

Population growth of a tropical tintinnid, *Metacyclis tropica* on different temperature, salinity and diet

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수온, 염분 및 먹이에 따른 열대 유충류, *Metacyclis tropica*의 성장

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Abstract This study investigated the effects of temperature, salinity, and algal diet to find the optimal conditions for 5 days for the mass culture of the tropical tintinnid, *Metacyclis tropica*. This tintinnid had a small, hyaline, and ovoid lorica. The oral diameter, length, and maximum width of the lorica were 36.7 μm , 49.5 μm , and 44.5 μm , respectively. In the temperature experiments, the highest maximum density and population growth rate were observed at 30°C with 340.7 cells/mL and 1.1/day, respectively. Lower salinities adversely affected the population growth of *M. tropica*. The maximum density was observed at 33 ppt (840 cells/mL). In the diet experiments, *M. tropica* fed *Isochrysis galbana* showed the highest density (413 cells/mL) and population growth rate (1.2/day). As a result, *M. tropica* is appropriate as a potential prey organism for early fish larvae with smaller mouths because the tintinnid has a relatively small size compared to the rotifer. In addition, the conditions of 30°C, 33 ppt and supplying *I. galbana* would be effective in the cultivation of *M. tropica*.

요약 본 연구는 열대 유충류인 *Metacyclis tropica*의 최적배양조건을 구명하기 위해 5일 동안 수온, 염분 및 먹이원인 미세 조류의 효과를 조사하였다. *M. tropica*는 작고 투명한 난형의 피갑을 가지며, 피갑의 구강부 지름, 갑장 및 갑폭의 크기는 각각 36.7 μm , 49.5 μm 및 44.5 μm 였다. 수온별 실험에서, *M. tropica*의 최고밀도와 성장률은 수온 30°C에서 각각 340.7 cells/mL와 1.1/day로 나타났다. 낮은 염분은 *M. tropica*의 성장에 악영향을 미쳤으며 염분 33 ppt에서 최고밀도가 840 cells/mL로 나타났다. 먹이실험에서, 먹이로 *Isochrysis galbana*를 공급하였을 때, 가장 높은 성장을 보였다(배양밀도, 413 cells/mL; 성장률, 1.2/day). 본 실험을 종합하여보면, *M. tropica*는 rotifer에 비해 작은 크기를 가지기 때문에 자어기에 입이 작은 어류의 초기먹이생물로 적합하며, 수온 30°C, 염분 33 ppt에서 *I. galbana*를 먹이로 공급하는 것이 *M. tropica*의 대량배양을 위한 최적배양조건인 것으로 판단된다.

Keywords : Diet, *Metacyclis tropica*, Optimum culture condition, Salinity, Temperature, Tintinnid

1. Introduction

Planktonic ciliate, tintinnids are important grazers of phytoplankton and are links between nanophytoplankton and larval fish or crustaceans in marine food web[1]. The organisms are found in the gut of marine

larval fish such as clupeids, gadids, flat fish, sciaenids, acanthurids and ammondityidae, particularly first-feeding larvae compared to old larvae prefer to feed on tintinnids[2]. In the study of Nagano et al. (2001), the aggregation of tintinnids in marine improved survival of first-feeding Japanese sand lance (*Ammodytes*

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personatus) larvae[3]. In addition, the survival of grouper (*Epinephelus septemfasciatus*) larvae was enhanced by presence of tintinnid as a live feed in laboratory study[4]. Thus, tintinnids have been considered promising candidates for mass production as main or additional live feeds for fish larvae in aquaculture[5]. However, little is known about the skill of mass culture of tintinnids.

We initially isolated the planktonic tintinnid, *Metacylis tropica* on a tropical coast located in Chuuk Lagoon, Micronesia near the equator. The tintinnid has relatively small size compared to rotifer (*Brachionus rotundiformis*, SS-type), provides advantages as being live food for marine fish larvae with small mouths[6]. Generally, suitable prey size to be ingested by larval fish is determined by larval mouth size[7].

The purpose of this study was to examine the effect of temperature, salinity and diet on the population growth of *M. tropica* to find optimum culture condition of the tintinnid which has never been reported before on its culture. In addition, we analyzed molecular taxonomy to identification of the tintinnid. These results provide basic information on the mass culture of the tintinnid which is used as initial livefeed for marine fish larvae with a small mouth.

2. Materials and Methods

2.1 Isolation, identification and maintenance of the tintinnid

M. tropica used in this study were collected using plankton net (mesh size: 80 μm) in Chuuk Lagoon, Micronesia (7°27'07"N, 151°53'52"E) with temperature 29-32°C, salinity 30-33 ppt. The collected samples were carried rapidly to the laboratory in Korea-South Pacific Ocean Research Center, KIOST and isolated. Observations were made through the use of an inverted phase contrast microscope equipped with an ocular micrometer and a microphoto system at a magnification of 400X. Thirty individuals were fixed in 4% neutral

buffered formalin solution and measured their sizes. The tintinnid was identified with morphological characteristics of lorica such as shape, length, maximal width and oral diameter[8].

For molecular taxonomy, total genomic DNA from the tintinnid was extracted using the DNeasy Blood & Tissue Kit (Qiagen). The partial sequence of 18S rDNA was determined using primers 18S-Tin3F: 5'-GCGGTATTTATTAGATAWCAGCC-3' and 28S-TinR1: 5'-TGGTGCACTAGTATCAAAGT-3' [9]. Polymerase chain reaction and DNA sequencing were performed using slightly modified methods described by Lee et al. (2014)[10]. The newly determined partial sequence was deposited in GenBank with accession number KP883283 and compared to GenBank database sequences. For species identification, nucleotide similarities with pairwise distance value were determined using MEGA 5.2[11].

Isolated *M. tropica* were cultured with the 250 mL Erlenmeyer flask contained 33 ppt filtered (0.2 μm) and autoclaved natural sea water. These were cultured with food mixture of *Isochrysis galbana* ($\sim 1 \times 10^5$ cells/mL) and *Tetraselmis tetrahele* ($\sim 2 \times 10^4$ cells/mL) at $30 \pm 1^\circ\text{C}$.

2.2 Preparation of algal diets

The prymnesiophyte *I. galbana* (ISO: 3.2×5.3 μm), Green *Isochrysis* sp. (GISO: 3.7×3.5 μm), *Pavlova lutheri* (PAV: 5.5×3.7 μm), the prasinophyte *Tetraselmis tetrahele* (TET: 10.2×6.6 μm), Eustigmatophyte *Nannochloropsis oculata* (NAN: 2.6×2.6 μm), *Synechococcus* sp. (SYN: 1.1×1.2 μm), the diatom *Chaetoceros simplex* (CHA: 4.2×6.1 μm), were used as feed for the tintinnid. These algae were cultivated in Walne's medium, using filtered (1 μm mesh of glass fiber filter) and autoclaved seawater. The cultures were incubated at room temperature (20-23°C), under continuous light (4000 lx). Algae were fed to the tintinnid at the mid-to-late logarithmic phase.

2.3 The culture of the tintinnid

This study was divided into 3 parts: the effect of temperature, salinity and food. In all parts of experiments, *M. tropica* was inoculated at 3 cells/mL into 250 mL Erlenmeyer flask contained 150 mL of filtered (1 μm mesh of glass fiber filter) and autoclaved seawater. They were cultured under natural illumination and daily replenished *I. galbana* at 2×10^5 cells/mL for 5 days. Firstly, to investigate the effect of temperature, temperature was adjusted at 22, 26, 30 and 34°C and salinity was fixed at 33 ppt. The salinity effect was secondly investigated. The salinity was adjusted at 10, 15, 20, 25, 30 and 33 ppt and temperature was fixed at 30°C. These salinities were prepared by diluting seawater with distilled water. In the last experiment, the diet effect was investigated using mentioned seven microalgae. Before the test began, tintinnids were placed in the fresh medium containing each diet to remove the impact of diet history for 24 hours using 20 μm sieve. The volume of ISO (2×10^5 cells/mL) was used as standard to keep the equal biomass of diets in the vessel. Each treatment was replicated three times and every 12 hour recorded the number of tintinnid in 3 mL sub-samples under a stereomicroscope. Population growth rate (r) was calculated from following equation:

$$r = (\ln N_t - \ln N_i) / t$$

where, t is culture days with maximum density when tintinnid density (cell/mL) was the highest, N_i and N_t are the initial and highest tintinnid density, respectively.

2.4 Statistical analysis

Data were analyzed by one-way ANOVA. If significant ($P < 0.05$) difference was found in the ANOVA test, Duncan's multiple range test was used to rank the groups. Data are presented as mean \pm SE (standard error). All statistical analyses were conducted using SPSS program Ver. 12.0 (SPSS Inc.,

Michigan Avenue, Chicago, Illinois, USA)

3. Results and Discussion

3.1 Morphological characteristics and molecular taxonomy of the tintinnid

The oral diameter, length and maximum width of the tintinnid used in this study were $36.7 \pm 0.8 \mu\text{m}$, $49.5 \pm 5.5 \mu\text{m}$ and $44.5 \pm 1.1 \mu\text{m}$, respectively ($n=30$). The collar was short (7–8 μm) with 2 annuli (Fig. 1A). The shape of the aboral end was slightly pointed (Fig. 1B). According to the morphological characteristics of Durán (1957), the tintinnid was identified as *M. tropica*[8].

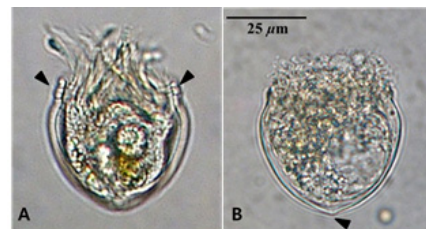


Fig. 1. *Metacylis tropica*. A, the focus is at the front of the tintinnid to find the annuli of collar (arrows indicate); B, fixed individual with formalin and an arrow indicate slightly pointed aboral end.

In addition, we analyzed the 18S rDNA gene to identify the tintinnid used in this study. The newly determined sequences (1490 bp long) were compared to GenBank sequences. The closest matches (99.6% similarity) in GenBank using BLAST search was the 18S rDNA sequence of *M. angulata* (GenBank accession numbers AY143568), followed by 99.4% similarity with *M. pithos* (JX101862).

3.2 The culture of the tintinnid

In the temperature experiments, the highest maximum density and population growth rate were found at 30°C with 340.7 cells/mL and 1.1/day ($P < 0.001$), followed by 26°C with 131.7 cells/mL and 0.8/day respectively, but the treatment at 22°C kept

low density with 1 to 7 cells/mL, even *M. tropica* at 34°C showed negative growth and eventually died on the 3.5th day (Table 1, Fig. 2A).

The population growth of tintinnids is regulated by not only temperature but also food availability. According to Stoecker et al. (1983) and Verity (1985), the growth rates of *Favella* sp., *Tintinnopsis vasculum* and *T. acuminata* increased with increasing temperature [12,13]. In the present study, *M. tropica* showed a similar pattern to the above.

Lower salinities negatively affected the population growth of *M. tropica*. The maximum density was shown at 33 ppt (840 cells/mL), and significantly higher than others. While the population growth rate was the highest at 30 ppt ($P < 0.001$), and the lowest at 20 ppt; they could not grow (Table 1, Fig. 2B). The maximum density and population growth rate of the tintinnid in this study were relatively higher than those of other tintinnid culture studies that were reported during the past several decades (Table 2).

Tintinnids are algivorous filter-feeder [4,14] but not all microalgae are used by the organisms. In this study, *M. tropica* fed ISO showed the highest density and population growth rate (413 cells/mL and 1.2/day, $P < 0.001$). These results are similar to those of Graham and Strom (2010), who reported that non-toxic prey ISO support the growth of *Metacylis* sp. [15]. However, the tintinnid fed NAN died on day 3.5 (Table 1, Fig. 2C). It is known that ciliates are not able to digest phytoplankton with hard cell walls such as NAN because ciliates have no jaw which can break hard cell walls as a rotifer [16]. Picoplankton is important as a food source for microzooplankton, in practice, the cyanobacteria SYN is the most frequently observed food in the tintinnid vacuoles [17].

On the contrary, *M. tropica* showed the lowest population growth when they fed on SYN. This can be attributed to the toxicity of SYN or food size preference of *M. tropica*. It is known that SYN have the toxicity to *Eutintinnus* sp. and *Metacylis* sp. [18].

Table 1. Growth of *Metacylis tropica* cultured at different conditions

Experiments	Maximum density (cells/mL)	Population growth rate (/d)
Temperature (°C)	22	7.0 ± 0.58 ^a
	26	131.7 ± 5.36 ^b
	30	340.7 ± 31.20 ^c
	34	8.0 ± 2.65 ^a
Salinity (ppt)	10	- ¹
	15	-
	20	5.3 ± 0.33 ^a
	25	183.3 ± 25.10 ^b
	30	596.7 ± 29.63 ^c
	33	840.0 ± 46.19 ^d
	ISO	413.3 ± 48.48 ^c
Diet	TET	129.3 ± 42.73 ^b
	GISO	84.7 ± 14.81 ^{ab}
	PAV	45.3 ± 11.68 ^{ab}
	CHA	5.3 ± 0.33 ^a
	NAN	-
	SYN	4.3 ± 0.33 ^a

¹Negative population growth rate.

ISO, *Isochrysis galbana*; GISO, Green *Isochrysis* sp.; PAV, *Pavlova lutheri*; TET *Tetraselmis tetrahele*; NAN, *Nannochloropsis oculata* SYN, *Synechococcus* sp.; CHA, *Chaetoceros simplex*.

^{a,b,c}Values (Mean±SE) within a column with different superscript letters are significantly different ($P < 0.05$).

On the other hand, tintinnids are known as a selective filter feeder based on prey size [17]. In case of the genus *Favella*, the organism tends to have a preference for larger sized prey. In the study of Stoecker et al. (1995), 10 µm particles (equivalent spherical diameter) were captured more efficiently than smaller particles (4 µm) by *Favella* sp. [19]. Moreover, Kamiyama and Arima (2001) reported that the prey selectivity of *F. taraikaensis* is higher for larger algae than the smaller algae such as PAV under mixed prey conditions [14]. Preferred prey size is related to lorica oral diameter [20] and the maximum ingested food size is up to 40-45% of the oral diameter of the lorica in tintinnids (Spittler 1973). Therefore, *M. tropica* can ingest the prey with the maximum size of 16.5 µm in this study. In the case of CHA, it has the suitable size of 4.2×6.1 µm to be ingested, while it would be difficult to be taken by *M.*

tropica due its long seta (approximately 24 μm) which is over the size limit.

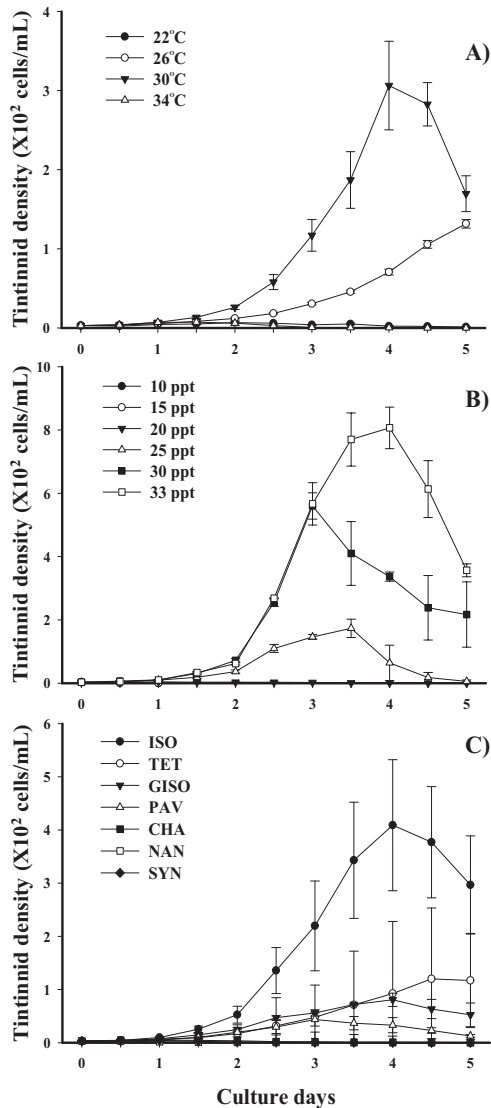


Fig. 2. Variation of population growth against temperature (A), salinity (B) and diet (C) in the tintinnid, *Metacylis tropica*. Vertical bars describe standard error.

Despite GISO and PAV have similar size to ISO, the growth of *M. tropica* fed the two diets were lower than that of the tintinnid fed ISO. This may be due to the difference of prey quality such as nutrition, digestibility and so on. Further study is required to

understand the cause of the difference.

Table 2. Growth rates (GR) and maximum densities (MD) of tintinnids in laboratory experiments

Species	GR (/d)	MD (ind./mL)	Diet	Temp. (°C)	Source
<i>Favella</i> sp.	1.39		<i>Heterocapsa triquetra</i>	20	[12]
<i>T. acuminata</i>	2.00 ^b	<10	<i>I. galbana</i>	20	[13]
<i>T. vasculum</i>	1.10 ^b	<10	<i>Dicrateria inornata</i>	15	[13]
<i>T. beroidea</i>	~0.46 ^a	720	<i>Gymnodinium</i> sp., <i>Paviova lutheri</i>	22	[22]
<i>Amphorella quadrilineata</i>	~0.46 ^a	437	<i>Gymnodinium</i> sp., <i>P. lutheri</i>	22	[22]
<i>F. taraiakaensis</i>	1.19		<i>Alexandrium tamarense</i>	15	[23]
<i>F. taraiakaensis</i>	1.31		<i>H. triquetra</i>	15	[23]
<i>Metacylis tropica</i>	1.70	840	<i>I. galbana</i>	30	present study

^a Estimated from figure of sources. ^b Recalculated from the value of source by the equation used in present study.

4. Conclusion

The conditions of 30°C, 33 ppt and supplying *I. galbana* would be effective in the cultivation of *M. tropica*. These results indicate that *M. tropica* is appropriate for being a test organism and a potential prey organism for early fish larvae with smaller mouths. Further studies including the effects of additional physical and biological factors are required to establish a stable method of mass culturing the tintinnid.

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