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How Long do the Plastids Retained by *Elphidium excavatum* (Terquem) Last in their Host?

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Abstract

Intact plastids are retained by *Elphidium excavatum* (Foraminifera). There is preferential retention of diatom plastids. Freshly collected specimens of *E. excavatum* were fed diets of three different axenic cultures of algae for a week, and then starved for 8 weeks to assess the longevity of plastids retained from their diets. The number of plastids retained per individual was assessed weekly with the aid of a confocal laser scanning microscope. Starved individuals served as controls. Since controls already had plastids they retained while they were feeding in the field before they were harvested, we subtracted control values from experimental values to estimate the experimental results. Results indicate that plastids remain in the cytoplasm of *Elphidium* for at least 8 weeks. When the foraminifera were incubated with 12/12h light/dark cycle, the half-life of diatom plastids was shorter. Although we observed plastids in foraminifera fed chlorophytes or dinoflagellates, the numbers retained were the same or less than starved controls, suggesting that no new plastids had been added to the "symbiont" population during the week of experimental feeding. The half-lives of all retained chloroplasts were longer for hosts that were incubated in the dark (diatom plastids 9.5 weeks; dinoflagellate plastids 4.7 weeks; chlorophyte plastids 8.2 weeks; and plastids [most probably from diatoms] in

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controls 10.7 weeks). The results are in general accordance with what is known about the kleptochloroplast phenomenon in the ascoglossan mollusk, *Elysia chlorotica*.

Keywords: Foraminifera, *Elphidium excavatum*, plastid retention, symbiosis, kleptoplastids

1. Introduction

Symbioses between larger foraminifera and diverse microalgae (diatoms, dinoflagellates, chlorophytes and rhodophytes) are well known (reviewed by Lee, 1998). Less well researched is the phenomenon of chloroplast husbandry (retention of chloroplasts or plastids from partially digested algae) by foraminifera (reviewed by Bernhard and Bowser, 1999). *Elphidium* is one of the genera of foraminifera that keeps isolated plastids obtained from ingested algae (kleptochloroplasts) in its cytoplasm (Lopez, 1979; Leutenegger, 1984; Lee and Lee, 1989; Cedhagen, 1991; Bernhard and Bowser, 1999). Previous studies (Lee and Lee 1989; Correia and Lee, 2000) suggested that the retention of plastids by *Elphidium* spp. is a selective process and that diatom plastids were preferentially selected, and/or retained. Isolated chloroplasts/plastids inside foraminifera eventually appear to undergo digestion, or self destruction, at a slow rate (Lee and Lee, 1989). Evidence for this gradual digestion, or self destruction was found at the ultrastructural level (Lee et al., 1988), but this aspect of the phenomenon was not studied in detail. In this present study, we assessed two aspects of chloroplast husbandry in *Elphidium excavatum*: 1) How long do sequestered chloroplasts/plastids last if the host is starved? 2) Are there qualitative aspects to retention (do the light regime, or the species of alga that was the donor of the plastid, effect the chloroplast/plastid longevity in the foraminifera)?

2. Materials and Methods

Sample collection

Samples of *Elphidium excavatum* were collected in the salt marsh at the southeast periphery of Lake Tashmoo, Martha's Vineyard, MA, USA (N41°32' W 70°40') in the first week of August 2000. The sampling protocol has been described by Correia and Lee (2000). Individual specimens of *E. excavatum* were picked from the sediment with sable artist brushes and divided into 8 aliquots of 50 foraminifera each. Each aliquot contained individuals of approximately the same size.

Algae

The three algae used as food in the experiments were isolated from littoral benthic communities and axenically cloned on agar using the methods described by Lee et al. (1975). Two of the three species, *Amphora coffeiformis* (diatom) and *Dunaliella salina* (green alga) were isolated from the sub-littoral epiphytic community of Lake Tashmoo. *Amphidinium* sp. (dinoflagellate) was isolated from the shallow benthos of sedimentation ponds at the National Center for Mariculture in Eilat, Israel. *Amphidinium* sp. was chosen for this experiment because it is a dinoflagellate which has a benthic and epibenthic habitat. It lives in the same habitat as the foraminifera.

Feeding protocol

Eight experimental groups (50 organisms each group) were set up. Two treatment groups of foraminifera were incubated in unsupplemented filter sterilized Woods Hole seawater after being harvested (starved controls). The other 6 groups, were divided into 3 treatment pairs and each group was fed an aliquot from an axenic culture of either *Amphora coffeiformis*, *Dunaliella salina* or *Amphidinium* sp. The final concentration of the algal cells in the seawater was 1×10^6 cells /ml. All cultures were incubated for a week at 25°C in front of a fluorescent light bank (60 µEin) with a 12 hour light/12 hour dark cycle. After one week the foraminifera in each treatment were individually brushed to remove adhering algae and transferred to 250 ml plastic tissue culture flasks with aseptically added filter sterilized seawater. From this point forward in the experiment all organisms were starved. One of the flasks in each paired treatment group was incubated in a 12 hour light/12 hour dark cycle; the other flask was incubated within a light-tight box in complete darkness. Each week, five individuals were randomly selected from each flask, placed on a slide with a drop of SlowFade® (Molecular Probes, Inc., P.O. Box 22010, Eugene, Oregon) and frozen at –80°C. The number of chloroplasts/plastids within each frozen individual was estimated with the aid of a confocal laser scanning microscope (Molecular Dynamics Multiprobe 2001 with an argon/krypton laser). Scans used an exciter filter with a wavelength of 590 nm. Initial optical sections, 1 µm thick were performed at 5 µm intervals. At least 3 serial sections were chosen to represent each individual and the number of chloroplasts/plastids in each section was counted and multiplied by an appropriate volumetric factor to calculate the number of chloroplasts in the entire volume of the organism. The volume of each individual specimen sampled was calculated by a program that was part of the software package of the microscope. This method was appropriate since the

chloroplasts/plastids retained are evenly distributed within the cytoplasm of the foraminifera.

Statistical analysis

All ANOVA calculations were done using the SAS® (version 6.12) statistical package. The values obtained were compared using a sequential BonFerroni analysis in order to minimize the inflation of error associated with multiple pair-wise comparisons (Rice, 1989). The a value used for the comparisons was 0.05. The half-lives of the chloroplasts were calculated by finding a regression curve that closely fit the data. The regression parameters were calculated using the statistical package included in the Microsoft Office Excel software.

3. Results

The number of plastids retained by the foraminifera decreased as a function of time (Fig. 1). The number per individual was always higher in those foraminifera fed *Amphora* (diatom), but the differences became statistically significant only after the 4th week of incubation in the light (Fig. 1a) and 6th in the dark (Fig. 1b). When the numbers of plastids retained by *E. excavatum* incubated in a light/dark cycle were compared to those incubated in the dark, two trends became evident (Fig. 2). With few exceptions, the number of plastids remaining in the cytoplasm of each individual foraminifera was significantly higher in the group of foraminifera fed *Amphora* and the starved controls incubated in the light/dark cycle, than it was in those which were incubated continuously in the dark (Figs. 2a and b). Except for the first 2 weeks of those fed *Dunaliella* (Fig. 2d), those fed *Amphidinium* and *Dunaliella*, retained approximately the same number of plastids regardless of whether they were incubated in a light/dark cycle or in complete darkness (Figs. 2c and d). Biweekly measurements of primary fixation using ^{14}C-labeled tracer showed that the kleptoplastids in the unfed controls were still functioning during the two months of the experiment (Correia and Lee, unpublished).

The half-lives of the plastids retained by the foraminifera were calculated from regression lines fitted to the data (Table 1). The half-life of the plastids was higher when the foraminifera were kept in the dark regardless of which type of algae they were fed. This was also the case for the starved controls which retained their chloroplasts longer when they were incubated in the dark.

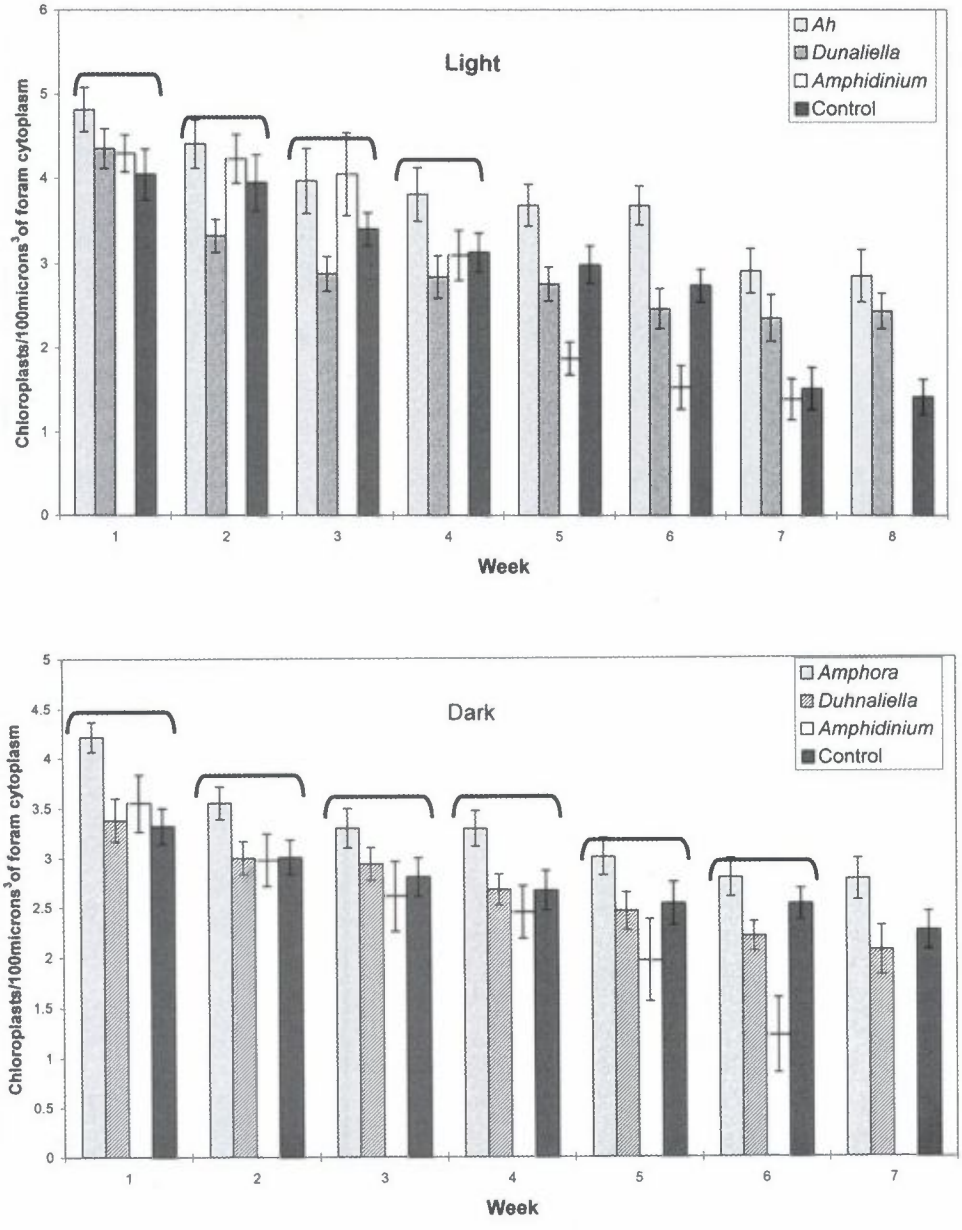

Figure 1. Change over time in the number of chloroplasts retained by *Elphidium excavatum* (Terquem) starved after being fed different algal diets. Square brackets indicate statistically identical values considering an α value of 0.05. The error bars represent the standard deviation. The absence of α value indicates that all individuals were dead. Controls refers to starved individuals.

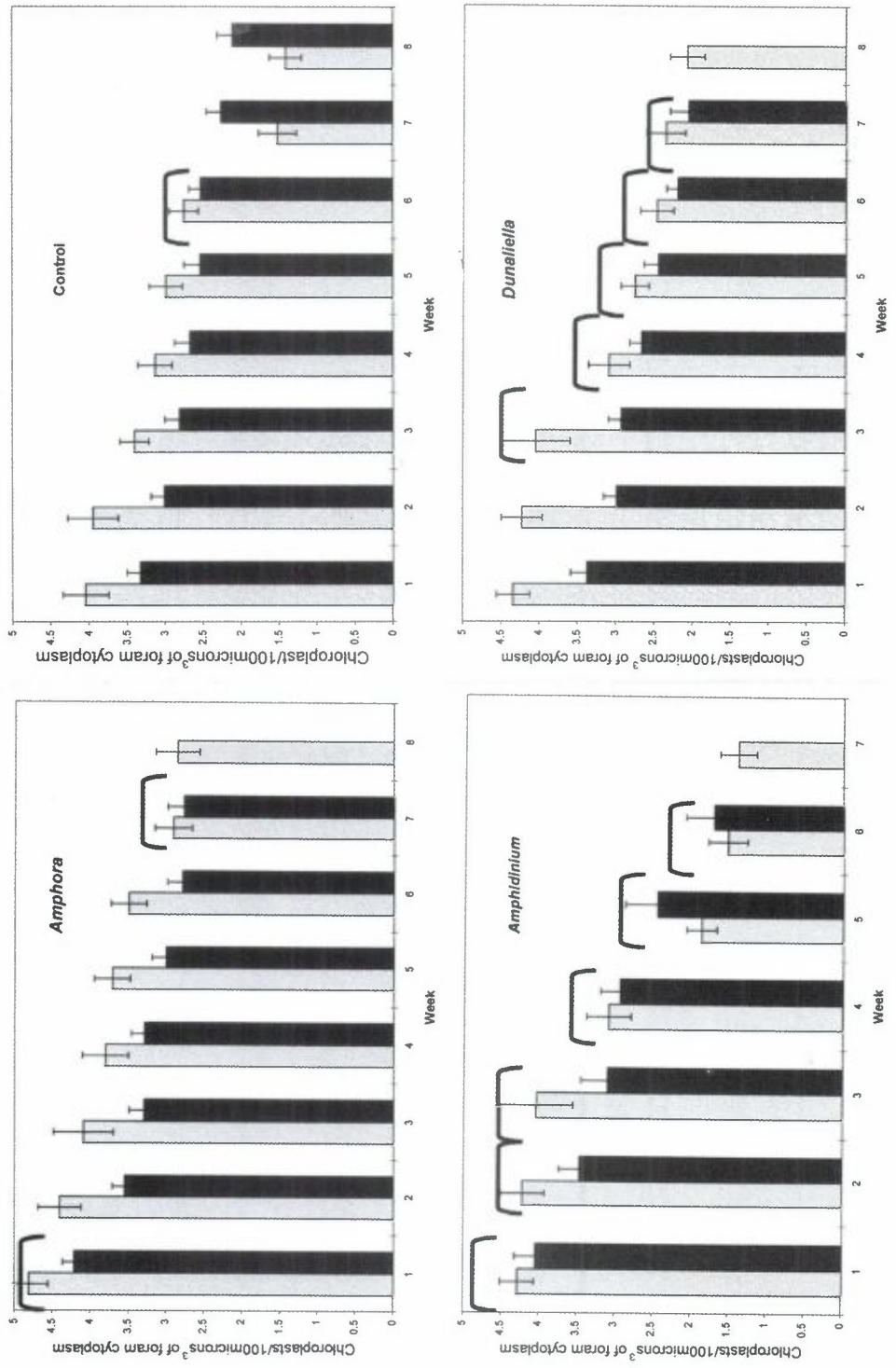
Control
Dunaliella
Amphora
Amphidinium
Week
Chloroplast/100microns³ of foram cytoplasm

Table 1. Half-life of chloroplasts retained by *Elphidium excavatum* (Terquem). Values are in weeks and based on observations over a period of 8 weeks.

Diet	Light regime	
	Light/Dark (12/12)	Dark
Amphora coffeiformis	9.1	9.5
Amphidinium sp.	4.5	4.7
Dunaliella salina	6.6	8.2
Starved control	6	10.7

4. Discussion

The results of our study on kleptochloroplast longevity within *Elphidium excavatum* are in agreement with the data that are emerging from the more intensively studied sea slug system (reviewed by Trench, 1975; Rumpho et al., 2000). In recent years it has been reported that photosynthetic sea slugs (*Elysia chlorotica*) could be maintained in the laboratory for at least 9 months in the absence of algal food (Rumpho et al., 2000). However, Green et al. (2000) note that the sea slugs lose pigmentation in a fashion similar to the senescence of a leaf. These workers suggest that as the natural life cycle of the sea slug proceeds, the hosts may no longer provide the cellular environment or proteins necessary to maintain plastid photosynthetic machinery. Plastids remain viable within *Elphidium excavatum* (Terquem) for at least 8 weeks, even when the foraminifera are starved. At 9 weeks, many foraminifera are pale in color and many have died. It would be misleading for us to draw conclusions about the viability of plastids in *Elphidium excavatum* compared to those of *Elysia chlorotica* because we may not have met all the requirements to optimally maintain these foraminifera in the laboratory at this time. The first chloro-

Figure 2. See opposite side.
Comparison between the number of chloroplasts retained by *Elphidium excavatum* (Terquem) fed different algal diets and incubated either in a light/dark cycle (light bars) or in complete darkness (dark bars). Square brackets indicate statistically identical values considering an α value of 0.05. The absence of a value indicates that all individuals were dead. Controls refers to starved individuals.

plast longevity experiments with *E. chlorotica* suggested that these animals could be maintained for only 4 months in the absence of algal food (*Vaucheria litorea*) (Gibson et al., 1986) but, as mentioned above, the time more than doubled as requirements for the maintenance of the host in the laboratory were better understood. The improvements in sea slug maintenance draw attention to a major obstacle in the advancement of the studies of chloroplast husbandry in foraminifera. If we could raise a species of *Elphidium*, or one of the other chloroplast-retaining genera, in continuous laboratory culture, then we would not have to rely on specimens collected in the field for experimentation. We then could use organisms whose chloroplast/plastid lineages are completely known rather than having to subtract unknown background lineages. Other environmental variables, not yet examined, such as seasonal differences in available food, temperature, irradiance etc. would no longer be background factors in experimental organisms. Another approach we have yet to apply, is to try to make an *Elphidium* species apochlorotic, or nearly so, by using photosynthetic inhibitors like DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea). This approach allowed us to render *Amphistegina lessonii*, a diatom-bearing foraminifera, nearly apochloritic so that we could test whether we could replace one species of diatom endosymbiont with another (Lee et al., 1983, 1986).

The results of our present study, a previous study (Correia and Lee, 2000), and fine structural studies (Correia and Lee, 2002), suggest that diatoms are the major (perhaps the only) contributors to the population of plastids that are retained by *E. excavatum*. Our experiments show that when the foraminifera were fed *Amphidinium* sp. (dinoflagellate). or *Dunaliella salina* (green alga) they retained no more plastids/chloroplasts per foraminifer than starved controls. The simplest explanation is that they do not retain any chloroplasts/plastids of these types of algae.

Nothing is known about the factors that sustain long term plastid activity in the *E. excavatum* system with which we have been working (Lopez, 1979; Leutenegger, 1984; Lee and Lee, 1989; Cedhagen, 1991; Bernhard and Bowser, 1999). While our own fine structural studies of various species of *Elphidium* have revealed that rarely some other algal organelles are retained within plastid vacuoles, such as nuclei and mitochondria (Lee et al., 1988; Correia and Lee, 2002), workers studying *E. chlorotica* have not made similar observations. In agreement with microscopic observations, Southern blot analysis and polymerase chain reaction did not detect an algal nuclear genome in the sea slug (Green et al., 2000). The key question then becomes how do the chloroplasts remain functional for several months in the absence of any algal nucleo-cytosolic influence? Studies of the largest chloroplast genomes known suggest that they have code for only ~1–5% of the gene products necessary for plastid function (Reith and Munholland, 1995; Martin and Herrmann, 1998).

Knowledge gleaned from studies of sea slugs and their kleptochloroplasts suggest many potential future studies of the phenomenon in foraminifera. Trench (1975) followed protein synthesis in the chloroplasts by using a radionuclide tracer ([^{3}H] leucine) to measure chloroamphenicol-sensitive plastid protein synthesis. Mujer et al. (1996) were able to demonstrate transcripts of two chloroplast genes, psbA and 16S rRNA in *E. chlorotica* cultured for 8 months in the absence of algae. Photosystem proteins D1, D2, CP43 and Rubisco LS which are synthesized by plastids were also present. Pierce et al. (1996) found that [^{35}S] labeled Methionine, as a tracer of protein synthesis, was translocated into the plastids. It would be desirable to do similar studies with foraminifera if the experiments could be designed to detect protein synthesis and translocation at a much smaller scale. Perhaps autoradiography has the sensitivity for the detection of ^{35}S labeled methione protein in the plastids of a pulse-chase experiment. In context with chloroplast gene autonomy, Rumpho et al. (2000) and Martin and Herrman (1998) suggest that proteins destined for mitochondria (glucogenic and pentose pathways) could be directed to and function in the plastids and could prolong chloroplast maintenance. They suggested that the proteins isolated from the chloroplasts of *Codium fragile* are much more stable than those isolated from spinach. By analogy, perhaps plastids from diatoms are more stable in the cytoplasm of *E. excavatum* than are those of green algae.

The plastids retained by *E. excavatum* seem to be preserved longer by foraminifera incubated in the dark. Could this be due to slower turnover of plastid proteins when the photosynthetic machinery is not active? This remains to be resolved. The fate of the chloroplasts could not be determined by the methods we used in our experiment. The chloroplasts either autofluoresce in the confocal microscope or they do not. Chloroplasts could be lost from the system by lack of maintenance leading to autolysis, or by host digestion. Clues may come from ultrastructural studies or from pigment analysis. Much work remains, we are just beginning to probe the kleptochloroplast phenomenon in foraminifera.

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