

Phototaxis of sacoglossan sea slugs with different photosynthetic abilities: a test of the ‘crawling leaves’ hypothesis

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Abstract Several sacoglossan sea slugs utilise chloroplasts ingested from algae for photosynthesis (kleptoplasty), a unique trophic strategy unknown in other animals. Its adaptive significance, especially the behavioural adaptations involved in this phenomenon, has not been fully explored. To address this issue, the effects of kleptoplasty on phototaxis were investigated, both across and within species, using sacoglossans collected along Japanese coasts in 2012 and 2013. First, the presence of phototaxis and preferred light intensity was studied in five sacoglossans with various photosynthetic capabilities using an I-maze with a light gradient (4–330 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Each individual was allowed to move for 30 min to choose the optimal light intensity. *Elysia hamatanii*, *E. trisinuata*, and *Plakobranthus ocellatus*, all with high photosynthetic activity (i.e. kleptoplastic), showed positive phototaxis. Among them, *E. hamatanii* preferred the highest light intensity followed by *P. ocellatus* and *E. trisinuata*, and the order corresponded with the shallowness of their habitats. Conversely, *Stiliger ornatus* and *Placida* sp., with virtually no photosynthetic activity (non-kleptoplastic), showed neutral and negative phototaxis, respectively. Next,

the phototaxis of *E. hamatanii* individuals with (fed) and without (starved) functional chloroplasts was compared to examine the effects of the presence of kleptoplasts on phototaxis within a species. Both fed and starved individuals showed positive phototaxis, but the preferred light intensity of starved individuals was lower than that of fed individuals. These results suggest that sacoglossans with functional chloroplasts exhibit positive phototaxis towards a preferred light intensity which may benefit photosynthesis efficiency.

Introduction

Sacoglossans (Mollusca: Opisthobranchia) are unique in the animal kingdom in that several members are known to incorporate chloroplasts from food algae into their digestive cells for photosynthesis (termed kleptoplasty; Clark et al. 1990; Evertsen et al. 2007). Many cytological, physiological, and genetic studies have been conducted to elucidate the mechanism that facilitates this unique trophic strategy (e.g. Evertsen et al. 2007; Rumpho et al. 2008; Schwartz et al. 2010; Wägele et al. 2011; Bhattacharya et al. 2013). However, its adaptive significance such as survival or growth increments has been less well addressed (Hinde and Smith 1975; Casalduero and Muniain 2008; Yamamoto et al. 2013; Akimoto et al. 2014). In particular, few studies have examined the behavioural adaptations that result from the acquisition of photosynthetic ability in sacoglossans (Cruz et al. 2013).

One behaviour that is clearly adaptive for photosynthetic animals is phototaxis. As with other animals with symbiotic algae (e.g. acoelomorphan; Serôdio et al. 2011), several kleptoplastic sacoglossans (*Elysia viridis*, *E. tuca*, *E. timida*, *E. crispata*, *Costasiella lilianae*, and *Thuridilla hopei*) have been observed to show positive phototaxis (Gallop

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et al. 1980; Weaver and Clark 1981; Schmitt and Wägele 2011), whereas sacoglossans without kleptoplasty (*Oxynoe antillarum* and *Berthelinia caribbea*) show negative phototaxis (Weaver and Clark 1981). Thus, kleptoplastic sacoglossans may be regarded as ‘crawling leaves’ that move towards light (Trench 1974; Cruz et al. 2013). However, in these previous studies, several individual sacoglossans were simultaneously tested in the same container, so the data cannot be considered statistically independent. This is especially important for sea slugs because at least some are known to gather by following the waterborne pheromones or mucus trails of conspecifics (e.g. Leonard and Lukowiak 1985; Cummins et al. 2004). In addition, there has been no study of the effect of starvation (i.e. deprivation of functional chloroplasts) on phototaxis in individual sacoglossans, although juveniles of *Elysia timida* show positive phototaxis prior to obtaining functional chloroplasts from their food (Schmitt and Wägele 2011). Furthermore, the current knowledge of photobehaviour is limited to only eight or so species (Gallop et al. 1980; Weaver and Clark 1981; Schmitt and Wägele 2011, Cruz et al. 2013), and phototaxis in other sacoglossans is unknown. Therefore, further studies are needed that statistically show the presence of phototaxis in sacoglossans, particularly in species that have not yet been investigated.

In this study, we addressed three questions relevant to the ‘crawling leaves’ hypothesis using five species commonly found in the Indo-Pacific that were previously untested for phototaxis. First, do kleptoplastic sacoglossans show positive phototaxis whereas non-kleptoplastic sacoglossans do not? Second, do kleptoplastic sacoglossans choose optimal light intensities according to their ability to photosynthesize? Third, does presence/absence of functional chloroplasts affect phototaxis or preferred light intensities even within a species? In the first experiment, we addressed the first and the second questions by investigating the presence

of phototaxis and light intensity preferences in three photosynthetic sacoglossans (*Elysia hamatanii*, *E. trisinuata*, and *Plakobranthus ocellatus* ‘black type’ sensu Krug et al. 2013; all in the superfamily Plakobranchoidea) and two sacoglossans with little or no photosynthetic ability (*Stiliger ornatus* and *Placida* sp. sensu Trowbridge et al. 2008; both in Limapontioidea). In the second experiment, we addressed the third question by comparing phototaxis in fed and starved *E. hamatanii*.

Materials and methods

Field collection and maintenance

Sacoglossans were either collected in Shirahama, Wakayama Pref., or Ginowan, Okinawa Pref., in Japan during the season when sacoglossans are most abundant (May to September) (Table 1). Study individuals were collected from upper tidal pools (for *E. hamatanii*) or in the shallow subtidal zone (<1 m deep for *P. ocellatus* and <2 m deep for *E. trisinuata* and the remaining species, relative to the mid-tide level). Their food algae were also collected whenever found.

Sacoglossans were brought to Nara Women’s University soon after collection. They were maintained in groups at 25 °C under fluorescent light (Clearlight CL601; Gex, Osaka) illuminated at 23 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (12-h light:12-h dark) in either 700 mL (for the small sacoglossan *E. hamatanii*) or 21 L (for the other species) of artificial seawater (Acute Premium Salt; Gex, Osaka). They were maintained with the algae on which they were found in the field (*Cladophoraceae* sp. for *E. hamatanii*; a mixture of *Codium fragile*, *C. subtubulosum*, and *C. cylindricum* for *E. trisinuata*, *S. ornatus*, and *Placida* sp.) until the experiment (within 15 day after collection). *P. ocellatus* was not fed, as

Table 1 Sampling sites of five sacoglossan species

Species	Sampling site	Latitude and longitude	Microhabitat	Date
<i>Elysia hamatanii</i>	Shirahama, Wakayama	33° 41’N; 135° 20–22’E	Tide pools in upper intertidal zone	2012/5/20 ^a 2012/9/21 ^b 2013/8/8 ^c
<i>Plakobranthus ocellatus</i>	Toguchi, Ginowan, Okinawa	26° 16’N; 127° 43’E	Sand bottom <1 m deep	2012/8/4
<i>Elysia trisinuata</i>	Shirahama, Wakayama	33° 41’N; 135° 20–22’E	On the <i>Codium</i> spp. <2 m deep	2012/8/19
<i>Stiliger ornatus</i>	Shirahama, Wakayama	33° 41’N; 135° 20–22’E	On the <i>Codium</i> spp. <2 m deep	2012/8/19
<i>Placida</i> sp.	Shirahama, Wakayama	33° 41’N; 135° 20–22’E	On the <i>Codium</i> spp. < 2 m deep	2012/5/28 ^d 2012/8/19 ^d

^a Individuals used in the phototaxis experiment

^b Those used for the first starvation experiment

^c Those used for the second starvation experiment

^d Four individuals collected in May and eight in August

we could not find their food algae at the sampling site (cf., Yamamoto et al. 2013). However, *P. ocellatus* is among the species that retain chloroplasts the longest (months: Evertsen et al. 2007; Yamamoto et al. 2013), and study individuals remained photosynthetically active throughout the experiment.

PAM fluorometry

The photosynthetic activities of sacoglossans and their food algae were examined by pulse-amplitude-modulated (PAM) fluorometry using MINI-PAM (Heinz Walz GmbH, Effeltrich, Germany). On each measurement occasion, an individual sacoglossan (dark-adapted for 30 min) was placed on a Petri dish (85 mm in diameter) without sea water, and a cover glass was gently placed on the sacoglossan to restrict the animal's movement. Then, the PAM probe (5 mm diameter) was set 8 mm above the cover glass, and the maximum quantum yield of Photosystem (PS) II ($=F_v/F_m$) was measured (Schreiber et al. 1995; Evertsen et al. 2007; Vieira et al. 2009). In the study of sacoglossans, F_v/F_m values >0.5 are generally regarded as indicating photosynthetic competence (Evertsen et al. 2007; Händeler et al. 2009; Yamamoto et al. 2009; Akimoto et al. 2014). Subsequently, the samples were dark-adapted again for at least 30 min, and the photosynthesis versus irradiance curves were obtained by subjecting each sample to various light intensities (0, 24, 38, 55, 81, 122, 183, 262, 367 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in this order at 1-min intervals) to estimate the relative electron transport rates (rETR) (Vieira et al. 2009). The rETR was calculated as $\Delta F/F_m'$ multiplied by E , where $\Delta F/F_m'$ is the effective quantum yield of PSII and E is the exposure irradiance (Vieira et al. 2009; Cruz et al. 2013). The photosynthetic activity of the food algae was also examined as described above, but cover glasses were not used. Relatively short (1 min) intervals for light acclimation were chosen to avoid excessive damage to the samples during measurements. Because of the insufficient light acclimation, the rETR values obtained here may be underestimations, but should be still informative in estimating light response characteristics of the photosynthetic activities, especially in the high-light zones where light activation should have been completed.

Phototaxis experiment

The presence of positive or negative phototaxis and preferred light intensity were determined for each sacoglossan species. An I-shaped acrylic container ($55 \times 5 \times 5$ cm) with 800 mL of artificial sea water was placed in the dark and maintained at 25 °C. An individual sacoglossan was then introduced to the middle of a maze

(light intensity: 28 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) with its head facing away from either end of the I-maze. A white LED lamp (LED desk light; Plata Inc., Osaka) was used to illuminate vertically one end of the maze to produce a light gradient of 4–330 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (measured at the water surface using the illuminometer LI-250A; LICOR, Nebraska, USA) over the 55-cm distance. A small electric fan (NPM-1081U; Doshisya, Tokyo) was used to diffuse the heat, so there was no significant difference in water temperature between both ends of the maze (Wilcoxon signed-rank test, $N = 9$ pairs, $T = 15$, $P = 0.43$). The position of the sacoglossan (distance from the illuminated end of the I-maze) and the light intensity at its location were recorded 30 min after the beginning of the experiment (i.e. the preferred light intensity). Seventeen *E. hamatanii* individuals were used along with 20 *P. ocellatus*, 12 *E. trisinuata*, 6 *S. ornatus*, and 12 *Placida* sp. Each individual was tested only once, and the I-maze was washed thoroughly and the water was changed before each trial.

Effect of starvation on phototaxis

The presence of phototaxis and preferred light intensity were determined for starved (experimental) and fed (control) individuals. Only *E. hamatanii* was used for this experiment because this species exhibited a strong preference for light (see the 'Results' section), and its preferred alga (Cladophoraceae sp.) was easy to maintain in the aquarium. During the first trial conducted in September and October 2012, 15 randomly selected individuals of *E. hamatanii* were maintained without food (starved), and seven individuals were maintained with Cladophoraceae sp. (control). Animals of both treatment groups were individually kept in 500 mL of artificial sea water at 25 °C with a light intensity of 23 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. More individuals were used for the starved group because higher mortality was expected (Akimoto et al. 2014). During maintenance, the F_v/F_m of each individual was monitored every 4–7 day. Once the average value decreased below 0.2 in the starved group (after 21 day), the presence of phototaxis and preferred light intensity were determined for both the starved and control individuals as in the previous experiment.

However, it is possible that the starved individuals were less active than the fed individuals due to their shortage of energy, and this might have resulted in smaller distances of moving towards the light source. Thus, we performed another experiment in September 2013 in which the animals were placed at the lightest end of the maze ($N = 11$ starved and 7 control *E. hamatanii* individuals, respectively). In addition to the position after 30 min, the distance

that each individual moved was followed for the first 5 min by visually tracking its trail using grid (1 mm) paper placed beneath the maze.

Statistical analysis

Whether each group of sacoglossans (species or individual *E. hamatanii* subjected to different food conditions) has positive or negative phototaxis was judged by comparing the numbers of individuals that moved towards the lighter or darker side of the I-maze using a binomial test against 1:1. The preferred light intensity, averaged for all the individuals for each species in each experiment, was compared using a Kruskal–Wallis test with a Bonferroni correction or a Mann–Whitney *U* test between individuals under two food conditions. All tests were two-tailed and conducted with JMP version 9.0 software (SAS Institute, Cary, NC, USA).

Results

Interspecific differences in phototaxis

All 17 *E. hamatanii* individuals moved towards the light side of the I-maze, indicating that they had positive phototaxis (binomial test, $P < 0.001$; Table 2). Similarly, *P. ocellatus* and *E. trisinuata* both showed positive phototaxis. In *S. ornatus*, three individuals moved towards the light side, and the other three moved towards the dark. *Placida* sp. showed negative phototaxis.

At the end of the 30-min experiment, the light intensity selected by the sacoglossans was highest for *E. hamatanii* followed, in order, by *P. ocellatus*, *E. trisinuata*, *S. ornatus*, and *Placida* sp. (Fig. 1). Significant differences were detected between species that preferred strong light (*E. hamatanii*) and the species that preferred an intermediate light intensity (*E. trisinuata*) as well as between that which preferred intermediate light and that which preferred the darker side (*Placida* sp.).

Table 2 Photosynthetic activities (F_v/F_m) and the number of individuals moved towards light or dark side in five sacoglossans

Species	F_v/F_m (mean \pm SD)	Light	Dark	<i>P</i> (binomial test)
<i>Elysia hamatanii</i>	0.58 ± 0.068	17	0	<0.001
<i>Plakobranthus ocellatus</i>	0.61 ± 0.037	19	1	<0.001
<i>Elysia trisinuata</i>	0.57 ± 0.031	11	1	0.003
<i>Stiliger ornatus</i>	0.0053 ± 0.0050	3	3	1
<i>Placida</i> sp.	0.0015 ± 0.0031	0	12	<0.001

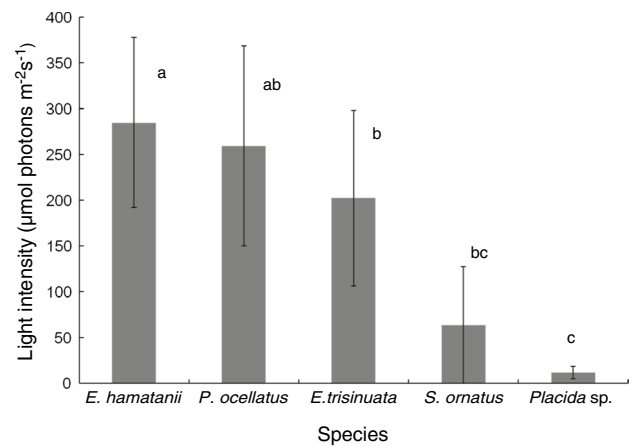


Fig. 1 Light intensities (mean \pm SD) selected by five sacoglossan species in the I-maze experiment. Same letters above the bars indicate no significant difference (Bonferroni correction after Kruskal–Wallis test). In general, kleptoplastic species (*E. hamatanii*, *P. ocellatus*, and *E. trisinuata*) preferred stronger light intensities than non-kleptoplastic ones (*S. ornatus* and *Placida* sp.)

PAM fluorometry

Elysia hamatanii, *P. ocellatus*, and *E. trisinuata* showed high (> 0.5) F_v/F_m values, indicating that they can photosynthesize (Table 2). In contrast, *S. ornatus* and *Placida* sp. showed quite low (≈ 0) F_v/F_m values, indicating that they are not photosynthetic.

The photosynthesis versus irradiance curves of the two of the three photosynthetic species, *E. hamatanii* and *E. trisinuata*, were similar to those of their food algae (Cladophoraceae sp. and *Codium subtubulosum*, respectively; Fig. 2). The maximum rETR values for *E. hamatanii* and *E. trisinuata* were obtained at light intensities of 268 and 183 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively, which coincided with the light intensities of their food algae. Although the food algae was not collected, the photosynthesis curve of *P. ocellatus* was more similar to *E. hamatanii* than to *E. trisinuata*, and it did not reach the maximum until 367 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 2).

Effect of starvation on phototaxis

At the beginning of the first trial, the F_v/F_m of *E. hamatanii* was 0.54 ± 0.05 (mean \pm SD, $N = 7$) for the control (fed) group and 0.54 ± 0.04 (mean \pm SD, $N = 15$) for the experimental (starved) group. After 21 day of being subjected to the food treatments, the F_v/F_m of the control individuals did not decrease (0.63 ± 0.01), but it decreased to 0.19 ± 0.11 ($N = 13$ due to the death of two individuals) for the starved individuals (Online Resource 1).

After the 21-day treatment period, all of the 13 starved individuals and seven control individuals showed

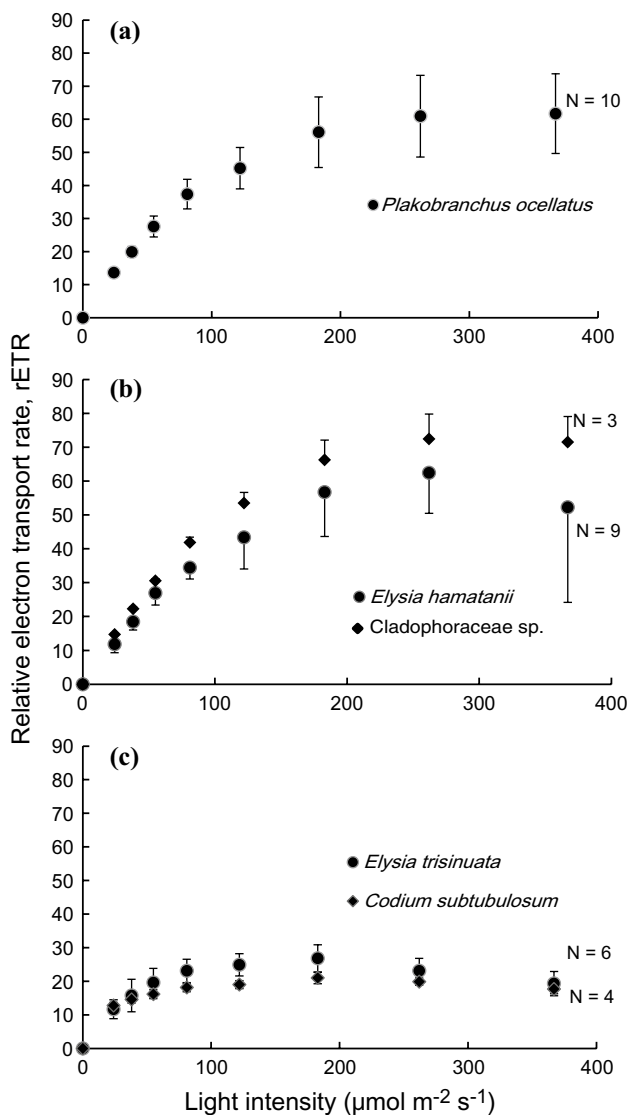


Fig. 2 Relative electron transport rate (rETR, mean \pm SD, or mean \pm or $-$ SD for graphical purposes) versus light intensity (E) curves of *Plakobranchus ocellatus*, *Elysia hamatanii*, *E. trisinuata*, and the food algae of the latter two species (*Cladophoraceae* sp. and *Codium subtubulosum*, respectively). The light response curve for *P. ocellatus* does not reach its maximum until the highest light intensity tested ($367 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), whereas the maximum was reached at 268 and $183 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for *E. hamatanii* (and its food alga) and *E. trisinuata* (and its food alga), respectively

positive phototaxis after 30 min (binomial test, $P < 0.001$ and $P = 0.008$, respectively) even when they were placed on the centre of the I-maze. However, starved individuals selected a lower light intensity ($157 \pm 106 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; mean \pm SD) than that selected by the control individuals ($265 \pm 67 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Mann–Whitney U test, $U = 20$, $P = 0.048$).

In the second trial, in which the sacoglossans were placed at the lightest end of the maze, starved individuals

actually moved less (14.1 ± 6.1 cm; mean \pm SD, $N = 11$) than the controls (22.9 ± 6.8 cm, $N = 7$) within 5 min following the start of the experiment. However, after 30 min, the starved individuals tended to choose slightly lower, although non-significant ($U = 25$, $P = 0.24$), light intensities ($261 \pm 65 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) than the control individuals ($301 \pm 21 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) even though they needed to move longer distances.

Discussion

Elysia hamatanii, *P. ocellatus*, and *E. trisinuata* all showed high photosynthetic capability (F_v/F_m) and positive phototaxis. In contrast, *S. ornatus* and *Placida* sp. showed virtually no photosynthetic capability and did not show positive phototaxis. Thus, the capacity for kleptoplasty seems to affect the differences in the phototaxis across species. Our results are in agreement with previous studies that have reported positive phototaxis in kleptoplastic sacoglossans (Gallop et al. 1980; Weaver and Clark 1981; Schmitt and Wägele 2011). These results strongly suggest that positive phototaxis is an adaptive behaviour of kleptoplastic species.

Moreover, the degree of the preferred light intensities of the three photosynthetic sacoglossans in this study matched the shallowness of their habitats (Fig. 1; Table 2). *E. hamatanii* preferred the highest light intensity ($285 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and lives in the shallowest zone; *P. ocellatus* ($259 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and then *E. trisinuata* ($203 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) followed, and the in situ light intensity of their habitats likely decreases in this order. Furthermore, the preferred light intensities of *E. hamatanii* and *E. trisinuata* corresponded roughly with the light intensities at which the maximum rETR values of their own and of their food algae are reached (268 and $183 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively). Likewise, the photosynthetic acoelomorphan *Symsagittifera roscoffensis* preferred a light intensity that is near the intensity at which its photosynthetic activity reaches the maximum (Seródio et al. 2011). However, in the present study the preferred light intensity for *P. ocellatus* was slightly lower than the light intensity for the highest rETR value ($367 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Both Gallop et al. (1980) and Weaver and Clark (1981) reported similar results for several sacoglossans and suggested possible disadvantages of being exposed to strong light including pigment destruction or predation risk. In fact, Vieira et al. (2009) showed that *E. viridis* individuals exposed to a strong light lose functional chloroplasts more rapidly than those exposed to a weaker light. Thus, kleptoplastic sacoglossans appear to select the optimal or slightly lower light intensities for photosynthesis.

Both *S. ornatus* and *Placida* sp. were non-kleptoplastic, but *S. ornatus* showed no phototaxis, whereas *Placida* sp. showed negative phototaxis under our experimental conditions. The lack of kleptoplasty observed in these species needs further investigation, as Yamamoto et al. (2009) suggested that they are kleptoplastic. It is possible that they can retain chloroplasts only for a very short term (e.g. <1 day), after which they lose their ability to photosynthesize. In any case, the different phototactic tendencies of these two species may be related to their antipredator behaviour. Although many sacoglossans produce toxic or irritating secretions for defence (Cimino and Ghiselim 1998; Marín and Ros 2004; Finn et al. 2009), some predators of these species, such as butterfly fish (Chaetodontidae), are known (Weaver and Clark 1981). The body surface of *S. ornatus* contains bright blue and yellow parts that are apparently aposematic, so it is possible that *S. ornatus* individuals have no phototaxis because they do not need to hide from predators. On the contrary, the body colour of *Placida* sp. is green and highly cryptic on *Codium* spp., so their negative phototaxis may play a role in hiding from predators.

This study also found a difference in the preferred light intensities between starved and fed *E. hamatanii* individuals, so the presence of functional chloroplasts affects the preferred light intensities. However, even starved *E. hamatanii* lacking functional chloroplasts showed positive phototaxis in this study. Likewise, juvenile *E. timida* show photobehaviour (exposure of parapodial lobes in response to light) before the sequestration of their first chloroplasts (Schmitt and Wägele 2011). These results indicate that the presence of functional kleptoplasts is not the direct cause of photobehaviour in sacoglossans. The preference for strong light by fed individuals (with functional chloroplasts) in the present study is adaptive, as they need light for photosynthesis, but it is not clear why starved individuals also show positive phototaxis. It is possible that positive phototaxis may have functions other than optimising photosynthesis, such as locating food algae (Cruz et al. 2013).

In summary, this study has suggested that sacoglossans generally show phototaxis in an adaptive way, both across and within species and thus provides support for the idea that kleptoplastic sacoglossans function as ‘crawling leaves’. However, further research is needed on the adaptive significance of such photobehaviour because they may have other functions.

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