Diel feeding rhythms in marine protistan grazers

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Ph.D. Thesis 2020

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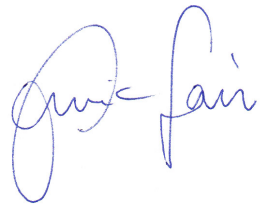

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Als meus *avis*, per ensenyar-me a ser forta.
Als meus *pares*, per fer-ho possible.
Al meu *germà*, per guiar-me.
Al *Ferran*, per creure en mi sempre.

PREFACE

"La naturaleza es grande en las cosas grandes, mas es grandísima en las cosas diminutas"

Jacques Henri de Saint Pierre

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Moltes gràcies...

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A tots vosaltres,

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TABLE OF CONTENTS

SUMMARY

RESUMEN

RESUM

Summary

Protistan grazers are a key component of marine planktonic food webs. These marine protists are the major grazers of pelagic primary production in the oceans and, therefore, they display a crucial role in marine biogeochemical cycles as pivotal intermediaries of the energy and mass flux from primary producers to higher trophic levels. Notwithstanding their relevant role in the global pelagic system, some key aspects related to their trophic behaviour remain still poorly understood. Among these features, diel feeding rhythms are of relevant importance as they represent the coupling between the cycles of primary production and the feeding cycles of their predators and, consequently, they strongly condition the carbon flux mediated by marine protistan grazers and the dynamics of planktonic food webs.

This Ph.D. Thesis aims to deepen our knowledge of the diel feeding rhythms in marine protistan grazers, exploring their occurrence and the mechanisms that generate and modulate this rhythmic behaviour. Accordingly, we first investigated the existence of diel feeding rhythms in diverse species of heterotrophic and mixotrophic protistan grazers (the dinoflagellates *Gyrodinium dominans*, *Oxyrrhis marina* and *Karlodinium armiger*, and the ciliates *Strombidium arenicola* and *Mesodinium rubrum*). Then, we evaluated how intrinsic characteristics of the prey (*Rhodomonas salina*), including the growth phase and the diel variations on its stoichiometric composition, as well as own characteristics of the grazers, such as their previous feeding history and the timing for cell division, may be causal factors or perhaps regulate the diel feeding activity of marine protists. We also assessed the effect of extrinsic factors, such as the prey concentration, the light and the risk of predation, on the feeding rhythm of marine protists. Finally, we conducted field experimentation to study the diel feeding rhythms of protistan grazers in a natural ecosystem, the Gullmar Fjord (Sweden).

As major conclusions of the present Ph.D. Thesis, we found that there might not exist a unique underlying mechanism causing the different patterns of diel feeding rhythms we observed in marine protistan grazers. Instead, it appears that marine protists species might have developed feeding rhythms largely conditioned by their

physiological and behavioural characteristics, as well as by the ecological conditions from their original habitat, which might determine the factors by which it is modulated.

Resumen

Los ramoneadores protistas son un componente clave de las redes tróficas planctónicas marinas. Estos protistas marinos son los principales consumidores de producción primaria pelágica en los océanos y presentan, por lo tanto, un papel crucial en los ciclos biogeoquímicos marinos como intermediarios fundamentales de los flujos de energía y masa desde los productores primarios hacia niveles tróficos superiores. A pesar de su relevante papel en el sistema pelágico global, algunos aspectos clave relacionados con su comportamiento trófico son todavía poco conocidos. Entre estas características, los ritmos diarios de alimentación son de gran importancia, ya que representan el acoplamiento entre los ciclos de producción primaria y los ciclos de alimentación de sus depredadores y, en consecuencia, condicionan en gran medida el flujo de carbono mediado por los ramoneadores protistas marinos y la dinámica de las redes alimentarias planctónicas.

Esta Tesis Doctoral tiene como objetivo profundizar en nuestro conocimiento de los ritmos de alimentación en los ramoneadores protistas marinos, estudiando su ocurrencia y los mecanismos que generan y modulan este comportamiento rítmico. Por ello, primeramente investigamos la presencia de ritmos diarios de alimentación en diversas especies de ramoneadores protistas heterotróficos y mixótrofos (los dinoflagelados *Gyrodinium dominans*, *Oxyrrhis marina* y *Karlodinium armiger*, y los ciliados *Strombidium arenicola* y *Mesodinium rubrum*). Luego, evaluamos como características intrínsecas de la presa (*Rhodomonas salina*), como son su fase de crecimiento y las variaciones diarias en su composición estequiométrica, así como también características propias de los ramoneadores, tales como su historia de alimentación previa y el momento de división celular, pueden ser factores causantes o bien reguladores de los ritmos diarios de alimentación en los protistas marinos. También investigamos el efecto de factores extrínsecos, como son la concentración de presas, la luz y el riesgo de depredación, sobre la actividad de alimentación rítmica de los protistas marinos. Finalmente, realizamos un estudio de campo sobre los ritmos de alimentación diarios de los ramoneadores protistas en un ecosistema natural, el Fiordo de Gullmar (Suecia).

Como conclusiones principales de la presente Tesis Doctoral, encontramos que quizás no exista un mecanismo causal único que explique los diferentes patrones de ritmos diarios de alimentación en los ramoneadores protistas marinos. Nuestro estudio parece indicar que las especies de protistas marinos desarrollan ritmos diarios de alimentación condicionados, en gran medida, por sus características fisiológicas y de comportamiento, además de por las particularidades ecológicas de su hábitat de origen, las cuales determinarían los factores por los que este patrón de actividad puede ser modulado.

Resum

Els pastors protistes són un component clau de les xarxes alimentàries planctòniques marines. Aquests protistes marins constitueix el principal consumidor de la producció primària pelàgica en els oceans i presenta, per tant, un paper crucial en els cicles biogeoquímics marins com intermediaris fonamentals en els fluxos d'energia i de massa des dels productors primaris cap a nivells tròfics superiors. Tot i el seu rol rellevant en el sistema pelàgic global, alguns aspectes clau relacionats amb el seu comportament tròfic són encara poc coneguts. Entre aquestes característiques, els ritmes diaris d'alimentació són de gran importància, ja que representen l'acoblament entre els cicles de producció primària i els cicles d'alimentació dels seus depredadors i, en conseqüència, condicionen en gran manera el flux de carboni mediat pels pastors protistes marins i la dinàmica de la xarxa alimentaria planctònica.

Aquesta Tesi Doctoral té com a objectiu aprofundir en el nostre coneixement sobre els ritmes d'alimentació diaris en els pastors protistes marins, estudiant la seva presencia i els mecanismes que generen i modulen aquest comportament rítmic. Així doncs, primerament vam investigar l'existència de ritmes diaris d'alimentació en diverses espècies de pastors protistes heterotròfics i mixòtrofs (els dinoflagel·lats *Gyrodinium dominans*, *Oxyrrhis marina*, i *Karlodinium armiger*, i els ciliats *Strombidium arenicola* i *Mesodinium rubrum*). Llavors, vam avaluar com característiques intrínseques de la presa (*Rhodomonas salina*), com són la fase de creixement i les variacions diàries en la seva composició estequiomètrica, així com també característiques pròpies dels pastors, com la seva història d'alimentació prèvia i el moment de divisió cel·lular, poden ser factors causants o bé reguladors dels ritmes diaris d'alimentació dels protistes marins. També vam avaluar l'efecte de factors extrínsecs, com són la concentració de presa, la llum i el risc de depredació, en l'activitat d'alimentació rítmica dels protistes marins. Finalment, vam portar a terme un estudi de camp per explorar els ritmes d'alimentació dels pastors protistes en un ecosistema natural (el Fiord de Gullmar, Suècia).

Com a conclusions principals de la present Tesi Doctoral, vam trobar que potser no existeix un mecanisme causant únic dels ritmes diaris d'alimentació en pastors

protistes marins. El nostre estudi sembla indicar que les espècies de protistes marins desenvolupen ritmes d'alimentació condicionats, en gran manera, per les seves característiques fisiològiques i de comportament, així com també de les particularitats ecològiques del seu hàbitat d'origen, les quals determinarien els factors pels quals el ritme és modulat.

GENERAL INTRODUCTION

1. The rhythms of life: a time for everything

The rotation of Earth around its axis every 24 hours leads to diel day-night and heat-cold cycles. The tilt of the Earth's axis causes seasonal variations annually that modulate the length of the day-night cycles. The rotation of the Earth and the gravitational pull of the sun and the moon disrupts the water masses from the oceans generating the ascent and descent of the sea levels (Bulla et al., 2017). These geophysical cycles, among others, drive the environmental periodic processes on our planet (Naylor, 2010). Organisms exposed to these periodical fluctuations have developed a broad variety of behavioural and physiological adaptive responses by generating their own temporal programs, known as *biological rhythms* (Fig. 1; Gamble and Keeble, 1903). By definition, a rhythm is an event that is repeated with a similar pattern (Aschoff, 1960). Rhythms may be one of the most obvious adaptative features of life on Earth, although frequently overlooked (Luce, 1970). Circadian rhythms (i.e., period about 24 h) are the most extensively documented, but ultradian (i.e., period less than 24 h) and infradian (i.e., period longer than 24h) rhythms also exist (Hildebrandt, 1967). Biological rhythms can be triggered by the environment (named *exogenous rhythm*) or might be endogenously generated in the organism itself by a timekeeper called the *biological clock* (Fig. 1; Kramer, 1952; Aschoff, 1960). This internal clock can be entrained to an external synchronizing factor, known as *Zeitgeber* (or time-giver; Aschoff, 1951), consequently equalising the period of the biological rhythm to that of the entraining cycle (Fig. 1; Harker, 1958; Dunlap et al., 2004; Refinetti, 2012). Under constant conditions deprived of diel cycles, rhythms controlled exogenously disappear, while endogenous rhythms persist free-running at periods approximating to that of the environmental cycles of reference (Fig. 1; Dunlap et al., 2004; Naylor, 2005; Aguzzi and Sardà, 2007). Possessing an endogenous timekeeper provides an internal temporal organization for metabolic, physiologic and behavioural processes, and enables the organisms to arrange these vital functions to occur at the optimal timing of the daily cycle (Suzuki and Johnson, 2001). Such timekeeper, hence, let organisms anticipate the onset of important changes befalling in a predictably and periodically manner in the

environment, allowing them to prepare for actual needs and thus enhancing their fitness and physiological functioning for survival (Naylor, 2010).

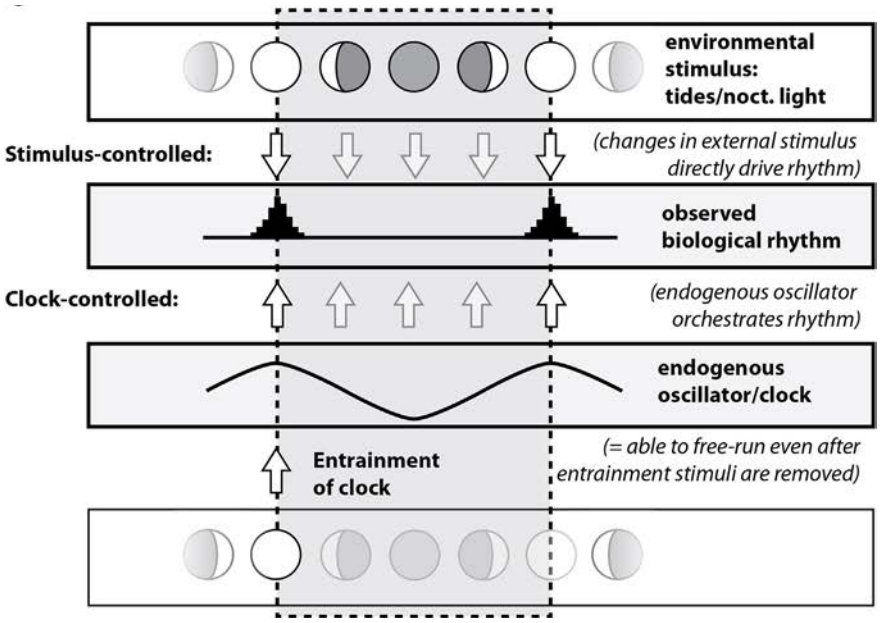


Figure 1. Biological rhythms can be either directly triggered by a change in the environmental cue (*stimulus-controlled*), or can be driven endogenously by an internal clock which is entrained by an environmental cue (*clock-controlled*); in the latter case, the rhythm may persist if the environmental cue is removed (from Raible et al., 2017).

1.1 Biological rhythms in the sea

Biological rhythms have been described in terrestrial and marine ecosystems, and exist in almost all living organisms, from prokaryotes to eukaryotes (Dunlap et al., 1999; Johnson and Golden, 1999; Paranjpe and Sharma, 2005). While terrestrial biological rhythms are well understood, limited research has studied in depth the temporal programs in marine organisms, often due to the challenge of characterizing communities' dynamics in a temporal and spatial scale, the expensive and intensive labour of sampling the organisms in the field, or the difficulty to maintain them under laboratory conditions (Tessmar-Raible et al., 2011; Häfker et al., 2017). Marine organisms are adapted to a diversity of complex temporal environments, including

daily, tidal, lunar and seasonal cycles (Bulla et al., 2017). Biological rhythms in the sea have been described in processes like cell division, photosynthesis, vertical migrations, gene expression, oxygen consumption, hatching process, locomotor activity, and so forth (Hastings et al., 1961; Arudpragasam and Naylor, 1964; Ennis, 1973; Kluge, 1982; Naylor, 1988; Bollens and Frost, 1991; Liu et al., 1995). Our knowledge of marine biological rhythms and their molecular basis is, however, often limited to few model organisms in simple laboratory settings (Bulla et al., 2017).

In the oceans, the functioning of pelagic food webs and the regulation of biogeochemical cycles are also linked to the behavioural rhythms of the planktonic organisms inhabiting them. In nature, numerous environmental factors oscillate over the daily cycle; however, only a few factors can serve as entraining cues. Among them, the 24 h solar cycle of light and darkness is the major environmental entraining agent for biological rhythms, although other factors like temperature cycles or food availability can act as entrainment cues (Dunlap et al., 2004). Because life evolved in the sea, marine organisms can provide fundamental knowledge about the origin of biological rhythms and their clocks, their diversification through evolution, and also know-how of ancient correlations between clocks and the species physiology (Tessmar-Raible et al., 2011). Throughout the present Ph.D. Thesis, we propose to go deep into one of the most widespread, but unknown, biological rhythm in the marine realm, *the diel feeding rhythm*.

2. The marine environment and food web structures

All living organisms inhabiting the marine environment are organized creating a complex network made of a large amount of interconnecting feeding relationships (trophic interactions) between resources and consumers, that assemble into *marine food webs* (Day, 1999; Rogers et al., 2010). The food web structure is strongly controlled by forces of bottom-up (i.e., resource limitation) and top-down (i.e., predation; Andersson et al., 2017). Although the structure of food webs may vary along environmental gradients (e.g., by changes in the species composition in relation

to habitat and region), the process of energy transfer through successive trophic levels is the same (Rogers et al., 2010). Among this structuration, the *pelagic food web* is of major importance as it has a central role in the regulation of CO_2 exchange between the ocean and the atmosphere, and in the carbon flux towards the deep sea (Fig 2; Steinberg and Landry, 2017).

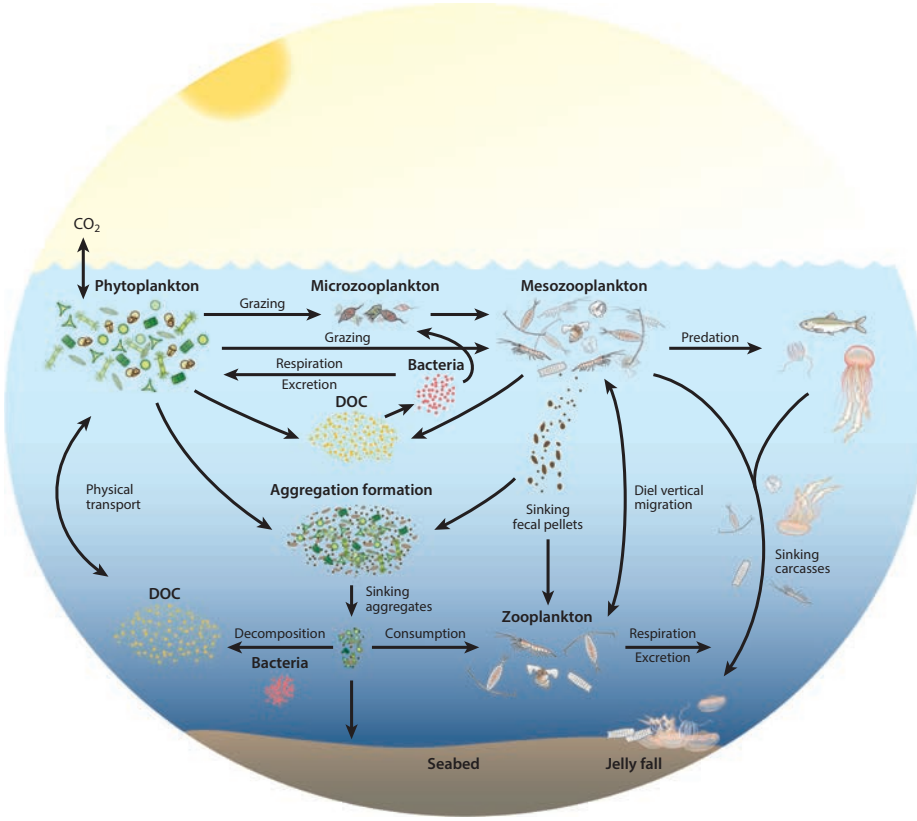


Figure 2. Carbon flux in the ocean: pathways of cycling and export of carbon by plankton (from Steinberg and Landry, 2017).

2.1 The pelagic ecosystem

A great deal of the biological activity within pelagic food webs is carried out by plankton (from Greek *planktos,* meaning *drifter*; Hensen, 1887; Fig. 3). This group encompasses organisms that live part (meroplankton) or all their life (holoplankton)

drifting in the water column. Planktonic organisms have limited capacity of locomotion, which is generally insufficient to allow them to swim against strong currents, although enough to move vertically within the water column during vertical migrations (Hamner, 1988; Ritz, 1994; Day, 1999; Hestetun et al., 2018). The plankton cover a wide diversity of taxonomic groups, including virus, bacteria, protists, and meatazoans, and encompass a wide diversity of functions and range of sizes, from micrometres to several meters (Fig. 3; Day, 1999; Hestetun et al., 2018). Plankton assemblages are traditionally classified according to their size into: femto- (<0.2 μm), pico- (0.2-2 μm), nano- (2-20 μm), micro- (20-200 μm), meso- (0.2-20 mm), macro- (2-20 cm) and mega- (>20 cm) plankton (Sieburth, 1972).

Figure 3. Artwork illustrating the diversity of marine plankton (from Sardet, 2013).

The basis of pelagic food webs in the oceans is formed by *phytoplankton*, the planktonic photosynthetic organisms that are responsible of the primary production (Fig 2; Ryther, 1969; Legendre and Rassoulzadegan, 1996; Field et al., 1998). Marine phytoplankton are composed of unicellular or colonial organisms, which mainly reproduce by asexual cell division, although some species present complicated life cycles, with resting stages formation and even sexual reproduction (Andersson et al., 2017; Raven, 2017). Encompassing about 4000 described marine species in the world's ocean, phytoplankton generate more than half of the oxygen produced annually by photosynthesis on the planet and largely contribute to the removal of CO_2 from the atmosphere; hence, phytoplankton play a crucial role in the regulation of Earth's climate (Hestetun et al., 2018).

Phytoplankton cells can die, among other causes, through sinking out of the euphotic zone, by viral, bacterial or parasitic infection or being consumed by grazers, such as *zooplankton* (Robinson, 2017). Zooplankton comprise a phylogenetically and functionally diverse assembly of both single-cell protozoa and multicellular animals (metazoans), expanding over a size range that can vary over more than 15 orders of magnitude, and occupying diverse trophic levels in the pelagic food webs (Hirst, 2017; Steinberg and Landry, 2017). Zooplankton, which include both prey and predators, exploit the primary producers and the members of the microbial food webs, while they become the source of food for small pelagic fishes (Alcaraz and Calbet, 2007). There is a strong trophic relationship between phytoplankton and zooplankton, and selective grazing by zooplankton becomes an important factor modulating the structure of phytoplankton communities (Gołdyn and Kowalczewska-Madura, 2008). Zooplankton are generally divided into the *microzooplankton*, protistan-dominated consumers group also comprising small metazoans, and the *mesozooplankton*, composed principally by metazoans, particularly copepods (Dussart, 1965; Paffenhöfer, 1998). This Ph.D. Thesis will specifically address the microplanktonic protistan grazers assemblage from the planktonic food web.

Finally, the planktonic components that express, or have the potential to express, phototrophy and phagotrophy (mixotrophs) are called *mixoplankton* (Flynn et al., 2019). Mixotrophs have been recognized as common and very important components

in the plankton community (Stoecker, 1998; Faure et al., 2019; Leles et al., 2019). The present Ph.D. Thesis will also consider some members of this group.

3. Protistan grazers, a key component in marine planktonic systems

Among planktonic organisms, protistan grazers occupy a key position in marine food webs as the main grazers of primary production (Landry et al., 1993; Calbet and Landry, 2004; Schmoker et al., 2013). As such, they display a pivotal role as essential intermediaries of matter and energy transfers between primary producers and the upper trophic levels in pelagic marine ecosystems and become a crucial component of the microbial loop (Sherr and Sherr, 2002; Calbet and Saiz, 2005). Marine protists had commonly received less attention than larger size fractions (e.g., mesozooplankton), and they have been traditionally placed to secondary contributors ranks when defining the marine ecosystems dynamics (Calbet, 2008). Nonetheless, increasing evidence has shown that protistan grazers are one of the most important groups in marine biogeochemical cycles, together with phytoplankton and bacteria (Sherr and Sherr, 2002; Calbet and Landry, 2004). Indeed, the estimated daily average consumption of primary production by protistan grazers in the oceans at a global scale accounts for 62.4% (31.3 Gt C $year^{-1}$; Schmoker et al., 2013), with relatively modest variances among seasons and regions. Specifically, protistan grazers consumption accounts for 60% of the total primary production daily grazed in coastal and estuarine environments and 70% in open oceans, and ranges from 59% in temperate-subpolar and polar systems to 75% in tropical-subtropical regions (Calbet and Landry, 2004). In fact, in most ecosystems marine protist herbivory outcompetes mesozooplankton, their main predators, whose daily average consumption has been estimated to range between 10% and 40% of the primary production in high-productivity and low-productivity regions, respectively (Calbet, 2001). Hence, acknowledging the substantial amount of carbon mediated by marine protistan grazers in the pelagic system, it urges the necessity to get a detailed comprehension of the factors that can affect the functionality of this group in planktonic food webs.

3.1 Components of marine protists

The assemblage of marine protists comprises taxonomically diverse organisms, represented by ciliates, dinoflagellates, flagellates, radiolarians, foraminiferans, among others (Gifford, 1991; Paffenhöfer, 1998; Quevedo and Anadón, 2000). Among them, *ciliates* and *dinoflagellates* are the main components of marine protistan grazers, commonly dominating the communities of protistan grazers worldwide in terms of biomass (Burkill et al., 1993; Neuer and Cowles, 1995; Levinsen et al., 1999; Sherr and Sherr, 2007; Lavrentyev et al., 2014). Given their importance, these two groups will constitute the main object of this Ph.D. Thesis.

Ciliates are unicellular eukaryotic organisms with a size range of 10 μm and 4500 μm, and more than 8000 morphospecies described (Lynn, 2008). They are characterized by a cytostome and cilia, which can be arranged in rows or clusters (Kraberg and Stern, 2017). Almost all ciliates are free-swimming planktonic species with a rapid swimming capacity. The majority of them are heterotrophic and feed on bacteria, phytoplankton, and other ciliates. However, few species temporally retain chloroplasts from their ingested autotrophic prey to undergo photosynthesis (mixotrophs). They generally proliferate by asexual division, but sexual reproduction can occur in unfavourable environmental conditions. Ciliates are globally distributed and inhabit nearly every environment, from freshwater to fully saline waters (Kraberg and Stern, 2017). In this Ph.D. Thesis, two target species of ciliates are used for the experiments: the heterotrophic ciliate *Strombidium arenicola* (Dragesco, 1960; Fig. 4A) and the mixotrophic ciliate *Mesodinium rubrum* (Lohmann, 1908; Fig. 4B). On the one hand, the genus of oligotrich ciliates *Strombidium* (Claparède and Lachmann, 1859) is a prominent component of marine protists (Montagnes, 1996), well recognized as a cosmopolitan and diverse group, with about 80 ubiquitous species described until now (Lee et al., 2012). *S. arenicola* was first discovered in the Brittany coast (Dragesco, 1960), and later in the Caspian Sea (Agamaliev, 1971) and the Black Sea (Petran, 1971). On the other hand, the mixotrophic *M. rubrum* is one of the most common planktonic ciliates in coastal marine and estuarine waters, it is broadly distributed inhabiting a wide range of environmental conditions and occurs almost all year round in plankton assemblages (Taylor et al., 1971; Satoh and Watanabe, 1991;

Johnson et al., 2004; 2013). *M. rubrum* has become a species of interest because of its fascinating ability to form outstanding reddish non-toxic blooms, described around the world (Darwin, 1840; Powers, 1932; Ryther, 1967; Proença, 2004). This species, additionally, is of very high physiological, cytological and evolutionary interest (Lindholm, 1985).

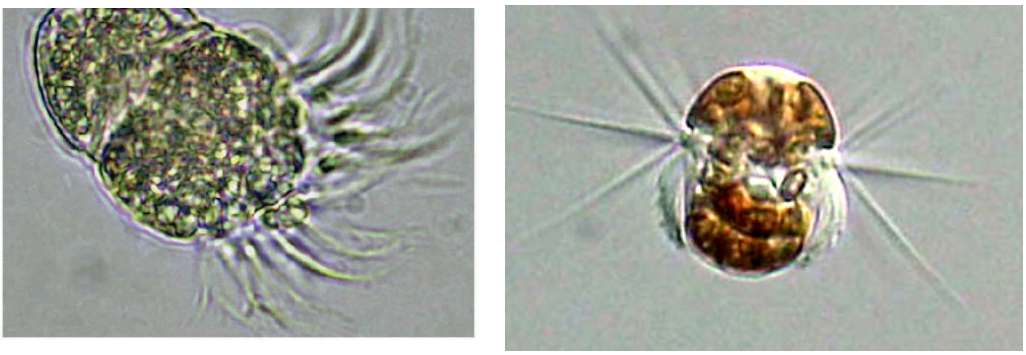
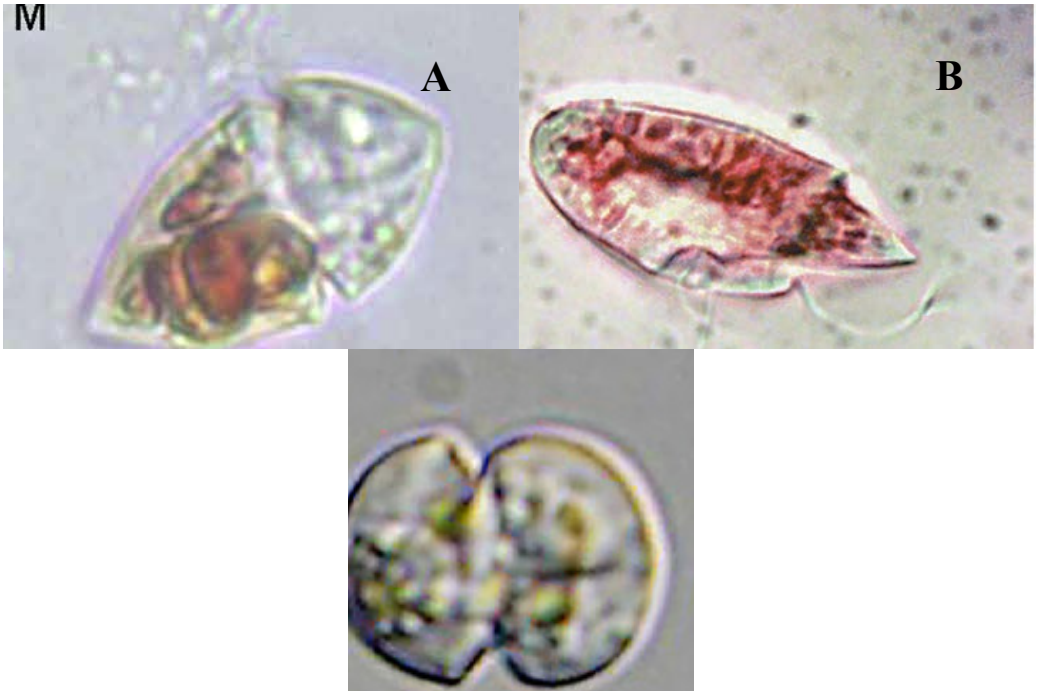


Figure 4. Target species of ciliate grazers studied in the present Ph.D. Thesis: (**A**) *Strombidium arenicola* (photo: Albert Calbet) and (**B**) *Mesodinium rubrum* (photo: University of New Hampshire).

Dinoflagellates are a diverse group of unicellular organisms with a size ranging from 5 µm to 2000 µm diameter, and about 2000 living described species (Kraberg and Stern, 2017). This group is characterized by possessing a pair of flagella morphologically differentiated, which give dinoflagellates a certain capacity to move in the water column (Fenchel, 2001). About 90% of dinoflagellate species are planktonic, and about half of the living species contain chloroplasts (Barbrook et al., 2019); thus, they can be autotrophic, heterotrophic or mixotrophic, with the last two presenting a complex modes of feeding mechanism: direct engulfment of prey, peduncle or pallium feeding (Jones, 1994; Hansen and Calado, 1999). Cell division typically occurs asexually by binary fusion. Dinoflagellates are widely distributed in aquatic environments all over the world (Barbrook et al., 2019), although the greatest diversity is marine (90%; Hansen and Calado, 1999; Taylor et al., 2008; Barbrook et al., 2019). Larger dinoflagellates typically reside nutrient-rich coastal waters while smaller organisms commonly inhabit open waters (Cullen et al., 2002). Numerous

species of dinoflagellates are harmful and synthesize potent toxins, with considerable consequences to other species of marine protists, animals, human health and the economy (Kraberg and Stern, 2017). As far as the experiments conducted in this Ph.D. Thesis, we tested the heterotrophic dinoflagellates *Gyrodinium dominans* (Hulburt, 1957; Fig. 5A) and *Oxyrrhis marina* (Dujardin, 1841; Fig. 5B), and the mixotroph *Karlodinium armiger* (Bergholtz et al., 2006; Fig. 5C). On the one side, *G. dominans* and *O. marina* are cosmopolitan species, living in different environments characterized by contrasting biological, physical and chemical properties, which has led to the development of different adaptive strategies by each species (Watts et al., 2011; Calbet et al., 2013; Goffredo and Dubinsky, 2014). While *G. dominans* is commonly abundant in coastal regions and open ocean waters (Kim and Jeong, 2004; Jeong et al., 2010), *O. marina* mainly inhabits intertidal pools and salt marshes (Johnson, 2000; Begun et al., 2004), and is scantily present in open waters (Watts et al., 2011). With extensive usage in experiments across the last 100 years and the exponential increasing applicability over numerous fields, *O. marina* has been recognized as an emerging model organism in evolution/genomics, ecology and biogeography, because of its widespread distribution, the small size, the applicability in assessing ecological issues, and because it is easily recognized, isolated from natural waters, cultivated in the laboratory and manipulated in experiments (Lowe et al., 2011; Montagnes et al., 2011). On the other side, *K. armiger* represents the first example of mixotrophic microalgae that immobilize, kill and feed live larger metazoans (Berge et al., 2012). This dinoflagellate produces karmitoxins involved in prey capture, and several studies have demonstrated mortality induced by *K. armiger* in fish, mussels, rotifers and copepods (e.g., Garcés et al., 2006; Rasmussen et al., 2017; Binzer et al., 2018). Hence, *K. armiger* has been recognized as a severe threat to aquaculture in the Mediterranean Sea and other sites, because of the devastating mortality effects of farmed fish and mussels (e.g., in Alfacs Bay, Spain. Fernández-Tejedor et al., 2007; Berge and Hansen, 2016).



3.2 Diel feeding rhythms of marine protistan grazers

Despite the relevant role of marine protistan grazers on the global pelagic system, as major grazers of planktonic primary producers, some key aspects related to their trophic behaviour remain still poorly understood. In this respect, the present Ph.D. Thesis will address one of the most little-known features of marine protist behaviour: their *diel feeding rhythms*. This represents an issue of major importance because the synchrony or lack of it between the cycles of primary production and the feeding activity of their predators can largely affect the carbon fluxes.

Feeding behaviour has been well studied among the main predators of marine protists, copepods, which typically exhibit a diel feeding rhythm with higher ingestion rates during the night-time (Haney, 1988; Tsuda and Nemoto, 1988; Atkinson et al., 1996; Steinberg and Landry, 2017). Traditionally, feeding rhythms in copepods were considered to be caused as a result of the animals' diel vertical migrations (i.e., residing food-enriched surface layers during the night-time and inhabiting deeper waters during the day-time; Gauld, 1953; Daro, 1980; Hayward, 1980; Simard et al., 1985). It is known, however, that an endogenous component might be present, and that other factors like predator threat, light and food concentration can modulate the diel feeding behaviour of copepods (Head et al., 1985; Stearns, 1986; Head and Harris, 1987; Bollens and Stearns, 1992; Cieri and Stearns, 1999).

Little is known about the rhythmic feeding activity of marine protists in nature, and the few assessments conducted *in situ* with natural assemblages have rendered inconsistent results (Litaker et al., 1988; Claustre et al., 1999; Neveux et al., 2003; Ng and Liu, 2016; Armengol et al., 2019). Instead, the few studies available with cultivated organisms in the laboratory have reported a diel feeding rhythm with higher ingestion rates during the day-time (Strom, 2001; Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011; Ng and Liu, 2015; Ng et al., 2017). Hence, the reason for diurnal (i.e., during the day-light hours) feeding behaviour in marine protists remains still poorly understood, although several mechanisms have been proposed for the observed feeding patterns in marine protistan grazers. On the one side, Strom (2001) suggested that light plays a direct role by enhancing the digestion of the phytoplankton prey fed by heterotrophic protists throughout the photooxidative breakdown of ingested organic matter, *ergo*, the formation of reactive oxygen species in the protist food vacuole. The mechanisms of a light-aided digestion, though, would only applicate to circumstances of high food concentration, where ingestion rates are governed by the rate of food vacuole processing; under low or limiting food concentrations instead, ingestions rates are constrained by the encounter rate between prey and grazer. The hypothesis of a direct effect of light on ingestion rates of heterotrophic protists was also supported by Tarangkoon and Hansen (2011). If this

was the case, however, one would expect that the diel differences should fade away under constant light conditions. Contrarily, experiments conducted with marine heterotrophic protists have proven that rhythms can still be present after 24h exposure to continuous darkness (Jakobsen and Strom, 2004). On the other side, Jakobsen and Strom (2004) proposed that the diel feeding activity, and also the cell division cycle, of most heterotrophic protists, were dictated by an endogenous circadian cycle modulated by light. Moreover, the same authors also justified higher ingestions during the day-time because of the benefits of grazing in photosynthetically active cells of phytoplankton. Nonetheless, should the photosynthetic state of the prey cells be exclusively responsible for the diurnal feeding pattern of marine protists, rates on dead fluorescently labelled algae (FLA) ingestion would not be higher in the light, as it was observed by Strom (2001). Finally, Ng and Liu (2015) and Ng et al. (2017) have recently stated that the diel variation in phytoplankton stoichiometry caused by differential patterns in carbon (C) and nitrogen (N) metabolism, could potentially affect the rhythmic feeding behaviour of their grazers. This hypothesis presents certain limitations notwithstanding, as it means that prey species without diel variations of the C:N ratio would not trigger a rhythmic feeding behaviour on their predators. Moreover, species not characterized by strong variations in stoichiometry during the diel cycle should be rare in nature because of the daily uncoupling of C and N assimilation (Kohata and Watanabe, 1989; Stramski and Reynolds, 1993; Anning et al., 2000; Lopez et al., 2016). Overall, all these previous hypotheses appear to be rather challenging among them and far from definitive. The underlining reasons for the presence of a diel feeding rhythm in marine protistan grazers are, therefore, still an open question, and it is also unclear whether this rhythm is endogenously controlled or triggered by an environment external cue. Thus, further research is needed to better understand the mechanisms behind the regulation of this trophic behaviour in nature.

In this Ph.D. Thesis, we propose to investigate the existence of diel feeding rhythms in marine protistan grazers and deepen our understanding of the causes that originate and modulate this rhythmic behaviour. Moreover, we intend to ascertain whether there is a universal mechanism underlying such rhythm in marine protists, or if there

are species-specific patterns according to the conditions from the habitats. Hence, we have put emphasis to study broad range of species, with different taxonomic and nutritious modes, to search within the diversity of tested grazers for common patterns or species-specific responses. Furthermore, new hypotheses for the regulation of diel feeding behaviour in protistan grazers are formulated. For instance, no previous approaches have assessed the role of predation risk as a possible mechanism unchaining diel feeding rhythms of marine protists. Considering that the main predators of marine protists, copepods, typically exhibit a reverse diel feeding rhythm to that of marine protists with higher nocturnal ingestion, it could be speculated that marine protists had developed this rhythmicity in their feeding behaviour as a strategy to avoid predation. In marine protistan grazers, and many other organisms, foraging implies motility and, hence, an increase in conspicuousness to visual predators, which increases the risk of predation (Titelman and Kiørboe, 2003; Visser, 2007). Therefore, for protistan grazers, increasing feeding during the day-time, when predators inhabit deeper waters, could be an advantage.

OBJECTIVES AND GENERAL STRUCTURE OF THE Ph.D. THESIS

Objectives of the Ph.D. Thesis

In this Ph.D. Thesis we aim to investigate the presence of diel feeding rhythms in marine protistan grazers and to analyse some of the potential underlying mechanisms that may regulate this rhythmic behaviour.

The overall objective of the thesis was divided into the following specific ones:

Objective 1:
To determine the effects of the growth phase (i.e., exponential *versus* stationary) of prey and, in turn, the diel variations in the nutritional properties (i.e., stoichiometric composition) of prey on the rhythmic feeding activity of marine protists.

We hypothesize that the feeding rhythms in marine protistan grazers are not the result of compensatory feeding due to diel variances in C and N metabolisms and that, instead, there is another ultimate factor unchaining these diel feeding rhythms.

Objective 2:
To evaluate whether the diel feeding rhythm of marine protists is affected by the feeding history of the grazer (i.e., well-fed *versus* starved).

Our hypothesis is that the feeding rhythm is affected by the nutritional status of the grazer, with well-fed grazers showing a diel feeding rhythm of higher amplitude compared to the feeding rhythm of starved grazers.

Objective 3:
To determine the effect of prey concentration on the diel feeding rhythms of protistan grazers.

We expect the rhythmic feeding activity of marine protists to be affected by prey availability, with rhythms of higher amplitude under non-limiting food conditions.

The reason supporting this hypothesis relies on the fact that, when facing saturated prey conditions, protistan grazers may reduce food-gathering during the night period, when copepods – their main predators – are more actively feeding; in consequence, differences between day and night feeding would become larger. On the contrary, when prey are scarce, grazers may require feeding both during the day and during the night to fulfill all metabolic demands.

Objective 4:
To evaluate the link between the processes of cell division and the diel feeding rhythm of protistan grazers.

We hypothesize that the presence of diurnal feeding behaviour in protistan grazers may be a consequence of a cell division process scheduled to the night-phase, which may result in the impairment of feeding activity during the duplication process.

Objective 5:
To investigate the effect of light as triggering factor and the existence of an endogenous component in the feeding rhythms of marine protists.

We advocate that the feeding rhythms of marine protists are governed by an endogenous rhythm, which is daily adjusted by light.

Objective 6:
To assess the effect of the risk of predation by copepods on the diel feeding rhythm of protistan grazers.

We hypothesize that diel differences in the feeding activity of marine protists may have evolved as a strategy to reduce predation. For protistan grazers, feeding involves motility and therefore becoming more conspicuous and increasing the probability of encounter with predators (e.g., copepods). As copepods mostly present nocturnal feeding behaviour, it can be speculated that it would be advantageous for protistan

grazers to display a diel feeding rhythm reversed to that displayed by copepods in order to reduce the probability of protistan grazers to be predated.

Objective 7:
To explore the existence of diel feeding rhythms of protistan grazers in a natural ecosystem.

We will study the presence of rhythmic feeding activity in natural assemblages of marine protistan grazers and analyse the results in relation to some of the causal mechanisms studied with cultured protists in the laboratory. We expect a general common pattern similar to the one found in the laboratory with cultivated species.

Structure of the Ph.D. Thesis

The research carried out in this Ph.D. Thesis to fulfill the above-mentioned objectives is arranged in four chapters.

The presence of diel feeding rhythms in several marine species of heterotrophic protists was first approached in **Chapter 1**, although new potential heterotrophic and mixotrophic species of protists exhibiting a rhythmic feeding activity were added to this collection in the next chapters. Objectives 1, 2 and 3 all together were also assessed in this chapter. This study has been published in a peer-reviewed journal: Arias, A., Saiz, E., and Calbet, A. 2017. Diel feeding rhythms in marine microzooplankton: effects of prey concentration, prey condition, and grazer nutritional history. *Marine Biology*, 164(10):205.

Objectives 4 and 5 were dealt with in **Chapter 2** and published in a peer-reviewed journal: Arias, A., Saiz, E., and Calbet, A. 2019. Towards an understanding of diel feeding rhythms in marine protists: consequences of light manipulation. *Microbial Ecology*, 79(1):64-72.

Objective 6 was addressed in **Chapter 3**, and also includes some experiments carried out during a short stay at the University of Gothenburg (Sweden) under the supervision of Dr. Erik Selander. The results from the study have been submitted to a peer-reviewed journal: Arias, A., Selander, E., Saiz, E., and Calbet, A. 2020. Predator chemical cue effects on the diel feeding behaviour of marine protists. [submitted]

The research to address Objective 7 was carried out in a two-month field study conducted at the Kristineberg Marine Research Station (Sweden), under the supervision of Dr. Peter Tiselius. This study is presented in **Chapter 4** and the corresponding article has been submitted to a peer-reviewed journal: Arias, A., Saiz, E., Tiselius, P., and Calbet, A. 2020. Trophic interactions and diel feeding rhythms of microzooplankton in a productive Swedish fjord. [submitted]

Finally, the memory of the Ph.D. Thesis also includes a General Discussion of the main results followed by a General Conclusions section.

CHAPTER 1

Diel feeding rhythms in marine microzooplankton: effects of prey concentration, prey condition, and grazer nutritional history

Arias, A., Saiz, E., and Calbet, A. 2017. Diel feeding rhythms in marine microzooplankton: effects of prey concentration, prey condition, and grazer nutritional history. *Marine Biology*, 164(10):205.

1.1 Abstract

In this study we aim at disentangling the causes and consequences of diel feeding rhythms in marine protistan grazers. We focused on the diel feeding activity of two heterotrophic dinoflagellate species, *Gyrodinium dominans* (one laboratory strain) and *Oxyrrhis marina* (laboratory cultivated and wild strains). We observed higher ingestion during the day in both dinoflagellate species. Feeding rhythms appeared to be independent of circadian changes in prey biochemical composition. Grazers fed with prey under stationary phase, with equivalent stoichiometric composition between day and night, showed 5 (*G. dominans*) and 10 (*O. marina*) times higher ingestion rates during the day. Previous grazer feeding history (starved *versus* well-fed) did not affect the feeding rhythm. However, prey concentration altered the rhythm; food limiting conditions reduced the amplitude of the rhythm. Our results establish a resource dependence of diel periodicity in microzooplankton grazing, which can have unanticipated consequences for standard field dilution grazing experiments.

1.2 Introduction

Light is a major driver of life in our planet and, as such, it regulates the production and distribution of phototrophic organisms; for instance, the vertical distribution and seasonal production peaks of plankton in aquatic ecosystems are dependent on light availability (Sverdrup, 1953; Margalef, 1978). It is known that light also drives the feeding rhythms of mesozooplankton both in marine and freshwater systems (e.g., Duval and Geen, 1976; Mackas and Bohrer, 1976). In most occasions, feeding rhythms in mesozooplankton are linked to daily patterns of vertical migration (i.e., surface during night, deeper during day-time; Gauld, 1938; Stearns, 1983; Saiz and Alcaraz, 1990; Saiz et al., 1992; Putzeys and Hernández-León, 2005), typically triggered by the presence of visual predators (i.e., fish) in the upper layers of the oceans (Bollens and Frost, 1991; Bollens, 1996). Nocturnal feeding has also been observed, however, in the absence of vertical migration in some copepod species (Peruyeva, 1977; Boyd et al., 1980; Calbet et al., 1999).

In the case of microzooplankton, a key group in the transfer of energy from primary producers to upper trophic levels in the marine pelagic environment (Calbet and Landry, 2004; Calbet and Saiz, 2005; Schmoker et al., 2013), less is known about their diel feeding rhythms and the triggering factors modulating them. This functional group is taxonomically diverse and overall encompasses organisms with limited migratory capacity. The few evidences available on microzooplankton diel feeding behaviour indicate, contrarily to mesozooplankton, that ingestion rates are higher during the day (Strom, 2001; Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011).

Several hypotheses have been suggested to explain this particular behaviour. Jakobsen and Strom (2004) advocated the presence of an endogenous circadian cycle, light-modulated, for feeding and growth of many protozoans. On the other hand, Strom (2001) proposed that light may enhance digestion by generating reactive oxygen species in the protozoan food vacuole, while promoting ingested material break down and increasing assimilation and gross growth efficiencies. By this process, digestion would not limit (or limit less) the incorporation of new items into

the food vacuoles, enhancing the feeding rates. Alternatively, Ng and Liu (2015) suggested that diel variations on phytoplankton stoichiometry (i.e., higher C:N ratio during day) can potentially influence the feeding behaviour of grazers. A diel periodicity of the C:N ratio has been detected in various phytoplankton groups (Stramski and Reynolds, 1993; Clark et al., 2002; Jauzein et al., 2011), and it is suggested to be regulated by a circadian clock (Edmunds, 1988). Bonded to this diel variation of the stoichiometric composition of prey are the changes of size result of synchronous division (Sweeney and Hastings, 1958; Edmunds, 1965; Eppley and Coatsworth, 1966; Paasche, 1968; Bruce, 1970). Most of the hypotheses above, however, are questioned by the facts that i) the presence of diel feeding rhythms seem to be preserved under either continuous light or darkness, and ii) diel feeding rhythms appear even when protists are fed on "inert", dead cells (Sweeney and Hastings, 1958; Chisholm and Brand, 1981; Jakobsen and Strom, 2004). We believe the presence of reverse diel feeding rhythms in microzooplankton, though endogenous, might have evolved as an adaptation to avoid being predated. Because feeding typically implies swimming, grazers become more conspicuous and increase encounter rates when feeding (Broglio et al., 2001); therefore, to reduce the probability of being predated protozoans should display diel feeding rhythms reverse to that of their major grazers (i.e., copepods; Saiz and Calbet, 2011). Until now, however, no experimental evidence confirms this plausible hypothesis, although in copepods such behavioural mechanisms have been reported (Saiz et al., 1993; Heuschele et al., 2014).

Here we studied the diel feeding activity of two heterotrophic dinoflagellate species, *Gyrodinium dominans* (a laboratory cultivated strain kept for many generations) and *Oxyrrhis marina* (both a laboratory cultivated and a wild strain) and examined the effect of several factors on their feeding behaviour. Considered cosmopolitan species, they inhabit different environments with contrasting biological, physical and chemical properties; this has lead to different adaptive ecological and physiological strategies (Calbet et al., 2013). We determined: (1) the presence of diel feeding rhythms in our target grazers and then checked whether nutritional properties of prey between day and night may explain the presence of rhythms; (2) whether the growth phase of the prey can evoke changes on the grazers' feeding behaviour; (3) the diel

response of well-fed *versus* starved grazers (i.e., 48h unfed) to prey; in this case, we expected rhythms to be influenced by the grazer previous feeding history, with well-fed grazers showing higher amplitude diel feeding rhythms than starved ones; (4) the effects of prey concentration on the amplitude of the diel feeding rhythms of microzooplankton. One could expect the diel activity to be influenced by prey availability. Under non-limiting food conditions, microzooplankton may "opt" to feed less during the night period, when potential predators may have a larger impact, and, therefore, the differences between day and night feeding would become higher; under food limitation, the grazers might be forced to search for food both during the day and during the night to cover their metabolic demands, as it occurs in more complex organisms, such as copepods (Huntley and Brooks, 1982; Calbet et al., 1999).

1.3 Materials and methods

1.3.1 Culture of the dinoflagellate predators and the algal prey

Laboratory cultures of the heterotrophic dinoflagellates *Gyrodinium dominans* (GYR-BCN), and *Oxyrrhis marina* (OXY-BCN and OXY-BCN-2016, a new strain recently isolated) were used to study diel cycles in feeding and growth under different conditions. All strains were isolated by A. Calbet off Barcelona coast (NW Mediterranean, 41° 23′ 0 N) in 2011, 1996 and 2016 respectively, and then kept in the laboratory at the Institut de Ciències del Mar in Barcelona.

For these experiments, the grazer cultures were grown in round flasks with metal-enriched autoclaved seawater (1mL metal stock solution per litre; Guillard, 1975) at 19±1ºC, 38 PSU under a 10L:14D light-darkness cycle. Grazer stocks were daily fed with a culture of the cryptophyte *Rhodomonas salina* grown on f/2 medium (Guillard, 1975) in 5 L Pyrex culture flasks provided with air and diluted daily to ensure exponential growing conditions. The grazer cultures were maintained in these conditions several weeks (>4) prior to conducting the feeding experiments.

1.3.2 Prey and grazer diel changes in size and stoichiometric composition

We assessed the morphological (size) and stoichiometric (C:N:P) changes during the different growing phases (i.e., exponential and stationary phases) of *R. salina* by following the development of a triplicated culture of *R. salina* since inoculation until the beginning of the decay phase. The cultures were sampled before the light and dark periods started to determine cell size and concentration with a Beckman Coulter Multisizer III particle counter (100 µm aperture tube). Concurrently, we also analysed the elemental composition (carbon, nitrogen and phosphorus) of *R. salina* during the exponential and stationary phases of growth. For C and N analysis, 5 mL aliquots of the *R. salina* culture were filtered onto 25-mm diameter pre-combusted GF/F filters (450 ºC, 6h), dried at 60°C during 48 h and kept in a desiccator until analysis with an elemental analyser FlashEA1112 (ThermoFinnigan). For P analysis, 2 mL aliquots were used and immediately frozen at -80 ºC after filtration; later, samples were digested with NaOH-$K_2S_2O_8$, and then analysed as inorganic P with an AA3HR autoanalyser (Seal Analytical). Following the same procedure, we analysed the stoichiometric composition of OXY-BCN and GYR-BCN before the light and dark periods after 2-days of starvation, when no prey was present in the suspension. For these samples we filtered from 20 to 50 ml, depending on the grazer concentration.

1.3.3 Experimental set-up

General set-up

The general procedure for the experiments was as follows. At each experiment, grazer and prey stock concentrations were determined with a Beckman Coulter Multisizer III particle counter (100 µm aperture tube) within 1-2 h before the beginning of, respectively, the light period (9:00 a.m.) and the night period (7:00 p.m.). Then, the desired predator-prey suspensions were prepared and distributed at intervals by filling one-third of experimental (both grazer and prey) and control (only prey) bottles (72 mL polyethylene culture flasks; 3-4 replicates). Extra bottles at each prey-predator

mixture were also prepared for determination of initial concentrations. Once set, the bottles were incubated on a plankton wheel (0.2 r.p.m.). About 1 h before the end of the respective light and night periods, the corresponding incubations were terminated and grazer and prey concentrations determined. During the light period the bottles were exposed to fluorescent lamps providing an irradiation that ranged between 80 and 290 μE m^{-2} s^{-1} through a complete rotation of the wheel. Grazer and prey concentrations (in cells and in biovolume) at the beginning and at the end of the incubations were determined with the Beckman Coulter Multisizer III particle counter.

Effect of prey growth condition on diel feeding rhythms

We designed a series of experiments to explore whether the diel differences in feeding and growth rates of the grazers were affected by the prey growth phase (i.e., exponential *versus* stationary), and at its turn, by its stoichiometric composition. For these experiments we used the strains GYR-BCN and OXY-BCN, previously starved for 48 h. All the experimental procedures are as described in the *General set-up* section. Based on the results obtained (see Results), we decided to use *R. salina* (7-8 x 10^4 cells mL^{-1}) in stationary phase for the rest of the experiments in order to minimize the day-night cells size variation.

Effect of the grazer feeding history on diel feeding rhythms

We investigated whether the previous feeding history of the grazer affected the amplitude of the diel feeding rhythm. Hence, we compared the grazing and growth rates of GYR-BCN and OXY-BCN fed ad libitum with *R. salina* with those of 48 h-starved grazers (i.e., unfed for two days). The experiments were conducted following the general procedures described above.

Effect of food concentration on diel feeding rhythms.

We evaluated the effect of different prey concentration on the feeding behaviour of GYR-BCN, OXY-BCN and OXY-BCN-2016. According to the results of the previous experiments we establish the experimental protocol to use prey on stationary

phase and to starve the grazers for 48 h prior to the experiments. Prey concentrations were chosen in order to encompass three different scenarios of the functional feeding response (based on data from Calbet et al., 2013): limiting, intermediate and saturated food conditions (Table 1.1). The prey:predator ratios at the beginning of the incubations were about 35:1 in *G. dominans* and 100:1 for *O. marina.* All the experiments were conducted in triplicate and followed the general procedures described above.

Table 1.1. Prey and grazer concentrations (cell mL^{-1}) used in the experiments on the effect of food concentration on diel rhythms. Cell concentrations were selected according to Calbet et al. (2013) to include limiting, intermediate and saturating food conditions.

Grazer	Treatment	Prey concentration (cell mL^{-1})	Grazer concentration (cell mL^{-1})
GYRO-BCN	Limiting	4000 – 10000	600 – 850
	Intermediate	$4x10^4$ – $6x10^4$	1500 – 2800
	Saturated	$10x10^4$ – $11x10^4$	3000 - 3500
OXY-BCN	Limiting	8000 – 12000	192 – 240
	Intermediate	15000 – 25000	257 – 330
	Saturated	$15x10^4$ – $20x10^4$	1607 - 2100
OXY-BCN-2016	Limiting	8000	192
	Medium	15000	257
	Saturated	$15x10^4$	1607

1.3.4 Calculation of feeding rates

The calculation of feeding rates followed the exponential equations of Frost (1977). The grazing coefficient g (h^{-1}) was estimated as:

$$g = \mu - k$$

where μ (h^{-1}) is the intrinsic prey growth in the control bottles (only prey), and k is the apparent prey growth determined in the experimental bottles (with predators). Clearance rate F (µl $grazer^{-1}$ h^{-1}) was estimated as:

$$F = \frac{g}{\hat{C}_{grazer}}$$

where $\hat{C}_{grazer}$ (cells mL^{-1}) is the average grazer concentration in the incubation, estimated as:

$$\hat{C}_{grazer} = \frac{C_{0,grazer} - C_{1,grazer}}{ln(C_{1,grazer}/C_{0,grazer})}$$

where $C_{0,grazer}$ and $C_{1,grazer}$ are, respectively, the grazer initial and final concentrations in the incubation and t (h) is the incubation time.

Ingestion rates I (cells grazer^{-1} h^{-1}) were estimated as:

$$I = F \times \hat{C}_{prey}$$

where $\hat{C}_{prey}$ (cells mL^{-1}) is the average prey concentration estimated according to the equations in Frost (1977).

Feeding rates were converted into prey biovolume consumption by multiplying cell ingestion rates by the (geometric) mean prey volume during the incubation.

1.4 Results

1.4.1 Prey and grazer diel changes in size and stoichiometric composition

Rhodomonas salina entered in exponential growth (μ = 0.38 d^{-1}) after a short (1 day) lag phase and remained exponentially growing for four days (Fig. 1.1A). Stationary phase reached densities of 1.6x10^6 cells mL^{-1}. Cells divided mostly during the night, which produced important differences in cell size between day and night. These differences were evident only during the exponential phase (Fig. 1.1B); cells were about 33% larger in volume during the day hours than during the night hours.

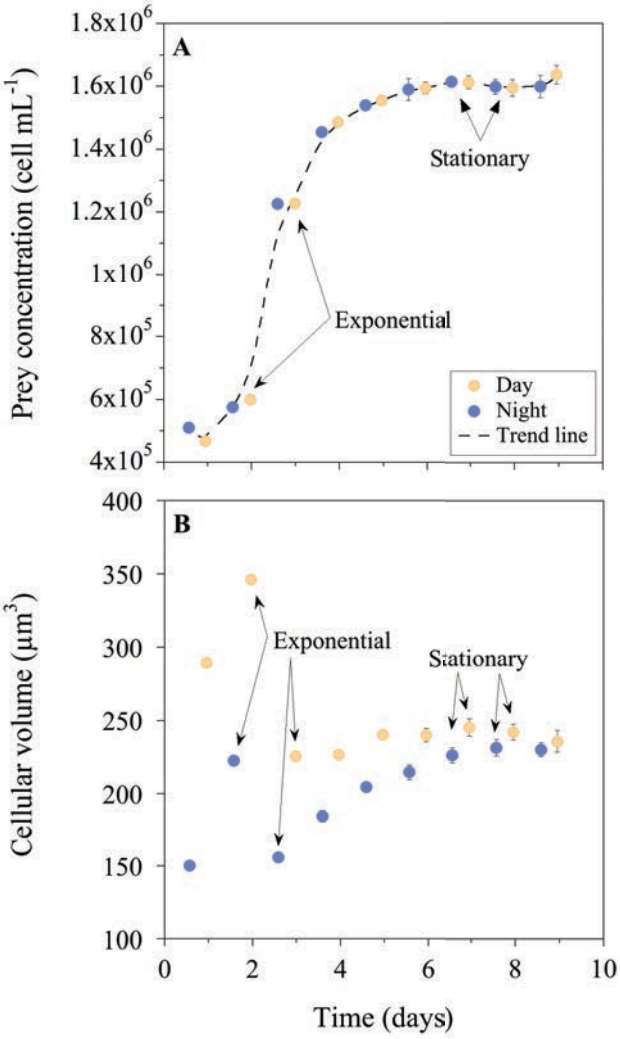


Figure 1.1. (A) Time course of *Rhodomonas salina* concentration (cell mL^{-1}) and **(B)** cellular volume (μm^3) after inoculum in batch culture. In both graphics, the days sampled as representative of exponential and stationary growth phases are highlighted. Yellow and blue dots indicate light and night hours, respectively. Error bars represent standard error.

We present in Table 1.2 the stoichiometric composition of *R. salina* under exponential (days 12-13, both day and night periods, in Fig. 1.1) and stationary (from day 16, day period, to day 18, night period, in Fig. 1.1) phases. All elemental ratios were significantly higher during day-time in the exponential phase of growth (C:N, 20% higher; C:P, 64%; N:P, 42%), whereas no difference between day and night composition were detected in stationary phase (Table 1.2). When comparing between

exponential and stationary phases, the C:N and C:P were more than double during stationary phase respect exponential, whereas for N:P values differences between growth phases were of much lower magnitude.

Table 1.2. Day and night C:N, C:P and N:P molar ratios (average ± SE) of *Rhodomonas salina* under **(A)** exponential phase and **(B)** stationary phase. Significance levels (p-value) of two-tailed *t*-test comparing day and night averages are also shown.

	C:N	**C:P**	**N:P**
(A) Exponential			
Day	7.7 ± 0.14	104.4 ± 20.24	13.6 ± 2.86
Night	6.4 ± 0.29	63.6 ± 8.26	9.56 ± 1.05
p-value	<0.001	0.003	0.01
(B) Stationary			
Day	16.2 ± 0.88	245.7 ± 11.30	15.2 ± 0.97
Night	15.7 ± 1.04	229.8 ± 14.49	14.7 ± 0.35
p-value	0.56	0.18	0.37

The stoichiometric ratios of *O. marina* and *G. dominans* did not overall differ significantly between day and night (Table 1.3); only the C:N ratios of *G. dominans* differed between day and night, but the magnitude of variation was rather small (4% higher during the day; Table 1.3).

Table 1.3. Day and night C:N, C:P and N:P molar ratios (averages ± SE) of two-day starved **(A)** *Gyrodinium dominans* (Gyr-BCN) and **(B)** *Oxyrrhis marina* (OXY-BCN). Significance levels (p-value) of two-tailed *t*-test comparing day and night averages are also shown.

	C:N	**C:P**	**N:P**
(A) *Gyrodinium dominans*			
Day	5.8 ± 0.07	46.0 ± 13.4	8.0 ± 2.4
Night	5.6 ± 0.06	39.4 ± 12.1	7.0 ± 2.2
p-value	0.01	0.55	0.64
(B) *Oxyrrhis marina*			
Day	5.8 ± 0.09	34.5 ± 8.2	6.0 ± 1.4
Night	5.7 ± 0.06	31.8 ± 8.0	5.6 ± 1.4
p-value	0.20	0.70	0.77

1.4.2 Effect of prey growth conditions on diel feeding rhythms

We compared the diel feeding response of GYR-BCN and OXY-BCN when feeding on *R. salina* in the exponential and stationary growth phases. The feeding rates obtained are presented in terms of cell (cells ind^{-1} hour^{-1}) and volume ingested (μm^3 grazer^{-1} hour^{1}) in Fig. 1.2. In both species prey on stationary phase induced feeding rhythms of higher amplitude than when on exponential phase, being the rates about 5 (GYRO-BCN) and 10 (OXY-BCN) times higher during the day ($p<0.001$, *t*-test). In contrast, the results obtained with prey growing at exponential rates did not result in such a clear outcome. Whereas for GYR-BCN ingestion rates were higher during the day ($p<0.001$ for cells and volume, *t*-test), OXY-BCN showed an opposite pattern, with higher cell-based ingestion rates during the night ($p<0.05$, *t*-test), and no statistically significant differences between day and night when on a volume basis.

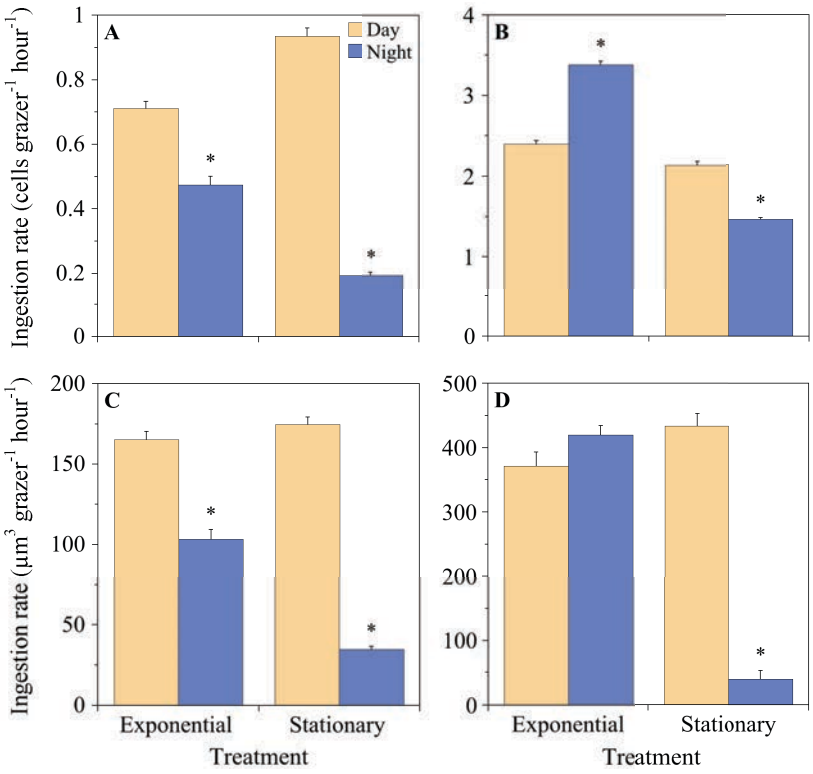


Figure 1.2. Day and night ingestion rates of (**A**, **C**) *Gyrodinium dominans* and (**B**, **D**) *Oxyrrhis marina*, expressed either in terms of prey number and of prey volume, for each experimental condition (exponential and stationary growth). Error bars indicate standard error and asterisks represent significant differences ($p<0.01$).

1.4.3 Effect of the feeding history on diel feeding rhythms

Diel ingestion rates of previously fed and starved GYR-BCN and OXY-BCN are presented in terms of cells and volume ingested in Fig. 1.3. In all cases ingestion rates were significantly higher during the light period ($p<0.05$, *t*-test). For GYR-BCN ingestion rates were about 55% (fed grazers) and 44% (starved grazers) higher during the day, whereas for OXY-BCN the increase was 13% and 34% for fed and starved grazers, respectively (Fig. 1.3).

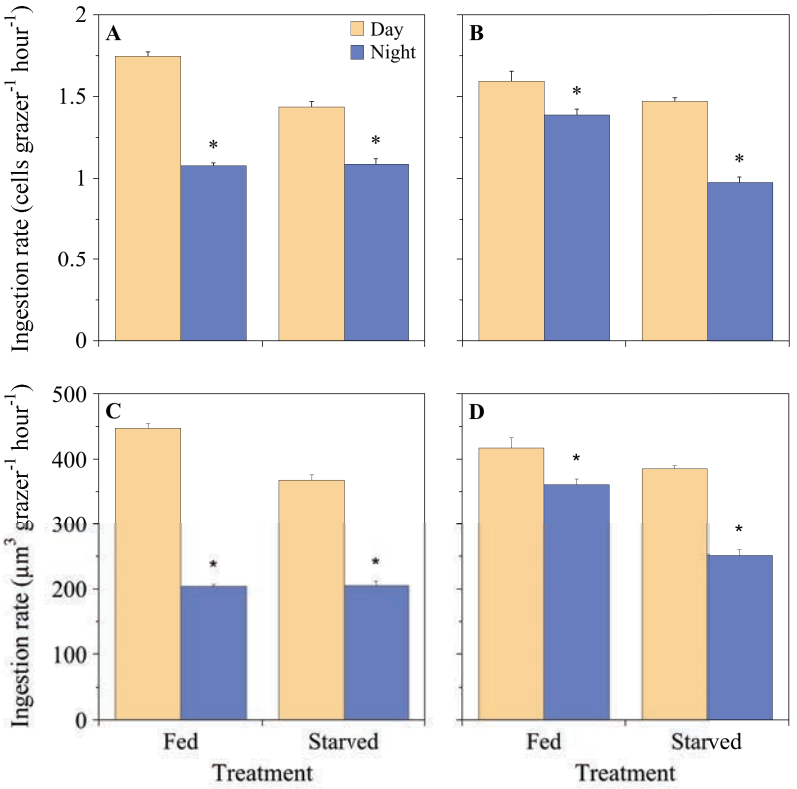


Figure 1.3. Ingestion rates (in cells and volume) of (**A**, **C**) *Gyrodinium dominans* and (**B**, **D**) *Oxyrrhis marina* as a function of previous feeding ($p<0.01$; either fed or starved), expressed either in terms of prey number and of prey volume. Error bars indicate standard error and asterisks represent significant differences ($p<0.01$).

1.4.4 Effect of food concentration on diel feeding rhythms

Food availability clearly modified the feeding rhythms of the 3 grazers studied (Fig. 1.4A,B). Under saturating food conditions all grazers showed the largest differences between day and night ingestion rates (in terms of prey volume consumed); these differences diminished as food concentration decreased. At limiting food conditions, the daily rhythms disappeared for OXY-BCN and it was poorly marked in the other strains. Out of the 3 strains studied, GYR-BCN showed the highest rhythm amplitude, followed by OXY-BCN-2016 (Fig. 1.4). Except for GYR-BCN, the slopes of the relationship between food concentration and the ratio between day and night ingestion rates were significantly different from zero ($p<0.05$; Fig. 1.4), indicating a significant effect of prey concentration; in the case of GYR-BCN, however, the removal of one outlier value made the regression turn out significant ($p<0.05$). The effects of food concentration on the diel feeding rhythms of the three grazers were similar, as indicated by the lack of significant differences among slopes (Fig. 1.4; $p=0.51$, ANCOVA test). However, the intercepts were significantly different between regression lines ($p<0.01$), which support that the magnitude of the rhythm is species/strain specific. In all treatments the grazers showed negligible growth rate (data not shown).

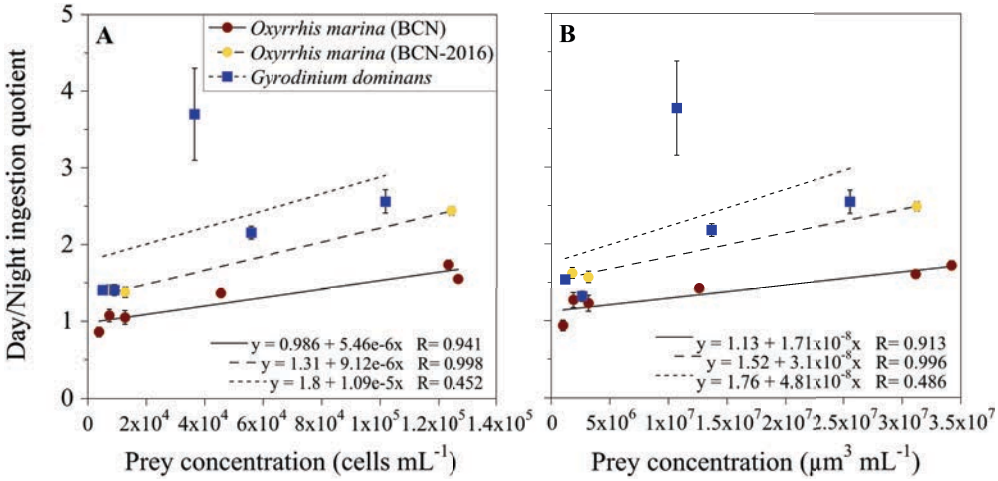


Figure 1.4. Feeding rhythm index (quotient between day and night ingestion rates) of *Oxyrrhis marina* (BCN and BCN-2016 strains) and *Gyrodinium dominans* as a function of prey availability. **(A)** Values in cells mL^{-1} and **(B)** Values in volume (μm^3 mL^{-1}). Error bars show standard error.

1.5 Discussion

Corroborating previous evidence on protozoan diel behaviour (Christoffersen, 1994; Liu et al., 1997; Dolan and Šimek, 1999; Strom, 2001; Binder and DuRand, 2002; Jakobsen and Strom, 2004) we found reverse circadian feeding rhythms (i.e., higher feeding rates during daytime) in two marine heterotrophic dinoflagellate species. The causes and consequences of this particular behaviour are discussed below.

1.5.1 Causes of microzooplankton diel feeding rhythms

Phytoplankton activity is affected by light, leading to a diel periodicity in cell division and cellular properties (including C:N ratio; Prézelin, 1992; Vaulot et al., 1995; Liu et al., 1997; Vaulot and Marie, 1999; Binder and DuRand, 2002; Ng and Liu, 2015). Synchronized cell division during night-time is confirmed for many phytoplankton groups and results in a cell size decrease during this period of the day (Prézelin, 1992; Binder and DuRand, 2002; Ng and Liu, 2015). Likewise, because of photosynthetic carbon fixation during light hours, C:N and C:P ratios increase during the day (Stramski and Reynolds, 1993; Clark et al., 2002; Jauzein et al., 2011; Ng and Liu, 2015). Diel variations of both cellular properties and cell size in phytoplankton should have ecological implications and could affect the dynamic of diel trophic interactions (Ng and Liu, 2015). Ng and Liu (2015) argued that the feeding behaviour of nanoflagellated grazers could be strongly induced by these diel stoichiometric variations of prey, as a result of compensatory feeding response by increasing grazing rates on low quality prey (i.e., high C:N) during the day-time. However, the same authors found also a distinct diel grazing pattern when flagellates were feeding on fluorescently-labelled dead bacteria. Also, Strom (2001) found that under saturating food conditions, the ingestion of dead fluorescently-labelled algae was 2.2 times higher in the light; she suggested a light-aided digestion mechanism. These two latter evidences seem to contradict the role of diel changes in algae composition as triggers of their grazers' diel feeding activity. Moreover, Jakobsen and Strom (2004) detected that the diel variations in growth and ingestion rates during day and night persisted

in 24 h continuous darkness (although the rhythm slowly eroded after a few days), challenging Ng and Liu (2015) and Strom (2001) hypotheses. It seems, then, that either particular rules apply to each species, or that there must be an alternative explanation for the presence of diel feeding rhythms in microzooplankton. In our experiments we also detected a diel well-marked difference in *R. salina* cell size and stoichiometric composition during exponential phase, but these differences faded away in early stationary phase. This fact allowed us to test the role of diel changes in size and elemental composition in causing the existence of feeding rhythms. We found that GYR-BCN and OXY-BCN showed higher ingestion rates during the day when fed on stationary phase prey, therefore corroborating that these two factors cannot explain the presence of feeding rhythms. Nevertheless, the smaller prey size during night under exponential growth may mask, in occasions, these feeding patterns, as we observed in OXY-BCN (Fig. 1.2); this effect disappeared under stationary phase conditions where prey size was similar. Our results, therefore, lead to the conclusion that other factors, not only related to prey characteristics, must play an important role in determining the diel feeding activity of microzooplankton.

Oceanic planktonic habitats are known to be often food limited (Conover, 1968). When prey are limiting, feeding behaviour of a grazer may be compromised as it is linked to swimming and, therefore, a grazer must increase its search effort. Hence, it increases the encounter rate with their own predators and, at the same time, they become more conspicuous to them. In this situation, a balance between feeding to maintain minimum nutritional requirements and the risk of being predated is necessary (Huntley and Brooks, 1982; Saiz et al., 1992; Calbet et al., 1999), in particular given the high preference for dinoflagellates and ciliates displayed by copepods, the major contributors to mesozooplankton (Saiz and Calbet, 2011). Several adaptive strategies of microzooplankton to famine have been proposed and demonstrated in laboratory studies. A reduction of metabolic rate in starving protozoans was suggested by Fenchel and Finlay (1983). Some species are also known to recur to resting cysts formation when prey concentration is low (Goodman, 1987; Fenchel, 1990). We suggest that microzooplankton may “opt” to diminish the feeding rhythm. As we initially hypothesised, the feeding rhythm of the studied

microzooplanktonic grazers was of higher amplitude when food was not limiting. It would be expected that at low food concentration, starved grazers would feed in an arbitrarily manner, without following a light-darkness cycle to fed. As a consequence, this would lead to a major mobility by the protozoans, which implies being more detectable by their own predators. This means that the threat of predation may be an important component explaining microzooplankton diel feeding activity. Backing up this hypothesis we found a greater diel response in the recently isolated *O. marina* (OXY-BCN-2016) compared to the long-term laboratory-cultivated one (OXY-BCN). It is expected, as it occurs in other planktonic organisms, that some of the natural behaviours (i.e., the risk of predation) might be partially lost after consecutive generations of cultivation in the laboratory (Tiselius et al., 1995; Calbet et al., 1999).

In our study, the magnitude of the feeding rhythm (Fig. 1.4) differed between species/strains, being GYR-BCN the species with the most marked rhythm. *G. dominans* is typically found in costal and oceanic waters where vertical migrations of mesozooplankton are common and predation risk is higher at night (Saiz et al., 2014). On the other hand, *O. marina* inhabits intertidal pools and salt marshes (Begun et al., 2004), being infrequent in open waters (Lowe et al., 2010; Watts et al., 2011), and has life history traits that allow it to quickly exploit resources whenever conditions are favourable (Calbet et al., 2013).

We have demonstrated so far that, at least for the species of microzooplankton studied here, feeding rhythms appear to be independent of circadian changes in prey stoichiometric composition or previous grazer feeding history, but are modified by prey concentration. It may be argued, however, that decreased feeding rates during the night may be consequence of synchronized division of the grazer at night, constraining feeding while dividing. Even though we cannot disregard this hypothesis, our data do not seem to confirm it. Growth rates after the two-day starvation period were negligible for both GYR-BCN and OXY-BCN in all the experiments. Therefore, it is unlikely that the arrangement of the cell organelles during division can explain the arrest of ingestion during night.

1.5.2 Consequences of microzooplankton diel feeding rhythms

Regardless of the ecological reasons behind the existence of diel feeding rhythms in microzooplankton, their consequences in natural ecosystems are important. Higher ingestions during day, on pre-dividing algal cells should have more impacts on phytoplankton populations than the same grazing activity on already divided (night) algae. Given the relationship between prey availability and intensity of the diel feeding activity we found, it can be hypothesized diel rhythms are more relevant in upwellings and productive systems than in oligotrophic ones. In all these systems microzooplankton appear as the major herbivore (Calbet and Landry, 2004; Schmoker et al., 2013). Unfortunately, there are not field data on diel microzooplankton herbivory. The only field study we are aware of dealt with bacterivory in coastal South China Sea and Hong Kong waters and found evidences of a higher diurnal grazing activity on bacteria (Ng and Liu, 2015).

A relevant outcome of our data is that it establishes a resource dependence of diel periodicity in microzooplankton grazing, which can have unanticipated consequences for the most common way to determine microzooplankton grazing rates in the field, the dilution grazing experiments (Landry and Hassett, 1982). Along the dilution series diel feeding activity will be artificially modified since food availability is modified. Moreover, the consideration of predator-prey growth and grazing coupling is of important relevance when choosing the starting point of the microzooplankton grazing experiments, as for any other grazing experiment with organisms displaying diel feeding rhythms (e.g., copepods), especially under non steady-state situations. From what has been shown here, it is expected that the outcome of 24 h microzooplankton grazing experiments will not be the same if started during the day or during the night. Theoretically, when the experiments are initiated during the day, the grazing during this period should impact more the phytoplankton populations than when started during the night, when phytoplankton are dividing and microzooplankton are less active. Field data also back up the differences on diel concentrations of phytoplankton and uneven grazing over the daily cycle (Neveux et al., 2003), although the microzooplankton grazing rhythms in that study did not clearly match defined day-night periods. The possible bias in dilution experiments

should depend on the prey concentration in the water, being productive areas the most likely affected. We advise, therefore, to indicate in the manuscripts the actual time the experiments begin. We hope future modelling/experimental efforts would provide a way to correct for this artefact, which nowadays reminds as an incognita.

In summary, the coupling or uncoupling of microzooplankton grazing activity with the rhythms of activity of their predators and prey may have relevant consequences for the carbon flow, and biogeochemical cycles in general, in marine ecosystems, and certainly deserves more attention in future studies. The proper integration of protist laboratory data into models of planktonic ecosystem functioning (Jakobsen and Strom, 2004) and the inadequate interpretation of this behaviour in field studies can lead to a biased approximation to the efficiency of matter and energy transfer in the trophic web and on the overall understanding of the functioning of the marine ecosystem.

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CHAPTER 2

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

Arias, A., Saiz, E., and Calbet, A. 2019. Towards an understanding of diel feeding rhythms in marine protists: consequences of light manipulation. *Microbial Ecology*, 79(1):64-72.

2.1 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 *Strombidium arenicola* 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.

2.2. 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 (Mittag, 2001). These rhythms determinate the optimal timing for metabolic, physiological and behavioural activities within the daily cycle (Suzuki and Johnson, 2001).

A broad variety of diel rhythms have been described in aquatic organisms (Duval and Geen, 1976). For instance, marine mesozooplankton commonly feed in a typical day-night cycle pattern, often coupled with daily vertical migrations, characterized by higher feeding rates during the night (Huntley and Brooks, 1982; Dagg et al., 1989; Visser et al., 2001). Contrarily to mesozooplankton, a reverse diel feeding rhythm with higher ingestion rates during the day-time has been reported for marine protistan grazers (Strom, 2001; Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011; Arias et al., 2017), with the exception of some mixotrophs that may reduce feeding under light (Porter, 1988; Berk et al., 1991; Chen and Chang, 1999). Although an endogenous component in the feeding behaviour of marine protists has been proposed (Jakobsen and Strom, 2004), other explanations for the presence of this feeding rhythm have been suggested as well, such as light-aided digestion (Strom, 2001), compensatory feeding in response to stoichiometric fluctuations (Ng and Liu, 2015; Ng et al., 2017), prey availability (Arias et al., 2017), an others. Yet, the reasons for this mostly diurnal feeding behaviour in protistan grazers remain still unclear.

In previous studies, we already explored the effect of the stoichiometric composition (i.e., food quality) of prey (*Rhodomonas salina*) and prey availability on the diel feeding rhythm of microzooplankton (see Arias et al., 2017). 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., Ng and Liu, 2015; Ng et al., 2017), 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. (2017) study, such as the interference of cell division during night on the feeding behaviour. It might be possible that individuals dividing during the night (which seems to be the common in many dinoflagellates; Sweeney and Hastings, 1958; Homma and Hastings, 1989; Yamaguchi, 1992; Van Dolah et al., 1995; Garcés et al., 1999; Katano et al., 2011) 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 (Kohata and Watanabe, 1986). For example, Baek et al. (2009) 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 arenicola*.

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

2.3. Material and methods

2.3.1. 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 arenicola* (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 mL metal stock solution per litre; Guillard, 1975) and maintained at 19 ± 1 °C, and 38 PSU under a 10:14 h Light-Darkness (L:D) cycle (irradiance of 60-90 μE m^{-2} 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 (Guillard, 1975) in Pyrex culture flasks provided with air to avoid cell sedimentation. Cultures were diluted daily to maintain them in an exponential phase of growth.

2.3.2 Evaluation of the effects of light on cell division

To address the role of cellular division cycle on the diel feeding rhythm of marine protistan grazers we first grew *G. dominans*, *O. marina*, *K. armiger* and *S. arenicola* 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., 2013; Martínez, unpublished; Arias, unpublished). At the beginning of the experiment, we determined the grazer and prey concentrations (Table 2.1) 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 μE m^{-2} 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.

Table 2.1. Initial (averages ± SE) prey and grazer concentrations (cell mL^{-1}) used for the experiments of growth under 10:14 L:D cycle (left columns) and under continuous darkness (right columns).

	L:D cycle		**Continuous darkness**	
Species	**Prey concentration (cell mL^{-1})**	**Grazer concentration (cell mL^{-1})**	**Prey concentration (cell mL^{-1})**	**Grazer concentration (cell mL^{-1})**
R. salina	152483 ± 424	-	310467 ± 1381	-
G. dominans	107733 ± 508	1265 ± 26	74720 ± 1201	851 ± 21
O. marina	117483 ± 593	1677 ± 86	125783 ± 4572	4686 ± 97
K. armiger	76444 ± 263	813 ± 44	56645 ± 316	811 ± 22
S. arenicola	182350 ± 581	631 ± 47	130783 ± 2382	1330 ± 109

2.3.3. 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 marine protists by measuring the grazing rates of *G. dominans*, *O. marina*, *K. armiger* and *S. arenicola* 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 $\mu E\ m^{-2}\ s^{-1}$ 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 *R. 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 stoichiometric composition differences (Arias et al., 2017). 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 (1972), using prey biovolume as currency instead of cells to neglect any differences in prey size that may occur.

2.4. Results

2.4.1. 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. 2.1). Regarding the prey, the chryptophyte *R. salina* presented higher growth rates during the night (0.047 h^{-1} ± 0.0007 h^{-1}) than during the day (-0.0006 h^{-1} ± 0.0004; *t*-test, $p<0.001$; Fig. 2.1A). 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. 2.1B,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. 24 h), 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 day-time (Fig. 2.1D; $p<0.05$). After several hours of continuous darkness exposure, growth rates decreased below zero and remain negative until the end of the experiment (day 3). *S. arenicola* showed the widest amplitude rhythms under the natural L:D cycle, with a maximum of 0.075 h^{-1} ± 0.0066 during the day and a minimum of 0.004 h^{-1} ± 0.0056 during the night (Fig. 2.1E). *S. arenicola* presented the same day-night pattern than the mixotrophic dinoflagellate *K. armiger*, with higher growth rates during the day-time. When exposed to continuous darkness, *S. arenicola* also experienced a phase of adaptation during the first hours; following, the day-night rhythm was recovered with smaller amplitude.

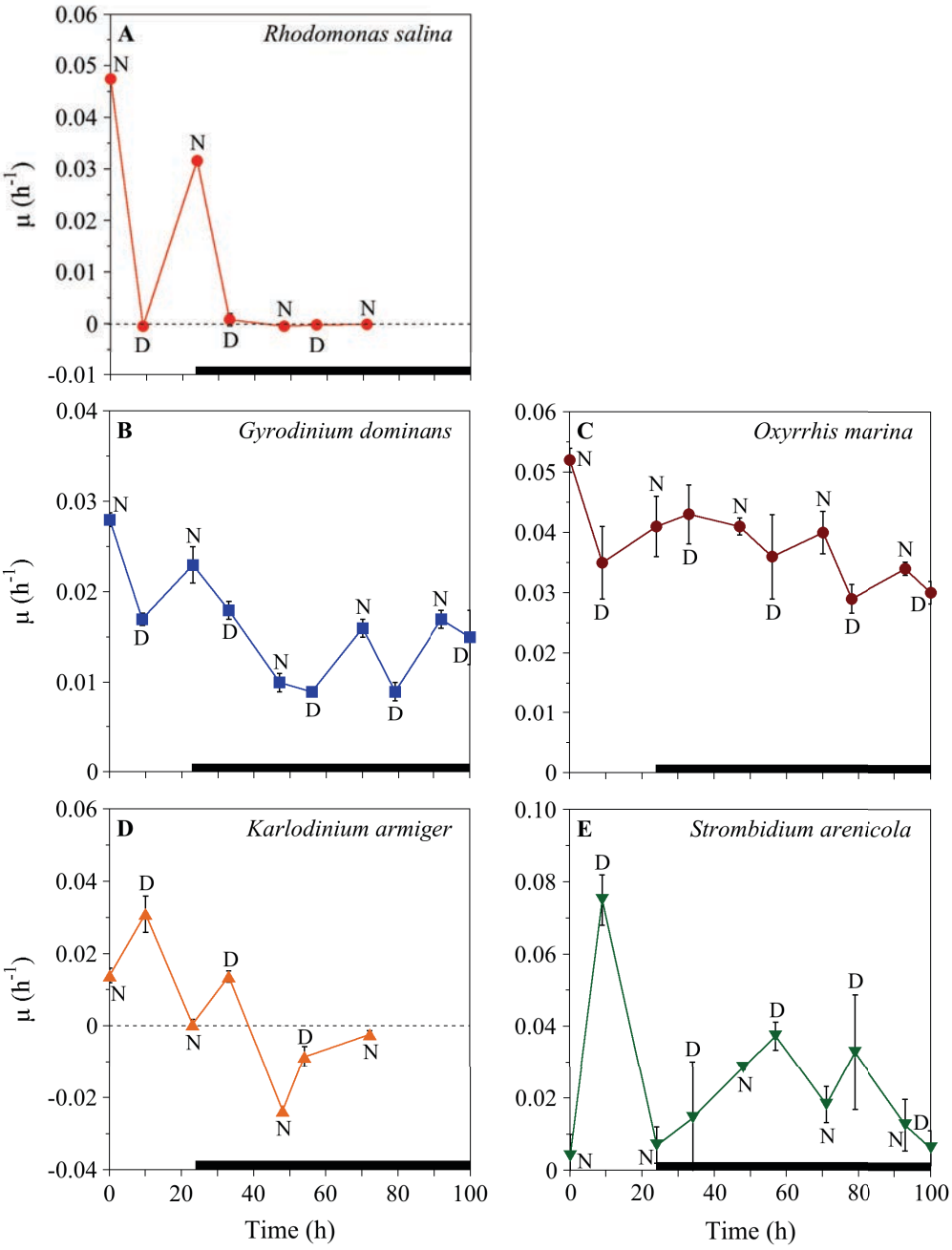

Figure 2.1. Growth rates (μ) as function of time of (**A**) *Rhodomonas salina*, (**B**) *Gyrodinium dominans*, (**C**) *Oxyrrhis marina*, (**D**) *Karlodinium armiger* and (**E**) *Strombidium arenicola*. Horizontal black bar indicates continuous darkness period. Error bars indicate standard error. D and N indicate day and night phases, respectively.

2.4.2. 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 day-time (Fig. 2.2). 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 arenicola* showed the lowest differences in day/night (*O. marina*: 1.61 ± 0.056; *t*-test, $p<0.05$; *S. arenicola*: 1.58 ± 0.04; *t*-test, $p<0.001$). Quotient value 1 indicates equal day and night ingestion rates, while values above 1 indic

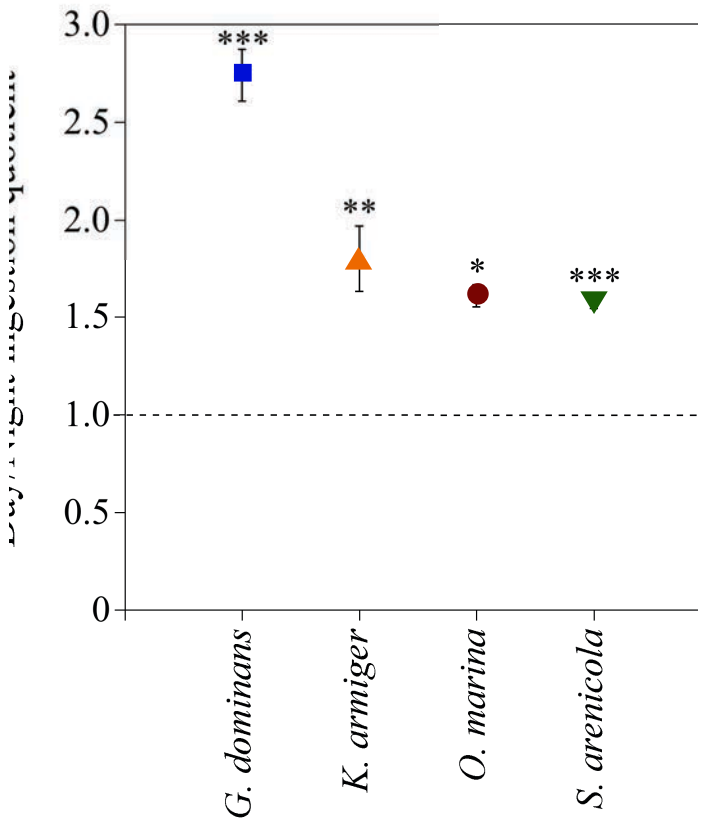

Figure 2.2. Feeding rhythm index (quotient between day and night ingestion rates) of *Gyrodinium dominans*, *Karlodinium armiger*, *Oxyrrhis marina* and *Strombidium arenicola*. Asterisks represent significant differences (*, p<0.05; **, p<0.01; ***, p<0.001). Dashed line represents equal day and night ingestions (nonexistence of feeding rhythm).

Under continuous light and continuous darkness, the amplitude of the feeding rhythm exhibited by *G. dominans*, *K. armiger* and *S. arenicola* reduced and eventually vanished or showed awkward patterns (Fig. 2.3), whereas *O. marina* kept the rhythm along the experiment (Fig. 2.3C,D). 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 (Fig. 2.3A,B); later, the diel rhythm slightly reversed. After 4 days of constant light conditions, the total daily (day+night) intake of *G. dominans* (616.91 μm^3 $grazer^{-1}$ h^{-1}) remained similar to that showed at the beginning of the experiment under natural L:D cycle (605.44 μm^3 $grazer^{-1}$ 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 (Fig. 2.3C,D); 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 day-time. 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 continuous light and sharply decreased under continuous darkness (Fig. 2.3E,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, *S. arenicola* grazing rhythm under constant light conditions was lost after 12 h (Fig. 2.3.G,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).

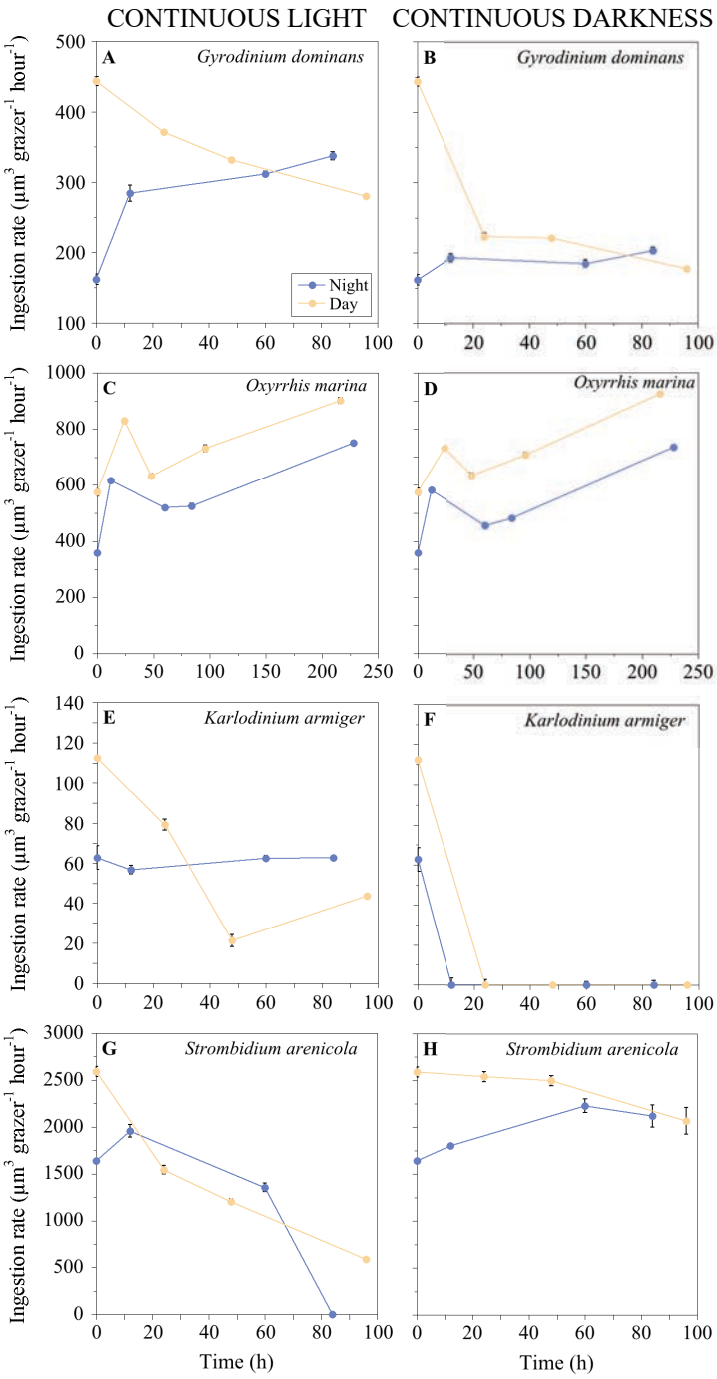

Figure 2.3. Day and night ingestion rates (in terms of volume consumed by grazer and hour) as function of time, under continuous light and under continuous darkness treatments, of (**A**, **B**) *Gyrodinium dominans*, (**C**, **D**) *Oxyrrhis marina*, (**E**, **F**) *Karlodinium armiger* and (**G**, **H**) *Strombidium arenicola*. Yellow line represents day ingestion rates and blue line represents night ingestion rates.

2.5. Discussion

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

2.5.1. The role of light in the diel feeding rhythm of marine protists

Diurnal feeding patterns have been confirmed for various protists species (Strom, 2001; Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011), although the causes of this behaviour are not well understood yet. In the introduction section we highlighted several not yet refuted hypotheses that could explain such a particular behaviour. For instance, Strom (2001) suggested that light might facilitate the digestion process in cell vacuoles, and this process might explain the higher diurnal grazing activity observed in protists. Should light enhance grazing rates, all species would have reduced their feeding under continuous darkness compared to continuous light. Our experiments rejected this hypothesis, except for the mixotrophic *K. armiger*, which manifested a clear need for light to keep grazing activity, as also found for other mixotrophic dinoflagellates (Li et al., 1999; Kim, 2008; Berge and Hansen, 2016). Although heavily depending on light for survival, *K. armiger* nutritional configuration has been described to be closer to the phagotrophy extreme within the mixotrophic spectrum (Berge and Hansen, 2016). Berge and Hansen (2016) observed that, in order to achieve high growth conditions, carbon fixation played an important role in prey-limitation environments; in this situation, *K. armiger* obtained most of the carbon required through photosynthesis. On the contrary, under prey-saturated conditions, as the ones used in our experiments, phagotrophy becomes the main source of *K. armiger* for carbon acquisition (between 60 to 90% of the total carbon gains; Berge and Hansen, 2016).

Contrarily, *O. marina*, recognised in many instances as a model species for microzooplankton (Montagnes et al., 2011), displayed a very distinct response to light

manipulation compared to the rest of protozoans. As a matter of fact, neither constant light or darkness seemed to affect the diel feeding behaviour of *O. marina*. This opportunistic species, inhabiting intertidal pools, salt marshes and embayments (Droop, 1953; Jonsson, 1994; Johnson, 2000; Begun et al., 2004), has a very complicated life history involving encystment and very high growth rates to survive tidal cycles and extreme conditions (Jonsson, 1994); because of these characteristics, it could be argued that its feeding and reproductive behaviour might be less dependent of other environmental factors (e.g., light) to optimize survival. The responses of *G. dominans* and *S. arenicola* 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 *S. arenicola* were negatively affected under continuous light and kept rather constant under continuous darkness. Therefore, it seems *G. dominans* needs light to keep feeding, while *S. arenicola* requires darkness, possibly to get rid of some oxidative subproducts (Slaveykova et al., 2016).

Whilst continuous light conditions are restricted to high latitudes during summer months, and one would expect local species showing particular adaptions, 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 (Agusti et al., 2015). Under such circumstances, our data suggest that ciliates, such as *S. arenicola*, will thrive better the adverse conditions than e.g., dinoflagellates such as *G. dominans*, or mixotrophs.

2.5.2. 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 (Bhaud et al., 2000). For instance, in

most species of phytoplankton, cell division is confined to the dark phase (Prison and Lorenzen, 1966; Suzuki and Johnson, 2001; Cross and Umen, 2015; Jong et al., 2017), being dinoflagellates clear examples of such a nocturnal physiological response (Heller, 1977; Weiler, 1979). However, phytoplankton cell division has also been observed, although less frequently, occurring during the light period (Eppley and Coatsworth, 1966; Leighfield and Van Dolah, 2001; Van Dolah et al., 2007), or unconnected to the light phase (Eppley et al., 1967; Paasche, 1968; Richman and Rogers, 1969; Chisholm et al., 1978).

The benefits of specific timing for cell division may rely on a strategy to cost minimization. Cook (1966) and Cohen and Parnas (1976) 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"; Johnson, 2010). 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 (Cross and Umen, 2015; Jong et al., 2017). 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 (Cross and Umen, 2015; Jong et al., 2017).

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. (1990) 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 *S. arenicola*, 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., Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011), although nocturnal cell division has also been observed (e.g., Rychert, 2016), and most ciliates are diurnal feeders (Jakobsen and Strom, 2004). Therefore, we cannot accept as universal the hypothesis of cell arrest during division being the cause of diel grazing cycles.

2.6 Final remarks

Our data shows that in general feeding rhythms in marine protists 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 (2004) 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 protistan grazers 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 (Sharma, 2003). 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 protistan grazers feeding rhythms.

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CHAPTER 3

Predator chemical cue effects on the diel feeding behaviour of marine protists

Arias., A, Selander, E., Saiz, E., and Calbet, A. Predator chemical cue effects on the diel feeding behaviour of marine protists. [submitted]

3.1 Abstract

We have assessed the effect of predator chemical cues on the diel feeding rhythms of heterotrophic and mixotrophic marine protists. All grazers studied exhibited relatively high diurnal feeding rates. The magnitude of the diel feeding rhythm, expressed as the quotient of day and night ingestion rates, was inversely related to the time that the predator was maintained in predator-free cultures in the laboratory. In the case of the recently isolated ciliate *Strombidium arenicola,* the rhythm was lost after a few months. When challenged with predator chemical cues (copepodamides) from the copepod *Calanus finmarchicus* at realistic concentrations (0.6-6 pM), *S. arenicola* partially re-established a diurnal feeding rhythm. Conversely, the amplitude of the diel feeding rhythm for the ciliate *Mesodinium rubrum* was not affected by copepodamides, although the 24 h integrated food intake increased by approximately 23%. For the dinoflagellates *Gyrodinium dominans* and *Karlodinium armiger*, copepodamides significantly reduced the amplitude of their diel feeding rhythms; significant positive effects on total daily ingestion were only observed in *G. dominans*. Finally, the dinoflagellate *Oxyrrhis marina,* isolated >20 years ago, showed inconsistent responses to copepodamides, except for an average 6% increase in its total ingestion over 24 h. Our results suggest a species-specific response to predation risk in marine protists.

3.2 Introduction

Protistan grazers are key components of marine planktonic food webs, representing a crucial trophic link between primary producers and mesozooplankton (Gifford, 1991; Calbet and Saiz, 2005). Despite their relevance, some key aspects of marine protist trophic behaviour and their impacts on planktonic food webs are still unclear. This is the case, for instance, for diel feeding rhythms. While laboratory-based studies with different protist species have repeatedly reported higher ingestion rates during the day-time (hereafter referred to as diurnal feeding; e.g., Strom, 2001; Jakobsen and Strom, 2004; Ng and Liu, 2015; Arias et al., 2017; Ng et al., 2017) than during the night-time, the reasons for the existence of these rhythms are not yet well understood. Arias et al. (2019) proposed that the diurnal feeding rhythm of marine protists could have evolved as a strategy to minimize the risk of predation, given that their main predators, copepods, typically exhibit nocturnal feeding (Fig. 3.1). Feeding by free-living protists involves motility, therefore increasing conspicuousness and encounter rates with predators (Broglio et al., 2001). Thus, an optimal protistan grazer might have developed an inverted feeding rhythm to that of its predator as a compromise between gathering food and avoiding predation (Lima and Dill, 1990; Tiselius et al., 1993; Titelman, 2001; Kiørboe et al., 2010). Indeed, a predation-avoidance strategy has already been proposed to drive diel rhythms in larger zooplankton (e.g., copepods; Bollens and Frost, 1991; Tiselius et al., 1997; Kiørboe, 2011), but such behavioural responses to predation have not yet been demonstrated in microplanktonic grazers.

Within this framework, we aim to experimentally test the effect of predation risk by copepods on the diel feeding rhythm of marine protists. We first explored how laboratory conditions affect the presence of diel feeding rhythms in several species of heterotrophic and mixotrophic protists. Then, we investigated the effects of predator chemical cues, simulating the threat of predation, on the rhythmic feeding activity of these grazer protists. To do that, we used copepodamides as a chemical cue to mimic the presence of predators.

Copepods release different types of chemical cues in the surrounding waters (Selander et al., 2016) that induce defensive traits in their prey (Fig. 3.1). The most well-known are copepodamides (Selander et al., 2015), which induce toxin production in the dinoflagellate *Alexandrium minutum* (Selander et al., 2015) and in the diatom *Pseudo-nitzschia seriata* (Selander et al., 2019), a reduction of the chain length in the diatom *Skeletonema marinoi* (Selander et al., 2019) and an increase in the bioluminescence capacity in various dinoflagellate species (Lindström et al., 2017; Prevett et al., 2019). Nevertheless, the effect of copepodamides on the feeding activity of marine protists remains unexplored.

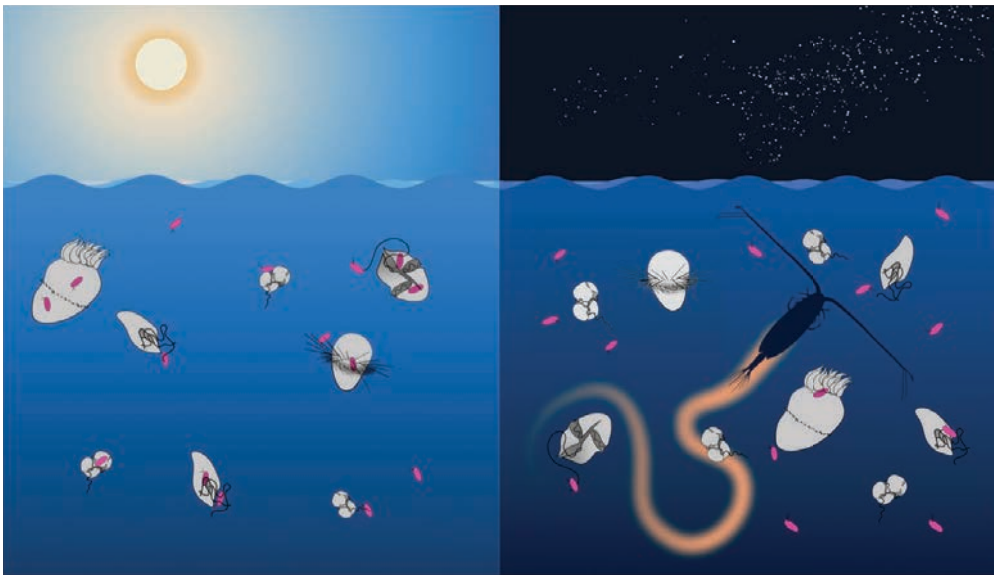

Figure 3.1. Illustration of protistan grazers' feeding during the day *versus* the night, together with the nocturnal risk of predation they are exposed to (illustrated by Jan Heuschele).

3.3 Materials and methods

3.3.1 Prey and grazer cultures

We used the heterotrophic ciliate *Strombidium arenicola* (strain ICM-ZOO-SA1-2017), the mixotrophic ciliate *Mesodinium rubrum* (strain DK-2009), the heterotrophic dinoflagellates *Gyrodinium dominans* (strain ICM-ZOO-GD1-2011)

and *Oxyrrhis marina* (strain ICM-ZOO-OM1-1995), and the mixotrophic dinoflagellate *Karlodinium armiger* (strain ICM-ZOO-KA1-2013) as grazers in our experiments. All strains were isolated from the NW Mediterranean Sea between 1995 and 2017, except for *M. rubrum*, which was isolated from Danish waters in 2009 (Dr. Per J. Hansen, University of Copenhagen). Stock cultures were kept in a cold room at 19 ± 1°C and grown on 38 PSU autoclaved filtered seawater enriched with metals (1 mL metal stock per litre; Guillard, 1975), provided with irradiance of 90 μE m^{-2} s^{-1} (white fluorescent) and a 10:14 h L:D cycle. Grazers were fed the cryptophyte *Rhodomonas salina* daily, except for *M. rubrum*, which was fed the cryptophyte *Teleaulax amphioxeia* every other day. Batch cultures of *R. salina*, provided with gentle air bubbling, were grown in f/2 medium and diluted daily to maintain exponential growth. *T. amphioxeia* was grown under the same conditions but without air supply.

3.3.2 Diel grazing rhythm experiments

We first analysed the permanence of diel feeding rhythms in the target species. These experiments consisted of single trials, except for the recently isolated *S. arenicola*, which was tested 6 (October 2017), 10 (February 2018), 19 (November 2018) and 20 (December 2018) months after the time when it was isolated (April 2017).

Grazing experiments were conducted under saturated prey conditions, specific to each studied species (Table 3.1; functional response data from Arias, unpublished; Calbet et al., 2013; Martínez, unpublished; Fig. S3.1). In the experiments, the prey *R. salina* was offered in stationary phase to avoid day/night size differences (see Arias et al., 2017). Prior to the experiments, the grazers were starved for 48 h (Arias et al., 2017). In the experiment setup, two suspensions were prepared: one only with the prey to serve as a control for prey growth and another with the same concentration of prey and the desired amount of grazers. The experiments were conducted in triplicate 72 mL polyethylene culture flasks, which were incubated on a plankton wheel (0.2 r.p.m) from the beginning (9:00 a.m.) until the end of the day (7:00 p.m.), at 19 ±

1°C, and 90 µE m^{-2} s^{-1} irradiation; the experiment was then repeated for the night-time incubation under complete darkness (from 7:00 p.m. to 9:00 a.m.). Concentrations of prey and grazers were determined with a Beckman Coulter Multisizer III particle counter (100 µm aperture tube) at the beginning and the end of each incubation period.

Table 3.1. Prey (*Rhodomonas salina*) and grazer concentrations (cells mL^{-1}) used in the feeding experiments. The period of time the grazer cultures were maintained under laboratory conditions is also shown.

Grazer	Time since isolation	Prey concentration (cell mL^{-1})	Grazer concentration (cell mL^{-1})
Strombidium arenicola	6 months	46079 – 48952	175 – 343
	10 months	75091 – 77118	259 – 387
	19 months	78094 – 80915	206 – 388
	20 months	81544 - 84929	272 - 462
Mesodinium rubrum	8 years	10570-12860	1510-2988
Gyrodinium dominans	6 years	100700 - 110500	3000 - 3580
Karlodinium armiger	4 years	100000-111800	6130-7500
Oxyrrhis marina	22 years	140010-160500	1705-2360

3.3.3 Effects of copepodamides on protist feeding behaviour

To test the effect of predation risk on the rhythmic feeding behaviour and the total daily ingestion (i.e., day and night ingestions sum) of the target grazers, we carried out diel feeding experiments using two copepodamide treatments, 1.4 and 18 pM initial concentrations (average effective concentrations during incubations of 0.6 and 6 pM, respectively; Table 3.2, Fig. S3.2; see Supplementary Materials for the determination of effective concentrations methodology). Copepodamides were extracted from freeze-dried *Calanus finmarchicus* through a series of chemical separation steps (see Selander et al., 2015 for further details). The lowest concentration used in our study was within the natural range of copepodamide concentrations (0.4-2 pM; Grebner et al., 2018; Selander et al., 2019). As concentrations may vary widely depending on the density of copepods or the

proximity to the source, we also included a higher concentration (average effective concentrations 6 pM) to cover this range.

Table 3.2. Initial, final and average effective concentrations of copepodamides during the feeding incubations. The half-life of the copepodamides is also provided.

Initial concentration (pM)	Final concentration (pM)	Average effective concentration (pM)	$T_{1/2}$ (h)
1.4	0.2	0.6	6.2
18	2	6	3.2

The feeding incubations were conducted similarly to those previously described, but in this case, the control treatments had methanol added at the highest concentration used as diluent for the copepodamide solution. Fresh copepodamide doses were prepared for each day and night incubation. We conducted the experiments twice, on different occasions, to ensure data robustness. The experiments with *S. arenicola* were conducted after 19 and 20 months of laboratory cultivation, when no diel feeding rhythm was apparent.

3.4 Results

3.4.1 Laboratory time-dependent diel feeding rhythm

For the whole group of protists studied, there was a negative relationship between the time from isolation and the amplitude of the diel feeding rhythm, defined as the quotient day/night ingestion rates (Fig. 3.2). In general, the magnitude of the rhythm ranged from 1.5 (*O. marina*) to 3 times (*S. arenicola* and *G. dominans*) higher ingestion rates during the day than during the night (Fig. 3.2). The rhythm was still detectable after 22 years of laboratory cultivation in *O. marina*.

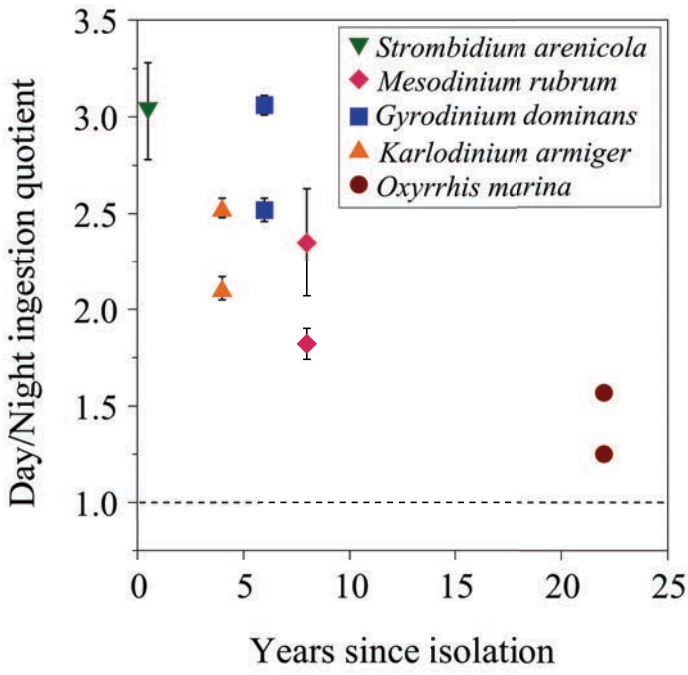

Figure 3.2. Diel feeding rhythms, as the quotient between day and night ingestion rates, of *S. arenicola*, *M. rubrum*, *G. dominans*, *K. armiger* and *O. marina* as a function of the time in culture since isolation. All day ingestion rates were significantly higher than the night ingestion rates (*t*-test, $p<0.01$). Dashed lines indicate the value of equal day and night ingestion rates (i.e., non-existence of diel feeding rhythm), and error bars show the standard errors.

Conversely, the diel feeding rhythm of the recently isolated ciliate *S. arenicola* decreased more rapidly over time in a predator-free laboratory environment (Fig. 3.3A); ingestion rates during day-time were 3 times significantly higher than during night-time when first measured (*t*-test, $p<0.001$; October 2017), but these diel differences completely disappeared after 19 months of maintenance in the laboratory (November 2018; *t*-test, $p>0.05$; Fig. 3.3A).

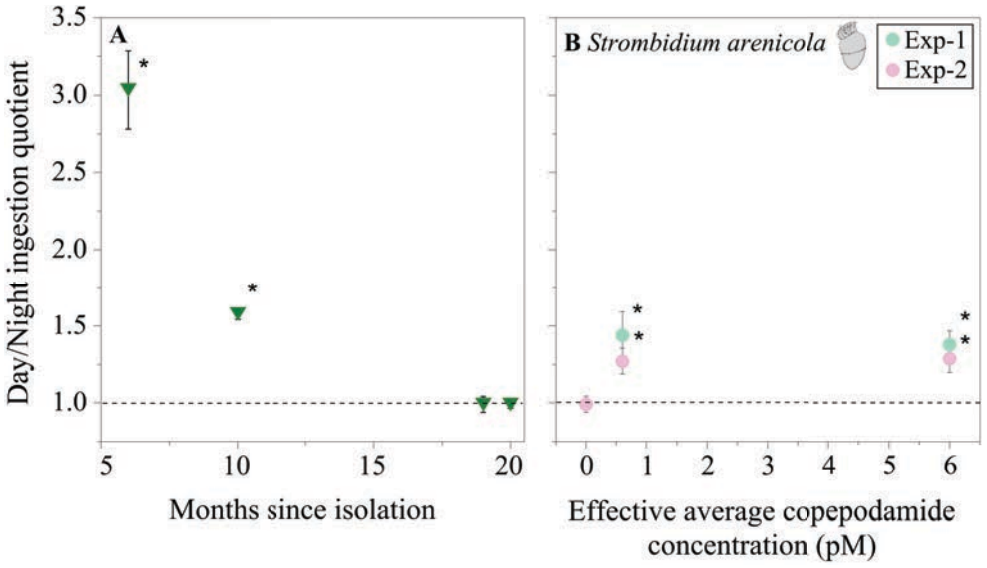

Figure 3.3 (**A**) Temporal evolution in the diel feeding rhythm of the ciliate *S. arenicola*, expressed as the quotient between day and night ingestion rates, from isolation (October 2017) until December 2018. Asterisks indicate significant differences between day and night ingestion rates (*t*-test, $p<0.001$). (**B**) Recovery of the diel feeding rhythm in *S. arenicola* as a function of copepodamide effective concentrations. Green and pink dots denote two independent experiments. Asterisks indicate significant differences between copepodamide treatments relative to the control (*t*-test, $p<0.05$). Dashed lines indicate the values of equal day and night ingestion rates (i.e., non-existence of diel feeding rhythm). Error bars show the standard errors.

3.4.2 Effect of predation risk on the diel feeding rhythm of laboratory-cultured protists

When exposed to grazer cues, under both concentrations of copepodamides, the diel feeding rhythm of *S. arenicola* was partially reinstated (27-45% recovery relative to the treatment without copepodamides; *t*-test, $p<0.05$ in all treatments; Fig. 3.3B; see Table S3.1 for actual day and night ingestion rates). This enhancement of the diel feeding rhythm did not consistently affect total daily ingestion (Table 3.3). Regarding the other ciliate, day and night ingestion rates quotient of *M. rubrum*, also showed a positive response to copepodamides (Fig. 3.4A), but it was weak (3-10% increase) and not significant (*t*-test, $p>0.05$ in all cases). However, in this case, the total daily ingestion increased by 23%, on average (Table 3.3).

Table 3.3 Total daily ingestion rates (day+night sum; μm^3 $grazer^{-1}$ day^{-1}) of the studied grazers under the different copepodamide concentrations. The percentage of variation with respect to the control treatments is also provided. Data from Experiment 1 and Experiment 2 are presented separately. ANOVA Dunnett test p-values are shown. *n.s.* indicates no significant differences.

		EXPERIMENT 1			EXPERIMENT 2		
Species	**Treatment**	**Ingestion (avg ± SE)**	**% variation**	**p**	**Ingestion (avg ± SE)**	**% variation**	**p**
	Control	51229 ± 1782	0	-	26285 ± 463	0	-
S. arenicola	1.4	46374 ± 2312	-9.5	*n.s.*	25422 ± 751	-3.3	*n.s.*
	18	44325 ± 443	-13.5	<0.05	24168 ± 392	-8.1	*n.s.*
	Control	228 ± 0.7	0	-	732 ± 14	0	-
M. rubrum	1.4	315 ± 9.1	38.1	<0.001	817 ± 16	11.6	<0.05
	18	275 ± 7.8	20.6	<0.01	892 ± 20	21.8	<0.001
	Control	6078 ± 68	0	-	7307 ± 91	0	-
G. dominans	1.4	6718 ± 53	10.5	<0.001	8061 ± 35	10.3	<0.001
	18	6997 ± 43	15.1	<0.001	7704 ± 17	5.4	<0.01
	Control	2448 ± 46	0	-	1807 ± 14	0	-
K. armiger	1.4	2305 ± 58	-5.8	*n.s.*	1753 ± 12	-3.0	*n.s.*
	18	2286 ± 23	-6.6	*n.s.*	2118 ± 23	17.2	<0.001
	Control	13305 ± 116	0	-	17035 ± 77	0	-
O. marina	1.4	14442 ± 253	8.5	<0.01	17749 ± 81	4.2	<0.05
	18	13509 ± 54	1.5	*n.s.*	18849 ± 192	11	<0.001

Dinoflagellates were less consistent and showed variable responses to copepodamides. The amplitude of the diel feeding rhythm of *G. dominans* decreased by approximately 13% and 8% in copepodamide exposures of 0.6 and 6 pM, respectively (*t*-test, $p<0.05$ in all cases; Fig. 3.4B; Table S3.1). Total ingestion over 24 h, on the other hand, increased by 10%, on average (Table 3.3). *K. armiger* also significantly reduced the feeding rhythm in a dose-dependent manner, 22% in 0.6 pM and 46% in 6 pM copepodamide exposure (*t*-test, $p<0.05$ in all treatments; Fig. 3.4C; Table S3.1). The total daily ingestion of this species was only significantly different from the control in the higher (6 pM) copepodamide exposure in one of the two

replicated experiments (Table 3.3). Finally, the *O. marina* response to copepodamides was inconsistent (Fig. 3.4D; Table S3.1); in the first experiment, the amplitude of the feeding rhythm decreased 2-23% when exposed to copepodamides (*t*-test, $p<0.05$ for the lowest copepodamide concentration), but in the second experiment, it increased significantly by 8%-12% (*t*-test, $p<0.05$ in all treatments). The effects of copepodamides on total ingestion (over 24 h) on this species ranged from non-significant to a 11% reduction (Table 3.3).

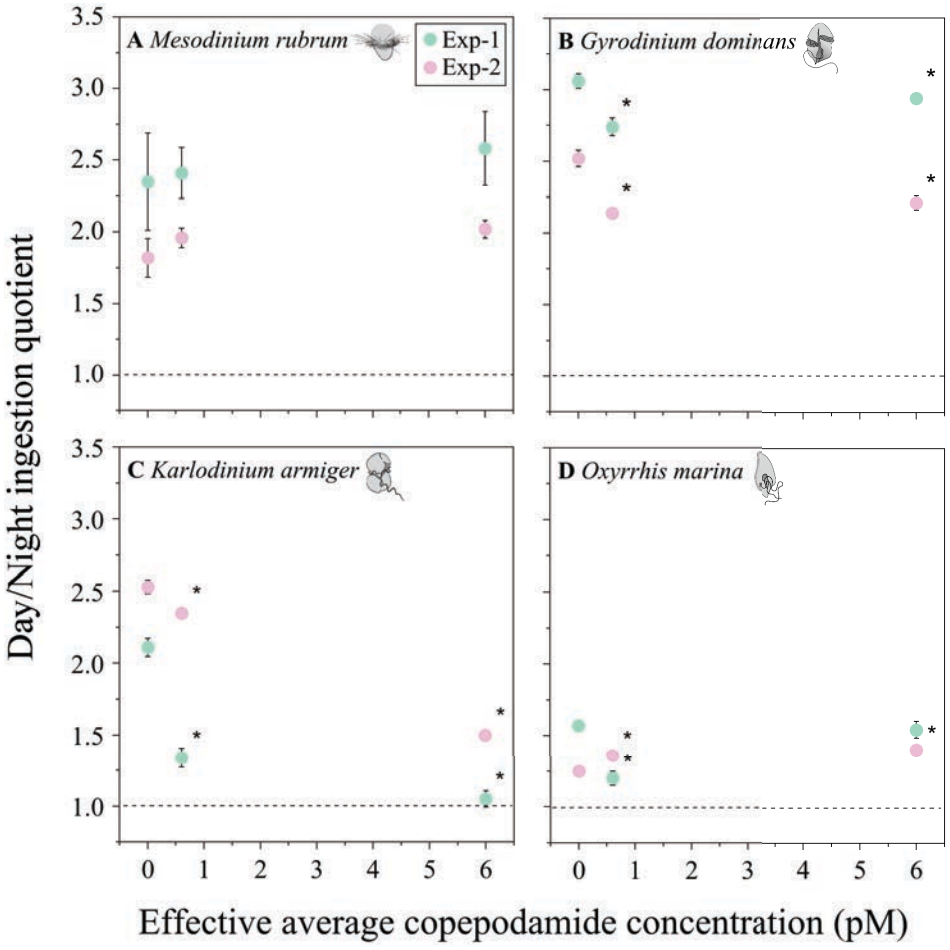

Figure 3.4 Diel feeding rhythms, as the quotient between day and night ingestion rates, of (**A**) *M. rubrum*, (**B**) *G. dominans*, (**C**) *K. armiger*, and (**D**) *O. marina* as a function of copepodamide effective concentrations. Green and pink dots denote two independent experiments. Asterisks indicate significant differences between copepodamide treatments relative to the control (*t*-test, * $p<0.05$). Dashed lines indicate the values of equal day and night ingestion rates (i.e., non-existence of diel feeding rhythm). Error bars show the standard errors.

3.5 Discussion

In this study, we provided the first evidence of a modulation in the diel feeding behaviour of marine protistan grazers in response to predator chemical cues. Moreover, we also showed that copepodamides have the potential to reinstate the diel feeding rhythm in a ciliate, whose inherent rhythmic behaviour was lost when reared under predator-free laboratory conditions.

3.5.1 Loss and recovery of the feeding rhythms in the laboratory: the particular case of ciliates

The absence of predators under laboratory rearing conditions appeared to be the most likely factor inducing the loss of the diurnal feeding rhythm in the ciliate *S. arenicola*, although we cannot disregard other unknown causes. Similarly, the remainder of the protists studied also seemed to show a time-dependent weakening of their diel feeding rhythm, although at a much longer scale (years). Similar results were observed by Arias et al. (2017) when comparing the feeding rhythm amplitude of two strains of the dinoflagellate *O. marina* isolated in different years (1995 and 2016), with the newest isolated strain showing the highest amplitude feeding rhythm.

The fading of a diel feeding rhythm in the absence of predators in the laboratory has already been documented for marine copepods (Calbet et al., 1999), and the presence of fish has also been reported to sharply enhance their diel feeding cycle (Bollens and Stearns, 1992), although chemical cues alone do not seem effective (Kiørboe et al., 2018). However, the physical presence of fish can induce changes in some behavioural and morphological traits of copepods. For example, fish presence has been reported to induce diapause in copepods from freshwater ecosystems (Hairston and Olds, 1987), as well as mating behaviour alterations (Jersabek et al., 2007), changes in body and clutch sizes (Svensson, 1997; Wasserman and Froneman, 2013), and variations in the pigmentation level used as photoprotection (Hansson, 2004). Other groups, such as freshwater rotifers and water fleas, however, are more prone to

respond to predator chemical cues. For instance, freshwater water fleas develop behavioural (e.g., Ringelberg, 1991; Dodson et al., 1995), morphological (e.g., Tollrian, 1990; 1995) and life-history trait (Weider and Pijanowska, 1993; Reede, 1995) responses as anti-predator defences to predator exudates or physical presence (Wojtal-Frankiewicz et al., 2010). Additionally, rotifers display morphological responses, involving the development and elongation of spines and appendages with the consequent increment in body size, to kairomones produced by copepods (Gilbert, 1999; 2013). Similar responses have been described in dinoflagellate defensive mechanisms as a response to copepod chemical alarm signals. Lindström et al. (2017) reported an increase in the total bioluminescence capacity of the long-term laboratory-cultivated (9-14 years) dinoflagellates *Lingulodinium polyedra* and *Alexandrium tamarense* when exposed to copepodamide dose treatments. Likewise, the production of toxic secondary metabolites in dinoflagellates (described as another defence mechanism against predators) is also reduced when organisms are cultivated in the laboratory (Lindström et al., 2017), but it is also restored under exposure to waterborne copepod cues (Selander et al., 2006) and copepodamides (Selander et al., 2015).

The recovery of the diel feeding rhythm in *S. arenicola* when exposed to copepodamides resulted in a significant decrease in ingestion rates during the night (see Table S3.1), supporting the hypothesis of a relationship between feeding rhythm and risk of predation. The effect of predation risk also translated into the decrease in the total ingestion rate observed in this species. In contrast, in *M. rubrum,* feeding rhythms were not significantly affected, and the total daily ingestion rate increased when exposed to copepodamides. Therefore, the two ciliates studied responded differently to predator chemical cues. The difference may have resulted from behavioural differences between species. It is known that predation risk to ciliates is determined by their escape ability (Broglio et al., 2001; Jakobsen, 2001). In our study, *S. arenicola*, such as other *Strombidium* species, was expected to have a relatively low escape ability (Jakobsen, 2001). Consequently, at night, when copepods ascent to surface layers and may overlap with ciliates, this species may benefit from reduced nocturnal feeding (which implies lower swimming activity) to reduce

conspicuousness and hence safeguard its survival. Conversely, when predators are absent, continuous feeding seems to be more advantageous. *M. rubrum*, on the other hand, exhibited a very different swimming behaviour based on a combination of long motionless periods interspersed with shorter periods of quick jumps (Tamar, 1979). Previous studies have highlighted the effective escape response of *M. rubrum* when surrounded by copepods, which substantially reduces its vulnerability to predator mortality in comparison to that of other planktonic ciliates (Jonsson and Tiselius, 1990). In fact, *M. rubrum* is characterized by an extremely high swimming speed for a protist (at over 5 mm s^{-1} and up to 8.5 mm s^{-1}, at least momentarily; Lindholm, 1981; 1985), approximately an order of magnitude faster than most other ciliates (Fenchel and Hansen, 2006). Therefore, the weak response of *M. rubrum* to copepodamides may be based on its high capability to escape from predators, which may make it less necessary for this species to largely modify its diel feeding behaviour.

3.5.2 Contrasting responses of dinoflagellates to copepodamides

The general response of dinoflagellates to copepodamide exposure was a decrease in the amplitude of the diel feeding rhythm, except for *O. marina*, which did not present a clear response. Regarding the heterotrophs *G. dominans* and *O. marina*, the variation in the amplitude of the diel feeding rhythm was caused by an unequal increase in both diurnal and nocturnal feeding and a consequent significant increase in total daily ingestion rates (Table S3.1). In contrast to ciliates, dinoflagellates are not able to escape from copepods due to their limited swimming capacity (Jakobsen, 2001). Thus, we believe that when threatened by predation, heterotrophic dinoflagellates may increase total daily prey uptake, independent of a dictated diel feeding rhythm, to maximize their energy intake for reproduction and ensure the rapid growth of the population, guaranteeing their survival. In environments with high predation risks, faster growth has been suggested as an adaptive response to outgrow the hunting impact of the predator in the population (Urban, 2007). An increase in

the prey growth rate as a defence response to predation risk has also been described in water fleas (Rose et al., 2001; Pauwels et al., 2010).

In the particular case of *O. marina*, the ambiguous results of the effect of predation risk on the diel feeding rhythm (increasing *versus* decreasing its amplitude) could also be associated with the habitat of the species. This dinoflagellate typically thrives in coastal habitats, shallow waters, intertidal pools (Droop, 1953; Jonsson, 1994; Johnson, 2000), salt marshes, and embayments (Begun et al., 2004), which might be environments less frequented by natural predators or be deadly traps without escape when predators are present. Hence, this dinoflagellate may not have experienced the necessity to evolve predator defence mechanisms.

Both *K. armiger* and *G. dominans* showed a reduction in the magnitude of feeding rhythms when exposed to copepodamides. However, in contrast to *G. dominans*, *K. armiger* did not consistently increase its total ingestion rate. We think that this particular behaviour might be related to the capability of *K. armiger* to produce karmitoxin, a toxin that can cause the rapid (within minutes; Berge et al., 2012) immobilization and mortality of copepods (Rasmussen et al., 2017). Toxin production in dinoflagellates has been reported to be induced by the presence of copepods and their chemical signals (Bergkvist et al., 2008; Wohlrab et al., 2010) and, recently, by copepodamides (Selander et al., 2015). Several dinoflagellates have efficient grazer deterrent traits that alone probably allow them to co-exist with copepods (Xu and Kiørboe, 2018; Prevett et al., 2019).

3.6 Final remarks

In this study, we have shown that predation risk can affect the feeding behaviour of several heterotrophic and mixotrophic protist species. The overall pattern of a gradual decrease in the diel feeding rhythm in long-term predator-free laboratory cultures may indicate, among other factors, the importance of predation risk in modulating feeding behaviour. Moreover, the diversity of the responses to copepodamides as a

proxy for predation risk by copepods, their main natural predator, suggests a species-specific response, depending on the physiological (e.g., deterrent production), behavioural (e.g., hydrodynamic conspicuousness and escape ability) and ecological (e.g., habitat) traits of the grazers. Nonetheless, we should consider that the risk of predation might not be the only trigger of the diel feeding rhythm in all marine protists.

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3.8 References

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CHAPTER 4

Trophic interactions and diel feeding rhythms of protistan grazers in a productive Swedish fjord

Arias, A., Saiz, E., Tiselius, P., and Calbet, A. (2020). Trophic interactions and diel feeding rhythms of microzooplankton in a productive Swedish fjord. [submitted]

4.1 Abstract

Protistan grazers play a pivotal role in the energy transfer between lower and upper trophic levels in marine planktonic food webs. While laboratory data suggest that marine protists exhibit higher feeding rates during the day-time, evidence from the field is scarce and contradictory. In this study, we first characterized the nano- and microplanktonic community of the Gullmar Fjord (Sweden) and its environmental conditions during July and August 2017. Then, we explored the grazing impact of marine protists on the phytoplankton community of this ecosystem and assessed their diel grazing activity using the dilution technique. Finally, we evaluated the impact of mesozooplankton at natural concentrations during the experiments. Marine protists removed 26% of the phytoplankton standing stock and 96% of the primary production daily, while mesozooplankton did not exert a significant impact on microplankton activity. We did not detect significant diel grazing rhythms in marine protists during the first experimental period; however, during the second part of the study, after an upwelling event, grazing rates were significantly higher during the night. Therefore, the grazing rhythm of protistan grazers in natural systems may vary according to the species composition and abundances of both grazer and prey communities.

4.2 Introduction

Protistan grazers are a key link between primary producers and higher trophic levels in marine systems (Calbet, 2008; Saiz and Calbet, 2011), where their daily grazing impact on phytoplankton primary production ranges from 50 to 75% (Calbet and Landry, 2004). Several laboratory studies with cultured organisms have observed that marine protist grazing is often more intense during the day hours (Strom, 2001; Jakobsen and Strom, 2004; Ng and Liu, 2015; Arias et al., 2017). However, little is known about the feeding patterns of marine protists in natural assemblages. The few attempts to quantify their diel (i.e., diurnal and nocturnal) feeding rhythms *in situ* rendered different results. Litaker et al. (1988) attributed the nocturnal reduction in chlorophyll *a* (Chl *a*) reported in the Newport River estuary (North Carolina, USA) during summer to the grazing activity of protistan grazers, based on the diel patterns of phaeopigment and cell abundance. Later, Claustre et al. (1999), also based on indirect evidence, deduced higher grazing impacts during the night in the equatorial Pacific. However, Neveux et al. (2003) advocated for rather constant grazing losses over the entire diel cycle. Conversely, in the South China Sea and Hong Kong coastal waters, Ng and Liu (2016) observed that heterotrophic nanoflagellates exhibited higher feeding rates during the day-time and related them to diel variations in the picoplankton C:N ratio. Finally, Armengol et al. (2019) reported different grazing patterns between oligotrophic areas and productive waters of the tropical and subtropical Atlantic Ocean, which may be related to differences in the microplanktonic grazers community composition. Thus, given the lack of agreement between studies, more research is needed to resolve the mechanisms determining marine protist diel feeding patterns in nature.

To conduct our study, we chose the Gullmar Fjord, a productive fjord in southwestern Sweden. While numerous studies have addressed the role of mesozooplankton in this coastal ecosystem, very little is known about the role of marine protists. The few studies on the subject highlighted heterotrophic dinoflagellates as the primary grazers of diatoms during spring blooms (Tiselius and Kuylenstierna, 1996) and ciliates as relevant in pelagic trophic cascades involving copepods and phytoplankton (Tiselius and Møller, 2017). Ciliates also seem to have the potential to control the

phytoplankton biomass of the fjord through grazing (Tiselius et al., 2015). However, the grazing and trophic role of marine protists in this region has not yet been assessed. Moreover, the presence of diel feeding rhythms in these grazers is still unexplored.

Our aim was threefold: (1) to determine the grazing impact of marine protists on the phytoplankton community in the Gullmar Fjord; (2) to assess the diel feeding activity of protistan grazers at that latitude, at which summer nights are relatively short and a good scenario exists for the study of diel variations; and (3) to determine the effect of the presence of mesozooplankton at natural concentrations on the diel feeding rhythms of protistan grazers and overall grazing impacts. Previous evidence in this fjord indicates that copepod grazing provides seasonal regulation of the system mediated through trophic cascades involving copepods, protistan grazers, and phytoplankton (Tiselius et al., 2015). Moreover, a recent study by Arias et al. (2017) highlighted the likely importance of predation risk in modulating the feeding rhythms of marine protists.

4.3 Materials and methods

4.3.1 Study site and general sampling procedures

This study was conducted weekly from 17 July to 30 August 2017 at the Coastal Buoy station in the Gullmar Fjord on the Swedish west coast. The water for the experiments was collected at a 2 m depth onboard the R/V Oscar von Sydow with a 30 L Niskin bottle. Temperature and salinity were simultaneously measured with an ADM Mini-CTD.

Every week, we conducted two different kinds of dilution grazing experiments: one with standard technique (Landry and Hassett, 1982) and a two-point modified version (Landry et al., 1984; Schmoker et al., 2016). For the standard dilution experiments, seawater was collected in the early morning (between 9:00-10:00 a.m., local time), whereas for the two-point modified experiments, water was collected in the early

afternoon (between 2:00-3:00 p.m., local time). In both cases, the seawater was gently transferred from the Niskin bottle into 20 L polypropylene carboys, filled all the way up to avoid splashing during transportation to the laboratory. The carboys were additionally covered with dark plastic bags to protect the water from exposure to surface sunlight. The experiments were set up just after arrival to the laboratory (Kristineberg Marine Research Station, Sweden) in a temperature-controlled room (19ºC). All the material used for the experiments was previously washed with 10% HCl and thoroughly rinsed with distilled water, and vinyl gloves were worn during the entire experimental process.

4.3.2 Standard dilution experiments

We followed the standard dilution technique from Landry and Hassett (1982) to assess the daily grazing impact by protistan grazers on the primary producers of the fjord. Part of the collected water was gravity filtered through 0.2 µm AcroPak cartridges to be used for the dilution series. The remaining water (i.e., whole seawater) was carefully siphoned out through a 100 µm mesh (reverse filtration; Kobari et al., 2019; Mayers et al., 2019) to remove the large zooplankton; this mesh size was selected to completely exclude adult and juvenile forms of zooplankton and hence ensure a grazing impact exclusively from microzooplankton, as previous trials using a 200 µm mesh did not retain small copepods. Then, the whole seawater was combined with the diluent (0.2 µm filtered seawater) in duplicate 2.5 L PC bottles in adequate proportions to generate a dilution series of 100%, 75%, 50%, 25% and 12% of whole seawater. Nitrogen (NH_4Cl) and phosphate ($NaH_2PO_4 \cdot H_2O$) were added to each of the bottles at final concentrations of 10 µM N and 0.7 µM P, respectively (Calbet and Saiz, 2018). In addition, four extra 100% seawater bottles without added nutrients were also prepared, two of them to serve as initial bottles to assess the initial Chl *a* concentration and the other two as non-nutrient-amended bottles for net phytoplankton growth determination. All bottles were carefully filled to the top to avoid air bubbles. The bottles used for the initial measurements were processed right away, whereas the dilution series bottles were incubated for 24 h *in situ* at a 2 m depth

in the pier at Kristineberg Marine Research Station, with similar light and water conditions to those at the seawater collection point. A HOBO Data Logger was incubated together with the bottles to monitor light and temperature.

Samples for Chl *a* analysis were taken at the beginning of the experiment from the duplicate initial bottles at all dilution levels and at the end of the incubation period (ca. 24 h) from the remaining bottles. For each dilution level, different sample volumes were filtered through Whatman GF/F filters (25 mm diameter) under low vacuum pressure (<100 mm Hg): 100 mL for initial and 100% bottles (nutrient-amended and non-amended treatments), 125 mL for 75%, 150 mL for 50%, 200 mL for 25% and 250 mL for 12% bottles. Chl *a* was then extracted from the filters in 5 mL of 96% ethanol for 9 h in darkness at room temperature (Wasmund et al., 2006). Chl *a* concentration was determined from the extracts after measurements before and after acidification on a calibrated Turner Design 10-AU fluorometer.

4.3.3 Two-point modified dilution experiments

To assess the diel grazing activity of protistan grazers, we carried out both day-phase and night-phase incubations using the two-point dilution technique (Landry et al., 1984; Calbet and Saiz, 2013; Schmoker et al., 2016). Differing from the standard dilution experiments, the two-point dilution method consists of a solely two-level dilution series, 10% and 100% whole seawater. Following the dilution technique, both dilution levels were nutrient amended (see section before), and extra replicates with 100% nutrient non-amended bottles were also set up. Moreover, we also prepared another set of four bottles with whole seawater (100%) without 100 μm prefiltration (i.e., allowing for the natural large zooplankton assemblage) to evaluate the top-down control of marine protist grazing by mesozooplankton (here defined as the >100 μm fraction). Therefore, each day/night incubation comprised a set of 16 experimental bottles, which were incubated *in situ* at the same site as the standard dilution experiments. General procedures were the same as described above for the standard dilution experiments, except that in the two-point experiments, four

replicates of each treatment and 5 L PC bottles were used. The 24 h experiments always started at sunset, and all bottles were sampled three times throughout the incubation: (1) samples were taken at the beginning of the night-phase, during the sunset of the initial day; (2) samples were taken at the end of the night-phase, simultaneously acting as initial samples for the following day-phase; and (3) samples were taken at the end of the day-phase, taken during the next sunset (i.e., ca. 24 h from the experimental setup). Samplings (1) and (2) were carried out outdoors at the incubation site, whereas sampling (3) was done in the laboratory. Sampling consisted of carefully mixing the bottles and then siphoning either 500 mL (10% bottles) or 100 mL (all other the treatments) of the water samples into dark bottles. Filtrations for Chl *a* determination were always performed immediately after sampling; Chl *a* analyses followed the same procedure described for the standard dilution experiments.

Data from this set of experiments were both managed as independent day-phase and night-phase incubations (to assess diel differences) and as an entire 24 h incubation (accounting for a full diel cycle). Thus, we first calculated the grazing rates during the day and night phases and then assessed the differences in the grazing activity of marine protists between both periods. Second, we used the data to compute the grazing mortality rates of protistan grazers and phytoplankton apparent growth rates over the integrated 24 h period (computed using the initial and the final measurements, i.e., after 24 h, of the experiments). These “24 h-integrated” values were later analysed together with the results from the standard dilution technique mentioned in the previous section. Finally, we also assessed the effect of mesozooplankton on the diel (day/night) and “24 h-integrated” feeding activities of protistan grazers by comparing the phytoplankton net growth rates in the bottles with and without >100-µm metazoans.

4.3.4 Quantitative analysis of the nano-, micro- and mesoplanktonic community

For microscopic analysis of the initial microplankton community composition, we took an additional 250 mL samples from the initial bottles (i.e., 100 µm-filtered whole water) of both standard and two-point modified dilution experiments. Samples were preserved in a final concentration of 2% acidic Lugol's solution and posteriorly settled for 48 h in Utermöhl chambers before analysis on an XSB-1A inverted microscope. Phytoplankton and microplanktonic grazers were identified at the species level when possible. Depending on abundance, either fields, transects or the entire chamber were processed at suitable magnifications (125x, 312x and 500x). The organism's volume (μm^3) was estimated from digital pictures employing approximated geometric shapes (cylinder for centric diatoms, hexahedron for non-centric diatoms, and ellipsoid for the remaining groups). Geometric means were used to compute average cell volumes.

Regarding mesozooplankton (>100 µm), we only took samples with the two-point modified dilution technique; at the end of the experiment, the remaining contents of the bottles with whole seawater (100%) without 100-µm prefiltration (i.e., containing the larger zooplankton) were sieved through a 90 µm sieve, and the collected zooplankton were preserved in a 4% formaldehyde. Once in the laboratory, we identified and quantified the most representative groups under a stereomicroscope.

4.3.5 Calculations

In all experiments, a Model I linear regression was fitted between the fraction of undiluted water (X-axis) and the apparent growth rate (Y-axis; k, d^{-1}) from the nutrient-amended treatments, calculated from changes in the Chl *a* concentration according to an exponential model. The slope of this regression analysis corresponds to the grazing mortality rate (m, d^{-1}) of protistan grazers, whereas the intercept represents the instantaneous phytoplankton growth rate in the nutrient-amended

treatments (μ_n, d^{-1}). Because this intercept would represent an overestimation of μ, the *in situ* (without added nutrients) phytoplankton instantaneous growth rate (μ_0) was determined as:

$$\mu_0 = k_0 + m$$

where k_0 is the net growth rate in the 100% non-nutrient amended bottles.

Primary production and the grazing impact of protistan grazers in terms of the primary production consumed were used to compute the percentage of phytoplankton standing stock consumed per day. These parameters were calculated according to Landry et al. (2003). Additionally, we used $100 \times m{:}\mu_0$ as a proxy for the % of the primary production grazed daily by marine protists.

To estimate the carbon content of unicellular organisms, we used a conversion factor of 0.19 pgC μm^{-3} for ciliates (Putt and Stoecker, 1989), the equations of Menden-Deuer and Lessard (2000) for dinoflagellates and diatoms, and the equations of Montagnes et al. (1994) for the other phytoplankton groups.

Finally, we applied NMDS analysis using the package vegan in R, coupled with the ANOSIM test, to explore differences in the nano- and microplanktonic community data through the experimental period.

4.4 Results

4.4.1 Environmental conditions at the study site

During the experimental period, the water temperature at the 2 m depth (i.e., sampling depth) ranged from 16.7 to 18.8 ºC, and the salinity ranged from 23.3 to 29.8 (Fig. 4.1A). The lowest temperature (16.7 ºC) and the maximum salinity (29.8) occurred on 9 August, indicating an upwelling event. The Chl *a* concentration at the sampling

depth ranged between 0.65 and 2.0 µg L^{-1} during the study period, with the lowest values found in July and the highest in August (Fig. 4.1B).

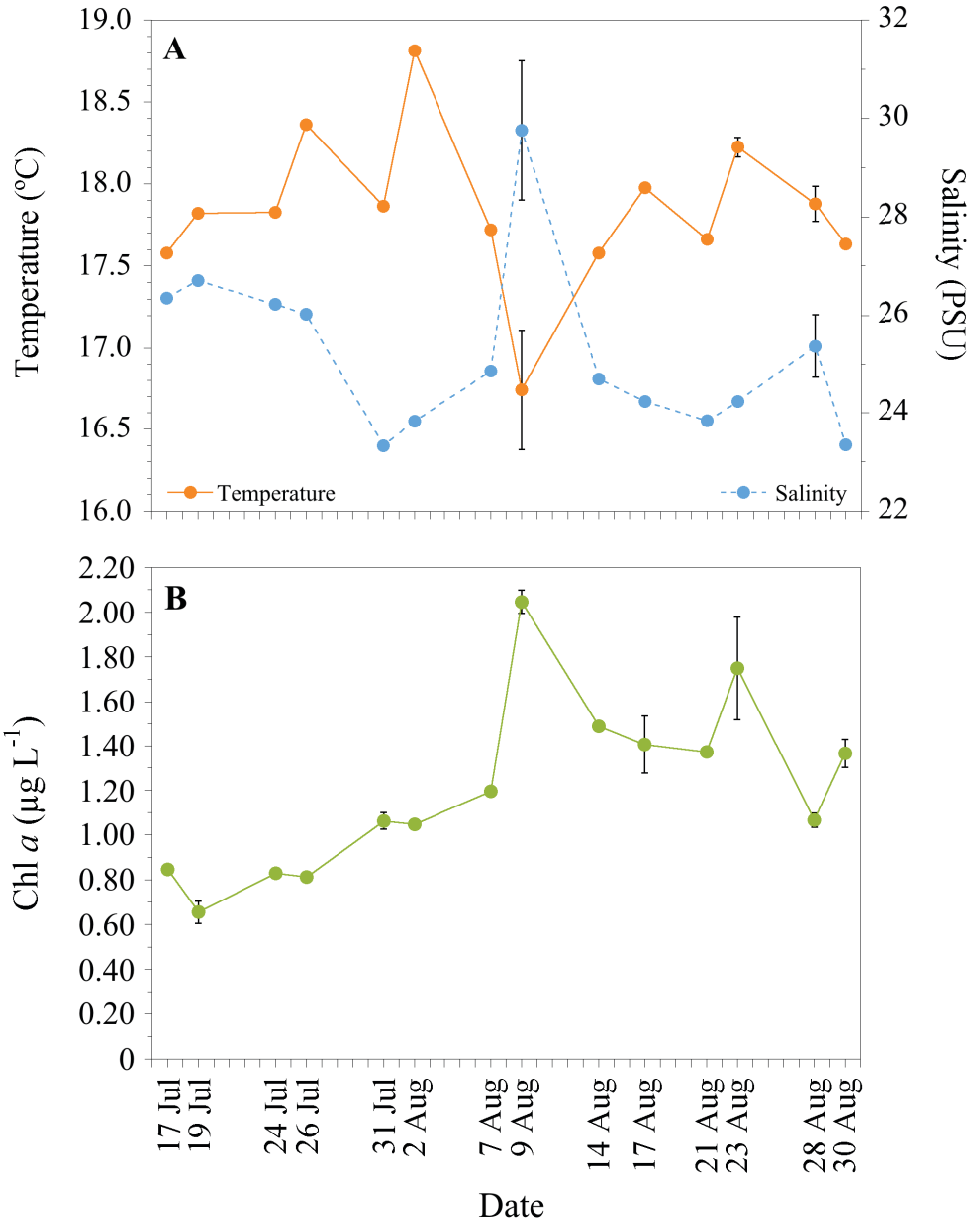

Figure 4.1. (**A**) Seawater temperature (ºC) and salinity (PSU) at a 2 m depth in the Gullmar Fjord from 17 July to 30 August. (**B**) Chlorophyll *a* concentrations (µg Chl *a* L^{-1}) in initial experimental bottles. Error bars show the standard deviations.

4.4.2 Plankton community

A total of 37 genera of nano- and microplankton were identified, including 15 genera of dinoflagellates, 14 of diatoms, 4 of ciliates and 3 other genera of phytoplankton (Table S4.1). In terms of abundance, dinoflagellates and diatoms dominated the nano- and microplanktonic communities, accounting for 35% (range 9-55%) and 41% (range 7-87%) of the microplankton assemblage, respectively (Fig. 4.2A). Dinoflagellates were the overall highest contributors to community biomass (57%, range 42-75%), whereas the contribution of diatoms to biomass was always low (7%, range 1-24%; Fig. 4.2B). Ciliates were quite stable components of the microplankton community, contributing to 14% (range 2-35%) of the microplankton abundance; however, in terms of biomass, they ranked second after dinoflagellates, accounting for 36% (range 15-56%) of the total microplankton biomass (Fig. 4.2A,B).

After the second week of August, diatoms peaked and became the most abundant group in the nano- and microplanktonic community (87% of the total abundance on 14 Aug; Fig. 4.2A), although their biomass remained relatively low on that day (11%; Fig. 4.2B). This diatom peak was mostly composed of *Skeletonema costatum* (peak concentration 197307 cell L^{-1} on 14 August) and was accompanied by other diatom species, such as *Asteronellopsis glacialis*, *Leptocylindrus danicus*, *Chaetoceros* spp. (<20 μm), *Guinardia delicatula* and other nonidentified pennate diatoms (Table S4.1). In parallel, on these days, the dinoflagellates *Ceratium furca, C. fusus, Prorocentrum micans*, and *Protoperidinium* spp. also increased in abundance (Table S4.1). NMDS analysis and ANOSIM test showed that the nano- and microplanktonic community differed between the two periods: before and after the upwelling event (ANOSIM statistic R=0.7263, p=0.002; Fig. 4.3). Thus, in the period before the upwelling event (17 July to 9 August; Fig. 4.2) the nano- and microplanktonic community was characterized with low abundance and biomass, dominated by ciliates and dinoflagellates (weekly average biomass during this period of 8 μgC L^{-1} and 11 μgC L^{-1}, respectively); after the upwelling event (14 to 30 August; Fig. 4.2) there was a peak in the number of diatoms and the biomass of ciliates and dinoflagellates also increased (weekly average biomass of 17 μgC L^{-1} and 33 μgC L^{-1}, respectively; Fig. 4.2A,B, and Table S1).

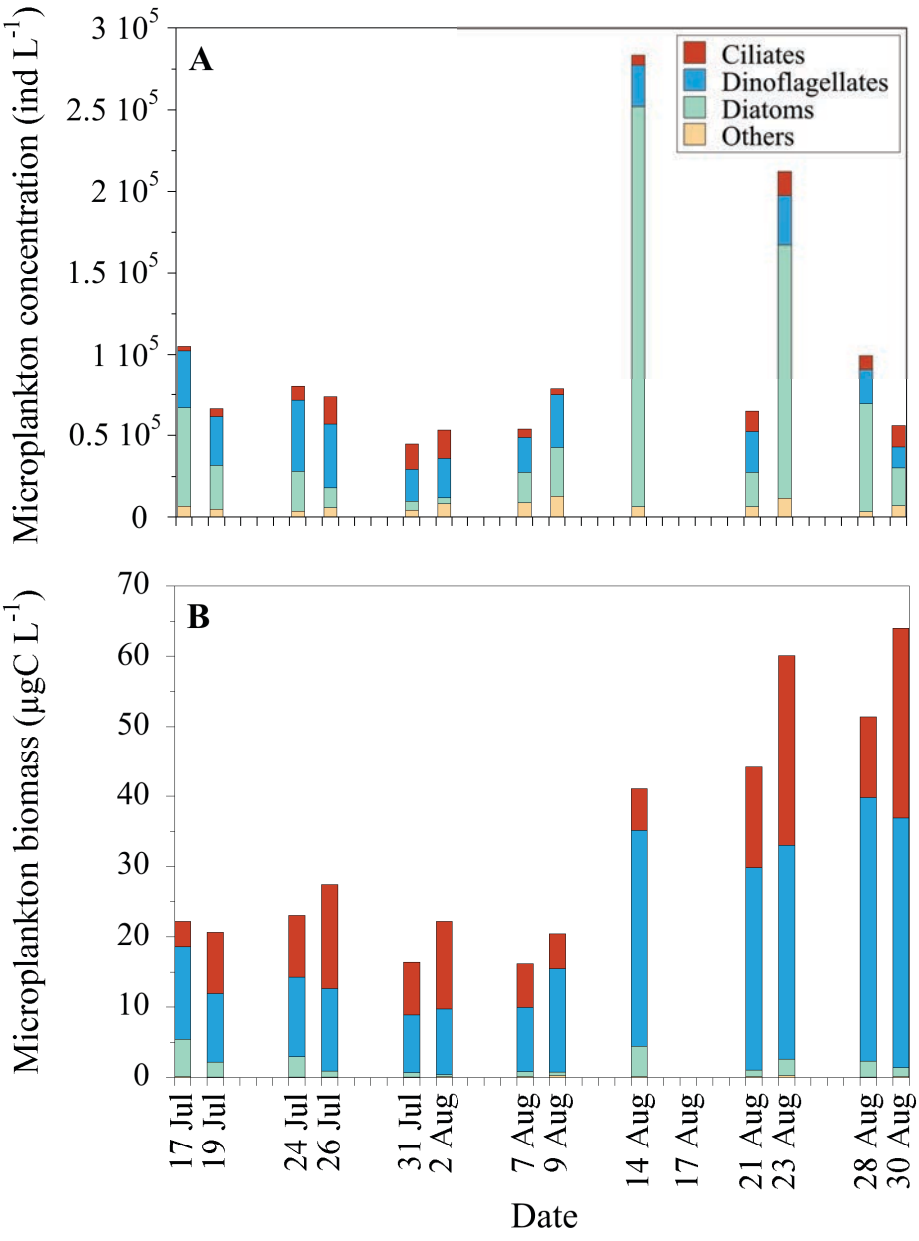

Figure 4.2. (**A**) Concentration (ind L^{-1}) and (**B**) biomass (µg C L^{-1}) of the characterized microplankton groups during the study period (17 July to 30 August).

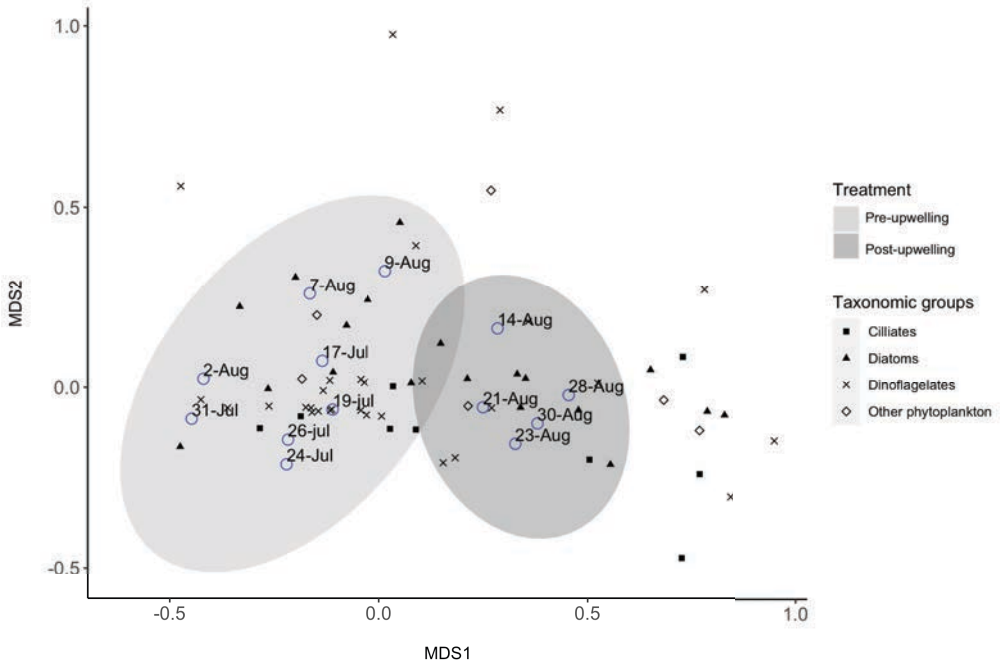

Figure. 4.3. Non-metric multidimensional scaling (NMDS) analysis comparing the variances in the composition of nano- and microplanktonic community in the Gullmar Fjord throughout the experimental period. Light-grey area corresponds to the period before the upwelling event (dates from 17 July to 9 August) and dark-grey area to the subsequent period (from 14 to 30 August).

The overall increase in ciliate and dinoflagellate biomass during the second period (*t*-tests, $p<0.05$ and $p<0.0001$, respectively) was not due to a change in abundance but to a large increase in individual cell volume. The average cell volume (in geometric means) during the first period was 32482 μm^3 for dinoflagellates and 3718 μm^3 for ciliates. During the second period, the average cell volumes remained similar for dinoflagellates (34469 μm^3) but sharply increased for ciliates (10096 μm^3). While during the pre-upwelling period the biomass of dinoflagellates and ciliates were similar, after the upwelling the biomass of dinoflagellates was much larger than that of ciliates (*t*-test, $p<0.05$).

The mesozooplankton (>100 μm metazoans) in our bottle samples were dominated by copepod nauplii, accounting for up to 77% of the total abundance, followed by copepodites at 15% (Table 4.1). Appendicularians and cnidarians were also present in the community but always accounted for <5% of the total abundance.

Table 4.1. Composition of the >100 μm zooplankton in the two-point modified dilution experiments in terms of individual abundance for each experimental day.

Date	Copepod nauplii (ind L^{-1})	Copepodites (ind L^{-1})	Apendicularians (ind L^{-1})	Cnidarians (ind L^{-1})	Others (ind L^{-1})
19 Jul	16.9	5.1	0.8	0.5	0.1
26 Jul	36.6	4.9	2.2	3.6	1.0
02 Aug	30.2	4.8	0.3	0.1	0.1
09 Aug	19.4	2.6	1.1	0.1	0.2
17 Aug	18.8	6.2	2.7	1.5	0.6
30 Aug	35.1	6.2	0.9	0.9	0.2

4.4.3 Daily phytoplankton growth and grazing rates of marine protists

The results from the standard dilution experiments and the two-point dilution experiments are all presented together in Table 4.2. The *in situ* phytoplankton instantaneous growth rates (μ_0, d^{-1}) ranged from -0.14 to 0.47 d^{-1} (Table 4.2). Throughout the experimental period, instantaneous growth appeared to be strongly limited by nutrient availability, as rates under no nutrient limitation (μ_n) were always higher than μ_0 (paired *t*-test, $p<0.0001$; Fig. 4.4). The highest nutrient limitation occurred on 19 July ($\mu_0:\mu_n=0.07$), whereas the lowest limitation occurred on 7 August ($\mu_0:\mu_n$ = 0.54; Fig. 4.4). The lowest (and negative) instantaneous growth rates, however, were observed on 9 August. Total marine protist grazing ranged between 0.11 d^{-1} and 0.48 d^{-1}, with a daily average of 0.25 d^{-1} (Table 4.2). The linear regression analyses were statistically significant for all dilution experiments, excluding those of 24 July and 30 August, which should be interpreted as nil grazing rates. As suggested

by Latasa (2014), we included in the table the actual results from all regression equations with their corresponding statistical significance.

The daily grazing activity of marine protists removed on average 26% of the phytoplankton standing stock, with values ranging between 11% (30 August) to 45% (17 July; Table 4.2). This corresponded to an average daily removal of 96% of the primary production (range 26 to 343%; Table 4.2).

Table 4.2. Summary of the results from both types of dilution grazing experiments, including the experiment date, sunrise and sunset times, type of experiment (STD: standard dilution experiment, 2-point: two-point modified dilution experiment), mortality rate from microplankton grazing (m, d^{-1}), phytoplankton apparent growth rate (μ_0, d^{-1}), the coefficient of determination of the linear regression (r^2) and its significance (p-value), the percentage of phytoplankton standing stock grazed per day (%SS) and the percentage of primary production grazed (%PP). P-values were obtained by testing the deviation of the slope from zero (F test) in the standard dilution experiments and comparing the growth rates under the diluted and non-diluted treatments (t-test) in the 2-point experiments. n.d. not determined.

Date	Type of experiment	Sunrise time	Sunset time	m (d^{-1}) ± SE	μ_0 (d^{-1}) ± SE	r^2	p-value	%SS	%PP
17 Jul	STD	-	-	0.48±0.12	0.33±0.32	0.73	0.007	44.6	146.2
19 Jul	2-point	4:41 am	9:59 pm	0.39±0.07	0.11±0.09	-	0.002	33.9	343.2
24 Jul	STD	-	-	0.13±0.07	0.44±0.08	0.33	0.086	0	0
26 Jul	2-point	4:55 am	9:46 pm	0.42±0.08	0.47±0.08	-	0.002	42.6	88.8
31 Jul	STD	-	-	0.28±0.09	0.32±0.09	0.57	0.012	28.8	87.0
02 Aug	2-point	5:09 am	9:31 pm	0.28±0.07	0.44±0.07	-	0.009	30.2	62.4
07 Aug	STD	-	-	0.12±0.02	0.45±0.03	0.84	<0.001	13.9	26.1
09 Aug	2-point	5:24 am	9:14 pm	0.18±0.02	-0.14±0.03	-	<0.001	15.0	n.d.
14 Aug	STD	-	-	0.17±0.04	0.15±0.04	0.73	0.007	16.9	117.7
17 Aug	2-point	5:42 am	8:53 pm	0.20±0.05	0.25±0.06	-	0.006	20.4	80.0
21 Aug	STD	-	-	0.33±0.03	0.38±0.05	0.92	<0.001	33.7	86.2
23 Aug	2-point	5:55 am	8:37 pm	0.29±0.08	0.31±0.11	-	0.009	29.6	92.7
28 Aug	STD	-	-	0.18±0.07	0.41±0.08	0.50	0.022	20.6	45.4
30 Aug	2-point	6:10 am	8:18 pm	0.11±0.06	0.24±0.06	-	0.105	0	0

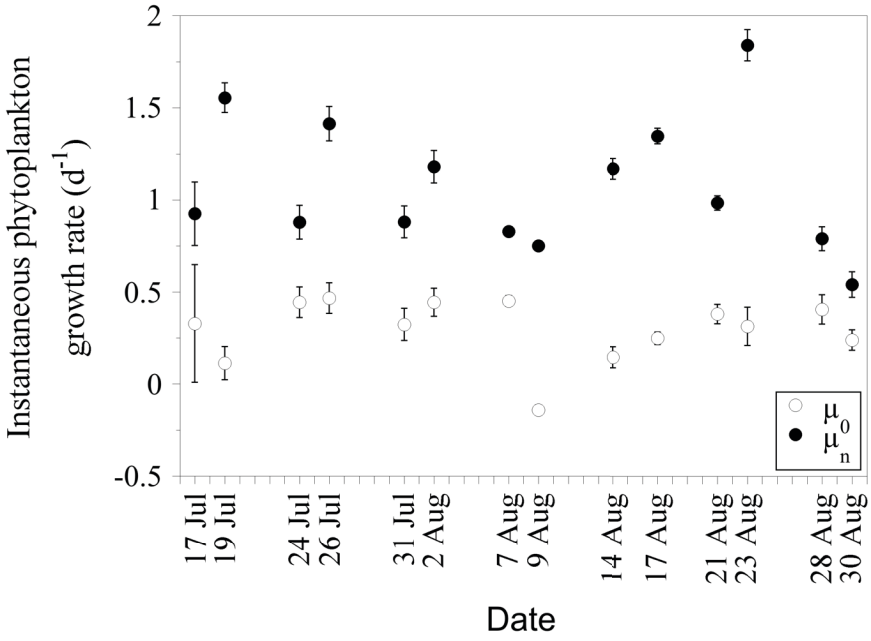

Figure 4.4. Instantaneous growth rate (d^{-1}) of phytoplankton under non-amended treatments (open circles) and nutrient-amended treatments (black circles) during the experimental period. Error bars represent standard error (n=2 for the standard experiments and n=4 for the modified experiments).

4.4.4 Diel grazing behaviour of marine protists

Figure 4.5A shows the microzooplankton grazing rates calculated separately for the day phase and the night phases. No significant day-night differences were detected in none of the experiments individually, mainly because of the variability within replicates. Nevertheless, when the experiments are grouped according to the periods before and after the water mass exchange, a differentiated grazing pattern was observed: while during the first period (from 19 July–2 August) there were no significant differences between day and night grazing rates (paired *t*-test, $p>0.05$), microzooplankton grazing rates turned to be significantly higher at night during the second period (from 17–30 August; paired *t*-test, $p<0.05$). Specifically, during the first period (before the upwelling event), we observed a certain tendency to higher diurnal (i.e., during the day-light hours) grazing with grazing rates between 17% and

65% higher during the day-phase, with the exception of 26 July, when the pattern reverse and grazing activity was higher during the night phase (about 22% higher). However, from 17–30 August, microzooplankton grazing rates were about 95% and 103% significantly higher at night, while diurnal grazing on the 30 August was nearly inexistent. This pattern is also observed when the feeding rhythms are expressed as the quotient between the day and night grazing rates, with a nocturnal grazing rhythm becoming apparent during the second period (17–30 August; Fig. 4.5B).

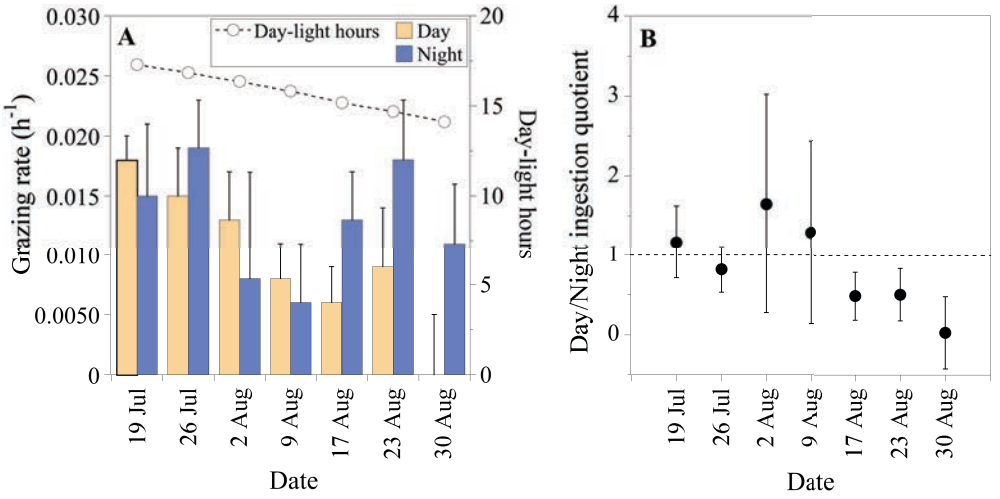

Figure 4.5. (**A**) Temporal variation in the grazing rates (h^{-1}) of marine protists during the day (yellow bars) and night (blue bars) computed per hour within each phase for the respective experimental day and (**B**) variation in the ratio between day and night grazing rates during the study period. Open circles in (**A**) represent daylight hours. The dashed line in (**B**) indicates equal day and night grazing rates (i.e., no grazing rhythm). Error bars represent standard errors.

The net impact of protistan grazers in the bottles with and without mesozooplankton was also assessed during the day and night phases (Fig. 4.6A,B). In this regard, no differences were detected between treatments either during the day (*t*-tests, $p>0.05$ in all cases; Fig. 4.6A) or the night (*t*-tests, $p>0.05$ in all cases except on 26 July, in which $p<0.05$; Fig. 4.6B). Hence, this indicates that the presence of mesozooplankton in the incubations did not significantly alter the diel grazing pattern of microplankton nor their overall impact.

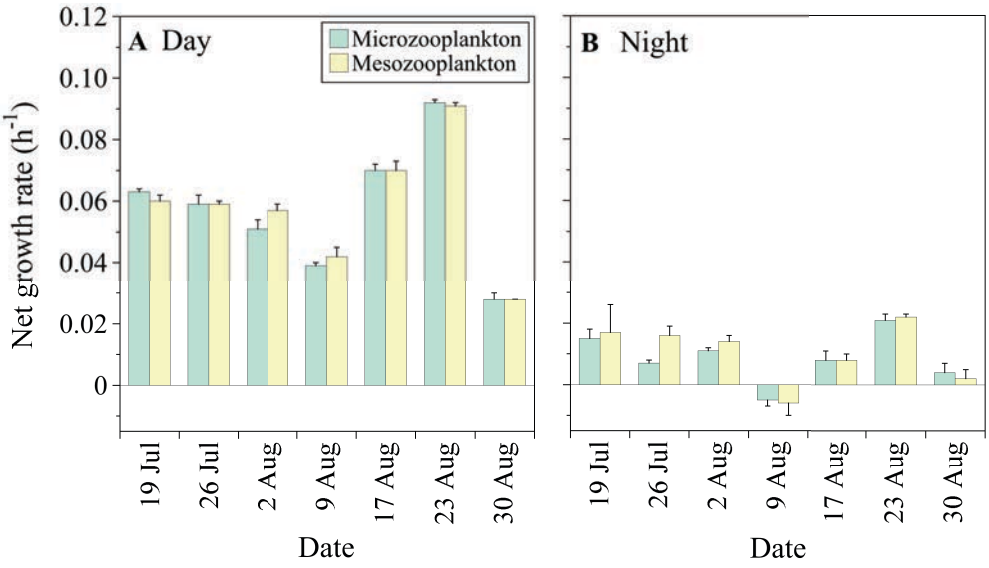

Figure 4.6. Phytoplankton net growth rates (h^{-1}) during the (**A**) day and (**B**) night in bottles with (yellow bars) and without (green bars) mesozooplankton. Only data from 2-point dilution experiments were used. Error bars represent standard errors.

Finally, we also explored the relationship between the monitored environmental variables (Chl *a* concentration and temperature) and the different estimated rates (phytoplankton growth, microplankton grazing mortality rates, and the % of primary production grazed daily) by simple linear regression. We only found significant relationships between μ_0 and Chl *a* ($p<0.05$) and temperature ($p<0.01$), but r^2 was low ($r^2=0.32$ and $r^2=0.51$, respectively), and when data for 9 August were removed, the relationships between μ_0 and Chl *a* or temperature disappeared.

4.5 Discussion

4.5.1 Hydrography and the plankton community of the Gullmar Fjord

The structure of pelagic ecosystems is determined by physical processes that are subjected to geographical and temporal variability. Thus, the species composition of

the community, food web interactions, and key functional features are affected by those processes (Calliari and Tiselius, 2009). The Gullmar Fjord is a very dynamic area where numerous associations between hydrodynamic processes and changes in the zooplankton community have been described (Lindahl, 1983; Lindahl and Hernroth, 1988; Jephson et al., 2012). In fact, Lindahl and Perissinotto (1987) highlighted the dominance of hydrodynamics over biotic processes as the main drivers of short-term variations in the fjord's zooplankton community, as occurs in other fjord systems (Matthews and Heimdal, 1980; Stone, 1980; Lie et al., 1983; Skreslet et al., 2000; Willis et al., 2006). On 9 August, we believe that an exchange of water masses occurred at our sampling site, coinciding with a minimum water temperature and a maximum salinity. Concurrently, there was a shift in the nano- and microplankton communities in terms of abundance and composition but no shift in the mesozooplankton community composition in our experimental bottles. One of the major biological changes associated with the water mass exchange was the peak of diatoms, which seemed to unchain a succession of modifications in the species composition of the community. In this sense, the increase in some dinoflagellate species could have been a direct response to diatom prey availability. The most pronounced identified response was observed in *Prorocentrum micans*, whose abundance was directly coupled to the increase in diatoms, as previously reported for other areas (Yoo et al., 2009). The average cell size in the ciliate community also increased.

4.5.2 Trophic interactions and grazing behaviour

We did not observe any substantial differences between the microplankton grazing rates obtained from the two different methodologies used (i.e., standard and two-point modified dilution experiments: *t*-test, p=0.92). Overall, microplankton grazers controlled primary producers of the Gullmar Fjord quite intensively during summer 2017, with daily removal of 96%. This impact is slightly higher than previous reports in the fjord based only on ciliates (40-70%; Tiselius et al., 2015) and agrees well with

the 67% impact on primary production by micrograzers expected for the coastal Atlantic (Schmoker et al., 2013).

High grazing impacts by marine protists on primary production can result not only from intense grazing activity but also from low phytoplankton production (growth). The latter seemed to be the case during our study, as demonstrated by the generally low grazing rates of marine protists and phytoplankton growth rates; low phytoplankton growth rates were most likely caused by severe nutrient limitation (Fig. 4.4). Although we did not quantify nutrient concentrations, depletion of inorganic nutrients during summer has previously been reported in the Gullmar Fjord (Tiselius et al., 2015) as well as in other similar Nordic fjords (Hjarbæk Fjord, Holmboe et al., 1999; Roskilde Fjord, Pedersen and Borum, 1996). The situation, however, is expected to be different in winter time, when nutrients are high, but light and temperature limit phytoplankton growth (Calliari and Tiselius, 2009; Tiselius et al., 2015). Under this scenario, microzooplankton are expected to exert higher impacts on the low biomass of phytoplankton (in fact, the lowest through the year; Tiselius et al., 2015). Likewise, during winter microzooplankton might be exposed to low predation pressure because most of mesozooplankton are concentrated in deep waters, and the biomass of mesozooplankton in the fjord might suffer a reduction because of deep water renewals (Lindahl and Hernroth, 1988). In spring, when the biomass and production of phytoplankton and microzooplankton achieve the highest values it is expected a much higher grazing activity of microzooplankton, although their impact should be not sufficient to control the development of the seasonal phytoplankton bloom.

4.5.3 Diel feeding rhythms in marine protists

The shift in the microplankton community we observed did not result in changes in the growth rates of phytoplankton, except on the day of the event. However, it did result in a behavioural modification of the diel grazing patterns of marine protists, likely caused by the establishment of a different microplankton community

associated with the water mass. Hence, on a per hour basis, day and night grazing rates of marine protists were similar before the water exchange episode; after it, night grazing rates were higher than the day grazing rates, and a diel rhythm appeared. Our field observations of marine protist diel grazing behaviour contrast with the frequent diurnal feeding activity reported from laboratory observations of ciliates and heterotrophic dinoflagellates (Strom, 2001; Jakobsen and Strom, 2004; Ng and Liu, 2015; Arias et al., 2017; Arias et al., 2019). However, previous field evaluations of marine protist grazing activity also provide a diversity of patterns found in natural assemblages. For instance, along a latitudinal transect in the tropical and subtropical Atlantic Ocean, Armengol et al. (2019) found that in oligotrophic areas, protistan grazers exhibited a diurnal feeding rhythm, whereas their grazing patterns were ambiguous in productive waters. The researchers suggested that the dominance of dinoflagellates (which are prone to diurnal activity) in oligotrophic waters and that of ciliates in productive systems (whose feeding behaviour may strongly depend on each particular species; Jakobsen and Strom, 2004) could partially explain the diverse feeding patterns of marine protists. Our data also support the existence of diversity among the grazing rhythm patterns of marine protists in natural assemblages according to the community composition. In this regard, the kind of prey ingested and particularly the digestion time may have consequences for the feeding rhythm of marine protists. In a recent study, Zhang et al. (2017) demonstrated that the silica content of diatom cells prolonged digestion by protists. The numerical dominance of diatoms after the water mass exchange event may be, hence, an important factor indirectly affecting the diel grazing behaviour of protistan grazers.

Grazing rhythms of marine protists have been proposed to act as a strategy for avoiding the risk of predation (Arias et al., 2019). Feeding involves motility and consequently being more conspicuous to rheotactic predators that feed mostly during the night (Broglio et al., 2001). In addition, previous laboratory studies have demonstrated that the feeding rhythms of the heterotrophic dinoflagellates *Gyrodinium dominans* and *Oxyrrhis marina* micrograzers are modified by prey concentration (Arias et al., 2017). Thus, Arias et al. (2017) reported that the above-mentioned micrograzers exhibited pronounced feeding rhythms under saturating prey

conditions, whereas the day-night differences decreased or even vanished when prey were scarcer. These authors justified their findings as the result of the fact that micrograzers have to spend more time searching for food when food is limiting. In the present study, prey concentrations during the experiments were extremely low (ranging from 0.45 μg C L^{-1} up to 5.45 μg C L^{-1}) in comparison to the saturating conditions (≈ 4200 μg C L^{-1}) used by Arias et al. (2017). It is therefore not surprising that the diel rhythm was not evident in part of the study. Nonetheless, after the water mass exchange, when prey availability increased, significant diel differences between day and night grazing rates were observed (although opposite than expected). Hence, although prey concentrations during the second period was also far from the laboratory conditions used in Arias et al. (2017), it might already be enough to trigger a response.

In our analysis we cannot discard the fact that our water sampling took place during the day-time, and the night microbial communities *within* the bottles we studied might not be the same that the ones actually found in the fjord at night. For logistical reasons, because the short duration of the dark period (6-10 hours) and the time required to sample and set up the experiment (between 4-5 hours, including sailing to the site), we opted for a single daily water collection and a 24h incubation divided in two phases. This procedure implies that the microplankton community obtained at the beginning of the experiment evolved throughout the incubations inside the bottles in a manner that we cannot guarantee it was equivalent to that in nature (Modigh and Franzè 2009). This methodological artefact would mostly affect the night part of the incubations, because of the larger number of hours passed. Nevertheless, when we quantified those possible changes by processing some night samples from the experiments on 5-Aug, 9-Aug, 23-Aug, 30-Aug and 4-Sept, preserved in 2% acidic Lugol's solution, we found little changes in the abundance of dinoflagellates and ciliates through the incubation (6-12% during the night-phase and ≤10% during the day-phase, on average). Therefore, it seems likely that any changes in the abundance or composition of grazers our during dilution experiments were modest and by far much lower than the changes associated to prey. There are events, however, that cannot be captured on a bottle incubation, e.g. microplankton vertical migration,

changes of the community result of water mass exchanges, mesozooplankton swarming and grazing on microplankton, among others. Regarding vertical migration, some studies have reported the occurrence of dinoflagellates vertical migration in the Gullmar Fjord (Olsson and Granéli, 1991; Jephson et al., 2012), with cells ascending to upper waters during the day and deepening at night. However, to occur, these displacements need very particular water stability conditions (Jephson et al., 2012). Moreover, given that the mixed layer depth was 8-10 m we can assume the community should not change much in the few hours apart between experiments. Similarly, because of the diel vertical migrations of mesozooplankton it could be that the effect of this group on the microplanktonic community might be stronger during the night-phase, when they inhabit surface layers. We should consider, however, that it is quite likely that by the beginning of a hypothetical night incubation with water from the fjord this effect would be not evident yet and, therefore, the communities in the fjord at night and in our bottles should be rather similar. This is also supported by the insignificant mesozooplankton grazing effect during the incubations. What we could not discard is an episodic water mass exchange. These episodes, however, seemed not to be very frequent during our study. In any case, even considering our communities during night differ somehow from those naturally occurring in the fjord, we can conclude that the 24 h integrated microzooplankton grazing rates are correct (or at least as correct as in any dilution grazing study) and that we show the diel grazing behaviour of the microplankton community captured at the beginning of the incubation.

4.5.4 Effects of mesozooplankton

The presence of mesozooplankton in our grazing experiments did not have any significant effects on phytoplankton net growth rates or microzooplankton grazing rates. Previous studies on copepod grazing in the Gullmar Fjord have also reported limited, although significant, daily impacts on primary production (5-13% removal in May 1987, Tiselius, 1989; 3-5% in October 2006 and July 2007, Tiselius et al., 2015). It seems that in our experiments, the abundance of >100 µm zooplankton

might have been rather low. For instance, the abundance of copepodites in our bottles (3-5 ind L^{-1}) was lower than previously reported values for the summer season at similar depths (15-16 copepods L^{-1}; Tönnesson and Tiselius, 2005). The cause for the relatively low abundance of copepods observed in our study may be related to the seawater collection depth and timing; water was collected from a 2 m depth with a Niskin bottle during the day-time when copepods shelter in the depth because of vertical migration (Frost, 1988; Chae and Nishida, 1995). Thus, we believe that the non-observed significant effect of mesozooplankton might be caused by their low concentrations during the incubations. We cannot discard the possibility of cascade effects counteracting the mesozooplankton impact within the bottles but, given the low mesozooplankton abundance, these cascade effects would be expected to be very low.

4.6 Conclusions

We have presented the first data, based on direct measurements, of the grazing activity of microplankton in the Gullmar Fjord. In general, the grazing rates were low, although they had a high impact on primary production because of the low growth rates of the nutrient-limited phytoplankton community. Mesozooplankton did not exert any significant net effect on phytoplankton either directly or, apparently, through trophic cascades involving microplankton. The study coincided with a water exchange episode that resulted in a different microbial community. These changes in species composition and abundance were reflected in the diel feeding behaviour of microplankton, which did not display any evident rhythm before the water exchange but showed nocturnal feeding after it. Our results indicate that the diel grazing activity of microplankton in the ocean may vary depending on the species composition and abundances of both grazers and prey, what has important implications for the cascade of mater and energy channelled by protistan grazers to upper trophic levels and the amount of carbon mediated by them.

4.7 Acknowledgements

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SYNTHESIS OF THE MAIN RESULTS

CHAPTER 1:

Diel feeding rhythms in marine microzooplankton: effects of prey concentration, prey condition, and grazer nutritional history

Higher feeding activity during the day-time was confirmed in the heterotrophic dinoflagellate grazers *Gyrodinium dominans* and *Oxyrrhis marina* (for the last case, in both laboratory and recently isolated strains). The diel feeding rhythm of all grazers was modified by prey concentration, with less acute or even inexistent (*O. marina*) rhythms under limiting prey concentrations. During exponential growth, the stoichiometric composition of the prey *Rhodomonas salina* showed marked diel differences, with significantly higher elemental ratios during the day-time (C:N, 20% higher; C:P, 64%; N:P, 42%), while these differences were inexistent in the stationary phase. When grazers were fed on prey at different phases of growth, feeding rhythms were of wider amplitude when feeding on prey at the stationary phase than when at exponential phase. Most likely feeding rhythms were less intense when fed prey in exponential growth because of the differences in cell size during day and night at this phase. When *G. dominans* and *O. marina* were fed prey at the stationary phase, however, they exhibited ingestion rates about 5 and 10 times higher during the day-time, respectively. This fact indicated that the rhythmic feeding activity was independent of the circadian changes in the stoichiometric composition of prey. Finally, previous grazer feeding history (starved *versus* well-fed) did not affect the feeding rhythm.

CHAPTER 2:

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

A diel cycle in the cellular division was observed in all the studied prey and grazer species. Cell division occurred at night for the prey *Rhodomonas salina* and also for the heterotrophic dinoflagellates grazers *Gyrodinium dominans* and *Oxyrrhis marina*, whereas the mixotrophic dinoflagellate grazer *Karlodinium armiger* and the

heterotrophic ciliate *Strombidium arenicola* divided during the day. Under continuous darkness, *R. salina* lost the diel rhythm in cellular division and eventually arrested growth. *G. dominans*, *O. marina*, and *S. arenicola* exhibited an initial period of adaptation to the constant darkness conditions with no day-night differences in the growth rates but recovered the diel rhythm within a short period (a few hours). *K. armiger* died quickly under continuous darkness. Regarding feeding, when exposed to continuous both light and darkness, the grazers *G. dominans*, *K. armiger* and *S. arenicola* lost the rhythmic feeding activity after some time (hours and even days for certain species) of exposure, and *O. marina* maintained the diel feeding rhythm during the whole experimental period despite the light conditions.

CHAPTER 3:
Predator chemical cue effects on the diel feeding behaviour of marine protists

Diurnal feeding rhythm was evident in the laboratory-maintained marine protists *Mesodinium rubrum*, *Gyrodinium dominans*, *Oxyrrhis marina*, and *Karlodinium armiger*, as well as in the recently isolated ciliate *Strombidium arenicola*. We found a negative relationship between the amplitude of the diel feeding rhythm and the time the grazers had been maintained in laboratory cultures under the absence of predators. In the particular case of the ciliate *S. arenicola*, we observed that the rhythm was completely lost after several months of being transferred into laboratory cultures. When copepodamides were used as chemical cues mimicking predator threat, we found that the diel feeding rhythm of *S. arenicola* was partially restored, suggesting a predator-risk induction of feeding rhythms in this ciliate species. When other grazers were tested for their response to copepodamides, the other ciliate, *M. rubrum,* showed a non-significant increase in the amplitude of the diel feeding rhythm. Regarding the dinoflagellates, *G. dominans* and *K. armiger* significantly reduced the amplitude of the diel feeding rhythm under the presence of copepodamides. Instead, the response of the dinoflagellate *O. marina* to copepodamides did not result in a consistent pattern.

CHAPTER 4:

Trophic interactions and diel feeding rhythms of protistan grazers in a productive Swedish fjord

A field study was carried out during July and August 2017 in the Gullmar Fjord (Sweden). The monitoring of the environmental conditions indicated that on 9 August there was a possible event of water masses exchange, with a concurrent change in the nano- and microplankton assemblages. Marine protist grazing activity daily removed about 26% of the phytoplankton standing stock and 96% of the primary production, on average. Instead, mesozooplankton did not significantly impact neither phytoplankton growth nor marine protist activity. Regarding the diel feeding rhythm of marine protists, no significant differences in the day and night grazing rates were detected in none of the experiments individually. Nonetheless, when the results were grouped in the period before and after the water mass exchange, grazing activity was significantly higher at night-time during the second period, whereas no significant differences between day and night grazing rates were observed during the first one.

GENERAL DISCUSSION

Protistan grazers are a key component in marine food webs and play a crucial role in marine pelagic ecosystems both as main grazers of pelagic primary production and also as a significant component of the diet of copepods, therefore enhancing the carbon flux prone to reach upper trophic levels. In this Ph.D. Thesis, we shed light on the causes and the factors involved in regulating the diel feeding rhythms of marine protistan grazers, which are still poorly studied. This subject is of relevance because of the important consequences that the rhythmic feeding behaviour of marine protists has in the dynamics of marine planktonic food webs and the biogeochemical cycles driven by them.

1. Do marine protists feed in a diel cycle?

Several physiological functions and behaviours in the marine realm have been described to follow a diel cycle, including photosynthesis activity, cellular division, vertical migration, and so on, marine protist feeding not being an exception. While diel rhythms on autotrophic protists have been long studied, very little is known about day-night differences in the feeding activity of heterotrophic and mixotrophic protists. Given their role as major grazers of planktonic primary producers, the presence of diel variations in their feeding rhythm strongly cascades throughout the planktonic food web.

Fulfilling the first aim of this Ph.D. Thesis, we corroborated that various species of heterotrophic and mixotrophic marine protists feed under a diel cycle, with higher feeding activity during the day-time, in agreement with earlier reported evidence (Strom, 2001; Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011; Ng and Liu, 2015; Ng et al., 2017). The amplitude of the diurnal feeding rhythm differed between species, suggesting a certain dependency on species-specific life-history traits and the habitats the grazers originated from. In general, even though several explanations for the higher diurnal feeding activity in marine protists have been previously proposed, they are somewhat contradictory and not fully demonstrated. Therefore, we start this discussion by exposing the actual hypotheses on the diel

feeding rhythms of marine protists, analysing their inconsistencies and suggesting new alternatives.

2. Which are the factors triggering diel feeding rhythms in marine protistan grazers?

Chapter 1 dealt with the effect of the **previous feeding history of the grazer** (i.e. well-fed *versus* starved) and the **growth phase of the prey** (i.e. exponential *versus* stationary), and therefore its **nutritional status** (i.e. stoichiometric composition), on the diel feeding rhythms of marine protists. While the rhythmic feeding activity of marine protistan grazers was not conditioned by the previous feeding history of the grazers, a clear effect was observed by the growth phase of prey. Nonetheless, diel changes in prey stoichiometric composition were only evident during the exponential phase of growth and did not exert an effect on the feeding rhythm.

The results presented in this chapter, showing diel feeding rhythms when the micrograzers were feeding on prey in the stationary phase of growth (i.e., with equal day and night C:N), did not support the mechanism proposed by Ng and Liu (2015) of an increment of ingestion rates on low-quality (i.e., high C:N) prey during the day-time as a compensatory feeding response. In fact, this compensatory feeding mechanism was already challenged by a later work from the same authors (Ng and Liu, 2016), where heterotrophic nanoflagellates showed diel feeding rhythms on fluorescently labelled bacteria. Also, Strom (2001) observed between 2.2 and 6.8 times higher ingestion rates under light conditions in two ciliate species fed on fluorescently labelled algae. Our research from Chapter 1, however, did not definitively unveil the factors triggering the rhythmic feeding activity of marine protists, but evaluated possible driving forces and made evidence that other factors not exclusively associated with the properties of prey might be very relevant.

Chapter 2 addressed the **role of light** as a causal mechanism for the diel feeding rhythms of marine protists, exploring the mechanisms proposed by both Strom (2001)

and Jakobsen and Strom (2004). Briefly, the diurnal feeding rhythm of nearly all grazers species studied in this chapter lost the rhythmic feeding activity after several hours/days of exposure to both continuous light and darkness, except for the heterotrophic dinoflagellate *O. marina*. This result first questioned the viability of Strom (2001) hypothesis, who proposed a mechanism based on light-aided digestion by which light would promote the extensive breakdown of the ingested prey, and therefore enhance grazing, during the day. However, should an enhancement of the digestion by light occur (1) the day-time intake would not have been different than the night one under continuous light, and (2) the rhythmic feeding activity would have been lost under continuous darkness in all the species, which was not the case for *O. marina*. However, the pattern of response from *O. marina* might support the endogenous control (circadian cycle) of the feeding rhythm proposed by Jakobsen and Strom (2004), although we cannot dismiss that this dinoflagellate would have eventually lost the rhythm in a longer time exposure to continuous light and darkness. Hence, while we rejected an enhancement of digestion by light and the control by an internal clock as underlying mechanisms of the rhythmic feeding activity, light seems to be required as an external synchronizing agent to sustain the feeding rhythm.

In addition, we also studied the possibility that diel feeding rhythms in marine protists might be the result of indirect effects due to **synchronized cellular division** in the grazer. Some rhythmic behaviours have been described to be synchronized to the cell division cycle, as is the case of diel vertical migrations in some species (e.g., Baek et al., 2009). The cessation of food intake during cellular division has been suggested for bacterivorous flagellates, to explain low grazing rates in coincidence with remarkable increments in the number of flagellate cells (Wikner et al., 1990). In our study, while the grazers *G. dominans* and *O. marine* divided at night, the pattern of diurnal division observed for *K. armiger* and *S. arenicola* was concurrent with higher feeding rates during the day-time. Consequently, we discard feeding activity impairment due to cellular duplication as a universal mechanism to explain diel feeding rhythms in protists.

Until here, although we have refuted some of the hypotheses previously proposed to explain the presence of diel feeding rhythms in marine protists, we still did not

establish a common mechanism triggering this diel behavior. Hence, we then propose that these rhythms may have found their origin on the risk of predation. Since diverse organisms exhibit differences in their periods of feeding activity within the daily cycle, it has been suggested that activity rhythms may have evolved to organize the time structure of environments (Kronfeld-Schor and Dayan, 2003). Therefore, temporal portioning between prey and predator might support their coexistence in the communities (Stiling, 1999). In fact, as the threat of predation is commonly experienced in predictable diel fluctuations, organisms might have developed temporal activity programs that balance mortality, foraging and reproduction to maximize their fitness (Kronfeld-Schor and Dayan, 2003; van der Veen et al., 2017). In the case of marine protists, given that their most direct predators (i.e., copepods) feed mostly during the night, the diurnal feeding of marine protists could have appeared as a mechanism to reduce predation risk. If our hypothesis were true, we would also expect that the response to predation risk (i.e., diel feeding rhythm) would be modulated by **prey availability**, as it has been suggested for copepods (Calbet et al., 1999). Hence, we suggest that under food limiting conditions, the feeding rhythm might be compromised as grazers are forced to increase the search effort and, therefore, increasing the encounter rates with predators. On the other hand, when food is not limiting, it may pay off for protistan grazers to reduce nocturnal feeding when copepods inhabit upper layers and can potentially have a larger impact on them. The results presented in Chapter 1 showed that the feeding behaviour of marine protists was widely modified by food resource availability, with all grazers exhibiting the greatest differences between day and night feeding activity under saturating food conditions. When food was limiting, instead, diel differences were scarcely marked or inexistent. These results, hence, indirectly suggest that the risk of predation could play an important role in the diel feeding behavior of protistan grazers. Moreover, given the positive relationship between prey availability and the amplitude of the diel feeding rhythm, it could be expected a major occurrence of diel feeding rhythms in upwelling and productive waters rather than oligotrophic areas.

Following up on these previous results, in Chapter 3 we directly addressed the effects of the **risk of predation** on the diel feeding activity of marine protistan grazers, using

chemical cues (copepodamides) from copepods as a mimic. We expected that the presence of chemical cues from predators would cause a reduction of nocturnal feeding and, consequently, feeding rhythms of major amplitude. Nonetheless, the outcome of the experiments was diverse, depending on the group of protists. While the response of the ciliate *Strombidium arenicola* to copepodamide presence was the triggering of diel rhythms, most of the dinoflagellates decreased the amplitude of their feeding rhythms. Moreover, within each group, the strategy to face the risk of predation resulted to be species-specific. The response of the ciliates highly relied on their swimming behaviour, with the ability to execute escape responses. Instead, because of their limited swimming capacity, the strategy of dinoflagellates might be based on the rapid growth of the population to guarantee its survival and, thus, opt to feed independently from a diel feeding rhythm to maximize its opportunities and cover their metabolic reproduction demands. In the case of *K. armiger*, however, the anti-predator strategy might completely rely on its capacity to produce toxins that have the potential to rapidly immobilize and kill copepods, probably allowing its co-existence with predators.

The insights provided here foster the understanding of how the diel feeding behaviour of marine protists is modulated by environmental factors occurring in the field. Deepening into the causes that originate such behaviour is an essential knowledge necessary to improve the predicting capability of phytoplankton dynamics and biogeochemical models. Apart from integration into models, data provided in this study can be collated with field abundances to gain insight into the role of marine protistan grazers in planktonic food webs.

3. The limitation of laboratory-based research to address biological rhythms

Typically, most of the studies on biological rhythms are laboratory-based, using model organisms reared in captivity (Kronfeld-Schor et al., 2013). However, natural environments are substantially more complex than laboratory conditions. Although

many environmental variables can be reproduced in the laboratory (e.g., light, temperature, prey presence), usually they cannot encompass the complexity, and temporal and spatial variability found in nature. As a consequence, on occasions, laboratory-reared organisms experience behavioural and physiological changes induced by the laboratory conditions (Madison et al., 2005). Somehow, the intensity of biorhythms can also be affected by laboratory conditions. In fact, several studies have demonstrated a decrease in the intensity of some behavioural rhythms in experiments conducted with laboratory-reared organisms compared to wild specimens. In this sense, Tiselius et al. (1995) observed the loss of the feeding rhythm in a 12-years-old laboratory culture of the copepod *Acartia tonsa*. Also, Calbet et al. (1999) reported that *Paracartia (Acartia) grani* exhibited feeding rhythms of lower intensity compared to wild specimens after successive generations rearing in the laboratory.

The present Ph.D. Thesis warns about this issue. In Chapter 3, we detected that the recently isolated ciliate *S. arenicola* originally exhibited a diel feeding rhythm shortly after isolation from the sea, but gradually reduced these day-night differences until the complete disappearance within months of being cultivated under laboratory facilities. This effect was further supported by a general trend of reduction in the amplitude of the diel feeding rhythm as a function of the time maintained under laboratory facilities in the whole group of protists studied. Thus, for instance, the two strains of the dinoflagellate *O. marina* isolated in different timings (years 1995 and 2016) shown in Chapter 1 differed in the amplitude of their feeding rhythm, with the more recently isolated strain presenting the more intense rhythm. Hence, while laboratory-based experimentation is required as a preliminary approach to study the mechanisms underlying biological rhythms, it is necessary to go further and expand the research of biological rhythms to natural environments.

4. Diel feeding rhythms of marine protists in a natural ecosystem

As previously stated, natural environments are highly complex and organisms are exposed to a great diversity of abiotic and biotic environmental factors, many of them uncontrollable (van der Veen et al., 2017). In Chapter 4, our observations of marine protist diel grazing rhythms in the field did not match the laboratory evidence of higher diurnal grazing found in this Ph.D. Thesis and in the literature (Strom, 2001; Jakobsen and Strom, 2004; Tarangkoon and Hansen, 2011; Ng and Liu, 2015; Ng et al., 2017). Yet, the previous scarce attempts to assess diel grazing activity of marine protists in natural assemblages rendered diverse and inconsistent patterns as well (Litaker et al., 1988; Claustre et al., 1999; Neveux et al., 2003; Ng and Liu, 2016; Armengol et al., 2019), what may reflect the complexity of nature in front of the standard laboratory conditions. Nevertheless, some patterns arose in our experiments, shedding some light on the mechanisms controlling the diel feeding behaviour of marine protistan grazers. We observed that a change in the grazing pattern of marine protists (from no significant diel differences to significantly higher ingestion rates during the night-time) occurred following a change in the microplankton community. This was consistent with the field observations by Armengol et al. (2019), who reported different feeding patterns in marine protists between oligotrophic and productive regions and associated them with differences in the species composition of both the phytoplankton and protistan grazers assemblages. Likewise, the remarkable dominance of diatoms after the change in the community composition could indirectly affect rhythmic grazing activity by lengthening the digestion process (e.g., the silica content of diatoms may involve higher digestion times; Zhang et al., 2017). Finally, prey availability has proved to be a factor of relevance in modulating diel feeding rhythms. In Chapter 1 we showed that the rhythmic feeding activity of marine protistan grazers was clearly modified by prey availability, reducing its amplitude, to even non-existent, under low prey concentration. In our field study, prey abundance in the natural ecosystem was far from saturating conditions (values ranging between 0.45 μg C L^{-1} up to 5.45 μg C L^{-1} in the field study *versus* ca. 4200 μg C L^{-1} in the laboratory-based experiments at saturating concentrations).

Considering the prey concentration in our field experiments, not observing feeding rhythms in protistan grazers in part of the study would be rather expected.

CONCLUSIONS

The main conclusions derived from this Ph.D. Thesis are listed below:

I. The feeding behavior of common heterotrophic and mixotrophic species of marine protistan grazers follows a diel rhythm characterized by higher feeding activity during the day-time.

II. The previous feeding history of marine protistan grazers does not affect their rhythmic feeding activity.

III. The growth phase of the prey can modulate the amplitude of the diel feeding rhythms of marine protists.

IV. The higher feeding activity of marine protistan grazers during the day-time is not caused by a compensatory feeding response, due to diel differences in prey stoichiometric composition.

V. Light appears as an external synchronizing agent necessary to sustain the rhythmic activity in numerous species of marine protistan grazers.

VI. The regulation of the feeding rhythm by an endogenous control (internal clock) is not a universal causal mechanism for the rhythmic feeding activity among marine micrograzers.

VII. The higher diurnal feeding rates of marine protists cannot be explained by a mechanism based on a light-aided enhancement of prey digestion.

VIII. The diurnal feeding behavior of marine protistan grazers is not generally caused by an impairment of the feeding activity during cellular division.

IX. The diel feeding rhythms of marine protists are strongly dependent on resource availability: the largest rhythm amplitudes occur under saturated prey conditions, while under limiting prey availability feeding rhythms lose intensity or even disappear.

X. The risk of predation can strongly modulate the feeding rhythm of several species of protistan grazers and can even reinstate such rhythmic activity when lost under laboratory conditions. Hence, it becomes a plausible trigger mechanism for these rhythms in marine protists, although the character of the response appears to be group- and species-specific.

XI. There might not be a unique underlying mechanism causing diel feeding rhythms in marine protistan grazers. Instead, protist species might have developed a characteristic diel feeding rhythm according to their behavioural and physiological traits, and also dependent on the ecological conditions from their original habitat, altogether largely determining the factors by which the rhythm is modulated and leading to a magnitude of the rhythm specific to the species.

XII. Different patterns of diel feeding rhythms in protistan grazers may exist among marine ecosystems, highly depending on the ecological characteristics of the site, as well as the species composition and abundances of both grazer and prey communities.

XIII. The magnitude of some natural diel behaviors in protistan grazers may be attenuated after several consecutive generations of rearing under laboratory conditions, and the absence of predators seem to be a potential cause for such fading.

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SUPPLEMENTARY MATERIAL

Supplementary material Chapter 3

Functional response of the recent isolated ciliate *Strombidium arenicola*

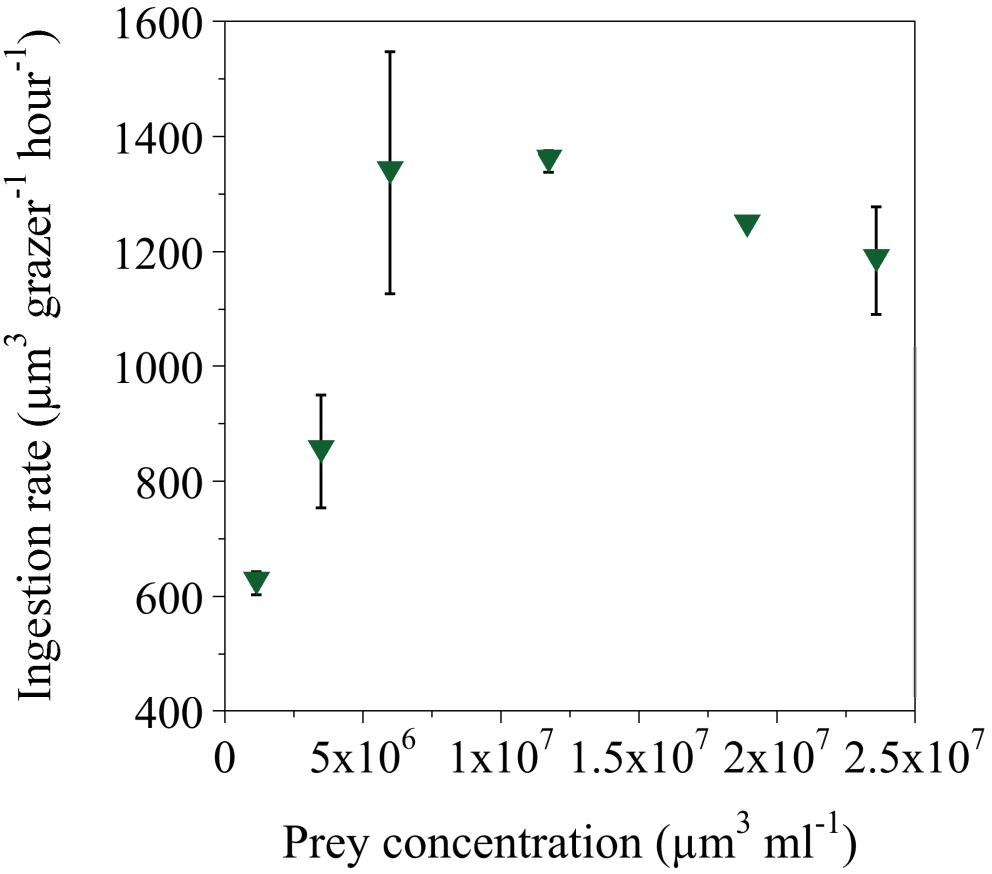

Figure S3.1. Ingestion rate of the ciliate *Strombidium arenicola* (μm^3 $grazer^{-1}$ h^{-1}) as a function of prey concentration (μm^3 mL^{-1}). Error bars show standard error.

Determination of copepodamides concentrations

As copepodamides are surface-active and degrade over time, a preliminar test was carried out to measure the effective concentrations in the experiments over time. Copepodamides were extracted from freeze-dried *Calanus finmarchicus*, both male and female, through a series of chemical separation steps (see Selander et al., 2015 for details). The experimental procedures to assess the losses of copepodamide were performed in identical conditions to that of the feeding experiments (see below section).

Four sets of suspensions in FSW medium were prepared with mixtures of the desired prey and grazer concentrations, with copepodamides added at the following nominal concentrations: 0 (only adding methanol, the diluent), 0.01, 0.1 and 1 nM. Each suspension was split into twelve 72 ml polyethylene culture flasks, to get three replicates per each copepodamide concentration at every sampling time: t=0 (initial samples), 2, 5, and 10 (final samples) hours. Flasks were all incubated on a plankton wheel (0.2 r.p.m) at 19 ± 1ºC, and an irradiation of 90 µE m^{-2} s^{-1}. The triplicate samples from each concentration removed at every sampling time were loaded onto solid-phase extraction (SPE) columns (Evolute Express ABN, 100 mg, 3ml, Biotage). The columns were de-salted with 1 column volume MilliQ water and the compounds eluted into 3 ml methanol. The methanol evaporated and the copepodamides were then resolved in a small (80 µl) volume before analysis on an Agilent 1260 Infinity HPLC system connected to an Agilent 6410 Triple Quad LC/MS (see Selander et al., 2015 for further details).

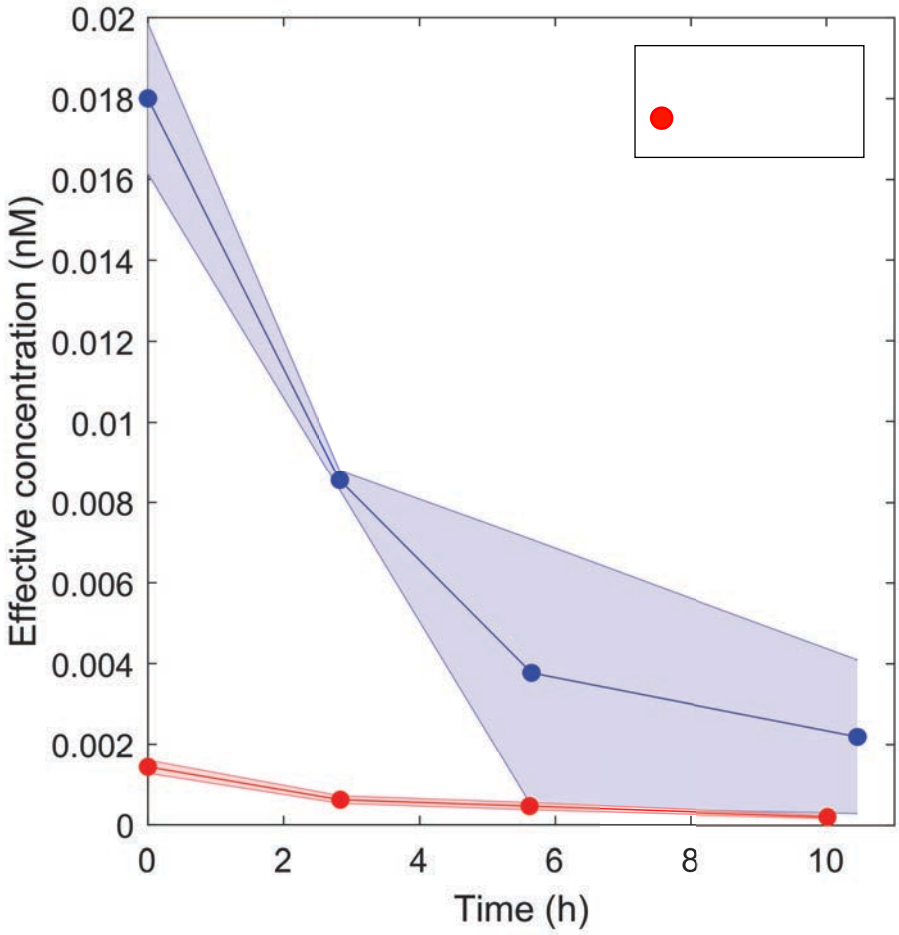

Figure S3.2 Effective concentration (nM) of copepodamides during 10h incubation. Closed circles represent the average data from the sampling time points and shaded area is the error interval (standard deviation).

Total daily ingestions of the target grazers

Table S3.1. Day and night ingestion rates (in terms of prey volume ingested, μm^3 $grazer^{-1}$ $hour^{-1}$) for each of the studied grazer species as a function of copepodamides treatments (Control, 0,01 nM and 0.1 nM). Rates are differentiated between Experiment 1 and Experiment 2. Average + standard error are shown. *p*-values from one-way ANOVA followed by a Dunnet's test are presented to show the significance level of each copepodamides treatment with respect to the correspondent control in each phase (day and night).

	EXPERIMENT 1									EXPERIMENT 2								
Species		**Night ±SE**			**Night ±SE**		**Day ±SE**			**p-value**		**Night ±SE**			**Night ±SE**		**Day ±SE**			**p-value**
S. arenicola (Nov-Dec18)		2144.5 ±111.6			1635.5 ±161.9		2199.1 ±83.8			<0.05		1100.4 ±17.8			953.7 ±48.3		1160.7 ±40.0			<0.05
G. dominans		136.2 ±0.3			162.2 ±3.4		474.0 ±5.2			<0.001		186.2 ±4.0			227.6 ±3.1		472.0 ±5.4			<0.01

'able S4.1. Abundance (in cells L^{-1}) of dinoflagellates, diatoms, other phytoplankton and ciliates identified on each experimental ay. *NI* means *not identified* species/genus. The average cell volume (as geometric mean, μm^3) is also provided.

mphidinium crassum	2833	0	0	0	0	0
)inophysis acuta	72986	0	0	0	0	20
Gyrodinium spp. >60µm	19981	35	0	20	15	45
)olykrikos schwartzii	83727	0	0	0	0	25
)yrocystis lanula	)4653	10	0	0	10	25

Scrippsiella trochoidea	6235	26	0	0	0	0	0	0	25	10	15	35	15	0
Torodinium robustum	9090	208	180	360	255	145	180	145	198	300	255	215	195	173
Dinoflagellates cysts	4445	98	50	185	103	55	40	70	70	210	90	145	110	103
NI dinoflagellates >40µm	42343	331	210	340	375	180	170	175	195	465	235	635	480	248
NI dinoflagellates 20-40 µm	5281	1089	1199	1987	1199	1028	1096	1781	1850	3562	1233	2569	2055	959
NI dinoflagellates <20µm	227	32281	27092	39662	35483	16577	20516	17194	25927	14248	16714	17810	10686	4658
DIATOMS														
Asteronellopsis glacialis	653	0	0	0	0	0	0	0	0	3494	1918	891	9316	4590
Chaetoceros spp. <20 µm	569	2238	582	617	651	0	0	137	240	10275	2809	6884	5617	4384
Chaetoceros spp. >20 µm	1754	0	8	0	10	5	0	0	0	20	15	150	485	483
Cylindrotheca closterium	440	3180	1165	891	685	1096	1267	1644	2637	3425	1507	2329	3288	891
Diitylum brightwelli	32162	0	0	0	0	0	0	0	10	30	40	13	35	33
Guinardia delicatula	1908	26	18	10	3	0	5	0	28	235	195	180	745	518
Leptocylindrus danicus	1377	0	0	0	0	0	0	0	0	475	170	118	3115	1065
Pleurosigma spp.	33032	20	8	0	0	0	3	20	3	5	0	0	0	5
Proboscia alata	3020	23584	9111	13495	3151	2535	1130	685	78	60	15	10	0	8
Pseudo-nitzschia spp.	663	34	58	25	15	0	0	105	93	300	190	300	605	490
Rhizosolenia spp.	3110	0	3	5	5	0	0	10	25	35	25	38	70	28
Skeletonema costatum	81	26947	13803	6234	5514	411	171	7467	5343	197307	2124	140768	36579	4042
Striatella unipunctata	198400	14	8	0	0	0	0	10	0	5	0	5	5	10
Thalassionema spp.	832	13	3	0	3	0	0	25	0	0	0	0	5	8
Thalassiosira spp. >20µm	14028	13	5	10	5	45	20	30	10	20	20	18	10	13
Thalassiosira spp. <20µm	2763	0	0	0	34	0	0	206	171	274	69	0	0	0
NI centric diatoms	844	2031	993	2535	1302	822	308	2877	1850	17399	6371	3185	5754	5480
NI pennates diatoms	813	281	206	0	0	69	0	274	69	0	0	0	10	8
NI benthic diatoms	43	1847	925	480	788	480	788	5206	19077	12467	5480	959	617	1028
OTHER PHYTOPLANKTON														
Cryptomonas spp.	161	6838	5069	3768	6097	4521	8220	8905	12775	6097	6439	10481	3014	7090

Filamentous cyanobacteria	712	0	3	0	0	0	0	0	10	300	155	288	330	140
Dictyocha fibula	8509	0	0	0	0	0	0	0	0	10	20	15	45	15
Dictyocha speculum	7700	20	3	0	0	10	5	30	33	5	10	10	0	23
Eutreptiella spp.	1206	38	8	15	3	15	0	5	90	95	25	993	30	23
Fungus (sporangia)	4469	0	0	0	0	0	0	0	183	0	5	3	0	18
CILIATES														
Laboea strobila	276707	7	3	0	0	0	0	0	0	0	45	25	5	43
Didynium nasutum	46649	0	0	0	0	0	0	0	0	0	0	10	0	0
NI ciliates <20um	1534	1749	2500	4864	11474	13837	12364	2603	1610	4247	8563	7706	5138	5960
NI ciliates 20-40um	8033	648	1781	3083	4555	1850	4829	2398	1884	1370	3425	5138	2466	5103
NI ciliates >40um	48233	178	545	290	490	65	158	190	173	235	460	1680	655	1660
Tiarina fusus	27018	0	0	0	0	0	0	0	0	85	0	20	5	8
Tintinnid sp.	41563	0	0	0	0	0	0	0	0	0	25	18	25	28
Tintinnids <100µm	2154	93	75	20	18	0	0	0	15	10	25	80	55	10
Tintinnids >100µm	52850	38	45	10	3	0	0	15	5	20	5	20	5	18

ANNEX

Published papers

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ORIGINAL PAPER

Diel feeding rhythms in marine microzooplankton: effects of prey concentration, prey condition, and grazer nutritional history

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Abstract In this study, we aim at disentangling the causes and consequences of diel feeding rhythms in marine microzooplankton. We focused on the diel feeding activity of two heterotrophic dinoflagellate species, *Gyrodinium dominans* (one laboratory strain) and *Oxyrrhis marina* (laboratory cultivated and wild strains). We observed higher ingestion during the day in both dinoflagellate species. Feeding rhythms appeared to be independent of circadian changes in prey biochemical composition. Grazers fed with prey under stationary phase, with equivalent stoichiometric composition between day and night, showed 5 (*G. dominans*) and 10 (*O. marina*) times higher ingestion rates during the day. Previous grazer feeding history (starved vs well-fed) did not affect the feeding rhythm. However, prey concentration altered the rhythm; food limiting conditions reduced the amplitude of the rhythms. Our results establish a resource dependence of diel periodicity in microzooplankton grazing, which can have unanticipated consequences for standard field dilution grazing experiments.

Introduction

Light is a major driver of life in our planet, and as such it regulates the production and distribution of phototrophic organisms; for instance, the vertical distribution and seasonal production peaks of plankton in aquatic ecosystems are dependent on light availability (Sverdrup 1953; Margalef 1978). It is known that light also drives the feeding rhythms of mesozooplankton both in marine and freshwater systems (e.g. Duval and Geen 1976; Mackas and Bohrer 1976). In most occasions, feeding rhythms in mesozooplankton are linked to daily patterns of vertical migration (surface during night, deeper during daytime; Gauld 1938; Stearns 1983; Saiz and Alcaraz 1990; Saiz et al. 1992; Putzeys and Hernández-León 2005), typically triggered by the presence of visual predators (i.e. fish) in the upper layers of the oceans (Bollens and Frost 1991; Bollens 1996). Nocturnal feeding has also been observed, however, in the absence of vertical migration in some copepod species (Peruyeva 1977; Boyd et al. 1980; Calbet et al. 1999).

In the case of microzooplankton, a key group in the transfer of energy from primary producers to upper trophic levels in the marine pelagic environment (Calbet and Landry 2004; Calbet and Saiz 2005; Schmoker et al. 2013), less is known about their diel feeding rhythms and the underpinning factors modulating them. This functional group is taxonomically diverse and overall encompasses organisms with limited migratory capacity. The few evidences available on microzooplankton diel feeding behaviour indicate, contrarily to mesozooplankton, that ingestion rates are higher during the day (Strom 2001; Jakobsen and Strom 2004; Tarangkoon and Hansen 2011).

Several hypotheses have been suggested to explain this particular behaviour. Jakobsen and Strom (2004) advocated the presence of an endogenous circadian cycle, light-modulated, for feeding and growth of many protozoans. On the other hand, Strom (2001) proposed that light may enhance digestion by generating reactive oxygen species in the protozoan food vacuole, while promoting ingested material break down and increasing assimilation and gross

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growth efficiencies. By this process, digestion would not limit (or limit less) the incorporation of new items into the food vacuoles, enhancing the feeding rates. Alternatively, Ng and Liu (2015) suggested that diel variations on phytoplankton stoichiometry (i.e. higher C:N ratio during day) can potentially in uence the feeding behaviour of grazers. A diel periodicity of the C:N ratio has been detected in various phytoplankton groups (Stramski and Reynolds 1993; Clark et al. 2002; Jauzein et al. 2011), and it is suggested to be regulated by a circadian clock (Edmunds 1988). Bonded to this diel variation of the stoichiometric composition of prey are the changes of size result of synchronous division (Sweeney and Hastings 1958; Edmunds 1965; Eppley and Coatsworth 1966; Paasche 1968; Bruce 1970). Most of the hypotheses above, however, are questioned by the facts that (1) the presence of diel feeding rhythms seem to be preserved under either continuous light or darkness, and (2) diel feeding rhythms appear even when protists are fed on inert , dead cells (Sweeney and Hastings 1958; Chisholm and Brand 1981; Jakobsen and Strom 2004). We believe the presence of reverse diel feeding rhythms in microzooplankton, though endogenous, might have evolved as an adaptation to avoid being predated. Because feeding typically implies swimming, grazers become more conspicuous and increase encounter rates when feeding (Broglio et al. 2001); therefore, to reduce the probability of being predated protozoans should display diel feeding rhythms reverse to that of their major grazers, i.e. the copepods (Saiz and Calbet 2011). Until now, however, no experimental evidence con rms this plausible hypothesis, although in copepods such behavioural mechanisms have been reported (Saiz et al. 1993; Heuschele et al. 2014).

Here we studied the diel feeding activity of two heterotrophic dino agellate species, *Gyrodinium dominans* (a laboratory cultivated strain kept for many generations) and *Oxyrrhis marina* (both a laboratory cultivated and a wild strain) and examined the effect of several factors on their feeding behaviour. Considered cosmopolitan species, they inhabit different environments with contrasting biological, physical, and chemical properties; this has lead to different adaptive ecological and physiological strategies (Calbet et al. 2013). We determined the following: (1) the presence of diel feeding rhythms in our target grazers and then checked whether nutritional properties of prey between day and night may explain the presence of rhythms; (2) whether the growth phase of the prey can evoke changes on the grazersí feeding behaviour; (3) the diel response of well-fed *vs* starved grazers (i.e. 48 h unfed) to prey; in this case, we expected rhythms to be in uenced by the grazer previous feeding history, with well-fed grazers showing higher amplitude diel feeding rhythms than starved ones; (4) the effects of prey concentration on the amplitude on the diel feeding rhythms of microzooplankton. One could expect the diel activity being in uenced by prey availability. Under non-limiting food conditions, microzooplankton may opt to feed less during the night period, when potential predators may have a larger impact, and, therefore, the differences between day and night feeding would become higher; under food limitation, the grazers might be forced to search for food both during the day and during the night to cover their metabolic demands, as it occurs in more complex organisms, such as copepods (Huntley and Brooks 1982; Calbet et al. 1999).

Materials and methods

Culture of the dinoag ellate predators and the algal prey

Laboratory cultures of the heterotrophic dino agellates *G. dominans* (GYR-BCN), and *O. marina* (OXY-BCN and OXY-BCN-2016, a new strain recently isolated) were used to study diel cycles in feeding and growth under different conditions. All strains were isolated by A. Calbet off Barcelona coast (NW Mediterranean, 41 23′0N) in 2011, 1996 and 2016, respectively, and then kept in the laboratory at the Institut de Ci ncies del Mar in Barcelona.

For these experiments, the grazer cultures were grown in round asks with metal-enriched autoclaved seawater (1 mL metal stock solution per litre; Guillard 1975) at 19 ± 1 C, 38 PSU under a 10L:14D light darkness cycle. Grazer stocks were daily fed with a culture of the cryptophyte *Rhodomonas salina* grown on *f*/2 medium (Guillard 1975) in 5 L Pyrex culture asks provided with air and diluted daily to ensure exponential growing conditions. The grazer cultures were maintained in these conditions several weeks (>4) prior to conducting the feeding experiments.

Prey and grazer diel changes in size and biochemical composition

We assessed the morphological (size) and biochemical (C:N:P) changes during the different growing phases (i.e. exponential and stationary phases) of *R. salina* by following the development of a triplicated culture of *R. salina* since inoculation until the beginning of the decay phase. The cultures were sampled before the light and dark periods started to determine cell size and concentration with the Coulter counter. Concurrently, we also analysed the elemental composition (carbon, nitrogen and phosphorus) of *R. salina* during the exponential and stationary phases of growth. For C and N analysis, 5 mL aliquots of the *R. salina* culture were ltered onto 25 mm diameter pre-combusted GF/F lters (450 C, 6 h), dried at 60 C for 48 h and kept in a desiccator until analysis with an elemental analyser FlashEA1112 (ThermoFinnigan). For P

analysis, 2 mL aliquots were used and immediately frozen at −80 C after ltration; later, samples were digested with NaOH $K_2S_2O_8$, and then analysed as inorganic P with an AA3HR autoanalyser (Seal Analytical). Following the same procedure, we analysed the biochemical composition of OXY-BCN and GYR-BCN before the light and dark periods after 2 days of starvation, when no prey was present in the suspension. For these samples we ltered from 20 to 50 mL, depending on the grazer concentration.

Experimental set-up

General set-up

The general procedure for the experiments was as follows. At each experiment, grazer and prey stock concentrations were determined with a Beckman Coulter Multisizer III particle counter (100 µm aperture tube) within 1 2 h before the beginning of, respectively, the light period (0900 hours) and the night period (1900 hours). Then, the desired predator prey suspensions were prepared and distributed at intervals by lling one-third of experimental (both grazer and prey) and control (only prey) bottles (72 mL polyethylene culture asks; 3 4 replicates). Extra bottles at each prey predator mixture were also prepared for determination of initial concentrations. Once set, the bottles were incubated on a plankton wheel (0.2 r.p.m.). About 1 h before the end of the respective light and night periods, the corresponding incubations were terminated and grazer and prey concentrations determined. During the light period the bottles were exposed to uorescent lamps providing an irradiation that ranged between 80 and 290 µE m^{-2} s^{-1} through a complete rotation of the wheel. Grazer and prey concentrations (in cells and in biovolume) at the beginning and at the end of the incubations were determined with the Beckman Coulter Multisizer III particle counter.

Effect of prey growth condition on diel feeding rhythms

We designed a series of experiments to explore whether the diel differences in feeding and growth rates of the grazers were affected by the prey growth phase (i.e. exponential *vs* stationary), and at its turn, by its biochemical composition. For this experiment we used the strains GYR-BCN and OXY-BCN, previously starved for 48 h. All the experimental procedures are as described in the General set-up section. Based on the results obtained (see Results), we decided to use *R. salina* (7 8×10^4 cells mL^{-1}) in stationary phase for the rest of the experiments in order to minimize the day night cellsí size variation.

Effect of the grazer feeding history on diel feeding rhythms

We investigated whether the previous feeding history of the grazer affected the amplitude of the diel feeding rhythm. Hence, we compared the grazing and growth rates of GYR-BCN and OXY-BCN fed ad libitum with *R. salina* with those of 48 h-starved grazers (i.e. unfed for 2 days). The experiments were conducted following the general procedures described above.

Effect of food concentration on diel feeding rhythms

We evaluated the effect of different prey concentration on the feeding behaviour of GYR-BCN, OXY-BCN and OXY-BCN-2016. According to the results of the previous experiments we established the experimental protocol to use prey on stationary phase and to starve the grazers for 48 h prior to the experiments. Prey concentrations were chosen in order to encompass three different scenarios of the functional feeding response (based on data from Calbet et al. 2013): limiting,

Table 1 Prey and grazer concentrations (cell mL^{-1}) used in the experiments on the effect of food concentration on diel rhythms

Grazer	Treatment	Prey concentration (cell mL^{-1})	Grazer concentration (cell mL^{-1})
GYR-BCN	Limiting	400010,000	600850
	Intermediate	$4 \times 10^4$6 $\times 10^4$	15002 800
	Saturated	$10 \times 10^4$11 $\times 10^4$	30003 500
OXY-BCN	Limiting	800012000	192240
	Intermediate	15,00025,000	257330
	Saturated	$15 \times 10^4$20 $\times 10^4$	16072 100
OXY-BCN-2016	Limiting	8000	192
	Medium	15,000	257
	Saturated	15×10^4	1607

Cell concentrations were selected according to Calbet et al. (2013) to include limiting, intermediate and saturating food conditions

intermediate and saturated food conditions (Table 1). The prey:predator ratios at the beginning of the incubations were about 35:1 in *G. dominans* and 100:1 for *O. marina.* All the experiments were conducted in triplicate and followed the general procedures described above.

Calculation of feeding rates

The calculation of feeding rates followed the exponential equations of Frost (1977). The grazing coefficient (h^{-1}) was estimated as follows:

$\mu -$

where μ (h^{-1}) is the intrinsic prey growth in the control bottles (only prey), and k is the apparent prey growth determined in the experimental bottles (with predators). Clearance rate F (µL grazer^{-1} h^{-1}) was estimated as

$\overline{\hat{\ }}$

where $\hat{\ }$ (cells mL^{-1}) is the average grazer concentration in the incubation, estimated as

$\hat{\ } \quad \overline{(\quad / \quad)}$

where $C_{0,grazer}$ and are, respectively, the grazer initial and nal concentrations in the incubation and t (h) is the incubation time.

Ingestion rates I (cells grazer^{-1} h^{-1}) were estimated as

$\times \hat{\ }$

where $\hat{\ }$ (cells mL^{-1}) is the average prey concentration estimated according to the equations in Frost (1977).

Feeding rates were converted into prey biovolume consumption by multiplying cell ingestion rates by the (geometric) mean prey volume during the incubation.

Results

Prey and grazer diel changes in size and biochemical composition

Rhodomonas salina entered in exponential growth ($\mu = 0.38$ day^{-1}) after a short (1 day) lag phase and remained exponentially growing for 4 days (Fig. 1a). Stationary phase reached densities of 1.6×10^6 cells mL^{-1}. Cells divided mostly during the night, which produced important differences in cell size between day and night. These differences were evident only during the exponential phase (Fig. 1b); cells were about 33% larger in volume during the day hours than during the night hours.

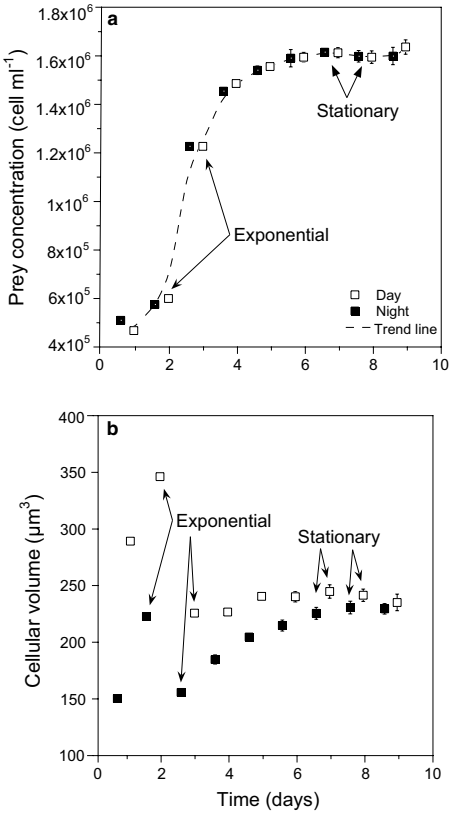

Fig. 1 **a** Time course of *Rhodomonas salina* concentration (cell mL^{-1}) and **b** cellular volume (μm^3) after inoculum in batch culture. In both graphics, the days sampled as representative of exponential and stationary growth phases are highlighted. White and black squares indicate light and night hours, respectively. Error bars represent standard error

We present in Table 2 the stoichiometric composition of *R. salina* under exponential (days 1213, both day and night periods, in Fig. 1) and stationary (from day 16, day period, to day 18, night period, in Fig. 1) phases. All elemental ratios were signi cantly higher during day time in the exponential phase of growth (C:N, 20% higher; C:P, 64%; N:P, 42%), whereas no difference between day and night composition were detected in stationary phase (Table 2). When comparing between exponential and stationary phases, the C:N and

Table 2 Day and night C:N, C:P and N:P molar ratios (average ± SE) of *Rhodomonas salina* under exponential phase (a) and stationary phase (b)

	C:N	C:P	N:P
(a) Exponential			
Day	7.7 ± 0.14	104.4 ± 20.24	13.6 ± 2.86
Night	6.4 ± 0.29	63.6 ± 8.26	9.56 ± 1.05
p value	<0.001	0.003	0.01
(b) Stationary			
Day	16.2 ± 0.88	245.7 ± 11.30	15.2 ± 0.97
Night	15.7 ± 1.04	229.8 ± 14.49	14.7 ± 0.35
p value	0.56	0.18	0.37

Signi cance levels (*p* value) of two-tailed *t* test comparing day and night averages are also shown

Table 3 Day and night C:N, C:P and N:P molar ratios (averages ± SE) of two-day starved (a) *Gyrodinium dominans* (GYR-BCN) and (b) *Oxyrrhis marina* (OXY-BCN)

	C:N	C:P	N:P
(a) *Gyrodinium dominans*			
Day	5.8 ± 0.07	46.0 ± 13.4	8.0 ± 2.4
Night	5.6 ± 0.06	39.4 ± 12.1	7.0 ± 2.2
p value	0.01	0.55	0.64
(b) *Oxyrrhis marina*			
Day	5.8 ± 0.09	34.5 ± 8.2	6.0 ± 1.4
Night	5.7 ± 0.06	31.8 ± 8.0	5.6 ± 1.4
p value	0.20	0.70	0.77

Signic ance levels (*p* value) of two-tailed *t* test comparing day and night averages are also shown

C:P ratios were more than double during stationary phase respect exponential, whereas for N:P differences were of much lower magnitude between both growth phases.

The stoichiometric ratios of *O. marina* and *G. dominans* did not overall differ signi cantly between day and night (Table 3); only the C:N ratios of *G. dominans* differed between day and night, but the magnitude of variation was rather small (4% higher during the day; Table 3).

Effect of prey growth conditions on diel feeding rhythms

We compared the diel feeding response of GYR-BCN and OXY-BCN when feeding on *R. salina* in the exponential and stationary growth phases. The feeding rates obtained are presented in terms of cell (cells ind^{-1} h^{-1}) and volume ingested (μm^3 $grazer^{-1}$ h^{-1}) in Fig. 2. In both species prey on stationary phase induced feeding rhythms of higher amplitude than when on exponential phase, being the rates about 5 (GYRO-BCN) and 10 (OXY-BCN) times higher during the day ($p < 0.001$, *t* test). In contrast, the results obtained with prey growing at exponential rates did not result in such a clear outcome. Whereas for GYR-BCN ingestion rates were higher during the day ($p < 0.001$ for cells and volume, *t* test), OXY-BCN showed an opposite pattern, with higher cell-based ingestion rates during the night ($p < 0.05$, *t* test), and no statistically signi cant differences between day and night when on a volume basis.

Effect of the feeding history on diel feeding rhythms

Diel ingestion rates of previously fed and starved GYR-BCN and OXY-BCN are presented in terms of cells and volume ingested in Fig. 3. In all cases ingestion rates were signi cantly higher during the light period ($p < 0.05$; *t* test). For GYR-BCN ingestion rates were about 55% (fed grazers) and 44% (starved grazers) higher during the day, whereas for OXY-BCN the increase was 13 and 34% for fed and starved grazers, respectively (Fig. 3).

Effect of food concentration on diel feeding rhythms

Food availability clearly modi ed the feeding rhythms of the three grazers studied (Fig. 4a, b). Under saturating food conditions all grazers showed the largest differences between day and night ingestion rates (in terms of prey volume consumed); these differences diminished as food concentration decreased. At limiting food conditions, the daily rhythms disappeared for OXY-BCN and it was poorly marked in the other strains. Out of the three strains studied, GYR-BCN showed the highest rhythm amplitude, followed by OXY-BCN-2016 (Fig. 4). Except for GYR-BCN, the slopes of the relationship between food concentration and the ratio between day and night ingestion rates were signi cantly different from zero ($p < 0.05$; Fig. 4), indicating a signi cant effect of prey concentration; in the case of GYR-BCN, however, the removal of one outlier value made the regression turn out signi cant ($p < 0.05$). The effects of food concentration on the diel feeding rhythms of the three grazers were similar, as indicated by the lack of signi cant differences among slopes (Fig. 4, $p = 0.51$; ANCOVA test). However, the intercepts were signi cantly different between regression lines ($p < 0.01$), which support that the magnitude of the rhythm is species/strain speci c. In all treatments the grazers showed negligible growth rate (data not shown).

Discussion

Corroborating previous evidence on protozoan diel behaviour (Christoffersen 1994; Liu et al. 1997; Dolan and Šimek 1999; Strom 2001; Binder and DuRand 2002; Jakobsen and Strom 2004) we found reverse circadian feeding rhythms (i.e. higher feeding rates during daytime) in two marine

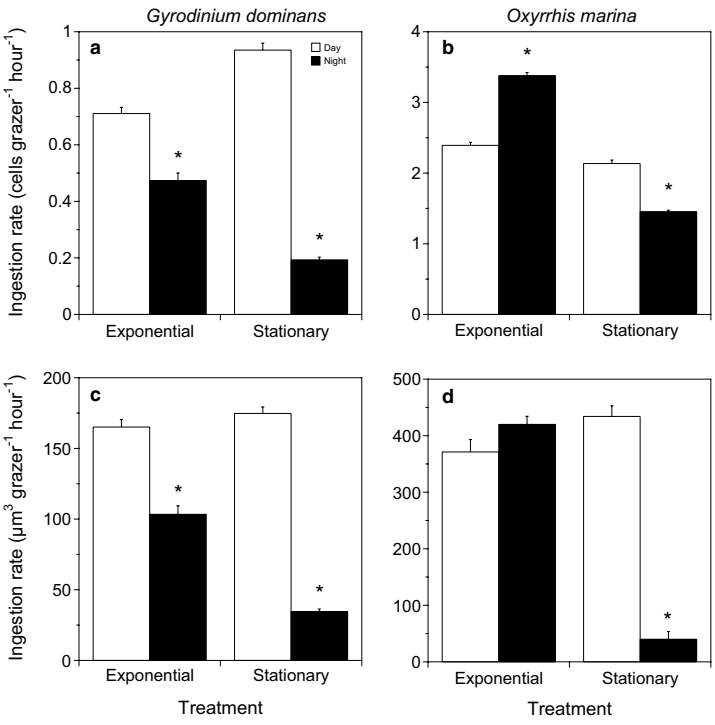

Fig. 2 Day and night ingestion rates of *Gyrodinium dominans* (**a**, **c**) and *Oxyrrhis marina* (**b**, **d**), expressed either in terms of prey number and of prey volume, for each experimental condition (exponential and stationary growth). Error bars indicate standard error and asterisks represent signi cant differences ($p < 0.01$)

heterotrophic dino agellate species. The causes and consequences of this particular behaviour are discussed below.

Causes of microzooplankton diel feeding rhythms

Phytoplankton activity is affected by light, leading to a diel periodicity in cell division and cellular properties (including C:N ratio) (Pr zelin 1992; Vaulot et al. 1995; Liu et al. 1997; Vaulot and Marie 1999; Binder and DuRand 2002; Ng and Liu 2015). Synchronised cell division during nighttime is con rmed for many phytoplankton groups and results in a cell size decrease during this period of the day (Pr zelin 1992; Binder and DuRand 2002; Ng and Liu 2015). Likewise, because of photosynthetic carbon xation during light hours, C:N and C:P ratios increase during the day (Stramski and Reynolds 1993; Clark et al. 2002; Jauzein et al. 2011; Ng and Liu 2015). Diel variations of both cellular properties and cell size in phytoplankton should have ecological implications and could affect the dynamic of diel trophic interactions (Ng and Liu 2015). Ng and Liu (2015) argued that the feeding behaviour of nano agellated grazers could be strongly induced by these diel stoichiometric variations of prey, as a result of compensatory feeding response by increasing grazing rates on low quality prey (i.e. high C:N) during the daytime. However, the same authors found also a distinct diel grazing pattern when agellates were feeding on uorescently-labelled dead bacteria. Also, Strom (2001) found that under saturating food conditions, the ingestion of dead uorescently labelled algae was 2.2 times higher in the light; she suggested a light-aided digestion mechanism. These two latter evidences seem to contradict the role of diel changes in algae composition as triggers of their grazerís diel feeding activity. Moreover, Jakobsen and Strom (2004) detected that the diel variations in growth and ingestion rates during day and night persisted in 24-h continuous darkness (although the rhythm slowly eroded after a few days), challenging Ng and Liu (2015) and Strom (2001) hypotheses. It seems, then, that either particular rules apply to each species, or that there must be an alternative explanation for the presence of diel feeding rhythms in microzooplankton. In our experiments we also detected a diel well-marked difference in *R. salina* cell size and stoichiometric composition during exponential phase, but these differences faded away in early stationary phase. This fact allowed us to test the role

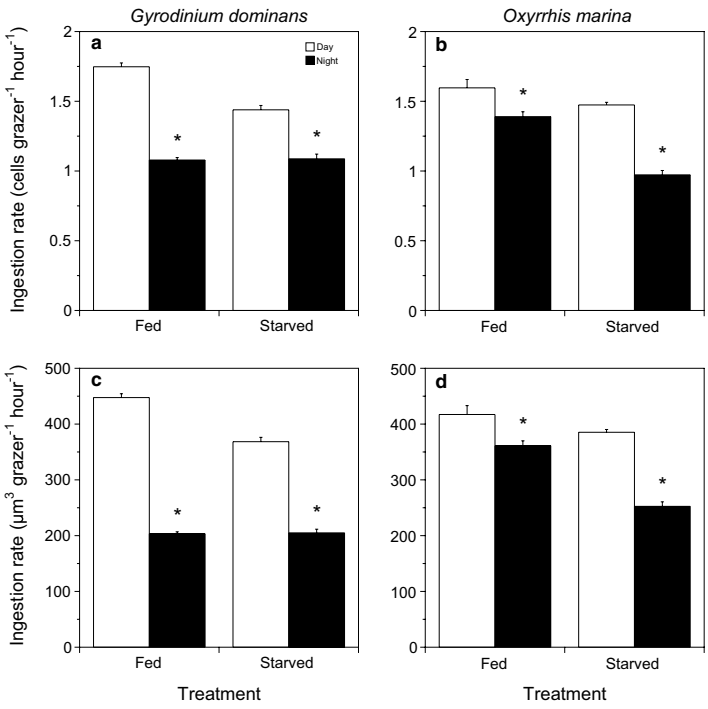

Fig. 3 Ingestion rates (in cells and volume) of *Gyrodinium dominans* (**a**, **c**) and *Oxyrrhis marina* (**b**, **d**) as a function of previous feeding ($p < 0.01$) (either fed or starved), expressed either in terms of prey number and of prey volume. Error bars indicate standard error and asterisks represent signi cant differences ($p < 0.01$)

of diel changes in size and elemental composition in causing the existence of feeding rhythms. We found that GYR-BCN and OXY-BCN showed higher ingestion rates during the day when fed on stationary phase prey, therefore, corroborating that these two factors cannot explain the presence of feeding rhythms. Nevertheless, the smaller prey size during night under exponential growth may mask, in occasions, these feeding patterns, as we observed in OXY-BCN (Fig. 2); this effect disappeared under stationary phase conditions where prey size was similar. Our results, therefore, lead to the conclusion that other factors, not only related to prey characteristics, must play an important role in determining the diel feeding activity of microzooplankton.

Oceanic planktonic habitats are known to be often food limited (Conover 1968). When prey are limiting, feeding behaviour of a grazer may be compromised as it is linked to swimming and, therefore, a grazer must increase its search effort. Hence, it increases the encounter rate with their own predators and, at the same time, they become more conspicuous to them. In this situation, a balance between feeding to maintain minimum nutritional requirements and the risk of being predated is necessary (Huntley and Brooks 1982; Saiz et al. 1992; Calbet et al. 1999), in particular given the high preference for dino agellates and ciliates displayed by copepods, the major contributors to mesozooplankton (Saiz and Calbet 2011). Several adaptive strategies of microzooplankton to famine have been proposed and demonstrated in laboratory studies. A reduction of metabolic rate in starving protozoans was suggested by Fenchel and Finlay (1983). Some species are also known to recur to resting cysts formation when prey concentration is low (Goodman 1987; Fenchel 1990). We suggest that microzooplankton may opt to diminish the feeding rhythm. As we initially hypothesised, the feeding rhythm of the studied microzooplanktonic grazers was of higher amplitude when food was not limiting. It would be expected that at low food concentration, starved grazers would feed in an arbitrarily manner, without following a light-darkness cycle to fed. As a consequence, this would lead to a major mobility by the protozoans, which implies being more detectable by their own predators. This means that the threat of predation may be an important component explaining microzooplankton diel feeding activity. Backing up this hypothesis we found a greater diel response in the recently isolated *O. marina* (OXY-BCN-2016)

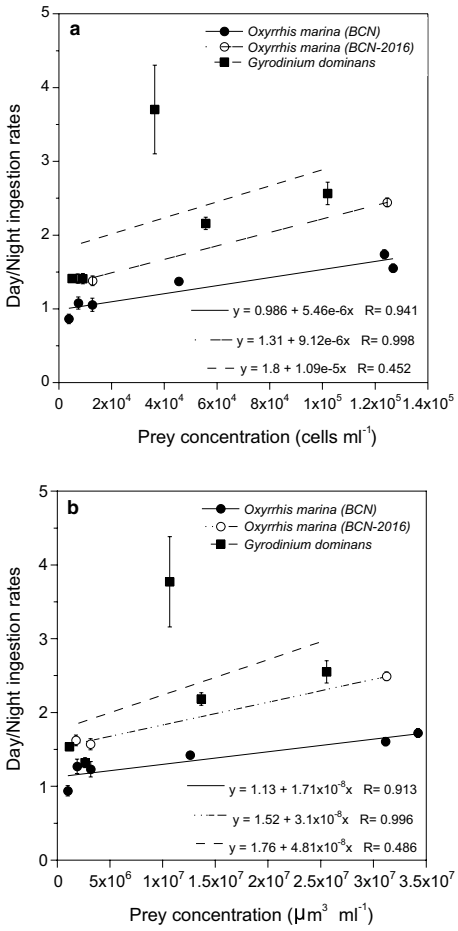

Fig. 4 Feeding rhythm index (quotient between day and night ingestion rates) of *Oxyrrhis marina* (BCN and BCN-2016 strains) and *Gyrodinium dominans* as a function of prey availability. **a** Values in cells mL^{-1}. **b** Values in volume (μm^3 mL^{-1}). Error bars show standard error

compared to the long-term laboratory-cultivated one (OXY-BCN). It is expected, as it occurs in other planktonic organisms, that some of the natural behaviours (i.e. the risk of predation) might be partially lost after consecutive generations of cultivation in the laboratory (Tiselius et al. 1995; Calbet et al. 1999).

In our study, the magnitude of the feeding rhythm (Fig. 4) differed between species/strains, being GYR-BCN the species with the most marked rhythm. *G. dominans* is typically found in costal and oceanic waters where vertical migrations of mesozooplankton are common and predation risk is higher at night (Saiz et al. 2014). On the other hand, *O. marina* inhabits intertidal pools and salt marshes (Begun et al. 2004), being infrequent in open waters (Lowe et al. 2010; Watts et al. 2011), and has life history traits that allow it to quickly exploit resources whenever conditions are favourable (Calbet et al. 2013).

We have demonstrated so far that, at least for the species of microzooplankton studied here, feeding rhythms appear to be independent of circadian changes in prey biochemical composition or previous grazer feeding history, but are modi ed by prey concentration. It may be argued, however, that decreased feeding rates during the night may be consequence of synchronised division of the grazer at night, constraining feeding while dividing. Even though we cannot disregard this hypothesis, our data do not seem to con rm it. Growth rates after the 2-day starvation period were negligible for both GYR-BCN and OXY-BCN in all the experiments. Therefore, it is unlikely that the arrangement of the cell organelles during division can explain the arrest of ingestion during night.

Consequences of microzooplankton diel feeding rhythms

Regardless of the ecological reasons behind the existence of diel feeding rhythms in microzooplankton, their consequences in natural ecosystems are important. Higher ingestions during day, on pre-dividing algal cells should have more impacts on phytoplankton populations than the same grazing activity on already divided (night) algae. Given the relationship between prey availability and intensity of the diel feeding activity we found, it can be hypothesised diel rhythms are more relevant in upwellings and productive systems than in oligotrophic ones. In all these systems microzooplankton appear as the major herbivore (Calbet and Landry 2004; Schmoker et al. 2013). Unfortunately, there are no e ld data on diel microzooplankton herbivory. The only eld study we are aware of dealt with bacterivory in coastal South China Sea and Hong Kong waters and found evidences of a higher diurnal grazing activity on bacteria (Ng and Liu 2015).

A relevant outcome of our data is that it establishes a resource dependence of diel periodicity in microzooplankton grazing, which can have unanticipated consequences for the most common way to determine microzooplankton grazing rates in the eld, the dilution grazing experiments (Landry and Hassett 1982). Along the dilution series diel feeding activity will be arti cially modi ed since food availability is modi ed. Moreover, the consideration of predator prey growth and grazing coupling is of important relevance when choosing the starting point of the microzooplankton

grazing experiments, as for any other grazing experiment with organisms displaying diel feeding rhythms (e.g. copepods), especially under non steady-state situations. From what has been shown here, it is expected that the outcome of 24-h microzooplankton grazing experiments will not be the same if started during the day or during the night. Theoretically, when the experiments are initiated during the day, the grazing during this period should impact more the phytoplankton populations than when started during the night, when phytoplankton are dividing and microzooplankton are less active. Field data also back up the differences on diel concentrations of phytoplankton and uneven grazing over the daily cycle (Neveux et al. 2003), although the microzooplankton grazing rhythms in that study did not clearly match de ned day night periods. The possible bias in dilution experiments should depend on the prey concentration in the water, being productive areas the most likely affected. We advise, therefore, to indicate in the manuscripts the actual time the experiments begin. We hope future modelling/experimental efforts would provide a way to correct for this artefact, which nowadays reminds as an incognita.

In summary, the coupling or uncoupling of microzooplankton grazing activity with the rhythms of activity of their predators and prey may have relevant consequences for the carbon ow, and biogeochemical cycles in general, in marine ecosystems, and certainly deserves more attention in future studies. The proper integration of protists laboratory data into models of planktonic ecosystem functioning (Jakobsen and Strom 2004) and the inadequate interpretation of this behaviour in eld studies can lead to a biased approximation to the efficiency of matter and energy transfer in the trophic web and on the overall understanding of the functioning of the marine ecosystem.

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Compliance with ethical standards

Ethical standards This study was funded by project FERMI (CGL2014-59227-R; MINECO/FEDER, UE). Anna Arias was funded with a FPI fellowship (BES-2015-074092) from the MINECO of Spain.

Coni ct of interest Anna Arias, Enric Saiz and Albert Calbet declare that they have no coni ct of interest.

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

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ENVIRONMENTAL MICROBIOLOGY

Towards an Understanding of Diel Feeding Rhythms in Marine Protists: Consequences of Light Manipulation

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Abstract
Temporal programs synchronised 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 *Strombidium* 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 regulated not 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.

Keywords Cell division · Continuous darkness · Diel rhythms · Feeding rhythms · Grazing · Microzooplankton · Marine protists

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, characterised 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] and prey availability [7]. 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

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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 the night on the feeding behaviour. It might be possible that individuals dividing during the night (which 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 the night. We can, therefore, hypothesise 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 in 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 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 synchronised cellular division cycle on diel feeding rhythms.

Material and Methods

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 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 60–90 μE m^{-2} s^{-1} of white fluorescent lights). The cryptophyte *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 and air was provided to avoid cell sedimentation. Cultures were diluted daily to maintain them in an exponential phase of growth.

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

Table 1 Initial (averages ± SE) prey and grazer concentrations (cell mL^{-1}) used for the experiments of growth under the 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})
R. salina	152,483 ± 424	–	310,467 ± 1381	–
G. dominans	107,733 ± 508	1265 ± 26	74,720 ± 1201	851 ± 21
O. marina	117,483 ± 593	1677 ± 86	125,783 ± 4572	4686 ± 97
K. armiger	76,444 ± 263	813 ± 44	56,645 ± 316	811 ± 22
Strombidium sp.	182,350 ± 581	631 ± 47	130,783 ± 2382	1330 ± 109

experiment and every 24 h (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 μE m^{-2} s^{-1}) for 24 h 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 months before conducting the experiment. At nearly the end of each day and night period, 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 did prey concentration fall down below the species-specific saturation level. In case the 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.

Evaluation of the Effects of Light Manipulations on Grazing Rates

We investigated the effect of light presence as a 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 the grazers and prey (experimental) and only prey (control) suspensions, and then we distributed them into 72-mL polyethylene culture flasks. Then, all the 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 concentrations 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 228 h for *O. marina*). Bottles from the continuous light experiment were exposed to an irradiation that ranged between 40 and 90 μE m^{-2} 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 minimise day-night cell 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 the 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 a 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 difference in prey size that may occur.

Results

Growth Rates

We detected clear differences between day and night growth rates (μ) under the 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 h^{-1} ± 0.0007 h^{-1}) than during the day (− 0.0006 h^{-1} ± 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 (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 a certain time (ca. 24 h), 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 daytime (Fig. 1d; $p < 0.05$). After several hours of continuous darkness exposure, growth rates decreased below 0 and remained 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 h^{-1} ± 0.0066 during the day and a minimum of 0.004 h^{-1} ± 0.0056 during the night (Fig. 1e). *Strombidium* sp. presented the same day-night pattern as the mixotrophic dinoflagellate *K. armiger*, but with higher growth rates during the daytime. When exposed to continuous darkness, *Strombidium* sp. also experienced a phase of adaptation during the first hours; afterwards, the day-night rhythm was recovered with smaller amplitude.

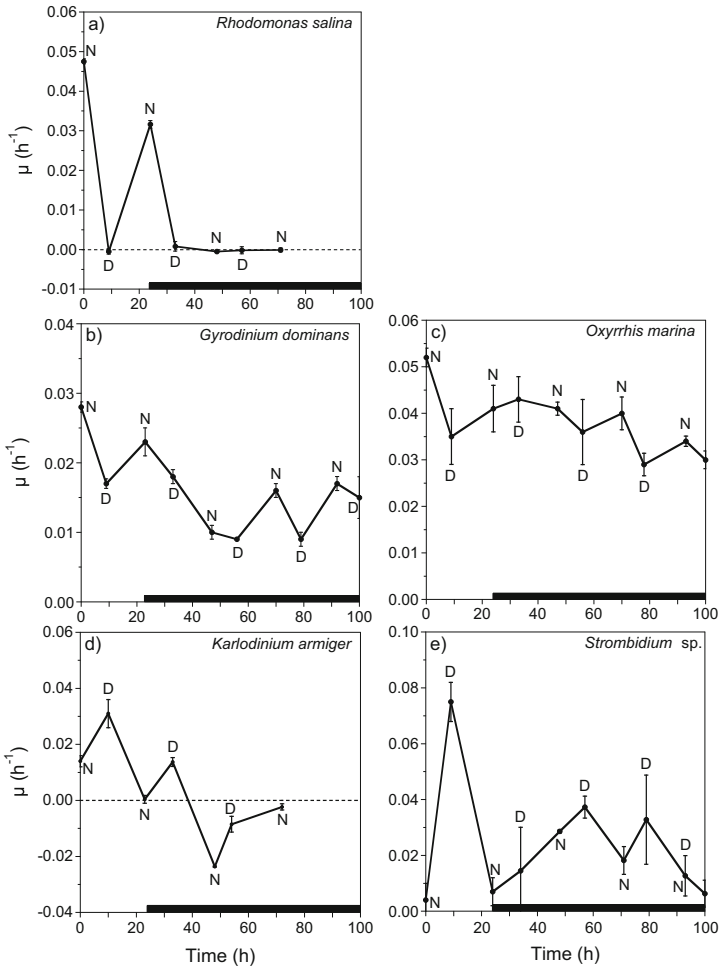

Fig. 1 Growth rates (μ) as function of time of *Rhodomonas salina* (a), *Gyrodinium dominans* (b), *Oxyrrhis marina* (c), *Karlodinium armiger* (d), and *Strombidium* sp. (e). The horizontal black bar indicates the continuous darkness period. The error bars indicate the standard error. D and N indicate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

Grazing Rates

All grazers displayed significant differences between day and night ingestion rates (as prey volume consumed by grazer and hour) under the natural L:D cycle, with higher ingestion rates during 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, $p < 0.001$). A quotient value of 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

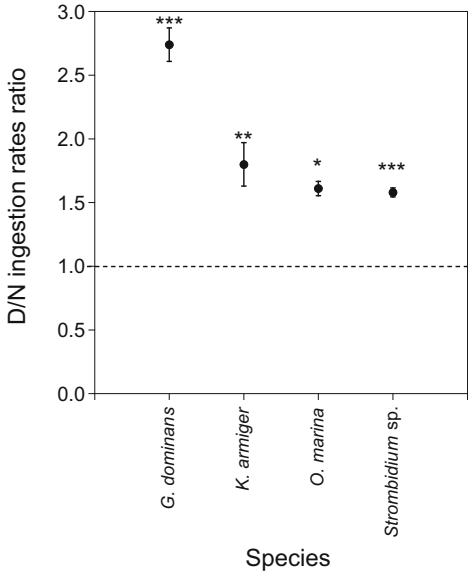

Fig. 2 Feeding rhythm index (quotient between day and night ingestion rates) of *Gyrodinium dominans*, *Karlodinium armiger*, *Oxyrrhis marina* and *Strombidium* sp. Asterisks represent significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The dashed line represents equal day and night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

(day + night) intake of *G. dominans* (616.91 μm^3 $grazer^{-1}$ h^{-1}) remained similar to that showed at the beginning of the experiment under the natural L:D cycle (605.44 μm^3 $grazer^{-1}$ 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 with 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).

Discussion

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

The Role of Light in Microzooplankton Diel Feeding Rhythm

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

Contrarily, *O. marina*, recognised in many instances as a model species for microzooplankton [30], displayed a very distinct response to light manipulation compared with the rest of protozoans. As a matter of fact, neither constant light nor darkness seemed to affect the diel feeding behaviour of *O. marina*. This opportunistic species, inhabiting intertidal pools, salt marshes and embayments [31–34], has a very complicated life history involving encystment and very high growth rates to survive tidal cycles and extreme conditions [34]; because of these characteristics, it could be argued that its feeding and reproductive behaviour might be less dependent on other environmental factors (e.g. light) to optimise survival. The responses of *G. dominans* and *Strombidium* sp. were particularly interesting. While both organisms lost their feeding rhythm under constant light or darkness, the way this loss was attained and the effects on the total daily intake after several days of light manipulation differed. Total daily intakes

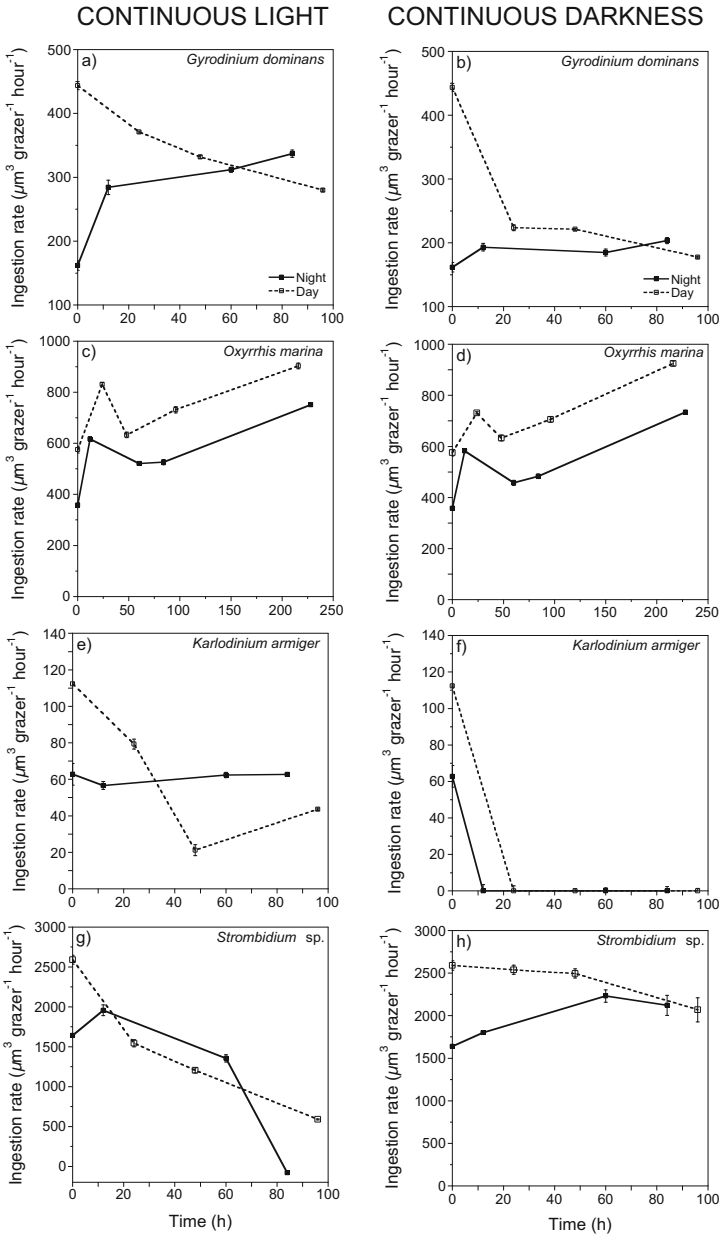

Fig. 3 Day and night ingestion rates (in terms of volume consumed by grazer and hour) as function of time, under continuous light and under continuous darkness treatments, of *Gyrodinium dominans* (a, b), *Oxyrrhis marina* (c, d), *Karlodinium armiger* (e, f) and *Strombidium* sp. (g, h). The dashed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

of *G. dominans* under continuous light were fairly constant, whereas under continuous darkness, they decreased; this diminution was a 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 adaptions, full darkness not only characterises 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 in adverse conditions than e.g. dinoflagellates such as *G. dominans* or mixotrophs.

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], dinoflagellates being clear examples of such a nocturnal physiological response [41, 42]. However, phytoplankton cell division has also been observed, although less frequently, to occur 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 minimisation. 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 been scheduled to optimise the use of resources and minimise energy costs in order to maximise 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, 39]. Flagella-dependent phototaxis is necessary in light conditions for optimisation of light absorption for photosynthesis; therefore, as there is no necessity of phototaxis during dark phase, cell division could mostly take place during the night [38, 39].

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. [53] 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. [54]), 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.

Final Remarks

Our data show that, in general, feeding rhythms in microzooplankton are most likely conditioned not by the cell division cycle, nor by the enhancement of digestion by light, nor 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 synchronising 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 of predators displaying rhythmicity as well [55]. 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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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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

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