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Direct Examination of the Dietary Preference of the Copepod *Calanus helgolandicus* Using the Colorimetric Approach

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Abstract – The food selectivity of tethered females of the copepod *Calanus helgolandicus* was examined by using the colorimetric approach. First, feeding behavior of the copepod did not show any differences between the red-color stained with neutral red and non-stained diets using the diatom *Coscinodiscus curvatulus*. Then, the copepods were fed a mixtures of two diets, the diatom *C. curvatulus*, stained with neutral red, and the dinoflagellate *Gymnodinium sanguineum* for 14~60 minutes of feeding duration. The foregut colors of females were examined using a stereo-microscope and a video monitor. The foreguts of animals fed with a high density of diatoms in mixed diets showed a dark red color, whereas those fed with a high density of dinoflagellate in mixed diets were a dark yellow. The results suggest that this species of copepod may not selectively feed either one of the diets used for this study. Their feeding activity may be more likely related to the density of available prey in their environment. Therefore, this quick and easy colorimetric approach could provide very useful information, like the pre-screening procedure before designing and conducting the time-consuming and complex feeding experiments to understand the feeding ecology of copepods.

Key words – food selectivity, *Calanus helgolandicus*, diatom, neutral red dye, foregut color

1. Introduction

Feeding is the main route for the transfer of energy and organic matter from lower to higher trophic levels within plankton communities. Therefore, the quantification of feeding ecology remains a key factor in the studies of phytoplankton-copepod trophic interactions (Båmstedt *et al.* 2000). Several methods are commonly used in the feeding studies of mesozooplankton (see Båmstedt *et al.* 2000), including gut fluorescence, food removal, radiotracers, digestive enzyme activity, fecal pellet production rate, and direct cinematographic observation. The direct observation method has been used primarily to study copepod feeding behavior. This method can be divided into two different approaches, depending on whether the observations are achieved with tethered (*e.g.* Gill and Poulet 1986; Sykes and Huntley 1987; Price *et al.* 1988) or with free-swimming copepods (*e.g.* Saiz 1994; Paffenhöfer *et al.* 1996; Paffenhöfer and Mazzocchi 2002). Advances in our understanding of copepod feeding behavior have been derived from high-speed microcinematography (Paffenhöfer *et al.* 1982; Strickler 1982; Price and Paffenhöfer 1986; Turner *et al.* 1993). Although the cinematographic technique is sophisticated and expensive and can be generally used only at well-equipped laboratories, this technique alone provides qualitative measurements.

Neutral red dye has been used to observe the egg cannibalism in *Calanus helgolandicus* in the laboratory by dying copepod eggs (Laabir *et al.* 1995). The egg cannibalism by *C. helgolandicus* was simply identified through the presence of the marker red dye in the copepod bodies. This colorimetric approach can save the experimental time and cost of equipment to understand the feeding habits of copepods, and provide useful information on dietary preference of copepods. Only a

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few studies, however, have been applied this colorimetric approach using neutral red dye for understanding the feeding ecology of copepods (Laabir *et al.* 1995).

In this study, we describe the procedure of a colorimetric approach using neutral red dye to stain diatom cells and to determine the food selectivity of copepods on selected algal species in the laboratory, and then evaluate the usefulness of the inexpensive and simple colorimetric approach as a pre-screening procedure for sophisticated and intensive feeding experiments seeking to understand the feeding ecology of *C. helgolandicus*.

2. Materials and Methods

Calanus helgolandicus were collected using 500 μm mesh plankton net towed obliquely offshore near Roscoff, France, in the western part of the English Channel. The specimens were kept in an insulated box until they were brought to the laboratory within 1 to 2 hours after collection. Adult females were sorted and placed in incubators filled with ambient seawater at 10.4±0.4 °C for 24 hours in dim light.

A fine copper wire (~50 μm in diameter) was glued to the dorsal side of the cephalothorax of each female with a minimal amount of cyanoacrylate glue (Sykes and Huntley 1987). The females were then allowed to rest in a 4 L beaker filled with 0.22 μm filtered seawater for 24 hours before the feeding experiments. After 24 hours of starvation, the females were carefully transferred into crystallized dishes (40 mL volume) filled with ~35 mL of seawater containing the mixture of two phytoplankton species, the diatom *Coscinodiscus curvatulus* (19.1 μm long, 32.2 μm wide) and the dinoflagellate *Gymnodinium sangineum* (50.1 μm long, 34.1 μm wide, and 26.4 μm thick). Cultures of the two phytoplankton species were achieved according to the method of Keller *et al.* (Keller *et al.* 1987). Diatom cells were stained with a neutral red solution prior to the experiments in order to identify their presence in the foregut of females, while non-stained diatoms and dinoflagellates displayed a normal yellowish color. The feeding responses of single female were tested at two different food concentrations and mixtures: high diatom-low dinoflagellate (Hdia-Ldin; diatom density: 9.3×10^2 cells mL^{-1}, dinoflagellate density: 3.7×10^2 cells mL^{-1}) and low diatom-high dinoflagellate concentrations (Ldia-Hdin; diatom density: 3.7×10^2 cells mL^{-1}, dinoflagellate density: 9.3×10^2 cells mL^{-1}). Three to four females of *C. helgolandicus* tethered were exposed to the mixed diets either Hdia-Ldin or Ldia-Hdin for 14~60 minutes of feeding duration depending on the feeding activity of the females. The algae in the incubators were manually re-suspended to prevent potential cell sinking during the feeding experiments. The position of the tethered females was controlled with a micromanipulator (Gill and Poulet 1986). At the end of the feeding experiment, the foregut contents and colors were observed under a stereo-microscope (Leica) equipped with a CCD camera (Olympus) that was linked to an ordinary video monitor. Comparisons of relative colors in the foregut indicated the food selectivity of the copepod. In addition, comparisons were made among females fed on the mixed diets (Hdia-Ldian or Ldia-Hdin) and starved in the filtered seawater for 24 hours as a control.

The neutral red solution was prepared by adding 250 mg neutral red (Sigma) to 100 mL of filtered seawater (0.22 μm). Cultures of the diatom *C. curvatulus* were gently filtered on a sieve with a 15 μm mesh and further soaked in the neutral red solution for 30 minutes. The red cells were then backwashed with filtered seawater three or four times to remove excess dye. All diatoms were quickly stained, and the intact cells kept their reddish color for at least several hours in the water. Before the feeding experiment with the dietary mixture, a single diet experiment was conducted with the non-stained and stained diatom *C. curvatulus* (cell density: 1.4×10^3 cells mL^{-1}) to test the feeding responses of *C. helgolandicus* on the non-stained and stained diatom. Seven to eight female *C. helgolandicus* tethered were exposed to the non-stained or stained diatom for 5~30 minutes of feeding duration, and the foregut color and ingestion activity were observed using the stereomicroscope.

In the single diet experiment, the difference of the feeding response of *C. helgolandicus* between the non-stained and stained diatoms was tested by Mann-Whitney U test. In the mixed diet experiment, the food selectivity of the copepod between the diatom and dinoflagellate was examined by Wilcoxon signed ranks test. All statistical analyses were conducted using the SPSS program (SPSS Inc. Version 11.5).

3. Results and Discussion

The single diet feeding experiment with stained and

Table 1. Direct stereomicroscopic observations of the feeding responses of tethered females of *Calanus helgolandicus* to diatom *Coscinodiscus curvatulus* (1.4×10^3 cells mL^{-1}) non-stained (A) and stained (B) with neutral red. Strong and weak in feeding movement indicate active and continuous, and non-active and intermittent feeding behaviors of copepod, respectively. +: low ingestion; ++: high ingestion

(A) Non-stained diatom

Test number	Duration of incubation (min)	foregut color	Feeding movement	Relative ingestion estimate
1	10	Yellowish	Strong	++
2	15	Yellowish	Strong	+
3	15	Yellowish	Strong	++
4	20	Yellowish	Strong	++
5	20	Yellowish	Strong	+
6	20	Yellowish	Weak	+
7	20	Yellowish	Weak	+

(B) Stained diatom

Test number	Duration of incubation (min)	Foregut color	Feeding movement	Relative ingestion estimate
1	5	Reddish	Strong	++
2	10	Reddish	Strong	++
3	10	Reddish	Strong	++
4	25	Reddish	Strong	+
5	25	Reddish	Weak	+
6	25	Reddish	Weak	+
7	25	Reddish	Weak	+
8	30	Reddish	Strong	+

non-stained diatom showed that *Calanus helgolandicus* females ordinarily ingested either of the diets, whether stained or not stained with neutral red dye (Table 1). Any apparent differences of the food selectivity by *C. helgolandicus* females were not observed between the non-stained and stained diatoms (Mann-Whitney U test; $P>0.05$; n=7 for non-stained diatom, n=8 for stained diatom; Table 1). Minor differences might be due to individual variability in feeding movement and relative feeding activity. Therefore, we concluded that the feeding behavior of the copepod (at least for the females of this species) is not affected by staining its diet, particularly diatom *C. curvatulus*. However, it is still uncertain if their feeding rates on these two (stained vs. non-stained) diets are different or not because we did not measure the feeding rates during this experiment.

For the feeding experiments with the mixture of two diets, *C. helgolandicus* females ingested both diatoms and dinoflagellates. The foregut color of females fed on the mixed diets with Hdia-Ldin showed deeper red color than that of females fed on the mixed diets with Ldia-Hdin and that of females in the control (Fig. 1). Reversely, the intensity of yellowish color, indicating the consumption of dinoflagellates, was apparently higher in the foregut of females incubated with Ldia-Hdin compared to those that consumed a low dinoflagellate diet and the control (Fig. 1). Analysis of video records (n=7; duration: 14-60 minutes, Table 2) showed that the females ingested both diatoms and dinoflagellates in proportion to the cell concentrations in the diets. However, no significant changes of their feeding behaviors were detected during the feeding duration applied in this study. The higher ingestion of diatoms was observed with Hdia-Ldin diets, but estimated ingestion activities of *C. helgolandicus* were not significantly different between diatoms and dinoflagellates (Wilcoxon signed ranks test; $P>0.05$; n=3; Table 2a) because of the low number of replication (n=3). In contrast, the lower ingestion of diatoms was observed for the feeding experiment with Ldia-Hdin diets. Furthermore, the statistical test indicated that estimated ingestion activities of *C. helgolandicus* between diatoms and dinoflagellates were significantly different for this Lida-Hdin feeding experiment (Wilcoxon signed ranks test; $P<0.05$; n=4; Table 2b). We still found that the feeding activities varied slightly among individuals. It

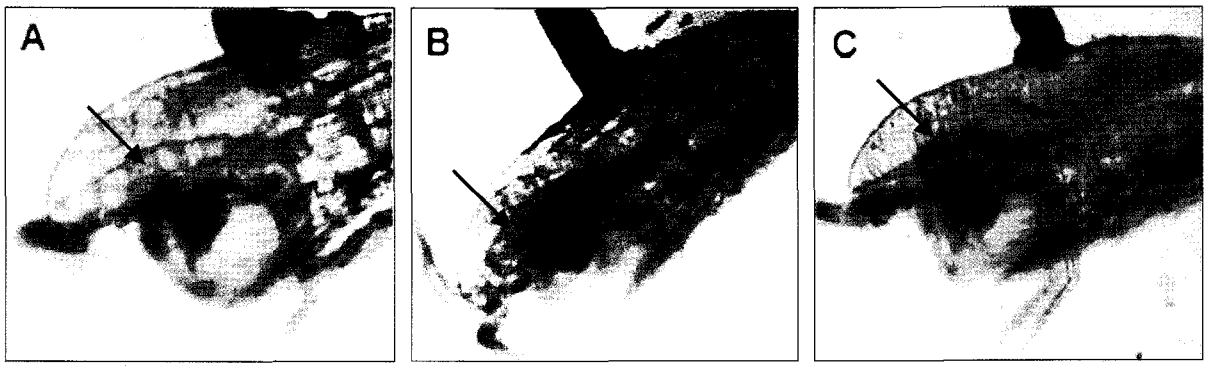

Fig. 1. Comparison between the foregut colors (arrows) of tethered females of copepod *Calanus helgolandicus* incubated in (A) filtered seawater (Control), (B) Hdia-Ldin diet: mixed high *Coscinodiscus curvatulus* (9.3×10^2 cells mL^{-1}) and low *Gymnodinium sanguineum* concentrations (3.7×10^2 cells mL^{-1}), and (C) Ldia-Hdin diet: mixed low *C. curvatulus* (3.7×10^2 cells mL^{-1}) and high *G sanguineum* concentrations (9.3×10^2 cells mL^{-1}). Diatoms were stained with neutral red.

Table 2. Results of video records of the food selectivity of single tethered females of *Calanus helgolandicus* fed on mixed diets: the stained diatom *Coscinodiscus curvatulus* and the non-stained dinoflagellate *Gymnodinium sanguineum* with two different cell concentrations; a) high diatom and low dinoflagellate concentrations (Hdia-Ldin diet) and b) low diatoms and high dinoflagellate concentrations (Ldia-Hdin diet). +: low ingestion; ++: high ingestion

Type of mixed diet and food concentration (cells mL^{-1})	Test number	Food type	Duration of incubation (min)	Major color in foregut	Relative ingestion estimate
a) Hdia-Ldin diatom: 9.3×10^2 dinoflagellate: 3.7×10^2	1	diatom	20	Red	++
		dinoflagellate			+
	2	diatom	14	Red	++
		dinoflagellate			+
	3	diatom	30	Red	++
		dinoflagellate			+
b) Ldia-Hdin diatom: 3.7×10^2 dinoflagellate: 9.3×10^2	1	diatom	40	Yellow	+
		dinoflagellate			++
	2	diatom	60	Yellow	+
		dinoflagellate			++
	3	diatom	25	Yellow	+
		dinoflagellate			++
	4	diatom	30	Yellow	+
		dinoflagellate			++

may be due to the individual variability and differences in the physiological conditions among animals used for the experiments (Table 2).

These results of the food selectivity of the copepod female were very similar to the previous study (Kang and Poulet 2000) where the food selectivity of free-swimming *C. helgolandicus* females, fed with the same phytoplankton species, was measured by the food removal method (Frost 1972; Båmstedt *et al.* 2000). The lack of a distinct difference in the feeding responses between tethered and free-swimming specimens (Kang and Poulet 2000) suggests that the food selectivity was not affected by tethering the copepods.

Neutral red, which can vividly stain live animals, has often been used to distinguish dead and live plankton and then to estimate the mortality of zooplankton; this is known as the vital staining technique (Dressel *et al.* 1972; Crippen and Perrier 1974). In our context, various diatoms having silica shell can be easily stained using this marker, which makes it an appropriate choice for other experiments with copepods. In fact, food uptake by copepods can be simply estimated through observation of

the foregut colors. This simple approach may permit reliable observations of copepod feeding on key stained diatom species under laboratory conditions. It can be applied to two or more diatom species using mixed diets and allows for a preliminary evaluation of the ingestion of diatoms without labor-intensive experiments (*e.g.* the food removal method), or the use of expensive equipment (*e.g.* the high-speed cinematographic method). Therefore, the results of this study suggested that the food selectivity of copepods can be simply estimated in the laboratory within short time periods (*i.e.* a few tenths of minutes) using tethered specimens and staining selective diets by neutral red.

Although we could not evaluate the color intensity in the foregut of the copepod quantitatively, nor the applicability of staining the dinoflagellate and other diatom species, it may be useful to determine the food preference of copepods before conducting time-consuming and more complex experiments to understand the feeding behaviors of the copepod *Calanus*, particularly in relation to diatom species. In the future, it may be possible to estimate the ingestion rate of copepods on diatoms using this colorimetric approach, if the color intensity of the foregut of copepods is quantitatively converted to the amount of stained diets ingested by the copepods.

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