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## Research

**Cite this article:** Christa G, Zimorski V, Woehle C, Tielens AGM, Wägele H, Martin WF, Gould SB. 2014 Plastid-bearing sea slugs fix CO<sub>2</sub> in the light but do not require photosynthesis to survive. *Proc. R. Soc. B* **281**: 20132493.  
<http://dx.doi.org/10.1098/rspb.2013.2493>

Received: 23 September 2013

Accepted: 25 October 2013

### Subject Areas:

evolution, plant science, biochemistry

### Keywords:

Gastropoda, Sacoglossa, Kleptoplasty, *Elysia*, photoautotroph, photosynthetic slugs

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# Plastid-bearing sea slugs fix CO<sub>2</sub> in the light but do not require photosynthesis to survive

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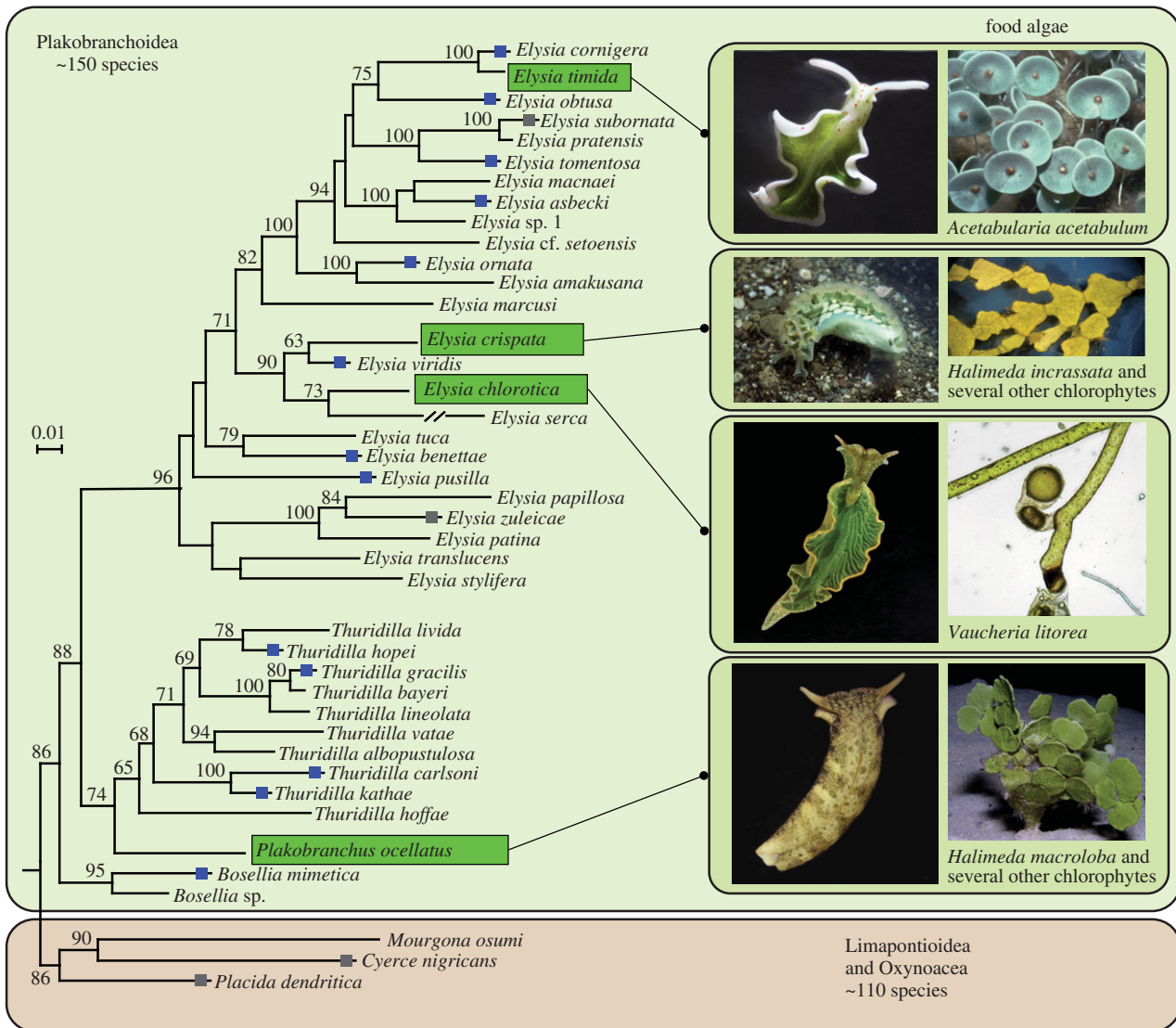
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Several sacoglossan sea slugs (Plakobranchoidea) feed upon plastids of large unicellular algae. Four species—called long-term retention (LtR) species—are known to sequester ingested plastids within specialized cells of the digestive gland. There, the stolen plastids (kleptoplasts) remain photosynthetically active for several months, during which time LtR species can survive without additional food uptake. Kleptoplast longevity has long been puzzling, because the slugs do not sequester algal nuclei that could support photosystem maintenance. It is widely assumed that the slugs survive starvation by means of kleptoplast photosynthesis, yet direct evidence to support that view is lacking. We show that two LtR plakobranchoidea, *Elysia timida* and *Plakobranchus ocellatus*, incorporate <sup>14</sup>CO<sub>2</sub> into acid-stable products 60- and 64-fold more rapidly in the light than in the dark, respectively. Despite this light-dependent CO<sub>2</sub> fixation ability, light is, surprisingly, not essential for the slugs to survive starvation. LtR animals survived several months of starvation (i) in complete darkness and (ii) in the light in the presence of the photosynthesis inhibitor monolinuron, all while not losing weight faster than the control animals. Contrary to current views, sacoglossan kleptoplasts seem to be slowly digested food reserves, not a source of solar power.

## 1. Introduction

Symbioses between animals or heterotrophic protist and algae are fairly common in nature [1]; prominent examples include the zoochlorellae of *Hydra viridis* [2,3] or dinoflagellates (zooxanthellae) of corals [4] and the many different species of algae found in ciliates [5]. A more curious kind of symbiosis is found among the sacoglossan molluscs (marine slugs) from the Plakobranchoidea. These animals establish a symbiosis with only a part of their algal partner: the plastid. Nearly 150 species of plakobranchoidea have been described to date, and similar to all sacoglossans they feed upon algae by sucking the cytoplasm out of the large, syncytial algal cells upon which they feed. While most sacoglossan species simply digest the plastids, plakobranchoidean species (termed plakobranchoidea for convenience) exhibit a delayed digestion, and four species—*Elysia chlorotica*, *Elysia timida*, *Elysia crispata* and *Plakobranchus ocellatus*—retain the ingested plastids, the kleptoplasts, in their digestive gland for several months [6–10]. This gland fills most of the animal's body and gives them a distinctive green colour (figure 1), which is why they are sometimes called 'leaves that crawl' [6] or 'solar-powered slugs' [9]. Because these four species can maintain plastids with functional photosystems for several months, they are designated as long-term retention (LtR) species in contrast to those plakobranchoidea that are only able to maintain functional plastids for up to two weeks, and which are hence termed short-term retention (StR) species [7]. In *E. timida*, the undigested plastids



**Figure 1.** Overview of phylogenetic relationships of Plakobranchioidea (Sacoglossa). Blue rectangles indicate photosynthetic activity over at least two weeks of starvation (based on pulse–amplitude–modulation (PAM) measurements), whereas grey squares indicate species that immediately digest plastids. Slugs showing photosynthetic activities over two months are highlighted in green and the respective pictures of the species and food algae are provided on the right. Photo of *E. chlorotica* with permission of M. Rumpho, and that of *Vaucheria litorea* with permission of C. F. Carter. Phylogeny based on Wägele *et al.* [8].

remain ultrastructurally intact and photosynthetically active, as determined by photosystem fluorescence, for more than two months [7,8]. Having acquired a load of plastids, the animals can be kept in the laboratory, in the light, for months without additional food [7–10].

How Ltr plakobranchids maintain their kleptoplasts for such long periods of time has been the subject of much speculation and considerable recent research. The predicted proteome of *Arabidopsis* plastids ranges from 1000 to approximately 3500 proteins [11], but plastid genomes only encode for 60 (higher plants) to 200 (red algae) protein-coding genes in the organelle's DNA [12]. The remaining plastid proteins (more than 90%) are encoded in the nucleus, synthesized as precursor proteins on cytosolic ribosomes and imported from the cytosol through the plastid-specific protein translocon machinery (reviewed in [13–15]). Because plakobranchids do not sequester algal nuclei, which can however be ingested for a short time during feeding [16], and because some proteins in higher plant chloroplasts can have turnover rates on the order of 30–120 min [17–19], it has been widely assumed that sequestered plastids of plakobranchids also require imported proteins to remain photosynthetically active. The

most popular theory for the source of those assumedly essential genes has been lateral gene transfer (LGT) from the algae to the slug, and some PCR-based reports provided evidence in favour of that view, for example involving the gene for the light-harvesting complex protein LHC [10].

The by far most prominent report for putative involvement of LGT in sacoglossans concerns a sequence for the manganese cluster-stabilizing protein PsbO of photosystem II in *E. chlorotica* [20]. The PCR amplification products for PsbO obtained from *E. chlorotica* were identical in sequence to those from *Vaucheria*, including a canonical bipartite targeting signal [21,22] that directs the PsbO precursor across the four membranes that surround the plastid in *Vaucheria*. However, the *Vaucheria* plastids that are sequestered in *E. chlorotica* are only surrounded by two membranes; the outer two are removed during sequestration [23]. As a consequence, were the *E. chlorotica* PsbO precursor protein [20] really expressed, the gene product would enter the secretory pathway, and thus be excreted from the cell because of its intact and highly conserved signal peptide, rather than being targeted to the remaining inner two membranes of the sequestered *Vaucheria* plastid [8]. This aspect of targeting

renders the case for LGT of PsbO in *E. chlorotica* very problematic and raises the question: is there LGT from algae to slugs in Ltr plakobranchids, or not?

To address this, Wägele *et al.* [8] sequenced expressed sequence tags (ESTs) from the Ltr species *E. timida* and *P. ocellatus* and found no evidence in either species for the expression of any genes of demonstrably green algal nuclear provenance. Similar results for *E. chlorotica* were subsequently obtained, with no transcripts for PsbO or any other *Vaucheria*-derived nuclear genes identified [24], leading to the conclusion that, contrary to earlier claims, LGT probably does not underpin photosynthetic activity of sequestered plastids in *E. chlorotica* after all. However, Pierce *et al.* [25] reported that among the 100 million *E. chlorotica* transcripts that they sequenced, about 100 reads might indicate LGT in *E. chlorotica*, although only one pointed to an essential function in photosynthesis (a light-harvesting complex protein). But photosynthesis requires the expression of thousands of nuclear genes [11,26], not 100. Moreover, transcripts for photosynthetic functions are generally abundant: for example, the small subunit of RuBisCO and LHC together constitute approximately 20% of all transcripts in *Arabidopsis* leaves [27]. The 100 genes that Pierce *et al.* [25] found comprise 0.000001% of the mRNA each, or 0.0001% of the total, so even if those 100 genes are LGTs, they cannot underpin a photosynthetic lifestyle. While Pierce *et al.* [25] interpret those 100/100 000 000 reads as evidence of LGT from alga to mollusc, we would interpret that same data to indicate that their sequencing substrate was 99.9999% free of contaminating algal nucleic acids.

The very expectation that some sacoglossans have undergone LGT stems from the inference that plastids require many proteins in order to support a photosynthetic lifestyle. As the genes for the proteins are missing, the next question is: how strong is the evidence that the slugs depend upon photosynthesis to begin with? The main evidence supporting the view that plakobranchids are photosynthetic (in the sense of being photoautotrophic) comes from earlier studies and is of two main types. First, Trench and co-workers [28,29] showed that *E. viridis* incorporates  $^{14}\text{C}$  from  $^{14}\text{CO}_2$ . A number of other studies also reported the incorporation of  $^{14}\text{C}$  from  $^{14}\text{CO}_2$  in plakobranchids that sequester plastids [30–33], but animals can also incorporate  $\text{CO}_2$  via carboxylation reactions. The second line of evidence for plakobranchids being photosynthetic comes from the observation that once the plastids have been incorporated into the digestive gland, Ltr species can survive for months in the absence of additional food [7,10,23,24,34,35]. Such plakobranchids are said to be ‘starved’ and are typically cultivated in the light [8,9].

However, a subtlety of such experiments that is not immediately evident to the observer (who is understandably fascinated by the sight of plastid-bearing slugs), but that has been pointed out in earlier work [36–40], is that starved animals become smaller as starvation progresses. Starved animals also tend to lose their green colour with time, getting pale as starvation progresses [37,38,41]. Here, we take a step back in the study of ‘photosynthetic slugs’—as many, including ourselves, have called them in the past—by re-inspecting the role of light. We test the light dependence of  $^{14}\text{CO}_2$  incorporation into acid-stable compounds in *E. timida* and *P. ocellatus*, the long-term starvation survival of plastid-bearing slugs in light versus dark, and the effect of the photosynthesis inhibitor monolinuron on the ability of *P. ocellatus* to survive starvation in the light. Surprisingly, photosynthesis was not essential for

the slugs to survive months of starvation, which explains the lack of gene transfer from alga to animal in these species and, more importantly, calls for a general rethinking of the ‘photosynthetic slug’ story.

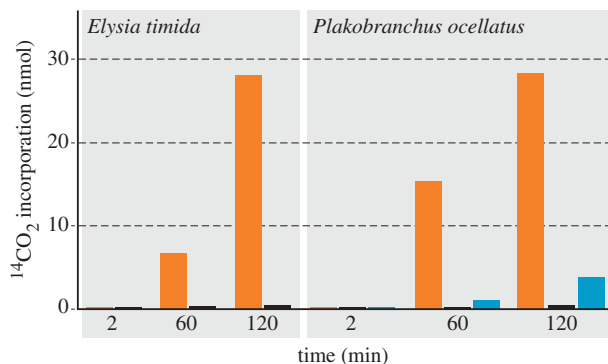
## 2. Results and discussion

The relationship between sacoglossans that perform Ltr of their sequestered plastids is now widely reported in the literature as an example of acquired photoautotrophy in animals [9,24,42], typically leading to questions of how many and what kinds of genes have been transferred to support this photoautotrophic lifestyle [20]. Critical of that view, we recently tested the gene transfer hypothesis in sacoglossans that perform Ltr and found no evidence for the expression of any genes of demonstrably green algal nuclear provenance to support plastid longevity in two of the four known Ltr species, *E. timida* and *P. ocellatus* [8]. That eyebrow-raising result prompted us to further re-inspect the degree to which plakobranchid sacoglossans exhibiting Ltr depend on photosynthesis in the first place.

### (a) *Elysia timida* and *Plakobranchus ocellatus* display light-dependent $\text{CO}_2$ fixation

Previous studies on several plastid-bearing sea slugs have shown that green animals can fix  $^{14}\text{CO}_2$  [29,32,33,43–45]. However, there are also exchange reactions and carboxylation steps in animal metabolism that would allow  $^{14}\text{CO}_2$  to be incorporated into animal tissue in a light-independent manner. For example, propionate is a main primary source of reduced carbon in many animals; it is absorbed from the gut, where it is released from ingested food by the gut microbial flora. Propionate is channelled into metabolism as propionyl-CoA, which is then carboxylated to methylmalonyl-CoA and rearranged in a vitamin B<sub>12</sub>-dependent reaction to the citric acid cycle intermediate succinyl-CoA and then succinate, which can be used either for biosynthetic (amino acids, haem, etc.) or for energetic purposes [45]. Thus, via succinate,  $^{14}\text{CO}_2$  can be incorporated into animal tissue, but in a light-independent manner. Furthermore, the fixation rates reported so far vary substantially between different species studied [23,32,33,43–45].

We investigated the ability of *E. timida* bearing *Acetabularia* plastids to fix  $^{14}\text{CO}_2$  in the absence and presence of light. In total, we used 24 slugs for three individual experiments. We analysed the light-dependent incorporation of [ $^{14}\text{C}$ ]-labelled  $\text{CO}_2$  after 2 min, 1 and 2 h. Four slugs were used for every time point and kept either in the light or in the dark. Afterwards, incorporation of labelled carbon was measured. We note that adult slugs lacking plastids cannot be used as a control here, because individuals of these sacoglossan species do not develop into adults unless they feed, at the larval stage, upon their specific algae, and because plastid-bearing adults die before they can be starved to the stage of lacking plastids altogether. After 2 min incubation with [ $^{14}\text{C}$ ]-labelled  $\text{CO}_2$ , incorporation in the light was slightly higher than that in the dark (0.05 nmol in the light versus 0.04 nmol in the dark). After 1 h, slugs in the light showed incorporation 23-fold higher than slugs in the dark (6.73 nmol incorporated  $^{14}\text{CO}_2$  in the light and 0.30 nmol in the dark). In the light,  $^{14}\text{CO}_2$  incorporation after 2 h was 60 times greater than for slugs kept in the dark (28.1 versus 0.46 nmol  $^{14}\text{CO}_2$ ; figure 2). Thus, we can



**Figure 2.** Light-dependent incorporation of  $^{14}\text{CO}_2$  by *E. timida* and *P. ocellatus*.  $\text{CO}_2$  incorporation in *E. timida* is almost completely blocked when the slugs were kept in the dark. In *P. ocellatus*, we additionally blocked photosynthesis using monolinuron, which led to an 87% decrease of  $\text{CO}_2$  incorporation. Owing to the size difference, four *E. timida* specimens (always representing an equal amount of weight) and only one *P. ocellatus* were used for each individual time point measured. Only for the 120 min values of *P. ocellatus*, the mean of two individual measurements is shown (values (nmol per incubation) of these 120 min incubations of *P. ocellatus* were D: 0.42 and 0.47; L: 24.5 and 32.2; M: 3.14 and 4.35); for all others a single measurement was carried out. Orange, light; black, dark; blue, light + monolinuron.

confirm that *Acetabularia* plastids in *E. timida* fix  $\text{CO}_2$  in a light-dependent manner, as has been reported for other plakobranchids [29,32,33,43–45]. That  $\text{CO}_2$  fixation is almost abolished in the dark demonstrates that light-independent carbon fixation reactions, although they can occur in slug metabolism, are overshadowed by light-dependent  $\text{CO}_2$  fixation in sequestered plastids.

For *P. ocellatus*, we obtained similar results (figure 2), showing further that in comparison to the untreated slugs, the incorporation of  $^{14}\text{CO}_2$  in the monolinuron-treated samples was 87% lower after 120 min, indicating that photosynthesis in the slugs is inhibited by the drug. Previous studies reported  $^{14}\text{C}$  in a variety of slug metabolites [29,33,43–45], but whether the label stems from photosynthate exported from intact plastids or simply from decomposing plastids is not known. That is, it is possible that sequestered plastids do not export reduced carbon, but are simply digested, a possibility that is supported by microscopic observations suggesting that kleptoplasts accumulate substrate under starvation conditions, rather than secreting it [46,47]. Notwithstanding many studies in the literature addressing the nature of plastid–slug metabolite interactions, it seemed that the more crucial question was whether light-dependent  $\text{CO}_2$  fixation was essential for survival of the animals grown without algal food.

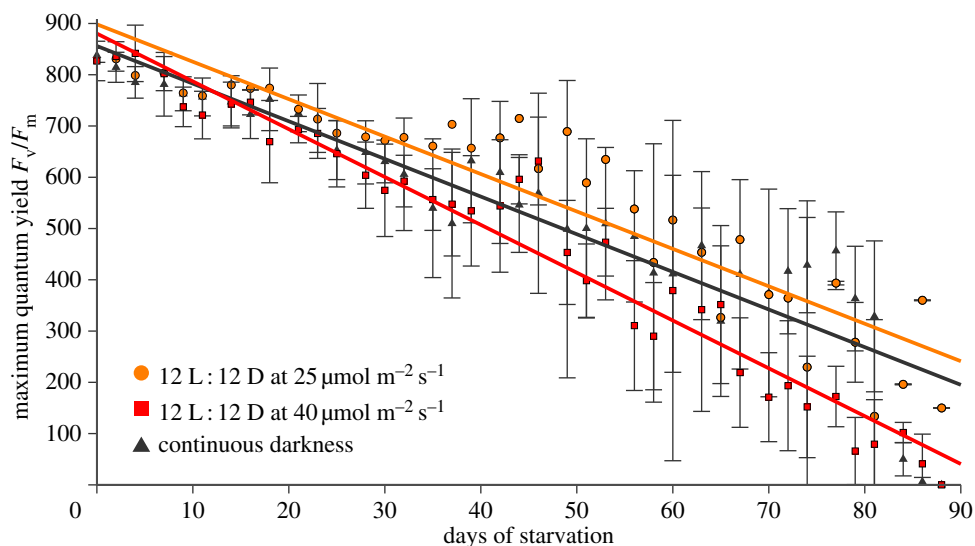
### (b) Blocking photosynthesis affects neither survival rate nor weight decrease during starvation

Earlier work on the LtR species *E. timida* and *P. ocellatus* delivered conflicting results with respect to the role of light during starvation. Some studies indicated that specimens starved in the dark lost weight faster and had a higher death rate than those starved in the light, from which it was concluded that photosynthesis is important for the survival of these LtR slugs [36–38]. However, in those experiments some slugs survived just fine in the dark, and conversely some kept in the light died. In the starvation experiments performed on *E. timida* [36], the survival rate was monitored for only three aquaria, which were chosen apparently randomly from a

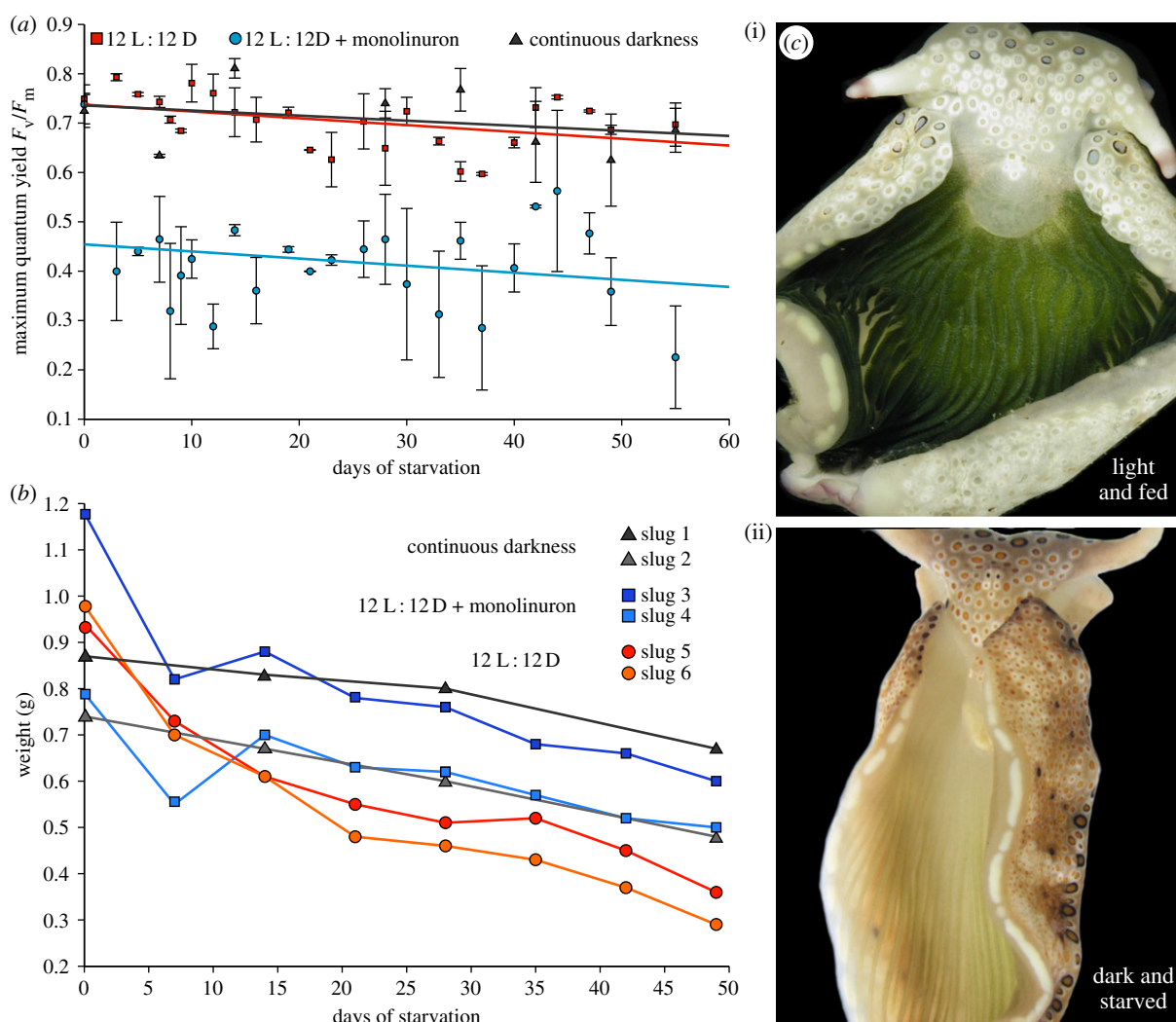
total of nine, and the exact survival rate across all aquaria was not reported. While Yamamoto *et al.* [40] also reported a higher death rate for those slugs kept in the dark, they noted that the higher dark death rate could be attributable to water fouling, as the survival rate even for those kept in the light was very low. From our experience, it is crucial to keep each experimental animal in a separate repository, while at the same time regularly monitoring water quality. In all of our experiments, only one animal (one *E. timida* kept at  $40 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) died on day 23 of starvation.

Recent results reported for *P. ocellatus* indicate that in the wild, the contribution of photosynthesis by sequestered plastids to the animal's carbon uptake is very minor, and raised the question of whether photosynthesis in kleptoplasts contributes significantly to nutrition during starvation [48]. To readdress the role of light, we blocked photosynthesis in two ways: first by simply culturing slugs in the dark and second by inhibiting photosynthesis with monolinuron. We kept six specimens of *E. timida* individually in total darkness over a time course of almost three months. Based on pulse–amplitude–modulation (PAM) fluorescence, the maximum quantum yield was better for the dark-kept animals than for those kept in the light ( $40 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ; figure 3). *Plakobranthus ocellatus* animals were weighed and their plastid photosynthetic capacity measured through PAM. Using  $2 \mu\text{g ml}^{-1}$  of monolinuron, slugs cultivated at  $40 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  revealed an average inhibition of photosynthesis by 42%, as determined by PAM-measurements (figure 4a). Yet, animals cultivated in the presence of monolinuron survived just as well as the control set over the 55 days analysed. Importantly, the control slugs, the monolinuron-treated slugs and those kept in the dark, all showed approximately the same degree of weight loss on day 49 when the experiment was ended (figure 4b).

In our hands, both LtR plakobranchids studied survived equally well in the dark as they did in the light. In fact, for *Plakobranthus*, comparing the individual regressions of weight loss with each other demonstrated that those kept in the dark lost weight the slowest, albeit only slightly slower than those kept in the light, which lost weight the fastest. Weight loss measurements for *E. timida* were unreliable because these animals are small (less than 100 mg each) and handling proved difficult. However, the results clearly indicate that photosynthesis as a core carbon source cannot be essential for slug survival in these two LtR sacoglossan species, because survival was not light-dependent. It remains a possibility that the slugs require specific compounds synthesized in plastids for proper development, for example the synthesis of pyrone-containing propionates [49], in particular because Ireland & Scheuer [30] suggested that a significant part of the fixed  $\text{CO}_2$  might be dedicated to the biosynthesis of pyrone-containing propionates. But as far as basic nutrition goes, the slugs are apparently not 'solar powered' at all. That readily explains why no algal genes essential for photosynthesis are expressed by slug nuclei: the gene products are not required and this is why algal nuclear genes are not required to support kleptoplast longevity [8,24]. Accordingly, recent nuclear genome data of egg DNA of *E. chlorotica* uncovered no evidence for putatively transferred algal genes [50]. Once a nuclear genome becomes available, it will need to provide evidence that genes of algal origin occur at the same copy number in the assembly as a single copy plakobranchid gene.



**Figure 3.** PAM measurements of *E. timida*. The maximum quantum yields of slugs kept in the dark (black) were compared to slugs kept under low- (orange) and high-light (red) conditions. Those kept under high light show the strongest decrease over the three months measured, whereas the linear regression of those kept in the dark runs in parallel to that of those kept under low-light conditions. Six specimens were used for each condition tested. The error bars present the standard deviation.



**Figure 4.** Influence of photosynthesis inhibition on *P. ocellatus*. (a) PAM measurements of monolinuron-treated slugs in a 12 L : 12 D cycle ( $25 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ; blue) in comparison to those kept in the dark (black) and under at a 12 L : 12 D cycle ( $25 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ; red). Two specimens were used for each condition tested and the error bars present the standard deviation. (b) Weight measurements of the *P. ocellatus* specimens shown in (a). (c) Exemplary images of *P. ocellatus* specimens. Image (i) shows a slug kept in the light and which was regularly fed, hence best representing natural conditions. Image (ii) shows a slug after 55 days of starvation in the dark.

The plastids of these LtR species remain capable of photosynthesis, as the PAM and  $^{14}\text{CO}_2$  incorporation results show (figures 3, 4 and 2, respectively), but the observation that light has no detectable effect on animal survival or weight loss during starvation indicates that whatever the plastids do, they do not have a life-extending effect on the animals whose survival does not depend upon plastid photosynthetic activity over the three-month period that we analysed. Photosynthesis in sequestered plastids of LtR species might be important for plastid longevity, but this has yet to be shown. Our experiments measured the survival of the slugs, not the survival of the plastids directly, although the data in figure 4a show that plastids maintained in the dark are, by the measure of PAM fluorescence, just as viable as those maintained under 12 L:12 D. Thus, the plastids of the LtR species *P. ocellatus* and *E. timida* appear to be a source of stored food, and although similar experiments have yet to be reported for the other two LtR species—*E. chlorotica* and *E. crispata*—the implication from our findings is that light is probably not required for long-term survival during starvation of those species either.

If plastid fitness had a direct impact on slug fitness, then blocking photosynthesis, whether through light deprivation or monolinuron, should influence the weight and survival rate of the animals. Yet, those *Plakobranchnus* specimens kept in the dark or treated with monolinuron lost weight at the same rate as the starving control specimens (figure 4b) and the *E. timida* slugs kept in the dark appeared as healthy as the control set, too. Furthermore, the linear regression of the maximum quantum yield of slugs kept in the dark (figure 3) and those treated with monolinuron (figure 4a) runs parallel to those of the control animals, rather than declining more rapidly over time as a proxy of reduced and essential plastid contribution to nutrition. Klochkova *et al.* [39] recently observed that specimens of the StR species *Elysia nigrocapitata* survived for five months without performing photosynthesis, during which time the starved animals dramatically lost weight. Notably, *E. nigrocapitata* animals go from 3 cm in length to 3 mm during starvation, but reversibly, if provided with food [39].

### 3. Conclusion

It has been established that LGT is not involved in kleptoplast maintenance following starvation either in slugs [8,24] or in Foraminifera [51]. The present findings go a step further by showing that sacoglossan slugs survive for months with the help of kleptoplasts, but without the help of photosynthesis. While the plastids are photosynthetically active, they do not confer a photoautotrophic lifestyle upon the slugs. It rather appears that the slugs sequester their plastids not directly as a source of photosynthetic capabilities, but as a source of stored food reserves, whose nutritional value does not depend on light subsequent to sequestration. Plastid longevity in LtR sacoglossan slugs remains an interesting phenomenon, but the present results prompt a shift in emphasis from viewing the kleptoplasts as green solar panels towards viewing them as green food reserves.

### 4. Experimental procedures

*Elysia timida* individuals were collected in Banyuls-sur-Mer (France) between July and September 2012 and transferred

to Bonn (Germany). Specimens were kept with food algae in Petri dishes with artificial seawater (Tropic Marin) at 20°C and water changed every 2 days. For acclimation to laboratory conditions, the slugs were illuminated at 25  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and a 12 L:12 D cycle under a 'day-light lamp' (Androv Medical, model AND1206-CH) for 6 days. Then six individuals of *E. timida* were separately starved in Petri dishes under 25 and 40  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  under a 12 L:12 D cycle and under complete darkness for a maximum of 88 days. Analyses of photosynthetic activity were performed with a pulse-amplitude-modulated fluorometer (Diving PAM, Walz, Germany) by measuring the maximum quantum yield of chlorophyll a fluorescence in photosystem II. Specimens kept under light conditions were dark acclimated for 15 min prior to the measurement.

*Plakobranchnus ocellatus* individuals were collected in the Philippines in November 2012 and transferred to Bonn (Germany). Two aquaria were set up with 20 l artificial seawater (Tropic Marin) at 22°C with two specimens of *P. ocellatus*, respectively, each in individual fishnets. Additionally, two specimens were placed in an aquarium in individual fishnets in 10 l artificial seawater at 22°C. One-third of the water in every aquarium was changed weekly and the best water quality established through the use of an internal filter (Eheim, Germany). To one 20 l aquarium, 2  $\mu\text{g}$  3-(4-Chlorophenyl)-1-Methoxy-1-Methylurea (monolinuron)  $\text{ml}^{-1}$  seawater was added. This and the 10 l aquarium were illuminated at 25  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  under a 12 L:12 D. The second 20 l aquarium was kept in the dark. All specimens were starved for 55 days and afterwards fixed in 4% formaldehyde for further analysis not addressed here. PAM-measurements were taken using a Diving PAM (Walz, Germany). Weights of all six specimens were measured on days 0, 14, 28 and 49 of the experiment by placing the slugs on a spoon, gently removing all remaining water with a paper towel and placing them into a preset water container on a scale. Measurements were taken three times and mean values determined. Data of each trial were pooled. For each individual, the weights were scaled to a maximum of 1 and the linear regression calculated. With  $-0.0059$ , 'dark' had the lowest slope followed by the monolinuron-treated slugs with  $-0.0067$  and the control set with  $-0.0114$ . Using a Tukey test, we tested pairwise whether the slopes of the linear regression lines were equal ( $H_0$ ). The resulting  $q$ -values with a significance level of 0.05 showed a significant difference between 'dark' and 'normal' conditions, while the remaining slopes did not significantly differ ( $p$ -values: control to monolinuron-treated  $\geq 0.0567$ ; monolinuron-treated to darkness  $\geq 0.9193$ ; and darkness to control  $\geq 0.023$ ).

*Elysia timida* used for incubations with [ $^{14}\text{C}$ ]-labelled  $\text{CO}_2$  were collected in the Mediterranean Sea (Banyuls-sur-Mer, France) in October 2012 and transferred to Düsseldorf (Germany). They were maintained for six weeks at 15°C and 33  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  in 12 l-aquaria containing 25 specimens, artificial seawater (37  $\text{g l}^{-1}$  hw-Marinemix professional (Wiegandt, Germany)) and *Acetabularia acetabulum* as food source. They were starved for several days before labelling. To test the light-driven incorporation of  $\text{CO}_2$  by *Elysia*, the slugs were incubated in 1.2 ml artificial seawater supplemented with 0.32 mM [ $^{14}\text{C}$ ]- $\text{NaHCO}_2$  (18  $\mu\text{Ci}$  per incubation, NEN-radiochemicals, MA, USA). For each measurement, four slugs were incubated together in a transparent plastic 1.5 ml tube. *Plakobranchnus ocellatus* individuals used for incubations with [ $^{14}\text{C}$ ]-labelled  $\text{CO}_2$  were collected in the Philippines in

April 2013 and transferred to Bonn (Germany). To test the light-driven incorporation of CO<sub>2</sub>, they were incubated in 5 ml artificial seawater supplemented with 0.16 mM [<sup>14</sup>C]NaHCO<sub>2</sub> (36 µCi per incubation). Each measurement contained one single organism in an 8 ml glass tube. The measurements for time point 120 were carried out twice and the mean values are shown in figure 2. All incubations of both species were performed at room temperature either in the dark or illuminated (72 µmol quanta m<sup>-2</sup> s<sup>-1</sup>). The incubations lasted 2 min, 1 or 2 h, and afterwards the slugs were separated from the radioactive incubation medium, rinsed five times with seawater, and then homogenized in a small glass teflon Potter-Elvehjem tissue grinder in 1 ml (*Elysia*) or 3 ml H<sub>2</sub>O (*Plakobranchnus*). The homogenates of *Elysia* were removed and the Potter tube was rinsed twice with 1 ml H<sub>2</sub>O. These 3 ml, containing the homogenized slugs, were

acidified with 150 µl 1M HCl and the open vial was then shaken overnight to remove all the substrate, that is (labelled) carbon dioxide. Afterwards, incorporation of labelled carbon atoms by the *Elysia* slugs was determined in a scintillation counter after the addition of 12 ml LUMA-Gel scintillation cocktail (LUMAC, The Netherlands). To the homogenates of *Plakobranchnus* 3 ml H<sub>2</sub>O and 300 µl 1M HCl were added, while the rest of the method to measure acid-stable incorporation of carbon dioxide was the same as for *Elysia*.

**Acknowledgement.** We thank Margarete Stracke and Valérie Schmitt for collecting and cultivating animals and algae.

**Funding statement.** This study was financially supported by an ERC grant to W.F.M. and DFG grants to H.W. and S.B.G.

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