

SHORT COMMUNICATION

Kleptoplast photoacclimation state modulates the photobehaviour of the solar-powered sea slug *Elysia viridis*

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ABSTRACT

Some sacoglossan sea slugs incorporate intracellular functional algal chloroplasts (kleptoplasty) for periods ranging from a few days to several months. Whether this association modulates the photobehaviour of solar-powered sea slugs is unknown. In this study, the long-term kleptoplast retention species *Elysia viridis* showed avoidance of dark independently of light acclimation state. In contrast, *Placida dendritica*, which shows non-functional retention of kleptoplasts, showed no preference over dark, low or high light. High light-acclimated (HL_{ac}) *E. viridis* showed a higher preference for high light than low light-acclimated (LL_{ac}) conspecifics. The position of the lateral folds (parapodia) was modulated by irradiance, with increasing light levels leading to a closure of parapodia and protection of kleptoplasts from high light exposure. Furthermore, closure of parapodia occurred at higher irradiance in HL_{ac} *E. viridis*. Our results strongly indicate that kleptoplast photoacclimation state modulates the photobehaviour of the solar-powered sea slug *E. viridis*.

KEY WORDS: Kleptoplasty, Light preference, Parapodia, Photoprotection, Sacoglossa

INTRODUCTION

The incorporation and maintenance of intracellular functional chloroplasts by host cells (kleptoplasty) occurs in a single taxon of metazoans – sacoglossan sea slugs (Rumpho et al., 2011; Serôdio et al., 2014). ‘Stolen’ plastids (kleptoplasts) may remain functional in cells of the digestive gland of these sea slugs for periods ranging from a few days to several months (Händeler et al., 2009). In accordance, different levels of kleptoplasty have been proposed, including long-term, medium-term, short-term and non-functional retention (Clark et al., 1990; Händeler et al., 2009). In sea slugs with higher levels of functional retention, often termed ‘solar-powered sea slugs’ (Rumpho et al., 2000), kleptoplast photosynthesis has been shown to be nutritionally relevant (Pierce et al., 2015; Cartaxana et al., 2017).

Positive phototaxis has been shown for several sea slugs displaying kleptoplasty and it has been suggested that this behaviour promotes light harvesting by stolen plastids (Gallop et al., 1980; Weaver and Clark, 1981; Schmitt and Wägele, 2011; Miyamoto et al., 2015). However, these animals seem to avoid high

light intensities, possibly as a strategy to prevent the premature loss of kleptoplast photosynthetic function (Weaver and Clark, 1981; Cruz et al., 2013). A distinguishable behaviour in response to light has been recorded in *Elysia timida*: a change in the position of its lateral folds (parapodia) from a closed position to a spread, opened leaf-like posture under lower irradiance and the opposite behaviour under high light levels (Rahat and Monselise, 1979; Jesus et al., 2010; Schmitt and Wägele, 2011). This behaviour in response to light changes was only recorded for this species and has been assumed to be linked to long-term retention of kleptoplasts (Schmitt and Wägele, 2011).

In this study, we determine whether the photobehaviour of the solar-powered sea slug *Elysia viridis* is linked to the photophysiological state of kleptoplasts (i.e. photoacclimation). In order to photoacclimate the kleptoplasts, *E. viridis* and its algal food source, *Codium tomentosum*, were maintained under low and high light levels. We describe a correlation between the position of *E. viridis* parapodia and irradiance and present evidence that light preference and parapodia closure are dependent on the photoacclimation state of the kleptoplasts.

MATERIALS AND METHODS

Sample collection and maintenance

The sea slugs *Elysia viridis* (Montagu 1804) and *Placida dendritica* (Alder and Hancock 1843), as well as 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). Both sea slugs were sampled from dense stands of *C. tomentosum*. Animals were kept in aerated water collected at the sampling site and transported to the laboratory within 2 h. Sea slugs and their macroalgal food were maintained in recirculated life-support systems operated with artificial seawater (ASW) at 18°C and a salinity of 32. Photoperiod was 14 h light:10 h dark at an irradiance of 40 μmol photons m⁻² s⁻¹ (low light acclimated, LL_{ac}) provided by T5 fluorescent lamps. *Elysia viridis* and *C. tomentosum* were also maintained in the same conditions as described above but under an irradiance of 200 μmol photons m⁻² s⁻¹ (high light acclimated, HL_{ac}). Chosen irradiance levels are within the range measured in the shaded intertidal rock pools where the macroalgae and animals were collected. The duration of the light-acclimation period was 2 weeks. Animals used for pigment analysis showed no significant differences in dry mass (*t*-test, *P*=0.129) between LL_{ac} and HL_{ac} treatments (2.64±0.63 and 3.20±0.38 mg, respectively; means±s.d.).

Light preference

Sea slugs were placed individually into 150 mm long×10 mm wide glass tubes filled with ASW. The tube was composed of sequential dark and transparent areas as follows: 15 mm dark–15 mm transparent–60 mm dark–60 mm transparent (Fig. S1). In the first light preference experiment, the tube was illuminated from above,

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resulting in a homogeneous light field of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (low light 1, LL₁) in the transparent areas and a gradient of $0\text{--}5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (dark) in the dark areas. In the second light preference experiment, the tube was illuminated, resulting in a homogeneous light field of $550 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (high light, HL) in the transparent areas and a gradient of $5\text{--}80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (low light 2, LL₂) in the dark areas. Irradiance was measured with a submersible spherical micro quantum sensor (US-SQS/L, Heinz Walz GmbH, Pfullingen, Germany). The animals were then monitored for 20 min and the time spent in each part of the tube was registered. Twenty-four specimens of *E. viridis* were monitored (12 LL_{ac} and 12 HL_{ac}). Six *P. dendritica* were also monitored as a negative control, as this sea slug feeds on the same macroalga but presents non-functional retention of kleptoplasts.

Opening/closure of parapodia as an effect of irradiance

Sea slugs were placed in Petri dishes with ASW for 30 min to 1 h at an irradiance of $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ under a digital microscope (DMS-300, Leica Microsystems, Wetzlar, Germany). When the animals were static and fully relaxed, they were subjected to increasing irradiance at steps of 30 s duration: 10, 30, 120, 350 and $650 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Light was delivered from an LED light source (KL-200 LED, Schott, Mainz, Germany). At the end of each light step, a photograph was taken with the digital microscope camera. The exposed dorsal area (EDA), excluding the pericardium, was then calculated from the photographs with open source software ImageJ (version 1.49). For each slug, the percentage EDA was calculated as $\text{EDA}_E/\text{EDA}_{\text{max}} \times 100$, where EDA_E was the EDA at irradiance E and EDA_{max} was the maximum EDA. Twenty-eight specimens of *E. viridis* were monitored (14 LL_{ac} and 14 HL_{ac}). As *P. dendritica* have cylindrical cerata extending from the dorsal surface and lack parapodia, they were not considered in this experiment.

PAM fluorometry

Fluorescence measurements were carried out on LL_{ac} and HL_{ac} macroalgal samples using a Junior PAM fluorometer (Heinz Walz GmbH). The distance between the fluorometer fibre optic and the surface of the sample was kept constant at 1 mm during all measurements. Steady-state light curves (SSLCs) were constructed with 11 incremental steps of actinic irradiance (E ; 0, 25, 45, 65, 90, 125, 190, 285, 420, 625 and $820 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). For each step, the effective quantum yield of photosystem II ($\Delta F/F_m'$) was monitored every 2 min and when a steady-state was reached the relative electron transport rate (rETR) was calculated as $\text{rETR} = E \times \Delta F/F_m'$. The light response was characterized by fitting the model of Eilers and Peeters (1988) to rETR versus E curves and by estimating the parameters α (initial slope of the light curve), ETR_{max} (maximum rETR) and E_k (light saturation coefficient). The model was fitted iteratively using MS Excel Solver. Curve fit was very good in all cases ($r > 0.98$).

HPLC pigment analysis

Pigment analysis of LL_{ac} and HL_{ac} sea slugs and macroalgae was done as described in detail by Cruz et al. (2014). Briefly, sea slugs and macroalgal samples were frozen in liquid nitrogen, freeze-dried and pigments extracted in 95% cold buffered methanol (2% ammonium acetate). After filtration, the extracts were injected into an HPLC system (Shimadzu, Kyoto, Japan) with a photodiode array detector (SPD-M20A). Pigments were identified from absorbance spectra and retention times and concentrations calculated in comparison with pure crystalline standards from

DHI (Hørslø, Denmark). Pigment concentrations were expressed per dry mass.

Statistical analysis

Significant differences of photoacclimation state (LL_{ac} and HL_{ac}) for measured parameters were tested using independent samples t -tests. Light preference was tested using one-sample t -tests for a test value of 10 min. Assumptions were verified using Levene's (homogeneity of variances) and Shapiro–Wilk (normal distribution) tests. A three-parameter logistic model was applied to EDA versus E data for LL_{ac} and HL_{ac} states. Significant differences in E_{50} (irradiance causing 50% closure of parapodia) between different photoacclimation states were evaluated using a generalized likelihood ratio test. All statistical analyses were carried out using IBM SPSS Statistics 24.

RESULTS AND DISCUSSION

The two irradiance levels under which *E. viridis* and *C. tomentosum* were maintained induced a clear difference in the photoacclimation state of the plastids, as demonstrated by differences between LL and HL treatments in both photophysiological parameters (SSLC parameters ETR_{max} and E_k) and pigment concentration (Fig. 1). Photosynthetic capacity at saturating irradiance (ETR_{max}) and light saturation coefficient (E_k) were significantly higher for HL_{ac} (42.8 ± 5.0 and $86.3 \pm 9.7 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively; mean \pm s.d.) than for LL_{ac} *C. tomentosum* (23.0 ± 9.8 and $45.8 \pm 9.4 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) (t -tests, $P = 0.036$ and 0.006 , respectively). These differences are characteristic of HL- versus LL-acclimated algae (Cruz and Seródio, 2008). The parameter related to photosynthetic efficiency at limiting irradiance, α (0.49 ± 0.11 and $0.50 \pm 0.08 \mu\text{mol}^{-1} \text{m}^2 \text{s}$ for LL_{ac} and HL_{ac}, respectively; mean \pm s.d.), did not show significant differences between light treatments (t -test, $P = 0.907$) (Fig. 1A).

Concentrations of pigments from the light harvesting complexes (LHCs) of siphonous green algae chloroplasts, chlorophylls *a* and *b* (Chl *a* and Chl *b*) and siphonoxanthin (Siph) were significantly higher in LL_{ac} *C. tomentosum* and *E. viridis* (t -tests, in all cases $P < 0.05$) (Fig. 1B,C). This is a consequence of adaptation to the available light conditions, with specimens reared under low irradiance requiring higher concentrations of pigments for light harvesting than individuals reared under high light. An opposite trend was observed for pigments all-*trans*-neoxanthin (*t*-Neo) and violaxanthin (Viola) with significantly higher concentrations found in HL_{ac} *C. tomentosum* and *E. viridis* (t -tests, in all cases $P < 0.05$). This is yet more evidence of photoacclimation, as these pigments are involved in photoprotection. Indeed, Uragami et al. (2014) observed the accumulation of *t*-Neo and Viola in *Codium intricatum* under high irradiance conditions and hypothesized that these pigments promote oligomerization of the LHCs in order to quench the excess amount of excitation energy. Pigment differences due to light acclimation were similar in sea slugs and the algal food source, indicating a fast turnover of kleptoplasts.

When allowed to choose between LL₁ ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and dark ($0\text{--}5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) conditions, the long-term kleptoplast retention species *E. viridis* showed a significant preference for LL₁ (t -tests, $P < 0.001$ in both LL_{ac} and HL_{ac}) (Fig. 2A). Choice of low light versus dark was independent of the photoacclimation state (t -test, $P = 0.634$). In contrast, *P. dendritica*, a non-functional kleptoplast retention sea slug, showed an extremely variable behaviour without any preference for dark, low or high light conditions (t -tests, $P = 0.586$ and $P = 0.996$ for dark versus LL₁ and HL versus LL₂, respectively) (Fig. 2A,B). Positive phototaxis

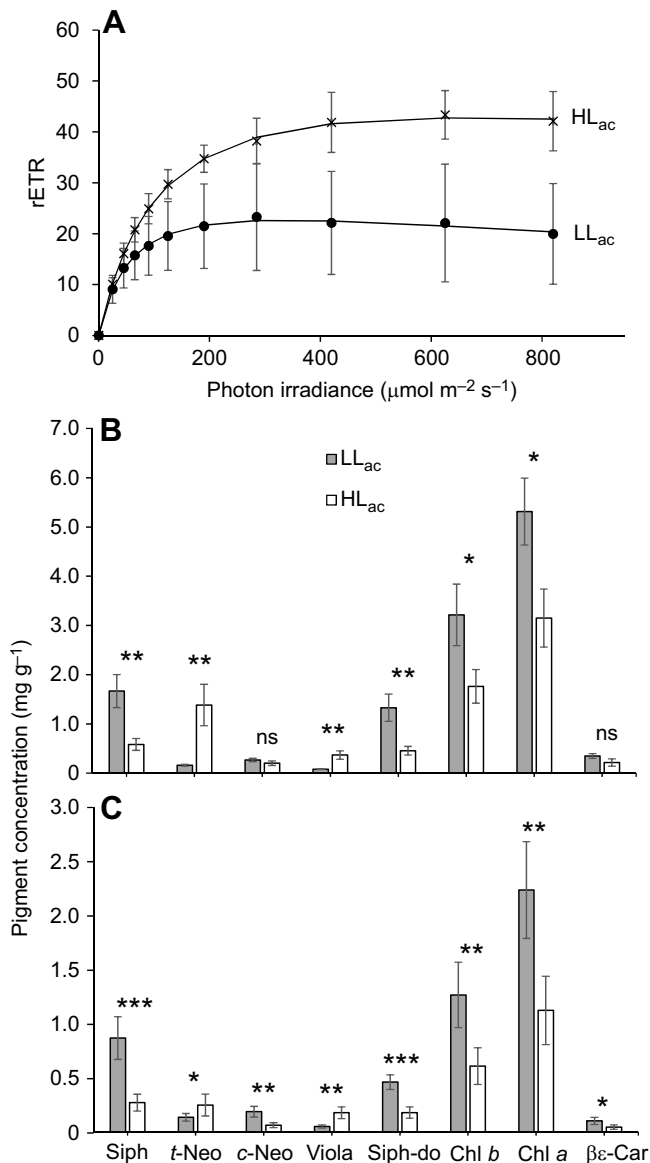


Fig. 1. Photoacclimation of *Codium tomentosum* and *Elysia viridis*. (A) Steady-state light-response curves of relative electron transport rate (rETR) versus photon irradiance in low light-acclimated (LL_{ac}, 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and high light-acclimated (HL_{ac}, 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) *C. tomentosum*. Lines represent curve fits according to the model of Eilers and Peeters (1988). (B,C) Pigment concentrations on a per dry mass basis of LL_{ac} and HL_{ac} *C. tomentosum* (B) and *E. viridis* (C). Siph, siphonaxanthin; t-Neo, all-trans-neoxanthin; c-Neo, 9'-cis-neoxanthin; Viola, violaxanthin; Siph-do, siphonaxanthin dodecenoate; Chl b, chlorophyll b; Chl a, chlorophyll a; $\beta\epsilon$ -Car, $\beta\epsilon$ -carotene. Values are means \pm s.d. ($n=3$ for *C. tomentosum* and $n=5$ for *E. viridis*). Asterisks indicate significant differences in pigment concentrations between LL_{ac} and HL_{ac} (t -tests): * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant.

has been shown for several sea slugs species hosting functional kleptoplasts (Gallop et al., 1980; Weaver and Clark, 1981; Schmitt and Wägele, 2011; Miyamoto et al., 2015). *Costasiella lilianae* and *Elysia crispata* preferred light intensities of up to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ over dark, while *Berthelinia caribbea* and *Oxynoe antillarum*, which do not retain algal chloroplasts, showed a preference for dark conditions (Weaver and Clark, 1981). Likewise, Miyamoto et al. (2015) reported positive phototaxis for photosynthetic sea slugs and neutral or negative

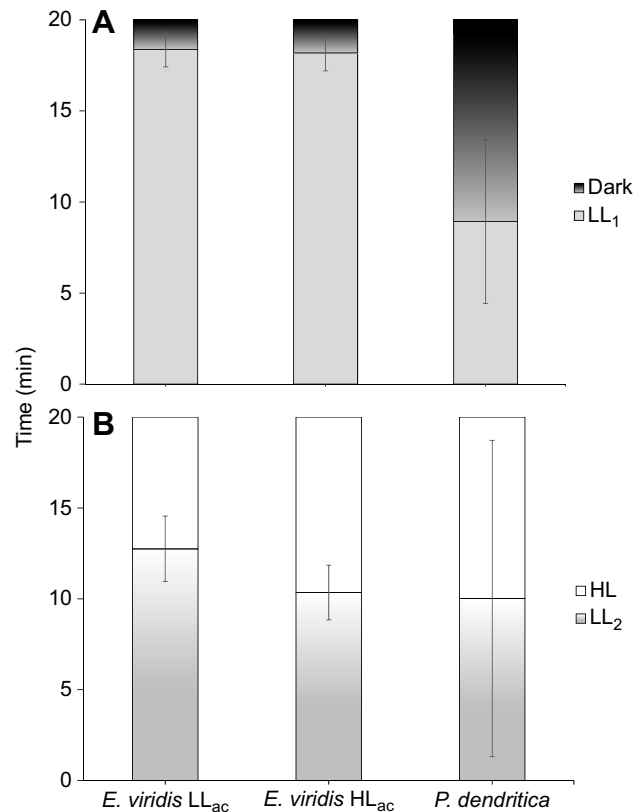


Fig. 2. Light preference of sacoglossan sea slugs. (A,B) Time spent by LL_{ac} (40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and HL_{ac} (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) *Elysia viridis* and by *Placida dendritica* in dark (0–5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) versus low light 1 (LL₁, 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) conditions (A); or in high light (HL, 550 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) versus low light (LL₂, 5–80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) conditions (B). Values are means \pm s.d. ($n=12$ for *E. viridis* and $n=6$ for *P. dendritica*).

phototaxis for species without functional kleptoplasts. However, light-driven behaviour in sacoglossan sea slugs may not be exclusively related to the presence of functional kleptoplasts. Juveniles of *E. timida* display a clear preference for light prior to the acquisition of kleptoplasts (Schmitt and Wägele, 2011). Indeed, phototaxis is a widespread animal behaviour and factors such as foraging might play an important role in the response of sacoglossan sea slugs to light.

When allowed to choose between LL₂ (5–80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and HL (550 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), *E. viridis* showed a significant preference for low light in LL_{ac} specimens (t -test, $P<0.001$), but no specific preference in HL_{ac} individuals (t -test, $P=0.441$). Light preference was dependent on the photoacclimation state: HL_{ac} specimens spent significantly more time in high light than LL_{ac} conspecifics (t -test, $P=0.002$) (Fig. 2B).

Closure of parapodia, with a consequent decrease in EDA, was observed under increasing irradiance for both LL_{ac} and HL_{ac} *E. viridis* (Fig. 3; Fig. S2). However, the pattern of parapodia closure was dependent on the photoacclimation state. E_{50} was significantly higher ($\chi^2_{df=1}=62.27$, $P<0.001$) for HL_{ac} *E. viridis* than for LL_{ac} conspecifics (486 ± 28.4 and 138 ± 15.4 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively; mean \pm s.e.m.). For LL_{ac} *E. viridis*, the relationship between EDA and E was exponential, with substantial closure of parapodia occurring even under low irradiance (from 30 to 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). HL_{ac} *E. viridis* closed their parapodia under higher irradiance levels (350 and 650 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) than LL_{ac} specimens (Fig. 3; Fig. S2).

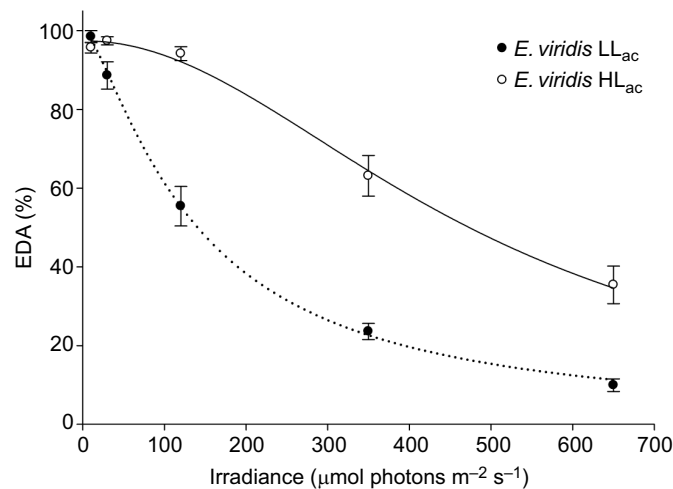


Fig. 3. Opening/closure of parapodia as an effect of irradiance in *E. viridis*. Variation of the percentage of exposed dorsal area (EDA) with irradiance in LL_{ac} (40 μmol photons m⁻² s⁻¹) and HL_{ac} (200 μmol photons m⁻² s⁻¹) *E. viridis*. Lines show the fit obtained with a three-parameter logistic model ($r=0.95$ and 0.90 for LL_{ac} and HL_{ac}, respectively). Values are means±s.e.m. ($n=14$).

These results are congruent with light acclimation at 40 μmol photons m⁻² s⁻¹ (LL_{ac}) and 200 μmol photons m⁻² s⁻¹ (HL_{ac}) and indicate that photobehaviour of *E. viridis* was modulated by the photoacclimation state of the kleptoplasts. In a light preference experiment, the sea slugs *Elysia hamatani*, *Elysia trisinuata* and *Plakobranthus ocellatus* were reported to choose light intensities that maximized photosynthesis (Miyamoto et al., 2015). Similarly, a clear avoidance of extreme low or high light levels and a preferential accumulation at irradiance levels coincident with the optimum for photosynthetic activity were reported for the symbiotic acopl flatworm *Symsagittifera roscoffensis* (Serôdio et al., 2011).

Avoidance of high irradiance through parapodia closure is a possible strategy to prevent photoinhibition and a premature loss of kleptoplast photosynthetic function (Weaver and Clark, 1981; Cruz et al., 2013). Rahat and Monselise (1979) observed that *E. timida* opened their parapodia wide under light intensities between 3×10^3 and 3×10^5 erg cm⁻² s⁻¹ (approximately 14–1400 μmol photons m⁻² s⁻¹) and closed them at lower or higher irradiance levels. We found that *E. viridis* opened their parapodia at an irradiance of 10 μmol photons m⁻² s⁻¹ and closed them at a lower irradiance than that reported for *E. timida*. These differences may be related to a distinct kleptoplast distribution in the two species: exclusively in the inner part of the parapodia in *E. timida* and in both inner and outer sides in *E. viridis*. This probably makes the closure of parapodia more efficient in protecting kleptoplasts from high light exposure in *E. timida*. Alternatively, differences may arise from higher sensitivity to light of *Codium*-derived chloroplasts hosted by *E. viridis* as a result of the absence of a photoprotective xanthophyll cycle (Cruz et al., 2015), which is present in *Acetabularia*- and *Vaucheria*-derived chloroplasts of *E. timida* and *Elysia chlorotica*, respectively (Jesus et al., 2010; Cruz et al., 2015). Changes in the position of the parapodia in response to light variations were not observed in *Thuridilla hopei*, a short-term (ca. 8 days) kleptoplast retention species (Schmitt and Wägele, 2011).

Plastid acquisition during evolution of eukaryotes imposed an increase in the production of reactive oxygen species (ROS) and the development of the redox-signalling network (Woehle et al., 2017). Hence, it is reasonable to hypothesize that kleptoplasty may be challenging to metazoan cells as a result of ROS produced from

chloroplast oxygen-based metabolic processes, particularly under high light conditions (Nishiyama et al., 2006). ROS generated as by-products of photosynthesis, especially singlet oxygen and H₂O₂, are signals involved in responses to excessive light (Li et al., 2009), but whether these molecules trigger light avoidance/closure of parapodia in solar-powered sea slugs remains to be investigated.

In conclusion, we describe a correlation between the position of *E. viridis* parapodia and irradiance. Our results indicate that the photobehaviour (light preference and parapodia closure) of this sea slug species is modulated by the photoacclimation state of the kleptoplasts.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.C., S.C.; Methodology: P.C., L.M., C.Q.; Formal analysis: P.C., L.M., C.Q., G.C., R.C., S.C.; Investigation: P.C., L.M., C.Q., S.C.; Writing - original draft: P.C., S.C.; Writing - review & editing: P.C., L.M., C.Q., G.C., R.C., S.C.; Supervision: P.C., S.C.; Project administration: S.C.; Funding acquisition: S.C.

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Supplementary information

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