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ORIGINAL PAPER

# Crawl away from the light! Assessing behavioral and physiological photoprotective mechanisms in tropical solar-powered sea slugs exposed to natural light intensities

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**Abstract**
Photosynthesizers face a trade-off regarding light: they need enough to maintain high photosynthetic rates, yet excess leads to oxidative stress. Despite this, light and its detrimental effects are chronically underestimated. Solar-powered sea slugs (Sacoglossa: Gastropoda) provide the ideal lens with which to study this trade-off, since they steal chloroplasts from algae but do not inherit photoacclimation and photorepair capacities. We collected three slug species in Curaçao during March and December 2022, comparing the amount of light they received in nature to their optimal light intensities for photosynthesis, and their preferred light intensities. We then investigated behavioral and physiological photoprotection mechanisms to determine if and how they limit light. Finally, we examined oxidative activity under optimal and excess light. All three species were naturally exposed to more light (> 1000 µmol $m^{-2}$ $s^{-1}$) than is optimal or preferred. *Elysia crispata* (kleptoplast retention for > 3 months) is fully exposed to light in nature but reduces the light reaching its kleptoplasts via parapodial shading. *Elysia velutinus* retains kleptoplasts for ~ 2 weeks and hides in its macroalgal food, limiting light exposure. Both species displayed low amounts of oxidative activity under optimal light, which increased slightly under excess light. *Elysia ornata* retained chloroplasts for ~ 3 days, lacked observable photoprotection and always displayed high levels of oxidative activity, potentially explaining its limited capacity for kleptoplast retention. Furthermore, both *E. velutinus* and *E. ornata* display strong light-avoidance behaviors. This study clearly demonstrates links between high light intensities, photoprotection, and oxidative stress, highlighting the need for future studies that examine aquatic photosynthesizers under natural lighting.

**Keywords** Elysia · Kleptoplasty · PAM fluorometry · Phototaxis · Sacoglossa

## Introduction

Photosynthesis sustains virtually all life on the planet, making light, specifically photosynthetically active radiation (PAR, hereafter referred to as light) a fundamental resource for photosynthetic organisms. Despite this, light can also be considered a cell-damaging, photosynthesis-inhibiting, and metabolism-regulating factor (D'Alessandro et al. 2020; Lima-Melo et al. 2021). On one hand, photoautotrophs need to acquire enough light to maximize their photosynthetic production; however, exposure to excess light can cause photoinhibition, a decrease in photosynthetic efficiency when the reaction centers are inhibited (Horton and Ruban 2005). High light intensities can also cause photodamage, which occurs when reactions that oxidize water to form molecular oxygen occur in proximity to electrons leaking from the electron transport chain following chlorophyll excitation, producing Reactive Oxygen Species (ROS) (Nugent 1996; Kozuleva et al. 2020). ROS, such as superoxide and hydrogen peroxide, can cause irreparable damage to DNA, lipid peroxidation as well as affect the function and structure of essential cellular components (Fridovich 1999); however, ROS also play an important role in redox signaling (Sies and Jones 2020). Excess ROS can disrupt cellular redox homeostasis, in a process termed oxidative stress, if a cell is unable to mitigate their reactivity through antioxidant activity (Fridovich 1999). To prevent ROS production due to excess light in the first place, photosynthetic organisms

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employ a variety of photoprotective mechanisms (Franklin and Forster 1997). Once damaged by ROS oxidation, cellular components must be repaired (photorepair) or replaced before the cell can return to optimal photosynthetic function (e.g., Goh et al. 2012).

Since preventing photoinhibition and photodamage are critical processes for maintaining high photosynthetic yield, photoautotrophs have evolved numerous ways to regulate the amount of light reaching their photosystems (termed photoacclimation) (Brunet et al. 2011). For example, many algal species contain pigments (e.g., xanthophylls and carotenes) in the light-harvesting complexes of both photosystems, which help regulate the light entering reaction centers and/or manage the detrimental products of excess excitation at the reaction centers (Goss and Latowski 2020). In algae, light-harvesting and photoprotective pigment pools are involved in coping with photodamage and can also play a key role in photoacclimation due to their high plasticity (Horton and Ruban 2005). This means that they can adapt to changes in the light field, mostly through changes in pigment concentration and composition (Escoubas et al. 1995; Rodriguez et al. 2006). This ability is fundamental for chloroplast optimization and continued function within the algae (Falkowski and Raven 2013). Photoautotrophs also employ a wide variety of biochemical mechanisms to mitigate the detrimental effects of excess light, such as quenching singlet/triplet excited states of chlorophyll that produce ROS, and by producing antioxidants to neutralize ROS that have formed (detailed in Pinnola and Bassi 2018).

Numerous studies have investigated the trade-off between getting enough light to sufficiently drive photosynthesis and receiving too much light causing photodamage in terrestrial organisms, where devices that can measure light intensity and photosynthetic performance are in widespread use and relatively inexpensive. Studying aquatic organisms in situ has proven more difficult, because measuring light intensity underwater presents several challenges. Light is highly variable, changing with time of day, atmospheric conditions, by season, with depth, and due to turbidity, since light attenuates with increasing depth and turbidity scatters photons (Kirk 1994). Very few photometers have been developed for use at depth and they are often (prohibitively) expensive, which has made measuring light intensity and photosynthetic performance in aquatic organisms difficult. Furthermore, these systems are often designed to be handled by a researcher rather than functioning as long-term monitoring setups following deployment in the field.

These complications mean that the vast majority of studies examining the effects of light on aquatic (and particularly marine) organisms are conducted in either outdoor or indoor laboratory settings, both of which have pros and cons. Outdoor studies provide the variability in light that organisms would experience in the field (i.e. sunrise/sunset, cloud cover), but often limit sunlight using materials that provide shade. Studies conducted in indoor settings under standardized lighting conditions do not account for natural variations in light intensity, since they rely on lamps that often have a limited light intensity. For example, McLachlan et al. (2020) reviewed 255 studies on corals, finding that the average light exposure was 229 µmol $m^{-2}$ $s^{-1}$ light intensity for indoor studies, and 429 µmol $m^{-2}$ $s^{-1}$ for outdoor studies (McLachlan et al. 2020). For shallow-dwelling species (0–10 m depth), these light intensities are a vast underestimation of the light intensity they actually receive in nature, which can reach 2000 µmol $m^{-2}$ $s^{-1}$ just below the surface in the tropics, dropping to 600 µmol $m^{-2}$ $s^{-1}$ at 6 m depth (e.g., Mantelatto et al. 2020), depending on turbidity, seasonality, etc. This chronic underestimation of light intensities for shallow-dwelling species makes it impossible to understand their natural photophysiology (McLachlan et al. 2020; Grottoli et al. 2021), a shortcoming that becomes particularly relevant when light is a key factor in the experimental design.

Experiments that manipulate light intensities are frequently used to examine the photophysiology of photosymbiotic organisms, i.e., species derived from heterotrophic lineages that have acquired photosynthetic capacities. In these systems, a metazoan or "protist" host (e.g., scleractinian corals) harbors photoautotrophic endosymbionts, such as cyanobacteria or unicellular algae (e.g., Venn et al. 2008; Kirk and Weis 2016; Melo Clavijo et al. 2018). One particular and highly derived type of photosymbiosis stands out as ideal for studying the trade-off between acquiring enough light to efficiently photosynthesize and minimizing excess light to avoid photodamage. Some species of sea slugs (Gastropoda: Sacoglossa) have acquired the ability to photosynthesize by isolating and retaining chloroplasts from their algal food sources in a process termed functional kleptoplasty (Rumpho et al. 2000; Serôdio et al. 2014). These acquired kleptoplasts do not divide but may remain photosynthetically active for periods ranging from a few days to several months providing nutritional benefits during periods of food scarcity (Hinde and Smith 1975; Casalduero and Muniain 2008; Händeler et al. 2009; Pelletreau et al. 2014; Laetz et al. 2017).

Numerous studies have investigated photoacclimation, photodamage, and photorepair in sacoglossan slugs under temperature and light controlled or uncontrolled laboratory conditions. These studies repeatedly demonstrate that kleptoplasts within sacoglossan slugs lack the ability to photoacclimate, meaning that the photoacclimation state found in the algae at the time it was consumed, is inherited by the slug, and may play a role in determining the duration with which kleptoplasts can remain photosynthetically functional (Serôdio et al. 2014; Cartaxana et al. 2018; Richards Donà et al. 2022). Likewise, photorepair capacities are likely limited, if functional at all, in sacoglossan slugs, meaning that

photodamage could also play a role in kleptoplast longevity once incorporated in a slug (Serôdio et al. 2014; de Vries et al. 2015; Christa et al. 2018; Richards Donà et al. 2022).

There are numerous behavioral and physiological strategies that could explain how some slugs retain kleptoplasts while lacking photoacclimation and photorepair mechanisms. Slugs with parapodia (dorsal extensions of the foot) have been observed covering their kleptoplasts to potentially limit light exposure (Cartaxana et al. 2018, 2019; Richards Donà et al. 2022) and slugs may restrict movement to shady areas when light intensity is high, a strategy that is more likely in "naked" slugs with highly visible kleptoplasts (Richards Donà et al. 2022). Some evidence for each of these strategies has been found in sacoglossan slugs; however, these observations were either made in the lab where lighting conditions were measured and controlled, or they were observed in the field and the light intensities were not measured. Moreover, the arrangement of chloroplasts in dense aggregations within the slug's digestive tubules serves as an additional defense mechanism, safeguarding the chloroplasts in the inner layers from photodamage (Havurinne et al. 2022). Some slug species may also produce photoprotective (sunscreen) pigments to reduce the light intensity reaching their kleptoplasts (Serôdio et al. 2014; Richards Donà et al. 2022), while others may contain kleptoplasts that dissipate excess light energy as heat, thereby reducing damage to the photosystems (Havurinne and Tyystjärvi 2020). Additionally, a slug's own tissue and/or mucus can serve to shield its kleptoplast against UV radiation (Havurinne et al. 2022). Individual or combinations of these behavioral and physiological photoprotective strategies may play a crucial role in shielding kleptoplasts from acute light stress and preventing ROS formation; however, this has not been studied in slugs exposed to natural light intensities.

To understand the photophysiology of kleptoplasts under natural conditions, we examined three congeneric species of sacoglossan slugs that have different kleptoplast retention capacities. *Elysia crispata (*Mörch 1863) (Fig. 1A) can retain functional kleptoplasts from various algal food sources for more than 40 days (Händeler et al. 2009; Middlebrooks et al. 2011), whereas *Elysia velutinus* (Pruvot-Fol 1947) (Fig. 1B) can retain chloroplasts from *Halimeda sp.* for up to 20 days (Christa et al. 2014; Laetz and Wägele 2017) and *Elysia ornata* (Swainson 1840) (Fig. 1D*)* retains kleptoplasts from *Bryopsis sp.* for less than 12 days (Händeler et al. 2009; Richards Donà et al. 2022). The habitat and depth at which each species was found differed (Table 1), so we conducted light profiles to measure the maximum amount of light to which each of these species could be exposed and examine how light attenuates with depth. We then measured the specific light intensity to which each specimen was exposed when it was collected, termed in situ

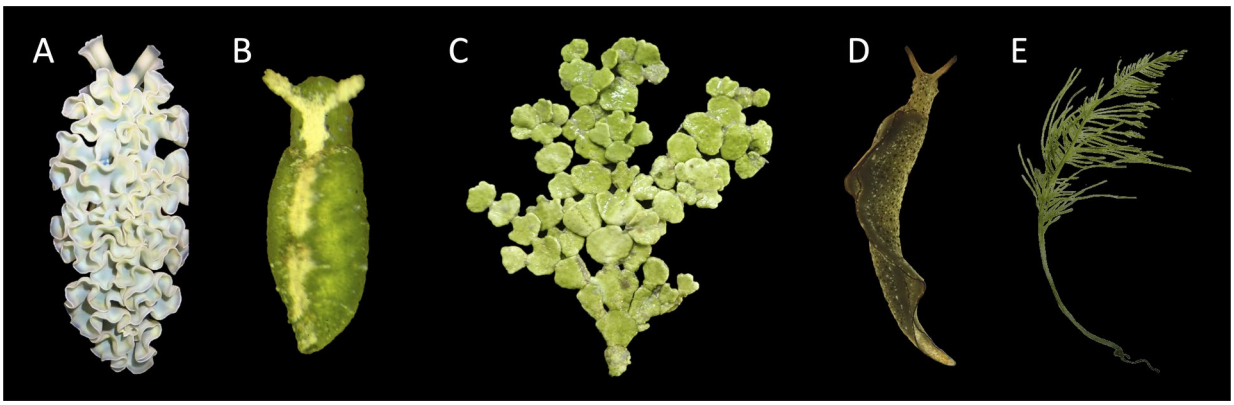


**Fig. 1** The sacoglossan and algal species used in this study. **A** *Elysia crispata*—specimens ranged from ~ 4–14 cm in length, **B** *Elysia velutinus*—specimens ranged from ~ 0.1–1 cm in length, **C** *Halimeda sp.*—thalli ranged from ~ 5–15 cm in height from the substrate, **D** *Elysia ornata*—specimens ranged from ~ 0.6–1.5 cm in length, and **E** *Bryopsis plumosa*—specimens ranged from 5 to 10 cm in height from the substrate

**Table 1** Collection information for each slug species

| Species | Location | Site coordinates | Depth (meters) | Associated algae |
|---|---|---|---|---|
| *E. crispata* | Playa Kalki | 12° 22′ 32.063″ N, 69° 9′ 27.873″ W | 5–10 | Unknown |
| *E. velutinus* | Piscadera Bay | 12° 7′ 20.085″ N 68° 58′ 8.289″ W | 4–6 | *Halimeda spp.* |
| *E. ornata* | Double reef | 12° 6′ 27.907″ N 68° 56′ 53.700″ W | 0–2 | *Bryopsis plumosa* |

($E_{in}$) light measurements here. These measurements were compared with their saturation light intensity ($E_{opt}$), used as a proxy for their optimal photosynthetic light intensities. $E_{opt}$ was obtained from Rapid Light Curves (RLCs), which are a measurement of photosynthetic relative Electron Transport Rate (rETR) versus the light intensity (E) under natural conditions, which reflects their natural light histories (the light exposure to which an organism has been exposed). Additionally, behavioral experiments were performed to determine their preferred light intensity ($E_{pref}$) and understand if they displayed positive or negative phototactic behaviors (moving toward or away from light, respectively). Finally, we utilized fluorescent staining to examine cellular redox homeostasis and oxidative activity in specimens exposed to light intensities both lower than and far exceeding their optima, to observe whether exposure to high light induced increased oxidative activity in each species.

## Materials and methods

### Specimen collection

Each of the sacoglossan species was collected from a different site on Curaçao, an island in the Southern Caribbean Sea (Table 1). *Elysia crispata* was found in light-exposed areas on coral reefs and coral rubble. Based on coloration and the reef locations they inhabited, the specimens used in this study are most likely the *crispata* ecotype (Middlebrooks et al. 2019). Since our specimens were never found feeding on, or in close proximity to the algal macrophytes on which this species is known to feed (Christa et al. 2014; Middlebrooks et al. 2019), we were unable to examine their associated algal food in the following experiments. While a variety of different color patterns can be found in the *E. crispata* populations inhabiting Curaçao's waters, we tried to limit the following investigations to similarly colored and sized individuals (Fig. 1A). *Elysia velutinus* (Fig. 1B) were always found hiding in their macroalgal food *Halimeda cf. opuntia* and *Halimeda cf. incrassata* (Fig. 1C) and *E. ornata* (Fig. 1D) were found in patches of *Bryopsis plumosa* (Hudson) C. Agardh, 1823 (Fig. 1E).

Light intensity measurements (PAR, measured in µmol $m^{-2}$ $s^{-1}$) were taken in situ with an SQ-500-SS Full-spectrum Quantum sensor attached to a 30 cm cable and micro-Cache Bluetooth micro-logger (Apogee Instruments Inc., USA), using corrections for underwater measurements as instructed by the manufacturer. The quantum sensor was placed in a watertight camera flash housing (Olympus PFL-E01 underwater housing for FL36 flash), the logging function was turned on to automatically register measurements at 10-s intervals and data were downloaded via the Apogee Connect app from Apogee Instruments Inc., USA (Supplementary Fig. 1). For underwater measurements, the part of the flash housing that was covering the sensor was covered with a light impermeable plastic, which was only removed when a measurement was taken. Preliminary testing in the lab ensured that this housing did not affect the light intensity and spectrum reaching the sensor, (i.e. the same light intensity was measured with and without the underwater housing).

Five light profiles were also conducted at Piscadera reef. A scuba diver held the light meter 5 cm above the substrate for 1 min to record the light intensity at increasing depths of 2 m from 0 to 10 m. The other diver monitored the depth and measurement time using an Aqualung i100 dive computer (Aqualung Inc., USA). These profiles were measured between 11:00 and 13:00 to ensure that the sun was directly overhead and a snorkeler on the surface ensured that no clouds were present during the measurement period. Light intensity measurements were also taken directly next to each specimen when it was found in the field (Supplementary Fig. 1). The number of specimens measured for each experiment can be found in Table 2. All analyses were conducted in RStudio (RStudio Team 2020) based on R version 3.6.1 (R Core Team 2022). Light profiles were first analyzed using a linear model ("lm" function in the *stats* package; R Core Team 2022) and subsequently outfitted with a second-degree polynomial function allowing light to vary non-linearly with increasing depth. Models were compared to determine how

**Table 2** The number of specimens used in each experiment presented in this study

| Experiment | *E. crispata* | *E. velutinus* | *E. ornata* | *Halimeda sp.* | *B. plumosa* |
|---|---|---|---|---|---|
| In situ light exposure ($E_{in}$) | $n=23$ | $n=11$ | $n=7$ | – | – |
| RLCs to determine optimal light intensity ($E_{opt}$) | $n=9$ | $n=6$ | $n=9$ | $n=9$ | $n=9$ |
| Phototaxis experiment ($E_{pref}$) | $n=9$ | $n=9$ | $n=6$ | – | – |
| Parapodial shading | $n=7$ | – | – | – | – |
| Oxidative activity | $n=3$ control<br>$n=3$ high light | $n=2$ control<br>$n=3$ high light | $n=3$ control<br>$n=3$ high light | – | – |

All specimens were euthanized following these experiments for use in other studies that we are currently conducting

much variation was explained using Akaike Information Criterion ("AIC" function in the R *stats* package; R Core Team 2022). The polynomial-containing model explained more of the variation in the data (adjusted $r^2 = 0.83$) and had a lower AIC value (418.32), so this model is presented here and visualized with the R package *visreg* (Breheny and Burchett 2017).

## PAM fluorometry

To avoid changes in their light history, Pulse Amplitude Modulated (PAM) fluorometry was performed on each of the three sea slug species and the two algal species directly after collection at each sampling site (within 30 min), using a Junior PAM with a blue measuring light (Walz GmbH, Germany). All samples were placed on the leaf clip to keep a constant distance of 1 mm between the fluorometer fiber optic and the surface of the sample. Rapid Light Curves (RLCs) were generated by measuring $F_m$' (the maximum fluorescence emitted by a light-adapted sample) and $F_s$ (steady-state fluorescence emitted by light-adapted sample) at each light step (see Supplementary Table 1 for complete PAM settings and light steps). The relative Electron Transport Rate (rETR; E) for each light intensity was characterized by fitting the model of Eilers and Peeters (1988) and estimating the parameters α, $rETR_{max}$ and $E_{max}$ (Eq. 1, see Table 3 for explanation of abbreviations/variables). The model was fitted iteratively using the R package *Phytotools* version 1.0 (Silsbe et al. 2015). To estimate the optimal light intensity, we calculated $E_{opt}$ using Eq. 2 as described by Richards Donà et al. (2022). Individual RLCs for every slug specimen were plotted using *ggplot2* (Wickham 2016). We also plotted averages of the slug and algae RLCs using generalized additive model smoothing via the "gam" function (R Core Team 2022). The calculated $E_{opt}$ values for *E. velutinus* and *E. ornata* were compared to their respective algal food sources using *t* tests, after normality and homogeneity of variances were confirmed with Shapiro–Wilk tests and Bartlett's tests, respectively (all conducted with the package stats; R Core Team 2022). The number of specimens used in each experiment is detailed in Table 2.

Equation 1: Calculating rETR (E) versus *E* curves according to the model presented by Eilers and Peeters (1988)

$$rETR(E) = \frac{E}{\frac{1}{\alpha \bullet {E_{max}}^2} \cdot E^2 + \left(\frac{1}{rETR_{max}} - \frac{2}{\alpha \cdot E_{max}}\right) \cdot E^2 + \frac{1}{\alpha}}. \quad (1)$$

Equation 2: Estimating $E_{opt}$ from rETR (E)

$$E_{opt} = \frac{rETR_{max}}{\alpha}. \quad (2)$$

## Phototaxis experiment

Specimens were collected as described above and immediately transported to the laboratory for the phototaxis experiment. The preferred light intensity ($E_{pref}$) of the sea slugs was calculated by placing individuals in rectangular trays (40 × 60 cm) illuminated by a gradient of light intensities provided by 30W full-spectrum light LED lamps (OSRAM GmbH, Germany) that were installed above each tray. Every tray was uniformly and constantly oxygenated from both ends and kept at room temperature, matching the temperature from which these specimens were collected (26 ± 1 °C). A grid was drawn on the bottom and the light intensity was measured for each cell with the quantum meter described above. The light intensity ranged from 0 to 1396 µmol $m^{-2}$ $s^{-1}$. Additionally, a hiding spot with no light was provided, covering four squares on the dark side of the tray (Supplementary Fig. 2).

After 5 min with the light off to avoid bias due to relocation stress, the lights were turned on and the position of the slug was recorded ($t_0$) together with its corresponding

**Table 3** Abbreviations and descriptions for relevant photosynthetic parameters used in this study

| Notation | |
|---|---|
| rETR | The relative Electron Transport Rate |
| rETR (E) | The relative Electron Transport Rate for each light intensity (dimensionless) |
| E | The ambient spectrally averaged photon light intensity of PAR (400–700 nm) |
| $F_m$´ | The maximum fluorescence emitted by a light-adapted sample (arbitrary units) |
| $F_s$ | The steady-state fluorescence emitted by a light-adapted sample (arbitrary units) |
| $E_{opt}$ also called $E_k$ in some literature | The light intensity at which the maximum rETR (E) would be reached if there was no inflection in the curve (i.e. extrapolating the initial linear part of the curve to intersect with $rETR_{max}$ (µmol $m^{-2}$ $s^{-1}$) |
| $E_{max}$ | The light intensity at $rETR_{max}$ (no extrapolation of the linear portion of the curve) (µmol $m^{-2}$ $s^{-1}$) |
| $E_{pref}$ | The preferred light intensity obtained from the last measurement from the phototaxis experiment (µmol $m^{-2}$ $s^{-1}$) |
| $E_{in}$ | The in situ light intensity, measurement taken in the field with (µmol $m^{-2}$ $s^{-1}$) |
| $rETR_{max}$ | The maximum relative electron transport rate of the rETR vs. E curve (dimensionless) |

light intensity in that position. Additional measurements were recorded every 5 min for a total of 30 min. The light was then turned off and moved to the opposite side of the tray to control for any effect of the side of each tank and the experiment was repeated. The hiding spot was also moved. The side of the tank where the light first was placed was randomly assigned for each trial. The final position after every 30-min trial ($t_{30}$) was determined to be their preferred light intensity ($E_{pref}$).

To assess the phototactic behavior of each specimen, we examined how the light intensity to which it was exposed changed throughout the phototaxis trial by building linear mixed-effects models using the "lmer" function from the *lme4* package (Bates et al. 2015). These models included "species", "time" and "light position" as fixed effects, "specimen ID" as a random effect to account for repeated measures of each individual and considered "light intensity" as the response variable. These models were compared by computing Akaike Information Criteria (AIC, package *stats*, R Core Team 2022). The best of these models contained an interaction between "species" and "time". This model was simplified using the "step" function from the package *stats* (R Core Team 2022), which confirmed that "light position" did not significantly explain variation, so it was excluded from the final model. This model explained 40% of the variation (conditional $r^2 = 0.40$, computed with the *MuMIn* package; Barton 2022) and displayed an AIC of 2371.33, explaining more variation and fitting the dataset better than the other models, so the results from this model were used for subsequent analyses. Post hoc analyses were conducted using pairwise comparison of estimated marginal means (package *emmeans*; Lenth et al. 2019), which consider the variation explained by all predictors while making pairwise comparisons. A full summary of the results from these analyses is found in Supplementary Table 2 for intraspecific variation and Supplementary Table 3 for interspecific variation in $E_{pref}$. Statistical significance was considered at $p < 0.05$ for all experiments conducted in this study.

## Comparing in situ, optimal, and preferred light intensities

To explore both intra- and interspecific differences among in situ, optimal, and preferred light intensities, linear mixed-effects models were generated using the *lme4* package (Bates et al. 2015). Both models used the fixed effects "category" (which indicated $E_{in}$, $E_{opt}$ or $E_{pref}$) and "species", the covariate "date" (since in situ light measurements were conducted in March and December, and we wanted to control for time of year) and "(specimen) id" as a random effect, since each specimen was measured twice in the phototaxis ($E_{pref}$) trials. The light intensity "E" was used as the response variable in all models. The best fit model was chosen based on AIC, and contained an interaction between "species" and "category" with the covariate "date" and random effect as additive effects. This model was then simplified with the function "step" in the *stats* package (R Core Team 2022), which indicated that "date" and "(specimen) id" did not significantly explain variation, so they were subsequently removed to produce the final model. The residuals in this model were determined to be normally distributed and homoscedastic via QQ- and residual vs. fitted plots, after a square-root transformation was applied. This model included an interaction between "species" and "category", and explained 61% of the variation (adjusted $r^2 = 0.61$), so it was chosen for subsequent analyses. A two-way ANOVA was run using the "Anova" function in the package *car* (Fox and Weisberg 2019; F-statistic$_{8,104} = 22.52$, $P < 2.2e{-}16$). This model was then used for post hoc testing using the package *emmeans* (Lenth et al. 2019) to determine differences in light intensity for each species and ($E_{in}$, $E_{opt}$ or $E_{pref}$). These results are detailed in Supplementary Tables 4 and 5.

## Shading experiments

Preliminary observations revealed that *E. crispata* contracts its parapodia when exposed to high light, potentially cloaking the kleptoplasts located in the digestive gland tubules, aligning with previous observations made in other species (Cartaxana et al. 2018; Richards Donà et al. 2022). Since the parapodial edges do not contain kleptoplasts, this tissue could function as a photoprotective layer, shielding the kleptoplast-containing tubules below. To investigate if this tissue limits the amount of light reaching the kleptoplasts and determine how much light is blocked, three *E. crispata* specimens were placed in glass containers with 25 ± 1 °C seawater under a high-intensity lamp (OSRAM 30W full-spectrum LED, OSRAM GmbH, Germany) that was initially turned off. Indirect light from small windows made the specimens visible on camera (< 20 PAR). Specimens were allotted 3 min of darkness before the experiment began to avoid measuring a response that was due to relocation stress. Turning on the light exposed each specimen to a light intensity of 1600 µmol $m^{-2}$ $s^{-1}$. Parapodial positioning was filmed using an Olympus Tough TG-6 camera (Olympus Worldwide). After 10 s, the light was turned off again and the video recording was stopped when the parapodia returned to a completely relaxed position. The time it took for slugs to contract their parapodia when the light was turned on, and the time they took to relax after it was turned off were determined from the videos (Supplementary Video 1).

To estimate the amount of light that the parapodial edges are blocking from reaching the kleptoplasts through absorbance and/or reflection, a small cup was made of parafilm (Sigma-Aldrich—Merck KGaA, Germany) and placed around the quantum sensor, with flared upper edges

to ensure that no light was blocked by the parafilm (Supplementary Fig. 4). This cup was filled with 3 ml of seawater and placed under a 30W LED light (see above) at a height that allowed 400 µmol $m^{-2}$ $s^{-1}$ to reach the sensor. As the quantum sensor has a diameter of 2 cm, slugs with a diameter of ~ 2 cm (the length was 4–5 cm when fully outstretched) when their parapodia are contracted were chosen for the following procedure to minimize differences due to specimen size and avoid overestimating the amount of light that was blocked due to larger amount of tissue covering the sensor than would cover an animal. These specimens were euthanized by decapitation before their white parapodial edges were removed, placed on top of the light meter sensor in 3 mL seawater, and the resulting light intensity was measured (Table 2). The difference between the initial light intensity (400 µmol $m^{-2}$ $s^{-1}$) and the light intensity when the parapodial edges were placed between the light and the sensor provides an estimation of the total light intensity that the parapodial tissue blocks.

### Kleptoplast abundance and light-dependent oxidative activity

To examine if exposure to high light intensities induced increases in oxidative activity that could indicate oxidative stress from photodamage, we stained specimens from each slug species with dihydrodichlorofluorescein diacetate ($H_2$DCF-DA). This cell-permeable non-fluorescent dye penetrates cells and where it can undergo hydrolysis, leading to its oxidation and conversion to DCF, a molecule that fluoresces bright green (λemission = 522 nm) when excited by blue light (λexcitation 492–495). While DCF fluorescence is routinely interpreted as the conversion of $H_2$CDF (non-fluorescent) to DCF (fluorescent) when oxidized by hydrogen peroxide, the actual mechanism is far more complicated and often overlooked (Wardman 2007). Once inside a cell, $H_2$DCF can be oxidized by a variety of oxidants (e.g., certain ROS, some reactive nitrogen species, etc.), but it is not oxidized by hydrogen peroxide without the help of a redox-active metal catalyst or cytochrome *c* (Karlsson et al. 2010; Winterbourn 2014). We therefore interpreted DCF fluorescence as an indicator of a tissue's general redox state, rather than as a direct measure of hydrogen peroxide activity (as recommended by Karlsson et al. 2010; Volk and Moreland 2014; Winterbourn 2014). Increased amounts of DCF fluorescence, therefore, demonstrate increased levels of oxidation, which can be interpreted as oxidative stress (potentially from ROS generated due to photodamage) and an inability for the cell to maintain cellular redox homeostasis.

Specimens were exposed to either 400 µmol $m^{-2}$ $s^{-1}$ of light or 1600 µmol $m^{-2}$ $s^{-1}$ for 30 min to examine a potential relationship between light exposure and oxidative activity in slug tissues. The number of specimens measured is recorded in Table 2. Each specimen was euthanized via decapitation. Five live tissue biopsies measuring ~ 4 $mm^2$ were taken from each specimen in different parapodial regions (near the head, middle, and end of the parapodia) to examine the cellular redox state throughout each specimen. Biopsies were then placed in the dark, stained with 12.5 µM of DCF dissolved in filtered seawater for 20 min and then washed three times with filtered seawater. They were then placed on a microscope slide and coverslipped. Imaging was conducted using a Leitz Orthoplan epifluorescence microscope (Leitz, Germany) and a Canon EOS digital camera (Canon, Global). Functional chloroplasts were observed via chlorophyll autofluorescence, which fluoresces red when exposed to blue light (λexcitation = 488 nm; λemission = 633 nm). Only the middle of each tissue biopsy was imaged to avoid capturing ROS activity that may stem from dissection rather than light, even though a difference between the edge of the tissue and the center was not actually observed.

## Results

### Light intensity profiles

The light intensity in Piscadera Bay, Curaçao peaked at 1835.7 ± 335.6 µmol $m^{-2}$ $s^{-1}$ directly below the water surface (0 m depth) and decreased rapidly with increased depth. At 10 m depth, the light intensity was 219.89 ± 90.49 µmol $m^{-2}$ $s^{-1}$ (Fig. 2).

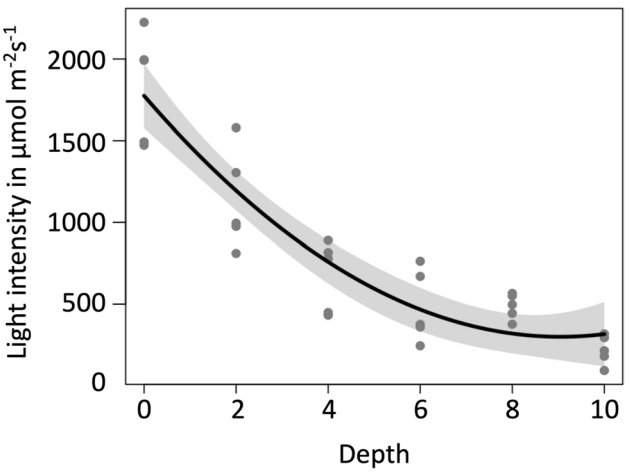


**Fig. 2** Light intensity vs. depth at Piscadera reef, Curaçao. Note that the model predicts a slight upturn with depth; however, this is almost certainly due to variation in the measurements and does not reflect reality

## Phototaxis experiment

All species exhibited distinct phototactic patterns, as evidenced by their trajectories and preferred light intensities. *Elysia crispata* moved in seemingly random trajectories during the experiment (Fig. 3A-B), contrasting *E. velutinus* and *E. ornata,* which exhibited consistent movement toward areas with lower light intensity (Fig. 3D-E, G-H). When the preferred light intensity for each species was compared to their locations at the beginning of the experiment (following the adjustment period in darkness), *E. crispata* presented no significant difference [Estimated Marginal Means (EMM), t331.1 = 0.64, $P = 0.99$; Fig. 3C]. Conversely, both *E. velutinus* and *E. ornata* presented a strong negative phototactic behavior over time (EMMs, t331.1 = 5.14, $P = 9.58e{-}6$ and EMMs, t331.1 = 5.07, $P = 1.38e{-}5$, respectively; Fig. 3F and I and detailed in Supplementary Table 2). Comparing between species revealed that 44.4% of *E. velutinus* and 66.6% of *E. ornata* specimens preferred the sheltered area where light intensity is zero, contrasting only 11.1% of *E. crispata* individuals. While *E. velutinus* and *E. ornata* did not differ from one another, both preferring low light intensities (EMMs, t64.1 = −1.027, $P = 0.5628$), *E. crispata* differed from both of these species, choosing a range of light intensities (EMMs, t64.1 = 3.623, $P = 0.0017$ and t64.1 = 4.267, $P = 0.0002$, when compared to *E. velutinus*

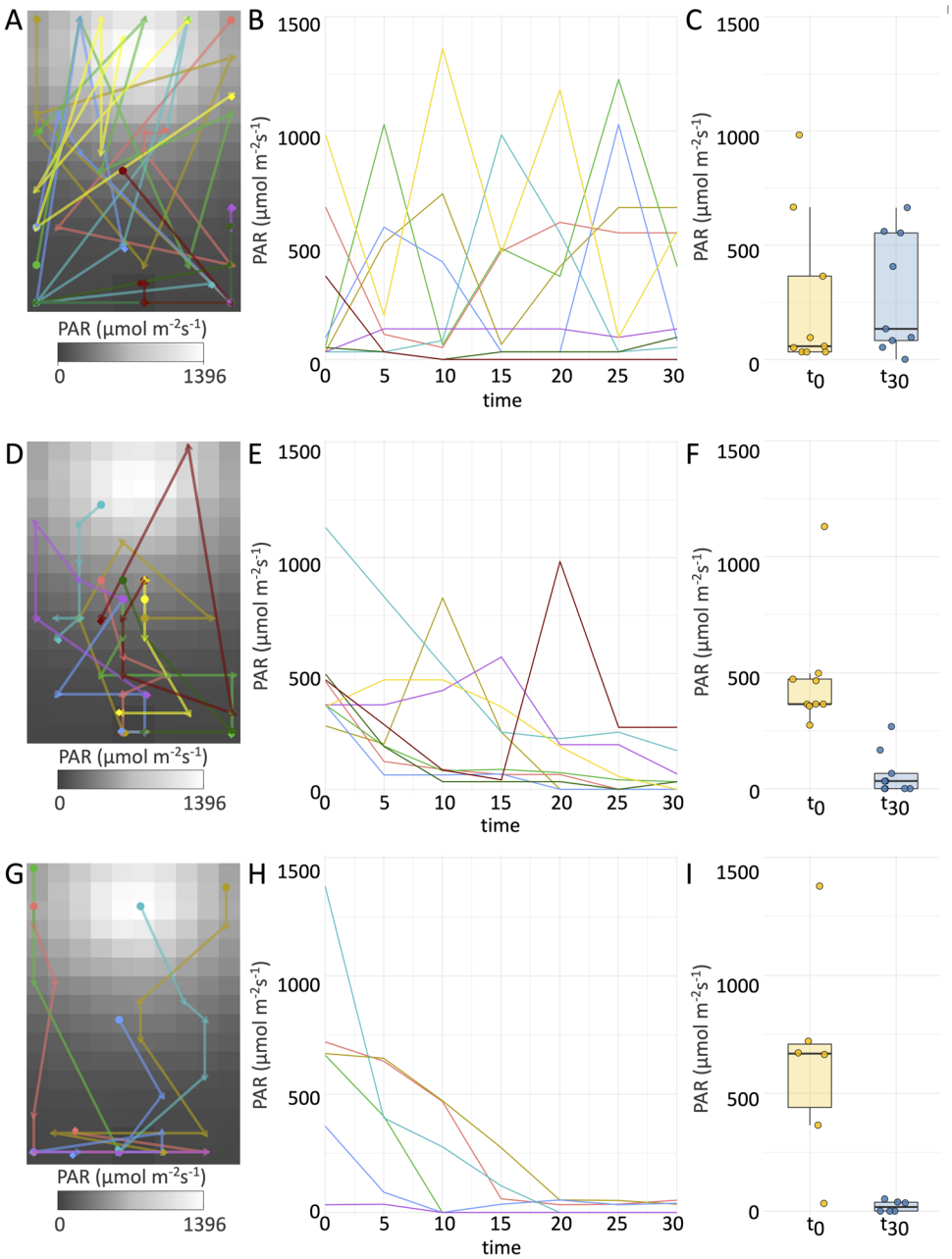


**Fig. 3** Phototactic responses in *Elysia crispata, E. velutinus,* and *E. ornata*. In all plots, colored lines indicate individual specimens that were tested. **A** the trajectories *E. crispata* individuals chose during the experiment as viewed from above the tank. Starting points are denoted by points, arrows indicate the direction of travel during the experiment, and diamonds indicate the final position after 30 min. The gray boxes in the background depict the light intensity in each square within the test chamber. **B** Change in light intensity exposure throughout the experiment for *E. crispata* individuals. **C** Comparing the light intensity to which specimens were exposed when the experiment began ($t_0$) and commenced after 30 min ($t_{30}$). **D-F** Trajectories, changes in light intensity over time and initial vs. final light intensity measured for *E. velutinus* individuals and **G-I** the same for *E. ornata.* Note that these plots only depict results from when the light was positioned at the back of the tank, for clarity, and because the position of the light did not end up explaining variation. Supplementary Fig. 3 contains the plots from the trials when the light was placed in the front of the tank

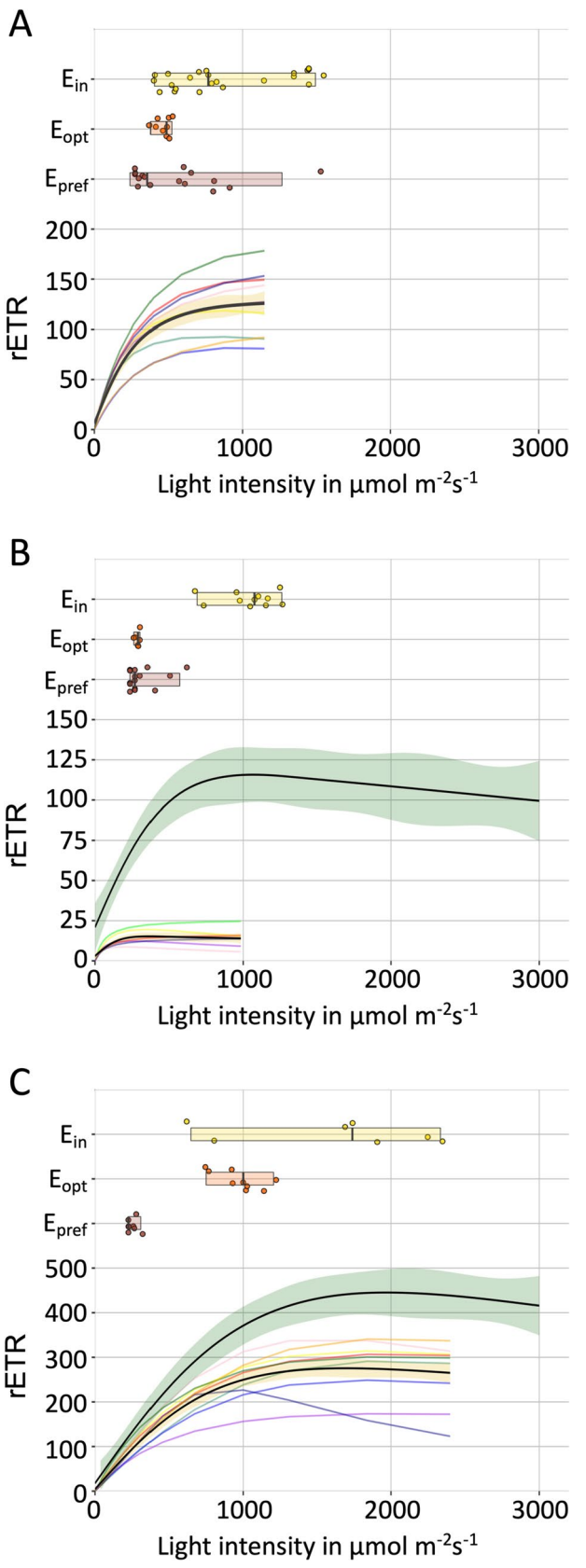


**Fig. 4** The preferred ($E_{pref}$), in situ ($E_{in}$), and optimal ($E_{opt}$) light intensities for all three *Elysia* species, their corresponding Rapid Light Curves (RLCs) and the RLCs from their algal food sources (*E. velutinus* and *E. ornata* only). Each plot contains $E_{pref}$, $E_{in}$ and $E_{opt}$ for each of the specimens that were examined, displayed as boxplots at the top of each panel. Rapid Light Curves (RLCs) generated for each specimen are depicted with colored lines in the bottom part of each panel. All smoothed RLC regressions are depicted in black and surrounded by yellow confidence bands (for slugs) or green confidence bands (for algae). **A** *Elysia crispata* $E_{pref}$, $E_{in}$, $E_{opt}$ & RLCs, **B** *E. velutinus* $E_{pref}$, $E_{in}$, $E_{opt}$ & RLCs and *Halimeda cf. opuntia* RLC, and **C** *E. ornata* $E_{pref}$, $E_{in}$, $E_{opt}$ & RLCs and *Bryopsis plumosa* RLCs

and *E. ornata*, respectively). A statistical summary of these tests is provided in Supplementary Table 3.

## Comparing in situ, optimal, and preferred light intensities

The in situ, optimal, and preferred light intensities within species were also compared using EMMs. The light intensity to which *E. crispata* was exposed in situ was significantly higher than the amount needed for optimal photosynthesis (EMM, t104 = 3.57, $P = 0.002$) and the amount it preferred (EMM, t104 = 5.00, $P = 6.83e{-}6$). Conversely, no differences were found between $E_{pref}$ and $E_{opt}$ (EMM, t104 = 0.42, $P = 0.91$) (Fig. 4A). Similarly, for *E. velutinus,* significant differences were observed between $E_{in}$ when compared to $E_{opt}$ (EMM, t104 = 6.09, $P = 5.66e{-}8$) and when compared to $E_{pref}$ (EMM, t104 = 8.92, $P = 1.16e{-}13$). No differences were found between $E_{pref}$ and $E_{opt}$ (EMM, t104 = 0.68, $P = 0.77$) for this species. *Elysia ornata* differed significantly in all three comparisons ($E_{in}$ vs. $E_{opt}$–EMM, t104 = 2.83, $P = 0.015$), $E_{in}$ vs. $E_{pref}$ EMM, t104 = 10.33, $P = 5.91e{-}14$, and $E_{pref}$ vs. $E_{opt}$ (EMM, t104 = 7.91, $P = 8.86E{-}12$). A full statistical summary is available in Table S4. $E_{opt}$ values for *E. velutinus* and *E. ornata* were compared to $E_{opt}$ values from their respective food algae, finding that $E_{opt}$ was higher for *Halimeda sp.*, (mean ± standard deviation, 153.04 ± 40.13) than *E. velutinus* (57.8 ± 17.1, Paired *t* test, t9.98 = −6.02, $P = 0.00013$; Fig. 4B). The optimal light intensities for *E. ornata* (676.7 ± 156.06) and *B. plumosa* (684.60 ± 255.76) did not differ though (Paired *t* test, t13.23 = −3.78, $P = 0.9377$; Fig. 4C).

When comparing between species, *Elysia ornata* was exposed to a significantly higher in situ light intensity than *E. crispata* (EMM, t104 = −3.83, $P = 0.00064$) and *E. velutinus* (EMM, t104 = 2.20, $P = 0.076$); however, no significant differences were found between *E. crispata* and *E. velutinus* (EMM, t104 = −1.61, $P = 0.25$). *Elysia ornata* also demonstrated a significantly higher optimal light intensity when compared to *E. crispata* (EMM, t104 = −3.45, $P = 0.0023$) and *E. velutinus* (EMM, t104 = 5.18, $P = 3.31e{-}6$); however, no significant difference was observed between the later species (EMM, t104 = 2.09, $P = 0.097$, Fig. 4). *Elysia crispata*

preferred a significantly higher light intensity ($E_{pref}$) than *E. ornata* (EMM, t104 = 4.53, $P = 4.71e{-}5$) and *E. velutinus* (EMM, t104 = 3.75, $P = 0.0008$). No significant difference was found between $E_{pref}$ for *E. ornata* and *E. velutinus* (EMM, t104 = −1.17, $P = 0.47$). A full statistical summary is available in Table S5.

### Shading experiments

*Elysia crispata* specimens presented relaxed or moderately relaxed parapodia in the field and when the light was off in the lab, sometimes exposing the kleptoplasts located below the parapodial edges (Fig. 5A-B). When light was turned on exposing them to of 1600 µmol $m^{-2}$ $s^{-1}$, they clenched their parapodia and kleptoplasts were no longer visible, a behavior that was also observed in the field when a cloud, which had blocked some light, moved and they were exposed to more light (Fig. 5C). This response took less than 1 s in our lab trials. While the light was still on, all the individuals started relaxing their parapodia, and after an average (mean) of 11.6 ± 6.19 s, but their parapodial tissue still blocked a majority of their kleptoplasts (Fig. 5B). When the light was on, they were never observed completely relaxed, exposing their kleptoplasts. The parapodial tissue that was dissected and placed between a light source providing 400 µmol $m^{-2}$ $s^{-1}$ light intensity and the quantum sensor dissipated 216.1 ± 21.5 µmol $m^{-2}$ $s^{-1}$ of light (50.9%).

### Kleptoplast abundance and light-dependent oxidant activity

The digestive gland tissue was easily distinguished by the presence of kleptoplasts, which are not found in non-digestive tissues. Both *E. crispata* and *E. velutinus* had digestive tubules that ended in blind sacs. The tubule itself comprised non-kleptoplast-containing cells and cells that were filled with dense aggregations of kleptoplasts. Most of the kleptoplast-containing cells were concentrated in the sacs at the end of each tubule (Fig. 6A-B). This contrasts *E. ornata* whose digestive gland contained larger tubules with cells that each contained a few kleptoplasts (i.e., separate kleptoplast-bearing and non-bearing cell types were not observed; Fig. 6C).

$H_2$DCF oxidation was observed in all of the species examined and this signal was localized to the digestive gland in every species, meaning that non-digestive tissues lacked fluorescent signal and appeared black. Under a lower light intensity (400 µmol $m^{-2}$ $s^{-1}$), *E. crispata* and *E. velutinus* displayed low levels of $H_2$DCF oxidation, which was evenly spread throughout the digestive tissues in both kleptoplast-bearing and non-kleptoplast-bearing cells (Fig. 6A-B). *Elysia ornata* contained large accumulations of DCF that were localized in cellular compartments within the digestive gland (Fig. 6C). Under 1600 µmol $m^{-2}$ $s^{-1}$, both *E. crispata* and *E. velutinus* displayed increased levels of DCF fluorescence diffused throughout their digestive glands (Fig. 6D-E), while *E. ornata* maintained high levels of compartmentalized DCF fluorescence (Fig. 6F).

## Discussion

The light intensity to which each of these species was exposed in situ was always higher than the amount needed for optimal photosynthesis. This indicates that all three species need to employ behavioral and/or physiological photoprotective strategies to limit the light reaching their kleptoplasts, if they are to avoid photoinhibition and photodamage. In this study, we characterized these photoprotective mechanisms, finding that each of these species employs a different combination of photoprotective mechanisms due to a variety of ecological factors and some species are more capable of photoprotection than others.

*Elysia crispata* specimens were found in a range of depths between 5 and 15 m on coral rubble and the coral reef, where they feed on a multitude of algal species, many

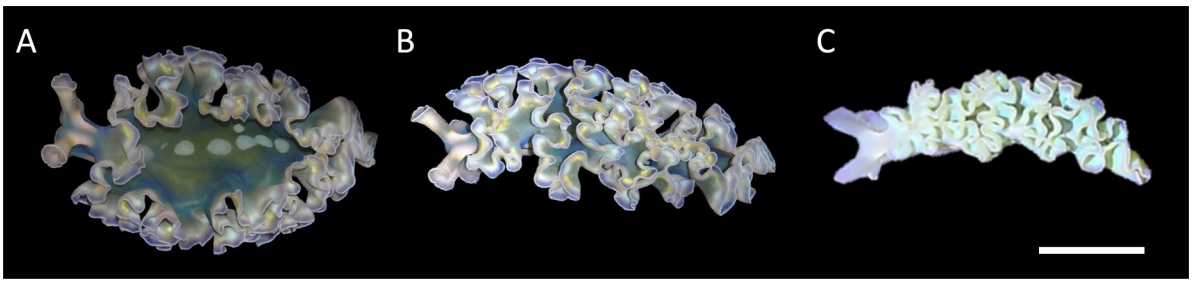


**Fig. 5** Three parapodial positions were observed in *Elysia crispata* specimens, **A** completely relaxed exposing its kleptoplasts (green coloration) under very low intensity lighting (~20 µmol $m^{-2}$ $s^{-1}$); **B** moderately relaxed parapodia—slugs were observed in this position in the field, under lab lighting (~20 µmol $m^{-2}$ $s^{-1}$) and again after 11.6 ± 6.19 s of high light exposure (1600 µmol $m^{-2}$ $s^{-1}$); **C** entirely contracted parapodia covering the kleptoplasts immediately (< 1 s) after exposure to high light. All photos were taken with the same camera settings. The scale bar indicates ~ 2 cm

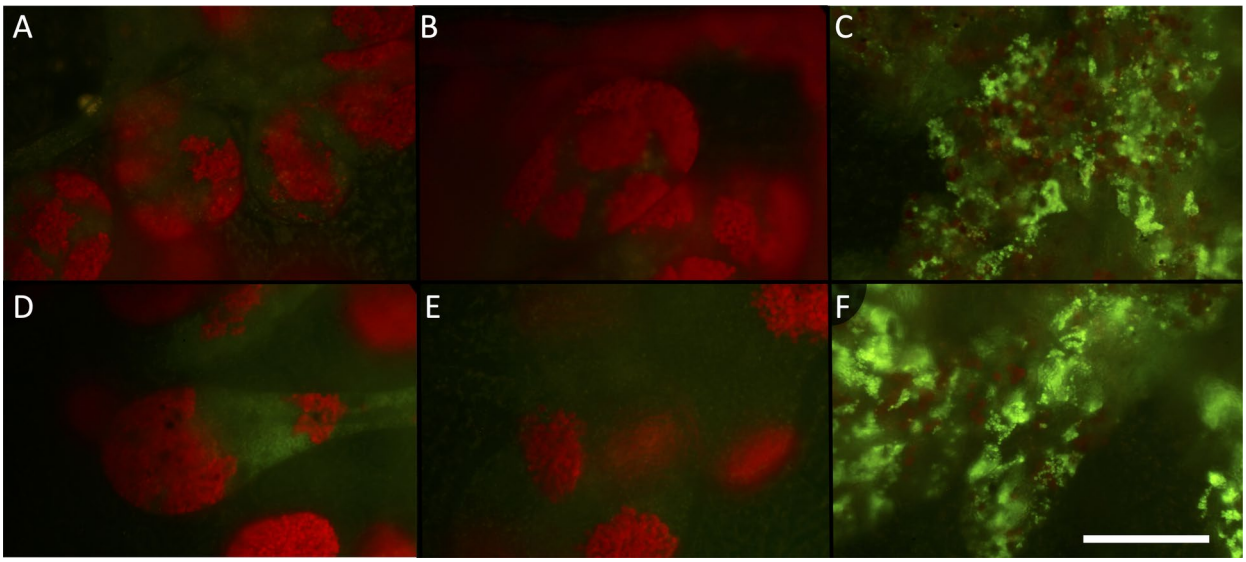


**Fig. 6** Oxidative activity in sacoglossan slugs. Chloroplasts are visible due to their red autofluorescence and oxidative activity is visible in green, because DCF fluoresces green when oxidized. **A–C** *Elysia crispata, E. velutinus*, and E. ornata exposed to 400 µmol $m^{-2}$ $s^{-1}$, respectively. **D–F** *E. crispata, E. velutinus,* and *E. ornata* exposed to 1600 µmol $m^{-2}$ $s^{-1}$), respectively. Scale bar indicates 75 µm for **A** and **D**, and 50 µm for **B–C**, **E–F**

of them macroscopic (summarized in Krug et al. 2016). The specimens observed in this study were not observed feeding on, nor in close proximity to any of their known algal food sources, observations that align with the previous studies on this species (Middlebrooks et al. 2014). In this habitat, *E. crispata* specimens were observed exposed to the full intensity of light penetrating to the seafloor ($E_{in} = 652.20 \pm 397.01$ µmol $m^{-2}$ $s^{-1}$, $n = 23$), which is ~ 3 times higher than the optimal light intensity for photosynthesis that we measured. Observing *E. crispata* exposed to incident light align with reports by numerous authors (e.g., Krug et al. 2016), but contrast reports of *E. crispata* in Florida, USA, which were described as hiding under rocks during the day, emerging at 16:00–17:00 and spreading out their parapodia to maximize light absorption (Weaver and Clark 1981). We frequently looked below pieces of coral rubble for other slug species and never observed *E. crispata* under the coral rubble, indicating that this population does not seek out shade to limit the light reaching its kleptoplasts, even if other populations do. This discrepancy is perhaps best explained by the high variability in parapodial coloration, the thickness of the parapodial edges and degree to which it is folded, that are characteristic of different individuals, ecotypes, and populations of this species (Krug et al. 2016). Asserting that the population of *E. crispata* that we surveyed do not hide from excess light is further supported by observations we made during the phototaxis experiments, where *E. crispata* demonstrated neither light-avoidance nor light-seeking behavior (Fig. 3A–C). This again contrasts findings by Weaver and Clark (1981) who reported that *E. crispata* prefers light intensities of 400 µmol $m^{-2}$ $s^{-1}$, avoiding locations with higher and lower light intensities, so we again interpret these differences as due to variability among different populations. The large variability we observed in *E. crispata* preferred light intensities ($E_{pref} = 279.80 \pm 330.50$ µmol $m^{-2}$ $s^{-1}$, $n = 9$) is similar to the variability of light to which it is exposed in the field ($E_{in} = 652.2 \pm 397.01$ µmol $m^{-2}$ $s^{-1}$, $n = 23$), again suggesting that this population can withstand a large range of light intensities and does not seek shelter to limit light.

Instead of moving to the shade, *E. crispata* specimens cover their kleptoplasts with their parapodia, as previously reported in other sacoglossan species (Cartaxana et al. 2018; Richards Donà et al. 2022). In the *E. crispata* we examined, this behavior blocked up to 200 µmol $m^{-2}$ $s^{-1}$ of light, reducing the light striking the kleptoplasts from ~ 650 to ~ 450 µmol $m^{-2}$ $s^{-1}$. Although this intensity is still higher than the optimal intensity we measured ($E_{opt} = 225.60 \pm 50.46$ µmol $m^{-2}$ $s^{-1}$, $n = 9$), it is close to the 400 µmol $m^{-2}$ $s^{-1}$ preferred light intensity reported by Weaver and Clark (1981). We also observed *E. crispata* further relaxing their parapodia and exposing their chloroplasts when provided low light intensities (< 100 µmol $m^{-2}$ $s^{-1}$), a behavior we only witnessed in the field, occasionally, at night, but was observed by Weaver and Clark (1981) close to sunset. These observations indicate that *E. crispata* changes its parapodial positioning to control the amount of light reaching its kleptoplasts. Parapodial shading as a means for photoprotection has been observed in other species such as *Plakobranchus ocellatus* (van Hasselt 1824) (Richards Donà et al. 2022) and *Elysia timida* (Risso, 1818)

(Giménez-Casalduero and Muniain 2006; Jesus et al. 2010; Schmitt and Wägele 2011). Interestingly, these species are all capable of long-term kleptoplasty and their food sources grow in areas exposed to high light intensities, indicating that the ability to regulate light via parapodial shading may be a factor that facilitates prolonged chloroplast function by limiting photodamage and the formation of ROS.

Parapodial tissue may help limit light from reaching kleptoplasts in a few ways. The opaque white color that dominates this tissue likely reflects some light and other pigments within the tissue could help absorb additional light. For example, mollusk-derived red and brown pigment-containing cells have been detected in *E. crispata,* and may confer photoprotective benefits (Yonge and Nicholas 1940; Pierce et al. 2006). Furthermore, many *E. crispata* specimens display small orange patches or lines along their white parapodial margins, which could contain carotenoids, which in turn could confer additional photoprotective mechanisms.

*Elysia crispata* displayed large accumulations of kleptoplasts within certain cells in the digestive gland, mostly located in the blind sacs at the end of each branch of the digestive gland tubules. Some oxidative activity (DCF fluorescence) was observed in slugs exposed to 400 µmol $m^{-2}$ $s^{-1}$, but considerably more was present in slugs exposed to 1600 µmol $m^{-2}$ $s^{-1}$ indicating that higher light intensities induce oxidation in the digestive gland. Whether or not this oxidative activity leads to oxidative stress and difficulty maintaining cellular redox homeostasis will require further investigation. It is also still unclear if this oxidative activity is actually due to chloroplast-related physiological processes such as ROS produced via photodamage, since other physiological processes can produce oxidants that can convert $H_2$DCF to DCF (e.g., mitochondrial respiration, NADPH oxidases, etc.; Karlsson et al. 2010; Winterbourn 2014; de Almeida et al. 2022). This study does, however, demonstrate that increased oxidative activity in the digestive gland is linked to high-light exposure and that *E. crispata* uses parapodial shading as a photoprotective mechanism to limit light entering its kleptoplasts when exposed to light intensities above its optimum; however, these results should be verified with additional methods to verify and refine our understanding of light-induced oxidative stress in sacoglossan slugs. Future investigations could include bioassays such as the ABTS and/or lipid peroxidation malondialdehyde assays or immunohistochemistry targeting specific antioxidants.

Although *Elysia velutinus* is also able to retain kleptoplasts in high densities and also exposed to light intensities that exceed its photosynthetic optimum, it employs a different photoprotective strategy than *E. crispata*. Our specimens were always found hiding in their macroalgal food *Halimeda sp.* at 4–6 m depth; however, other studies have also found them feeding on *Caulerpa sp.* (J.V. Lamouroux, 1809) (Christa et al. 2014). In the field, *Halimeda sp.* was exposed to light intensities approximately four times higher than its photosynthetic optimum (Fig. 4B). Likewise, if exposed to direct sunlight, *E. velutinus* would receive 14 times more light than needed for optimal photosynthesis. Some of this light could be blocked via white tissue along their parapodial margins (Fig. 1B; (Marcus and Marcus 1967); however, the relatively small areas covered by white tissue and highly reduced parapodial size suggest this would have little photoprotective effect for a majority of their kleptoplasts. Since the algae form dense bushes, their thalli stand upright and *E. velutinus* positions itself flat against the algae, often perpendicular to overhead illumination, it is unlikely that *E. velutinus* receives a significant amount of direct sunlight, suggesting that excess light exposure in situ is probably not a regular occurrence for this species. Weaver and Clark (1981) report that *E. velutinus* (then called *Elysia tuca*) displays a daily vertical migration in the algae, only coming to the top of an algal thallus at night to feed. In *Halimeda spp.* and other calcifying algae, the upper part of the thallus contains younger and more ingestible algal tissue (Clark and DeFreese 1987; Marín and Ros 1992). Migrating up the thalli was not observed in this study, as specimens were always observed buried deep in the clumps of algae. In our phototaxis trials, *E. velutinus* demonstrated a light-avoidance response, which is unsurprising considering its preference for hiding among algal thalli. This response could also be due to non-photoprotective-related reasons such as predator avoidance or a dislike for the exposed nature of the container in which they were tested. Nevertheless, crawling into the shade provided by its macroalgal food remains the most likely photoprotective mechanism utilized by this species, even if they display this behavior for reasons other than photoprotection.

Because *E. velutinus* and *Halimeda sp.* could receive far more light than is optimal for photosynthesis, we expected a higher amount of oxidative activity when exposed to light intensities above their optima, which we did observe with DCF staining in the slug (Fig. 6E). Considering their propensity to hide in their algal food which likely limits their light exposure in situ, we expected that *E. velutinus* would display more oxidative activity than *E. crispata* and *E. ornata* under both light intensities, but it displayed less. Photoprotection provided by the algae would have to be chloroplast-encoded for transmission and continued function in the slug. One of the main chloroplast-encoded photoprotective mechanisms observed in many ulvophyceaen algae, the xanthophyll cycle (i.e. the high-energy quenching component of Non-Photochemical Quenching—NPQ), has not been observed in *Halimeda spp.*, so this does not explain the lack of ROS we observed in *E. velutinus* (Christa et al. 2017). Furthermore, while *Halimeda spp.* do contain carotenoids such as siphonein and siphonoxanthin which are

sometimes linked to photoprotection, these pigments have been linked to light-harvesting in *Halimeda spp.* rather than light limitation (Beach et al. 2003), although other functions remain possible. For example, a link between siphonoxanthin extracted from green algae and reduced lipid peroxidation (a common consequence of ROS activity), oxidative stress, and an increase in ROS gene expression was recently demonstrated in mice (Zheng et al. 2020). While these findings cannot be extrapolated to mollusks and algae, they do suggest that these pigments could have ROS-mitigation-related functions, which could explain the low oxidant activity observed in *E. velutinus* in this study. Confirming or refuting this will require further investigation, as will the possibility that the slug's own antioxidant activity successfully mitigated potential ROS that is generated by high light exposure. This study does, however, demonstrate that there is a discrepancy between the amount of light needed to maintain optimal photosynthetic activity and the far higher amount this species receives if exposed to direct sunlight. It also demonstrates that *E. velutinus* likely avoids photodamage by hiding in *Halimeda sp.*, and may also utilize algal-derived photoprotective mechanisms and/or display a high capacity for scavenging any oxidants that are produced via antioxidant activity.

*Elysia ornata* was observed living in and around small patches of *Bryopsis plumosa* algae at a depth of 0–1 m. While *E. ornata* displayed the highest $E_{opt}$ out of the three species we measured, its $E_{opt}$ was still significantly lower than the mean in situ irradiance to which it was exposed, indicating that the *E. ornata,* like *E. crispata* and *E. velutinus*, is exposed to more light than would be photosynthetically optimal and thus likely suffers from photoinhibition and photodamage if photoprotective measures are not utilized. *Elysia ornata* also displayed a strong negative phototactic response, preferring darkness over light exposure, which could indicate that they try to limit light by crawling to areas with less light intensity such as between the algal thalli. However, numerous specimens were observed on top of the algal thalli in situ suggesting that they do not utilize hiding as a primary defense against photodamage. Furthermore, the algae certainly reduce some light from reaching the slug, but the strong wave action at this site causes the algae to move back and forth, likely limiting its shading capacity. Therefore, we conclude that the strong negative phototactic reaction observed in the lab is due to other factors such as predator avoidance or a dislike for the exposed nature of the container in which they were tested.

Compared to the other species, *E. ornata* has a very low kleptoplast abundance, yet it demonstrates the highest amount of $H_2$DCF oxidation within the digestive gland tissues, indicating that these cells are suffering from high oxidative activity and potentially oxidative stress (Fig. 6C, F). The increased amount of oxidation could be due to the low number of chloroplasts observed in this species. Chloroplasts are packed tightly in specific cells in *E. crispata* and *E. velutinus*, meaning that the outermost chloroplasts could shield the innermost from excess light and the photodamage/oxidants it produces, as has been observed in *E. timida* (Havurinne et al. 2022). This is accordingly absent in *E. ornata*, which does not contain chloroplast aggregations in specific cells but rather contains far fewer chloroplasts that are dispersed throughout the digestive tissue leaving them exposed to light that penetrates the tissue. Furthermore, *Bryopsis spp.* lack a xanthophyll cycle that would dissipate at least some excess light via NPQ (Christa et al. 2018), so slugs incorporating kleptoplasts from *Bryopsis spp.* are also unable to dissipate excess light via NPQ. *Bryopsis spp.* likely manage the light entering their photosystems via other photoacclimation mechanisms, including lowering the amount of light-harvesting pigments and altering the ratios of the light-harvesting pigments they produce (Brunet et al. 2011; Giossi et al. 2021). Since kleptoplastic slugs inherit the photoacclimation status of the kleptoplasts they ingest, *E. ornata* should acquire kleptoplasts that are photoacclimated to the high light intensities to which they are exposed in the field. Despite this, the high levels of oxidation we observed in *E. ornata* regardless of the light exposure prior to measurement indicate that this species struggles to maintain cellular redox homeostasis in its digestive gland.

Recent studies on Pacific populations of *E. ornata* detected numerous pigments associated with photoprotection mechanisms including neoxanthin, β ε-carotene, and violaxanthin (Niyogi et al. 1997; Richards Donà et al. 2022); however, the potential presence of these pigments in our specimens did not prevent the accumulation of oxidants in the digestive gland, suggesting that they offer limited photoprotective value against oxidation for this population. Therefore, the high levels of oxidative activity we observed are likely an ongoing challenge for *E. ornata* that are not mitigated by the photoacclimation status of the kleptoplasts it ingests, behavioral photoprotection mechanisms it employs, photoprotective pigments it produces, or efficient antioxidant activity. This could explain why *E. ornata* has a limited capacity to retain chloroplasts and explain why only one (*E. crispata*) of the other eight tropical sacoglossan species that are known to feed on *Bryopsis spp.* is capable of retaining kleptoplasts for more than 2 weeks (Christa et al. 2014; Curtis et al. 2015).

The distinct DCF-filled cellular compartments located within the digestive gland we observed in *E. ornata* differ in size and DCF intensity when compared to the diffuse signals we observed in *E. crispata*, *E. velutinus*, and non-digestive *E. ornata* tissue. Numerous different pathways can lead to DCF oxidation and they can occur in different cellular structures (Karlsson et al. 2010; Winterbourn 2014). While it is unclear which cellular compartment(s) contained

such high levels of oxidation in *E. ornata,* lysosomes offer a likely possibility. Most of the pathways suggested for $H_2$DCF oxidation have been observed in the mitochondria (where cytochrome *c* is localized), cytoplasm (following lysosomal recycling of redox-active metals like iron ions) or in the lysosomes themselves, where redox-active metal catalysts are highly abundant (Karlsson et al. 2010). The diffuse signal we observed in *E. crispata, E. velutinus,* and non-digestive tissues in *E. ornata* could be due to cytochrome *c*-assisted $H_2$DCF oxidation or cytoplasmic oxidation via metal catalysts, but the distinct compartments containing high levels of $H_2$DCF oxidation we observed in *E. ornata* are most likely lysosomes. This is due to the abundance of redox-active metals they contain and the observation that *E. ornata* digest their kleptoplasts within days of acquisition, indicating that they have high lysosomal activity in their digestive tissues. High lysosomal activity has been observed in other sacoglossan species that immediately digest their kleptoplasts (Laetz et al. 2016). We therefore propose that the strong DCF signal we observed in *E. ornata* exposed to both light intensities is likely caused by an abundance of oxidants (potentially ROS), which may or may not be produced via the kleptoplasts but is certainly localized in the digestive gland, coupled with the redox-active metal catalysts contained in the lysosomes produced for kleptoplast digestion; however, subsequent studies with lysosome specific fluorescence markers will be needed to verify this hypothesis.

The absence of large cellular compartments filled with DCF in *E. crispata* and *E. velutinus* could indicate that they lack aggregations of lysosomes and their associated redox-activated metal catalysts, which is unsurprising, since both species retain kleptoplasts for ~ 2 weeks and ~ 4 months, respectively (Händeler et al. 2009; Middlebrooks et al. 2011; Laetz and Wägele 2018), meaning that lysosomal activity is likely lower in their digestive gland tissues, as has been observed in other kleptoplast-retaining sacoglossans (Laetz et al. 2016). The capacity to retain kleptoplasts has been linked to increased $H_2$DCF oxidation (interpreted as increased hydrogen peroxide abundance) in two other sacoglossan species, *Elysia cornigera* (Nuttall 1989) and *Elysia timida* (de Vries et al. 2015), species that can retain kleptoplasts for more than 3 months and a few weeks, respectively (e.g., Laetz and Wägele 2018, 2019). Increased DCF fluorescence was observed in *E. cornigera* but not in *E. timida,* leading de Vries et al. (2015) to hypothesize that ROS mitigation facilitates extended kleptoplast retention in *E. timida*, the species capable of longer kleptoplast retention. This hypothesis oversimplifies the various mechanisms by which $H_2$DCF can be oxidized and directly connects DCF fluorescence to ROS activity which is problematic (Wardman 2007); however, if this hypothesis is restated without these assumptions, it could still prove valid. Both *E. cornigera* (de Vries et al. 2015) and *E. ornata* (this study) are species that are unable to retain functional kleptoplasts for more than a few weeks and both displayed increased oxidative activity in the digestive gland cells when compared to species capable of longer kleptoplast retention. This may indicate that the ability to maintain cellular redox homeostasis and avoid oxidative stress plays a role in determining a slug's ability to retain kleptoplasts for extended time periods. To properly establish a pattern between oxidative activity and the duration kleptoplasts can be retained, a variety of sacoglossan species with various kleptoplast retention capacities will need to be surveyed, ideally with redox biomarkers that can identify specific oxidants (e.g., dihydroethidium which specifically targets superoxide). Further investigations will also be needed to determine if the oxidative activity observed in these species is actually induced via photodamage or if it occurs mainly in the digestive gland for other reasons.

The variety and efficacy of behavioral and physiological photoprotective measures detailed in this study demonstrate the complex interactions photosymbiotic animals have with light in their environments and some of the consequences they face when exposed to excess light. The high light intensities we recorded in the field (> 1800 µmol $m^{-2}$ $s^{-1}$ at 0 m depth) demonstrate the disparity between actual conditions in nature and the light intensities that almost all experimental studies use when studying the effects of light on photosynthetic organisms. For example, in sacoglossan slugs alone, 20 + studies have included high-light treatments, almost always ranging from 80 to 200 µmol $m^{-2}$ $s^{-1}$ (e.g., Vieira et al. 2009; Christa et al. 2013; Baumgartner et al. 2015), yet > 200 µmol $m^{-2}$ $s^{-1}$ was observed at 10 m depth in this study, well below the depths many of these sacoglossans inhabit. This disparity has also been observed in other photosymbiotic animals such as corals (McLachlan et al. 2020; Grottoli et al. 2021). For many research questions, exposing specimens to light intensities far lower than they would receive in nature is not problematic; however, properly understanding how these animals interact with light in their environments requires researchers to measure the light intensity in the field and to design laboratory experiments with natural light intensities in mind.

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**Author contributions** All authors were involved in preliminary testing of the equipment, data collection in the field, editing the manuscript. and acquiring funding to conduct this research. LBP and EMJL wrote the first draft, conducted data analysis, and prepared the figures and tables. EMJL conceived of the study, supervised the project, and managed the administration.

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**Data availability** All data is available at https://doi.org/10.34894/61V18I.

## Declarations

**Conflict of interest** The authors declare there is no conflict of interest.

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



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