Marine Biology (2018) 165:82
https://doi.org/10.1007/s00227-018-3329-8

ORIGINAL PAPER

The ability to incorporate functional plastids by the sea slug *Elysia viridis* is governed by its food source

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Received: 9 December 2017 / Accepted: 20 March 2018 / Published online: 7 April 2018

Abstract

Functional kleptoplasty in sacoglossan sea slugs is among the most curious photosynthetic associations known. One member of these marine molluscs, *Elysia viridis*, is known to incorporate plastids from a variety of different algae food sources, but with apparently different outcomes and differences in the time span of the retention of functional kleptoplasts. While it was previously shown that kleptoplasts that stem from *Codium tomentosum* are kept functional for several weeks (long-term retention, LtR), those that stem from *Bryopsis hypnoides* or *Cladophora rupestris* are thought to be of limited use regarding photosynthetic capacity (short-term retention, StR). This is important, because it touches upon the popular yet controversial question of how important photosynthesis is for the thriving of these slugs. The aim of the present study was to determine to what degree the plastid source determines retention time. We, therefore, compared *E. viridis* feeding on either *Cladophora* sp. or *B. hypnoides*. We show that kleptoplasts of *B. hypnoides* incorporate $^{14}CO_2$, but with rapidly declining efficiency throughout the first week of starvation, while the plastids of *Cladophora* sp. are, surprisingly, not incorporated to begin with. The radulae of the different samples showed adjustment to the food source, and when feeding on *Cladophora* sp., *E. viridis* survived under laboratory conditions under both starvation and non-starvation conditions. Our results demonstrate that (i) the ability to incorporate plastids by *E. viridis* differs between the food sources *B. hypnoides* and *Cladophora* sp., and (ii) photosynthetic active kleptoplasts are not an inevitable requirement for survival.

Introduction

Animals are heterotrophs. Some may, however, tap the benefits of photosynthetic biochemistry through a symbiotic relationship with phototrophic organisms such as cyanobacteria or unicellular algae (Johnson 2010; Venn et al. 2008). Well-known examples of animals establishing photosynthesis-based symbioses include sponges (Riesgo et al. 2014), corals (Baker 2003), acoelomorphs (Serôdio et al. 2011), molluscs (Rumpho et al. 2011), tunicates (Hirose 2015) and even a vertebrate (Graham et al. 2013), although for the latter the role of photosynthesis is questioned (Burns et al. 2017). Theory has it that the photosynthetic symbionts provide their hosts with additional nutrients or, as in the case of the egg masses of the spotted salamander, predominantly with oxygen (Pinder and Friet 1994; Graham et al. 2013). In turn, the host shelters the symbionts from biotic and abiotic factors and provides sufficient inorganic nutrients such as CO_2 to ensure a high rate of photosynthesis (Venn et al. 2008). In any case, the symbionts are not able to completely satisfy the nutritional demands of the host, and so the animals need to feed irrespectively (Barnes and Hughes 1999).

Responsible Editor: K. Bischof.

Reviewed by H. Waegele and an undisclosed expert.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00227-018-3329-8) contains supplementary material, which is available to authorized users.

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The animals are never phototrophic, but may be mixotrophic if they do not remain heterotrophic. Some sacoglossan sea slugs present yet another unique case, as they acquire photosynthetic plastids not by means of a symbiotic relationship, but by specifically sequestering the plastids from the algae food source, a process known as kleptoplasty (Händeler et al. 2009).

Sacoglossa cut the cell wall of their macroalgal prey using their highly specialized radula, upon which they suck out the cellular content (Händeler et al. 2009). The majority of sacoglossan slugs digest the plastids rapidly together with the remaining cytosolic components, but some members specifically sequester the plastids into the cytosol of epithelial cells that line the digestive gland system (Händeler et al. 2009; de Vries et al. 2014b). Here, the then-called kleptoplasts reside intracellularly with apparently no phagosomal membrane separating them from the cytosol (Rumpho et al. 2011; Wägele and Martin 2013; de Vries et al. 2014a). Based on the period of time the slugs can house the functional kleptoplasts, they are assigned to three main categories based on chlorophyll *a* fluorescence measurements of the maximum quantum efficiency of photosystem II (F_v/F_m) (Händeler et al. 2009). One distinguishes between non-retention forms (NR; no F_v/F_m), short-term-retention forms (StR; F_v/F_m of at least 0.4 over 14 days of starvation), and long-term-retention forms (LtR; F_v/F_m of over 0.4 for more than 21 days of starvation) (Händeler et al. 2009). For five LtR species, data suggest the kleptoplasts can be retained photosynthetically active—that is, they continue to fix CO_2 in a light-dependent manner (Wägele and Martin 2013; de Vries et al. 2014b)—for a few weeks or months to come (Händeler et al. 2009). Until recently, six LtR species were thought to have been identified (de Vries et al. 2014b), but a recent taxonomic revision united the once separate *Elysia clarki* and *Elysia crispata* (Krug et al. 2016). Retaining functional kleptoplasts is taxonomically restricted to the Sacoglossa among Metazoa and otherwise only known to occur in Foraminifera, dinoflagellates and ciliates (Bernhard and Bowser 1999; Johnson et al. 2007; Minnhagen et al. 2008; Rumpho et al. 2011). The sustained activity of the kleptoplasts in Sacoglossa is thought to benefit the sea slugs throughout starvation, which they may naturally experience in some of their habitats such as the Mediterranean (Marín and Ros 1992). How kleptoplasts benefit the animals has been controversially discussed (Christa et al. 2014a; Cartaxana et al. 2017).

We do not know how the plastids are sequestered from the digestive tract's lumen and released into the cytosol of an animal cell. Considering the underlying complexity of such a process and that phagotrophy is not how animals usually absorb food particles from their digestive tract's lumen (Karasov and Martínez del Rio 2007), we can assume it happens deliberately. Common perception is that functional kleptoplasts are retained due to profiting from photosynthetic carbon fixation. Some studies indeed show that photosynthates are used by the sea slug *Elysia viridis* during starvation (Trench et al. 1974; Hinde 1978), and some draw similar conclusions based on indirect measurements (Schwartz et al. 2010; Maeda et al. 2010). Moreover, starch accumulates upon food deprivation in the kleptoplasts in one LtR species (Laetz et al. 2017)—the starch is apparently not actively released from intact organelles—and is thought to become available only downstream during starvation, when the kleptoplasts are degraded or digested (Laetz et al. 2017). This would support the idea that kleptoplasts serve more as a kind of food storage vessel than as active photosynthesizers, which was suggested upon observing that blocking photosynthesis (chemically or by rearing them in the dark) had no measurable effect on weight decrease in *Plakobranchus ocellatus* van Hasselt, 1824 (Christa et al. 2013a). This is not to say that photosynthesis is not important, but the amount of CO_2 fixed by the kleptoplasts might only provide about 1% (Rauch et al. 2017b) to a maximum of 36% (Hinde 1978) of the animal's total carbon requirement. However, a report by Raven et al. (2001) suggested that up to 60% of the carbon of the NR form *Oxynoe viridis* comes from the kleptoplast while the slugs are feeding. Hence, it is more likely that carbon incorporation is achieved through feeding rather than through kleptoplast photosynthesis.

Some opinions have brought forward the possibility that plastid biochemistry, besides carbon fixation, might be beneficial to the slugs (Cueto et al. 2005; Díaz-Marrero et al. 2008; Casalduero and Muniain 2006; de Vries et al. 2014a, b). This possibility is supported by limapontiodean species such as *Placida dendritica* or *Cyerce nigricans*, which also sequester plastids but in a non-photosynthetic active state (Evertsen and Johnsen 2009; Christa et al. 2015). What complicates the matter and hinders broader, generalizing statements is that slug species differ with regard to their preferred food source (Jensen 1997; Christa et al. 2013b, 2014a; Middlebrooks et al. 2014; Baumgartner and Toth 2014) and the time span they retain functional kleptoplasts (Händeler et al. 2009), a paramount example being *E. viridis* (Montagu, 1804).

E. viridis is found along the Atlantic and Mediterranean coastline and forages on a variety of different algal food sources. Among others, the food algae include siphonaceous species such as *Codium tomentosum* Stackhouse, 1797 and *Bryopsis hypnoides* Lamouroux, 1809 and cellular organized species such as *Cladophora dalmatica* Kützing, 1843 and *Chaetomorpha* spp. (Händeler et al. 2009; Baumgartner et al. 2009; Baumgartner and Toth 2014; Baumgartner et al. 2014). Due to diverse habitats between the different geographical populations, ranging from Denmark (Evertsen and Johnsen 2009) to the Mediterranean (Händeler et al. 2009), *E. viridis* might actually represent a species complex.

With regard to the duration kleptoplasts can remain photosynthetically active, different reports exist for *E. viridis*. When fed on *C. tomentosum*, the sea slug was categorized as an LtR species (Evertsen and Johnsen 2009; Evertsen et al. 2007; Cruz et al. 2014; Serôdio et al. 2010; Teugels et al. 2008; Trench et al. 1974; Vieira et al. 2009), but individuals from the Mediterranean that had fed on *B. hypnoides* and specimens from the North Atlantic that had fed on *C. rupestris* were found to behave more like StR species, because initial F_v/F_m values did not exceed 0.6 (Baumgartner et al. 2015; Händeler et al. 2009). These differences might actually be based on different light acclimation states and different experimental light conditions (Serôdio et al. 2014), but are nonetheless informative as they shed light on the importance of photosynthesis regarding the vitality of slugs during starvation. F_v/F_m ratios, however, are not suitable to evaluate the true contribution of kleptoplasts to the slug's overall physiology. Parameters such as CO_2 incorporation throughout starvation, and after the slugs were fed on different algae food sources, are currently lacking for *E. viridis*.

In this study, we wanted to understand whether the differences of plastid longevity in the sea slug *E. viridis* are based on the algal food source. For this we analysed 47 individuals of *E. viridis* in total that were reared either solely on *Bryopsis hypnoides* or *Cladophora* sp. Animals were starved for 21 days and F_v/F_m values, $^{14}CO_2$ incorporation and chlorophyll *a* fluorescence were monitored while we also observed morphological changes. We observed stark differences associated with the choice of algae and, for instance, were not able to detect any photosynthetically active kleptoplasts in *E. viridis* individuals fed on *Cladophora* sp. Our results demonstrate that the ability to incorporate functional kleptoplasts depends on the food source.

Materials and methods

Collection and culturing of sea slugs and algae

Specimens of *Elysia viridis* were collected at the intertidal zone in Aguda, Portugal (41°02′53.1″N, 8°39′12.0″W) together with their native food source *Codium tomentosum* and *Cladophora* sp. All specimens were transferred to the laboratory and a maximum of 3 slugs were kept in large glass Petri dish (ø of 120 mm) in artificial sea water (ASW, Tropic Marine) with a salinity of 35 PSU under a 12-h light/12-h dark cycle at a light intensity of 25 µmol photons m^{-2} s^{-1} (SolarStinger®, Econlux) and at a temperature of 15 °C. The water was changed at minimum of three times a week. The food algae *Cladophora* sp. and *Bryopsis hypnoides* were grown under the same conditions but in ASW enriched with Guillard's F/2 medium (Guillard and Ryther 1962; Guillard 1975). All slugs collected on *Cladophora* sp. ($n = 12$) were exclusively fed on this algae species, while only the specimens collected from *C. tomentosum* ($n = 35$) were transferred onto *B. hypnoides* and solely fed on the latter alga. Prior to any experiment, the slugs were fed the respective food source for a minimum of 1 month. After this laboratory acclimation period, the slugs were separated into individual Petri dishes and deprived of their food for 21 days under the same conditions.

Gene amplification and phylogenetic reconstruction

To exclude the possibility of having collected cryptic *Elysia viridis* species, we performed a phylogenetic reconstruction for one sample collected from each food source using partial *COI*. DNA was extracted using DNeasy® Plant Mini Kit (Qiagen) and stored at − 20 °C. Amplifications were performed using standard PCR protocols and as described in more detail in Christa et al. (2014b). Annealing temperature of 48 °C for *COI* was used. Samples were sequenced by Eurofins Genomics (Ebersberg, Germany) and analysed with Geneious (v. R7.1, Biomatters, New Zealand). Sequences of several other closely related *Elysia* species were obtained from GenBank and included to investigate the relationship between *E. viridis* samples from different collection sites with *Plakobranchus ocellatus* van Hasselt, 1824 used as the out-group (Supplementary Table 1). COI alignment was performed using the G-INSI-mode in MAFFT (Katoh et al. 2002), inspected and, if required, manually edited. Phylogenetic reconstruction was performed using PhyML (v. 3.0; Guindon et al. 2010) and 1000 bootstraps with the GTR+G+I model as proposed by jModeltest (Guindon and Gascuel 2003).

Measuring of photosystem II activity and carbon fixation

During the experiments, slugs in the presence or absence of the photoinhibitor monolinuron (2 µg ml^{-1} final concentration; JBL GmbH) were measured in regular intervals (0, 3, 7, 11, 14, 17, 21 days of starvation) with regard to the activity of the photosystem II (PSII) of the kleptoplasts using a FluorCam FC 800MF (Photo Systems Instruments, Brno, Czech Republic). For carrying out the measurements, the slugs were transferred from culturing light conditions to 25 µmol photons m^{-2} s^{-1} modulated red light (emission peak at 625 nm) and acclimated for 5 min in the FluorCam chamber. Chlorophyll *a* fluorescence of light-acclimated kleptoplasts was measured and the effective quantum yield of PSII was determined by applying a saturating pulse (> 7500 µmol photons m^{-2} s^{-1}, red light) as $\Delta F/F'_m$ ($\Delta F = F'_m - F_s$; F_s, F'_m: minimum and maximum fluorescence emitted by light adapted samples, respectively). Subsequently, the slugs were dark acclimated for 5 min and the maximum PSII quantum

yield F_v/F_m was determined ($F_v = F_m - F_o$; F_o, F_m: minimum and maximum fluorescence emitted by dark adapted samples, respectively). Images (512 × 512 pixels) were processed using the FluorCam7 software (Photon System Instruments), by defining areas of interest (AOI) including the whole dorsal surface of each slug. The values of the fluorescence parameters were calculated by averaging all pixel values in each AOI, and by averaging the fluorescence intensity during the 2 s immediately before (F_o, F_s) the saturating pulse and during 0.6 s (F_m, F'_m) of exposure to the saturating pulse (total duration: 0.8 s). All measurements were performed in biological triplicates.

We determined the $^{14}CO_2$ incorporation for specimens feeding on *Bryopsis hypnoides* and on *Cladophora* sp. using a slightly modified protocol based on Christa et al. (2014a). One—or when available two—slugs were incubated in a 2-mL plastic tube that contained 1.2 ml ASW supplemented with 0.4 mM [^{14}C]-$NaHCO_3$ (25 µCi per incubation, NEN-radiochemicals, MA, USA) for 2 h at room temperature and illuminated with 72 µmol photons m^{-2} s^{-1} for reasons of compatibility with previous studies (Christa et al. 2014b; de Vries et al. 2015). After the incubation, the radioactive medium was removed, the samples were washed 5 times with seawater and then homogenized in a 1-mL glass Potter–Elvehjem tissue grinder. The homogenates were removed and the potter was washed twice with 1 ml H_2O. The total of 3 ml homogenized sample was acidified with 150 µl 1 M HCl and the open vial was incubated overnight on a shaker to remove all vaporizable substrate. The next day, 12 ml of LUMA-Gel scintillation cocktail (LUMAC, The Netherlands) was added and incorporated carbon was determined with a scintillation counter. For each data point, measurements were performed for biological triplicates.

Imaging

Whole-mount images were made with a Canon EOS6D (macro lens MP-E 65 mm 1:2.8 and a Canon macro twin lite MT-24EX flashgun). Detailed chlorophyll *a* fluorescence images of the slugs' parapodial rims were taken with a Zeiss LSM710 microscope; those of entire slugs and of algae filaments were taken with a Nikon Eclipse Ti-E. We used Fiji (version 2.0.0) for image processing. At two different time points (0 and 10 days of starvation) we sampled slugs to screen for kleptoplasts in the animals' tissue by transmission electron microscopy (TEM). Samples of slugs and algae were washed twice with PBS before fixation with 2.5% glutaraldehyde + 0.1 M cacodylate buffer (pH 7.4) overnight at 4 °C. After incubation, the samples were washed four times in 0.1 M cacodylate buffer. Samples were stored in the buffer for no more than 2 weeks and stained with 2% osmium tetraoxide supplemented with 0.8% potassium hexacynoferrate for 1 h. Subsequently, the samples were washed multiple times with 0.1 M cacodylate buffer until the OsO_4 solution was entirely removed. The samples were then suspended by gently shaking in preheated 3.5% agar. The agar was hardened on ice and then separated from the samples with a guillotine reaction vessel tip and poured into small glass vessels containing 1 ml 0.1 M cacodylate buffer. Samples were dehydrated by an ascending series of ethanol (60, 70, 80%, two times 90% and finally 100% ethanol). All dehydration steps were performed on ice and for a minimum of 10 min. Afterwards the samples were transferred to glass jars with epoxy resin consisting of freshly prepared EPON mixture with propylene oxide. The EPON–propylene oxide samples were kept in vacuum glass vessels overnight to fully polymerize. Afterwards, the samples were removed from the EPON–propylene oxide mixture and cut to approximately 2-mm-thick slides with razor blades. A single disc was placed in a plastic mould and filled with EPON. The EPON blocks and samples were polymerized in the oven for 24 h at 40 °C and 24 h at 60 °C. The samples were then embedded for sectioning using the Ultracut E-microtome (Reichert-Jung, New York, USA) and subsequently visualized on a Zeiss 902.

For scanning electron microscopy (SEM) we successfully isolated six times the radulae from slug tissue either fed on *Codium tomentosum*, *Cladophora* sp. and *Bryopsis hypnoides* by incubating the slug heads in 5–40% KOH for 18 h. The cleaned radulae were fixed in 2.5% glutaraldehyde for 1 h at room temperature. The samples were then washed four times for 10 min in 0.1 M phosphate-buffered saline (PBS, pH 7.2) before dehydration by an ascending series of ethanol (50, 70, 80, 90, 96 and 100%; each step 10 min). Afterwards, the samples were washed twice with 100% acetone for 10 min and chemically dried by pre-infiltration in 1:1 acetone–tetramethylsilane (TMS) for 30 min and in 1:2 TMS for another 30 min with a final incubation of 30 min in pure TMS. A small amount of TMS was exchanged and the samples were left drying overnight. The next day, dried samples were glued onto SEM specimen-mounts by conductive adhesive tabs (Plano), coated with gold and imaged using a Zeiss Leo 1430 VP at an accelerating voltage of 18.00 kV.

Results

Elysia viridis is a widely distributed species, occurring along the coast of the Atlantic Ocean from the north in Denmark down to Portugal in the south, and also throughout the Mediterranean Sea. Hence, we first conducted a phylogenetic analysis of our sampled slugs to compare them to already available data and to screen for a potential species complex. In our phylogenetic analyses, specimens collected from the coastline around Portugal cluster together with those from the collection spots at Ferrol,

Spain and Roscoff, France—this cluster forms a sister clade with the Mediterranean species sampled at Banyuls, France (Fig. 1). Yet, based on the low differences of the patristic distances of the COI gene between the *E. viridis* populations of Banyuls and the remaining collection places of 0.021 ± 0.005 (Table 1), we exclude a species complex. The distances to the closest other *Elysia*, *E. chlorotica* is 0.252 ± 0.01 (Table 1).

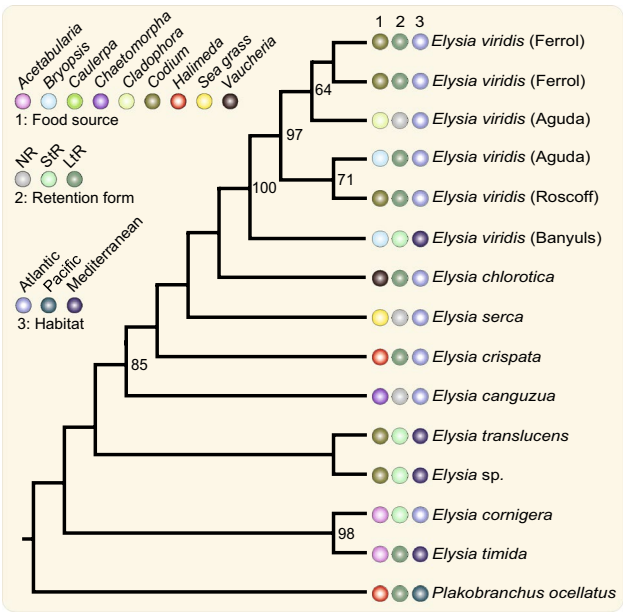

Fig. 1 Phylogenetic relationship of the *Elysia viridis* individuals collected at different sampling sites. Depending on the food source it consumes and according to the literature, *E. viridis* is either considered a long-term (LtR) or a non-incorporation (NI) form. For details on the latter, please refer to the main text. Node label represents bootstrap support values of the maximum likelihood tree reconstruction. Only values above 50 are displayed

The radula of *E. viridis* differs depending on the food source

In its natural habitat, specimens from the Atlantic Ocean feed predominantly on *Codium tomentosum* and on *Cladophora* sp., while those from the Mediterranean feed on *Bryopsis hypnoides*. Earlier reports indicate that Sacoglossa can change the morphology of their radula according to the algae upon which they primarily feed (Jensen 1993). We collected our specimens from *Codium tomentosum* and *Cladophora* sp., but for those collected on *Codium* the diet was changed to *B. hypnoides* in the laboratory. This allows monitoring a change in radula morphology. Slugs feeding on *B. hypnoides* ($n = 4$) and *Cladophora* sp. ($n = 5$) were found to have similar, blade-like radulae, while specimens collected in the field on *C. tomentosum* ($n = 1$) had falcate-shaped radulae (Fig. 2).

Elysia viridis displays different forms of kleptoplast functionality dependent on the food source

Specimens that were fed on *Bryopsis hypnoides* displayed chlorophyll *a* fluorescence over their entire body (Fig. 3), with an initial F_v/F_m value of 0.729 ± 0.013 (Fig. 4a). Throughout starvation, the fluorescence declined steadily (Fig. 3) and after 21 days of starvation the F_v/F_m value had significantly dropped to 0.491 ± 0.043 (Fig. 4a, ANOVA, $p < 0.05$). For these specimens, the $\Delta F/F'_m$ declined equally and also significantly from 0.664 ± 0.016 to 0.380 ± 0.22 (Fig. 4b, ANOVA, $p < 0.05$). When exposed to the photosynthesis blocker monolinuron, the fluorescence throughout the slug was observed to be similar to the specimens starved without the blocker (Fig. 3) and the kleptoplasts had comparable initial F_v/F_m values of 0.698 ± 0.038 (Fig. 4a). During starvation the overall fluorescence declined similarly, but the F_v/F_m value was reduced significantly to 0.242 ± 0.187 after 11 days of starvation (Fig. 4a; ANOVA, $p < 0.05$). We could not measure any F_v/F_m value for the remaining starvation period, although the chlorophyll fluorescence was still

Table 1 p-distances of partial COI sequences between the specimens of *Elysia viridis* and *Elysia chlorotica*

	Elch	Elvi (BY)	Elvi (AG 1)	Elvi (FE 1)	Elvi (FE 2)	Elvi (AG 2)	Elvi (RO)
Elch		0.234	0.252	0.252	0.252	0.263	0.257
Elvi (BY)	0.234		*0.018*	*0.018*	*0.018*	*0.029*	*0.023*
Elvi (AG 1)	0.252	*0.018*		*0.000*	*0.000*	*0.014*	*0.009*
Elvi (FE 1)	0.252	*0.018*	*0.000*		*0.000*	*0.014*	*0.009*
Elvi (FE 2)	0.252	*0.018*	*0.000*	*0.000*		*0.014*	*0.009*
Elvi (AG 2)	0.263	*0.029*	*0.014*	*0.014*	*0.014*		*0.017*
Elvi (RO)	0.257	*0.023*	*0.009*	*0.009*	*0.009*	*0.017*	

Elch E. chlorotica, *Elvi (BY) E. viridis* (Banyuls, France), *Elvi (AG) E. viridis* (Aguda, Portugal), *Elvi (FE) E. viridis* (Ferrol, Spain), *Elvi (RO) E. viridis* (Roscoff, France). The number in the species name indicates different individuals. Highlighted in italics are all *E. viridis* species

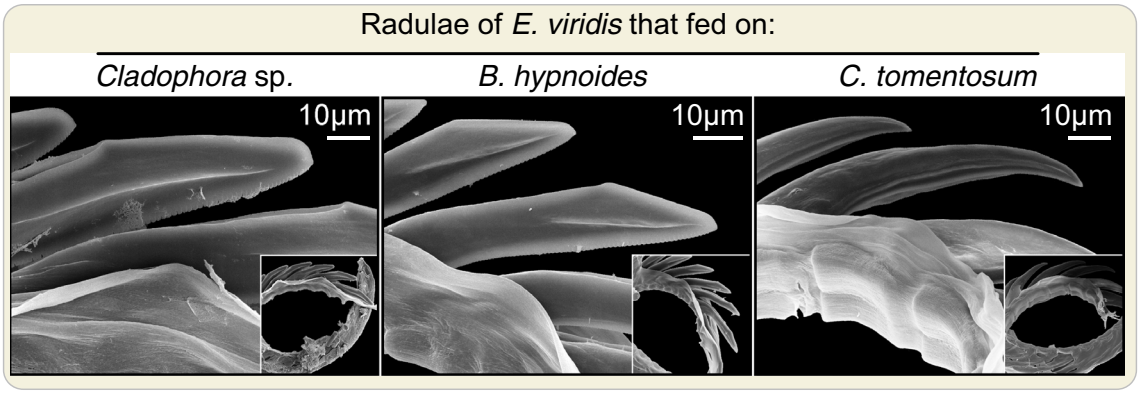

Fig. 2 The radulae, in Sacoglossa specialized teeth organized in a row, of *Elysia viridis* are highly variable and differ depending on the food source

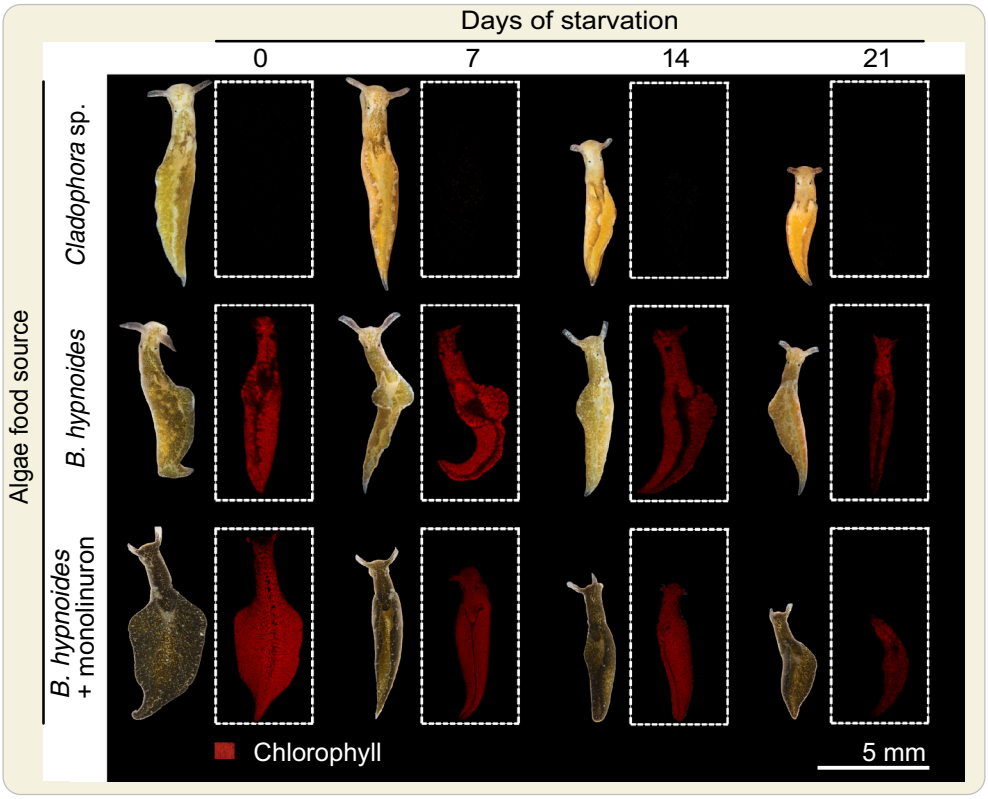

Fig. 3 Macroscopic chlorophyll fluorescence images of specimens of *Elysia viridis* fed for 1 month on either *Bryopsis hypnoides* or *Cladophora* sp. (T0) and after the number of days of starvation. Kleptoplasts of *Bryopsis hypnoides* are present throughout the body in *Elysia viridis* which give the slugs their distinct green coloration. During starvation, the overall fluorescence signal declines in slugs fed with *B. hypnoides*, which happens to the same degree (based on visual inspection) in the specimens reared in the presence of the photosynthesis blocker monolinuron. No measurable chlorophyll fluorescence was detected in specimens fed on *Cladophora* sp.

detectable (Fig. 3). In contrast to the control, the $\Delta F/F'_m$ did not exhibit any value that was above the background noise of less than 0.2 (Fig. 4b). Surprisingly, for specimens that were fed on *Cladophora* sp. we could not determine any chlorophyll *a* fluorescence (Fig. 3). For animals starved for 4 days, and even those freshly fed, F_v/F_m values did not exceed values of 0.25 (Fig. 4a). After 4 days of starvation F_v/F_m ratios were no longer measurable. With regard to $\Delta F/F'_m$ we were not able to measure any notable value either, even in slugs that were freshly fed (Fig. 4b).

Next, we investigated the capacity of slugs fed on *B. hypnoides* to fix $^{14}CO_2$ through the kleptoplasts. We measured

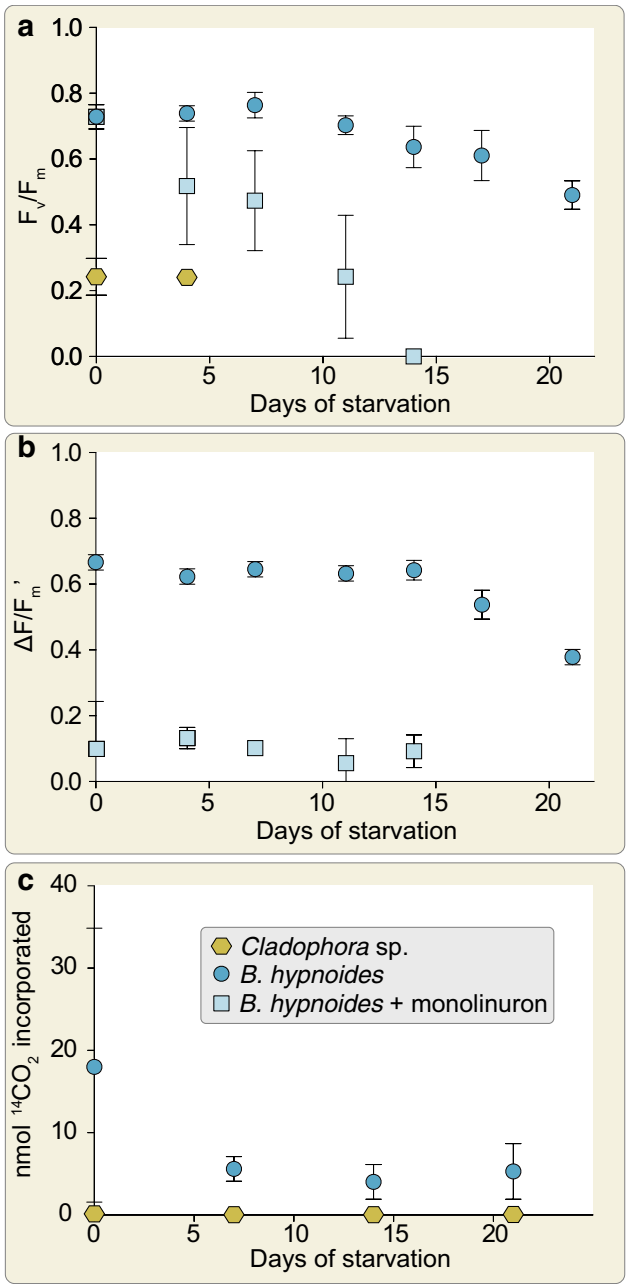

Fig. 4 Maximum quantum yield (F_v/F_m), photosynthetic yield and $^{14}CO_2$ fixation of kleptoplasts in *Elysia viridis* during a 21-day starvation period. **a** The maximum quantum yield F_v/F_m of kleptoplasts derived from *Bryopsis hypnoides* (blue circles) starts to decline after 11 days of starvation, while the F_v/F_m values of kleptoplasts of monolinuron-treated animals (blue squares) decline immediately during starvation and was no longer measurable after 14 days of starvation. In animals fed on *Cladophora* sp. (yellow hexagons), F_v/F_m values where only measurable for 4 days (and which never exceeded background noise), before no more signals were detectable. **b** The photosynthetic yield $\Delta F/F'_m$ of kleptoplasts derived from *Bryopsis hypnoides* (blue circles) started to decline after 17 day of starvation, while the $\Delta F/F'_m$ of monolinuron treated animals (blue squares) never reached beyond background noise. No $\Delta F/F'_m$ of animals fed with *Cladophora* sp. could be measured. **c** After only a few days of starvation, the level of $^{14}CO_2$ fixation by kleptoplasts that stem from *Bryopsis hypnoides* drops to about 30% of the initial value. Specimens fed with *Cladophora* sp. showed no measurable levels of $^{14}CO_2$ incorporation that exceeded those of background noise

$^{14}CO_2$ incorporation of freshly fed slugs and for those starved for 7, 14 and 21 days. In freshly fed specimens, we found $^{14}CO_2$ incorporation of 18.0 ± 17.5 nmol that had already declined to 5.6 ± 1.5 nmol, i.e. a drop of almost 70%, after only 7 days of starvation (Fig. 4c). In the following 2 weeks of starvation the $^{14}CO_2$ incorporation stabilized (4.0 ± 2.1 and 5.3 ± 3.3 after 14 and 21 days, respectively; Fig. 4c). Furthermore, and in line with the fluorescent measurements, we could not detect any $^{14}CO_2$ incorporation for specimens that had solely fed on *Cladophora* sp. (Fig. 4c).

Elysia viridis does not sequester functional plastids of *Cladophora* sp.

The results of the photosynthetic measurements and the $^{14}CO_2$ incorporation showed that the kleptoplasts gained from *Bryopsis hypnoides* are fully functional in the cytosol of *Elysia viridis*, at least for a few days after starvation commences (Fig. 4). Plastids that stem from *Cladophora* sp., however, are not functionally incorporated. To investigate whether the differences with regard to F_v/F_m and CO_2 fixation between kleptoplasts from *B. hypnoides* and *Cladophora* sp. are due to a more pronounced degradation or digestion of the latter, we imaged the algae and slugs by TEM and compared the morphology of the incorporated kleptoplasts in the cells lining the digestive tubules. In the alga, the plastids of *B. hypnoides* are oval shaped and distributed throughout the coenocytic organized algae (Fig. 5; *B. hypnoides*). In the sea slugs, the kleptoplasts lose their oval-shaped morphology and appear more roundish (Fig. 5; freshly fed). A similar kleptoplast phenotype was detected in slugs treated with monolinuron (Fig. 5; 10 days starvation + monolinuron). In *Cladophora* sp., the plastids are reticulated and organized parietally (Fig. 5; *Cladophora* sp.). We could not identify any structures resembling intact kleptoplasts in *E. viridis*, when the specimens had solely been feeding on *Cladophora* sp. (Fig. 5; freshly fed), consistent with the lack of detectable $^{14}CO_2$ fixation (Fig. 4c). The large, round structures identified in some epithelial cells of freshly fed and starved animals, lack the structures resembling kleptoplasts, in particular thylakoids, and are most likely digestive vacuoles (Fig. 5; *Cladophora* sp, freshly fed and 10 d starvation).

Discussion

Elysia viridis represents an important species when exploring functional kleptoplasty in sacoglossan sea slugs (Cruz et al. 2013; Serôdio et al. 2014; de Vries et al. 2014a). Its polyphagous lifestyle, which includes diets from *Codium* spp. (Hinde and Smith 1972; Trench and Gooday 1973; Trench et al. 1973; Evertsen and Johnsen 2009; Christa et al.

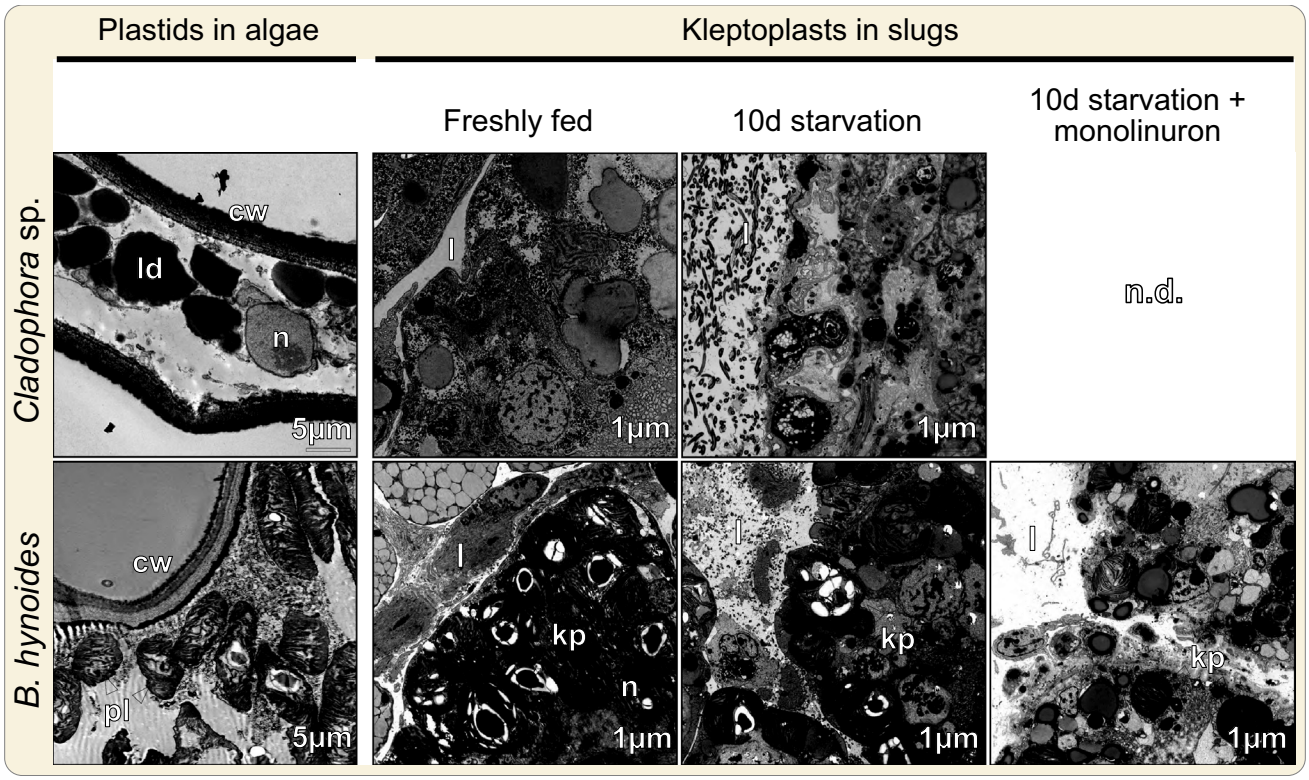

Fig. 5 Transmission electron microscopy (TEM) images of the food algae *Bryopsis* and *Cladophora* and of the digestive tract cells of the sea slugs that had fed on the respective algae. Note the distinct difference between slugs that had fed on *Cladophora* and those that had fed on *Bryopsis*. Only from the latter, kleptoplasts are sequestered and released into the cytosol of the sea slug's cells, where the stroma of the kleptoplasts is separated from the cytosol by two membranes, that is identical to what it is like in the alga itself. Also note that no starch is detectable in the kleptoplasts of slugs treated with monolinuron, as seen in Laetz et al. (2017). *Ld* lipid droplet, *cw* cell wall, *n* nucleus, *pl* plastid, *kp* kleptoplast, *l* digestive tract lumen

2014a), *Bryopsis hypnoides* (Händeler et al. 2009), *Chaetomorpha* spp. and *Cladophora* spp. (Baumgartner and Toth 2014; Baumgartner et al. 2014), and its wide geographical distribution makes it a suitable model to study the effects different algal food sources (and their plastid biology) have on the retention rate of kleptoplasts and its effect on enduring starvation. Hence, *E. viridis* complements the already established system of the two congener species *Elysia timida* Risso, 1818 and *Elysia cornigera* Nuttal, 1989, which feed on the same alga (*Acetabularia acetabulum*) but with only the former enduring starvation long term (de Vries et al. 2015; Rauch et al. 2017a; Laetz et al. 2017; Jerschabek Laetz and Wägele 2017).

Previous studies based on *E. viridis* speculated that the food source of the slug is what determines the retention form, that is, the span of time the kleptoplasts remains photosynthetically active within slug cells. With regard to PSII activity, the kleptoplasts of the slugs fed on *Codium tomentosum* were found to retain activity 20–80 days after the slugs were deprived of their food source (Hinde and Smith 1972, Vieira et al. 2009; Cruz et al. 2014). Kleptoplasts of *B. hypnoides* were suggested to only have a short-term functionality (Händeler et al. 2009). The sequestered kleptoplasts of *Cladophora rupestris* were found to be of limited functionality altogether (Baumgartner et al. 2015), although *E. viridis* was predominantly found on *Cladophora* in the wild (where they were also observed to be larger in size than those found on *Codium*) and from which it was concluded that "*Cladophora* might represent a superior host relative to *Codium*" (Baumgartner et al. 2015).

Our F_v/F_m values from *E. viridis* feeding on *Bryopsis hypnoides* were on average about 30% above those previously published (Händeler et al. 2009). The sequestered plastids from *B. hypnoides* were, furthermore, found to remain functional similarly long with regard to PSII activity during starvation, as those that stem from *Codium tomentosum* (Serôdio et al. 2010; Cruz et al. 2014). Hence, when fed on *B. hypnoides*, *E. viridis* is, during starvation under our light conditions by the definition of retention forms (Händeler et al. 2009), a LtR species. In contrast, specimens that we fed on this particular *Cladophora* sp. did not retain any functional kleptoplasts, as demonstrated by F_v/F_m values of 0.25 and less [i.e. background noise and even lower than what was described for *C. rupestris* (Baumgartner et al.

2015)], $^{14}CO_2$ incorporation measurements and TEM imaging (Figs. 4a, 5). No functional kleptoplasts of *Cladophora* sp. were found to be sequestered by *E. viridis*, letting us to conclude that this sacoglossan slug is kleptoplast free, or non-incorporating (NI), when feeding on our species of *Cladophora* sp. This does not exclude, however, that other species of *Cladophora* might very well be incorporated in a functional state. Moreover, small *E. viridis* specimens of approximately 2 mm in length that we collected in the wild, grew to the size of 1–1.5 cm under our laboratory conditions by solely feeding on *Cladophora* sp. and in the absence of photosynthesizing kleptoplasts—an outcome similar to that of specimens feeding on *B. hypnoides*. From what we can tell, this is the first report that demonstrates that a Sacoglossa can be classified either as a LtR or a NI form, first and foremost depending on the food source it consumes.

The non-invasive measurement of PSII activity by means of PAM fluorometry is a valuable tool to determine the presence of functional kleptoplasts in Sacoglossa. By now it is arguably the most commonly used tool to study kleptoplasty in Sacoglossa (Evertsen and Johnsen 2009; Händeler et al. 2009; Serôdio et al. 2010; Cruz et al. 2013; Serôdio et al. 2014; Cruz et al. 2015) and it also provides the values that are used to determine the retention class (Händeler et al. 2009). Due to its ease of use, it almost completely superseded measuring $^{14}CO_2$ incorporation since it was used for the first time some 15 years ago to investigate the photobiology of sacoglossan kleptoplasts (Rauch et al. 2017b). PAM fluorometry, however, without the measurement of the fraction of absorbed incident light (or absorption cross section) cannot provide absolute estimates of electron transport rates at PSII and thus no information directly relatable to the quantity of photosynthetically fixed carbon. Only direct CO_2 incorporation measurements provide a reading that one can use to interpret the nutritional benefit of kleptoplast photosynthesis, which is surprisingly low for Sacoglossa (Rauch et al. 2017b). Independent of the method used, the categorization of sacoglossan slugs into different forms of retention (i.e. LtR versus StR) should be treated with caution. Many other factors, such as the food source (this study, Baumgartner and Toth 2014) or the light conditions (Vieira et al. 2009), likely influence kleptoplast longevity more than the slugs itself. Thus, although the categories are helpful, they might be misleading and alternative definitions should be considered in the future.

In *E. viridis*, the kleptoplast-dependent fixation of $^{14}CO_2$ was determined a few times already, but with varying results and in at least one case higher values were measured in the dark than in the light (Hinde and Smith 1972; Trench and Gooday 1973; Trench et al. 1973; Hinde and Smith 1975). We found that kleptoplasts of *B. hypnoides* fix about 18 nmol of $^{14}CO_2$ in freshly fed slugs, but once deprived of their food source, this value dropped significantly within the first couple of days (Fig. 4a). These results are similar to what was observed for two other sacoglossan species that had fed on *A. acetabulum* (de Vries et al. 2015). Here, too, $^{14}CO_2$ fixation dropped within the first few days to approximately 40% of the initial values, before a further decline decelerated. It might take a few days before starvation triggers a change in the slug's metabolism and in how also kleptoplasts are handled. When feeding on *Cladophora* sp., no $^{14}CO_2$ fixation was measurable, which is consistent with the PAM fluorometry measurements (Fig. 4) and the absence of any kleptoplasts in the cells of the slug's digestive tract (Fig. 5). Maybe due to the reticulated parietal morphology of the plastids in *Cladophora* sp., the slugs are not able to sequester intact single plastids to begin with. The initial fluorescence signals detected likely trace back to free chlorophylls or to chlorophyll still connected to fragmented thylakoid membranes, but not intact plastids.

Symbiosis for the reason of profiting from products of photosynthesis can be quite beneficial for an animal host. In corals, up to 95% of the total energy budget stems from the phototrophic symbionts such as the dinoflagellate *Symbiodinium* (Muscatine et al. 1981; Edmunds and Davies 1986). But the situation is not always that clearly cut and the case of the endosymbiotic alga *Oophila amblystomatis* (F.D. Lambert ex N. Wille, 1909) that resides in cells of the spotted Salamander *Ambystoma maculatum* (Shaw, 1802) (Kerney et al. 2011) serves as a good example. Although these amphibians are sometimes referred to as "solar salamanders" (Petherick 2010), the endosymbionts likely fix no carbon for the animal and might even experience a metabolic shift from phototrophy to fermentation (Burns et al. 2017).

From reading the literature on Sacoglossa—the "photosynthetic slugs"—one can quickly gain the impression that these marine molluscs live photoautotrophically like a plant (Pelletreau et al. 2012; Rumpho et al. 2011; Cruz et al. 2013; Pierce et al. 2015). It appears a kind of default expectation regarding the interaction of a phototroph with a heterotroph, but (i) the contribution of kleptoplasts to the nutrition of sea slugs is only poorly understood and (ii) a comparison to the aforementioned animal–alga symbiosis needs to be treated with caution. Sacoglossa do not engage in a symbiosis with algae, but only sequester their plastids. Neglecting these differences for now, for *E. viridis* it was postulated that 36% of the photosynthetically fixed carbon is used by the slugs (Hinde 1978) and incorporated into various metabolites (Trench et al. 1970; Greene and Muscatine 1972; Hinde 1978). Those numbers, unfortunately, tell us little about how much of the total carbon requirement is satisfied by kleptoplasts in LtR Sacoglossa. Recent calculations for *Elysia timida* provide an estimate that it is less than 1% (Rauch et al. 2017b)—a rather bleak estimation. Considering that the quantity of fixed carbon that was measured for *E. viridis* feeding on *B. hypnoides* is comparable, it explains

why *E. viridis* is able to survive and grow when feeding on *Cladophora*: carbon fixation of the kleptoplasts alone is not the reason to overcome starvation periods, at least in the case of this LtR Sacoglossa.

Some Sacoglossa such as *Elysia chlorotica* (LtR), *E. timida* (LtR) or *E. cornigera* (StR) feed on a single alga species (Christa et al. 2014b; Pierce et al. 2015; de Vries et al. 2015), while others such as *Plakobranchus ocellatus* (LtR), *Elysia crispata* (LtR) and *E. viridis* (StR/LtR) are polyphagous (Christa et al. 2014b; Baumgartner et al. 2015). Thus, feeding on a single species does not necessarily correlate with the long-term retention of kleptoplasts as the case of *E. timida* and *E. cornigera* illustrates (de Vries et al. 2015). The different forms of retention are maybe associated with food preferences and differences in habitats (Christa et al. 2013b; de Vries et al. 2015). In some geographical locations, the seasonal changes are more pronounced than in others: *E. cornigera* (StR) has its sole food source, *Acetabularia crenulata*, available the whole year round in the Caribbean, while *E. timida* (LtR) has to deal with seasonal absence of its food source *A. acetabulum* in the Mediterranean during winter (Marín and Ros 1992). However, *Costasiella ocellifera* and *Elysia crispata* are both LtR species, and they are occurring sympatric in the Caribbean with *Elysia cornigera*, an StR species (Christa et al. 2015), despite the fact that their food sources are available throughout the year. Thus, to what degree the habitat shapes the retention form during the evolution of functional kleptoplasty in Sacoglossa is still elusive.

Polyphagy might also depend on the ability to adapt the radula to different food sources like shown for *E. viridis* before (Jensen 1993), but it was also shown that juvenile *E. viridis* raised on *Codium* were not able to switch to *Cladophora* (Trowbridge and Todd 2001). Further, polyphagy might be limited in other species such as *P. dendritica* due to high energetic costs that come along with switching food (Trowbridge 1998). We collected our specimens in the field from either *C. tomentosum* or *Cladophora* sp. and observed that depending on which single food source they were fed under laboratory conditions, the radulae differed (Fig. 2). The radulae from those feeding on *Cladophora* sp. and *Bryopsis hypnoides* showed very similar morphologies (thicker and short, tanto knife-like), while radulae from slugs feeding on *C. tomentosum* (thinner and longer, hawksbill or talon blade-like) differed (Fig. 2). These differences in radulae morphology most likely reflect the differences in algae morphology: *Bryopsis hypnoides* and *Cladophora* sp. have elongated, flattened siphons that can probably only be pierced laterally, while the thalli of *C. tomentosum* consist of several filaments that are densely branched towards the periphery where they form the utricles. If these results reflect true adaption to a specific sort of food alga, then it occurred within the period of 4 weeks, i.e. the time between sampling (and the transfer to the lab and a single food source) and the isolation of the radulae. However, it remains to be shown if a radula adaptation might work in both directions, that is, from *Cladophora* or *Bryopsis* to *Codium*, or if the reduced growing and feeding capacity of specimens from *Codium* fed with *Cladophora* (Trowbridge and Todd 2001) are based on other factors such as the "learning" to feed on a specific food source after a switch as proposed by Jensen (1989).

Conclusion

Unravelling the reasons of plastid sequestration in Sacoglossa and the contribution of photosynthesis to slug survival during starvation is no easy undertaking. On these grounds, it is important to identify both slug and algae species that allow a comparative analysis to systematically identify the factors that mediate long-term retention and provide a rationale for why plastids are sequestered to begin with. Based on PAM fluorometry measurements, TE and SE microscopy, and $^{14}CO_2$ incorporation measurements, we demonstrate that whether *Elysia viridis* is able to incorporate and retain kleptoplasts, it depends first and foremost on the food source. Importantly, *E. viridis* is able to live and reproduce in the presence and absence of photosynthesizing kleptoplasts and can furthermore adapt its radula to an available food source within weeks. These results underscore the complexity of sacoglossan biology and that the contribution of kleptoplasts to the physiology of the slugs is likely not explained by photosynthesis and carbon fixation alone. Through *E. viridis*, we have access to a system that allows a detailed study dedicated to comparing the survival rate during starvation and dependence on the food source and its associated plastid biology.

Acknowledgements Funding through the DAAD (P.R.I.M.E.) and FCT to GC (SFRH/BPD/109892/2015), DFG to S.B.G. (GO1825/4-1), and through the ERC to Prof. William F. Martin (ERC 666053) is gratefully acknowledged. For financial support, thanks are due to Centre for Environmental and Marine Studies (UID/AMB/50017), FCT/Ministry of Science and Education through national funds, and the co-funding by European Fund For Regional Development, within the PT2020 Partnership Agreement and Compete 2020.

Author contributions CR, SBG, JS and GC planned the experiments, which were conducted by CR, AGMT and GC. CR, SBG and GC wrote the manuscript, whose final version was approved by all authors. We thank Steffen Köhler (CAi, HHU) for imaging of the slugs and for his help with SEM imaging, and Marion Nissen (CAi, HHU) for her help with the TEM.

Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no competing interests.

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