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Ultrastructure of the digestive gland of larval and adult stages of the sacoglossan *Elysia patagonica*

**Abstract** Chloroplast retention in the sacoglossan gastropod *Elysia patagonica* Muniain and Ortea, 1997 was investigated. Intact chloroplasts of the green algae *Bryopsis plumosa* (Chlorophyta: Bryopsidaceae) were observed by transmission electron microscopy in vacuoles of the digestive cells of benthic adults starved for 20 days. Intact chloroplasts from the microalgal *Nannochloropsis* sp. (Heterokontophyta: Eustigmatophyceae) were found in the digestive cells of veliger larvae feeding for 8 days. Similar digestive cells were observed in adults and planktonic larvae of *E. patagonica*. The retention ability is confirmed in both stages.

**Introduction**

Feeding specialization is not uncommon among marine herbivores. The sacoglossans (=ascoglossans) are similar to terrestrial insects in that 80% of the species feed from no more than one family of plants (Hay 1992), with most species feeding on only one genus of seaweed (Marin and Ros 1988, 1989). Evolution in the order Sacoglossa has been closely linked to their specialized sectorial herbivorous habits (Jensen 1997). However, recent studies showed that the sacoglossan *Elysia viridis* (Montagu, 1804) preferred to associate with and consume the introduced macroalga *Codium fragile* (Chlorophyta: Codiaeace) over the native species to Scottish shores, *C. tomentosum* (Trowbridge 1998, 2000).

Sacoglossans appear to be one of the few metazoan groups to retain functional chloroplasts as intracellular organelles in the cells of the digestive gland, although this capacity has been recorded from ciliates and foraminifera (Stoecker et al. 1987;Clark 1992). This phenomenon has been described as “chloroplast symbiosis” by several authors, and later, more appropriately, as “kleptoplasty”. Kleptoplasty appears among shelled and non-shelled sacoglossan species. Some shelled families (Cylindrobullidae, Volvatellidae, Juliidae and Oxynoiidae) maintain structurally intact plastids for several days, but without photosynthetic function (Clark et al. 1990), while functional kleptoplasty occurs only in the non-shelled families Polybranchiidae, Hermaeidae and Limapontiidae (see Jensen 1997; Williams and Walker 1999).

Elysoids (Plakobranchiidae) are known to retain chloroplasts, acquired from algal food, in the cells of the digestive gland, where they remain functional for various periods of time depending on the species involved. *E. viridis* can survive 2 months of starvation under light conditions because it retains functional chloroplasts during this period of time (Hinde and Smith 1975). The molluscs can utilize organic carbon released from their chloroplasts. The role that the chloroplasts play in the metabolic requirements of sacoglossans seems to be of considerable importance; their retention represents an evolutionary strategy that provides a net energy supply to the sacoglossan host (Ros and Marin 1991).

The presence of sequestered plastids is not hereditary; the plastids must be obtained anew by each generation of molluscs because they are not transmitted in the spawn or embryos (Greene 1970; Marin and Ros 1989,
1993; Muniai 1997). Previous chloroplast retention studies of digestive cells within the Sacoglossa have dealt with adult specimens or benthic juveniles from species with direct development. In *Elysia subornata* Verril, 1901, chloroplasts are not retained by the digestive gland until the digestive diverticula is differentiated in the crawling juvenile, about 5 days after hatching (Clark et al. 1979). The Mediterranean *Elysia timida* (Risso, 1818), with direct development (cited as a poecilogonic species by Marin and Ros 1993), has intact chloroplasts for 7 days after hatching (Marin and Ros 1993). In *Elysia chlorotica* (Gould) the plastids stay photosynthetically functional for 8–9 months, many months longer than those of any other species yet described (Pierce et al. 1996, 1999).

*Elysia patagonica* Muniai and Ortea, 1997 was the first sacoglossan described from Argentina, and its ecological and chemical characteristics have recently been investigated (Muniai et al. 1995, 1999; Muniai 1997). The present study compares and discusses the digestive cells and mechanisms of plastid retention in planktrophic veliger larvae versus those in benthic adults.

**Materials and methods**

Numerous specimens of *Elysia patagonica* were collected on the chromophytic alga *Bryopsis plumosa*, in tidal pools of the intertidal rocks at Punta Marqués (45°58’S; 67°34’O), San Jorge Gulf, Argentina. The plants of *B. plumosa* contained the sacoglossans *E. patagonica* and *Placia* sp. The holotype and paratypes of *E. patagonica* are deposited in the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” (MACN 33780, MACN 33800, see Muniai and Ortea 1997).

Transmission electron microscopy

The collected adults were immediately fixed in 2.5% Millonig’s phosphate-buffered glutaraldehyde (pH 7.2–8.2) for 1 h. The pieces were washed in 2.5% NaHCO₃ in distilled water (60 min at 25°C), postfixed in 2% OsO₄ in 1.25% NaHCO₃ for 1 h, dehydrated through an ethanol series, and then embedded in Epon. The larvae obtained from egg masses deposited in an aquarium were fixed in Millonig’s phosphate-buffered glutaraldehyde for 15 min at room temperature. Samples were centrifuged into soft agar which was then allowed to gel, post-fixed for 1 h at 10°C in 2% OsO₄ in 1.25% NaHCO₃ rinsed with NaHCO₃, and then stained with uranyl acetate and lead citrate. After electron dehydration, all samples were embedded in Spurr’s low-viscosity resin, thinly sectioned and stained with uranyl acetate and lead citrate.

Larval culture

Adult sacoglossans were maintained in a refrigerated and aerated aquarium with a constant supply of *B. plumosa*. Egg masses of *E. patagonica* were removed from the algae and the aquarium wall, and then transferred to clean culture chambers containing 20 ml of 0.22 μm Millipore filtered seawater (MFSW). Egg masses were maintained in seawater controlled with antibiotics (800 mg penicillin-G, 180 mg dihydrostreptomycin and 8 mg chloramphenic in 100 ml of MFSW) until hatching of the planktrophic larvae. Larvae cultures were incubated at 11°C and 17°C and kept on a 12 h light:12 h dark cycle at an irradiance of about 300 μE m⁻² s⁻¹.

Veiger larvae of *E. patagonica* were hatched with the microalgal culture of *Nannochloropsis* sp. (Heterokontophyta: Eustigmatophyceae). Algae were transferred with a Pasteur pipette in order to maintain a constant algal density 0.5–1×10⁶ cells ml⁻¹. Culture chambers were directly examined with a Nikon (SMZ-U, zoom 1:10) inverted microscope, equipped for transmitted light and epifluorescence microscopy. Egg capsule diameters, number of embryos per capsule, larval development and feeding were recorded using a video camera system and printed on photograph paper. The microalgal culture (*Nannochloropsis* sp.) was obtained from the fish culture laboratory of the Instituto Español de Oceanografía, Mar Menor, Spain.

**Results**

Larval development

Characteristic of *Elysia patagonica* are the high incidence of multiple ova within the egg capsules and the exclusive presence of planktrophic larvae (Muniai 1997; Muniai and Ortea 1997; Muniai and Penchas- zadeh 2000).

The shell length of newly hatched larvae was 251–323 μm (n = 15, mean = 279.7, SD = 21.80). Green algae were observed in the digestive tract of larvae within 2 h of hatching (Fig. 1A). One week after hatching, veliger shells ranged from 260 to 377 μm in length (n = 15, mean = 305.8, SD = 36.02). In the planktrophic veligers of *E. patagonica*, algal cells were captured and conveyed to the mouth by the ciliated velar lobes. The algal food was conducted to the stomach by the activity of esophageal cilia; it was accumulated in the ventral portion of the stomach, where the cilia continued to keep the food in motion (Fig. 1B–C). The veligers were very active during feeding and swimming. The larval stomach was divided into a ventral chamber and a style sac (Fig. 2a). The style sac was characterized by a wide band of densely packed cilia. The larval intestine was divided into two main regions, an anterior and a posterior intestine. The anterior intestine was a densely ciliated pouch with a wide lumen beginning at the posterior end of the style sac. Some of the larvae had completed metamorphosis within 14 days after hatching at 17°C, and empty shells from which metamorphosed animals had emerged were observed in the culture chambers. During the last period before metamorphosis the larvae were inactive, not feeding and the oral cilia had been absorbed. The only regular movement observed was the activity of the conspicuous retractor muscle while shedding the shell (Fig. 1D).

Larval digestive system

Transmission electron microscopy sections were prepared of larvae maintained for 8 days with an algal culture. The sagittal section through the wall of the larval stomach showed the dorsal chamber (style sac) presenting a densely ciliated wall and numerous microalgae stored free in the vestibule. Lipid granules were
Fig. 1A–D Veliger larvae of *Elysia patagonica*. Photographs from video image. A High activity during hatching. Scale bar = 5.50 μm. B,C Details of the stomach portion: ventral chamber and style sac during food ingestion. Scale bars = 4.50 μm. D Pre-metamorphosis: velum absorbed, indistinguishable stomach, well-developed eyespot and conspicuous retractor muscle and opercul. Scale bar = 6 μm

Fig. 2a,b Planktotrophic larva of *Elysia patagonica*. a Diagram of the digestive system (*E* esophagus; *F* foot; *G* gastric shield; *IA* anterior intestine; *IP* posterior intestine; *M* retractor muscle; *O* operculum; *SH* shell; *SS* style sac; *V* vestibule; *VE* velum). b Diagram of the digestive cells observed in the intestinal epithelium (*ci* cilia; *cp* intact chloroplast; *er* endoplasmic reticulum; *h* planktonic alga; *l* lysosomes; *m* mitochondria; *mv* microvillus; *n* nucleus)

present at the peripheral wall of the ventral chamber of the stomach. Electron micrographs of the digestive gland showed only one cell type (Fig. 2b). The digestive cells contained some intact chloroplasts of *Nannochloropsis* sp. Plastids occurred at the periphery rather than clumped together at the center of the digestive cell. In one vacuole, we observed a cyanobacterium that was being digested (Fig. 4b). The cytoplasm contained some electron-dense bodies, which were probably algae or plastids undergoing digestion. There were also small
electron-transparent vacuoles in the cytoplasm of the digestive cells. The luminal border showed an unkempt array of microvilli and several cilia.

Adult digestive system

As previous studies have shown, adults of *E. patagonica* feed on the green algae *B. plumosa*. They cut and puncture the algal wall by means of blade-shaped teeth with denticulate edges and the help of the developed pharyngeal dorsal muscle. A pair of long and narrow salivary glands open into the esophagus immediately behind the buccal pump. A conspicuous muscular esophageal pouch exists. The stomach consists of a dorsal and ventral portion (see Fig. 5a in Muniain and Ortea 1997). From the ample ventral portion emerges the major duct of the digestive gland, which extends in a parapodial caudal direction. The dorsal portion of the stomach is a single, elongated muscular bag. The wall of the digestive gland is a single-layered epithelium separated from the surrounding connective tissue and muscle cells by basal lamina of varying thickness.

In the adult, the epithelium was composed of two different cell types: digestive cells and secretary cells (Fig. 3; Fig. 4a, c). The digestive cells occurred more abundantly than secretary cells, and were variable in shape and size. Many appeared cubical or cylindrical. The height varied from 9.5 to 12.9 µm. The luminal border contained many microvilli and few cilia. Digestive cells exhibited chloroplast size selectivity. The digestive gland showed two morphological types of digestive cells. In the first type, the cytoplasm contained numerous intact chloroplasts, together with vacuoles containing chloroplasts in different states of decomposition. This cell was more abundant in the digestive branches of larger lumen. The second cellular type contained one large chloroplast which took up most of the cell volume. The cytoplasm showed one or two extremely degenerated chloroplasts. This second type of digestive cell was common in the small digestive branches. The structure of the chloroplasts in both digestive cells was virtually identical to *B. plumosa* plastids. Plastid degeneration in *E. patagonica* was similar to that in other sacoglossans. Chloroplasts in a state of degeneration were progressively more electron dense and showed thylakoid delimitation and interthylakoid vesicle formation. All of the chloroplasts were surrounded by a membrane which appeared to form a continuous envelope around the plastids. In molluscs starved for 2 weeks, most digestive cells contained still intact plastids, but some cells contained electron-dense bodies. The electron-dense bodies were smaller than intact chloroplasts and some still accommodated recognizable thylakoids (Fig. 5A).

The secretory cells were rectangular in shape, and up to 16 µm in height. The apical border showed characteristic short microvilli, with numerous small and light vesicles. The luminal border had many cilia. Apically, the vacuolized cytoplasm contained many electron-transparent vacuoles; the cytoplasm was denser in the basal half of the cell. The oval, lobular nucleus lay near the base of the cell. Rough endoplasmic reticulum and mitochondria were common. The Golgi complex of these cells was characterized by the presence of electron-transparent vesicles (Fig. 5B).

**Discussion**

Larval development and digestive system

The development of most sacoglossans includes a planktonic larval stage, which facilitates dispersion of these species (Thompson 1967; Mileikovsky 1972). The South Atlantic species *Elysia patagonica* exhibits only planktotrophic development (Muniain 1997; Muniain and Penchasadeh 2000).

The digestive gut of planktotrophic veligers is composed of various types of specialized cells that act in concert to facilitate ingestion, transportation and digestion of unicellular algae (Thompson 1959; Bickell and Chia 1979; Bickell et al. 1981). The functional significance of retention of plastids in phytoplanktotrophic larvae

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**Fig. 3a–d** Diagram of the epithelium lining the digestive diverticula of the adult *Elysia patagonica*. Digestive cells: a showing early degenerative changes; b containing numerous electron-dense bodies; and c with intact chloroplasts. d Secretory cell (cd chloroplast with degenerative changes; ci cilia; cp intact chloroplast; er endoplasmic reticulum; l lysosomes; m mitochondrion; mv microvillus; n nucleus; v vacuoles)
could be similar to that in benthic adults or in some planktonic ciliates and foraminifers. Retention of plastids is a common phenomenon in marine planktonic ciliates, through ingestion of phytoplankton (Blackbourn et al. 1973; Jonsson 1987; Stoecker et al. 1987; Stoecker and Silver 1990; Cedhagen 1991). It appears that the sequestered plastids are required because they provide an essential nutrient (organic carbon) during the starvation periods characteristic of the highly fluctuant planktonic environment. In this process the uptake of *Nannochloropsis* spp., specifically the chlorophyll a found in the chloroplasts of these microalgae, is integral. *Nannochloropsis* spp. are present in the picoplankton throughout most of the world’s oceans (Maruyana et al. 1986).

The phagocytosis of planktonic unicellular algae by digestive cells proceeds as a result of the same mechanisms in both larvae and adults of *E. patagonica*. Our observations have demonstrated that feeding habits and
the structure of digestive cells are similar in adults and in planktonic larvae, and that, in sacoglossans, the retention occurs in the veliger larvae. Some authors have asserted that chloroplast retention is a primitive character of the order Sacoglossa, which possibly originated as early as the Cylindrobullidae, but at least as early as the Oxyloidae (Clark and Busacca 1978; Clark et al. 1990). Recently, by phylogenetic analysis, Mikkelsen (1998) reconfirmed the genus Cylindrobulla among the shelled Sacoglossa; Jensen (1996) had mistakenly excluded this genus from the Sacoglossa to form a new order, the Cylindrobullacea.

Adult digestive system

The digestive tract of E. patagonica contains two cell types, while a third cell type has been described in Elysia viridis by Griebel (1993). This third cell type is characterized by the presence of large vacuoles that contain electron-dense material with a concentric structure. Also, digestive cells containing concentric granules have been described in the cephalaspids Runcina coronata and R. ferruginea. X-ray microanalysis of the granules indicates that the concentric structures contain mainly magnesium phosphate (Kress et al. 1994). A variety of
functions have been proposed for these mineralized concretions, but their exact function remains unclear. This cellular type is not found in the digestive tract of adults or larvae of *E. patagonica*.

The ultrastructure of the digestive cells of *E. patagonica* is analogous to other species of the genus *Elysia*: *E. viridis*, *E. chlorotica* and *E. timida* (Taylor 1968; Graves et al. 1979; Marin and Ros 1989; Grieben 1993). However, Trench et al. (1973) found that about half the chloroplasts in the digestive cells of *E. viridis* lay free in the cytoplasm, while the others were surrounded by a host-produced membrane. In the digestive cells of *E. patagonica*, all chloroplasts are engulfed by a vacuolar membrane. The disappearance of this phagosome membrane has been considered a mechanism to avoid phagosome digestion (Hinde 1983). However, in the Mediterranean *E. timida*, a species of long-term functional retention, with all kleptoplastids surrounded by host membranes, photosynthesis persists for more than 3 months during starvation (Marin and Ros 1989). All plastids of *E. patagonica* are enclosed in vacuoles. In *E. patagonica* starved for 20 days, digestive cells contained intact chloroplasts. This suggests that the presence or absence of host membranes does not correlate with the period of functional retention of kleptoplastids. The selectivity of chloroplast size by digestive cells has not been previously described in sacoglossans with kleptoplastid retention. In *E. patagonica*, the digestive cells with small chloroplasts seem to have a high plastid turnover, because usually these cells simultaneously contain both intact and decomposing plastids. The large-sized digestive cells, on the other hand, contain only one big chloroplast, occupying almost the entire cell volume, and one or two vacuoles containing degenerated plastids; this would indicate low plastid turnover.

**Kleptoplasty and chloroplast retention**

Chloroplasts are acquired after metamorphosis in most sacoglossans (Trench 1969; Clark et al. 1979; Marin and Ros 1993). Chloroplasts are not absorbed by the digestive gland until the diverticula is differentiated, several days after larval metamorphosis, but the ontogeny of thanatocresis has only been studied in several elysoids with direct development. The veliger larvae of the sacoglossan *E. subornata* retain chloroplasts for about 5 days after hatching, when the digestive gland changes to an extensively ramified system of diverticula (Clark et al. 1979).

If plastid symbiosis occurs in adult Elysidae, does it also occur among their phytoplanktrophic veliger larvae? Adult sacoglossans suck out the cytoplasmatic cell contents of Siphonales, Siphonocladales and Cladophorales. Once ingested, the plastids are moved by ciliate currents into the diverticula of the digestive gland, where they are phagocytized by the digestive cells. The anatomy and physiology of sacoglossans, including a large internal surface for digestion by absorption, absence of macerating structures such as a gizzard, and a reduced intestine, are adapted to a fluid diet (Marin and Ros 1988; Clark 1992; Jensen 1997).

The digestive cells of herbivorous *Runcina* spp., shell-less cephalaspids, retain chloroplasts, but plastids are digested immediately after phagocytosis (Kress et al. 1994). The more primitive sacoglossans (Volvatellidae, Julidae and Oxynoidae) feed on siphoncalean algae of the genus *Caulerpa*, and they exhibit shorter non-functional chloroplast retention. Structurally intact plastids are found during the 24 h after ingestion but no photosynthetic activity is detectable (Clark et al. 1990).

Long retention of functional chloroplasts seems to indicate that kleptoplasty is a pleiomorphic character associated with branching of the digestive gland. Branching of the digestive gland is a necessary feature for photosynthetic function; the Limapontiidae (= Stiligeroidae) species, which exhibit functional chloroplast retention, also have a densely branched system (Jensen 1991, 1992). Functional kleptoplasty probably evolved in the common placobranchaceous ancestor, as it occurs to varying degrees in both major lineages (Clark et al. 1990; Jensen 1996).

Recently, an interesting line of inquiry investigated by Pierce et al. (1999) demonstrated that sacoglossan populations are regulated by cyclical infections of protozoa or viruses, and, in *E. chlorotica*, these viruses may be involved in the maintenance of symbiotic chloroplasts within the molluscan cells.

**Diet and chemical responses**

Many sacoglossans are also able to retain photosynthetically functional algal chloroplasts in their tissues and utilize them to produce secondary metabolites, including defensive compounds, resulting in the isolation of sesquiterpenoids and diterpenoids. These compounds are sequestered from green algae and either accumulated or chemically modified, and, along with polypropionates, are biosynthesized de novo (Cimino et al. 1999).

Chemical studies on adult specimens of *E. patagonica* and their algal hosts (*B. plumosa*) showed that the sacoglossan is capable of synthesizing polypropionates de novo, and that these compounds are completely absent in the algal extracts of *B. plumosa*. The retention of algal chloroplasts has been confirmed in the present study; this affirms the mollusc's capacity to produce polypropionates as secondary metabolites, probably with a defensive function (Muniaín 1997; Muniaín et al. 1999, unpublished data). Further ecological investigations are required to examine seasonal differences in the presence and diet of sea slugs.

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