

Colour morphotypes of *Elysia timida* (Sacoglossa, Gastropoda) are determined by light acclimation in food algae

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ABSTRACT: *Elysia timida* (Risso, 1818) colonizing the shallow waters of the Mar Menor Lagoon (Spain) exhibit a brown and a green morph. It was hypothesised that these morphs were the result of feeding preferentially on brown and green algae, respectively. *E. timida* and its potential food sources, *Acetabularia acetabulum* (Chlorophyta) and *Halopteris filicina* (Heterokontophyta) were collected by snorkelling during April 2010. Photosynthetic pigments were analysed by HPLC, photo-physiological parameters were estimated by PAM fluorometry and body colour was characterized by spectral reflectance. Digital photography was used to count the number and area of red spots (small red dots on the slug's surface) on the parapodia of the 2 morphs. In the laboratory, green *E. timida* was fed with *A. acetabulum* cultured under 2 light treatments (high light, 600 $\mu\text{mol E m}^{-2} \text{s}^{-1}$ and low light, 40 $\mu\text{mol E m}^{-2} \text{s}^{-1}$), and digital photography was used to monitor colour alterations in *E. timida*. Spectral reflectance confirmed the colour differences, but both morphs showed a pigment composition similar to the green alga *A. acetabulum* and showed none of the pigments present in the brown alga *H. filicina*, neither immediately after collection of the slugs *in situ*, nor after the feeding experiment. *A. acetabulum* grown under high light intensity changed from green to brown colour and *E. timida* changed to brown colour when fed with high-light acclimated *A. acetabulum*. Thus, *E. timida* colour differences could not be attributed to feeding on different algae groups but was likely the result of feeding on *A. acetabulum* growing under different light intensities.

KEY WORDS: *Acetabularia acetabulum* · *Elysia timida* · Food source · Light acclimation · Morphotypes · Pigments · Symbiosis · Mar Menor lagoon

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INTRODUCTION

Sacoglossan gastropods (suborder Placobranchacea, Rang 1829) are benthic herbivore molluscs. Most of them feed on syphonalean green algae using an uniserial radula, and some are able to host functional chloroplasts within specialized cells (Jensen 1996, Marín & Ros 2004, Giménez-Casaldueiro 1999, Raven et al. 2009). The 'symbiont' is not a genetically autonomous, complete organism but rather an isolated,

semi-autonomous organelle (chloroplast) (Rumpho et al. 2001). This has led some authors to rename the captured chloroplasts as kleptoplasts, instead of symbionts and the event, kleptoplasty, in substitution of the term symbiosis (Clark et al. 1990, Raven et al. 2001).

Elysia timida (Risso, 1818) developed kleptoplasty by feeding on the chlorophyte alga, *Acetabularia acetabulum* (Linnaeus) P.C. Silva, 1952 (Chlorophyta, Polyphysaceae). It can retain functional kleptoplasts for more than 40 d without food input (Marín & Ros

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1993, Jesus et al. 2010), despite a strong drop in PSII quantum efficiencies after 15 to 20 d, and has photo-regulation mechanisms, particularly the xanthophyll cycle (using zeaxanthin, antheraxanthin, and violaxanthin pigments) (Jesus et al. 2010)

Elysia timida is a Mediterranean sacoglossan mollusc that lives in well-lit shallow littoral habitats (Thompson & Jaklin 1988). These slugs developed a chemical defence producing mucus that contains polypropionate metabolites (Gavagnin et al. 1994). Additionally, the colour given by the sequestered algal plastids confers a cryptic colouration to the animals that should also play an important role in defence against predation (Giménez-Casalduero et al. 2002, Marín & Ros 2004). The animals possess red spots along the body (Rahat & Monselise 1979) and it is possible that a density difference can lead to color differences. Specimens from Mar Menor exhibit 2 different morphs, green (similar to green algae) and brown (or dun—a colouration closer to brown algae) (Giménez-Casalduero 1999) that are always present in Mar Menor (González-Wangüemert et al. 2006). González-Wangüemert et al. (2006), testing 10 enzyme loci concluded that these morphs were not genetically different. Some authors speculate that this difference is the result of feeding on algae with different pigment contents (González-Wanguemert et al. 2006), since this species is frequently observed crawling over algae (Rahat 1976) such as the brown alga *Halopteris filicina* (Grateloup) Kützinger (Heterokontophyta, Stypocaulaceae). Although there are frequent references to observations of the animal on top of potential food sources, the feeding process itself is difficult to observe (e.g. Ros & Rodríguez 1985). Methods used to confirm sacoglossan food sources include microscopy morphology, never tried with *E. timida* (Wägele & Johnsen 2001, Evertsen & Johnsen 2009) and spectrophotometry (Ros & Rodríguez 1985), but these methods do not always offer precise information about the algae pigments present in the kleptoplasts. Amplifying chloroplast genes (e.g. *rbcL* or *tuf A*) from sea slug total DNA using PCR (Curtis et al. 2006, 2007, Handeler et al. 2010) is a good and precise method that could potentially be used to infer the animal food source but that would not yield any information about pigment content; namely it would not be a useful technique if the differences in *E. timida* colour are caused by pigment synthesis outside the kleptoplast. High performance liquid chromatography (HPLC) allows the separation and quantification of pigments. Recently, Jesus et al. (2010) provided HPLC results from Mar Menor specimens suggesting that *E. timida* feed only on *A. acetabulum*.

Pulse amplitude modulated (PAM) fluorescence is a non-destructive and non-invasive technique that can be used to evaluate photosystem (PS) II photosynthetic quantum efficiency and a variety of other photo-physiological parameters. This has been used in the past to evaluate whether a given sea slug contains active chloroplasts and to quantify their PSII quantum efficiency (Wägele & Johnsen 2001, Burgardt et al. 2005, Evertsen et al. 2007, Evertsen & Johnsen 2009, Jesus et al. 2010). PAM fluorescence can also be used for determining sea slug food sources, based on the fact that chloroplasts from different algae groups exhibit different PAM fluorescence parameters (e.g. PSII quantum efficiency).

The objective of the current study was to investigate possible reasons for the presence of 2 different colorations in *Elysia timida* from Mar Menor Lagoon, a salt water lagoon in southeast Spain.

MATERIALS AND METHODS

Sampling

The present study was divided into 2 parts. For the first part, adult individuals of *Elysia timida* were collected randomly during April 2010 at the Mar Menor lagoon (37° 44' N, 00° 46' W), separated from the Mediterranean Sea by La Manga, a sandbar 22 km in length and with a variable width from 100 to 1200 m. Samples were collected by hand while snorkelling at depths ranging from 0.5 to 1.0 m; 10 adult specimens of similar size of each colour morph (Fig. 1) were selected by visual discrimination (according to Giménez-Casalduero 1999) and kept in aerated containers under low light, until further analysis; 3 replicates of the algae where the slugs were seen (*Acetabularia acetabulum* and *Halopteris filicina*) were also collected randomly. The same 10 animals of each morph and the same 3 samples of each alga were used in all experiments.

For the second part, the feeding experiments, 10 green morph slugs were picked randomly from our aquariums stock to be fed *Acetabularia acetabulum* with 2 different light treatments; 3 samples from each alga light treatment then were used to run HPLC pigment analysis.

Parapodia red spot analysis

The number and area of the parapodia red spots on each *Elysia timida* were determined with ImageJ

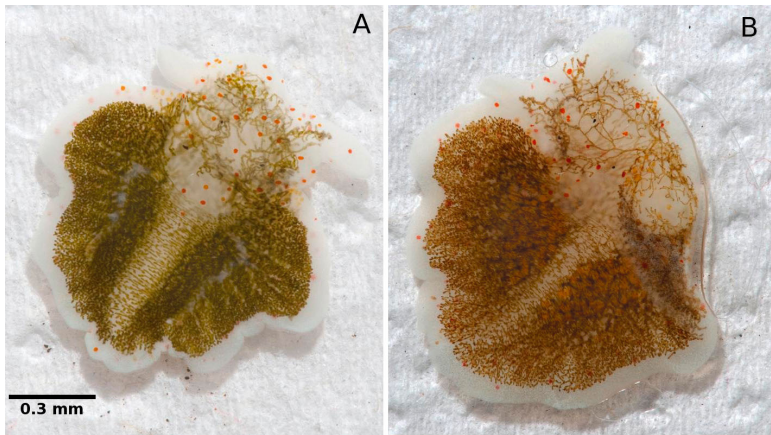


Fig. 1. *Elysia timida* morphs. (A) Green and (B) brown

software (Rasband 1997–2009), using high-resolution digital photographs (Nikon D700 with a Nikon 105 mm macro lens) calibrated with a photograph of a ruler. Data are given as no. of spots per mm² and total area of spots per mm² on the parapodia.

Spectral reflectance

Spectral reflectance of *Elysia timida*, *Acetabularia acetabulum* and *Halopteris filicina* was measured with a USB2000 (Ocean Optics) with a VIS-NIR optical configuration controlled by OObase32 software (Ocean Optics). Spectra were determined for each animal under a halogen light source (Phillips 13163/5H) complemented with an infra-red light source (Phillips infra-red, R95E). Reflectance spectra of the animal surface was calculated by dividing the upwelling spectral radiance from the animal surface with the reflectance of a clean polystyrene plate from which we subtracted the machine dark noise (Dn) (electronic signal measured at total darkness). The polystyrene plates differed <3% from a calibrated 99% reflectance standard plate (Spectralon) (Forster & Jesus 2006). Spectra were measured in the range 338 to 1023 nm with 0.3 nm bandwidth. Spectra were reduced to 1 nm bandwidths using a moving average and cut between 400 and 800 nm to reduce noise at the extremities. The spectroradiometer sensor was positioned so that the surface was always viewed from the nadir position at a fixed distance of approximately 2 mm from the subject. A concave microscopy slide was used to flatten the slugs before any reflectance measurements, following the methodology of Vieira et al. (2009). This non-destructive procedure did not damage the animal and was carried

out in less than 30 s in order to minimize suffering. Fragments of alga were measured in petri dishes, with a thin layer of water. Normalized difference vegetation index (NDVI) was calculated to estimate chlorophyll content; 10 spectral measurements from each morph and from fragments of both algal taxa were averaged.

Pulse amplitude modulated fluorometry

Rapid light curves (RLC) were carried out after 15 min dark adaptation using a PAM fluorometer (DIVING PAM), with a red measuring light (0.6 kHz), and a halogen saturating pulse (sampling frequency 20 Kz, 800 ms, 9500 $\mu\text{mol E m}^{-2} \text{s}^{-1}$). RLC were set up with 8 incremental light steps (0, 25, 91, 202, 380, 550, 750, 1100, 1500 $\mu\text{mol E m}^{-2} \text{s}^{-1}$) at 30 s intervals. Slugs were set inside a petri dish and covered with a small layer of sea water, the PAM probe was set up on a stand holder at a fixed distance of approximately 2 mm from the slug and the petri dish moved to compensate the slug's movements, thus keeping the slug underneath the PAM sensor, as described by Jesus et al. (2010). Previous tests showed that this methodology returned similar results to holding and squashing the slug on a microscopy slide. The RLC parameters, photosynthetic rate (α) in the light-limited region of RLC, the minimum saturating irradiance (E_k) and the maximum relative electron transport rate (rETRmax), were then calculated.

Feeding experiment

Acetabularia acetabulum also showed colour variation and we hypothesized that this might be the result of pigment shifts caused by different light acclimation states. To test this hypothesis, *A. acetabulum* was cultured under low light (LL, 40 $\mu\text{mol E m}^{-2} \text{s}^{-1}$) and high light (HL, 600 $\mu\text{mol E m}^{-2} \text{s}^{-1}$); 2 small rocks colonized by green *A. acetabulum* were incubated inside a culture chamber (Fytoscope fs130, PSI) at 20°C on a 12:12 h (dark:light) period. After 1 wk, it was observed that *A. acetabulum* grown under HL changed from green to red/brownish while the LL *A. acetabulum* remained green (Fig. 2). Then, 5 green *Elysia timida* were fed with HL *A. acetabulum* and 5 with LL *A. acetabulum*. Changes in *E. timida* colour were registered by digital photography

throughout 5 d. All slugs were kept at LL conditions at 20°C from the beginning until the end of the experiment. Images were acquired with the animals flattened on a concave microscopy slide. Care was taken to keep the light conditions constant and an 18% gray card (FotoWand 4963) was placed inside the frame to normalize the images using Photoshop CS3 software, and *E. timida* images were extracted from the background and aligned in a single image showing colour changes from Day 1 to 5 (Fig. 3, Fig. S1 in the supplement at www.int-res.com/articles/suppl/b017p081_supp.pdf).

HPLC pigment analysis

High performance liquid chromatography (HPLC) pigment analysis was carried out to determine if the slugs and algae contained different pigments (which would indicate different food sources) or different pigment concentrations.

Slugs and algae were freeze-dried after being frozen in liquid nitrogen 3 d after being collected in the field. A subsample of 4 mg was used to extract pigments in 1 ml of 95% cold buffered methanol (2% ammonium acetate). Samples were sonicated (1 min) and left for

15 min at -20°C , in the dark. Extracts were filtered with $0.2\ \mu\text{m}$ Whatman membrane filters just before HPLC analysis. A Shimadzu HPLC containing solvent delivery module (LC-10ADVP), with system controller (SCL-10AVP), and a photodiode array (M10AVP), was used. Pigments were separated with a C18 column for reverse phase chromatography (Supelcosil, 25 cm long, $4.6\ \mu\text{m}$ diameter, and $5\ \mu\text{m}$ particles). Chromatographic separation was carried with 0.5 M ammonium acetate in methanol and water (85:15, v:v), acetonitrile and water (90:10, v:v), and 100% ethyl acetate. The solvent gradient followed Kraay et al. (1992), adapted by Brotas & Plante-Cuny (2003), with a flow rate of $0.6\ \text{ml}\ \text{min}^{-1}$ and an injection volume of $100\ \mu\text{l}$. Identification and calibration of the HPLC peaks was confirmed with chl *a*, chl *b*, and β -carotene standards from Sigma, and chl *c*, fucoxanthin (fuco), diadinoxanthin (DD), diatoxanthin (DT), violaxanthin (vio), antheraxanthin (ant), lutein (lut), zeaxanthin (zea), and pheophythin *a* standards from DHI Laboratory Products. Pheophorbide *a* and chlorophyllide *a* standards were prepared from a green alga, *Ulva* cf. *intestinalis*, following Brotas & Plante-Cuny (2003). Chlorophylls and carotenoids were identified and quantified with photodiode array. Pheophorbides and pheophytins were identified and quantified with the fluorescence detector. Pigments were expressed as μg (pigment) g^{-1} (dry wt) and as pigment:chl *a* ratios.

The same procedure was applied to 3 samples of each *Acetabularia acetabulum* light treatment.

Statistical analysis

Comparisons using pigment and ratio concentrations, total area covered by red spots (mm^2), number of red spots per area and NDVI were made between brown and green morphs. Photoregulation pigment comparisons were made between the 2 *Acetabularia acetabulum* light treatments. Zeaxanthin + lutein, antheraxanthin, and violaxanthin – lutein could not be separated from zeaxanthin by HPLC.

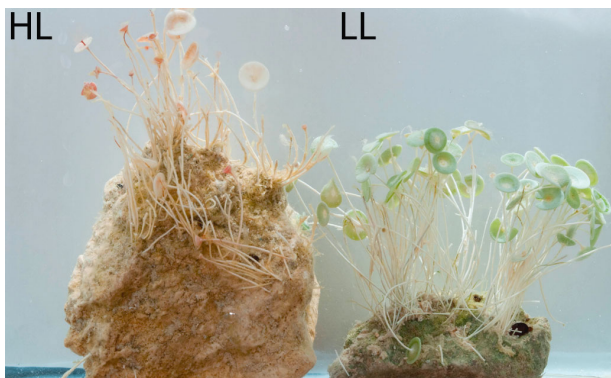


Fig. 2. *Acetabularia acetabulum* after 1 wk of light treatment. HL: high light ($600\ \mu\text{mol}\ \text{E}\ \text{m}^{-2}\ \text{s}^{-1}$), LL: low light ($40\ \mu\text{mol}\ \text{E}\ \text{m}^{-2}\ \text{s}^{-1}$)

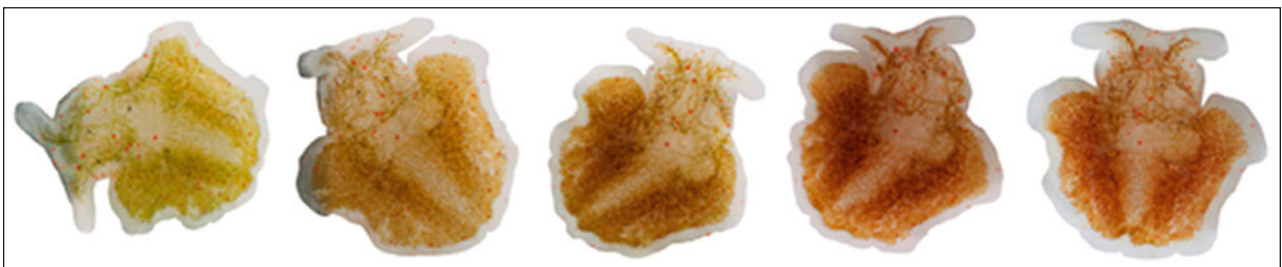


Fig. 3. *Elysia timida* fed with high-light acclimated *Acetabularia acetabulum* from Days 1 to 5 (left to right)

When normality and homoscedasticity were observed (Shapiro-Wilks test, Levene's test), Student's *t*-test was used. The corresponding non-parametric test, Wilcoxon-Mann-Whitney, was used when normality and homoscedasticity were not observed ($p < 0.05$ for all tests).

Non-parametric multivariate techniques were used to compare pigment patterns in algae and morphs, using the PRIMER statistical package (Clarke & Warwick 1994). A triangular matrix of similarity was calculated using the Bray-Curtis similarity coefficient based on standardized values of pigment concentration (Clarke & Warwick 1994). Graphical representation of multivariate patterns of pigments was obtained by non-metric multidimensional scaling (nMDS).

RESULTS

Pigments

Elysia timida HPLC pigment analysis showed a pigment signature (Fig. 4) characteristic of green algae (Chlorophyta), with the presence of chl *b*, zeaxanthin and antheraxanthin. Both *E. timida* morphs exhibited the same pigment composition as *Acetabularia acetabulum*. Neither *E. timida* nor *A. acetabulum* contained fucoxanthin, which was observed only in *Halopteris filicina*. Pigment concentrations in brown and green morphs were never significantly different ($p > 0.1$, *t*-test and Wilcoxon-Mann-Whitney *U*-test). Pigment concentration and pigment ratios of both morphs were more similar to *A. acetabulum* than to *H. filicina* (Figs. 4 & 5); particularly obvious were the lack of chl *b* and the presence of fucoxanthin in *H. filicina*. Results from the non-parametric multivariate analysis showed that pigment pattern in *H. filicina* was different from *A. acetabulum* and from both *E. timida* morphs (Supplement Fig. S2).

Photoregulation pigment ratio (zeaxanthin + lutein, antheraxanthin and violaxanthin) analysis showed a statistically significant difference between the 2 *Acetabularia acetabulum* treatments (*t*-test, $p = 0.00474$), where these pigments were always higher in the HL algae (Fig. 6).

In brown morph *Elysia timida*, the pigment:chl *a* ratio showed a relative increase in carotenoids related to photoregulation (zeaxanthin, lutein, violaxanthin).

The number of parapodia red spots per area showed no significant differences between the 2 morphs (*t*-test, $p = 0.188$). Spot size per area in the brown morph was marginally higher than in the green morph (Table 1) (*t*-test, $p = 0.062$).

Spectral reflectance

Elysia timida morphs showed similar reflectance spectra (Fig. 7A) that differed mainly in a red shift exhibited by the brown morph. Both *E. timida* morphs showed more similarities with *Acetabularia acetabulum* than with *Halopteris filicina* reflectance

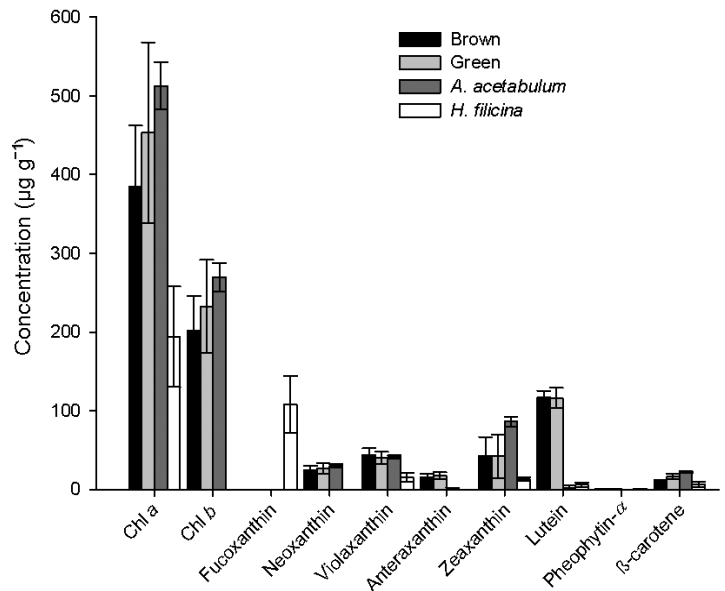


Fig. 4. *Elysia timida*, *Acetabularia acetabulum* and *Halopteris filicina*. Pigment concentration in brown and green morphs, and in algae (mean \pm SD; $n = 10$ for slugs, and $n = 3$ for algae)

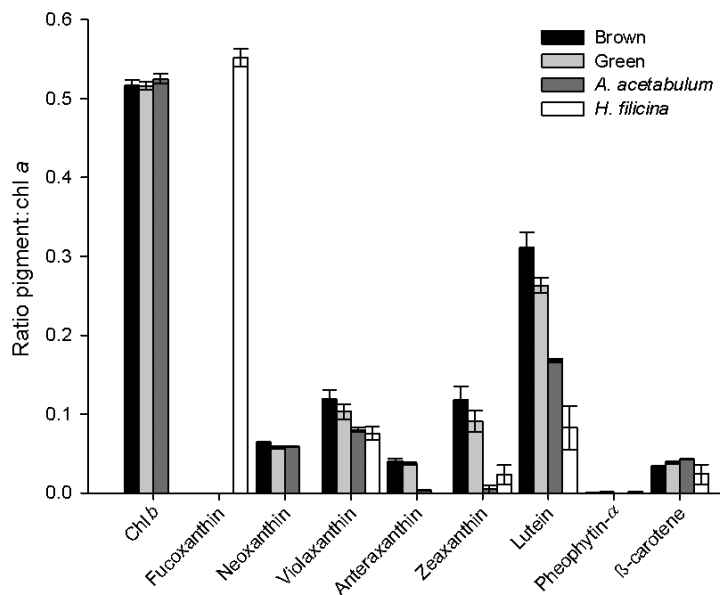


Fig. 5. *Elysia timida*, *Acetabularia acetabulum* and *Halopteris filicina*. Ratios of pigment to chl *a* concentration in brown and green morphs, and in algae (mean \pm SD; $n = 10$ for slugs, and $n = 3$ for algae)

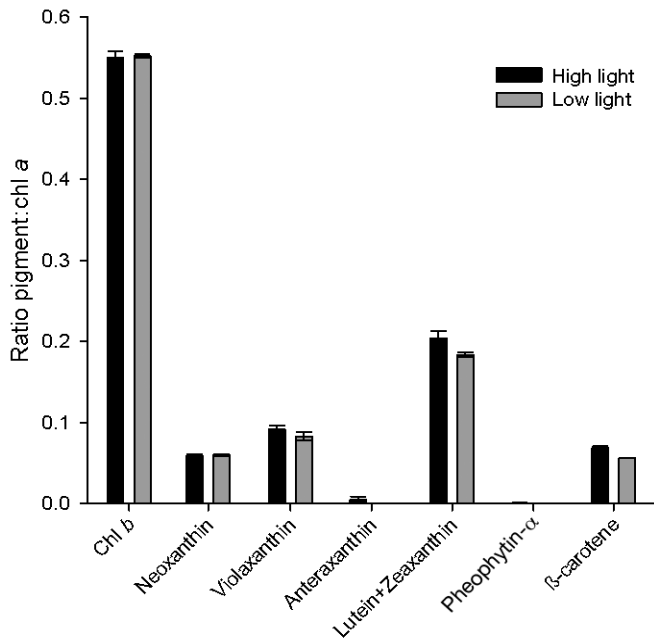


Fig. 6. *Acetabularia acetabulum*. Ratios of pigment to chl a concentration in algae grown under high light (HL; 600 $\mu\text{mol E m}^{-2} \text{s}^{-1}$) and low light (LL; 40 $\mu\text{mol E m}^{-2} \text{s}^{-1}$) conditions (mean \pm SD; n = 3)

Table 1. *Elysia timida*. Number and area of red spots per mm^2 in the parapodia region of brown and green morphs (mean \pm SD; n = 10)

Morph	Number	Area
Brown	7.693 \pm 0.655	0.0708 \pm 0.0057
Green	6.232 \pm 0.306	0.0531 \pm 0.0043

Table 2. *Elysia timida*, *Acetabularia acetabulum* and *Halopteris filicina*. Normalized difference vegetation index (NDVI), PAM fluorescence rapid light curve (RLC) parameters (mean \pm SD; n = 10) extracted after adjustment to the model of Platt et al. (1980), in brown and green morphs, and in algae (mean \pm SD; n = 10 in all cases). α : photosynthetic rate in the light-limited region of the RLC; Ek: minimum saturating irradiance; rETRmax: maximum relative electron transport rate

	NDVI	α	Ek	rETRmax
<i>E. timida</i> Brown	0.741 \pm 0.082	0.174 \pm 0.034	847 \pm 256	146 \pm 47
<i>E. timida</i> Green	0.747 \pm 0.059	0.170 \pm 0.062	875 \pm 418	131 \pm 36
<i>A. acetabulum</i>	0.620 \pm 0.039	0.266 \pm 0.036	356 \pm 53	95 \pm 28
<i>H. filicina</i>	0.690 \pm 0.143	0.356 \pm 0.058	367 \pm 37	131 \pm 30

spectra (Fig. 7B), i.e. *H. filicina* showed an absorption feature at 628 nm that was not present in either *A. acetabulum* or *E. timida* morphs. The 2 *E. timida* morph spectra differed between 550 to 650 nm (Fig. 7): the green morph showed a peak at 565 nm (a green/

yellow wavelengths), and the brown morph showed a peak at 587 nm (a yellow/red wavelength). NDVI (Table 2) showed no significant differences between morphs (*t*-test, $p = 0.7913$).

PAM fluorometry

RLCs showed very similar patterns among all taxa (Fig. 8), with only *Halopteris filicina* exhibiting a slightly different pattern at lower light intensities but converging to similar relative electron transport rate (rETR) values at higher light intensities; relative maximum electron transport rate (rETRmax) showed no differences among taxa ($F_{3,28} = 2.519$, $p = 0.08$). None of the RLC parameters, i.e. rETRmax, Ek, and photosynthetic rate in the light-limited region of the RLC (α) showed significant differences between the *Elysia timida* colour morphs (Table 2). *E. timida* showed lower α (Tukey HSD, $p < 0.05$), and higher Ek (Tukey HSD, $p < 0.05$) values than both algae.

Feeding experiment

Elysia timida fed with HL *Acetabularia acetabulum* changed colour from green to brown after less than 5 d, acquiring a similar colour to the *A. acetabulum* grown under high light. *E. timida* fed with LL *A. acetabulum* remained green throughout the 5 d (Figs. 3 & S1).

DISCUSSION

Sacoglossan green coloration commonly results from the chlorophylls present in the tissues after feeding on green algae. Thus, it is reasonable to assume that slugs feeding on different algal classes exhibit different colours as a consequence of ingesting different pigment types, e.g. fucoxanthin from brown algae. Giménez-Casalduero (1999) hypothesized that the brown morph feeds on brown algae while the green morph feeds on *Acetabularia acetabulum*. HPLC analysis of slugs captured *in situ* showed that neither *E. timida* morph fed on *Halopteris filicina*, and that both fed on *A. acetabulum*. Feeding experiments showed that the 2 morphs in Mar Menor probably result from feeding on *A. acetabulum* exposed to different light conditions.

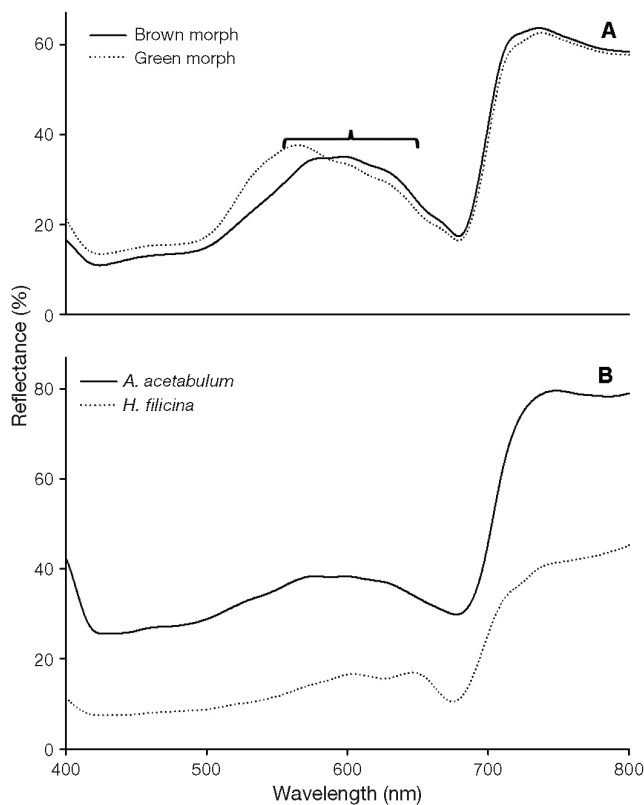


Fig. 7. *Elysia timida*, *Acetabularia acetabulum* and *Halopteris filicina*. Reflectance (400 to 800 nm) in (A) brown and green morphs, and (B) algae (mean \pm SD; $n = 10$ in all cases): The bracket in (A) indicates the reflectance shift between morphs

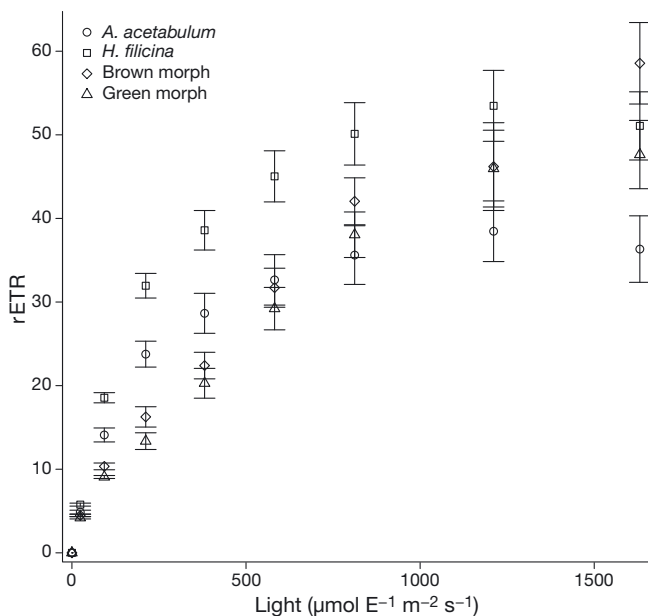


Fig. 8. *Elysia timida*, *Acetabularia acetabulum* and *Halopteris filicina*. PAM fluorescence rapid light curves (RLC) in brown and green morphs, and in algae

Pigments

Fucoxanthin is the major accessory pigment in the brown alga *H. filicina* (Fig. 4); thus the absence of this pigment in both *E. timida* morphs suggests that this algae was not being used as food. This conclusion is also supported by the MDS analysis (Fig. S2, www.int-res.com/articles/suppl/b017p081_supp.pdf).

Neither *Elysia timida* morph showed significant evidence of pigment degradation, showing levels of pheophytins similar to their food source *Acetabularia acetabulum*. This showed that no obvious kleptoplast degradation took place during the duration of the experiment, even in the animals fed with high-light acclimated kleptoplasts, further supporting previous data showing that kleptoplasts in this species are highly efficient for long time periods (Marín & Ros 1993, Giménez & Muniain 2006, 2008, Jesus et al. 2010).

The significant differences observed in the photo-regulation pigments (Fig. 7) between both *Acetabularia acetabulum* light treatments indicate that these are the pigments responsible for the colour change in the algae.

Further, the differences in pigment concentration (although not significant) are responsible for the colour shift between the 2 morphs. The lack of significance probably results because the slugs do not exclusively have one particular type of chloroplast in their body, although they differ in chloroplast concentration from HL or LL *Acetabularia acetabulum*, as they can retain the chloroplasts for several days after eating HL or LL algae.

Colouration

Spectral reflectance analysis (Fig. 7) confirmed the colour differences visible with the naked eye (Fig. 1) as reported by Giménez-Casalduero (1999), and supports the HPLC data, showing that the difference in colouration of the 2 morphs was not caused by the presence of brown algal pigments in the brown morph.

The feeding experiments support the hypotheses that slug colouration reflects the light-acclimation status of *Acetabularia acetabulum* and, consequently, that *Elysia timida* feeds exclusively on this alga. Thus, slugs that feed on *A. acetabulum* exposed to HL tend to become brownish. This has been observed in *E. clarki* which exhibit colour differences depending on the species of *Bryopsidales* they feed on (Curtis et al. 2007). We conclude that the pigments in *A. acetabulum* are transferred to the slugs.

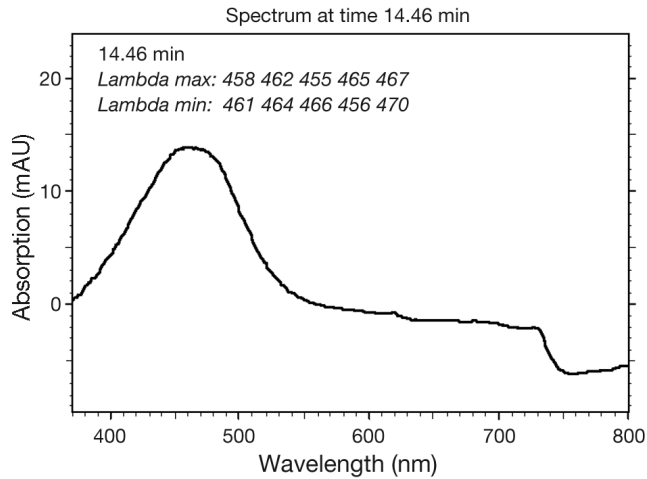


Fig. 9. Unidentified carotenoid. HPLC absorption spectrum

The number and density of red spots were always higher in the brown morph. Red spots play a role in the response to light variations (Rahat & Monselise 1979), and this suggests that the brown morph occupies higher light environments, not only while feeding on *Acetabularia acetabulum* exposed to HL. This hypothesis is supported by higher concentrations of an unidentified carotenoid (Fig. 9) in the brown morph.

Fluorometry

RLC parameters suggest that both morphs are adapted to similar light regimes. This seems to contradict the hypothesis that higher carotenoid content in the brown morph indicates higher light adaptation. However, the RLC technique captures photosynthetic light response on very short time periods (4 min in the current work), which mainly reflects the current photosynthetic state. Thus, it is possible that the hypothetically different light-acclimation states of the 2 morphs will be detected only by using steady state light response curves, which reflect more effectively the long-term light adaptations. RLC analysis also confirmed that pigment degradation is not a determinant factor to differentiate morphs, since efficiencies along the curves were similar in both.

The data collected in the current study support the observations by Jesus et al. (2010) that *Elysia timida* typically exhibited higher E_k and lower α than *Acetabularia acetabulum*, suggesting that the animals are better adapted to high light exposure than the algae, probably by combining movements (e.g. parapodia closure) with algal photophysiological mechanisms (e.g. the xanthophyll cycle).

Conclusions

Field observations are useful for setting up ecological hypotheses, but they are not sufficient to accurately determine animal feeding habits. The 2 *Elysia timida* morphs in the Mar Menor lagoon feed on the same algal species and still exhibit different colour patterns. The brown alga *Halopteris filicina* was excluded as a possible food source, since the pigments present in both slug morphs were common only to *Acetabularia acetabulum*. Nevertheless, *E. timida* may feed on other chlorophyta. *A. acetabulum* produces higher concentrations of photoregulation pigments when exposed to higher light intensities. This promotes a change from green to brown in the algae that is transferred to the slugs after feeding. The number and density of red spots on the parapodia might also help to differentiate the colours. The possible effect of ultrastructural changes in the plastids on the colour of the slugs remains to be studied.

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LITERATURE CITED

- Brotas V, Plante-Cuny MR (2003) The use of HPLC pigment analysis to study microphytobenthos communities. *Acta Oecol* 24:S109–S115
- Burghardt I, Evertsen J, Johnsen G, Wägele H (2005) Solar powered seaslugs—mutualistic symbiosis of aeolid Nudibranchia (Mollusca, Gastropoda, Opisthobranchia) with *Symbiodinium*. *Symbiosis* 38:227–250
- Clark KB, Jensen KR, Stirts HM (1990) Survey for functional kleptoplasty among West Atlantic Ascoglossa (= Sacoglossa) (Mollusca: Opisthobranchia). *Veliger* 33:339–345
- Clarke KR, Warwick RM (1994) Change in the marine communities: an approach to statistical analysis and interpretation. Natural Environment Research Council, Plymouth
- Curtis NE, Massey SE, Pierce SK (2006) The symbiotic chloroplasts in the sacoglossan *Elysia clarki* are from several algal species. *Invertebr Biol* 125:336–345
- Curtis NE, Pierce SK, Massey SE, Schwartz JA, Mangel TK (2007) Newly metamorphosed *Elysia clarki* juveniles feed on and sequester chloroplasts from algal species different from those utilized by adult slugs. *Mar Biol* 150: 797–806
- Evertsen J, Johnsen G (2009) *In vivo* and *in vitro* differences in chloroplast functionality in the two north Atlantic sacoglossans (Gastropoda, Opisthobranchia) *Placida dendritica* and *Elysia viridis*. *Mar Biol* 156:847–859
- Evertsen J, Burghardt I, Johnsen G, Wägele H (2007) Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. *Mar Biol* 151: 2159–2166

- Forster RM, Jesus B (2006) Field spectroscopy of estuarine intertidal sediments. *Int J Remote Sens* 27:3657–3669
- Gavagnin M, Spinella A, Castelluccio F, Cimino G, Marín A (1994) Polypropinates form the Mediterranean mollusk *Elysia timida*. *J Nat Prod* 57:298–304
- Giménez-Casalduero F (1999) Estudio comparativo en diferentes poblaciones de *Elysia timida* Risso, 1818 (Gastropoda, Opisthobranchia, Sacoglossa) en mar abierto y ambiente lagunar. *Iberus* 17:137–146
- Giménez-Casalduero F, Muniain C (2006) Photosynthetic activity of the solar-powered lagoon mollusc *E. timida* (Risso, 1818) (Opisthobranchia: Sacoglossa). *Symbiosis* 41:151–158
- 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 Biol Ecol* 357:181–187
- Giménez-Casalduero F, Muniain C, García-Charton J (2002) *Elysia timida* (Risso, 1818) (Gastropoda, Opisthobranchia): relationship and feeding deterrence to a potential predator on the south-western Mediterranean coast. *Mar Biol* 141:1051–1057
- González-Wangüemert M, Giménez-Casalduero F, Pérez-Ruzafa A (2006) Genetic differentiation of *Elysia timida* (Risso, 1818) populations in the Southwest Mediterranean and Mar Menor coastal lagoon. *Biochem Syst Ecol* 34:514–527
- Händeler K, Wägele H, Wahrmund U, Rüdinger M, Knoop V (2010) Slugs' last meals: molecular identification of sequestered chloroplasts from different algal origins in Sacoglossa (Opisthobranchia, Gastropoda). *Mol Ecol Resour* 10:968–978
- Jensen KR (1996) Phylogenetic systematics and classification of the Sacoglossa (Mollusca, Gastropoda, Opisthobranchia). *Philos Trans R Soc Lond B* 351:91–122
- Jesus B, Ventura P, Calado G (2010) Behaviour and a functional xanthophyll cycle enhance photo-regulation mechanisms in the solar-powered sea slug *Elysia timida* (Risso, 1818). *J Exp Mar Biol Ecol* 395:98–105
- Kraay GW, Zapata M, Veldhuis M (1992) Separation of chlorophylls c1, c2, and c3 of marine phytoplankton by reversed-phase-C18-high-performance liquid chromatography. *J Phycol* 28:708–712
- Marín A, Ros J (1993) Ultrastructural and ecological aspects of the development of chloroplast retention in the sacoglossan gastropod *Elysia timida*. *J Molluscan Stud* 59:95–104
- Marín A, Ros J (2004) Chemical defenses in sacoglossan opisthobranchs: taxonomic trends and evolutive implications. *Sci Mar* 68:227–241
- Platt T, Gallegos CL, Harrison WG (1980) Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. *J Mar Res* 38:687–701
- Rahat M (1976) Direct development and symbiotic chloroplasts in *Elysia timida* (Mollusca: Opisthobranchia). *Isr J Zool* 53:186–193
- Rahat M, Monselise EBI (1979) Photobiology of the chloroplast hosting mollusc *Elysia timida* (Opisthobranchia). *J Exp Biol* 79:225–233
- Rasband WS (1997–2009) ImageJ, US. National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>
- Raven J, Walker D, Jensen K, Handley L, Scrimgeour C, McInroy S (2001) What fraction of the organic carbon in sacoglossans is obtained from photosynthesis by kleptoplastids? An investigation using the natural abundance of stable carbon isotopes. *Mar Biol* 138:537–545
- Raven JA, Beardall J, Flynn KJ, Maberly SC (2009) Phagotrophy in the origins of photosynthesis in eukaryotes and as a complementary mode of nutrition in phototrophs: relation to Darwin's insectivorous plants. *J Exp Bot* 60:3975–3987
- Ros J, Rodríguez J (1985) La simbiosis algal en *Elysia timida* Risso 1818. Primeros resultados. *An Biol Biol Ambiental* 1985:37–47
- Rumpho ME, Summer E, Green B, Fox T, Manhart J (2001) Mollusc/algal chloroplast symbiosis: how can isolated chloroplasts continue to function for months in the cytosol of a sea slug in the absence of an algal nucleus? *Zoology* 104:303–312
- Thompson TE, Jaklin A (1988) Eastern Mediterranean Opisthobranchia: Elysiidae (Sacoglossa = Ascoglossa). *J Molluscan Stud* 54:59–69
- Vieira S, Calado R, Coelho H, Serodio J (2009) Effects of light exposure on the retention of kleptoplastic photosynthetic activity in the sacoglossan mollusc *Elysia viridis*. *Mar Biol* 156:1007–1020
- Wägele H, Johnsen G (2001) Observations on the histology and photosynthetic performance of 'solar-powered' opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae. *Org Divers Evol* 1:193–210

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