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Pigment profile in the photosynthetic sea slug *Elysia viridis* (Montagu, 1804)

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ABSTRACT

Some sacoglossan sea slugs are capable of retaining functional chloroplasts 'stolen' from macroalgae (kleptoplasts). The present study surveyed the pigment composition of the sea slug *Elysia viridis* (Montagu, 1804) and its food source *Codium tomentosum* from three different locations along the Portuguese coast. The pigments siphonaxanthin, *trans* and *cis*-neoxanthin, violaxanthin, siphonaxanthin dodecenoate, chlorophyll (Chl) *a* and Chl *b*, ε,ε- and β,ε-carotenes and an unidentified carotenoid were observed in all *E. viridis* analysed. With the exception of the unidentified carotenoid, the same pigment profile was recorded for the macroalga *C. tomentosum*. Pigments characteristic of other macroalgae present in the sampling locations (*Ulva* sp. or the epiphyte *Ceramium* sp. present on *C. tomentosum*) were not detected in the slugs (Chl *c*, fucoxanthin, lutein, β,β-carotene). These results suggest that *E. viridis* retained chloroplasts exclusively from *C. tomentosum*. The differentiation between sea slugs and respective food source from different locations indicated that the site of collection was less relevant to the separation of groups than differences between the macroalgae and the sea slugs. In general, the carotenoids to Chl *a* ratios were significantly higher in *E. viridis* than in *C. tomentosum*. Further analysis using starved individuals suggests carotenoid retention over Chls during the digestion of kleptoplasts. Finally, despite a loss of 80% of Chl *a* in *E. viridis* starved for 2 weeks, measurements of maximum quantum yield of photosystem II (F_v/F_m) using variable Chl *a* fluorescence indicated a decrease of only 5% of the photosynthetic capacity of kleptoplasts.

INTRODUCTION

Some sacoglossan sea slugs (Opisthobranchia) are capable of incorporating macroalgal chloroplasts in tubule cells of their digestive diverticula (e.g. Greene, 1970; Hinde & Smith, 1972; Clark *et al.*, 1981; Green *et al.*, 2000; Curtis, Massey & Pierce, 2006; Evertsen *et al.*, 2007; Händeler *et al.*, 2009; Jesus, Ventura & Calado, 2010). These 'stolen' plastids (commonly termed kleptoplasts) remain functional within the animal cells, a process known as kleptoplasty (recently reviewed by Rumpho *et al.*, 2011; Pierce & Curtis, 2012; Cruz *et al.*, 2013). Kleptoplasts are able to fix carbon (e.g. Greene, 1970; Hinde & Smith, 1972; Clark *et al.*, 1981; Clark, Jensen & Stirts, 1990) and transfer photosynthates to the sea slug (Trench, Boyle & Smith, 1973b), although the nutritional value of photosynthesis in these organisms is largely unknown (Christa *et al.*, 2014).

The retention of functional kleptoplasts in sea slugs is quite variable among species (e.g. Clark *et al.*, 1990; Evertsen *et al.*, 2007). For instance, *Elysia chlorotica* (Gould, 1870) feeds on the siphonous macroalga *Vaucheria litorea* and retains photosynthetically active chloroplasts for several months (Green *et al.*, 2000). Green *et al.* (2005) concluded that chloroplasts of *V. litorea* were more robust than typical land plant chloroplasts and related this stability to their long-term functioning in the cytosol of *E.*

chlorotica cells. The link between robust plastids and their prolonged survival inside animal cells had already been suggested (Trench, Boyle & Smith, 1973a; Trench & Ohlhorst, 1976; and references therein). Furthermore, this 'robustness' may be related to the kleptoplasts' capability of photodamage repair at the photosystem II level (de Vries *et al.*, 2013). Therefore, the algal origin of kleptoplasts may be an important factor determining their functional longevity.

Early works identifying the source of retained kleptoplasts have relied on observations of the crawling activity of sea slugs on macroalgae, along with feeding experiments (e.g. Clark & Busacca, 1978; Jensen, 1980). Due to the high level of uncertainty of these approaches, the use of molecular (sequencing of *tufA* and/or *rbcL* genes, e.g. Curtis *et al.*, 2006; Wägele *et al.*, 2011; Maeda *et al.*, 2012; Christa *et al.*, 2013) and biochemical (pigment profiles using HPLC; Evertsen & Johnsen, 2009; Costa *et al.*, 2012; Ventura, Calado & Jesus, 2013) tools has become more common when identifying the macroalgal source of retained kleptoplasts.

In this study, we present a detailed description of the pigment (Chls and carotenoids) profile of *Elysia viridis* (Montagu, 1804) from three different locations along the Portuguese coast. We investigated if there are any differences between populations from different sites, and between the sea slugs and their respective food source. To further explore the differences found between sea slugs and their macroalgal food source, we analysed changes in pigment profiles of sea slugs deprived of any exogenous food source.

MATERIAL AND METHODS

Sampling

Adults of the sea slug *Elysia viridis* were collected, during summer 2012, in intertidal rock pools on the Portuguese west coast: Aguda beach, Vila Nova de Gaia (41° 02′ 39.99″ N, 8° 39′ 09.20″' W), Barra beach, Aveiro (40° 38′ 37.67″ N, 8° 44′ 56.75″ W) and Baleal beach, Peniche (39° 22′ 40.74″ N, 9° 20′ 25.26″ W). All sea slugs collected were always found on the macroalga *Codium tomentosum*. This species and another putative food source of *E. viridis* from the same sampling sites, *Ulva* sp. (Hawes, 1979; Jensen, 1989; Händeler & Wägele, 2007), were also sampled. In the laboratory and for each location, five individuals of *E. viridis* measuring at least 8 mm in total length were frozen in liquid nitrogen on the same day of collection. Samples of collected macroalgae were also frozen in liquid nitrogen, including the epiphyte *Ceramium* sp. found on *C. tomentosum* collected at Barra.

Starvation experiment

Specimens of *E. viridis* collected in Barra beach (Aveiro), measuring between 9 and 12 mm, were maintained in recirculating seawater under low light (incident light at water surface: 20 μmol photons m^{-2} s^{-1}) at 18°C and on a 14:10 h light:dark photoperiod. Stocked specimens were able to feed on *C. tomentosum* for 2 weeks, after which three individuals of *E. viridis* were frozen for pigment analysis (T0). *Codium tomentosum* was then removed from the system (depriving stocked sea slugs from feeding on any macroalgae) and three animals were sampled after 1 (T1) and 2 weeks (T2) of starvation. Immediately before sampling for pigment analysis, animals were dark-incubated for 30 min and the maximum quantum efficiency of photosystem II ($F_v/F_m = (F_m - F_o)/F_m$, where F_m and F_o are the maximum and minimum fluorescence, respectively) was determined. Fluorescence measurements were performed using a Pulse Amplitude Modulation (PAM) fluorometer comprising a computer operated PAM-Control Unit (Walz, Effeltrich, Germany) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments GmbH, Würzburg, Germany) (further details in Serôdio, 2004). The light delivered by the fluorometer and the fluorescence emitted by the sample were conducted by a 6 mm-diameter Fluid Light Guide fibre optics bundle in direct contact with a coverslip covering the animals as described by Cruz *et al.* (2012).

Pigment analysis

Sea slugs and macroalgal samples were freeze-dried and pigments extracted in 95% cold buffered methanol (2% ammonium acetate). Samples were ground with a glass rod, sonicated for 30 s and briefly vortexed. Samples were then transferred to −20°C for 20 min in the dark. Extracts were filtered through 0.2-μm Fluoropore membrane filters (Millipore, Billerica, MA, USA) and immediately injected into a HPLC system (Shimadzu, Kyoto, Japan) with a photodiode array detector (SPD-M10AVP). Chromatographic separation was carried out using a Supelcosil C18 column (25 cm length; 4.6 mm diameter; 5 μm particles; Sigma-Aldrich, St. Louis, MO, USA) for reverse phase chromatography and a 35 min elution programme. The solvent gradient followed Kraay, Zapata & Veldhuis (1992), with an injection volume of 100 μl and a flow rate of 0.6 ml min^{-1}. Pigments were identified from absorbance spectra and retention times and concentrations calculated from the signals in the photodiode array detector in comparison with pure crystalline standards from DHI (Hørsolm, Denmark). The fucoxanthin standard was used for the quantification of siphonaxanthin and siphonaxanthin dodecenoate because no purified standard or specific absorption coefficients were available (Egeland *et al.*, 2011). Concentration of each pigment was normalized by the individual Chl *a* concentration (P/Chl *a*) or by the individual dry weight (P/dw).

Statistical analyses

A resemblance matrix was calculated using Euclidean distances measurements between transformed ($\mathrm{Log}(X+1)$) values of P/dw or P/Chl *a* of the different samples. Spatial projection of the three populations of *E. viridis* and *C. tomentosum* were evaluated using principal coordinates analysis (PCO). Based on the same resemblance matrix, a two-way crossed analysis of similarities (ANOSIM) using all possible permutations was performed. To identify which pigments provided the main differentiation between groups, a two-way similarity percentage (SIMPER) analysis was performed based on transformed pigment data. PCO, ANOSIM and SIMPER were performed using Primer 6.1.11 and PERMANOVA + (PRIMER-E, UK). Differences (1) in individual pigment concentrations between the different populations of *E. viridis* and *C. tomentosum* (Aguda, Barra and Baleal), (2) in pigments to chlorophyll *a* ratios between *E. viridis* and *C. tomentosum* in the three studied locations, and (3) between fed and starved animals were tested using one-way analysis of variance (ANOVA). Post-hoc comparisons were made with Tukey HSD tests. ANOVA and post-hoc comparisons were performed using Statistica 10 (StatSoft, USA).

RESULTS

Pigments recorded in *Elysia viridis* from all three sampled populations were the carotenoids siphonaxanthin (Siph), *trans* and *cis*-neoxanthin (*t*-Neo and *c*-Neo), violaxanthin (Viola), siphonaxanthin dodecenoate (Siph-do), ε,ε- and β,ε-carotenes (εε-Car and βε-Car) and chlorophylls *a* and *b* (Chl *a* and Chl *b*) (Fig. 1; Table 1). An unidentified carotenoid (unid-Carot) was also found in *E. viridis* (Peak 6, Fig. 1; Table 1). The pigment profile of *Codium tomentosum* matched that of *E. viridis* (Fig. 1), with the exception of the unid-Carot that was never detected in *C. tomentosum*. Pigments lutein (Lute) and β,β-carotene

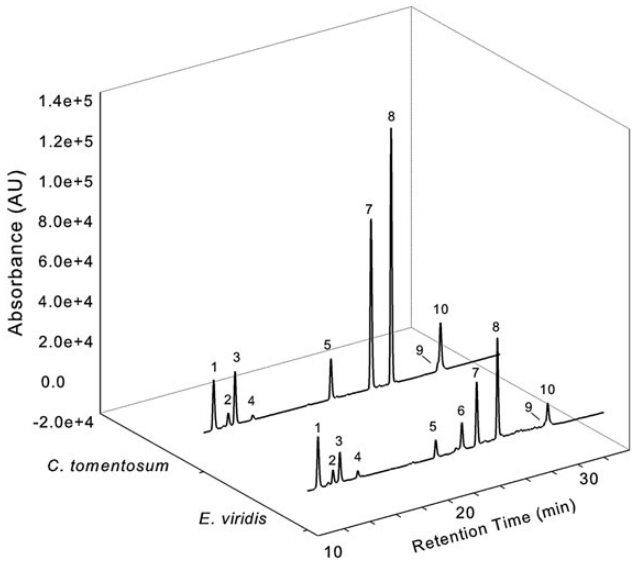

Figure 1. Typical HPLC absorbance (440 nm) chromatograms for *Elysia viridis* and *Codium tomentosum*. Numbers indicate the pigments listed in Table 1.

Table 1. List of pigments found in *Elysia viridis*, with average retention times and absorption maxima (λ max).

	Retention time (min)	λ max (nm)
1. Siphonaxanthin (Siph)	11.50	449
2. all-*trans*-Neoxanthin (*t*-Neo)	12.73	418,442,471
3. 9′-*cis*-Neoxanthin (*c*-Neo)	13.28	414,438,467
4. Violaxanthin (Viola)	14.77	417,441,471
5. Siphonaxanthin dodecenoate (Siph-do)	21.10	456
6. *E. viridis* unidentified carotenoid (unid-Carot)	23.23	460
7. Chlorophyll *b* (Chl *b*)	24.41	458,597,646
8. Chlorophyll *a* (Chl *a*)	26.06	431,617,663
9. ε,ε-Carotene (εε-Car)	29.81	417,441,471
10. β,ε-Carotene (βε-Car)	30.04	425,448,476

(ββ-Car) found in *Ulva* sp. were not observed in the sea slugs. Furthermore, pigments of *Ceramium* sp. (epiphytic on *C. tomentosum*) such as chlorophyll *c* (Chl *c*), fucoxanthin (Fuco) and ββ-Car were also not observed in *E. viridis*. The unid-Carot was not detected in any of the macroalgae analysed.

Pigment concentrations per dry weight found in *E. viridis* and *C. tomentosum* are shown in Table 2. The differentiation between sea slugs and respective food source from different locations indicated that there was a much stronger separation between species when evaluating both the ratios P/Chl *a* (Fig. 2A and ANOSIM: $R = 0.848$, $P = 0.006\%$) and P/dw (Fig. 2B and ANOSIM: $R = 0.825$, $P = 0.006\%$) than when evaluating the differentiation between locations (P/Chl *a*: global $R = 0.458$, $P = 0.01\%$; P/dw: global $R = 0.541$, $P = 0.006\%$). In general, macroalgal samples were more closely related to each other, regardless of their original location, than the sea slugs (Fig. 2A). When P/dw were evaluated (Fig. 2B) instead of the ratio P/Chl *a*, sea slugs collected in Baleal appear close to their food source while sea slugs collected in Barra or Aguda appear clearly separated from their food sources.

The SIMPER analysis to the P/dw values identified Chls *a* and *b* as the main source for differentiation between species

Table 2. Pigment concentrations (mg g^{-1} dw) in *Elysia viridis* and *Codium tomentosum* from three different locations along the Portuguese coast (mean ± standard deviation, $n = 5$).

	Barra (Aveiro)	Baleal (Peniche)	Aguda (V.N. Gaia)
Siph			
E. viridis	0.246 ± 0.048^{a}	0.340 ± 0.029^{b}	0.343 ± 0.053^{b}
C. tomentosum	0.290 ± 0.039^{a}	0.238 ± 0.033^{a}	0.497 ± 0.059^{b}
t-Neo			
E. viridis	$0.137 \pm 0.040^{a,b}$	0.156 ± 0.027^{a}	0.091 ± 0.025^{b}
C. tomentosum	0.145 ± 0.071^{a}	0.139 ± 0.049^{a}	0.173 ± 0.047^{a}
c-Neo			
E. viridis	0.152 ± 0.024^{a}	0.268 ± 0.025^{b}	0.218 ± 0.050^{b}
C. tomentosum	0.229 ± 0.026^{a}	0.181 ± 0.025^{a}	0.407 ± 0.041^{b}
Viola			
E. viridis	0.035 ± 0.012^{a}	0.037 ± 0.005^{a}	0.018 ± 0.003^{b}
C. tomentosum	$0.031 \pm 0.009^{a,b}$	0.021 ± 0.003^{a}	0.035 ± 0.003^{b}
Siph-do			
E. viridis	0.118 ± 0.030^{a}	0.227 ± 0.018^{b}	0.160 ± 0.045^{a}
C. tomentosum	0.212 ± 0.015^{a}	0.197 ± 0.027^{a}	0.408 ± 0.048^{b}
Chl *b*			
E. viridis	0.806 ± 0.237^{a}	1.547 ± 0.113^{b}	$1.156 \pm 0.335^{a,b}$
C. tomentosum	1.827 ± 0.182^{a}	1.748 ± 0.244^{a}	3.372 ± 0.395^{b}
Chl *a*			
E. viridis	1.197 ± 0.365^{a}	2.214 ± 0.189^{b}	$1.719 \pm 0.515^{a,b}$
C. tomentosum	2.633 ± 0.172^{a}	2.520 ± 0.343^{a}	5.002 ± 0.579^{b}
εε-Car			
E. viridis	0.009 ± 0.002^{a}	0.010 ± 0.002^{a}	0.010 ± 0.003^{a}
C. tomentosum	0.005 ± 0.001^{a}	0.010 ± 0.002^{a}	0.023 ± 0.006^{b}
βε-Car			
E. viridis	0.120 ± 0.030^{a}	0.140 ± 0.023^{a}	0.102 ± 0.028^{a}
C. tomentosum	0.094 ± 0.007^{a}	0.122 ± 0.024^{a}	0.234 ± 0.079^{b}

Different letters indicate significant differences ($P < 0.05$) between locations. Pigment abbreviations according to Table 1.

(93%). In addition, Figure 3A shows that total carotenoids (Siph + *t*-Neo + *c*-Neo + Viola + Siph-do + εε-Car + βε-Car) to Chl *a* ratios were significantly ($P < 0.001$) higher in *E. viridis* than in the respective food source, while no significant differences were observed for Chl *b* to Chl *a* ratio between *E. viridis* and *C. tomentosum* (Fig. 3B).

When animals were deprived from their food source, a significant decrease in P/dw values was observed after 1 or 2 weeks of starvation (Table 3). Chl *a* decreased to 75 and 20% in individuals starved for 1 and 2 weeks, respectively. Total carotenoids to Chl *a* ratio significantly ($P < 0.001$) increased from 0.55 ± 0.02 to 0.94 ± 0.08 after the 2 weeks starvation period, while Chl *b* to Chl *a* ratios remained constant (Fig. 4). There was also a significant ($P < 0.05$) effect of starvation on maximum photosynthetic rates: F_v/F_m values decreased from 0.814 ± 0.007 to 0.778 ± 0.017 after 2 weeks of starvation. However, this represented only a small decrease of 5% from the initial values.

DISCUSSION

Specific pigments present in the profile of *Ulva* sp. and in the epiphytes (*Ceramium* sp.) of *Codium tomentosum* were not recorded in *Elysia viridis*. On the other hand, with the exception of one unid-Carot, the pigment profile of sea slugs matched that of *C. tomentosum*. The pigment profile of the siphonaceous marine alga *C. tomentosum* was similar to that reported for *Codium fragile* (Benson & Cobb, 1981), with a spectrum of carotenoids notable by the absence of ββ-Car and the presence of εε-Car, βε-Car,

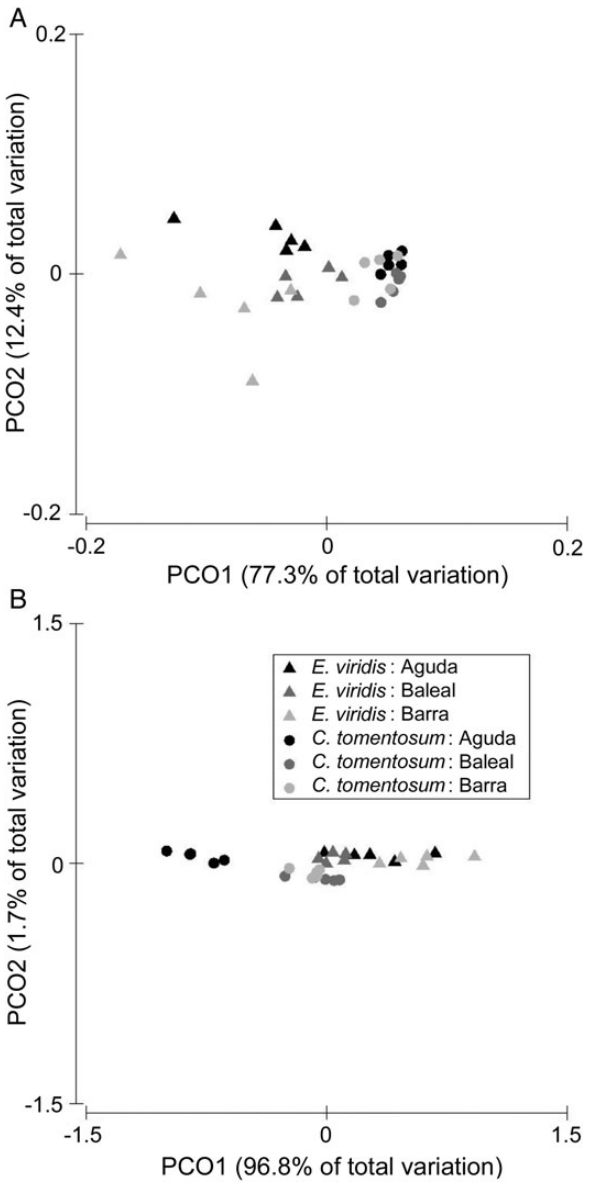

Figure 2. Two-dimensional principal coordinates analysis (PCO) based on photosynthetic pigments profile displayed by *Elysia viridis* and *Codium tomentosum* from three different locations (Aguda, Baleal and Barra). **A.** PCO plot based on the ratio pigment per Chl *a* (P/Chl *a*). **B.** PCO plot based on the pigment concentration per dry weight (P/dw). Both P/Chl *a* and P/dw values refer to each individual pigment identified and quantified using HPLC methods.

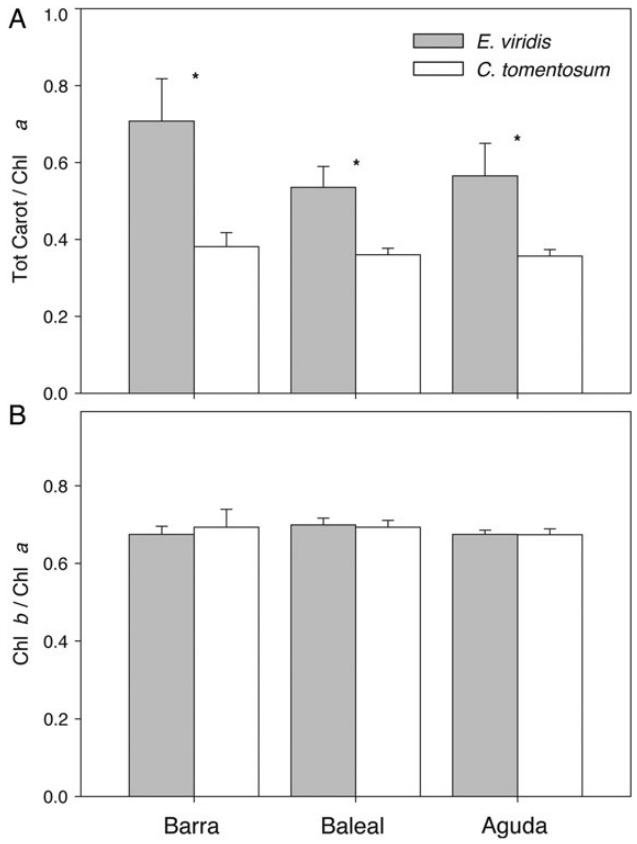

Figure 3. Pigment to chlorophyll *a* ratios (mean ± standard deviation, $n = 5$) in *Elysia viridis* and *Codium tomentosum* in the three studied locations (Aguda, Baleal and Barra). **A.** Total carotenoids. **B.** Chlorophyll *b*. * indicates significant differences between *E. viridis* and *C. tomentosum* at each location (one-way ANOVA, $P < 0.001$).

Table 3. Pigment concentrations (mg g^{-1} dw) in satiated *Elysia viridis* (T0) and in individuals starved for 1 (T1) and 2 (T2) weeks.

	T0	T1	T2
Siph	0.290 ± 0.047[a]	0.149 ± 0.051[b]	0.147 ± 0.062[b]
t-Neo	0.140 ± 0.021[a]	0.090 ± 0.037[a]	0.025 ± 0.003[b]
c-Neo	0.222 ± 0.017[a]	0.127 ± 0.039[b]	0.075 ± 0.010[b]
Viola	0.029 ± 0.002[a]	0.022 ± 0.008[a]	0.005 ± 0.000[b]
Siph-do	0.161 ± 0.014[a]	0.115 ± 0.032[a]	0.025 ± 0.004[b]
Chl *b*	1.207 ± 0.119[a]	0.920 ± 0.266[a]	0.223 ± 0.023[b]
Chl *a*	1.790 ± 0.216[a]	1.341 ± 0.419[a]	0.337 ± 0.038[b]
εε-Car	0.009 ± 0.002[a]	0.004 ± 0.001[b]	0.002 ± 0.002[b]
βε-Car	0.130 ± 0.029[a]	0.062 ± 0.029[b]	0.038 ± 0.005[b]

Different letters indicate significant differences ($P < 0.05$) between T0, T1 and T2. Pigment abbreviations as in Table 1.

Siph, Siph-do, Neo and Viola. The presence of a light-harvesting Siph-Chl *a*/*b* protein complexes allow enhanced absorption of blue-green and green light (Anderson, 1983). This may be important in this species due to the high thickness and density of the thallus, where the available light to most chloroplasts is likely to be predominantly green and reduced on blue and red. In a similar manner, the presence of these light-harvesting complexes in kleptoplasts of *E. viridis* will enable the sea slugs to photosynthesize while dwelling on dense *Codium* sp. fronds.

Hawes (1979) described the ultrastructure of kleptoplasts of *E. viridis* as derived from *C. fragile*, but also reported the existence of non-*Codium* chloroplasts, possibly originating from *Ulva*. In our study, the absence of Lute and ββ-Car, pigments found in *Ulva* sp., show that in the three locations *E. viridis* did not retain kleptoplasts from this macroalga. Evertsen & Johnsen (2009) reported a similar pigment profile for *E. viridis* on the coast of Norway feeding on *C. fragile*, with the exception of the presence of a Chl *c*-like pigment, which was interpreted by the authors as remains of phaeophytes (brown algae) in the sea slugs. No Chl *c* or Fuco pigments were present in *E. viridis*, excluding the possibility of brown algae being a source of the kleptoplasts present in the specimens surveyed in the present work. The food sources of photosynthetic sea slugs may be an important factor determining the longevity of kleptoplasts in the animal host (Green *et al.*,

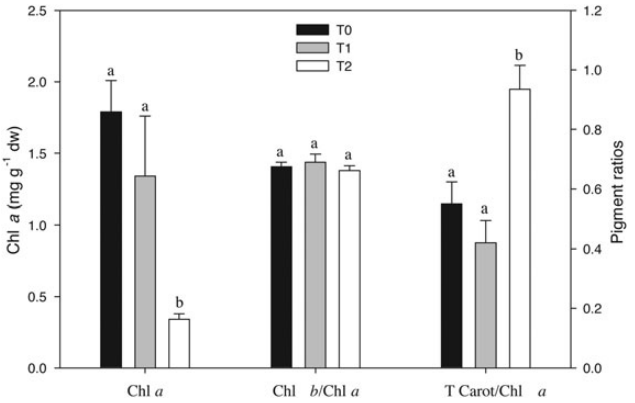

Figure 4. Concentrations of chlorophyll *a* (mg g^{-1}), chlorophyll *b* and total carotenoid to chlorophyll *a* ratios (mean ± standard deviation, $n = 3$) in satiated *Elysia viridis* (T0) and in individuals starved for 1 week (T1) and 2 weeks (T2). Different letters indicate significant differences between T0, T1 and T2 (Tukey HSD, $P < 0.01$).

2005) and it is therefore important to investigate their source. Although HPLC pigment analysis cannot be used to distinguish between algae with exactly the same pigment signature, pigments of adult *E. viridis* in the three studied locations were sufficiently specific to identify *Codium* as the sole origin of acquired kleptoplasts.

In general, carotenoids found in animals are either directly accumulated from food or partially modified through metabolic reactions (see Maoka, 2011 and references therein). Therefore, the unid-Carot was most likely a product modified by the sea slugs since it was not present in their food source. For example, apocarotenoids derived from β-carotene, Lute and zeaxanthin (Zeax) were observed in the sea hare *Aplysia kurodai* (Yamashita & Matsuno, 1990). Retention time and absorption spectrum of unid-Carot matched those of trans-β-apo-8′-carotenal, commonly used as an internal standard in HPLC pigment analysis. The same pigment (unid-Carot) was present in *E. chlorotica* and not in its food source *Vaucheria litorea*. Moreover, this unid-Carot was retained in starved *E. chlorotica* individuals when all other (kleptoplast) pigments were degraded (K. N. Pelletreau & M. E. Rumpho, unpubl.). A similar unidentified carotenoid was also found in *E. timida* (Costa *et al.*, 2012), which the authors correlated with the presence of red spots and their properties in the response to light variations (Rahat & Monselise, 1979).

Recently, Jesus *et al.* (2010) identified the presence of the pigments antheraxanthin (Anth) and Zeax in *Elysia timida* (Risso, 1818) and described the occurrence of a functional violaxanthin cycle involved in photoprotection. In this study, concentrations of Viola were low in *C. tomentosum* and *E. viridis* and no detectable levels of Anth or Zeax were observed in either organism. Therefore, the occurrence of a similar photoregulatory mechanism in *E. viridis* seems unlikely, but requires further research.

The differentiation between sea slugs and their respective food source from different locations showed that the sea slugs are clearly different from their food source as evaluated by the ratio P/Chl *a*. When P/dw values are taken into account, other dynamics emerge but overall the differentiation between sea slugs and algae is always stronger than the differentiation between sites of collection. Based on P/dw values, *C. tomentosum* collected in Aguda was discriminated from the other two locations by the significantly higher values of Chls *a* and *b* (approximately twice the values found in Baleal or Barra), suggesting photoacclimation to lower light levels in this population (Murchie & Horton, 1997). Surprisingly, the differentiation between *E. viridis* populations from different locations does not match that of *C. tomentosum*. As discussed below, sea slugs may retain carotenoids from digested kleptoplasts. Therefore, we suggest that different life history such as age, exposure to the food source and its quality are all factors that influence the pigment composition in each *E. viridis* population, resulting in a differential profile from the respective food source collected at a single point in time.

The separation of species using P/dw values mostly derived from the Chls *a* and *b*. Together with the observation that the ratio of carotenoids/Chl *a* was always higher in *E. viridis* than their food source, we hypothesized that this trend is a result of selective retention or simply a slower degradation of carotenoids over time. To investigate how pigment composition fluctuates after plastids retention, we examined the effect of food deprivation on the pigment fingerprint of *E. viridis*. The results showed that the same pigment signature was maintained but that the ratio of carotenoids/Chl *a* increased in starved animals, confirming a retention of carotenoids over Chls by the sea slugs. This result was contrary to that reported by Evertsen & Jonhsen (2009) showing similar levels of photosynthetic pigments per Chl *a* in *E. viridis* and *C. fragile*. One possible explanation for differences between our work and that of Evertsen & Johnsen (2009) could be the age of the sea slugs used in each study. Considering our assumption that *E. viridis* is able to perform a selective retention of carotenoids, it would be expected to find a higher carotenoid content in older specimens. This higher carotenoid content in older sea slugs would likely act as a source of bias for this type of analysis of the ratio of carotenoids/Chl *a*. Unfortunately, as the size of *E. viridis* is not directly related to their age, using sea slugs with similar sizes does not control for potential variability. Unless laboratory cultured specimens are employed (Dionísio *et al.*, 2013), the comparison of results from different studies should always be performed with caution.

The higher carotenoids/Chl *a* ratios in *E. viridis* described in the present work may be explained by a faster decay of Chls over carotenoids upon chloroplast acquisition. Ventura *et al.* (2013) have previously shown that in the sea slug *Thuridilla hopei* (Vérany, 1853) carotenoids seem to degrade at slower rates than Chls. Carotenoids are known to play numerous roles in marine animals, namely camouflage, photoprotection and signalling, as well as acting as antioxidants, enhancers of immune activity and reproductive output and larval survival (see review by Maoka, 2011). Similar roles for carotenoids in *E. viridis* may explain the specific retention/synthesis of these pigments in the sea slugs.

Kleptoplasts of *E. viridis* were shown to remain functional in starved specimens for different periods of time (Hinde & Smith, 1972; Hawes & Cobb, 1980; Evertsen & Johnsen, 2009; Vieira *et al.*, 2009). It is important to note that a direct comparison between different studies on the longevity of sea slug kleptoplasts may be severely compromised by environmental factors and laboratorial artefacts (Cruz *et al.*, 2013). In the experimental conditions used in this study a significant loss (80%) in Chl *a* was observed after 2 weeks of starvation, while the decrease of maximum photosynthetic capacities in the same period was considerably lower (5%). Not surprisingly, these results show that photosynthetic activity measured by variable Chl *a* fluorescence can be high despite only a reduced number of photosystems remaining active. Similar to what has been observed in previous studies (Vieira *et al.*, 2009; Jesus *et al.*, 2010), we expect that given enough time and with more severe loss of Chl *a* the F_v/F_m values would decrease drastically following a biphasic response.

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