Towards an understanding of diel feeding rhythms in marine protists: consequences of light manipulation

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Abstract

Temporal programs synchronized with the daily cycle are of adaptive importance for organisms exposed to periodic fluctuations. This study deepens into several aspects of the exogenous and endogenous nature of microbial grazers. We investigated the diel rhythms of cell division and feeding activity of four marine protists under different light regimes. In particular, we tested if the feeding cycle of protistan grazers could be mediated by a light-aided enhancement of prey digestion, and also explored the consequences of cell division on diel feeding rhythms. Cell division occurred at night for the heterotrophic dinoflagellates *Gyrodinium dominans* and *Oxyrrhis marina*. In contrast, the mixotrophic dinoflagellate *Karlodinium armiger* and the ciliate *Strombidum* sp. mostly divided during the day. Additionally, a significant diurnal feeding rhythm was observed in all species. When exposed to continuous darkness, nearly all species maintained the cell division rhythm, but lost the feeding cycle within several hours/days (with the exception of *O. marina* that kept the rhythm for 9.5 days). Additional feeding experiments under continuous light also showed the same pattern. We conclude that the feeding rhythms of protistan grazers are generally not regulated by cell division, nor by the enhancement of digestion by light. Our study, moreover, indicates that the cell division cycle is under endogenous control, whereas an external trigger is required to maintain the feeding rhythm, at least for most of the species studied here.

1. Introduction

Daily periodicity in light and darkness, associated with the Earth's rotation, governs many known environmental processes in our planet. Consequently, the organisms exposed to this daily periodicity have developed rhythms as an adaptive response to sunlight fluctuations [1]. These rhythms determinate the optimal timing for metabolic, physiological and behavioural activities within the daily cycle [2].

A broad variety of diel rhythms have been described in aquatic organisms [3]. For instance, marine mesozooplankton typically feed in a typical day-night cycle pattern, often coupled with daily vertical migrations, characterized by higher feeding rates during the night [4-6]. Contrarily to mesozooplankton, a reverse diel feeding rhythm with higher ingestion rates during the daytime has been reported for microzooplankton [7-10], with the exception of some mixotrophs that may reduce feeding under light [11-13]. Although an endogenous component in the feeding behaviour of microzooplankton has been proposed [8], other explanations for the presence of this feeding rhythm have been suggested as well, such as light-aided digestion [9], compensatory feeding in response to stoichiometric fluctuations [14, 15], prey availability [7], etc. Yet, the reasons for this mostly diurnal feeding behaviour in microzooplankton remain still unclear.

In previous studies, we already explored the effect of the stoichiometric composition (food quality) of prey (*Rhodomonas salina*) and prey availability on the diel feeding rhythm of microzooplankton (see Arias et al. [7]). We concluded that while low prey availability drastically altered the feeding rhythm (i.e. decreasing its intensity), changes in prey stoichiometric composition did not result in any significant effects on the rhythm. These results challenged some of the previous theories on the mechanisms driving feeding rhythms in protozoans (e.g. [14, 15]), but were not conclusive enough to fully explain the triggers of such rhythms; the contribution of an underlying endogenous component in the feeding rhythm was not evaluated and the light-aided digestion hypothesis was not tested. Moreover, other hypotheses rose from the Arias et al. [7] study, such as the interference of cell division during night on the feeding behaviour. It might be possible that individuals dividing during the night (as seems to be the common in many dinoflagellates, [16-21]) might have their feeding impaired during the duplication process, diminishing by this way the ingestion rates compared with those

during light hours. Actually, several rhythmic events seem to be linked to the cell division cycle [22]. For example, Baek et al. [23] observed a dependency of the diel vertical migration of *Ceratium furca* on the cell division cycle, with mitosis taking place at the bottom layer during night. We can, therefore, hypothesize that diurnal feeding patterns in marine protistan grazers may be a consequence of the nocturnal cell division cycle. To address this question, we have conducted laboratory experiments to determine the cell division timing and the grazing rate response to continuous light and darkness, and then analyse the coupling between cell cycle and grazing behaviour. Likewise, this sort of experiments serves to validate the existence of an endogenous control of the feeding rhythm and to determine the possibility of an enhancement of digestion by light. If the feeding rhythm is endogenously controlled, it should remain both under continuous light or continuous darkness. On the other hand, if digestion is strengthened by light, the feeding rhythm is expected to be lost under continuous darkness. As target species we chose the heterotrophic dinoflagellates *Gyrodinium dominans* and *Oxyrrhis marina*, the mixotrophic dinoflagellate *Karlodinium armiger*, and the heterotrophic ciliate *Strombidium* sp..

Our specific aims were to (1) determine whether the diel division and feeding rhythms in heterotrophic and mixotrophic microzooplankton are endogenously controlled; (2) validate the enhancement of microzooplankton digestion by light; (3) explore the possible role of synchronized cellular division cycle on diel feeding rhythms.

2. Material and methods

2.1. Grazer and prey cultures

We conducted grazing and growth experiments with laboratory cultures of *Gyrodinium dominans* (strain ICM-ZOO-GD001), *Oxyrrhis marina* (strain ICM-ZOO-OM001), *Karlodinium armiger* (strain ICM-ZOO-KA001) and *Strombidium* sp. (strain ICM-ZOO-SSP001). All species were isolated by A. Calbet in coastal waters of the Catalan Sea (NW Mediterranean), between 1995 and 2017, and kept in a culture collection under laboratory conditions at the Institut de Ciències del Mar – CSIC in Barcelona. Grazer cultures were grown in round flasks with metal-enriched autoclaved seawater (1

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Comentario [1]: As suggested by Reviewer#1, more details regarding the species used in the experiments have been added to this section.

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Comentario [2]: *Gyrodinium dominans* was misspelled; we have corrected the error.

mL metal stock solution per litre; [24]) and maintained at 19 ± 1 °C, and 38 PSU under a 10:14 h Light-Darkness (LD) cycle (irradiance of $60\text{--}90 \mu\text{E m}^{-2} \text{s}^{-1}$ of white fluorescent lights). The chryptophyte *Rhodomonas salina* was used as prey to daily feed grazer stocks. Prey was laboratory grown in batch culture in f/2 medium [24] in Pyrex culture flasks provided with air to avoid cell sedimentation. Cultures were diluted daily to maintain them in an exponential phase of growth.

2.2. Evaluation of the effects of light on cell division

To address the role of cellular division cycle on the diel feeding rhythm of microzooplankton we first grew *G. dominans*, *O. marina*, *K. armiger* and *Strombidium* sp. under a standard 10:14 L:D cycle and then we transferred them into constant darkness. In these experiments we used feeding-saturating food concentrations of prey (*R. salina*) to ensure that organisms were not food limited (data of feeding functional responses from Calbet et al. [25]; Martínez, unpublished; Arias, unpublished). At the beginning of the experiment, we determined the grazer and prey concentrations (Table 1) with a Beckman Coulter Multisizer III particle counter (100 μm aperture tube) and then the prey-predator mixtures were distributed into 1-L Pyrex bottles (three replicates per species and treatment). Three more bottles were added with only prey to monitor *R. salina* growth. Nutrients were added at the beginning of the experiment and every 24 hours (10 mL f/2 per litre). Both experimental (prey and predator mixtures) and control (only *R. salina*) bottles were incubated in an acclimated room at 19 ± 1 °C, under a 10:14 L:D cycle (approximate irradiance of $90 \mu\text{E m}^{-2} \text{s}^{-1}$) for 24h and then incubated in total darkness by wrapping them in aluminium foil. All the organisms used had been previously grown under a standard L:D cycle for > 6 month before conducting the experiment. At nearly the end of each day and night periods stock samples of each culture were taken and prey and grazer concentrations were measured with the Multisizer III particle counter to assess the changes in prey and predator concentrations. For the dark-period incubations, we took special care of working under very dim light. We ensured that in none of the cases prey concentration fell down below the species-specific saturation level. In case that prey concentration decreased below the saturated condition, new prey was added in order to maintain the aimed prey concentration (i.e.

feeding saturating conditions). The instantaneous growth rates were calculated assuming exponential growth.

2.3. Evaluation of the effects of light manipulations on grazing rates

We investigated the effect of light-presence as trigger of the diel feeding rhythm in microzooplankton by measuring the grazing rates of *G. dominans*, *O. marina*, *K. armiger* and *Strombidium* sp. under three experimental conditions: natural diel cycle (10:14 L:D), continuous light and continuous darkness. Experiments were initiated with a full cycle under the natural L:D cycle, followed by a period of either continuous light or continuous darkness; both treatments were run in parallel. For each treatment we prepared 4.5 L Pyrex bottles with grazers that were daily fed to maintain feeding-saturating food conditions in the stock cultures, and kept under the selected light conditions during the whole experiment. From these bottles we took aliquots that were used to estimate the grazing rates. We used the Multisizer III particle counter to prepare suspension of the selected concentrations of grazers and prey (experimental) and only prey (controls) and distributed them in triplicated 72 mL polyethylene culture flasks. Then, all bottles were incubated on a plankton wheel (0.2 r.p.m.) inside a controlled temperature room (19 ± 1 °C). About 1 h before the end of the light and dark periods, the incubations were finished and the concentration of prey and grazer from experimental and control bottles were determined. We left a 12 h interval between each day-night cycle of experiments, and repeated them for a total of 96 h (or 228h for *O. marina*). Bottles from the continuous light experiment were exposed to an irradiation that ranged between 40 and 90 $\mu\text{E m}^{-2} \text{s}^{-1}$ through a complete rotation of the plankton wheel. Bottles under continuous darkness were wrapped with several layers of aluminium foil to isolate them from light. The culture of *Rhodomonas salina* used as prey was kept under a 10:14 L:D cycle and in stationary growth condition in order to minimize day-night cells size and biochemical composition differences [7]. As mentioned above, special care was taken to conduct the experiments under saturation food conditions in order to make sure that ingestion rate was controlled by the rate of food vacuole processing and not by the encounter rate between prey and grazer. Prey cultures were started at different times to warrant that prey at stationary phase would be available at the start of each incubation. Each culture was monitored every day to examine the phase of growth. Ingestion rates were calculated according to the equations

of Frost [26], using prey biovolume as currency instead of cells to neglect any differences in prey size that may occur.

3. Results

3.1. Growth rates

We detected clear differences between day and night growth rates (μ) under natural 10:14 L:D cycle in all the studied species (Fig. 1). Regarding the prey, the chryptophyte *R. salina* presented higher growth rates during the night ($0.047 \text{ h}^{-1} \pm 0.0007 \text{ h}^{-1}$) than during the day ($-0.0006 \text{ h}^{-1} \pm 0.0004$; t-test, $p < 0.001$; Fig. 1a). When *R. salina* was exposed to continuous darkness, growth rates were nil and the day-night rhythm disappeared after 24 h.

G. dominans and *O. marina* also presented significant higher growth rates during the night-time (Fig. 1b, c; $p < 0.05$). When transferred into continuous darkness, both species exhibited an initial period of adaptation where the day-night growth rhythm vanished; after certain time (ca. 24h), the rhythms reappeared. Nevertheless, growth rates were always of lower magnitude under continuous darkness. Contrarily, under a natural L:D cycle *K. armiger* showed higher growth rates during the daytime (Fig. 1d; $p < 0.05$). After several hours of continuous darkness exposure, growth rates decreased below zero and remain negative until the end of the experiment (day 3). *Strombidium* sp. showed the widest amplitude rhythms under the natural L:D cycle, with a maximum of $0.075 \text{ h}^{-1} \pm 0.0066$ during the day and a minimum of $0.004 \text{ h}^{-1} \pm 0.0056$ during the night (Fig. 1e). *Strombidium* sp. presented the same day-night pattern than the mixotrophic dinoflagellate *K. armiger*, with higher growth rates during the daytime. When exposed to continuous darkness, *Strombidium* sp. also experienced a phase of adaptation during the first hours; following, the day-night rhythm was recovered with smaller amplitude.

3.2. Grazing rates

All grazers displayed significant differences between day and night ingestions rates (as prey volume consumed by grazer and hour) under the natural L:D cycle, with higher ingestion rates during the daytime (Fig. 2). Out of the four species studied, *Gyrodinium*

dominans presented the highest day/night ingestion rates quotient (2.74 ± 0.13 ; t test, $p < 0.001$), followed by *Karlodinium armiger* (1.80 ± 0.17 ; t-test, $p < 0.01$); *Oxyrrhis marina* and *Strombidium* sp. showed the lowest differences in day/night (*O. marina*: 1.61 ± 0.056 ; t-test, $p < 0.05$; *Strombidium* sp.: 1.58 ± 0.04 ; t-test, $p < 0.001$). Quotient value 1 indicates equal day and night ingestion rates, while values above 1 indicate the existence of diel feeding rhythm.

Under continuous light and continuous darkness, the amplitude of the feeding rhythm exhibited by *G. dominans*, *K. armiger* and *Strombidium* sp. reduced and eventually vanished or showed awkward patterns (Fig. 3), whereas *O. marina* kept the rhythm along the experiment. In particular, *G. dominans* night ingestion rates gradually increased, whereas day ingestion rates decreased, resulting in a loss of the feeding rhythm in near 60 and 75 h under continuous light and darkness, respectively; later, the diel rhythm slightly reversed. After 4 days of constant light conditions, the total daily (day+night) intake of *G. dominans* ($616.91 \mu\text{m}^3 \text{ grazer}^{-1} \text{ h}^{-1}$) remained similar to that showed at the beginning of the experiment under natural L:D cycle ($605.44 \mu\text{m}^3 \text{ grazer}^{-1} \text{ h}^{-1}$). However, under constant darkness *G. dominans* experienced a reduction of 37% in the total daily ingestion rate. *O. marina* displayed the same pattern under continuous light and continuous darkness; after a slight fluctuation in ingestion rates, the amplitude of the rhythm remained similar during the whole experimental period (9.5 days) with higher ingestion rates during the daytime. At the end of the experiment, there was an actual increase of 77% in total daily ingestion rates in both treatments compared to the natural L:D cycle condition. *K. armiger* experienced a decrease in day ingestion rates in both treatments, whereas night ingestion rates remained constant under constant light and sharply decreased under darkness (Fig. 3e, f). This caused *K. armiger* to lose the rhythm after nearly 30 h of incubation under constant light, and under darkness ceased feeding in < 24 h. Finally, *Strombidium* sp. grazing rhythm under constant light conditions was lost after 12 h; both day and night rates drastically decreased along the experiment. Under constant darkness the rates gradually decreased during the day and increased during the night, to become equal by the end of the experiment (after 4 days).

4. Discussion

215 Our data demonstrate that several well-studied marine protists divide with a diel cycle
216 and present a diurnal feeding pattern. Moreover, we have showed that the diel feeding
217 rhythm seems to be strongly dependent on an external synchronizing agent in most of
218 the species studied.

219 4.1. The role of light in microzooplankton diel feeding rhythm

220 Diurnal feeding patterns have been confirmed for various protists species [8-10],
221 although the causes of this behaviour are not well understood yet. In the introduction
222 section we highlighted several not yet refuted hypotheses that could explain such a
223 particular behaviour. For instance, Strom [9] suggested that light might facilitate the
224 digestion process in cell vacuoles, and this process might explain the higher diurnal
225 grazing activity observed in protists. Should light enhance grazing rates, all species
226 would have reduced their feeding under continuous darkness compared to continuous
227 light. Our experiments rejected this hypothesis, except for the mixotrophic *K. armiger*,
228 which manifested a clear need for light to keep grazing activity, as also found for other
229 mixotrophic dinoflagellates [27-29]. Although heavily depending on light for survival,
230 *K. armiger* nutritional configuration has been described to be closer to the phagotrophy
231 extreme within the mixotrophic spectrum [29]. Berge and Hansen [29] observed that, in
232 order to achieve high growth conditions, carbon fixation played an important role in
233 prey-limitation environments; in this situation, *K. armiger* obtained most of the carbon
234 required through photosynthesis. On the contrary, under prey-saturated conditions, as
235 the ones used in our experiments, phagotrophy becomes the main source of *K. armiger*
236 for carbon acquisition (between 60 to 90% of the total carbon gains; [29]).

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238 Contrarily, *O. marina*, recognised in many instances as a model species for
239 microzooplankton [30], displayed a very distinct response to light manipulation
240 compared to the rest of protozoans. As a matter of fact, neither constant light or
241 darkness seemed to affect the diel feeding behaviour of *O. marina*. This opportunistic
242 species, inhabiting intertidal pools, salt marshes and embayments [31-34], has a very
243 complicated life history involving encystment and very high growth rates to survive
244 tidal cycles and extreme conditions [34]; because of these characteristics, it could be
245 argued that its feeding and reproductive behaviour might be less dependent of other
246 environmental factors (e.g. light) to optimize survival. The responses of *G. dominans*

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Comentario [3]: We have added an additional paragraph in order to discuss the balance of carbon incorporation on mixotrophic organisms.

and *Strombidium* sp. were particularly interesting. While both organisms lost their feeding rhythm under constant light or darkness, the way this lost was attained and the effects on the total daily intake after several days of light manipulation differed. Total daily intakes of *G. dominans* under continuous light were fairly constant, whereas under continuous darkness they decreased; this diminution was result of reducing day ingestion rates and keeping the night ones constant. Contrarily, total daily ingestion rates of *Strombidium* sp. were negatively affected under continuous light, and kept rather constant under continuous darkness. Therefore, it seems *G. dominans* needs light to keep feeding, while *Strombidium* sp. requires darkness, possibly to get rid of some oxidative subproducts [35].

Whilst continuous light conditions are restricted to high latitudes during summer months, and one would expect local species showing particular adaptations, full darkness, characterize not only winter in high latitudes but may also occur in deep layers of the ocean. Sudden water displacements, anticyclonic eddies, etc. may temporarily drag organisms to the deep [36]. Under such circumstances, our data suggest that ciliates, such as *Strombidium* sp., will thrive better the adverse conditions than e.g. dinoflagellates such as *G. dominans*, or mixotrophs.

4.2. Connecting cell division to diel feeding rhythms

Many unicellular organisms present circadian rhythms of cell division, following the characteristic eukaryotic G1-S-G2-M cell cycle [37]. For instance, in most species of phytoplankton, cell division is confined to the dark phase [2, 38-40], being dinoflagellates clear examples of such a nocturnal physiological response [41, 42]. However, phytoplankton cell division has also been observed, although less frequently, occurring during the light period [43-45], or unconnected to the light phase [46-49].

The benefits of specific timing for cell division may rely on a strategy to cost minimization. Cook [50] and Cohen and Parnas [51] suggested that algae grown under a L:D cycle store energy during the day from photosynthesis to use it afterwards for cellular division during the night. As a general idea, division patterns in a L:D cycle may have scheduled to optimize the use of resources and minimize energy costs in order to maximize population growth. From an evolutionary point of view, it has been argued that an initial driving force for a night division would have been the advantage of

concentrating during the night-period those cellular processes vulnerable to light (“Escape from light hypothesis” [52]). Other theories consider the involvement of the flagella in the division process: the flagellum is reabsorbed before cell division, which allows the cell to use its basal bodies for chromosome segregation and cytokinesis [38, 53]. Flagella-dependent phototaxis is necessary in light conditions for optimization of light absorption for photosynthesis; therefore, as there is no necessity of phototaxis during dark phase, cell division could mostly take place during night [38, 53].

One may wonder whether these theories for autotrophs may also apply to microbial grazers, assuming that organisms cannot feed during mitosis. In this line of reasoning, Wikner et al. [54] postulated that higher diurnal grazing of bacterivorous flagellates was caused by the cessation of ingestion during the flagellate cell division. It is evident from our results, however, that this was not the case for most of the species we studied. Actually, the rather surprising division behaviour of *K. armiger* and *Strombidium* sp., which divided during the day, was coincident with the remarkably diurnal feeding behaviour. These diurnal patterns seem not to be exceptional in microbial grazers, Previous literature support ciliate higher growth rates during the day (e.g. [8, 10]), although nocturnal cell division has also been observed (e.g. [55]), and most ciliates are diurnal feeders [8]. Therefore, we cannot accept as universal the hypothesis of cell arrest during division being the cause of diel grazing cycles.

4.3. Final remarks

Our data shows that in general feeding rhythms in microzooplankton are most likely not conditioned by the cell division cycle, nor by the enhancement of digestion by light, or by an internal clock. In this regard, Jakobsen and Strom [8] suggested a light-modulated endogenous circadian cycle both in cell division and feeding for heterotrophic protists. Although our results support the endogenous control of the cell division cycle in protistan grazers (i.e. the diel growth differences persisted under continuous darkness), in general light appears to be needed as an external synchronizing agent to maintain the feeding rhythm. Nevertheless, the underlying mechanisms responsible for diurnal feeding rhythm in microzooplankton remain still not well understood. As an alternative hypothesis to be validated in future works we propose that the feeding rhythms in

marine protists evolved as a strategy to avoid predation by nocturnal feeders, such as copepods. It has been argued that timing in some physiological processes could confer some advantage in front predators displaying rhythmicity as well [56]. Feeding is linked to swimming and, consequently, organisms become more conspicuous and increase their encounter rate with predators while nourishing. Therefore, further insights considering the effect of the presence of predators are needed to clarify the triggers of microzooplankton feeding rhythm.

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365 **Compliance with Ethical Standards**

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369 **Conflict of Interest:**

370 Anna Arias, Enric Saiz and Albert Calbet declare that they have no conflict of interest.

371 **Ethical approval:**

372 This article does not contain any studies with animals performed by any of the authors.

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Figure captions

Graphics program used: KaleidaGraph

Fig. 1 Growth rates (μ) as function of time of *Rhodomonas salina* (a), *Gyrodinium dominans* (b), *Oxyrrhis marina* (c), *Karlodinium armiger* (d) and *Strombidium* sp. (e). Horizontal black bar indicates continuous darkness period. Error bars indicate standard error. D and N indicate day and night phases, respectively

Fig. 2 Feeding rhythm index (quotient between day and night ingestion rates) of *Gyrodinium dominans*, *Karlodinium armiger*, *Oxyrrhis marina* and *Strombidium* sp.. Asterisks represent significant differences (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Dashed line represents equal day and night ingestions (nonexistence of feeding rhythm)

Fig. 3 Day and night ingestion rates (in terms of volume consumed by grazer and hour) as function of time, under continuous light and under continuous darkness treatments, of *Gyrodinium dominans* (a-b), *Oxyrrhis marina* (c-d), *Karlodinium armiger* (e-f) and *Strombidium sp.* (g-h). Dashed line represents day ingestion rates and continuous line represents night ingestion rates

Table 1 Initial (averages $\pm$ SE) prey and grazer concentrations (cell mL⁻¹) used for the experiments of growth under 10:14 L:D cycle (left column) and under continuous darkness (right column)

	L:D Cycle		Continuous darkness	
	Prey concentration (cell mL ⁻¹)	Grazer concentration (cell mL ⁻¹)	Prey concentration (cell mL ⁻¹)	Grazer concentration (cell mL ⁻¹)
<i>R. salina</i>	152483 $\pm$ 424	-	310467 $\pm$ 1381	-
<i>G. dominans</i>	107733 $\pm$ 508	1265 $\pm$ 26	74720 $\pm$ 1201	851 $\pm$ 21
<i>O. marina</i>	117483 $\pm$ 593	1677 $\pm$ 86	125783 $\pm$ 4572	4686 $\pm$ 97
<i>K. armiger</i>	76444 $\pm$ 263	813 $\pm$ 44	56645 $\pm$ 316	811 $\pm$ 22
<i>Strombidium</i> sp.	182350 $\pm$ 581	631 $\pm$ 47	130783 $\pm$ 2382	1330 $\pm$ 109