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RESEARCH ARTICLE

REVISED Food shaped photosynthesis: Photophysiology of the sea slug *Elysia viridis* fed with two alternative chloroplast donors

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Abstract

Background

Some Sacoglossa sea slugs steal and integrate chloroplasts derived from the algae they feed on into their cells where they continue to function photosynthetically, a process termed kleptoplasty. The stolen chloroplasts – kleptoplasts – can maintain their functionality up to several months and support animal metabolism. However, chloroplast longevity can vary depending on sea slug species and algal donor. In this study, we focused on *Elysia viridis*, a polyphagous species that is mostly found associated with the macroalga *Codium tomentosum*, but that was reported to eat other macroalgae, including *Chaetomorpha* sp.

Methods

We have investigated the changes in *E. viridis* physiology when provided with the two different food sources to evaluate to which extent the photosynthetic and photoprotective mechanisms of the algae chloroplasts matched those of the plastids once in the animal cells. To perform the study, we rely on the evaluation of chlorophyll *a* variable fluorescence to study the photophysiological state of the integrated kleptoplasts and high-performance liquid chromatography (HPLC) to study variations in the photosynthetic pigments.

Results

Open Peer Review

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We observed that the photosynthetic efficiency of *E. viridis* is lower when fed with *Chaetomorpha*. Also, significant differences were observed in the non-photochemical quenching (NPQ) abilities of the sea slugs. While sea slugs fed with *C. tomentosum* react similarly to high-light stress as the alga, *E. viridis* hosting *Chaetomorpha* chloroplasts were unable to properly recover from photoinhibition or perform a functional xanthophyll cycle (XC).

Conclusions

Our results showed that, even if the sea slugs fed with the two algae show photosynthetic activities like the respective algal donors, not all the photoprotective mechanisms present in *Chaetomorpha* can be maintained in *E. viridis*. This indicates that the functionality of the kleptoplasts does not depend solely on their origin but also on the degree of compatibility with the animal species integrating them.

Plain language summary

Sea slugs known as Sacoglossa (also called "solar-powered sea slugs") have a fascinating ability to steal and use chloroplasts from the algae they eat. This process is called kleptoplasty. These stolen chloroplasts, also called kleptoplasts, can remain functional for several months and help the sea slugs with their metabolism by performing photosynthesis like plants. However, the time of chloroplast maintenance can vary depending on the species of sea slug and the type of algae they feed on. In this study, we focused on a species called *Elysia viridis*, which eats various types of algae, including *Codium tomentosum* and *Chaetomorpha* sp. These two algae have different characteristics when it comes to photosynthesis and protection against excessive light. We investigated how the physiology of *E. viridis* changed when it was given these two different food sources. Our results show that sea slugs had similar photosynthetic activities to their respective food alga. However, not all photoprotective mechanisms of *Chaetomorpha* algae could be maintained in *E. viridis*. This suggests that the functionality of the stolen chloroplasts depends not only on their source but also on how well they match the sea slug species that incorporate them.

Keywords

Kleptoplasty, NPQ, photoprotection, Sacoglossa, xanthophyll cycle.

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REVISED **Amendments from Version 1**

In this new version of the manuscript we have addressed the specific comments made by the reviewers. In particular, we provided explanations of our color choices in the graph and corrected several typos evidenced by the reviewer 1. Regarding the concerns addressed by the reviewer 2, we have addressed them in this way: we have corrected the specific typos, we have included more accurate bibliographic references that better aid to focus the point of our discussion, in particular trying to add more references not belonging to the authors' specific work. In the discussion we have rephrased several sentences to avoid statements that could be misleading or not appropriately supported by the data showed in the manuscript. Finally, we have corrected several typos in the reference list and in the figures. We hope to have delivered a more readable and clear version.

Any further responses from the reviewers can be found at the end of the article

Introduction

Photosynthesis is a process used by phototrophic organisms to harness chemical energy from light. Generally attributed to plants and algae, photosynthesis may occur in metazoans that establish endosymbiotic associations with algae. A particular case is that of Sacoglossa sea slugs that feed suctorially on macroalgae and maintain functional chloroplasts in their digestive glands, a process termed kleptoplasty (Greene, 1970; Greene & Muscatine, 1972; Kawaguti & Yamasu, 1965) as the animal effectively "steals" the organelle for its own use. After having pierced the algal cell wall with its radula, the animal sucks and digests all the cellular components, including the nucleus, while the chloroplasts are maintained intact and are integrated in the animal cells. In some species, the stolen chloroplasts – kleptoplasts – can continue to function for several weeks to a few months, despite the absence of algal nuclear genes necessary to encode most chloroplast proteins. The process is often considered an example of endosymbiosis, although the chloroplast itself does not constitute an organism (Dorrell & Howe, 2012).

While in some species of Sacoglossa sea slugs (e.g., *Elysia timida* and *Plakobranchus ocellatus*) kleptoplasts are mostly visible only in some body regions (e.g., the dorsal area), in other species such as *E. viridis*, they are distributed evenly and not concentrated in a specific part of the animal (Gallop *et al.*, 1980). In *E. viridis*, the presence of ramifications of digestive tubules, throughout most of the sea slug's body, contributes to a colour resembling that of its algal food and helps the animal to blend with the environment to escape predators (Hirose, 2005). Moreover, the presence of kleptoplasts could provide additional advantages such as the translocation of photosynthates to other animal cells that do not host kleptoplasts. Recent studies, for example, demonstrated that carbon derived from photosynthesis is translocated to the reproductive organs in two different sea slugs (i.e., *Elysia timida* and *Elysia viridis*) (Cartaxana *et al.*, 2021; Cruz *et al.*, 2020). The availability of "autotrophic powered cells" has multiple advantages for the animals as active photosynthesis can help in minimizing the loss of weight during food shortage, supporting the reproductive output of the sea slugs or increasing mucus production (Cartaxana *et al.*, 2021; Donohoo *et al.*, 2020; Evertsen & Johnsen, 2009; Lopes *et al.*, 2022; Maeda *et al.*, 2021; Shiroyama *et al.*, 2020; Yamamoto *et al.*, 2013).

Acquiring chloroplasts, however, may pose some problems to the animal cells: photosynthesis, especially under stronger light, is often associated with the production of reactive oxygen species (ROS) that inhibit the *de novo* synthesis of proteins (i.e., impairing the repair of photosystem II (PSII)), but also damage DNA and non-photosynthetic host cells (Murata *et al.*, 2007). To tackle damages, photosynthetic organisms are equipped with multiple photoprotective mechanisms that shield the photosynthetic apparatus from damages after light is absorbed by the light-harvesting systems. The prevalent mechanism is the conversion and dissipation of the excess excitation energy as heat, defined as the energy-dependent component of non-photochemical quenching (NPQ), qE. This fast reversible NPQ component depends on an irradiance-dependent proton gradient across the thylakoid membrane that decrease the lumenal pH. In land plants and algae, PsbS protein serves as the sensor that detects these changes and that activates qE quenching in PSII antenna by interacting with light harvesting complexes (LHCII) and inducing conformational changes (Christa *et al.*, 2017; Dall'Osto *et al.*, 2005). qE is further modulated by the activation of a xanthophyll cycle (XC) (i.e., a process where violaxanthin (Vx) is reversibly converted to zeaxanthin (Zx) under high light through the intermediate conversion into antheraxanthin (Ax) by the enzymes violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP) (Goss & Jakob, 2010; Müller *et al.*, 2001). Apart from qE, authors hypothesized the presence of a pH-independent quenching mechanism, based on the direct binding of Zx to the periphery of antenna complexes. This NPQ component, named qZ, would appear later than qE and would use the Zx already synthesized and, as such, would be independent from lumen acidification and subsequent activation of VDE enzyme (Dall'Osto *et al.*, 2005). A fast reversible NPQ component and functional XCs has been reported in kleptoplasts of *E. timida* and *E. chlorotica* (Cartaxana *et al.*, 2019; Cruz *et al.*, 2015) as they are one of the main mechanisms present in the algal donors (i.e., *Acetabularia acetabulum* and *Vaucheria litorea*, respectively). Not all the macroalgae, however, have a functional XC and most of the protoprotective mechanism in this class of organisms are currently unclear. For example, reports suggest that some members of the order Bryopsidales lack a functional XC and do not show qE-type quenching during light stress, but instead accumulate other carotenoids (e.g., all-*trans*-neoxanthin) as a way to shield the reaction centers or to contribute to an efficient energy transfer between photosystem components (Benson & Cobb, 1983; Giossi *et al.*, 2020; Giossi *et al.*, 2021; Li *et al.*, 2023; Zuo *et al.*, 2006).

E. viridis is a polyphagous sacoglossan sea slug (i.e., can eat a wide array of different macroalgae). In the Swedish coast, for example, it has been found foraging on multiple species such as

the septate algae *Cladophora sericea*, *Cladophora rupestris*, *Chaetomorpha melagonium*, and *Ceramium virgatum* (a rhodophyte, differently to all the other mentioned taxa, that are chlorophytes), as well as the siphonaceous species *Codium fragile* and *Bryopsis plumosa* (Baumgartner & Toth, 2014). On the Portuguese coast, *E. viridis* can be found eating the alga *C. tomentosum* and this association, together with its photophysiology, has been extensively characterized (Cruz *et al.*, 2014; Cruz *et al.*, 2015; Rauch *et al.*, 2018). *C. tomentosum* is one of the species that does not show a functional XC and, as such, *E. viridis* has never been reported to be able to functionally replicate such mechanism. Recently, in laboratory conditions, we could observe an active feeding behaviour of *E. viridis* on samples of *Chaetomorpha* sp. associated with the retention of functional chloroplasts. *Chaetomorpha* is structurally very different from *Codium* as it is a filamentous alga that can grow up to several centimeters in length. The thallus has a cylindrical shape and is usually unbranched, loosely entangled and stiff, made by the junction of several squared cells separated by septa (Blair *et al.*, 1982). This characteristic affects the feeding pattern of the animals that must move and pierce every single cell to suck the cytoplasm. *Chaetomorpha* is typically of a bright green colour but can sometimes appear darker or even blackish depending on the growth environment. Interestingly, the toughness of the outer cell wall seems also to be influenced by the intensity of the environmental light, assuming a rigid consistence when the alga is cultured under low irradiance (Krause-Jensen *et al.*, 1996). Moreover, *Chaetomorpha* is reported to have a XC cycle (although its functionality has not been tested) and grown under high irradiances accumulates lutein, showing then a pigment composition different from *C. tomentosum* (Bischof *et al.*, 2006; Christa *et al.*, 2017).

Experimental studies on polyphagous sea slugs fed with specific algal chloroplast donors show that functionality and longevity of kleptoplasts depend on plastid origin (Cartaxana *et al.*, 2023; Curtis *et al.*, 2015). In this work, we compared the photophysiology of the sea slug *E. viridis* fed with the two different algae, investigating to which extent the xanthophyll cycle of the algal donor is conserved in the animals.

Methods

Animals and algae collection and maintenance

Wild sea slug *Elysia viridis* (Montagu, 1804) and the macroalga *Codium tomentosum* Stackhouse, 1797, were collected during low tide in the intertidal rocky area of Aguda beach, Vila Nova de Gaia, Portugal (41°02′50.2″N, 8°39′15.2″W). A minimum of 100 animals were collected in several expeditions, depending on the tide and on the animal availability. The number was decided based on the dedicated space in the laboratory to ensure the best possible maintenance conditions. After collection, the animals and the algae were transported to the laboratory and maintained in 150 L recirculated life support systems (LSS) operated with artificial seawater (ASW) at 18 °C and a salinity of 35 ensured by a refrigerating system (Ultra Titan 200; HAILEA, GuangDong, China). A laboratory adaptation period of 2 weeks was chosen to ensure replicability in feeding and the light history of the animals at the beginning of the experiment. The photoperiod was set to 12 h light:12 h dark, with a photon scalar irradiance of 60–80 µmol photons m^{-2} s^{-1}, at the water surface, being provided by T5 fluorescent lamps. Irradiance was measured in the water with a US-SQS Spherical Micro Quantum Sensor and a ULM-500 Universal Light Meter (Walz, Effeltrich, Germany). *Chaetomorpha* sp. strands were obtained from coral life support systems existing in the same laboratory. Exact origin is unknown. The alga of this study was specifically cultivated in 2 L flasks with f/2 medium (without silica) and constant aeration at 20 °C and an irradiance of 60–80 µmol photons m^{-2} s^{-1} provided by LED lamps (Valoya 35 W, spectrum NS12), for 2 weeks before time zero of the experiment and up to the final feeding moment.

70 animals chosen to be used for the following evaluations were isolated in fish maternity nets in the same LSS with the above-described light conditions to ensure homogeneity of water conditions and a high degree of monitoring. The animals chosen among the original 100 sea slugs collected were the ones visually similar in size and color. Specimens were then provided with a constant supply of food (35 fed with either *C. tomentosum* or *Chaetomorpha* sp.) for 1 month. Approximately 2 g of new algae were provided at least once every 5 days. Variables such as light distribution across the LSS and in different depths of the maternity net were considered providing that the two experimental settings were having the same characteristics. Every day, animals were monitored to ensure that no specimen was isolated in zones of the maternity net without algal food. The animals were weighed at the start and at the end of the feeding phase to estimate the daily weight increase by placing them on a nylon mesh with absorbent paper below, for gently removing the excess of water before weighing on an FH-200 laboratory scale (Waagenet AF GmbH, Berlin, Germany).

Photosynthetic measurements

Fluorescence measurements were carried out on the samples by using an Imaging-PAM fluorometer (Mini version, Heinz Walz GmbH). To efficiently perform the measures on the sea slugs avoiding movements, the animals were blocked in a 0.2% agar solution in ASW before starting the measurements. The solution was prepared by mixing the appropriate concentration of agar in ASW, boiling the mixture, and letting it cool on ice. The solution was collected in an ice-cooled Pasteur pipette, slowly poured on the animals in a petri dish and solidified on a bed of ice before taking the measurements. At the end of the experiment, the animals were carefully removed from the embedding agar by using a Pasteur pipette and moved to a container with ASW to let them recover. They took approximately 15 min to recover full mobility. Effective quantum yield of photosystem II (ΦPSII) was measured as ΔF/Fm', where ΔF corresponds to Fm'- F (the maximum minus the minimum fluorescence of light-exposed organisms). Maximum quantum yield of PSII, Fv/Fm, was calculated as (Fm-Fo)/Fm, where Fm and Fo are, respectively, the maximum and the minimum fluorescence of dark-adapted samples.

For dark acclimation, samples were incubated for at least 30 min in darkness to allow the full relaxation of photosystems. Light curves were constructed with 12 incremental steps of actinic irradiance (E; 0, 15, 37, 55, 82, 136, 176, 238, 371, 446, 538, 649 µmol photons m^{-2} s^{-1}). For each step, ΦPSII was monitored every minute and electron transport rate (ETR) was calculated as E x ΦPSII x 0.84 x 0.5 (where 0.84 is the light absorptance by an average green leaf and 0.5 is the fraction of absorbed quanta available for PSII). The light response and associated parameters ETRm (maximum electron transport rate), Ek (light saturation coefficient) and alpha (photosynthetic rate in light limited region of the light curve) were characterized by fitting iteratively the model of the rETR versus E curves using MS Excel Solver (Platt *et al.*, 1980). The fit was very good in all the cases (r>0.98). The NPQ kinetics were obtained by recording chlorophyll *a* fluorescence values in samples exposed to a light stress and recovery (LSR) protocol: sequential periods of 15 min dark, 20 min high light (HL, 1200 µmol photons m^{-2} s^{-1}) and 40 min recovery under low light (LL_{Rec}, 40 µmol photons m^{-2} s^{-1}). NPQ was calculated as (Fm-Fm')/Fm' where Fm is the maximum fluorescence obtained in dark adapted samples while Fm' is the maximum fluorescence recorded in any subsequential saturating light pulse. qE, qZ (qM) and qI components of NPQ were estimated as described in Sello *et al.* (2019) by fitting the relaxation kinetic with a biexponential decay ($y=y0+A1\ e^{-x/t1}+ A2\ e^{-x/t2}$) in the SigmaPlot software. Y0, the minimum value reached by the fitting, represented the NPQ that is not relaxed after 20 min of dark recovery and thus represents an estimation of the slow qI component. A1 and A2 instead corresponded to the fastest qE and qZ (or qM) components.

Pigments analysis

Pigment analysis was performed as described by Cruz *et al.* (2014). Briefly, sea slugs and algae were sampled and immediately frozen in liquid nitrogen. Samples were freeze-dried, powdered by using a fine metal rod and pigments extracted in 95% cold buffered methanol (2% ammonium acetate) by adding the buffer, sonicating the samples for 2 min and then incubating them at -20°C for a minimum amount of 20 min. Sample debris were then removed by passing the samples through a 0.2 mm Fluoropore membrane filters (Millipore, Billerica, MA, USA). After filtration, the extracts were injected into a Prominence i –LC-2030C 3D Plus HPLC system (Shimadzu, Kyoto, Japan) equipped with the proprietary inbuilt photodiode array detector (PDA). Pigments were identified from absorbance spectra and retention times and concentrations were calculated in comparison with pure crystalline standards (DHI, Hørsolm, Denmark). The activity of the xanthophyll cycle and the sequential de-epoxidation of the pigments violaxanthin (Vx) to antheraxanthin (Ax) and zeaxanthin (Zx), was estimated by calculating the de-epoxidation state (DES) as: DES=([Zx]+0.5×[Ax])/([Zx]+[Ax]+[Vx]).

Softwares and statistical analysis

Raw data obtained from the ImagingWin (WALZ, chlorophyll *a* variable fluorescence) and from Shimadzu LabSolution softwares were exported and analysed in Microsoft Excel (Microsoft Corporation, 2018. Available at: https://office.microsoft.com/excel). The same software has been used to build the figures and to calculate statistically significant differences by using independent samples t-tests.

Results and discussion

Data underlying the results can be found on BioStudies (Morelli, 2023).

Elysia viridis mirrors the photosynthetic performance and the pigment profile of the different algal donors

Elysia viridis is a species able to feed on multiple algae, contrary to other members of the same genus such as *E. timida* or *E. chlorotica* that can feed and retain chloroplasts only from one specific algal food (Baumgartner & Toth, 2014). Among the wide range of algal food, the sea slug is predominantly found on *C. tomentosum* or *C. fragile*, two coenocytic algae whose cells lack septation facilitating ingestion of cytoplasm by the animals. Sea slugs collected in the wild on the Portuguese coast are typically associated to *C. tomentosum*, thus, to carry out the experiments, we maintained them either on this alga or we provided them with abundant *Chaetomorpha* to induce a diet switch. In sacoglossans, the radula features a single row of teeth that is adapted to the group's suctorial feeding habits. Since the algal food exhibit a wide range of characteristics, such as toxic substances or heavy calcification, polyphagous species, such as *E. viridis*, have a high level of plasticity in form and dimension of teeth depending on the food source (Jensen, 1993). When an animal is transferred from one alga to another, there is a learning span of at least one week, during which the morphology of the radular teeth is not distinguishable from that present with the previous food source (Jensen, 1989; Rauch *et al.*, 2018). This learning process involves increased efficiency in grasping the food, piercing the algal cell, and sucking out the cytoplasm. Accordingly, when the animals were transferred from their natural habitat (where they mostly feed on *C. tomentosum*) to *Chaetomorpha*, the animals spent between 3 to 5 days only crawling on the algal filaments. The sea slugs became then more static between 7 and 10 days after being transferred, likely coinciding with the change of teeth and the process of learning how to pierce the cell wall, and feed properly. This plasticity corroborates previous observations (Jensen, 1989), where specimens of *E. viridis* found on *Chaetomorpha* and moved on to *Codium* were able to efficiently learn how to feed on the latter alga. The animals, however, required a relatively longer time to learn again how to properly handle *Chaetomorpha* once transferred back to the original food source (Jensen, 1989).

After one month of continuous feeding, the specimens fed on *Chaetomorpha* (Ev-Ch) showed a clearly different colour compared to the ones fed on *C. tomentosum* (Ev-Ct) (Figure 1A). Chromatographic analysis associated the different shades of green with an altered pigment profile: Ev-Ct specimens showed high levels of siphonoxanthin and β,ε-carotene, typical of Bryopsidales algae (Cruz *et al.*, 2014; Giossi *et al.*, 2021),

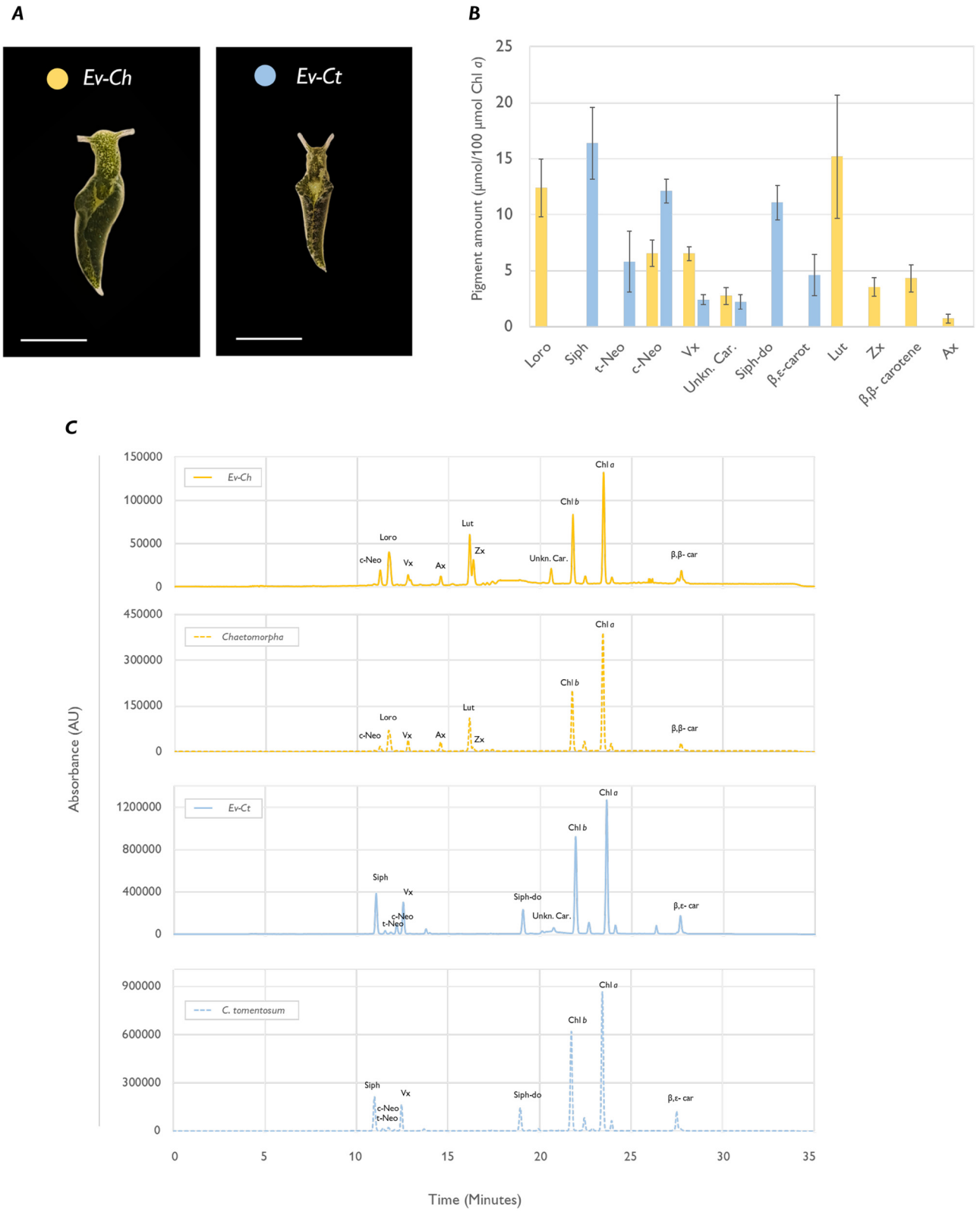

Figure 1. Pigment profile of *Elysia viridis* and its algae food sources. (**A**) Representative pictures of *E. viridis* specimens fed with *Chaetomorpha* sp. (Ev-Ch) or with ***C.*** *tomentosum* (Ev-Ct). The scale bar corresponds to 0.5 cm; (**B**) Carotenoid amount in sea slugs fed with the different algae. Data corresponds to mean and standard deviation (n=4) and values are expressed as relative to 100 µmol of Chl *a* in the same samples; (**C**) HPLC chromatograms extracted at 440 nm, representative of sea slugs (straight line) and macroalgae (dotted line) pigment profiles. Siph, siphonaxanthin; *t*-Neo, all- *trans*-neoxanthin; *c*-Neo, 9′-*cis*-neoxanthin; Vx, violaxanthin; Ax, antheraxanthin; Zx, Zeaxanthin; Lut, Lutein; Siph-do, siphonaxanthin dodecenoate; Chl *b*, chlorophyll *b*; Chl *a*, chlorophyll *a*; β,ε-Car, β,ε-carotene; β,β-car, β,β-carotene; Unkn. Car., unidentified *Elysia viridis* carotenoid.

while specimens fed with *Chaetomorpha* had no trace of the abovementioned carotenoids and accumulated instead high levels of lutein, β,β-carotene and loroxanthin (Figure 1B). Moreover, we found on both Ev-Ct and Ev-Ch an unknown carotenoid in animal samples already reported in other studies (Cruz *et al.*, 2014) with the maximum absorption of 460 nm, with a retention time of about 23 min (Figure 1C).

While the nature of this pigment is currently unclear, it could be a compound synthesized by the animal and necessary for its own metabolism that needs a common carotenoid precursor present in *C. tomentosum* and in *Chaetomorpha*. The carotenoid biosynthetic pathway is well characterized in plants and algae, occurring mainly in the chloroplasts, but it is absent in the animals that must obtain these compounds through their diets (Maoka, 2011; Morelli & Rodriguez-Concepcion, 2023). Indeed, the use of algae-derived carotenoids as precursors for keto-carotenoids necessary for animal metabolism is not so uncommon in molluscs. Sea slugs such as *Clione limacina* and *Paedoclione doliiformis*, oxidatively metabolize dietary carotenoids and accumulate them in their gonads to protect against oxidative stress (Borodina & Zadorozhny, 2021; Maoka *et al.*, 2014), while specimens of *E. timida* were hypothesized to accumulate carotenoid photoreactive pigments in the eyespots used for light perception (Rahat & Monselise, 1979).

Apart from the pigmentation, sea slugs photosynthetic performance was differently influenced by the algal food. The maximum quantum yield (Fv/Fm) of Ev-Ch specimens was significantly lower compared to the one observed in Ev-Ct animals. Interestingly, while the Fv/Fm in Ev-Ct slugs was higher than the one of *C. tomentosum* itself, Ev-Ch samples showed a lower value compared to the one of *Chaetomorpha* sp. (Figure 2A). As already reported in other works, photosynthetic performance of sea slugs tends to be better than that of algae they are fed on, likely because of an intracellular supply of respired inorganic carbon and nutrients produced by the metabolism of the host (Serôdio *et al.*, 2014). In the case of *Chaetomorpha* chloroplasts, they could be functional but losing stability very fast, hindering their performance once integrated in the animal host cells. The lifespan of *Chaetomorpha*-derived kleptoplasts in *E. viridis* tissues is unknown. However, a link between the different algal food and the retention performance in this species has already been described, with sea slugs being photosynthetically competent once fed with *Bryopsis hypnoides* but unable to incorporate chloroplasts from *Cladophora* sp. (Rauch *et al.*, 2018). When fed with the former, or with *C. tomentosum*, in fact, *E. viridis* is classified as a long-term retention (LtR) species while it does not show traces of photosynthetic activity (non-retention, NR) while fed with *Cladophora* sp. (Rauch *et al.*, 2018). We can then hypothesize that the incorporation process and the subsequential kleptoplast features could have a critical impact on the stability of the kleptoplasts.

To classify the photosynthetic state of the kleptoplasts, we calculated light curves (LCs) in *E. viridis* specimens and in their respective algal foods (Figure 2C). Photosynthetic parameters estimated by plotting the data according to the model of Platt (Platt *et al.*, 1980) showed a significantly higher maximum electron transport rate (ETRm) and light saturation coefficient (Ek) for Ev-Ct individuals and *C. tomentosum*, while the photosynthetic rate in the light-limited region of the light curve (alpha) was higher in Ev-Ch sea slugs and *Chaetomorpha* (Figure 2D). More importantly, the LCs obtained from the animals were similar to the ones obtained from the algal food, confirming that chloroplasts are functional in the animal cells and that their photoacclimation state is inherited by the algae and maintained in the animal, as observed in other similar studies (Cartaxana *et al.*, 2018). Curiously, Ev-Ch sea slugs shown a higher daily weight increase compared to Ev-Ct ones (Figure 2B). We could speculate that sea slugs continuously feeding on *Chaetomorpha* could retain chloroplast for less time in their body (likely because of the poor photosynthetic performance) and would eat more algae when provided with unlimited food. In presence of *Chaetomorpha*, sea slugs could adopt a binge eating habit to have a constant caloric intake instead of focusing on the integration of intact plastids with low functionality in their body similarly to what happens in juveniles of *E. timida* that before establishing kleptoplasty feed continuously and digest chloroplasts to support their own body growth (Laetz & Wägele, 2017).

Elysia viridis fed with *Codium tomentosum* can maintain chloroplast photoprotective mechanisms, while animals fed with *Chaetomorpha* sp. cannot

One of the main unresolved questions when investigating kleptoplasty is how plastids can continue to function in animal cells for long periods even if they are exposed constantly to wide range of stress conditions and without the access to many fundamental algal nuclear-encoded proteins. In particular, the conservation of mechanisms to tackle light stress in these photosynthetic animals is still puzzling (Cruz & Cartaxana, 2022). Sacoglossan sea slugs are known to contribute to chloroplast shielding, either by folding their parapodia under high irradiances (i.e., to shield internal kleptoplasts while permitting filtered light energy to reach kleptoplasts on the parapodial undersides) or by simply crawling away from excessive light (Cartaxana *et al.*, 2018; Donà *et al.*, 2022). Furthermore, physiological mechanisms involved in chloroplast protection can be maintained when the plastids are transferred to the animal cells. Some of the algae currently used as a food by sacoglossan sea slugs, indeed, are very resilient and show robust chloroplasts able to survive in harsh conditions. For example, isolated thylakoids of *V. litorea* were found to be more resilient to photoinhibition of PSII than spinach thylakoids, likely because of a lower $^1O_2$ production and higher rates of ROS detoxification (e.g., through the action of α-tocopherol and carotenoids) (Havurinne *et al.*, 2022). In some algae, the active conversion of Vx to Zx under high irradiances (i.e., the xanthophyll cycle) has been reported as one of the main mechanisms to protect against light stress and in some animals fed with these algae (e.g., *E. timida* and *E. chlorotica*) the cycle is maintained during medium periods of starvation and actively participate in kleptoplast protection (Cartaxana *et al.*, 2019). However, other sea slugs (i.e., *E. cornigera*), fed with the same *A. acetabulum* were reported to show very different degree of kleptoplast compatibility being unable to cope with ROS

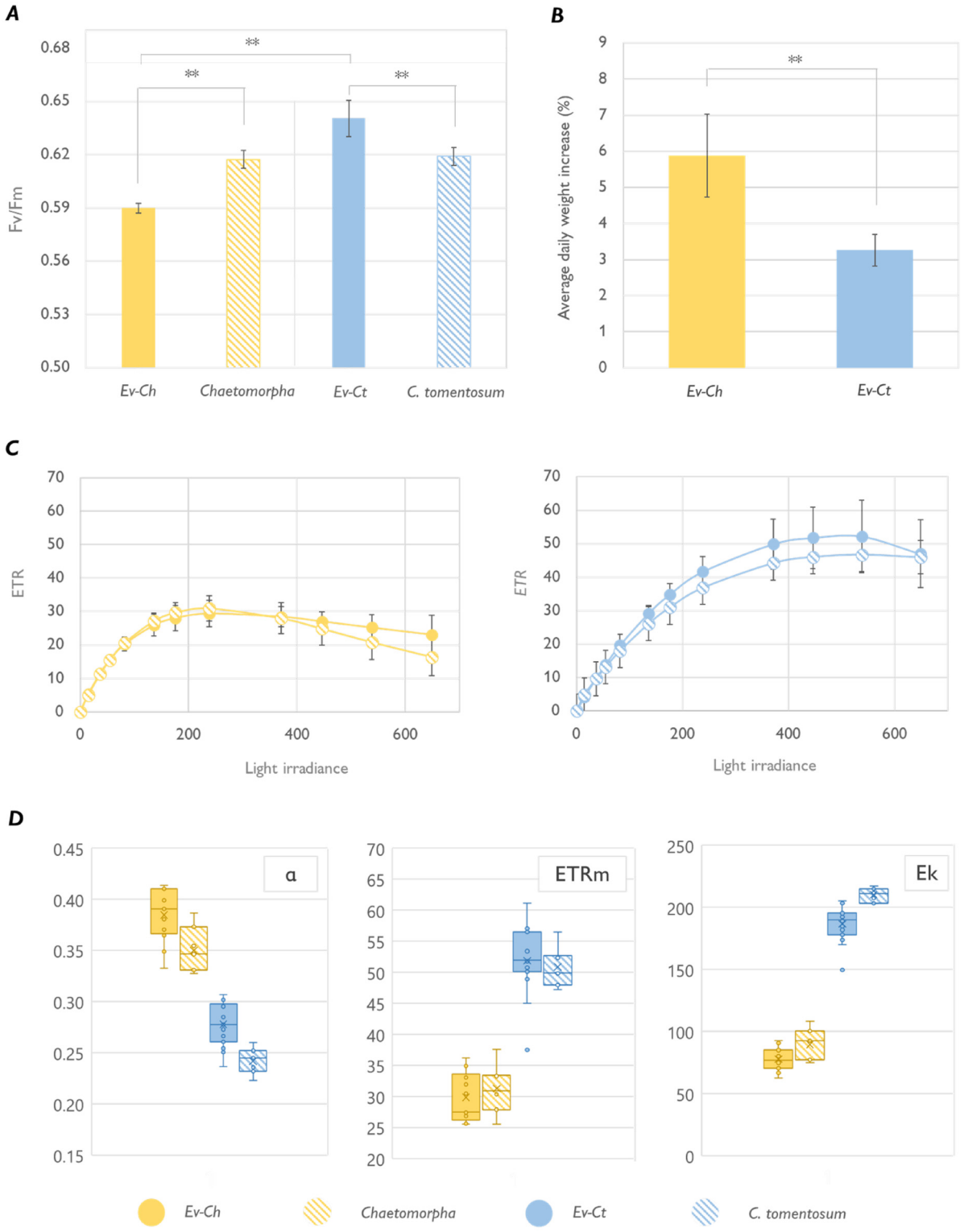

Figure 2. Photosynthetic performance of *Elysia viridis* and its algae food sources. (**A**) Maximum quantum yield (Fv/Fm) in *E. viridis* fed with *Chaetomorpha* sp. (Ev-Ch), *Chaetomorpha* sp., *E. viridis* fed with *Codium tomentosum* (Ev-Ct) and *C. tomentosum.* Data correspond to mean and standard deviation (n=5); (**B**) Averaged daily weight increase estimated for feeding animals, data corresponds to mean and standard deviation (n=10) measured after 1 month of continuous feeding; (**C**) Light curves of sea slugs and the respective algal food. Data corresponds to mean and standard deviation (n=10); (**D**) alpha, ETRm and Ek parameters calculated from the LCs represented in panel **C**. Boxes show the values between the upper and the lower quartile, the cross represents the mean and the horizontal line the median. Whiskers (the upper and lower extremes) and circles represent single data and the ones located outside of the whiskers limit are the outliers (data with the same numerical value are visualized as a single point). Asterisks mark statistically significant differences (t-test, ** $p < 0.01$).

accumulation with the same efficiency as *E. timida* (de Vries *et al.*, 2015). We hypothesized, thus, that the efficacy of the photoprotective mechanisms is not depending only on the physiologic characteristics of the algal donor but also on the individual compatibility of the stolen plastids with the sea slugs species.

To test our hypothesis, we evaluated the response to short term light stress in Ev-Ct and Ev-Ch specimens and in the respective food sources. Under high light (HL, 1200 µmol photons m^{-2} s^{-1}), the photosynthetic yield ΦPSII of the sea slugs reached values very near 0, showing an expected photoinhibition state, regardless of the algal food (Figure 3A).

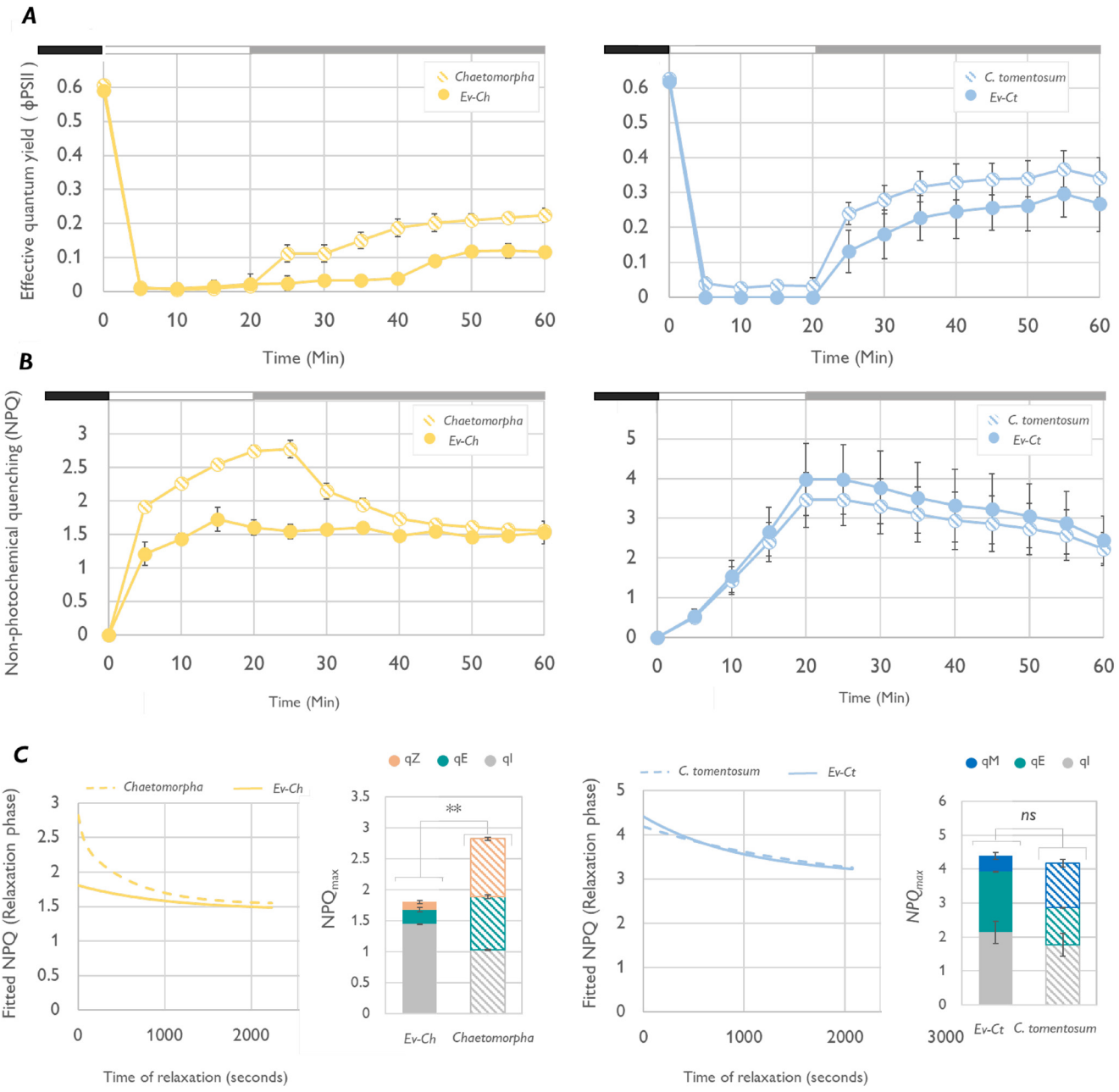

Figure 3. Light stress and recovery of *Elysia viridis* and its algae food sources. (**A**) Effective quantum yield (ΦPSII) calculated in *E. viridis* fed with *Chaetomorpha* sp. (Ev-Ch), *Chaetomorpha* sp., *E. viridis* fed with *Codium tomentosum* (Ev-Ct) and *C. tomentosum* during a light stress-recovery experiment. In the chart, the different phases of the protocol are identified by the black box (15 min, previous dark acclimation), the white box (light stress, exposure to 1200 µmol photons m^{-2} s^{-1} for 20 min) and the grey box (low light recovery, 40 µmol photons m^{-2} s^{-1} for 40 min). Data corresponds to mean and standard deviation (n=5); (**B**) Non-photochemical quenching (NPQ) kinetics obtained in the same conditions as described for panel A; (**C**) Graphs representing the relaxation phase of the NPQ kinetic fitted to the equation of a bi-exponential decay in SigmaPlot. The program is used also to calculate the components of the NPQ represented in the two bar charts as part of the total NPQ level. Asterisks mark statistically significant differences (t-test, ** $p < 0.01$, ns: no significant differences) between the values of total NPQ_{max} in the indicated samples.

At the same time NPQ level increased to be then slowly dissipated under low irradiances (LL $_{Rec}$, 40 µmol photons m^{-2} s^{-1}) in concomitance with a recovery of photosynthetic yield (Figures 3A, B). The relaxation and recovery kinetics followed the same pattern in sea slugs and algae and as observed in other studies, 40 min of LL incubation were not enough to recover the maximum quantum yield (Cruz *et al.*, 2015). The slow relaxation of the NPQ in *C. tomentosum* could be influenced by chloroplast movement inside the algal tissue as chloroplasts can redistribute and reorientate in the cell as a response to the light direction resulting in typical patterns for light of low or high fluence rate. In this condition, plastids take a "high intensity arrangement" designed to protect the chloroplasts against excessive radiation by stacking the plastids on the cell borders and piling them to provide self-shading (Davis & Hangarter, 2012). In *C. tomentosum*, which has no functional XC, chloroplast movement could have a prevalent role on preventing excess energy absorption. In siphonous algae such as *Halimeda distorta*, the piling of chloroplasts to one side of the algae branch following prolonged exposure to very high irradiance or darkness is so intense to change the green color intensity (Drew & Abel, 1992). Moreover, the kinetic of NPQ relaxation under low light in *C. tomentosum* and Ev-Ct is strikingly like the one observed in *A. thaliana npq4* mutants. The latter lack the PsbS protein and are unable to carry on a functional xanthophyll cycle thus rely on plastid movement to develop a slow, qE-type, energy quenching mechanism named qM (Dall'Osto *et al.*, 2014). Accordingly, we could mathematically calculate a high contribution of qM component to the quenching by fitting the relaxation kinetic to the equation of a biexponential decay (Figure 3C). In fact, during NPQ relaxation, a pivotal role is played by the fast component qE that act in the first minutes of low light/dark exposure, but a slower component, associated with Zx binding to LHC proteins named qZ is the responsible for energy quenching after 10–15 min of relaxation (Dall'Osto *et al.*, 2005). In organisms where this pigment is not present, such as *C. tomentosum* or the *npq4 A. thaliana* mutant, the dissipation of energy is related to chloroplast movement, hence the qM definition (Dall'Osto *et al.*, 2012). On another hand, differently from *Codium*, *Chaetomorpha* accumulates Zx under high irradiances and its NPQ kinetic is very similar to the ones of other XC-performing algae such as *V. litorea*, suggesting a more marginal role for chloroplast movement-based photoprotective mechanisms over the XC. Despite this, as observed for *Codium*, also in *Chaetomorpha* the recovery of ΦPSII is not complete after 40 min in low light. However, the fast qE-quenching component in the first minutes is evident and, considering the presence of Zx, the subsequent slower recovery could be related to the qZ component. Interestingly, Ev-Ch animals showed the ability to build up NPQ up to a certain extent but were unable to relax it and show little recovery of the photosynthetic activity during LL_{Rec} (Figures 4A, B).

When investigating the functionality of the XC, we observed that dark-acclimated *Chaetomorpha* sp. showed no traces of Zx, accumulated it after 20 min of HL exposure, and about 75% of Zx was converted back to Vx at the end of the LL $_{Rec}$ (Figure 4). The cycle resulted in a significantly higher de-epoxidation state (DES) during the HL exposure compared to the LL $_{Rec}$. On the other hand, Ev-Ch sea slugs showed traces of Zx already upon dark acclimation, under HL the amount of this pigment increased, but did not decrease under LL $_{Rec}$, accordingly with the inability to quench the built up of NPQ. As previously observed, the photosynthetic competence of *Chaetomorpha* chloroplasts diminished when integrated into *E. viridis*. Consequently, excessive light exposure could lead to severe photoinhibition. During this state, the proton gradient across the thylakoidal membranes might be altered, preventing the recovery of the cycle back to violaxanthin due to the unavailability of a suitable environment for the responsible enzymes to function (Tjus & Andersson, 1993). In algae, this situation is usually overcome by plastid-targeted proteins encoded by nuclear genes likely lacking in *E. viridis*. Experimental evidence from other kleptoplastic organisms has demonstrated that kleptoplasty is supported by nucleus-encoded genes for plastid-targeted proteins, which possess specific signals for targeting these proteins to the kleptoplasts. Plastid transporters are also present to facilitate the targeting of proteins and metabolites into the kleptoplasts (Hehenberger *et al.*, 2019; Lekieffre *et al.*, 2018).

In certain kleptoplastic organisms, such as Ross Sea dinoflagellates (RSD), photosynthetic functions are divided between an ancestral plastid (peridinin plastid) and the kleptoplasts. During plastid sorting, electron flow from PSII to PSI is disrupted, but it is subsequently restored through proteins encoded by the dinoflagellate nucleus, which are then targeted to the kleptoplasts via a specific signal (Hehenberger *et al.*, 2019). We hypothesize that when *E. viridis* consumes an alga that is not its "preferred" donor (e.g., *Chaetomorpha*), compatibility is only partial. Even if the kleptoplasts within animal cells can maintain certain functionalities (e.g., basal photosynthetic activity), they might struggle to manage stress due to the necessity for specific protein coding and import.

It is worth noting that transit peptide diversity across the plant kingdom is significant, and sometimes proteins encoded by one plant species cannot enter the chloroplasts of another organism without minor changes in the coding sequence (Patron & Waller, 2007). A kleptoplast-targeted protein encoded by *E. viridis*, but designed to enter *Codium* chloroplasts, may not be able to interact effectively with *Chaetomorpha* plastids. Consequently, fundamental functions in this chloroplast, such as repair or metabolic regulation, might be compromised, leading to rapid decay. Furthermore, the mechanism of kleptoplast integration in animal cells is a subject of debate. Kleptoplasts integrated into various sacoglossan sea slug species have been reported to exhibit diverse ultrastructures compared to their original forms within the algal donors. For example, in the LtR species *E. timida*, chloroplasts were found to have functional thylakoids and stroma, but no inner and outer envelopes, being surrounded only by the phagosome membrane while, in the NR form *Thuridilla hopei,* kleptoplasts showed the

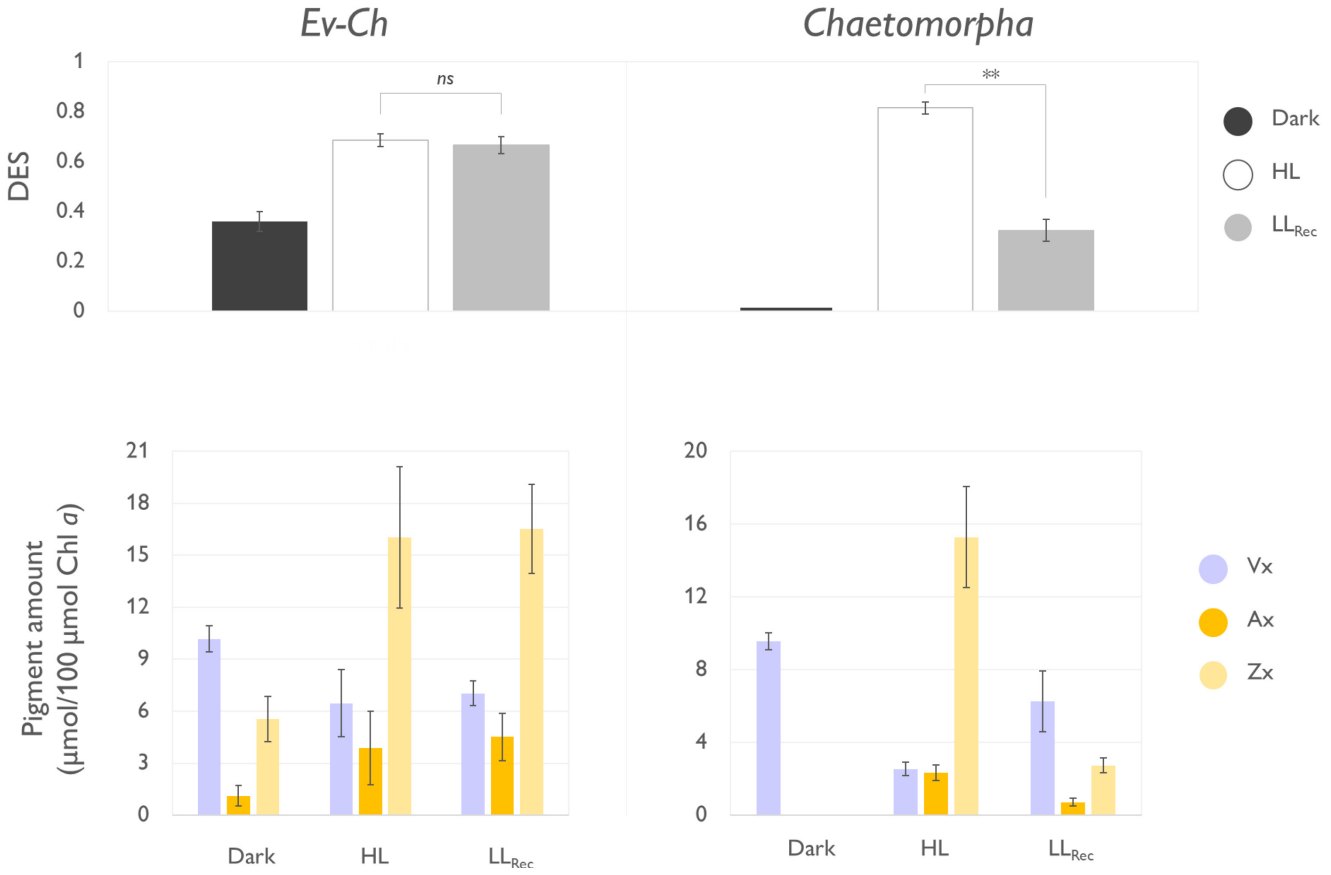

Figure 4. Operation of the xanthophyll cycle in *Elysia viridis* fed with *Chaetopmorpha* sp. (Ev-Ch) and in the individual algae. The top panels show the de-epoxidation state (DES) expressed as (Zx+0.5*Ax)/(Vx+Ax+Zx) in Ev-Ch sea slugs and *Chaetomorpha* sp. subjected to the light stress and recovery protocol represented in Figure 3; Vx, violaxanthin; Ax, antheraxanthin; Zx, zeaxanthin. The lower panel represent the levels of the single xanthophylls expressed as µmol of pigment relative to 100 µmol of Chl *a* observed in the same samples. Data corresponds to mean and standard deviation (n=4). Asterisks mark statistically significant differences between the high light (HL) and the recovery in low light (LL_{Rec}) phases (t-test, ** $p < 0.01$, ns: no significant differences).

whole set of membranes (Martin *et al.*, 2013). In *E. viridis*, chloroplasts derived from different algal donors could preserve different features that could impact the actual photosynthetic function of the kleptoplast and the possibility of specific proteins to enter the plastids. The association between *E. viridis* and *Codium* likely evolved specifically, resulting in the encoding of all necessary subunits of a functional translocon complex capable of importing useful proteins to maintain chloroplast functionality. However, in the case of *Chaetomorpha* chloroplasts, certain components may be absent or interact differently with the slug's nuclear-encoded proteins, resulting in a loss of function. To test these hypotheses, further molecular studies will be required.

Conclusions

This study assesses the photophysiology of the sea slug *E. viridis* fed with algae with different photoprotective and photosynthetic capabilities (*C. tomentosum* and *Chaetomorpha* sp.). In this work, we investigated which photoprotective mechanisms present in the algae are found in the animal host. Our results show that specific photoprotective mechanisms of algal chloroplasts (e.g., the xanthophyll cycle) are not necessarily maintained once the plastids are transferred to the sea slug cells and suggest that plastid functionality depends on the sea slug-alga combination. Algae chloroplasts have their individual robustness but, once part of the host animal cell, require a support that only some can provide. Thus, only in the case of specific associations, such as those between *E. timida* and *A. acetabulum* or *E. chlorotica* and *V. litorea*, it is possible for the kleptoplasts to maintain all their functionalities.

Ethics and consent

The use of *E. viridis* specimens was not subjected to ethical approval considering that the use of lower-level invertebrates species (e.g., brine shrimp, fruit flies, nematodes, mosquitoes, etc.) is not restricted for research purposes. After the collection, animals were maintained in optimal conditions using the

LSS in controlled environment to ensure the maximum fitness. Animals were subjected to starvation only when required by the experimental setting.

Data availability

Underlying data

BioStudies: Datasets accompanying the manuscript: "Food shaped photosynthesis: Photophysiology of the sea slug *Elysia viridis* fed with two alternative chloroplast donors". Accession number S-BSST1106, https://www.ebi.ac.uk/biostudies/studies/S-BSST1106 (Morelli, 2023).

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PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 08 May 2024

https://doi.org/10.21956/openreseurope.17955.r38910

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✔ **Rachael M Wade**
The University of British Columbia, Vancouver, British Columbia, Canada

I believe the authors have responded well to the revisions and made them satisfactorily.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Algal evolution and biology; plant physiology; sacoglossan kleptoplasty

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 12 April 2024

https://doi.org/10.21956/openreseurope.17955.r38911

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✔ **Yoichi Yusa**
Faculty of Science, Nara Women's University, Nara, Nara Prefecture, Japan

I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquatic ecology

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 25 July 2023

https://doi.org/10.21956/openreseurope.17448.r33598

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?

Rachael M Wade
[1] The University of British Columbia, Vancouver, British Columbia, Canada
[2] The University of British Columbia, Vancouver, British Columbia, Canada

General comments:

The authors consistently cite their own papers, when there are often a multitude of other papers by other authors that could be cited. I would appreciate seeing the paper revised through this lens. For example, recent work by the authors is cited when discussing the proposed lack of the xanthophyll cycle in some siphonous greens, but this was supported by Benson and Cobb (1983)[1]. There are also consistent issues with incorrectly crediting and citing papers, please carefully review each paper referenced and its findings.

Introductory summary sections:

Background:

- I think the first sentence could be reworded to be more clear – Some sacoglossan sea slugs integrate chloroplasts from the algae they feed on into their cells (perhaps add here "where they continue to function photosynthetically"), a process termed kleptoplasty. Chloroplasts are inherently intracellular, so the current wording does not truly describe the process of kleptoplasty.

- 'Associated with' instead of 'associated to'.

Results:

- Add 'similarly' or 'comparably' after "While sea slugs fed with *C. tomentosum* react..."

- Change 'algae' to 'alga'.

Conclusions:

- This conclusion is too strong given the methods used in this study; no attempts to understand the role of the slug as a new host plays in interacting with the different chloroplasts, only how the chloroplasts are functioning in the host. There are significant evolutionary and biological differences between *Codium* and *Chaetomorpha*, they are from two completely different Orders. This taxonomic difference in host species is reflected in the

genomes of the algae – the cladophoralean genome is a fragmented assemblage of hairpin chromosomes (see Del Cortona *et al.* 2017 *Cell*[2]). This may be playing a significant role in how the chloroplasts behave independent of their algal cells. Additionally, siphonous greens have siphonein and siphonoxanthin, which may play a role in photoprotection (although they're largely described as antennae accessory pigments). Your conclusion is entirely plausible, but the possibility of evolutionary history and biology is also equally plausible and need to be discussed as such. This is abundantly clear when so much of this theory is based on reorganization of chloroplasts as a strategy to reduce photodamage, but this strategy is common in higher land plants, it is not clear to me why the authors assume that *Chaetomorpha* would not be able to exhibit similar phototactic responses. I do feel that the Conclusion section does a better job of supporting some of the theories I question here than other parts of the manuscript and could be used to strengthen those sections in question.

Introduction:

First paragraph:

- Change 'associated' to 'attributed'.

- Your citing of your own 2022 paper for the definition of kleptoplasty and its occurrence in Sacoglossa is not correct, you should be citing the papers by those first witnessed the phenomenon in sacoglossans, which is most often attributed to Kawaguti and Yamasu (1965)[3] and Greene (1970)[4], Green & Muscatin (1972)[5]. If you want to say the topic is reviewed by your paper, great, but give credit where credit is due. Particularly because, surprisingly, none of those papers above are cited in that review.

- Your description of the extent of the digestive tissue and the crypsis it results in only applies to some taxa. Please either revise to be more inclusive of the diversity of Sacoglossa morphologies, or narrow your discussion entirely to your study organism.

- I have never heard this theory before, that kleptoplasty arose to achieve crypsis. To me, this does not make sense in reflection of endosymbiotic theory, in which most people describe kleptoplasty as a sort of steppingstone to true endosymbiosis. I've re-read the Evertsen & Johnsen 2009 publication you cited and nowhere does this paper test this theory, nor even suggest it. The next sentence is even more confusing when you say that a sacoglossan with non-functional kleptoplasts is still benefiting from photosynthate! How? This is perhaps a transition issue, please revise for clarity.

Second paragraph:

- Donohoo *et al.* 2020[6] and Evertsen & Johnsen 2009[7] should also be cited in your last sentence.

Fourth paragraph:

- Would be helpful to note that *Ceramium* is a rhodophyte, while all other taxa mentioned are chlorophytes. Similarly, in the description of *Chaetomorpha*, it would be helpful to reiterate that while multinucleate, it is not siphonous, like *Codium.*

Methods:

Second paragraph:

- Change 'weighted' to 'weighed'.

Results:

- Defined polyphagous in its second appearance, rather than the first in the intro. Please revise.

- Change 'sap' to 'cytoplasm'.

- I'm confused by your discussion of *Placida*, I'm assuming in review of Evertsen & Johnsen (2009)[7] and describing them has having non-functional yet incorporated plastids. The abstract and results state that the chloroplasts are digested immediately, not maintained, yet non-functional. They are phagocytized first, but are immediately digested. The way their study is described in some (but not all) of this manuscript, it suggests the chloroplasts are somehow hanging out in the slugs tissues, alive, but not photosynthesizing. Additionally, this paper is credited with the observation that up to 75% of kleptoplasts are overturned, but this was actually studied by Gallop *et al.* 1980[8].

Paragraph 5:

- Increased weight/decreased mortality at low-moderate light was also shown in Donohoo *et al.* 2020[6].

Paragraph 6:

- RE: siphonous greens exhibiting phototaxis in regard to plastid rearrangement: I don't doubt it occurs, but I think it is just as likely to occur in a large-celled, septate green, like *Chaetomorpha*. This is also seen throughout higher land plants.

Paragraph 7:

- In my opinion, the HGT debate has been put to bed by the thorough work in Maeda *et al.* 2021[9] but is missing from your discussion of this topic.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Algal evolution and biology; plant physiology; sacoglossan kleptoplasty

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jul 2023

Luca Morelli

Dear Dr. Wade, We really appreciate your comments and the constructive discussion that they arose. We will work on a version considering all your reviews and suggestions and we will upload it as soon as possible.

Luca Morelli

Competing Interests: No competing interests were disclosed.

Author Response 17 Feb 2024

Luca Morelli

General comments: **C1**: The authors consistently cite their own papers, when there are often a multitude of other papers by other authors that could be cited. I would appreciate seeing the paper revised through this lens. For example, recent work by the authors is cited when discussing the proposed lack of the xanthophyll cycle in some siphonous greens, but this was supported by Benson and Cobb (1983)1. There are also consistent issues with

incorrectly crediting and citing papers, please carefully review each paper referenced and its findings. **R1**: We appreciate and thank the reviewer for all the comments and feedback. We have checked the chosen references to correct any inconsistencies and provided new references to credit other authors. Introductory summary sections:

Background: C2: I think the first sentence could be reworded to be more clear – Some sacoglossan sea slugs integrate chloroplasts from the algae they feed on into their cells (perhaps add here "where they continue to function photosynthetically"), a process termed kleptoplasty. Chloroplasts are inherently intracellular, so the current wording does not truly describe the process of kleptoplasty. 'Associated with' instead of 'associated to'.
R2: We have rephrased the sentence for improved clarity and done the requested corrections. It reads, “Some Sacoglossa sea slugs steal and integrate chloroplasts derived from the algae they feed on into their cells where they continue to function photosynthetically, a process termed kleptoplasty”

Results: C3:

- Add 'similarly' or 'comparably' after "While sea slugs fed with C. tomentosum react..."
- Change 'algae' to 'alga'.

R3: We have done the requested corrections.

Conclusions: C4: This conclusion is too strong given the methods used in this study; no attempts to understand the role of the slug as a new host plays in interacting with the different chloroplasts, only how the chloroplasts are functioning in the host. There are significant evolutionary and biological differences between Codium and Chaetomorpha, they are from two completely different Orders. This taxonomic difference in host species is reflected in the genomes of the algae – the cladophoralean genome is a fragmented assemblage of hairpin chromosomes (see Del Cortona et al. 2017 Cell2). This may be playing a significant role in how the chloroplasts behave independent of their algal cells. Additionally, siphonous greens have siphonein and siphonoxanthin, which may play a role in photoprotection (although they’re largely described as antennae accessory pigments). Your conclusion is entirely plausible, but the possibility of evolutionary history and biology is also equally plausible and need to be discussed as such. This is abundantly clear when so much of this theory is based on reorganization of chloroplasts as a strategy to reduce photodamage, but this strategy is common in higher land plants, it is not clear to me why the authors assume that Chaetomorpha would not be able to exhibit similar phototactic responses. I do feel that the Conclusion section does a better job of supporting some of the theories I question here than other parts of the manuscript and could be used to strengthen those sections in question.
R4: We have modified the Results and Discussion and Conclusion sections for clarity and to downgrade the conclusions of our study. In its current form, the manuscript focuses on the characterization of the photophysiology providing some ideas for future works. Any speculations are clearly identified as such.

Introduction: First paragraph: **C5**: Change 'associated' to 'attributed'.
R5: We have performed the required correction.

C6: Your citing of your own 2022 paper for the definition of kleptoplasty and its occurrence in Sacoglossa is not correct, you should be citing the papers by those first witnessed the phenomenon in sacoglossans, which is most often attributed to Kawaguti and Yamasu (1965)3 and Greene (1970)4, Green & Muscatin (1972)5. If you want to say the topic is reviewed by your paper, great, but give credit where credit is due. Particularly because, surprisingly, none of those papers above are cited in that review.
R6: We have inserted additional references in the text.

C7: Your description of the extent of the digestive tissue and the crypsis it results in only applies to some taxa. Please either revise to be more inclusive of the diversity of Sacoglossa morphologies or narrow your discussion entirely to your study organism.
R7: we have modified the text to increase the clarity. Now it raeds, "While in some species of Sacoglossa sea slugs (e.g., *Elysia timida* and *Plakobranchus ocellatus*) kleptoplasts are mostly visible only in some body regions (e.g., the dorsal area), in other species such as E. viridis, they are distributed evenly and not concentrated in a specific part of the animal (Gallop et al., 1980). In E. viridis, the presence of ramifications of digestive tubules, throughout most of the sea slug's body, contributes to a colour resembling that of its algal food and helps the animal to blend with the environment to escape predators (Hirose, 2005)"

C8: I have never heard this theory before, that kleptoplasty arose to achieve crypsis. To me, this does not make sense in reflection of endosymbiotic theory, in which most people describe kleptoplasty as a sort of steppingstone to true endosymbiosis. I've re-read the Evertsen & Johnsen 2009 publication you cited and nowhere does this paper test this theory, nor even suggest it. The next sentence is even more confusing when you say that a sacoglossan with non-functional kleptoplasts is still benefiting from photosynthate! How? This is perhaps a transition issue, please revise for clarity.
R8: We have rephrased the section for clarity (see R7). Second paragraph:

C9: Donohoo et al. 20206 and Evertsen & Johnsen 20097 should also be cited in your last sentence.
R9: The references suggested were added to the text. Fourth paragraph:

C10: Would be helpful to note that Ceramium is a rhodophyte, while all other taxa mentioned are chlorophytes. Similarly, in the description of Chaetomorpha, it would be helpful to reiterate that while multinucleate, it is not siphonous, like Codium.
R10: We have modified the text to include a short description of Chaetomorpha to offer a better overview of its morphology. Now it reads, "*Chaetomorpha* is structurally very different from *Codium* as it is a filamentous alga that can grow up to several centimeters in length. The thallus has a cylindrical shape and is usually unbranched, loosely entangled and stiff, made by the junction of several squared cells separated by septa (Blair et al., 1982). This characteristic affects the feeding pattern of the animals that must move and pierce every single cell to suck the cytoplasm".

Methods: C11: Second paragraph: Change 'weighted' to 'weighed'.
R11: Corrected. Results:

C12: Defined polyphagous in its second appearance, rather than the first in the intro. Please

revise. Change 'sap' to 'cytoplasm'.
R12: We have made the required corrections.

C13: I'm confused by your discussion of Placida, I'm assuming in review of Evertsen & Johnsen (2009) and describing them has having non-functional yet incorporated plastids. The abstract and results state that the chloroplasts are digested immediately, not maintained, yet non-functional. They are phagocytized first, but are immediately digested. The way their study is described in some (but not all) of this manuscript, it suggests the chloroplasts are somehow hanging out in the slugs tissues, alive, but not photosynthesizing. Additionally, this paper is credited with the observation that up to 75% of kleptoplasts are overturned, but this was actually studied by Gallop et al. 1980.
R13: The discussion of *Placida* was removed to avoid confusion as no actual data from experiments involving *Placida* were used for this work. Paragraph 5:

C14: Increased weight/decreased mortality at low-moderate light was also shown in Donohoo et al. 2020.
R14: The additional reference was inserted in the text Paragraph 6:

C15: siphonous greens exhibiting phototaxis in regard to plastid rearrangement: I don't doubt it occurs, but I think it is just as likely to occur in a large-celled, septate green, like Chaetomorpha. This is also seen throughout higher land plants.
R15: We have rearranged the text to make our statements clearer. Now it reads, **"**In *C. tomentosum*, which has no functional XC, chloroplast movement could have a prevalent role on preventing excess energy absorption. In siphonous algae such as *Halimeda distorta*, the piling of chloroplasts to one side of the algae branch following prolonged exposure to very high irradiance or darkness is so intense to change the green color intensity (Drew & Abel, 1992). Moreover, the kinetic of NPQ relaxation under low light in *C. tomentosum* and Ev-Ct is strikingly like the one observed in *A. thaliana* npq4 mutants. The latter lack the PsbS protein and are unable to carry on a functional xanthophyll cycle thus rely on plastid movement to develop a slow, qE-type, energy quenching mechanism named qM (Dall'Osto et al., 2014). Accordingly, we could mathematically calculate a high contribution of qM component to the quenching by fitting the relaxation kinetic to the equation of a biexponential decay (Figure 3C). In fact, during NPQ relaxation, a pivotal role is played by the fast component qE that act in the first minutes of low light/dark exposure, but a slower component, associated with Zx binding to LHC proteins named qZ is the responsible for energy quenching after 10–15 min of relaxation (Dall'Osto et al., 2005). In organisms where this pigment is not present, such as *C. tomentosum* or the npq4 *A. thaliana* mutant, the dissipation of energy is related to chloroplast movement, hence the qM definition (Dall'Osto et al., 2012). On another hand, differently from *Codium*, *Chaetomorpha* accumulates Zx under high irradiances and its NPQ kinetic is very similar to the ones of other XC-performing algae such as *V. litorea* suggesting a more marginal role for chloroplast movement-based photoprotective mechanisms over the XC (Cruz et al., 2015)." Paragraph 7:

C16: In my opinion, the HGT debate has been put to bed by the thorough work in Maeda et al. 20219 but is missing from your discussion of this topic.
R16: the last part of the text has been modified to avoid overinterpretation of data and to provide a better fit between our conclusions and the experimental set-up. The part about

HGT has been removed.

Competing Interests: No competing interests were disclosed.

Reviewer Report 19 July 2023

https://doi.org/10.21956/openreseurope.17448.r33325

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Yoichi Yusa
[1] Faculty of Science, Nara Women's University, Nara, Nara Prefecture, Japan
[2] Faculty of Science, Nara Women's University, Nara, Nara Prefecture, Japan

This is an interesting study reporting the different performance of the sacoglossan sea slug *Elysia viridis* that fed on two algal species. Overall, I feel that the manuscript is well written and scientifically sound. Thus, I have only a few comments.

Minor comments:

1. In the introduction, similar studies in which a sea slug is fed two different algae are not referred to. I think that such references are needed to make the originality of your study clearer (as you did in the discussion).

2. Figures: Orange and blue colors with different degrees of paleness are difficult to see. I suggest the authors to use clearer patterns.

3. P.2, paragraph 1, 5 lines from the bottom: Italicize “Chaetomorpha”.

4. P.2, paragraph 2, 4 lines from the bottom: Although it may sometimes be considered an example of endosymbiosis, it is not, in my opinion, because the chloroplast is not an organism as you wrote.

5. P.7, in the middle of paragraph 3: “hindering their performance once integrated in the host cells”. Please clarify that the “host” is the sacoglossan here.

6. Figure 4 legend: Use the bold letters to the end of the first line.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquatic ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 29 Jul 2023

Luca Morelli

Dear Dr. Yusa, We kindly appreciate your comments on the manuscript. We will start now to elaborate a version including your changes and suggestions.

Best regards Luca

Competing Interests: No competing interests were disclosed.

Author Response 17 Feb 2024

Luca Morelli

R1 Yoichi Yusa **C1**: This is an interesting study reporting the different performance of the sacoglossan sea slug Elysia viridis that fed on two algal species. Overall, I feel that the manuscript is well written and scientifically sound. Thus, I have only a few comments.
R: We thank the reviewer for the positive feedback and for pointing out some mistakes in the manuscript. In the new version, we have addressed and corrected them.

Minor comments: In the introduction, similar studies in which a sea slug is fed two different algae are not referred to. I think that such references are needed to make the originality of your study clearer (as you did in the discussion).
R: We have included reference to studies comparing photosynthetic sea slugs fed with specific algae. It reads, “Experimental studies on polyphagous sea slugs fed with specific algal chloroplast donors show that functionality and longevity of kleptoplasts depend on plastid origin (Curtis et al. 2015; Cartaxana et al. 2023).”

Figures: Orange and blue colors with different degrees of paleness are difficult to see. I suggest the authors to use clearer patterns.
R: Different degrees of paleness are not used in the figures. We opted for solid versus pattern fill for the comparison of alga and sea slug (fed with that specific alga) and different colours (blue versus yellow) for the comparison of different sea slugs (or different algae).

P.2, paragraph 1, 5 lines from the bottom: Italicize “Chaetomorpha”.
R: Corrected.

P.2, paragraph 2, 4 lines from the bottom: Although it may sometimes be considered an example of endosymbiosis, it is not, in my opinion, because the chloroplast is not an organism as you wrote.
R: We agree with the reviewer, but the fact is that it is sometimes referred in the literature as endosymbiosis, although, as referred in the sentence, the chloroplast does not constitute an organism.

P.7, in the middle of paragraph 3: “hindering their performance once integrated in the host cells”. Please clarify that the “host” is the sacoglossan here.
R: Clarified. It reads, “hindering their performance once integrated in the animal host cells.”

Figure 4 legend: Use the bold letters to the end of the first line.
R: Corrected.

Competing Interests: No competing interests were disclosed.