Enhancement of polyunsaturated fatty acid production under low-temperature stress in *Cylindrotheca closterium*

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Abstract

Marine microalgae synthetize great amounts of essential metabolites such as fatty acids and amino acids. In addition, their exposure to stress factors can induce the overproduction of these desirable metabolites. Thus, in this work, we assessed the effect of low-temperature stress on lipid production and composition of the diatom *Cylindrotheca closterium*, in order to evaluate its potential as an alternative feedstock of essential polyunsaturated fatty acids (PUFAs). The alga was first cultured in a photobioreactor at 20 °C (control), and stressed by suddenly lowering the culture temperature, from 20 °C to 11 °C, at the exponential (LTEP) or stationary (LTSP) growth phases. Neutral lipids (NLs) were the main lipid fraction of all conditions assayed, and their production was maximal at LTSP. Fatty acid analysis also showed that the greatest production of PUFAs was observed in the NL fraction at LTSP condition. In terms of essential fatty acids, the production of the omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid was similar in both treatments. The production of omega-6 fatty acids was significantly higher in the LTSP condition due to an increase in arachidonic acid content. Sterols increased under both stress conditions, with a predominance of cholesterol. Considering that the LTSP condition elicited the best PUFA production, the amino acid composition was determined. The 46.81% of total amino acids were essential components for aquatic animals. These findings provide evidence of the potential of *C. closterium* as an alternative, sustainable source of sterols, essential fatty acids, and amino acids.

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Introduction

Microalgae are the main producers of eicosapentaenoic acids (EPA), arachydonic (ARA) and docosahexaenoic (DHA) in natural food chains (Adarme-Vega et al., 2012) and have therefore generated great interest in the food industries, nutraceuticals, and aquaculture foods (Miller et al. 2008; Bozarth et al. 2009; Yi et al. 2017). The main commercial source of these fatty acids is fish oil (Ward and Singh 2005; Maisashvili et al. 2015; Lazzarotto et al. 2018). The need to reduce this dependence on fish oil to avoid overfishing in the world's oceans and fluctuations in supply and cost is increasingly recognized (Turchini et al. 2009; Tocher 2015; FAO 2018). Efforts are therefore directed towards the assessment of renewable and sustainable alternative sources of essential fatty acids EPA, ARA, and DHA.

Diatoms are natural producers of essential omega-3 (ω-3) fatty acids such as EPA and DHA (Hamilton et al. 2014).

During the exponential growth stage, these essential fatty acids are commonly found in the polar lipid fraction, being a part of the phospholipid structure (López-Alonso et al. 2000). However, during the stationary growth phase, PUFAs are stored as triacylglycerides (TAGs) in cytosolic lipid droplets (Boelen et al. 2017; Camacho-Rodríguez et al. 2018; Sanaa et al. 2018; Zulu et al. 2018). It has been reported that TAG synthesis also occurs when cell homeostasis is disrupted due to the action of a stressor (Borowitzka 2018; Wang et al. 2019). In addition to TAGs, some microalgae can synthetize other high-value products as proteins containing an important amount of essential amino acids (Brown 1991; Gong et al. 2019) and sterols (Rampen et al. 2010).

The biochemical composition of diatoms not only depends on the strain but also on the culture conditions employed. Their FA, protein, and sterol composition varies according to the growth stage, culture medium, and environmental conditions (salinity, light, and temperature) (Luo et al. 2015; Qiao et al. 2016; Gong et al. 2019). In particular, adaptation to low temperatures has been shown to increase the synthesis of poly-unsaturated long-chain FAs in some diatoms (Qiao et al. 2016). However, there is little evidence showing how sudden changes in the culture temperature can affect PUFA production by diatoms.

Cylindrotheca closterium is a marine diatom with a high growth rate (Liang et al. 2002; Duong et al. 2015) that has been proposed as a promising candidate for biofuels (Wang et al. 2015) and for the production of high value pigments (Wang et al. 2018). In this species, PUFA-containing lipids have been shown to increase under nitrogen starvation (Wang et al. 2019). However, the effect of low-temperature stress on PUFA production has not been evaluated. The aim of this study was therefore to evaluate the effect of low-temperature stress at two different growth stages on essential fatty acid production in *C. closterium*. The composition of sterols and amino acids was also analyzed in order to assess the nutritional value of the biomass.

Materials and methods

Algal strain

The marine diatom *Cylindrotheca closterium* (Ehrenberg) Reimann & J. C. Lewin was isolated from Bahía Blanca Estuary (38° 45′ S, 62° 22′ W). This is a tychopelagic diatom, which was identified according to Hasle and Syvertsen (1997) and Popovich and Marcovecchio (2008). Unialgal non-axenic cultures were established and maintained as culture stock, in f/2 culture medium (McLachlan 1973) at 20 °C, under 37 µmol photons m^{-2} s^{-1} irradiance provided by fluorescent tubes, in a 12:12-h light/dark cycle.

Culture in photobioreactor

An initial inoculum containing 1.5×10^5 cells of *C. closterium* at the exponential growth stage was transferred to a cylindrical photobioreactor (Figmay 15L, FIGMAY S.R.L, Córdoba, Argentina) of 0.25 m diameter and 0.70 m height, containing f/2 culture medium to a final volume of 10 L. The temperature of the culture was regulated by recirculating a cooling fluid through a glass coil, supplied by a thermostatic bath (Alpha RA 8, LAUDA, Germany).

Low-temperature stress experiments were performed as follows: (a) for the control condition, the temperature was maintained at 20 ± 1 °C for 10 days; (b) for the low temperature at the stationary phase (LTSP) condition, the temperature was maintained at 20 ± 1 ° C for 7 days and then lowered to 11 ± 1 °C for the 3 remaining days; and (c) for the low temperature at the exponential phase (LTEP) condition, *C. closterium* cells was exposed to 20 ± 1 °C for 3 days, and then kept at 11 ± 1 °C for 7 days. Light intensity of 60 µmol photons m^{-2} s^{-1} was provided by LED lamps, with a 12:12-h light/dark cycle. Cultures were supplied with 1% CO_2 and maintained under continuous stirring at 9 rpm by means of central paddles. Samples were taken every day to determine growth parameters. The biomass was harvested on day 10 through centrifugation at 3500×g for 15 min, washed with phosphate saline buffer, lyophilized and stored at − 80 °C.

Specific growth rate, doubling time, and biomass dry weight determinations

Cell density was determined by daily counts using a Sedgwick-Rafter chamber. The growth rate (µ; div. day⁻¹), was estimated during the period of exponential growth by least squares fit to a straight line of the logarithmically transformed data (Guillard 1973). The time it takes a cell to double, doubling time (DT) (hours), was calculated as DT: $(1/\mu) \times 24$. Cell volume was calculated using the formula of an elliptic prism (Hillebrand et al. 1999).

For biomass dry weight (DW), 10 mL of sample were collected daily, filtered through pre-conditioned and pre-weighed filters (Whatman GF/C) and washed with 10 mL distilled water. Then, filtered biomass was dried for 12 h at 70 °C, cooled in a desiccator and weighed. Dry weight was obtained as the difference between the two values (Martín et al. 2016).

Quantification of triacylglycerols and sterols

Intracellular lipid droplets were detected through Sudan IV staining and observed in an optical microscope (DM2000, Leica Microsystems, Germany). Neutral lipids were detected by the fluorescent lipophilic probe, Nile Red (9-diethylamino-

5H-benzo[alpha]phenoxazine-5-one), using a fluorescence microscope (Eclipse E800, Nikon, Japan) (Martín et al. 2016). In addition, TAG and sterol accumulation kinetics were evaluated according Scodelaro Bilbao et al. (2016). Briefly, lipids were extracted from *C. closterium* with chloroform:methanol (2:1, *v*/v) (Folch et al. 1957). A biphasic system was created through the addition of 0.05% $CaCl_2$ and centrifuged at 2000×*g* for 10 min. The lower phase was recovered and dried under N_2 atmosphere. TAG and sterols were separated through thin layer chromatography (TLC) and identified using the commercial standards triolein (2 g L^{-1}) (Wienner Lab, Rosario, Argentina) and β-sitosterol (100 μg mL^{-1}) (Sigma-Aldrich, USA), respectively. TLC was performed using silica gel plates (silica gel 60 G Merck, Germany). The mobile phase was n-hexane: diethyl ether (80:20 *v*/v). The resulting bands were visualized with 2,7-dichlorofluorescein. TAGs and sterols were scraped and eluted with chloroform/methanol/water (5:5:1, *v*/*v*/*v*). Finally, through addition of 100 μL of isopropyl alcohol, samples were quantified spectrophotometrically using commercial kit TAG color GPO/PAP and Colestat enzimático (Wienner Lab, Argentina). All reagents were of analytical grade and used without further purification.

Gas chromatography analysis

Lipid fraction and fatty acid profile determination

Lipid fractionation was performed from total lipid extracts (obtained as detailed in the "Gas chromatography analysis" section), using chloroform activated Silica Extraction Cartridges (PKG30 1 g, Enviro-Clean, USA) (Popovich et al. 2012). Neutral lipid (NL), glycolipid (GL), and phospholipid (PL) fractions were eluted, recovered, and evaporated to dryness under nitrogen. Finally, the weight of each fraction was determined.

To determine the fatty acid composition of total lipid extracts and lipid fractions, FAs were hydrolyzed and subjected to methylation. The resulting FA methyl esters (FAMEs) were analyzed through gas chromatography by a gas chromatograph (HP 4890D, Hewlett Packard, USA) equipped with a capillary SP2560 column (100 m, 0.25 mm, and 0.2 μm) (Supelco Inc., USA). The equipment also contained a split/splitless injector and a flame-ionization detector, both at 260 °C. Data were analyzed using the HP3398A GC Chemstation Software (Hewlett Packard, USA). The Supelco FAME 10 mix 37 (CRM47885, Supelco Inc., USA) certified standard was used to identify FAMEs. Fatty acids content (g (100 g biomass)$^{-1}$) was estimated from % of total FAMEs (Batista et al. 2013; Bonfanti et al. 2018). The conversion factors F were calculated according to Weihrauch et al. (1977), based on published information by Yu et al. (2009), Yao et al. (2015) and Yang et al. (2017).

Sterol extraction and identification

Sterols were extracted and identified as previously described in the "Gas chromatography analysis" section. The resulting dried extract was silylated with SIGMA SIL-A following the manufacturer's instructions (Sigma-Aldrich, USA). Separation and identification of sterols was carried out according to methods described by the International Olive Council (IOC, 2001) in a gas chromatograph (7820A, Agilent, USA) equipped with a 30 m capillary column of 0.25 mm i.d. and 0.2 μm film thickness (SE 54, Supelco Inc., USA) as in Scodelaro Bilbao et al. (2016). An internal standard of 5-α-cholestan-3-β-ol (Sigma-Aldrich, Switzerland) (2 mg mL^{-1}) was used.

Protein extraction

Proteins from *C. closterium* were extracted using a lysis buffer (100 mM Tris HCl (pH 7.5), 300 mM NaCl, 4 mM EDTA, 4 mM EGTA, 0.2% Triton X-100, 2% Nonidet P-40). Samples were vortexed vigorously and centrifuged for 15 min at 14,000×*g* to remove insoluble material and unbroken cells. The colorimetric Bradford reaction (Bradford, 1976) was performed in an aliquot of the resulting supernatant using bovine serum albumin as standard.

Amino acid profile determination

Cylindrotheca closterium samples consisting of 6 mg of lyophilized material were hydrolyzed by the addition of 1 mL of methanesulfonic acid 4 N (containing 0.2% (*w*/v) tryptamine) at 115 °C for 22 h in vacuum-sealed tubes. The resulting free-amino acids were separated through cationic exchange chromatography using an automatic amino acid analyzer (Biochrom 30, GE-Healthcare Life Sciences, UK), sodium citrate buffers of different final pH (pH = 2.2, 3.2, 4.25, and 6.45) and 0.4 M of sodium hydroxide (pH = 13.6) following the manufacturer's instructions. The resulting extract was then re-derivatized using of ninhydrin and products were detected through visible spectrophotometry at 570 nm and 440 nm. Finally, amino acids were identified through comparison of retention times of amino acid commercial standards (18AA-Sigma and individual standards for L-methionisulfone, L-norleucine, L-methioninsulfoxide, L-tryptophan, and L-ornithine). In addition, quantification was performed using L-norleucine as internal standard (Simpson et al. 1976; Brown 1991).

Statistical analysis

One-way ANOVA and Tukey tests were used to analyze differences among treatments using software INFOSTAT (Di Rienzo et al. 2018). The significance level was set at $p < 0.05$.

Results

The growth rate and biomass kinetics of *Cylindrotheca closterium* at 20 °C and under the two low-temperature stress conditions (LTSP and LTEP) are shown in Fig. 1 and Table 1. As can be seen in Fig. 1a, *C. closterium* followed the same growth kinetics under all the conditions assayed. Cells grew exponentially up to day 4, when they reached the stationary growth phase, lasting until day 10. Although the final cell density of control and LTSP conditions was similar ($p > 0.05$), it was significantly lower when low-temperature stress was applied at the exponential growth phase (LTEP) ($p < 0.05$) (Fig. 1a). In terms of cell volume, the highest values were observed under LTEP and LTSP conditions ($p < 0.05$) (Table 1). The growth rates (μ) and doubling times (DT) revealed no statistically significant differences between the conditions assayed ($p > 0.05$) (Table 1).

Biomass kinetics showed a two-day lag phase under all the conditions tested. The largest biomass production occurred at day 6 (LTEP) and day 8 (control), followed by a marked decrease up to day 10. Under the LTSP condition, however,
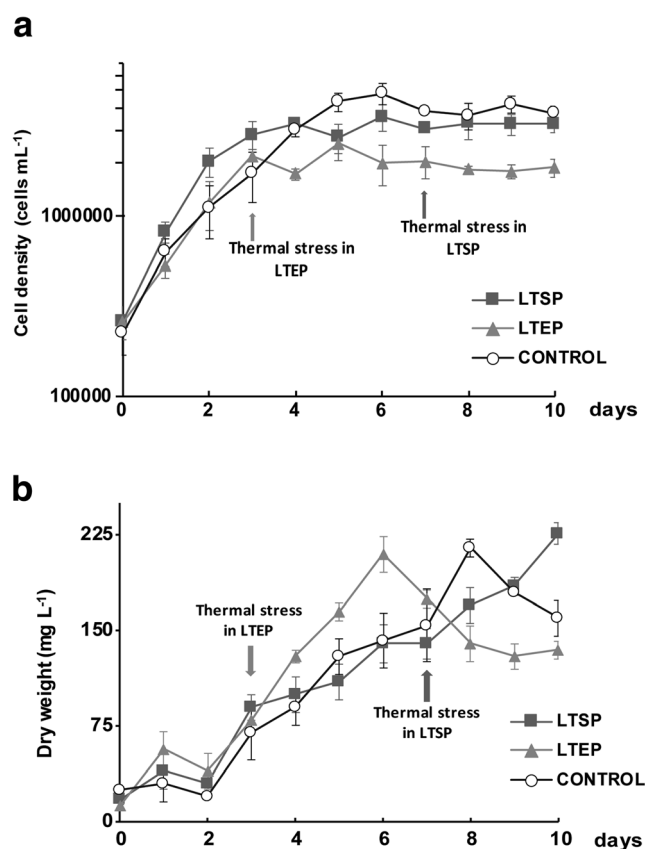
the largest biomass production was observed on day 10 with no subsequent decrease in biomass production (Fig. 1b and Table 1).

Total lipids reached their maximum values (28% dry weight) under control and LTSP conditions, and ≈20% dry weight under the LTEP condition. With respect to lipid fractions (% of total lipids), the NL was quantitatively the main fraction, followed by GL and PL under all conditions (Fig. 2a). The different temperature stress conditions did not affect the lipid fraction distribution with respect to total lipids ($p > 0.05$) (Fig. 2a). However, when the values were expressed as mg L^{-1}, NL production was significantly higher under the LTSP condition (Fig. 2b). TAG accumulation kinetics showed the highest TAG production (in mg L^{-1}) at the end of the stationary growth phase. However, the highest TAG values were obtained under LTSP stress condition on day 10 (Fig. 3a). This effect was also evidenced through the observation of cells under an optical microscope, where *C. closterium* cells exhibited numerous small lipid droplets dispersed in the cytoplasm by day 4 (exponential growth phase), whereas two or three larger lipid droplets were observed on day 10 of culture (stationary growth phase) (Fig. 3 b and c).

The lipid composition of *C. closterium* cells in terms of total fatty acids is shown in Table 2. Gas chromatography analysis revealed an increase in PUFAs, at the expense of a reduction in the relative percentage of SFA in response to low temperature stress, regardless of the phase in which it was applied. In addition, the percentage of PUFAs was significantly higher under the LTEP condition than under the LTSP condition, attributable mainly to eicosapentaenoic (EPA), whereas arachidonic (ARA) only increased under the LTSP condition. The decrease in the total percentage of SFA was mainly due to palmitic and stearic acids. The total percentage of mono-unsaturated fatty acids (MUFAs) only increased under the LTSP condition, owing to the role of palmitoleic acid. However, when these results were expressed in terms of production (g (100 g biomass)$^{-1}$), both temperature-stress conditions showed a similar increase in the amount of PUFA that was above the level found in the control (Fig. 4a). The amount of ω-3, increased from 1.92 to 2.96 g (100 g biomass)$^{-1}$ under both LTSP and LTEP conditions (Fig. 4b), mainly due to an increase in EPA and DHA (Fig. 4c). In terms of essential FAs, EPA and DHA production was similar under the two stress conditions. In the case of ω-6, its production (g (100 g biomass)$^{-1}$) was significantly higher under the LTSP condition. This was due to an increase in the level of ARA detected under this temperature condition.

The FA composition of each lipid fraction was analyzed and changes in the relative proportions (% of total FAMEs) of SFAs, MUFAs, and PUFAs were observed (Table 3). For the LTSP condition, the amount of SFAs and PUFAs in the NL fraction decreased with respect to the control, at the expense of an increase in MUFAs. In the NL fraction of the LTEP

Fig. 1 Growth and biomass production of *C. closterium* in photobioreactor. **a** Cell density (cells mL^{-1}) and **b** biomass kinetics (mg of dry weight L^{-1}) for control, LTSP (low-temperature stress applied at stationary phase), and LTEP (low-temperature stress applied at exponential phase). Values are represented as the average ± standard deviation ($n = 3$)

Table 1 Specific growth rate (μ), doubling time (DT), and biomass production of *C. closterium* under control, LTSP (low-temperature stress at stationary phase) and LTEP (low-temperature stress at exponential phase) conditions. Values are expressed as an average ± standard deviation (*n* = 3). Statistically significant differences (*p* < 0.05) in rows are indicated with different letters. *Final values at day 10 of culture

	Control	LTSP	LTEP
Maximum growth rate (div day^{-1})	1.13 ± 0.40 a	1.30 ± 0.20 a	1.05 ± 0.30 a
Doubling time (h)	23.75 ± 10.47 a	18.75 ± 2.64 a	24.11 ± 6.36 a
Cell density (cells mL^{-1})*	3,723,040 ± 334,905 c	3,269,280 ± 204,404 b	1,864,667 ± 210,308 a
Cell volume (μm^3)*	86.03 ± 31.84 a	144.29 ± 34.61b	153.38 ± 44.89 b
Biomass production (mg dry weight L^{-1})*	160 ± 14 b	226 ± 08 c	135 ± 07 a

condition, the proportion of SFAs also decreased but was accompanied by an increase in MUFAs and PUFAs. The maximum values of PUFAs were found in the GL fraction, with values of 26.61% and 22.71% of total FAMEs for the LTEP and LTSP conditions, respectively. Nevertheless, when these values were expressed as mg (100 g biomass)$^{-1}$, the results clearly revealed that neutral lipids were quantitatively the most important FA source. The maximum production of PUFAs was observed in the NL fraction of the LTSP

Fig. 2 Effect of culture temperature on *C. closterium* lipid fractions. **a** Relative percentage (% of total lipids) of neutral lipids (NL), glycolipids (GL), and phospholipids (PL) and **b** *C. closterium* lipid fractions (mg L^{-1}). Values are shown as averages (± SD) (*n* = 3). Different letters (per lipid fraction) represent significant differences (*p* < 0.05)

condition, reaching a value of 2554 mg (100 g biomass)$^{-1}$. Table 4 shows the essential FA content of each lipid fraction. When the results were expressed as % of total FAMEs (left column), the highest increases of ω-3 and ω-6 with respect to control were detected in the GL fraction of the LTEP condition. However, in terms of production (mg (100 g biomass)$^{-1}$), the most important source of ω-3 and ω-6 FAs was the NL fraction, in all the conditions tested. EPA content increased significantly under both temperature treatments, reaching values of 1252 and 1339 mg (100 g biomass)$^{-1}$ for LTSP and LTEP, respectively, with no significant differences between these two conditions (*p* > 0.05). The highest DHA and ARA contents were detected in the dominant lipid fraction (NL) under LTSP.

Finally, the ω-3 and ω-6 FA contents (% of total FAMEs) obtained from *C. closterium* cells grown under the LTSP condition with the values reported for different commonly used fish oils were compared (Table 5). In this way, *C. closterium* showed the highest value of ARA, a similar content of EPA and a lower value of DHA than those reported in fish oils.

With respect to *C. closterium* sterol content, their spectrophotometric quantification showed that low-temperature stress induced either at the exponential or stationary growth phases induced a similar increase in sterol content (Fig. 5a). Cholesterol and the phytosterols brassicasterol, stigmasterol, Δ5-23-stigmastadienol, clerosterol, β-sitosterol, Δ5-24-stigmastadienol, and Δ-stigmastenol were the major sterols found under all the conditions assayed. Cholesterol was the main sterol found, representing 41, 76, and 57% of total sterols under control, LTSP and LTEP conditions, respectively (Fig. 5b). The major increase thus being under the LTSP condition. Although brassicasterol was the main phytosterol found under the control condition representing 32% of total sterols, its content was lower under the two thermal stresses assayed (Fig. 5b).

The effect of low-temperature stress on the protein content of *C. closterium* biomass was determined spectrophotometrically. The LTSP condition exhibited similar protein content (mg g^{-1} biomass) as the control, whereas when the stress was applied at the exponential growth phase, the amount of proteins was significantly higher than under the other two
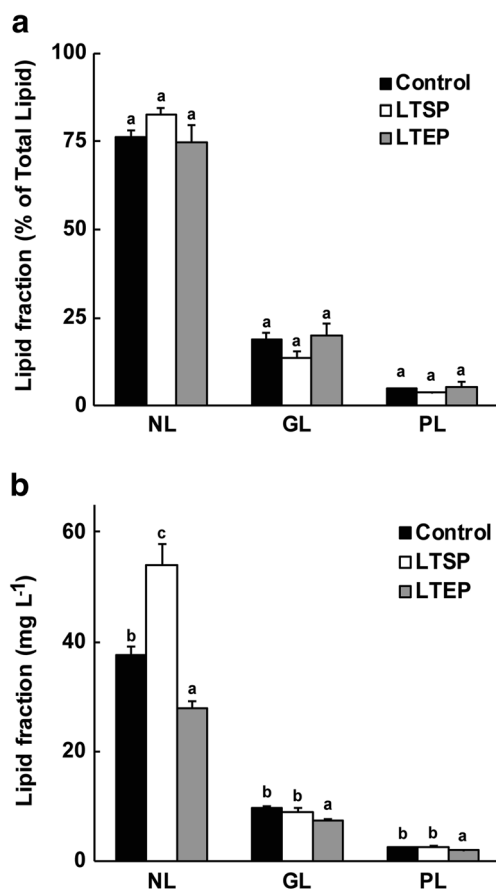
Fig. 3 Time-response analysis of *C. closterium* lipid production. **a** Spectrophotometric quantification of TAG (mg L^{-1}) after 2, 4, 7, and 10 days of culture under control, LTSP, and LTEP temperature conditions. Values are represented as the averages ($\pm$ SD) ($n = 3$). Different letters represent statistically significant differences ($p < 0.05$) between conditions. Intracellular lipid droplets of *C. closterium* cells exposed to low-temperature stress at the stationary growth phase (LTSP) and stained with **b** SUDAN IV and with **c** Nile Red
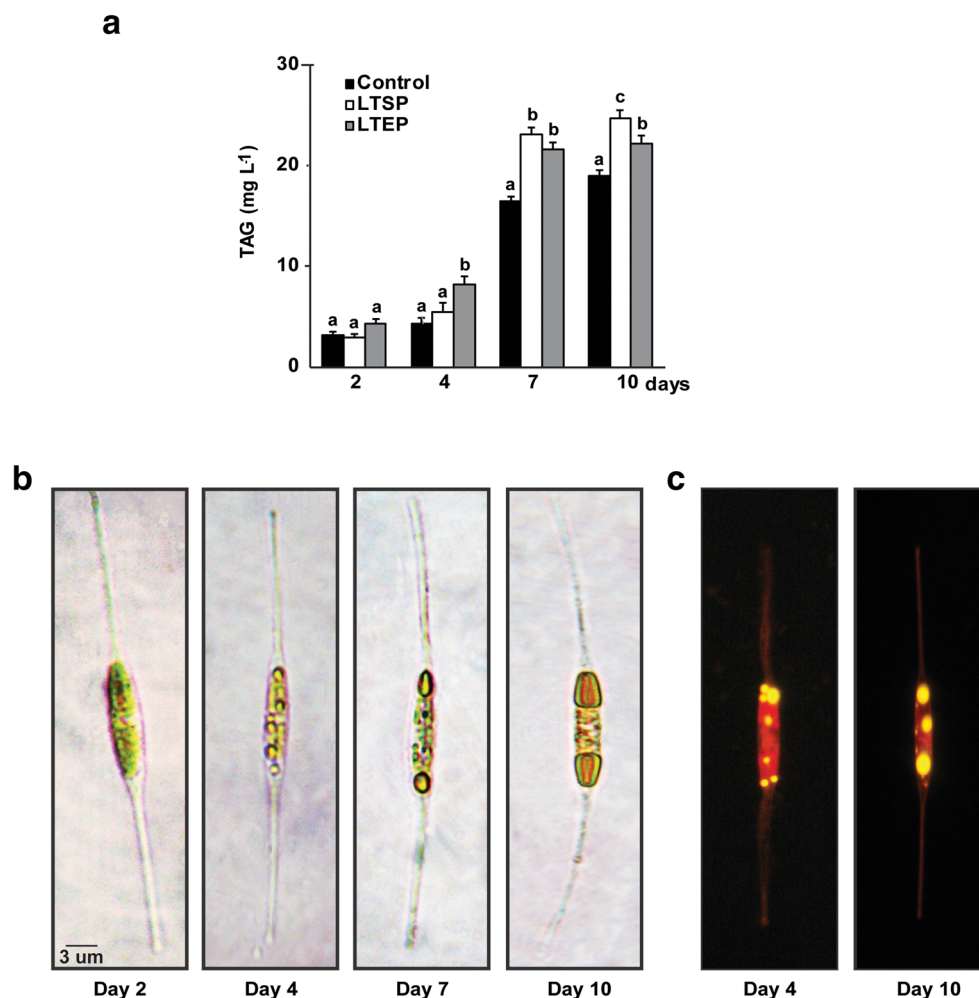
conditions assayed (Fig. 6a). Taking into account that under the LTSP condition elicited the best production in terms of essential FAs, this condition was selected for the analysis of the amino acid profile (AA). Total AA content was 16.5 g (100 g biomass)$^{-1}$. Essential amino acids (EAA) for aquatic animals represented 46.81% of the total AA (Fig. 6b). Except for tryptophan, all essential amino acids were present in *C. closterium*, the major ones being leucine, valine, phenylalanine and lysine (Table 6). The highest AA relative amounts corresponded to glutamate and aspartate, with values of 11.90% and 9.84% of total AA, respectively (Table 6).

Discussion

The marine diatom *C. closterium* has been reported to have good growth performance and higher biomass production than other diatom species (Liang et al. 2002; Duong et al., 2015; Wang et al. 2018). In the present study, the specific growth rate was in the range reported for other strains of *C. closterium* (Wang et al. 2015; Demirel 2016; Erdoğan et al., 2016) and

higher than that reported for other native marine diatoms from Bahía Blanca Estuary (Popovich et al. 2012). In addition, although low-temperature exposure decreased cell density, it rapidly increased cell volume, resulting in an increased biomass production under both stress conditions. These results agree with those found by Montagnes and Franklin (2001), who reported that low temperatures can induce a decrease in the growth rate of diatoms together with an increase in their cell volume.

Diatoms induce the synthesis and storage of lipids under diverse stress conditions (e.g., high light intensity, nutrient deficiency, and temperature) (Lombardi and Wangersky 1995; Zulu et al. 2018). *Cylindrotheca closterium* showed higher lipid content when low-temperature stress was applied at the stationary phase than when it was applied at the exponential phase, where the total lipid yield was negatively affected. In addition, the amount of lipids produced by *C. closterium* under the LTSP condition was higher than that reported for other strains of *Cylindrotheca* cultured in f/2 medium at 22 °C and harvested in the stationary growth phase (Liang et al. 2002) or at the end of exponential growth phase

Table 2 Fatty acid profiles (as % of total FAMEs) of *C. closterium*. Control, LTSP (low-temperature stress at stationary phase), LTEP (low-temperature stress at exponential phase). Table shows means ± standard deviation (*n* = 3), and the different letters in rows stand for statistically significant differences (*p* < 0.05) between temperature conditions

Fatty acids			
	Control	LTSP	LTEP
Saturated (SFA)			
C 14:0	10.46 ± 0.46 ab	10.90 ± 0.15 b	10.18 ± 0.27 a
C 15:0	0.38 ± 0.04 a	0.38 ± 0.01 a	0.73 ± 0.02 b
C 16:0	31.56 ± 0.48 c	24.78 ± 0.11 b	21.76 ± 1.19 a
C 17:0	0.09 ± 0.02	nd	nd
C18:0	3.86 ± 0.15 c	0.50 ± 0.05 b	0.35 ± 0.03 a
C 20:0	0.38 ± 0.02 c	0.14 ± 0.01 b	0.10 ± 0.01 a
C 22:0	0.19 ± 0.03	nd	nd
C 24:0	0.10 ± 0.07 b	nd	0.04 ± 0.01 a
Mono-unsaturated (MUFA)			
C 14:1	nd	nd	0.10 ± 0.01
C 16:1	25.68 ± 0.55 a	33.94 ± 1.11 c	30.16 ± 1.63 b
C 17:1	0.88 ± 0.01 a	1.63 ± 0.13 b	2.47 ± 0.07 c
C 18:1 ω-9 t	1.20 ± 0.07 a	1.91 ± 0.14 b	nd
C 18:1 ω-9c	8.60 ± 0.13 b	3.37 ± 1.01 a	4.31 ± 0.12 a
C 24:1 ω-9	0.40 ± 0.15 a	0.81 ± 0.20 b	0.59 ± 0.07 ab
Polyunsaturated (PUFA)			
C 18:2 ω-6c	2.36 ± 0.14 a	3.10 ± 0.10 a	5.78 ± 1.00 b
C 18:3 ω-6	0.87 ± 0.06 a	1.17 ± 0.15 b	0.91 ± 0.04 a
C 20:2 ω-6	0.82 ± 0.04 a	1.50 ± 0.02 b	1.75 ± 0.17 c
C 20:3 ω-6	0.21 ± 0.01 a	0.32 ± 0.11 b	0.17 ± 0.02 a
C 20:4 ω-6 (ARA)	3.00 ± 0.36 a	4.20 ± 0.21 b	3.11 ± 0.18 a
C 20:5 ω-3 (EPA)	7.05 ± 0.95 a	10.20 ± 0.77 b	15.46 ± 1.51 c
C 22:2 ω-6	0.10 ± 0.01 a	nd	0.15 ± 0.02 a
C 22:6 ω-3 (DHA)	0.69 ± 0.14 a	1.23 ± 0.08 b	1.52 ± 0.30 b
∑ SFA	47.97 ± 1.49 b	37.07 ± 0.17 ab	33.51 ± 1.36 a
∑ MUFA	36.70 ± 0.21 a	41.35 ± 0.43 b	37.64 ± 1.69 a
∑ PUFA	15.28 ± 1.70 a	21.64 ± 0.80 b	28.85 ± 3.04 c
∑ ω 3	7.74 ± 1.08 a	11.43 ± 0.80 b	16.98 ± 1.79 c
∑ ω 6	7.50 ± 0.68 a	10.21 ± 0.10 ab	11.72 ± 1.27 b
∑ ω 9	10.19 ± 0.35 b	6.08 ± 1.14 a	4.91 ± 0.06 a

Fig. 4 Analysis of the fatty acid production of *C. closterium*. The results, expressed as gram fatty acid (g) in 100 g of biomass, are shown as **a** saturated (SFA), mono-unsaturated (MUFA) and polyunsaturated (PUFA) fatty acids; **b** omega 3, 6, and 9 fatty acids; and **c** eicosapentaenoic (EPA), arachidonic (ARA), and docosahexaenoic (DHA) fatty acids for *C. closterium* cells under control, LTSP (low-temperature stress at stationary phase), and LTEP (low-temperature stress at exponential phase). Conversion factor (F) used were 0.88 for control and LTEP conditions and 0.90 for LTSP condition. Values are presented as an average ± standard deviation (*n* = 3). Different letters represent significant differences (*p* < 0.05) between growth conditions for each fatty acid mentioned

(Wang et al. 2015). Contrary to our results, temperature stress did not affect the lipid content of the diatom *Phaeodactylum tricornutum* (Sayanova et al. 2017), although this different behavior could be due to the fact that *P. tricornutum* stress experiments were ended when the cells were at the exponential growth phase.

TAGs are the major lipid constituent in several microalgae (Zulu et al. 2018). In diatoms, their maximum production occurs under adverse environmental conditions, such as nutrient deficiency or low temperature (Yu et al. 2009; Boelen et al. 2017; Wang et al. 201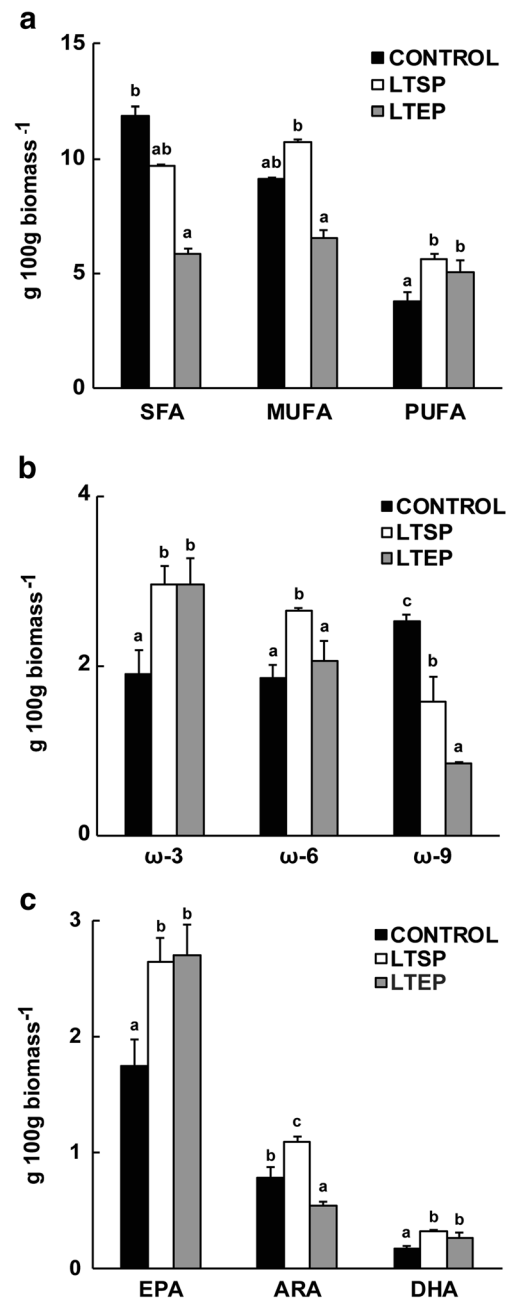9). TAG accumulation in *C. closterium* followed the expected behavior, reaching the highest lipid value at the stationary growth phase. In addition, low temperature induced TAG accumulation over the control condition.

Table 3 Saturated (SFAs), mono-unsaturated (MUFAs), and polyunsaturated (PUFAs) fatty acid composition of *C. closterium* lipid fractions. Lipid fraction: NL (neutral lipid); GL (glycolipids); PL (phospholipids). Control, LTSP (low-temperature stress at stationary phase), LTEP (low-temperature stress at exponential phase). Values are presented as average ± standard deviation ($n = 3$). To estimate fatty acid production (expressed as mg $(100 \text{ g biomass})^{-1}$) the conversion factor (F) used for control, LTSP, and LTEP conditions, respectively, were LN: 0.73, 0.79, and 0.71; GL: 0.12, 0.09, and 0.13; and PL: 0.04, 0.03, and 0.04. Different letters (per lipid class) represent statistically significant differences ($p < 0.05$)

Control		LTSP		LTEP	
% of total FAMEs	mg $(100 \text{ g biomass})^{-1}$	% of total FAMEs	mg $(100 \text{ g biomass})^{-1}$	% of total FAMEs	mg $(100 \text{ g biomass})^{-1}$
NL					
SFA	46.09 ± 0.27 c	7120 ± 42.25b	41.74 ± 0.34b	7770 ± 63.54c	36.52 ± 0.52a	3860 ± 55.25a
MUFA	38.76 ± 0.29 a	5990 ± 45.02b	44.51 ± 0.22c	8290 ± 41.42c	42.17 ± 0.25b	4460 ± 27.18a
PUFA	15.14 ± 0.03 b	2340 ± 5.25a	13.74 ± 0.51a	2554 ± 86.23b	21.31 ± 0.69c	2250 ± 73.58a
GL						
SFA	50.52 ± 0.93c	327 ± 6.01c	42.53 ± 0.37b	149 ± 1.20a	40.32 ± 1.64a	203 ± 8.25b
MUFA	30.02 ± 0.66a	195 ± 4.25c	33.51 ± 0.62b	116 ± 2.19a	30.07 ± 1.06a	151 ± 5.35b
PUFA	19.45 ± 0.53a	126 ± 3.44ab	22.71 ± 0.32ab	79 ± 1.02a	26.61 ± 2.68c	149 ± 13.47b
PL						
SFA	47.32 ± 2.68a	24 ± 1.48b	42.67 ± 1.83a	14 ± 0.58a	43.82 ± 0.54a	18 ± 0.23ab
MUFA	39.29 ± 1.51a	20 ± 0.77b	39.31 ± 1.29a	11 ± 0.31a	36.61 ± 0.42a	15 ± 0.18ab
PUFA	13.40 ± 1.18a	7 ± 0.60a	21.35 ± 1.45b	7 ± 0.45a	19.57 ± 0.12b	8 ± 0.05b

Table 4 Essential fatty acid content of *C. closterium* lipid fractions at control, LTSP, and LTEP conditions. Essential fatty acids: ω-3 (omega-3); ω-6 (omega-6); EPA (eicosapentaenoic); ARA (arachidonic); DHA (docosahexaenoic). Lipid fraction: NL (neutral lipid); GL (glycolipids); PL (phospholipids). To estimate fatty acid production (expressed in mg $(100 \text{ g biomass})^{-1}$) the conversion factor (F) used for control, LTSP and LTEP conditions, respectively, were LN: 0.73, 0.79, and 0.71; GL: 0.12, 0.09, and 0.13; and PL: 0.04, 0.03, and 0.04. Values are the average ± standard deviation ($n = 3$). Different letters represent significant differences ($p < 0.05$) between conditions

Control		LTSP		LTEP	
% of total FAMEs	mg $(100 \text{ g biomass})^{-1}$	% of total FAMEs	mg $(100 \text{ g biomass})^{-1}$	% of total FAMEs	mg $(100 \text{ g biomass})^{-1}$
NL					
ω-3	7.72 ± 0.11a	1190 ± 16.98a	7.34 ± 0.38a	1370 ± 71.07ab	13.80 ± 0.72b	1440 ± 75.48b
ω-6	7.42 ± 0.08b	1150 ± 12.46b	6.40 ± 0.16a	1190 ± 23.02b	7.55 ± 0.18b	810 ± 20.89a
EPA	7.10 ± 0.11a	1097 ± 13.77a	6.73 ± 0.33a	1252 ± 62.15b	12.67 ± 0.68b	1339 ± 72.25b
DHA	0.59 ± 0.05a	91 ± 3.20a	0.60 ± 0.04a	111 ± 6.54b	0.93 ± 0.07b	98 ± 7.14ab
ARA	2.92 ± 0.08c	451 ± 1.37b	2.70 ± 0.07b	502 ± 13.12b	2.18 ± 0.18a	230 ± 19.46a
GL						
ω-3	6.34 ± 0.29a	41 ± 1.88b	8.70 ± 0.27b	30 ± 0.93a	12.12 ± 0.95c	61 ± 4.78c
ω-6	13.12 ± 0.30a	85 ± 1.91b	14.01 ± 0.14ab	49 ± 0.39a	17.48 ± 1.89b	88 ± 9.50b
EPA	6.08 ± 0.29a	39 ± 1.79b	8.39 ± 0.19b	29 ± 0.65a	11.70 ± 0.88c	59 ± 4.42c
DHA	0.26 ± 0.16a	2 ± 0.13b	0.31 ± 0.09a	1 ± 0.30a	0.42 ± 0.07a	2 ± 0.37b
ARA	2.12 ± 0.30b	7 ± 0.49b	1.90 ± 0.04b	7 ± 1.32a	1.38 ± 0.17a	7 ± 0.83a
PL						
ω-3	6.00 ± 0.89a	3 ± 0.53a	11.81 ± 0.58b	4 ± 0.18ab	12.32 ± 0.03b	5 ± 0.02b
ω-6	7.39 ± 0.32a	4 ± 0.19b	9.54 ± 0.89b	3 ± 0.30a	7.25 ± 0.08a	3 ± 0.03a
EPA	4.27 ± 0.89a	2 ± 0.38a	7.68 ± 0.47b	2 ± 0.15a	8.54 ± 0.09b	4 ± 0.04b
DHA	1.73 ± 0.76a	1 ± 0.08a	4.13 ± 0.69b	1 ± 0.22a	3.79 ± 0.13b	2 ± 0.05b
ARA	3.81 ± 0.32a	2 ± 0.15b	4.84 ± 0.58a	1 ± 0.01a	3.83 ± 0.21a	1 ± 0.09a

Table 5 Comparison between the fatty acids of *C. closterium* stressed at stationary growth phase and fish oils. Fatty acids are expressed as % of total FAMEs. Values are presented as average ± standard deviation (*n* = 3). *From De Silva et al. (2011)

	Anchovy*	Herring*	Capelin*	Menhaden*	*C. closterium*
EPA	7.6–22.0	3.9–15.2	6.1–8.0	11.1–16.3	10.2
ARA	0.1	< 1	0.2	0.2	4.2
DHA	9.0–12.7	2.0–7.8	3.7–6.0	4.8–13.8	1.2

Therefore, low temperature acted as a stressor as defined by Borowitzka (2018). Moreover, the temperature did not affect *C. closterium* lipid fraction distribution, as neutral lipids continued to be the main lipid fraction. These results agree with those reported for other diatoms grown under different stress conditions, in which neutral lipids were the most abundant (Popovich et al. 2012; Yang et al. 2017).

An increase in PUFAs such as EPA and DHA is able to reverse the reduction in cell membrane fluidity that occurs with exposure to low temperatures (Aussant et al. 2018). In this connection and in line with other reports on marine diatoms (Mortensen et al. 1988; Chen 2012; Pasquet et al. 2014), in *C. closterium*, low temperature induced a significant rise in long-chain PUFAs. This effect was also observed in *Thalassiosira pseudonana*, *Chaetoceros calcitrans*, *Chaetoceros simplex*, *Chaetoceros gracilis*, and *P. tricornutum*. These diatoms showed higher PUFA and lower SFA contents when cells were adapted to grow at 10 °C than when they grew at 25 °C (Thompson et al. 1992). In addition, Jiang and Gao (2004) reported in *P. tricornutum* that the relative amount of PUFAs significantly increased from 12 to 20% in response to low-temperature stress. This effect was observed after 3 days of thermal stress, suggesting that the mechanisms involved in the response to low-temperature stress are very fast (Sayanova et al. 2017). Ryckebosch et al. (2014) reported in *P. tricornutum* and *T. pseudonana* higher EPA and DHA contents in the NL fraction (% of total FAMEs) than those found in *C. closterium* in this study. Our results show that both temperature treatments (LTSP and LTEP) induced an increase in the level of EPA (% of total FAMEs) in the polar lipid fraction. This is consistent with the report of Chen et al. (2008), suggesting that EPA is the main FA of the

Fig. 5 Analysis of *C. closterium* sterols. **a** Spectrophotometric determination of the total content of sterols in *C. closterium* (expressed as mg L^{-1}) at 2, 4, 7, and 10 days of culture under control, LTSP, and LTEP temperature conditions. **b** Gas chromatography analysis of the sterol profile of *C. closterium* cells at the end of each temperature condition (control, LTSP, and LTEP) expressed as % of sterols. Insert: relative amounts of total phytosterols and cholesterol at different growth conditions. Values are presented as average ± standard deviation (*n* = 3). Different letters represent significant differences (*p* < 0.05)
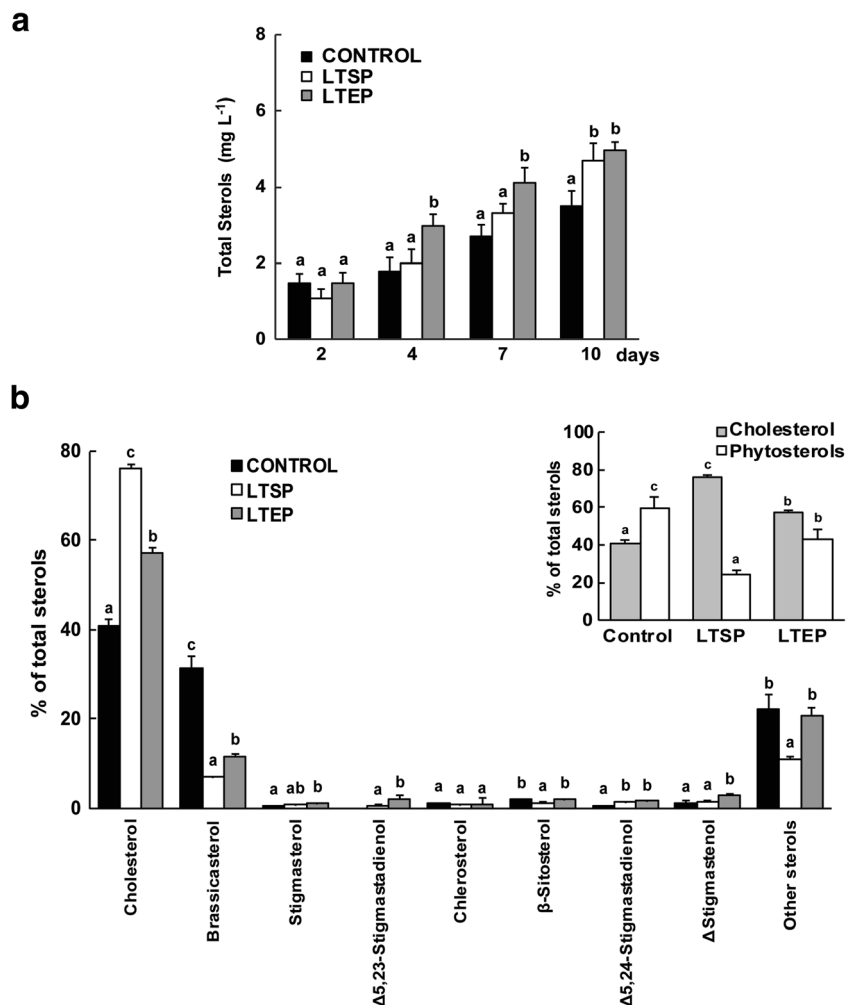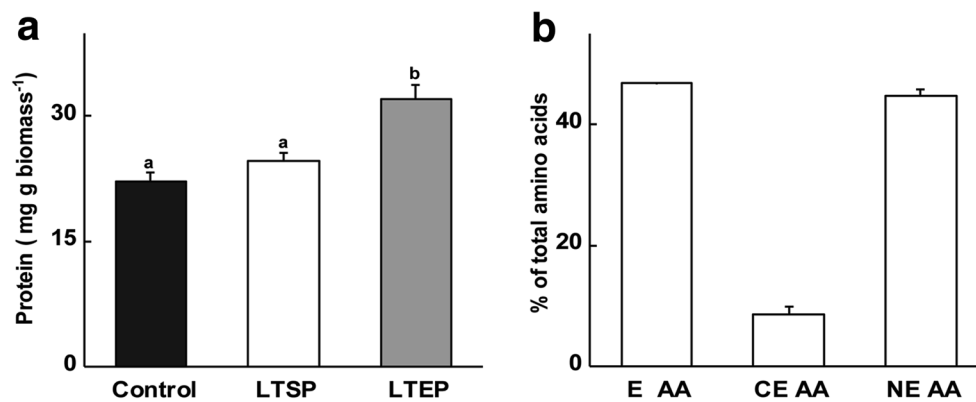
Fig. 6 Protein content and amino acid composition of *C. closterium*. **a** Protein content (mg g^{-1} biomass) for control, LTSP and LTEP growth conditions. Different letters represent statistically significant differences (p < 0.05) between conditions. **b** Percentage of essential (EAA), conditionally essential (CEAA), and non-essential amino acids (NEAA) for aquatic animals (Li et al. 2008) of *C. closterium* under LTSP growth condition. Values are presented as average ± standard deviation (n = 3)

polar fraction when cells are exposed to low culture temperature. However, since polar lipids are a minor lipid fraction, they are not relevant in terms of PUFA production. In *C. closterium* we detected an increase in the relative amount of EPA in the NL fraction only when the temperature was lowered at the exponential growth phase. This may be due to the fact that TAG-containing lipid droplets are synthetized

during low-temperature stress using unsaturated FAs. However, when the temperature is lowered at the stationary growth phase, lipid droplets have already been formed so their EPA content cannot be modified.

An increase in the % of total FAMEs does not necessarily imply an increase in FA production in terms of biomass. Boelen et al. (2017) reported that although *Skeletonema costatum* showed higher EPA content than *T. pseudonana* (≈ 20% of total FAMEs against ≈ 8% of total FAMEs), normalizing these values to take into account the biovolume (fg μm^{-3}) showed EPA to be 9 times more abundant in *T. pseudonana* than in *S. costatum.* In this regard, when we expressed the FA contents of *C. closterium* in terms of biomass, we arrived at the conclusion that neutral lipids were the main fraction contributing to EPA, DHA, and ARA production. It thus follows that the best cultivation strategy for achieving essential FA production would be to lower the temperature at the beginning of the stationary growth phase. The results obtained in *C. closterium* with respect to ω-3 FA content are similar to those reported for *P. tricornutum*, with values of 2960 and 2945 mg (100 g biomass)$^{-1}$, respectively. Nevertheless, ω-6 FAs were more abundant in *C. closterium* (2648 mg (100 g biomass)$^{-1}$) than in *P. tricornutum* (mg (100 g biomass)$^{-1}$) (Matos et al. 2016). Among microalgae, diatoms contain a large amount of ARA ω-6 FAs (Sanaa et al. 2018). Owing to its role in growth, development and immune function, broodstock nutrition and egg and larval quality PUFA are essential for vertebrates (Bell and Sargent 2003; Sanaa et al. 2018). The production of EPA and DHA in *C. closterium* is consistent with that reported for other microalgal species. However, the production of ARA in *C. closterium* was much higher than that reported for *Chlorella vulgaris* (12 mg (100 g biomass)$^{-1}$), *Haematococcus pluvialis* (292 mg (100 g biomass)$^{-1}$) *Isochrysis galbana* (69 mg (100 g biomass)$^{-1}$) and *P. tricornutum* (83 mg (100 g biomass)$^{-1}$) (Batista et al. 2013; Matos et al. 2016; Bonfanti et al. 2018). On the other hand, EPA production in *C. closterium* was higher than that reported for *I. galbana* (276 mg (100 g biomass)$^{-1}$) and similar to that

Table 6 Amino acid composition (% of total amino acids) of *C. closterium* for the LTSP thermal stress condition. Values are presented as average ± standard deviation (n = 3). *Essential (EAA), conditionally essential (CEAA), and non-essential (NEAA) amino acids for aquatics animals (Li et al. 2009)

Amino acids (AA)	% of total AA
Essential AA*	
Arginine	5.40 ± 0.08
Histidine	3.10 ± 0.01
Isoleucine	4.49 ± 0.35
Leucine	7.83 ± 0.17
Lysine	6.01 ± 0.01
Methionine	2.67 ± 0.04
Phenylalanine	6.07 ± 0.17
Threonine	5.04 ± 0.23
Tryptophan	nd
Valine	6.19 ± 0.57
Conditionally essential AA*	
Cysteine	1.52 ± 0.01
Glutamine	nd
Hydroxyproline	nd
Proline	6.62 ± 1.30
Taurine	nd
Non-essential AA*	
Alanine	6.38 ± 0.06
Asparagine	nd
Aspartate	9.84 ± 0.40
Glutamate	11.90 ± 0.47
Glycine	5.59 ± 0.14
Serine	6.56 ± 0.32
Tyrosine	4.43 ± 0.04

reported for *P. tricornutum* (2753 mg (100 g biomass)$^{-1}$), while DHA production was higher than in *P. tricornutum* (80 mg (100 g biomass)$^{-1}$) and much lower than in *I. galbana* (2146 mg (100 g biomass)$^{-1}$) (Matos et al. 2016; Bonfanti et al. 2018). Our findings therefore support previous studies proposing diatoms as sources of EPA and ARA FAs (Sanaa et al. 2018; Shah et al. 2018). Furthermore, EPA and ARA values of *C. closterium* under the LTSP growth condition were found to be very similar to those reported for fish oils (De Silva et al. 2011). Induction of *C. closterium* thermal stress during the stationary growth phase would therefore indicate this to be the strategy of choice for achieving the highest production of total lipids enriched in PUFAs.

Diatoms, when grown under stress conditions, can produce diverse amounts of sterols (Ballantine et al. 1979; Ryckebosch et al. 2014; Volkman 2016). In the present study, a decrease in culture temperature was shown to give rise to an increase in the synthesis of sterols in *C. closterium*. Véron et al. (1996) reported similar results related to the effect of temperature on the sterol content in *P. tricornutum*. However, Piepho et al. (2012) observed in *Cyclotella meneghiniana* that sterol concentrations were higher at 25 °C than at 10 °C.

In terms of sterol composition, the main sterol found in C. closterium was cholesterol (75% of total sterols in the LTSP condition), while small amounts of phytosterols were detected. These results are in agreement with those obtained in *Nitzschia closterium* and the eustigmatophyte *Nannochloropsis oculata*, where the dominant sterol was cholesterol (Volkman et al. 1992; Barrett et al. 1995). Although the presence of cholesterol in the human diet is associated with an increased risk of atherosclerosis and coronary thrombosis (Attia et al. 2015), its presence in fish diets is necessary (Barrett et al. 1995; Norambuena et al. 2013; Zhu et al. 2014, 2018). Rampen et al. (2010) characterized the sterol profile of more than 100 diatoms harvested at the end of the logarithmic growth phase. The results reported for *C. closterium* were similar to ours, as they identified the following sterols: Cholest-5-en-3β-ol (cholesterol), 24-methylcholesta-5,24(28)-dien-3β-ol (brassicasterol) and 24-ethylcholesta-5,24(28Z)-dien-3β-ol (Δ-5-24 stigmastadienol), representing 92%, 5%, and 3% of the total sterol content, respectively.

Proteins are essential nutrients for fish as a source of nitrogen and dietary protein is one of the major and most expensive components of formulated aquafeeds (Li et al. 2009). Amino acids are the building blocks of proteins and some of them, known as essential amino acids, cannot be synthetized by fish and so must be provided by the diet (Cowey 1994). We observed that under the LTEP condition, the protein content in *C. closterium* was higher than that under the LTSP condition. However, as the LTSP condition exhibited the best production in terms of essential FAs, we selected this condition for the amino acid profile analysis. Nine of the ten essential amino acids for aquatic animals were found, suggesting that

C. closterium proteins produced after lowering culture temperature at the stationary growth phase are of good quality (Li et al. 2009). This amino acid profile resulted is very similar to that reported by Brown (1991) for different species of diatoms.

Conclusion

Our results show that low-temperature stress induced during the stationary growth phase of *Cylindrotheca closterium* is an appropriate strategy to (1) increase biomass production, (2) increase total lipid production, and (3) increase the production of essential fatty acids. Furthermore, the rapid response of *C. closterium* to low-temperature stress suggests that this could be an appropriate strategy for productive processes. The basic information provided in this paper constitutes a valuable input in the search for the alternative and sustainable production of PUFAs, sterols and essential amino acids. One of the remaining challenges is to develop larger scale productive systems which are economically viable and have an effective temperature control for inducing thermal stress.

Author contributions All authors participated in the conception and design of experiments. MDA, DC, and CAP performed the temperature experiments in the photobioreactor. MDA, PSB, and DC performed the biochemical analysis. All authors made the interpretation of the data of the article and discussed their results. MDA and PSB wrote the article. PIL, CAP, and DC made the critical revision of the article contributing with intellectual content. All authors finally approved the article submission.

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