The Effect of Different Feed Types on the Growth Rate and Biochemical Composition of the Marine Ciliate, *Euplotes* sp.

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ABSTRACT

This research determined and compared growth rates and chemical composition of the marine ciliate, *Euplotes* sp. cultured with different diets under laboratory conditions. The different diets consisted of algae (*Isochrysis galbana, Tetraselmis suecica*), baker's yeast (*Saccharomyces cerevisiae*) and marine thraustochytrid, *Aurantiochytrium limacinum*, assigned as mono- or mixed diets with an initial concentration of 60,000 cells·ml^{-1}. Twenty single-cell *Euplotes* sp. were cultured in each treatment. Results revealed that growth rates of *Euplotes* sp. varied significantly ($p<0.05$) among different feed types. There were no significant differences for the highest mean growth rates of *Euplotes* sp. fed on the monoalgal diet *T. suecica* and the mixed diet consisting of *T. suecica, S. cerevisiae* and *A. limacinum*. Examination of the biochemical composition of *Euplotes* sp. fed on the mono diet of *S. cerevisiae* and *A. limacinum* showed that oleic acid (C18:1n9c) was dominant in the *S. cerevisiae* group, while palmitic acid (C16:0) was dominant in the *A. limacinum* group. The *A. limacinum* diet contained docosahexaenoic acid (C22:6n-3, DHA), but only a small percentage of this fatty acid was found in the ciliates fed with this diet. Eicosapentaenoic acid (C20:5n-3, EPA) was present in *Euplotes* sp. although it was not present in the *A. limacinum* diet. The amino acids cystine and tryptophan were present in the *S. cerevisiae* diet but were not detected in *Euplotes* sp. fed with it. Tryptophan was found in *Euplotes* sp. fed with the *A. limacinum* diet but was not found in the *A. limacinum* diet. The most abundant non-essential amino acids found in *Euplotes* sp. and in the assigned diets (*S. cerevisiae* and *A. limacinum*) were glutamic acid and serine.

Keywords: *Euplotes* sp., growth rate, biochemical composition

INTRODUCTION

The marine aquaculture industry can contribute considerably to economies of many countries (Naylor and Burke, 2005). The success of aquaculture industry is based on several factors and controls, including the use of an appropriate live feed during the early stage larvae (Jones *et al*., 1993; Watanabe and Kiron, 1994). Highly unsaturated fatty acids including eicosapentaenoic acid (20:5, n−3, EPA) and docosahexaenoic acid (22:6, n−3, DHA), play important roles in the development of the brain and central nervous system, maintenance of cell membrane structure and function, stress tolerance, and proper development and function of neural and visual systems in fish (Sargent *et al*., 1997). Since fish larvae show very high growth rates, primarily accomplished by increasing body

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muscle mass through protein synthesis and accretion, they require more dietary amino acids (Rønnestad *et al*., 1999). Traditionally, rotifers have been used as the initial food for marine fish larvae (Lubzens and Zmora, 2003). However, a diet consisting solely of rotifers as live feed does not fulfill the optimal requirements for growth or survival of the fish larvae (Conceição *et al*., 2010).

Marine ciliates are diverse and vary in sizes. They are important components of estuarine and coastal marine systems as they function as both bacterial consumers and prey for metazoan grazers such as fish larvae (Harvey *et al*.,1997; Figueiredo *et al*., 2007). Additionally, some ciliates perform the function of "essential lipid upgrading" and are reported to have de novo biosynthesis abilities, synthesizing fatty acids that are essential for aquatic animals (Harvey *et al*., 1997; Klein Breteler *et al*., 1999; Chu *et al*., 2008; Bec *et al*., 2010). The hypotrich ciliate, *Euplotes*, is commonly found in a broad range of habitats in both marine and freshwater ecosystems (Foissner *et al*.,1999). *Euplotes* is a relatively small protozoa, ranging from 60–110 µm (Madhu and Madhu, 2014; Mukai *et al*., 2016). Previous research have shown that *Euplotes* spp. can be successfully cultured using unialgal diets, for e.g. *Tetraselmis tetrathele* (Cheng *et al*., 2004), *Isochrysis galbana* (Dhanker and Hwang, 2013; Cheng *et al*., 2004), *Rhodomonas salina* (Drillet and Dutz, 2014); a rotifer culture diet (Selco, 3000); baker's yeast (Côrtes *et al*., 2013) and rice and starch gel (Zhukova and Kharlamenko, 1999). A few studies have reported on the biochemical composition of *Euplotes* spp. (Zhukova and Kharlamenko, 1999). Nagano *et al*. (2000) demonstrated that when the early larval stage of grouper, *Epinephelus septemfasciatus* was fed with *Euplotes* sp., the grouper showed a higher survival rate at 4–6 days after hatching. These results suggested that this ciliate could be an important alternate feed source, which could improve survival rates, especially for small-mouth marine fish larvae. Additionally, the biochemical composition of ciliates is of prime importance in understanding the nutritional benefits for growth and reproduction that metazoans obtain by consuming these protozoa. However, few studies have investigated the effects of nutrition on this ciliate (Cheng *et al*., 2004). The purpose of this study was to investigate the effect of different diets on the growth rate and biochemical composition of *Euplotes* sp.

MATERIALS AND METHODS

Culture condition

Marine ciliates *Euplotes* sp. were isolated from a rotifer culture. The ciliates were then kept in sterile filtered seawater (salinity 25±1 psu) at a temperature of 25±1˚C under a photon irradiance of 100 µmol m^{-2} sec^{-1} provided by a cool light fluorescent lamp with a 12:12 hour light and dark cycle. *Saccharomyces cerevisiae* was used as the diet for the ciliates.

Diet preparation

Cultures of microalgae, *Isochrysis galbana* and *Tetraselmis suecica* were kept in a Conway medium (Walne, 1974) under the same conditions as the *Euplotes* sp. culture. *Saccharomyces cerevisiae* (0.1 g in weight) was then put into 250 ml sterile filtered seawater (salinity 25±1 psu), at a temperature of 25±1˚C, at least 30 minutes before the start of the experiment. A commercial grade solution of marine thraustochytrid, *Aurantiochytrium limacinum* was used, which was diluted with sterile filtered seawater (salinity 25±1 psu) to obtain the desired density.

Growth rate experiment

A growth rate experiment was carried out for 24 hours. The selected individual single-cell *Euplotes* sp. were incubated in a microplate which contained 2 ml sterile filtered seawater. Twenty single-cell *Euplotes* sp. were assigned to each diet (Table 1).

The growth rate calculated by the equation followed Tarangkoon and Hansen (2011). Briefly, the growth/mortality rate of *Euplotes* sp. (µy) and diet cells (µx) were calculated assuming exponential growth/mortality:

Table 1. Diet, assignment of initial diet concentration and size (diameter)

Diet code	Diet	Concentration (cell·ml^{-1})	Diameter (µm)
TR 1	*I. galbana*	60,000	1.83±0.49
TR 2	*T. suecica*	60,000	3.43±0.44
TR 3	Baker's yeast (*S. cerevisiae*)	60,000	1.68±0.41
TR 4	*A. limacinum*	60,000	3.38±1.18
TR 5	*I. galbana* + *T. suecica*	30,000+30,000	
TR 6	*I. galbana* + *S. cerevisiae* + *A. limacinum*	20,000+20,000+20,000	
TR 7	*T. suecica* + *S. cerevisiae* + *A. limacinum*	20,000+20,000+20,000	

$$\mu_{y,x}\,(d^{-1}) = \frac{\ln N_1 - \ln N_0}{t}$$

where N_0 and N_1 are cell numbers at the start (t_0) and at the end (t_1) of the experiment respectively, and t is the duration of the experiment (d).

At the end of the experiment, 0.1 ml of 5% neutral formalin was added to each experimental well. The number of *Euplotes* sp. in each well was counted under an Olympus 70 IX inverted microscope.

Biochemical analysis

The mass cultures of *Euplotes* sp. were fed solely with *S. cerevisiae* or solely with the marine thraustochytrid, *A. limacinum* for biochemical composition analysis. The diet concentrations were set at 100,000 cells·ml^{-1} and *Euplotes* sp. were inoculated into a 1,000-liter fiberglass container at room temperature for two days. Later the ciliates were harvested by flocculation (Brown and Robert 2002). The flocculated *Euplotes* sp. were centrifuged at 3000 rpm for 10 minutes, and supernatant was drained. The flocculated concentrates in the centrifuge tubes were collected and stored at -18˚C for biochemical composition analysis. The flocculated samples and diets were transferred to the Central Laboratory (Thailand) Co. Ltd. Songkla Branch for biochemical analysis. Unsaturated fatty acids were obtained by modified AOAC method. and Compendium of Methods for Food Analysis (DMSc 2003). Amino acid compositions were analyzed according to Sarwar *et al*. (1988).

Data analysis

The growth rates of *Euplotes* sp. under different diets were tested by One-Way ANOVA. If significant differences ($p<0.05$) were found, Tukey's multiple comparisons test was used to determine specific differences among the treatments.

RESULTS AND DISCUSSION

Our study clearly indicated that *Euplotes* sp. was able to ingest all the different diet types because all diet sizes in this study (Table 1) were within the acceptable ranges as reported in previous studies. *Euplotes* spp. could capture and ingest food with diameters between 0.57–10 µm (Wilks and Sleigh, 1998; Drillet and Dutz, 2014). The growth rates of *Euplotes* sp. fed with different diets were significantly different (ANOVA, $p< 0.05$; Table 2). The highest growth rate, (5.12±0.32 d^{-1}) was achieved with the mixed diet of *T. suecica, S. cerevisiae* and *A. limacinum*, and there was no significant difference in ciliate growth rate when compared with the *T. suecica* diet (Tukey-HSD, $p<0.05$, n = 20). The lowest growth rate (2.15± 0.36 d^{-1}) occurred in *Euplotes* sp. fed only on *I. galbana*, and there was no significant difference in comparison with the ciliates fed on *S. cerevisiae* (Tukey-HSD, $p<0.05$, n = 20). Higher growth rates for *Euplotes* sp. were achieved by diets used in this study than from the following diets used in other studies, namely: *Nannochloropsis* spp. (Ushilo *et al*., 1998), *Dunaliella* sp. (Goltz *et al*.,

Table 2. Mean (±SD) growth rate of *Euplotes* sp. fed on different diets. Data labelled with different superscript are significantly different (Tukey's HSD, $p<0.05$ following ANOVA). Data are means of 20 replicates per treatment.

Diet code	Diet	Growth rate (d^{-1})
TR 1	*I. galbana*	2.15 ± 0.36^{d}
TR 2	*T. suecica*	5.12 ± 0.32^{a}
TR 3	Baker's yeast *(S. cerevisiae)*	2.24 ± 0.30^{d}
TR 4	*A. limacinum*	3.54 ± 0.91^{c}
TR 5	*I. galbana* + *T. suecica*	4.58 ± 0.28^{b}
TR 6	*I. galbana* + *S. cerevisiae* + *A. limacinum*	3.87 ± 0.23^{c}
TR 7	*T. suecica* + *S. cerevisiae* + *A. limacinum*	4.99 ± 0.28^{a}

2015), bacterium *Pseudomonas* sp. (Turley *et al*., 1986), and yeast (Côrtes *et al*., 2013).

The biochemical composition of ciliates is an important element in understanding the nutritional benefits for growth and reproduction that metazoans gain from the consumption of ciliates (Harvey *et al*., 1997). Therefore, this research aimed to identify an alternative food for ciliate culture which would be easy to prepare and would have a low production cost. Baker's yeast (*S. cerevisiae*) has a high protein content and can be used for rotifer culture, while marine thraustochytrid (*A. limacinum*) has a high level of docosahexaenoic acid (C22:6n-3, DHA). Research findings from Arafiles *et al*. (2011) showed that *Aurantiochytrium* sp. produced a high amount of docosahexaenoic acid (C22:6n-3, DHA), comprising up to 22.5% of its fatty acid content. Therefore, we chose the following two diets, *S. cerevisiae* and *A. limacinum*, instead of phytoplankton for the mass culture of *Euplotes* sp. The major fatty acids of *Euplotes* sp. and its diets (*S. cerevisiae* and *A. limacinum*) contained saturated and monounsaturated fatty acids (Table 3). Among these, oleic acid (C18:1n9c) was dominant in *S. cerevisiae* and *Euplotes* sp. grown in *S. cerevisiae* culture, while palmitic acid (C16:0) was dominant in *A. limacinum* and *Euplotes* sp. grown in *A. limacinum* culture. *Aurantiochytrium limacinum* contained docosahexaenoic acid (C22:6n-3, DHA), but *Euplotes* sp. fed on *A. limacinum* exhibited a low percentage of this fatty acid. Eicosapentaenoic acid (20:5, n−3, EPA) was not present in *A. limacinum* but in *Euplotes* sp. fed on *A. limacinum*. Our findings were similar to Harvey *et. al*. (1997), who found that the marine ciliates *Pluoronema* sp. and *Farea salina* showed a fatty acid composition similar to their diets in general. *Euplotes* sp. contains essential fatty acids, and the results revealed that some polyunsaturated fatty acids, i.e. eicosapentaenoic acid and arachidonic acid (C20:4n6, ARA), were found in *Euplotes* sp. fed on *A. limacinum* but were not found in its diet (Table 3). Zhukova and Kharlamenko (1999) and Drillet and Dutz (2014) found that the ratio of DHA to EPA significantly affects the survival of marine fish larvae. *Euplotes* sp. fed with *A. limacinum* showed a DHA to EPA ratio of 1.44, which was in the preferred range (1–2) for fish larval nutrition (Rodríguez *et al*., 1998). An EPA/ARA ratio of 1.38 was found in *Euplotes* sp. fed on *A. limacinum*. However, Bell *et al*. (2003) suggested that improvements in dorsal pigment in turbot and halibut can be achieved by providing an EPA/ARA ratio >5:1. Although the exact requirements and effects of ARA in relation to EPA and DHA are not fully understood, they are likely to be species specific (Dhont and van Stappen, 2003). These indicate that *Euplotes* sp.

Table 3. Fatty acid composition (% total fatty acids) in diets (*S. cerevisiae* and *A. limacinum*) and diet-depleted cultures of *Euplotes* sp. Data are averages from two analyses (n=2).

Fatty acid	% Content			
	S. cerevisiae	*Euplotes* sp. fed *S. cerevisiae*	*A. limacinum*	*Euplotes* sp. fed *A. limacinum*
Myristic acid (C14:0)	0.22	0.60	4.46	4.74
Myristoleic acid/ Tetradecenoic (C14:1)	0.00	1.33	0.00	1.17
Pentadecanoic acid (C15:0)	0.21	0.53	5.14	3.96
cis-10-Pentadecenoic acid (C15:1)	0.13	0.36	0.00	0.00
Palmitic acid (C16:0)	17.41	16.32	80.53	73.11
Palmitoleic acid/ Hexadecenoic (C16:1)	24.71	18.60	0.00	2.47
Heptadecanoic acid/Margaric (C17:0)	0.55	1.16	2.17	1.62
cis-10-Heptadecenoic acid/Margaroleic (C17:1)	0.93	1.11	0.00	0.00
Stearic acid (C18:0)	13.27	10.71	2.81	2.87
Elaidic acid (C18:1n9t)	0.20	0.00	0.00	0.00
Oleic acid (C18:1n9c)	34.01	41.68	0.19	3.92
Linoleic acid/Octadecadienoic (C18:2n6c)	7.11	1.40	0.00	0.00
Linolenic acid (ALA) (C18:3n3)	0.11	0.00	0.00	0.00
Arachidic acid (C20:0)	0.13	0.86	0.35	0.73
cis-11,14-Eicosadienoic acid (C20:2)	0.67	0.00	0.00	0.00
Arachidonic acid (C20:4n6) (ARA)	0.00	0.00	0.00	0.67
cis-5,8,11,14,17-Eicosapentaenoic acid (EPA) (C20 : 5n3)	0.00	0.00	0.00	0.92
Behenic acid (C22:0)	0.10	0.68	0.00	1.19
Erucic acid/Docosaenoic (C22:1n9)	0.00	3.45	0.00	1.28
cis-13,16-Docosadienoic acid (C22:2)	0.18	0.00	0.00	0.00
Lignoceric acid (C24:0)	0.05	0.00	0.17	0.00
Nervonic acid (C24:1)	0.00	1.21	0.00	0.00
cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA) (C22:6n3)	0.00	0.00	4.19	1.33
DHA/EPA ratio				1.44
EPA/ARA ratio				1.38
Saturated fatty acid	31.95	30.86	95.62	88.22
Monounsaturated fatty acid	59.98	67.74	0.19	8.85
Polyunsaturated fatty acid	8.07	1.40	4.19	2.92

is capable of assimilating and possibly synthesizing some fatty acids. However, Bec *et al*. (2010) suggested that environmental factors (such as temperature, O_2, salinity, auto-, mixo-, or heterotrophic nutrition) could also profoundly alter the fatty acid composition of protists.

A few studies have focused on the importance of amino acids in zooplankton (e.g., Kleppel *et al*., 1998; van der Meeren *et al*., 2008). Our findings show that Euplotes sp. have higher concentrations of essential amino acids than of nonessential amino acids. There are high concentrations of lysine and arginine in *Euplotes* sp. and in diets consisting of *S. cerevisiae* and *A. limacinum*. Lysine is a substrate for the synthesis of carnitine, which is required for the transport of long-chain fatty acids from the cytosol into the

mitochondria for oxidation (Li *et al*., 2008), while arginine appears in the protein and tissue fluid of fish as phosphoarginine, a major reservoir of ATP with limited or completely absent de novo synthesis (Li *et al*., 2008). The composition of essential amino acids in *Euplotes* sp. basically resembles the composition of its diet. Previous researches have revealed that the amino acid composition of mesozooplankton predators appears rather constant, and more or less independent of the amino acid composition of their diet (Frolov *et al*., 1991; Cowie and Hedges, 1994; Guisande *et al*., 1999; Guisande *et al*., 2000; Helland *et al*., 2003a, b). Our findings reveal that cystine and tryptophan were absent in the ciliates but present in the *S. cerevisiae* diet. Tryptophan was found in *Euplotes* sp. culture but was absent in the *A. limacinum* diet (Table 4). The most abundant non-essential amino acids in *Euplotes* sp. and its diets were glutamic acid and serine. Moreover, glutamine was found in *Euplotes* sp. although it was not found in the *S. cerevisiae* diet. These findings indicate that *Euplotes* sp. is capable of assimilating and possibly synthesizing some amino acids. However, the de novo biosynthesis abilities of *Euplotes* sp. require further investigation.

Table 4. Amino acid profile and content of *S. cerevisiae* and *A. limacinum* used as diets for *Euplotes* sp. and diet-depleted cultures of *Euplotes* sp. Data are averages from two analyses (n=2).

Amino acid	**Amino acid content (mg·100g^{-1} DW)**			
	S. cerevisiae	***Euplotes* sp. fed *S. cerevisiae***	***A. limacinum***	***Euplotes* sp. fed *A. limacinum***
Essential amino acids				
Valine	1,520.9	110.61	131.02	117.47
Methionine	308.78	168.11	104.44	78.12
Lysine	4,316.49	172.05	156.31	103.12
Isoleucine	1,337.03	146.81	155.11	148.75
Leucine	2,257.7	151.18	179.44	136.88
Phenylalanine	1,332.55	150.60	164.98	156.93
Threonine	1,035.45	124.62	137.24	114.02
Cystine	240.76	0.00	0.00	0.00
Cysteine	0.00	0.00	0.00	0.00
Tryptophan	332.13	0.00	0.00	178.60
Histidine	776.08	128.40	132.62	119.45
Arginine	1,029.35	493.21	560.77	361.95
Total essential amino acids	14,487.20	1,645.57	1,721.92	1,515.27
Non-essential amino acids				
Aspartic acid	1,570.28	118.53	126.45	103.01
Serine	939.86	172.85	189.4	165.62
Glutamic acid	2,122.31	158.16	245.95	142.9
Glycine	1,058.06	121.89	144.77	121.1
Alanine	1,501.03	123.23	171.21	128.25
Proline	1,088.28	162.09	183.745	155.46
Tyrosine	1,046.91	130.37	145.81	143.8
Glutamine	0.00	158.16	0.00	0.00
Total non-essential amino acids	9,326.715	1,145.25	1,207.38	960.14
Total amino acids	23,813.91	2,790.82	2929.29	2,475.41

CONCLUSION

The findings from the present study indicate that the marine ciliate *Euplotes* sp. can feed on a variety of diets, including microalgae (*I. galbana* and *T. suecica*), baker's yeast (*S. cerevisiae*), and marine thraustochytrids (*A. limacinum*). *Euplotes* sp. shows a capacity for de novo biosynthesis of some amino acids and some essential fatty acids. A mixed diet of *T. suecica, S. cerevisiae* and *A. limacinum* is the most suitable diet for culturing *Euplotes* sp.

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