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A sea slug's guide to plastid symbiosis

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Abstract

Some 140 years ago sea slugs that contained chlorophyll-pigmented granules similar to those of plants were described. While we now understand that these "green granules" are plastids the slugs sequester from siphonaceous algae upon which they feed, surprisingly little is really known about the molecular details that underlie this one of a kind animal-plastid symbiosis. Kleptoplasts are stored in the cytosol of epithelial cells that form the slug's digestive tubules, and one would guess that the stolen organelles are acquired for their ability to fix carbon, but studies have never really been able to prove that. We also do not know how the organelles are distinguished from the remaining food particles the slugs incorporate with their meal and that include algal mitochondria and nuclei. We know that the ability to store kleptoplasts long-term has evolved only a few times independently among hundreds of sacoglossan species, but we have no idea on what basis. Here we take a closer look at the history of sacoglossan research and discuss recent developments. We argue that, in order to understand what makes this symbiosis work, we will need to focus on the animal's physiology just as much as we need to commence a detailed analysis of the plastids' photobiology. Understanding kleptoplasty in sacoglossan slugs requires an unbiased multidisciplinary approach.

Keywords: kleptoplasty; sacoglossan slugs; photosynthesis; plastid biology; photosynthetic slugs; evolution

Of leaves that crawl

Plastids are the main difference that distinguishes a plant or algal cell from animal cells. However, in 1876 de Negri and de Negri [1] described some green sea slugs to harbor granules that appeared to be stained green through chlorophyll pigments, similar to those of plant and algae plastids. It took almost another century before Kawaguti and Yamasu [2] could demonstrate that the globular chlorophyll bodies were identical to the plastids of the slug's algal food source (Fig. 1). Due to the nature by which the slugs acquire the plastids from their algal food source, these stolen organelles were termed kleptoplasts: stolen plastids. In his work on *Elysia crispata* (at that time known as *Tridachia crispata*), Trench [3] was one of the first to suggest that the slugs might specifically sequester the organelles for their ability to photosynthesize. Trench [3] not only demonstrated the incorporation of $^{14}CO_2$ through the plastids that are embedded within the epithelial cells that form the digestive glandular tubules, but also analyzed the perpetuation of the kleptoplast-slug relationship by separating the slugs from their algal prey. Ever since, starving the slugs has been a common approach to determine the slug's capacity to maintain functional kleptoplasts [4]. Along these lines the presence of photosynthesizing kleptoplasts was generally associated with the ability of some sacoglossans to survive starvation periods that can last many months [5]. This led researchers to coin the term of "leaves that crawl" [6].

In contrast to plants and algae, plastids of slugs are not vertically inherited; kleptoplasts have to be acquired by each new slug generation. Sacoglossan sea slugs have a highly specialized radula that consists of individual, serially organized teeth [7]. Only one tooth is used at a time and, when idle, stored in an autapomorphic structure called "saccus" [8], eponymous for the sacoglossan group. Some slug species can feed only on a single algal species and this might be associated with a specialized radula of mature animals [7]. In other cases, such as *Elysia viridis* that can feed for instance on *Codium* and *Bryopsis*, animals seem to have a more generally adapted radula allowing them to feed on a variety of different species [7]. However, this remains an observed correlation and it is difficult to imagine how one could provide empirical evidence at this point. Feeding experiments in the laboratory alone do not do the trick and radula mutation is far from feasible. Currently we do not know where food source selection actually begins. From what we know it could very well be that the "selective" animals can penetrate different siphonaceous algae and selection occurs downstream, not mechanically (hard and soft or small and large), but biochemically (sweet and sour or fresh and putrid).

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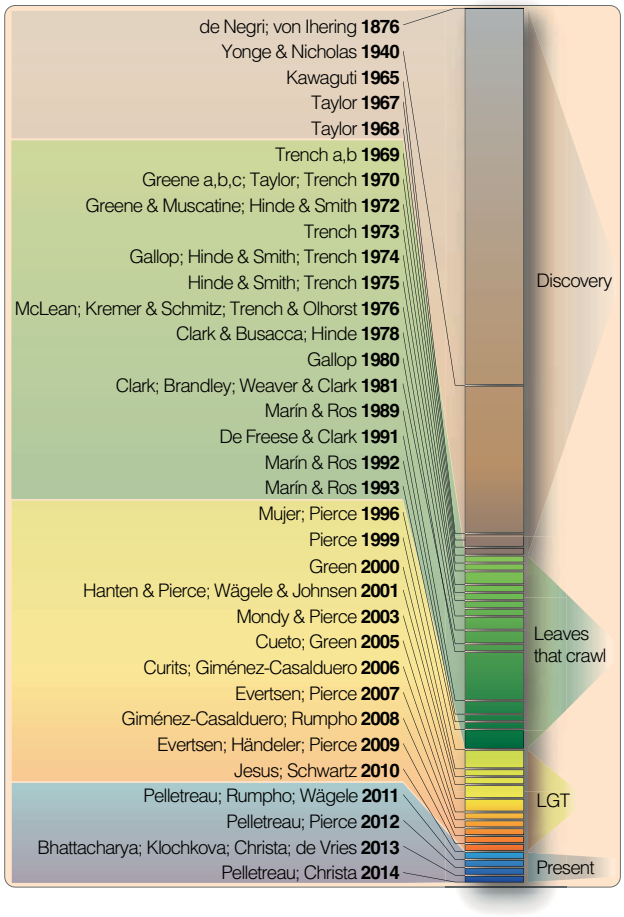

Fig. 1 Overview of about 140 years of research on green sacoglossan slugs. The timeline highlights key publications [1–6,8,10–17,20–23,27,33,40–43,46–49,52,53,56,61,63–94] on sacoglossan slugs since 1876. Four main periods (on the right) can be distinguished: the discovery phase, in which slugs and "chlorophyll-pigmented granules" were morphologically described (**a**), evidence for the incorporation of CO_2 suggested that slugs are "leaves that crawl" (**b**), and at a time where in general more and more gene transfers from one genome to another were identified, the concept was born that lateral gene transfer (LGT) from algae to slugs could support kleptoplasty in sacoglossans (**c**). The LGT concept dominated the field for 15 years until it was challenged for the first time in 2011 [12], which changed the way kleptoplasty in sacoglossan slugs is now viewed and studied. The listed manuscripts all refer to primary data manuscripts except the 1975 review by Trench, in which the term 'leaves that crawl' was coined. If an article has more than two authors, only the first author is listed.

The slugs do not feed on the entire alga, but rather use the radula's tooth to penetrate the cell wall of siphonaceous algae. They then suck out the entire cytosolic content of the algae including the organelles and all other compartments. This is not yet special, but in a few sacoglossan species only the plastids are selectively sequestered from the phagocytosed material. Individual food vacuoles can initially contain several kleptoplasts [9], but these are subsequently released into the cytosol after the vacuole is degraded. Considering that the vast majority of sacoglossans appear to treat incorporated plastids just like any other food particle, we can assume that kleptoplast retention happens on purpose. Still, the molecular mechanism of how plastids are initially recognized and subsequently released into the cytosol remains entirely unknown.

The ability of some kleptoplasts to remain functional inside the animal cells was long spearheaded by the idea that the slugs express genes they obtained through lateral gene transfer (LGT) from the algal nuclei, and which encode proteins that maintain plastid functionality [10,11]. When the first slug transcriptomes emerged that concept was challenged [12,13]. It has been discussed elsewhere in detail why LGT cannot support the stolen plastids [12,14], but, in brief, the reasons are (***i***) the meagre amount of photosynthesis-related transcript identified among slug messenger RNA – "one in a million reads" – and (***ii***) that algal genes have never been identified within in the genomic context of slug nuclear DNA. It now seems that intrinsic properties the stolen plastid bring along render some algal plastids more robust (that is "longer-living" in a foreign environment outside the algal/plant cytosol) than others [15–19]. How exactly is not known, but it is important to note that plastid transcription and translation can continue for months in some species and that their genomic coding capacity varies from that of land plastids [11,17].

The topsy-turvy of functional plastid retention among sacoglossan slugs

Based on pulse amplitude modulation (PAM)-fluorometry, and the determined maximum quantum yield (F_v/F_m) of photosystem II (which is commonly used to determine the photosynthetic capacity of slugs [20]), the majority of sacoglossans do not retain any functional plastids (non-retention species, NR) or for only a few days and up to two weeks (short-term retention species, StR). Seven non-monophyletic species are currently known to retain kleptoplasts with F_v/F_m values that remain on a level that is generally considered to account for the presence of a functional PSII for several months: *Elysia chlorotica*, *E. timida*, *E. crispata*, *E. clarki*, *E. viridis*, *Plakobranchus ocellatus* and *Costasiella ocellifera* [3,4,19,21–25]. All latter species are referred to as long-term retention (LtR) slugs.

But what really makes an LtR species? Genotyping incorporated algal plastids shows that the algal food sources of NR species sometimes matches to those of StR and LtR species [26]. The amount of plastids sequestered and stored in the digestive tubules also does not seemingly differ between long- and short-term retainers (Fig. 2; [19]). To make the matter even more complicated, some LtR species feed on several algae simultaneously [27–29]. What that means is that the food source alone cannot explain the long-term retention of functional kleptoplasts in LtR species. Interestingly though, plastid genome barcoding throughout starvation in the two polyphagous LtR species *E. clarki* and *P. ocellatus* suggest that the speed with which kleptoplasts are digested differs and depends on the plastid source [27,30,31]. It demonstrates that it takes two to tango: the right slug and the right plastid source.

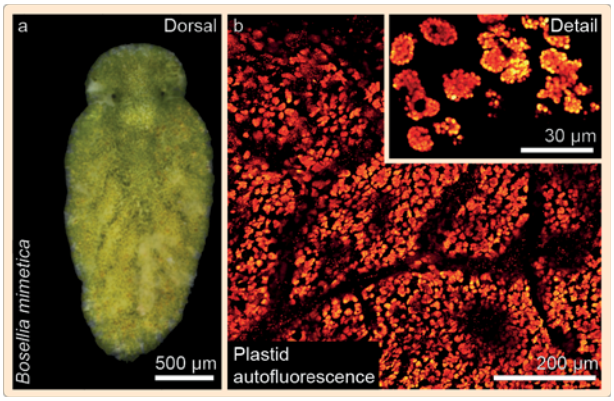

Fig. 2 Quantity does not equal quality. **a** The sacoglossan slug *Bosellia mimetica* harbours numerous kleptoplasts in its digestive tubular network, which (**b**) appear much denser in comparison to some LtR species (see [19,91]). Yet, *B. mimetica* is classified as a StR species; within a few days of starvation F_v/F_m values drop below those that are considered to represent functional photosynthesis [4].

Phylogenetic and photosynthetic analyses recently provided evidence that functional kleptoplasty evolved multiple times within the Plakobranchacea [25,32]. In turn, all basal shelled species are NR forms, but these species do not form a monophyletic group [4,19]. Virtually no molecular study has yet addressed the issue of uncovering the animals' mechanisms that determine the mode of retention. Comparative analyses of different species, in particular the molecular differences between NR, StR and LtR species, represent a promising tool to do so. For example, the trend that NR slugs engulf plastids in phagosomal membranes was noticed before [33,34]. NR species seem to keep the incorporated plastids inside phagosomes for immediate degradation all the time; they do not appear to ever release them into the cytosol [33,34]. StR and LtR forms on the contrary are known to retain kleptoplasts – that are released from phagosomes – in a similar fashion throughout starvation [19]. That is, StR do not appear to degrade kleptoplasts quicker than LtR species, but empirical evidence (for example based on $^{14}CO_2$ incorporation data) is currently lacking. Both, StR and LtR species, should have, in theory, the same "point of departure" when starvation commences and should have the same potential to make use of the kleptoplasts sequestered. Yet they do not. Notably, all LtR species have in common that cytosolic kleptoplast are left surrounded by two membranes only; they are those that constitute the canonical two membranes we are familiar with from land plant plastids [35–37]. This is even true for *E. chlorotica* that feeds on the stramenopile *Vaucheria*, but which houses complex plastids that in the alga are surrounded by four membranes [36,38,39]. That always these two membranes remain – the two that trace back to outer membrane and plasma membrane of the original cyanobacterial endosymbiont [37] – might hint at how, and in fact that, substrate and metabolites are actively exchanged between animal and plastid.

Darkness is more than the absence of photosynthesis

Although it is commonly accepted that the slugs benefit from photosynthesis, direct evidence is surprisingly scarce. Various studies on kleptoplastic slugs have analyzed survival rates or demonstrated that $^{14}CO_2$ is fixed by the acquired plastids [40–45]. To what degree the quantity of such carbon compounds is then physiologically relevant has, however, not yet been satisfactorily shown. Photosynthates sustaining the slugs might not be the sole necessity for sacoglossans to survive (long lasting) starvation periods. Note that for example the NR species *Costasiella nonatoi* survives starvation for about a month without showing any measurable PSII activity [25]. Similar observations have been made for *Elysia nigrocapitata* [46]. The question remains to what degree carbon fixation in sacoglossan slugs is needed to endure starvation (Fig. 3) and whether there is maybe another primary reason for starvation survival. If so, benefiting from functional kleptoplasts comes second.

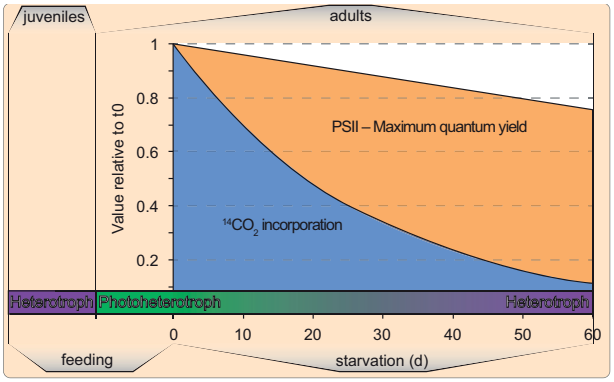

Fig. 3 Schematic overview of kleptoplast performance in LtR species. When the slugs hatch, they immediately need to feed on algal cytosol and begin to sequester their first plastids. During this phase, called transient kleptoplasty, juveniles are basically heterotrophic. Adult slugs are photoheterotrophic animals: they graze upon algae as long as these are available, but at the same time house CO_2-fixing kleptoplasts. When deprived of their food LtR species likely benefit from photosynthesis during the early phases of starvation, but the amount of incorporated carbon cannot sustain the animal. They switch back to heterotrophy through efficiently digesting their own tissue and degrading kleptoplasts. The latter is the reason why all starving LtR species are observed to shrink. Published CO_2 fixation rates for *C. ocellifera*, *E. viridis*, *P. ocellatus* and *E. timida* (blue curve; [14,22,40,43,69]) and photosystem II maximum quantum yield (F_v/F_m) values for *C. ocellifera*, *P. ocellatus* and *E. timida* (orange curve; [14,25]) of various studies on LtR species were pooled and plotted in relation to the values measured for freshly fed animals. The contrast between the two curves highlights that caution is warranted when kleptoplast productivity is evaluated on F_v/F_m values alone.

Previous studies reported the incorporation of photosynthetically fixed carbon (stemming from [^{14}C] bicarbonate) in a variety of slug metabolites [47,48]. Neglecting for now that we do not know enough about what photosynthate

supports the animal (and to what degree), it is important to ask: how do the slugs acquire products of photosynthesis? Kleptoplast-synthesized substrate will inevitably end up being metabolized, no matter whether that substrate was actively provided by an intact kleptoplast or whether it stems from an organelle degraded (e.g. through an autophagosome). It is important to determine which initial route the labeled CO_2 took to be incorporated into slug-specific metabolites. Microscopic analyses of *E. viridis* suggest that kleptoplasts that originate from *Codium fragile* accumulate substrate such as starch during starvation [49,50], which raises the question if any is actively secreted by the stolen organelles. Apart from any energy support the plastids might provide for the slugs, the animals might benefit from various other biochemical pathways the stolen plastids bring along [19,51]. It is also possible that photosynthates and kleptoplast-derived metabolites play a more crucial role for the proper development of juveniles than for the maintenance of mature adult slugs as recently suggested [14]. This is supported by recent observations on juveniles of *E. chlorotica* [52]. The data provided evidence with regard to the need of kleptoplast-derived lipid production for the proper development of the animals.

Animals are often kept in the dark, as a control for the slugs' dependency on photosynthates [52–54]. This is a good time to remember that the absence of light translates into more than just the absence of plastid photosynthesis. A few essential biochemical processes, such as the synthesis of vitamin D [55], occur in a light-dependent manner. Sacoglossans can synthesize a series of unique secondary metabolites including elysiapyrones [56] and tridachione [44] and it has been suggested that their synthesis is light dependent. Their synthesis could still be linked to the presence of kleptoplasts, but not photosynthesis. It has been hypothesized that an essential function of these polypropionates is linked to the quenching of ROS [56,57], hereby protecting the kleptoplasts' photosystems and/or the slugs' own tissue from oxidative stress.

Morphology does not equal function

The lateral foot expansions – the parapodia – are the morphological foundation from which the term "leaves that crawl" originates. In many plastid-bearing slugs the parapodia are intervened with numerous digestive tubules that harbor the green kleptoplasts; the wing-shaped structures are reminiscent of a leaf (Fig. 2). Some studies claim that the opening and closing of the parapodia is a controlled response to different light intensities, to either expose or shield the kleptoplasts to and from sunlight, respectively [58,59]. But these observations are to be interpreted cautiously [60]. There is no question that the position of the parapodia affects the amount of light reaching the kleptoplasts [61], but the response of some species is slow and it takes many minutes for the slugs to close the parapodia. Moreover, the LtR species *P. ocellatus* does not appear to alter the position of its parapodia at all. Even species that completely lack parapodia can retain functional plastids for a long time, as the case of the LtR species *C. ocellifera* demonstrates [25]. Parapodia are furthermore not limited to sacoglossan slugs. A wide range of heterobranchs have evolved parapodia that are used for various purposes including swimming and digging. In a nutshell, there is currently no evidence, or reason to assume, that sacoglossan parapodia have evolved as a consequence of housing kleptoplasts.

Two steps forward, one step back

Sacoglossan sea slugs puzzle researchers, as much as they fascinate. Ed Yong said it best when he commented on a recent analysis, which provided evidence that some adult slug species survive in the dark and do not loose weight faster than those kept in the light [14]: "Good science is about resisting the pull of easy conclusions. It's about testing stories that seem like they should be right to see if they actually are right. This is no easy task. Consider the case of the 'solar-powered' slugs" [62]. Several concepts have been put forward trying to explain how an organelle, adapted to plant cells, remains functional inside the cytosol of a eumetazoan cell. Two key concepts, slugs become photoautotrophs through kleptoplasty and kleptoplasts are supported by laterally transferred genes, are currently challenged [12,14,46], in particular the latter [12,13]. Research on the animal-plastid symbiosis in sacoglossan sea slugs is at a turning point. We know that some slugs sequester plastids through a sophisticated phagocytic mechanism whose details remain currently unknown. The kleptoplasts can continue to photosynthesize in the cytosol of the slugs' epithelial cells, but adults do not strictly depend on ongoing photosynthesis to survive starvation periods long-term. Laterally transferred algal genes do not support stolen plastids: the kleptoplasts' very own

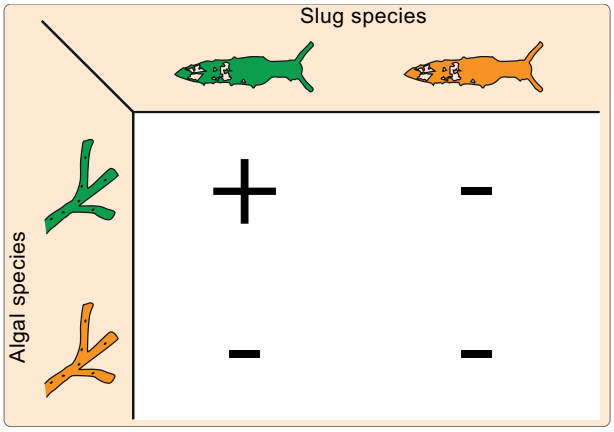

Fig. 4 Animal-plastid compatibility is determined by a two component system. In order to profit from functional kleptoplasts long-term (+), a slug species – component A – with an adapted physiology and digestive mechanism must acquire plastids from an algal species – component B – that naturally has robust plastids (both in green). The multifaceted factors that determine the compatibility of both partners in the plastid-slug system are yet to be discovered, but they could include dealing with plastid-derived toxins in case of the slugs and an adapted photobiology in case of the plastids. If any of the two partners lacks these requirements (orange), only short- or non-functional kleptoplast retentions are established (–).

biochemistry continues to function more independently than previously anticipated. From what we can tell, animal-plastid symbiosis depends on a combination of a robust plastid and a slug species whose physiology has evolved to tolerate (and likely service through substrate and metabolite exchange) an alien organelle (Fig. 4).

From the animals' perspective the following questions are hence key to better understand this unique symbiosis and how functional kleptoplasty has evolved multiple times in different slug species: (***i***) What are the benefits for the slugs next to carbon fixation? (***ii***) What underpins kleptoplast compatibility in the few species so far identified that house functional plastids for months? (***iii***) What are the molecular details of how plastids are recognized by the animals and released into the cytosol? A multidisciplinary approach will contribute to our understanding of how an organelle functions in a cytosol for which it did not evolve, and how some algae plastids have evolved to become as robust as they are. Through recent developments we now have the opportunity to make progress on this unique biological system of general evolutionary interest, for the purpose of better understanding plant animal symbioses.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: all authors have contributed equally to the review of literature. JV and SBG drafted the manuscript, whose final version was written and approved by all four authors.

Competing interests

No competing interests have been declared.

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