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SHORT COMMUNICATION

The photon menace: kleptoplast protection in the photosynthetic sea slug *Elysia timida*

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ABSTRACT

Absorption of excessive light by photosymbiotic organisms leads to the production of reactive oxygen species that can damage both symbiont and host. This is highly relevant in sacoglossan sea slugs that host functional chloroplasts 'stolen' from their algal foods (kleptoplasts), because of limited repair capacities resulting from the absence of algal nuclear genes. Here, we experimentally demonstrate (i) a host-mediated photoprotection mechanism in the photosynthetic sea slug *Elysia timida*, characterized by the closure of the parapodia under high irradiance and the reduction of kleptoplast light exposure; and (ii) the activation of a reversible xanthophyll cycle in kleptoplasts, which allows excessive energy to be dissipated. The described mechanisms reduce photoinactivation under high irradiance. We conclude that both host-mediated behavioural and plastid-based physiological photoprotective mechanisms can mitigate oxidative stress induced by high light in *E. timida*. These mechanisms may play an important role in the establishment of long-term photosynthetically active kleptoplasts.

KEY WORDS: Kleptoplasty, Light stress, Photobehaviour, Photoprotection, Photoinactivation, Violaxanthin cycle

INTRODUCTION

Kleptoplasty is the capacity of a non-photosynthetic host to incorporate functional algal chloroplasts into their own cells, and has been reported in dinoflagellates, ciliates, foraminiferans and a single taxon of metazoans, sacoglossan sea slugs (Serôdio et al., 2014). Although kleptoplasty does not fit the definition of symbiosis *sensu stricto* (Raven et al., 2009), it is commonly referred to as chloroplast symbiosis (Rumpho et al., 2000). While kleptoplasty facilitates crypsis in sacoglossan sea slugs, its highest adaptive value is probably the nutritional benefit derived from kleptoplast photosynthesis (Händeler et al., 2009; Rumpho et al., 2011; Cartaxana et al., 2017).

Stolen plastids may remain functional in the cells of the digestive gland of sacoglossan sea slugs for periods ranging from only a few days to several months (Händeler et al., 2009). The long-term maintenance of kleptoplast photosynthetic activity in the absence of the algal nucleus is still a puzzling feature, as the chloroplast genome solely encodes a fraction of the proteins required for general maintenance of the photosynthetic apparatus (Keeling, 2013). Accumulation of experimental evidence that contradicts the horizontal gene transfer hypothesis in photosynthetic sea slugs (Wägele et al., 2010; Bhattacharya et al., 2013) has led to the search for alternative mechanisms that can help to explain the longevity of functional kleptoplasts.

Acquiring chloroplasts is potentially dangerous for host organisms, as damage generated by reactive oxygen species (ROS) may extend beyond the organelle to the rest of the host cell (Dorrell and Howe, 2012). Photosynthetic organisms trigger photoprotection mechanisms when exposed to high irradiance in order to dissipate the excess absorbed energy or reduce light absorption, consequently limiting ROS production (Takahashi and Badger, 2010). Studies addressing the maintenance of the physiological photoprotective potential of chloroplasts following incorporation into sea slug cells are scarce, but it has been hypothesized that it plays a significant role in the longevity of kleptoplast photosynthetic activity by reducing light-induced oxidative stress (Cruz et al., 2015). Furthermore, host-mediated photoprotection through the folding of dorsal body flaps (parapodia) in sacoglossan sea slugs can reduce kleptoplast exposure and light absorption (Cartaxana et al., 2018). Analogous behavioural (tissue retraction) and physiological (xanthophyll cycle) photoprotective mechanisms have been described in corals exposed to high irradiance levels (Brown et al., 1999, 2002).

In this study, we characterized host-mediated behavioural and kleptoplast physiological photoprotection mechanisms in the sea slug *Elysia timida* and provide new insights into the role that these mechanisms play on the long-term maintenance of kleptoplast photosynthetic activity in sacoglossan sea slugs.

MATERIALS AND METHODS

Sample collection and maintenance

Specimens of *Elysia timida* (Risso 1818) were collected in March 2018 in Puerto de Mazarrón in the Mediterranean Sea, Spain. Sampling of *E. timida* and its algal food, *Acetabularia acetabulum*, was done by SCUBA diving at a depth of ca. 2 m. Animals were kept in aerated water collected at the sampling site and transported to the laboratory within 48 h. Sea slugs and macroalgae were maintained for 2 weeks in a 150 l recirculated life support system operated with artificial seawater (ASW) at 18°C and salinity 35. Photoperiod was maintained at 14 h light:10 h dark, with an irradiance of 40 µmol photons m^{-2} s^{-1} provided by T5 fluorescent lamps.

Behavioural (host-mediated) photoprotection: opening/closure of parapodia as an effect of irradiance levels

Sea slugs were placed inside 83 mm diameter Petri dishes with 40 ml of ASW for 30 min to 1 h at an irradiance of 10 µmol photons m^{-2} s^{-1} under a digital microscope (DMS-300, Leica Microsystems, Wetzlar, Germany). When the animals were static and fully relaxed, they were exposed to increasing irradiance at steps of 30 s duration: 10, 70, 250, 700 and 1200 µmol photons m^{-2} s^{-1}. Light was delivered by an LED light source (KL-200 LED, Schott, Mainz, Germany) and measured

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using a spherical micro quantum sensor (US-SQS/L, Heinz Walz GmbH, Pfullingen, 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, percentage of EDA was calculated as $EDA_E/EDA_{max}\times100$, where EDA_E is the EDA at irradiance E and EDA_{max} is the maximum EDA (Cartaxana et al., 2018). A total of 18 animals were individually monitored.

Physiological photoprotection: light stress and operation of the xanthophyll cycle

Sea slugs were placed inside 83 mm diameter Petri dishes with 40 ml of ASW and exposed to a light stress and recovery (LSR) protocol: sequential periods of 15 min dark, 20 min high light (HL, 1000 µmol photons m^{-2} s^{-1}) and 40 min recovery under low light (LL_{rec}, 40 µmol photons m^{-2} s^{-1}). Light was delivered by the same LED light source described above. Photosynthetic efficiency was assessed by measuring chlorophyll *a* fluorescence with a JUNIOR-PAM fluorometer (Heinz Walz GmbH). At the end of each LSR step, the optical fibre of the fluorometer was placed in direct contact with the animal's parapodia, a saturating pulse was applied and minimum and maximum fluorescence levels were registered. Maximum quantum yield of photosystem (PS)II (F_v/F_m) was calculated as $(F_m-F_o)/F_m$, where F_m and F_o are, respectively, the maximum and the minimum fluorescence of dark-adapted slugs (Murchie and Lawson, 2013). Effective quantum yield of PSII ($\Delta F/F_m'$) was measured as $(F_m'-F_s)/F_m'$, where F_m' and F_s are, respectively, the maximum and the minimum fluorescence levels measured under steady-state irradiance. The LSR protocol was performed for fed, 1 week-starved and 3 week-starved specimens with seven independent replicates for each period (dark, HL and LL_{rec}; for a total of 63 sea slugs).

Pigment analysis was performed as described in detail by Cruz et al. (2014). Briefly, sea slugs monitored during the LSR protocol were immediately frozen in liquid nitrogen. Samples were freeze-dried and pigments extracted in 95% cold buffered methanol (2% ammonium acetate). After filtration, the extracts were injected into a HPLC system (Shimadzu, Kyoto, Japan) with a photodiode array detector (SPD-M20A). Pigments were identified from absorbance spectra and retention times and concentrations were calculated in comparison with pure crystalline standards (DHI, Hørsolm, Denmark). The operation of the xanthophyll cycle, comprising the sequential de-epoxidation of the pigments violaxanthin (Vx) to antheraxanthin (Ax) and zeaxanthin (Zx), was followed by calculating the de-epoxidation state (DES) as: DES=([Zx]+0.5×[Ax])/([Zx]+[Ax]+[Vx]). The xanthophyll cycle is a ubiquitous mechanism in photosynthetic organisms with the accumulation of Ax and Zx providing photoprotection by the dissipation of excess absorbed light energy as heat, a process called non-photochemical quenching (NPQ) (Demmig-Adams and Adams, 1996; Brown et al., 1999).

Statistical analysis

EDA data failed to meet the assumptions of ANOVA with repeated measures regarding normality and/or homogeneity of variances. Hence, the effects of irradiance on EDA were tested with the non-parametric Friedman's test (χ^2 value presented), followed by a *post hoc* analysis using Wilcoxon signed-rank tests with a Bonferroni correction. Effects of light (dark, HL and LL_{rec}) or feeding (fed, 1 week starved and 3 week starved) treatments on F_v/F_m, $\Delta F/F_m'$, DES and pigment pools were tested using one-way ANOVA (F-values presented). Normality was checked using Shapiro–Wilk test and homogeneity of variance using Levene's test. Multiple comparisons were performed using Tukey HSD. If data failed to meet ANOVA assumptions of normality and/or homogeneity of variance, Kruskal–Wallis non-parametric tests were performed (H-values presented), followed by Dunn's *post hoc* tests. Significance levels were adjusted by the Bonferroni correction for multiple tests. All statistical analyses were performed using IBM SPSS Statistics 24.

RESULTS AND DISCUSSION

There was a statistically significant effect (χ^2_4=58.489, P<0.001) of irradiance on the EDA of *E. timida* (Fig. 1). The animals opened the parapodia significantly (P=0.020) when exposed to a light transition of 10–70 µmol photons m^{-2} s^{-1}. Further light transitions led to a progressive closure of parapodia, which was statistically significant (P<0.001) for high irradiance levels (700 and 1200 µmol photons m^{-2} s^{-1}). These results experimentally demonstrate that the position of *E. timida* parapodia is dependent on irradiance: (i) parapodia opened under low light levels, exposing kleptoplasts and promoting light harvesting as these organelles are distributed in the inner part of the parapodia; (ii) parapodia closed under high light levels, shielding kleptoplasts from excessive irradiance and reducing absorbed light energy. Light is attenuated while crossing the more stratified and denser tissue of the animal in the closed position, as in corals and other thick photosynthetic tissues (Wangpraseurt et al., 2019). This light-induced behaviour was previously observed for *E. timida* (Rahat and Monselise, 1979), but quantitative measurements of kleptoplast exposure to light were missing.

Continuous exposure to high light has been shown to significantly reduce functional kleptoplast longevity in sacoglossan sea slugs (Vieira et al., 2009; Klochkova et al., 2013). Hence, avoidance of high irradiance through the closure of parapodia may constitute an efficient strategy to prevent inhibition of kleptoplast photosynthesis and further damage to the host metabolism (Cruz et al., 2013). This adaptation is functionally equivalent to tissue retraction recorded in corals hosting photosynthetic endosymbionts or leaf folding in plants, which has been shown to provide effective protection against photoinactivation (Brown et al., 2002; Pastenes et al., 2005).

The photoacclimation state of kleptoplasts was shown to modulate the photobehaviour response in the sea slug *Elysia viridis*, indicating

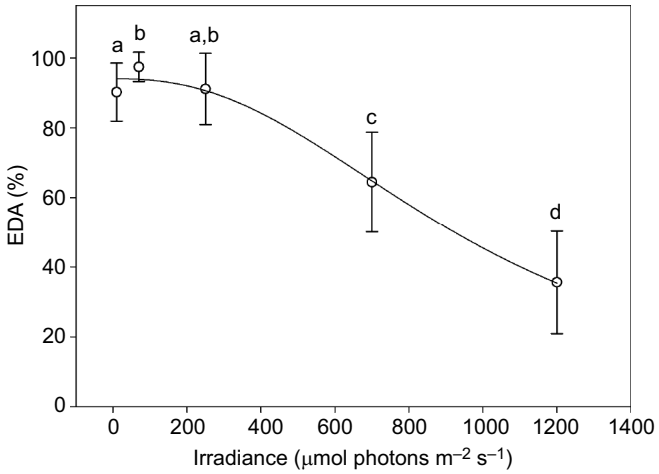

Fig. 1. Opening/closure of parapodia in *Elysia timida* as an effect of irradiance. Variation of exposed dorsal area (EDA) in animals exposed to a sequential increase in irradiance. Values are means±s.d. (n=18). Line shows the fit obtained with a three-parameter logistic model (r=0.99). Different letters indicate significant differences (P<0.05).

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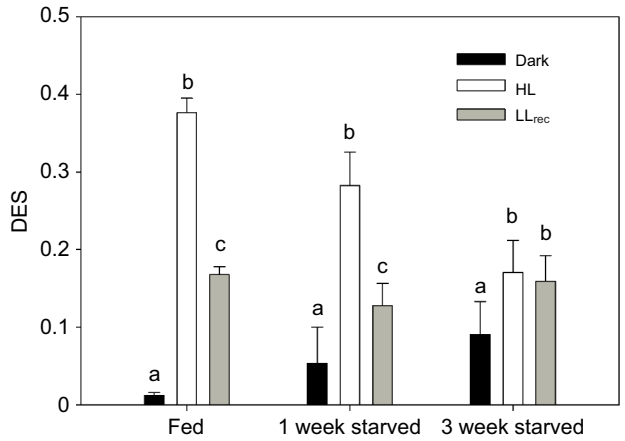

Fig. 2. De-epoxidation state (DES) in kleptoplasts of *E. timida*. Variation of DES in animals stocked under different feeding conditions (fed, 1 week starved and 3 week starved) during a light stress and recovery (LSR) protocol: 15 min of dark; 20 min of high light (HL, 1000 µmol photons m^{-2} s^{-1}); and 40 min of recovery under low light (LL_{rec}, 40 µmol photons m^{-2} s^{-1}). Values are means±s.d. (*n*=7). Different letters indicate significant differences between dark, HL and LL_{rec} (*P*<0.05).

host–kleptoplast communication (Cartaxana et al., 2018). Recently published transcriptomic data of *Elysia chlorotica* show that chloroplast sequestration leaves a significant signature on host gene expression, similar to that occurring during symbiosis establishment in corals, and indicate the engagement of cell communication between host and kleptoplasts (Chan et al., 2018). Petrou et al. (2017) describe a host-mediated relocation of *Symbiodinium* within the calcium carbonate test of the photosymbiotic foraminifera *Marginopora vertebralis*, a mechanism controlled by symbiont signalling under high light stress.

The LSR protocol caused pronounced changes in the DES of *E. timida* kleptoplasts (Fig. 2). The general trend was an increase in the DES of kleptoplasts from dark to high light caused by an increase in pigments Ax and Zx and a decrease in Vx, which was partially reversed after a 40 min recovery in low light (Fig. 2; Fig. S1). These results indicate a functional Vx cycle in kleptoplasts of *E. timida*, comprising the sequential de-epoxidation of Vx to Ax and Zx under high light conditions and the reverse reaction under low irradiance. Pigment conversion under high light led to the dissipation of excess absorbed light energy as heat (Demmig-Adams and Adams, 1996). In accordance with our results, Jesus et al. (2010) observed the accumulation of Zx in *E. timida* exposed to high irradiance. A functional Vx cycle and rapidly reversible NPQ was reported for *Vaucheria litorea* chloroplasts in the sea slug *E. chlorotica*, which was absent in *Codium*-derived chloroplasts of *E. viridis* (Cruz et al., 2015).

The degree of DES under high light was negatively correlated with the level of starvation ($F_{2,17}$=52.891, *P*<0.001; Fig. 2). Under high light, DES was significantly higher in fed *E. timida*, intermediate in 1 week-starved specimens, and lower in 3 week-starved specimens (in all cases, *P*<0.001). A significant effect of starvation ($F_{2,18}$=3.803, *P*=0.042) was also observed on the quantum yield of PSII ($\Delta F/F_m'$) of *E. timida* kleptoplasts after 20 min of high light (Table 1). The photosynthetic yield under this light level was significantly higher (*P*=0.033) in fed *E. timida* than in 3 week-starved specimens. A significant effect of starvation was observed on $\Delta F/F_m'$ after recovery under low light (H_2=14.524, *P*=0.001). The quantum yield upon recovery was significantly higher in fed *E. timida* than in 1 week- and 3 week-starved specimens (*P*=0.022 and *P*= 0.001, respectively; Table 1), and correlated with DES under high light exposure

Table 1. Photosynthetic efficiency of *Elysia timida* kleptoplasts

E. timida treatment	Dark	HL	LL_{rec}
Fed	0.80±0.01[a]	0.22±0.09[a]	0.72±0.01[a]
1 week starved	0.80±0.02[a]	0.16±0.08[a,b]	0.66±0.03[b]
3 week starved	0.80±0.02[a]	0.10±0.04[b]	0.63±0.03[b]

Maximum (dark-acclimated animals) and effective quantum yield (light-acclimated animals) of photosynthesis in animals stocked under different feeding conditions (fed, 1 week starved and 3 week starved) during a light stress and recovery (LSR) protocol: 15 min of dark; 20 min of high light (HL, 1000 µmol photons m^{-2} s^{-1}); and 40 min of recovery under low light (LL_{rec}, 40 µmol photons m^{-2} s^{-1}). Values are means±s.d. (*n*=7). Different superscript letters in the same column indicate significant differences (*P*<0.05).

(Fig. S2). These results indicate that the efficiency of photoprotection via the Vx cycle decreased as kleptoplasts aged with animal starvation, indicating that the kleptoplasts lose the capacity for physiological photoprotection and suggesting that Vx-cycle enzymes are nuclear encoded in *A. acetabulum*, as already reported for other green algae (Zorin et al., 2017). Our results contradict recent findings by Christa et al. (2018) reporting an equally active photoprotective component of NPQ (qE) in kleptoplasts of *E. timida* during starvation. These authors further argue that NPQ does not protect the kleptoplasts from net photoinactivation because *E. timida* and *E. viridis* showed comparable decreases in length and F_v/F_m when starved for 21 days under continuous high light. In contrast, we show that the presence of a fully active Vx cycle in kleptoplasts of fed *E. timida* positively affected the rapid recovery of photosynthetic efficiency after high light stress, probably by reducing the photoinhibitory component of NPQ (qI).

The absence of algal nuclear genes may strongly limit chloroplast repair capacities once these organelles are incorporated into sacoglossan sea slug cells (Serôdio et al., 2014). However, in an earlier study, it was shown that chloroplasts of *V. litorea* sequestered by the sea slug *E. chlorotica* continue to synthesize D1, the core protein of PSII and the main target of photoinhibition (Mujer et al., 1996). More recently, de Vries et al. (2013) hypothesized that the plastid-encoded FTSH, a D1 quality control protease that is essential for PSII repair, could rescue kleptoplasts from photodamage in photosynthetic sea slugs. More recently, Christa et al. (2018) estimated the rate constant of PSII inactivation (k_{PI}) in kleptoplasts of *E. viridis* and *E. timida* and concluded that kleptoplasts have a reduced PSII repair capacity when compared with chloroplasts in their natural host algae.

Overall, we conclude that both host-mediated behavioural and plastid-based physiological photoprotective mechanisms mitigate high light-induced oxidative stress in *E. timida* and probably contribute to the long-term maintenance of photosynthetic activity in kleptoplasts of sacoglossan sea slugs.

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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., B.J., G.C., R.C., S.C.; Formal analysis: P.C., L.M., S.C.; Investigation: P.C., L.M., B.J., G.C., R.C., S.C.; Writing - original draft: P.C., S.C.; Writing - review & editing: P.C., L.M., B.J., 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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Figure S1. Violaxanthin, antheraxanthin and zeaxanthin pigment pools in kleptoplasts of *Elysia timida*. Animals were stocked under different feeding conditions (fed, 1week-, and 3 week-starved) during a LSR protocol: 15 min of dark; 20 min of high light (HL, 1000 µmol photons m^{-2} s^{-1}); and 40 min of recovery under low light (LL_{REC}, 40 µmol photons m^{-2} s^{-1}). Values are means ± standard deviations (n=7). Different letters indicate significant differences between Dark, HL and LL_{REC} ($p < 0.05$).

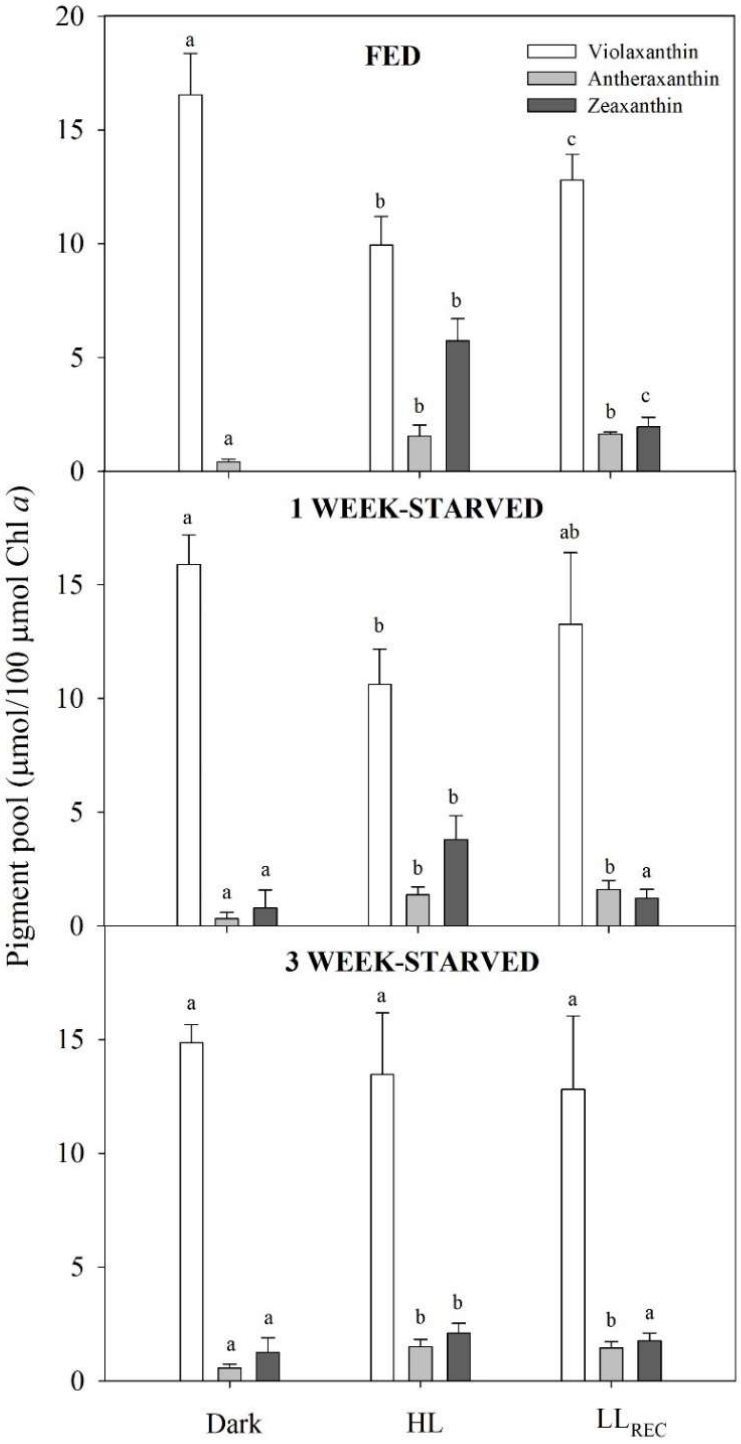

Figure S2. Relationship between de-epoxidation state (DES) under high light (HL, 1000 µmol photons m^{-2} s^{-1}) and effective quantum yield of photosystem II ($\Delta F/F_m'$) upon recovery (LL_{REC}, 40 µmol photons m^{-2} s^{-1}) in kleptoplasts of *Elysia timida* stocked under different feeding conditions (fed, 1 week-, and 3 week-starved). Values are means ± standard deviations (n=7). Line shows a linear fit (r=0.96).