



The role of parapodia and lack of photoacclimation in kleptoplasts of the sacoglossan sea slug *Plakobranthus ocellatus*

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Abstract The sacoglossan sea slug *Plakobranthus ocellatus* is a pantropical gastropod that pilfers and incorporates algal chloroplasts (kleptoplasts) into its digestive cells and benefits from the production of photosynthate. It is a mobile, reef forager with mottled, wing-like parapodia that provide good camouflage in sand and are typically observed closed over the kleptoplast-filled digestive tubules. Functional kleptoplasts continue to photosynthesize but are separated from the algal nuclei and are unable to divide within host cells. The mechanisms that enable kleptoplasts to endure are poorly understood and the extent and limitations of functionality have not yet been fully characterized. We investigated kleptoplasts in three tropical sacoglossan species, *Elysia ornata*, *Thuridilla gracilis*, and *P. ocellatus*, collected from different depths and light fields to identify pigments, quantify retention times, and determine photosynthetic parameters. We found that *P. ocellatus* had the highest estimated retention time and maintained the highest ratio of photoprotective to photosynthetic pigments. A subsequent manipulative experiment on *P. ocellatus* specimens collected at the same site, depth,

and time involved exposure to three different irradiances and showed that kleptoplasts did not photoacclimate over the course of 7 d. No significant changes in *in vivo* kleptoplast photosynthetic parameters or corresponding spectral reflectance occurred when measuring kleptoplasts directly with open parapodia. Reflectance of closed parapodia, however, showed significant increases in the medium- and high-light treatments on day seven indicating localized kleptoplast degradation. Our results suggest that closed parapodia play an important role in kleptoplast protection by shielding internal kleptoplasts while permitting filtered light energy to reach kleptoplasts on the parapodial undersides. The cryptically patterned parapodia assume the role of photoprotectant, compensating for kleptoplast inability to photoacclimate. This allows *P. ocellatus* to forage in high-light, exposed, sandy areas and exploit algal food resources that may be unavailable or too risky for other sacoglossans.

Keywords Photoacclimation · Sacoglossans · Kleptoplast · Pulse-amplitude-modulated fluorometry · Light saturation parameter (E_k)

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Introduction

Sacoglossan gastropods (Mollusca, Opisthobranchia) are suctorial marine herbivores that remove functional chloroplasts from a variety of algal food sources (Christa et al. 2014; Wade and Sherwood 2017) and relocate them to digestive cells without digestion. This remarkable ability (Händler et al. 2009; Raven et al. 2009) permits the sea slugs to utilize the photosynthate produced by the stolen chloroplasts (“kleptoplasts”) to fuel their own metabolism (Trench et al. 1969; Cruz et al. 2020). Once separated from

their algal cells and nuclei, kleptoplasts continue to absorb light energy and produce photosynthate (Walker 1970; Trench and Ohlhorst 1976) but experience a decrease in chlorophyll *a* (Chl *a*) fluorescence and photosynthetic activity over time, consistent with an inability to divide (Trench and Ohlhorst 1976; Evertsen et al. 2007). Previous investigations on the retention of functional chloroplasts (RFC) in sacoglossans estimated that kleptoplasts remain *in hospite* and functional for periods ranging from 1 h to over 3 months (Evertsen et al. 2007; Pierce et al. 2015). This wide range indicates different retention strategies from prompt chloroplast consumption to the long-term optimization of kleptoplast functionality. We do not yet understand, however, the factors that determine retention times and survivability, or whether these factors are driven by the host, the kleptoplasts, or both. Nor do we have a good understanding of the ecological importance of kleptoplasts in sacoglossans, the drivers that determine sacoglossan foraging preferences, or their abilities to travel between different light intensity regimes.

Intact algal chloroplasts have highly plastic pigment pools and are able to photoacclimate to changes in the light field largely through concentration changes in pigments from two functional categories: light-harvesting (photosynthesis) and photoprotective (Escoubas et al. 1995; Sakshaug et al. 1997; Rodríguez et al. 2006; Brunet et al. 2011). This ability to photoacclimate is fundamental for chloroplast optimization and survival in intact algal systems (Falkowski and Raven 2007) and is presumed to be similarly important in *in hospite* kleptoplasts. The original light acclimation status of the algal chloroplasts has important consequences for functionality within the new host and may restrict hosts to similar light gradients (Cartaxana et al. 2018). Previous research suggests that once separated from the algal cellular components, kleptoplasts are unable to photoacclimate to changes in irradiance (Serôdio et al. 2014). Neither division nor pigment synthesis was observed in exposed kleptoplasts of *E. viridis* (Evertsen and Johnsen 2009), whereas exposed kleptoplasts in *Elysia chlorotica* appear to synthesize chlorophyll *a* (Chl *a*; Pierce et al. 2009) and some important PSU proteins (Pierce et al. 1996). We sought to better understand kleptoplast functionality by investigating whether kleptoplasts in three tropical sacoglossans (with exposed and cloaked kleptoplasts) can photoacclimate.

Photoacclimation is a process of reversible physiological alteration in the number, size, and/or position of chloroplasts and/or their light harvesting and photoprotective pigments in photosynthetic units (PSU) within cells (Escoubas et al. 1995; Rodríguez et al. 2006). Long-term photoacclimation processes involve structural and compositional changes, whereas short-term photoacclimation can be accomplished through activation of the xanthophyll

cycle (XC), which is a primary method of non-photochemical fluorescence quenching (NPQ) in most photosynthetic organisms (Brunet et al. 2011). Pigment or size changes within the PSU affect spectral light absorption and utilization and the rate of electron transfer and may occur on timescales of minutes to days (Escoubas et al. 1995; Falkowski and Chen 2003; Hamel and Smith 2020). Photoacclimation in functional chloroplasts allows optimization of growth rates (through division) under a wide range of irradiances while maintaining relatively steady rates of photosynthesis (Brunet et al. 2011). Chloroplast pigment concentrations are modulated by light, resulting in a larger pool of photoprotective pigments produced in high-light-acclimated chloroplasts (Kirk 2011). If relative concentrations of photoprotective and light harvesting pigments in kleptoplasts are “frozen” in their original light-acclimated state, and long-term RFC is the goal, sacoglossans may be limited to light fields matching the original algal chloroplast acclimation status. Slugs that can shield their kleptoplasts with parapodia may be able to circumvent irradiance boundaries and achieve long-term RFCs without photoacclimation.

Most sacoglossans feed on and retain chloroplasts originating from coenocytic or siphonous algal species (DeWreede 2006; Leliaert et al. 2012) within the Bryopsidales (= Caulerpales; Jensen 1997), and the Dasycladales (Marín and Ros 1992). Recent studies in Hawai'i showed that *P. ocellatus* incorporates chloroplasts from 23 different siphonous green algal sources, preferring epilithic, diminutive species (Wade and Sherwood 2017, 2018), many of which grow beneath the sand (RM Wade, pers. comm). Algal pigments include major chlorophylls (e.g., Chl *a*, Chl *b*, and divinyl *a* and *b*, and the Chl *c* group), > 30 major carotenoids (carotenes and xanthophylls), and three major phycobiliprotein groups (allophycocyanins, phycocyanins, and phycoerythrins (Roy et al. 2011). Siphonaxanthin and its ester, siphonein, are exclusive to the green algal order Bryopsidales (Yokohama 1981; Roy et al. 2011), which is characterized by coenocytic and siphonous thallus construction, ideal for the sacoglossan feeding strategies (Jensen 1993; Hirose 2005). These carotenoids are generally accepted to serve light harvesting and energy transfer functions for photochemistry (Rowan 1989). Various other carotenoids harvest light but have lower energy transfer efficiency and thus serve more commonly in the photoprotective role, dissipating heat through quenching of excited-state chlorophylls and radical oxygen species (ROS) or through cyclic de-epoxidation and epoxidation of xanthophylls (xanthophyll cycle or XC; Krause and Weis 1991; Baker 2008; Taiz and Zeiger 2010). Carotenoids, like chlorophylls and other photosynthetic pigments, are located in the light-harvesting complexes of both photosystems and help regulate light energy flow to

reaction centers and/or manage the damaging products of excess excitation at the reaction centers (Taiz and Zeiger 2010).

We took a multifaceted approach—employing photochemical, chemotaxonomic, and spectroscopic measurement techniques—to better understand the photoacclimatory capability (and by extension, the importance of full functionality) of kleptoplasts in these enigmatic solar-powered sea slugs.

Materials and methods

Pulse-amplitude-modulated (PAM) fluorometry, which imposes minimal disturbance on the experimental organisms, was used to examine several photosynthetic parameters in sacoglossans to determine their kleptoplast capacity for photoacclimation. The light saturation parameter (E_k), photosynthetic efficiency parameter (α), optimal quantum yield (F_v/F_m), and relative maximum electron transport rate ($rETR_{max}$) were analyzed to determine whether changes in irradiance would induce a corresponding photoacclimation response. High-precision liquid chromatography (HPLC) was used to isolate diagnostic pigments from known algal groups to identify the kleptoplast constituents and compile a composition inventory divided into photosynthetic and photoprotective groups. Modular spectrometers are highly effective at obtaining rapid, noninvasive *in vivo* spectral reflectance (R) measurements to identify pigment absorbance peaks. We examined known kleptoplast pigment absorbance peaks (or reflectance troughs) and compared these over time to determine whether shifts in pigment composition occurred. Optimization of photoprotective to photosynthetic pigment ratios paired with corresponding changes in photosynthetic parameters may be interpreted as signs of photoacclimation to a new irradiance regime (Brunet et al. 2011).

During initial mensurative studies conducted at Lizard Island Research Station, Great Barrier Reef, Australia (LIRS; 14°40'S, 145°28'E), we identified the constitutive pigments in kleptoplasts and measured *in vivo* photosynthetic function over time to determine host RFC levels in three tropical sacoglossan species *Plakobranthus ocellatus* (van Hasselt 1824) and *Thuridilla gracilis* (Risbec 1928), which are rarely observed with kleptoplasts exposed, and *Elysia ornata* (Swainson 1840; Fig. 1). Notably, *Thuridilla* (Bergh 1872) has recently been described as a species complex (Martín-Hervás et al. 2021), and the species complex *Plakobranthus ocellatus* is under taxonomic revision. *Plakobranthus ocellatus* remains the currently accepted name for the Australian and Hawaiian specimens (Krug et al. 2013; WoRMS; Yonow and Jensen 2018).

LIRS specimens were collected at two separate time points in Austral winter (June 2003–Group 1) and Austral summer (January 2006–Group 2), representing a difference of roughly 2 h of total daylight. A subsequent manipulative experiment testing capacity for photoacclimation to low- (LL) and high-light (HL) conditions was conducted over a 7-d period using *P. ocellatus* specimens collected at the same time, and from the same depth and 10 m² reef area. This second experiment, run at Hawai'i Institute of Marine Biology at Kāne'ohe Bay, Hawai'i (HIMB), sought to resolve the issue of variability in depth-related original light acclimation states. Several investigations have reported an apparent lack of photoacclimation capacity in primarily temperate sacoglossan kleptoplasts (Vieira et al. 2009; Jesus et al. 2010; Serôdio et al. 2014), but none have tested it directly in tropical specimens from the same size group, collected from the same depth and area, within the same 30-min period. These unique collection conditions permitted direct testing of photoacclimation in similarly light-acclimated kleptoplasts without confounding biological or habitat-induced variability. Physical changes that comprise long-term photoacclimation occur within days and would be detectable in the HL and LL treatments before the conclusion of the 7-d experiment (Falkowski and Owens 1980; Sukenik et al. 1990; Escoubas et al. 1995). *In vivo* PAM fluorometry and spectral reflectance measurements were used to determine whether kleptoplasts could respond to new light regimes.

Mensurative study

LIRS sacoglossans were collected in shallow water (1–8 m depth) within the reef barriers and lagoons. Slugs were kept in aquaria with running seawater with *in situ* temperatures ranging from 24–25 °C in June to 30–31 °C in January under natural light conditions (maximum 250–300 μmol photons m⁻² s⁻¹). No access to food was provided.

Rapid light-response curves (RLCs) were measured with a Diving-PAM (Heinz Waltz GmbH,

Germany) on slugs that had been dark-acclimated for 15 min (Wägele and Johnsen 2001). A slide projector with a 150-W halogen lamp and slides made from layered plankton-net were used to create 12–15 neutral density filters with increasing irradiances levels. Levels were verified with a RAMSES ACC underwater spectroradiometer at 350–800 nm (Trios GmbH, Germany). Irradiance during experiments was determined from inside the vials with the Diving-PAM E_{PAR} 2π irradiance sensor. Measurements took place between 18:00 and 20:00 to estimate photosynthetic performance during the end of the illumination phase each day. Individual sacoglossans were placed into separate 24-mL glass vials inserted in white Styrofoam wells for even light distribution. Black rubber barriers were

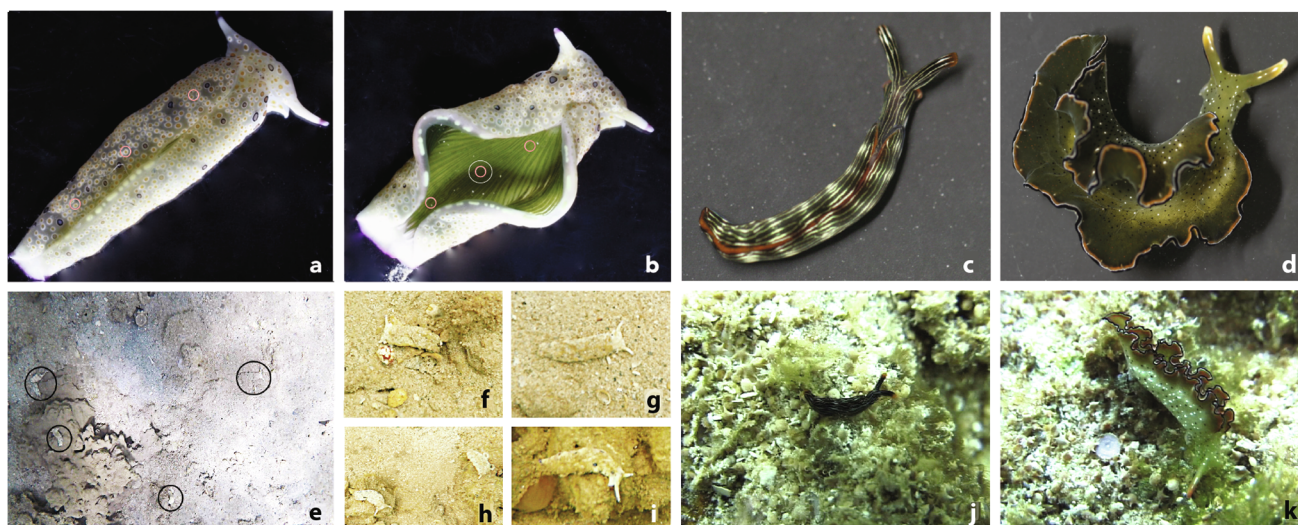


Fig. 1 Three tropical sacoglossan species: *Plakobranthus ocellatus* (a, b, e–i) with closed parapodia except (b) with manually opened parapodia exposing internal lamellae and kleptoplast-filled digestive tubules. a Body length ~50 mm, b ~25 mm. *Thuridilla gracilis* (c,

j; ~30 mm), and *Elysia ornata* (d, k; ~45 mm). Top photographs taken in laboratory, bottom at collection sites. Small pink circles a, b indicate targeted areas for reflectance probe (6.35 mm) and larger white circle (b) for PAM probe (5 mm)

positioned between vials to prevent stray light beyond the active measuring site. The dark-acclimated and illuminated fluorescence minima (F_o and F_o' , respectively) were obtained with a weak non-actinic measuring pulse ($0.15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) from a light-emitting diode (LED with peak emission at 650 nm; 0.6 KHz in dark (Wägele and Johnsen 2001). Saturating pulses of light ($\sim 10,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 0.8 s duration) from a halogen lamp oxidized all RC_{PSII} to obtain measurements of F_m (dark-acclimated fluorescence maximum) and F_m' (illuminated fluorescence maximum) during the nine-step RLC with increasing values of actinic light at each step. 2-min incubation times were used between saturating pulses and placement of the PAM probe assured minimal movement and the parapodia remained open during measurements. The relative electron transport rate (rETR) is defined as Φ_{PSII} multiplied by the incident irradiance at each saturating pulse: $\text{rETR} = \Phi_{\text{PSII}} * E_{\text{PAR}}$, and was used to obtain information on kleptoplast photoacclimation ability. Curve fitting was done in Sigmaplot version 10.0 according to the equation by Webb et al. (1974). We also calculated E_k ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) as the ratio of $\text{rETR}_{\text{max}}/\alpha$ (initial slope of light curve; Genty et al. 1989; Kroon et al. 1993; Sakshaug et al. 1997).

RFC was estimated for the Group 1 (June) specimens, which were kept for up to 12 d without access to food. Rapid light-response curves for Group 2 (January) specimens were run only once before each was sacrificed for pigment extraction. RLCs were conducted 1–2 d after the slugs were collected to permit acclimation to aquaria conditions. The initial Φ_{PSII} was calculated from the

regression line intercepting the y-axis and the daily decrease was estimated by linear regression.

All *in vitro* pigment preparations were analyzed under low light to avoid pigment degradation. At LIRS, Group 2 slugs and algae were measured for wet weight, then gently dried with adsorbent paper to avoid a reduction in extraction efficiency. Each was wrapped in aluminum foil and stored in liquid nitrogen for pigment extraction and HPLC analysis at Trondhjem Biological Station (TBS). Frozen specimens were transferred to glass vials with 2–4 ml pre-cooled methanol bubbled with N_2 to avoid oxidation. Tissue was ground with a glass rod allowing 24 h of pigment extraction in darkness at 4 °C. After extraction, the samples were filtered through a 0.45- μm Millipore filter to remove debris, and 150 μl pigment extract was injected into the HPLC system (Hewlett-Packard HPLC Series 1100), following published separation and quantification protocol (Rodríguez et al. 2006). Chlorophylls and carotenoids were detected by absorbance at 420, 440, 460, and 480 nm and identified by a diode array absorbance detector (350–750 nm with 1.2 nm spectral resolution). HPLC pigment calibration was performed using Chl *a* and β , ϵ -carotene standards from SIGMA (Aldrich, UK) and chlorophyll and carotenoid standards (Chl *b*, Chl *c*¹, violaxanthin, neoxanthin, siphonaxanthin, and siphonein) developed in TBS laboratory. The extinction coefficient of 250 $\text{L g}^{-1} \text{cm}^{-1}$ at 445 nm in acetone for β , β -carotene (Jeffrey et al. 1997) was used for the calculation of μg siphonaxanthin and siphonein, adjusting for differences in molecular weight between carotenoids (Johnsen and Sakshaug 2007). Additional specific extinction coefficients were also used (Roy et al. 2011).

Manipulative experiment

Forty-eight specimens of *Plakobranthus ocellatus* were collected from the fringing reef off Kokokahi in south Kāneʻohe Bay, Oʻahu (21°414'N, -157°78'W). All similar-sized specimens were collected from a ~10 m² area of the reef at ~1.5 m depth. Individual slugs were placed in water-filled Whirl-Pak sampling bags (Nasco, WI, USA) for transportation to the Coral Reef Ecology Laboratory (CREL) at the Hawai'i Institute of Marine Biology (HIMB). Irradiance (E) measurements were taken in full sun at approximately solar noon at the site of collection above the water surface, ~5 cm below the surface, and ~1.5 m depth with a LI-250A light meter attached to an underwater cosine sensor (LI-COR, NE, USA). E_{PAR} (Photosynthetic Active Radiance, 400–700 nm) values measured ~1800, ~1250, and ~900 μmol photons m⁻² s⁻¹, respectively.

All specimens were placed in individual tricorner polypropylene beakers in a flow-through water table at the laboratory. Foam cubes affixed to each corner of the eighteen 250-mL beakers provided flotation, and three small holes were punctured into the beakers to permit inward water flow and uniform *in situ* temperature and to prevent osmotic stress due to evaporation. Six beakers were placed in each of three treatments in the aerated water table filled to approximately 12 cm from the flow-through system. Each treatment was separated with PVC pipes that functioned as dividers and float support and each was flanked by water-filled aquaria. From the bulk specimen collection, 18 individuals were randomly placed—one in each beaker—and assigned to a treatment: high light (H), medium light (M), and low light (L). The remaining specimens were divided equally into the three treatments and placed in the corresponding aquaria as alternates in case of mortality. The H treatment beakers and aquaria remained unshaded, the M treatment was covered in shade cloth reducing ambient light ~71%, and the L treatment reduced light ~96%. Irradiance maxima in partial and full sun ranged from ~880–2100 (H), ~255–600 (M), and ~25–80 (L) μmol photons m⁻² s⁻¹. The specimens were kept without food for the entire 8-d (D1–8) experiment.

On D1, nine specimens from the bulk collection were dark-acclimated for 15 min for initial *in vivo* Chl *a* fluorescence kinetics measurements with Diving-PAM and WinControl-3.25 software. One rapid light-response curve (RLC) per specimen was run with saturating pulses at 30-s intervals and increasing actinic irradiances to 1000 μmol photons m⁻² s⁻¹. The intensity of the blue light-emitting measuring probe (470 nm, LED, 0.05 μmol photons m⁻² s⁻¹) did not induce photosynthetic activity. Three specimens from this group were randomly assigned, with an additional three specimens, to each treatment (*n* = 6). All

specimens were acclimated to their new light environments for 48 h before subsequent PAM and reflectance measurements. The external surface of parapodia produced low fluorescence signal and high noise; thus, only internal kleptoplasts in digestive lamellae were measured.

In vivo reflectance (R) spectra were collected in random order on D3, D5, and D7 with an R400-7-SR 400 μm reflectance probe attached to a Flame S-UV-VIS-ES 200-850 nm modular spectrometer and a Mikropack Deuterium-Halogen DH-2000 UV-VIS-NIR light source (all Ocean Insight—formerly Ocean Optics, Dunedin, FL, USA). Prior to measurements, 100% reflectance was determined using the OceanView version 4.1 software wizard and the WS-1 reflectance standard, to provide a baseline. Three spectral reflectance measurements—adjacent to the pericardial swelling, central, and distal—were recorded per specimen to properly represent variable kleptoplast density on the internal lamellae and external parapodial surface (Fig. 1). Relevant chlorophyte wavelengths 438 (Chl *a*, *b* and carotenoids), 470 (Chl *b* and carotenoids), 550 (low absorption window of all pigments), 645 (Chl *b*), and 678 nm (Chl *a*) were used for statistical comparison per Beach et al. (2006) and Serôdio et al. (2010). All specimens were alive and mobile when returned to the reef at the conclusion of the experiment.

Statistical analysis

Photosynthetic parameter and spectral reflectance data were extracted and sorted using Python 3 scripts in PyCharm version 11.0.5. After verification that all initial specimens (*n* = 9) had similar rates of photosynthesis, we used Welch's one-way ANOVA (α = 0.05) allowing for unequal variances in JMP Pro version 16 (SAS) to compare final day (D7) photosynthetic parameters by treatment (*n* = 6). Relative electron transport rate (rETR) curves (from PAM RLCs) were plotted in RStudio version 1.2.5042 "Double Marigold" using the Phytotools package version 1.0 and the model "fitWebb" (Webb et al. 1974). Mean spectral reflectance data for the three measurements each of open and closed parapodia were smoothed with the Savitzky-Golay filter and compared D7 to D3 using non-parametric Wilcoxon/Kruskal-Wallis rank sums tests at five wavelengths, including 438 nm (Chl *a* maximum absorption in blue portion of spectrum), 470 nm, 550 nm, 645 nm, and 678 nm (Chl *a* maximum absorption in red). Figures were produced in RStudio, JMP, and Adobe Illustrator.

Results

LIRS Australia—mensurative study

In vitro Pigments—chemotaxonomy and function

The pigment composition of the three sacoglossan species showed that all contained Chl *a*, Chl *b*, siphonaxanthin, siphonin, neoxanthin, violaxanthin, and β,ϵ -carotene, indicative of the green Bryopsidales. Additionally, *P. ocellatus* and *E. ornata* contained 19'hexanoyloxyfucoxanthin, diatoxanthin and a mix of unknown carotenoids potentially from microphytobenthos, i.e., diatoms or epiphytic haptophytes on the food algae. *Plakobranthus ocellatus* also contained lutein, and an anthera-like carotenoid, not found in *E. ornata* or *T. gracilis*. The ratio (W:W) between photosynthetic and photoprotective pigments was 4.31 in *P. ocellatus*, 7.09 in *E. ornata*, and 15.62 in *T. gracilis* when considering violaxanthin as a photoprotective carotenoid. *Plakobranthus ocellatus* has the highest number of photoprotective to photosynthetic pigments among the three species even when violaxanthin is tallied with photosynthetic pigments (Table 1).

Retention of functional chloroplasts (RFC)

RFC values for the three sacoglossan species were estimated based on a daily decrease in Φ_{PSII} of 4–6% in *P. ocellatus*, 67–100% decrease in *E. ornata*, and 76–94% decrease in *T. gracilis* (Fig. 2). RFC for *P. ocellatus* ($n = 3$) was 197–277 d based on Φ_{PSII} decrease of 0.003–0.004 d^{-1} (y-intercept 0.79 Φ_{PSII} estimated from regression line, $R^2 = 0.33$). RFC for *E. ornata* ($n = 3$) was 8–12 d based on Φ_{PSII} decrease of 0.039–0.042 d^{-1} (y-intercept 0.67 Φ_{PSII} estimated from regression line, $R^2 = 0.47$), and 12–14 d for *T. gracilis* ($n = 2$) based on Φ_{PSII} decrease of 0.037–0.048 d^{-1} (y-intercept 0.57 Φ_{PSII} estimated from regression line, $R^2 = 0.61$).

An inability for several specimens of each species to reach light saturation—even at actinic irradiances up to 1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ —hindered estimations of rETR_{max} , and wide value ranges in the calculated photosynthetic parameters rETR_{max} , α , and E_k , made analysis and interpretation of these results problematic (Table S1). For instance, E_k in *P. ocellatus* ranged from 100 to 2219, a 20-fold difference that highlights the importance of original kleptoplast acclimation status within the slugs collected at different depths and light fields.

HIMB Hawai'i—photoacclimation experiment

In vivo photosynthetic parameters and spectral reflectance in *Plakobranthus ocellatus*

Relative ETR_{max} , E_k , α , and F_v/F_m were analyzed as photosynthetic parameters for inter- and intraspecific comparison between specimens from the three light level treatments (Table 2). It was impossible to obtain reliable RLC data on closed parapodia due to a low Chl *a* fluorescence signal to noise ratio; thus, the photosynthesis results reflect status of internal kleptoplasts only. We found no statistically significant changes in any of the treatments for any of the parameters as a function of time. Plots of rETR vs irradiance curves for all treatments and times demonstrate a generally homogeneous response among the treatments with most rETR values between 200 and 400 (dimensionless) at the final saturating pulse and maximum actinic value of 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 3). Only ~48% of all specimens reached saturation prior to the final saturating pulse. Notably, the maximum actinic irradiance applied during RLCs was ecologically relevant since it was close to the ambient maximum downwelling irradiance measured (~900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in the slug habitat.

Reflectance measurements were taken for internal kleptoplasts of open parapodia and the external surface of closed parapodia. We looked for lateral shifts in peaks, which indicate changes in photoprotective to photosynthetic ratios, as well as basic increases or decreases in *in vivo* reflectance by analyzing several wavelengths over time. In open parapodia, no significant differences in kleptoplast reflectance were found at any wavelength in any of the treatments. In closed parapodia, we observed statistically significant increases in reflectance from D3 to D7 at all analyzed wavelengths in the H and at two wavelengths in the M treatments (Table 3). There were no significant changes in the L treatment. Figure 4 illustrates reflectance at 678 nm of internal and external slug surfaces revealing low and mostly stable reflectance in internal versus higher and more dynamic reflectance on the external surface of parapodia as a function of time. Higher reflectance is equivalent to lower absorbance (transmittance is considered negligible), consistent with a loss or degradation of Chl *a* and other pigments in kleptoplasts. Reflectance spectra for the six specimens per day per treatment were plotted as mean values for open (Fig. 5a, c, e) and closed parapodia (Fig. 5b, d, f). These spectra show clear increases in reflectance in the closed but no clear peak shifts in either open or closed parapodia. All spectra remained similarly shaped over time.

Table 1 Pigment composition identified and quantified by HPLC, in the three LIRS sacoglossan species with most observed parapodial state (open or closed) denoted.

		<i>Plakobranchnus ocellatus</i> Parapodia closed		<i>Thuridilla gracilis</i> Parapodia closed		<i>Elysia ornata</i> Parapodia open	
Wet weight (g) mean/CV%		0.324/42		0.029/38		0.366/54	
Total pigment composition		µg pigm/g slug		µg pigm/µg Chl a		µg pigm/g slug	
Pigments	<i>In vitro</i> absorption maxima	Mean/CV%	Mean/CV%	Mean/CV%	Mean/CV%	Mean/CV%	Mean/CV%
Chl a	411,430,536,580,616,662 [1.22]	73.44/53	1.00/0	127.28/169	1.00/0	45.61/66	1.00/0
Chl a-like	411,431,535,582,617,663 [1.30]	2.47/127	0.03/97	0	0	2.20/173	0.04/173
Chl b	(425),457,596,645 [2.79]	26.20/41	0.37/12	56.84/153	0.54/27	18.97/64	0.45/22
Chl b-like	(430),457,595,646 [3.65]	1.33/77	0.02/50	0	0	0	0
Pheophytin a	411,507,536,608,666 [2.49]	1.21/245	0.01/245	0	0	0	0
Siphonaxanthin	449,(470)	0.63/118	0.01/145	1.88/197	0.01/104	3.84/54	0.10/40
Siphonein	456,(476)	1.74/31	0.03/32	13.61/58	0.27/105	1.44/73	0.04/40
<i>Neoxanthin</i>	415,439,467 [0.91]	7.50/28	0.12/43	0.45/283	0.01/283	6.69/34	0.20/66
<i>Neo-like</i>	414,436,464 [0.66]	0.48/94	0.01/114	0	0	0	0
*Violaxanthin	417,442,471 [0.97]	9.34/12	0.15/35	10.52/159	0.10/53	0.99/30	0.04/116
*Viola-like	418,443,472 [0.89]	0	0	0	0	1.99/28	0.08/108
<i>Lutein</i>	(423),448,476 [0.63]	3.97/49	0.06/59	0	0	0	0
<i>Anthera-like</i>	(423),448,476 [0.62]	2.39/39	0.04/57	0	0	0	0
<i>β,ε-carotene</i>	424,448,475 [0.52]	0.41/139	0.01/110	1.81/214	0.01/109	0.50/88	0.01/93
<i>β,β-carotene</i>	454,482 [0.17]	0.44/110	0.00/78	0	0	0	0
19' HOF	448,471 [0.04]	0.22/51	0.00/63	0	0	0.24/95	0.01/149
<i>Diatoxanthin</i>	(430),454,481 [0.36]	0.28/245	0.01/245	0	0	0	0
Carotenoid mix	(410),432,460 [0.72]	0.62/111	0.01/122	0	0	0.76/45	0.02/43
Total pigments/slug		132.68/44		212.40/157		83.23/47	
Total PS (w, w/o viola)		115.15, 105.81		210.13, 199.61		75.58, 72.06	
Total PC (w, w/o viola)		24.53, 15.19		12.78, 2.26		10.17, 7.19	
PS:PC (viola in PS, viola in PC)		7.58, 4.31		92.92, 15.62		10.51, 7.09	

Mean µg pigment per gram slug (wet weight) and mean µg pigment per µg chl a. The mean µg pigment per gram slug is also presented as a total µg pigments per gram slug with corresponding ratios of Chl a to other important pigments or pigment groups. Square brackets indicate ratios between peaks and minima for all pigments, whereas chlorophyll values represent the blue: red ratio. CV%=coefficient of variation of the mean standard error. 19'HOF=19'hexanoyloxyfucoxanthin. Photosynthetic (PS) pigments are in bold, photoprotective/carotenoids with low energy transfer efficiency (PC; xanthophyll cycle) are in italic, unassigned (multi-function or unclear) are in bold-italic. Total PC ratios calculated with (w) and without (w/o) violaxanthin since its function varies

Discussion

In situ observations of *Plakobranchnus ocellatus* indicate that this species prefers open and well-lit, fine grain soft substrates at 1–2 m depth, often close to coral habitats. The macroalgal food sources for this species are not usually observed since they tend to be small and often hidden

beneath the sand (Wade and Sherwood 2017). This contrasts with the other species we examined, which are often found on or close to visible detectable macroalgae (>1 cm length) and on coral substrates. These contrasts are important when comparing kleptoplast functionality among species and highlight the problems associated with obtaining slugs from dissimilar light habitats. The unique

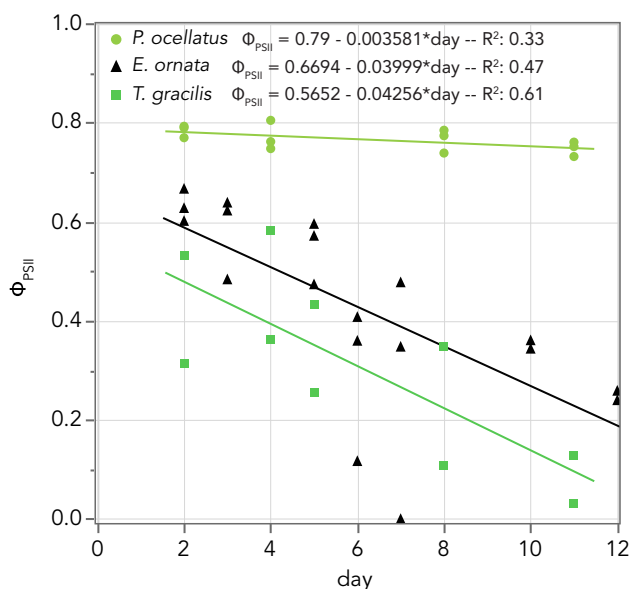


Fig. 2 RFCs for three sacoglossan species during experimental period (11 d, *P. ocellatus*, *T. gracilis*; 12 d, *E. ornata*) at LIRS. RFC values were estimated by plotting the Φ_{PSII} against time (Evertsen et al. 2007) as per the equation: $RFC = initial\Phi_{PSII}/decreasein\Phi_{PSII}d^{-1}$

specimen collection for the experiment in Hawai'i eliminated variability in light habitat, specimen size, seasonal effects, and available food algae, which can greatly influence the original chloroplast photoacclimation status. The results of the *P. ocellatus* light treatment experiment at HIMB provided no evidence that photoacclimation occurred in any of the specimens. We expected signs of photoacclimation to occur most prominently in the low- and/or high-light treatments because these light ranges diverged most from the *in situ* slug habitat maximum ($\sim 900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

A solar insolation curve illustrates the approximate daily light habitats the specimens experienced during the experiment and puts these in context with the habitat from which they were collected (Supplemental Fig. 1). If kleptoplasts were capable of photoacclimation we would have

observed significant signs of photoprotective to photosynthetic ratio shifts and optimization of photosynthetic parameters by day 7 (Hamel and Smith 2020). We found no significant differences in photosynthetic parameters of internal kleptoplasts in any of the treatments during the 7-d period. No significant shifts occurred at numerous known chlorophyte photosynthetic pigment reflectance troughs (absorbance peaks) in the internal kleptoplasts in any of the treatments during the experimental period. Since RLCs were only conducted on internal kleptoplasts, photosynthetic parameters of kleptoplasts lining the undersides of the closed parapodia are unknown. We did, however, measure reflectance of closed parapodia and found a significant effect of increased light intensity exposure over time at all analyzed wavelengths in the H treatment and at two wavelengths in the M treatment (Fig. 5; Table 3). The increase in reflectance of closed parapodia suggests the kleptoplasts underneath experienced light-induced change consistent with degradation or pigment decrease as a function of time. The increase in the M treatment indicates a constant requirement for new kleptoplasts even under sub-saturating or natural light conditions. This result was somewhat surprising since the M kleptoplasts should have been acclimated to irradiances above the experimental values. Together with the remarkably stable, low reflectance values of internal kleptoplasts over time, the changes indicate parapodia serve an important photoprotective role for the non-photoacclimating internal kleptoplasts. These results also highlight the importance of burrowing in *P. ocellatus* foraging behavior since this may serve to reduce incident light energy on parapodia, particularly when light is supersaturating. The camouflaging parapodia appear to serve a defensive role, protecting slugs from predation and light intensity and/or prolonged exposure. They permit *P. ocellatus* (RFC=197–203 d) to forage in open, well-lit environments, with access to high-light-acclimated chloroplasts, which contribute to the long-term retention of functional chloroplasts in the species.

The chemotaxonomy results from the Australian LIRS specimens showed that pigment composition and ratios

Table 2 Welch's ANOVA for *in vivo* photosynthetic parameters ($rETR_{max}$, E_k , α , and F_v/F_m) of internal kleptoplasts comparing effects of treatment at conclusion of experiment (D7; $n = 6$).

Day	Light	$rETR_{max}$ mean \pm SD	p	df F	E_k mean \pm SD	p	df F	α mean \pm SD	p	df F	F_v/F_m mean \pm SD	p	df F
D1		290 \pm 25			639 \pm 309			0.66 \pm 0.16			0.59 \pm 0.08		
D7	L	426 \pm 144	0.09		634 \pm 343	0.10		0.82 \pm 0.16	0.055		0.65 \pm 0.07	0.09	
D7	M	349 \pm 30		2	892 \pm 132		2	0.60 \pm 0.07		2	0.60 \pm 0.05		2
D7	H	298 \pm 50		3.19	561 \pm 346		3.18	0.65 \pm 0.17		4.17	0.53 \pm 0.12		1.44

Means for baseline measurements (D1; $n = 9$) included specimens that were later assigned to each treatment. Light treatments; L = low, M = medium, H = high. Mean, standard deviation (SD), and p -value reported.

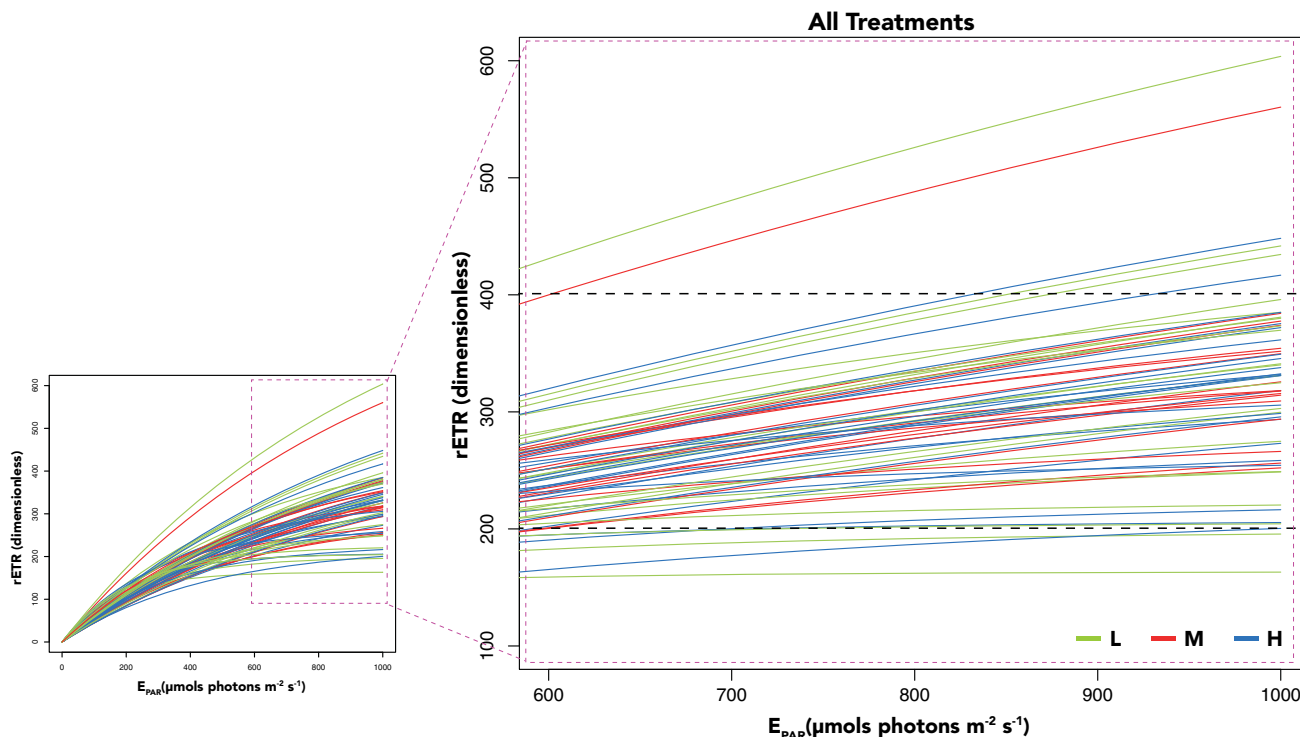


Fig. 3 Relative ETR (rETR) curves detailing 600 to 1000 E_{PAR} ($\mu\text{mols photons m}^{-2} \text{s}^{-1}$) with full plot (small, left). Curves for all specimens from three treatments over 7-d experimental period. Most $rETR_{max}$ (at E_{PAR} 1000) ranged between 200 and 400 (dimensionless; black dashed lines) with no apparent trends. Area of detail delimited by pink dotted box

Table 3 Means and standard deviation for *in vivo* reflectance (%) at several important Chlorophyte wavelength peaks across the visible spectrum for D3 and D7

	Day	Light	438 nm	<i>P</i>	470 nm	<i>P</i>	550 nm	<i>P</i>	645 nm	<i>P</i>	678 nm	<i>P</i>
			mean/std dev	ChiSq	mean/std dev	ChiSq	mean/std dev	ChiSq	mean/std dev	ChiSq	mean/std dev	ChiSq
External	D3	L	10.39/5.29	0.08	13.13/5.89	0.34	23.09/6.74	0.63	22.33/7.10	0.52	20.26/6.66	0.87
	D7		14.75/5.43	3.10	16.6/5.20	0.92	24.71/6.35	0.23	23.09/7.19	0.41	20.42/6.68	0.03
	D3	M	8.37/5.16	0.01	10.49/5.53	0.01	20.81/10.46	0.08	21.22/11.55	0.08	19.32/10.24	0.055
	D7		22.28/8.04	6.56	24.11/7.65	6.56	33.75/7.18	3.10	33.53/7.34	3.10	30.62/7.61	3.69
	D3	H	8.52/2.76	0.01	10.98/3.11	0.03	20.09/3.79	0.01	20.24/4.39	0.01	18.63/4.32	0.01
	D7		16.07/4.93	6.56	18.73/5.65	5.03	29.69/5.32	6.56	30.29/5.05	6.56	27.45/4.02	6.56
Internal	D3	L	5.71/4.28	1.0	7.21/5.83	0.63	19.57/9.07	0.20	9.52/5.71	0.26	5.42/3.82	0.52
	D7		4.60/1.75	0.0	4.53/1.77	0.23	12.94/5.05	1.64	5.64/3.08	1.26	3.45/1.48	0.41
	D3	M	4.24/1.74	0.52	5.18/2.1	0.15	13.43/5.90	0.34	6.53/3.26	0.75	3.78/2.03	0.87
	D7		3.68/0.97	0.41	3.79/1.20	2.08	16.49/7.18	0.92	7.72/4.17	0.10	3.59/1.46	0.03
	D3	H	2.89/0.74	0.055	3.54/1.60	0.20	17.25/7.83	0.34	6.96/3.07	0.26	2.90/1.26	0.11
	D7		4.51/1.59	3.69	4.58/1.67	1.64	17.17/3.60	0.92	7.80/2.19	1.26	4.02/1.37	2.56

P-values and chi square approximations from the results of nonparametric tests for external kleptoplasts in closed (top) and internal kleptoplasts in open (bottom) parapodia reflectance comparing specimen change from D3 to D7 ($n = 6$). Degrees of freedom = 1. Abbreviations as in Table 2

varied greatly between the species. *Plakobranthus ocellatus* had the highest concentration of photosynthetic pigments per wet weight and the highest corresponding concentration of photoprotective pigments among the species, resulting in the lowest photosynthetic: photoprotective (PS:PC) pigment ratios of 4.3 with violaxanthin considered as PC and 7.6 when calculated with PS pigments. These low PS/PC ratios, relative to those for *T.*

gracilis (15.6 and 93.0) and *E. ornata* (7.1 and 10.5), indicate a stronger preference by *P. ocellatus* for high-light-acclimated kleptoplasts (Table 1). Additionally, an antheraxanthin-like carotenoid was present only in *P. ocellatus*, consistent with high-light-acclimated algae. Violaxanthin is strongly light-harvesting and may transfer light energy to reaction center Chl *a* under low-light conditions. In high light, decreased luminal pH will promote

de-epoxidation of violaxanthin to initiate the xanthophyll cycle (Brunet et al. 2011). Chemotaxonomy reveals no functional traits in pigments; therefore, we made no definitive determination regarding the primary function of violaxanthin in kleptoplasts. Zeaxanthin was not present in any of the species but this does not necessarily indicate a lack of photoprotective capacity (Goss et al. 1998; Brunet et al. 2011; Christa et al. 2017). Additionally, lutein, a major carotenoid in the Chlorophyta, is considered to be the light-dependent product of conversion from siphonaxanthin and is believed to quench triplet excited chlorophyll (^3Chl ; Croce et al. 1999; Baroli et al. 2000; Jahns and Holzwarth 2012). Lutein comprised 6% of pigments in *P. ocellatus* but was absent in the other two slug species. Its presence in *P. ocellatus* likely augments the VA xanthophyll cycle for photoprotection (Raniello et al. 2006; Christa et al. 2017) while photoprotection in the kleptoplasts of the other two species may not be as vital. Neoxanthin, present in all three species, scavenges the highly destructive singlet oxygen ($^1\text{O}_2$) and thus also plays a role in photoprotection (Niyogi et al. 1997; Croce et al. 1999; Baroli et al. 2000). The apparent preference for kleptoplasts with a lower PS:PC ratio could partially explain the extended kleptoplast functionality in *P. ocellatus* relative to the other species and is a strong indication that these slugs spend time in high-light environments.

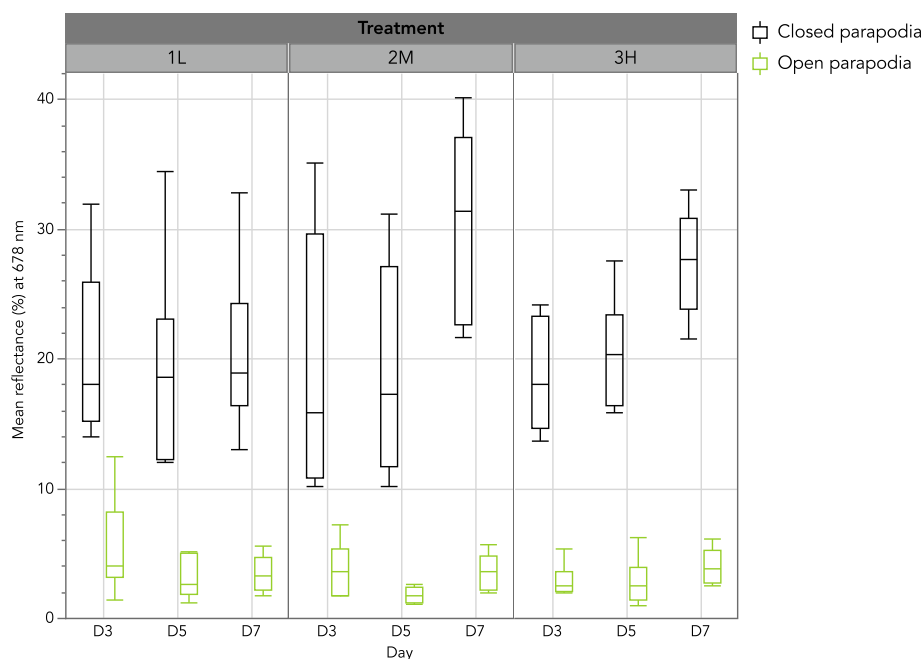
Photosynthetic parameter differences among the three LIRS species revealed the importance of the original light conditions on sacoglossan food algae preferences. The light saturation parameter (E_k) values show vast differences in prior photoacclimation status of the kleptoplasts among

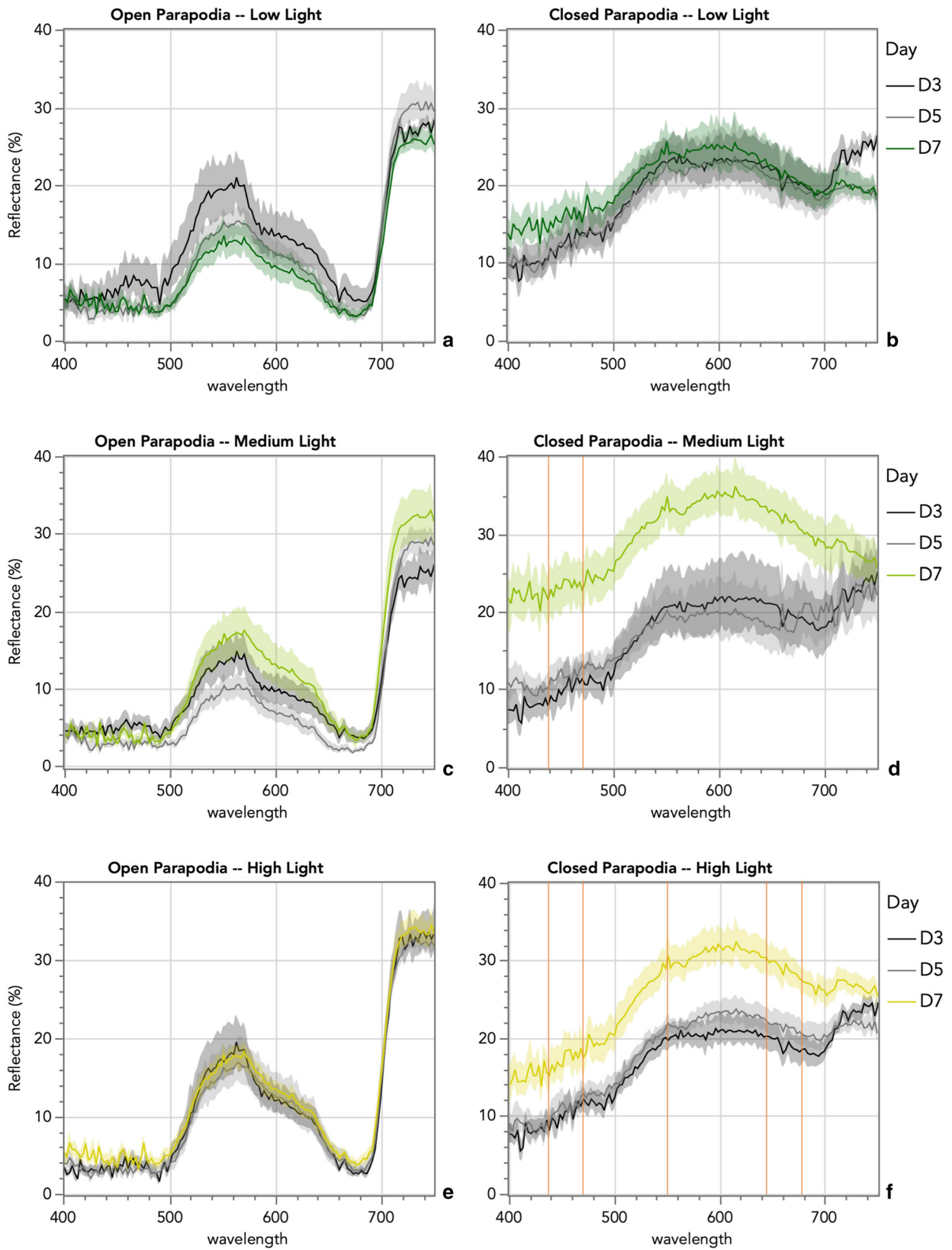
Fig. 5 Mean reflectance spectra over time are contrasted between open (a, c, e) and closed parapodia (b, d, f), sorted by low (a, b), medium (c, d), and high (e, f) light treatments ($n = 6$ per treatment) per day. Shaded confidence intervals are superimposed on spectrum data smoothed with the Savitzky–Golay filter to reduce noise. Red vertical lines indicate wavelengths where significant differences were found

and within the three species (Table S1), indicating food algae origins from a wide range of light habitats. *Thuridilla gracilis* individuals (RFC=12–14 d) collected from the same habitat and depth (reef flat, sand; 1–2 m) had $r\text{ETR}_{\text{max}}$ values ranging from 356 to 1115, indicating the consumption of chloroplasts from both bright and more shaded microhabitats. A surprising 20-fold difference in $r\text{ETR}_{\text{max}}$ occurred between specimens collected in <2 m versus 2–8 m depth. Comparing E_k values in kleptoplasts across all species from 1 m depth, we observed that *T. gracilis* and *E. ornata* (E_k 450–694) accessed lower-light-acclimated kleptoplasts relative to those in *P. ocellatus* (E_k 675–2219). We also observed that light saturation was not reached in several individuals from all three LIRS species and in *P. ocellatus* specimens in Hawai‘i and interpreted this as the result of immensely high numbers of kleptoplasts per unit area, a reduction in absorbing cellular components, and minimal effects of self-shading or the “package effect” (Fig. 6).

The concept that *P. ocellatus* keeps its parapodia closed to protect kleptoplasts from excess irradiance is not novel. Indeed, other functional kleptoplast sacoglossans respond to high irradiance by closing their parapodia (Cartaxana

Fig. 4 a *In vivo* mean reflectance values at 678 nm (red peak absorption of Chl *a*) comparing *within* treatment response over time for open (green) and closed (black) parapodia. Light values: L = low ~ 25 –80, M = medium ~ 255 –600, and H = high ~ 880 –2100 in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. D = day





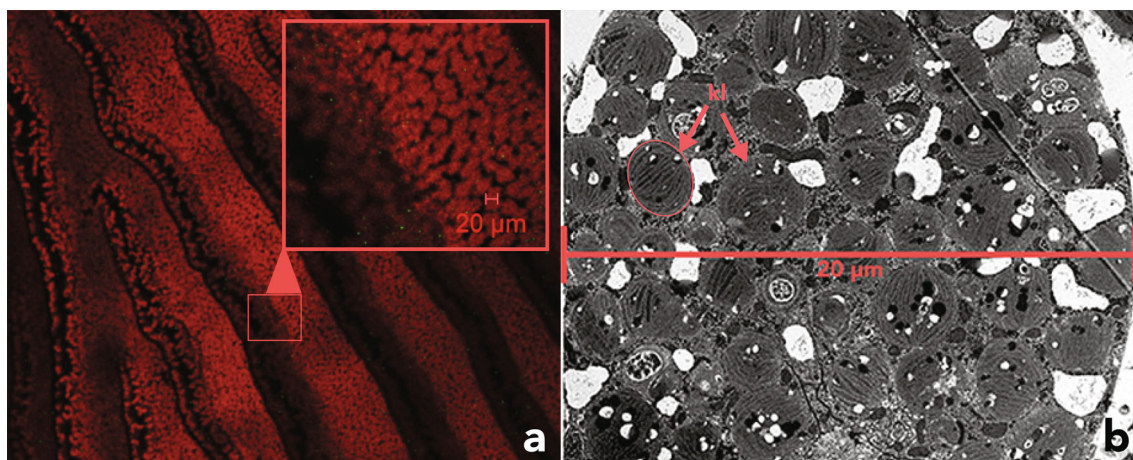


Fig. 6 Kleptoplasts in *P. ocellatus*. **a** Confocal laser scanning microscopy scan of digestive tubules at $2.5\times$ magnification with zoomed in area inset. Red color represents fluorescence emission in

the 568–702 nm range. **b** Transmission electron microscopy photomicrograph showing numerous kleptoplasts (kl) per unit area. 20 μm size bar for comparison

et al. 2018, 2019). The novelty lies in the storage containers where copious internal kleptoplasts are located in dorsal lamellae along the central portion of the body, in digestive tubules radiating outward from directly below the heart and stomach toward the tail (Hirose 2005). These unique structures allow the transfer of materials along their length via luminal microvilli to the distal edges of the digestive glands, located on the undersides of the parapodia (Hirose 2005). These distal kleptoplasts are more exposed and more likely to experience light-induced degradation. The reflectance results strongly indicate these kleptoplasts may be sacrificed and subsequently assimilated to protect the internal kleptoplasts, which may persist and provide food security for up to an estimated 277 d. It is important to note that these long-term estimates are based on a weak linear decline in kleptoplast functionality during short-term experiments and should be tested to verify their accuracy.

Since no evidence of kleptoplast photoacclimation has ever been observed and retention of functional kleptoplasts is presumed important, slugs must avoid light regime change or provide some form of photoprotection. There are two likely strategies that may best explain long-term kleptoplast functionality without photoacclimation. (1) Slugs may restrict movement to shady areas when irradiance is high, remaining close to reef structure, which would also provide cover from predation. This is more likely in “naked” slugs with highly visible kleptoplasts. (2) Slugs with parapodia may cloak their kleptoplasts to avoid high-light exposure. This ability to protect kleptoplasts presumably provides the advantage of unlimited range on reef structure (i.e., coral colonies and coral rubble), sand, or other open spaces. *Plakobranthus ocellatus* appears to employ this second strategy, as it is commonly found in open, sandy areas, and maintains closed parapodia. The

authors estimate observing over 100 specimens in the field and laboratory (both in Australia and Hawai‘i) but never with open parapodia. Unlike the brightly colored or green sacoglossans, the external surface of *P. ocellatus* parapodia are speckled and drab and provide good camouflage in the sand where they have also been observed to burrow. In this manner, *P. ocellatus* can forage in open areas using crypsis to avoid predators while protecting their internal kleptoplasts from exposure to light. This study provides additional evidence that photosynthetically functional kleptoplasts are sensitive to light intensity changes but do not photoacclimate. We also propose an additional functional role for the camouflaging parapodia, which likely gives *P. ocellatus* an ecological advantage over other reef algae foragers.

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