Zoological Studies **59:**65 (2020)
doi:10.6620/ZS.2020.59-65

Zoological Studies

Open Access

Seasonality and Longevity of the Functional Chloroplasts Retained by the Sacoglossan Sea Slug *Plakobranchus ocellatus* van Hasselt, 1824 Inhabiting A Subtropical Back Reef Off Okinawa-jima Island, Japan

Shu Chihara, Takashi Nakamura, and Euichi Hirose*

*Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan. *Correspondence: E-mail: euichi@sci.u-ryukyu.ac.jp (Hirose)*
E-mail: ringoamemh@icloud.com (Chihara); takasuke@sci.u-ryukyu.ac.jp (Nakamura)

Received 18 July 2020 / Accepted 12 October 2020 / Published 30 November 2020
Communicated by Yoko Nozawa

Plakobranchus ocellatus is a sacoglossan sea slug that feeds on multiple algal species and retains chloroplasts as kleptoplasts for several months. The seasonal differences in the photosynthetic properties of kleptoplasts were examined in sacoglossans collected from a subtropical back reef off of Okinawa-jima (26°21'55"N 127°44'10"E) in 2017–2018. The effective quantum yield of photosystem II in kleptoplasts indicated that stronger ambient light causes more stress in kleptoplasts. The maximum quantum yields (QY) at 20°C, 30°C, and 40°C indicated that kleptoplasts were more functional in photosynthesis in winter than in spring or summer, whereas kleptoplasts may have the highest tolerance to high temperatures in summer. In the long-starvation experiment (LSE), the relative ratio of body weight (rel$W$) linearly decreased and the sacoglossans died within 2 months in the total dark condition, whereas in the LSE with illumination, the animals survived up to 5 months. The time course for the decrease in the relative ratio of the QY (relQY) in the LSE indicated that the photosynthetic function was almost normal for 2 months, regardless of the presence or absence of illumination, after which time relQY gradually decreased to zero. In the field, *P. ocellatus* continuously took up new kleptoplasts that have suitable properties of photosynthetic ability for each season. In a subtropical environment, in which water temperatures vary from below 20°C to above 30°C, seasonal changes could cause a temporary shortage of algal food and affect the photosynthetic activity of *P. ocellatus* kleptoplast. Our results, however, indicated the kleptoplasts of *P. ocellatus* functioned normally for several months and maintained the presence of this sacoglossan in a subtropical environment throughout the year.

Key words: Kleptoplasty, Long-starvation experiment, Long-term retention, Pulse-amplitude-modulated fluorescence, Total darkness.

BACKGROUND

Sea slugs in the order Sacoglossa usually feed on algal cytoplasm and some retain the chloroplasts of the food algae in their digestive gland cells as functionally photosynthetic organelles; these are termed kleptoplasts (Muscatine et al. 1975; Williams and Walker 1999; Pierce et al. 2015). The duration for which these chloroplasts are retained varies among species from 1 day to several months (Clark et al. 1990). In long-term

Citation: Chihara S, Nakamura T, Hirose E. 2020. Seasonality and longevity of the functional chloroplasts retained by the sacoglossan sea slug *Plakobranchus ocellatus* van Hasselt, 1824 inhabiting a subtropical back reef off Okinawa-jima Island, Japan. Zool Stud **59:**65. doi:10.6620/ZS.2020.59-65.

retention species, the animal can survive months without any food in the light, suggesting that the kleptoplasts provide enough nutrients for the sea slug to survive. For example, *Elysia chlorotica* Gould, 1870 survived for 8–9 months (Mujer et al. 1996), and *E. timida* (Risso, 1818) and *Plakobranchus ocellatus* van Hasselt, 1824 have been estimated to survive for up to 3 and 11 months, respectively (Evertsen et al. 2007). In long-starvation experiments (LSEs), the relative size and survival rate of sea slugs under light were remarkably higher than those under total dark conditions in *E. timida*, *E. trisinuata* Baba, 1949, *E. viridis* (Montagu, 1804), and *P. ocellatus* (Giménez Casalduero and Muniain 2008; Yamamoto et al. 2013; Cartaxana et al. 2017; Akimoto et al. 2014), indicating that kleptoplasts contribute to photosynthesis, which in turn helps the hosts survive in LSEs. Moreover, Donahoo et al. (2020) pointed out the importance of light intensity in LSEs; moderate light conditions were better than both near-dark and field light conditions in reducing the decline of the body weight and photosynthetic efficiency of the kleptoplasts in *Plakobranchus* cf. *ianthobaptus* Gould, 1852. However, some studies showed that the time course of the decreases in photosynthetic capacity and relative size of sea slugs was not different between illuminated and total dark conditions (*e.g.*, Christa et al. 2014 2018) or the photosynthetic capacity of the sea slugs kept in the dark was always higher than that in the light (Christa et al. 2015). Some authors, therefore, doubt the idea of phototrophic sacoglossans and suggested that the sacoglossans retain kleptoplasts for reasons other than carbon fixation (reviewed in de Vries et al. 2014; Rauch et al. 2017).

The observation that sea slugs retain kleptoplasts for a long time begs the question of how kleptoplasts can be maintained in animal tissues without algal nuclei, because the genes of many chloroplast proteins are encoded in the nuclear genome of the food algae. One possible explanation is the horizontal gene transfer from the algal nuclear genome to the sea slug genome (*e.g.*, Mujer et al. 1996; Rumpho et al. 2008). However, this idea is supported by neither transcriptomic (reviewed by Pelletreau et al. 2011) nor genomic analyses in sea slugs (Bhattacharya et al. 2013; Cai et al. 2019). Even without algal nuclear genes, Rauch et al. (2015) suggested that kleptoplasts are more robust than isolated plastids of land plants. Moreover, the sea slug may mitigate stress on the kleptoplasts, because the genes involved in the oxidative stress-response mechanisms are up-regulated during the development of kleptoplasty (Chan et al. 2018).

Many sacoglossan species feed on multiple species of algae (Jensen 1980; Williams and Walker 1999; Christa et al. 2015; Middlebrooks et al. 2019). The kleptoplasts in *Elysia* sp. (cf. *E. furvacauda* Burn, 1958) are seasonally different in algal species (Brandley 1984) and those within the same sacoglossan species, *e.g.*, *E. crispata* Mörch, 1863, are locally different among algal species (Christa et al. 2015; Middlebrooks et al. 2019), whereas the occurrence of kleptoplasty and retention periods in *E. viridis* are different among the food algal species (Rauch et al. 2018). The optimum irradiance for the kleptoplasty in LSEs may be different among the sacoglossan species and populations, probably due to the source species of the kleptoplasts (Donahoo et al. 2020). It is possible that the sacoglossans exhibit seasonal and local adaptation in their dietary choices.

Plakobranchus ocellatus belong to a species group comprising several cryptic species (Adachi 1991; Krug et al. 2013; Meyers-Muñoz et al. 2016). The kleptoplasts within a single individual of *P. ocellatus* are composed of the chloroplasts of multiple algal species (Maeda et al. 2012; Christa et al. 2013; Wade and Sherwood 2017 2018), and the sacoglossan retains kleptoplasts over several months, even in LSEs. Based on stable nitrogen isotope analysis results, the trophic position of this species in nature is a primary consumer (*i.e.*, algivore), suggesting that the sea slug continuously acquires kleptoplasts (Maeda et al. 2012). Additionally, the population density of *P. ocellatus* has been shown to decrease during cold (≤ 21°C) and hot (≥ 27°C) periods in the subtropical coral reefs in the Ryukyus (Tanamura and Hirose 2016a). Therefore, summer and winter are probably unfavorable seasons for this species, and the kleptoplasts probably differ in species composition and photosynthetic properties across seasons. This may suggest that the photosynthetic efficiency in the kleptoplasts depends on the season. For instance, Hayashi et al (2013) reported seasonal fluctuations of the maximum quantum yield (*QY*) in the symbiotic algae of a coral *Porites australiensis* in Okinawa. In contrast, the individuals act as producers after 5 months of starvation; therefore, the animal relies on the kleptoplasts to provide all the nutrients necessary for survival (Maeda et al. 2012). Therefore, the host animal can depend on the photosynthetically functional kleptoplasts during starvation periods. *Plakobranchus ocellatus* was estimated to survive for 11 months without food algae based on the relative decrease in *QY* in kleptoplast photosystem II in the animal tissue obtained from the pulse-amplitude-modulated fluorescence in an LSE for 2–3 months (Evertsen et al. 2007; Händeler et al. 2009; Wägele et al. 2011). This estimation hypothesized a proportional decrease in *QY*, although this hypothesis should be verified in a longer starvation experiment to better understand the longevity of kleptoplasts in *P. ocellatus.* In the present study, the effective quantum yield (*QY’*) in the field and *QY* at

20°C, 30°C, and 40°C in the laboratory were compared among the *P. ocellatus* individuals collected in spring, summer, and winter to reveal any seasonal differences in the photosynthetic properties of kleptoplasts. We also measured the change in weight and *QY* of the sacoglossans in LSEs for up to 5 months to verify the responses of the animals and kleptoplasts to starvation with and without illumination.

MATERIALS AND METHODS

Animals

Plakobranchus ocellatus was collected from the sandy back reef at Toguchi Beach, Okinawa-jima Island, Japan (26°21'55"N 127°44'10"E) from June 2017 to August 2018. The animals on the sandy bottom at 1 m deep or less were often covered with fine sand and a snorkel-diver gently collected the individuals of 0.9–1.1 g in wet weight by hand (Fig. 1A). Multiple types (or cryptic species) in the *P. ocellatus* species group are recognized in Okinawa (Trowbridge et al. 2011); however, the majority of individuals in the sandy back reef were 'var. F' *sensu* Meyers-Muñoz et al. (2016), and this type of *P. ocellatus* is also referred to as 'type 1' *sensu* Adachi (1991) and 'black-type' *sensu* Krug et al. (2013). We used only this type of individuals in the present study, following the identification based on the color/spot patterns in the field (Fig. 1B, C).

The water temperature at the sampling site varied from 17°C to 33°C. We measured the water temperature at each sampling time; we regarded the individuals collected in March–May (23–27°C) as the spring individuals, those in June–October (28–33°C) as the summer individuals, and those in December–February (17–22°C) as the winter individuals. Light intensity around noon was measured with a quantum meter (MQ-210, Apogee) on the sandy bottom of the sampling site.

Measuring photosynthetic quantum yield

We examined 98 individuals of *P. ocellatus* comprising 25 spring, 46 summer, and 27 winter individuals. We held each individual by hand, opened the parapodia, and gently placed an Aquapen sensor (AP-100, Photon System Instruments) on the postero-dorsal surface of the body where digestive glands containing kleptoplasts radially extend from the stomach (Hirose 2005). We measured the operating efficiency of photosystem II in the kleptoplasts as the *QY'* (or $\Delta F/F_m'$: the quantum efficiency of photosystem II electron transport in the light) for each individual at the sampling site without any dark adaptation; *QY'* values are reversibly down-regulated responding to environmental stress and usually smaller in more stressed chloroplasts under field conditions. In order to minimize the acute photoinhibition caused by the strong ambient light in the field condition, *QY'* was measured immediately after sand was removed from the individual. Next, the animals were brought into a dark room in the laboratory and each individual was placed into a cup containing 100 mL seawater collected from the individual's sampling site. The *QY* values at 20°C, 30°C, and 40°C were measured for each individual, following a 20-min acclimation in the incubators (VS-404, Versos) to the dark and temperature before each measurement. *QY* (or F_v/F_m; the maximum quantum yield of photosystem II (Demmig and Bjorkman 1987)) obtained as the relative value of the ground fluorescence

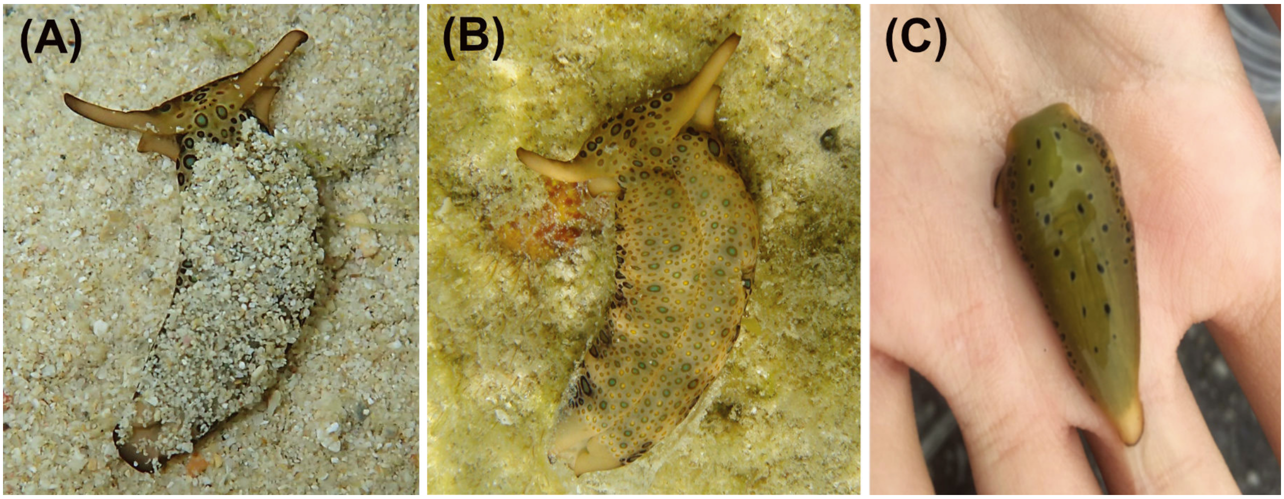

Fig. 1. Field photographs of *Plakobranchus ocellatus* at the sampling site (A, B) showing the color/spot patterns on the ventral side. The dorsal surface is often covered with fine sand (A). The body length is 4–5 cm.

and the maximum fluorescence after a saturating pulse, and the values are smaller in more damaged chloroplasts (Murchie and Lawson 2013). Although the damage and recovery processes of photosystem II occur simultaneously under light-induced stress, irreversible damage to photosystems may accumulate when the rate of damage to the photosynthetic apparatus exceeds the rate of recovery. Therefore, the decline in QY can be interpreted as an indicator for semi-chronic stress.

Post-hoc pairwise comparisons were conducted using Wilcoxon's rank sum test with an adjusted p-value (Holm's methods) in R version 3.5.1 (R Core Team 2018) and RStudio (RRID:SCR_000432). The QY' values were compared among seasons (spring, winter, and summer). In addition, the QY values were compared among seasons at each temperature (20°C, 30°C, and 40°C). Because the QY values of each individual at 20°C, 30°C, and 40°C were paired values, the paired QY values among temperature were compared for each season.

Long-starvation experiment with and without illumination

Nine individuals (ID i–ix) were collected in January 2019. The average and standard deviation of the wet weight for the nine individuals were 1.01 ± 0.054 g. Five and four individuals were reared in two different aquaria containing 600 mL of artificial seawater (Marine Art SF-1, Osaka Yakken) with gentle aeration. We identified and recognized the individual sea slugs from their color/spot patterns. The bottoms of the aquaria were covered with autoclaved sands collected from the sampling site. The aquarium with five individuals (i–v) was wrapped in plastic film and incubated at approximately 25°C under a 12-h day/12-h night cycle (50 µmol photons m^{-2} s^{-1} at the bottom of the aquaria) in an illuminated incubator (FLI-2000H, EYELA), *i.e.*, the illuminated LSE. The aquarium with four individuals (vi–ix) was wrapped with aluminum foil and incubated at approximately 25°C in total darkness in an incubator, *i.e.*, the dark LSE. The artificial seawater was exchanged at approximately 7-day intervals. The QY and wet weight values of each sea slug were measured at 4- to 16-day intervals. Measurements were performed in the dark during the night cycle period of the illuminated LSE. The seawater on the animal bodies was blotted with a paper towel and the weight of each individual was measured using an electronic balance (HF-400, A&D Co.). When the sea slugs showed no body-contraction during the weight measurement, the individuals were considered to be dead. The experiments were continued until all the individuals had died. The day of the last measurement was regarded as the duration of survival for each individual, and the duration was compared between illuminated LSE and dark LSE using a Student's t-test following the Shapiro-Wilk test of normality and F-test of equality of two variances in R.

We obtained the relative ratio of the measured values against the values at the beginning of the experiment as the relative ratio of the QY (relQY) and the relative ratio of body weight (rel$W$) as follows:

$$\mathrm{rel}QY = \frac{\text{Measured } QY}{QY \text{ at the beginning of the experiment}}$$

$$\mathrm{rel}W = \frac{\text{Measured wet weight}}{\text{wet weight at the beginning of the experiment}}$$

All the animals were alive on the 38th day and all the animals in the illuminated LSE were alive on the 127th day (see RESULTS). We calculated the decrease per day in rel$W$ and relQY for days 0–38 in dark and illuminated LSE and for days 54–127 in illuminated LSE. They were compared using the pairwise Wilcoxon's rank sum test with adjusted p-value (Holm's methods) in R. The data for the five individuals (i–v) were combined into a dataset of illuminated LSE and those of the four individuals (vi–ix) were combined into a dataset of dark LSE. Correlation between relQY and rel$W$ were tested using Spearman's rank correlation test in R.

RESULTS

Seasonal difference in photosynthetic properties of kleptoplasts

The maximum light intensities were 2630 µmol m^{-2} s^{-1} in spring, 3120 µmol m^{-2} s^{-1} in summer, and 1550 µmol m^{-2} s^{-1} in winter on the sandy bottom at the sampling site. The QY' values measured in the field were highest in winter, whereas the lowest QY' were measured in summer; the average QY' (± standard deviation) value was 0.403 (± 0.052) for the spring, 0.321 (± 0.069) for the summer, and 0.623 (± 0.110) for the winter individuals. The QY' values were significantly different among all pairwise combinations of the three seasons (pairwise comparisons using Wilcoxon's rank sum test, $p < 0.01$). The QY values measured after the dark adaptation were also different among the seasons and temperatures (Fig. 2). The QY values in the winter individuals were significantly higher than those of the spring and summer individuals in each temperature group (Fig. 2A). The QY values at 40°C were significantly lower than those at 20°C and 30°C in the winter and spring individuals, whereas no significant differences were found among the temperature groups in the summer individuals (Fig. 2B).

Time course of the body weights and *QY* in LSE with and without illumination

The durations of individual survival were significantly longer in the illuminated LSE (127–155 d) than the dark LSE (38–77 d) (Student's *t*-test, $p < 0.001$, $t = 7.1229$, *d.f.* = 5.2521). The two individuals (ID ii and v) that were alive on the 155th day in the illuminated LSE died before the last measurement on the 162nd day. At the beginning of the experiments, the body weights were 0.927–1.074 g and *QY* values were 0.64–0.74 for the nine individuals. The rel*W* values in the dark LSE gradually decreased from the beginning of the experiment (Fig. 3A). In the illuminated LSE, the decrease in rel*W* appeared slow or unclear during the first 2 months, after which point it decreased quickly (Fig. 3A). The time courses of rel*QY* in the illuminated LSE almost overlapped the time course in the dark LSE (Fig. 3C) and the decrease in rel*QY* appeared slow or unclear during the first 2 months. Then, the sea slugs

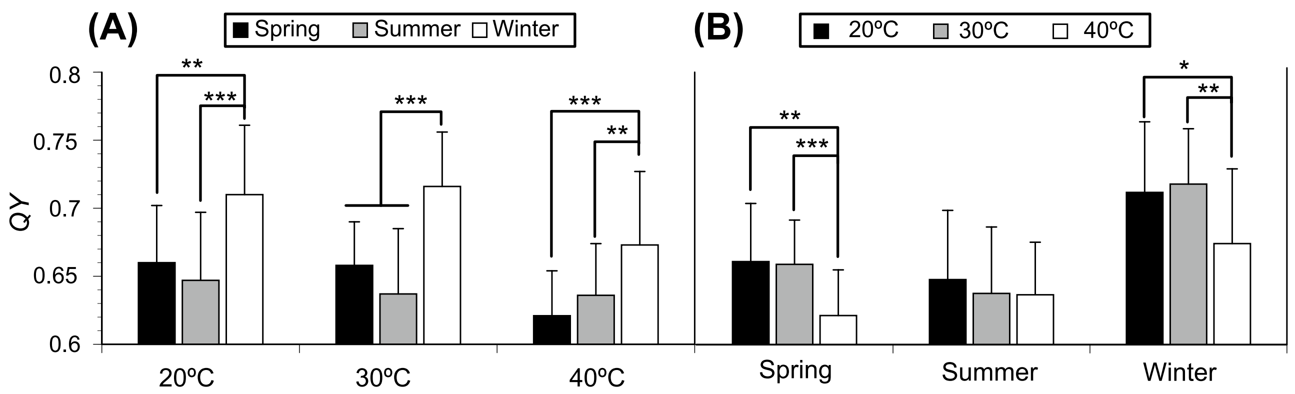

Fig. 2. Post-hoc comparisons of the maximum quantum yields (*QY*) among the seasons (spring, summer, and winter) for each temperature group (20°C, 30°C, and 40°C) (A) and post-hoc comparisons of the paired *QY* values among temperature groups for each season (B). Asterisks indicate levels of significant difference from the pairwise comparison of Wilcoxon's rank sum test with adjusted *p*-value (Holm's methods): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

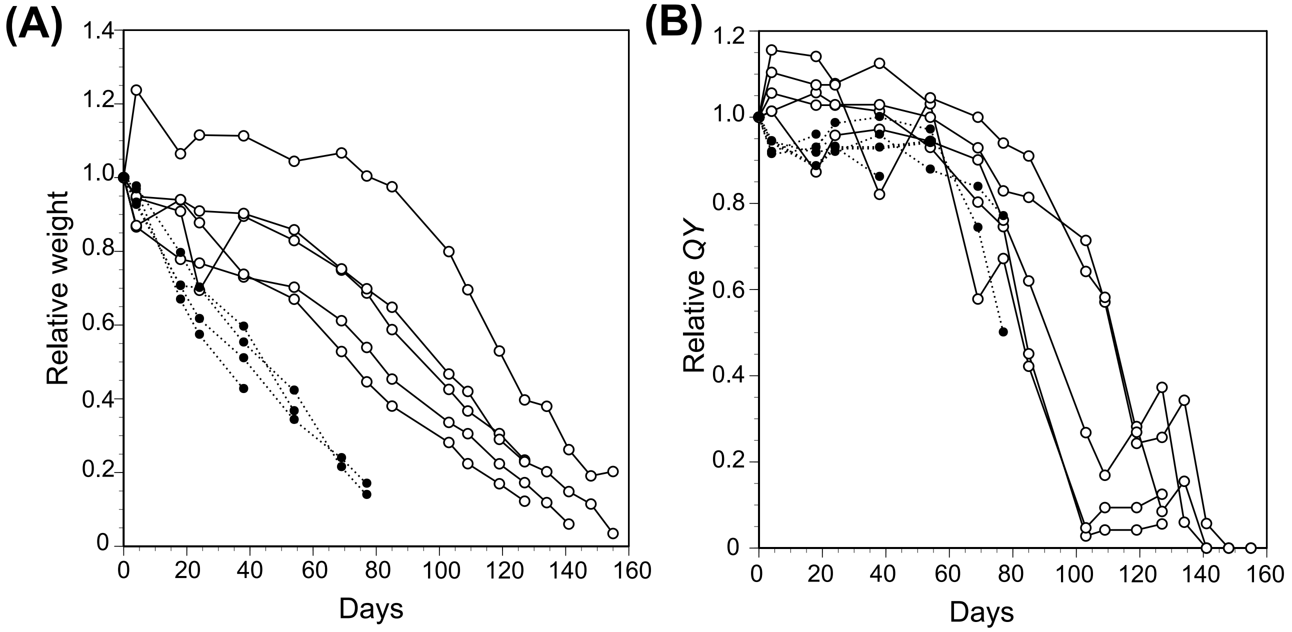

Fig. 3. Time course of the relative weight (rel*W*) of *P. ocellatus* individuals (A) and the relative maximum quantum yield (rel*QY*) of the kleptoplasts (B) in the long-starvation experiment (LSE) under illumination (open circle, solid line) and total dark (closed, dotted line). Each animal died during the period between the last plot and the next measurement day.

died in the dark LSE; the relQY values were 0.5 or higher for the last measurement (38–77 d). In contrast, the decrease in relQY became larger in the illuminated LSE after the first 2 months, and the relQY values were 0–0.125 at the last measurements (127–155 d). In the illuminated LSE, three of the five individuals survived for 7 days or more even after the relQY was zero. The digestive gland was green in all the individuals at the beginning of the experiment; however, it became transparent in the three individuals who had relQY values of zero (Fig. 4A and B). On the 103rd day, we found a greenish material at the bottom of the aquarium in the illuminated LSE, and its QY was 0.22 (Fig. 4C). We did not find similar material in the dark LSE.

Figure 5 shows the decrease per day in rel$W$ and relQY in the dark LSE during days 0–38 and in the illuminated LSE during days 0–38 and 54–127. The decrease per day in rel$W$ (Fig. 5A) was largest in the dark LSE during days 0–38 and the smallest in the illuminated LSE during days 0–38 (pairwise comparisons using Wilcoxon's rank sum test, $p < 0.05$). The decrease per day in relQY (Fig. 5B) was small and not significantly different between the dark and illuminated LSE during days 0–38, while that during days 54–127 was significantly larger (pairwise comparisons using Wilcoxon's rank sum test, $p < 0.05$).

Correlation between relQY and rel$W$ was significantly supported in the illuminated LSE (Spearman's rank correlation, $p < 0.001$, rho = 0.873554) (Fig. 6A). In the dark LSE, there was no support for a significant correlation between relQY and rel$W$ (Spearman's rank correlation, $p = 0.1027$, rho = 0.3489241) (Fig. 6B).

DISCUSSION

Kleptoplasts retained in the digestive gland of *P. ocellatus* were seasonally different in photosynthetic properties. In the field, the QY' values were significantly different among the seasons (winter > spring > summer), which is consistent with the light intensities measured in the field. Kleptoplasts were under greater stresses in stronger ambient light. In *P. ocellatus*, the parapodia were always closed, and fine sand grains often covered the body in the field (Fig. 1), which protect the sacoglossan body from severe solar radiation in the shallow back reef. Similarly, *P.* cf. *ianthobaptus* often burrows in the sand, probably to shade the kleptoplast from high irradiance (Dohahoo et al. 2020). In *Elysia* spp., the sea slug closes the parapodia under high irradiance to reduce light exposure to kleptoplasts (Cartaxana et al. 2018 2019). In the laboratory measurements, the QY values in winter were significantly higher than those in spring or summer for each temperature group. The kleptoplasts retained in the winter individuals were more functional in photosynthesis (or less damaged) than those in the spring and summer individuals. The QY values were significantly lower at 40°C than those at 20°C and

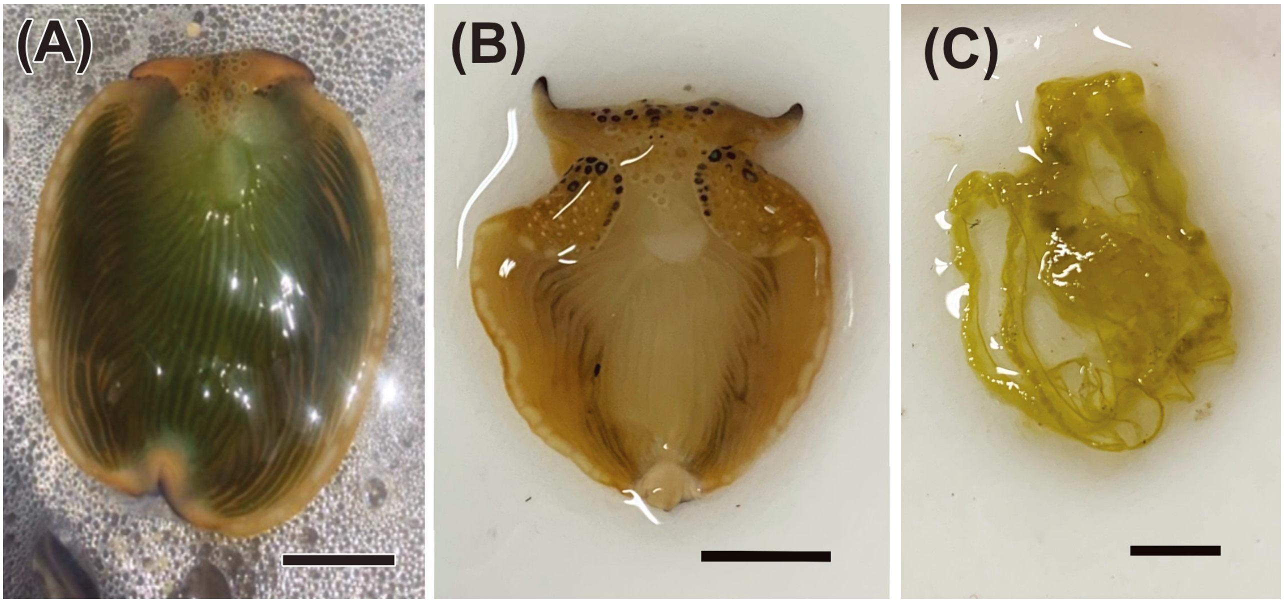

Fig. 4. Live individuals of *P. ocellatus* (A, B) and the greenish material during the long-starvation experiments (C). Parapodia of the sea slugs were forced open. An individual with green digestive glands before the starvation experiment (A). An individual with transparent digestive glands on the 141st day (B). The greenish material expected to contain excreted kleptoplasts (C). Scale bars: A = 10 mm, B and C = 5 mm.

30°C in the spring and winter individuals, whereas the differences among the temperatures were not supported in the summer individuals. 40°C is considerably higher than the natural water temperature regime at the sampling site, and it should thus induce thermal stress on the animals, but it is also the probable temperature for the potential food algae inhabiting the intertidal zone in summer. On the other hand, 20°C and 30°C are within the natural temperature range of the back reef in winter and summer, respectively. The kleptoplasts in summer may have a higher tolerance than those in spring and winter. The optimum irradiance for kleptoplasts retained

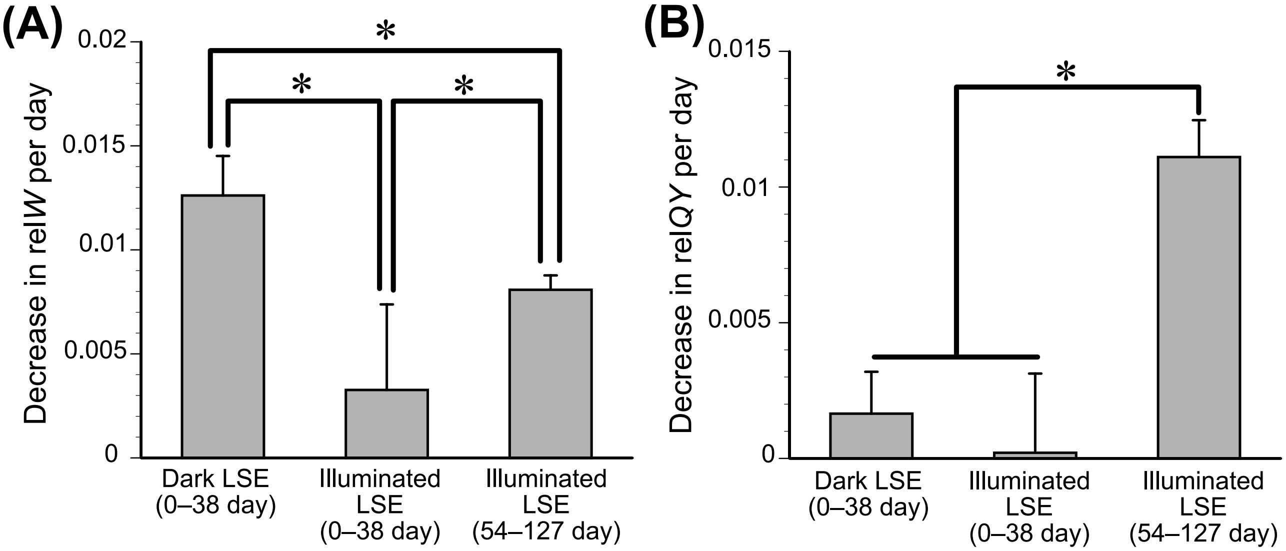

Fig. 5. The decrease per day in relative weight (rel*W*) and relative *QY* (rel *QY*) in the dark LSE during 0–38 day and in the illuminated LSE during 0–38 day and 54–127 day. Error bars indicate standard deviations. * $p < 0.05$ in the pairwise comparison of Wilcoxon's rank sum test with adjusted *p*-value (Holm's methods).

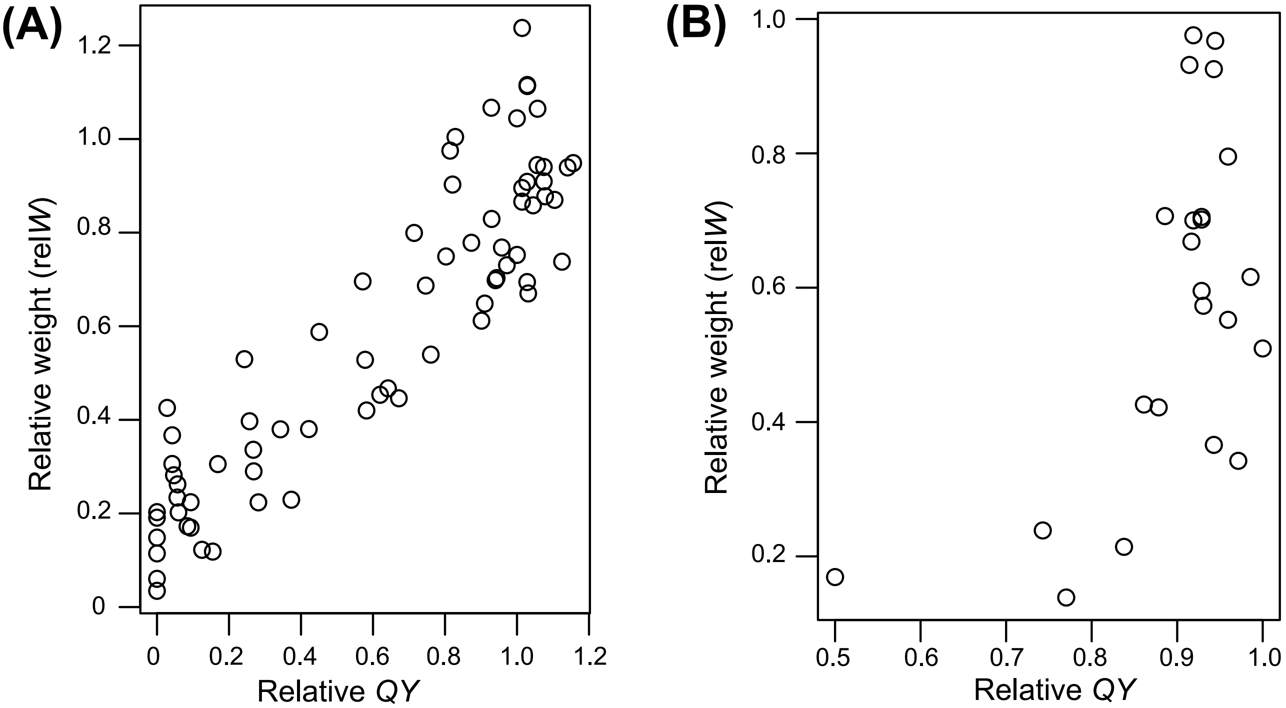

Fig. 6. Scatter plot of the relative maximum quantum yields (rel*QY*) against the relative weights (rel*W*) in the long-starvation experiment (LSE) with illumination (A) and total dark (B).

in *Plakobranchus* species probably differs among the species and habitats (Donahoo et al. 2020), suggesting a diversity of kleptoplasts in physiological properties. The diversity of kleptoplasts from various food algae is likely reflected in the diversity of the properties. Maeda et al. (2012) demonstrated that *P. ocellatus* in Okinawa retained the chloroplasts that had originated from multiple algal species and the trophic position of this sacoglossan was a primary consumer, suggesting continuous feeding in the field. There were also seasonal changes in the relative abundance of the kleptoplast species (Maeda et al. 2012). The chloroplasts in the food algae have different photosynthetic properties during different seasons, such as tolerance to strong light and high temperature, depending on the algal species. Brandley (1984) also reported seasonal differences in algal species of the kleptoplasts retained in *Elysia* sp. (cf. *E. furvacauda* Burn, 1958). Therefore, the kleptoplasts of *P. ocellatus* are constantly renewed by the ingested algae and potentially optimized for better performance responding to the dynamic shifts in the environment.

In the LSE, *P. ocellatus* survived for ~77 days in the total dark and ~155 days under illuminated conditions, supporting the theory that kleptoplasts contribute nutrients to photosynthesis. The time courses of rel*QY* showed that the stress of long-starvation had different effects on the sea slugs and kleptoplasts. The decrease in rel*QY* was small during the first 2 months in the LSE regardless of the illumination; therefore, the kleptoplasts were functional. Thereafter, the rel*QY* values gradually decreased to zero in the illuminated LSE, indicating that the proportion of malfunctional kleptoplasts gradually increased in the sea slug. A comparison to the decrease in rel*QY* per day support this scenario (Fig. 5B). In the LSE, the damaged kleptoplasts would not recover due to the shortage of essential molecules, such as chloroplast proteins, to maintain the functional kleptoplasts. Christa et al. (2018) reported that non-photochemical quenching does not protect kleptoplasts from photoinactivation in *E. timida* and *E. viridis*. However, the animals died before the values reached below 0.5 in the dark LSE, and thus the sea slugs died with functional kleptoplasts in the total dark. This is probably the reason why the correlation between rel*QY* and rel*W* was not supported in the dark LSE. The weights of the sea slugs linearly decreased from the beginning of the experiment in the dark LSE because the kleptoplasts did not supply nutrients by photosynthesis but might have consumed some nutrients for their maintenance. In contrast, the decrease in rel*W* per day in the illuminated LSE was significantly smaller than the decrease in the dark LSE (Fig. 5A). Therefore, photosynthesis in kleptoplasts could maintain the sea slug, although it might not allow for positive growth of the animal under the present experimental conditions. The decreases in both rel*QY* and rel*W* were small during the first 2 months in the illuminated LSE, after which point the rate of decrease became higher. The time course of rel*W* is consistent with the comparison of the decrease per day between days 0–38 and 54–127 (Fig. 5A). Therefore, the photosynthetic ability of kleptoplasts was important for the survival of the sacoglossans. This was supported by the significant correlation between rel*QY* and rel*W* in the illuminated LSE. The present results were consistent with the LSE for 21 days in *P. ocellatus* (Yamamoto et al. 2013) and 28 days in *E. timida* (Giménez Casalduero and Muniain 2008), *i.e.*, longer survival and slower weight loss in illuminated LSE than those in dark LSE.

In the present study, the survival period was ~5 months in the illuminated LSE; however, the survival period was estimated to be ~11 months in several other studies (Evertsen et al. 2007; Händeler et al. 2009; Wägele et al. 2011). It is difficult to simply compare the survival periods and time courses of rel*QY* and rel*W* with other studies because the survival and physiological response in LSE depends on the culture conditions of the organisms. For instance, the light intensity in the present study (50 µmol m^{-2} s^{-1} at the bottom of the aquaria) was much lower than that of the natural light condition (300 µmol m^{-2} s^{-1} at maximum: Evertsen et al. 2007; Wägele et al. 2011) or the illuminated condition (200 µmol m^{-2} s^{-1}: Christa et al. 2013); however, it was comparable to the illuminated conditions, *i.e.*, 40 and 25 µmol m^{-2} s^{-1} (Christa et al. 2013 2014), 28 µmol m^{-2} s^{-1} at the water surface (Yamamoto et al. 2013), and 30 µmol m^{-2} s^{-1} (Cartaxana et al. 2017). The survival for 11 months in the LSE was estimated from the linear decrease in *QY* in LSE for 2 months; however, the rate of decrease might change during a starvation period longer than 3 months. In the present study, the decrease in rel*QY* was slow or unclear during the first 2 months in the illuminated LSE and then became faster (Figs. 3B, 5B). This time course of *QY* is reasonable if the kleptoplasts are mostly functional for 1–2 months and then gradually malfunction because of the shortage of molecules essential for their maintenance. This may also indicate that a 2-month LSE is too short to recognize the real time course of *QY* of the kleptoplasts in *P. ocellatus*.

The digestive gland became transparent when the rel*QY* value was zero in the illuminated LSE (Fig. 4B). Although Christa et al. (2014) reported a similar result in a *P. ocellatus* individual in the dark LSE, we did not observe a transparent digestive gland among individuals in the dark LSE in the present study. The occurrence of a greenish material with some *QY* indicated that keleptoplasts were mulfunctioning in the illuminated

LSE and occasionally excreted from the sea slugs. It might also be possible that the sea slug digested malfunctioning kleptoplasts in the gland cell. The disintegration of kleptoplasts has been ultrastructurally described for *P. ocellatus* (Hirose 2005).

CONCLUSIONS

The annual change in water temperature in subtropical coral reefs is much greater than that in tropical coral reefs, and several sea slug species occur seasonally in the subtropical coral reefs in Okinawa, where water temperatures fluctuate approximately 15°C over the course of a year (Tanamura and Hirose 2016b 2017). However, *P. ocellatus* occurs year-round, with seasonal fluctuations in population density (Tanamura and Hirose 2016a). Kleptoplasty is supposed to alleviate the effects of seasonal changes in food availability (Marín and Ros 1992); therefore, the nutritional contributions from the kleptoplasts may be important for *P. ocellatus* to occur throughout the year. The increasing temperature shortened the longevity of kleptoplasts and the survival period of *E. timida* in LSE (Laetz and Wägele 2018) because the photosynthetic properties, such as tolerance to excessive light and high temperature, are different among the food algal species. The present study revealed seasonal difference in the photosynthetic properties of kleptoplasts, suggesting that their constitution is continuously optimized and maintained for the seasonal conditions. Seasonal fluctuations in environmental factors may cause temporal shortages of algal food species. The LSE in the present study showed that the kleptoplasts were mostly functional for 1–2 months or more, which would be enough time for *P. ocellatus* to survive the unfavorable period. Kleptoplasty in *P. ocellatus* maintained the occurrence of this sacoglossan throughout the year in a subtropical environment in which water temperatures change from lower than 20°C to higher than 30°C.

Acknowledgments: This study was partly supported by the Okinawa Research Core for Highly Innovative Discipline Science Project from University of the Ryukyus to TN and EH. We thank the anonymous reviewers for their valuable comments.

Authors' contributions: SC and EH designed the study and prepared the manuscript. SC performed the field study. SC and TN performed the measurements in the laboratory. All the authors gave approval for publication.

Competing interests: The authors declare that they have no competing interest.

Availability of data and materials: The dataset analyzed in this study is available from the corresponding author.

Consent for publication: Not applicable.

Ethics approval consent to participate: This work complies with the ethical guidelines of the University of the Ryukyus Animal Experimentation Committee.

REFERENCES

Adachi A. 1991. Morphological study on sacoglossan opisthobranch *Plakobranchus* spp. Dissertation (Master of Science), University of Ryukyus. (in Japanese)

Akimoto A, Hirano YM, Sakai A, Yusa Y. 2014. Relative importance and interactive effects of photosynthesis and food in two solar-powered sea slugs. Mar Biol **161:**1095–1102. doi:10.1007/s00227-014-2402-1.

Bhattacharya D, Pelletreau KN, Price DC, Sarver KE, Rumpho ME. 2013. Genome analysis of *Elysia chlorotica* egg DNA provides no evidence for horizontal gene transfer into the germ line of this kleptoplastic mollusc. Mol Biol Evol **30:**1843–1852. doi:10.1093/molbev/mst084.

Brandley BK. 1984. Aspects of the ecology and physiology of *Elysia* cf. *furvacauda* (Mollusca: Sacoglossa). Bull Mar Sci **34:**207–219.

Cai H, Li Q, Fang X, Li J, Curtis NE, Altenburger A, Shibata T, Feng M, Maeda T, Schwartz JA, Shigenobu S, Lundholm N, Nishiyama T, Yang H, Hasebe M, Li S, Pierce SK, Wang J. 2019. Data descriptor: A draft genome assembly of the solar-powered sea slug *Elysia chlorotica.* Sci Data **6:**1–13. doi:10.1038/sdata.2019.22.

Cartaxana P, Morelli L, Jesus B, Calado G, Calado R, Cruz S. 2019. The photon menace: Kleptoplast protection in the photosynthetic sea slug *Elysia timida*. J Exp Biol **222:**3–6. doi:10.1242/jeb.202580.

Cartaxana P, Morelli L, Quintaneiro C, Calado G, Calado R, Cruz S. 2018. Kleptoplasts photoacclimation state modulates the photobehaviour of the solar-powered sea slug *Elysia viridis*. J Exp Biol **221:**1–23. doi:10.1242/jeb.180463.

Cartaxana P, Trampe E, Kühl M, Cruz S. 2017. Kleptoplast photosynthesis is nutritionally relevant in the sea slug *Elysia viridis*. Sci Rep **7:**7714. doi:10.1038/s41598-017-08002-0.

Chan CX, Vaysberg P, Price DC, Pelletreau KN, Rumpho ME, Bhattacharya D. 2018. Active host response to algal symbionts in the sea slug *Elysia chlorotica*. Mol Biol Evol **35:**1706–1711. doi:10.1093/molbev/msy061.

Christa G, Händeler K, Kück P, Vleugels M, Franken J, Karmeinski D, Wägele H. 2015. Phylogenetic evidence for multiple independent origins of functional kleptoplasty in Sacoglossa (Heterobranchia, Gastropoda). Org Divers Evol **15:**23–36. doi:10.1007/s13127-014-0189-z.

Christa G, Pütz L, Sickinger C, Melo Clavijo J, Laetz EMJ, Greve C, Serôdio J. 2018. Photoprotective non-photochemical quenching does not prevent kleptoplasts from net photoinactivation. Front Ecol Evol **6:**1–11. doi:10.3389/fevo.2018.00121.

Christa G, Wescott L, Schäberle TF, König GM, Wägele H. 2013. What remains after 2 months of starvation? Analysis of sequestered algae in a photosynthetic slug, *Plakobranchus ocellatus* (Sacoglossa, Opisthobranchia), by barcoding. Planta **237:**559–72. doi:10.1007/s00425-012-1788-6.

Christa G, Zimorski V, Woehle C, Tielens AG, Wagele H, Martin WF, Gould SB. 2014. Plastid-bearing sea slugs fix CO_2 in the light but do not require photosynthesis to survive. Proc R Soc B Biol Sci **281:**20132493. doi:10.1098/rspb.2013.2493.

Clark KB, Jensen KR, Stirts HM. 1990. Survey for functional kleptoplasty among West Atlantic Ascoglossa (Sacoglossa) (Mollusca: Opisthobranchia). Veliger **33:**339–345.

Demmig B, Bjorkman O. 1987. Comparison of the effect of excessive light on chlorophyll fluorescence (77K) and photon yield of O_2 evolution in leaves of higher plants. Planta **171:**171–184. doi:10.1007/BF00391092.

de Vries J, Christa G, Gould SB. 2014. Plastid survival in the cytosol of animal cells. Trends Plant Sci **19:**347–350. doi:10.1016/j.tplants.2014.03.010.

Donohoo SA, Wade RM, Sherwood AR. 2020. Finding the sweet spot: Sub-ambient light increases fitness and kleptoplast survival in the sea slug *Plakobranchus* cf. *ianthobaptus* Gould, 1852. Biol Bull **238:**154–166. doi:10.1086/709371.

Evertsen J, Burghardt I, Wägele GJH. 2007. Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. Mar Biol **151:**2159–2166. doi:10.1007/s00227-007-0648-6.

Giménez Casalduero F, Muniain C. 2008. The role of kleptoplasts in the survival rates of *Elysia timida* (Risso, 1818): (Sacoglossa: Opisthobranchia) during periods of food shortage. J Exp Mar Bio Ecol **357:**181–187. doi:10.1016/j.jembe.2008.01.020.

Händeler K, Grzymbowski YP, Krug PJ, Wägele H. 2009. Functional chloroplasts in metazoan cells - a unique evolutionary strategy in animal life. Front Zool **6:**28. doi:10.1186/1742-9994-6-28.

Hayashi E, Suzuki A, Nakamura T, Iwase A, Ishimura T, Iguchi A, Sakai K, Okai T, Inoue M, Araoka D, Murayama S, Kawahata H. 2013. Growth-rate influences on coral climate proxies tested by a multiple colony culture experiment. Earth Planet Sci Lett **362:**198–206. doi:10.1016/j.epsl.2012.11.046.

Hirose E. 2005. Digestive system of the sacoglossan *Plakobranchus ocellatus* (Gastropoda: Opisthobranchia): light- and electron-microscopic observations with remarks on chloroplast retention. Zool Sci **22:**905–916. doi:10.2108/zsj.22.905.

Jensen KR. 1980. A review of sacoglossan diets, with comparative notes on radular and buccal anatomy. Malacol Rev **13:**55–77.

Krug PJ, Vendetti JE, Rodriguez AK, Retana JN, Hirano YM, Trowbridge CD. 2013. Integrative species delimitation in photosynthetic sea slugs reveals twenty candidate species in three nominal taxa studied for drug discovery, plastid symbiosis or biological control. Mol Phylogenet Evol **69:**1101–1119. doi:10.1016/j.ympev.2013.07.009.

Laetz EMJ, Wägele H. 2018. How does temperature affect functional kleptoplasty? Comparing populations of the solar-powered sister-species *Elysia timida* Risso, 1818 and *Elysia cornigera* Nuttall, 1989 (Gastropoda: Sacoglossa). Front Zool **15:**17. doi:10.1186/s12983-018-0264-y.

Maeda T, Hirose E, Chikaraishi Y, Kawato M, Takishita K, Yoshida T, Verbruggen H, Tanaka J, Shimamura S, Takaki Y, Tsuchiya M, Iwai K, Maruyama T. 2012. Algivore or phototroph? *Plakobranchus ocellatus* (Gastropoda) continuously acquires kleptoplasts and nutrition from multiple algal species in nature. PLoS ONE **7:**e42024. doi:10.1371/journal.pone.0042024.

Marín A, Ros JD. 1992. Dynamics of a peculiar plant-herbivore relationship: the photosynthetic ascoglossan *Elysia timida* and the chlorophycean *Acetabularia acetabulum*. Mar Biol **112:**677–682. doi:10.1007/BF00346186.

Meyers-Muñoz MA, van der Velde G, van der Meij SET, Stoffels BEMW, van Alen T, Tuti Y, Hoeksema BW. 2016. The phylogenetic position of a new species of *Plakobranchus* from West Papua, Indonesia (Mollusca, Opisthobranchia, Sacoglossa). Zookeys **594:**73–98. doi:10.3897/zookeys.594.5954.

Middlebrooks ML, Curtis NE, Pierce SK. 2019. Algal sources of sequestered chloroplasts in the sacoglossan sea slug *Elysia crispata* vary by location and ecotype. Biol Bull **236:**88–96. doi:10.1086/701732.

Mujer CV, Andrews DL, Manhart JR, Pierce SK, Rumpho ME. 1996. Chloroplast genes are expressed during intracellular symbiotic association of *Vaucheria litorea* plastids with the sea slug *Elysia chlorotica*. Proc Natl Acad Sci USA **93:**12333–12338. doi:10.1073/pnas.93.22.12333.

Murchie EH, Lawson T. 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J Exp Bot **64:**3983–3998. doi:10.1093/jxb/ert208.

Muscatine L, Pool RR, Trench RK. 1975. Symbiosis of algae and invertebrates: Aspects of the symbiont surface and the host-symbiont interface. Trans Amer Microsc Soc **94:**450–469. doi:10.2307/3225518.

Pelletreau KN, Bhattacharya D, Price DC, Worful JM, Moustafa A, Rumpho ME. 2011. Sea slug kleptoplasty and plastid maintenance in a metazoan. Plant Physiol **155:**1561–1565. doi:10.1104/pp.111.174078.

Pierce SK, Curtis NE, Middlebrooks ML. 2015. Sacoglossan sea slugs make routine use of photosynthesis by a variety of species-specific adaptations. Invertebr Biol **134:**103–115. doi:10.1111/ivb.12082.

R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rauch C, de Vries J, Rommel S, Rose LE, Woehle C, Christa G, Laetz EM, Wägele H, Tielens AGM, Nickelsen J, Schumann T, Jahns P, Gould SB. 2015. Why it is time to look beyond algal genes in photosynthetic slugs. Genome Biol Evol **7:**2602–2607. doi:10.1093/gbe/evv173.

Rauch C, Jahns P, Tielens AGM, Gould SB, Martin WF. 2017. On being the right size as an animal with plastids. Front Plant Sci **8:**1–8. doi:10.3389/fpls.2017.01402.

Rauch C, Tielens AGM, Serôdio J, Gould SB, Christa G. 2018. The ability to incorporate functional plastids by the sea slug *Elysia viridis* is governed by its food source. Mar Biol **165:**1–13. doi:10.1007/s00227-018-3329-8.

Rumpho ME, Worful JM, Lee J, Kannan K, Tyler MS, Bhattacharya D, Moustafa A, Manhart JR. 2008. Horizontal gene transfer of the algal nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica*. Proc Natl Acad Sci USA **105:**17867–17871. doi:10.1073/pnas.0804968105.

Tanamura D, Hirose E. 2016a. Population dynamics of the sea slug *Plakobranchus ocellatus* (Opisthobranch: Sacoglossa: Elysioidea) on a subtropical coral reef off Okinawa-jima Island, Ryukyu Archipelago, Japan. Zool Stud **55:**42. doi:10.6620/ZS.2016.55-43.

Tanamura D, Hirose E. 2016b. Seasonal fluctuation of opisthobranchs in the shallow reef lagoon at Zanpa (Okinawajima Island, Ryukyu Archipelago, Japan). Biol Mag Okinawa **54:**17–25. (in Japanese)

Tanamura D, Hirose E. 2017. Seasonal occurrence of gastropterids (Gastropoda: Cephalaspidea) and their habitat selection in a subtropical back-reef on Okinawajima Island (Ryukyu Archipelago, Japan). Zool Stud **56:**34. doi:10.6620/ZS.2017.56-34.

Trowbridge CD, Hirano YM, Hirano YJ. 2011. Inventory of

Japanese sacoglossan opisthobranchs: Historical review, current records, and unresolved issues. Am Malacol Bull **29:**1–22. doi:10.4003/006.029.0201.

Wade RM, Sherwood AR. 2017. Molecular determination of kleptoplast origins from the sea slug *Plakobranchus ocellatus* (Sacoglossa, Gastropoda) reveals cryptic bryopsidalean (Chlorophyta) diversity in the Hawaiian Islands. J Phycol **53:**467–475. doi:10.1111/jpy.12503.

Wade RM, Sherwood AR. 2018. Updating *Plakobranchus* cf. *ianthobapsus* (Gastropoda, Sacoglossa) host use: Diverse algal-animal interactions revealed by NGS with implications for invasive species management. Mol Phylogenet Evol **128:**172–181. doi:10.1016/j.ympev.2018.07.010.

Wägele H, Deusch O, Handeler K, Martin R, Schmitt V, Christa G, Pinzger B, Gould SB, Dagan T, Klussmann-Kolb A, Martin W. 2011. Transcriptomic Evidence that longevity of acquired plastids in the photosynthetic slugs *Elysia timida* and *Plakobranchus ocellatus* does not entail lateral transfer of algal nuclear genes. Mol Biol Evol **28:**699–706. doi:10.1093/molbev/msq239.

Williams SI, Walker DI. 1999. Mesoherbivore-macroalgal interactions: Feeding ecology of sacoglossan sea slugs (Mollusca, Opisthobranchia) and their effects on their food algae. Ocean Mar Biol Annu Rev **37:**87–128.

Yamamoto S, Hirano YM, Hirano YJ, Trowbridge CD, Akimoto A, Sakai A, Yusa Y. 2013. Effects of photosynthesis on the survival and weight retention of two kleptoplastic sacoglossan opisthobranchs. J Mar Biol Assoc United Kingdom **93:**209–215. doi:10.1017/S0025315412000628.