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Polyploid polynuclear consecutive cell-cycle enables large genome-size in *Haematococcus pluvialis*

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ABSTRACT

Studies revealed and keep revealing a staggering diversity of life-cycle patterns and genome-sizes in microalgae. Even within a single order like the Volvocales with *Volvox carteri*, *Chlamydomonas reinhardtii*, and *Dunaliella salina*, differences are significant. Additional knowledge gaps in microalgal physiology and genetics hinder our understanding of their evolution and valorization. Herein, we present for the first time, the multiple fission cell-cycle with corresponding DNA quantities of the economically important Volvocales *Haematococcus pluvialis*. Further, we provide two straightforward protocols for efficient culture synchronization, based on filtration or skimming of zoospores. The cell-cycle and nuclear DNA content where monitored by flow cytometry and fluorescence microscopy after staining with dsDNA dye PICO- or SYBR-green®. The average nuclear DNA content was 3.6–4.1 and 22.6–25.2 pg in zoospores/coenobial daughter-cells and aplanospores, respectively. We found a single DNA replication step for all coenobial daughter cells prior mitosis, followed by one consecutive cytokinesis. Thus, our results suggest a new *Haematococcus*-type C_n cell-cycle with unusual polyploid and polynuclear phases. This cell-cycle regime might allow for the large genome-size of *Haematococcus* and is consistent with the mutational-hazard hypothesis for Volvocales microalgae.

1. Background

The green unicellular microalga *Haematococcus pluvialis* (class Chlorophyceae, order Volvocales) is intensively studied and commercialized, due to its outstanding ability to synthesize and accumulate large quantities of the red pigment astaxanthin [1–4]. Natural astaxanthin is sold as nutraceutical and cosmeceutical for human consumption, while the synthetic astaxanthin is mostly supplemented to aquaculture and poultry feed [5–13].

The life-cycle of *H. pluvialis*, like other Volvocales, is complex and includes single- and multi-cellular as well as motile, sessile and encysted stages. Five cell types can be distinct by their maturity and morphology: gametospores (microzooids), motile zoospores, non-motile aplanospores, daughter cell coenobia, and resting cysts [1, 14–17]. Synchronization of *H. pluvialis* cultures was first described by Kafka [18]. Thiamine (Vitamin B_1) in combination with high-light triggered *H. pluvialis* cultures to omit the resting cyst stage and enter directly into the non-motile aplanospore stage [18]. Göhde & Berger [19] showed that synchronized *H. pluvialis* cells can periodically divide into four to eight daughter cells. This complex life-cycle and morphological changes have hindered studies to *Haematococcus* DNA content and cell-cycle. Studies in closely related Chlorophyceae microalgae, like *Volvox carteri*, *Chlamydomonas reinhardtii* and *Scenedesmus* sp., found a wide range of DNA quantities and varying cell-cycles of the C_n-type, Fig. 1. C_n cell-cycles (C_nCC) are characterized by multiple partially overlapping DNA replications, nuclear and cellular divisions. The number of simultaneously released daughter cells (2^n) from a coenobium, also known as the division number, depends on the environmental conditions and n can range between 1 and 15 [20–22]. Zachleder and co-workers described two major C_nCC types, the clustered *Chlamydomonas*-type [23] and the consecutive *Scenedesmus*-type [24], Fig. 1. In a clustered C_nCC, multiple commitment points can be passed throughout growth, leading to a sequence of repetitive DNA duplications, mitosis and cytokinesis events compiled at the end of the cycle, Fig. 1. Whereas in a consecutive C_nCC, multiple commitment points lead to a series of DNA replication and mitosis, followed by one cytokinesis, Fig. 1. Key difference between these two C_nCC patterns is the nuclearity of their protoplast prior cytokinesis; clustered C_nCC are binuclear while consecutive C_nCC can be polynuclear.

The main objectives of our study were to specify the DNA content and cell-cycle pattern of *H. pluvialis*, and to develop a temporal and structural refined growth-division-model. Herein, we developed two new orthogonal and independent protocols for culture synchronization and DNA measurement and identified a novel *Haematococcus*-type C_nCC.

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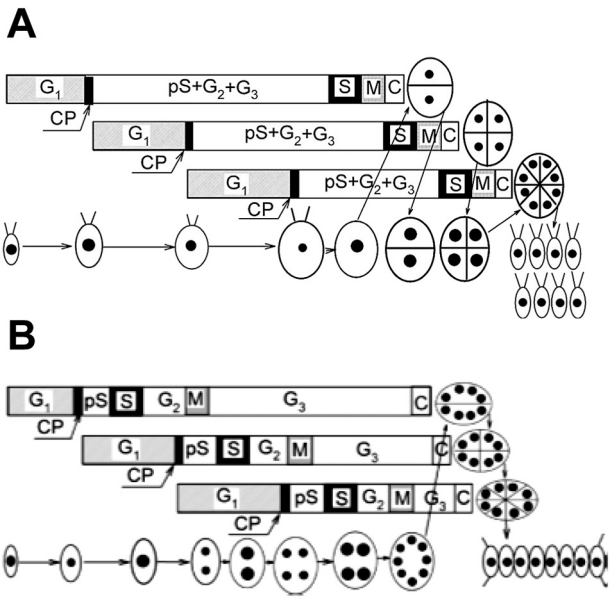

Fig. 1. Chlorophyceaen microalgae differ in their multiple fission C_n-type cell-cycles.
Multiple fission cell-cycle models show three partially overlapping growth and DNA replication–fission sequences in maternal cells when dividing into eight daughter cells. A. Illustration of the clustered *Chlamydomonas*-type C_nCC with binary DNA replication, nuclear and cellular fission. B. Illustration of the consecutive *Scenedesmus*-type C_nCC with binary DNA replication and nuclear fissions, and one consecutive cellular fission. Cell-cycle phases: CP; commitment point, G; gap, S; DNA synthesis, M; mitosis, C; cytokinesis, pS, prereplication. Arrows indicate the order of events.
Modified from Zachleder et al. [22].

2. Results

2.1. Physiological characterization

Following synchronization by "size-filtration" (Method 1), cultures showed their typical multiple fission cell-cycle of small zoospores maturing to large coenobia containing 4–8 daughter cells. Flow cytometric analysis confirmed the increase in cell diameter (CD) and in calculated volume (CV) from 16 to 26 µm and from ~2100 to 9200 µm^3 for zoospores (t_0) and mitotic aplanospores (t_{48}), respectively, Fig. 2A. Compare to zoospores, coenobia tripled their chlorophyll (Chl, 18.7 to 58.8 pg per cell) and carotenoid (TC, 4.0 to 12.3 pg per cell) contents, and dry weight (DW, 1.6 to 4.2 ng per cell), Fig. 2B. The cell number (CN) remained constant during the coenobia development up to 45 h, Fig. 2B. Between 45 and 70 h, coenobia released their zoospores and the CN increased by 9-fold ($\mu = 0.026 \cdot h^{-1}$), Fig. 2B. At 60 h, the average CD and CV dropped to 13 µm and 1200 µm^3 in newly released zoospores, Fig. 2A. Likewise, the cellular Chl and TC content, and DW normalized to 26.9 and 6.4 pg, and 1.6 ng per cell, respectively, Fig. 2B.

2.2. Culture synchronization

Flow cytometric analysis of "size-filtered" cells (Method 1), identified three *Haematococcus* sub-populations after 45 and 68 h, Fig. 3. The histograms of forward scattering (cell size) and side scattering (cell complexity) indicated a bimodal cell distribution of small zoospores and big aplanospores populations. Bivariate dot plot analysis identified a third subpopulation of unstained cells, which were identified by microscopic observations as non-flagellated zoospores. Zoospore populations generated a sharp peak of fluorescence at 109 on the Fluorescence Scale (FS) at both time points, Fig. 3. In contrast, aplanospores generated a broad fluorescent signal (109–739 FS) at 68 h, indicating DNA-rich cells in various mitotic-phases, Fig. 3. The unstained non-flagellated zoospores generated always a sharp peak of background-fluorescent (18 FS), Fig. 3. Between 45 and 68 h, the aplanospores matured to coenobia and underwent division, and their ratio of the culture decreased by 4-fold (68 to 17%). Simultaneously, the zoospore and non-flagellated zoospore populations increased by 3.5-fold (19 to 64%) and 5.5-fold (2 to 11%), respectively, Fig. 3.

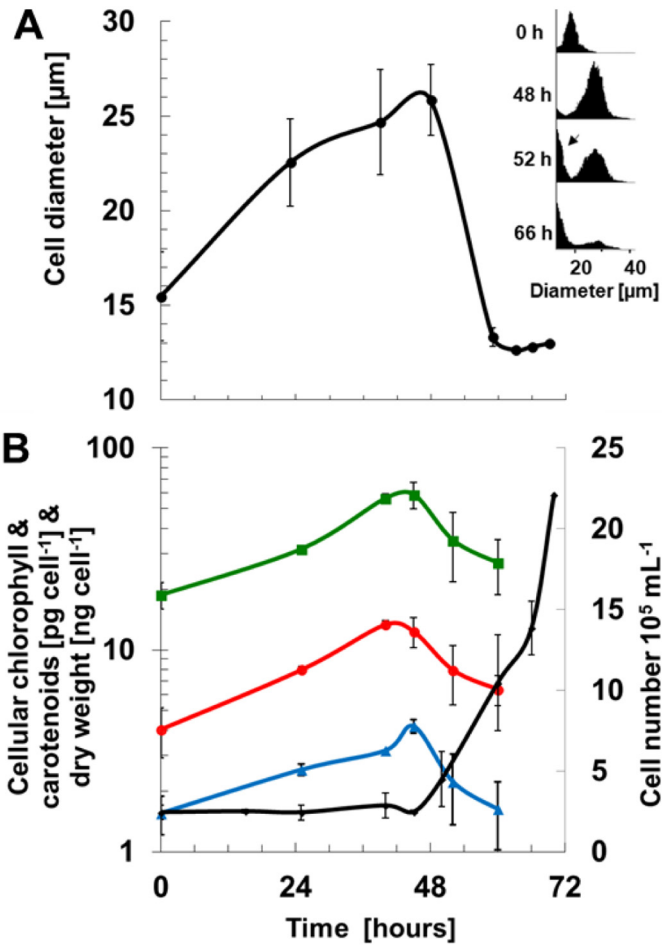

Fig. 2. Synchronized *H. pluvialis* cultures (size-filtered cells) gain size and macromolecules during the pre-commitment phase of the C_nCC.
A. Flow-cytometric analysis of cell diameter in PICO-Green® stained cells ($n = 4$, $N = 10.000$ cells). Inset: Histograms of cell size distribution. B. Changes in cell number (♦), dry weight (▲), and cellular chlorophyll (■) and carotenoids (●). Values represent means with standard deviation (SD). n = biological repeats, N = cells per biological repeat.

2.3. Interdependence of DNA quantity and cell-cycle pattern

2.3.1. Identifying DNA quantities throughout the cell-cycle

Cultures of "skimmed-off" cells (Method 2) revealed a highly homogenous population of flagellated zoospore at zero-time, Fig. 4. Based on flow cytometric analysis of "skimmed-off" cells and control chicken erythrocytes, the calculated nuclear dsDNA contents were 4.1 pg in zoospores (t_0) and ~25.2 pg in mitotic aplanospores (t_{36}) (data not shown). Complimentary observation by fluorescence microscopy after staining with SYBR-Green® dye clearly identified the different mitotic CC-phases, Fig. 5, and their corresponding DNA-quantities, Fig. 6. We found in *H. pluvialis* a C_n-type cell-cycle constituting of: G_1-flagellated zoospore (3.6 ± 0.6 pg DNA), S_1-non-flagellated zoospore (7.1 ± 1.1 pg), G_2/S_2-mononuclear aplanospores (22.6 ± 5.8 pg), Mitotic polynuclear aplanospores (14.2 ± 4.9 pg per nucleus), Cytokinesis of polynuclear aplanospores (each daughter nucleus