

# Food Effect on the Diel Variations and Starvation of the Melania Snail *Semisulcospira gottschei* Using RNA/DNA Ratios

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## Abstract

We investigated the nutritional status of the melania snail (*Semisulcospira gottschei*) using RNA/DNA ratios to evaluate the effect of feeding conditions (artificial versus natural) on the reaction times of the snails in a time course following starvation. In the short experiments (48 h), the RNA/DNA ratios of the artificial feeding groups were significantly higher than those of the natural groups. While two RNA/DNA ratio peaks were observed in the artificial food group during daytime, the natural food group showed a higher ratio at night. Under starvation conditions, the RNA content decreased whereas the DNA content was constant. The RNA/DNA ratios of the freshwater snail in both groups dramatically decreased after starvation and remained constant until the end of the experiment. We verified that the RNA/DNA ratio serves as an index of nutritional condition with respect to the effect of dietary differences. These results are important for understanding optimized aquaculture rearing conditions for this important commercial freshwater snail.

**Key words:** Nutrition, Melania snail, RNA/DNA ratio, Starvation, *Semisulcospira gottschei*

## Introduction

Body condition indices and morphometric measurements (length and weight) are relatively insensitive to and unreliable for describing the physiological or nutritional status of an organism (Fathallah et al., 2010). One can assess the physiological and nutritional status of bivalves by examining a variety of biochemical parameters or indices of instantaneous growth, usually expressed as ratios of nucleic acids (RNA/DNA). Nucleic acids play a major role in growth and development. While DNA content is stable under changing environmental conditions, and is thus used as an indicator of biomass and cell number (Dortch et al., 1983), RNA is directly involved in protein biosynthesis and growth is dependent on the amount of available RNA (Clemmesen, 1987). Food availability tends to be the main factor affecting growth and thus the RNA/DNA ratio in bivalves. Several studies have reported that the ratio of RNA/DNA is a reliable and useful index of nutritional condition and environmental stress in mollusks (Joyner-Matos

et al., 2007; Heilmayer et al., 2008), crustaceans (Anger and Hirche, 1990; Chicharo et al., 2007), and fish. Furthermore, the RNA/DNA ratio is correlated with food quality (Naimo et al., 2000; Vrede et al., 2002) and nutrition level (Whyte et al., 1990), and it decreases rapidly during periods of starvation (Bracho et al., 2000; Chicharo et al., 2001).

Freshwater snails of the genera *Semisulcospira*, *Koreanomelania*, and *Koreoleptoxis* are widely distributed in rivers, streams, and lakes (Davis, 1969). They have long been used as a healthy food source and provide an economically valuable fishery resource in Korea (Lee et al., 2005). Currently, wild populations of this species are declining due to over-exploitation as well as environmental deterioration caused by sewage, pesticides, and the restructuring of riverbeds. Three freshwater snail genera comprising nine species in the family Pleuroceridae are reported from Korea (Choi and Yoon, 1997). They are commonly classified as Gastropoda, Proso-

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branchia, Mesogastropoda. The genus *Semisulcospira* comprises the most common species, which are distinguishable by their brood pouch, in which embryos are raised (Lee et al., 2007). These species ingest epilithic algae, microorganisms, and particulate organic matter derived from other organisms. Invertebrates including snails play an important role in freshwater ecosystems by providing food for fish and other aquatic organisms, as well as for the cycling of organic matter. Also, studies have suggested that freshwater invertebrates can serve as water-quality indicators (Duft et al., 2003; Wade et al., 2008). Therefore, recent efforts initiated by the government of Korea have aimed to enhance the natural stocks of freshwater invertebrates, including snails, by releasing young snails to several river systems.

The goals of this study were to gain information regarding suitable rearing conditions for a valuable aquaculture species, *S. gotteschei*. Our specific aims were to evaluate whether the RNA/DNA ratio is suitable for determining the nutritional conditions of the freshwater snail *S. gotteschei*, examine diel periodicity on the RNA/DNA ratio of the snails, and evaluate the impact of starvation on snail growth.

## Materials and Methods

### Short-term rearing experiments

Short-term rearing experiments for the melania snail, *S. gotteschei*, were designed for two different culture conditions. The average body weight was  $567 \pm 81$  mg ( $n = 200$ ). The artificial food group was fed an artificial pellet diet (freshwater snail feed EP-1S; Suhyup Feed, Uiryeong, Korea) daily each morning (09:00 h) and supplied with 10  $\mu$ m filtered freshwater (20 cycles/day). This group was termed the “artificial group.” The natural food group was offered natural food by supplying fresh river water (20 cycles/day) without additional artificial food. Each group was reared in duplicate tanks. Other environmental conditions such as water temperature ( $20 \pm 0.5^\circ\text{C}$ ), dissolved oxygen (8.0-9.0 mg/L), and photoperiod (12 L:12 D) were identical between groups. Five snails were sampled from each group at 4-h intervals for 48 h. The wet weight was measured, after which the snails were stored at  $-80^\circ\text{C}$  (#907; Thermo Fisher Scientific, Marietta, OH, USA) until further analysis.

### Starvation experiment

A starvation experiment was performed in parallel with the rearing experiment to investigate the effect of fed vs. starved snails. In total, 200 snails were randomly transferred into each plastic tank (100 L) with filtered freshwater (10  $\mu$ m) from each rearing tank (maintained with artificial food or with the natural food organism assemblages from a pond). Each treatment consisted of two replicate tanks. All experimental condi-

tions were maintained equally. Sampling from the starvation treatments was performed daily each morning (10:00 h) for 25 days. Five snails were taken from each aquarium and their wet weight determined. The snail samples were stored at  $-80^\circ\text{C}$  for biochemical analysis.

### Nucleic acid extraction

For biochemical analysis, muscle tissue was dissected from the thawed snails, and the total nucleic acids were extracted from each sample individually by homogenization with a tissue grinder. During homogenization, the glass tube was kept cold by immersion in a plastic beaker with wet-ice. The SPRI-TE Nucleic Acid Extractor (Beckman Coulter, Fullerton, CA, USA) system was used for total nucleic acid extraction following the manufacturer’s instructions ([http://www.beckmangenomics.com/documents/products/SPRI\\_TE\\_OpMan.pdf](http://www.beckmangenomics.com/documents/products/SPRI_TE_OpMan.pdf)). This is a fully automated system capable of extracting nucleic acids from a variety of sample types including plasma, serum, viral transport media, formalin-fixed paraffin-embedded tissue, and entire blood samples. The instrument is equipped with magnetic filtration technology. Reagent cartridges contain a lysis solution, a binding reagent, a washing buffer, and an elution buffer.

### Quantification of nucleic acids

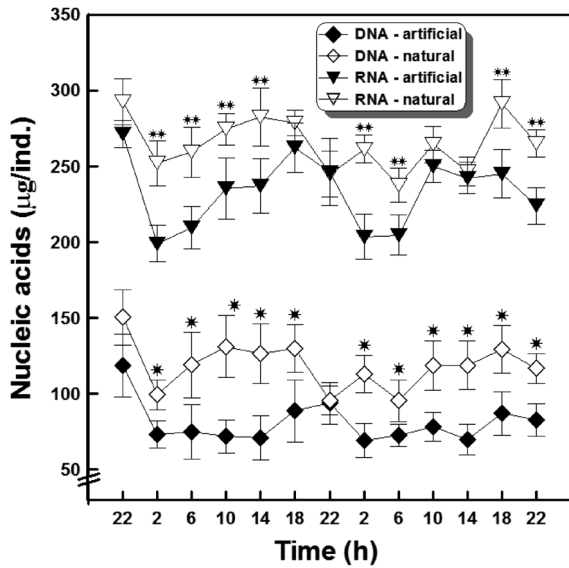
Fluorometric assays for the quantification of nucleic acids were based on the method described by Schmidt and Ernst (1995). Two aliquots (RNA and DNA) of the nucleic acid solution were processed in parallel. To the RNA aliquot (5  $\mu$ L), 5  $\mu$ L DNase solution (DNase I, RNase-free, 10  $\mu$ g/ $\mu$ L; Roche, Darmstadt, Germany) and 40  $\mu$ L DNase buffer (40 mM Tris-HCl, pH 7.9, 19 mM NaCl, 6 mM  $\text{MgCl}_2$ , 10 mM  $\text{CaCl}_2$ ) were added. To the DNA aliquot (5  $\mu$ L), 2  $\mu$ L RNase buffer (Roche), 5  $\mu$ L RNase solution (RNase, DNase -free, 0.5  $\mu$ g/ $\mu$ L; Roche) and 33  $\mu$ L nuclease-free water were added. After incubation for 1 h at  $37^\circ\text{C}$ , the reactions were terminated by cooling the solutions on ice. The concentrations of both RNA and DNA were estimated using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ, USA).

### Statistical analysis

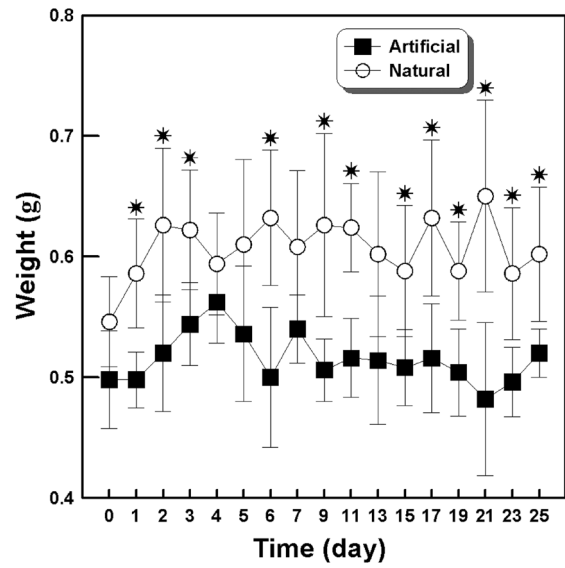
Sample means were compared using an unpaired independent sample *t*-test between the two experimental groups. Differences were considered significant at  $P < 0.05$ . All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

## Results

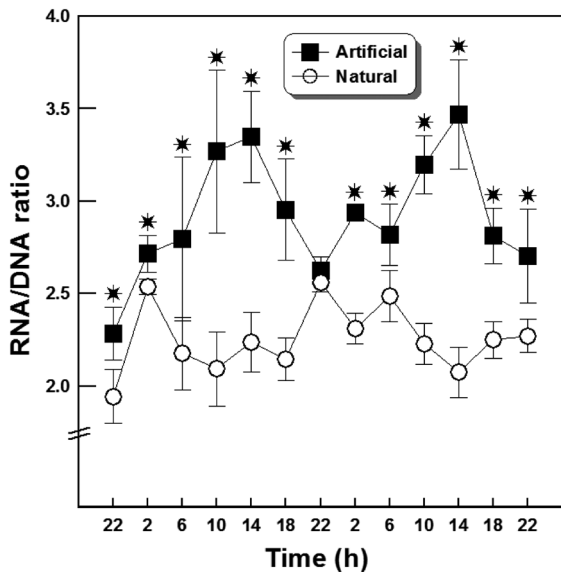
### Rearing experiment



**Fig. 1.** Change of the RNA/DNA content of melania snail during a 2 day rearing experiment. Values are given as means  $\pm$  SD of 5 snails. Significant differences between the experimental and natural group are indicated with \* for DNA or \*\* for RNA ( $P < 0.05$ ).



**Fig. 3.** Change of mean body weight of melania snail during a starvation experiment. Values are given as means  $\pm$  SD of 5 snails. Significant differences between the experimental and natural group are indicated with \* ( $P < 0.05$ ).



**Fig. 2.** Change of the RNA/DNA ratios of melania snail during a 3 day rearing experiment. Values are given as means  $\pm$  SD of 5 snails. Significant differences between the experimental and natural group are indicated with \* ( $P < 0.05$ ).

The average weight of the snails in the artificial group was  $509 \pm 44$  mg. The DNA contents of both groups were lower than the RNA contents and showed small variations between  $75 \mu\text{g}$  and  $150 \mu\text{g}$  (Fig. 1). The DNA contents of the natural group were significantly higher than those of the artificial group at most time points except 22:00 h ( $P < 0.05$ ).

The RNA contents of both groups ranged between  $200 \mu\text{g}$

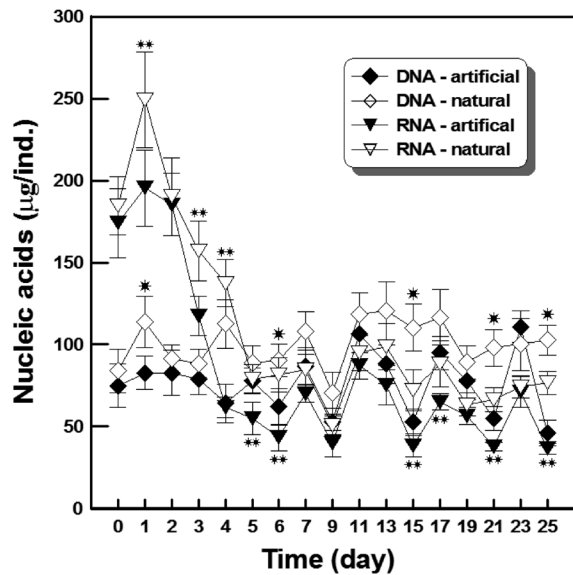
and  $300 \mu\text{g}$  (Fig. 1). Total RNA content differed significantly among most time points, with the natural group exhibiting a higher total RNA content than the artificial group ( $P < 0.05$ ). Both nucleic acids showed the lowest levels between 02:00 and 06:00 h.

The RNA and DNA levels of the freshwater snails calculated over the 3-day trial were 1.94 and 3.5, respectively (Fig. 2). In contrast to the total nucleic acid contents, the RNA/DNA ratio of the artificial group was significantly higher than that of the natural group at all time points except 22:00 h ( $P < 0.05$ ). While the artificial group showed two peak RNA/DNA ratios during the day (10:00 and 14:00 h), the highest ratios for the natural group occurred at night (22:00 and 02:00 h).

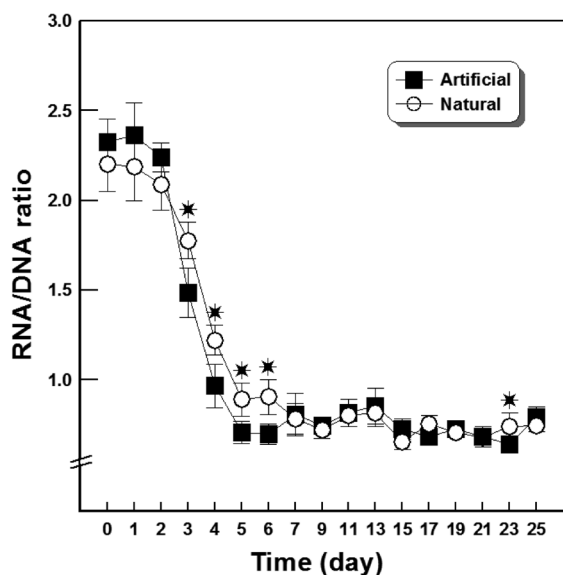
**Starvation experiment**

The body weights of freshwater snails showed minor variations in both of the starved groups (Fig. 3). Overall, snails in the natural group were heavier than those in the artificial group ( $P < 0.05$ ). The DNA contents of both groups were maintained with minor variations when starved (Fig. 4). However, the RNA contents of both groups dramatically decreased beginning 3 days after starvation was initiated until the 4th and 5th day, when the RNA and DNA contents leveled out.

The RNA/DNA ratios of the freshwater snails in both groups dramatically decreased from days 3 to 5, after which that of the starved group remained constant at the end of the experiment (Fig. 5). Significant differences in the RNA/DNA ratios between the artificial and natural group were detected from the 3rd day to the 6th day of starvation.



**Fig. 4.** Change of the RNA and DNA content of melania snail during a starvation experiment for 25 days. Values are given as means  $\pm$  SD of 5 snails. Significant differences between the experimental and natural group are indicated with \* for DNA or \*\* for RNA ( $P < 0.05$ ).



**Fig. 5.** Change of the RNA/DNA ratios of melania snails during a starvation experiment for 25 days. Values are given as means  $\pm$  SD of 5 snails. Significant differences between the experimental and natural group are indicated with \* ( $P < 0.05$ ).

## Discussion

### Diel variation of the nutritional status of freshwater snails

Growth measurements based on shell size and body weight are technically simple and inexpensive, but require costly

long-term growth experiments to detect change (Wo et al., 1999). Growth is directly linked to protein synthesis, which is related to the amount of RNA in cells. Given that the amount of DNA in a cell remains relatively constant, and can thus serve as an index of cell number or biomass (Clemmesen, 1994), the RNA/DNA ratio should provide a good indication of the rate of protein synthesis and therefore the growth of organisms of different sizes.

In our short-term rearing experiments, the DNA and RNA contents in the natural group were higher than those in the artificial group, probably because the snails in the natural group were larger (heavier) than those in the artificial group. We also found that the RNA/DNA ratio of the artificial group was significantly higher than that of the natural group at most time points. Bulow (1987) suggested that for the golden shiner (*Notemigonus crysoleucas*), fish body mass decreased when the RNA/DNA ratio was less than 2.0, whereas it increased quickly when the ratio was greater than 4.0. Similarly, Kim et al. (2008) reported that when the RNA/DNA ratio exceeded 2.0, larvae of the river pufferfish (*Takifugu obscurus*) maintained minimum growth during feeding experiments. Although we measured RNA/DNA ratios greater than 2.0 in both study groups, that of the artificial group was consistently the highest.

For the artificially fed group, the RNA/DNA ratio was highest during the day, which may have been a result of the assigned feeding schedule whereby food was provided at 09:00 h. The subsequent increase in the RNA/DNA ratio was likely due to a dramatic increase in the RNA content while the DNA content remained constant, indicating that the culture conditions were ideal and continuous growth was occurring. These results highlight the importance of suitable feeding conditions as well as of including an additional feeding at night.

In contrast to the artificially fed group, the RNA/DNA ratio of the naturally fed group was highest at night. Chicharo et al. (2001) reported similar results in two mollusks (*Crassostrea angulata*, *Ruditapes decussates*). These results suggest that an endogenous rhythm is involved in the production of RNA. If such a diel variation in RNA/DNA ratios exists, it follows that the average RNA/DNA ratios may not adequately represent the condition of an organism if any temporal bias in sampling occurs (Chicharo et al., 2001). The influence of circadian periodicities in the RNA/DNA ratio has been investigated previously (Mugiya and Oka, 1991; Rooker and Holt, 1996), and diel periodicity would mainly be expected to affect individuals that are in good nutritional condition.

### Effect of starvation on RNA/DNA ratios

The goal of this study was to gain an understanding of the effect of starvation and dietary source on the growth of the melania snail by measuring the quantitative change in the ratio of RNA to DNA. Starvation is one of the most important factors affecting the recruitment of larvae (Tripathi and Verma, 2003).

Negative growth during starvation may be the result of snails utilizing their body reserve to fuel vital processes at times of energy shortage. We observed decreases in the RNA content and no change in the DNA content throughout our study period. Given that cellular RNA is essential for the biosynthesis of proteins, the amount of bulk RNA increases rapidly in growing tissues while the amount of cellular DNA remains fairly constant. Clemmesen (1987) and Raae et al. (1988) reported similar results in fish larvae. They explained the higher DNA content in starved larvae as residual cellular energy being used for rapid unscheduled DNA synthesis due to the lack of sufficient nutrition. Herein, the RNA/DNA ratios ranged from 0.7 to 1.0 under starved conditions. Although the validity of direct comparisons among results of different experimental setups has been argued (Caldarone and Buckley 1991), several studies have reported that RNA/DNA ratios less than 2.0 indicate starvation conditions in fish and mollusks (Sika and Layman, 1995; Chícharo et al., 2001; Vidal et al., 2006).

The RNA/DNA ratio of the artificial group decreased substantially 3 days after starvation. This result is consistent with those of a previous report that demonstrated the validity of using the RNA/DNA ratio as an indicator of nutritional condition in the Japanese turban shell (*Turbo cornutus*) (Okumura et al., 2002). This report also verified that the RNA/DNA ratio is a relatively rapid indicator of nutritional stress compared to other indices (glycogen content and C:N ratio). In addition, significant differences were observed between the two groups, suggesting that artificially fed snails show less tolerance to starvation, thus highlighting the importance of suitable feed during cultivation.

In conclusion, we demonstrated that the RNA/DNA ratio can serve as a useful, fast, and sensitive indicator of starvation stress. Specifically, we showed that the RNA/DNA ratio can be used as an index of the growth and the starvation state of freshwater snails. Further studies are needed to apply these laboratory results to natural populations. Such studies could assess the growth and the nutritional status of the most valuable snail species in Korean freshwater aquaculture.

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