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CHLOROPLAST SYMBIOSIS IN A NON-ELYSIID MOLLUSC, *COSTASIELLA LILIANAE* MARCUS (HERMAEIDAE: ASCOGLOSSA (=SACOGLOSSA): EFFECTS OF TEMPERATURE, LIGHT INTENSITY, AND STARVATION ON CARBON FIXATION RATE

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Abstract

The hermaeid ascoglossan slug *Costasiella lilianae* possesses functional symbiotic chloroplasts derived from its algal food, *Avrainvillea nigricans*. Symbiotic plastids continued to fix carbon after 65 days starvation, though efficiency of fixation declined about 87%. Chlorophyll level did not decline during this period.

Degeneration of symbiotic plastids involved swelling and delamination of thylakoids, increase in electron density of plastids, and decrease in pyrenoid electron density. Plastids within single cells degenerate at about the same time, suggesting that individual cells phagocytize the entire complement of plastids during a brief period.

Temperature strongly influenced carbon fixation, both in rate of net fixation and in production of alcohol-insoluble photosynthates. The optimum temperature for fixation was 25°C. Photosynthetic rate exhibited saturation at about 500 $\mu\epsilon$ (microeinsteins)$\cdot m^{-2} \cdot s^{-1}$ and substantial fixation occurred at intensities as low as 25 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$. No inhibition occurred in full sunlight (1500 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$).

Introduction

The opisthobranch molluscan order Ascoglossa (=Sacoglossa) appears to be unique among Metazoa in its ability to symbiotically retain functional chloroplasts within cells of the digestive tract (see Trench, 1975, for a recent review). However, there is disagreement on how widespread the symbiosis is within the order. There are three major morphological types of Ascoglossa: the tectibranch (primitively shelled) families (Cylindrobullidae, Volvatellidae, Juliidae, Oxynoidae, and Lobigeridae); the elysioids (Elysiidae), which are shell-less and have parapodia (lateral extensions of the body wall); and the stiligeroids (Caliphyllidae, Hermaeidae, Stiligeridae), which are also shell-less, but lack parapodia and have dorsal papillae (cerata). Of the three types, chloroplast symbiosis has been thought to be limited to the Elysiidae (Muscatine and Greene, 1973; Hinde and Smith, 1974; Trench, 1975; Muscatine *et al.*, 1975; Graves *et al.*, 1979). However, other workers (Taylor, 1967, 1971; Kremer and Schmitz, 1976; Clark and Busacca, 1978) have shown that forms of chloroplast symbiosis do occur in some non-elysiids, though these symbioses are often of short duration. We report here evidence of long-term chloroplast symbiosis in *Costasiella lilianae* (Marcus and Marcus), the second genus and species of Hermaeidae known to retain symbiotic plastids.

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Abbreviations: $\mu\epsilon$, microeinsteins; CPM, counts per minute; DPM, disintegrations per minute; LSC, liquid scintillation counting; AS, alcohol soluble; AI, alcohol insoluble; Chl, chlorophyll.

Materials and Methods

Initial stocks of *Costasiella lilianae* were obtained from Bermuda and Key Largo, Florida, from the alga *Avrainvillea nigricans* Decaisne. All experimental stocks were drawn from aquarium cultures maintained with a photoperiod of 18 h light: 6 h dark, irradiance of 50–100 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$, and temperatures of 23–25°C.

Carbon fixation

Photosynthetic rates were estimated by uptake rate of radioisotopic carbon. Each animal was incubated 1 h in 4 ml of membrane-filtered seawater with $NaH^{14}CO_3$ at an activity of 2.0–2.5 μCi/ml. Vials were sealed with slotted stoppers to exclude air (and thus prevent animals from crawling out of the medium). Dark control animals were incubated in foil-wrapped vials. After incubation, animals were washed three times in sea water and homogenized in cold 100% methanol. Chlorophyll was extracted by phase separation into diethyl ether and measured spectrophotometrically (Strain and Svec, 1966). The ether extract, examined for radioactivity by liquid scintillation counting (LSC), contained negligible activity. The remaining homogenate was centrifuged and a 0.1 ml subsample of the alcoholic/aqueous supernatant fluid was removed for LSC. The centrifuged pellet was suspended in 1 ml tissue solubilizer for 24 h, and 0.1 ml of the solubilized fraction was removed for LSC. CPM rates were corrected for counting efficiency, background, quenching, and dilution factors, and uptake rates calculated as DPM $\cdot \mu$g chlorophyll$^{-1} \cdot$ h.

Chlorophyll retention

Animals were removed from stock cultures, placed in a newly-prepared (alga-free) aquarium, and maintained as described above. Animals withdrawn at intervals were relaxed in 8% $MgCl_2 \cdot 6H_2O$ and measured. After a recovery period in seawater, carbon fixation rates and chlorophyll levels were estimated as described above. Chlorophyll concentrations were calculated as milligrams per gram dry weight of animal tissue, using an empirically derived equation for animal weight: $\text{Log}_{10}W = 3.166\ \text{Log}_{10}L + 0.928$ ($r = .94$), where W = dry weight in micrograms and, L = relaxed length in millimeters.

Effect of temperature on fixation rate

Fixation rates were measured over a range of 15–35°C, corresponding to the temperature range measured at the Key Largo habitat over 5 years. Animals were acclimated at the test temperature for 24 h before isotope incubation (irradiation rate 325 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$.) Six animals were used for each light incubation and for each dark control.

Total carbonate content of seawater was measured by the acidometric method (Strickland and Parsons, 1972), and carbon fixation rates were estimated from the equation (after Colijn and Van Buurt, 1975): μg C fixed $= (A_l\text{-}A_d)/(A_a) \times C \times 1.06$ where A_l = light fixation activity (DPM), A_d = dark fixation activity, A_a = activity added to sample, C = total carbon available in incubation medium; and 1.06 = isotope discrimination ratio.

Effect of irradiation rate on fixation rate

Animals were incubated at varied light intensities at 25°C, using layers of gray fiberglass window screening as neutral density filters. Incandescent lamps were used for irradiation rates of 25–100 $\cdot m^{-2} \cdot s^{-1}$; sunlight was used for higher rates.

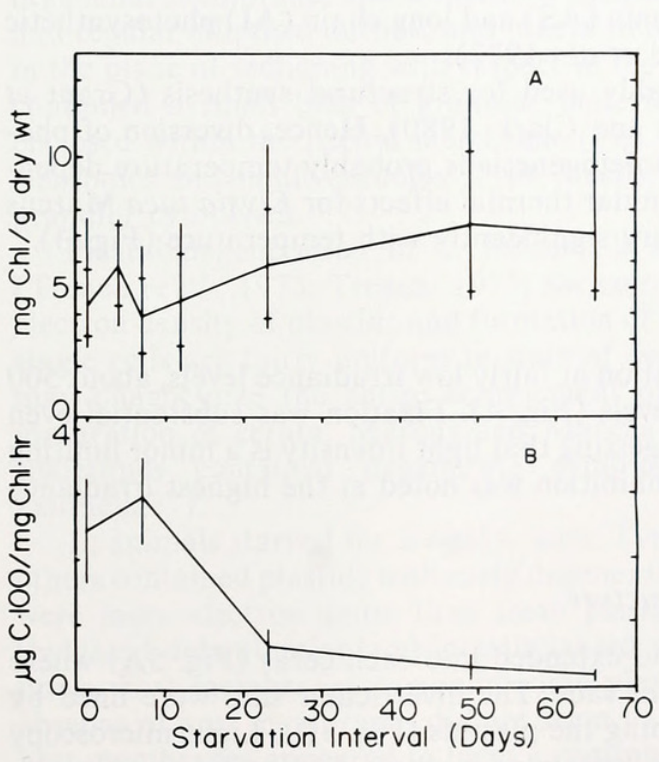
Results

Starvation

Costasiella lilianae retained photosynthetically active chloroplasts, as evidenced by significant carbon fixation in both freshly fed and starved animals (Fig. 1). Chlorophyll levels increased somewhat after long-term starvation (65 days), but carbon fixation rate gradually declined after starvation periods longer than a week. Light fixation was significantly higher than dark fixation ($p < 0.05$) even after 65 days starvation (Fig. 1).

Temperature effects

Carbon fixation by *C. lilianae* varied widely as a function of temperature (Figs. 2, 3). Net (light minus dark) fixation increased nearly threefold from 20° to 30°C but declined somewhat at 35°C. Alcohol soluble (AS) and alcohol insoluble (AI) fraction were differentially affected by temperature: at suboptimal temperatures, most of the total fixation is in the AS fraction, but nearly equal levels of AI and AS components were fixed at temperatures optimal for total fixation. These frac-



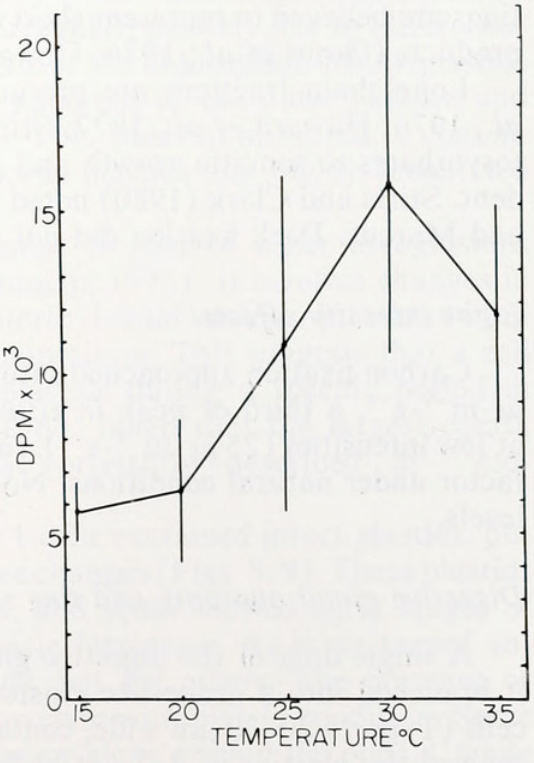


FIGURE 1. (Left) A. Effect of starvation on chlorophyll level (mean, standard deviation, and range shown; n = 10). B. Effect of starvation on net fixation rate (mean ± standard deviation, n = 10).

FIGURE 2. (Right) Effect of temperature on net total (alcohol soluble ± alcohol insoluble) carbon fixation by *Costasiella lilianae* (mean ± standard deviation; n = 6).

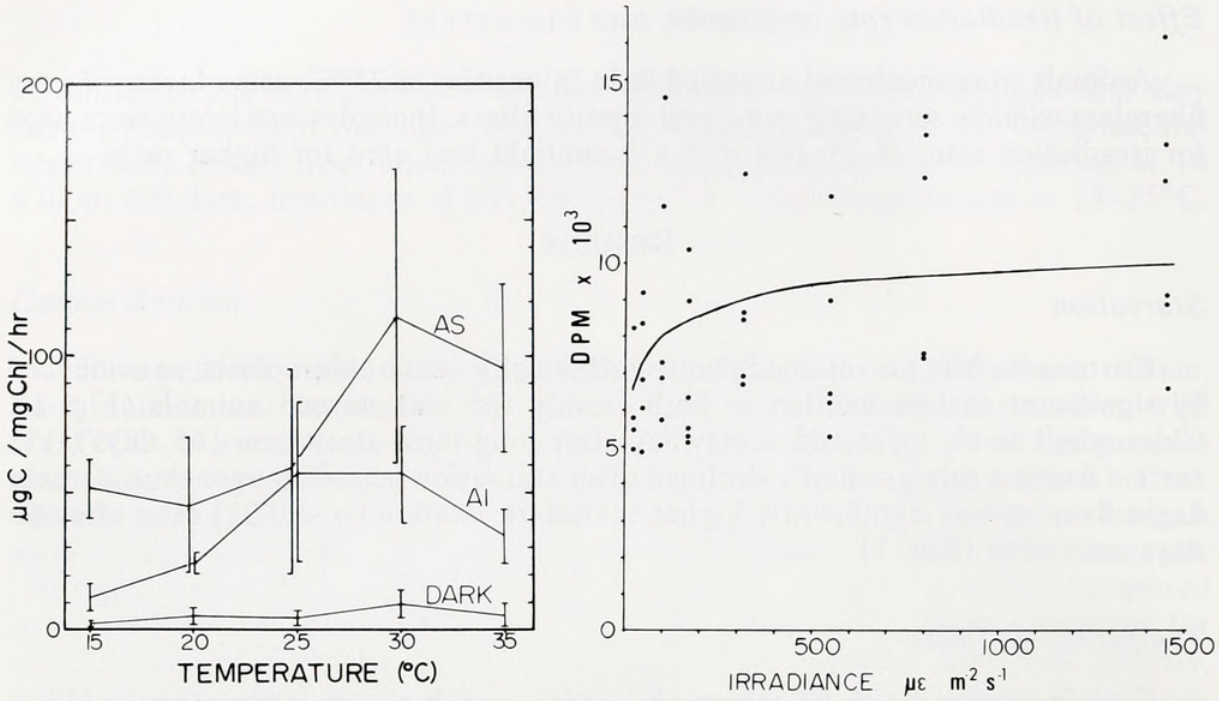


FIGURE 3. (Left) Effect of temperature on net alcohol-soluble (AS), net alcohol insoluble (AI) and dark (AI + AS) carbon fixation by *Costasiella lilianae*. (mean ± SD, n = 6).

FIGURE 4. (Right) Effect of light intensity on carbon fixation rate in *Costasiella lilianae*. Regression line calculated for interval of 25–15,000 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$ by method of least squares with semilogarithmic transformation: y = 2608 log x + 2750, where x = DPM, y = intensity; r = .47. Relationship highly significant at $p < .01$.

tions are believed to represent short chain (AS) and long chain (AI) photosynthetic products (Grant *et al.*, 1976; Howard *et al.*, 1977).

Long-chain fractions are presumably used for structural synthesis (Grant *et al.*, 1976; Howard *et al.*, 1977; Stirts and Clark, 1980). Hence, diversion of photosynthates to somatic growth and gametogenesis is probably temperature dependent. Stirts and Clark (1980) noted similar thermal effects for *Elysia tuca* Marcus and Marcus. Dark fixation did not vary significantly with temperature (Fig. 3).

Light intensity effects

Carbon fixation approached saturation at fairly low irradiance levels, about 500 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$, a third of peak *in situ* levels (Fig. 4). Fixation was substantial even at low intensities (25 $\mu\epsilon \cdot m^{-2} \cdot s^{-1}$), suggesting that light intensity is a minor limiting factor under natural conditions. No inhibition was noted at the highest irradiance levels.

Digestive gland anatomy and fine structure

A single duct of the digestive gland extended into each ceras (Fig. 5A) where it branched into a grape-like cluster of sacs. The diverticular sacs were lined by cells (Type 1) 20–30 µm wide, containing the plastids (Fig. 5B). Light microscopy showed the plastids as 3–7 µm bodies containing a single dark-staining pyrenoid. A second cell type (Type 2) lacked plastids and had a granular cytoplasm.

Electron micrographs of digestive gland diverticula of unstarved animals (Fig. 6) also showed the two cell types. Type 1 cells contained abundant chloroplasts 3–

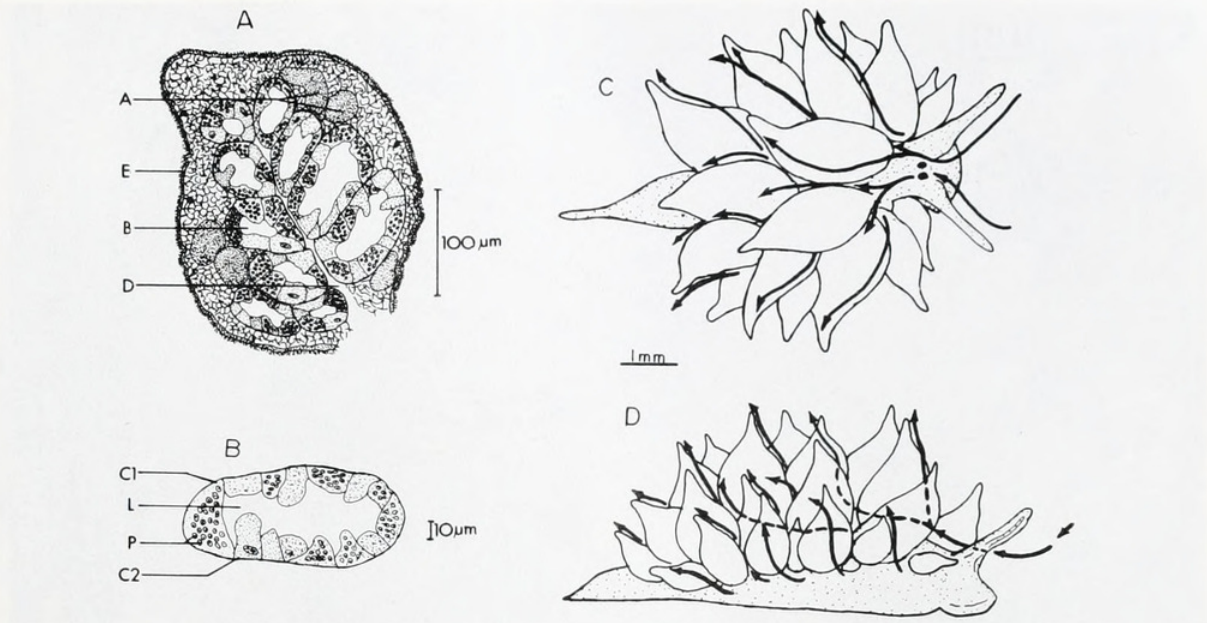


FIGURE 5. A. Sagittal section through ceras of *Costasiella lilianae*. B. Enlargement of single digestive diverticular pouch. C. Dorsal view of animal showing ciliary currents. D. Right lateral view. Abbreviations: A—Albumen gland; B—Lumen of diverticulum; C1—Type 1 cell; C2—Type 2 cell; D—Digestive gland duct; E—ciliated epithelium; L—Lumen of diverticulum; P—plastids.

7 µm long, with abundant thylakoids and plastoglobuli, and a large (1–1.5 µm) central pyrenoid composed of an electron-dense core surrounded by a starch ring. The structure of the chloroplast was virtually identical to *Avrainvillea* plastids described by Hori and Ueda (1967). The chloroplast was bounded by an intact organellar membrane, and sometimes enclosed by a host membrane. Some plastids had regular elliptical outline, and others were irregular, possibly due to differences in the plane of sectioning with respect to the axis of the chloroplast. Many plastids contained a small bleb of granular material adherent to the outer surface and enclosed within the plastid membrane (Fig. 9). This material appeared to contain ribosomes and small vacuoles. Type 2 cells lacked plastids and highly vacuolated granular cytoplasm.

Plastid degeneration in *C. lilianae* is similar to that of other ascoglossans (Trench *et al.*, 1973; Trench, 1975; Switzer-Dunlap, 1975). It involves changes in electron density of plastids and formation of interthylakoid vesicles. Plastids within single cells are fairly uniform in state of decomposition. This suggests that a cell may phagocytize the entire complement of plastids during a specific period in maturation of cerata. Marcus and Marcus (1969) noted that the largest cerata sometimes contained degenerate symbionts (originally described as "zooxanthellae").

In animals starved for 2 weeks, some Type 1 cells contained intact plastids, but others contained plastids with early degenerative changes (Figs. 8, 9). These plastids were more electron dense than fresh plastids, and some showed early stages of thylakoid delamination and interthylakoid vesicle formation. As in unstarved animals, host membranes surround some plastids, but not others. The presence or absence of host membranes did not seem to correlate with plastid condition. Some host membranes appeared to form a continuous envelope around the plastid, while membranes formed from several vesicles enclose other plastids (Figs. 7, 8).

In animals starved for 4 weeks, some cells show further plastid degeneration (Fig. 10), though other cells contained intact plastids. Degenerating plastids had

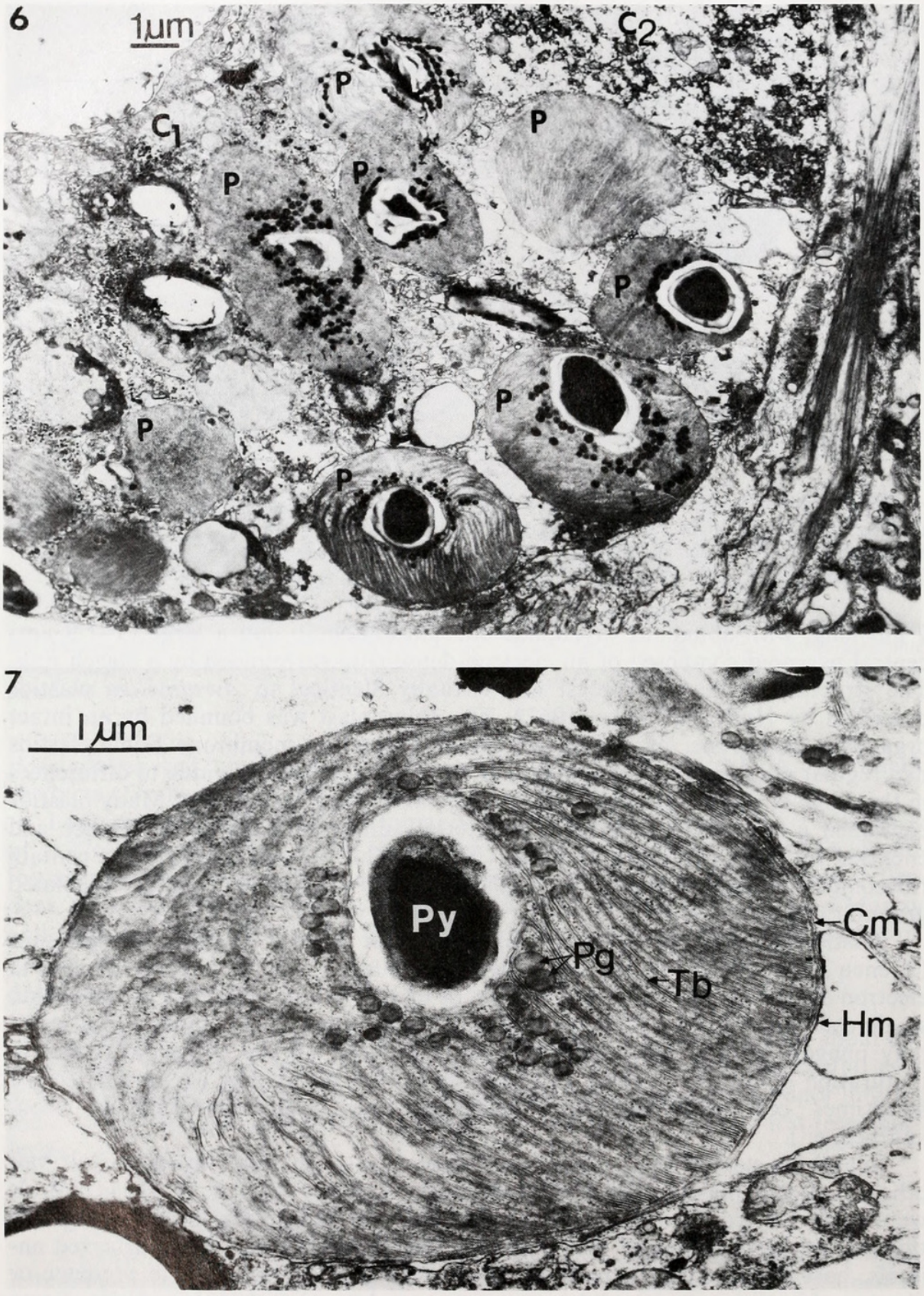
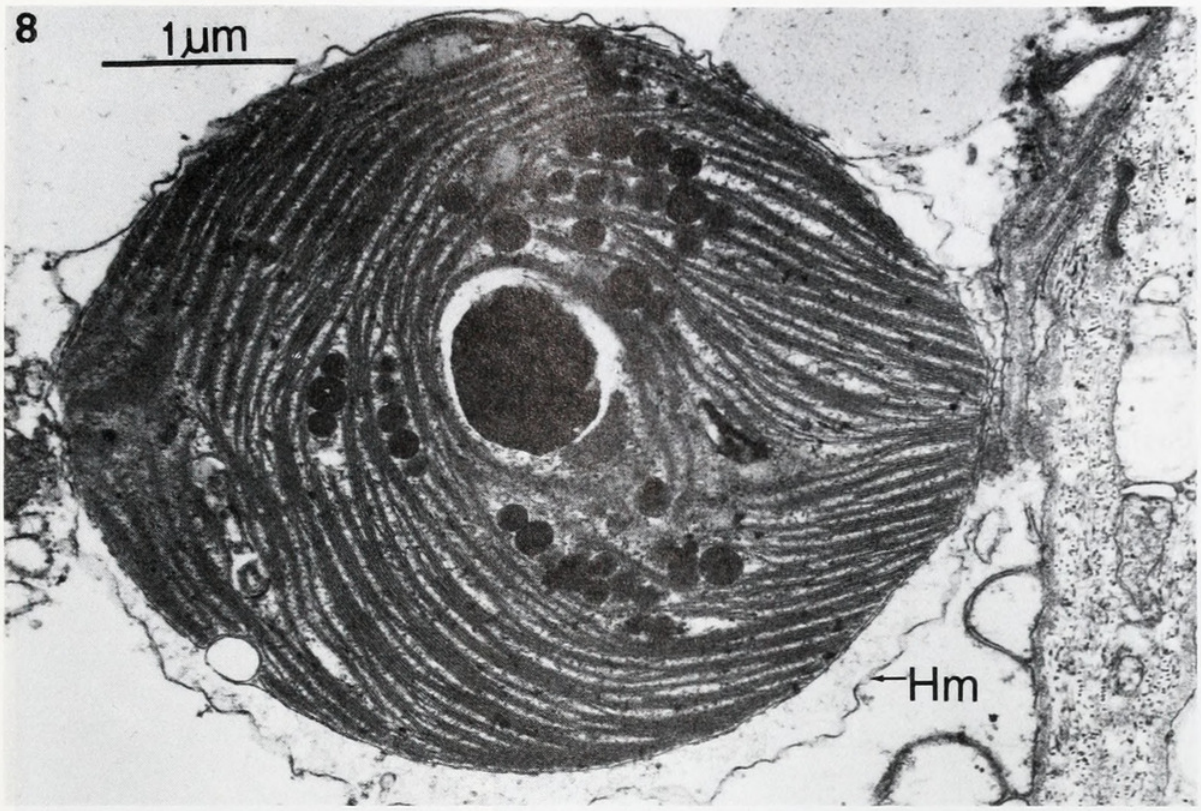


FIGURE 6. Section through diverticulum of digestive gland of unstarved *Costasiella lilianae*. C_1—type 1 cell; C_2—type 2 cell; P—plastid.

FIGURE 7. Symbiotic chloroplast from digestive gland cell of unstarved *Costasiella lilianae*. Cm—chloroplast membrane; Hm—host membrane; Pg—plastoglobuli; Py—pyrenoid; Tb—Thylakoid band.



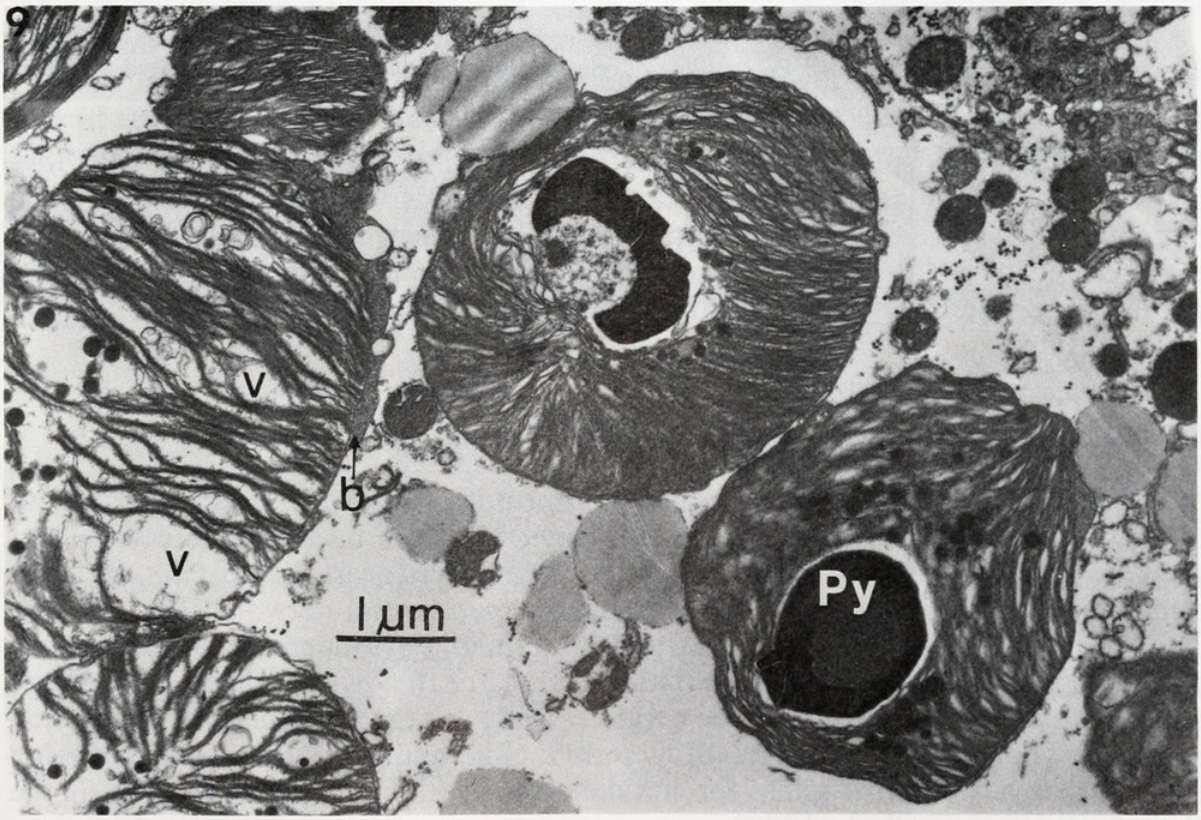


FIGURE 8. Intact chloroplast from digestive gland cell of 2-week-starved *C. lilianae*. Hm—host membrane.

FIGURE 9. Chloroplasts from digestive gland cell of 2-week-starved *C. lilianae*, showing early degenerative changes. b—cytoplasmic bleb enclosed within plastid membranes; Py—pyrenoid; v—interthylakoid vesicle.

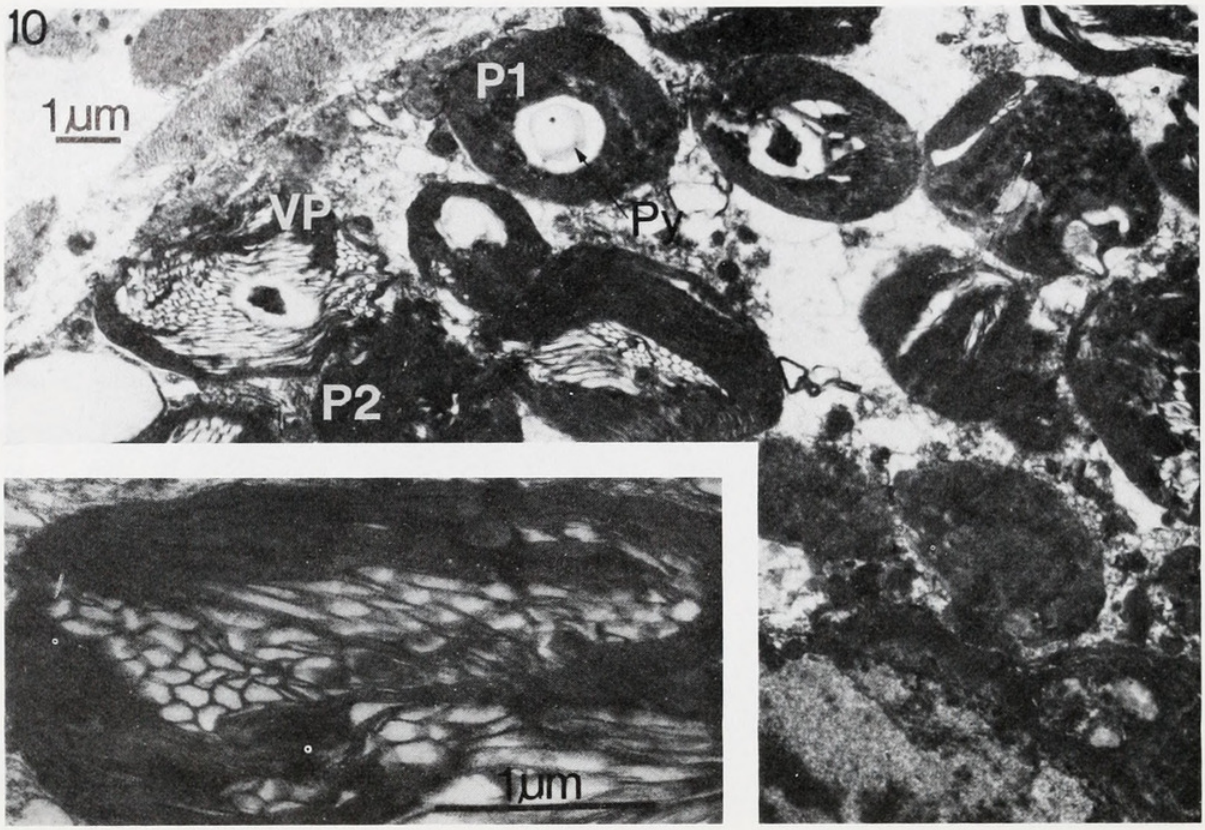


FIGURE 10. Digestive gland cell from 4-week-starved *C. lilianae*. P1, P2—electron dense plastids; Py—pyrenoid (note low density); VP—vesiculated plastid; inset—enlargement of vesiculated plastid.

highly vesicularized plastids, though outer membranes were still intact and thylakoids were recognizable in some plastids. Thylakoids were electron dense, plastoglobuli were indistinct, and some pyrenoids were electron transparent. Vacuoles enclosed most plastids. The cytoplasm contained numerous electron-dense bodies, somewhat smaller than intact plastids. These may have been final stages of plastid degeneration.

Functional morphology

Abundant saccate digestive diverticula almost completely fill the cerata and tail of *C. lilianae*. A pericardial vein complex occurs at the bases of the cerata (not shown). A dense layer of cilia covers the ceratal surfaces. These cilia create a strong circulation over ceratal surfaces and the pericardial network (Fig. 5C, D). This morphology differs from the flattened parapodial arrangement of the elysiids, but is functionally similar. The internal branching of the diverticula creates a large area for storage of plastids, and the cerata provide a large area for light absorption and gas exchange. The arrangement of cerata, cilia, and pericardial veins probably functions as a "negative gill" (for uptake of CO_2 and release of O_2), as in the elysiids (Stirts and Clark, 1980).

These apparent adaptations for gas exchange suggest that CO_2 transport strongly limits symbiotic photosynthesis. The effect of light on photosynthetic rate (Fig. 4) was rather weak at all but the lowest irradiance rates, suggesting that some other factor is limiting. The irradiance effect was measured near the thermal optimum, so CO_2 is the most probable limiting factor. Under optimal conditions,

about 0.3–0.9 μg C was fixed per incubation vial, about 1% of the total CO_2 available. This is enough to noticeably raise pH (Sverdrup *et al.*, 1942), possibly removing CO_2 by carbonate precipitation. This would be an artifact of small experimental water volume, but similar effects in host tissues may explain the "lime spherules" in digestive gland cells of plastid-retentive species (Kawaguti and Yamasu, 1965; Taylor, 1968; Switzer-Dunlap, 1975; Graves *et al.*, 1979). These "lime spherules" are apparently absent in aposymbiotic species (Graves *et al.*, 1979).

Discussion

Chlorophyll retention time, carbon fixation rate, structural integrity of chloroplasts, and stability of photosynthetic efficiency of *C. lilianae* are qualitatively and quantitatively equivalent to those of the most efficient elysiids. *Elysia viridis* (Montagu) and *Tridachia crispata* Mörch retain plastids up to 3 months (Trench and Ohlhorst, 1976), but retention varies with maintenance conditions. Chlorophyll level in *T. crispata* decreased 80% in 16 days (Clark and Busacca, 1978) under the same conditions as the present study. Retention in *E. viridis* is temperature dependent, with a Q_{10} of about 2 (calculated from data of Hinde and Smith, 1975) to 3.4 (from data of Trench *et al.*, 1973). Switzer-Dunlap (1975) found that light quality affected plastid retention in *Plakobranchus ocellatus* (van Hasselt). Another possible influence is the plastid source and its natural plastid turnover rate; algae with rapid plastid turnover may provide plastids that hosts do not retain long. This might explain variability in *T. crispata*, which feeds on and retains plastids from a variety of Chlorophyta (Clark and Busacca, 1978; Jensen, in press).

Few data are available for interfamilial comparison of photosynthetic efficiencies (Table I). Light:dark fixation ratios vary widely, and must be interpreted with caution, because light fixation rates may be measured at suboptimal conditions.

Table I

Comparison of light:dark fixation ratios and net carbon fixation rates in ascoglossan molluscs.

Species	Family	Light/dark ratio	Net fixation ($\mu gC \cdot mg\ Chl^{-1} \cdot hr^{-1}$)	References
Costasiella lilianae (Marcus)	Hermaeidae	18–90	200–300	Present study
Limapontia depressa A & H	Stiligeridae	9.5		Hinde & Smith, 1974
Elysia viridis (Montagu)	Elysiidae	33	95	Trench *et al.*, 1973 Hinde & Smith, 1974, 1975
Elysia hedgepethi Marcus	Elysiidae	9.0		Greene & Muscatine, 1972
Elysia tuca Marcus	Elysiidae	100	60	Goetzfried, 1977 Stirts & Clark, 1980
Elysia cauze Marcus	Elysiidae	22		Clark *et al.*, 1979
Plakobranchus ocellatus van Hasselt	Elysiidae	6.3		Greene & Muscatine, 1972

Carbon mass fixation rates are available only for three species (Table I). Maximum rates for *C. lilianae* are 2 to 6 times those of elysiid species.

Long-term, highly efficient chloroplast symbiosis in a ceratiform ascoglossan clearly shows that the symbiosis is not limited to the Elysiidae as previously proposed (Muscatine and Greene, 1973; Hinde and Smith, 1974; Trench, 1975; Muscatine *et al.*, 1975; Graves *et al.*, 1979). Other less pronounced and possible examples of stiligeroid symbioses were summarized by Clark and Busacca (1978) (also see Taylor, 1967; Kremer and Schmitz, 1976).

If plastid symbioses occur within stiligeroid families as well as in Elysiidae, some may also occur among the common ancestors of the two evolutionary lines, the tectibranch Ascoglossa. Several tectibranchs have not shown functional plastids (Muscatine and Greene, 1973; Clark *et al.*, unpublished data), but some evidence of functional plastids exists (Stirts, 1980; Clark, in press).

Poorly understood factors affect plastid maintenance. Symbiotic capacity varies within populations, as in *Limapontia depressa* (Alder and Hancock) (see Hinde and Smith, 1974). Fixation rates also vary widely within populations. Coefficients of variation in fixation rate range from 0.16 to 0.65 in this study, and from 0.35 to 0.41 for two elysiids (Greene and Muscatine, 1972; our calculations).

Several major factors probably determine whether an ascoglossan retains chloroplasts as symbionts. Diet appears to be one such factor, as only certain types of algal plastids seem to be retained as symbionts. Siphonalean plastids resist treatments which destroy other plastids (Giles and Sarafis, 1974), and hence may resist digestion by host cells (Trench *et al.*, 1973; Trench, 1975; Gallop *et al.*, 1980). However, this cannot be the only factor, because many tectibranch species are aposymbiotic despite siphonalean diet (Muscatine and Greene, 1973), and nonsiphonalean plastids support well-developed symbioses (Taylor, 1971; Hinde and Smith, 1974; Graves *et al.*, 1979). Variable melanism, influenced by diet, is common among stiligerids (see Clark, 1975); the results of Hinde and Smith (1974) suggest that melanism is associated with non-functional plastids. Other factors may relate to cost-benefit aspects of symbiosis—whether photosynthetic yield to the host exceeds costs of plastid maintenance. Rates at which substances "leak" from plastids may also affect net benefit to the host (see Gallop, 1974). Climatic factors, especially temperature and light, obviously are important determinants of photosynthetic yield (Hinde and Smith, 1975; Goetzfried, 1977; Clark *et al.*, 1979; Stirts and Clark, 1980; present study). Intersections of these several "sets" of factors may greatly reduce the total number of symbiotic Ascoglossa, and give a false impression of disjunct distribution of symbiosis when few species have been examined. However, there does not appear to be a major phylogenetic determinant of symbiotic capacity within the non-tectibranch Ascoglossa.

Acknowledgments

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