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PLASTID ENDOSYMBIONTS IN THE FRESHWATER CRUSTACEAN *DAPHNIA OBTUSA*

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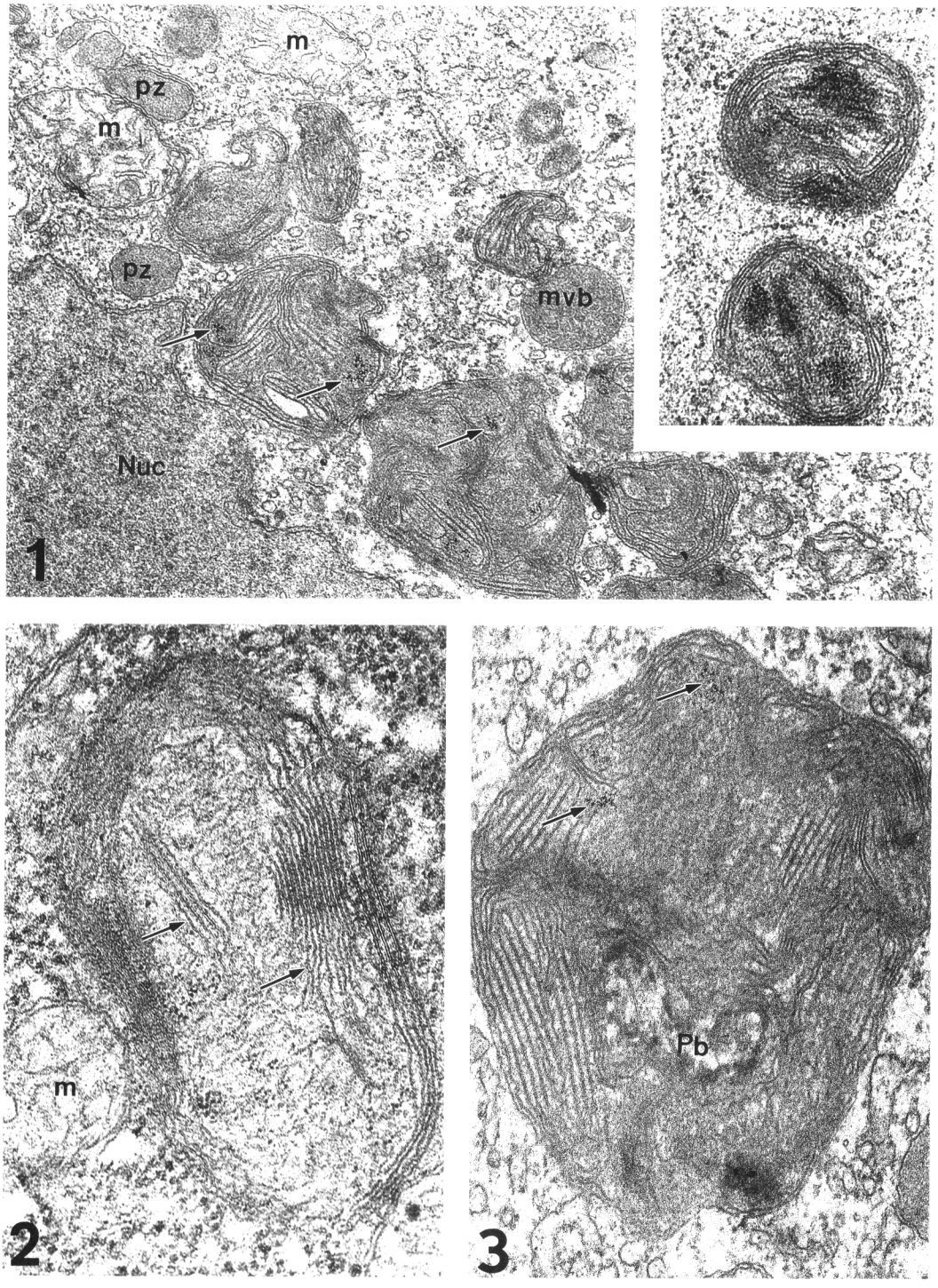
ABSTRACT

Symbioses between cyanobacteria (or eukaryotic algae and their plastids) and protists or invertebrates are well known and are important in evolution and ecology. However, no such symbioses have been previously reported in arthropods. An ultrastructural study of a branchiopod crustacean (*Daphnia obtusa*) from temporary ponds consistently revealed plastids inside gut endocytes. Plastids were most frequent in animals from shaded woodland ponds. Ultrastructure indicated that plastids in *D. obtusa* (a) are sequestered cyanobacteria and plastids from eukaryotic algae, and (b) senesce, suggesting that these generalist grazers have not closely coevolved with a plastid source. *Daphnia obtusa* (and other cladocerans?) in temporary ponds may benefit from plastid presence in several ways (nutrition, oxygen, calcium availability); daphnids inhabiting permanent waters may be less selected for plastid uptake. This paper represents an extension of plastid endosymbiosis into the Arthropoda, and indicates more sophisticated evolutionary and ecological interactions between these important crustacean herbivores and their food than previously recognized.

Photosynthetic endosymbionts are well known in some protists and invertebrates, but have not been described in arthropods (Douglas, 1994). Most such symbioses involve whole chlorophyte or cyanobacteria cells, and 80% of hosts are marine animals (Reisser, 1992). However, plastid symbionts (Hinde and Smith, 1974; Trench, 1982; Clark, 1992; Laval-Peuto, 1992; Pierce *et al.*, 1996) are known in protists and marine sacoglossan molluscs (Trench, 1975; Laval-Peuto, 1992; Kohler *et al.*, 1997).

"Chloroplast symbiont" has long been used to describe plastids sequestered by sacoglossan molluscs from their chlorophyte foods and retained in animal tissues (Hinde and Smith, 1974). We adopt the less-specific term "plastid symbiont" (Laval-Peuto, 1992) when discussing our results because we have yet to identify the sources of the endocytic plastids we studied. In addition, we include endosymbiotic cyanobacteria (cyanelles) in our discussion of plastids: cyanobacteria and plastids from "higher" plants are similar in some ultrastructural features and may be evolutionarily related (Trench, 1982). Although the terms "sequestered chloroplast" (Rogerson *et al.*, 1989) or "kleptoplastid" (Clark, 1992) aptly represent the uptake of plastids by a host, and may be appropriate, we do not adopt them here because (a) cyanobacteria are taxonomically distinct (i.e., not an organelle from a chlorophyte cell), and (b) it remains possible that hosts are themselves regulated to some extent by plastid presence (Pierce *et al.*, 1996). In addition, some discussion of coevolution is relevant if cyanobacteria are a source for plastid endosymbionts.

In studying temporary-pond crustaceans, we noticed that the digestive tract of *Daphnia obtusa* Kurz, 1874 (Crustacea: Branchiopoda) was often green, despite low phytoplankton density in the water. This observation itself is not novel: *Daphnia* are effective filter-feeders of phytoplankton (Wetzel, 1983; Dodson and Frey, 1991), and the simplest explanation for pigmented gut endocytes is absorption of chlorophyll during digestion of phytoplankton cells. Nonetheless, we posed an alternative (though not mutually exclusive) hypothesis: that gut endocytes harbor photosynthetic endosymbionts. As a test of this hypothesis, we conducted an ultrastructural study on *D. obtusa* gut tissue, with the rationale that endosymbiont presence would most clearly be tested with structural evidence. Additional studies to evaluate function of endosymbionts (e.g., photosynthesis, sugars or oxygen production, etc.) would be justified only upon positive structural evidence, and were beyond the scope of this initial study.



Figs. 1–3. Dark plastids in midgut endocytes of *Daphnia obtusa*. 1, Plastids seen near the nucleus (Nuc) vary in size, but most are larger than mitochondria (m). They show parallel arrays of single membranes, dense stroma, and rare, small clusters of granular material (arrows). Peroxisomes (pz) are common, whereas multivesicular bodies (mvb) are rarely seen. Abundance of rough endoplasmic reticulum and nuclear euchromatin attest to metabolic activity of the cell. ×25,200. The inset shows two dark plastids seen in a different plane of the same cell. ×33,000; 2, In addition to peripheral lamellae, short thylakoid stacks (arrows) are common in dark plastids. Clusters of granules deposited in stoma are rare. Note a relatively large size of the plastid as compared to the mitochondrion (m). ×70,000; 3, Larger dark plastids sometimes contain a pyrenoid body (Pb). Also note multiple stacks of single thylakoid membranes and isolated clusters of granular material (arrows). ×43,100.

Table 1. Summary of conditions in contrasting habitats of *Daphnia obtusa* examined for plastids. Data were derived from other studies of the ponds, including Nix and Jenkins (in press). Values are expressed as the average of all measurements during 1997 ± 1 standard deviation (SD). Average hydroperiod for woodland ponds = 103 ± 12 days. Chlorophyll *a* is a measure of phytoplankton biomass: shaded ponds typically had lower values than the open pond, but exhibited high levels when ponds were drying down (therefore greater average and much greater SD than for the open farm pond). Seston C was estimated as ash-free dry weight for seston particles that may be ingested by *D. obtusa* (operationally defined as > 3 μm and < 35 μm in size). Shaded woodland ponds were also high in dissolved organic matter, as indicated by color (data not presented).

Variable	Shaded woodland ponds (3)	*n*	Open farm pond	*n*
pH	5.8 ± 0.4	44	7.7 ± 1.2	18
DO	5.4 ± 3.6	42	10.7 ± 3.2	17
Temp (C)	14.8 ± 5.0	44	18.8 ± 7.1	18
Chl *a* (μg/L)	19.4 ± 51.4	43	4.2 ± 8.4	16
Total P (mg/L)	0.8 ± 0.8	42	0.9 ± 1.0	17
TotN (mg/L)	3.3 ± 2.6	39	1.4 ± 0.6	16
Seston C (mg/L)	56.0 ± 117.0	45	11.4 ± 12.0	18

We also compared *D. obtusa* from several ponds to test the prediction that endosymbiont presence is related to environmental quality. Other invertebrates found to have photosynthetic endosymbionts inhabit systems that vary in resource availability or that have generally low resource levels (Marin and Ros, 1992; Reisser, 1992; Douglas, 1994). The animals we studied inhabit temporary ponds; compared to permanent waters, temporary ponds can impose severe selection pressures due to short hydroperiod (interval that pond exists), and low food quality or quantity (Kenk, 1949; Moore, 1970; Wiggins *et al.*, 1980). We predicted that *D. obtusa* in shaded, temporary ponds with short hydroperiods may retain endosymbionts, while animals in an open farm pond with longer hydroperiod may not.

Materials and Methods

Daphnia obtusa were collected in central Illinois from three shaded woodland temporary ponds with short hydroperiods (months) and a nearby, open farm pond with a hydroperiod greater than one year. Animals were transported in pond water on ice to the laboratory, and their gut was dissected and fixed in 3.5% glutaraldehyde (in 0.2 M cacodylate buffer, pH 7.4) within 4 h after collection. An hour-long fixation was followed by postfixation in 2% osmium tetroxide, dehydration in a graded series of ethanol, followed by propylene oxide, and embedding in epoxy resin mixture. Six to eight animals were examined from each of the four ponds studied ($n = 27$). Thin sections of caecae, midgut, and hindgut were stained with uranyl acetate and lead citrate and examined with a Hitachi H600 transmission electron microscope. Gonads and embryos were also studied in selected samples.

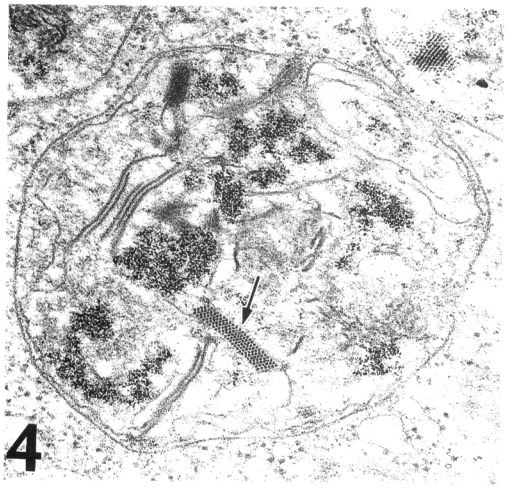
Results

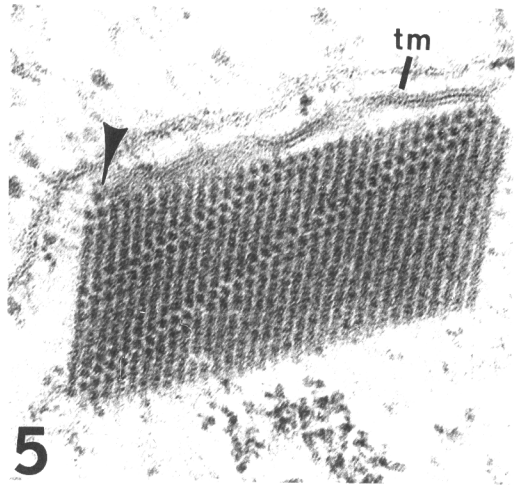
Endocyte fine structure was generally similar to that previously reported for *Daphnia*. Cuboidal to columnar endocytes were lined with microvilli and commonly showed ultrastructural correlates of intense metabolic activity: nuclei rich in euchromatin, prominent nucleoli, dilated Golgi bodies, an abundance of ribosomes and mitochondria, and well-developed endoplasmic reticulum (Fig. 1). Peroxisomes were common, but lysosomes and multivesicular bodies were only occasionally seen (Fig. 1). We found little evidence of storage of lipid droplets and carbohydrate granules in endocyte cytosol.

Plastids were a consistent feature of endocytes in the midgut and caecae of all specimens studied, and were more frequent in *D. obtusa* from shaded, short-hydroperiod ponds than in those collected from an open, long-hydroperiod farm pond. Plastids were not seen in the hindgut or in other tissues (gonads, eggs). Ponds differed in physical-chemical conditions (Table 1).

Two types of plastids were observed: dark and light. Dark plastids were most common and were found throughout the endocyte cytosol. Dark plastids varied in size but were usually larger than mitochondria, were bound by a single-unit membrane, and had an electron-dense stroma and numerous thylakoids (Figs. 1–3). Some of the larger dark plastids contained a pyrenoid (Fig. 3). Thylakoid membranes in dark plastids were arranged as peripheral concentric lamellae and centrally placed stacks composed of multiple single membranes (Fig. 2). Most dark plastids contained a few small clusters of fine electron-dense granules (arrows, Figs. 1, 3).

Light plastids (Fig. 4) were typically larger and less frequent than dark ones. Bound by a single membrane, light plastids had pale





Figs. 4, 5. Light plastids in midgut endocytes of *Daphnia obtusa*. 4. Light plastids show pale stroma, abundant large clusters of granular material, and at least one crystalline body (arrow). Scant thylakoid stacks are represented by two to three short membranes which show a trilaminar structure. ×31,900; 5. Crystals in light plastids are composed of a lattice of electron-dense particles which often shows continuity (arrowhead) with the nearby trilaminar thylakoid membrane (tm). ×128,000.

stroma and scant, more centrally placed thylakoids represented by two to three stacked membranes. Thylakoid membranes had a trilaminar composition (Figs. 4, 5). Prominent features of light plastids were large clusters of granular material and one or a few crystalline inclusions (Fig. 4). These inclusions were composed of a lattice of electron-dense particles, and thylakoid membranes in the vicinity of the crystal showed a continuity with the crystalline lattice (Fig. 5).

Discussion

General features of *Daphnia obtusa* caecae and midgut endocytes in our study resembled ultrastructure previously reported for various *Daphnia* species (Hudspet and Revel, 1971; Schultz and Kennedy, 1976; Schlecht, 1979; Elendt, 1989; Elendt and Storch, 1990; Bodar *et al.*, 1990), including the study of Quaglia *et al.* (1976) on *D. obtusa* ultrastructure from a temporary pond. However, none of these studies noted plastids, nor did we observe plastids in published micrographs of these studies.

The consistent presence of plastids in our specimens is novel, and is the first evidence of photosynthetic endosymbionts in an arthropod. We hypothesize that plastids have not been observed previously because most animals were from laboratory cultures and/or relatively high-quality habitats (e.g., permanent ponds). In addition, the apparent absence of plastids in previous ultrastructural observations of temporary-pond animals (Quaglia *et al.*, 1976) suggests that plastids are not obligate in *D. obtusa*. We discuss below the details of our results, some implications of those results, and directions for future research.

Plastid membrane ultrastructure provides clues to the origin of plastids in *D. obtusa*. The number of membranes enveloping plastids varies among endosymbioses and may indicate both origin of the endosymbiont and the extent of transmembrane interchange between plastid and host (Reisser, 1992; Douglas, 1994). Plastid symbionts are commonly thought to "leak" photosynthates to host cells (Muscatine and Lenhoff, 1963; Hinde and Smith, 1974; Trench, 1975). A leaky membrane facilitates both enhanced release of glucose from the endosymbiont and efficient uptake of nitrate from host cytosol (Trench, 1975), thus maximizing the benefits of symbiosis for both partners. Photosynthetic endosymbionts of eukaryotic origin in invertebrates and protists are usually bound by a double membrane, indicating uptake by phagocytosis followed by retention of a modified phagosomal membrane (symbiosome) surrounding the plastid (Smith, 1973; Rands

et al., 1993; Douglas, 1994). Plastids in apicomplexans are surrounded by four membranes, suggesting an ancestral ingestion of a eukaryote that itself contained a plastid (Köhler *et al.*, 1997). Endosymbiotic cyanobacteria in various unicellular green algae either show a reduced cell envelope or are surrounded only by a single membrane (Fogg *et al.*, 1973; Kies, 1984). The single membrane surrounding *D. obtusa* plastids is consistent with a cyanobacterial origin for the plastid symbionts, and may reflect an adaptation to maximize host-symbiont metabolite exchange while minimizing metabolic expense of producing and maintaining symbiosomes.

Internal ultrastructure of plastids provides both a clue to plastid metabolic activity and additional evidence of a cyanobacterial origin. Although chlorophyte plastids and cyanobacteria often contain trilaminar thylakoids, loosely packed single-thylakoid membranes can be present in both endosymbiotic (Fogg *et al.*, 1973; Kies, 1984) and free-living (Fogg *et al.*, 1973) cyanobacteria. This arrangement of thylakoid membranes resembles that observed in our study.

Whereas a cyanobacterial source of plastids is consistent with ultrastructural features of most *D. obtusa* plastids we observed, variation in plastid size and structure indicates that cyanobacteria were not an exclusive source for *D. obtusa* plastid symbionts. Pyrenoids are not found in cyanobacteria (Fogg *et al.*, 1973; Kies, 1984), so pyrenoid-containing plastids that we observed may have originated from green algae. This is particularly likely if one takes into account that chloroplasts of some unicellular green algae, like plastids in our study, also contain concentric, peripheral thylakoid lamellae composed of single membranes (Dodge, 1973). Because we have not observed intact algal cells in endocytes, we assume that ingested algae are lysed in the gut lumen and plastids are phagocytosed by endocytes. In summary, *D. obtusa* may take up and retain plastids from several sources, rather than a single plastid donor.

The longevity of plastid symbionts seems to be affected by host life history, food availability, and features of the host's habitat. Sacoglossan molluscs maintain plastid symbionts for months (Pierce *et al.*, 1996), because the molluscs live more than one year and are obligate grazers of algae that are available for predictably short intervals (Marin and Ross, 1992). In contrast, *D. obtusa* are generalist filter-feeders and live several weeks in unpredictable habitats that select for mixed life-history strategies (Stearns, 1976). We interpreted the ultrastructure of light plastids as evidence of senescence, which indicates that *D. obtusa* are facultative in their plastid uptake, depending on environmental conditions, and do not maintain plastid symbionts for extended periods. This would suggest a lesser degree of coevolution between these short-lived animals and their plastids than in other photosynthetic symbioses (Trench, 1975; Douglas, 1994; Pierce *et al.*, 1996).

The majority of *D. obtusa* plastids were metabolically active, based on the abundance of dark plastids, and presumably released photosynthetic products to the host. The scarcity of thylakoid membranes in light plastids suggests a decrease in plastid metabolic activity. Membranes may have disassembled or condensed into crystalline lattice. Crystals similar to those seen in *D. obtusa* light plastids are sometimes seen in free-living cyanobacteria and chlorophyte algae (Dodge, 1973; Jensen and Brown, 1970). These crystals show continuity with thylakoid membranes in aged cyanobacteria, as in our study (Fogg *et al.*, 1973).

Accumulation of granules in light plastids also suggests senescence. Free-living cyanobacteria sometimes store the enzyme ribulose 1-5-bis-phosphate carboxylase as carboxysome particles (Kies, 1984). Assuming that endosymbiotic plastids are in the host cell because photosynthetic products are exported to the host, stores of enzyme reserves in productive endosymbionts would be minimal. Accordingly, carboxysomes are uncommon in endosymbiotic cyanobacteria (Fogg *et al.*, 1973; Kies, 1984). As plastids senesce, their photosynthetic enzymes may condense into granules. Alternatively, granules may be accumulated carbohydrates, not released to the host as plastid membrane function declined. Free-living cyanobacteria store carbohydrates as polyglucoside granules, but cyanelles have enhanced sugar transport to the host and do not produce similar granules (Kies, 1984).

The scarcity of granular material in metabolically active dark plastids suggests export of organic product to the host. In addition, the paucity of lipid and glycogen in endocyte cy-

tosol (relative to that observed by Elendt, 1989) may indicate that animals we collected from temporary ponds were rapidly dedicating energy derived from plastids to growth and reproduction. Elendt (1989) observed lipid droplets and glycogen in endocytes of cultured *Daphnia*, but numbers of these energy stores decreased substantially after one day of starvation (Elendt and Storch, 1990). Quaglia *et al.* (1976) observed only occasional lipid droplets in endocytes of temporary pond *Daphnia* and made no mention of glycogen granules. Clearly, experiments on animal-endosymbiont energetics are needed to evaluate the activity and importance of plastids in *D. obtusa*.

Daphnia can select foods, but are generalist filter feeders and feed upon multiple algal species, including cyanobacteria (Porter, 1973, 1977; Porter and Orcutt, 1980). It is likely that *D. obtusa* do not obtain plastids from a single plastid donor, but instead take up and retain plastids from several sources. *Daphnia obtusa* typically live less than five weeks and probably do not maintain plastids for long periods of time as do other, longer-lived invertebrates. We did not find evidence of plastids in eggs and embryos; plastids are probably not vertically transmitted.

Our prediction that photosynthetic endosymbionts would be found in animals from woodland temporary ponds only (and not in animals from the open farm pond) was too absolute: plastids were more common in woodland-pond animals, but also present in farm-pond animals. Woodland temporary ponds were shaded, and had large quantities of decaying leaves, high dissolved organic matter, low pH, and low dissolved oxygen. Woodland ponds usually had low phytoplankton density, although chlorophyll *a* concentration increased markedly as ponds dried down or rewetted (Nix and Jenkins, in press). In addition, algae in woodland ponds were often dominated by filamentous (e.g., *Spirogyra*) or colonial (e.g., *Volvox*) forms that are less edible for *Daphnia* than smaller, single-cell forms (Wetzel, 1983). Finally, the temporary ponds form only in Spring, precluding a second *Daphnia obtusa* population peak in Autumn, as commonly occurs in permanent ponds (Wetzel, 1983).

We hypothesize that woodland temporary-pond conditions combine to provide lower habitat quality of shorter duration, and therefore stronger selection for plastid uptake, compared to permanent habitats with more consistent phytoplankton density, circumneutral pH, greater dissolved oxygen, lower dissolved organic matter, and opportunity for both Spring and Autumn populations. The apparent relationship between plastid frequency and habitat quality suggests an inverse relationship between selection for plastid endosymbiosis and resource availability for the host, consistent with endosymbionts in other invertebrates (Marin and Ross, 1992; Douglas, 1994; Pierce *et al.*, 1996). By extension, we expect that *Daphnia* (and perhaps other crustaceans) in temporary ponds may more commonly exhibit plastid endosymbiosis, whereas *Daphnia* in most lakes would not be expected to have plastid symbionts.

Clearly, our results point to further inquiry, including studies to address the following questions:

1. Is plastid endosymbiosis a general (habitats, taxa, timing) phenomenon in crustaceans, and what conditions promote it? We expect plastid endosymbionts to be most likely in small, transparent crustaceans (e.g., cladocerans) that inhabit waters with relatively low habitat quality and duration (e.g., temporary ponds).
2. How much photosynthesis occurs in plastid endosymbionts, and what selective advantage is conferred by plastid endosymbiont presence? Does plastid photosynthesis contribute labile carbohydrates to host cells in a habitat dominated by refractory detritus? Does plastid photosynthesis provide needed oxygen in the nearly anaerobic waters? Does plastid carbonate uptake promote host calcium uptake (as in corals) in acidic waters? Or does plastid phagocytosis (intracellular digestion) simply represent a more efficient means to capture nutrition from partially digested foods (extracellular digestion)? Some combination of the above?
3. What algal species serve as plastid donors? We predict that cladocerans (generalist filter feeders) are not restricted to a single species for plastid uptake, but can sequester ingested cyanobacteria as well as plastids from macerated eukaryotic algae.
4. What is the mechanism of plastid recognition and uptake? We expect that plastids are phagocytosed by midgut and/or cecae cells, but we did not observe this process,

and we observed no phagosomal membranes surrounding intracellular plastids in *D. obtusa*. In addition we expect that plastid uptake is facultative, depending on the environmental conditions, host, and algal cells available during seasonal succession.

5. Can plastid endosymbionts be transported among host cells or tissues? We observed no plastids in gonads and eggs, so we expect that plastids are taken up by each individual during feeding.
6. How long can plastids be maintained in short-lived daphnids? Our observation of dark (active) and light (degraded) plastids suggests less maintenance of plastids than observed in other, longer-living organisms (e.g., sacoglossan molluscs). We think it is unlikely that short-lived cladocerans exhibit long-term plastid maintenance.
7. Do the crystals and granules observed in light plastids indicate degraded function of the plastid membrane, photosynthetic pathways, or both?

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