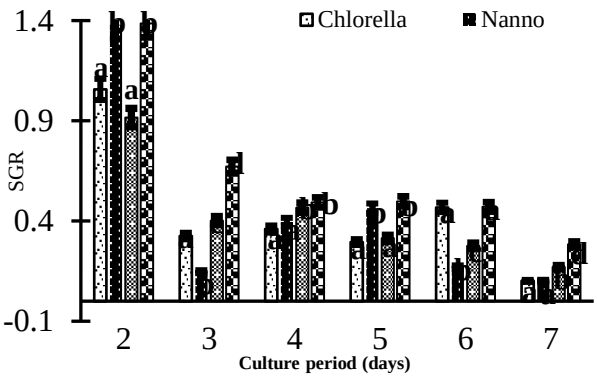

Figure 1: The SGR day^{-}1 during the 7-day culture period. Data are presented as mean ± S.E based on three replicates. Different superscript letters on a particular culture day indicate a significant difference among treatments with Tukey's multiple comparison test; $p < 0.05$. Chlorella = C. vulgaris, Nanno = N.

oculata, Chaeto = C. calcitrans and Mixed = Mixture of the three algae species.

The proportion of the egg-carrying female rotifers to the entire culture period ranged between 46.61 ± 1.93 and 87.98 ± 6.29 % (Table 2). The mixed diet showed the highest proportion of egg-carrying female rotifers on the 6th day while the lowest ration of egg-carrying females was observed on C. vulgaris on the 2nd day. The ratio of egg-carrying females remained relatively similar in the four treatments between day 3 to 5 and on the last day ($p > 0.05$) but the mixed diet showed a significantly higher ration of egg-carrying females than all other treatments on the 6th day ($p < 0.05$) (Table 2).

Feeding Treatment

Day	*C. vulgaris*	*N. oculata*	*C. calcitrans*	*Mixed*
2	46.61 ± 1.93^{a}	57.26 ± 3.58^{ab}	61.60 ± 4.94^{ab}	69.64 ± 3.49^{b}
3	64.20 ± 1.56^{a}	69.29 ± 2.86^{a}	79.94 ± 4.34^{a}	48.76 ± 1.92^{a}
4	65.31 ± 1.00^{a}	65.09 ± 1.38^{a}	61.63 ± 3.73^{a}	61.92 ± 0.80^{a}
5	72.22 ± 1.06^{a}	63.58 ± 3.07^{a}	69.43 ± 4.84^{a}	74.08 ± 5.07^{a}
6	69.42 ± 3.26^{a}	66.43 ± 1.28^{ab}	60.37 ± 3.53^{b}	82.58 ± 3.48^{c}
7	81.51 ± 3.35^{a}	80.70± 0.52^{a}	81.77 ± 7.33^{a}	87.98 ± 6.29^{a}

Table 1: The mean ratios (%) of egg-carrying female B. rubens from rotifers fed on four different treatment diets over the 7-day culture period. Data are presented as mean ± S.E based on three replicates. Different superscript letters in the same row indicate a significant difference among treatments with Tukey's multiple comparison test; $p < 0.05$.

The egg densities depicted a progressive increase from day 2 to day 7 (Table 3). A significant difference in egg density was observed among all the diets on the 2nd day. On the third day, C. vulgaris and N. oculata showed significantly lower egg densities compared to C. calcitrans and the mixed diet ($p < 0.05$), which were also not significantly different among themselves ($p > 0.05$). The results obtained from the mixed diet remained significantly higher than the rest of the treatments from the 5th day to the last day ($p < 0.05$). On the final day, the highest egg density (653.9 ± 3.6 eggs ml^{-}1) was observed in the rotifers fed on the mixed algal diet while the lowest (359.9 ± 14.4 eggs ml^{-}1) was observed in rotifers fed on C. vulgaris algae diet on the same day. It was clear that the mixed diet maintained high egg densities throughout the experiment.

Feeding Treatment

Day	*C. vulgaris*	*N. oculata*	*C. calcitrans*	Mixed
2	45.9 ± 1.4^{a}	84.2 ± 3.4^{b}	59.6 ± 1.5^{c}	137.2 ± 16.3^{d}
3	98.5 ± 8.3^{a}	120.9 ± 3.1^{b}	178.6 ± 1.6^{c}	186.6 ± 1.4^{c}
4	110.7 ± 14.4^{a}	172.4 ± 4.3^{b}	233.8 ± 4.3^{c}	179.9 ± 49.4^{b}
5	147.2 ± 3.7^{a}	271.6 ± 7.5^{b}	284.6 ± 5.0^{b}	390.0 ± 5.5^{c}
6	318.5 ± 21.1^{a}	327.0 ± 27.1^{a}	346.7 ± 6.3^{b}	552.2 ± 15.5^{c}
7	359.9 ± 14.4^{a}	481.9 ± 6.5^{b}	362.7 ± 7.2^{a}	653.9 ± 3.6^{c}

Table 2: The egg densities (eggs.ml^{-}1) of rotifers fed on the four different diets over the 7-day culture period. Data are presented as mean ± S.E based on three replicates. Different superscript letters in the same row indicate a significant difference among treatments with Tukey's multiple comparison test; $p < 0.05$.

The egg ratios observed from the 2nd day to the final day were higher than 70 % (Table 4). There was a progressive increase in % egg in the monodiets on the first 3 days. *C. calcitrans* had an egg ratio of 239.33 ± 10.97 % on the 3rd day, which was significantly higher than the other treatments ($p < 0.05$), but the egg ratio in this diet showed a consistent drop from the 4th day to the last. Rotifers fed on the mixed diet had a significantly higher final egg ratio (208.45 ± 2.82 %), than all other treatments, while the lowest egg ratio (133.34 ± 9.33 %) was observed in *C. vulgaris* diet on the same day (Table 4). The mixed diet showed a rapid increase in the egg ratios from day 5 until the end of the experiment. In all treatments, except for *C. calcitrans*, the final egg ratios were the highest ones observed during the entire culture period.

Feeding Treatment

Day	*C. vulgaris*	*N. oculata*	*C. calcitrans*	*Mixed*
2	79.92 ± 4.18^{a}	106.88 ± 10.57^{b}	120.18 ± 11.23^{c}	172.56 ± 23.49^{d}
3	123.44 ± 8.53^{a}	131.32 ± 1.92^{a}	239.33 ± 10.97^{b}	119.25 ± 3.24^{a}
4	96.27 ± 9.90^{a}	126.27 ± 5.45^{b}	196.20 ± 5.40^{b}	73.86 ± 18.82^{a}
5	96.04 ±	124.97 ±	174.59 ±	141.24 ±

	2.55[a]	5.36[b]	3.02[c]	2.83[b]
6	130.20 ± 8.90[a]	127.25 ± 12.68[a]	161.36 ± 5.12[b]	191.31 ± 2.83[c]
7	133.34 ± 9.33[a]	168.82 ± 2.37[b]	154.71 ± 3.36[b]	208.45 ± 2.82[c]

Table 3: The egg ratio (%) of rotifers fed on the four different diets over the 7-day culture period. Data are presented as mean ± S.E based on three replicates. Different superscript letters in the same row indicate a significant difference among treatments with Tukey's multiple comparison test; $p < 0.05$.

DISCUSSION

The type of microalgae species used as rotifer diet significantly influenced the culture performance, i.e. rotifer densities, specific growth rates, egg-carrying female ratio, egg density and egg ratio of the freshwater rotifers, *B. rubens*. when rotifers were fed on a blended diet of microalgae, the performance was outstanding, in comparison to when monodiets alone were used. The mixed diet had significantly high rotifer density than the other treatments on the last day (313.8 ± 3.6 rotifers ml^{-1}). *C. vulgaris* and *N. oculata* observed a non-significant difference in rotifer densities which were higher than what was obtained with *C. calcitrans*. The blended diet was assumed to have presented a diverse alternative in terms of not only food size but also exposure to a better nutritional regime, hence increasing the chances of rotifer's functional response to better nutritionally endowed diets. Sometimes mixed food types support zooplankton growth better than when individual feed types are offered separately (Lavens and Sorgeloos, 1996; Loka et al., 2016), because even the best of the feed types may have its own limitations (Hirayama et al., 1989; Schmidt and Jónasdóttir, 1997; DeMott, 1999). Spolaore et al. (2006) found that offering a combination of several microalgae species to rotifer cultures could have a comparative advantage as it avails the various nutrients into the diet pool, thus covering for the losses if monospecific diets were used.

Similar observations on the effect of microalgae diets on rotifer densities were also made by Wang et al. (1998), Xi and Huang (1999), Xi et al. (2000, 2002, 2011) and Hu and Xi (2008) who studied the effects of algal diets on the growth performance of *B. calyciflorus* and *B. urceolaris* although they used *Scenedesmus obliquus* which is a different algae species from the ones used in the present study. Bae and Hur (2011) found the best specific growth rate (0.68 day^{-1}) of *B. plicatilis* when fed on *N. oculata* but lower specific growth rate (0.53 day^{-1}) when they were fed on *C. vulgaris*, offering the algae diets at a density 1.5×10^5 ml^{-1} and 2.2×10^5 ml^{-1} for *C. vulgaris* and *N. oculata* respectively. However, the growth rates reported by Bae and Hur (2011) were higher than the ones reported in the present study for the two algal diets (0.42 day^{-1} for *C. vulgaris* and 0.45 day^{-1} for *N. oculata*). The results in the present study were also higher than those obtained by Brown (1998), who found 0.21 day^{-1} as the highest SGR using *N. oculata* to feed *B. plicatilis* and similar to those obtained by Arimoro (2006). The lower growth performance obtained from feeding rotifers on *C. calcitrans* may be attributed to the large size of this algal species as compared to the other two microalgae species. This might have possibly affected the filtration and ingestion rates of the algae cells by the rotifers (Štrojsová et al., 2009).

Microalgae have been used as the main food for rotifers, and as very important live food for larvae of marine fish, filter-feeding invertebrates, etc. (Muller-feuga, 2000; Chakraborty et al., 2007; Converti et al., 2009; Xavier et al., 2016). In order to be used in aquaculture, a microalgal strain has to meet various criteria including easy to be cultured, nontoxic, be of the correct size and shape to be ingested and have high nutritional qualities and a digestible cell wall to make nutrients available (Brown et al., 1999; Renaud et al., 2002; Muller-Feuga et al., 2003; Spolaore et al., 2006; Murugesan et al., 2010). Algal preference by different rotifer species has been documented (Ajah, 2008; Kennari et al., 2008). Rotifers exhibit variable selectivity to different algae species, notably as a result of morphological characteristics, cell wall structure, or the biochemical composition of individual algae species (Chotiyaputta and Hirayama, 1978; Salt, 1987; Tomaselli, 2004; Abou-Shanab et al., 2016).

Freshwater *Chlorella* was introduced as a substitute for *Nannochloropsis* in the early 1990s (Yoshimura et al., 1996) due to the latter's seasonal growth variation (Sukenik et al., 1993; Converti et al., 2009; Bae and Hur, 2011). It has a spherical microscopic cell with varied cell diameter (between 2 -10 µm) (Yamamoto et al., 2004; Peña-Aguado et al., 2005; Havlik et al., 2013; Vandergheynst et al., 2013) and a dry weight of 27.0 pg $cell^{-1}$. *C. vulgaris* can be cultured in an organic medium (e.g. acetic acid as a carbon source), it grows faster, and it reaches a higher density (Maruyama and Hirayama, 1993). *N. oculata* on the other hand has been widely used in many aquaculture hatcheries to establish the initial step of an artificial food chain (Watanabe et al., 1979; Watanabe et al., 1983; Lubzens

et al., 1995; Ferreira et al., 2009) considering its comparative nutritional advantages over *C. vulgaris* or *C. calcitrans*. Some of these advantages are primarily high nutritional value, especially relatively large amounts of valuable lipids (Sukenik, 1991; Sukenik et al., 1993; Krienitz and Wirth, 2006; Kandilian et al., 2013; Santhosh and Anil, 2013) and its unique fatty acid composition, especially EPA, ARA and DHA (Lubzens et al., 1995; Low and Toledo, 2015). It consists of spheroidal or slightly ovoid cells (Gwo et al., 2005) approximately 2–3 µm in diameter (Heng and Pilon, 2014) and an estimated dry weight of to be 6.1 pg cell^{-1}. *C. calcitrans* is a single-cell microalgae species between 4 to 6 µm in diameter (Timmermans et al., 2001). Its dry weight is estimated to be 11.3 pg cell^{-1}

According to Rothhaupt (1990a, b), *B. calyciflorus* prefers microalgae of about 10 µm equivalent spherical diameter (ESD) (which is the diameter of a sphere which has a volume equivalent to the volume of the microalgae), while the smaller *B. rubens* prefers algae with an ESD of 5 µm but also ingests particles from 3.5 – 12 µm ESD equally. Therefore, the growth of rotifers is dependent on the size of the microalgae used to feed them (Hirayama et al., 1979; Cho et al., 2007). Moreover, the effect of different microalgal species on the proximate biochemical composition of the rotifer species *B. plicatilis* has been reported by various authors (Ben-Amotz et al., 1987; Whyte and Nagata, 1990; Whyte et al., 1994), demonstrating the importance of the biochemical composition of the algal diet in modulating the biochemical composition of the filter-feeders.

Feed types have been cited to have effects on the reproductive features of rotifers. Studies have suggested that algal food type could yield substantially different reproductive rates of rotifers (Hirayama et al., 1979; Yufera et al., 1983; Snell and Hawkinson 1983; James and Abu-Rezeq 1988; Korstad et al., 1989; Rothhaupt et al., 1990; Xi and Huang 1999; Xi et al., 2000; Garcia-Rodríguez and De La Cruz-Aguero, 2008). In this study, rotifers fed on the blended algal diet were exposed to a better dietary regime that enhanced egg production, hence the high proportion of egg-carrying rotifers and egg ratio observed. Özba et al. (2006) reported that egg-carrying female rotifer numbers increased continuously by feeding fresh microalgae (*Nannochloropsis* spp.) but experienced lower ratio when other algae species and yeast diets were used. The increase in the rotifer population depends on the initial production of eggs and is determined to a large extent by the quality of the food (Planas et al., 1989).

The reproductive performance observed in all the treatments were on a higher side in comparison to many studies done with the freshwater rotifers *B. calyciflorus*. Arimoro (2006) found that the reproductive rates of *B. calyciflorus* ranged between 27 – 52 % depending on the nutrient source. James (1989) found a reproductive rate between 2 – 78 % d^{-1}. In his study, freshwater *C. vulgaris* had the highest reproductive rate (52 %) while a mixed algal diet had the lowest reproductive rate (27 %). In contrast to Arimoro (2006) who only observed single eggs attached on the female rotifers, the present study included eggs which were suspended in the water column as well as eggs which were still attached on the female rotifers in the calculation of egg ratio. It was further observed that one single female rotifer, in many instances, carried more than one egg at the same time. It could be for these reasons that the egg ratios obtained in the present study were extremely high.

Although the current study did not study the microbial environment of the rotifer cultures, studies have established that the cultivation diet may also affect the microbial conditions of the live feed (Reitan et al., 1997; Sperfeld et al., 2012). Nour (2004) concluded that the application of microalgae as rotifer feeds bolstered rotifers with selected probiotics and/or antibiotics. Sorgeloos (1994) opined that the application of microalgae in rotifer feeding also improved the quality of rotifers, reducing bacterial contamination in fish larvae, and increasing their survival rate.

In conclusion, the mass culture of the freshwater rotifer, *B. rubens* is possible when they are fed on three microalgae species. The rotifers have a better growth performance when fed on a mixture of *N. oculata*, *C. vulgaris* and *C. calcitrans* as opposed to when these algae are offered as monodiets.

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