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RESEARCH NOTE

# Kleptoplasts are continuously digested during feeding in the plastid-bearing sea slug *Elysia viridis*

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Photosymbiosis is a widespread form of mutualistic symbiosis found among many metazoan lineages (Melo Clavijo *et al.*, 2018) and is particularly beneficial for the animal host. The assimilates translocated by the symbionts to the host can meet or even supersede the host's nutritional demands, and this is particularly important for reef-building corals (Stanley & Lipps, 2011). A rather unusual form of photosymbiosis is found among some sacoglossan sea slugs (Trench, 1975; Rumpho, Summer & Manhart, 2000) and in two rhabdocoel flatworms (Van Steenkiste *et al.*, 2019). In these taxa, only chloroplasts, ingested from algal prey, occur intracellularly as some sort of symbiont. In some Sacoglossa species, these prey-derived chloroplasts (referred to as kleptoplasts) remain photosynthetically active for periods ranging from weeks to months (Evertsen *et al.*, 2007; Händeler *et al.*, 2009; Christa *et al.*, 2015). Due to the unique nature of this photosymbiosis, the term functional kleptoplasty was introduced (Rumpho *et al.*, 2011). Even though functional kleptoplasty has been intensively investigated in Sacoglossa, it is still not clear to what extent assimilates can be translocated by kleptoplasts to host cells and to what degree this nutritional support is beneficial for the host (Wägele & Martin, 2014; Cartaxana *et al.*, 2017; Rauch *et al.*, 2017).

To understand potential nutritional benefits for Sacoglossa bearing functional kleptoplasts, two different physiological states of the host slugs have to be distinguished, feeding and starving. Most of the research conducted to date has focused on starving Sacoglossa, with some species being able to maintain functional kleptoplasts for 2–4 weeks (referred to here as short-term retention) or several months (long-term retention), without any additional food supply (Händeler *et al.*, 2009) and without any genomic support by the host algae (Rauch *et al.*, 2015). During starvation, slugs with long-term retention of kleptoplasts seem to reduce the digestion of kleptoplasts (Laetz *et al.*, 2017b), while simultaneously assimilates accumulate in the alien organelle (Laetz *et al.*, 2017a) or in the host cytosol (Pelletreau *et al.*, 2014). Thus, active export of assimilates by the kleptoplasts is probably highly reduced or does not occur, and assimilates may be accessible only through digestion or degradation of the kleptoplasts.

Whether kleptoplasts are maintained or digested in feeding slugs remains unknown. It has been shown that about 60% of the metabolized carbon originates from feeding on the algae. However, this applies to species in which functional kleptoplasts are not retained and are immediately digested (Raven *et al.*, 2001). Furthermore, electron microscopy-based (Evertsen & Johnsen, 2009) and physiological (Laetz *et al.*, 2017a) studies, and molecular investigations of individuals collected in different seasons (Maeda *et al.*, 2012) have shown that in some species kleptoplasts are digested during feeding. These results contrast with electron microscopy-based work that has shown that kleptoplasts are not digested while feeding (Cruz *et al.*, 2020) and that assimilates are actively excreted into the slug's cytosol (Trench, Boyle & Schmidt, 1973; Gallop, 1974).

In an effort to shed further light on this issue, we investigated kleptoplasty in *Elysia viridis* (Montagu, 1804) (Fig. 1A), a sacoglossan sea slug that is able to retain functional kleptoplasts for *c.* 4–12 weeks (i.e. depending on the collection site; Hinde & Smith, 1972; Cartaxana *et al.*, 2017; Christa *et al.*, 2018). Our approach involved conducting a food-switch experiment to examine potential kleptoplast digestion during feeding. In September 2018, live individuals of *E. viridis* were collected from the alga *Codium tomentosum* (Fig. 1B) in intertidal habitat in Figueira da Foz, Portugal. The slugs were transferred to the laboratory and cultured in filtered seawater (obtained from the field site) at 18 °C, under a day/night cycle of 12 h/12 h and at a light intensity of 40 $\mu$mol quanta $m^{-2}$ $s^{-1}$. To understand whether kleptoplasts are digested during feeding, we induced a food switch by providing the alga *Bryopsis hypnoides* (Fig. 1C) as the sole food source to the polyphagous *E. viridis* (Baumgartner & Toth, 2014; Christa *et al.*, 2014). *Bryopsis hypnoides* was cultured under the same conditions as *E. viridis*, except that the filtered and autoclaved seawater was supplemented with Guillard's F2 medium (Guillard, 1975). Three specimens of *E. viridis* were sampled randomly after 0, 1, 2, 5, 7, 14, 21 and 60 d of feeding on *B. hypnoides* and fixed in 70% ethanol. Examples of *C. tomentosum*, the food source from the field site, were also fixed in 70% ethanol. All ethanol-preserved samples were stored at −20 °C. Total DNA was extracted using TRIzol (Thermo Fisher, USA) following the

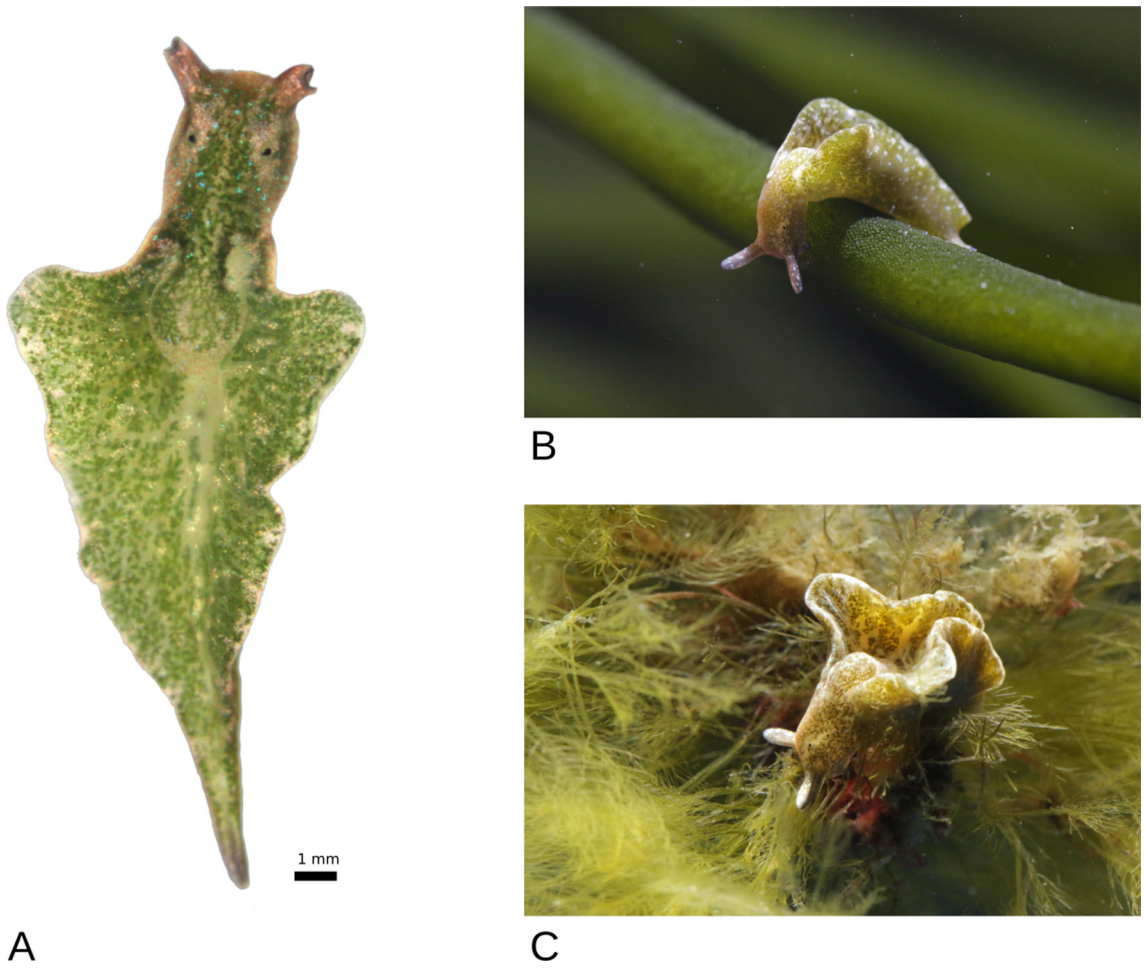


**Figure 1. A.** General morphology of *Elysia viridis* with opened parapodia (lateral appendices of the foot). The green coloration derives from the ingested chloroplasts in the digestive gland system. *Elysia viridis* feeding on a siphon of the alga *Codium tomentosum* (**B**) and on the alga *Bryopsis hypnoides* (**C**). Images and equipment used: **A**, G. Christa (Olympus SZX12 stereo microscope with a mounted DP21 camera); **B**, **C**, C. Brandão (Canon 70D with a Canon EF 100 mm f/2.8L Macro IS USM objective).

manufacturer's protocol and quantified using Qubit fluorometric quantification (Thermo Fisher, USA).

In order to generate algal-specific primers, we first amplified and sequenced a 526-bp fragment of the large subunit of *rbcL*, the gene encoding the enzyme ribulose-1,5-bisphosphate carboxylase–oxygenase of *C. tomentosum* (GenBank acc. no. MT993751), following the method described by Christa *et al.* (2014). Two species-specific primer pairs for the amplification of short fragments of *rbcL* for *C. tomentosum* and *B. hypnoides* (GenBank acc. no. AY942169) were then generated using primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and available *rbcL* sequences for *C. tomentosum* (see above) and *B. hypnoides* (GenBank acc. no. AY942169) as templates. The length of the amplified *rbcL* fragment was 84 bp for *C. tomentosum* and 160 bp of *rbcL* for *B. hypnoides* (Table 1). To test the reliability of the two primer pairs, a first amplification of each DNA fragment was carried out. For this, a standard PCR reaction was performed using the GoTaq® Green Master Mix (Promega, USA) in a 20-$\mu$l reaction volume including 10 $\mu$l 2× concentrated PCR mix, 0.1 $\mu$M final concentration of each primer, 6.4 $\mu$l PCR-grade water and 4 ng template DNA. For the amplification of the *rbcL* fragment with the species-specific primers, we used the following reaction conditions: an initial denaturation of 15 min at 95 °C; 40 cycles of 1 min at 95 °C, 1 min at 50 °C (*C. tomentosum*) or 54 °C (*B. hypnoides*) and 1 min at 70 °C; and a final elongation of 10 min at 70 °C. PCR reactions were performed for randomly chosen slug specimens fed for 0, 7, 14, 21 and 60 d on *B. hypnoides* and for both algae. The success of amplifications was assessed by running PCR products on a 1.5% agarose gel, stained with Stain G (SERVA Electrophoresis GmbH, Germany), and visualizing under a UV light imager. The samples fed for 60 d on *B. hypnoides* were used as a long-term control sample but not included in the qPCR analysis. The qPCR reactions were performed for slug species fed on *B. hypnoides* for 0, 1, 2, 5, 7, 14 and 21 d using both primer pairs in turn; qPCRs were done on an Agilent AriaMx4000 (Agilent, USA) platform using 4 ng as input DNA in a 20-$\mu$l reaction volume containing 4 $\mu$l of 5× concentrated my-Budget EvaGreen® QPCR-mix (BioBudget, Germany) and 0.1 $\mu$M final concentration of each primer. The amplification was performed as a three-step reaction: 5 min initial denaturation at 95 °C; 40 cycles of 1 min

**Table 1.** Primers used for amplifying fragment of the *rbcL* gene from the algae *Codium tomentosum* (CodF, CodR) and *Bryopsis hypnoides* (BryF, BryR).

| Primer name | Primer sequence (5′–3′) | Fragment size (bp) |
|---|---|---|
| CodF | CGC ATT TCA CCA GCT TAT GCA A | 84 |
| CodR | AGC ACG TCC ATA ATT TTT AGC G | |
| BryF | CGG GTA TGT TGC GTA TCC ACT T | 160 |
| BryR | GTC ACG CTC GAC TTC AAT ACC A | |

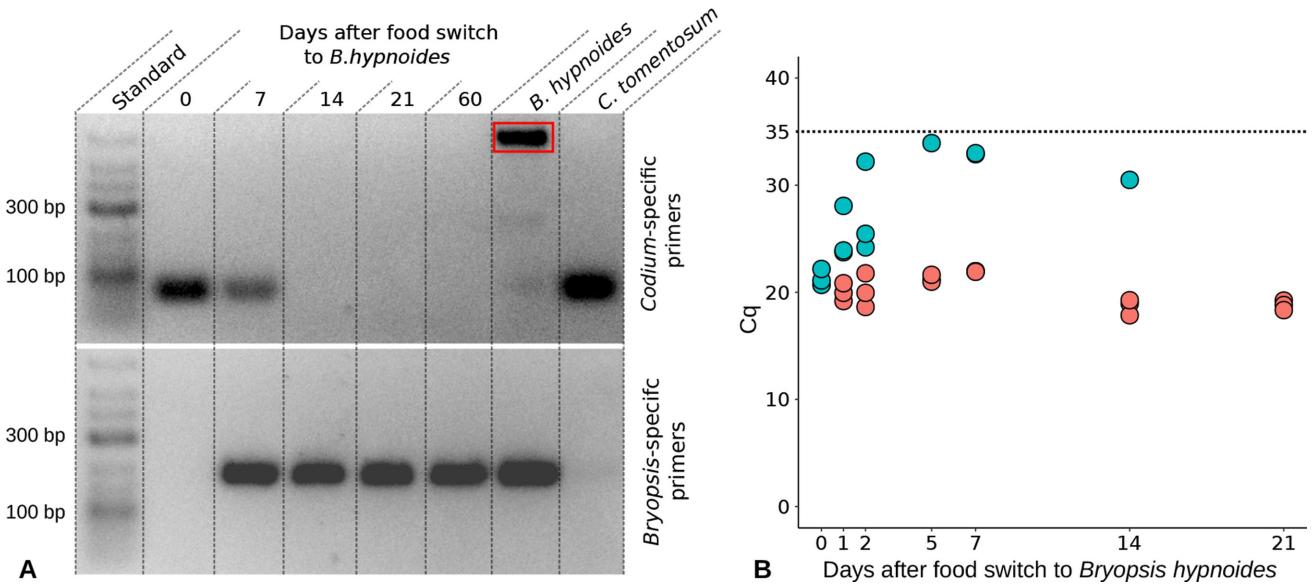


**Figure 2. A.** PCR results for specimens of *Elysia viridis* fed 0, 7, 14, 21 and 60 d after food switch from *Codium tomentosum* to *Bryopsis hypnoides*. Highlighted in red is the large fragment obtained using the *Codium*-specific primers for DNA from *B. hypnoides*. A corresponding fragment was not obtained for any of the slug DNA samples analysed. **B.** Results of qPCR analyses for specimens of *E. viridis* fed for 0, 1, 2, 5, 7, 14 and 21 d after food switch from *C. tomentosum* to *B. hypnoides*. Only samples with a Cq value <35 (cut-off value for successful amplification) are displayed. Samples amplified using primers specific to *B. hypnoides* and *C. tomentosum* are indicated in blue and red, respectively.

denaturation at 94 °C and 1 min annealing at 50 °C (*C. tomentosum* primers) or 54 °C (*B. hypnoides* primers); and 1 min of elongation at 70 °C. For each sample, technical duplicates were performed, and non-template control (NTC) samples were used to exclude any unspecific amplification. For analysis, the cycle threshold (Cq), which is the number of amplification cycles needed to exceed a certain fluorescence signal, was set to a fluorescence signal of 180 for both primer pairs. Cq values exceeding 35 amplification cycles were not considered as a successful amplification because the NTC samples did not show an amplification until this cycle. Thus, samples with a Cq value >35 were considered to be lacking kleptoplasts.

Our results showed that both primer pairs can be used for a species-level identification of *C. tomentosum* and *B. hypnoides* kleptoplasts in *E. viridis* (Fig. 2A), although an additional large fragment was obtained during amplification of *B. hypnoides* DNA using the primer pair specific for *C. tomentosum*. This issue did not arise for DNA from *E. viridis*, so can be ignored. It was not possible to amplify any *B. hypnoides* kleptoplasts from field-collected specimens of the slug (Fig. 2A, B) and this is consistent with the view that *E. viridis* was not feeding on *B. hypnoides* at the site where the slugs were collected. During the first 2 d of feeding on *B. hypnoides*, chloroplasts of *C. tomentosum* were detected in all the slugs analysed by qPCR, using the primer pair specific for this species of alga (Fig. 2B). The qPCR detected *C. tomentosum* chloroplasts in one out of three specimens fed in the laboratory for 5 d on *B. hypnoides* (Fig. 2B; Cq = 33.93), in two specimens fed for 7 d on *B. hypnoides* (Fig. 2B; Cq = 32.51 and 33.97, respectively) and in one specimen fed for 14 d on *B. hypnoides* (Fig. 2B; Cq = 30.38). No chloroplasts of *C. tomentosum* were found 21 d after food switch to *B. hypnoides* in any of the samples analysed by qPCR at the chosen sensitivity threshold (Fig. 2A, B). Chloroplasts of *B. hypnoides* were detected in all specimens 1 d after the food switch and this was also the case for all the remaining specimens (i.e. those fed 2, 5, 7, 14 and 21 d after the switch) (Fig. 2B).

These results provide evidence that following a food switch, kleptoplasts are digested or expelled in continuously feeding specimens of *E. viridis*. Digestion of symbionts is common among animals (Hoang, Morran & Gerardo, 2019), with apoptosis in corals being used to remove heterologous symbionts (Dunn & Weis, 2009) and to maintain the symbiosis (Fransolet, Roberty & Plumier, 2012). Besides digestion, damaged symbionts are often expelled from the host's cytosol to prevent damage to the cell (Fujise *et al.*, 2014). Since assimilates are found to be metabolized in Sacoglossa (Kremer, 1976; Hinde, 1978; Cruz *et al.*, 2020), it is more likely that the kleptoplasts are usually digested, rather than expelled. This also implies that a substantial fraction of the assimilates produced by kleptoplasts might be directed to the slugs through the digestion of the kleptoplasts. Nonetheless, translocation of assimilates from the kleptoplasts to the host, as suggested elsewhere (Gallop, 1974; Cruz *et al.*, 2020), should not be ruled out. However, it does appear that if both processes are present, they co-occur; a differentiation of these two processes does seem complicated. Thus, at least in *E. viridis*, the process of nutrient exchange differs from that observed in corals; in corals, the algal symbionts and the animal host are connected through an exchange cycle of nutrition (Matthews *et al.*, 2017) and this prevents the digestion of the symbionts. Whether the kleptoplasts of sacoglossan sea slugs are connected in a similar way to their host is unknown; even if such a cycle is present in *E. viridis*, it does not seem to save kleptoplasts from being digested while the slugs are feeding. A future comparison of initial kleptoplast maintenance following a food switch with species that are able to maintain their plastids for several months (e.g. *E. timida* or *E. crispata*) would help us to understand whether differences in the digestion of chloroplasts during feeding reflect variation in the capacity to retain kleptoplasts.

Our results also have implications for the hypothesis that an increased nutritional value of feeding slugs is obtained by an acclimation to higher light and thus an increased photosynthetic activity of the kleptoplasts (Baumgartner, Pavia & Toth, 2015; Shiroyama *et al.*, 2020). As our study suggests, kleptoplasts are continuously digested. Therefore, it is more likely that the cytosol of algae grown under high light conditions has a higher nutritional value compared to algae grown under low light conditions, so that the expectation would be that slugs grow faster when feeding under high light conditions. The photophysiology of kleptoplasts and their potential nutritional contribution have been intensively studied in *E. viridis*. In some studies, *E. viridis* has been field collected from *C. tomentosum* and then transferred to the laboratory and fed with *B. hypnoides*

or other algae (Christa *et al.*, 2018; Rauch *et al.*, 2018). This food switch was conducted because *B. hypnoides*, unlike *C. tomentosum*, can be easily cultured in the laboratory, enabling relatively straightforward maintenance of the algae and the slugs under controlled conditions. Prior to the experiments, the slugs were usually fed with *B. hypnoides* for 4 weeks. However, it has never been experimentally verified whether the period of this food switch is sufficient to ensure that all the kleptoplasts from *C. tomentosum* are replaced, so that the experiment is not influenced by a mixture of kleptoplasts from different food sources. Our results strongly suggest that in order to ensure a complete replacement of kleptoplasts before experimental studies begin, *E. viridis* should be fed for at least 3 weeks with a new food source. An important related consideration is that differences in size, age or health between individuals might influence the speed with which all initial kleptoplasts are replaced.

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