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Light modulates the lipidome of the photosynthetic sea slug *Elysia timida*

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ABSTRACT

Long-term kleptoplasty, the capability to retain functional stolen chloroplasts (kleptoplasts) for several weeks to months, has been shown in a handful of Sacoglossa sea slugs. One of these sea slugs is *Elysia timida,* endemic to the Mediterranean, which retains functional chloroplasts of the macroalga *Acetabularia acetabulum.* To understand how light modulates the lipidome of *E. timida*, sea slug specimens were subjected to two different 4-week light treatments: regular light and quasi-dark conditions. Lipidomic analyses were performed by HILIC-HR-ESI-MS and MS/MS. Quasi-dark conditions caused a reduction in the amount of essential lipids for photosynthetic membranes, such as glycolipids, indicating high level of kleptoplast degradation under sub-optimal light conditions. However, maximum photosynthetic capacities (F_v/F_m) were identical in both light treatments (≈0.75), showing similar kleptoplast functionality and suggesting that older kleptoplasts were targeted for degradation. Although more stable, the phospholipidome showed differences between light treatments: the amount of certain lipid species of phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylglycerol (PG) decreased under quasi-dark conditions, while other lipid species of phosphatidylcholine (PC), PE and lyso-PE (LPE) increased. Quasi-dark conditions promoted a decrease in the relative abundance of polyunsaturated fatty acids. These results suggest a light-driven remodelling of the lipidome according to the functions of the different lipids and highlight the plasticity of polar lipids in the photosynthetic sea slug *E. timida.*

1. Introduction

Macroalgae are not just a food source for some Sacoglossa sea slug species, as these animals sequester functional algal chloroplasts (kleptoplasts) while digesting other cellular components [1,2]. This evolutionary strategy allows these animals to obtain energy through heterotrophic – digestion of the algae – and autotrophic – kleptoplast photosynthesis – pathways [3,4]. The relevance of photosynthesis for sacoglossan sea slugs is a highly debated topic among researchers studying kleptoplasty [4–6]. Several studies have reported strong evidence for the important role of photosynthesis in sea slug metabolism [3,7], reproduction [4,8] and survival [9]. However, other authors argue that kleptoplast photosynthesis is not relevant to these sea slugs [5].

The sacoglossan sea slug *Elysia timida* (Risso, 1818) is a Mediterranean endemic species, which retains functional chloroplasts of the macroalga *Acetabularia acetabulum* (Linnaeus) P.C. Silva, 1952, a morphologically complex unicellular organism. This alga presents a life cycle marked by a calcification stage which reduces the grazing capacity of *E. timida* and shapes its developmental strategy [10]. Chloroplasts sequestered by *E. timida* maintain their activity for about 45 days after sea slugs are placed in starvation (with no access to new chloroplasts), although with a significant reduction in their photosynthetic activity in the first 5–10 days [11,12].

Lipids are the main constituents of biological membranes, playing an essential role as structural molecules, as well as in energy metabolism

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Table 1
Polar lipid classes identified in *Elysia timida* under two different irradiance treatments: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. Data represent sum of normalized extracted ion chromatogram (XIC) area for each lipid class. Mean ± SD, regular light, $n = 6$; quasi-dark, $n = 5$.

	Light	Dark
Phospholipids		
PC	14.39 ± 3.41	18.81 ± 4.02
LPC	0.05 ± 0.03	0.08 ± 0.04
PE	8.14 ± 1.28	8.43 ± 1.68
LPE	0.22 ± 0.03	0.37 ± 0.17
PI	0.49 ± 0.08	0.37 ± 0.09
LPI	0.02 ± 0.00	0.02 ± 0.01
PG	0.37 ± 0.07	0.18 ± 0.07
LPG	0.01 ± 0.01	0.01 ± 0.01
PS	1.55 ± 0.20	1.38 ± 0.28
PA	0.13 ± 0.09	0.11 ± 0.03
Glycolipids		
MGDG	2.61 ± 0.38	1.31 ± 0.43
MGMG	0.02 ± 0.01	0.01 ± 0.01
DGDG	0.72 ± 0.10	0.38 ± 0.11
SQDG	0.41 ± 0.09	0.22 ± 0.09
Betaine Lipids		
DGTS	32.15 ± 4.48	19.07 ± 5.05
MGTS	0.25 ± 0.22	0.17 ± 0.07
Sphingolipids		
CPE	0.08 ± 0.01	0.08 ± 0.02
CAEP	0.29 ± 0.05	0.30 ± 0.06

Abbreviations: CAEP – Ceramide aminoethylphosphonate; CPE – Ceramide phosphoethanolamines; DGDG – Digalactosyl diacylglycerol; DGTS – Diacylglyceryl-*N*,*N*,*N*-trimethyl homoserine; LPC – Lyso-phosphatidylcholine; LPE – Lyso-phosphatidylethanolamine; LPG – Lyso-phosphatidylglycerol; LPI – Lyso-phosphatidylinositol; MGDG – Monogalactosyl diacylglycerol; MGMG – Monogalactosyl monoacylglycerol; MGTS – Monoacylglyceroltrimethylhomoserine; PA – Phosphatidic acid; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PI – Phosphatidylinositol; PS – Phosphatidylserine; SQDG – Sulfolipid sulfoquinovosyl diacylglycerol.

and cell signalling processes. The lipid composition of photosynthetic membranes has been preserved from cyanobacteria to chloroplasts [13], which are characterized by the presence of galactoglycerolipids (monogalactosyl diacylglycerol – MGDG and digalactosyl diacylglycerol – DGDG) and sulpholipids (sulfoquinovosyl diacylglycerol – SQDG) [14]. These lipids have been identified and/or quantified in sea slugs with functional kleptoplasty (e.g., *E. viridis*, *E. crispata*) [15,16], but not addressed yet in *E. timida*. Light energy is essential to photosynthesis, driving the production of chemical energy (ATP and NADPH) which is used to drive the assimilation of CO_2 through the Calvin-Benson-Bassham cycle. Therefore, light is a relevant factor in the regulation of lipid metabolism in photosynthetic organisms.

The present study aimed to investigate how light shapes the lipidome of the photosynthetic sea slug *E. timida*. We identified for the first time the polar lipidome (i.e., phospholipids, glycolipids and betaine lipids) of the sea slug *E. timida* and report the effects of quasi-dark conditions, which severely limit photosynthesis, on the lipidome and fatty acid (FA) profile, and on the photosynthetic capacity of this sea slug species.

2. Materials and methods

2.1. Sampling and maintenance conditions

Samples of *E. timida* and *A. acetabulum* were collected in Puerto de Mazarrón (Mediterranean Sea, Spain) by scuba diving (depth of approximately 2 m). The samples were transported to the laboratory within 48 h in chilled aerated seawater collected from the sampling site. Sampled sea slugs and algae were conditioned for 14 days in a 150 L recirculated life support system with artificial seawater at 18 °C, a salinity of 35 and photoperiod of 14 h:10 h, light:dark (a photon scalar irradiance of 60 μmol photons m^{-2} s^{-1} being provided by T5 fluorescent lamps). Photon scalar irradiance was measured with a Spherical Micro Quantum Sensor and a ULM-500 Universal Light Meter (Heinz Walz GmbH, Germany).

2.2. Experimental design

Six pairs of adult sea slugs were placed in different wells (56 mm diameter × 60 mm depth) of a floating tray in the recirculating life support system, with an internal re-circulating water pump. The water exchange inside the wells was done through the mesh (0.5 mm) covering the bottom of the wells. Pairs of *E. timida* were randomly distributed in the wells and subjected to two different treatments: regular light treatment – the specimens were subjected to a photon scalar irradiance of 40–160 μmol photons m^{-2} s^{-1}, depending on the position inside the well; quasi-dark treatment – specimens were subjected to a photon scalar irradiance of only 5 μmol photons m^{-2} s^{-1}. Specimens from both treatments were fed every day the same amount of *A. acetabulum* grown at a photon scalar irradiance of 60 μmol photons m^{-2} s^{-1} under a 14 h:10 h, light:dark photoperiod. During the experimental period, one animal died in the quasi-dark treatment, reducing the number of replicates in this treatment to $n = 5$. After 28 days, *E. timida* specimens were gently washed in ultrapure water, flash-frozen in liquid nitrogen and stored at −80 °C before lyophilization.

2.3. Lipid analysis

2.3.1. Lipid extraction

The total lipid extracts from *E. timida* samples were obtained using the modified Bligh and Dyer method [17]. Freeze-dried samples of sea slugs were macerated and vigorously homogenized with 600 μL of methanol and 300 μL of dichloromethane, sonicated for 1 min and incubated on ice for 30 min on an orbital shaker. After centrifugation at 568 x *g* for 10 min, the organic phase was collected in a new glass tube and mixed with 300 μL of dichloromethane and 300 μL of ultrapure water. The tubes were centrifuged at 568 x *g* for 10 min and the organic phase was collected in a new tube. The aqueous phase was reextracted with 300 μL of dichloromethane, centrifuged at 568 x *g* for 10 min, and the recovered organic phase was collected. The two organic phases were mixed and dried under a stream of nitrogen and stored at −20 °C until further analysis. Total lipid extracts were determined by gravimetry.

2.3.2. Hydrophilic interaction liquid chromatography–mass spectrometry (HILIC-LC-MS)

Total lipid extracts were analysed by hydrophilic interaction liquid chromatography on a HPLC Ultimate 3000 Dionex (Thermo Fisher Scientific, Germering, Germany) with an autosampler coupled online to a Q-Exactive hybrid quadrupole mass spectrometer (Thermo Fisher, Scientific, Bremen, Germany). The solvent system used for this analysis consisted of two mobile phases: Mobile phase A consisted of water, acetonitrile and methanol (25 %, 50 %, 25 %), with 5 mM ammonium acetate, and mobile phase B was a mixture of acetonitrile and methanol (60 %, 40 %), with 5 mM ammonium acetate. Elution started with 5 % of mobile phase A, which was held isocratically for 2 min, followed by a linear increase to 48 % of mobile phase A within 8 min. A new linear increase to 65 % of mobile phase A within 5 min held for 2 min. After this procedure, the conditions were returned to the initial settings in 13 min (3 min to decrease to 5 % of phase A and a re-equilibration period of 10 min prior next injection). To perform HILIC-LC-MS analyses, 10 μL of total lipid extract (corresponding to 10 μg of total lipid extract), 8 μL of phospholipid standards mix (dMPC - 0.04 μg, dMPE - 0.04 μg, LPC - 0.04 μg, dPPI - 0.08 μg, dMPG - 0.024 μg, dMPS - 0.08 μg, tCL (14:0) - 0.16 μg, SM (d18:1/17:0) - 0.04 μg, Cer (d18:1/17:0) - 0.08 μg- 0.01 μg, dMPA - 0.16 μg) and 82 μL of eluent (5 % of mobile phase A and 95 % of mobile phase B) were mixed and a 5 μL of this mixture was injected into the Ascentis Si column HPLC Pore column (10 cm × 2.1 mm, 2.7 μm, Sigma-

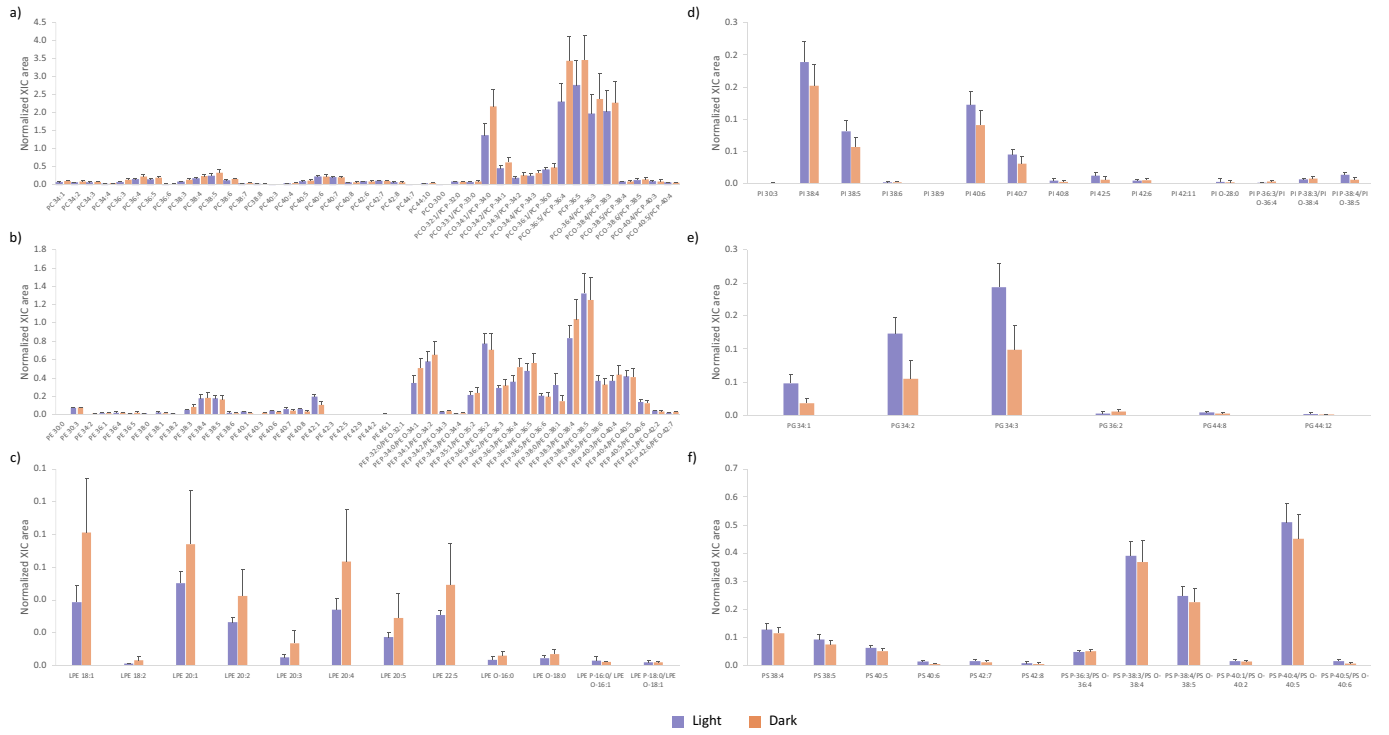

Fig. 1. Phospholipid species identified in *Elysia timida*. Phospholipid classes containing the phospholipid species that contributed significantly to the different between treatments. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. Normalized extracted-ion chromatogram (XIC) area of a) Phosphatidylcholine (PC), b) Phosphatidylethanolamine (PE), c) Lyso PE (LPE), d) Phosphatidylinositol (PI), e) Phosphatidylglycerol (PG) and f) Phosphatidylserine (PS). Mean ± standard deviation (SD), regular light, $n = 6$; quasi-dark, $n = 5$.

Aldrich), with a flow rate of 200 μL min^{-1} at 35 °C. Acquisition in the Orbitrap® mass spectrometer was performed in positive (electrospray voltage 3.0 kV) and negative (electrospray voltage −2.7 kV) modes, with a resolution of 70,000 and AGC target of 1e6. The capillary temperature was 350 °C, and the sheath gas flow was 20 U. For MS/MS determinations, a resolution of 17,500 and an AGC target of 1e5 were used, and cycles consisted of one full scan mass spectrum, and ten data-dependent MS/MS scans were repeated continuously throughout the experiments, with the dynamic exclusion of 60 s and an intensity threshold of 2e4. The normalized collision energy™ (CE) ranged between 25, 30 and 35 eV. Data acquisition was performed using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA).

2.3.3. Data analysis

The polar lipid species were identified by the retention time and *m/z* of the ions observed in the LC-MS spectra. Ion peak integration and assignments were performed using MZmine version 2.42 [18], based on an in-house lipid database. During data processing, all peaks with raw intensity less than 1e4 were excluded. Lipid species were confirmed by interpretation of HILIC-ESI-MS/MS fragmentation [19] (Supplementary Figs. S1-S10) and/or mass accuracy ≤5 ppm (Supplementary Table S1). The lipid species were quantified by exporting the integrated peak areas and the data were normalized by dividing the peak areas of the extracted ion chromatograms (XIC) of each lipid species by the peak area of the selected internal standard for the lipid class as following: PC with dMPC; LPC with LPC; PE, LPE, DGTS, MGTS, CPE and CAEP with dMPE; PI and LPI with dPPI; PG, LPG and SQDG with dMPG; PS with dMPS; PA with dMPA; MGDG, MGMG and DGDG with Cer (d18:1/17:0)

2.3.4. Fatty acid analysis by GC–MS

The FA profile of *E. timida* was analysed by gas chromatography–mass spectrometry (GC–MS). FA methyl esters (FAME) were obtained after transesterification of 100 μg of total lipid extract, using the FA 19:0 as internal standard (1.03 μg mL^{-1} in *n*-hexane, CAS number 1731-94-8, Merck). The lipid extract was mixed with 1 mL of internal standard and 200 μL of potassium hydroxide in methanol (2 M). The mixture was homogenized and 2 mL of an aqueous solution of sodium chloride (10 mg mL^{-1}) were added. The sample was centrifuged at 568 x *g* for 5 min to separate the phases. The FAME were transferred to a new tube and dried under a stream of nitrogen. The samples were dissolved in *n*-hexane and 2 μL were injected on an Agilent Technologies 6890 N Network chromatograph equipped with a DB-FFAP column (length: 30 m, internal diameter: 0.32 mm, film thickness: 0.25; J&W Scientific, Folsom, CA, USA). The GC was connected to an Agilent 5973 Network Mass Selective Detector operated with an electron impact (EI) source at an electron energy of 70 eV and scanning the mass range *m/z* 50–550 in 1 s cycle in a full scan mode acquisition. The initial oven temperature was 80 °C for 3 min and increased linearly to 160 °C at 25 °C min^{-1}, followed by a linear increase to 210 °C at 2 °C min^{-1} and 250 °C at 30 °C min^{-1}. This temperature was maintained for 10 min. The injector and detector were programmed at temperatures of 220 °C and 250 °C, respectively. The carrier gas was helium at a flow rate of 1.4 mL min^{-1}. FAME present in the sample were identified by retention time and MS fragmentation using a commercial FAME standard mixture (Supelco 37 Component FAME Mix, ref. 47885-U, Sigma-Aldrich) and confirmed by comparison with the spectral library from 'The Lipid Web' [20]. The calibration curves of the FAME standards acquired under the same instrumental conditions were used to quantify the FAME identified in the sample.

2.4. Chlorophyll *a* fluorescence

The photosynthetic activity of *E. timida* individuals was evaluated weekly by measuring chlorophyll *a* (Chl *a*) variable fluorescence using a JUNIOR-PAM fluorometer (Heinz Walz). Specimens were placed individually in Petri dishes with a thin layer of artificial seawater. The

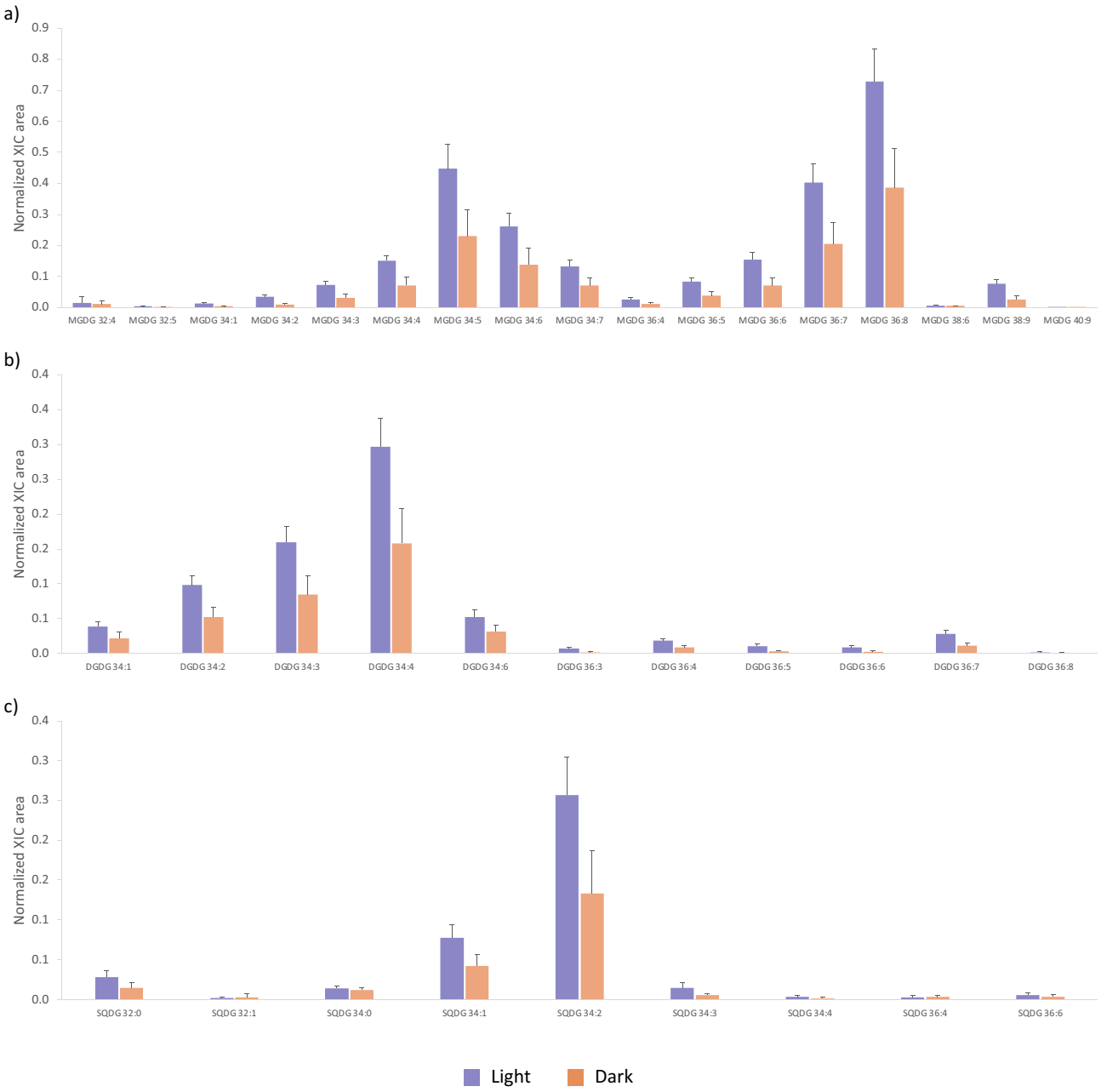

Fig. 2. Glycolipid species identified in *Elysia timida*. Glycolipid classes containing the glycolipid species that contributed significantly to the different between treatments. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. Normalized extracted-ion chromatogram (XIC) area of a) Monogalactosyl diacylglycerol (MGDG), b) Digalactosyl diacylglycerol (DGDG) and c) SQDG.

individuals were dark-adapted for 30 min and the optical fibre of the fluorometer was placed in contact with the sea slug's parapodia. A saturating pulse was applied, and the minimum and maximum fluorescence levels were recorded (F_o and F_m, respectively). The maximum quantum yield of photosystem (PS) II (F_v/F_m) was calculated as ($F_m - F_o)/F_m$ [21]. The parameter F_v/F_mfm was used as an indicator of photosynthetic capacity.

2.5. Statistical analysis

Data from the two sea slugs from each experimental unit (well) were averaged to avoid pseudoreplication, and the averages were treated as independent replicates [22].

Polar lipid species were grouped by lipid classes into phospholipids, glycolipids and betaine lipids (Supplementary Table S1). Zero values recorded for a particular lipid species were substituted by 1/2 times the minimum positive value for the respective lipid species. Significant differences in the log-transformed amount of the different identified lipid species were calculated using permutation tests. To control for the false discovery rate at $p = 0.05$ for these multiple tests, the Benjamini-Hochberg step-up adjustment was applied [23]. Log-transformed data of polar lipid species were used as variables in a principal component analysis (PCA) to identify the major dimensions in the data. A PCA for each lipid class dataset was performed to visualize the differences between treatments.

Additional statistically significant differences between *E. timida* specimens submitted to different treatments were investigated using one-way ANOVAs. The following data were tested: dry weight, the total

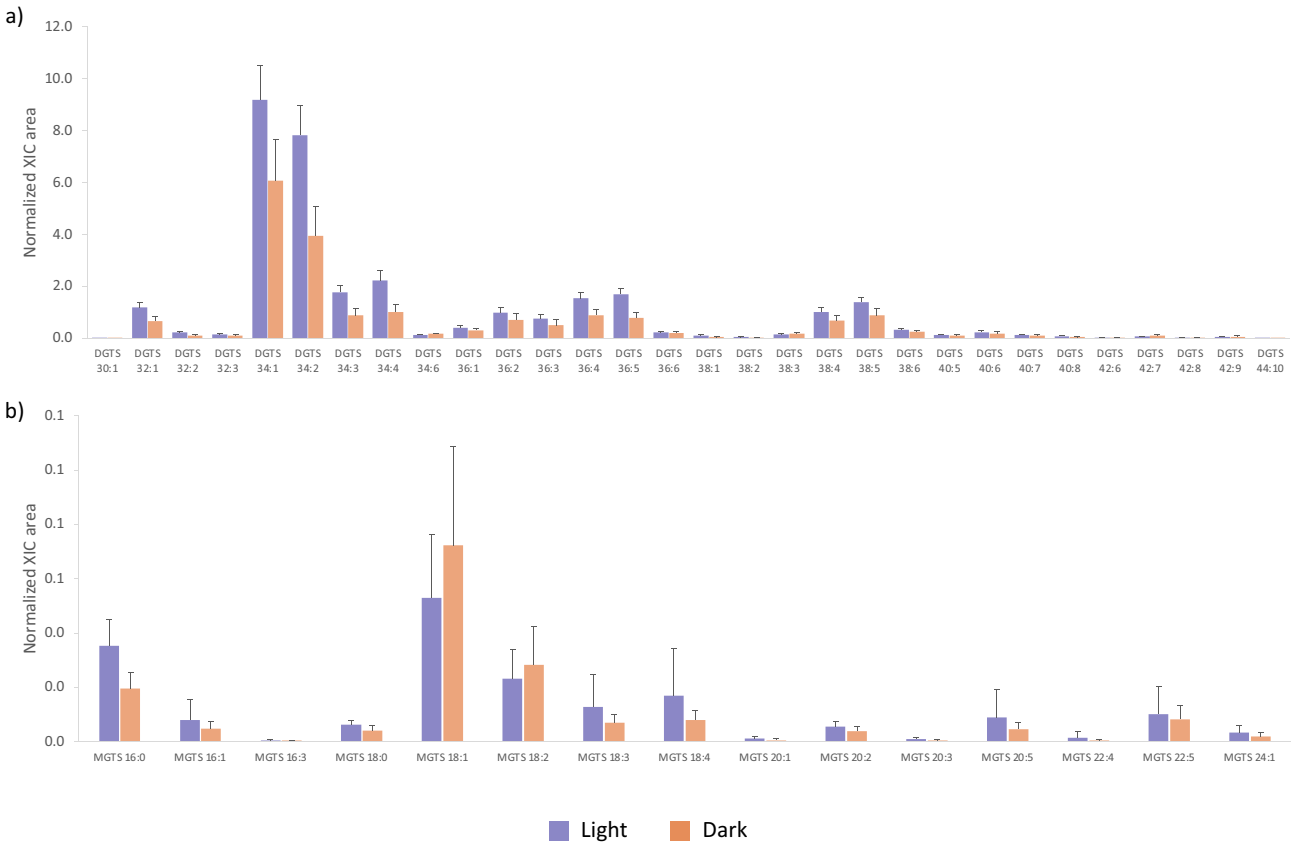

Fig. 3. Betaine lipid species identified in *Elysia timida*. Betaine lipid classes containing the betaine lipid species that contributed significantly to the different between treatments. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. Normalized extracted-ion chromatogram (XIC) area of a) Diacylglyceryl-*N*,*N*,*N*-trimethyl homoserine (DGTS) and b) monoacylglyceryl-*N*,*N*,*N*-trimethyl homoserine (MGTS).

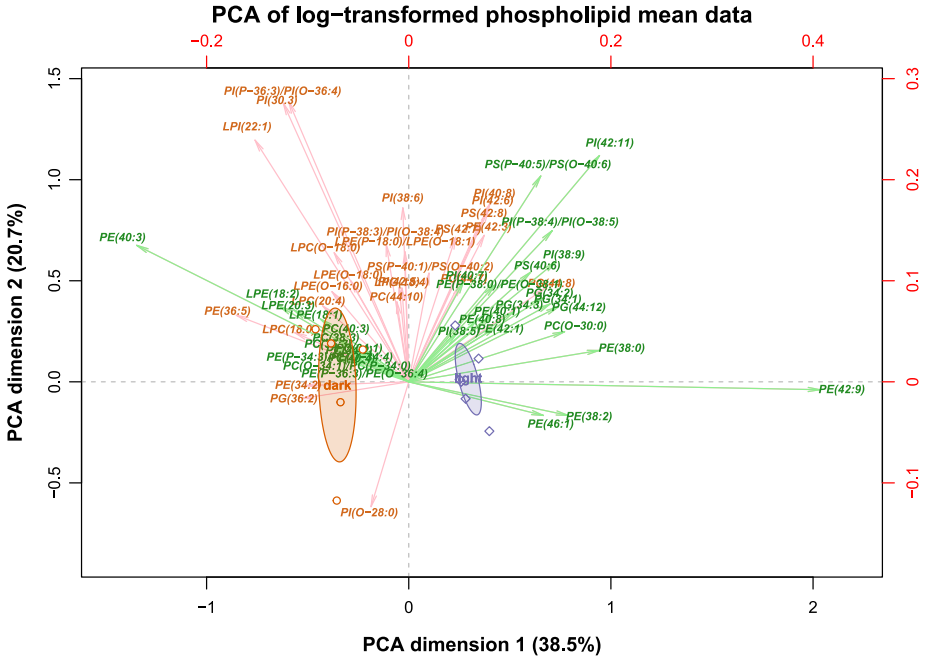

Fig. 4. PCA of log-transformed normalized extracted-ion chromatogram (XIC) areas of phospholipid species identified in *Elysia timida*. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. The light vs. quasi-dark difference in multivariate space lines up almost exactly with the first dimension. Lipid species in green are both high contributors to the solution as well as significantly different between treatments.

amount of lipids, relative abundances of the most abundant FA, and FA classes (i.e., saturated [SFA], monounsaturated [MUFA], polyunsaturated [PUFA], branched [BrFA] fatty acids). Normality was checked using a Shapiro-Wilk test, and homogeneity of variances using Levene's test. Whenever these assumptions were not verified, the nonparametric Kruskal-Wallis test was used.

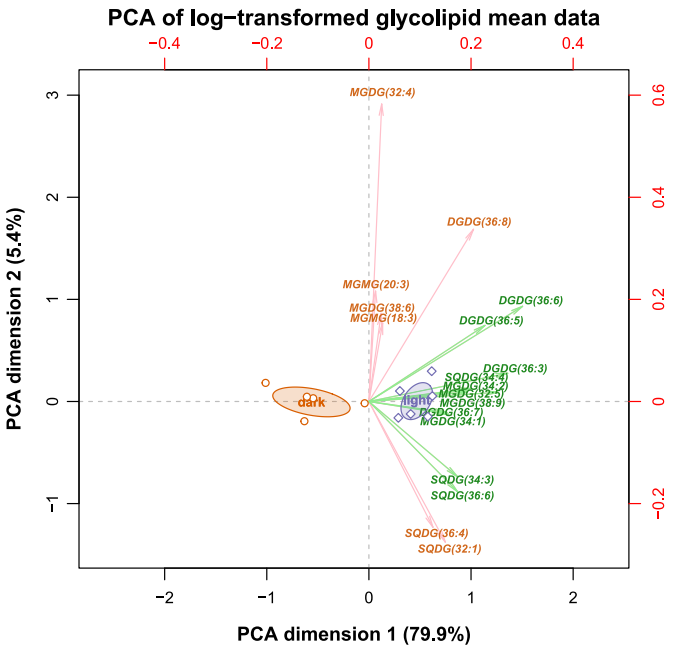

Fig. 5. PCA of log-transformed normalized extracted-ion chromatogram (XIC) areas of glycolipid species identified in *Elysia timida*. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. The light vs. quasi-dark irradiance difference in multivariate space lines up almost exactly with the first dimension. Lipid species in green are both high contributors to the solution as well as significantly different between treatments.

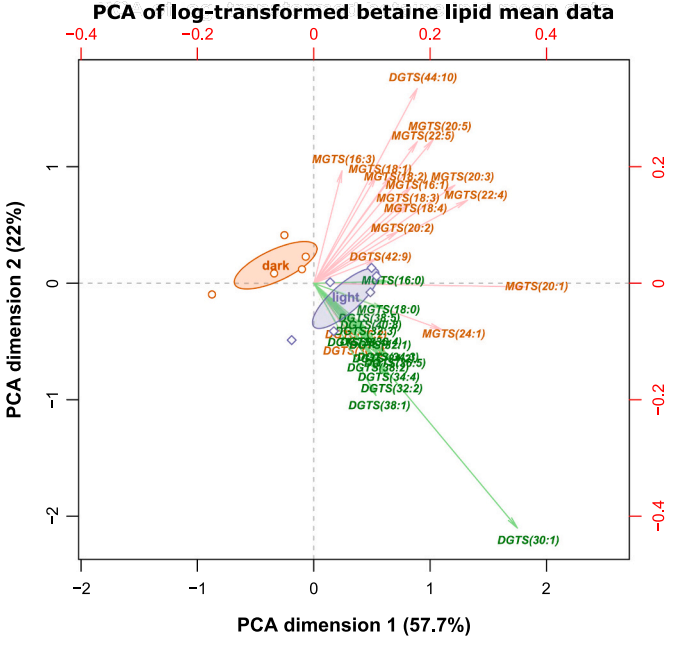

Fig. 6. PCA of log-transformed normalized extracted-ion chromatogram (XIC) areas of betaine lipid species identified in *Elysia timida*. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. The light vs. quasi-dark difference lines up diagonally in multivariate space. Lipid species in green are both high contributors to the solution as well as significantly different between treatments.

3. Results

Sea slugs from the regular light treatment had significantly higher dry weight than conspecifics from the quasi-dark treatment (4.59 ± 0.33 mg versus 3.95 ± 0.49 mg, respectively) (ANOVA, F value = 6.41 p = 0.03). However, there were no significant differences in the total amount of lipids (1.02 ± 0.14 and 1.06 ± 0.20 mg $slug^{-1}$, regular and quasi-dark light treatments, respectively).

The lipidomic analysis identified 233 lipid species (Supplementary Table S1), which were categorized into 18 polar lipid classes (Table 1). Within the phospholipid group, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) had the highest number of identified lipid species (41 and 44, respectively), followed by 15 phosphatidylinositol (PI), 12 phosphatidylserine (PS), 12 lyso-PE (LPE), 8 lyso-PC (LPC), 6 phosphatidylglycerol (PG), 3 lyso-PI (LPI), 3 phosphatidic acid (PA) and 1 lyso-PG (LPG) lipid species. Four classes of glycolipids have been identified, including: 17 lipid species of monogalactosyl diacylglycerols (MGDG), 11 of digalactosyl diacylglycerols (DGDG), 9 of sulfolipid sulfoquinovosyl diacylglycerols (SQDG) and 3 of monogalactosyl monoacylglycerols (MGMG). Within the betaine lipid group, we identified 30 diacylglyceroltrimethylhomoserines (DGTS) and 15 monoacylglyceroltrimethylhomoserines (MGTS) lipid species. Two classes of sphingolipids were identified: 2 lipid species of ceramide aminoethylphosphonates (CAEP) and one lipid species of ceramide phosphoethanolamine (CPE).

The statistical analysis of the levels of the lipid species showed that 80 out of 233 had significant differences between treatments (Supplementary Table S2). The lipid species that contributed to discriminate between treatments were phospholipids (PE: 11; PC: 8; PI: 5; PG: 4: LPE:3; PS: 2 lipid species), glycolipids (MGDG: 14; DGDG: 10; SQDG: 6 lipid species) and betaine lipids (DGTS: 15; MGTS: 2 lipid species) (Supplementary Table S2 and Fig. S11). Most of these lipid species (67) showed a higher amount in sea slugs subjected to regular light than under quasi-dark treatment, corresponding to 30 lipid species of glycolipids (MGDG: 14, DGDG: 10, SQDG:6), 20 phospholipids (PE: 8, PI: 5, PG: 4, PS: 2, PC: 1) and 17 betaine lipids (DGTS: 15, MGTS: 2) (Supplementary Table S2). However, 13 species of phospholipids exhibited a higher amount under quasi-dark conditions: 7 lipid species of PC (PC 38:3, PC 40:3, PC 36:3, PC 40:4, PC 34:2, PC 34:1, PC O-34:1/PC P-34:0), 3 of PE (PE 40:3, PE P-34:3/PE O-34:4, PE P-36:3/PE O-36:4) and 3 of LPE (LPE 18:2, LPE 20:3, LPE18:1) (Supplementary Table S2).

Normalized XIC area of the lipid classes containing the lipid species that contributed significantly to the differences between treatments is shown in Fig. 1 (phospholipids), Fig. 2 (glycolipids) and Fig. 3 (betaine lipids).

The first and second dimensions of the PCA of phospholipid species explained 59.2 % of the variance (PCA-1: 38.5 %, PCA-2: 20.7 %) (Fig. 4). The PCA of glycolipids variables explained 85.3 % of the variance (PCA-1: 79.9 %, PCA-2: 5.4 %) (Fig. 5). The difference between regular light and quasi-dark conditions in multivariate space lined up almost exactly with the first dimension. The PCA of betaine lipids explained 79.7 % of the variance (PCA-1: 57.7 %, PCA2: 22 %) (Fig. 6). The treatment differences lie on a diagonal line in the two-dimensional PCA solution. In all the PCA, the lipid species in green represent both high contributors to the respective PCA solution and significant differences between treatments.

In the lipid extracts of *E. timida,* we identified 43 FA (Supplementary Table S3), corresponding to SFA, MUFA, PUFA and BrFA classes (Fig. 7a). Levels of SFA ere significantly higher in specimens subjected to quasi-dark treatment (ANOVA, F value = 15.53 $p < 0.01$), while PUFA showed a higher relative abundance in sea slugs from regular light treatment (Kruskal-Wallis test, KW chi-squared = 5.63, $p = 0.02$). There were no significant differences between regular and quasi-dark treatments in the relative abundance of the FA classes of MUFA and BrFA. The most abundant FA have been summarized in Fig. 7b. In these FA, significant differences were observed in the relative abundance of 18:0,

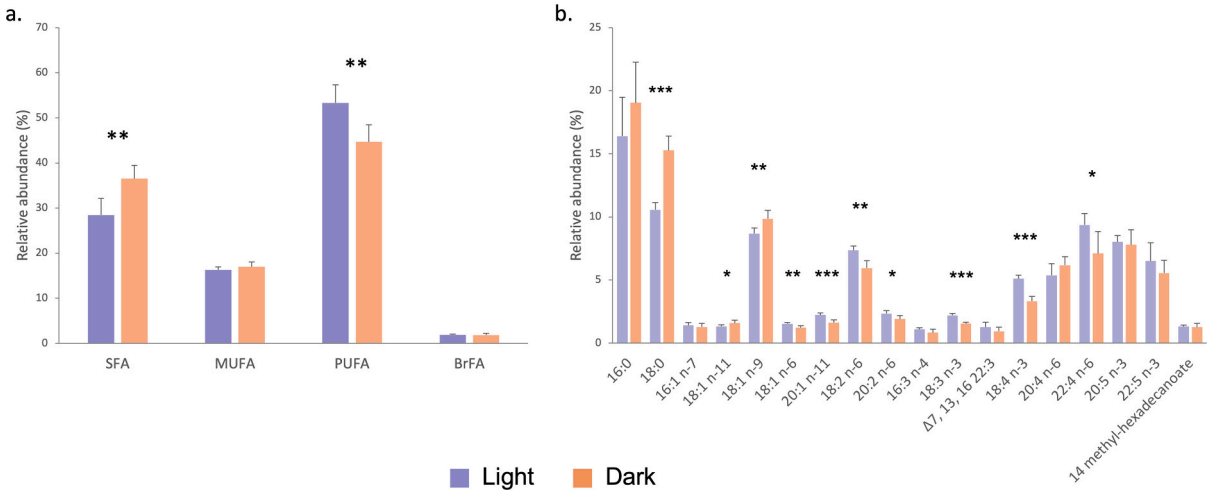

Fig. 7. Fatty acid profile of *Elysia timida*. a) Fatty acid classes and b) most abundant fatty acids identified in *Elysia timida*. The specimens were subjected to two different irradiance treatments for 4 weeks: regular light (Light, 40–160 µmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 µmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. Data represent relative abundances. Mean ± SD, regular light, $n = 6$; quasi-dark, $n = 5$. * $p < 0{,}05$; ** $p < 0.01$; *** $p < 0.001$.

Table 2
Photosynthetic capacity (F_v/F_m) of *Elysia timida*, under two different irradiance treatments: regular light (Light, 40–160 µmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 µmol photons m^{-2} s^{-1}) during the 4-week experimental period. Animals were fed continuously with *Acetabularia acetabulum*. Mean ± SD, regular light, $n = 6$; quasi-dark, $n = 5$.

	Light	Dark
Week 0	0.77 ± 0.02	0.77 ± 0.02
Week 1	0.77 ± 0.01	0.74 ± 0.01
Week 2	0.77 ± 0.01	0.73 ± 0.01
Week 3	0.77 ± 0.01	0.74 ± 0.01
Week 4	0.76 ± 0.01	0.74 ± 0.01

18:1 *n*-11, 18:1 *n*-9, 18:1 *n*-6, 20:1 *n*-11; 18:2 *n*-6, 20:2 *n*-6, 18:3 *n*-3, 18:4 *n*-3 and 22:4 *n*-6 (Fig. 7b).

The photosynthetic capacity of kleptoplasts (F_v/F_m) in *E. timida* was monitored throughout the experimental period with no significant differences between light treatments (Table 2). After 4 weeks, the F_v/F_m values were 0.76 ± 0.01 and 0.74 ± 0.01 for regular light and quasi-dark conditions, showing the presence of healthy kleptoplasts in both treatments.

4. Discussion

4.1. Lipidome

The *E. timida* lipidome is composed of classes of lipids synthesized in different organelles and playing different physiological roles. Glycolipids (e.g., MGDG, DGDG, SDQG) and the phospholipid PG are synthesized in chloroplasts and are essential for photosynthesis [14,24], while phospholipid classes such as PC, PE, PI, CPE and betaine lipids (e.g., DGTS) are synthesized in the endoplasmic reticulum [25,26], playing structural and signalling roles.

The several differences in the levels of the lipid classes found between treatments, and the lack of significant differences in the total amount of lipids, suggest a remodelling of lipid composition according to the distribution and function of the different lipids. Specimens of *E. timida* enduring quasi-dark conditions showed lower levels of chloroplast envelope membrane lipids such as MGDG, DGDG, SQDG and PG, as well as betaine lipids (DGTS). On the other hand, higher levels of some lipid species of phospholipids such as PC, PE and LPE (e.g., PC 40:3, PE 40:3, LPE 20:3) were observed under quasi-dark conditions. These results corroborate that the lipidome of *E. timida* is influenced by light intensity as was observed in other photosynthetic organisms [27,28]. A study on *Nannochloropsis gaditana* growing under different light intensities, demonstrated that low light caused a reduction in free fatty acids and diacylglycerol (DAG, precursor of galactoglycerolipids) [27]. A similar trend was also observed in DGTS and DGDG of this marine alga, while MGDG, PC, PE and PG incremented in low light conditions (10 µmol photons m^{-2} s^{-1}) compared to medium light (100 µmol photons m^{-2} s^{-1}) [27].

Lipid content in photosynthetic membranes has a highly conservative composition [29,30]. Glycolipids are specific lipids of chloroplast membranes, such as envelop and thylakoids, playing an essential role to maintain an efficient photosynthetic activity (e.g., photosystem machinery stabilization) [31,32]. Although glycolipids are the main lipids in chloroplasts, the phospholipid PG is also relevant in photosynthesis. SQDG and PG are responsible for maintaining the anionic charge on thylakoids, preserving structural and functional integrity of the photosynthetic machinery [33].

Phospholipids and glycolipids were the lipid classes with a greater number of lipid species contributing to the explained variance between treatments, although with different expressions. The significant reduction of all lipid classes with relevant roles in chloroplasts, namely all glycolipid classes (i.e., MGDG, DGDG, SQDG), as well as the phospholipid PG in specimens under quasi-dark treatment suggests that kleptoplasts are more rapidly targeted for degradation under this light condition. Furthermore, the maximum photosynthetic capacities (F_v/F_m) were identical in the two light treatments (≈0.75). These results suggest a degradation of older kleptoplast membranes, while the most recently acquired kleptoplasts maintain their photosynthetic capacity. Thus, sea slugs may obtain usable metabolites from kleptoplast degradation, while compensating for inhibited photosynthetic activity under quasi-dark conditions. Older kleptoplasts are expected to have higher accumulated amounts of starch and may serve as reservoir (e.g., polysaccharides) to use under less favourable periods [6]. A quantification analysis of chloroplast abundance will contribute to test this hypothesis.

The phospholipidome was more resilient under suboptimal light conditions. Only 33 out of the 145 identified phospholipid species changed significantly between treatments, in some cases with opposite trends. Normalized XIC area of lipid species mainly from the PE, PI and PG classes decreased under quasi-dark conditions, while some phospholipids belonging to the PC, PE and LPE classes increased under this

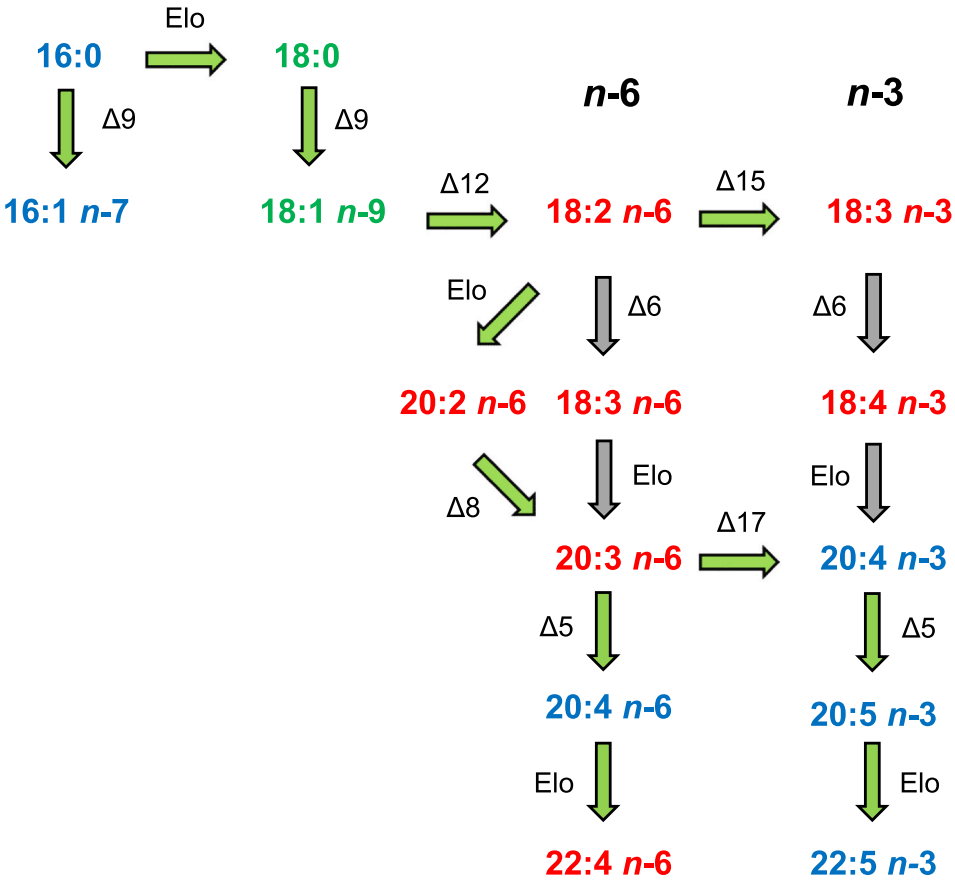

Fig. 8. Biosynthetic pathway of polyunsaturated fatty acids in *Elysia timida* samples. The specimens were subjected to two different irradiance treatment for 4 weeks: regular light (L ight, 40–160 μmol photons m^{-2} s^{-1}) and quasi-dark (Dark, 5 μmol photons m^{-2} s^{-1}). Animals were fed continuously with *Acetabularia acetabulum*. Blue fatty acids indicate no significant differences between treatments, green fatty acids indicate a significant increment under dark conditions, and red fatty acids indicate a significant decrease under dark conditions. Green arrows indicate light-dependent fatty acid biosynthesis. Grey arrows indicate no light-dependent fatty acid biosynthesis. One way ANOVA, $p < 0.05$. Desaturase enzymes are denoted with "Δ" and elongases with "Elo". Adapted from Cartaxana et al. (2021).

treatment. These lipid classes are the most abundant in animal tissues [34,35]. These results suggest that light promotes the remodelling of phospholipids with structural (e.g., PE and PC) and signalling functions (e.g., PI).

The lipidome profile of *E. timida* revealed the presence of several lipids recognized by their antioxidant properties and protective agents against enzymatic hydrolysis. The main classes of phospholipids (e.g., PE, PC, PS had a high proportion of plasmalogens (ether phospholipids) which have been identified as protective membrane agents against oxidation [36–38]. CAEP sphingolipids, also known as sphingophosphonolipids (due to the presence in their structure of a very stable P—C bond) have been suggested to contribute to membrane protection against hydrolytic enzymes, protecting cell integrity against environmental changes [39,40]. Sphingophosphonolipids are widely distributed among marine invertebrates, and were specially studied in molluscs and symbiotic cnidaria [39,41–43].

Betaine lipids, such as DGTS, have been described to increase in response to environmental factors, such as phosphate starvation and low temperatures [44,45]. DGTS are non-phosphorus polar glycerolipids, analogous to PC in extraplastidial membranes, but there is a lack of knowledge of the functions and location of betaine lipids. A study using nine species of marine eukaryotic phytoplankton demonstrated that replacement of phospholipids for betaine lipids was different between species of algae [46]. Only four microalgae (*Picochlorum atomus*, *Tetraselmis suecica*, *N. gaditana* and *Phaeodactylum tricornutum*) showed a marked increase in betaine lipids under phosphate deprivation, suggesting that the physiological exchange between betaine lipids and phospholipids is not a common phenomenon in the marine eukaryotic phytoplankton [46]. Betaine lipids are known to be more dynamic in response to external factors in the lipid metabolism of primary endosymbionts (more similar to land plants) than in secondary endosymbionts (e.g., Chromalveolate) [46]. These differences could be related to dissimilar lipid dynamics according to primary and secondary endosymbiosis. In the *E. timida* profile, the most abundant lipid species of DGTS (DGTS 34:1) and PC (PC P-36:5) showed different fatty acyl composition and different degrees of unsaturation. These results suggest that the role of DGTS in the lipid metabolism of kleptoplasts is not a replacement for PC. Further studies are needed to clarify the role of betaine lipids in kleptoplasty.

The molecular species of glycolipids with higher amounts in *E. timida* were MGDG 18:4_18:4, DGDG 18:2_16:2 and SQDG 18:2_16:0. These results contrast with those observed in the lipidome of *E. viridis*, which presented MGDG 18:3_16:3, DGDG 18:3_16:3 and SQDG 18:3_16:0 as the main glycolipid species [15,35]. Additionally, the glycolipid profile of *E. viridis* showed that SQDG was the glycolipid class with the highest normalized XIC area [35], which differs from the prevalence of MGDG observed in *E. timida*. Both *E. timida* and *E. viridis* exhibit functional kleptoplasty but retain functional chloroplasts from different macroalgae (*E. timida* from *A. acetabulum* and *E. viridis* from *Codium tomentosum*). This dissimilarity in the profile of glycolipid species reveals that sacoglossans retain chloroplasts with different glycolipid composition and profile. This result shows a dissimilar composition in the plastid membranes of acquired chloroplasts, probably due to the retention of chloroplast with different algal origin.

4.2. Fatty acids

Specimens under quasi-dark treatment significantly increased the relative abundance of some SFA and MUFA (e.g., 18:0, 18:1 *n*-9, 18:1 *n*-11) and decreased the relative abundance of PUFA (e.g., 18:2 *n*-6, 18:3 *n*-3, 18:4 *n*-3, 20:2 *n*-6, 22:4 *n*-6). The decrease in PUFA under quasi-dark conditions can be caused by a direct effect of light on the expression and/or activity of desaturase enzymes involved in PUFA biosynthetic pathways. However, an effect of kleptoplast photosynthesis on the

levels of PUFA cannot be ruled out. Cartaxana et al. [4] have shown incorporation of labelled inorganic ^{13}C into several long-chain PUFA of *E. timida*, including PUFA that are not found in its algal food source (e.g., 22:4 *n*-6). In the case of 22:4 *n*-6 and 22:5 *n*-3, an increase in the ^{13}C signal was observed in the last elongation steps from 20:4 *n*-6 and 20:5 *n*-3, respectively, indicating that the carbon donor (malonyl-CoA) during this elongation process was ^{13}C-enriched and thereby preferentially provided by kleptoplasts [4]. In our study, SFA would not be as affected by inhibited photosynthesis because the slugs could be obtaining them directly from ingestion of the alga. Torres et al. [47] reported that methylmalonyl-CoA incorporating kleptoplast fixed-carbon is used by sacoglossan sea slugs in the synthesis of polypropionate pyrones by the action of FA synthase-like proteins. The light dependence was observed in both *n*-3 and *n*-6 PUFA biosynthetic pathways (Fig. 8). However, it was more marked in the *n*-6 pathway, due to several FA identified in this pathway, such as 20:2 *n*-6, 20:3 *n*-6 and 22:4 *n*-6, are not present in the FA profile of *A. acetabulum* [4]. This pathway contains the main substrate for the synthesis of prostaglandins (a subclass of eicosanoids), the FA 20:4 *n*-6. Prostaglandins are produced by oxidation of the PUFA 20:4 *n*-6 (which is released from phospholipids by phospholipase) through the action of cyclooxygenase (COX) and lipoxygenase (LOX) pathways in response to external stimuli [48,49]. The FA 20:3 *n*-6 and 20:5 *n*-3 have also been identified as precursors of these molecules [49]. In invertebrates, prostaglandins have been recognized to play relevant physiological roles during oogenesis and spermatogenesis, and as ion transport and defence molecules [48].

4.3. Conclusion

This study demonstrated the influence of light on the lipidome of *E. timida*. Quasi-dark conditions and limited photosynthetic activity promoted a decline in the relative abundance of PUFA, suggesting a decrease in the biosynthetic capacity of the sea slug to assemble these fatty acids. The lower relative abundance of essential lipids for photosynthesis under quasi-dark conditions, such as glycolipids and PG, and the maintenance of maximum photosynthetic capacities (F_v/F_m) suggest that the older chloroplasts were targeted for degradation. Quasi-dark also promoted a decrease of betaine lipids, while the phospholipidome exhibited a more stable composition, although remodelling of the PE, PC, PI and LPE lipid species was observed.

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CRediT authorship contribution statement

Study design: Felisa Rey and Paulo Cartaxana; Sampling: Paulo Cartaxana; Experimental work: Felisa Rey and Paulo Cartaxana; Data analysis and interpretation, Felisa Rey, Paulo Cartaxana, Susana Aveiro and Michael Greenacre; Statistical analysis: Felisa Rey and Michael Greenacre; LC-MS setup: Tânia Melo; Writing – original draft preparation: Felisa Rey; Funding acquisition: Pedro Domingues, Maria do Rosário Domingues and Sónia Cruz; Critical revision of the manuscript: all authors; Editing: Felisa Rey. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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