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

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Plakobranchus ocellatus is a sacoglossan sea slug that feeds on multiple algal species and retains chloroplasts as kleptoplasts for several months. The seasonal difference in photosynthetic properties of kleptoplasts was examined in sacoglossans collected from a subtropical back reef off Okinawa-jima (26°21′55″ N 127°44′10″ E) in 2017–2018. The effective quantum yield of photosystem II of kleptoplasts indicated greater stress on kleptoplasts under stronger ambient light. The maximum quantum yield (QY) at 20°C, 30°C, and 40°C indicated that kleptoplasts were more functional in photosynthesis in winter than in spring and summer, whereas kleptoplasts in summer may have a greater tolerance to high temperature. 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 and then relQY gradually decreased to zero. In the field, *P. ocellatus* continuously uptakes new kleptoplasts that have suitable properties of photosynthetic ability for each season. In a subtropical environment, where 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 occurrence of this sacoglossan in a subtropical environment throughout the year.

BACKGROUND

Sea slugs of 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, which are termed kleptoplasts (Muscatine et al. 1975, Williams and Walker 1999, Pierce et al. 2015). The duration of the retention of these chloroplasts varies among species from 1 day to several months (Clark et al. 1990). In long-term retention species, the animal can survive months without any food under light, suggesting that the kleptoplasts provide enough nutrients for survival of the sea slug. 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 the contribution of kleptoplasts to photosynthesis for the survival of the hosts in LSE. Moreover, Donahoo et al. (2020) pointed the importance of light intensity in LSEs; moderate light conditions were better than both near-dark and field light conditions to reduce 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 decrease of 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, doubted 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 long-term retention of kleptoplasts poses the question as to why 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 the sea slug (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 the 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. 2019).

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 in 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 in nature of this species 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 in cold ($\leq 21°C$) and hot ($\geq 27°C$) 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 would be seasonally different in species composition and photosynthetic properties. This may suggest that the photosynthetic efficiency in the kleptoplasts is seasonally different. For instance, Hayashi et al (2013) reported the seasonal fluctuation of the maximum quantum yield (*QY*) in the symbiotic algae of a coral, *Porites australiensis* in Okinawa. In contrast, the trophic position of the individuals after 5 months of starvation is a producer; therefore, the animal relies on the kleptoplasts to provide their entire nutrients 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 on the basis of the relative decrease in *QY* in

photosystem II of the kleptoplasts in the animal tissue obtained from the pulse-amplitude-modulated fluorescence in a LSE for 2–3 months (Evertsen et al. 2007; Händeler et al. 2009; Wägele et al. 2011). This estimation hypothesized the proportional decrease in *QY*, although this hypothesis should be verified in a longer starvation experiment for better understanding of 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 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) of *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 assigned 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, with the individuals collected in March–May (23–27°C) regarded as the spring individuals, those in June–October (28–33°C) the summer individuals, and those in December–February (17–22°C) the winter individuals. Light intensity around noon was measured with a quantum meter (MQ-210, Apogee) on the sandy bottom at the sampling site.
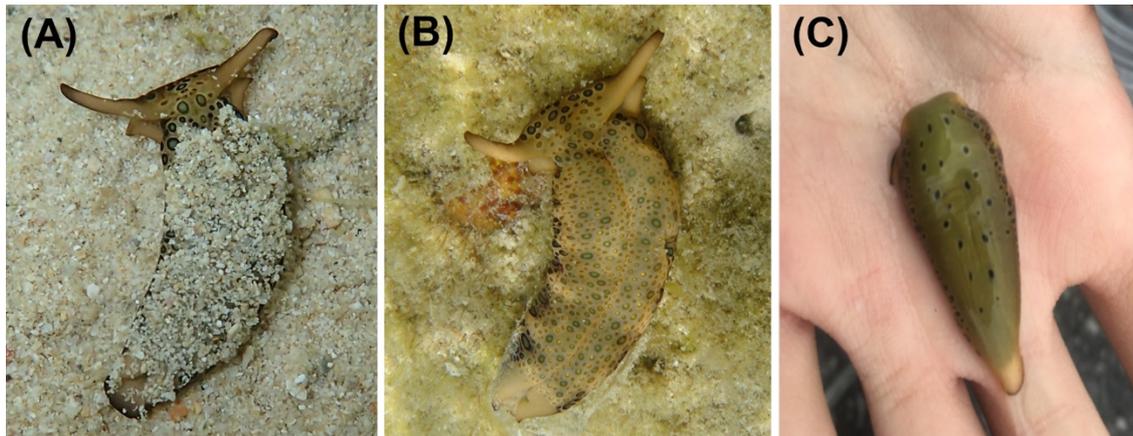
Fig 1. Field photograph of *Plakobranchus ocellatus* at the sampling site (A, B) and the color/spot patterns on the ventral side. The dorsal surface is often covered with fine sand (A). The body length is 4-5 cm.

Measurement of the photosynthetic quantum yield

We examined 98 individuals of *P. ocellatus* that comprised 25 spring, 46 summer, and 27 winter individuals. We held each individual by hand, opened the parapodia, and gently placed the 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 the removal of sand from the individual. Next, the animals were brought to a dark room in the laboratory and each individual was placed in a cup containing 100 mL seawater collected at the sampling site of the animals. 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 and the maximum fluorescence after a saturating pulse, and the values are smaller in more damaged chloroplasts (Murchie and Lawson 2013). Although damaging 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 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 at 20°C, 30°C, and 40°C of each individual were paired values, the paired QY values among temperature were compared in 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 in 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 with a 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 regarded 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 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:

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

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

The all animals were alive on the 38th day and the all animals in the illuminated LSE were alive on the 127th day (see Results). We obtained the decrease per day in rel$W$ and relQY during 0–38 day in dark and illuminated LSE and during 54–127 day 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 of the five individuals (i–v) were combined as the dataset of illuminated LSE and those of the four individuals (vi–ix) were combined as the 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' ($\pm$ standard deviation) value was 0.403 ($\pm$ 0.052) for the spring, 0.321 ($\pm$ 0.069) for the summer, and 0.623 ($\pm$ 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 significant differences were not found among the temperature groups in the summer individuals (Fig. 2B).
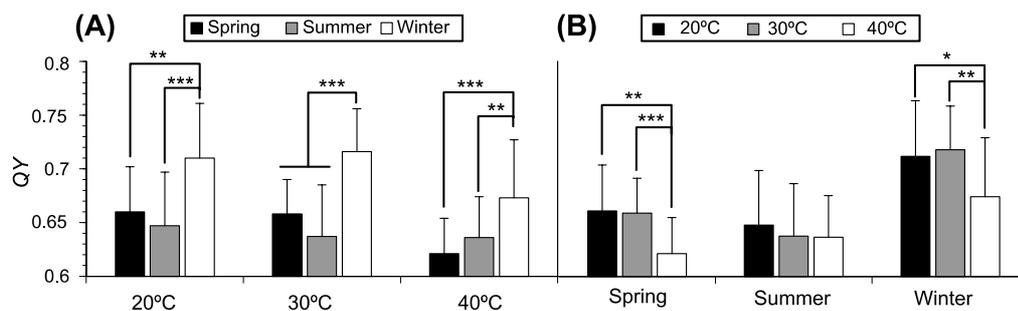
Fig. 2. Post-hoc comparisons of the maximum quantum yields (*QY*) among the seasons (spring, summer, and winter) at each temperature group (20°C, 30°C, and 40°C) (A) and post-hoc comparisons of the paired *QY* values among temperature groups in each season (B). Asterisks indicate the significant differences supported by pairwise comparison of Wilcoxon's rank sum test with adjusted *p*-value (Holm's methods): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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 those in the dark LSE (38–77 d) (Student's *t*-test, $p < 0.001$, $t = 7.1229$, df = 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* were 0.64–0.74 in 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 and then rel*W* decreased faster (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 died in the dark LSE; the rel*QY* values were 0.5 or higher at the last measurement (38–77 d). In contrast, the decrease in rel*QY* became larger in the illuminated LSE after the first 2 months, and the rel*QY* 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 rel*QY* 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 rel*QY* values of zero (Fig. 4A and B). On the 103rd day, we found a greenish material on the bottom of the aquarium in the illuminated LSE and its *QY* was 0.22 (Fig. 4C). We did not find similar ones in the dark LSE.

Figure 5 shows the decrease per day in rel*W* and rel*QY* in the dark LSE during 0–38 day and in the illuminate LSE during 0-38 day and 54–127 day. The decrease per day in rel*W* (Fig. 5A) was the largest in the dark LSE during 0-38 day and the smallest in the illuminated LSE during 0-38

day (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 0-38 day, while that during 54–127 day 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, a significant
correlation was not supported between relQY and rel$W$ (Spearman's rank correlation, $p = 0.1027$,
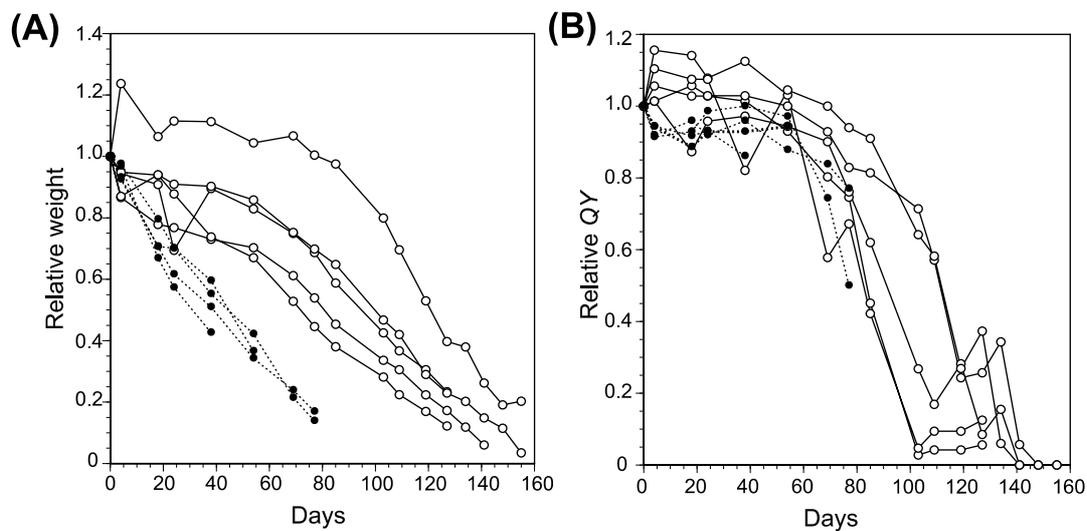rho = 0.3489241) (Fig. 6B).

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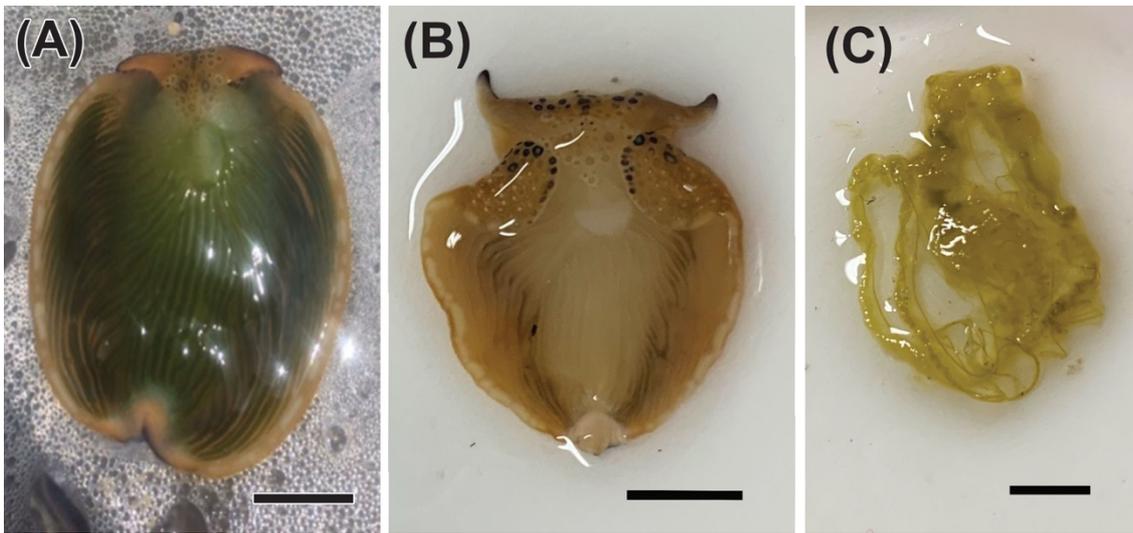the period between the last plot and the next measurement day.

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: 10 mm in A, 5 mm in B and C.
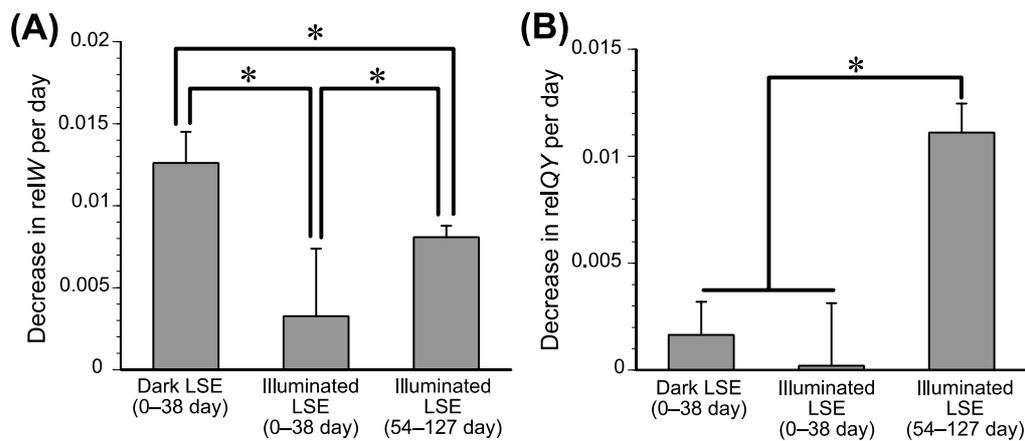

Fig. 5. The decrease per day in relative weight (rel*W*) and relative *QY* (rel *QY*) in the dark LSE during 0–38 day and in the illuminate LSE during 0-38 day and 54–127 day. Error bars indicate standard deviations. *, $p < 0.05$ in the pairwise comparison of Wilcoxon's rank sum test with adjusted *p*-value (Holm's methods).
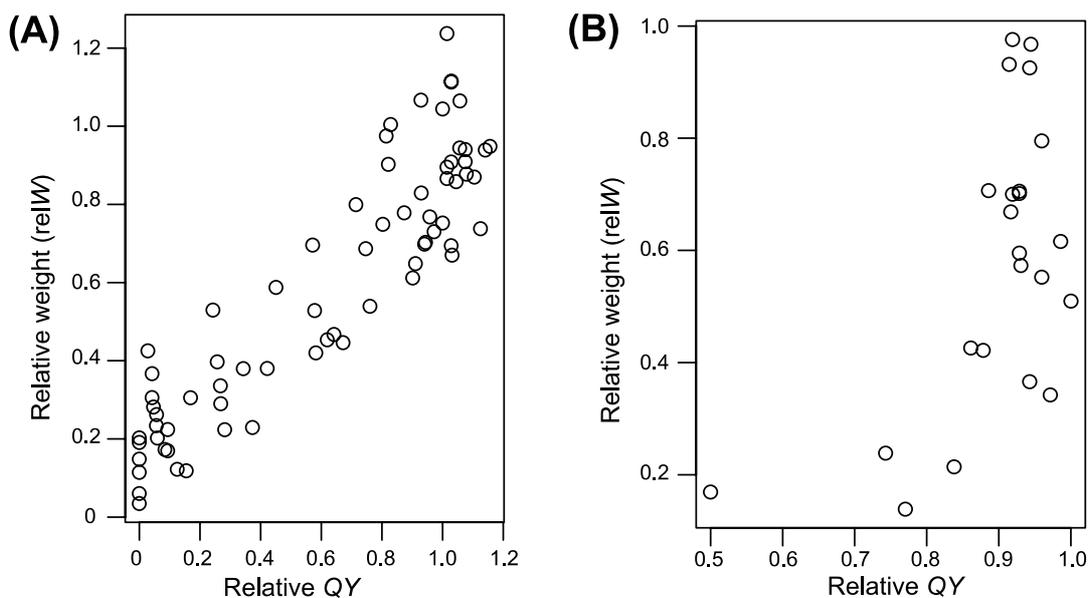
Fig. 6. Scatter plot of the relative maximum quantum yields (rel*QY*) against the relative weights (rel*W*) in the long-starvation experiment (LSE) with illumination (A) and total dark (B).

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 as winter > spring > summer, which was consistent with the light intensities measured in the field. Kleptoplasts are 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 the severe solar radiation in the shallow back reef. Similarly, *P.* cf. *ianthobaptus* often burrows 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 for reduction of 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 and summer in 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 30°C in the spring and winter individuals, whereas the differences among the temperatures were not supported in the summer individuals. Considering the natural water temperature regime at the sampling site, 40°C should be an extraordinarily high temperature condition inducing thermal stress for the animals but 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 in 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 kleptoplast retained in *Plakobranchus* species is probably different among the species and habitats (Donahoo et al. 2020), suggesting the diversity of kleptoplasts in physiological properties. The diversity of kleptoplasts originated 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. They also showed the seasonal change in the relative abundance of the kleptoplast species (Maeda et al., 2012). The chloroplasts in the food algae would be seasonally different in photosynthetic properties, such as tolerance to strong light and high temperature, depending on the algal species. Brandley (1984) also reported seasonal difference 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 shift of the environment.

In the LSE, *P. ocellatus* survived for ~77 days in the total dark and ~155 days under illuminated conditions, supporting the nutritional contribution of kleptoplasts to photosynthesis. The time courses of relQY showed that the stress of long-starvation had different effects on the sea slugs and kleptoplasts. The decrease in relQY was small during the first 2 months in the LSE regardless of the illumination; therefore, the kleptoplasts were functional. Thereafter, the relQY values gradually decreased to zero in the illuminated LSE, indicating that the proportion of malfunctional kleptoplasts gradually increased in the sea slug. The comparison of the decrease in relQY 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 relQY 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 decrease in both relQY and rel$W$ was small during the first 2 months in the illuminated LSE and

then both values decreased faster. The time course of rel$W$ is consistent with the comparison of the decrease per day between the period of 0-38 day and 54-127 day (Fig. 5A). Therefore, the photosynthetic ability of kleptoplasts was important for the survival of the sacoglossans. This was supported by the significant correlation between relQY 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 that 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 relQY 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 decrease rate might have changed during a longer starvation period than 3 months. In the present study, the decrease in relQY was slow or unclear during the first 2 months in the illuminated LSE and then became faster (Fig. 3B, 5B). This time course of QY is reasonable if the kleptoplasts can be mostly functional for 1–2 months and then gradually malfunction because of the shortage of essential molecules 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 relQY 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 the excretion of malfunctioning kleptoplasts in the illuminated LSE. 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 of 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 of food availability (Marín and Ros 1992); therefore, the nutritional contribution 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 the seasonal difference in the photosynthetic properties of kleptoplasts, suggesting that their constitution would be continuously optimized and maintained for the seasonal conditions. Seasonal fluctuations of environmental factors may cause the temporal shortage 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 where water temperatures changed from lower than 20°C to higher than 30°C.

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