Plastid longevity in sacoglossan sea slugs

Inaugural dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

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from Terneuzen

Düsseldorf, April 2018

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Faculty of Mathematics and Natural Sciences at
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Date of the oral examination: 10.04.2018

Statement of declaration

I hereby declare that this dissertation is the result of my own work. No other person's work has been used without due acknowledgment. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.

Düsseldorf, 22.02.2018 Cessa Rauch

In memory of Opa Jaap Wegner,
because thanks to him I could become the Biologist who I want to be..

what else could I have given you,

than what you already had,

you've had it all your life;

a piece of my heart

- René P. Rauch

**15.07.1955 - 31.10.2017†*

Publications included in this thesis

I. de Vries, J., **Rauch, C.**, Christa, G., Gould, S. B. (2014). A sea slug's guide to plastid symbiosis. Acta Soc. Bot. Pol., 83, 415 – 421. doi: 10.5586/asbp.2014.042

II. **Rauch, C.**, de Vries, J., Rommel, S., Rose, L. E., Woehle, C., Christa, G., Laetz, E. M., Wägele, H., Tielens, A. G. M., Nickelson, J., Schumann, T., Jahns, P., Gould, S. B. (2015). Why it is time to look beyond algal genes in photosynthetic slugs. Genome Biol. Evol., 7, 2602 – 2607. doi: 10.1093/gbe/evv173

III. **Rauch, C.**, Jahns, P., Tielens, A. G. M., Gould, S. B., Martin, W. F. (2017). On being the right size as an animal with plastids. Front. Plant Sci., 8, 1402. doi: 10.3389/fpls.2017.01402

IV. **Rauch, C.**, Christa, G., de Vries, J., Woehle, C., Gould, S. B. (2017). Mitochondrial genome assemblies of *Elysia timida* and *Elysia cornigera* and the response of mitochondrion-associated metabolism during starvation. Genome Biol. Evol., 9, 1873 – 1879. doi: 10.1093/gbe/evx129

V. **Rauch, C.**, Tielens, A. G. M., Serôdio, J., Martin, W. F., Christa, G., Gould, S. B. (2018). The ability to incorporate functional plastids by the sea slug *Elysia viridis* is governed by its food source. Mar. Biol., 165, 82. doi: 10.1007/s00227-018-3329-8

Other publications (not in this thesis)

VI. Zimorski, V., **Rauch, C.**, van Hellemond, J. J., Tielens, A. G. M., Martin, W. F. (2017). The Mitochondrion of *Euglena gracilis*. In Euglena: Biochemistry, Cell and Molecular Biology, 19 – 37, Springer International Publishing Berlin, Germany.

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1. Abstract

Some species of marine gastropods within the Sacoglossa family are the only Metazoa known to sequester active plastids (called kleptoplasts) from algae into their own cells. These 'green slugs' were discovered some 140 years ago and later described as 'leaves that crawl' due to their leaf-like appearance and photosynthetically active kleptoplasts. Science was unsuccessful in explaining the true contribution and benefits of functional kleptoplasty within Sacoglossa. Therefore, this dissertation tries to explain (i) what are the contributions of the slugs and kleptoplasts to maintain kleptoplasts photosynthetically active for months, separated from algal nuclear genes? And (ii) what benefit do slugs truly reap from kleptoplasts?

One topic that received a lot of attention, also in the popular press, was the presumed presence of laterally transferred genes from algae to slug (LGT). LGT was considered as the cause for autonomous kleptoplast functionality within the slugs' cells, despite the absence of algae nuclei. Many manuscripts were published that dealt with what genes had been transferred and what the frequency might be. However, as described in this dissertation, later work has proved beyond doubt that the reads for genes of algae origin were founded upon RNA and DNA remaining from the original food source. In recent years, the idea of LGT in slugs to explain kleptoplast longevity has been replaced with the idea of a more general 'algae plastid robustness' — some isolated algal plastids are able to continue to fix CO_2 much longer than plastids isolated from land plants.

Various studies measured CO_2 fixation of the slugs and upon starvation in all cases the levels of CO_2 fixed decreased. In Rauch et al. 2017b it was calculated how much the slugs truly benefit from ongoing CO_2 fixation by their kleptoplasts. This depends on the developmental time, size, weight and carbon turnover time of the slugs. It turned out that, for the species *Elysia timida*, a mere 0.25% of the slugs carbon stems from the kleptoplasts' CO_2 fixation. Which explains the slugs decrease in body size and loss of weight during starvation, despite presence of functional kleptoplasts.

This independency on kleptoplast CO_2 fixation was confirmed by feeding the polyphagous sea slug *Elysia viridis* with an algal species from which no kleptoplasts are incorporated — it had no influence on the slugs' survival rate during starvation. It seems that the actual contribution of kleptoplast photosynthesis to the energy demand

of the slugs is not a prerequisite to survive periods of starvation. However, this does not imply that kleptoplasts are not somehow beneficial for the slugs. In Rauch et al. 2017b available RNA-seq data of the two congenic slug species *Elysia cornigera* and *Elysia timida* was analysed. The findings revealed how the oxidative phosphorylation pathways are notably upregulated during starvation in *E. timida*. With these results, it is hypothesized how the kleptoplasts could act as alternative providers of reducing equivalents to prolong the slugs' survival from cellular starvation. It seems that the actual contribution of kleptoplast photosynthesis to the energy demand of the slugs is not a prerequisite to survive periods of starvation.

The dissertation at hand offers new insights into the interplay of kleptoplasts and slugs and provides new avenues from which one can explore Sacoglossa in the future.

2. Zusammenfassung

Einige Arten von Meeresschnecken innerhalb der Familie Sacoglossa sind die einzigen bekannten Metazoen, die in der Lage sind, funktionsfähige, aktive Plastiden aus ihrer Algennahrung zu isolieren und in ihre eigenen Zellen zu integrieren (Kleptoplasten). Diese grünen Nacktschnecken wurden vor etwa 140 Jahren entdeckt und später aufgrund ihres blattartigen Aussehens und ihren photosynthetisch aktiven Kleptoplasten als "leaves that crawl" beschrieben. Es war bislang noch nicht möglich, den wahren Beitrag und den Nutzen der funktionalen Kleptoplasten innerhalb der Sacoglossa zu entschlüsseln. Daher versucht diese Dissertation zu erklären, was (i) die Beiträge der Schnecken und Kleptoplasten sind, um die Kleptoplasten auch getrennt vom Zellkern der Algen über Monate hinweg photosynthetisch aktiv zu halten und (ii) welchen Nutzen die Schnecken wirklich aus den Kleptoplasten ziehen.

Ein möglicher Grund, der viel Aufmerksamkeit, auch in der Presse, erzielte, war ein mutmaßliches Vorhandensein lateral von der Alge zur Schnecke transferierter Gene. Der laterale Gentransfer (LGT) wurde als Ursache dafür angesehen, dass der Kleptoplast trotz Abwesenheit der Zellkerne der Algen über einen langen Zeitraum funktionstüchtig blieb. Über Jahrzehnte wurden viele Manuskripte veröffentlicht, die sich damit beschäftigten, welche Gene übertragen wurden und wie hoch die Häufigkeit der Übertragungen sein könnte. Eine Arbeit dieser kumulativen Dissertation hat jedoch zweifelsfrei gezeigt, dass die Lesevorgänge für Gene, die aus Algen stammen, auf RNA und DNA beruhten, die von der ursprünglichen Nahrungsquelle übriggeblieben sind (neben möglicher Kontamination). In den letzten Jahren wurde die Idee des LGT in Schnecken zur Erklärung der Langlebigkeit von Kleptoplasten durch die Idee einer allgemeineren "Algenplastid-Robustheit" ersetzt – einige aus Algen isolierte Plastiden können CO_2 länger fixieren als Plastiden, die aus Landpflanzen isoliert wurden.

In verschiedenen Studien wurde die CO_2-Fixierung der Nacktschnecken gemessen und bei Hunger war die Menge an fixiertem CO_2 in allen Fällen verringert. Wie stark die Schnecken von der fortlaufenden CO_2-Fixierung durch ihre Kleptoplasten wirklich profitieren, wurde in einer weiteren Arbeit dieser Dissertation untersucht. Dieses hängt von der Entwicklungszeit, der Größe, dem Gewicht und der Kohlenstoffumsatzzeit der

Schnecken ab. Es stellte sich heraus, dass für die Art *Elysia timida* nur 0,25% des Kohlenstoffs der Schnecken aus der CO_2-Fixierung der Kleptoplasten stammen, was die Abnahme der Körpergröße und den Gewichtsverlust trotz der Anwesenheit von funktionalen Kleptoplasten während des Hungers erklärt.

Dass die Schnecken nicht von der CO_2-Fixierung der Kleptoplasten abhängig sind, wurde durch Fütterungen der polyphagen Schnecke *Elysia viridis* mit einer Algenart bestätigt, aus der die Schnecken keine Kleptoplasten erlangen können – die Überlebensrate der Schnecken während des Hungerns unterschied sich nicht von der Schnecken mit Kleptoplasten. Es scheint, dass der tatsächliche Beitrag der Kleptoplast-Photosynthese zum Energiebedarf der Nacktschnecken keine Voraussetzung für das Überleben von Hungerperioden ist. Dies impliziert jedoch nicht, dass Kleptoplasten nicht auf irgendeine Art für die Nacktschnecken vorteilhaft sind. In einer weiteren dieser Dissertation zugrundeliegenden Arbeit wurden RNA-Seq-Daten der beiden kongenen Schneckenarten *Elysia conigera* und *Elysia timida* analysiert. Die Ergebnisse zeigen, dass die oxidativen Phosphorylierungswege während des Hungerns in *E. timida* bemerkenswert hoch reguliert sind. Dieses ließ Hypothesen zu, dass die Kleptoplasten als alternativer Anbieter von Reduktionsäquivalenten agieren können, um das Überleben durch zellulären Hunger zu verlängern.

Die vorliegende Arbeit bietet neue Einblicke in das Zusammenspiel von Kleptoplasten und Nacktschnecken und eröffnet neue Wege, um Sacoglossa in Zukunft zu erkunden.

3. Introduction

3.1 Animals with plastids

There are only a few things in the world of biology that spark the interest of not only scientists from other disciplines but also the broader public. One of those examples are photosynthetic sea slugs. Some 140 years ago, these remarkable animals were first described by de Negri and de Negri (1876), who discovered that these sea slugs were green coloured due to foreign 'bodies' that were reminiscent to those known from plants. Roughly 100 years later, in 1965, the "*granule bodies*" were identified to be plastids from the algae the slugs feed on (Kawaguti and Yamasu 1965).

Initially, this remarkable association was described as a symbiosis (Kawaguti and Yamasu 1965). When organisms have a symbiosis with phototrophic algae, the individual species profit from this relationship. This phenomenon is not uncommon and many marine examples are known: anemones and dinoflagellates (La Jeunesse and Trench 2000), corals and Zooxanthellae (Little et al. 2004), jellyfish and Symbiodinium (like *Cassiopea*; Mellas et al. 2014), or molluscs (like *Pteraeolidia ianthina*; Hoegh-Guldberg and Hinde 1986 and the giant clam *Tridacnidae*; Klumpp and Griffiths 1994), and sponges (Frost and Williamson 1980). One rare example among vertebrates is the Mole salamander, in which the eggs of the animals contain many algae (Graham et al. 2013; Graham et al. 2014), but it remains unclear what the true benefits from this complex relationship are (Burns et al. 2017).

These previous examples clarify that the special ability of the slugs of harbouring green plastids is not to be confused with symbioses. The slugs specifically *steal* individual plastids from their algae food source, which is why this ability is termed kleptoplasty (Trench 1969). After sequestration by the gut cells of the slug, the stolen plastids (then called kleptoplasts) remain photosynthetically active, that is they continue to fix CO_2 in a light-dependent manner (functional kleptoplasty; figure 3.1). Händeler and colleagues (2009) described for the first time three levels of photosynthetic activity of the kleptoplasts based on Pulse Amplitude Modulation (PAM) measurements. There is no functional retention (non-retention or NR), short-term retention (StR; kleptoplasts are active for about one week) and long-term retention (LtR) in which the kleptoplasts stay active for over a month (Händeler et al. 2009).

The sacoglossan slugs are the most famous example that executes kleptoplasty, as they are the only Metazoa able to do so, but they are much certainly not the only organisms. The ciliate *Myrionecta rubra* steels its plastids from the Geminigera clade, a cryptomonad (Johnson 2011). Another example of kleptoplasty is within some species of dinoflagellates; *Nusuttodinium sp. (= Gymnodiunium)* (Onuma and Horiguchi 2015) and *Pfiesteria piscicida* (Lewitus et al. 1999) and *Dinophysis* sp. (Minnhagen et al. 2011). And some species of foraminifera like the genera *Bulimina* (Bernhard and Bowser 1999), *Elphidium* (Pillet et al. 2011), *Haynesina* (Pillet et al. 2011; Jauffrais et al. 2016), *Nonionella* (Grzymski et al. 2002), *Nonionellina*, *Nonion*, *Reophax* and *Stainforthia*, which steal the plastids of different diatom species (Bernhard and Bowser 1999). These examples of unicellular organisms show the unique case dealt with when it comes down to plastid stealing multicellular slugs.

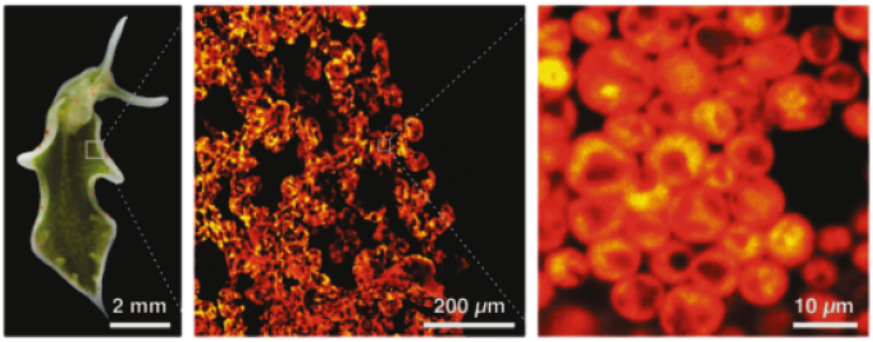

Figure 3.1. Chlorophyll auto-fluorescence of the functional kleptoplasts in the sea slugs *Elysia timida* (modified with permission after Rauch et al. 2015). The hot-red colouration indicates individual kleptoplasts that are located in the cells of the digestive tract of the slug, where they can stay photosynthetically active for weeks.

3.2 Sap-Sucking Slugs

3.2.1 Taxonomy

The sea slugs' ability of kleptoplasty is found across a variety of species of the same clade called the Sacoglossa (figure 3.2). The Sacoglossa consists of about 250–300 (Jensen 1997) described species and are part of the Opisthobranchia (Heterobranchia, or different gilled). They are roughly divided into two main morphologically distinct

classes; the shelled, or Oxynoacea and the shell-less, or Plakobranchacea. Oxynoacea consists of the families Volvatellidae, Juliidae, and Oxynoidae and Plakobranchacea consists of Plakobranchidae, Boselliidae, Platyhedylidae, Polybranchiidae, Hermaeidae, and Limapontiidae (Jensen 1996; Maeda et al. 2010). The Oxynoacea form the base of the tree and its sister taxon, the Plakobranchacea, covers the subclasses Limapontioidea and the Plakobranchoidea (de Vries 2013; Jensen 1996; Händeler and Wägele 2007).

Jensen (1997) pioneered the reconstruction of the Sacoglossa phylogeny on genus level based on morphological characters (Hennigian principles) (Jensen 1997). Later, in 2007, Händeler and Wägele published the first Sacoglossa phylogeny based on 16S rRNA (Händeler and Wägele 2007). Both phylogenies had *Cylindrobulla beauii* on the base of the tree with right next to it the Oxynoacea. Major differences between the two trees occur within the Oxynoacea and Limapontioidea (Händeler and Wägele 2007). However, they describe the phylogeny analyses as preliminary mainly because of the use of only one gene. Three years later, Maeda and colleagues published an updated sacoglossan phylogeny based on molecular markers (18S rRNA, mitochondrial CO1, trnV and 16S rRNA) (Maeda et al. 2010). Here they also discuss the gain and loss of kleptoplasty in order to reconstruct the appearance of this trait within some Sacoglossa species.

Only about 75 species out of 250–300 described species (Jensen 1997; de Vries et al. 2014a) dispersed throughout the Sacoglossa clade are able to perform kleptoplasty and even fewer of those are able to perform functional kleptoplasty (Maeda et al. 2010). According to Maeda et al. 2010 functional kleptoplasty was either lost (Volvatellidae) or gained (Plakobranchacea) within the phylogenetic tree. However, in a more recent study of Christa and colleagues (2014a) it is suggested that functional kleptoplasty probably evolved multiple times independently (two times for short-term retention and at least five times for long-term retention (LtR)) and is therefore considered a non-monophyletic trait (Christa et al. 2014a). That functional kleptoplasty evolved multiple times makes it very difficult to explain its presence and the possible benefits even more. According to this model, many species that did not evolve the trait are as successful in proliferating compared to species with functional kleptoplasty (figure 3.2).

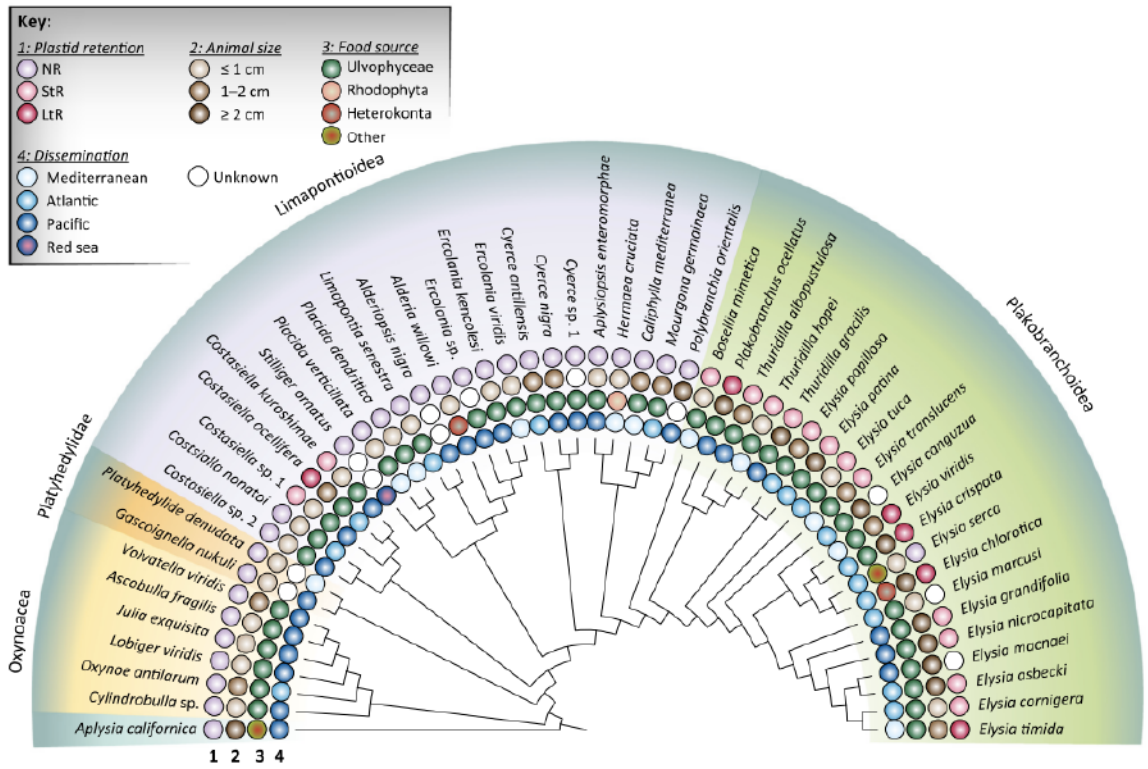

Figure 3.2. Phylogenetic tree of the Sacoglossa (de Vries et al. 2014a). A reconstruction of the phylogenetic tree from the Sacoglossa published by de Vries and colleagues in 2014(a). With clearly the Oxynoceae positioned at the base of the tree, followed by the Plathylidea and the two larger presented Limapotonidea and Plakobranchoidea that also include the most LtR species (red dots). The tree also represents the diversity of the group with regard to habitat, sizes and food sources (which will be explained in more detail in the following chapters).

3.2.2 Description

Sacoglossa are known as the *sap-sucking* slugs because of the way they eat their algae; they pinch a hole in the algal cell wall and *suck* out the cytosolic content. In order to do so, they are equipped with a special tooth-like organ: the radulae (figure 3.3). The teeth are uniseriate and only the leading tooth is being used (Gascoigne and Sartory 1974). The mechanisms are similar to the revolver teeth of a shark; it continues growing and replaces the older teeth when necessary. Other reports mentioned morphological adaptations to the food source for some species that feed on a variety of algae, like *Elysia viridis* (Jensen 1993; Rauch et al. 2018). However, unlike other Opisthobranchia, the used teeth are being kept during the slugs' lifetime in a dedicated structure called

the saccus (or ascus). This structure is what makes the Sacoglossa unique (Gascoigne and Sartory 1974; Jensen 1993).

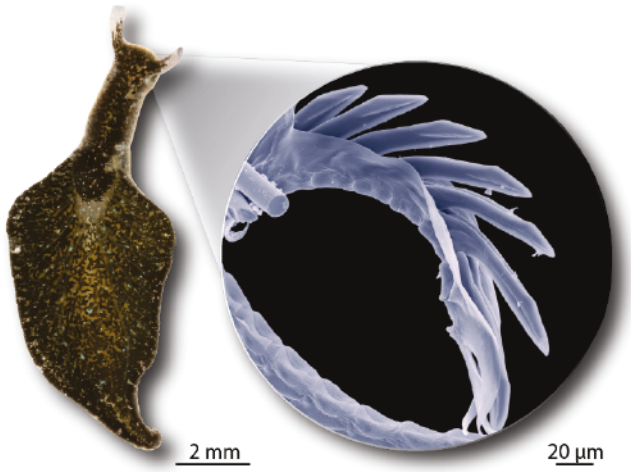

Figure 3.3. *Elysia viridis* with zoomed in panel of its radula (modified with permission after Rauch et al. 2017b*).* *E. virids* (left), like other Sacoglossa, uses its special uniserate radula (right) for pinching the algal cell wall.

Sacoglossan species are overall tiny, often less than 100 mm, with a few exceptions larger than 200 mm (Clark 1994; de Vries et al. 2014a). Besides being small, they are also often very well camouflaged when on their algae food source. Both Limapontioidea and Plakobranchoidea consist of species that contain either cerata (which are the lateral appendices dorsal of the body) or parapodia (the lateral appendices of the foot of the animal; figure 3.4) for increasing surface area in order to diffuse oxygen from the water. They do not have external gills (like Nudibranchia) or 'half' internal gills (like the sea hare *Aplysia* sp. (Leonard et al. 1989)). Neither species within the Oxynoacea nor species of the Limapontioidea have the ability to retain functional plastids (with exception of the genus *Costasiella*), which makes the species within the Plakobranchoidae an exception to the rule. This is something many researchers seem to overlook when studying photosynthetic sea slugs; the number of slug species able to perform functional kleptoplasty (75 described species) is by far outnumbered by the ones who do not perform functional kleptoplasty (roughly 250 described species) (Jensen 1997; de Vries et al. 2014a).

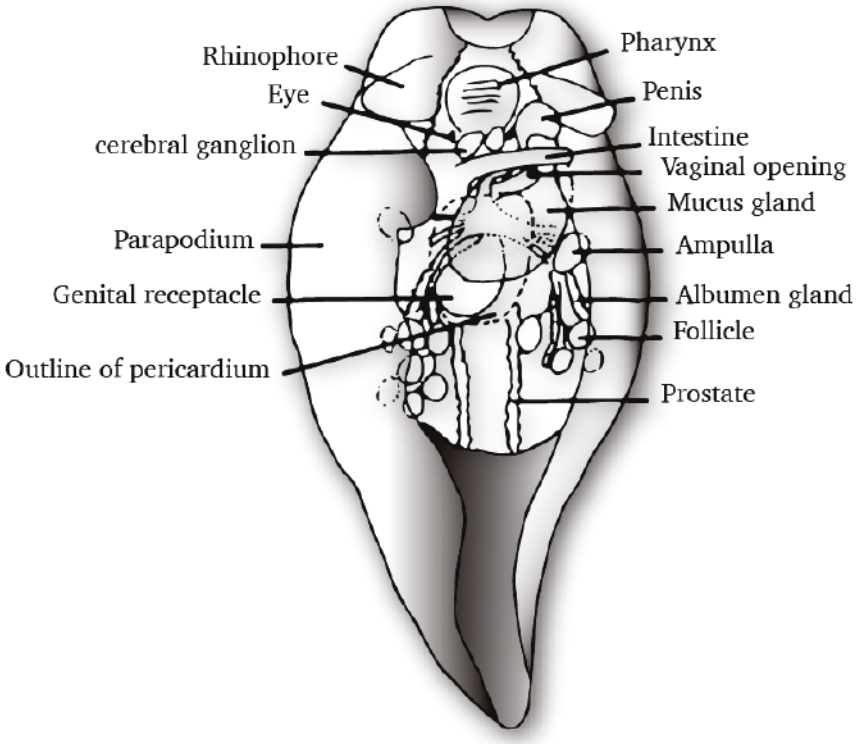

Figure 3.4. Basic anatomy of the sacoglossan *Elysia australis* (modified with permission after Jensen 1992). This basic body plan represents many of the Elysiidae. *E. australis* belongs to the Plankobranchoidea and is one of the species that is able to perform functional kleptoplasty (Evertsen et al. 2007).

3.2.3 Distribution

Sacoglossa are a very diverse species, especially when it comes to its distribution, which is, with exceptions of the arctic regions, worldwide (Jensen 2007). Particularly high diversity can be found around areas where their food source is abundant, like the Indo-pacific and the Caribbean, and generally not deeper than the algae growth zone of less than 100m (Jensen 2007). Furthermore, the highest species diversity of cold to temperate regions is in South-Eastern Australia and Japan (Jensen 2007). Although Jensen (2007) gave a very detailed overview of the Biogeography of the Sacoglossa, it is still questionable what the true distribution is; as such data is dependent on the collection sites of researchers.

The evolution of the trait of kleptoplasty within Sacoglossa is often explained by their habitat; some studies suggest that starvation tolerance and the ability of keeping

functional kleptoplasts could be correlated to seasonal abundance of their algae food source (Cruz et al. 2013; Wägele and Martin 2013; de Vries et al. 2015; Rauch et al. 2018). One of those examples is the comparison of two sister species *Elysia cornigera* with *Eylsia timida*, which both feed on the algae *Acetabularia,* however, only *E. timida* is able to keep the kleptoplasts long-term (de Vries et al. 2015). This could be explained because *E. timida* lives in the Mediterranean were the *Acetabularia* grows seasonal (Marín and Ros 1992) (figure 3.5). However, at the same time, species as *Costasiella ocellifera* and *Elysia crispata* have food source availability throughout the year and are still measured as long-term retention species (Christa et al. 2015).

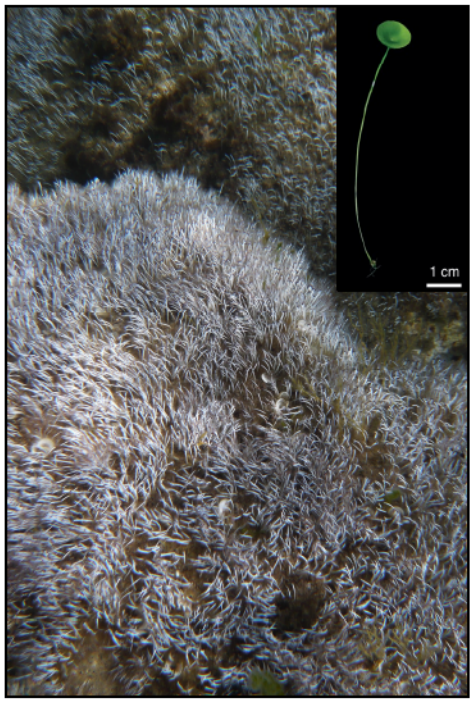

Figure 3.5. Fields of calcified *Acetabularia acetabulum* on the coast of the Mediterranean island Elba, Italy and a single decalcified specimen (upper right corner; modified with permission after de Vries et al. 2013). A field of *A. acetabulum* photographed by the author just after a cold winter season, in April 2015. On the picture, it is clearly visible how the algae are without reproductive caps and completely calcified (identified by the white colouration) which makes it impossible for the sacoglossan *Elysia timida* to feed on it. Therefore, the animals are forced into starvation for several months until the algae go into a new cycle with fresh reproductive caps and decalcified stalks (example in the upper right corner) (Marín and Ros 1992; de Vries et al. 2013).

3.2.4 Behaviour

Sacoglossan feeding behaviour and food preferences are very strongly linked to the division of the genera. None of the Oxynoacea (shelled Sacoglossa) feeds on anything else than the algae genus *Caulerpa* (Jensen 1997). The rest of the family tree has a more diverse diet, but it concentrates mainly on septate and siphonaceous green algae (Ulvophyceae). Because Oxynocea forms the base of the phylogeny of the Sacoglossa (see chapter 3.2.1 of this dissertation), its food *Caulerpa* is seen as the ancestral food

source. It suggests that from there the slugs radiated their specialisms by adaptation to other algae food sources (Jensen 1997).

Most Sacoglossa are specialists when it comes to their diets. For example, the species *Elysia timida* is known to feed only on the green siphonaceous algae *Acetabularia acetabulum*. And only a few other species are known to feed on other algae species besides the Ulvophyceae; *Elysia chlorotica* feeds on the heterokont algae *Vaucheria litorea* (Rumpho et al. 2000), *Elysia serca* feeds on certain species of seagrasses (Jensen 1982; Jensen 1993) and *Hermaea bifida* feeds on Rhodophyta (figure 3.6; Christa et al. 2014a). However, some reports describe a more general feeding behaviour in which a single slug species is able to feed on several species of algae within the same genus (polyphagous) (Jensen 1983, Jensen 1991, Trowbridge 1991). Examples of this are the species *Elysia viridis*, *Elysia crispata*, *Elysia clarki* and *Plakobranchus ocellatus* that are able to feed on multiple algae (Händeler et al. 2010; Christa et al. 2014a; Rauch et al. 2018).

With regard to functional kleptoplasty, there seems to be no consistency in the algae groups. Genetic barcoding experiments tried to categorize the different retention forms regarding to their algae food source, but it is completely indiscriminate (figure 3.6; Christa et al. 2014a). De Vries and colleagues (2015) used the sister species *Elysia cornigera* and *Elysia timida* as their model system, because they feed on the same algae species (*Acetabularia acetabulum*) and yet, they have completely different retention forms; short and long-term retention respectively (de Vries et al. 2015). Their conclusion was having functional kleptoplasts long-term is not necessarily correlated with the algae food source, but has more to do whether the species is able to cope with the stress that comes with starvation (de Vries et al. 2015).

But if the algae were of such minimum importance, why is it that feeding experiments with *E. viridis* indicated different results? The less picky feeder, *E. viridis*, feeds on different algae, i.a. *Bryopsis hypnoides* and *Cladophora* sp. However, when fed on Bryopsis, functional kleptoplasts are measured for several days in a row but when fed on *Cladophora* sp. there is no photosynthetic activity measured at all (Rauch et al. 2018). Taken together it seems that the functionality of the kleptoplasts is not decided by merely the algae source.

Another possibility is that keeping active kleptoplasts could be an inexpensive way of camouflage against predators. Some species of the Sacoglossa are extremely cryptic and by incorporating functional kleptoplasts they become even more obscure (Trowbridge 1994; Rumpho et al. 2000; Wägele 2004). Being green, on a green background is even for human eyes hard to detect. *Bosellia mimetica* (hence the name) has taken this to its full advantage by having its flat body pressed against the flat *Halimeda tuna* blades it eats from (Cimino and Ghiselin 1998; Marín and Ros 2004).

Some studies also suggest that the kleptoplasts could function as a defence against predation; by means of secondary metabolites from the kleptoplasts (Jensen 1994; Jensen 1997; Cimino and Ghiselin 1998; Marín and Ros 2004). But it is difficult to support these theories, not to the least extent because natural predation on the slugs is very rarely reported in the literature. Some field observations suggested that crabs, some fish (Trowbridge 1994), and even a mushroom coral (figure 3.7) are predating on the slugs (Mehrotra et al. 2015). But the actual evidence for consumption remains undetected; the slugs do not have any hard tissue that can remain recognizable in the stomach of its predators for proper identification.

Like all other opisthobranchs, Sacoglossa are hermaphrodites, and while mating, both partners can simultaneously – often in competition with each other – fertilize and be fertilized (Schmitt et al. 2007). Adults lay their eggs in a string, resembling a somewhat circular shape, mostly on their algae food source. Depending on the species, the egg hatchlings are either miniature representatives of their adult parents (juveniles) or are planktotrophic larvae (Trowbridge 2000; Gianguzza et al. 2005). The larvae have an alternative food source before it makes the final transition to algae and it is not until then, that the slugs start to incorporate kleptoplasts (Trowbridge 2000; Schmitt et al. 2014). These complex life cycles can make culturing the slugs particularly difficult (Gianguzza et al. 2005). Culturing the slugs in the laboratory is often necessary for controlled experiments. A lot of the studies conducted were based on slugs collected from the wild, which can come with complications. But fully closed laboratory cultured slugs, do not always represent the natural state, which can lead to wrong conclusions (e.g. feeding experiments with algae food sources the slugs do not encounter in their natural habitat). It is a discussion worth to keep in mind.

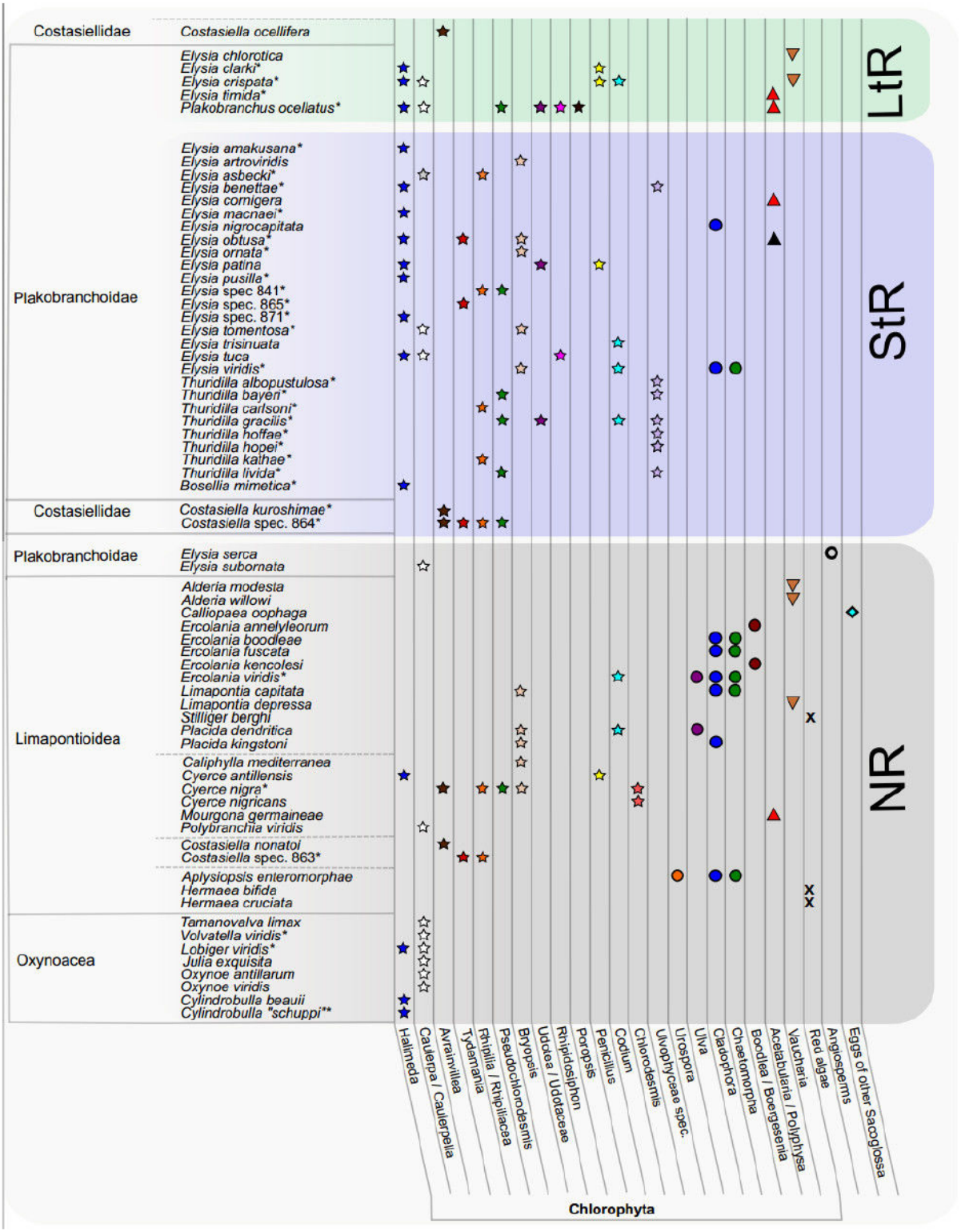

Figure 3.6. Overview of the food sources of Sacoglossa accompanied by their retention forms (Christa et al. 2014a). LtR = Long-term retention, StR = Short-term retention, NR = No retention.

Figure 3.7. Field observation of sacoglossan predation by a mushroom coral (modified with permission after Mehrotra et al. 2015). The mushroom coral *Pleuractis paumotensis* is observed ingesting the Sacoglossa *Plakobranchus* sp. at Koh Tao, Thailand.

3.3 The Science of Sacoglossa

3.3.1. Bird's-eye view of sacoglossan research

De Negri and de Negri (1876) were de first to describe that the Sacoglossa *Elysia viridis* harbours green bodies like that of plants. But it took another 100 years before Kawaguti and Yamasu (1965) with the help of electron microscopy could describe these green bodies as plastids. 1965 basically marked the beginning of scientific research of Sacoglossa, with special emphasis on the ones that perform functional kleptoplasty. The history of sacoglossan research marked four different periods, described in de Vries et al. 2014: the discovery of the slugs, the kleptoplasts fix CO_2 ('leaves that crawl'), the hunt for laterally transferred genes from algae to slug (lateral gene transfer; LGT) and present-day studies (figure 1 of de Vries et al. 2014).

Trench was the first one that counted radioactive labelled carbon in the slugs' tissue back in 1969 and started starvation experiments as a way to measure the kleptoplasts' contribution to the slug (Trench 1969). Since then, throughout the seventies till the nineties, it was applied almost like a standard when studying photosynthetic sea slugs (Trench 1969; Hinde and Smith 1972; Trench et al. 1973; Hinde and Smith 1975; Gallop et al. 1980; Marín and Ros 1992).

When taken a closer look to the techniques used throughout these years, there is a sudden shift from CO_2 fixation experiments to the introduction of Pulse Amplitude Modulation (PAM) measurements and manuscripts publishing about slug LGT (halfway the nineties; see table 1 of Rauch et al. 2017a). Suddenly the photosynthetic activity of the kleptoplasts alone was enough proof that the slugs are able to live from photosynthates produced by the kleptoplasts (Wägele and Johnsen 2001; Laetz et al. 2017a,b). These claims are often made without the necessary support (CO_2 fixation measurements and photosynthesis inhibition) (Cartaxana et al. 2017; Laetz et al. 2017a,b). Some of these studies were published synchronise with LGT manuscripts; the question was not anymore *what* the contribution of the kleptoplasts was (if any at all), one assumed it. This made the focus shift to the issue of *how* the kleptoplasts were kept transcriptionally active in the slugs (Pierce et al. 2009; Rumpho et al 2009; Schwartz et al. 2010; Pelletreau et al. 2011). And throughout the nineties and earlier two thousands, many conflicting reports are published, which weakened the LGT theory. At long last, lack of strong evidence finally made more and more studies vacate LGT theories altogether.

Recently, research in Sacoglossa has changed and is currently at a turning point. Newly published studies compared species such as the congenic sea slugs *Elysia timida* and *Elysia cornigera* (de Vries et al. 2015; Rauch et al. 2017b; Laetz et al. 2017a,b) and once again performed starvation experiments with the slugs, with controls by blocking photosynthesis and measuring CO_2 fixation (Christa et al. 2014b; de Vries et al. 2015; Rauch et al. 2018).

All studies previously done on Sacoglossa show one thing; that studying these green sea slugs has many challenges. Not rarely because of small sample sizes or inter- and intra-species differences. However, these challenges should not translate into conflicting data because of easily pulled conclusions. Currently there is still a lot to be done and this dissertation will tackle down some of the present-day questions.

3.3.2. The aims of this dissertation

In this dissertation some of the present-day questions about Sacoglossa and their ability of retaining kleptoplasts are being challenged. In order to find cues for why some species of slugs are able to retain kleptoplasts for a longer time than others, the gene expression of different species was compared and analysed. Furthermore, in order to understand the benefits the slugs gain from their kleptoplasts, radiolabelled carbon fixated by the kleptoplasts was measured and slugs fed on different diets were being starved and observed. A variety of slug species, kleptoplast sources, different molecular, radiolabelling, photobiology and observational techniques were used in order answer the following objectives:

Objective (i) *What keeps kleptoplasts functional?*
What are the slugs and kleptoplasts contributions to maintain kleptoplasts photosynthetically active for months of separation from algal nuclear genes?

Objective (ii) What benefit do slugs truly reap from kleptoplasts?
When the slugs incorporate kleptoplasts, do they have better survival rates than slugs that do not incorporate kleptoplasts?

Publication I: A sea slug's guide to plastid symbioses

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The presented manuscript was peer reviewed and published in the Journal 'Acta Societatis Botanicorum Poloniae' with impact factor 1.4, on 31 December 2014.

Contribution as second author minor: Contributed to the review of the literature and reviewed and approved the final version.

Official publication of the Polish Botanical Society

Acta Societatis Botanicorum Poloniae

Since 1923

Wrocław
Volume 83 Issue 4 (Winter 2014) Pages 257–462

Acta Societatis Botanicorum Poloniae

INVITED REVIEW Acta Soc Bot Pol 83(4):415–421 DOI: 10.5586/asbp.2014.042
Received: 2014-10-29 Accepted: 2014-11-26 Published electronically: 2014-12-31

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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Handling Editor: Andrzej Bodył

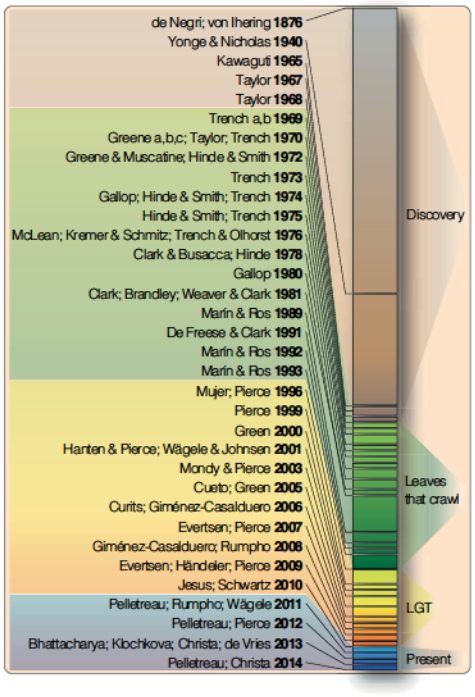

Fig. 1 Overview of about 140 years of research on green sacoglossan slugs. The timeline highlights key publications [1–6,8,10–17,20–23,27,33,40–43,46–49,52,53,56,61,63–94] on sacoglossan slugs since 1876. Four main periods (on the right) can be distinguished: the discovery phase, in which slugs and "chlorophyll-pigmented 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.

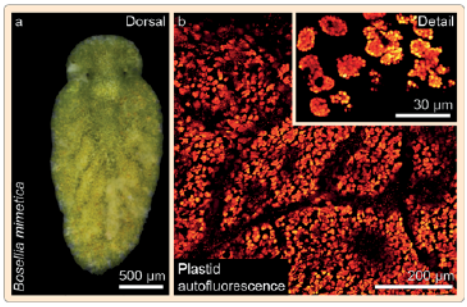

Fig. 2 Quantity does not equal quality. **a** The sacoglossan slug *Bosellia mimetica* harbours numerous kleptoplasts in its digestive tubular network, which (**b**) appear much denser in comparison to some LtR species (see [19,91]). Yet, *B. mimetica* is classified as a StR species; within a few days 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.

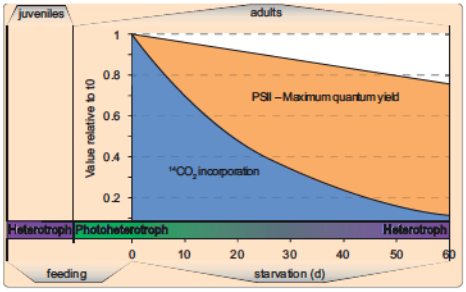

Fig. 3 Schematic overview of kleptoplast performance in LtR species. When the slugs hatch, they immediately need to feed on algal cytosol and begin to sequester their first plastids. During this phase, called transient kleptoplasty, juveniles are basically heterotrophic. Adult slugs are photoheterotrophic 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

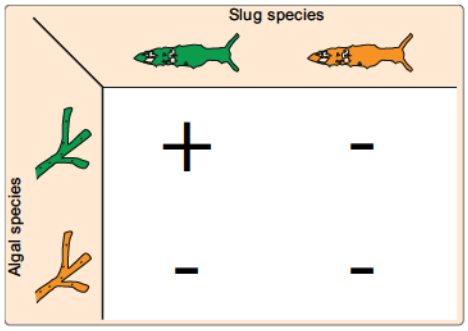

Fig. 4 Animal-plastid compatibility is determined by a two component system. In order to profit from functional kleptoplasts long-term (+), a slug species – component A – with an adapted physiology and digestive mechanism must acquire plastids from an algal species – component B – that naturally has 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.

Acknowledgments

Funding through the Deutsche Forschungsgemeinschaft to SBG (DFG; GO1825/4-1) and the DAAD to GC (P.R.I.M.E. program) is gratefully acknowledged. We thank Steffen Köhler (CAi, HHU) for photography and Bill Martin for suggesting Fig. 4.

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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4. Part I: No lateral transferred genes and not photoautotrophic

4.1 No laterally transferred genes from algae nuclei to photosynthetic slugs

The first analysis on laterally transferred genes (lateral gene transfer (LGT) or Horizontal gene transfer (HGT)) from algae to slug was done by Pierce and colleagues back in 1996 (Pierce et al. 1996). It was part of a long and intensive research period in which one of the more popular theories on slug kleptoplasty was being tested (Green et al. 2000; Pierce et al 2003; Pierce et al. 2007; Rumpho et al. 2008; Schwartz et al. 2010; Rumpho et al. 2011; Wägele et al. 2010; Piere et al. 2011; Schwarz et al. 2014; de Vries et al. 2015). While LGT from prokaryote to eukaryote is heavily debated (Boothby et al. 2015; Arakawa et al. 2016; Bemm et al. 2016; Koutsovoulos et al. 2016; Martin 2017), with regard to the slugs, it is another level of complexity. How are some slug species able to retain their kleptoplasts for such a long time? When isolated plastids are being kept isolated from the mother nuclei, they quickly degrade (Leegood and Walker 1983; Seftor and Jensen 1986; Polanská et al. 2004; Green et al. 2005). Approximately 10 years after the Pierces' 1996 LGT paper the LGT theory was debunked. Stricter analyses revealed that the genes were most likely contaminants and present in such low quantities that they could never support complete autonomous transcription and translation machineries for photosystem required proteins (Wägele et al. 2010; de Vries et al. 2015).

Before the LGT 'hype', in 1973, Trench and colleagues suggested that the kleptoplast autonomy in Long-term Retention (LtR) slugs could be linked to the 'robustness' of algae plastids (Trench et al. 1973; Giles and Sarafis 1972). In 2000 Green et al. sequenced one of the slugs' food sources plastid genome (*Vaucheria litorea*) that turned out to contain many more genes (119.1 kb) for photosynthesis than plastid genomes of land plants (Rumpho et al. 2011). Which confirmed the algae plastids' robustness. In 2014 de Vries and colleagues tested the theory by looking into the sequenced data of the algae food source *Acetabularia acetabulum*.

They found that the kleptoplasts of *A. acetabulum* encoded *ftsH* in their plastid genome (figure 3.8; de Vries et al. 2013), contrary to land plant plastids, which have *ftsH*

encoded in their nuclei (de Vries et al. 2013). FtsH is responsible for the repair of the D1 protein that breaks down because of photodamage during photosynthesis. This could very well be the key to plastid longevity for some LtR slug species, rather than laterally transferred genes. However, in autumn 2014 Schwartz and colleagues published a new paper. They claimed to have localized LGT in the slugs' chromosomes by means of fluorescent *in situ* hybridization (Schwartz et al. 2014). This paper was the inspiration to write a review (Rauch et al. 2015) in an attempt to clear up the LGT discussion and to move the field truly forward.

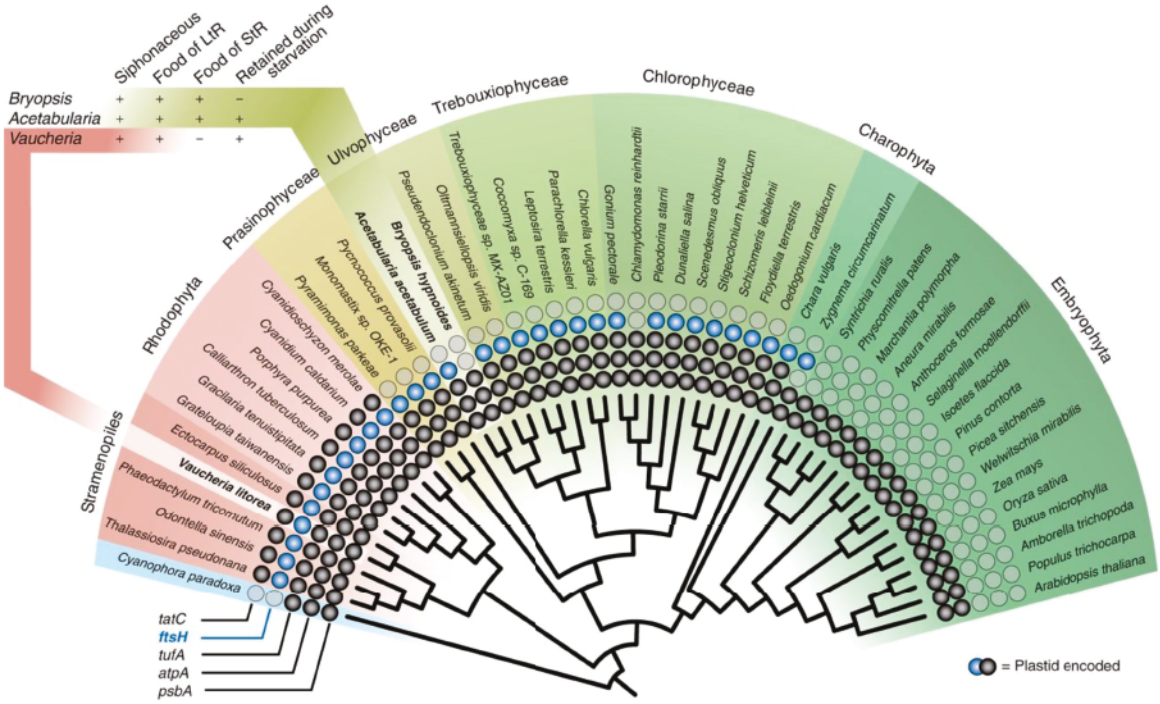

Figure 3.8. *ftsH* is encoded in the plastids' genome (de Vries et al. 2013). Many of the algae that are food sources for Sacoglossa share the trait of having *ftsH*, *tufA* and many other genes involved in photosynthesis still encoded in their plastid genome in comparison to many of the land plant species, this gives the algae plastids a higher level of autonomy (robustness).

Publication II: Why it is time to look beyond algae genes in photosynthetic slugs

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The presented manuscript was peer reviewed and published in the Journal 'Genome Biology and Evolution' with impact factor 4.3, on 29 August 2015.

Contribution as first author medium: Wrote parts of the manuscript, prepared the figures, contributed to the review of the literature and reviewed and approved the final version.

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Genome Biology and Evolution

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Why It Is Time to Look Beyond Algal Genes in Photosynthetic Slugs

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Accepted: August 26, 2015

Abstract

Eukaryotic organelles depend on nuclear genes to perpetuate their biochemical integrity. This is true for mitochondria in all eukaryotes and plastids in plants and algae. Then how do kleptoplasts, plastids that are sequestered by some sacoglossan sea slugs, survive in the animals' digestive gland cells in the absence of the algal nucleus encoding the vast majority of organellar proteins? For almost two decades, lateral gene transfer (LGT) from algae to slugs appeared to offer a solution, but RNA-seq analysis, later supported by genome sequencing of slug DNA, failed to find any evidence for such LGT events. Yet, isolated reports continue to be published and are readily discussed by the popular press and social media, making the data on LGT and its support for kleptoplast longevity appear controversial. However, when we take a sober look at the methods used, we realize that caution is warranted in how the results are interpreted. There is no evidence that the evolution of kleptoplasty in sea slugs involves LGT events. Based on what we know about photosystem maintenance in embryophyte plastids, we assume kleptoplasts depend on nuclear genes. However, studies have shown that some isolated algal plastids are, by nature, more robust than those of land plants. The evolution of kleptoplasty in green sea slugs involves many promising and unexplored phenomena, but there is no evidence that any of these require the expression of slug genes of algal origin.

Key words: lateral gene transfer, kleptoplasty, photosynthesis, plastid biology, photosynthetic sea slugs.

Introduction

Sacoglossa are considered one of nature's curiosities. Inside some of these sea slugs, plastids sequestered from algae can continue to photosynthesize for weeks, or even months, in the absence of algal nuclei (Greene 1970; Rumpho et al. 2001; Händeler et al. 2009). That is conspicuous, because when land plant plastids are isolated and removed from their cellular context they rapidly degrade (Leegood and Walker 1983; Seftor and Jensen 1986; Polanská et al. 2004; Green et al. 2005). With the description of endosymbiotic gene transfer (EGT; Martin et al. 1993) and the concomitant genome reduction the organelles experienced (Timmis et al. 2004), the prime cause for the instability of isolated plastids quickly became apparent: the majority of proteins working in plastids are nuclear-encoded and posttranslationally imported from the cytosol (McFadden 2014). Hence, the duration with which kleptoplasts are kept functional in animal cells in the absence of algae nuclei encoding a 1,000+ plastid proteins presents an obvious contradiction. This required an explanation and in 1996 (Pierce et al. 1996) it was proposed that slugs had

acquired algal genes that encode proteins servicing the plastids through lateral gene transfer (LGT). Once the idea was presented, it was destined to be tested.

There Is No Evidence for Lateral Transfer of Algal Genes in Slugs

Let us first take a look at what we should expect if genes of algal origin were to play a role in kleptoplast maintenance. The slugs are sometimes referred to as "crawling leaves," because the entire appearance of the species in question (e.g., *Elysia chlorotica*, *Elysia timida*, and *Elysia viridis*) is greenish and in a few cases indeed leaf-like. Scientists noticed this already more than 150 years ago. They were particularly intrigued by the digestive tubules that pervade almost the entire body, sometimes including the head, and whose cells house the kleptoplasts (fig. 1*a*). Based on what we know about the biology of photosynthesizing cells (in plants, algae, and cyanobacteria), we must predict that transcripts of algal origin, which are supposed to maintain kleptoplast integrity in the slugs, are abundant. Yet, among all RNA sequencing data available for several species (Rumpho et al. 2011; Wägele et al. 2011; Pierce et al. 2012; de Vries et al. 2015), sequencing reads pointing toward transcripts from genes of LGT origin remain close to zero. In fact, they remain well below the counts representing obvious contamination (fig. 2*a* and *b*).

Single reads can easily be artifacts. In RNAseq analysis, it is common practice to filter for only those genes that are supported by a reliable number of independent reads. In the RNA-seq analysis by Pierce et al. (2012)—the last RNA-seq report published to claim expression of genes of LGT origin is relevant for kleptoplast performance—the highest read count for a single algal nuclear gene of potential LGT origin was two. Two among 98,238,204 reads. A favored argument to explain why only such few reads are detected is that "the symbiotic chloroplasts resides in only a few cells within the slugs" (Pierce et al. 2012) or that "only a relatively few cells in the slug contain plastids" (Pierce et al. 2015).

Both the appearance of the animal (fig. 1*a* and *b*) and factual numbers tell a different story. The chlorophyll content in a 6 mm long *Elysia cornigera* is around 3.1 nmol/mg dry weight and in a 10-day-old tomato seedling with fully developed green cotyledons it is about 22.4 nmol/mg dry weight (fig. 2*c*). A single chloroplast contains 2.5×10^8 chlorophyll (Chl) molecules (Stolz and Walz 1988). One nanomole of Chl thus corresponds to about 2 million chloroplasts, and hence about 6 million chloroplasts are found per mg dry weight in *E. cornigera*. Three nanomoles of Chl further translates into a mass of about 2.7 µg, and therefore accounts for about 3% of the total dry weight of slugs. Impressive, even if the estimations would be lower by a factor of 10. Also, if only a few cells of a photosynthetic sacoglossan slug would harbor kleptoplasts, then how would that match up with the concept that photosynthesis continues almost unabated for up to a year to support animal growth (Pierce et al. 2012)? Furthermore, among the RNA-seq data of 2012 from *E. chlorotica*, 4,234 reads for the plastid-encoded *psbA* were detected (Pierce et al. 2012). That is noteworthy, because the samples sequenced were enriched for poly(A)-tailed mRNA prior to sequencing and the plastid mRNA was copurified only as a contamination due to the high AT-content of the plastid transcripts. There is probably even more mRNA encoding *psbA* present than sequenced and even if not, the number of reads for this single *psbA* gene by far exceeds the total number of reads (111) found for the 52 genes of suggested LGT origin. Furthermore, all the reads interpreted to be of LGT origin are ≥99% identical in sequence to the algal transcripts and that would mean they are exempt from evolutionary codon adaptation in the slug's nucleus.

Slugs analyzed are mostly collected from the wild and then grown on their food alga in open aquaria in the lab. The cultures are not axenic; they cannot be and they do not have to be for the kind of experiments that are currently performed. Contamination of the isolated RNA is unavoidable, but not a problem as long it is monitored. In the most recent transcriptomic analysis on two slug species (de Vries et al. 2015), the number of reads obtained for genes of heterotrophic protists was much higher than those for any algal nuclear gene and they were hence omitted from downstream analysis. We predict the amount of contamination in the data set of *E. chlorotica* (Pierce et al. 2012) to be comparable. The entire sequence data have never been made publicly available, rather only that of the few dozen genes discussed and therefore it was not possible to assess this issue in *E. chlorotica*. But a thought experiment is possible: we 1) do not screen the slug RNA-seq data for potential contamination and we 2) accept the presence of a few mRNA reads as evidence for functional LGT that support kleptoplast maintenance. We could then conclude that hundreds of ciliate genes support kleptoplasty in *E. timida*, but fail to do the same for the short-retaining *E. cornigera* albeit present (fig. 2*a*). The most rational conclusion that remains is that RNA-seq offers no support for the expression of slug nuclear genes that originate from the food alga. These slugs are not what they eat, and they eat a lot (Christa et al. 2014).

Recently, evidence for algal LGT in Sacoglossa other than sequencing data emerged: a study by Schwartz et al. (2014) used fluorescence in situ hybridization (FISH) to localize genes of algal origin among slug chromosome spreads. That report was quickly picked up by the popular press and it currently scores among the top 5% of all articles so far evaluated by Altmetric.com. Evidently, the public cares a lot about LGT in slugs. The public, however, is likely less aware that FISH analysis can be quite deceptive, for example consider the case of *Chlamydomonas* "basal body DNA" (Hall et al. 1989). The recent FISH analysis on *E. chlorotica* (Schwartz et al. 2014) also provided no controls for the specificity of the probes used (*prk*, *actin*, and *rbcL*) in form of Southern blots. Apart

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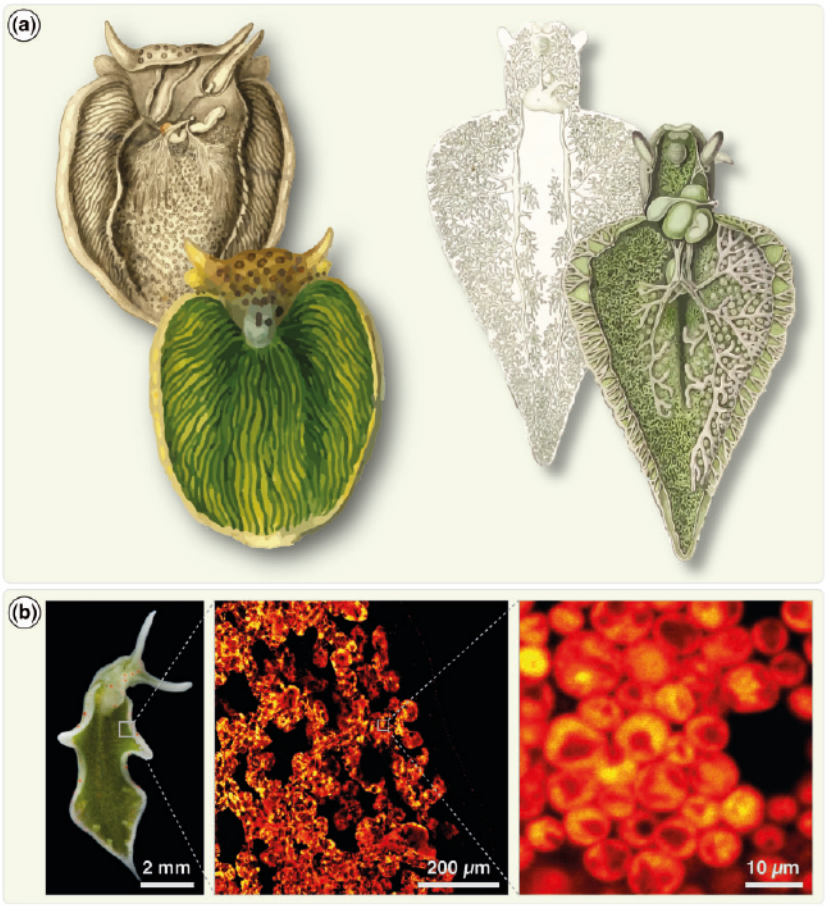

Fig. 1.—Sacoglossan slugs can house millions of kleptoplasts. (*a*) Shown are two of the earliest depictions of sacoglossan slugs and their "green" digestive tubules that can pervade the entire body. On the left a drawing by van Hasselt from 1824 showing *Plakobranchus ocellatus* and on the right a drawing of *Elysia viridis* by J. Thomas from 1852. Both demonstrate that an extensive digestive system, able to house millions of kleptoplasts, is not limited to *Elysia chlorotica*. Note how the digestive tubules of *E. viridis* pervade even the head of the animal. (*b*) The extent of stored plastids becomes apparent when viewing the slugs (here *Elysia timida*) under the microscope and filtering for the chlorophyll autofluorescence of the kleptoplasts (red-orange hue). In the middle, a detail of a region of the parapodia, with the individual digestive tubules being visible through the kleptoplasts' fluorescence. Zooming in further reveals the density with which the kleptoplasts are packed into the cytosol of the cells forming the digestive tubules.

from these technical issues, FISH analysis is not a suitable tool for providing evidence for LGT. The only reliable evidence for LGT would be to demonstrate the integration of algal DNA into the context of slug chromosomes (through DNA sequencing), from where it is expressed to support the stolen organelles by the product being specifically targeted to kleptoplasts. And although independent genome data of *E. chlorotica* is available (Pierce et al. 2012) to the authors of the FISH analysis, it has not been used to support their concept and also challenges published slug genome data that found no evidence for algal LGTs in *E. chlorotica* (Bhattacharya et al. 2013).

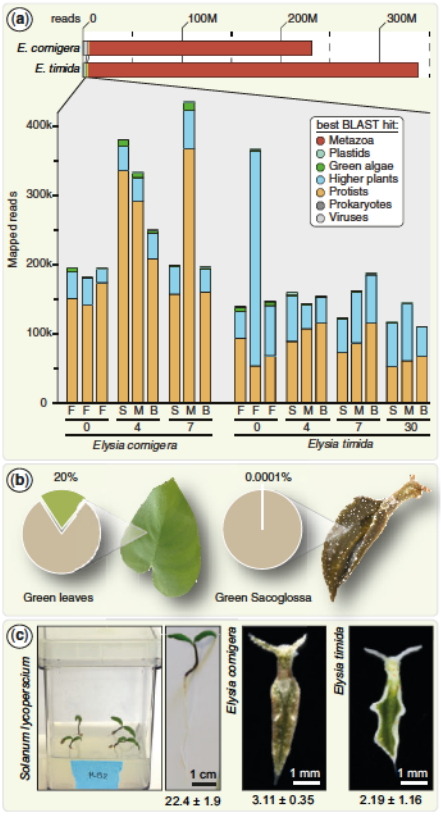

Fig. 2.—Among RNA-seq data, contaminating reads exceed reads of algal origin. (*a*) At the top the total number of reads (in million, M) recently sequenced for *Elysia cornigera* and *Elysia timida* (de Vries et al. 2015) are shown. Those reads were assembled into contigs and all contigs subjected to a BLAStx-based distribution analysis against RefSeq to determine their distribution among the taxonomic groups listed. Note that 1) the number of reads of protist origin in all cases exceeds those of green algal origin and that 2) in the LtR species *E. timida*, the amount of green algal reads declines with progressing starvation, while one would expect an elevated expression of genes supporting kleptoplasts. Slugs were freshly fed (F) and starved (S) for 4, 7, and 30 days under different conditions including monolinuron treatment blocking photosynthesis (M; 2 µg ml^{-1}) and high-light bleaching (B; 1 h of 1,000 µE $m^{-2}s^{-1}$ once per day). (*b*) While the percentage of nuclear mRNA transcripts associated with photosynthesis in a green leaf ranges around 20% (Bhalerao et al. 2003), slug transcriptomes return on average around 0.0001%. If the 52 genes described by Pierce et al. (2012) were truly transferred to the slug nuclear genome, they are expressed at a level that is 200,000 times too low to support photosynthesis. (*c*) The chlorophyll a+b concentrations of two slug species (from de Vries et al. 2015) versus those of entire 10-day-old tomato seedlings in nmol/mg dry weight.

As a last word on LGT, it should be mentioned that LGT to eukaryotes is manifest in two fundamentally different forms. First, there is gene transfer from organelles to the nucleus, or also called EGT. EGT is a continuous, ongoing process, and incontrovertibly documented in all sequenced genomes of eukaryotes (Timmis et al. 2004; Hazkani-Covo et al. 2010; Boto 2014). Second is outright LGT, where the donors are not chloroplasts or mitochondria. Newer findings show that latter, though it does occasionally occur, is extremely rare and does not manifest itself in the bigger picture of eukaryotic evolution (Ku et al. 2015). In this context, it is important to note that long-term retention of kleptoplasts evolved several times independently in sacoglossan sea slugs (Maeda et al. 2010; de Vries, Christa, et al. 2014; Christa et al. 2015). Hence, if the expression of nuclear genes of algal origin is the reason for robust kleptoplasts in one species, then the same should apply to other species as well. If all Sacoglossa retained functional kleptoplasts in a LGT-dependent manner, then they would have to be the record holders for LGT among animals, their LGT events outnumbering all other cases in animals thus far reported. Are Sacoglossa LGT magnets? Neither genome nor transcriptome data from these animals indicate that to be the case. Occam's razor dictates favoring a less assumptive scenario.

Stable Kleptoplasts in the Absence of LGT

Since the 1970s, it is known that some plastids sequestered by the sea slugs show a remarkable independent robustness (Giles and Sarafis 1972; Trench and Ohlhorst 1976; Green et al. 2005). The best explanations we have for robust plastids are effective photoprotection mechanisms (Serôdio et al. 2014; Cruz et al. 2015), a different coding capacity of the plastid genomes in question (Rumpho et al. 2000; de Vries et al. 2013) and maybe an overall difference in the stability (half-life) of essential proteins. That plastids sequestered by the slugs are intrinsically robust is, based on current information, the most parsimonious scenario. It explains how such a broad range of slug species can perform kleptoplasty (Christa et al. 2015) and why plastids of the same source can behave identically in slug species that differ in their ability to survive food deprivation (de Vries et al. 2015). Slugs acquiring robust plastids will not automatically retain them long-term and endure starvation as recently interpreted (Pierce et al. 2015). It is of equal importance that the slugs are physiologically adapted and require to retain them functionally (de Vries, Rauch, et al. 2014). This likely depends on whether they experience food deprivation in their habitat due to seasonal variation or not (Cruz et al. 2013; Wägele and Martin 2013; de Vries et al. 2015).

The ability to sequester and maintain an entire heterologous structure of foreign origin is not restricted to sacoglossa and their plastids. For the purpose of using them as a

defensive organ, some aeolidoidean sea slugs incorporate cnidocysts from their cnidarian prey to expose them on their surface (Obermann et al. 2012). Similar to the kleptoplasts, cnidocysts are first incorporated through oral feeding and as part of the regular diet. The peculiar thing is the release of the kleptoplasts from the phagosomes into the cytosol of the digestive epithelial cells. The latter appears more common when only organelles and not entire symbiotic organisms are retained by a host. The ciliate *Myrionectra rubra* releases transcriptionally active nuclei and plastids of its prey algae into the cytosol (Johnson et al. 2007), whereas symbiotic *Chlorella* algae of *Paramecium bursaria* or *Hydra viridis* remain inside a specialized digestive vacuole and isolated from the host's cytosol (Nowack and Melkonian 2010; Fujishima and Kodama 2012). It is not known how the plastids are specifically sorted from other food particles and then released into the cytosol or really why. Does it facilitate the easier exchange of substrate and metabolites? These observations, together with how Sacoglossa deal with kleptoplast-produced toxins such as reactive oxygen species and the general differences in starvation tolerance, remain promising research topics. All of these, however, are not associated with LGTs of algal origin.

Acknowledgments

Financial support through the Deutsche Forschungsgemeinschaft to S.B.G. (GO1825/4-1) and H.W. (WA618/8 and WA618/12) and the DAAD to G.C. (P.R.I.M.E. program) is gratefully acknowledged. The authors thank the Library of the Naturalis Biodiversity Centre (Netherlands) for the rasterized scans of van Hasselt's *Plakobranchus*.

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Associate editor: John Archibald

4.2 Photosynthetic slugs are not photoautotrophic with regard to CO_2 fixation

Some published manuscripts give the impression that certain Sacoglossa species are able to live photoautotrophically (Trench 1974b; Clark et al. 1990; Mujer et al. 1996; Rumpho et al. 2000; Rumpho et al. 2007; Evertsen et al. 2007; Händeler et al. 2009; Cruz et al. 2013). That is, the active and fully functional kleptoplasts provide the slugs with energy in the form of photosynthates during periods of food shortage (for example because of a seasonal disappearance of their algae food source (see chapter 3.2.3 of this dissertation; Marín and Ros 1992). But conclusions in these studies are often ill-considered. For example, some studies performed Pulse Amplitude Modulation (PAM) measurements and based on these results, determined that the slugs have a photoautotrophic lifestyle (Wägele and Johnsen 2001; Händeler et al. 2009; Laetz et al. 2017a,b; Cartaxana et al. 2017). But PAM measurements on its own are not sufficient to support the idea of a photoautotrophic slug, as it does not demonstrate a clear connection between the functional kleptoplasts and slug survival. Besides, there are also opposing reports, in which some studies describe a continued growth of the 'photosynthetic' slugs because of functional kleptoplasts during starvation (Rumpho et al. 2011; Pelletreau et al. 2011). While in our own and recent studies such results were never reproduced. In all cases, with different species of slugs, the weight and size of the animals decreased over time during starvation (Christa et al. 2014b; de Vries et al. 2015; Rauch et al. 2018).

Since slug research started to take off during the sixties it was generally accepted that slugs performed functional kleptoplasty for the same reason that plants have plastids. The term 'leaves that crawl' was coined during those days (Trench 1974a) and, when Christa and colleagues in 2014 published a paper called '*Plastid-bearing sea slugs fix CO_2 in the light but do not require photosynthesis to survive*' the popular press quickly picked it up (i.a. science.org, phys.org, Nature, Scientific American, National Geographic (Christa et al. 2014c)). This was unheard-of, despite the clear cases that the slugs did not really seem to do quite well in the absence of their algae food and did not significantly do better in the dark in comparison to their relatives kept in the light. These results indicated that the slugs obviously were not photoautotrophic.

A follow-up paper by the same authors (Christa et al. 2014c) challenged years of sacoglossan research by questioning why some species of Sacoglossa keep fully functional kleptoplasts, if they do not require photosynthesis to survive? There is still no clear answer. But there are various theories published, some substantial (camouflage, primary and secondary metabolites (Green et al. 2000; Wägele and Johnsen 2001; Cueto et al. 2005; Casalduero and Muniain 2006, 2008)), some less definite (nutrition and oxygen supply (Taylor 1971; Hinde and Smith 1972; Pelletreau et al. 2012; Laetz et al. 2017a,b)), but until this day, no convincing evidence has been published.

Besides missing scientific proof for a photoautotrophic lifestyle, it is also energetically impossible for a metazoan, like these slugs, to live just from sunlight (Rauch et al. 2017a). Not rarely people ask themselves if one day there will be interspecies chimaeras[1] between farm animals and plants to save up production costs. And while this is (economically) a wonderful idea, it is (scientifically) impossible to have a fully photosynthetic farm animal, like a cow. To make the point come across of why there is no such thing as photoautotrophic sea slugs and no photoautotrophic cows, it was calculated – with help of earlier published data – how much surface area a cow would need to fully support its energy demand (figure 4.9) and that 'photosynthetic' slugs only have their kleptoplasts contribute 0.25% to the total carbon. It is clear that you need to be the right size when it comes to an animal with plastids (Rauch et al. 2017a).

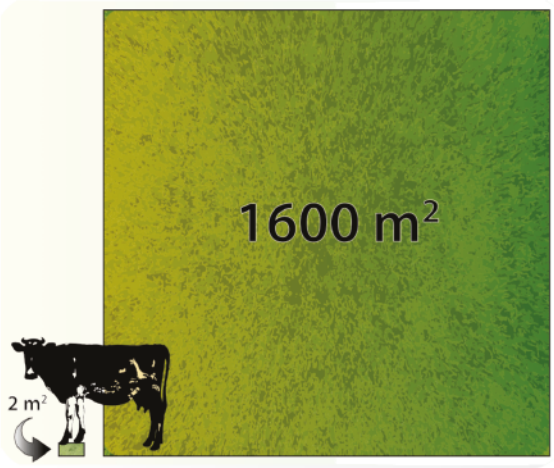

Figure 4.9. The impossible photobovid. An average 'beef' cow needs roughly 500 kg of maize corn; maize is able to produce 0.3 kg of corn per m². With these numbers, a cow, with the photosynthetic efficiency of maize, would need a surface of one-third of a football field (1600 m²) in order to produce 180 kg of beef (Rauch et al. 2017a).

[1] Chimaera as known in modern bioscience; entities made up of cells from different organisms (Wu et al. 2016).

Publication III: On being the right size as an animal with plastids

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The presented manuscript was peer reviewed and published in the Journal 'Frontier in Plant Science' with impact factor 4.5, on 17 August 2017.

Contribution as firsts author major: Wrote parts of the manuscript, prepared the figure and table, contributed to the review of literature and reviewed and approved the final version.

frontiers in Plant Science

MINI REVIEW
published: 17 August 2017
doi: 10.3389/fpls.2017.01402

On Being the Right Size as an Animal with Plastids

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Plastids typically reside in plant or algal cells—with one notable exception. There is one group of multicellular animals, sea slugs in the order Sacoglossa, members of which feed on siphonaceous algae. The slugs sequester the ingested plastids in the cytosol of cells in their digestive gland, giving the animals the color of leaves. In a few species of slugs, including members of the genus *Elysia*, the stolen plastids (kleptoplasts) can remain morphologically intact for weeks and months, surrounded by the animal cytosol, which is separated from the plastid stroma by only the inner and outer plastid membranes. The kleptoplasts of the Sacoglossa are the only case described so far in nature where plastids interface directly with the metazoan cytosol. That makes them interesting in their own right, but it has also led to the idea that it might someday be possible to engineer photosynthetic animals. Is that really possible? And if so, how big would the photosynthetic organs of such animals need to be? Here we provide two sets of calculations: one based on a best case scenario assuming that animals with kleptoplasts can be, on a per cm^2 basis, as efficient at CO_2 fixation as maize leaves, and one based on $^{14}CO_2$ fixation rates measured in plastid-bearing sea slugs. We also tabulate an overview of the literature going back to 1970 reporting direct measurements or indirect estimates of the CO_2 fixing capabilities of Sacoglossan slugs with plastids.

Keywords: *Elysia*, Sacoglossa, photosynthetic slugs, photosynthetic animal, growth rate, life cycle

OPEN ACCESS

Edited by:
Robert Edward Sharwood, Australian National University, Australia

Reviewed by:
Ben M. Long, Australian National University, Australia
Vivien Rolland, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

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Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *28 April 2017*
Accepted: *27 July 2017*
Published: *17 August 2017*

Citation:
Rauch C, Jahns P, Tielens AGM, Gould SB and Martin WF (2017) On Being the Right Size as an Animal with Plastids. Front. Plant Sci. 8:1402. doi: 10.3389/fpls.2017.01402

INTRODUCTION

The group of sea slugs belonging to the order Sacoglossa really know how to keep biologists busy. The group comprises about 400 species of small, soft-bodied marine animals (**Figure 1A**) that feed upon algae (Jensen, 2007; Wägele et al., 2011). The algal food is what mainly keeps the biologists busy. Adult sacoglossans feed upon not just any algae, but upon siphonaceous algae (**Figure 1B**). Siphonaceous describes large, tube-like filamentous algae, often having a reduced, hardly visible vacuole, meaning that they are filled with a rich cytosol, sometimes containing many hundreds of plastids. The sacoglossans possess a highly-specialized radula, a molluscan version of teeth (**Figure 1A**; zoom-in panel), that allows them to puncture their algal prey and suck out the cytoplasmic contents, which they then digest (Trench, 1969; Wägele et al., 2011).

A small number of sacoglossan species perform a very special trick: they do not digest the plastids, but they sequester them instead as kleptoplasts within the cells of their digestive gland (Rumpho et al., 2001; Händeler et al., 2009; de Vries et al., 2014a), giving the slugs their distinct

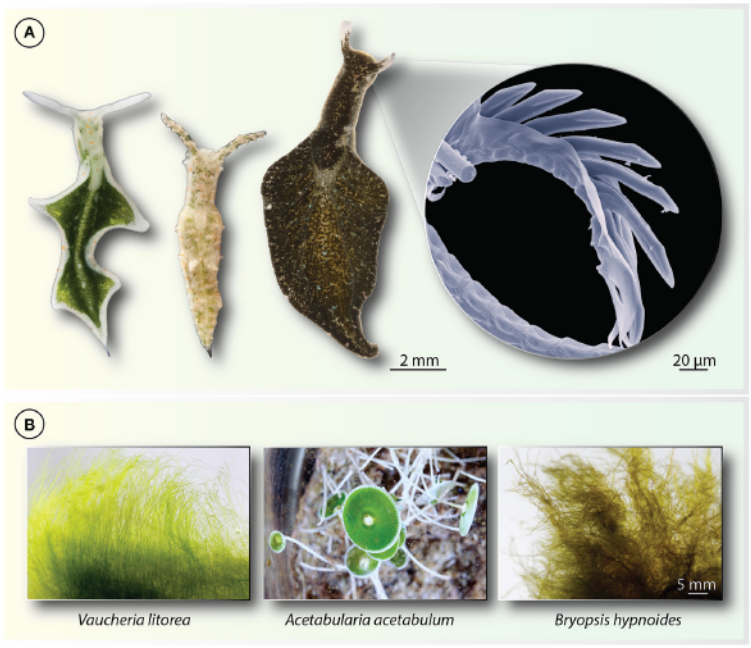

FIGURE 1 | Examples of sacoglossan sea slugs and their algal food. **(A)** From left to right the three Sacoglossa *Elysia timida*, *Elysia cornigera*, and *Elysia viridis*. All use a radula to penetrate siphonaceous algae from which they suck out the cytosolic content (hence the name sap sucking slugs) that includes compartments such as mitochondria, nuclei, and plastids. Plastids are specifically sequestered (the kleptoplasts) and incorporated into the cells that line the digestive tubules, in some cases giving the slugs their characteristic green color. The radula is a chitinous feeding structure of molluscs comparable teeth. A scanning-electron micrograph of one of *E. viridis* is shown on the very right, revealing the individual teeth-like structures the animals use to penetrate the algal cell wall. **(B)** Examples of macroalgae on which Sacoglossa feed and that are siphonacous. Some Sacoglossa are highly specialized and feed on single alga species, such as *E. timida* that only feeds on *Acetabularia*, while others such as *E. viridis* can feed on a larger variety of macroalgae.

green color. Electron microscopy experiments showed that the plastids can stay morphologically intact for days, weeks, or even months (Greene, 1970; Rumpho et al., 2001; Händeler et al., 2009). Early investigations in the 1970s indicated that the sequestered plastids remain photosynthetically active (Giles and Sarafis, 1972; Hinde and Smith, 1972; Trench and Ohlhorst, 1976). Accordingly, these sacoglossans have been described as "photosynthetic" for many decades. Indeed, the slugs could survive in the light for weeks without food, just with plastids (Pierce et al., 1996). More recent investigations, however, showed that the slugs with plastids survive in the dark, too, and that the presence of photosynthesis inhibitors also does not significantly, if at all, increase the rate with which they lose weight (Christa et al., 2014; de Vries et al., 2015). It seems that some sacoglossan species are simply more starvation tolerant than others and that has to do with better coping with reactive oxygen species (de Vries et al., 2015). Indeed, all slugs shrink during starvation and some can go from centimeters to millimeters in length when deprived of a food source. The green sacoglossans are no exception if starved (Mujer et al., 1996; Pelletreau et al., 2012; Klochkova et al., 2013; Christa et al., 2014; de Vries et al., 2015), that is, if they are forced to turn to autophagy for energy generation (de Vries et al., 2015). It thus appears that what we once thought were animals that are good at photosynthesis are first and foremost good at fasting. However, the concept of "photosynthetic animals" has considerable inertia in the literature. The media in particular regularly want to know about the status and future of photosynthetic animals.

Such discussions inevitably lead to debates of whether it might someday be possible to construct photosynthetic farm animals, given all that modern science can do with gene technology and the like. Thus, there is a need to critically inspect the concept of photosynthetic animals. The first and most obvious issue is that they would need a lot of surface area to harvest that light (Smith and Bernays, 1991). How much area would they need and, conversely, what portion of a green slug's food requirements can be covered from sequestered plastids? In the spirit of Haldane et al. (1926) essay "On being the right size,"

let us take a look, using some rough but robust and very conservative estimates.

THE SIZE OF PHOTOSYNTHETIC ANIMALS

If we go into the literature, we can readily find most of the values that we need. Fast growing dairy cows gain weight at a rate of about 1 kg per day, slow growing cows gain roughly 0.5 kg per day (Brody and Ragsdale, 1930). Let's use the 0.5 kg per day value, to which we will return later. Another value we need is how much food is required to generate that weight. An old rule of thumb is that roughly 5–6 kg of maize are needed to generate 1 kg of beef, though modern numbers provide a range of 3–10 kg of maize per kg of beef (Tilman et al., 2012). Cows consist of more than beef, though, so let's be generous and assume that only 3 kg of maize are needed per kg of cow bodyweight. In order to get our cow through one growing season, that is, through 1 year (and hence 180 kg of weight gain), 540 kg of maize would be required—let's grant another 10% in improved feed conversion efficiency and call it 500 kg of maize seed that is needed to grow our cow for a year. This is deliberately an underestimate, to be conservative.

How much leaf area is needed to produce those 500 kg of maize seed? Under excellent field conditions (good soil, good sunlight, sufficient water, and fertilizer) maize can yield about 160 bushels per acre (http://cropwatch.unl.edu/corn/yieldtrends), which converts to about 10 tons per hectare, or roughly 1 kg/m^2. Maize has a leaf area index of about 3, meaning 3 m^2 leaf area per m^2 field area (Watson, 1947; Wilhelm et al., 2000), such that yield, expressed in units of leaf area, corresponds to roughly 0.3 kg of maize seed per square meter of maize leaf area. Since we need 500 kg of maize per season, that means that our cow would need about 1,600 m^2 of surface area with the photosynthetic efficiency of maize (Tollenaar, 1983), one of the top-efficiency photosynthesizers known, to sustain that 0.5 kg per day growth rate, averaged over the year. Thus, under optimal field conditions and with the photosynthetic efficiency of maize, our photosynthetic cow needs a leaf roughly 40 × 40 m in size, about a third the size of a football field. We note that unless our leaf-bearing cow has the phenotypic appearance of a maize plant, it is going to have a leaf area index very close to 1, which is 3-fold less efficient per square meter than maize, even if it has the same photosynthetic efficiency.

Of course, our estimate so far assumes that the biogenesis and maintenance of the photosynthetic organ itself consumes no photosynthates. Were the organ is on average 1 cm thick, it would weigh 10 kg/m^2, which corresponds to 16,000 kg total "leaf" weight because we need 1,600 m^2 of photosynthetic surface area to generate 500 kg of maize seed to support 180 kg of weight gain per year (growing season). That is going to pose insurmountable problems, because the cow's "leaf" requires 88 times more body mass synthesis than the 180 kg of weight gain it is supposed to support. So let us assume that molecular biologists and gene engineers can find ways to make it 1 mm thick on average, adding only 1,600 kg of extra weight. That leaf will require 9 growing seasons to synthesize at a growth rate of 180 kg per year, and we have still not generated the 180 kg per year net surplus cow material for harvesting. Thirty months is a late "terminal age" for cattle (Bouton et al., 1978), meaning our photoengineered cow would have to spend three lifetimes growing its leaf before it gets started growing the body (at 180 kg per year). It is rapidly becoming clear that the photosynthetic cow project (a photobovid) is not going to work out very well, and we furthermore are beginning to understand more clearly why cows eat grass all day, leaving it to plants to do the heavy lifting of photosynthesis and leaving it to their anaerobic gut flora to break cellulose down into short chain fatty acids (acetate, propionate, and butyrate) that the cows can resorb as food (Bauchop and Mountfort, 1981; Dijkstra et al., 1993).

Let's be unrealistically generous, however, and say that our cow does not have to invest any photosynthates at all in the synthesis of its leaf—it gets the leaf for free. This would then be equivalent to the situation of the saccoglossan slugs, which grow up without photosynthesis and incorporate plastids into a ready-made leaf, their parapodia. If we generously gauge the shadow-generating surface area of the cow without the leaf as 2 m^2, then the photosynthesizing animal needs to have an organ with 800 times its own photosynthetically effective surface area. Put another way, with its own surface, the animal could cover about 0.1% of its growth needs (2/1,600), if it was, on a per cm^2 basis, as photosynthetically efficient as maize.

We have also left another important limiting factor—water demands—out of the calculation. The situation at the water trough for those engineered farm animals opens up a whole new set of problems into which we do not delve here. That brings us back to the slugs. The slugs are different from the cows, of course. They are a lot smaller, they are not warm-blooded so they do not need to generate lost heat, and they have a different growth rate.

THE PLASTID CONTRIBUTION TO PHYSIOLOGY IN SLUGS

A well-studied slug species is *Elysia timida* (**Figure 1A**), which under good laboratory conditions can grow to a fresh weight of roughly 100 mg, corresponding to a dry weight of ca. 10 mg per animal. In our own *E. timida* laboratory cultures and controlled conditions (Schmitt et al., 2014), the time from egg hatching to the juvenile stage is about 20 days. They start feeding on siphonaceous algae at day 3 after egg hatching (Schmitt et al., 2014). By day 20 they are ~0.1 mg in weight and 0.5 mm long. From that point, they require an additional 7–8 weeks to reach their final size with a dry weight of roughly 10 mg. In total, it takes about 12 weeks for *E. timida* to develop from eggs to maturity (Schmitt et al., 2014).

How much of that dry weight can come from photosynthesis in sequestered plastids? The average eukaryotic cell has a dry weight formula of roughly $C_5H_7O_2N$, meaning that eukaryotes are roughly 50% carbon by dry weight (Heldal et al., 1985). A slug of ca. 10 mg dry weight therefore contains 5 mg of carbon (5 mg C). From Christa et al. (2014) we know that the experimentally determined CO_2 incorporation rates for *E. timida* are 30 nmol of CO_2 in 2 h for four animals. This translates to 45 nmol C per animal per 12-h photosynthetic period, which converts to 540 ng of carbon per animal per 12-h-daylight day.

The adult animal contains 5 mg C; photosynthesis in fully grown animals can provide 0.54 µg per day, which corresponds to 0.011% incorporation of the final C content per day. If that rate were to continue for 84 days (the development period) the slug could, theoretically, fix 0.91%, or about 1% of its carbon.

Being able to fix 1% of one's carbon is not very photosynthetic one might say, but 1% is still a far too generous estimate, we need to correct for at least two more factors. First, the young adults are only about 500 µm long with an area of 0.25 mm^2 (Schmitt et al., 2014), or 0.3% the area of the fully-grown animal, which has a plastid-containing surface area of roughly 1 cm^2 (**Figure 1A**). Over the 84-day development period, on average half the surface area of the adult is available for photosynthetic activity, so that perhaps 0.5% of the animal's C can be contributed by photosynthesis.

But a contribution of 0.5% total C from photosynthesis to slug body weight is still too generous, because there is also the issue of carbon turnover. That is, the animals respire some of the C that they assimilate, whether from heterotrophic feeding (which is already >99% of total C, as we see from our calculation so far), or from the plastid contribution. The turnover time, or half-life, of C has not been measured so far in *E. timida*. There are, however, very good numbers available for C half-life in animals of many different sizes (van der Zanden et al., 2015), where it is seen that C-turnover times in animals scale tightly with body size. From the published curves of van der Zanden et al. (2015), we can estimate that in the case of invertebrates that weigh as much as *E. timida*, the C half-life should fall in the range of 12 days. That means that at constant weight, half of the carbon atoms present in the animal at time zero are still present after 12 days. But we do not have to worry too much about the carbon half-life, because the measured value of 540 ng C per animal per 12-h day is net incorporation, it already takes respiration into account. In the 12 h of dark, there is still respiration, so that the value of 0.5% of the animal's C needs to be halved once more (continued respiration but no photosynthesis in the 12-h dark phase) and we arrive at an estimate that roughly 0.25% of the adult's C can be contributed by photosynthesis, based on the most recent experimentally measured $^{14}CO_2$–based CO_2 incorporation rates for slugs available in the literature (Christa et al., 2014).

Thus, *E. timida* might be able to cover about 0.25% of its body weight increase during development from CO_2 fixation in plastids. That explains why some plastid-harboring sacoglossan slugs have been observed to lose weight in the light at the same rate as in the dark or with chemically inhibited photosynthesis (Christa et al., 2014).

However, as in any field of active scientific inquiry, there are conflicting reports, of course. For example, Laetz et al. (2017) recently reported that starch provided by plastids increases in *E. timida* during starvation before it decreases, and that this impacts slug survival. Those are important observations, but quantitative estimates of CO_2 fixation based on $^{14}CO_2$ incorporation were lacking. Earlier reports of carbon fixation by plastids in sacoglossans based on stable carbon isotope ratios ($^{13}C/^{12}C$) gave very high estimates of net photosynthetic contribution by slug-sequestered plastids (Raven et al., 2001), but quantitative estimates of CO_2 fixation based on $^{14}CO_2$ incorporation were again lacking. Some of the earliest $^{14}CO_2$ incorporation measurements from the 1970s delivered fairly high values (Hinde and Smith, 1975), but were also marked by considerable variation from measurement to measurement and in at least one case more $^{14}CO_2$ incorporation measured in the dark than in the light.

When we embarked upon measuring $^{14}CO_2$ incorporation in slugs, we also noticed disconcerting variation across experiments and puzzlingly high dark $^{14}CO_2$ incorporation values (AGMT and WFM, unpublished observations). We subsequently found that prolonged (overnight) and very strong acid treatment (1 M HCl) was needed to gasify and purge unincorporated $^{14}CO_2$ from animal homogenates after incubation experiments. If photosynthesis is the reason that sacoglossan slugs keep plastids, then we should be able to quantify that contribution via $^{14}CO_2$ incorporation measurements in other species that, like *E. timida*, sequester plastids.

LOOKING INTO THE LITERATURE: EXPECTATIONS AND EVIDENCE

If we dig into the literature on the possible function of photosynthesis in plastids during starvation in Sacoglossa, it is apparent that only very few studies carried out $^{14}CO_2$ incorporation measurements that would allow one to directly determine CO_2 incorporation (**Table 1**). Furthermore, many studies lacked the appropriate controls that would permit a clear causal connection between photosynthetic CO_2 fixation and animal survival, for example testing the effect of chemical inhibition of photosynthesis. There is a crucial difference between (a) the inference that sequestered, morphologically intact, plastids are important for enduring starvation (for possibly unknown reasons), and (b) the inference that sequestered, morphologically intact, plastids are providing CO_2 fixation at rates that would provide significant nutrition to the animals. No previous work, nor the present calculations at hand, call into question the view that sequestered plastids in long term retention Sacoglossa have some kind of biological significance. But we are also not aware of any previous work that experimentally justifies interpretations or claims that the quantity of fixed carbon provided by plastids is sufficient to support the idea of a "photoautotrophic animal," as pervades the literature on plastid-sequestering Sacoglossa, not seldom in the title or abstract. Our present calculations serve to underscore the point that measured $^{14}CO_2$ fixation rates for plastids sequestered in the cytosol of an animal cell cannot support animal growth.

CONCLUSION

The purpose of this paper is to provide a reference to which one might turn in the event that the media or an interested high school class calls, wanting to know whether the engineering of photosynthetic farm animals "like the slugs" might be a worthwhile avenue of scientific pursuit. The answer we obtain is that by weight, about 0.3% of the slug might be able to

TABLE 1 | Overview of relevant literature on Sacoglossa and experimental details.

References	# spec. (1)	# indv. (2)	wgt/len (3)	$^{14}CO_2$ (4)	Fv/Fm (5)	dark ctrl. (6)	DCMU (7)	Chl. (8)	Starved (9)	O_2 (10)
Trench, 1969	1			+		+		+	+	+
Greene, 1970	2	8		+		+		+	+	
Trench et al., 1970	1			+						
Taylor, 1971	1	6						+		+
Greene and Muscatine, 1972	3	11		+		+		+		
Hinde and Smith, 1972	1	39	+	+		+		+	+	
Trench et al., 1973	1			+		+		+	+	
Gallop, 1974	1			+				+	+	
Hinde and Smith, 1974	4	68	+	+		+		+	+	
Trench et al., 1974	2			+						
Hinde and Smith, 1975	1		+			+		+	+	
Kremer and Schmitz, 1976	1			+						
McLean, 1976	1								+	
Trench and Ohlhorst, 1976	2			+						
Clark and Busacca, 1978	4		+					+	+	
Gallop et al., 1980	1	27	+	+		+		+	+	
Clark et al., 1981	1			+		+		+	+	
Weaver and Clark, 1981	5									
Marín and Ros, 1989	4		+	+				+	+	
de Freese and Clark, 1991	3	24		+		+				
Marín and Ros, 1992	1		+	+						
Marín and Ros, 1993	1		+							
Mujer et al., 1996	1	220	+						+	
Green et al., 2000	1	25				+		+	+	+
Wägele and Johnsen, 2001	4	10			+					
Cueto et al., 2005	1									
Casalduero and Muniain, 2006	1	184	+						+	+
Curtis et al., 2006	1	5							+	
Evertsen et al., 2007	7	17			+				+	
Casalduero and Muniain, 2008	1	357	+			+		+		+
Rumpho et al., 2008	1									
Evertsen and Johnsen, 2009	2		+		+			+	+	
Händeler et al., 2009	29	186			+				+	
Pierce et al., 2009	1							+	+	
Jesus et al., 2010	1	20			+	+		+	+	
Maeda et al., 2010	18									
Schwartz et al., 2010	1								+	
Pierce et al., 2011	1								+	
Wägele et al., 2011	2	16			+				+	
Devine et al., 2012	1	45							+	
Pelletreau et al., 2012	1	80	+						+	
Bhattacharya et al., 2013	1								+	
Christa et al., 2013	1	41			+	+			+	
de Vries et al., 2013	1									
Klochkova et al., 2013	1	400	+		+	+		+	+	
Baumgartner et al., 2014	1		+							
Pelletreau et al., 2014	1		+			+			+	
Schmitt et al., 2014	1	179			+				+	
Serôdio et al., 2014	3		+		+					
Baumgartner et al., 2015	1		+		+					
Christa et al., 2015	105				+	+			+	

(Continued)

TABLE 1 | Continued

References	# spec. (1)	# indv. (2)	wgt/len (3)	$^{14}CO_2$ (4)	Fv/Fm (5)	dark ctrl. (6)	DCMU (7)	Chl. (8)	Starved (9)	O_2 (10)
Cruz et al., 2015	2				+	+		+		
Curtis et al., 2015	3				+				+	
de Vries et al., 2015	2		+	+	+	+	+	+	+	
Martin et al., 2015	4							+		
Laetz et al., 2017	1	40	+		+		+		+	
Wade and Sherwood, 2017	1	69			+				+	

The switch in methods used in particular regarding CO_2 fixation experiments and using pulse amplitude modulation (PAM) measurements (i.e., Fv/Fm ratios). Columns from left to right: (1) number of different species reported; (2) total number of slugs used; (3) weight (wgt) and/or length (len) measured; (4) $^{14}CO_2$ incorporation studies; (5) photosynthetic activity measured by means of Pulse Amplitude Modulation (PAM) measurements; (6) group of slugs was also kept in the dark; (7) chemical blocking of photosynthesis by DCMU; (8) chlorophyll/pigment concentration measurements; (9) a group of slugs were starved throughout the experiments; (10) O_2 evolution was measured.

survive from photosynthetic activity through its sequestered plastids. The other 99.7% has to live from normal ingested food, like the rest of us animals. Somewhat more bleak prospects arise for photosynthetic farm animals, because of their size and warm-blooded nature. In principle, the concept of photosynthetic animals is interesting. In practice, it underscores the observation that among all animals known so far; only seven species of sacoglossan slugs steal plastids from siphonaceous algae and sequester them long-term (de Vries et al., 2014b), apparently for reasons other than carbon fixation.

AUTHOR CONTRIBUTIONS

CR, SG, and WM drafted the MS. CR designed the figure and the table. CR, PJ, AT, SG, and WM all made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

We thank the DFG GO1825/4-1 and GO665/9-1 (SG and PJ) the NWO (AT), and the ERC 666053 (WM) for financial support.

ACKNOWLEDGMENTS

We thank Steffen Köhler (CAi, HHU) for help with slug and algae photography and we thank Mimi Maurer and her biology classmates at the Hohenstaufen-Gymnasium in Göppingen (Baden-Württemberg, Germany) for prompting us to perform a few calculations about the bounds on possible quantitative contribution of plastids in animal cells to photosynthesis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer BML and handling Editor declared their shared affiliation, and the handling Editor states that the process met the standards of a fair and objective review.

5. Part II: The influence of ROS and living without kleptoplasts

5.1. The influence of reactive oxygen species on the survival of starving slugs

Kleptoplastic sea slugs face a vast problem when it comes to retaining fully functional plastids; an extra source for the accumulation of reactive oxygen species (ROS). Plastids are very prone to photosynthesis caused photodamage and this can result in levitated H_2O_2 levels (Apel and Hirt 2004). When de Vries et al. (2015) conducted a transcriptome analyses of the sister species *Elysia cornigera* and *Elysia timida*, they noticed how different the short-term retention species, *E. cornigera*, and the long-term retention species, *E. timida*, reacted on the accumulation of ROS (de Vries et al. 2015). In short, during starvation, *E. cornigera* dies because of the accumulation of ROS while *E. timida* seems to be less affected by ROS and endures starvation (de Vries et al. 2015). This pointed towards decease due to cellular starvation and not due to the absence of active kleptoplasts. Especially because *E. cornigera* still harboured many active plastids, even after it deceased (de Vries et al. 2015). With this study, a follow-up analysis occurred, but this time the focus was on the mitochondria of the slugs, rather than its kleptoplasts.

Mitochondria are more than mere producers of ATP for the cells. They control Ca^{2+} levels in the cell (Clapham 2007), detoxify ammonia in liver cells (Soria et al. 2013), determine cell fate (Bhola and Letai 2016), house one of the two eukaryotic disulfide bond relay systems (Herrmann and Riemer 2012), synthesize Fe-S clusters (Lill and Kispal 2000) and are on the front line of a process that triggers and forms autophagosomes (Chen and Gibson 2008). The latter is formed with starvation in order to recruit nutrients by degrading components of the cytoplasm (Scott et al., 2004). One initial signal that induces autophagy is ROS, which is generated also through the activity of the electron transport chain of the inner mitochondrial membrane (Chen et al. 2007). The enzymatic and non-enzymatic antioxidant system, including e.g. glutathione peroxidase, glutathione, catalases and superoxide dismutases, have the ability to control ROS levels (Filomeni et al. 2015; Poljsak 2011). However, environmental stress such as starvation, disrupts the balanced metabolism of the cell and therefore increases ROS to

a degree that can be detrimental (Rejeb et al. 2014; Devasagayam et al. 2004). The increased ROS especially damages the mitochondria (Filomeni et al. 2015; He and Klionsky 2009; Scherz-Shouval and Elazar 2007), because of the delicate balance between ROS production and elimination that keeps the cell homeostasis going (Das and Roychoudhury 2014). The ability to suppress problematic ROS levels is suggested as important to endure starvation for Sacoglossa sea slugs that bear functional kleptoplasts (de Vries et al. 2015). However, the underlying mechanisms and role of the mitochondria during starvation remains unknown. Therefore, the gene expressions of *E. timida* and *E. cornigera* for the main energy metabolic pathways were mapped (like pentose phosphate, glycolysis, fatty acid synthesis, the citric acid cycle (TCA) and oxidative phosphorylation (OXPHOS)) (figure 5.10; Rauch et al. 2017b).

In order to understand what happens to the slugs during starvation, it is helpful to know the common process of starvation and how it generally consists of three phases. Phase I is fasting, in which the glycogen stores are not yet depleted and what distinguishes it from downstream starvation (Cherel et al. 1992; Lenaerts et al. 2006; Wang et al. 2006). This phase was also detected in *E. cornigera* and *E. timida* as in the first few days not much change in body size and gene expression was observed. In phase II fatty acid oxidation commences and ketone bodies are produced that contribute tremendously to the generation of energy during starvation (Cherel et al. 1992; Lenaerts et al. 2006; Wang et al. 2006). This results in a slow reduction of body weight and size. When also the lipid stores are depleted, autophagy is induced and phase III enters, which results in a more rapid decrease of body size and weight (Lenaerts et al. 2006; Wang et al. 2006). This was observed as the slugs reduced in size when starvation was prolonged and gene expression levels were either up or down regulated in comparison to the fed state (see figure 10; Rauch et al. 2017b).

Especially phase II and III demand a considerable metabolic change, from which the mitochondria are not spared. For instance, during phase III of starvation, the Electron Transport Chain (ETC) plays a critical role; when TCA cycle can no longer sufficiently provide the ETC with reducing equivalents, the ETC starts to release ROS, that on its turn triggers autophagy (Scherz-Shouval and Elazar 2007; Chen et al. 2007; Albayrak et al. 2003; Wolvetang et al. 1994). This can prolong the survival of an organism by

providing new sources of energy through the degradation of cellular material, including organelles, but not infinitely (Gibson 2013; Codogno and Meiher 2005; Scherz-Shouval and Elazar 2007; Chen et al. 2007; Albayrak et al. 2003; Wolvetang et al. 1994).

Starvation in photosynthetic slugs is special due to the presence of kleptoplasts that occur in the same cytosol as the animal's own mitochondria. In algae and plants, the metabolism of plastids and mitochondria are linked in a variety of ways besides the provision of energy-rich carbon compounds through photosynthesis (Hoefnagel et al. 1998). In the following paper the gene regulation of mitochondria-targeted proteins in the congenic sacoglossan slugs *E. timida* and *E. cornigera* was analysed during starvation, to shed light on how starvation-induced stress influences mitochondrial metabolism in the presence of kleptoplasts, light-stressed kleptoplasts and kleptoplasts whose photosynthesis was chemically blocked by monolinuron. Focussing on the main metabolic pathways involved, such as the pentose phosphate pathway, TCA and OXPHOS, a difference was noticed in regulation of genes encoding proteins of these pathways between the two species.

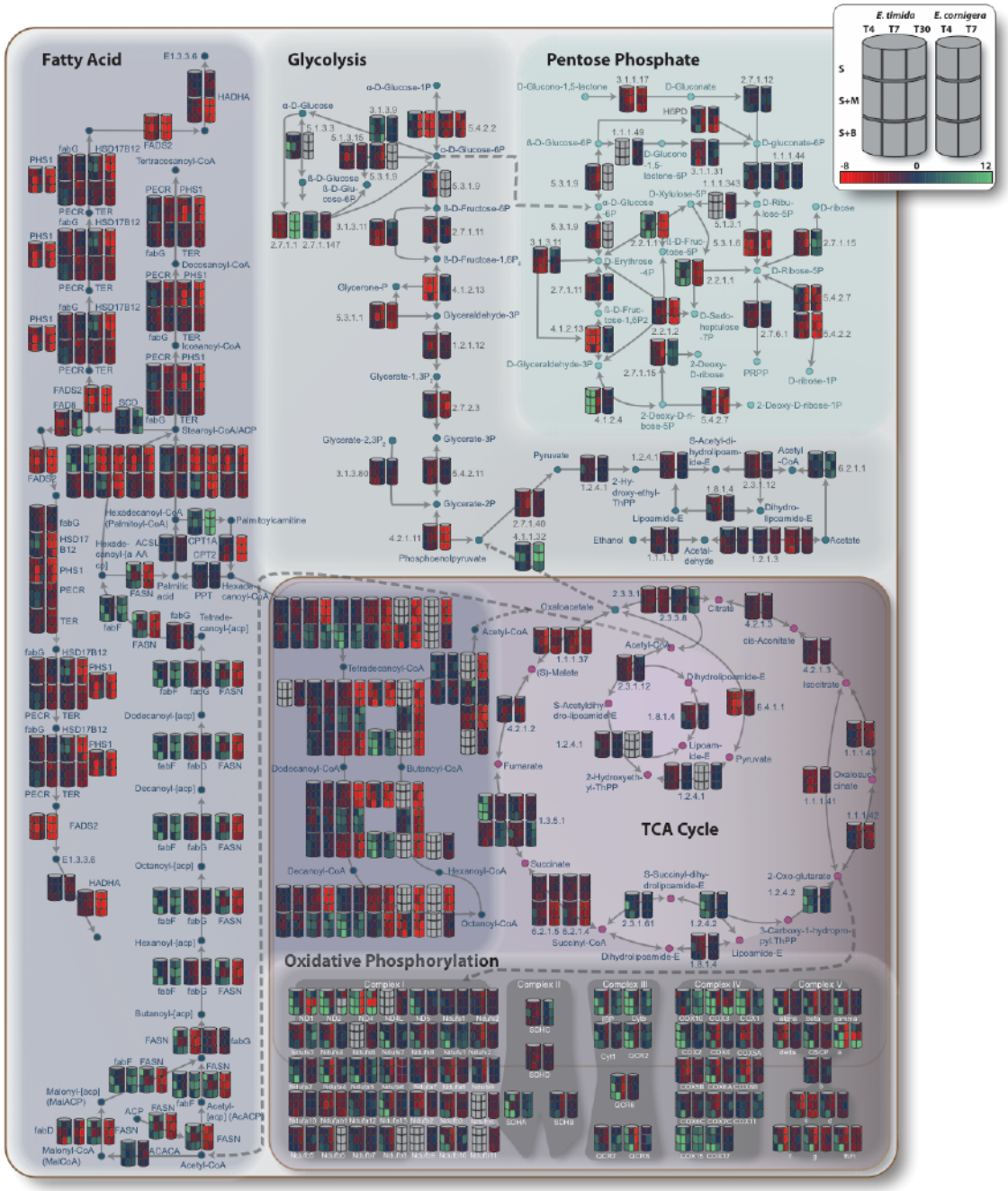

Figure 5.10. Gene expression shifts of mitochondria-associated metabolic pathways (supplementary figure of Rauch et al. 2017b). A reconstruction based on KEGG of the main energy metabolic pathways. Gene expression levels relative to the fed state for starving *E. cornigera* and *E. timda* (for 4 (T4), 7 (T7) and 30 (T30) days) are presented in the coloured barrels; with red indicating down regulation and green upregulation. Grey coloured barrels indicated no detectable gene expression. Individual barrels represent enzymes; multiple barrels represent a protein complex (e.g. fabf/fabG/FASN in the fatty acid pathway). S, starvation; S+M, starvation plus 2 µg/ml monolinuron; S+B, starvation plus bleaching at 1 mmol quanta m^{-2} s^{-1} for 1 hour each day.

Publication IV: Mitochondrial genome assemblies of *Elysia timida* and *Elysia cornigera* and the response of mitochondrion-associated metabolism during starvation

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The presented manuscript was peer reviewed and published in the Journal 'Genome Biology and Evolution' with impact factor 4.1, on 01 July 2017.

Contribution as first author major: Wrote parts of the manuscript, analysed the data, prepared the figures, contributed to the review of the literature and reviewed and prepared the final version.

The supplementary material is available online.

Mitochondrial Genome Assemblies of *Elysia timida* and *Elysia cornigera* and the Response of Mitochondrion-Associated Metabolism during Starvation

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Accepted: July 8, 2017

Data deposition: This project has been deposited at Genbank under the accession numbers KU174945 and KU174946.

Abstract

Some sacoglossan sea slugs sequester functional plastids (kleptoplasts) from their food, which continue to fix CO_2 in a light dependent manner inside the animals. In plants and algae, plastid and mitochondrial metabolism are linked in ways that reach beyond the provision of energy-rich carbon compounds through photosynthesis, but how slug mitochondria respond to starvation or alterations in plastid biochemistry has not been explored. We assembled the mitochondrial genomes of the plastid-sequestering sea slugs *Elysia timida* and *Elysia cornigera* from RNA-Seq data that was complemented with standard sequencing of mitochondrial DNA through primer walking. Our data confirm the sister species relationship of the two Sacoglossa and from the analysis of changes in mitochondrial-associated metabolism during starvation we speculate that kleptoplasts might aid in the rerouting or recycling of reducing power independent of, yet maybe improved by, photosynthesis.

Key words: photosynthetic slugs, mitochondrial genomes, energy metabolism, starvation, ROS stress.

Introduction

Sacoglossan sea slugs, with very few exceptions, feed on siphonaceous algae by piercing their cell walls and sucking out the cytosolic content. Most species can specifically sequester the plastids, known as kleptoplasts, from the nutriment mix (Trench 1969). Of the roughly 400 Sacoglossa species that are described (Jensen 2007; Martin and Wägele 2014), about 75 species are able to retain kleptoplasts that continue to fix CO_2 in a light-dependent manner in the cytosol of cells that form the digestive tract (Trench et al. 1973, 1974; Hinde 1978; Christa, de Vries, 2014; Christa, Zimorski, et al. 2014; de Vries et al. 2015). How the kleptoplasts stay active remains a key question, as it occurs in the absence of algal nuclear support (Wägele et al. 2011; Bhattacharya et al. 2013; Rauch et al. 2015) commonly thought to be essential.

The motive behind kleptoplasty in sacoglossan sea slugs remains elusive and the importance of on-going photosynthesis and CO_2 fixation is under debate (Christa, Zimorski, et al. 2014; Pierce et al. 2015; de Vries et al. 2015; Laetz et al. 2017). It remains uncertain to what degree CO_2 fixation contributes to the overall energy budget of the slugs and alternative reasons are rarely considered (Christa, de Vries, et al. 2014; de Vries et al. 2014). A comparison of the sister species *Elysia timida* and *Elysia cornigera*, which both feed on *Acetabularia acetabulum*, demonstrated that *E. cornigera* dies in the presence of CO_2 fixing kleptoplasts, and therefore in the presence of accumulated ROS, while *E. timida* endured starvation possibly through the suppression of reactive oxygen species (ROS) (de Vries et al. 2015).

Investigating kleptoplasty in Sacoglossa usually focuses on the performance of the kleptoplasts. From work on plants it is known that plastid and mitochondrial function are connected (Hoefnagel et al. 1998), but the effect of kleptoplasts and food deprivation on sea slug mitochondria has not yet been

explored. Here, we present the mitochondrial genomes of two sister species of Sacoglossa and a set of nuclear-encoded, mitochondrion targeted proteins whose expression changes we monitored simultaneously under different conditions. Our data seek to offer new resources and a new angle from which to study how kleptoplasts are being kept active by photosynthetic sea slugs.

Materials and Methods

Cultivation and Microscopy

Elysia timida was collected on Giglio (Italy; 42°22′ N, 10°52′ E and 42°21′ N, 10°52′ E) between 3 and 6 m depth and *Elysia cornigera* was collected on Spanish Harbor Key (Florida Keys, USA 24°38′ N, 81°18′ W) at up to 1m depth. Both *E. timida* and *E. cornigera* were reared at 21 °C under a 12hL:12hD rhythm at 25 µmol quanta m^{-2} s^{-1} in artificial sea water (ASW; 3.7% salinity, Tropic Marine) including water change every other day. For imaging, 1 week starved specimens of *E. timida* and *E. cornigera* were stained for 45 min with 2 µM MitoTracker Red CMXRos (excitation/emission HeNe 543/599 nm; LifeTechnologies) in 3.7% ASW, rinsed twice with ASW and decapitated. Confocal laser scanning microscopy was carried out with a Zeiss LSM 710. Images were processed with Fiji/ImageJ 1.48f (Schindelin et al. 2012).

Mitochondrial Genome Assembly

Mitochondrial (mt) genomes were primarily assembled from RNA-Seq data (de Vries et al. 2015) using Sequencher (Sequencher v. 5.3, Gene Codes Corporation, USA) and standard assembly settings. In addition, continuous stretches of ~6.5 kb of *E. timida* and ~12 kb of *E. cornigera* mitochondrial DNA were sequenced by primer walking to close gaps and compare RNA and DNA sequences. Genomic DNA was extracted with Plant DNAzol (ThermoFisher) and Phusion High-Fidelity DNA polymerase (New England Biolabs) used for standard PCR reactions. Amplification products were sequenced and fed into the Sequencher assembly. The sequences were found to be close to identical—but to 100% in terms of contiguity—to those of the sequenced RNA, with only occasional differences in base calls (about 3 per 1kbp), but no larger gaps indicating potential introns. Due to the nature of the samples it is not possible to distinguish between, for example, single-nucleotide polymorphisms or RNA editing, but if the latter is occurring at all, frequencies would be marginal. The average sequence coverage was 508,168 and 210,540 for *E. timida* and *E. cornigera*, respectively. Gene annotation was performed using MITOS (Bernt et al. 2013) and Geneious 8.0.3 (Biomatters, New Zeeland, Kearse et al. 2012) with the mt genomes of *Elysia chlorotica* (Rumpho et al. 2008), *Thuridilla gracilis* (Medina et al. 2011), and *Placida* sp. (Fan 2013) as references. Mitochondrial genome maps were generated using Organellar GenomeDRAW (Lohse et al. 2013).

Phylogenomics

For every protein-coding gene of the mitochondria we performed individual amino acid sequence alignments with Geneious 8.0.3 (Biomatters, New Zeeland, Kearse et al. 2012) and using *Siphonaria pectinata* (AY345049) as the outgroup. Alignments were performed using Fast Fourier Transform (MAFFT; Katoh and Standley 2013) with the G-INSI mode, inspected by Aliscore (Misof and Misof 2009) and conspicuous sites removed. All individual alignments were concatenated and a phylogenetic reconstruction performed using RaxML (Stamatakis 2006) with the LG + I + G + F model (four discrete gamma categories and sites), as suggest by ProtTest analyses (Abascal et al. 2005), and 1,000 bootstrap replicates.

Metabolic Pathway Mapping

Data of expressed genes involved in the mitochondrial metabolism of both *E. timida* and *E. cornigera* were extracted from a previous study (de Vries et al. 2015), based on their KEGG annotations (Ogata et al. 1999). In cases where KEGG IDs (e.g., K02262 representing COX3) represented multiple unigene IDs (Eti019163, Eti001642, and Eti000008), the sum of the expression values (in FPKM) were taken and fold changes calculated by $\log_2$ of the FPKM expression value divided by the control (the average of triplicates). Hence, the unigene ID with the highest expression has the most influence on the average expression.

Results and Discussion

The Mitochondrial Genomes of *E. cornigera* and *E. timida* and Phylogenomic Analysis

We assembled the mitochondrial (mt) genomes of *E. cornigera* and *E. timida* based largely on transcriptome data. For both species, three gaps of about 50 bp, 500 bp, and 1 kbp were closed by PCR and Sanger sequencing, but in principle it demonstrates the expression of almost the entire mt genomes, which is common for genomes of both plastids and mitochondria (Smith and Lima 2016; Tian and Smith 2016). We obtained 14,118 and 14,088 bp of assembled, circular mt genomic sequences for *E. cornigera* and *E. timida*, respectively (fig. 1a). The two fully assembled slug mt genomes fit well within the conserved nature of the mt genomes of all animals (Boore 1999): both contain two ribosomal RNAs, 22 transfer RNAs and 13 protein-encoding genes (see supplementary table 1, Supplementary Material online). The mt genomes of the slugs are syntenic (fig. 1a) and match the general pattern of mt genome arrangement among other Sacoglossa (Medina et al. 2011; Greve et al. 2017). On a nucleotide level, the mt genomes of *E. cornigera* and *E. timida* have a pairwise identity of 86.2% and *E. timida* a pairwise identity of 71.1% to *Elysia ornata*, which brachnches basal to

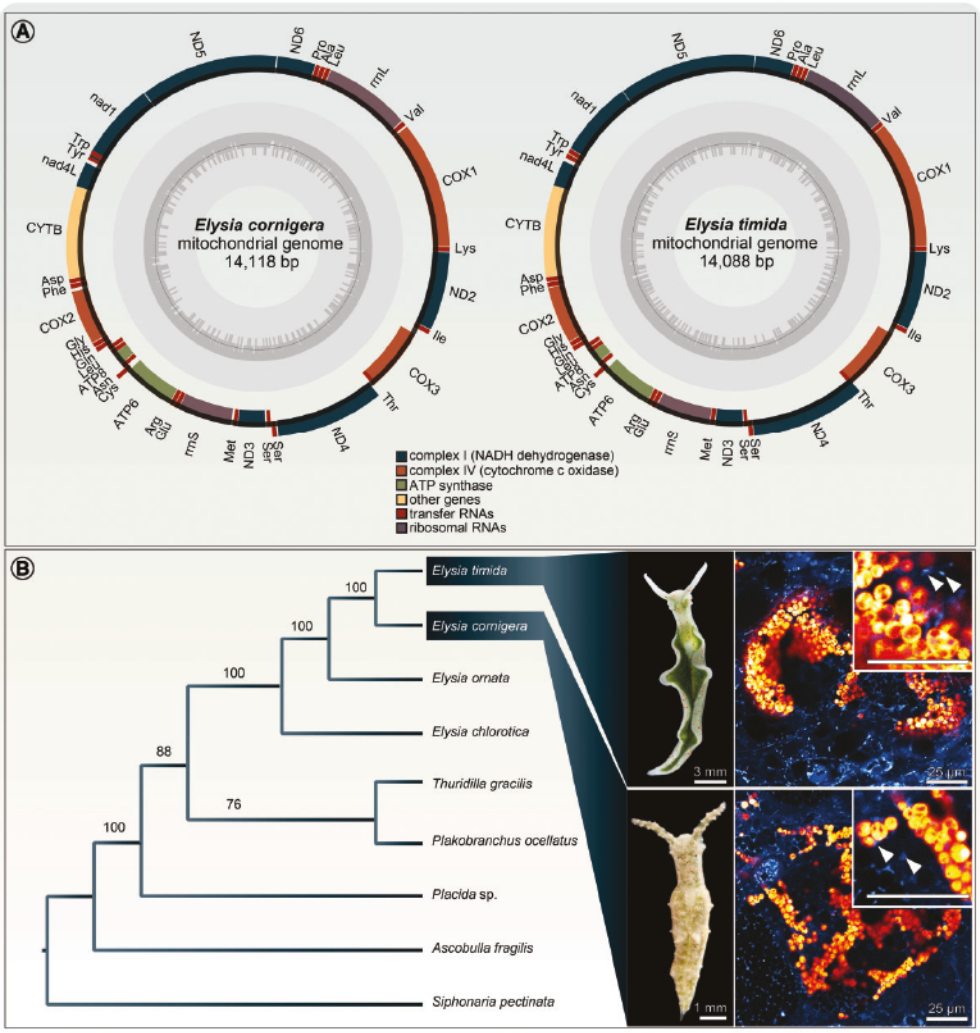

Fig. 1.—Mitochondrial genomes and sacoglossan phylogeny. (*a*) Maps of the two circular and syntenic mitochondrial genomes of *Elysia cornigera* and *Elysia timida* (accession numbers KU174945 and KU174946, respectively). The grey inner circles show the GC skew and the dark grey line marks the 50% GC threshold. (*b*) Phylogenetic tree of eight Sacoglossa and the false limpet *Siphonaria pectinata* as the outgroup, which corroborates the sister species relationship of *E. cornigera* and *E. timida*. Numbers indicate bootstrap values. The right panel shows of freshly fed slugs and confocal laser scanning micrographs of digestive tubules in which the kleptoplasts (false-coloured red-hot) are sequestered. Top right boxes are blow ups of digestive tubules with arrowheads pointing at mitochondria (blue) that often reside in close proximity to the kleptoplasts.

the two congener species in the phylogenetic tree (fig. 1*b*). Based on the 13 mt protein-coding genes and including all available sequences of complete Sacoglossa mt genomes, we performed a phylogenetic analysis. This supports the sister taxon relationship of *E. timida* and *E. cornigera* (fig. 1*b*) and is in line with a previous study (Christa et al. 2015). Our phylogenetic data furthermore supports the observed relationship of *Plakobranchus* and *Thuridilla* as a sister group to *Elysia* sp.

(Greve et al. 2017), which stands in contrast to an earlier study also based on mt genome analysis (Fan 2013).

Regulation of the Main Energy Pathways during Starvation

To study the effects of starvation on the core energy metabolism of plastid-housing slugs, we made use of global gene expression profiles of *E. timida* and *E. cornigera* specimens that had been exposed to three different conditions: starvation alone (S), starvation with the addition of the photosynthesis inhibitor drug monolinuron (S + M; cf. Arrhenius et al. 2004), and starvation with a 1-h daily high light treatment (S + B) with 1,000 µmol m^{-2} s^{-1}. From this data set, we extracted major pathways associated with mitochondrial physiology and metabolism such as the TCA cycle, the glycolytic pathway and oxidative phosphorylation pathway (OXPHOS). For the analysis only those genes were taken into account that were upregulated ($log_2[FC_{condition/t0}] \geq 1$) in comparison to the unstressed, fed state of the slugs. The trend for the gene regulation of the pentose phosphate pathway, the glycolysis pathway, the fatty acid pathway, and the tricarboxy acid cycle (TCA) all showed a similar pattern for both species and among the three different starvation treatments. In general, in *E. timida* the upregulating of these pathways tended to be more pronounced during the first days of starvation, while during prolonged starvation this upregulation ceased (supplementary fig. 1, Supplementary Material online).

High Light Stress Alters the Expression of the OXPHOS Pathway in *E. timida*

When *E. timida* is deprived of its food source, 35% of the proteins of the OXPHOS machinery are up-regulated after 4 days of starvation (fig. 2). For the up-regulation of the OXPHOS pathway to be functional, it requires a constant influx of reducing equivalents. Simultaneously, however, we observed a downregulation of the TCA cycle. This raises the question of the source of alternative reducing equivalents in a starving and plastid-housing slug. In many eukaryotes, reducing equivalents can be imported via the malate-aspartate and glycerol phosphate shuttle (Eto et al. 1999). In the slugs, most genes coding for proteins involved in these shuttles were downregulated after 4 days of starvation (dos) (supplementary table 2, Supplementary Material online), but it remains a possibility that the import of additional reducing equivalents from functional kleptoplasts further delays the shutdown of the OXPHOS pathway, like it occurs in plant cells (Hoefnagel et al. 1998). The latter is important, because any kind of OXPHOS pathway destabilization fosters the generation of ROS (Zorov et al. 2014).

Blocking of photosynthesis through cadmium (Cd) in *Arabidopsis* leads to an outburst of ROS (Bi et al. 2009). Like the Cd treatment, the treatment with monolinuron manipulates the flow of the electron-transport-chain (ETC), likely leading to the generation of problematic ROS levels. The latter could trigger the upregulation of the OXPHOS pathway of the mitochondria, which has been described to curb ROS-induced damage (Tanaka and Hanaoka 2013). In *E. cornigera*, the increased regulation of the OXPHOS pathway is maintained when the kleptoplasts are high light-stressed (23% and 27% after 4 and 7 dos, respectively; fig. 2b) and *E. timida* sustains a high level of regulation for the first week of starvation that ceases at the end of the starving period (78% and 81% after 4 and 7 dos, respectively, and 10% after 30 dos; fig. 2*b*).

When the kleptoplasts experience abiotic stress such as high light or a interference of photosynthesis function through monolinuron, it likely increases H_2O_2 levels like it does in plant cells, in which it is known that high light-derived ROS is a major hub in stress signalling (Miller et al. 2010; Apel and Hirt 2004; Shapiguzov et al. 2012). H_2O_2 is also indirectly generated through the ETC of the mitochondrial OXPHOS pathway (Murphy 2009) and perceived as a signal by the cell (Lee et al. 2011). It might be that the epithelial cells which harbour kleptoplasts cannot distinguish between H_2O_2 originated from the stolen organelle and its own mitochondria in the cell the same way plants can (Sewelam et al. 2014); they might solely receive it as a sign of a damaged mitochondria ETC. As a result, the turnover rate of the main ETC enzymes complexes is increased (Sharma et al. 2012), which in *E. timida* results in less ROS production from the mitochondria, because damaged ETC components are being replaced faster (D'Autreaux and Toledano 2007). The cell might register the ROS released by the kleptoplasts as retrograde signals from the mitochondria (Plecitá-Hlavatá and Ježek 2016). The ROS of two sources might booster the repair of one critical pathway through mechanisms common to eukaryotic cells (Sharma et al. 2012; Choudhury et al. 2016).

Conclusion

Research on sacoglossan sea slugs has focused to a large degree on the photosynthetic capacity of the sequestered kleptoplasts and their potential contribution to tolerate starvation through providing a source of energy-rich carbon compounds. Starvation tolerance in Sacoglossa, however, is evidently more complex and other physiological processes should be considered. Our mitochondrial genome data confirm the phylogenetic relationship of the two Sacoglossa, whose comparative analysis has proven useful in the past (de Vries et al. 2015). Based upon that data, our mitochondrion-focused screen for gene expression changes suggests that starving Sacoglossa uphold and even increase the expression of genes encoding for the OXPHOS pathway. Here, kleptoplasts might come into play by providing reducing equivalents for the animal's mitochondria, herby supporting ongoing ATP production and furthermore suppressing ETC-induced ROS stress. ROS production is likely also higher due to the presence of photosynthesising kleptoplasts, which might

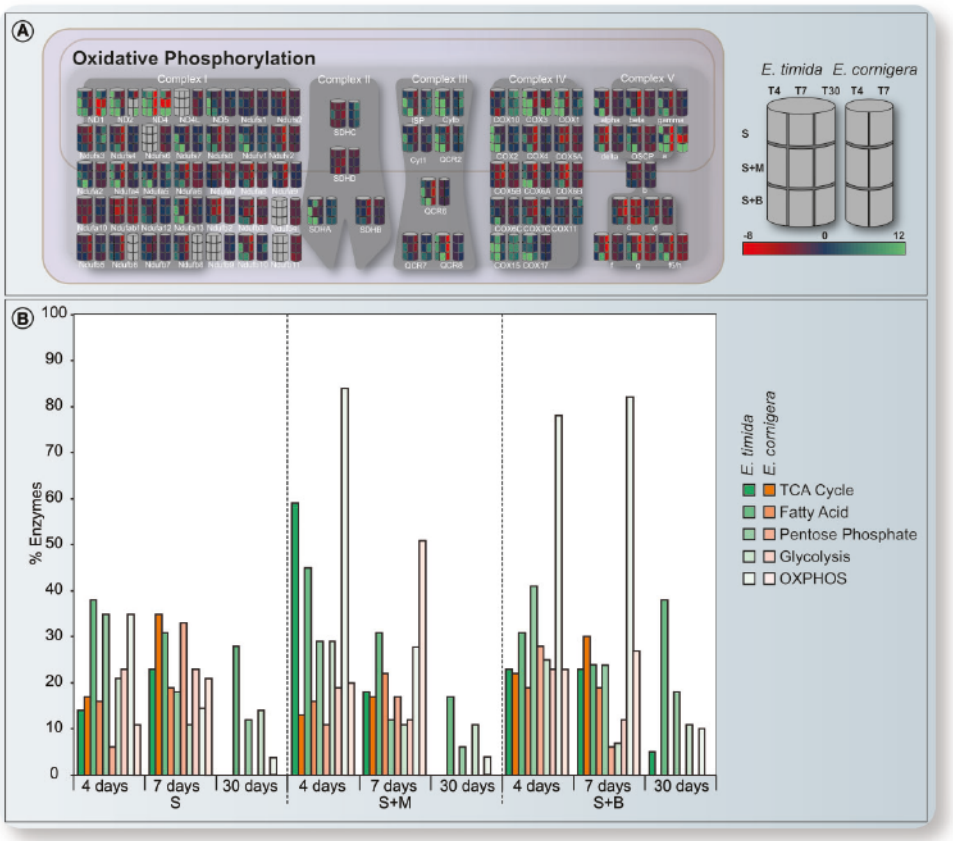

Fig. 2.—Gene expression changes in the OXPHOS and other mitochondrion-related pathways. (*a*) Gene expression shifts of the OXPHOS metabolic pathway. KEGG-based map of oxidative phosphorylation. Coloured "barrels" displays gene expression levels for starving *E. cornigera* and *E. timida* starved for 4, 7, and 30 days (T4, T7, T30) relative to the freshly fed state; upregulation is shown from green to blue, downregulation from blue to red, and grey boxes indicate no detectable gene expression for the given condition. Each barrel represents an enzyme, with the wider barrels representing *E. timida* due the extra data point of day 30 (T30). Multiple barrels at one step in the metabolic pathway represent a protein complex (e.g., complex I); note that one enzyme (or complex) can act at multiple steps in the pathway and in different complex compositions. For every enzyme (complex) the two species are shown adjacent to each other for comparison. Relevant substrates are indicated by dots and labelled with their common name. Note some steps of the fatty acid pathway occur in the mitochondrion. (*b*) Frequency of upregulation among metabolic pathway-associated gene expression. Bardiagram of the percentage of upregulation (relative to T_0: $\log_2[FC_{condition/T0}] \geq 1$) of gene expression associated with the 5 main mitochondrial energy metabolism pathways; all enzymes of one pathway are added up to represent 100%. Note that only in the case of oxidative phosphorylation (OXPHOS) in *E. timida* (and with either monolinuron or bleaching treatment), 80–100% of the associated enzymes are observed to be upregulated. This might reflect a response of the animal to an increase of reactive oxygen species actually stemming mainly from abiotically stressed kleptoplasts and not the mitochondria themselves. For details please refer to the text. TCA, tricarboxy acid cycle; S, starvation alone; S + M, starvation with additional 2 µg/ml of the photosynthesis inhibitor drug monolinuron; S + B, starvation with a 1-h daily high light (1,000 µmol m^{-2} s^{-1}) treatment.

enhance the endogenous response in the animal's cells that respond to alleged mitochondrial stress. The latter possibility is supported by the similar, but enhanced response observed upon abiotic stress. Our results suggest that the integration of kleptoplasts is maybe more involved then so far considered. This encourages to further investigate to which degree the interplay of kleptoplasts and animal mitochondria are linked in ways that resemble those studied in algae and plant cells.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

We thank Margarete Stracke for assistance in culturing, Steffen Köhler (CAi, HHU) for slug photography. Funding through the Deutsche Forschungsgemeinschaft (GO1825/4-1 to S.B.G., VR132/1-1 to J.d.V.), Foundation of Science and Technology (SFRH/BPD/109892/2015 to G.C.), and the European Research Council (ERC 666053 to Prof William F. Martin) is gratefully acknowledged.

Ethical Approval

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

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Associate editor: John McCutcheon

5.2 Living and surviving without retaining active kleptoplasts

Studies within sacoglossan research often focused on how kleptoplasts could benefit the slugs. These studies tested a broad spectrum of different ideas; i.a. for the synthesis of mucus or the benefits of produced photosynthates, oxygen, nitrogen, lipids, nutrients, metabolites and/or glucose or for aiding development and reproduction etc. (Trench et al. 1970; Taylor 1971; Hinde and Smith 1972; Trench et al. 1974b; Marín and Ros 1992; Wägele and Johnson 2001; Cueto et al 2005; Casalduero and Muniain 2006; Baumgartner et al. 2014; Pelletreau et al. 2014). Thereby a variety of experimental setups was used; starving the slugs, analysing their pigments, keeping slugs in the dark, blocking photosynthesis, measuring photosynthetic activity by pulse amplitude modulation measurements, track the slugs weight and size, measured radiolabelled fixed carbon, etc. (Trench 1969, Taylor 1971, Hinde and Smith 1972; Gallop et al. 1980, Händeler et al. 2009; Christa et al. 2014; de Vries et al. 2015; Laetz et al. 2017). Despite the variety of experiments and ideas, none of these studies were able to pinpoint out the exact benefits of the functional kleptoplasts for the slugs. But they have one thing in common; they lacked a proper comparison between different species of slugs.

That is why in 2015 de Vries and colleagues conducted a study in which the short-term retention (StR) species *Elysia cornigera* was compared with the long-term retention (LtR) species *Elysia timida*. These two are special in more than one-way: they are sister taxa, feed on the same algae food source and both species are easy to culture under laboratory conditions. This way the researchers could have a controlled environment and have the same conditions for all the biological replicates. When starvation was induced they found why the StR species died earlier of starvation than the LtR species (de Vries et al. 2015). This was likely due to the way the two species responded differently to starving cells that triggered the accumulation of reactive oxygen species. By using two different species sequestering plastids from the same algae, researchers could observe how much the kleptoplasts possibly influenced the survival rate of the slugs during starvation and their retention form.

Different from *E. cornigera* and *E. timida*, that feed on one single algae species, there is also an opposite model, in which one species of slug feeds on multiple algae species. *Elysia viridis* is such a polyphagous species and feeds on algae from a variety of genera;

like *Codium*, *Chaetomorpha*, *Cladophora* and *Bryopsis* (Baumgartner et al. 2014; Baumgartner et al. 2015; Rauch et al. 2018). In the following paper, *E. viridis* was fed with either *Cladophora* sp. or *Bryopsis hypnoides*. Whilst it was still unknown whether the slugs could incorporate the plastids from different algae species, what the longevity of the kleptoplasts would be and whether it had any influence on the slugs' ability to survive starvation. The results showed us that *E. viridis* is able to either bear functional kleptoplasts or does not incorporate the plastids of its food source at all, however, the starvation period is not influenced. Apparently, the nutritional contribution of the kleptoplasts to the slugs' overall energy demands during starvation is not an inevitable requirement to survive (Rauch et al. 2018).

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

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The presented manuscript was peer revieuwed and published in the Journal 'Marine Biology' with impact factor 2.4, on 07 April 2018.

Contribution as the first author major: Wrote the manuscript, produced and analysed the data, prepared the figures, contributed to the review of literature and reviewed and approved the final version.

Supplementary material available online.

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

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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Published online: 07 April 2018

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).

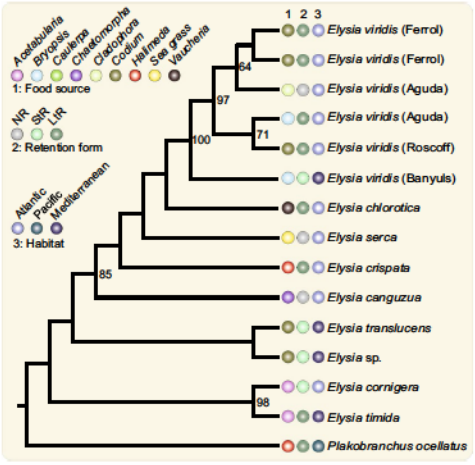

Fig. 1 Phylogenetic relationship of the *Elysia viridis* individuals collected at different sampling sites. Depending on the food source it consumes and according to the literature, *E. viridis* is either considered a long-term (LtR) or a non-incorporation (NI) form. For details on the latter, please 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

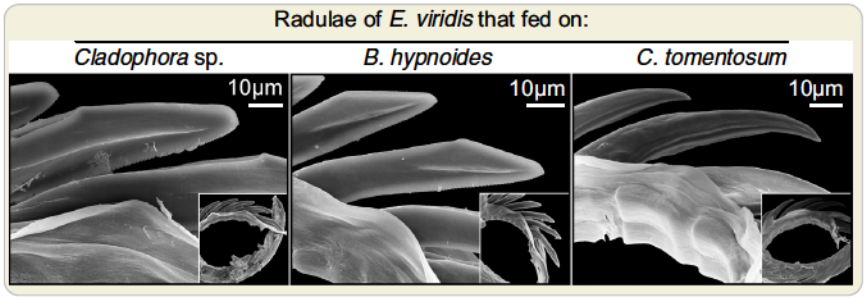

Fig. 2 The radulae, in Sacoglossa specialized teeth organized in a row, of *Elysia viridis* are highly variable and differ depending on the food source

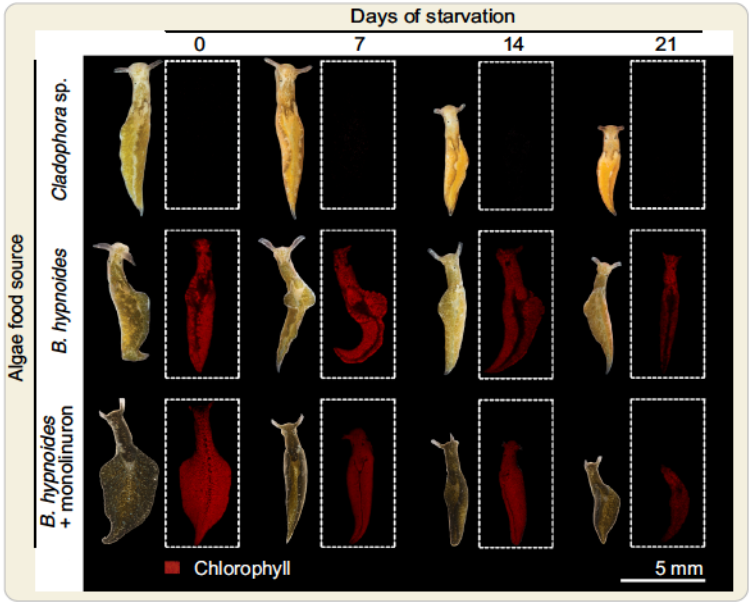

Fig. 3 Macroscopic chlorophyll fluorescence images of specimens of *Elysia viridis* fed for 1 month on either *Bryopsis hypnoides* or *Cladophora* sp. (T0) and after the number of days of starvation. Kleptoplasts of *Bryopsis hypnoides* are present throughout the body in *Elysia viridis* which give the 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

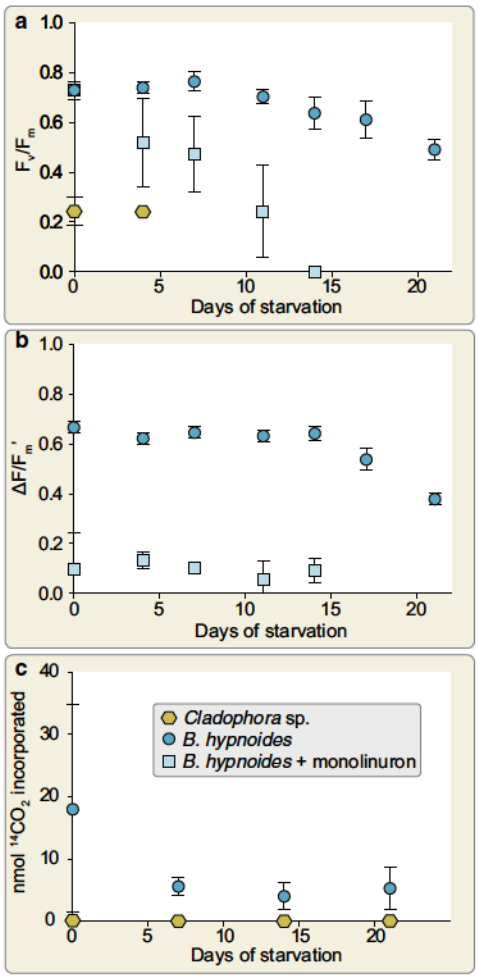

Fig. 4 Maximum quantum yield (F_v/F_m), photosynthetic yield and $^{14}CO_2$ fixation of kleptoplasts in *Elysia viridis* during a 21-day starvation period. **a** The maximum quantum yield F_v/F_m of kleptoplasts derived from *Bryopsis hypnoides* (blue circles) starts to decline after 11 days of starvation, 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.

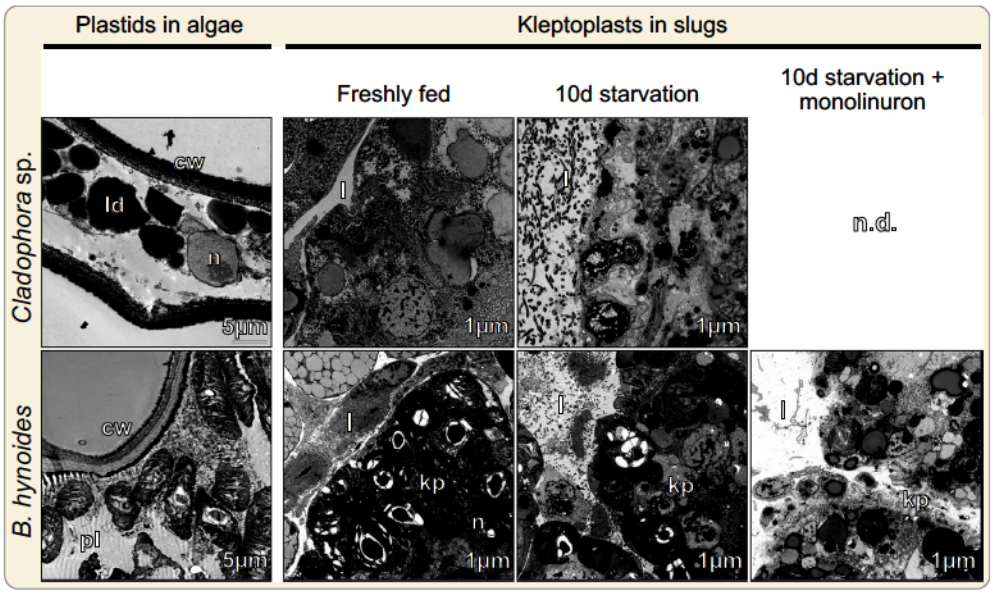

Fig. 5 Transmission electron microscopy (TEM) images of the food algae *Bryopsis* and *Cladophora* and of the digestive tract cells of the sea slugs that had fed on the respective algae. Note the distinct difference between slugs that had fed on *Cladophora* and those that had fed on *Bryopsis*. Only 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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6. Summary of results

Part I, chapter 3.1: RNA sequencing data are available for several species of slugs (Rumpho et al. 2011; Wägele et al. 2010; Pierce et al. 2012 and de Vries et al. 2015). In all cases, the sequencing reads indicate transcript of laterally transferred genes (lateral gene transfer; LGT) that are close to zero. For example, Pierce et al. (2012) performed RNA-seq data analyses of the long term retention species *Elysia chlorotica* and reported two among the 98,238,204 reads for a single algal nuclear gene that were probably the results of LGT. In relation to the percentage of nuclear mRNA transcript linked with photosynthesis in plants, which is 20% (Bhalerao et al. 2013) as compared to 0.0001% in the here studied slugs. de Vries and colleagues (2015) sequenced the reads for the species *Elysia cornigera* and *Elysia timida* and this resulted in an output of protist reads exceeding the reads of algal origin. Besides the algal reads declined over the period of starvation (de Vries et al. 2015; Rauch et al. 2017b).

Part I, chapter 3.2: The absence of algae genes in the slugs' nuclear genome questioned the photosynthetic ability of the 'crawling leaves'. By comparing the CO_2 fixation data from former studies the percentage of carbon contribution of the kleptoplasts to the slugs were calculated (Rauch et al. 2017a). The example species used here, *E. timida*, has on average developmental time of 84 days (Schmitt et al. 2014). During that time the incorporated kleptoplasts contribute about 1% to the overall slugs' carbon (540 ng of carbon a day or 0.01% of total carbon) (figure 6.11; Christa et al. 2014b). Including the size of *E. timida* (0.5%) and the carbon turnover rate of 12-days (van der Zanden et al. 2015), it turns out that roughly 0.25% carbon is contributed by the CO_2 fixating kleptoplasts (figure 6.12).

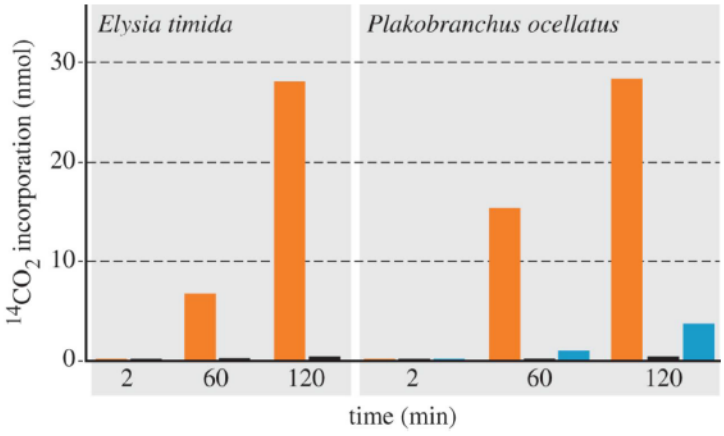

Figure 6.11. CO_2 fixation data of the LtR species *Elysia timida* and *Plakobranchus* ocellatus (Christa et al. 2014b). For *E. timida* this reads roughly 30 nmol $^{14}CO_2$ incorporation in 2 hours for four animals. This translates into 45 nmol C per animal, which is 540 ng of Carbon per 12 hour-daylight days (Rauch et al. 2017a).

Part II, chapter 4.1: Studies on kleptoplasty in sea slugs are mostly from the plastids perspective, however, from plant research it is known that plastids and mitochondria are coupled (Hoefnagel et al. 1998). Rauch and colleagues (2017b) studied the effects of starvation on the sea slugs' energy metabolism. With help of the earlier published transcriptome data of the species *E. cornigera* and *E. timida* by de Vries et al. (2015) the expressed genes involved in the energy metabolism based on KEGG were mapped (de Vries et al. 2015; Rauch et al. 2017b). It was observed how high- light stress increased the gene expression of the oxidative phosphorylation (OXPHOS) pathway in *E. timida*. In *E. cornigera* the OXPHOS pathway had a slightly increased gene expression during starvation (23 – 27% increase). However, *E. timida* reacted with a much higher level of expression (78 – 81%) in the same starvation period.

Part II, chapter 4.2: With regard to other species, besides *E. timida* and *E. cornigera*, the polyphagous slug *Elysia viridis* was also studied while feeding on different algae. It was observed how the slugs' kleptoplasts Fv/Fm values declined over time (dropped to 0.491±0.043) and CO_2 fixation decreased (70%) when fed on *Bryospis hypnoides*. This trend resembles that of other species (*E. timida* and *E. cornigera*; de Vries et al. 2015). However, when fed on *Cladophora sp.* there were no Fv/Fm values and CO_2 fixation data measured during starvation. The pulse amplitude modulation measurements, CO_2 incorporation levels and transmission electron microscopy images revealed in this study, show no sequestration of *Cladophora* sp. plastids in *E. viridis* (Rauch et al. 2018).

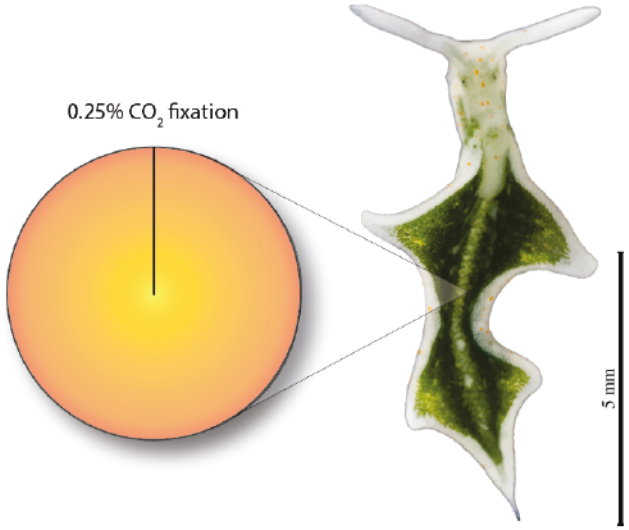

Figure 6.12. Total kleptoplasts carbon contribution. A total of approximately 0.25% carbon was contributed by the CO_2 fixating kleptoplasts to the slug (Rauch et al. 2017a).

7. Concluding remarks

Part I, chapter 3.1: The RNA sequencing results of *Elysia chlorotica*, *Elysia timida* and *Elysia cornigera* (Pierce et al. 2012; de Vries et al. 2015) resulted in sequencing reads of algae lateral gene transfer (LGT) origin close to zero. Furthermore, the slugs collected and analysed are from the wild and kept in open aquaria with their algae food source; the slugs are everything except axenic. These factors point clearly towards a result of contamination rather than proof of LGT. The 52 genes described by Pierce and colleagues (2012), if transferred to the slugs' nuclear genome, are expressed 200,000 times lower than the minimum needed for photosynthesis (Bhalerao et al. 2003; Pierce et al. 2012; Rauch et al. 2015). Another study of the same species (*E. chlorotica*; Bhattacharya et al. 2013) likewise found no evidence of algal LGT to the slugs' genome. These results explain that there are no laterally transferred genes of algae origin in the slugs' nuclear genome (Rauch et al. 2015).

Part I, chapter 3.2: The previous chapter goes together with the observation that the slugs are not photoautotrophic when it comes down to the carbon contribution of its kleptoplast; a mere 0.25% of carbon (Rauch et al. 2017a). This very well explains why plastid-harbouring slugs have been observed to lose weight in the light at the same rate as in the dark or when chemically inhibited during starvation (Christa et al. 2014b). It seems that the research focus on the photosynthetic capacity of the kleptoplasts and their contribution as a source of carbon is gradually changing.

Part II, chapter 4.1: Starvation involves more than just the kleptoplasts; since it was observed that the gene expression of the oxidative phosphorylation (OXPHOS) pathway in some slug species is being upregulated during starvation (de Vries et al. 2015; Rauch et al. 2017b). This points towards kleptoplasts that could function as an extra source of reducing equivalents or as an extra source of cell signalling reactive oxygen species (ROS). It seems that *E. timida* is able to recognize ROS of both the kleptoplasts and its own mitochondria. After recognition it either catalases the ROS and/or reacts by increasing the protein turn over of its OXPHOS pathways, which on its turn results in reduced ROS production by the mitochondria. By controlling elevated ROS levels caused by stress, *E. timida* is therefore able to endure cellular starvation longer than its sister species *E. cornigera* (de Vries et al. 2015; Rauch et al. 2017b).

Part II, chapter 4.2: With the most recent study, it was observed how the sea slug *Elysia viridis* is only able to incorporate plastids depending on the algae food source it consumes. When feeding on *Cladophora* sp. no Fv/Fm and CO_2 fixation values were detected. This was later confirmed by the use of transmission electron microscopy; no kleptoplasts from *Cladophora* sp. were observed. Which made it clear that *E. viridis* is unable to sequester plastids from *Cladophora* sp. This makes it the first sacoglossan species that is able to survive and proliferate with and without kleptoplasts. These results show that the reason for the sequestration of functional kleptoplasts is not explained by photosynthesis and carbon fixation alone (Rauch et al. 2018).

The aim of this dissertation was to (i) investigate what the kleptoplast contributions are to maintain photosynthetically active and (ii) address the question of what benefits the slugs reap from the kleptoplasts. The first chapter presented a general introduction to the topic (chapter 2, de Vries et al. 2014a), including a discourse concerning the many misconceptions inherent to the topic when it comes to LGT. The following chapter demonstrated that only 0.25% of the carbon needs of the slugs are satisfied by CO_2 fixation in kleptoplasts (chapter 3, Rauch et al. 2015; Rauch et al. 2017a).

The last chapter of the dissertation dealt with the possible contributions of the kleptoplasts to the biology of the slugs (chapter 4, Rauch et al 2017b) by comparing mRNA transcript abundance under different physiological conditions. It was observed that the slugs' capability to reduce ROS by increasing protein turnover of its OXPHOS pathway resulted in a longer lifespan during starvation. In this last chapter it is also shown that the polyphagous species *Elysia viridis* is able to live and proliferate without incorporated kleptoplasts. Taken together the findings of this dissertation suggest that functional kleptoplasty of the Sacoglossa during starvation is not a necessity for survival (Rauch et al, 2018).

For future studies, it is important to focus on new species in comparison. Species such as *E. viridis*, which only incorporates kleptoplasts in a food source dependent manner, show clear differences to species such as *E. cornigera* and *E. timida*, which feed on the same plastids and sequester them as kleptoplasts. Sacoglossa are fascinating, but therein lies a risk: The sight of a beautiful animal filled with bright green intact plastids makes one want to believe they are photosynthetic. But we have to remain critical, not

the least towards our own observations and the temptation to draw sensational conclusions, because in science still today the conclusion need to be supported by the data.

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Acknowledgements

*During the writing process of my dissertation, my dear father very sudden passed away. I know that he was always extremely proud of me and I wish I could have celebrated my defence with him. I will forever miss him, especially during all other proud moments throughout my life. 'This man is my creator, he is my God, but I always thought that a creator-God can not die' (from Huub Oosterhuis funeral speech). Dear father, I wish that one day we could meet again; rest in peace - René P. Rauch *15.07.1955 - 31.10.2017†*

I would like to thank all the people directly or indirectly involved with my PhD. Foremost I would like to gratefully thank my supervisors Sven Gould and Bill Martin for the opportunity they gave me. I want to thank them for their endless patience and guidance to me as a starting scientist.

Special thanks to Jan de Vries, Gregor Christa and Sriram Garg for their wisdom, advices, inspiration and laughter, to Maria Handrich for always being ready to help out and tolerate me in our office, Verena Zimorski for her (very much appreciated) organizational support. Many thanks, to Mayo Röttger, for our shared taste in liquorice and computer help. And Madeline, thank you for being such a wonderful travel companion and a very helpful bioinformatician. And finally, I would like to thank all my colleagues and friends of the molecular institute for the past three years for their support and the fun I had to be around you.

Of course, I would like to thank my family; Mom, Elte, Marlen, Artur; thank you for your everlasting support and patience. And my dear Amando, thank you for your serenity and calm attitude. I would also like to thank my old team of Naturalis and Leiden University for their inspiration and the yearly charge up I had with our courses and excursions in France. Thank you Charles H.J.M Fransen, Rinny E. Kooi, Hans A.J. Metz, Herre Stegema, Jan R. Wijbrans and all the others involved.

The list goes on and on and all the people involved during my PhD, you are not forgotten and thank you very much!