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Nutritional state determines reproductive investment in the mixotrophic sea slug *Elysia viridis*

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ABSTRACT: Parental size and nutritional state have been identified as important interrelated parameters determining reproductive investment in marine gastropods. However, studies on reproductive investment of sacoglossan sea slugs capable of incorporating intracellular functional chloroplasts (kleptoplasts) from their food sources are scarce. In the present work, we investigated the effects of different levels of availability of the macroalga *Codium tomentosum* on the reproductive investment of the mixotrophic sea slug *Elysia viridis*. Limited food availability decreased sea slug size (dry weight), kleptoplast abundance (chl *a* concentration), and photosynthetic capacity (PSII maximum quantum yield, F_v/F_m). Furthermore, sea slugs with limited access to food spawned significantly smaller egg masses and displayed a reduced number of eggs per egg mass. Intermittently fed sea slugs spawned a lower number of egg masses than continuously fed and starved sea slugs, indicating a trade-off between feeding and spawning activity under limited resources. No detectable effects of food limitation were observed on the size of individual eggs and total fatty acid content per egg. However, starved sea slugs produced eggs richer in saturated fatty acids, namely stearic acid (18:0). On the other hand, sea slugs with unlimited access to food spawned eggs richer in polyunsaturated fatty acids, such as linoleic, eicosatrienoic, and eicosapentaenoic acids (18:2 *n*-6, 20:3 *n*-3, and 20:5 *n*-3, respectively). In conclusion, nutritional state significantly affected resource allocation to reproductive traits in *E. viridis*.

KEY WORDS: *Codium tomentosum* · Egg mass · Fatty acid · Fecundity · Kleptoplasty · Parental size · Sacoglossa

1. INTRODUCTION

Sacoglossan sea slugs are unique in that they are the only metazoans for which incorporation and maintenance of intracellular functional chloroplasts (kleptoplasty) has been described (Serôdio et al. 2014). Several sacoglossan sea slug species use their radular teeth to penetrate the cell wall of algal filaments, suck and digest the cellular content, while incorporating functional algal chloroplasts into tubular cells of their digestive gland. Most studies support the assumption that these organisms are mixotrophic, obtaining organic carbon via both heterotrophic and phototrophic metabolisms (e.g. Hinde & Smith 1975, Giménez Casalduero & Muniain 2008, Yamamoto et al. 2013, Cartaxana et al. 2017). Recently published transcriptomic data further indicate that kleptoplasts are integrated energy powerhouses supporting animal development (Chan et al. 2018).

The sacoglossan sea slug *Elysia viridis* (Montagu, 1804) is found along the coasts of Scandinavia (Evert-

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sen & Johnsen 2009), the British Isles (Trowbridge 2000), and the Iberian Peninsula (Cruz et al. 2013). Along the Portuguese coast, it feeds on the coenocytic green macroalga *Codium tomentosum* Stackhouse, 1797, from which it acquires functional kleptoplasts (Cruz et al. 2014). Similarly, *C. tomentosum* occurs along the entire Portuguese coast (Pereira et al. 2006), with maximum growth during the summer months. As in other *Codium* species, macroscopic thalli generally disappear during winter, and perennial holdfasts regenerate new plants when environmental conditions improve (Burrows 1991). Hence, seasonal variation in food availability may be a key parameter determining the abundance of *E. viridis*. Despite the importance of nuclear-encoded proteins for plastid function, *Codium* chloroplasts may remain functional in *E. viridis* for several weeks (Evertsen et al. 2007). We have recently shown that kleptoplast photosynthesis is nutritionally relevant in *E. viridis* in the absence of food, although photosynthetic activity decreases if the macroalgal food source is unavailable and kleptoplasts are not replaced (Cartaxana et al. 2017). Therefore, food availability has an impact on both heterotrophic and autotrophic nutrition in this sea slug species.

E. viridis is a simultaneous hermaphrodite, each individual possessing both male and female sexual systems, reciprocally exchanging sperm between partners (Jensen 2001). After fertilization, round egg masses are deposited on *Codium* spp. thalli or surrounding benthic substrata. Planktotrophic larvae hatch from spawned eggs and must feed on plankton in the water column in order to successfully metamorphose into the juvenile stage (Trowbridge 2000, Jensen 2001).

Parental size and nutritional state have been identified as important parameters determining reproductive investment in marine gastropods, with larger and better nourished mothers having more resources to be allocated to reproduction (Chester 1996, Ito 1997, Gianguzza et al. 2005, Allen et al. 2009). Individuals that are stressed by limited resources or other environmental parameters tend to respond by reducing resource allocation to reproductive traits, which may include the reduction of spawning events, egg number, and size and energy content of individual eggs (e.g. Chester 1996, Allen et al. 2009, Dionísio et al. 2017).

Embryonic development of most marine invertebrates relies on yolk, where lipids play a central role as energetic resources and structural components of cell membranes (Meyer et al. 2007, Rey et al. 2015). Phospholipids, cholesterol, and triacylglycerols (TAGs) represent the most abundant lipid classes in marine invertebrate embryos (Benkendorff et al. 2005, Byrne et al. 2008, Prowse et al. 2017), being responsible for energy homeostasis, architecture of cellular membranes, cellular signaling, and the trafficking of yolk to the embryo (Fraher et al. 2016). Most of these functions are defined by the fatty acid (FA) composition of the lipids. In general, saturated and monounsaturated FAs (SFAs and MUFAs) have been associated with energetic functions, while polyunsaturated FAs (PUFAs) are related to structural and signaling lipids (Byrne et al. 2008, Rey et al. 2018). PUFAs such as arachidonic (20:4 *n*-6, ARA) and eicosapentaenoic (20:5 *n*-3, EPA) acids play a relevant role in embryonic development and early larval fitness. Several studies on marine invertebrates have associated a high abundance of these FAs with hatching success and early larval performance (Leonardos & Lucas 2000, Hendriks et al. 2003, Leroy et al. 2013). Therefore, embryonic lipid reserves represent an indicator of physiological conditions and could be used as a proxy of offspring fitness (Gallager et al. 1986, Tenore et al. 1995).

In the present work, we investigated changes in the reproductive investment of *E. viridis* exposed to different levels of *C. tomentosum* availability. We tested whether the nutritional state affected resource allocation to the following reproductive traits: egg mass size and dry weight (DW), egg number and volume, and FA profile. We hypothesized that decreased food availability will reduce resource allocation for reproduction.

2. MATERIALS AND METHODS

2.1. Sample collection and experimental set-up

The sea slug *Elysia viridis* and the macroalga *Codium tomentosum* were collected in September 2016 during low tide in the intertidal rocky area of Aguda beach, Vila Nova de Gaia, Portugal (41° 02′ 50.2″ N, 8° 39′ 15.2″ W). The timing of the experiment was chosen based on previous seasonal observations of higher abundance of bigger animals and number of egg masses deposited on *Codium* thalli. Animals were kept in aerated water collected at the sampling site and transported to the laboratory within 2 h. Sea slugs and their macroalgal food were maintained in a 150 l recirculated life support system (LSS) operated with artificial seawater (ASW; Red Sea Salt) at the temperature and salinity measured at the sampling site (temperature: 18°C, salinity: 32).

Individuals were acclimated to laboratory conditions for 2 wk before the beginning of the experiments, exposed to a light:dark photoperiod of 14:10 h and to irradiance levels of 80 µmol photons m^{-2} s^{-1} measured at the water surface using a ULM-500 Universal Light Meter and a Spherical Micro Quantum Sensor (Heinz Walz). The acclimation period was chosen to ensure replicability concerning the animals' nutritional state and light history at the beginning of the experiment. The chosen irradiance level was an average of the range measured in the shaded intertidal rock pools where the animals were collected (Cartaxana et al. 2018).

A floating PVC tray with wells (56 mm diameter × 60 mm depth) was placed in the LSS described above. The bottom of the wells allowed water exchange through a 1 mm mesh. A recirculating water pump was placed below the experimental tray to increase water renewal inside the wells. Animals were measured for body length at full extension, while crawling on a Petri dish with ASW. Thirty sexually mature sea slugs of ca. 20 mm were selected, and pairs of individuals were placed inside individual wells (1 pair $well^{-1}$). Sea slugs inside the wells were exposed to an average irradiance of 80 µmol photons m^{-2} s^{-1}. The 15 pairs were randomly separated in 3 feeding treatments (5 replicates $treatment^{-1}$): (1) 'Continuously fed,' in which sea slugs could feed on fresh *C. tomentosum* during the whole experiment (macroalgae were changed in these wells twice a week); (2) 'Intermittently fed,' in which, each week, the stocked sea slugs were able to feed on freshly collected *C. tomentosum* for 24 h followed by 6 d without food; and (3) 'Starved,' in which the stocked sea slugs were deprived of food during the whole experimental period (35 d). At the beginning of the experiment, there were no significant differences in sea slug length between treatments ($F_{2,27}$ = 0.301, p = 0.742). At the end of the experimental period, sea slugs were frozen in liquid nitrogen and freeze-dried, and their individual DW was determined.

2.2. Chlorophyll *a* fluorescence

The photosynthetic capacity of sea slug specimens was assessed weekly measuring chlorophyll *a* (chl *a*) variable fluorescence with a JUNIOR-PAM fluorometer (Heinz Walz). Each sea slug was placed in a Petri dish with a thin layer of ASW and dark-adapted for 30 min. After this period, the optical fiber of the fluorometer was placed in direct contact with the animal's parapodia, a saturating pulse was applied, and minimum and maximum fluorescence levels were registered (F_o and F_m, respectively). Maximum quantum yield of photosystem (PS) II (F_v/F_m) was calculated as $(F_m - F_o)/F_m$ (Murchie & Lawson 2013). The parameter F_v/F_m was used as an indicator of photosynthetic capacity.

2.3. Chl *a* content

Total chl *a* content of sea slug specimens was quantified as described in detail by Cruz et al. (2014). Briefly, freeze-dried sea slugs were extracted in 95% cold-buffered methanol (2% ammonium acetate). After filtration, the extracts were injected into an HPLC system (Shimadzu) with a photodiode array detector (SPD-M20A). Chl *a* was identified from absorbance spectra, and retention times and concentrations were calculated using a calibration curve of 5 dilutions of a pure crystalline standard from DHI. Chl *a* concentrations were expressed per DW (µg mg^{-1}) and used as an indicator of kleptoplast abundance.

2.4. Mating behavior and egg mass characterization

Pairs of *E. viridis* initiate mating by protruding their transparent penises and entangling their bodies (Fig. 1A, and Video SV1 in Supplement 1 at www.int-res.com/articles/suppl/m611p167_supp/). The penis is located on the right side of the head, while the female genital aperture is located at the base of the right parapodium. Simultaneous insertion of the penises in the female apertures was observed followed by reciprocal insemination (Video SV2). If insertion was unilateral, the individual that did not insert the penis forced separation. After sperm transfer, one of the slugs removes the penis from the partner's female aperture and forces the end of copulation (Video SV3). Egg masses were laid through the female aperture and deposited on the walls of the wells and occasionally on the mesh at the bottom of the wells and on the macroalgae (when present). Egg masses produced by sea slugs were counted daily during the experimental period (35 d) and collected using a scalpel (Fig. 1B).

Egg mass characteristics were assessed in the last 2 egg masses produced by each of the 5 sea slug pairs per treatment in the 35 d period. Egg masses were photographed using a Leica DMS300 digital microscope. One photograph was taken with the

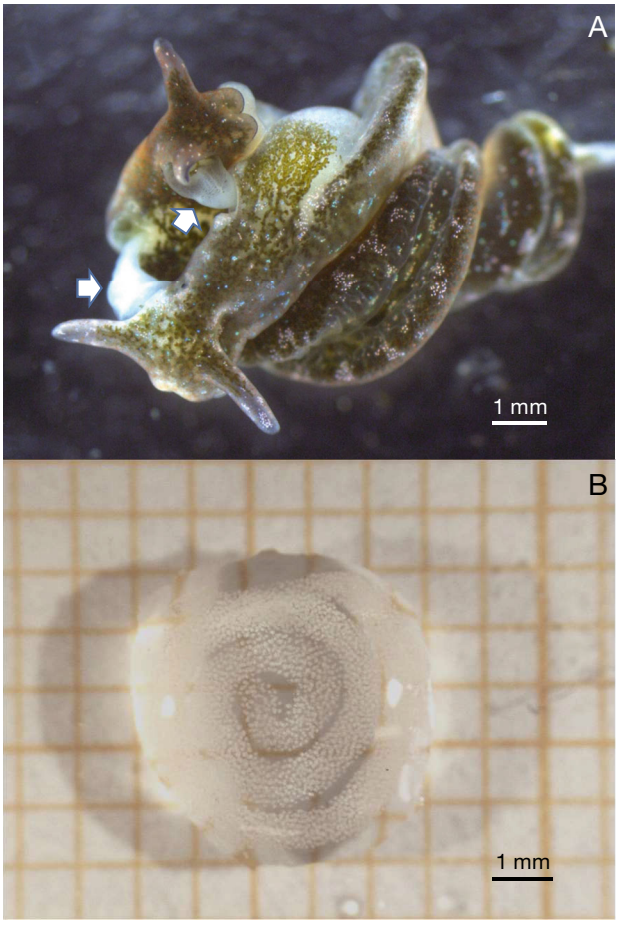

Fig. 1. (A) Two specimens of the hermaphrodite sea slug *Elysia viridis* copulating. Arrows indicate the transparent and elongated penis inserted in the partner's female genital aperture. (B) Egg mass spawned after internal fertilization

lowest magnification (15×, to view the entire egg mass), and several photographs were taken at the highest magnification (120×). Egg mass areas were calculated from the photographs with the lowest magnification using open source software ImageJ (version 1.49). The number of eggs and individual egg volume were estimated using the photographs with the highest magnification. Egg mass areas were selected using the freehand tool in ImageJ, whereas egg areas were estimated selecting circle areas around individual eggs. In each egg mass, 10 rectangles with a known area were defined and eggs inside that area were counted. Approximately 200 eggs were counted per egg mass, and the number of eggs in each egg mass was then extrapolated. The area of 10 random uncleaved eggs per rectangle was determined, and the volume of individual eggs was estimated from the measured area assuming a spherical shape. After photographing, egg masses were gently washed in ultrapure water, frozen at –80°C, and freeze-dried, and DW was determined prior to FA analysis.

2.5. FA analysis

Total lipids from egg mass samples were obtained using a solid-liquid extraction adapted from Bligh & Dyer (1959). Freeze-dried samples were transferred to glass centrifuge tubes and homogenized with a pestle. We added 200 µl of chloroform and 400 µl of methanol, following homogenization and incubation on ice for 30 min. An additional volume of 200 µl of chloroform was added and the mixture was homogenized. Samples were centrifuged at 568 × *g* (10 min at room temperature). The liquid phase containing lipids was transferred to glass tubes and dried with a nitrogen flow. Samples were kept at –20°C for further analysis.

FAs were analyzed through gas chromatography–mass spectrometry (GC-MS) after transmethylation (Aued-Pimentel et al. 2004). FA methyl esters (FAMEs) were obtained as follows: 1 ml of C19:0 internal standard (0.54 µg ml^{-1} in n-hexane, CAS number 1731-94-8, Merck) was added to the dried lipid extract, followed by 200 µl of a methanolic solution of potassium hydroxide (2M), and the mixture was well homogenized. Finally, 2 ml of an aqueous solution of sodium chloride (10 mg ml^{-1}) were added, and the sample was centrifuged for 5 min at 568 × *g* to separate the phases. The organic (upper) phase containing the FAMEs was transferred to a microtube and completely dried under nitrogen. FAMEs were then redissolved in 50 µl n-hexane, and 2 µl of this solution were used for GC-MS analysis on an Agilent Technologies 6890 N Network chromatograph equipped with a DB-FFAP column (123-3232, J&W Scientific) with 30 m length, an internal diameter of 0.32 mm, and a film thickness of 0.25 µm. The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the mass range m/z 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was set at an initial temperature of 80°C, standing at this temperature for 3 min and increasing linearly to 160°C at 25°C min^{-1}, followed by linear increases to 210°C at 2°C min^{-1} and 250°C at 30°C min^{-1}. Following this ramp, temperature was maintained at 250°C for 10 min. The injector and detector temperatures were 220 and 280°C, respectively. Helium was used as carrier gas at a flow rate of 1.4 ml min^{-1}. FAME

identification was performed by comparing retention times and mass spectra with those of commercial FAME standards (Supelco 37 Component FAME Mix, ref. 47885-U, Sigma-Aldrich) and confirmed by comparison with the Wiley library and the spectral library from 'The Lipid Web' (Christie 2018). FA quantification was performed using calibration curves obtained from FAME standards under the same instrumental conditions. FAs were expressed as: (1) µg per whole egg mass (µg FA egg mass^{-1}) and (2) µg FA egg^{-1} (FA content in whole egg mass divided by the number of eggs).

2.6. Statistical analysis

Data on the 2 slugs of each experimental unit (well) were averaged to avoid pseudoreplication, and the averages were treated as 5 independent replicates (Hurlbert 1984). Similarly, the last 2 egg masses produced in each experimental unit were averaged before statistical analysis. The existence of significant differences between treatments (continuously fed, intermittently fed, and starved) on measured parameters (sea slug DW and chl *a*, egg mass size and DW, number of eggs per egg mass, volume of individual eggs, and FA content) was tested using 1-way ANOVA. Normality was checked using a Shapiro-Wilk test, and homogeneity of variances using Levene's test. Multiple comparisons were performed using Tukey's HSD test. If data failed to meet ANOVA assumptions, Kruskal-Wallis non-parametric tests (H) were performed, followed by Dunn's post hoc tests. Significance levels were adjusted by the Bonferroni correction for multiple tests. The existence of significant differences in F_v/F_m was tested using a mixed ANOVA with time as a within-subjects factor and feeding treatment as a between-subjects factor. These statistical analyses were carried out using IBM SPSS Statistics 24. Furthermore, multivariate statistical analyses were performed with the most abundant FAs (>75% of total FA content). Prior to statistical analysis, in order to down-weight the contributions of quantitatively dominant FAs, the raw data matrix was log(x + 1) transformed. Following this transformation, a new matrix was assembled using the Bray-Curtis similarity coefficient (Clarke & Gorley 2006). The differences in FA profiles of eggs were analyzed by ordination analysis, using principal coordinates (PCO) analysis. To quantify the effect of each FA on potential differences recorded among feeding treatments, Spearman correlations were calculated. Only the FAs with a correlation coefficient of R > 0.70 were considered. Multivariate statistical tests were performed with Primer v6.1 with PERMANOVA + add-on (Clarke & Gorley 2006).

3. RESULTS

3.1. Sea slug characteristics

General characteristics of *Elysia viridis* after 35 d in 3 different feeding treatments are shown in Table 1. There was a significant effect of food availability ($F_{2,12}$ = 39.740, p < 0.001) on *E. viridis* DW. Sea slug DW was significantly higher for continuously fed, intermediate for intermittently fed, and lower for starved animals (in all cases p < 0.01) (Table 1). There was also a significant effect of food availability ($F_{2,12}$ = 40.543, p < 0.001) on *E. viridis* chl *a* concentrations, which were significantly higher in continuously and intermittently fed animals than in starved *E. viridis* (in both cases p < 0.001; Table 1). Although the mean chl *a* concentration was higher in continuously fed specimens, no significant differences (p = 0.052) were observed between continuously and intermittently fed animals. During the experimental period, 1 animal died on Day 35 in the starvation treatment. No additional mortality was recorded.

Table 1. Characteristics of *Elysia viridis* after 35 d in 3 different treatments: fed continuously with *Codium tomentosum* (Continously fed); fed 1 d per week with *C. tomentosum* (Intermittently fed); and deprived of food for the entire experimental period (Starved). Mean ± SD (n = 5) is shown. Different letters indicate significant differences between treatments (p < 0.05)

	Continuously fed	Intermittently fed	Starved
Sea slug dry weight (mg)	8.4 ± 1.5^a	5.3 ± 1.1^b	2.2 ± 0.3^c
Sea slug chl *a* (µg mg^{-1})	2.90 ± 0.58^a	2.18 ± 0.31^a	0.54 ± 0.33^b
Sea slug F_v/F_m (dimensionless)	0.73 ± 0.02^a	0.67 ± 0.01^a	0.20 ± 0.16^b
Sea slug mortality (n)	0	0	1
Average number of spawned egg masses ind.$^{-1}$	6.1	5.2	6.1
Average time for each slug to produce an egg mass (d)	5.7	6.7	5.7

The variation of *E. viridis* photosynthetic capacities (F_v/F_m) with time in the 3 feeding treatments is shown in Fig. 2. There was a significant effect of food availability ($F_{2,12}$ = 58.048, p < 0.001) on F_v/F_m, as well as a significant interaction ($F_{10,60}$ = 30.775, p < 0.001) between time and feeding treatments on this parameter. While F_v/F_m remained relatively constant in sea slugs of the continuously fed treatment during the 35 d experiment, it dropped 13% in the intermittently fed and 77% in starved animals. No significant differences between feeding treatments were registered at the beginning of the experiment, but after 35 d, F_v/F_m was significantly higher in continuously and intermittently fed than in starved *E. viridis* (in both cases p < 0.001; Table 1). After 14 d of treatment, F_v/F_m was already significantly higher (p = 0.001) in continuously fed than in starved sea slugs (Fig. 2).

3.2. Egg mass characteristics

Average egg mass production by *E. viridis* was 6.1 egg masses ind.$^{-1}$ in both continuously fed and starved treatments (Table 1). The number of egg masses produced by intermittently fed sea slugs was only 5.2 ind.$^{-1}$. Indeed, in the latter treatment, we registered no spawning activity during the 24 h in which the animals were feeding. Consequently, the average time for each sea slug to produce an egg mass was 6.7 d in the intermittently fed treatment and 5.7 d in both continuously fed and starved treatments (Table 1).

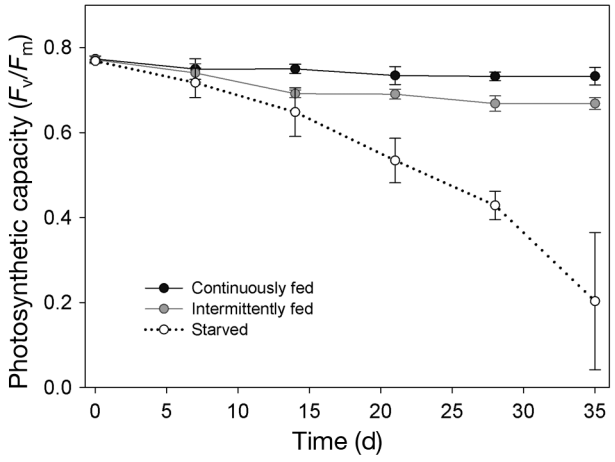

Fig. 2. Changes in photosynthetic capacity (F_v/F_m) of *Elysia viridis*. Animals were fed continuously with *Codium tomentosum* (Continuously fed), fed 1 d per week with *C. tomentosum* (Intermittently fed), or deprived of food for the 35 d experimental period (Starved). Mean ± SD, n = 5

The characteristics of the last egg masses produced by *E. viridis* in each feeding treatment are shown in Fig. 3. There was a significant effect of food availability (H_2 = 12.522, p = 0.002) on egg mass size (Fig. 3A). Significantly larger egg masses were produced by continuously fed *E. viridis* than by starved animals (p = 0.001). No significant differences were observed in the size of egg masses produced by intermittently fed sea slugs and animals of the other 2 treatments (in both cases p = 0.231). Similarly, there was a significant effect of food availability ($F_{2,12}$ = 84.181, p < 0.001) on egg mass weight (Fig. 3B). Egg mass DW was significantly higher in continuously fed, intermediate in intermittently fed and lower in starved *E. viridis* (in all cases p < 0.05). There was a significant effect of food availability ($F_{2,12}$ = 25.062, p < 0.001) on the number of eggs per egg mass (Fig. 3C). Significantly more eggs per egg mass were produced by continuously fed *E. viridis* than by intermittently fed (p = 0.001) and starved animals (p < 0.001). No significant differences (p = 0.164) were observed in the number of eggs per egg mass produced by intermittently fed and starved sea slugs. No significant effect of feeding treatment (H_2 = 0.740, p = 0.691) was observed on the volume of individual eggs (Fig. 3D).

3.3. Egg mass FA composition

FA analysis allowed the identification of 33 FAs (Tables S1 & S2 in Supplement 2 at www.int-res.com/articles/suppl/m611p167_supp/), although 2 FAs (18:3 *n*-6 and 18:4 *n*-3) were not detected in egg masses of starved individuals. Significant effects (in all cases p < 0.05) of feeding treatment were observed on the content of FA classes per egg mass, with SFAs, MUFAs, PUFAs, and total FAs decreasing with food limitation (Fig. 4A). When expressing FA content per egg, no significant effects of feeding treatment were observed on MUFAs, PUFAs, and total FAs ($F_{2,12}$ = 0.537, p = 0.598, $F_{2,12}$ = 2.983, p = 0.089, and $F_{2,12}$ = 0.029, p = 0.972, respectively; Fig. 4B). However, a significant effect of feeding treatment ($F_{2,12}$ = 4.880, p = 0.028) was observed on SFA content per egg. Significantly higher SFA content per egg (p = 0.024) was observed in egg masses produced by starved *E. viridis* than by continuously fed animals (Fig. 4B).

Individual FA content per egg revealed a trend of increased levels of SFAs and decreased levels of PUFAs with food limitation (Fig. 5, Table S2). Statistical analysis on individual FA content per egg showed significant differences (p < 0.05) in 4 of the 9 most abundant FAs: stearic (18:0), linoleic (18:2 *n*-6),

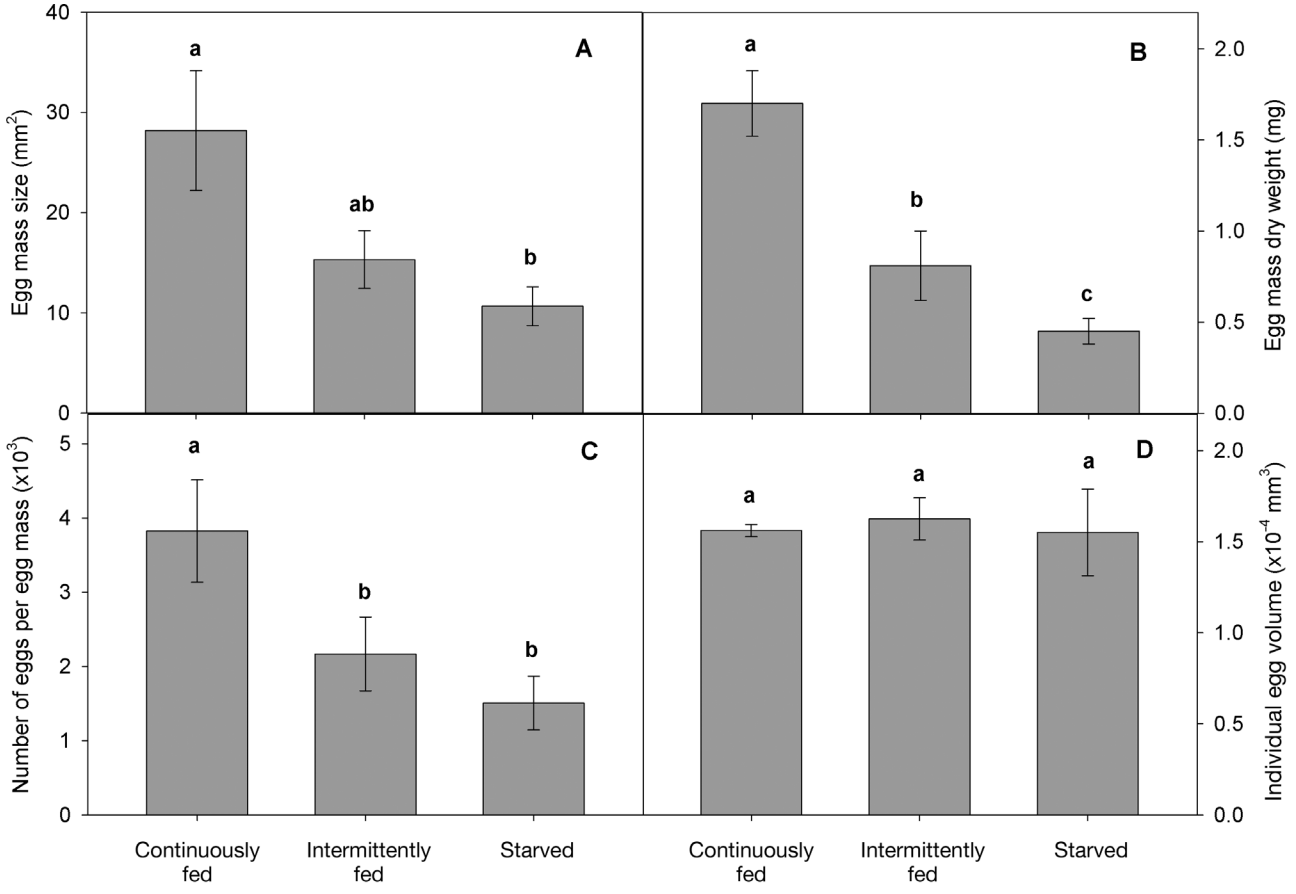

Fig. 3. Egg mass characteristics of *Elysia viridis*. Animals were fed continuously with *Codium tomentosum* (Continuously fed), fed 1 d per week with *C. tomentosum* (Intermittently fed), or deprived of food for the 35 d experimental period (Starved). (A) Egg mass size (mm). (B) Egg mass dry weight (mg). (C) Number of eggs per egg mass ($\times 10^3$). (D) Individual egg volume ($\times 10^{-4}$ mm^3). Mean ± SD, n = 5. Different letters above bars indicate significant differences between treatments ($p < 0.05$)

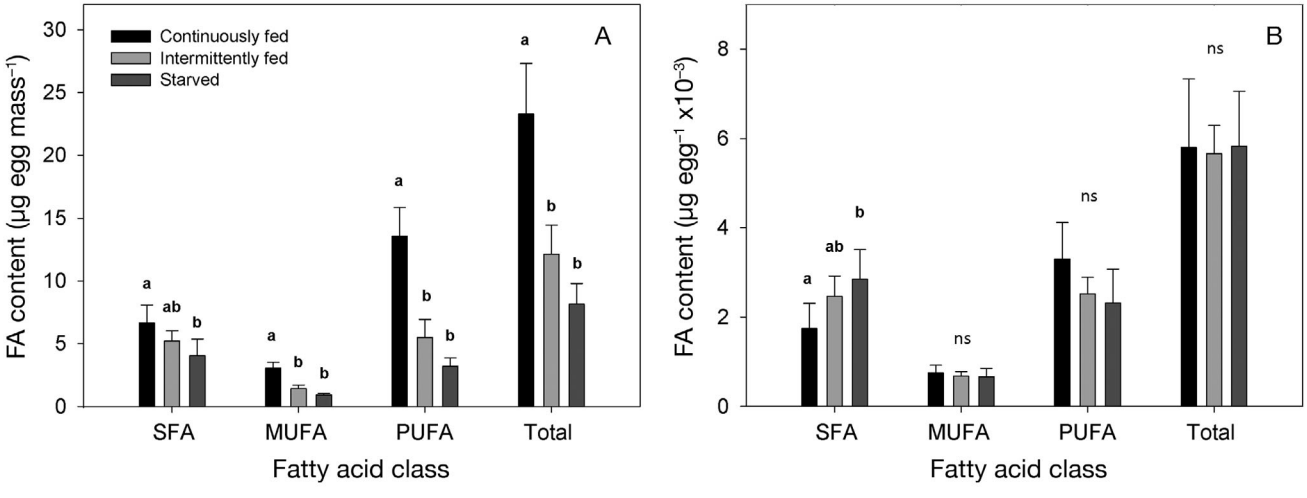

Fig. 4. Fatty acid (FA) class profile of *Elysia viridis* eggs. Animals were fed continuously with *Codium tomentosum* (Continuously fed), fed 1 d per week with *C. tomentosum* (Intermittently fed), or deprived of food for the 35 d experimental period (Starved). (A) FA content (µg) per whole egg mass. (B) FA content (µg) per egg ($\times 10^{-3}$). SFA: saturated FAs; MUFA: monounsaturated FAs; PUFA: polyunsaturated FAs; and Total: total FAs. Mean ± SD, n = 5. Different letters above bars indicate significant differences between treatments ($p < 0.05$); ns: no significant differences among treatments

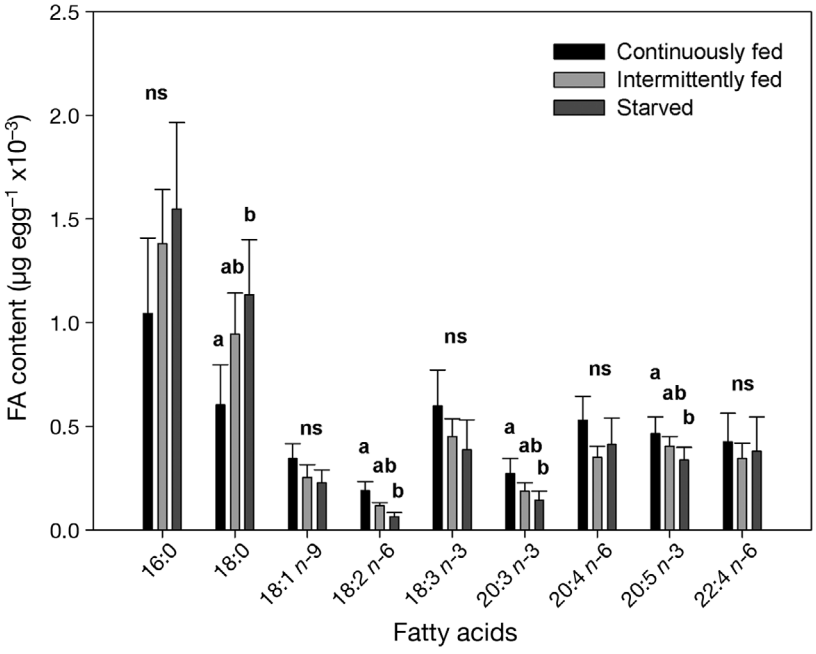

Fig. 5. Most abundant fatty acids (FAs) identified in *Elysia viridis* eggs. Animals were fed continuously with *Codium tomentosum* (Continuously fed), fed 1 d per week with *C. tomentosum* (Intermittently fed), or deprived of food for the 35 d experimental period (Starved). Contents are expressed as µg egg^{-1} (×10^{-3}). Mean ± SD, n = 5. Different letters above bars indicate significant differences between treatments ($p < 0.05$); ns: no significant differences among treatments

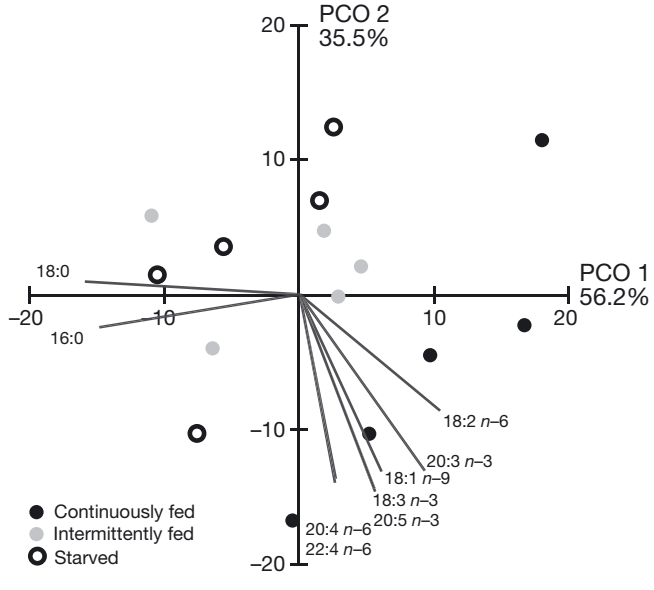

Fig. 6. Principal coordinate (PCO) analysis comparing the content ([µg egg^{-1}] × 10^{-3}) of the most abundant fatty acids (FAs) in *Elysia viridis* eggs. Animals were fed continuously with *Codium tomentosum* (Continuously fed), fed 1 d per week with *C. tomentosum* (Intermittently fed) or deprived of food for the 35 d experimental period (Starved). Vectors represent individual FAs with a Spearman correlation coefficient R > 0.7

eicosatrienoic (20:3 *n*-3), and EPA (20:5 *n*-3) acids (Fig. 5). PCO analysis showed a high variability in egg FA profiles, distinguishing 2 main groups (Fig. 6): eggs from continuously fed animals and from animals submitted to feeding restrictions, the latter including eggs from intermittently fed and starved animals. The first 2 PCO axes explained 91.7% of FA variation (PCO axis 1, 56.2%; PCO axis 2, 35.5%). The FAs that contributed to this separation between groups were the most abundant SFAs (16:0 and 18:0) in eggs from animals submitted to feeding restrictions, and PUFAs (18:2 *n*-6; 18:3 *n*-3; 20:3 *n*-3; 20:4 *n*-6; 20:5 *n*-3; 22:4 *n*-6) and the MUFA 18:1 *n*-9 in eggs from continuously fed animals (Fig. 6).

4. DISCUSSION

Previous studies outlining the importance of kleptoplast photosynthesis to nutrition of *Elysia viridis* in periods of food shortage (e.g. Hinde & Smith 1975, Cartaxana et al. 2017) already pointed out that the photosynthetic metabolism was not sufficient to sustain growth. If kleptoplasts are not replaced by the ingestion of macroalgal material, photosynthetic capacity collapses due to the need for production, repair, and replacement of plastid components that require nuclear-encoded proteins (Serôdio et al. 2014). In our study, limited food availability affected the size of *E. viridis* leading to a DW loss, as well as a significant decrease in kleptoplast abundance (chl *a*) and photosynthetic capacity (F_v/F_m). Sea slugs in the starved treatment showed almost no measurable variable fluorescence and very low chl *a* concentration after 35 d. Significantly lower photosynthetic capacity was observed just 14 d after the starvation treatment started.

Kleptoplasts of intermittently fed sea slugs showed a reduction of only 9% of photosynthetic competence (F_v/F_m) compared to continuously fed animals. However, it is important to note that these photosynthetic activity measurements are not related to the abundance of kleptoplasts but rather show how efficient the remaining kleptoplasts are. Indeed, intermittently feeding sea slugs were not capable of sustaining the same level of kleptoplast abundance, as chl *a*

on a DW basis was 25% lower in comparison to that recorded for continuously fed conspecifics. Additionally, there was a DW loss of 35% in intermittently fed sea slugs relative to continuously fed animals. These findings imply a significant loss of total available kleptoplast-derived photosynthates to intermittently feeding sea slugs. Applying the same rational to starved individuals, we conclude that almost no photosynthates were available to these sea slugs after 35 d.

Egg size was not related to nutritional state and body size of *E. viridis*. An average egg diameter of 67 µm was recorded in the present study, which is in line with the diameter range of 60–70 µm reported for *E. viridis* by Jensen (2001). Hence, egg size seems to be a conservative trait in this sea slug species, independent of the individuals' nutritional state. Similarly, Yusa (1994) reported no egg-size variation related to body size in the sea hare *Aplysia kurodae*. In contrast, Allen et al. (2009) observed that the maternal body size of the sea slug *E. stylifera* significantly affected egg size, with larger specimens producing larger eggs. In the opisthobranch *Haloa japonica*, egg size declines during the reproductive season with reduced food intake and decreasing maternal size (Ito 1997). In the nudibranch *Tenellia adspersa*, Chester (1996) also found egg size to be a plastic parameter related to parental nutritional state, with starved specimens producing significantly smaller eggs. An extreme case of plasticity, i.e. poecilogony, was described for the estuarine sacoglossan *Alderia modesta*, with planktotrophic and lecithotrophic larvae hatching from eggs of different sizes (Krug 1998). When starved, adults which previously produced only lecithotrophic larvae switched to producing planktotrophic larvae or mixed clutches (Krug 1998).

Equal numbers of egg masses were spawned by continuously fed and starved sea slugs, suggesting that food availability was not a determining parameter for the number of spawning events. This similarity occurred even though the body weight of sea slugs starved for 35 d was ca. 4 times lower than that of continuously fed animals, indicating that after maturity, the majority of energy was directed towards reproduction. However, intermittently fed sea slugs spawned a lower number of egg masses than continuously fed and starved animals, indicating a trade-off between feeding and spawning activity under limited resources in *E. viridis*.

The number of eggs produced by *E. viridis* decreased with food limitation, expressing a compromise between fecundity and nutritional state. This is in agreement with other studies on marine gastropods showing that larger, better nourished individuals have more resources to allocate to reproduction and consequently display a higher fecundity, expressed as the number of eggs produced (e.g. Yusa 1994, Chester 1996, Ito 1997, Gianguzza et al. 2005, Allen et al. 2009). Fecundity and total investment per egg mass are affected by food limitation, as animals use their internal reserves to maintain metabolism and to keep up with the highly energetic demanding reproductive activity (Ramirez Llodra 2002). In the case of *E. viridis* under restricted food availability, fecundity and total investment per egg mass could be further affected by a decrease in the availability of kleptoplast-derived photosynthates.

Total FA profiles in marine gastropods are related to their dietary regime and nutritional state (Martínez-Pita et al. 2005, Leal et al. 2012). In our study, total FA content per egg was very similar in all treatments, which could suggest that egg quality was not affected by the nutritional state of *E. viridis*. However, a closer look at the FA profile of spawned eggs revealed differences in SFA and PUFA levels, reflecting different egg qualities between feeding treatments.

SFAs and MUFAs are known to play energetic roles in embryonic metabolism, being the principal esterified FAs in neutral lipids such as TAGs (Rey et al. 2018). TAGs are recognized as the most common lipid storage molecules, which can be distributed as lipid droplets throughout the animal's body (Iverson 2009). A recent study in juveniles of *E. chlorotica* has suggested that lipid droplets, resulting from photosynthesis, provide an appropriate cell environment to stabilize and maintain plastids, playing a relevant role in achieving permanent kleptoplasty and storing carbon reserves (Pelletreau et al. 2014). These lipids resulting from autotrophic activity are stored as part of cellular fat reserves and are available to be used (Bachar et al. 2007). The higher levels of SFA displayed in the eggs of starved *E. viridis* likely result from the mobilization of the most readily available reserve of FAs existing in parental organisms to somehow mitigate the lack of other important FAs, namely PUFAs.

PUFAs are involved in specific biological functions, such as signaling, the maintenance of cell membrane architecture, or protection due to their antimicrobial activity (Benkendorff et al. 2005, Parrish 2009). These unsaturated FAs are essential during embryonic development, including ARA (20:4 *n*-6), a precursor of prostaglandins, or EPA (20:5 *n*-3), an FA known to increase hatching success and larval survival in mar-

ine invertebrates (Soudant et al. 1996, Hendriks et al. 2003). Additionally, within the most abundant FAs recorded in the present work, we identified 2 essential FAs: linoleic acid (18:2 *n*-6, LA) and α-linolenic acid (18:3 *n*-3, ALA). Both FAs are synthesized by primary producers, and most animals must obtain them from their diets (Dalsgaard et al. 2003, Kelly & Scheibling 2012), while autotrophic organisms can biosynthesize them (Dörmann 2007, Brown et al. 2009). Therefore, the decrease in LA observed in eggs from starved individuals can be a consequence of food deprivation and/or decreased kleptoplast photosynthetic activity during the 35 d of treatment. The comparison of the lipidome of *E. viridis* and its main macroalgae food *Codium tomentosum* revealed that exclusive lipids of plastid membranes were preserved during the process of kleptoplasty, suggesting that there is a conservative mechanism that assists in the preservation of plastid membranes and chloroplast functionality inside sea slug cells (Rey et al. 2017).

The amount of resources that organisms invest in their offspring is an important life-history trait. In this study, food limitation significantly reduced *E. viridis* fecundity (number of eggs produced per egg mass) and affected FA composition of individual eggs. Like most temperate species of sacoglossans, this sea slug has planktotrophic larvae, and culturing the sea slugs through their planktotrophic larval stages is a challenging task (Trowbridge 2000, Jensen 2001). Future studies should investigate the consequences of varying parental nutritional state on larval survival and offspring success in sacoglossan sea slugs.

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